NOVEL ANTIBACTERIAL STRATEGIES WITH EMPHASIS ON
THE CHARACTERIZATION OF β-LACTAMASES
AND β–LACTAMASE INHIBITORS

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DECLARATION

I declare that this thesis is my own composition. With the exception of papers 1-12 which describe work carried out as part of my PhD thesis at Edinburgh, no part of this thesis has been submitted for a higher degree.

David J Payne, 2001
ABSTRACT

This Thesis records my involvement and contributions to the discovery, evaluation and characterization of novel antibacterial strategies. My Ph.D. project focussed on the characterization of a series of novel extended spectrum TEM/SHV \(\beta\)-lactamases and plasmid mediated Class C enzymes. This, coupled with research which I conducted in India on the prolific widespread carriage of resistant organisms, provided me with an excellent insight into the need for novel antibacterial agents to combat the rising tide of resistant organisms. Subsequently, on joining SmithKline Beecham in 1990 I performed research that led to the discovery and characterization of a variety of novel inhibitors of serine \(\beta\)-lactamases. I also led a research initiative which successfully characterized a variety of new metallo-\(\beta\)-lactamases and identified novel inhibitors of this group of \(\beta\)-lactamases. Furthermore, these compounds demonstrated some potential for combating this emerging resistance mechanism. In 1995 my interests focussed on the exploitation of bacterial genomics for the identification and characterization of novel antibacterial targets. I initiated projects to evaluate fatty acid biosynthesis, cell wall biosynthesis, aromatic amino acid biosynthesis, cell division and protein secretion as antibacterial strategies. This work provided the first ever identification and characterization of the Gram positive cocci homologs of FabI (enoyl ACP reductase, fatty acid biosynthesis), MurA (UDP-N-acetylglucosamine enolpyruvyl
transferase, peptidoglycan biosynthesis), EPSP synthase (5-enolpyruvylshikimate-3-phosphate synthase, aromatic amino acid biosynthesis), FtsA, FtsZ (cell division) and SRP (signal recognition particle, protein secretion). It is my hope that this work will either directly or indirectly facilitate the discovery of pioneer antibacterial agents urgently required for our battle against bacterial pathogens in the next millennium.
ACKNOWLEDGEMENTS

First and foremost my thanks go to Professor Sebastian Amyes who supervised me through my Ph.D. and started me off in my research career. The majority of the research which makes up this Thesis was conducted during my employment at SmithKline Beecham Pharmaceuticals and demonstrates the company’s quest to conduct pioneer research and encourage their employees to further their scientific career. I thank Ian Chopra, David Knowles and David Winstanley (ex SmithKline Beecham) for enthusing me to lead teams of outstanding researchers to tackle a variety of exciting projects whilst at Brockham Park, UK and Marty Rosenberg (Collegeville PA, USA) for entrusting and supporting me to lead my current group and introducing me to the forefront of bacterial genomics and genomic technologies. Sincere thanks also to all the members of Anti Infectives Research Department at SmithKline Beecham (Collegeville PA, USA), and more specifically, to the exceptionally talented members of my current Group. Not only have many of these scientists played an integral role in the publications in this thesis but I am also grateful for their unique depth of support and friendship. Many thanks also to all the many other gifted and talented scientists that I have had the pleasure of interacting and collaborating with. Last and by no means least I thank my family and Kathy for their continued support and encouragement, especially Carole and Adrian for the photocopying.
INTRODUCTION

My interest in antibacterial strategies began in the summer of 1986 when I spent a 6 month sandwich training period at the Department of Chemotherapy, Glaxo Group Research, Greenford, UK as part of my Biochemistry Degree at Brunel University. I worked with Dr Ian Simpson on the characterization of plasmid mediated β-lactamases, this began my fascination with bacteria and antibacterial agents. Following graduation I was given the opportunity to do a CASE funded Ph.D. under the supervision of Professor Amyes (University of Edinburgh) and Dr Mike Marriott (Department of Chemotherapy, Glaxo Group Research, Greenford, UK). The focus of this project was to identify and characterize plasmid mediated β-lactamases which conferred resistance to third generation cephalosporins such as ceftazidime and cefotaxime. Under the superb supervision of Professor Amyes I discovered and characterized the novel extended spectrum plasmid mediated β-lactamases, TEM-E1, TEM-E2, TEM-E3, TEM-E4 and DJP-1 [1-8]. In all cases these enzymes were shown to confer transferable resistance to third generation cephalosporin antibiotics and hydrolyze representative compounds from this group of antibiotics. Of particular significance was TEM-E2 [8]. It was documented in 1982 that a patient was originally infected with a ceftazidime sensitive Klebsiella oxytoca which produced TEM-1, however during therapy with a third generation cephalosporin (3GC) the strain became resistant to ceftazidime. Upon initial
analysis it was erroneously documented that "the acquired resistance was not enzymatic since only the presence of TEM-1 could be demonstrated". However, my subsequent analysis 5 years later revealed that this strain produced a plasmid mediated extended spectrum TEM β-lactamase (TEM-E2) which hydrolyzed ceftazidime. This enzyme was latter sequenced and designated TEM-12. As this strain dates back to 1982 we believe that this may have been one of the very first extended spectrum β-lactamases to have been identified. I was then intrigued to investigate if these extended spectrum β-lactamases evolved from the TEM-1 wild type strain via selective pressure of 3GCs. Publications [9, 10] illustrate these enzymes could indeed be obtained spontaneously from TEM-1 and this is thought to explain the occurrence of extended spectrum β-lactamases in clinical isolates. When I initiated this work these enzymes were not widespread [11], yet today most hospitals around the world has been confronted with a clinical isolate producing an extended spectrum TEM or SHV β-lactamase.

In 1989 the Division of Hospital Infection, PHLS Colindale sent me a clinical strain of *E.coli* isolated from a patient who suffered severe acid burns in Pakistan and who was subsequently transferred to Queen Mary's University Hospital where the strain was isolated. This strain exhibited transferable resistance to 3GCs yet unlike the extended spectrum TEM and SHV β-lactamases the β-lactamase responsible for this transferable resistance was not inhibited by clavulanic acid [12].
The β-lactamase was designated BIL-1 after the name of the patient (Shafiq Bilal) and subsequent biochemical and DNA sequencing analysis of this enzyme revealed that this was one of the first reports of a plasmid mediated Class C β-lactamase [13, 14]. When I moved to SmithKline Beecham in 1990 I initiated a project to clone and sequence BIL-1 and this illustrated that BIL-1 appeared to originate from the Citrobacter freundii ampC gene [15]. Since this discovery many other examples of plasmid mediated β-latamases have been identified [16]. The work on BIL-1 was part of a very productive collaboration with PHLS Colindale along with Andy Fosberry, Elizabeth Lawlor and John Hodgson from SmithKline Beecham who taught me cloning and sequencing techniques.

The above research projects provided me with a grasp of the ingenuity of how bacteria can circumvent the challenge of β–lactam based antibacterial therapies. However, it was not until I took part in a Medical Research expedition to South India headed by Professor Amyes that I gained a comprehension of the true impact and prevalence of bacterial resistance. This expedition was organized outside of my Ph.D project and the goal was to compare the carriage of antibiotic resistant bacteria from town and village dwelling populations. To our surprise virtually all the subjects included in our study, irrespective of location, carried commensal bacteria resistant to trimethoprim, ampicillin and chloramphenicol [17-20].
From this point onwards my interests moved towards identifying and characterizing novel agents to combat multi resistant bacteria. The first of these was the work I performed shortly after completing my Ph.D. on the evaluation of cefdinir (a novel oral cephalosporin antibiotic). In this study the antibacterial activity of cefdinir was compared with cefixime, cephalexin, cefuroxime and amoxicillin against recently isolated strains of *Moraxella* spp., *H.influenzae*, *Streptococcus* spp., *Staphylococci* spp. and a selection of Gram negative rods. Cefdinir was as effective as cefixime against *H.influenzae* and *Moraxella* spp. and both were more active than cefuroxime. Cefdinir had the lowest MIC$_{50}$ of all the drugs tested against *Staphylococcus* [21, 22]. Further analysis demonstrated that cefdinir was more stable to 14 extended spectrum $\beta$-lactamases than either cefuroxime or cefixime, however ceftazidime was more stable than cefdinir to hydrolysis by eight of the 14 enzymes tested [23, 24]. Interestingly, even though cefdinir represents a new class of more potent oral cephalosporins it is still compromised by pre-existing antibiotic resistance mechanisms and thus exemplifies the need for novel antibiotics acting on novel antibacterial targets.

In 1990 I joined SmithKline Beecham Pharmaceuticals and here I begun research into the evaluation of novel and established $\beta$–lactamase inhibitors. Some of the first work that I performed was to develop a novel microtitre plate assay for the rapid evaluation of $\beta$–lactamase inhibitors. This assay enabled 8 IC$_{50}$s to be
determined simultaneously (using 10 different concentrations) employing approx. 20 times less reagents than established spectrophotometric techniques [25-27]. This enabled significant numbers of β-lactamase inhibitors to be tested against a wide range of different clinically relevant β-lactamases [28]. This technique was used to compare the potency of the three clinically used β-lactamase inhibitors (clavulanic acid, tazobactam and sulbactam) against >30 different plasmid mediated β-lactamases [29, 30]. This was the first time such a study was performed under the same experimental conditions, and consequently this is a frequently quoted paper. All the proteins and reagents were generated specifically for this study. Further to our comparative analysis of these three serine β-lactamase inhibitors we established an approach for evaluating the relative permeability of each of these compounds across the outer membrane of *E.coli, K.pneumoniae* and *E.cloacae*. In these studies we demonstrated for the first time that clavulanic acid penetrated these Gram negatives more readily than the other β-lactamase inhibitors [31].

IC₅₀'s and permeability analysis are both useful approaches for the relative evaluation of established β-lactamase inhibitors. However, when characterizing novel β-lactamase inhibitors more in-depth analysis of the mechanism of β-lactamase inhibition is required to facilitate the generation of structure activity relationships (SAR) and providing directions for improvements in inhibitor design. BRL 42715 is an experimental β-lactam based β-lactamase inhibitor, designed by
SmithKline Beecham scientists and paper [32] illustrates the first in-depth kinetic and physical analysis of β-lactamase inhibition by this molecule. BRL42715 was one of the most potent β-lactamase inhibitors so far discovered and our analysis demonstrated that the stoichiometry of inhibition was 1:1 for the TEM, P99 and S.aureus β-lactamases. This work also describes one of the early applications of electrospray mass spectrometry to study enzyme: inhibitor interactions. This technique provided a rapid confirmation of the stoichiometry and covalent interaction of BRL42715 with β-lactamases. Similarly paper [33] describes the discovery of a series of novel phosphonamidate serine β-lactamase inhibitors. These compounds were synthesized by John Bateson’s group of the Department of Medicinal Chemistry at SmithKline Beecham, Harlow, UK and was the start of a long and cherished collaboration with him and his Group. These were some of the most potent non-β-lactam based β-lactamase inhibitors so far discovered achieving μM inhibitory activity against the E.cloacae P99 β-lactamase. In addition, antibacterial synergy in combination with a β-lactam, was achieved against strains producing β-lactamases illustrating the therapeutic potential of these agents. Our use of electrospray mass spectrometry in this instance eloquently demonstrated both the potency and the mechanism of inhibition. The increase in mass of P99 resulting from exposure to these inhibitors demonstrated that the compounds irreversibly inhibited P99 via phosphonylation of the active site serine with the phosphonamidate bond as the scissile bond and the amino acid as the
leaving group [34]. These studies also initiated a long term collaboration and association with Jean Marie Frère's Group at Liege University, Belgium who assisted with the kinetic evaluation of these inhibitors [35]. My interest and application of ESMS for studying enzyme:inhibitor interactions was inspired by Robin Aplin, University of Oxford. Our initial collaborative work in this area demonstrated that satellite bands observed on isoelectric focusing gels (IEF) of \( \beta \)-lactamases were in some cases "ragged ends" caused by the loss of subsequent N-terminal amino acids [36]. Our other work in this area illustrated that \( \beta \)-lactam antibiotics could also induce satellite bands on IEF gels [37]. Previous to these two studies the nature of satellite bands on IEF remained the subject of much conjecture.

Other novel serine \( \beta \)-lactamase inhibitors that I have been involved in identifying include some natural product derived lipopeptide leads, one of these compounds was shown to be a time dependent inactivator of TEM-2 [38].

During the 1990s metallo-\( \beta \)-lactamases were beginning to emerge as a serious threat to the successful use of \( \beta \)-lactam based antibiotics. My review "Metallo-\( \beta \)-lactamases – a New therapeutic challenge" illustrated the potential of this threat and reviewed the occurrence of these enzymes on plasmids and in common clinical pathogens [39]. In 1992 John Bateson and I were appointed by Dr Ian Chopra to
head a multi-disciplinary team to explore the feasibility of designing a broad spectrum metallo-β-lactamase inhibitor. Such an inhibitor could be used in combination with a β-lactam agent as an effective approach to tackling strains producing metallo-β-lactamases. My role was to head the biological aspects of this research.

The initial focus of this project was to generate more reliable information on the occurrence of metallo-β-lactamases in different pathogens. Consequently, I initiated research to generate diagnostic approaches to identify the presence of metallo-β-lactamases in clinical pathogens. This was successfully achieved for *Stenotrophomonas maltophilia* by running crude extracts with and without EDTA and BR42715. Levels of these compounds were identified that would selectively inhibit the metallo-β-lactamase and serine β-lactamase bands on the IEF gel, respectively [40]. For the first time this technology enabled us to illustrate that the majority of clinical isolates of *S. maltophilia* produced both a metallo-β-lactamase and a serine β-lactamase. This analysis alone demonstrated that this species produced at least 7 different types of metallo-β-lactamases and up to 8 different types of serine β-lactamases [41]. We purified the majority of these different enzymes and characterized their activity with a variety of different β-lactam substrates and inhibitors. These studies suggested subtle yet significant differences between the active sites of the different enzymes [42, 43].
In addition, I performed a variety of other studies to determine the incidence of metallo-β-lactamases in clinical isolates. In collaboration with Professor Amyes and Dr. R. Stunt, a PhD student we jointly supervised, we assessed the incidence of metallo-β-lactamase production by carbapenem-resistant strains of *P. aeruginosa* from Japan [44] and characterized a novel carbapenem hydrolyzing enzyme from *Aeromonas veronii* biovar *sobria* [45, 46]. We also developed an IEF/microbiological assay approach for the detection and characterization of carbapenemases on IEF gels [47]. Dr. John Munn was another PhD student jointly supervised by Professor Amyes and myself and in this work we characterized the metallo-β-lactamases produced by clinical isolates of *Flavobacterium* and *Bacteroides fragilis* [48-50]. With Dr. Tim Walsh, University of Bristol, we were the first to determine the nucleotide and peptide sequence of the metallo-β-lactamase from *A. veronii* bv. *sobria* [51, 52]. This publication further demonstrated that the active site sequence of Asn-Tyr-His-Thr-Asp was unique to metallo-β-lactamases from *Aeromonas* spp. With T. Khushi and A. Fosberry of SmithKline Beecham we sequenced the CfIA metallo-β-lactamase from *B. fragilis* ED262 and demonstrated the presence of the CfIA enzyme in strains of *B. fragilis* isolated before 1987, predating the widespread use of carbapenems [53]. Finally, in collaboration with C. Betriu (University Hospital, San Carlos, Madrid) we illustrated the presence of the CfIA metallo-β-lactamase in six clinical isolates of
*B. fragilis* [54]. These studies made a significant contribution to enabling the microbiology community in track and understand the prevalence of metallo-β-lactamases.

The above research coupled with data generated by other workers, particularly the emergence of the IMP-1 metallo-β-lactamase in Japan, began to emphasize that metallo-β-lactamases were an increasing potential threat to the future successful use of β-lactam agents. Consequently, this further emphasized the need to discover broad spectrum metallo-β-lactamase inhibitors.

Natural product screening identified two novel phenazine metallo-β-lactamase inhibitors extracted from a strain of *Streptomyces* sp. which was isolated from a "garden path". Unfortunately our further analysis of these compounds demonstrated that they were non specific inhibitors of metallo-β-lactamases as their mechanism of inhibition was via non specific chelation of the active site metal ion and thus of no therapeutic utility [55]. Cross screening initiatives then identified a series of mercaptoacetic acid thiol esters as possessing IC\textsubscript{50}s as low as 2μM against certain metallo-β-lactamases. Here we used a combination of complex kinetics, tryptic digestion, amino acid sequencing and electrospray mass spectrometry to illustrate these compounds were specifically inhibiting metallo-β-lactamases via the mechanism based delivery of mercaptoacetic acid to form a disulphide linkage with
the active site cysteine residue. This represented the first mechanism based inhibitors of metallo-\(\beta\)-lactamases to be discovered [56]. This discovery lead us to synthesize a series of related mercaptophenylacetic acid thiol esters. Again our analysis demonstrated that the mode of inhibition of these compounds was not via non specific chelation of the active site zinc. Interestingly, unlike the previous series, these compounds were shown to be competitive inhibitors of metallo-\(\beta\)-lactamases [57]. However, neither the mercaptoacetic nor the mercaptophenylacetic acid thiol esters demonstrated any antibacterial synergy with a \(\beta\)-lactam antibiotic against strains producing metallo-\(\beta\)-lactamases. Therefore, although these novel inhibitors provided further information about approaches for inhibiting metallo-\(\beta\)-lactamases our search for a lead that would provide clinical utility continued.

I then instigated the screening of a variety of known metalloenzyme inhibitors against the *B.cereus* II and CfiA metallo-\(\beta\)-lactamases. This led to the discovery that thiophan (known inhibitor of neutral endopeptidase) and captopril (commercially available inhibitor of angiotensin converting enzyme, ACE) were low potency, competitive inhibitors of metallo-\(\beta\)-lactamases. Consequently, the chemistry team synthesized a series of mercaptocarboxylate inhibitors incorporating substrate recognition features from the \(\beta\)-lactam substrates. To our immense delight this resulted in a molecule (SB225666) with a >30 fold
improvement in metallo-β-lactamase inhibitory potency and a 2000 fold improvement in selectivity compared to thiorphan. In addition, this molecule exhibited specific inhibition (Ki values <1μM) of all the clinically relevant metallo-β-lactamases (CfiA, IMP-1 and L-1) and demonstrated antibacterial synergy with meropenem against strains of *B.fragilis* producing CfiA [58]. This discovery represented the precursor to a substantial novel inhibitor design and synthesis program lead by Dr John Bateson and I [59-61]. My responsibilities included the inhibitory & synergy evaluation of the lead molecules along with the generation of the biological tools, such as production of proteins and crystal structures, required to resource a competitive pharmaceutical research program.

Substantial improvements in inhibitory potency and antibacterial synergy were achieved with this initial lead largely through a rational inhibitor design utilizing crystal structures of metallo-β-lactamases. To this end we generated a high quality structure of the *B.cereus* II metallo-β-lactamase via a proactive collaboration with Dr Brian Sutton and Dr Stella Fabiane (Post Doc) of The Randall Institute, Kings College London, and this structure had a significant impact on our initial inhibitor design strategies [62, 63]. Later in the program we were the first group to generate a structure of the IMP-1 metallo-β-lactamase which has become the most clinically relevant of all the metallo-β-lactamases [64]. This structure provided crucial data to enhance our understanding of the active site interactions of these types of
compounds as illustrated by reference [64] which describes the crystal structure of IMP-1 with one of our lead mercaptocarboxylate inhibitors complexed in the active site. Furthermore, we have demonstrated that some of these molecules also possess inhibitory activity against serine β-lactamases [65]. Optimization of this series of metallo-β-lactamase inhibitors continues and of all the metallo-β-lactamase inhibitors so far reported, these mercaptocarboxylate molecules clearly show the greatest therapeutic promise to tackle this emerging resistance threat.

My interest in β-lactamases has also resulted in several reviews and book chapters and peripheral studies on β-lactamase producing strains [66-79].

Although, the design and optimization of β-lactamase inhibitors remains a validated and extremely successful approach to tackling multi resistant pathogens there is a widely recognized unmet medical need for novel antibacterial agents targeting novel antibacterial targets. Consequently, in 1995 I embarked on research strategies to utilize the power of bacterial genome data to identify and novel antibacterial targets [80]. I began by leading a team to investigate the enzymes involved in bacterial fatty acid biosynthesis as antibacterial targets [81]. Prior to 1995 the majority of the research on this pathway and the component enzymes focused on E.coli and at that time almost nothing was known about fatty acid
biosynthesis in Gram positives pathogens. Sequencing of entire bacterial genomes enabled rapid identification of the pathway enzymes in Gram positive pathogens for the first time [82-88]. Initial focus was on FabI, an enoyl ACP reductase, and I led a team to characterize and exploit this enzyme as a novel antibacterial target. We were the first group to identify, clone, express and purify this enzyme from *Staphylococcus aureus* [89] and we conducted the first detailed kinetic evaluation of this enzyme [90]. During our evaluation of this target it came to light that the molecular target of triclosan was FabI. Triclosan is a widely used biocide used in a variety of consumer products ranging from children’s toys to soap. Previously its mode of action was thought to be via the non specific dissolution of bacterial membranes so it was alarming to find that it had a specific protein target and thus potentially prone to evolution of resistance. We then decided to see if the inhibition of FabI by triclosan would provide any insight into approaches for inhibiting FabI. Consequently, we generated a crystal structure of triclosan complexed in the active site of the *E.coli* FabI. Although other workers had generated similar structures of FabI and triclosan our structure was the first to illustrate that the Ile192- Ser 198 loop adopts a closed conformation forming interactions with the bound triclosan and the NAD+ co factor. In all previous structures this loop remained either disordered or 'open'. The opening and closing of this flipping loop is likely to be an important factor in substrate recognition and inhibitor design [91]. Up until now all of the work illustrating the molecular target of triclosan was FabI centered on
analysis performed with *E.coli* and the *E.coli* FabI. However, we were the first group to demonstrate that the mode of antibacterial action of triclosan was also Fab I in *S.aureus* [92]. I felt it was more relevant to analyze the effect of this compound on *S.aureus* as the majority of triclosan use is focussed, certainly in the hospital environment, at the eradication of multi-resistant *S.aureus*. Our endeavors to identify potent developable FabI targeted antibiotics continues.

Another novel antibacterial target that I championed and led a small team to further exploit was aromatic amino acid biosynthesis and bacterial genomics enabled rapid identification of some of the component enzymes in Gram positive pathogens [93-97]. 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) is the penultimate enzyme in the chorismate biosynthetic pathway. This enzyme and pathway has been proposed as an antibacterial target for sometime, however, much of this enthusiasm is based on work performed on *E.coli* and *Salmonella* spp. with little work being performed with Gram positives. Analysis of bacterial genomes enabled us to now demonstrate that this is a conserved target present in many key clinical pathogens [98]. As it is imperative that any novel antibacterial agent is active against Gram positive pathogens, such as penicillin resistant *S.pneumoniae* and MRSA, we focussed our work on the characterization of the EPSP synthase from *S.pneumoniae*. Much of the work on this enzyme was performed by Wensheng Du who was an outstanding Post Doc under my supervision for 2 years.
His initial work described the identification, cloning, expression, purification and kinetic characterization of this enzyme [99]. In these studies he observed that the enzyme was activated by univalent cations, a phenomenon not explored by other workers. Interestingly, the concentrations causing a significant change in enzyme characteristics are physiologically relevant and thus we concluded that this was an important aspect of the characterization of bacterial EPSP synthase enzymes. Further research in this area focussed on detailed studies of the mechanism of this enzyme [100, 101] and an investigation of the thermodynamic linkages of the shikimate-3-phosphate substrate and univalent cations on the binding interaction of glyphosate with the enzyme [102, 103]. This extensive evaluation enabled us to run a high throughput screen to look for novel inhibitors of this target. As part of this work we identified aurin tricarboxylic acid as a competitive inhibitor of the S.pneumoniae EPSP synthase [104].

Peptidoglycan biosynthesis has been exploited as a source of antibacterial targets for some time. MurA is the antibacterial target of the antibiotic fosfomycin and I lead a team to further exploit MurA as an antibacterial target. Again much of the previous work on MurA had focused on the characterization of the enzymes from E.coli. As our focus was to design antibacterials that would be effective against multi-resistant Gram positive pathogens we evaluated this target in Gram positives and specifically S.pneumoniae [105]. Our initial analysis demonstrated for the first
time that Gram positive cocci exhibit two copies of Mur A whereas Gram negatives possess only one copy. Consequently, we set out to perform the first ever characterization of both Mur A enzymes from *S. pneumoniae*. The majority of this work was performed by Wensheng Du and he showed that both enzymes were kinetically very similar and both were inhibited by fosfomycin. Individual gene deletions showed that neither were essential yet a double gene deletion resulted in non-viability [106, 107]. As yet we have no rational reason why Gram positives possess two copies of this enzyme. However, this observation impacts on our discovery of novel Mur A antibacterials as to achieve activity against Gram positive pathogens any chemical leads we obtain in the future will need to inhibit both enzymes. I also initiated a small research program on the evaluation of the antibacterial clofoctol which was proposed as targeting cell wall biosynthesis. Clofoctol is an unexploited chemical structure and the goal of this project was to make clofoctol derivatives. Paper [108] characterizes the effects of clofoctol on bacterial membranes.

Cell division is one of the most critical events in the life cycle of a bacterial cell. Although many proteins have been implicated with this process [109], relatively little is known about their orchestration and interactions with each other. *FtsZ* is one of the key bacterial cell division proteins forming a ring at the cell division septum [110]. It has been known for some time that *FtsA* and *FtsZ* co-localize at
the septum in *E.coli* and this is proposed as a critical step for successful cell division and thus this interaction is a potential antibacterial target. Work in this area was performed by Dr Kang Yan of my group who demonstrated that this interaction occurred in Gram positive pathogens [111], furthermore by using the yeast two hybrid system he identified a specific residue that was responsible for the interaction [112]. Consequently, this work infers that the surface of interaction between these two proteins is likely to be small and thus it is feasible that a small molecule could inhibit the interaction. This study represents some of the first work ever to be performed on cell division enzymes from Gram positive cocci and demonstrates that the design of small molecule antagonists or agonists of the FtsA-FtsZ interaction may be a viable approach to tackling multi-resistant Gram positive pathogens.

I have also been involved in exploring the signal recognition pathway (SRP) as a source of antibacterial targets. This process is involved in bacterial protein secretion and consists of two essential protein components Ffh and FtsY and a small cytoplasmic RNA (4.5S RNA). Again this is a well studied system in *E.coli* with little work conducted in Gram positives. In this work, performed by Dr Angela Steel of my group, the Ffh, FtsY from *S.aureus* were identified, cloned, expressed and purified. Site directed mutagenesis on Ffh demonstrated residues involved in RNA binding yet did not effect the GTPase activity of Ffh [113]. This
is the first time any work has been conducted on the S.aureus SRP and provides the possibility that interrupting the 4.5SRNA: Ffh interaction could be a viable antibacterial approach.

In conclusion, my initial research focused on characterising and identifying the ingenious methods that bacteria implement to resist the challenge of antibiotics and I have seen first hand the extent of the problem of bacterial resistance. This provided me with a very useful knowledge of the problems associated with the use of antibiotics and the need for new antibacterial agents. My work at SmithKline Beecham Pharmaceuticals focused on approaches to overcome bacterial resistance using the established strategies of β–lactam: β–lactamase inhibitor combinations. However, in the last 4 years I have witnessed and played a part in the incredible impact that genomics is having on the antibacterial therapeutic area. Genomics is clearly enabling the identification and validation of novel antibacterial targets at an unprecedented rate and this quantum jump in technology will provide new antibacterial targets for the future [114-116]. Moreover, it is my hope that my research into both genomic based targets, β-lactamase inhibitors and β–lactamases will at some point in my career facilitate the discovery of a novel antibacterial agent to help overcome the increasing human suffering associated with infections caused by multi resistant pathogens.
LIST OF PUBLICATIONS


2. PAYNE, D. J., MARRIOTT, M. S., CHRISTODOULOU, C. & AMYES S. G. B. (1990). TEM-E4: A β-lactamase which confers transferable resistance to ceftazidime. *Journal of Pharmacy and Pharmacology* 42, 61P. *Payne performed majority of scientific experimentation; Chistodoulou provided molecular biology expertise; Amyes and Marriott were Ph.D. supervisors.*


7. PAYNE, D. J., MARRIOTT, M. S. & AMYES, S. G. B. (1990). Comparison of TEM-E3 and TEM-10 β-lactamases. Abst. A-70. 90th American Society for Microbiology, Anaheim, California, USA. Payne performed all practical work; Marriott and Amyes were Ph.D. supervisors.


12. WOODFORD, N., PAYNE, D. J., JOHNSON, A. P., WEINBREN, M. J.,
PERINPANAYAGAM, R. M., GEORGE, R. C., COOKSON, B. D. &
by clavulanic acid in *Escherichia coli*. Lancet 336, 8709, 253. Payne provided
practical work to illustrate that this enzyme (BIL-1) was a Class C like enzyme;
Woodford and Johnson performed conjugations and MICs; Weinbren and
Perinpanayagam isolated strain; Cookson, Amyes and George provided
intellectual input and supervision.

of the plasmid mediated β-lactamase BIL-1. Journal of Antimicrobial
Chemotherapy 30, 119-127. Payne performed majority of practical work;
Woodford provided strains for the study; Amyes was PhD supervisor.

mediated Class C β-lactamase. The Fifth β-lactamase Workshop, Holy Island.
Payne and Fosberry each provided 50% practical input.

Cloning and sequence analysis of *bla*BIL-1: a plasmid mediated Class C
β-lactamase gene in *Escherichia coli* BS. Antimicrobial Agents and Chemotherapy 38, 1182-1185. Payne and Fosberry each provided 50% practical input; Lawlor and Hodgson provided intellectual and editorial input.


19. AMYES, S. G. B., TAIT, S., THOMSON, C. J., PAYNE, D. J., MUKUNDAN, U. & JESUDASON, M.V. (1992). Reservoirs of antibiotic resistance genes. Conference on Hospital Acquired Infections, Christian Medical College Hospital, Vellore, Tamil Nadu, India. Payne, Tait and Thomson all participated equally in the collection and characterization of the clinical isolates from South India; Amyes, Jesudason and Mukundan coordinated the project.


Munich, Germany. Payne performed all scientific experimentation; Amyes was Ph.D. supervisor.


28. Payne performed 90% of practical work; Coleman provided computing expertise; Cramp performed 10% of the practical work.


27. **PAYNE, D. J. & PRADHANANGA, S. L.,** (1996). A microtitre-based assay for the determination of ID$_{50}$'s of β–lactamase inhibitors employing reporter substrates detected at UV or visible wavelengths. Application Note 1-3, Molecular Devices. **Payne developed methodology; Pradhananga generated inhibition data.**

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29. PAYNE, D. J., CRAMP, R., WINSTANLEY, D. & KNOWLES, D. J. (1994). Comparative activities of clavulanic acid, sulbactam, and tazobactam against clinically important β-lactamases. Antimicrobial Agents and Chemotherapy 38, 767-772. Payne designed approach and performed 50% of practical work; Cramp performed 50% of practical work. Winstanley and Knowles provided editorial and intellectual input.


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assistance; Knowles, Clarke and Bateson provided intellectual and editorial input.

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Stunt and Thomson performed practical work; Amyes and Payne supervised Stunt.


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Amyes instigated and coordinated project; Munn performed characterization; Thomson provided intellectual input.


Payne instigated project & performed IEFs and biochemical characterizations; Khushi assisted Payne; Fosberry performed sequencing; Reading provided editing input on the manuscript.


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Payne instigated and coordinated work, performed kinetic analysis and generated samples for mass spec. analysis; Bateson, Frere, Gasson, Ghosez, Marchand-Brynaet synthesized compounds, Skett, Tolson, Bell performed mass spec. analysis; Marshall and Reid performed ITC analysis.


Niconovich generated antibacterial analysis; Pearson and Cheever generated inhibition data (supervised by Payne).


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1 and co-ordinated & instigated all biological aspects of this work. Concha, Janson and Clarke performed crystallography and modeling; Gasson synthesised compounds; Cheever and Pearson performed kinetic analysis; Rawling and Lewis generated IMP-1 protein. Abdel-Meguid was lab head of the crystallography group, Bateson coordinated chemical aspects of this work.


*Coleman 40%, Payne 30%, Simpson 15%, Thorburn 15%.*

Payne invited to write review, equal contributions from Payne, Lonsdale and Pearson.


Payne provided $\beta$-lactam, $\beta$-lactamase inhibitors and efflux sections (approx 40% of review)

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Authors alphabetical, all authors made an equal contribution.


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performed bioinformatics; Sylvester performed cloning and purification of \textit{MurA1}.


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PUBLICATIONS


Plasmid-mediated ceftazidime resistance identified in a strain of Serratia marcescens isolated in Belgium

Sir,

There have been many reports of β-lactamases responsible for transferable resistance to third generation cephalosporins (Payne, Marriott & Amyes, 1989a, 1990; Philippon, Labia & Jacoby, 1989; Woodford et al., 1990) including three different types from Belgium (Payne et al., 1989a; Vuye, Verschraegen & Claeys 1989a). It has also been shown that some of the β-lactamases, responsible for this transferable resistance, can be obtained by spontaneous mutation from TEM-1/2 or SHV-1 enzymes (Gutmann et al., 1988; Sougakoff et al., 1988; Payne, Marriott & Amyes, 1989b). We would like to report the characterisation of another novel TEM-derived β-lactamase, designated TEM-E4, which shows similarities with a mutant β-lactamase (designated Mutant β-lactamase D) that was derived from spontaneous mutation of an Escherichia coli 153-2 strain encoding the RP4 plasmid, which produced the TEM-2 β-lactamase.

The TEM-E4 β-lactamase was produced by the ceftazidime-resistant Serratia marcescens 7919, a clinical strain which was isolated from a patient hospitalized in Belgium during 1987. When the ceftazidime-resistant S. marcescens 7919 was conjugated with the ceftazidime-sensitive E. coli J53-2, ceftazidime-resistant E. coli transconjugants were obtained (transfer frequency of 1·3x10−6 transconjugants per donor cell). Analysis of the plasmid DNA in these transconjugant strains showed a single plasmid band of 56 Kbp and this was designated pUK724. This plasmid was also visualized in the S. marcescens strain.

The spontaneous mutation procedure described by Payne et al., (1989a) was used to obtain Mutant β-lactamase D from the TEM-2 enzyme. The TEM-2 β-lactamase was encoded by the 51 kb plasmid RP4, which was carried by E. coli strain J53-2. Mutants encoding β-lactamase D were obtained at a frequency of approximately 1 in 109 cells. The ceftazidime resistance expressed by Mutant β-lactamase D was transferred to E. coli K12 strain SA10 (methionine deficient) when either ceftazidime or kanamycin was used as the selection agent. EcoRI restriction analysis of the plasmid DNA encoding the β-lactamases revealed that the plasmids encoding Mutant β-lactamase D and TEM-2 were identical and confirmed they were distinctly different from pUK724. These results confirm that the gene encoding Mutant β-lactamase D was indeed located on the RP4 plasmid.

The clinical S. marcescens 7919 strain was resistant to all the antibiotics tested, with the exception of imipenem (Table I). However, the E. coli transconjugant of this strain was resistant to ceftazidime but sensitive to all the other third and second generation cephalosporins tested. The levels of ceftazidime and ampicillin resistance, expressed by the E. coli transconjugant, were diminished by the addition of clavulanic acid (2 mg/l) to the media. This supports the view that the β-lactam resistance of the E. coli J53-2 transconjugant of S. marcescens...
### Table I. MICs conferred by TEM-E4 and the Mutant β-lactamase D (10^5 cfu inoculum)

<table>
<thead>
<tr>
<th>β-Lactam antibiotic</th>
<th>E. coli J53-2 (TEM-2)</th>
<th>E. coli J53-2 producing β-lactamase D</th>
<th>Serratia 7919</th>
<th>Serratia 7919 x E. coli J53-2</th>
<th>E. coli J53-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>4</td>
</tr>
<tr>
<td>+ clav</td>
<td>8</td>
<td>8</td>
<td>&gt; 32</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>8</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>32</td>
<td>16</td>
<td>&gt; 250</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>4</td>
<td>8</td>
<td>&gt; 250</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1</td>
<td>4</td>
<td>125</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>&lt; 1</td>
<td>4</td>
<td>&gt; 125</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cefazidime + clav</td>
<td>&lt; 0.06</td>
<td>0.25</td>
<td>2</td>
<td>0.25</td>
<td>0.13</td>
</tr>
<tr>
<td>Cefotaxime + clav</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>&gt; 4</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>16</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>8</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Imipenem</td>
<td>&lt; 0.06</td>
<td>0.13</td>
<td>2</td>
<td>&lt; 0.25</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

+ clav, above antibiotic in combination with 2 mg/l clavulanic acid.

**Figure.** Isoelectric focusing gel comparing the focusing positions of TEM-E4, Mutant β-lactamase D and TEM-6. A, TEM-1; B, TEM-2; C, S. marcescens 7919; D, E. coli J53-2 transconjugant of S. marcescens 7919; E, TEM-2; F, E. coli J53-2 producing Mutant β-lactamase D; G, E. coli J53-2 producing CTX-I; H, E. coli J53-2 producing TEM-E4; I, E. coli J53-2 producing TEM-6 and SHV-1; J, E. coli J53-2 producing TEM-E4; K, E. coli K12 strain SA10 producing Mutant β-lactamase D.
Table II. Relative \( V_{\text{max}} \) values* for TEM-E4 and Mutant \( \beta \)-lactamase D

<table>
<thead>
<tr>
<th>( \beta )-Lactamase</th>
<th>( \beta )-Lactam substrate</th>
<th>AMP</th>
<th>CARB</th>
<th>CER</th>
<th>CXM</th>
<th>CAZ</th>
<th>CTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant ( \beta )-lactamase D</td>
<td>100</td>
<td>30</td>
<td>82</td>
<td>2.5</td>
<td>7.4</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>TEM-E4</td>
<td>100</td>
<td>38</td>
<td>98</td>
<td>1.3</td>
<td>6.3</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>TEM-1</td>
<td>100</td>
<td>11</td>
<td>23</td>
<td>UM</td>
<td>UM</td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

*Values expressed as a percentage of the value for ampicillin. UM, Unmeasurable because hydrolysis of substrate was too low; AMP, ampicillin; CARB, carbenicillin; CER, cephaloridine; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime.

Table III. \( K_{m} \) values (\( \mu M \)) for various \( \beta \)-lactam substrates with TEM-E4 and Mutant \( \beta \)-lactamase D

<table>
<thead>
<tr>
<th>( \beta )-Lactamase</th>
<th>( \beta )-Lactam substrate</th>
<th>AMP</th>
<th>CARB</th>
<th>CER</th>
<th>CXM</th>
<th>CAZ</th>
<th>CTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant ( \beta )-lactamase D</td>
<td>37</td>
<td>139</td>
<td>100</td>
<td>250</td>
<td>1000</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>TEM-E4</td>
<td>29</td>
<td>73</td>
<td>80</td>
<td>200</td>
<td>1500</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>TEM-1</td>
<td>167</td>
<td>100</td>
<td>167</td>
<td>UM</td>
<td>UM</td>
<td>286</td>
<td></td>
</tr>
</tbody>
</table>

Table IV. Relative efficiency of hydrolysis (rel \( V_{\text{max}}/K_{m} \)) values TEM-E4 and Mutant \( \beta \)-lactamase D

<table>
<thead>
<tr>
<th>( \beta )-Lactamase</th>
<th>( \beta )-Lactam substrate</th>
<th>AMP</th>
<th>CARB</th>
<th>CER</th>
<th>CXM</th>
<th>CAZ</th>
<th>CTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant ( \beta )-lactamase D</td>
<td>100</td>
<td>8</td>
<td>30</td>
<td>0.37</td>
<td>0.27</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>TEM-E4</td>
<td>100</td>
<td>15</td>
<td>36</td>
<td>0.19</td>
<td>0.12</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>TEM-1</td>
<td>100</td>
<td>18</td>
<td>23</td>
<td>UM</td>
<td>UM</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

7919 must be \( \beta \)-lactamase mediated. It was also noted that the \( E. coli \) J53-2 strain, producing Mutant \( \beta \)-lactamase D, exhibited a similar profile of \( \beta \)-lactam resistance to the \( E. coli \) transconjugant of \( S. marcescens \) 7919 (Table I). Both the original \( S. marcescens \) isolate and the \( E. coli \) transconjugant produced the TEM-E4 \( \beta \)-lactamase. Isoelectric focusing determinations showed that TEM-E4 focused marginally above TEM-2 (pI 5.61) and aligned directly with Mutant \( \beta \)-lactamase D (Figure). In addition, the pl of TEM-E4 was below that of TEM-6 (Figure). Further concurrent isoelectric focusing determinations (data not shown) have demonstrated that no other extended spectrum \( \beta \)-lactamase with similar properties, focuses between TEM-2 and TEM-6 and this confirms the novelty of the TEM-E4 \( \beta \)-lactamase.

Table II and III show the \( K_{m} \) and \( V_{\text{max}} \) values determined for the hydrolysis of six different \( \beta \)-lactam substrates by TEM-E4, mutant \( \beta \)-lactamase D and TEM-1. Mutant \( \beta \)-lactamase D and TEM-E4 have similar kinetic constants for all the \( \beta \)-lactams tested, they exhibit lower rates of hydrolysis for cefotaxime than ceftazidime (Table II) although they have a much higher affinity for cefotaxime (Table III). TEM-E4 and Mutant \( \beta \)-lactamase D hydrolyse cefotaxime more efficiently than ceftazidime (Table IV), which was paradoxical as these enzymes conferred only a small degree of resistance to cefotaxime. This phenomenon has also been reported with TEM-E1, TEM-E2 and TEM-7 (Gutmann et al., 1988; Payne et al., 1989a, 1990). Mutant \( \beta \)-lactamase D, TEM-E4 and TEM-1 had similar ID\(_{50}\) values for clavulanic acid (0.4, 0.3 and 0.5 \( \mu M \) respectively) and were of similar size (23.5, 24.5 and 22.0 Kdal, respectively), when measured by gel filtration. Indeed, we believe that this variation is an artefact of the method. In addition, a 8.5 kb EcoRI restriction fragment of
pUK724 hybridised with a TEM-E1 probe. This probe hybridises only with TEM genes (Payne et al., 1989a) and not with SHV-I or OXA-1 genes. In addition to this, as the TEM-E4 enzyme can be obtained spontaneously from a TEM-2-producing organism it is highly probable that the gene encoding TEM-E4 differs from the nucleotide sequence of TEM-1/2 by only a few nucleotide residues.

The β-lactamases TEM-7, TEM-E1, TEM-E2, and TEM-E4 can all be obtained spontaneously from strains producing either TEM-1 or TEM-2 (Gutmann et al., 1988; Payne et al., 1989b). Consequently, any clinical isolate which produces either of these enzymes is a potential producer of a third generation cephalosporin hydrolysing β-lactamase. Considering recent surveys have shown that more than 80% of ampicillin-resistant E. coli produce TEM-1 or 2 (Simpson et al., 1986; Jouverot et al., 1987; Huovinen, Huovinen & Jacoby, 1988; Cooksey et al., 1990) it is surprising that TEM-7, TEM-E1, TEM-E2 or TEM-E4 are found so rarely. In fact, we believe that some of these enzymes have been found to be produced by only one clinical isolate. Conversely, the SHV-2 β-lactamase can be obtained directly from the SHV-1 enzyme and this may explain how SHV-2 has been identified in clinical bacteria from patients from such a diversity of countries such as West Germany (Kiebe et al., 1985), Chile, China, Tunisia, Greece (Jacoby et al., 1988), Egypt (Shannon et al., 1990), Switzerland (Labia et al., 1988), France (Jarlier et al., 1988) and the USA (Thomson et al., 1990).

Therefore, although these SHV-derived and TEM-derived β-lactamases can occur spontaneously from the wide distribution of TEM or SHV genes, it appears that their success and spread depends on other factors such as the pathogenicity of the strains concerned, the consistency of the selective pressure (not necessarily a third generation cephalosporin), or the stability of the plasmids encoding the β-lactamases.

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References


β-Lactamase production in human and animal isolates of Campylobacter jejuni in Turkey

Sir,

During the past decade, several reports have described the susceptibility of Campylobacter jejuni to antimicrobial agents (Shanker & Sorrell, 1983; Fliegelman et al., 1985; Lariviere, Gaudreau & Turgeon, 1986). In approximately 15% of clinical isolates of C. jejuni ampicillin resistance has been observed and associated with β-lactamase production (Fleming et al., 1982; Lariviere et al., 1986). Between 2% and 92.5% of C. jejuni isolates, from several countries, have been shown to produce β-lactamase (Wright & Knowles, 1980; Shanker & Sorrell, 1983; Fliegelman et al., 1985). Four distinct β-lactamases have been identified in C. jejuni based on various criteria (Lucain, Goossens & Pechere, 1985). Unfortunately, there is no information concerning the incidence of β-lactamase producing C. jejuni isolated from animals.

A total of 100 C. jejuni strains were used in this study, 50 human isolates from faecal specimens from diarrhoeic children (no strains were isolated from outbreaks and no patients were epidemiologically linked); 50 faecal isolates from animals, 25 from cattle and 25 from sheep. The animal strains were from different flocks. All the strains were isolated on Preston Selective Medium (Oxoid).

The minimum inhibitory concentration (MIC) of ampicillin for C. jejuni was determined by 10 μL calibrated loop-inoculation (10⁴ cfu) on to Mueller–Hinton Agar (Oxoid) containing two-fold concentrations of ampicillin (0.13–256 mg/L). The plates were incubated at 37°C under microaerobic conditions (5% O₂, 10% CO₂, 85% H₂) for 48 h. The endpoint was taken as complete inhibition of growth.

The presence of a β-lactamase was detected with the chromogenic cephalosporin, nitrocefin (Oxoid). Nitrocefin was rehydrated as directed by the manufacturers and used as follows: (i) direct plate method; one drop of nitrocefin solution was added onto the surface of a colony. The development of a deep orange-red colour within 30 min indicated the presence of β-lactamase: (ii) broken cell method; a heavy suspension of organisms was prepared in saline and sonicated on ice for 3 min. Aliquots of cell lysate (100 μL) were added to sterile wells in a microtitre tray containing 10 μL of nitrocefin solution. A deep-orange to red colour within 30 min indicated the presence of β-lactamase. A penicillin sensitive and a β-lactamase producing Staphylococcus aureus were included as controls.

The overall frequency of β-lactamase producing strains of C. jejuni was 49% (28/50) 56% and (21/50) 42% for the human and animal isolates respectively. In other studies, the incidence of β-lactamase production in human strains of C. jejuni has ranged from 2% in Australia (Shanker & Sorrell, 1983) to 92.5% in the United States of America (Fliegelman et al., 1985).

The broken cell method was a more sensitive technique for detecting β-lactamase in C. jejuni than adding nitrocefin to a colony on a plate (direct plate method). Shannon & Phillips (1980) have reported that β-lactamase is often undetected in suspensions of Gram-negative bacteria, unless subjected to ultrasonic disintegration. The necessity to disrupt the cell wall to detect β-lactamase was also evident for our isolates of C. jejuni.

The distribution of MICs of ampicillin for human and animal isolates (MIC₅₀ = 8 mg/L, MIC₇₀ = 32 mg/L, range = 0.3–64 mg/L) was identical. The lowest MIC of all β-lactamase producing strains was 8 mg/L. Most of the isolates that were resistant to ampicillin (MIC > 16 mg/L) produced β-lactamase (Figure). Negative results with nitrocefin...
TEM-E4: A \( \beta \)-LACTAMASE WHICH CONFRS TRANSFERABLE RESISTANCE TO CEFTAZIDIME

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When third generation cephalosporins (3GCs), such as ceftazidime and cefotaxime, were first introduced they were resistant to hydrolysis by all the plasmid-mediated \( \beta \)-lactamases known at that time. However, in the last four years a number of plasmid mediated \( \beta \)-lactamases have evolved which have the ability to hydrolyse ceftazidime and cefotaxime and, consequently, promote transferable resistance to 3GCs (Philippon et al 1989). Many of the 3GC hydrolysing \( \beta \)-lactamases have been shown to have evolved from the ubiquitous TEM-1/2 and SHV-1 resistance genes.

The ceftazidime resistant isolate of Serratia marcescens 7919 was isolated in Belgium in 1987. Conjugation of strain 7919 with Escherichia coli J53-2 resulted in ceftazidime-resistant E. coli J53-2 transconjugants. Analysis of the plasmid DNA in these transconjugant strains revealed a single plasmid band of 56kb, which was designated pUK724. This plasmid was also visualized in the S. marcescens strain. With the exception of imipenem, the S. marcescens 7919 strain was resistant to all penicillins as well as first, second and third generation cephalosporins but the E. coli J53-2 transconjugant was resistant only to ceftazidime and remained sensitive to all the other third and second generation cephalosporins tested. The ceftazidime and ampicillin resistances, expressed by the E. coli transconjugant, were diminished with the addition of clavulanic acid (2mg/L). This illustrated that the \( \beta \)-lactam resistance of the E. coli J53-2 transconjugant of S. marcescens 7919 must be \( \beta \)-lactamase mediated. Iso-electric focusing of bacterial extracts and visualisation with the chromogenic cephalosporin, nitrocephin revealed that both the original S. marcescens isolate and the E. coli J53-2 transconjugant produced a novel \( \beta \)-lactamase, designated TEM-E4. This enzyme focused marginally above the TEM-2 (pl 5.6) and below the TEM-6 (pl 5.85) enzyme. In comparison with standards, TEM-E4 was allocated a pI of 5.61. The TEM-E4 \( \beta \)-lactamase exhibited low rates of hydrolysis for ceftazidime and cefotaxime although it hydrolysed cefotaxime more efficiently than ceftazidime. It was also shown that TEM-E4 and TEM-1 had similar molecular weights and ID\(_50\) values for clavulanic acid.

In separate experiments, challenging E. coli J53-2 (RP4), a TEM-2 \( \beta \)-lactamase producing strain, with ceftazidime (2mg/L) resulted in spontaneous mutants capable of resisting the drug. In these mutants, the TEM-2 enzyme had mutated so that it was now capable of hydrolysing ceftazidime (Mutant \( \beta \)-lactamase D). Close comparison of Mutant \( \beta \)-lactamase D and TEM-E4 showed that they had similar resistance profiles to \( \beta \)-lactam antibiotics, they had identical pIs and similar kinetic constants for five different \( \beta \)-lactam substrates. These results infer that TEM-E4 resulted from direct mutation of the TEM-2 gene. Further evidence for this has been obtained by DNA-DNA hybridization studies which showed that the TEM-E4 gene hybridised with a radiolabelled TEM gene probe.

In conclusion, the TEM-E4 \( \beta \)-lactamase is a unique plasmid-encoded enzyme, capable of hydrolysing 3GCs, which is present in clinical bacteria. TEM-E4 could be obtained by spontaneous mutation of the common TEM-2 gene and seems to have derived directly from it.

We thank the SERC for the CASE studentship to DJP.

Separation of plasmid-mediated extended spectrum β-lactamases by fast protein liquid chromatography (FPLC system)

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1. SUMMARY

We have devised a reliable procedure for the separation of three β-lactamases of isoelectric focusing points (pI), 5.4, 6.5, and 7.9 by Fast Protein Liquid Chromatography (FPLC System). All of these enzymes were transferable and originated from a ceftazidime and cefotaxime resistant Klebsiella pneumoniae isolated in Bombay, India. The complete separation of the enzymes, achievable by this method, allowed each of the different individual β-lactamases to be characterised biochemically. This analysis revealed that the enzymes of pI 6.5 and pI 7.9 hydrolysed ceftazidime and cefotaxime, and were responsible for the resistance of K. pneumoniae, and its Escherichia coli J53-2 transconjugant to third generation cephalosporins. The enzyme of pI 5.4 was the TEM-1 β-lactamase. The β-lactamase of pI 7.9 appears quite different from any previously reported third generation cephalosporin hydrolysing β-lactamase, and consequently given the preliminary designation DJP-1. This is also the first example of extended spectrum hydrolysing β-lactamases found in Asia.

2. INTRODUCTION

There have been recent reports of transferable β-lactamases which confer resistance to broad spectrum cephalosporins [1–8]. These enzymes have been derived from the SHV and TEM β-lactamase genes [1]. In some cases these new enzymes are produced along with other transferable β-lactamases of different isoelectric points and sometimes these distinct β-lactamases are encoded by the same plasmid. Consequently, the investigation and characterisation of these enzymes would prove to be very difficult as they must be completely separated from the other β-lactamases. As many of these enzymes have similar molecular sizes, separation techniques based on the enzyme’s ionic charge have been employed. This can be
achieved by techniques such as preparative iso-electric focusing (IEF) which was used to isolate TLE-2 from TEM-1 and SHV-1 [9] and electro-dialysis which was used to separate TEM-E2 from TEM-1 [8]. However, these methods are time consuming and the amount and purity of the enzyme recovered is unsatisfactory. We have developed a Fast Protein Liquid Chromatography (FPLC System) technique for the rapid separation of three different β-lactamases produced by a ceftazidime and cefotaxime resistant Escherichia coli J53-2 transconjugant of a Klebsiella pneumoniae strain. In the past reverse phase High Performance Liquid Chromatography (HPLC) techniques have been used to purify β-lactamases such as SHV-1 [10], and FPLC System has been implemented to purify an inducible β-lactamase produced by Proteus vulgaris [11], and a β-lactamase from Clostridium butyricum [12], but neither procedure has ever been used to separate three plasmid encoded β-lactamases produced by the same strain.

3. MATERIALS AND METHODS

3.1. Strains

Klebsiella pneumoniae 8825 was isolated from a lymphoma patient at the Tate Memorial Hospital, Bombay, India. The rifampicin resistant E. coli J53-2 was used as the recipient strain in conjugation experiments.

3.2. Conjugation experiments

One ml of an overnight nutrient broth (Oxoid) culture of E. coli J53-2 and 0.1 ml of an overnight culture of K. pneumoniae were incubated with 4.5 ml of nutrient broth for 6 h at 37°C. The E. coli J53-2 transconjugants were selected on agar containing ceftazidime (1 mg/l) and rifampicin (50 mg/l) or carbenicillin (100 mg/l) and rifampicin (50 mg/l).

3.3. Antibiotic susceptibilities

Minimum inhibitory concentrations (MIC) of antibacterial drugs were determined on Diagnostic Sensitivity Test Agar (Oxoid) at a concentration of 10^5 cfu as described previously by Amyes and Gould (1984) [13]. The MICs of ampicillin, cefotaxime and ceftazidime were additionally measured in the presence of clavulanic acid (2 mg/l).

3.4. β-lactamase preparation

One litre of nutrient broth containing ceftazidime (4 mg/l) was inoculated with the E. coli J53-2 transconjugant of K. pneumoniae 8825 and grown overnight at 37°C. The cells were harvested by centrifugation for 15 min at 6000 × g. The bacterial pellets were washed in 25 mM sodium phosphate buffer (pH 7.0) and the centrifugation was repeated as described before. One ml of 25 mM sodium phosphate buffer (pH 7.0) was then added to the final pellet and the cells were resuspended to give 3 ml of cell suspension which was disrupted by ultrasound [14]. The cell lysate was clarified by centrifugation for one hour at 32,000 × g. All of this crude β-lactamase preparation was applied to a Sephadex G-75 gel filtration column (2 cm² × 90 cm) and eluted with 25 mM sodium phosphate buffer (pH 7.0) at 15 ml/h [15]. The column was calibrated with chymotrypsinogen, cytochrome c, and ovalbumin. The fractions which exhibited β-lactamase activity were pooled and dialysed against 50 mM Tris-HCl buffer (pH 8.2) overnight.

3.5. β-lactamase identification

The β-lactamases were identified by analytical IEF on polyacrylamide gels [16] containing a 1:1 ratio of pH 3.5–10 and pH 4–6 ampholines (LKB). The substrate profiles of the β-lactamases were determined by assaying their hydrolytic activity against fixed concentrations of six different β-lactam drugs [14]. The rates of hydrolysis were expressed relative to ampicillin.

3.6. Fast protein liquid chromatography

Separation of the β-lactamases was carried out with the Pharmacia FPLC System consisting of LCC 500 Plus Controller, UV-M Monitor, FRAC 100 with an HR5/5 Mono Q column. The column was equilibrated with 50 mM Tris-HCl buffer (pH 8.2). Two ml of the β-lactamase sample (from the pooled peak fractions obtained by gel filtration with the Sephadex G-75 column) were added to the column. Separation was achieved by elution
with a linearly increasing concentration of sodium chloride (in the above buffer) to a maximum concentration of 1 M. Approximately, 34 fractions (1 ml) were collected from each 30 min separation. Each aliquot was tested for β-lactamase activity by the chromogenic cephalosporin nitrocefin spot test [17].

3.7. Preparation of plasmid DNA

The method of Takahashi and Nagano [18] was used to isolate and visualize the plasmid DNA of the E. coli J53-2 transconjugant of K. pneumoniae 8825. The plasmid samples were run on an 0.5% agarose gel with plasmids Rl (89 kb), R6K (38 kb) as standards for large plasmids.

4. RESULTS

4.1. Conjugation experiments and plasmid analysis experiments

E. coli J53-2 transconjugants of K. pneumoniae 8825 were detected on agar plates containing ceftazidime or carbenicillin. The frequency of transfer on the ceftazidime and rifampicin was $1.18 \times 10^{-6}$ per donor cell, and the transfer frequency for the selection on carbenicillin and rifampicin was $3.53 \times 10^{-6}$ per donor cell. Analysis of the plasmid DNA in the E. coli J53-2 transconjugant revealed plasmids of 100 kb and 2.5 kb (Fig. 1). This suggested that the β-lactamase genes conferring resistance to carbenicillin and ceftazidime were either one and the same or they were located on the same plasmid.

4.2. Antibiotic susceptibilities

The K. pneumoniae 8825 strain was resistant to all the penicillins and first, second, and third generation cephalosporins, which were tested, with the exception of cefoxitin. All these resistance determinants were transferred to the E. coli J53-2 in the conjugation experiments (Table 1). In addition, the clinical strain and the transconjugant were both sensitive to imipenem. The K. pneumoniae 8825 and its transconjugant were sensitive to ampicillin, ceftazidime and cefotaxime in the presence of clavulanic acid (2 mg/l), inferring that the

<table>
<thead>
<tr>
<th>Antibiotic susceptibilities of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-lactam</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Amoxicillin + clav *</td>
</tr>
<tr>
<td>Carbenicillin</td>
</tr>
<tr>
<td>Cephaloridine</td>
</tr>
<tr>
<td>Cephalexin</td>
</tr>
<tr>
<td>Cefoxitin</td>
</tr>
<tr>
<td>Cefuroxime</td>
</tr>
<tr>
<td>Ceftazidime</td>
</tr>
<tr>
<td>+ clav *</td>
</tr>
<tr>
<td>Cefotaxime</td>
</tr>
<tr>
<td>+ clav *</td>
</tr>
<tr>
<td>Ceftriaxone</td>
</tr>
<tr>
<td>Aztreonam</td>
</tr>
<tr>
<td>Imipenem</td>
</tr>
</tbody>
</table>

* In combination with clavulanic acid (2 mg/l).
β-lactamases responsible for resistance to these drugs were either of the TEM or SHV groups.

4.3. Identification of β-lactamases
The original *K. pneumoniae* isolate produced four β-lactamases which focused at pI 5.4, pI 6.5, pI 7.7 and pI 7.9. The band at pI 5.4 aligned with TEM-1, the enzyme of pI 6.5 focused between TEM-3 (pI 6.5) and SHV-3 (pI 7.0), and the enzyme of pI 7.7 aligned with SHV-1/2. Crude enzyme preparations of the *E. coli* J53-2 transconjugants selected on either carbenicillin and rifampicin or ceftazidime and rifampicin possessed the β-lactamases which focused at pI 5.4, pI 6.5, and pI 7.9. However, they additionally produced another β-lactamase which focused at pI 8.1 (Fig. 2). This high pI band aligned directly with the *E. coli* J53-2 chromosomal β-lactamase.

When the crude preparation of the transconjugant strain selected on rifampicin and ceftazidime was separated through the Sephadex G-75 column the intensities of the β-lactamases of pI 7.9 and pI 8.1 were reduced.

4.4. Fast protein liquid chromatography
Two ml of the Sephadex G-75 purified β-lactamase sample were separated through the Mono Q column in each run. This process was repeated until sufficient quantities of the different enzymes were obtained. In each FPLC System separation, β-lactamase activity was detected in the following fractions: 2, 3 and 4 (the void volume), 10/11, and 13/14. When these fractions were examined by IEF the β-lactamases of pI 7.9 and pI 8.1 were eluted in fractions 2, 3 and 4, the enzyme of pI 6.5 in fractions 10/11, and the β-lactamase of pI 5.4 in fractions 13/14 (Fig. 3). All the fractions, from the individual separations, containing the same β-lactamase, were combined so that sufficient enzyme could be characterised biochemically.

4.5. Characterisation of β-lactamases
The hydrolytic activity of each of the three enzymes against six different β-lactam antibiotics are shown in Table 2. The β-lactamase which focused at pI 5.4 aligned with TEM-1, it also had no hydrolytic activity against ceftazidime or cepotaxime and was therefore assumed to be TEM-1. The β-lactamase of pI 6.5 had hydrolytic activity against cefturoxime, cepotaxime, ceftazidime.

### Table 2

<table>
<thead>
<tr>
<th>β-lactam</th>
<th>Fraction: 2–4</th>
<th>10–11</th>
<th>13–14</th>
</tr>
</thead>
<tbody>
<tr>
<td>pI of β-lactamases:</td>
<td>7.9</td>
<td>6.5</td>
<td>5.4</td>
</tr>
<tr>
<td>and 8.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>29</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>114</td>
<td>125</td>
<td>20</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>4.3</td>
<td>17</td>
<td>UM</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>17</td>
<td>9.2</td>
<td>UM</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>4.6</td>
<td>14</td>
<td>UM</td>
</tr>
</tbody>
</table>

* Rate for ampicillin = 100%; UM = unmeasurable due to insufficient hydrolysis.
dime, and had a greater activity for cephaloridine than TEM-1. The fractions containing enzymes of pI 7.9 and pI 8.1 had a similar pattern of hydrolytic activities but the activity against the newer cephalosporins must result from the enzyme of pI 7.9 and not the upper E. coli J53-2 chromosomal β-lactamase band, as control studies have shown that this chromosomal enzyme has no hydrolysing activity against these β-lactam antibiotics.

5. DISCUSSION

These results have shown that Fast Protein Liquid Chromatography has been the most powerful method to date in the separation of multiple β-lactamases produced by the same strain. Its rapid, complete separation of the enzymes enabled the biochemical characterisation of each individual β-lactamase and permitted an assessment of how each β-lactamase contributed to the range of β-lactam resistances expressed by the host strain. We were thus able to show that two of the enzymes produced by the clinically derived K. pneumoniae 8825 strain confer resistance to cefuroxime, cefotaxime, ceftriaxone, and ceftazidime. Evaluation of the biochemical profile of each of these enzymes strongly suggests that the β-lactamase pI 7.9 (β-lactamase DJP-1) is a novel third generation cephalosporin hydrolysing enzyme. However, the plasmid mediated β-lactamase of pI 6.5 may be the same as CAZ-hi [4], or CAZ-6 [2], both of which have a reported pI of 6.5. The majority of transferable third generation cephalosporin resistance has emergence in France, Germany, and the U.K. where broad spectrum cephalosporin usage is high. However, these two broad spectrum enzymes were found to be produced by a strain isolated in India where the use of these newer cephalosporins is much lower. The occurrence of these two β-lactamases is the first report of transferable third generation cephalosporin resistance in India and Asia, and also the first example of two broad spectrum β-lactamases encoded by the same plasmid.

ACKNOWLEDGEMENTS

We thank the Science and Engineering Research Council for the CASE award for DJP, Michaela Torrance of Pharmacia LKB Biotechnology for the FPLC System, and Dr Kelker for sending the strains from the Tate Memorial Hospital, Bombay, India.

REFERENCES

Correspondence

Comparison of TEM-E3 and TEM-5 β-lactamas

Sir,

There have been recent reports of TEM derived plasmid mediated β-lactamases that confer resistance to third generation cephalosporins: TEM-E1 (Payne, Marriott & Amyes, 1989b), TEM-E2 (Payne, Marriott & Amyes, 1989a), TEM-7 (Gutmann et al., 1988), RHH-1 (Spencer et al., 1988), and TEM-5 (Sirot, Labia & Thabaut, 1989) all of which have pl values that lie between those of TEM-1 (pI 5.4) and TEM-2 (pI 5.6). Consequently, all these enzymes focus over a range of 0.2 pI units, which causes confusion if the enzymes are not compared side by side on isoelectric focusing. In addition some of these enzymes appear to confer similar resistance profiles on the host strain, and without amino acid sequencing, isoelectric focusing remains one of the most potent methods of distinguishing these β-lactamases. In a recent letter to the Journal, Professor Phillips and his colleagues (Phillips, King & Shannon, 1989) suggested that the enzyme described by Drabu et al. (1989) was not sufficiently different from the TEM-5 β-lactamase to be considered a novel enzyme. We should like to report further studies on this enzyme which demonstrate that it is indeed different from the TEM-5 β-lactamase. The enzyme identified by Dr Drabu and her colleagues was encountered in the North Middlesex Hospital, but it has also been found in an Enterobacter cloacae strain isolated at a south London hospital, and we have now assigned this β-lactamase the provisional name TEM-E3. The MICs of the Escherichia coli J53-2 transconjugants of the two TEM-E3 and TEM-5 β-lactamase producing strains are compared in Table I. All the transconjugants showed similar susceptibilities to cephalexin, cefotaxin, cefotaxime, ceftriaxone, ceftizoxime and imipenem. However, the TEM-5 transconjugant was significantly more resistant to cefturoxime than either of the TEM-E3 transconjugants. The transconjugants expressing the TEM-E3 enzymes also were more resistant to ceftazidime and more than 16 times more resistant to aztreonam than the same strain expressing the TEM-5 β-lactamase. In common with other TEM-derived enzymes, the MICs of ceftazidime and cefotaxime of the TEM-E3 transconjugants were also dramatically reduced in the presence of clavulanic acid (2 mg/l) or sulbactam (2 mg/l) (Table I). The TEM-5 and TEM-E3 β-lactamases were compared side by side on an isoelectric focusing gel over a narrow pH range. The isoelectric focusing gel (Figure 1) shows the relative focusing positions of TEM-5 and TEM-E3 and, although both these enzymes have been assigned a pI of 5.55 (Sougakoff et al., 1988; Drabu et al., 1989), this gel reveals that TEM-E3 focuses just above TEM-5 but marginally below TEM-2. In common with other β-lactamases (Simpson & Plested, 1983),

Figure 1. Isoelectric focusing of TEM-E3 compared with TEM-5 over a narrow pH range.
Table I. MICs of *Esch. coli* J53-2 transconjugants expressing TEM-5 or TEM-E3

<table>
<thead>
<tr>
<th>β-lactam</th>
<th>TEM-E3 from North Middlesex Hospital</th>
<th>TEM-E3 from a south London Hospital</th>
<th><em>Esch. coli</em> J53-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>&gt; 500</td>
<td>&gt; 500</td>
<td>4</td>
</tr>
<tr>
<td>Cephalixin</td>
<td>8</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>64</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>+ clav</td>
<td>*</td>
<td>0.13</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>+ sulb</td>
<td>*</td>
<td>0.25</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Ceftriaxime</td>
<td>32</td>
<td>125</td>
<td>0.13</td>
</tr>
<tr>
<td>+ clav</td>
<td>*</td>
<td>0.25</td>
<td>0.13</td>
</tr>
<tr>
<td>+ sulb</td>
<td>*</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Cefixoxime</td>
<td>2</td>
<td>1</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Cefixoxime</td>
<td>1</td>
<td>0.5</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>2</td>
<td>32</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

+ clav, in combination with 2 mg/l clavulanic acid; + sulb, in combination with 2 mg/l sulbactam; *not compared directly

Table II. Relative rates of hydrolysis and molecular weights of the TEM-1, TEM-5, and the two TEM-E3 β-lactamases

<table>
<thead>
<tr>
<th>β-Lactamase</th>
<th>Mr</th>
<th>AMP</th>
<th>CARB</th>
<th>CER</th>
<th>CXM</th>
<th>CAZ</th>
<th>CTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-5</td>
<td>24000</td>
<td>100</td>
<td>68</td>
<td>286</td>
<td>57</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>TEM-E3 (NMH)</td>
<td>25500</td>
<td>100</td>
<td>22</td>
<td>50</td>
<td>1.2</td>
<td>39</td>
<td>2.9</td>
</tr>
<tr>
<td>TEM-E3 (SLH)</td>
<td>23500</td>
<td>100</td>
<td>34</td>
<td>38</td>
<td>0.8</td>
<td>29</td>
<td>1.6</td>
</tr>
<tr>
<td>TEM-1</td>
<td>22000</td>
<td>100</td>
<td>11</td>
<td>18</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Rate of hydrolysis for ampicillin = 100%; AMP, ampicillin; CARB, carbenicillin; CER, cephaloridine; CXM, cefuroxime; CAZ, cefazidime; CTX, cefotaxime; Mr, molecular weight; NMH, TEM-E3 from North Middlesex Hospital; SLH, TEM-E3 from a south London hospital.

The TEM-E3 β-lactamases show a number of satellite bands associated with the main band. As previously demonstrated (Drabu et al., 1989), this gel also distinguishes the RHH-1 enzyme from the TEM-E3 β-lactamase.

β-lactamase preparations were made from sonicated extracts of *Esch. coli* J53 transconjugant strains known to possess only the β-lactamase gene in question. Each extract was partially purified by gel filtration on a Sephadex G-75 column (2 cm x 90 cm). The relative substrate profiles of TEM-5, TEM-1, and the two TEM-E3 β-lactamases were compared under the same experimental conditions at fixed substrate concentrations (Table II). The two TEM-E3 enzymes obtained from the different hospitals had very similar profiles, but they were substantially different from the substrate profile of TEM-5 β-lactamase. The TEM-5 enzyme had a greater relative rate of hydrolysis of carbenicillin (two-fold), cephaloridine (six-fold), cefuroxime (45-fold), and cefotaxime (eight-fold) than TEM-E3, suggesting that the enzymes were significantly different. The molecular weights of the β-lactamases were determined on the same Sephadex G-75 column calibrated with molecular weight markers. Not surprisingly the TEM-5 and the two TEM-E3 β-lactamases all had molecular weights similar to TEM-1 (Table II).

Isoelectric focusing is one of the most potent biochemical tools for demonstrating that two proteins are different from one another and two β-lactamases which do not co-focus when placed side-by-side cannot be considered as identical. The parameters of isoelectric focusing are such that the magnitude of difference in the focusing patterns cannot be corre-
lated with the degree of dissimilarity between
the enzymes and no such conclusion should be
made. The focusing patterns of the two
London enzymes suggest they are identical and
different from the TEM-5 \( \beta \)-lactamase.
However, the substrate profiles most readily
show the difference between TEM-3 and
TEM-5. The substrate profiles of the two
London enzymes cannot be distinguished but
are both substantially different from the TEM-
5 enzyme profile.

Although caution certainly should be exer-
cised in classifying a novel enzyme, we should
be aware that many of these new third genera-
tion cephalosporin hydrolysing \( \beta \)-lactamases
could be present in our clinical population and
that, as they are so similar to TEM-1 and
TEM-2, they are easily missed. Indeed we have
already shown that these enzymes existed first
in England as long ago as 1982 (Payne et al.,
1989a) and that they were overlooked at the
time. The assignment of the provisional name
as TEM-3, based on the similarity of its
biochemical characteristics to the TEM-like
enzymes, is currently being confirmed by both
dNA/DNA hybridizations and gene
sequencing.

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In-vitro activity of the quinolone, PD 127,391

Sir,

I was interested to read the paper by King,
activity of PD 127,391 (Warner Lambert). It
may be useful for your readers to know of two
other code names for the same compound,
AM-1091 (Kyorin Pharmaceutical Co. Ltd)
and Bay v 3545 (Bayer AG, Leverkusen).

A meeting presentation by Hirai et al. (1986)
revealed in-vitro activity similar to that
reported by King et al.

P. M. SHAH  
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Zentrum der Inneren Medizin,  
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Main 70, FRG

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41.
TEM-E1: a novel β-lactamase conferring resistance to ceftazidime

D.J. Payne, M.S. Marriott * and S.G.B. Amyes

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Key words: β-lactamase; TEM; Ceftazidime; Resistance

1. SUMMARY

A novel β-lactamase, conferring resistance to ceftazidime, has been identified to be encoded by a 31 kb plasmid (pUK720) in a clinical E. coli strain isolated in Belgium. The β-lactamase, new designated TEM-E1, has a pI of approximately 5.4 and lies in between the iso-electric focused bands of the β-lactamases TEM-1 and TEM-7. The TEM-E1 β-lactamase has a similar molecular weight of 22,000 to the TEM-1 and it is also inhibited by clavulanic acid. However, the TEM-E1 enzyme differs from TEM-1 by its low rates and efficiency of hydrolysis for ceftazidime and cefotaxime, TEM-E1 has similar efficiency of hydrolysis values for ceftazidime and cefotaxime, but only confers resistance to ceftazidime.

2. INTRODUCTION

There have been a number of recent reports on the novel plasmid-mediated β-lactamases TEM-3 to TEM-7, RRH-1, and CAZ-2 [1,2,3]. Although some of these enzymes are known, by nucleotide sequence analysis, to have evolved from TEM-1/TEM-2 β-lactamases [1,4,5], they differ from these two prototype enzymes because they confer resistance to third generation cephalosporins. This communication reports a novel TEM-like enzyme, identified in a clinical E. coli strain isolated in Belgium, which confers resistance to ceftazidime and is distinct from the other 3GC hydrolysis enzymes previously reported.

3. MATERIALS AND METHODS

Antibiotic sensitivity tests and plasmid transfers were determined on solid media as before [6]. The minimum inhibitory concentrations (MIC) for ampicillin, ceftazidime and cefotaxime were also measured in the presence of clavulanic acid (2 mg/l) or sulbactam (2 mg/l). The method of Takahashi and Nagano [7] was used to extract, separate and visualize plasma DNA. β-lactamase preparations were obtained from 1 litre cultures of the ceftazidime resistance transconjugant strain E. coli J53-2. The enzyme was identified by analytical isoelectric focusing [8] with sonicated bacterial extracts. β-lactamase activity, substrate profile, Michaelis-Menten kinetics and the effect of inhibitors were determined by spectrophotometric assays [9–11]. The molecular weight of the β-lactamase was determined on a calibrated Sephadex G075 column (2 cm² × 90 cm) eluted with 25 mM sodium phosphate buffer (pH 7.0) at 16 ml/h [12].
4. RESULTS

The clinical strain *E. coli* 7891 was isolated from the urine of a patient attending a hospital in Belgium in 1987. The ceftazidime resistance gene could be transferred easily into the rifampicin-resistant strain *E. coli* J53-2. This transconjugant was resistant to ceftazidime (MIC = 32 mg/l at 10^5 cfu) but sensitive to all the other third generation cephalosporins tested.

4.1. Conjugation experiments

*E. coli* J5302 transconjugants were selected on agar containing ceftazidime (4 mg/l) and rifampicin (50 mg/l) after overnight mixed incubation of the donor and recipient strains. When the original clinical isolate, *E. coli* 7891, was examined for plasmic DNA, to DNA bands were identified (sizes 31 kb, and 3 kb) and these two bands were also seen in the ceftazidime resistant transconjugant. The ceftazidime-resistance gene was believed to be located on the 31 kb plasmid which we have designated pUK720.

4.2. Antibiotic sensitivity

The MIC data shows that both the clinical strain and the *E. coli* J5302 transconjugant were ceftazidime resistant, which is the only additional resistance conferred when compared to a TEM-1 producing strain (Table 1). The strains expressing the novel enzyme were seen to be sensitive to ceftazidime in the presence of clavulanic acid (2 mg/l) or sulbactam (2 mg/l). Clavulamic acid was the more efficient inhibitor when used in combination with ampicillin, but the two inhibitors seemed to be equally effective when third generation cephalosporins were used as substrates.

4.3. Isoelectric Focusing

Then β-lactamase produced by *E. coli* 7891 and the *E. coli* J5302 transconjugant were examined by analytical isoelectric focusing (IEF) employing a broad range ampholine (pH 3.5–10). Both strains produced identical enzymes of low pi which were barely distinguishable from the TEM-1 β-lactamase. The β-lactamase band was, however, quite clearly distinguishable from the other transferable third generation cephalosporin hydrolysing en-

<table>
<thead>
<tr>
<th>Antibiotic susceptibilities of strains</th>
<th>E. coli</th>
<th>E. coli</th>
<th>E. coli</th>
<th>E. coli</th>
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</thead>
<tbody>
<tr>
<td>β-lactam</td>
<td>7891</td>
<td>7891</td>
<td>J53-2</td>
<td>J53-2</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>4</td>
</tr>
<tr>
<td>A + clav</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>B + sulb</td>
<td>32</td>
<td>8</td>
<td>&lt; 250</td>
<td>4</td>
</tr>
<tr>
<td>Methicillin</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>8</td>
</tr>
<tr>
<td>Carbenicillo</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>4</td>
</tr>
<tr>
<td>Cefetoridine</td>
<td>64</td>
<td>32</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Cefatexin</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Cefotaxin</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>16</td>
<td>32</td>
<td>&lt; 0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>A + clav</td>
<td>0.5</td>
<td>0.25</td>
<td>&lt; 0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>B + sulb</td>
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<td>0.25</td>
<td>&lt; 0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.25</td>
<td>0.13</td>
<td>&lt; 0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>A + clav</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
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<tr>
<td>B + sulb</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
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</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.25</td>
<td>0.13</td>
<td>&lt; 0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>1</td>
<td>0.13</td>
<td>&lt; 0.06</td>
<td>0.06</td>
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<tr>
<td>Imipenem</td>
<td>0.25</td>
<td>0.5</td>
<td>0.13</td>
<td>0.25</td>
</tr>
<tr>
<td>Augmentin</td>
<td>32</td>
<td>32</td>
<td>16</td>
<td>8</td>
</tr>
</tbody>
</table>

A + clav = in combination with clavulanic acid (2 mg/l); B + sulb = in combination with sulbactam (2 mg/l). * E. coli J53-2 2136E produces TEM-1.

zymes. The enzyme from strain 7891 was re-examined by IEF with a 1:1 mixture of pH 4–6 and pH 3.5–10 ampholines to increase the sensitivity. The TEM-1, TEM-2 and TEM-7 β-lactamases were used as standard pi markers and the novel β-lactamase was found to focus between TEM-1 and TEM-7 enzymes (Fig. 1).

4.4. Molecular weight determination

A crude extract of the ceftazidime resistant transconjugant was applied to a sephadex G-75 column, calibrated with the standard proteins ovalbumin, chymotrypsinogen and cytochrome C. The size of the novel enzyme was 22,000 which was indistinguishable from the TEM-1 nβ-lactamase when estimated by the same method.

4.5. Kinetic characteristics

The substrate profiles of the TEM-1 β-lactamase and that from the *E. coli* J53-2 transconjugant of 7891 are shown in Table 2. The profiles
A B C D E F

Fig. 1. Iso-electric focusing pattern of the plasmic encoded ß-lactamase derived from strain 7891, compared with the enzymes TEM-1, TEM-2 and TEM-7 over a narrow pH range. A. E. coli 153-2 producing TEM-1; B. E. coli 7891 producing TEM-E1; C. E. coli 153-2 transconjugant of E. coli 7891 producing TEM-E1; D. E. coli BM694 producing TEM-7; E. as C. F. E. coli 153-2 producing TEM-2.

Table 2
*Relative rates of hydrolysis of ß-lactamases TEM-E1 and TEM-1

<table>
<thead>
<tr>
<th>ß-lactam substrate</th>
<th>ß-lactamase</th>
<th>TEM-E1</th>
<th>TEM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>29.6</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>42.1</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>0.25</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1.48</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.31</td>
<td>0.017</td>
<td></td>
</tr>
</tbody>
</table>

* Rate for ampicillin = 100%.

Table 3
*Efficiency values for TEM-E1 and TEM-1 (relative $V_{\text{max}}/K_m$)

<table>
<thead>
<tr>
<th>ß-lactam substrate</th>
<th>ß-lactamase</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-E1</td>
<td>TEM-1</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>18</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>134</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>2.5</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1.1</td>
</tr>
</tbody>
</table>

N.D. not done because break down was insufficient.

* Efficiency with ampicillin = 100%.

are similar for carbenicillin, ampicillin, and cephaloridine. However, the novel enzyme showed a low but significant rate of hydrolysis of ceftazidime and cefotaxime, whereas the TEM-1 enzyme showed no hydrolysis of either substrate. The $K_m$, $V_{\text{max}}$ and the relative efficiency of hydrolysis ($V_{\text{max}}/K_m$, ampicillin = 100%) values for TEM-1 and TEM-E1 for five ß-lactam substrates are shown in Table 3. The efficiency of hydrolysis of ceftazidime and cefotaxime by TEM-E1 were very similar; equal to or less than 2.5% of the rate for ampicillin.

4.6. Inhibition studies

When assayed for the hydrolysis of nitrocefin, the novel ß-lactamase was sensitive to inhibition by clavulanic acid ($ID_{50} = 0.63 \mu M$).

5. DISCUSSION

The properties of the enzyme from strain 7891 and, in particular, its isoelectric focusing pattern confirm that this plasmid-mediated enzyme from Belgium is not identical to any of the other transferable ß-lactamases responsible for third generation cephalosporin resistance [1]. The kinetic parameters of enzyme TEM-E1 were very similar to those of the TEM-7 ß-lactamase [13] and the rates and efficiency of hydrolysis of ceftazidime and cefotaxime observed for both these enzymes are low but very similar. However, both the enzyme derived from 7891 and the TEM-7 ß-lactamase confer much higher resistance to ceftazidime than cefotaxime. It was found that TEM-7 enzyme and the ß-lactamase from strain 7891 could be effectively distinguished by their different isoelectric focusing positions and thus we have called the new enzyme TEM-E1.
ACKNOWLEDGEMENTS

We thank the Science and Engineering Research Council for the CASE studentship for DJP and F. Goldstein for the TEM-7 producing strain.

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Tn3701, PROTOTYPE OF A CLASS OF COMPOSITE CONJUGATIVE GENETIC ELEMENTS IN STREPTOCOCCI.

Laboratoire des Staphylocoques et des Streptocoques, Institut Pasteur, Paris, France.

The plasmid-free Streptococcus pyogenes strain A454 contains a conjugative element, Tn3701, encoding resistance to erythromycin (Em), tetracycline (Tc), and minocycline (Mn) (1). We have mapped a 50-kilobase (kb) chromosomal region of A454 corresponding to the internal part of Tn3701. Tn3701 includes a 19.7-kb structure, designated Tn3703, on which the EmTcMn determinants were localized. Tn3703 which is very similar in structure to the conjugative transposon Tn916 (2), is capable of transposition but is not conjugative. By hybridization experiments, we revealed strong homology between Tn3701 and the conjugative chromosomal elements Ω (cat-tet) (3) and Tn3951 (4) described in Streptococcus pneumoniae strain BM6001 and in Streptococcus agalactiae strain BI09, respectively. Like Tn3701, Ω (cat-tet) and Tn3951 are composite elements containing in their internal region a structure similar to Tn916. Moreover, among eleven antibiotic-resistant plasmid-free clinical isolates tested (10 streptococci of groups A, B and G and one S. pneumoniae) three showed homology to Tn3701 and four to Tn916, suggesting that these two types of conjugative chromosomal elements are common in plasmid-free antibiotic resistant streptococci.

References

TWO NOVEL PLASMID MEDIATED B-LACTAMASES WHICH CONFER RESISTANCE TO CEFTAZIDIME

D.J. Payne*, M.S. Marriott+, and S.G.B. Amyes
Bacteriology Department, Medical School, University of Edinburgh, Edinburgh & +Chemotherapy Department, Glaxo Group Research Ltd, Greenford, U.K.

There have been a number of recent reports on the plasmid mediated B-lactamases TEM-3 to TEM-7. These enzymes confer resistance to third generation cephalosporins (3GC) and are believed to have evolved from TEM-1/TEM-2. We have identified a further two novel plasmid mediated B-lactamases which confer resistance to 3GC.

The first was derived from Klebsiella pneumoniae and isolated in 1982 at a Liverpool hospital. This is believed to be the earliest example of a plasmid mediated B-lactamase confering resistance to a 3GC. This strain contains a 103 kb plasmid which encodes TEM-1 (pI 5.4) and the novel B-lactamase (doublet at pI 5.3). The second B-lactamase was expressed by an E.coli strain isolated in Belgium. It is encoded by a 60 kb plasmid and has a pI between TEM-1 (pI 5.4) and TEM-7 (pI 5.41). Both the original isolates and their respective E.coli J53-2 transconjugants had much higher MICs of ceftazidime (32mg/L at 10^5 cfu) than for cefotaxime (0.25-0.5mg/L at 10^5 cfu). However, the relative efficiency of hydrolysis (Vmax/Km [ampicillin = 100%]) of ceftazidime and cefotaxime were in the order of 1-2x for both enzymes. The rates and efficiency of hydrolysis observed for these two enzymes for ampicillin, carbenicillin, and cephaloridine were also similar to TEM-1. Both enzymes were inhibited by clavulanic acid and had molecular weights similar to TEM-1. This enforces the view that these two enzymes (along with the 3GC hydrolysing...
A-70 Comparison of TEM-E3 and TEM-10 β-lactamases.


Transferable resistance to ceftazidime has been found in a South London Hospital (SLH) and the North Middlesex Hospital (NMH). The β-lactamases responsible (TEM-E3) were shown to have identical isoelectric points (pI) and similar Vmax, Km and efficiency of hydrolysis values for six β-lactam substrates. These assays were performed in parallel with TEM-10, a β-lactamase recently identified in the USA, and demonstrated that TEM-10 and TEM-E3 were identical. MIC data showed that E.coli J53-2 trans-conjugants producing TEM-10, TEM-E3 (NMH), or TEM-E3 (SLH) were resistant to ceftazidime (64-125 mg/L), aztreonam (32-64 mg/L), and only slightly resistant to cefotaxime (0.5-1 mg/L). TEM-E3 and TEM-10 had a greater affinity for cefotaxime (Km 11-21 μM), than for ceftazidime (Km 100-167 μM) although they hydrolyse cefotaxime at a much slower rate (2-3% relative to the Vmax for ampicillin) than ceftazidime (30-40%). However, the efficiency of hydrolysis values for cefotaxime were relatively high (12-16% relative to ampicillin and 20-33% for ceftazidime) considering the enzyme confers no significant cefotaxime resistance. This study confirms that the same gene is now present in clinical bacteria in the USA and Europe and this β-lactamase differs from both TEM-5 and TEM-9.
Characterisation of a unique ceftazidime-hydrolysing β-lactamase, TEM-E2

D. J. PAYNE, M. S. MARRIOTT* and S. G. B. AMYES†

Introduction

When cephalosporins and ceftazidime were first introduced, they were resistant to hydrolysis by most of the plasmid-mediated ß-lactamases that were known at that time, including the TEM-1 and TEM-2 enzymes.1,2 However, there have since been several reports of β-lactamases, which differ from TEM-1 and TEM-2 by only a few amino acids, that are able to hydrolyse these and related cephalosporins.3 Between 1984 and June 1987, 490 strains producing TEM-3 (which exhibits high activity against cephalaxime) were isolated in France.4 In 1987 and 1988 the novel enzymes TEM-4, 5, 6, 7, 9 and CAZ-2 were identified,5-7 all appear to be related to the TEM group of β-lactamases. We now describe a ceftazidime-resistant strain of Klebsiella oxytoca, isolated in 1982, which produces a β-lactamase that hydrolyses ceftazidime and cefotaxime and shows strong similarities with TEM-1.

Materials and methods

Bacterial strains

K. oxytoca strain 5445 was isolated from the blood and cerebrospinal fluid of a baby in the Neonatal Intensive Care Unit of Liverpool Maternity Hospital on Feb. 5, 1982. Rifampicin-resistant Escherichia coli J53-2 was used as the standard recipient strain in the conjugation experiments.8 E. coli J53-2 transconjugants were selected on agar containing rifampicin 100 mg/L and ceftazidime 4 mg/L after overnight incubation of a mixture of the donor and recipient strains.9

Antibiotic sensitivities and plasmid analysis

Minimum inhibitory concentrations (MICs) of ß-lactam antibiotics were determined by the agar incorporation method in Iso-Sensitest Agar (Oxoid), with a bacterial inoculum of 10^6 cfu. In certain experiments, clavulanic acid or sulbactam was included in the medium at a concentration of 2 mg/L. The method of Takahashi and Nagano10 was used to extract, separate and visualise plasmid DNA.

ß-Lactamase studies

Bacteria were grown overnight in 1 L of Nutrient Broth No. 2 (Oxoid) containing ceftazidime 4 mg/L, and ß-lactamase preparations were obtained from sonicated extracts of the concentrated bacterial pellet.11 The ß-lactamases were identified by analytical iso-electric focusing (IEF) of these extracts;12 ß-lactamases PSE-4, TEM-1, TEM-2 and TEM-7 were used as standard PI markers. ß-Lactamase activity, substrate profile, Michaelis-Menten kinetics and the effect of inhibitors were determined by spectrophotometric assays according to

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† Correspondence should be sent to Dr S. G. B. Amyes.
published methods.13–15 The relative molecular mass (M,) of the β-lactamase was determined on a Sephadex G-75 column (2 cm × 90 cm) eluted with 25 mM phosphate buffer (pH 7.0) at a flow rate of 16 ml/h.16 Ovalbumin, chymotrypsinogen and cytochrome C were used as standard M, markers.

Purification of the TEM-E2 β-lactamase by electrodialysis

A crude β-lactamase solution, prepared from the E. coli J53-2 transconjugant of K. oxytoca 5445, was spread on to an analytical IEF polyacrylamide gel containing pH 6–8 and pH 4–6 amphetamine (LKB Pharmacia) in a 1:1.5 ratio. Care was taken not to cover the area of the gel where the β-lactamase bands focused. After focusing, a 1-cm wide strip of filter paper soaked in a solution of nitrocefin (0.5 mg/ml) was placed from the cathode to the anode of the gel to identify the β-lactamase bands. The portion of the gel containing the TEM-E2 enzyme was cut out and placed in dialysis tubing with a minimal amount of 25 mM sodium phosphate buffer (pH 7.0). The dialysis sack was placed in the cathode reservoir of a Bio Rad Mini Sub Cell with 25 mM phosphate buffer (pH 7.0) as running buffer. A charge of 150 V was applied for 10 min. The dialysis sack was then removed and the polyacrylamide gel was discarded. The purity of the enzyme remaining in the sack was analysed by IEF.

Results

Conjugation experiments

The ceftazidime-resistant transconjugants of E. coli J53-2 were obtained by conjugation with K. oxytoca 5445 at 37°C for 6 h. Analysis of the plasmid DNA in the transconjugant strains showed a single large plasmid band of 103 kb, designated pUK721, similar to that found in the original clinical strain.

Antibiotic sensitivities

K. oxytoca 5445 and the E. coli J53-2 transconjugant were resistant to ceftazidime but sensitive to cefotaxime and ceftriaxone. Indeed, ceftazidime resistance was the most significant additional β-lactam resistance conferred by plasmid pUK721 when compared with a TEM-1 β-lactamase-producing strain (table I). Susceptibilities to ceftazidime were restored in the presence of clavulanic acid (2 mg/L) or sulbactam (2 mg/L). However, clavulanic acid was more efficient than sulbactam in reducing MICs of ampicillin.

<table>
<thead>
<tr>
<th>Table I. Antibiotic sensitivities of test strains</th>
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<td>Antibiotic</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
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<tr>
<td>+clav</td>
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<tr>
<td>+sulb</td>
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<td>Carbenicillin</td>
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<td>Aztreonam</td>
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</table>

*Strain 2136E produces TEM-1.

β-Lactamase studies

Analytical IEF with a broad range amphetamine (pH 3-5-10) revealed that K. oxytoca 5445 and the E. coli J53-2 transconjugant produced β-lactamases of low pI. IEF with a 1:1 mixture of pH 4-6 and pH 3-5-10 amphetamines showed that the clinical isolate and the E. coli transconjugant each produced a band which co-focused with TEM-1 and a novel β-lactamase which focused as a doublet band at pI 5-3 (figure). This novel β-lactamase, TEM-E2, was clearly distinguishable from any of the other cephaloside-hydrolysing β-lactamases.17 The TEM-E2 doublet band produced by K. oxytoca 5445 was separated from the TEM-1 band by electrodialysis from the IEF polyacrylamide gel (figure).

The Vmax and Km values of the TEM-E2 and TEM-1 enzymes are shown in table II. Neither enzyme had any measurable activity against cefuroxime. TEM-1 had no activity against ceftazidime but showed some affinity for cefotaxime. TEM-E2 showed activity against both substrates but had a greater affinity for ceftaxime. Both enzymes showed similar relative efficiency of hydrolysis of ampicillin, carbenicillin and cephaloridine (table II). However, the novel enzyme paradoxically hydrolysed ceftazidime less efficiently than cefotax-
The novel $\beta$-lactamase, TEM-E2, is different from any of the previously reported enzymes that hydrolyse cephalosporins of the cefotaxime type. Its kinetic characteristics appear similar to those of other TEM-like enzymes such as TEM-E1\textsuperscript{18} and TEM-7\textsuperscript{19} in that it hydrolysed cefotaxime and ceftazidime with similar, low efficiencies although it conferred resistance only to ceftazidime. The novel enzyme showed similarities with TEM-1, from which it could be separated by electrodialysis. An enzyme with very similar properties to TEM-E2 has been obtained from a TEM-1 producing strain by spontaneous mutation.\textsuperscript{17} This strongly suggests that TEM-E2 has been derived from the TEM-1 $\beta$-lactamase. Moreover, as the mutant strain exhibited virtually no TEM-1 activity, it is apparent that the TEM-1 enzyme did not contribute towards ceftazidime resistance. The first example of plasmid-mediated resistance to cefotaxime was identi-

Table II. Kinetic constants of TEM-E2 compared to those of TEM-1

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>TEM-E2 (µM)</th>
<th>$V_{\text{max}}$*</th>
<th>$V_{\text{max}}/K_m$†</th>
<th>TEM-1 (µM)</th>
<th>$V_{\text{max}}$*</th>
<th>$V_{\text{max}}/K_m$†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>119</td>
<td>100</td>
<td>100</td>
<td>167</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>83</td>
<td>23</td>
<td>32</td>
<td>100</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>500</td>
<td>87</td>
<td>20</td>
<td>167</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>181</td>
<td>0.84</td>
<td>0.55</td>
<td>286</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>500</td>
<td>0.87</td>
<td>0.21</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
</tr>
</tbody>
</table>

UM, unmeasurable.

*Relative to ampicillin as 100%.
†Efficiency of hydrolysis relative to ampicillin as 100%.
fied in Germany in 1983.2,20 This transferable resistance was later found to be conferred by the SHV-2 β-lactamase.21 Since the K. oxytoca 5445 strain discussed in this study was isolated in 1982, TEM-E2 is the earliest example of a TEM-like enzyme which can hydrolyse ceftazidime, and also the first plasmid-encoded β-lactamase to confer resistance to any of the new broader-spectrum cephalosporins. The study of this enzyme has also illustrated the use of a simple technique for the separation and purification of β-lactamases. The method may be particularly useful when the host strain produces enzymes with similar PI values.

We thank the Science and Engineering Research Council for the CASE studentship for D.J.P.

REFERENCES

Mutants of the TEM-1 \( \beta \)-lactamase conferring resistance to ceftazidime

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Spontaneous ceftazidime resistant mutants were obtained from an Escherichia coli K12 J62-2 expressing the TEM-1 \( \beta \)-lactamase (mutation frequency \( = 10^{-9} \)). These mutants produced \( \beta \)-lactamases with similar molecular weights, kinetic parameters and isoelectric points (pI) to the \( \beta \)-lactamases produced by ceftazidime resistant clinical isolates which have recently been identified in this laboratory. Mutant enzyme A focused as a doublet band at pI 5.3 with an additional weak pI 5.4 band. The doublet co-focused with the TEM-E2 \( \beta \)-lactamase, produced by a ceftazidime resistant \( Klebsiella \) oxytoca isolate, which was originally obtained in a Liverpool hospital. Mutant enzyme B had a pI identical to the TEM-E1 \( \beta \)-lactamase produced by a ceftazidime resistant clinical isolate of \( E. coli \) found in Belgium. These results suggest that the two \( \beta \)-lactamases in the clinical strains may have come from simple mutations of the TEM-1 \( \beta \)-lactamase gene.

Introduction

Plasmid mediated \( \beta \)-lactamases conferring resistance to third generation cephalosporins were present as early as 1982 (Payne, Marriott & Amyes, submitted for publication). Since then TEM-3 to TEM-7, RHH-1 and CAZ-2 \( \beta \)-lactamases have been reported (Chanal et al., 1988; Sougakoff et al., 1988; Spencer et al., 1988). Kinetic analyses of these enzymes have shown that they have distinct similarities to the TEM-1 and TEM-2 \( \beta \)-lactamasces. Moreover, amino-acid sequencing performed on some of these enzymes has provided evidence that they differ from TEM-1 and TEM-2 enzymes by only a few amino-acid residues (Collatz et al., 1988; Petit et al., 1988a; Sougakoff et al., 1988). It has been reported that TEM-1 and -2 enzymes can give spontaneous mutants that hydrolyse third generation cephalosporins (Gutmann et al., 1988; Sougakoff et al., 1988). In this study two novel mutant enzymes have been obtained directly from TEM-1 \( \beta \)-lactamase. These mutants differ from all those found in other studies but are identical to two novel \( \beta \)-lactamases which we have recently found in clinical isolates.

Materials and methods

Bacterial strains

\( Klebsiella \) oxytoca 5445 was isolated in 1982 at a Liverpool hospital and produces TEM-E2 \( \beta \)-lactamase. This is believed to be the earliest example of a plasmid mediated
β-lactamase conferring resistance to third generation cephalosporins (Payne, Marriott & Amyes, submitted for publication). *Escherichia coli* 7891 was isolated from the urine of a patient attending a hospital in Belgium and produces the novel third-generation cephalosporin hydrolysing enzyme TEM-E1 (Payne, Marriott & Amyes, 1989). Rifampicin resistant *E. coli* K12 J53-2 (pro^- met^+) and *E. coli* K12 J62-2 (pro^- his^- trp^-) strains were used as recipients in conjugation experiments (Bachmann, 1972). An *E. coli* J62-2 transconjugant of Klebsiella pneumoniae CF504 (Petit et al., 1988b) which produced only TEM-1 β-lactamase and not the TEM-5 enzyme present in the *K. pneumoniae* strain was used as the parent in the spontaneous mutation experiments.

**Spontaneous mutation experiments**

A 10-ml overnight nutrient broth culture (Oxoid No.2) of *E. coli* K12 J62-2, harbouring the TEM-1 β-lactamase coding plasmid was harvested by centrifugation and resuspended in 1 ml of 25 mM phosphate buffer (pH 7.0). Aliquots (0.1 ml) of this suspension were inoculated on to ten Isosensitest Agar (Oxoid) plates containing 0.7 mg/l ceftazidime. This was the lowest ceftazidime concentration that eradicated all the ceftazidime sensitive bacteria at this abnormally high inoculum. Ceftazidime resistant colonies were purified on fresh ceftazidime-containing plates and the growth requirements of each selected colony were checked to verify that they were *E. coli* J62-2. The identity of the mutants was further checked by API 20E strips. These strains were then examined for any change in β-lactamase profile.

**Antibacterial drug susceptibilities and plasmid analysis**

Minimum inhibitory concentrations (MIC) of antibacterial drugs were determined on solid media at a cell concentration of 10^5 cfu as described previously by Amyes & Gould (1984). The MICs of ampicillin, ceftazidime and cefotaxime were additionally measured in the presence of clavulanic acid (2 mg/l) and sulbactam (2 mg/l). The method of Takahashi & Nagano (1984) was used to extract, separate and visualise plasmid DNA.

**Conjugation experiments**

In all mating experiments 0.1 ml of an overnight culture of the donor strain was mixed with 1 ml of an overnight culture of the recipient strain in 4.5 ml of nutrient broth, as described previously by Amyes & Gould (1984). This mixture was then incubated at 37°C for 6 h. *E. coli* J53-2 transconjugants of the two clinical strains were selected on agar containing rifampicin (50 mg/l) and ceftazidime (4 mg/l). *E. coli* J53-2 transconjugants of the TEM-1 mutants were selected on Davis-Mingioli minimal agar (Davis & Mingioli, 1950) containing proline (50 mg/l), methionine (50 mg/l) and ceftazidime (4 mg/l). All kinetic studies were performed on enzymes isolated from the ceftazidime-resistant *E. coli* J53-2 transconjugants.

**β-Lactamase studies**

β-Lactamase preparations were obtained from ultrasonicates of overnight Oxoid Isosensitest broth cultures containing ceftazidime (4 mg/l) (Simpson, Harper & O'Callaghan, 1980). The β-lactamases were identified by analytical isoelectric focusing (IEF)
(Matthew et al., 1975) of bacterial sonicates. The \(\beta\)-lactamases TEM-1, TEM-2, TEM-7 and PSE-4 were used as standard pI markers. The enzymes were first examined on polyacrylamide gels containing a broad range ampholine (pH 3.5–10). They were subsequently re-examined on similar gels containing a 1:1 mixture of pH 4–6 and pH 3.5–10 ampholines, to provide a narrow pH range and increase sensitivity. Enzyme substrate profile, \(V_{max}\), \(K_m\), and inhibitor studies were determined by spectrophotometric assay (Sykes et al., 1981; Eliasson & Kamme, 1985; Reid & Amyes, 1986). The relative molecular masses (\(M_r\)) of the \(\beta\)-lactamases were determined on a calibrated Sephadex G-75 column (2 cm\(^2\) x 90 cm) eluted with 25 mM phosphate buffer (pH 7.0) at 16 ml/h (Andrews, 1964).

Results

Preparation of TEM-1 \(\beta\)-lactamase parent strain

*K. pneumoniae* CF504 was a clinical strain isolated in Clermont-Ferrand, France. It was resistant to ceftazidime and cefotaxime and the gene encoding these resistances was located on a 150 kb plasmid (pCFF14) (Petit et al., 1988b). Isoelectric focusing showed that this strain also produced TEM-1 \(\beta\)-lactamase as well as the TEM-5 enzyme responsible for the cephalosporin resistances. This strain was conjugated with the rifampicin-resistant *E. coli* J62-2 and transconjugants were selected on Isoosensitest agar plates containing rifampicin and ceftazidime. The transconjugants were purified and their purity checked. Analysis of the plasmid DNA revealed that the transconjugant had lost the 150 Kb plasmid (pCFF14), which encodes the TEM-5 \(\beta\)-lactamase (Petit et al., 1988b), and possessed only a 100 Kb plasmid along with two smaller plasmids. This transconjugant reverted to the normal characteristics of a TEM-1 \(\beta\)-lactamase producing *E. coli* J62-2. This strain was used as the parent in the spontaneous mutation experiments. The rationale for this was that there should be a higher chance of obtaining a third generation cephalosporin-hydrolysing mutant enzyme from a TEM-1 \(\beta\)-lactamase gene in a strain that was host to a TEM-like third generation cephalosporin-hydrolysing \(\beta\)-lactamase than a standard laboratory strain hosting only the TEM-1 \(\beta\)-lactamase gene.

Selection of mutants

The ceftazidime-resistant mutants, which appeared at a frequency of 1 in \(10^9\), were purified and examined for any change in \(\beta\)-lactamase profile. In all the colonies studied, ceftazidime resistance was associated with the mutation of the TEM-1 \(\beta\)-lactamase gene to produce one of two different \(\beta\)-lactamases (mutant enzymes A and B). We examined 20 colonies and the mutant \(\beta\)-lactamases they produced. This revealed that the ratio of occurrence of enzyme A to enzyme B was 4:1. The genes encoding the mutant \(\beta\)-lactamases were transferred by conjugation into *E. coli* J53-2, with selection of the transconjugants on ceftazidime-containing plates. The transfer of the plasmids elevated the MIC of ceftazidime for the *E. coli* J53-2 recipient more than 60-fold, from 0-13 to 8–16 mg/l (Table I). The transferability of the resistance determinant confirmed that the mutation had occurred to a plasmid encoded gene and not on the chromosome. Both the original mutants and their J53-2 transconjugants possessed the 100 Kb plasmid, but the two smaller plasmid bands were seen only in the original mutant strains.
Table I. MICS of β-lactams for *E. coli* strains harbouring mutant β-lactamases and β-lactamases from clinical strains

<table>
<thead>
<tr>
<th>β-lactam antibiotic</th>
<th>Mutant A</th>
<th>Mutant B</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>J62-2</td>
<td>J53</td>
<td>7891*</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Amp + clav&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Amp + sulb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>8</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Cephalixin</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>2</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>4</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Cezax + clav&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>0.13</td>
<td>ND</td>
</tr>
<tr>
<td>Cezax + sulb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>&lt;0.06</td>
<td>ND</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.06</td>
<td>0.06</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Cezax + clav&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>&lt;0.06</td>
<td>ND</td>
</tr>
<tr>
<td>Cezax + sulb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>&lt;0.06</td>
<td>ND</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>&lt;0.06</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0.25</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.13</td>
<td>0.5</td>
<td>0.25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amoxycillin/clavulanate</td>
<td>8</td>
<td>16</td>
<td>32</td>
</tr>
</tbody>
</table>

<sup>a</sup> + clav = in combination with 2 mg/l clavulanic acid.
<sup>b</sup> + sulb = in combination with 2 mg/l sulbactam.
<sup>c</sup> Produces TEM-E2.
<sup>d</sup> Produces TEM-E1.
ND Not done.
Amp, Ampicillin; Cezax, Ceftazidime; Cez, Cefotaxime.
**Isoelectric focusing**

Mutant enzyme A focused as a doublet band at pH 5.3 with an additional weak band that cofocused with TEM-1 enzyme. The TEM-E2 β-lactamase expressed by the ceftazidime resistant isolate, *K. oxytoca* 5445 produced an identical doublet band at pH 5.3, but exhibited a much stronger TEM-1 cofocusing band. Mutant enzyme B showed distinct similarities to enzyme TEM-E1 produced by the clinical isolate *E. coli* 7891. Mutant enzyme B and TEM-E1 co-focused and had a pH marginally above the TEM-1 enzyme but below the TEM-7 β-lactamase (Figure 1).

**Minimum inhibitory concentrations**

*E. coli* J53-2 transconjugants expressing the mutant enzymes and the transconjugants expressing the clinically obtained extended spectrum β-lactamases were all resistant to ampicillin, carbenicillin, cephaloridine, ceftazidime, and aztreonam. However, they were sensitive to all other cephalosporins tested including cefotaxime (Table I). Clavulanic acid and sulbactam were similar in their activity when ceftazidime was used as the principal antibiotic. However, clavulanic acid was seen to be the more efficient β-lactamase inhibitor when used in combination with ampicillin (Table I).

![Figure 1. Isoelectric focusing patterns of mutant enzymes and β-lactamases from clinical isolates over a narrow pH range. A, PSE-4; B, mutant A; C, TEM-E2; D, TEM-1; E, mutant B; F, TEM-E1; G, TEM-7; H, TEM-2.](image-url)
Table II. Relative rates of hydrolysis and molecular masses of β-lactamases TEM-1, TEM-E1, TEM-E2, and mutant enzymes A and B

<table>
<thead>
<tr>
<th>β-lactamase</th>
<th>M, a</th>
<th>ampicillin</th>
<th>carbenicillin</th>
<th>cephaloridine</th>
<th>cefuroxime</th>
<th>ceftazidime</th>
<th>cefotaxime</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-E1</td>
<td>22.0</td>
<td>100</td>
<td>29.8</td>
<td>42.1</td>
<td>0.25</td>
<td>0.31</td>
<td>1.48</td>
</tr>
<tr>
<td>Enzyme B</td>
<td>24.0</td>
<td>100</td>
<td>17.4</td>
<td>68</td>
<td>0.55</td>
<td>1.11</td>
<td>1.36</td>
</tr>
<tr>
<td>TEM-E2</td>
<td>25.5</td>
<td>100</td>
<td>19.3</td>
<td>41.6</td>
<td>0.22</td>
<td>0.23</td>
<td>0.58</td>
</tr>
<tr>
<td>Enzyme A</td>
<td>23.5</td>
<td>100</td>
<td>26.3</td>
<td>38.2</td>
<td>0.52</td>
<td>0.92</td>
<td>2.27</td>
</tr>
<tr>
<td>TEM-1</td>
<td>22.0</td>
<td>100</td>
<td>10.9</td>
<td>18</td>
<td>0.014</td>
<td>0.017</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*Rate for ampicillin = 100%.

Biochemical profiles of the mutant β-lactamases, TEM-E1, and TEM-E2

The two mutant enzymes and the two enzymes from clinical isolates all had molecular masses indistinguishable from the TEM-1 β-lactamase (Table II). The substrate profiles of these four enzymes are compared to TEM-1 in Table II. The profiles were similar to TEM-1 for ampicillin, carbenicillin and cephaloridine. However, both the mutant enzymes and the β-lactamases from clinical strains showed a low but significant rate of hydrolysis of ceftazidime and cefotaxime, whereas the TEM-1 enzyme showed no hydrolysis of either of these substrates. The relative \( V_{max} \) and the \( K_m \) values for these enzymes against a number of β-lactams were determined. These values were combined to give the relative efficiency of hydrolysis values (relative \( V_{max}/K_m \), compared with ampicillin = 100%) which takes into account both the binding and the hydrolysis capabilities of the enzyme for a particular β-lactam. The relative efficiency of hydrolysis values for ceftazidime and cefotaxime by TEM-E1, TEM-E2, mutant A, and mutant B were very similar (Table III). All the enzymes were inhibited by clavulanic acid with \( I_{50} \) in the region of 0.5-0.8 μM.

Discussion

Previous reports of third generation cephalosporin resistant mutants arising spontaneously from TEM-1, have detailed only one type of mutant, called TEM-101 by Gutmann et al. (1988) and TEM-121 to TEM-124 by Sougakoff et al. (1988), for which the MJC of ceftazidime was 4 mg/l and the β-lactamase pI 5.3. This enzyme had a

Table III. Relative efficiency values for TEM-E1, TEM-E2, and the mutant enzymes A and B

<table>
<thead>
<tr>
<th>β-Lactam antibiotic</th>
<th>TEM-E1</th>
<th>TEM-E2</th>
<th>TEM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>18</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>134</td>
<td>76</td>
<td>78</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>1.1</td>
<td>2.1</td>
<td>0.69</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>2.5</td>
<td>0.9</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Efficiency with ampicillin = 100%.

ND, Not done because breakdown was insufficient.
similar pI to mutant enzyme A but it did not focus as two distinct bands of equal intensity. Other reported mutant enzymes, derived from TEM-2, were called TEM-201 by Gutmann et al. (1988) and TEM-221 to TEM-225 by Sougakoff et al. (1988). These enzymes had the same pI as TEM-7. Mutant enzyme B differed in having a pI below TEM-7 and in being obtained from TEM-1. Thus mutant enzyme B was clearly different from other mutant enzymes previously reported. The mutant enzymes and the clinically derived enzymes all hydrolysed both ceftazidime and cefotaxime at similar rates and efficiencies but, surprisingly, only conferred resistance to ceftazidime and not cefotaxime. Amino-acid sequencing has shown that TEM-4 and TEM-5 are more similar to TEM-1 than TEM-2 (Petit et al., 1988a) and, consequently, these two enzymes are believed to have been derived from TEM-1. However, the sequences of the TEM-3 and TEM-7 enzymes (Collatz et al., 1988; Sougakoff et al., 1988) include the one amino-acid change which distinguishes TEM-2 from the TEM-1 enzyme and they are therefore thought to have evolved from the TEM-2 β-lactamase. This study has shown how two β-lactamases, indistinguishable from similar enzymes present in clinical strains, have been obtained in vitro from a TEM-1 producing organism, suggesting that TEM-E1 and TEM-E2 had similarly evolved from TEM-1.

Acknowledgements
We thank the Science and Engineering Research Council for the CASE studentship for DJP, and F. Goldstein for a TEM-7 producing strain and D. Sirot for the TEM-5 producing organism.

References


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Mutants of the TEM-1 β-lactamase conferring resistance to third generation cephalosporins

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There have been recent reports that a new series of plasmid-mediated β-lactamases, which confer resistance to third generation cephalosporins (3GC), have been derived from the TEM-2 enzyme. In this study, two types of ceftazidime (CAZ) resistant mutants have been obtained spontaneously from an E. coli J62-2 expressing the TEM-1 enzyme (mutation frequency = 10⁻⁹). These mutants produce β-lactamases with similar molecular weights, kinetic parameters and iso-electric points (pI) to novel β-lactamases produced by CAZ resistant clinical isolates which have recently been identified in this laboratory. The first mutant enzyme focuses as a doublet band at pI 5.3 with an additional weak TEM-1 band (pI 5.4). This doublet co-focuses with a β-lactamase produced by a CAZ resistant Klebsiella pneumoniae, which was originally isolated at a Liverpool hospital, although the clinical strain had a stronger TEM-1 band. The second mutant produced an enzyme with a pI identical to an enzyme produced by a CAZ resistant E. coli strain isolated in Belgium; both these β-lactamases focus between TEM-1 and TEM-7. These results purport the view that some plasmid-encoded β-lactamases, which confer resistance to 3GC, have evolved from the simple mutation of the TEM-1 β-lactamase resistance genes rather than the TEM-2 gene.
LACK OF TRANSFERABLE THIRD GENERATION CEPHALOSPORIN RESISTANCE IN SCOTLAND

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Four hundred and thirteen Gram-negative strains, which appeared resistant to cefuroxime by disk sensitivity testing, were isolated from patients of the Royal Infirmary, Edinburgh, Scotland over a period from January 1986 to January 1989. This collection of strains excluded Pseudomonas spp.. Seventy-nine of these strains had MICs of ceftazidime, cefotaxime or ceftiraxone of 4 mg/L or greater. The β-lactamases produced by 54 of these third generation cephalosporin (3GC) resistant strains were examined by analytical isoelectric focusing. This same group of 3GC resistant organisms were also tested to see if they exhibited transferable resistance to either ceftazidime or cefotaxime.

This study revealed that none of the 54 3GC resistant strains conferred transferable resistance to either ceftazidime or cefotaxime. Indeed, none of these strains produced any of the TEM or SHV derived β-lactamases which have recently been reported to confer transferable resistance to 3GCs. Consequently, the 3GC resistance conferred by strains in this survey most probably resulted from the production of chromosomal cephalosporinas. This survey yielded only one novel chromosomal β-lactamase which was produced by Xanthomonas maltophilia and focused as 3 distinct bands of pl 5.2 to 5.6 and possessed different characteristics to the L-1 or L-2 β-lactamases of X. maltophilia. In conclusion this survey has illustrated that TEM or SHV derived β-lactamases which mediate transferable resistance to 3GCs do not appear to be threatening the future use of 3GCs in Scotland.
Transferable cephalosporin resistance not inhibited by clavulenate in *Escherichia coli*

Sir,—We report the isolation of a strain of *Escherichia coli* resistant to penicillin and cephalosporins because of a plasmid-encoded beta-lactamase that was not inhibited by clavulenate. The organism was isolated in London from raw meat swabs and a biopsy specimen from a patient with 35% burns. The patient had been treated in Pakistan with cefotaxime and amikacin for at least 15 days before transfer to the UK. The strain was resistant to all beta-lactams tested (table), except imipenem (sensitive by disc test), and to chloramphenicol, tetracycline, and trimethoprim. On disc testing, addition of clavulenate did not restore susceptibility to cefotaxime. The strain was susceptible to aminoglycosides and cefotaxime. Following grafting, the patient made steady progress. Two episodes of clinical sepsis were treated with imipenem and cefotaxime, respectively, although no organisms were isolated from blood cultures. The resistant strain was not isolated subsequently from his burns. The patient was in isolation throughout his two-month stay and secondary spread to other patients was not observed.

The strain contained three plasmids of 80, 45, and 35 MD. Resistance to beta-lactams was transferred readily to, and maintained stably in, *E. coli*, *Enterobacter cloacae*, and *Klebsiella oyxoxa* (table). These transconjugants acquired the 80 MD plasmid together with resistance to chloramphenicol and tetracycline. In addition, *Ent cloacae* transconjugants acquired the 35 MD plasmid and *E. coli* transconjugants acquired plasmids which varied from 25 to 45 MD. These plasmids may have been deletions of plasmids carried by the original (donor) strain. These results suggest that the resistance to beta-lactams was encoded on the 80 MD plasmid. No transfer was detected to strains of *Proven mirabilis* or *Serratia marcescens*.

The *E. coli*, *K. oxyoxa*, and *Ent cloacae* transconjugants all produced an identical beta-lactamase of pl 8.8. This beta-lactamase (designated BIL-1) hydrolysed nitrocefin and cephaloridine, but not ampicillin, carbenicillin, cefuroxime, cefotaxime, and cefazidime. Unlike the TEM-1 beta-lactamase, the hydrolytic activity of BIL-1 was inhibited by ampicillin, cefaclor, and cefazidime. BIL-1 was not inhibited by clavulenate since this compound did not render the *E. coli* transconjugants sensitive to ampicillin, cefotaxime, or cefazidime (table). BIL-1 was almost 4000 times more resistant to inhibition by clavulenate than TEM-1 (data not shown).

There have been reports of plasmid-mediated beta-lactamases that confer resistance to the third generation cephalosporins, but these are all derivatives of the ubiquitous TEM-1/2 and SHV-1 resistance genes and are very sensitive to inhibition by clavulenate. Our data indicate that BIL-1 is unrelated to these enzymes. The characteristics of BIL-1 resemble more closely those of the chromosomally mediated beta-lactamases of *Ent cloacae*and *E. coli*. We are unaware of other reports of a plasmid-encoded class C-like extended-spectrum cephalosporinase. In addition, BIL-1 is the first example of a beta-lactamase confering transferable resistance to all penicillins, to first, second, and third generation cephalosporins, and to clavulenate.

The transferability in vitro of this resistance to bacteria of different genera raises the possibility that similar dissemination might occur in vivo. This importation of an organism with transferable resistance to beta-lactams lends further weight to the view that patients from abroad should be screened microbiologically, and that appropriate control measures should be instituted until they prove to be clear of such carriage.

We thank Ms J. A. Clarke for permission to report this case.


Cyclophosphamide versus ifosfamide in paediatric oncology

Sir,—We concur with Dr Shaw and Dr Eden’s (April 28, p 1022) comments about ifosfamide and cyclophosphamide use in paediatric oncology. For 15–20 years cyclophosphamide dose in widely used regimens in paediatric oncology has been 0·9–1·5 g/m² per course,

\[
\text{Typical ifosfamide doses are 6–10 g/m² per course, which is the equivalent of 1·5–3·0 g/m² of cyclophosphamide. In 3–5 times the "standard" cyclophosphamide dose. It is well established that tumours that do not respond to low doses of alkylating agents regress when the dose of the same agents is increased. Hence, it should be no surprise that results are encouraging when ifosfamide 6–10 g/m² per course is substituted for cyclophosphamide 1·5 g/m² per course in otherwise equivalent regimens.}
\]

Dr Jurgen and Dr Gobell (June 9, p 1999), commenting on Shaw and Eden’s report, state that ifosfamide has an advantage over high-dose cyclophosphamide in that the latter produces greater bone-marrow toxicity and necessitates supportive treatment with cytokines.

We would point out that studies in children and young adults from our institution have demonstrated the feasibility of administration of multiple courses of very high dose cyclophosphamide in conjunction with other cytotoxic agents. Thus, on the basis of previous experience, we initiated in 1987 an induction protocol for patients with poor-risk neuroblastoma that included four consecutive courses of cyclophosphamide 140 mg/kg (ie, 4200 mg/m²), doxorubicin 45 mg/m², and vincristine. This cyclophosphamide dose is 2·3 times the intensive dose and up to 4·7 times the standard dose. To achieve maximum dose intensity (high doses, short term interval), courses were started when the neutrophil count reached 3·5×10⁹/μl and platelets were over 100×10⁹/μl. Most courses began by day 21. Grade 3–4 myelosuppression occurred, yet, as evidence of the relative stem-cell sparing effect of cyclophosphamide, bone-marrow harvested after the four courses constituted haemopoiesis after subsequent myeloablative therapy with autologous bone-marrow rescue. Extramedullary toxicities were mild. This last finding, as well as analyses demonstrating the

**Susceptibility to beta-lactam antibiotics of *E. coli***

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amp</th>
<th>Carb</th>
<th>Cefo</th>
<th>Cefi</th>
<th>Cefu</th>
<th>Pip</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>(&gt; 16)</td>
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</tr>
<tr>
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<td></td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>(&gt; 4)</td>
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<td>(&gt; 4)</td>
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</tr>
</tbody>
</table>

* MIC/minimum inhibitory concentration; ** MIC in presence of 2 mg/l clavulenate and 16 mg/l ampicillin; ** Amp = ampicillin, Carb = carbenicillin, Cefo = cefotaxime, Cefi = cefazidime, Cefu = ceftazidime, Pip = piperacillin.
Characterization of the plasmid mediated \( \beta \)-lactamase BIL-1

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A multi-resistant strain of Escherichia coli isolated from a patient from Pakistan was shown to possess a new plasmid-mediated \( \beta \)-lactamase. This enzyme, designated BIL-1, conferred resistance to extended-spectrum cephalosporins such as cefotaxime and ceftazidime. However, unlike other plasmid-mediated \( \beta \)-lactamases capable of conferring resistance to these drugs, the BIL-1 was not a member of the TEM or SHV group of plasmid-mediated \( \beta \)-lactamases and it also conferred resistance to \( \beta \)-lactam drugs in combination with \( \beta \)-lactamase inhibitors (i.e. clavulanic acid). The biochemical properties of the enzyme suggest that BIL-1 is related to the Richmond & Sykes Class I chromosomal \( \beta \)-lactamases. Its inhibition properties by various \( \beta \)-lactam drugs are similar to the inhibition properties of the chromosomally-encoded P99 enzyme of Enterobacter cloacae.

Introduction

The development of \( \beta \)-lactam antibiotics has been a continuous saga of the design of new molecules to withstand hydrolysis by an ever-increasing diversity of \( \beta \)-lactamase enzymes. The extended spectrum cephalosporins, introduced in the early 1980s, were heralded as the ultimate \( \beta \)-lactam antibiotics because they were stable against hydrolysis by most of the plasmid mediated \( \beta \)-lactamases known at that time (Richmond, 1980; Simpson, Plested & Harper, 1982).

In 1982, the first strain to produce a plasmid mediated extended spectrum cephalosporin hydrolysing \( \beta \)-lactamase was isolated, but not reported. In England (Payne, Marriott & Amyes, 1990). The problem of \( \beta \)-lactamases hydrolysing extended spectrum cephalosporins was first identified in Germany when, in 1983, a series of Klebsiella and Serratia spp. was isolated that could transfer cefotaxime resistance (Knothe et al., 1983). This plasmid-encoded \( \beta \)-lactamase was a modification of the ubiquitous SHV-1 enzyme and was called SHV-2 (Kliebe et al., 1985). The first modification of a TEM \( \beta \)-lactamase was first reported in France four years after the identification of SHV-2 (Sirot et al., 1987). These observations heralded a plethora of reports of plasmid-encoded extended-spectrum \( \beta \)-lactamases, whose properties have been identified; they have been classified by their abilities to hydrolyse and confer resistance to cefotaxime and ceftazidime (Payne & Amyes, 1991). Throughout the 1980s, at least 26 extended spectrum cephalosporin hydrolysing plasmid mediated \( \beta \)-lactamases were identified and characterized.

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lysate was

sodium

The Enzyme was

Antibiotic

K12

Group isolates. TEM conjugants designated Klebsiella transconjugants of E.

Bacterial subject biochemically similar to a pI responsible for Minimum USA three acid originally also cephalosporin hydrolyse change only a Medeiros, been 120 In acid residues. These point mutations had therefore caused a critical change in the active site of the TEM-1/2 or SHV/1 enzymes which enabled them to hydrolyse extended spectrum cephalosporins. Most of the reported extended spectrum cephalosporin hydrolysing enzymes are sensitive to inhibition by clavulanic acid, and also hydrolyse a range of other β-lactam antibiotics.

In 1989, a multi-resistant strain of Escherichia coli was isolated from a patient originally treated in Pakistan. The extended spectrum cephalosporin and clavulanic acid resistance expressed by this strain could be transferred to recipient strains from three different genera. Further investigations revealed that the resistance determinant responsible for this phenomenon was an 80 mDa plasmid mediated β-lactamase of high pl designated BIL-1 (Woodford et al., 1990). This suggested that this enzyme might be similar to a plasmid-encoded β-lactamase, MIR-1, identified at the same time in the USA (Pananicolaou, Medeiros & Jacoby, 1990).

In this study, BIL-1 was characterized and shown to differ both physically and biochemically from the TEM and SHV derived β-lactamases which are currently the subject of worldwide discussion.

Materials and methods

Bacterial strains

E. coli BS was the original clinical isolate producing the BIL-1 β-lactamase. The transconjugants of E. coli BS were derived from E. coli J53-2 and two clinical isolates designated Klebsiella oxytoca 478 (rif') and Enterobacter cloacae 471 (nal'). All transconjugants produced the BIL-1 enzyme. The control strains producing TEM-1, TEM-E3 or SHV-3 were all E. coli J53-2 transconjugants of the original clinical isolates. E. cloacae P99 produced the chromosomal P99 β-lactamase, which is a classic Group I chromosomal β-lactamase (Bush, 1989). The CEP-1 producing strain E. coli K12 58-161sp (R22K) (Bobrowski et al., 1976) was obtained from Professor J. T. Smith (London School of Pharmacy).

Antibiotic susceptibilities

Minimum inhibitory concentrations (MICs) of antibiotics were determined on nutrient agar containing the appropriate concentrations of antibiotic. The MIC of ceftazidime was also measured in the presence of 2 mg/L of clavulanic acid.

Enzyme preparation

The β-lactamases were prepared from 11 overnight cultures of bacteria grown in nutrient broth at 37°C. The cells were harvested by centrifugation at 6000 g for 15 min at 4°C. The resultant pellet was washed in 25 mM sodium phosphate buffer (pH 7.0) and the centrifugation repeated. The final pellet was resuspended in 1 mL of 25 mM sodium phosphate buffer (pH 7.0) and subjected to two 30 sec treatments of ultrasonication (8 microns) at 0°C, with a 1 min cooling period between treatments. The resultant lysate was cleared by centrifugation at 32,000 g for 30 min at 4°C.
Isoelectric focusing

The \( \beta \)-lactamases were examined by analytic isoelectric focusing (Matthew et al., 1975) of the bacterial sonicates. The enzymes were examined on polyacrylamide gels employing a 1:1 ratio of pH 3.5–10 and pH 9–11 amphotiles. The BIL-1 enzyme was focused along with the following \( \beta \)-lactamases of known pI: SHV-5 (pI 8.2) (Gutmann et al., 1989); SHV-1 (pI 7.7); E. cloacae chromosomol \( \beta \)-lactamase type A (pI 8.8) and B (pI 7.8) (Seeberg, Tolxdorf-Neutzling & Wiedemann, 1983) and CEP-1 \( \beta \)-lactamase (pI 8.2) (Bobrowski et al., 1976).

Biochemical analysis of \( \beta \)-lactamases

The rates of hydrolysis of cephalosporins were measured at concentrations between 10 and 100 \( \mu \)M, whereas the rates of hydrolysis of penicillins were measured at concentrations between 100 and 1 mM. The \( K_m \) and \( V_{\text{max}} \) values for the hydrolysis of each \( \beta \)-lactam by each \( \beta \)-lactamase were obtained by the method of Lineweaver & Burk (1934). The results were expressed as efficiency of hydrolysis values (\( V_{\text{max}}/K_m \)) and normalized with respect to ampicillin (Bush & Sykes, 1986). The ID\(_{50}\) values for the \( \beta \)-lactamases were also determined by spectrophotometric assay. Firstly, the rate of hydrolysis of 0.2 mM nitrocefin by the \( \beta \)-lactamase was measured at 37°C. This procedure was then performed in the presence of a range of inhibitor concentrations (10 nM–1 mM). These assays were performed following a 5 min 37°C pre-incubation of inhibitor and enzyme and the reaction was initiated by the addition of nitrocefin. The ID\(_{50}\) values were calculated from plots of percentage inhibition versus the logarithm of the inhibitor concentration. \( K_i \) values were determined with nitrocefin as the substrate and the addition of the \( \beta \)-lactam at a concentration which provided between 50–70% inhibition. Specific activities were measured as nmoles nitrocefin hydrolysed/min/mg protein. Protein concentrations were determined by the method of Waddell (1956). The molecular sizes of the \( \beta \)-lactamases were determined on a calibrated Sephadex G-75 column (2 cm\(^2\) x 90 cm) eluted with 25 mM sodium phosphate buffer (pH 7.0) at 16 mL/h (Andrews, 1964).

Results

The TEM-1 \( \beta \)-lactamase conferred no resistance to any of the second or third generation cephalosporins tested (Table 1). The TEM-E3 and SHV-3 \( \beta \)-lactamases conferred increased MICs to most of the \( \beta \)-lactams tested, and cause substantially elevated MICs for a few antibiotics. However, BIL-1 conferred broad resistance. The MIC of ceftazidime was reduced by the addition of clavulanic acid for E. coli J53-2 harbouring TEM-E3 and SHV-3, but clavulanic acid did not alleviate the ceftazidime resistance conferred by BIL-1.

The isoelectric focusing of the \( \beta \)-lactamases produced by the transconjugants of E. coli BS along with the \( \beta \)-lactamases produced by the original recipient strains used in the conjugation experiments are shown in the Figure. The K. oxytoca, E. coli and E. cloacae transconjugants of E. coli BS all produced BIL-1, which focused in the same vicinity as the Type A E. cloacae chromosomal \( \beta \)-lactamase. The E. cloacae 471 transconjugant of E. coli BS produced BIL-1 and the E. cloacae chromosomal \( \beta \)-lactamase. Interestingly, the K. oxytoca transconjugant produced only the BIL-1 \( \beta \)-lactamase, and there was no evidence of the chromosomal (no plasmid detected) \( \beta \)-lactamase.
<table>
<thead>
<tr>
<th></th>
<th>TEM-1</th>
<th>TEM-E3</th>
<th>SHV-3</th>
<th>BIL-1</th>
<th>E. coli J53-2</th>
<th>E. cloacae P99</th>
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</thead>
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<tr>
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<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>4</td>
<td>&gt;128</td>
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<td>16</td>
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<td>&gt;64</td>
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<td>8</td>
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<td>2</td>
<td>64</td>
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<td>1</td>
<td>2</td>
<td>&lt;0·06</td>
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<td>&lt;0·06</td>
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<td>0·25</td>
<td>4</td>
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<td>2</td>
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</tbody>
</table>

* Measured as minimum inhibitory concentration (mg/L) at a bacterial inoculum of $10^5$ cells/mL.
+clav., MIC of ceftazidime measured in the presence of 2 mg/L clavulanic acid.

**Table 1.** Susceptibilities of *E. coli* J53-2 transconjugants producing TEM-1, TEM-E3, SHV-3 or BIL-1

**Figure 1.** IEF of novel enzyme and standard plasmid beta-lactamases of known pI. A. *E. coli* J53-2 producing SHV-1; B. Original clinical isolate (*E. coli* SB) producing BIL-1; C. *E. coli* J53-1 producing, SHV-5; D. *E. coli* J53-2 producing BIL-1; E. *K. oxytoca* 478; F. *K. oxytoca* 478 transconjugant producing BIL-1; G. CEF-1 (pl 8·2); H. *E. cloacae* 471; I. *E. cloacae* 471 transconjugant producing BIL-1; J. *E. cloacae* chromosomal β-lactamase type A (pl 8·8); K. *E. cloacae* chromosomal β-lactamase type B (pl 7·8).
produced by the original recipient strain. It should be noted that the CEP-1 β-lacta-
mase focused at a higher pI than previously reported (Broborowski et al., 1976).

The TEM-1 β-lactamase has no measurable hydrolytic activity against any of the
new oral cephalosporins or ceftazidime (Table II). The TEM-E3 enzyme had measur-
able hydrolytic activity against all the cephalosporins studied, in particular, it efficiently
hydrolysed ceftazidime and cefotaxime. Likewise, SHV-3 also had hydrolytic activity
against all of the second and third generation cephalosporins, but it hydrolysed
cefotaxime more efficiently than ceftazidime. The BIL-1 β-lactamase had measurable
hydrolytic activity against cephalothin, cephalaxin and nitrocefin, but no measurable
activity could be detected against any of the other β-lactams tested.

The ID_{50} determinations in Table III show that the BIL-1 enzyme was 1000, 12 and
35 times more resistant to inhibition by clavulanic acid, sulbactam and tazobactam,
respectively, than TEM-E3 or SHV-3 when nitrocefin was used as the substrate. Both
these β-lactamases were susceptible to inhibition by tazobactam, sulbactam and clavu-
lanic acid at concentrations of less than 1.5 μM. The hydrolysis of nitrocefin by the
BIL-1 β-lactamase was far more susceptible to binding by different β-lactams than
either of the TEM or SHV derived β-lactamases (Table IV). The only β-lactams which
did not have a substantial relative binding effect on BIL-1 were cephaloridine and
cephalothin. Both BIL-1 and P99 β-lactamases showed considerable ability to bind
cefotaxime, cefixime and cefuroxime.

Overall, both these enzymes had similar inhibition profiles which were dissimilar
from the profiles of either of the TEM or SHV β-lactamases. Table V shows K_{i} values

### Table II. Relative efficiency of hydrolysis (relative V_{max}/K_{m}). Values for
β-lactam antibiotics by TEM-1, TEM-E3, SHV-3, P99 and BIL-1

<table>
<thead>
<tr>
<th></th>
<th>TEM-1</th>
<th>TEM-E3</th>
<th>SHV-3</th>
<th>BIL-1</th>
<th>P99</th>
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<td>7:3</td>
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<td>9:3</td>
<td>19</td>
<td>98</td>
<td>51:2</td>
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<td>Cefuroxime</td>
<td>UM</td>
<td>1:6</td>
<td>20</td>
<td>UM</td>
<td>UM</td>
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<tr>
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<td>51</td>
<td>1:1</td>
<td>UM</td>
<td>UM</td>
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<td>23</td>
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</tr>
</tbody>
</table>

* Values are expressed as a percentage of the value for cephaloridine.

UM: Unmeasurable because hydrolysis of substrate was too low.

### Table III. Inhibition profile of β-lactamase inhibitors. Concentration (μM)
required to inhibit the hydrolysis of nitrocefin by 50% (ID_{50})

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>TEM-E3</th>
<th>SHV-3</th>
<th>TEM-1</th>
<th>P99</th>
<th>BIL-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clavulanic acid</td>
<td>0:3</td>
<td>&lt;0:1</td>
<td>0:4</td>
<td>513</td>
<td>362</td>
</tr>
<tr>
<td>Sulbactam</td>
<td>1:4</td>
<td>1:6</td>
<td>15:1</td>
<td>13:5</td>
<td>18:0</td>
</tr>
<tr>
<td>Tazobactam</td>
<td>0:9</td>
<td>0:3</td>
<td>0:6</td>
<td>3:6</td>
<td>3:2</td>
</tr>
</tbody>
</table>
Table IV. Relative binding of \(\beta\)-lactam substrates. Concentration (\(\mu\)M) of \(\beta\)-lactam antibiotic required to inhibit the hydrolysis of nitrocefin by 50\%.

<table>
<thead>
<tr>
<th>(\beta)-Lactam</th>
<th>TEM-E3</th>
<th>SHV-3</th>
<th>TEM-I</th>
<th>P99</th>
<th>BIL-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>245</td>
<td>500</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>96.8</td>
<td>254</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>468</td>
<td>305</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>0:11</td>
<td>0:04</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>3:0</td>
<td>22:3</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Cefixime</td>
<td>92:4</td>
<td>958</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>11:7</td>
<td>0:4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>316</td>
<td>544</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

for four \(\beta\)-lactams which inhibit the hydrolysis capabilities of BIL-1. Cefotaxime has the lowest \(K_i\) and thus must have the greatest affinity for the BIL-1 \(\beta\)-lactamase. Table VI shows that BIL-1 enzyme has a molecular size which is substantially larger than either of the TEM or SHV enzymes investigated. The specific activity of BIL-1 (166 \(\mu\)moles/min/mg protein) was compared with the specific activities of TEM-3, TEM-5, TEM-6, TEM-9 and TEM-10, and TEM-E1 to TEM-E4 (range 15-79 \(\mu\)moles/min/mg protein). The crude samples used in these assays all originated from *E. coli* J53-2 transconjugants of the original clinical isolates. These data revealed that the specific activity of BIL-1 was between two and eleven times greater than any of the other \(\beta\)-lactamases tested (results not shown).

Discussion

BIL-1 is a plasmid mediated \(\beta\)-lactamase which confers transferable resistance to extended spectrum \(\beta\)-lactam antibiotics. However, this study has shown that BIL-1 is

Table V. \(K_i\) (\(\mu\)M) for BIL-1 with different \(\beta\)-lactam antibiotics

<table>
<thead>
<tr>
<th>(\beta)-Lactam</th>
<th>BIL-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>8:7</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>1:5</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>20:0</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0:01</td>
</tr>
</tbody>
</table>

Table VI. Molecular sizes of TEM-1, TEM-3, SHV-3 and BIL-1

<table>
<thead>
<tr>
<th>(\beta)-Lactamase</th>
<th>Molecular size ((\times) 1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>22:0</td>
</tr>
<tr>
<td>TEM-E3</td>
<td>23:5</td>
</tr>
<tr>
<td>SHV-3</td>
<td>20:5</td>
</tr>
<tr>
<td>BIL-1</td>
<td>36:5</td>
</tr>
</tbody>
</table>
biochemically very different from all other reported extended spectrum \( \beta \)-lactamases except, perhaps, MIR-1 (Papanicolaou et al., 1990). This has been exemplified by the fact that BIL-1 is dissimilar from TEM-E3 and SHV-3, which are respective representatives of the TEM and SHV series of extended spectrum cephalosporin hydrolysing \( \beta \)-lactamases. This study has shown that BIL-1 is more similar to the traditionally chromosomally encoded \( \beta \)-lactamases of Group I in the Bush (1989) classification scheme. For example, the BIL-1 enzyme confers a broad resistance to all generations of cephalosporins; it has a high pl and a molecular weight of 36,000 which are all features of Group I chromosomal \( \beta \)-lactamases. In addition to this, like other Group I chromosomal \( \beta \)-lactamases (Minami, Inoue & Mitsuhashi, 1980; Seeberg et al., 1983), BIL-1 has no measurable hydrolytic activity against the third or second generation cephalosporins which it confers resistance to. It is postulated that the method implicated in this study could not detect the hydrolytic activity of BIL-1 against these \( \beta \)-lactam antibiotics. It is probable that BIL-1 hydrolys these antibiotics at very low rates which are undetectable by our methodology (Vu & Nikaido, 1985). The \( \text{ID}_{50} \) and \( K_i \) determinations do suggest that those substrates which did not appear to be hydrolysed by BIL-1 do have significant affinity for the active site of BIL-1. This affinity, along with the slow hydrolysis of the extended spectrum cephalosporins, is probably the mechanism which enables BIL-1 to confer resistance to the wide range of \( \beta \)-lactams listed in Table 1.

Like other Group I \( \beta \)-lactamases, BIL-1 was more resistant to inhibition by tazobactam, sulbactam and clavulanic acid than either TEM-E3 or SHV-3. Moreover, the BIL-1 \( \beta \)-lactamase exhibited an inhibition profile which was very dissimilar from that of either TEM-E3 or SHV-3. However, it closely resembled the inhibition profile of the E. cloacae P99 chromosomal \( \beta \)-lactamase which is a classic Group I chromosomal \( \beta \)-lactamase.

Lastly, many Group I chromosomal \( \beta \)-lactamases have very high specific activities because their regulation has been stably derepressed (Lindberg, Lindquist & Normark, 1988). BIL-1 is one of the first examples of a \( \beta \)-lactamase which confers transferable resistance to all generations of cephalosporins and selected \( \beta \)-lactamase inhibitors. Consequently, this paper illustrates the migration of a gene encoding a Class C-like (Ambler, 1980) or a Group I chromosomal \( \beta \)-lactamase (Bush, 1989) into plasmid DNA. The migration of bacterial chromosomal \( \beta \)-lactamase genes to a plasmid location may have occurred a number of times before. The first example of this phenomenon was believed to be the CEP-1 \( \beta \)-lactamase, which was considered to be a plasmid-mediated E. coli Class I \( \beta \)-lactamase (Bobraowski et al., 1976). So similar were the properties of the CEP-1 \( \beta \)-lactamase to the E. coli enzyme that it was thought that the plasmid did not actually carry the \( \beta \)-lactamase gene, but rather provided the means of increasing the expression of the host enzyme. Further potential candidates for the migration of class I \( \beta \)-lactamase genes have been CEP-2 (Levesque et al., 1982), CMY-1 (Bauernfeind, Grimm & Schweighart, 1989) and CMY-2 (Bauernfeind et al., 1990). All these enzymes have different enzymic properties from the TEM and SHV enzymes, but there are no sequence data to suggest that any of these enzymes are Class I \( \beta \)-lactamases. The MIR-1 \( \beta \)-lactamase is the latest example of a plasmid-mediated Class I \( \beta \)-lactamase (Papanicolaou et al., 1990). This enzyme has been partially sequenced and confirmed that it is a Class I enzyme. It has similar but not identical properties to the BIL-1 enzyme. The MIR-1 \( \beta \)-lactamase was found in some strains isolated in the USA, whereas the BIL-1 \( \beta \)-lactamase has only been found in one clinical isolate from a
patient transferred to the UK from Pakistan, so the full clinical significance of these two enzymes is as yet unknown. However, it is possible that BIL-1 could represent the precursor for a series of BIL-like β-lactamases reminiscent of the TEM and SHV series of β-lactamases.

References


American Society for Microbiology, Washington, DC.


Characterization of the plasmid mediated β-lactamase BIL-1


(Received 21 October 1991; revised version accepted 10 March 1992)
Class C β-lactamases are classically chromosomally mediated enzymes. In 1989 a multiple resistant strain of E.coli was isolated from a patient hospitalised in Pakistan. This strain was shown to exhibit transferable resistance to a wide range β-lactam antibiotics (Lancet 1990: 336; 253). Preliminary biochemical studies revealed that the β-lactamase (BIL-1) responsible for this transferable resistance was a Class C - like β-lactamase. The β-lactamase was cloned into a pACYC 184 vector and sequenced. The DNA sequence of BIL-1 was approximately 70% homologous to the E.coli and the Enterobacter cloacae Class C β-lactamases but 90% homologous to the Citrobacter freundii OS60 ampC gene.

MIR-1 is the only other previously identified plasmid mediated Class C β-lactamase (AAC 1991: 34; 2200-2209) and the partial sequence of MIR-1 is 40% homologous with BIL-1. Consequently, BIL-1 is an unique plasmid mediated ClassC enzyme which appears to be derived from the Citrobacter freundii Class C β-lactamase.
BIL-1: an example of a plasmid mediated Class C β-lactamase

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Introduction
Class C β-lactamases (Group I; Bush and Sikes) mediate resistance to many β-lactam antibiotics in clinically relevant organisms such as Escherichia coli, Enterobacter cloaceae and Pseudomonas aeruginosa. The Class C β-lactamases have traditionally been referred to as chromosomally mediated β-lactamases. However, in 1989 clinically derived strains of Klebsiella pneumoniae were isolated which exhibited transferrable resistance to a broad range of β-lactam antibiotics including cephalosporins. The β-lactamase (MIR-1) responsible for this resistance was partially sequenced and shown to be a plasmid mediated Class C β-lactamase (Payne, Fosberry and Park, Betchworth 1990).

In the same year, a multiple resistant strain of E.coli was isolated in London from raw area swabs and a biopsy specimen from a patient with 35% burns. The patient had originated in Pakistan before transfer to the UK. This strain was shown to produce a plasmid mediated β-lactamase (BIL-1) which exhibited similar characteristics to MIR-1. Therefore BIL-1 was sequenced to investigate its similarity with chromosomally mediated Class C β-lactamases.

Methods
Cloning Strategy

1. Large scale plasmid preparations were made from E.coli J92.2 (5446.89) which contained a BIL-1 β-lactamase producing transconjugant of the original clinical isolate. A plasmid in excess of 70kb was visualized by agarose gel electrophoresis.

2. The 70kb plasmid isolated from E.coli J92.2 (5446.89) was partially digested with Sau 3A I and then size fractionated on sucrose gradients. Fragments of 2-4kb were ligated into the Bam H1 site of the vector pACYC184. Recombinant clones were introduced into the host strain E.coli E44(ampc) by electroporation. Transformsants expressing the BIL-1 β-lactamase gene were selected for by growth on media containing ampicillin.

3. The presence of BIL-1 β-lactamase in a number of transformants was confirmed by IEF and by resistance to cefazoline. Plasmids from these transformants were subjected to preliminary restriction enzyme analysis. However, inconsistencies in the restriction mapping data obtained on these plasmids indicated an instability problem.

4. This problem of instability was rectified by introducing plasmids from BIL-1 expressing transforms into the neo-host E.coli DH1. The presence of BIL-1 β-lactamase was again confirmed by IEF and resistance to cefazoline.

Localization of the Gene Encoding BIL-1 β-lactamase

1. Further restriction enzyme analysis of three plasmids identified a common region of DNA which contained an EcoRV site.

2. A subcloning strategy was then implemented: Digestion of the plasmid pBROC442 with EcoRV generated two fragments of 6.0 and 2.9kb. Re digestion of the 6kb fragment generated pBROC443 (effectively a derivative of pBROC442 in which the 2.9kb fragment has been deleted). pBROC443 is unable to confer resistance to cefoxitin suggesting that the unique EcoRV site was situated in the BIL-1 β-lactamase gene. (Fig 1)

Results

1. The BIL-1 β-lactamase gene was found to be 1146 nucleotides in length. (Fig 2)

2. The putative promoter region for the BIL-1 β-lactamase gene was identified by comparison to the consensus E. coli promoter. (Fig 2)

3. The BIL-1 β-lactamase gene was sequenced on both strands.

Promoter Region BIL-1 β-Lactamase

<table>
<thead>
<tr>
<th>CTGTCGCTCAATTTACGCGGTTCCCGACCGGACG</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>TACGAACCTTTACCGTTGCGG</td>
<td>31</td>
</tr>
<tr>
<td>GCGGGACACCTTTACGTTGCG</td>
<td>36</td>
</tr>
</tbody>
</table>

Consensus E.coli Promoter

-45 to -25 region: TTGACA
-10 region: TAATTC

Ribosome Binding Site

AAAGG

Fig. 2

Discussion

The sequence of BIL-1 showed greater than 70% homology with four other Class C β-lactamases (Fig 3) which confirmed that BIL-1 was a plasmid mediated Class C β-lactamase. Furthermore, comparison of the BIL-1 amino acid sequence with that of the partial sequence of MIR-1 illustrated that these two enzymes were different. The amino acid sequence of BIL-1 was most homologous with the Citrobacter freundii Class C β-lactamase. In contrast MIR-1 appeared to have originated from the E.coli class C enzyme. (Fig 3)

In conclusion, BIL-1 was reported in one clinical isolate from Pakistan in 1989 and organisms which produced MIR-1 were isolated in 11 patients from a single hospital in the USA between 1988 and 1989. At present, these are the only two proven examples of plasmid mediated Class C β-lactamases worldwide. Consequently, it is premature to predict the true clinical impact of plasmid mediated Class C β-lactamases.

References

2. Woodford, N., Payne, D.J., Johnson, N., Amies, B.G. J. Antimicrobial Chemotherapy; Accepted for publication.
Cloning and Sequence Analysis of \( \text{bla}_{\text{BIL-1}} \), a Plasmid-Mediated Class C \( \beta \)-Lactamase Gene in *Escherichia coli* BS

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The extended-spectrum, plasmid-borne \( \beta \)-lactamase gene \( \text{bla}_{\text{BIL-1}} \), which was discovered in *Escherichia coli*, has been cloned. Unusually for a plasmid-borne \( \beta \)-lactamase, \( \text{bla}_{\text{BIL-1}} \) encodes a novel class C enzyme and appears to have originated from the chromosomal amp\(C\) gene of *Citrobacter freundii*.

\( \beta \)-Lactamases are microbial enzymes which inactivate \( \beta \)-lactam antibiotics by hydrolyzing the \( \beta \)-lactam ring. Comparison of the amino acid sequences of \( \beta \)-lactamases creates four molecular classes, A, B (1), C (10), and D (4). This paper is primarily concerned with class C \( \beta \)-lactamases, which together with class A \( \beta \)-lactamases are thought to derive from cell-wall-synthesizing enzymes (12). The class C \( \beta \)-lactamases are primarily cephalosporinases, but because they are often produced at high levels, they can mediate resistance to both cephalosporins and penicillins. Most gram-negative bacteria produce class C \( \beta \)-lactamases, and therefore, these enzymes create one of the most serious problems for the clinical use of \( \beta \)-lactams and in particular broad-spectrum cephalosporins (11).

Class C \( \beta \)-lactamases have previously been referred to as chromosomally mediated enzymes (25). However, in 1989 clinically derived strains of *Klebsiella pneumoniae* which exhibited transferrable resistance to a broad range of \( \beta \)-lactam antibiotics including cephamycins were isolated.

The \( \beta \)-lactamase (MIR-1) responsible for this resistance was partially sequenced and shown to be a plasmid-mediated class C \( \beta \)-lactamase (23).

In the same year, a multiply resistant strain of *Escherichia coli* was isolated in London, United Kingdom, from raw area swabs and a biopsy specimen from a patient with 35% burns. This strain was shown to produce a plasmid-mediated \( \beta \)-lactamase (BIL-1) (29) which exhibited biochemical characteristics similar to those of MIR-1 (24). Here we present the cloning and primary sequence of \( \text{bla}_{\text{BIL-1}} \) and by homology studies suggest the likely origin of this gene.

*E. coli* K-12 J62.2(5446.89) (24), a transconjugant of *E. coli* BS (29), the original clinical isolate producing BIL-1, was shown to produce BIL-1 by isoelectric focusing (19). A plasmid in excess of 70 kbp was isolated from *E. coli* J62.2(5446.89) by the alkaline lysis method of Timmis et al. (25) and partially digested with Sau3AI, generating fragments with an average size of 2 to 4 kbp. These fragments were ligated into the *BamH*I site of the plasmid pACYC184 (3).

Recombinant plasmids were introduced into the \( \beta \)-lactamase-deficient strain *E. coli* 44 (21) by electroporation (Bio-Rad Gene Pulser), and transformants producing BIL-1 were selected by resistance to ampicillin (50 \( \mu \)g/ml). A number of transformants were further characterized by resistance to cefazidime (3 \( \mu \)g/ml) and by identification of BIL-1 production by isoelectric focusing. Preliminary restriction endonuclease analysis of recombinant plasmids revealed plasmid instability with plasmids increasing in size after subculture of transformants. Primary recombinant plasmids containing \( \text{bla}_{\text{BIL-1}} \) were therefore transferred to the *E. coli* recA1 host

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* Corresponding author.
FIG. 2. Nucleotide sequence of the bla_{BIL-1} gene. The deduced amino acid sequence of the BIL-1 protein is also presented. SD, ribosome binding site; ▲, limit of sequence homology to ampC genes; △, signal sequence cleavage site.

DH1 (9, 17, 20). Further restriction enzyme analysis of several plasmids containing bla_{BIL-1} identified a common region of DNA flanked by an EcoRV site. Deletion of the EcoRV fragment in pBROC442 produced the plasmid pBROC443 (Fig. 1), which no longer conferred ceftazidime resistance on E. coli DH1. The EcoRV site was therefore inferred to lie within the bla_{BIL-1} gene.

An initial sequencing primer derived from pACYC184 sequences adjacent to the EcoRV site was used to sequence (Sequenase kit, version 2 [U.S. Biochemical Corp.]) one end of the cloned DNA in pBROC434.

Comparison of this preliminary nucleotide sequence with the sequence data base confirmed that the EcoRV site did indeed lie within a β-lactamase gene. Subsequently, sequencing primers were derived from the known bla_{BIL-1} sequence, and the sequence of the entire bla_{BIL-1} gene was determined on both strands in pBROC442. The complete sequence of bla_{BIL-1} together with the upstream flanking sequence is shown in Fig. 2. The DNA sequence was analyzed with the Genetics Computer Group programs of the University of Wisconsin (5), and the bla_{BIL-1} open reading frame (ORF), comprising 1,146 nucleotides and encoding a protein of 381 amino acids, was identified. After cleavage of the predicted signal sequence, the mature protein of 361 residues has a molecular mass of 39,936 Da and a predicted pI of 10.04. This is in agreement with the high value of the experimentally determined pI (24).

Two of the characteristic amino acid fingerprints of penicillin-binding proteins and β-lactamases are present in the bla_{BIL-1} ORF: the active-site serine (serine 70 in the Ambler classification scheme [1]) in the motif SXK at residues 64 to 67 of the mature protein and the motif KTG at residues 315 to 317 (12). This second motif plays an essential role in the formation of the tertiary structure of the active site. A third motif, SXN, which is present approximately 80 residues carboxy-terminal to the active-site serine in class A β-lactamases and penicillin-binding proteins, is not present in this ORF. However, the tyrosine residue at position 150, which forms part of the motif YAN, has been postulated to perform the same function as the serine in the SXN motif (22).

Comparison of both the nucleotide sequence of bla_{BIL-1} and the predicted amino acid sequence of BIL-1 with the appropriate data bases revealed extensive homology to class C β-lactamases. bla_{BIL-1} showed greatest homology to the chromosomally encoded class C β-lactamases of Citrobacter freundii with 93% DNA identity to the ampC genes of C. freundii OS60 (14) and C. freundii GN346 (27) and 94% identity at the amino
acid level, suggesting that bla_{\text{BIL}-1} originated from this species. Therefore, it appears that BIL-1 could have original been a C. freundii class C enzyme which migrated from the chromosome to a plasmid. This gene migration could have been mediated by transposable elements, which are normal constituents of most bacterial genomes and of many extrachromosomal plasmids and bacteriophages (13). Transposons promote DNA rearrangements and are known to play a role in the dissemination of antibiotic resistance genes (8, 18). However, preliminary studies were unable to demonstrate transposition of an element containing bla_{\text{BIL}-1}, indicating a transposition frequency of less than $10^{-6}$ for the putative bla_{\text{BIL}-1}-containing element (6).

The ampC gene of C. freundii encodes an inducible β-lactama- mase. Induction is regulated by AmpR, a trans-acting protein encoded by the ampR gene which lies immediately upstream of, and is transcribed in the direction opposite to that of, the ampC gene (15). The ampR gene has been sequenced, and the divergent ampC and ampR promoters have been shown to overlap (16). AmpR is a transcription activator, binding to a DNA region which is immediately upstream of the ampC promoter and which overlaps the ampR promoter (16). Sequences upstream of bla_{\text{BIL}-1}, homologous to sequences upstream of C. freundii OS60 AmpC extend for 117 bp and include both ampC and ampR promoter sequences. However, only 31 bp of the 38-bp region protected by the AmpR protein are present upstream of bla_{\text{BIL}-1}, and there is no evidence for an ORF homologous to the C. freundii ampR ORF. The absence of an ORF with homology to ampR implies that bla_{\text{BIL}-1} is not regulated in the same way as C. freundii ampC. Indeed, biochemical evidence points towards the constitutive production of BIL-1 β-lactamase (24).

Only 150 nucleotides of bla_{\text{MIR}-1} have been published (23); however, comparison of this limited sequence data reveals that BIL-1 and MIR-1 are related but distinct enzymes: bla_{\text{BIL}-1} and bla_{\text{MIR}-1} are 75% identical, but whereas bla_{\text{MIR}-1} shows greatest homology to the ampC genes of C. freundii, bla_{\text{BIL}-1} shows 90 to 91% identity to the ampC genes of Enterobacter cloacae (7). Until recently, these were the only proven examples of plasmid-mediated class C enzymes worldwide. However, two more cases have recently been reported (2, 28), both isolated from K. pneumoniae. It is probable that more plasmid-mediated class C β-lactamases exist, albeit currently undetected. Whilst not a current clinical problem, the existence of these plasmid-mediated class C β-lactamases must be a prime consideration for the design of new β-lactam antibiotics and β-lactamase inhibitors.

The nucleotide sequence of the bla_{\text{BIL}-1} gene has been deposited in the EMBL data library under accession number X74512.

REFERENCES


BACTERIAL EFFECTS OF CLAVULANIC ACID AND AMOXICILLIN/CLAVULANIC ACID AGAINST GRAM-NEGATIVE BACILLI. IN AN IN VITRO MODEL SIMULATING URINARY CONCENTRATIONS.


The majority of urinary isolates of Escherichia coli which produce TEM-1 \beta-lactamase are susceptible to amoxicillin/clavulanic acid (AMX/CA), whereas 'hyper-producers' are less susceptible. Organisms such as Enterobacter cloacae, which produces TEM-1 in vivo, are resistant to AMX/CA. However, concentrations of CA and AMX achieved in the urine of man following oral dosage of 125mg and 250mg respectively are high (approx. 500-1500µg/ml) and these were simulated in an in vitro bioplot model to demonstrate bactericidal activities against E.coli NCTC 11506, which produces TEM-1 \beta-lactamase (AMX/CA MIC=8-16µg/ml), E.coli J4T, a hyper-producer of TEM-1 (AMX/CA MIC=8µg/ml) and the AMX/CA-susceptible E.coli strain NCTC 10005 (AMX/CA MIC=128-64µg/ml), a Class I \beta-lactamase producer. Against both strains of E.coli, CA showed a marked bactericidal effect, with an inoculum of 10^8cfu/ml killing 99.9% of the organisms after 24h at 37°C, although AMX/CA was more effective. Against E.coli NCTC 10005, CA was less bactericidal, with 10^8cfu/ml at 8 hours but, surprisingly, a synergistic effect was seen using AMX/CA with 10^8cfu/ml by 4 hours. Inhibition of the Class I \beta-lactamase by CA was not seen but bladder shaped forms and spheroplasts were observed at 4 hours in the CA and AMX/CA treated cultures of E.coli NCTC 10005 respectively. Thus, urinary concentrations of AMX/CA showed bactericidal synergy against a resistant culture in this in vitro model.
Plasmid Mediated Class I β-lactamas

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Abstract

The purpose of this investigation was to compare the characteristics of MIR-1 and BIL-1 with other Class I enzymes. Both enzymes confer transferable resistance to a broad range of cephalosporins and BIL-1 had a different isoelectric point to MIR-1. IDSO and Ki determinations have shown that BIL-1 has high affinity for cephalosporins which is typical of Class I enzymes. In contrast, MIR-1 had low affinity for these substrates. This is an unusual characteristic of conventional Class I β-lactamas even though MIR-1 had appreciable homology at the amino acid sequence level.

This study shows that MIR-1 and BIL-1 are different plasmid mediated Class I β-lactamas and, unlike BIL-1, MIR-1 has a biochemical profile which is atypical of other Class I β-lactamas.

Introduction

Class I β-lactamas can cause serious drug resistance problems associated with β-lactam antibiotics. Such enzymes have previously been referred to as chromosomally mediated cephalosporinases. However, during the last 1980s BIL-1 and MIR-1 (β-lactamas) were identified in clinical isolates from Pakistan and USA respectively. Biochemical characterisation and DNA sequencing of these enzymes revealed that they were plasmid mediated Class I β-lactamas (6, 8).

The aim of the current study was to compare the biochemical characteristics and reported amino acid sequences of these two enzymes and to assess their similarity to chromosomal Class I β-lactamas.

Methods

K.pneumoniae 96D and E.coli BS were the original clinical isolates which produced MIR-1 and BIL-1 respectively. E.coli K12 pmg231 produced a cloned copy of the MIR-1 gene. Crude β-lactam samples were prepared and visualised as described previously (6). The BIL-1 and MIR-1 preparations obtained from the E.coli K12 transconjugants were purified by isoelectric focusing (IEF). IDSO values for 8 substrates were determined using nitrocefin as the reporter substrate and Ki values were determined from Dixon Plots.

Results

The organisms which produced BIL-1 or MIR-1 were resistant to all the cephalosporins tested (table 1) and figure 1 distinguishes the two enzymes by IEF. The IDSOs of cefuroxime, cefotaxime, cefazidime and cefoxitin were between 60-2000 times higher for MIR-1 than for the other three enzymes in table 2. In addition the Ki values of cefoxitin, cefotaxime and cefazidime for MIR-1 were many orders of magnitude higher than those for BIL-1 (table 3). The Ki values of cefazidime and cefotaxime for BIL-1 were similar to those reported for other Class I β-lactamas (table 3) which illustrates that BIL-1 is a typical Class I enzyme. However, MIR-1 has low affinity for cephalosporins which is atypical of Class I enzymes.

Conclusions

In conclusion, BIL-1 and MIR-1 are different enzymes. BIL-1 has a substrate profile characteristic of Class I chromosomal β-lactamas whereas MIR-1 has low affinity for all the later generation cephalosporins tested which is atypical of Class I enzymes. Comparison of the previously reported sequence data reveals that both enzymes have high homology to other Class I β-lactamas (6, 3). Paradoxically, the 296-344 amino acid sequence of MIR-1 has a higher homology (only non-conserved amino acids match) with P99 and is biochemically distinct from it, whereas BIL-1 has a lower homology to this region (3) but behaves as a typical Class I β-lactamae (6). These observations may be explained if crucial amino acids elsewhere.

Acknowledgements

We thank Professor Medeiros for the BIL-1 producing strains, P.HLS Cologne for the BIL-1 transconjugants and R. Moore for the MIC determinations. We also acknowledge A. Fosberry, E. Lawlor and J. Hodgson, for the BIL-1 nucleotide sequence data.

References


FIG. 1. Isoelectric focusing of BIL-1 and MIR-1. A, pH 6.77; B, pH 6.5 (pH 5.4); C, MIR; D, MIR and E, BIL-1.

The organisms which produced BIL-1 or MIR-1 were resistant to all the cephalosporins tested (table 1) and figure 1 distinguishes the two enzymes by IEF. The IDSOs of cefuroxime, cefotaxime, cefazidime and cefoxitin were between 60-2000 times higher for MIR-1 than for the other three enzymes in table 2. In addition the Ki values of cefoxitin, cefotaxime and cefazidime for MIR-1 were many orders of magnitude higher than those for BIL-1 (table 3). The Ki values of cefazidime and cefotaxime for BIL-1 were similar to those reported for other Class I β-lactamas (table 3) which illustrates that BIL-1 is a typical Class I enzyme. However, MIR-1 has low affinity for cephalosporins which is atypical of Class I enzymes.

Conclusions

In conclusion, BIL-1 and MIR-1 are different enzymes. BIL-1 has a substrate profile characteristic of Class I chromosomal β-lactamas whereas MIR-1 has poor affinity for all the later generation cephalosporins tested which is atypical of Class I enzymes. Comparison of the previously reported sequence data reveals that both enzymes have high homology to other Class I β-lactamas (6, 3). Paradoxically, the 296-344 amino acid sequence of MIR-1 has a higher homology (only non-conserved amino acids match) with P99 and is biochemically distinct from it, whereas BIL-1 has a lower homology to this region (3) but behaves as a typical Class I β-lactamae (6). These observations may be explained if crucial amino acids elsewhere.

Acknowledgements

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References

The incidence of antibiotic resistance in aerobic faecal flora in South India


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During a field study in South India in 1989, faecal specimens were collected from residents in villages and the town of Vellore in South India. Examination of the faecal specimens revealed that virtually the whole population carried commensal bacteria resistant to trimethoprim, ampicillin and chloramphenicol. Most specimens contained more than one type of bacterium resistant to each antibiotic. There was less resistance to nalidixic acid, with a higher proportion in the town (33%) than in the villages (13%). Although there was little cross-resistance of the ampicillin-resistant strains to later generation cephalosporins, 50% were resistant to the combination of amoxycillin and clavulanic acid. There was no significant cross-resistance of the nalidixic acid-resistant strains to fluorinated 4-quinolones, despite the free availability of ciprofloxacin and norfloxacin in the area. The probable reason for the high incidence of resistance to first generation antimicrobials is the extensive use of these agents, coupled with continuous exposure to large numbers of faecal micro-organisms.

Introduction

For many years it has been suggested that the incidence of antibacterial drug resistance in pathogenic bacteria isolated in developing countries is higher than in Northern Europe. Unfortunately, there is a lack of reliable data outlining the incidence and type of resistance in all types of bacteria from developing countries.

Co-trimoxazole resistance in pathogenic urinary Gram-negative bacteria from tropical areas has been reported from a number of centres (Amyes & Young, 1987), varying from 34% in Chile and 38% in Brazil to 40% in Thailand and 44% in Chile (Murray et al., 1985; Urbina, Prado & Canelo, 1989). Unfortunately, some of these studies employed a combined disc of trimethoprim and sulphamethoxazole, so the true incidence of resistance to trimethoprim alone was probably somewhat higher. Other studies have examined the incidence of resistance to trimethoprim alone in pathogenic enterobacteria. In Kuwait, the incidence of trimethoprim resistance in Salmonella spp. was 14.8%, but this rose to 43% in enteropathogenic Escherichia coli, and as high as 59% in Shigella spp. (Chugh, 1985). Almost all the strains were highly-resistant to this...
drug, and such an observation has often been associated with plasmid carriage of the resistance gene. In Nigeria, the incidence of trimethoprim resistance in urinary enterobacteria was shown to be > 63% (Lamikanra & Ndep, 1989), and similar levels have been reported in South African hospitals (Wylie & Koornhof, 1989). In Western countries, incidences of trimethoprim resistance in equivalent pathogenic bacteria have remained between 10% and 20% (Amyes, 1986; Oldfield et al., 1991; C. J. Thomson & S. G. B. Amyes, unpublished results).

In 1984, we examined the incidence and types of trimethoprim resistance in urinary pathogens from the Christian Medical College Hospital (CMCH) in Vellore, South India. When the strains were tested against trimethoprim at 10 mg/L, 64% of the strains were found to be resistant. When the strains were retested against trimethoprim at 1000 mg/L, 57% were resistant (Young et al., 1986). This indicated that most of the trimethoprim resistance was associated with genes that had originally been plasmid-derived. This was also the highest incidence of trimethoprim resistance amongst urinary pathogens that had been reported at that time. In Vellore at CMCH, the recent incidence of trimethoprim resistance in *Shigella* spp. and *Vibrio cholerae* has been > 80% (Jesudason, Lalitha & Koshi, 1985; Jesudason, Joseph & Koshi, 1989; Jesudason & Jacob John, 1990a), and around 11% in *Salmonella typhi* (Jesudason & Jacob John, 1990b). In the latter species, the incidence is now approaching the figure reported in South America (Taylor, Chumpitaz & Goldstein, 1985). In each case, the resistance in India is probably plasmid-derived as it is almost all of a high level. A high incidence of resistance has been observed to other antibacterial drugs in these species.

A similar story has been reported throughout the world for ampicillin resistance (Young, Nandivada & Amyes, 1989). In countries bordering the Persian Gulf, the incidence of ampicillin resistance has recently been shown to be between 60% and 70% (Gasser et al., 1991). When the strains from Vellore were tested for their susceptibility to ampicillin at 10 mg/L, 84% were resistant (Nandivada & Amyes, 1990). As was the case with trimethoprim resistance, this did not result from a unusual distribution of strains towards those that were naturally resistant because 77% of *E. coli* strains were also resistant. Very few studies have examined the incidence of ampicillin resistance in the developing world, and this was the highest incidence of resistance ever reported. This resistance was unusual because there was little concurrent resistance to cephaloridine, as might have been expected from experience with European strains (Reid et al., 1988).

As elsewhere in the world, the reservoirs for these resistance genes have not been identified. This paper outlines a study in which the incidence and types of resistance in the commensal faecal flora of healthy individuals from the town of Vellore and the surrounding area in Tamil Nadu, South India, were examined.

**Materials and methods**

**Collection of bacterial isolates**

The Community Health Unit at CMCH identified regions in the town of Vellore (population 150,000) where the population would be willing to provide specimens. In the 15 day period before sampling, the hospital staff identified potential volunteers. These volunteers were questioned rigorously by the hospital staff, both verbally and as the result of filling in questionnaires, in collaboration with ourselves, to establish what previous therapy they had been taking. In most cases this entailed an examination of
the tablets or medicines that the volunteer had been consuming. We were thus able to exclude all those who were taking antibacterial therapy; approximately 35% of the potential study group were excluded for this reason, and these people were not sampled subsequently. Each remaining volunteer was provided with a sterile container and asked to provide a faecal specimen for collection the following morning. The volunteer was asked a few simple questions, including whether meat was included in the diet, the number living in the household, and the sanitary arrangements. The Rural Unit for Health and Social Affairs (RHUSA) of the hospital, some 40 km from the town itself, recruited volunteers from three villages within its region. The same screening procedure was followed by the social workers from RHUSA, in conjunction with ourselves, and the same questions were asked about previous therapy. Approximately the same proportion (35%) of the potential study group were excluded from further study as they had consumed antibacterial drugs in the preceding 15 days. Specimens were taken as described for the urban group. In both the rural and urban studies, all children under five years of age were excluded automatically. All specimens were taken immediately to the laboratory for processing.

Identification of resistance

All faecal specimens were plated on to Oxoid MacConkey Agar plates containing either ampicillin (10 mg/L), nalidixic acid (10 mg/L), chloramphenicol (10 mg/L), or no antibiotic. The specimens were plated also on to a modified Difco Mueller-Hinton Agar containing trimethoprim (10 mg/L). This agar allows sensitivity to this drug and lactose-fermenting ability to be tested simultaneously (Amyes & Gould, 1984). The number of volunteers with positive resistant cultures on these plates after incubation for 24 h at 37°C was recorded.

Examination of resistance

Lactose-fermenting colonies from the antibiotic plates were purified and identified. They were then grown in overnight Oxoid Nutrient Broth cultures, and diluted subsequently in Davis-Mingioli medium (Davis & Mingioli, 1950). Approximately 10^5 cfu were placed on to Oxoid Iso-Sensitest agar plates containing other drugs of the same family. For example, strains found to be resistant to nalidixic acid were tested against ciprofloxacin, norfloxacin, etc., while the ampicillin-resistant strains were tested for resistance to penicillins and cephalosporins.

Results

Incidence of resistance

The samples for the urban part of the study were taken from volunteers in various parts of the town of Vellore (Figure). There was so much movement between various parts of the town that sampling and comparing individual areas seemed unreasonable. On the other hand, for the rural portion of the study, three villages were identified as representative of those in an area 40 km from Vellore. Kilvayattaranuppam was a small roadside village, with some traffic through it, and a small but itinerant population. Kavanur was a more remote village, near the Palar river, and with less day-to-day contact with the outside world. Melmoil was the most remote of the villages studied;
located in the foothills, there was virtually no day-to-day contact with other areas (Figure). Each village had a population of about 500 people.

The results (Table I) show that an extremely high proportion of the population carried commensal bacteria resistant to trimethoprim, ampicillin or chloramphenicol. Most of the organisms isolated were resistant to all three drugs. Surprisingly, there was little difference between the resistance proportions in the villages in the rural study, except that the remote village Melmoil had only one person carrying bacteria resistant to nalidixic acid. Similarly, as almost all the volunteers tested in the urban population carried ampicillin, chloramphenicol and trimethoprim-resistant bacteria, there was no significant difference between this group and the rural group. Again, the difference lay with the proportion of bacteria resistant to nalidixic acid, which was about twice as great in the urban group compared with the rural group.

These results mask the fact that most of the volunteers carried more than one

Table I. Proportion of volunteers with resistant bacteria

<table>
<thead>
<tr>
<th>Location</th>
<th>No. of volunteers</th>
<th>Volunteers with resistant bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Tmp</td>
</tr>
<tr>
<td>Rural study</td>
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<td></td>
</tr>
<tr>
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<td>43</td>
<td>42</td>
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<tr>
<td>Kavanur</td>
<td>46</td>
<td>44</td>
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<td>Melmoil</td>
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<td>37</td>
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<td>Combined</td>
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<td>123</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vellore</td>
<td>95</td>
<td>95</td>
</tr>
</tbody>
</table>

Tmp, Trimethoprim; Amp, ampicillin; Nal, nalidixic acid; Chl, chloramphenicol.
Resistance in commensal bacteria from India

Table II. Average number of resistance commensal bacteria/specimen

<table>
<thead>
<tr>
<th>Location</th>
<th>Tmp</th>
<th>Amp</th>
<th>Nal</th>
<th>Chl</th>
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</tr>
<tr>
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<td>2.1</td>
<td>1.6</td>
<td>0.3</td>
<td>1.6</td>
</tr>
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<td>1.8</td>
<td>1.8</td>
<td>0.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Melmoil</td>
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<td>1.9</td>
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<td>1.6</td>
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<tr>
<td>Combined mean</td>
<td>1.7</td>
<td>1.8</td>
<td>0.2</td>
<td>1.6</td>
</tr>
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<td>Urban study</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vellore</td>
<td>1.8</td>
<td>2.1</td>
<td>0.5</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Abbreviations as in Table I.

bacterial strain with a particular resistance phenotype. Indeed, in some cases, four different resistant strains were identified. The average number of resistant strains carrying each phenotype is shown in Table II. On average, just under two different strains of each resistant phenotype were identified/specimen.

One resistant isolate was retained for each antibacterial drug and tested further. Strains resistant to nalidixic acid did not show significant cross-resistance to ciprofloxacin, norfloxacin, ofloxacin or pefloxacin. Most of the strains resistant to nalidixic acid from both the urban and rural studies were E. coli; the MICs of the fluorinated 4-quinolones for these strains were higher than those for the sensitive E. coli strain KL16, but were still lower than the break-points normally regarded as indicative of resistance (Working Party of the British Society for Antimicrobial Chemotherapy, 1991).

Of the trimethoprim-resistance group, 75% were resistant to > 1000 mg/L, a level normally associated with certain types of plasmid-mediated resistance genes.

A representative proportion of the strains resistant to ampicillin were tested further for their resistance to mixtures of amoxycillin plus clavulanic acid, and for resistance to first, second and third-generation cephalosporins. There was very little resistance to the cephalosporins (Table III); however, 50% of the strains were resistant to a mixture of amoxycillin (10 mg/L) and clavulanic acid (5 mg/l).

Contributory factors

The social conditions were examined to determine if any of these could be eliminated as contributory factors. In both the urban and rural study groups, the incidence of resistance was very high; therefore, if one factor was considerably different, it might be eliminated. The proportion of males : females was 1 : 1.3 for the rural population, compared with 1 : 3 for the urban population (Table IV). This did not seem to have any significant effect.

It has been speculated that the eating of meat might permit greater ingestion of resistant bacteria. This did not appear to be the case. There was a minority of vegetarians, and in the remote village of Melmoil, with the lowest incidence of resistance, there were none. As the average number of meat meals per month was less than five (Table IV), the influence of bacteria from this route seems very limited. The age of the urban population was higher, but this seemed to have little effect, as did the
Table III. Resistance to β-lactam antibiotics amongst selected ampicillin-resistant strains (total of 58 strains)

<table>
<thead>
<tr>
<th>β-lactam antibiotic</th>
<th>Concentration (mg/L)</th>
<th>% of resistant strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Amoxycillin + clavulanic acid</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>10</td>
<td>24</td>
</tr>
<tr>
<td>Cephalexin</td>
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</tr>
<tr>
<td>Cefuroxime</td>
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<td>17</td>
</tr>
<tr>
<td>Ceftazidime</td>
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<td>0</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>4</td>
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</tr>
</tbody>
</table>

number of inhabitants per household (Table IV). In the rural study, there was a large number of animals associated with the human population, often living under the same roof. These included cats, dogs, goats and poultry. These were not so evident in the urban study and were probably not a contributory factor.

The water supply is likely to have had more influence. In each of the villages, the water supply was well-water from boreholes. In all three villages, only two of the volunteers drank boiled water. Examination of the well-water supply revealed large numbers of different antibiotic-resistant bacteria. In the town, the majority of volunteers drank the municipal water supply from the tap (73/95), although a few of these also drank well-water. The rest drank only well-water. The municipal water supply also contained a number of different antibiotic-resistant bacteria. In the villages, sanitary arrangements were non-existent and the population used the fields. In at least one village, Kavanur, some attempt had been made not to contaminate the water supply as the population was no longer allowed to excrete near the boreholes. However, the boreholes were close to a river and may have been contaminated from another village upstream.

Perhaps the most significant feature was the number of reported illnesses in the month before the donation of each specimen (Table V). Only a minority of volunteers

<table>
<thead>
<tr>
<th>Location</th>
<th>total</th>
<th>males</th>
<th>Vegetarians</th>
<th>age</th>
<th>mean figures no./house meat/month</th>
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<td>38</td>
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<td>4.8</td>
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<tr>
<td>Vellore</td>
<td>95</td>
<td>22</td>
<td>9</td>
<td>33.4</td>
<td>5.4</td>
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Table IV. Social conditions of volunteers at the collection sites
Table V. Reported illnesses in the month before samples were obtained

<table>
<thead>
<tr>
<th>Location</th>
<th>No. of volunteers</th>
<th>Therapy(^\circ)</th>
<th>none</th>
<th>diarrhoea</th>
<th>cough</th>
<th>fever</th>
<th>pain</th>
<th>vomiting</th>
<th>dysuria</th>
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<td>43</td>
<td>18</td>
<td>3</td>
<td>9</td>
<td>20</td>
<td>9</td>
<td>21</td>
<td>3</td>
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<td>13</td>
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<tr>
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<td>14</td>
<td>7</td>
<td>10</td>
<td>8</td>
<td>18</td>
<td>5</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Melmoil</td>
<td>38</td>
<td>15</td>
<td>14</td>
<td>5</td>
<td>9</td>
<td>16</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Combined</td>
<td>127</td>
<td>52</td>
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<td>21</td>
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<td>33</td>
<td>45</td>
<td>12</td>
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<tr>
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<td>22</td>
<td>21</td>
<td>6</td>
<td>6</td>
<td>15</td>
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</tbody>
</table>

\(^\circ\)Number of volunteers who received non-antibiotic therapy in the seven days before samples were obtained.
reported no symptoms at all in the previous month. According to the local community workers, this incidence of illness is quite typical in the urban and rural areas used in this study; however, we can find no similar studies from India to establish whether this is representative of the country as a whole. Most symptoms could be grouped in a few defined patterns, such as fever, vomiting, etc. The remaining group contained symptoms ranging from those associated with diabetes mellitus to backache. Approximately 50% of the volunteers in each group had received some therapy, other than antibiotics, in the previous seven days. Much of this therapy derived from 'over the counter' sales without prescription and, surprisingly, was equally prevalent in the rural and urban populations.

Local antibacterial drugs
Pharmaceuticals were purchased 'over the counter' in pharmacies in Vellore in order to establish what type of therapy the commensal bacteria might have been challenged with, and the potency of the local products. It was possible to purchase all antibacterial drugs except second and third generation cephalosporins, but including the latest fluorinated 4-quinolones. These compounds were analysed and were found to be virtually identical to their British counterparts, both in potency and composition (data not shown).

Discussion
These results show the highest incidence so far reported for the carriage of resistant bacteria within any human population. There have been few similar examinations of resistance in the commensal faecal flora from populations in Western countries. When the incidence of trimethoprim resistance in faecal bacteria from hospitalized patients in Scotland was examined, only 20% of the patients were found to carry trimethoprim-resistant bacteria (Amyes & Gould, 1984), while Reves et al. (1990) found < 10% carriage in the USA of trimethoprim-resistant bacteria in the general population. In contrast, there has been a growing incidence of resistance in pathogenic bacteria in countries outside Europe and North America (Farrar, 1985), and this presumably results partly from the increased use of antibiotics in these areas (Young et al., 1986; Wylie & Koornhof, 1989; Moellering, 1990). These incidences may be mirrored by the level of resistance in commensal bacteria and, within the area of South India studied, there appeared to be a correlation between the incidence of resistance in pathogenic strains (Young et al., 1986; Nandivada & Amyes, 1990) and the proportion of resistant commensal bacteria. Such a level of resistant bacteria in the commensal population is significant and poses a threat because resistance genes can pass readily from *E. coli* to be more pathogenic bacteria, as has been shown recently with the transmission of a 35 MDa plasmid conferring trimethoprim and ampicillin resistance to an invading strain of *Shigella flexneri* in native American women in the Western USA (Tauxe, Cavanagh & Cohen, 1989).

Why should the incidence of resistance be so high in India? It appears that the spread of resistance is associated strongly with living patterns and may be favoured particularly by the life-styles in the study region. Taylor *et al.* (1989) reported an outbreak of *Shigella dysenteriae* infection in Thailand in 1986, and showed that the epidemic organism spread rapidly through the population, particularly amongst the village communities. Although only 10% of the villagers actually demonstrated clinical
We found resistance the genes specimens from the 60% clinical carriage country, Western compliance only by the high ability to uncontrolled ingestion a could that pressure. into the day-care shown recently world, Elsewhere symptoms of infection, 76% were shown by serology to have harboured the epidemic bacterium. Elsewhere in South-East Asia, it has been shown that resistance genes have entered the faecal flora of wild mammals in the clear absence of selective pressure (Graves et al., 1988). However, it is not just within village life, nor in the developing world, that resistant commensal bacteria might spread easily. Reves et al. (1990) have shown recently that there is rapid spread of resistant E. coli between children attending day-care centres in the USA. As many as 30% of the children were found to carry trimethoprim-resistant bacteria, compared with 6% in the general population. Entry into the day-care centre was often associated with acquisition of resistant faecal bacteria, which could persist for many months in the absence of any obvious selective pressure. Interestingly, within each day-care centre there were bacteria of different resistance phenotypes, which were harboured by many of the attendees. This suggests that the centre was the source of resistant bacteria in the community. These bacteria could then spread to the families of the children, and such day-care centres may thus be a more important reservoir of bacteria harbouring resistance genes than hospitals or farm animals (Reves et al., 1990).

It is likely that high usage of antibacterial drugs, perhaps coupled with daily ingestion of high numbers of faecal bacteria in contaminated water supplies, is responsible for the initiation and maintenance of the high levels of resistance in South India. Prescriptions are often not mandatory for antibiotic purchase in this area, and uncontrolled access to antibacterial drugs may be a contributory factor; however, the ability to obtain antibacterial drugs 'over the counter' is unlikely to be the only cause for the high incidence of resistance. In South Africa, where the incidence of resistance to trimethoprim in urinary bacteria has ranged up to 63.6%, antibiotics are obtainable only by prescription. In this area, the major factor may be the living conditions or compliance with therapy (Wylie & Koornhof, 1989).

The extensive reservoirs of resistance genes in commensal bacteria from developing countries may also serve to introduce resistance genes into the bacterial populations of Western countries. In a study of travellers to Mexico from the USA, of those who had no trimethoprim-resistant E. coli in their commensal faecal flora on entering the country, 57% possessed commensal bacteria harbouring these genes by their return (Murray et al., 1990). Thus, the potential threat from India is immense. Increased carriage of resistance genes into the Western population is particularly likely when the flow of people increases rapidly, as has been predicted in the aftermath of the Gulf War of 1991. Recent reports from the Gulf have shown that the incidence of resistance in clinical bacteria to tetracycline, ampicillin, trimethoprim or chloramphenicol may be 60% or above (Gasscr et al., 1991). The existence of such large reservoirs of resistance genes not only poses substantial problems for the areas themselves, but also threatens the successful use of antibiotics throughout the world. It has been shown that new resistance genes to trimethoprim and ampicillin which emerged in Asia have later been found in the West (Nandivada & Amyes, 1989; Amyes & Towner, 1990).

Acknowledgements

We thank the British Society for Antimicrobial Chemotherapy and the Royal Society for providing the funds to enable a research group to work in India. We are also very grateful to Dr S. Pancharatnam and Dr R. Abel for arranging for the collection of specimens from the urban and rural reception areas.
References


Resistance in commensal bacteria from India


(Received 10 September 1991; revised version accepted 18 November 1991)
RESISTANCE TO ANTIMICROBIAL DRUGS IN COMMENSAL BACTERIAL FLORA IN SOUTH INDIA


In 1984 a study was carried out at the Christian Medical College Hospital (CMCH) in Vellore, Tamil Nadu, South India to investigate resistance to antibiotics in pathogens causing urinary tract infections. This investigation reported high levels of resistance to ampicillin and trimethoprim. Resistance to ampicillin (Minimum Inhibitory Concentration (MIC) >10mg/L) was found in 77% of Escherichia coli isolated (Nandivada & Amyes 1987) and 64% were resistant to trimethoprim (MIC >10mg/L) (Young et al 1986).

In 1989, we returned to CMCH to establish if the reservoirs of the antibiotic resistance genes were actually located in the commensal faecal flora of the healthy population. Two cohorts of volunteers were recruited: an urban group (U) from the people of the town of Vellore (pop. 150,000) and a rural group (R) from villages situated about 40km from the town. Three villages were used; Kilvayattarankuppam - a roadside village with good access to the town, Kavanur - a Riverside village with moderate access and Melmoil - a village in the foothills with poor communications. In both study groups faecal specimens were taken from volunteers who were at least five years old, apparently healthy and had received no antibacterial chemotherapy in the previous week. Each specimen (92 from the urban study and 122 from the rural group) was initially examined for the presence of large Gram-negative rods (not Pseudomonas) which were resistant to trimethoprim, ampicillin, chloramphenicol or nalidixic acid. The faecal specimens were plated onto a Macokey-like agar containing each of these drugs (at 10mg/L). There was almost universal carriage of enterobacteria resistant to trimethoprim (U=98.3%, R=100%), ampicillin (U=98.9%; R=97.5%) and chloramphenicol (U=96.8%; R=97.5%). There was neither any significant difference between the proportions in the urban and rural study areas nor between proportions from the individual villages, despite the varying degree of remoteness. On the other hand, the carriage of nalidixic acid resistance was considerably lower. However, it was significantly more common in the urban population (29.7%) than in the rural (11.2%) ($X^2 = 7.84; 0.01 > p > 0.001$).

Further study on the trimethoprim resistant strains has revealed that 86% of them were highly resistant (MIC >1000mg/L), which is indicative of a plasmid origin of the gene. However, only a minority of these highly resistant strains were capable of transferring trimethoprim resistance. None of the nalidixic acid resistant strains tested were resistant to ciprofloxacin (1mg/L) despite the free availability of this drug in India.

These results provide evidence that the commensal gut flora of the human population, in this part of India, acts as a reservoir for antibiotic resistance genes which may then be acquired by pathogens. Such high carriage rates of resistant commensals may be stimulated by the free availability of antibiotics "over the counter" without prescription. Antibacterial drugs, bought in Vellore, were examined by bio-assay and absorbance spectrum analysis and found to be virtually identical to equivalent drugs obtainable in the UK and USA.

We are grateful to the Royal Society and the British Society for Antimicrobial Chemotherapy for funding this research visit.

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*Department of Medical Microbiology, University of Edinburgh, Department of Biological Sciences, University of Dundee, Scotland and *Department of Microbiology Christian Medical College & Hospital, Vellore, India.

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Antimicrobial Section I

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Comparison of the Sensitivity of the Oral Cephalosporin Cefdinir (CI-983, FK-482), with Related Beta-Lactams on Clinical Strains Isolated in Scotland

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The MIC of cefdinir (CI-983), augmentin, cephalaxin, cefuroxime, cefixime and ceftazidime were determined against clinical isolates. Cefdinir was as effective as cefixime against Haemophilus and Branhamella strains and both were more effective than cefuroxime. Against the Streptococci, cefdinir was much more effective than cefixime and had similar efficacy to cefuroxime and augmentin. Against the Staphylococci, cefdinir had the lowest MIC50 of all of the drugs tested. The effectiveness of these antibiotics were tested against E. coli K12 strains harbouring 16 of the new extended-spectrum plasmid-mediated beta-lactamases and cefdinir was more effective than ampicillin, cephalaxin, cefuroxime, ceftazidime and aztreonam.

Cefdinir is an aminothiazolyl hydroxyimino cephalosporin with a C3 vinyl group. This combination makes it suitable for oral administration. The in vitro activity of cefdinir has been examined in the United States where it was shown to be noticeably more effective than cefixime against oxacillin-sensitive staphylococci. Against other Gram-positive bacteria cefdinir appeared more effective than cefixime but it was less effective against Gram-negative bacteria. We chose to examine the MICs of beta-lactam antibiotics against a range of strains isolated in the United Kingdom including extended-spectrum beta-lactamases.

MATERIALS AND METHODS

To determine MICs, bacterial isolates (obtained from Scottish hospitals, 1990) taken from an overnight nutrient broth suspension, were washed and resuspended in Davis-Mingioli minimal medium. A 2μl inoculum of 10^5 to 10^7 CFU was delivered by a multipoint inoculator to an Oxoid Diagnostic Sensitivity Test Agar plate, appropriately supplemented. MICs, the lowest concentration of drug capable of inhibiting visible growth, were determined. All beta-lactamases were prepared then assayed for activity by measuring a decrease in spectrophotometric absorbency related to the decrease in substrate concentration.

RESULTS

The minimum inhibitory concentrations, of a range of beta-lactam antibiotics were determined at cell concentrations of 10^5 and 10^7, except for the strains conferring the extended-spectrum beta-lactamases which were studied just at the lower cell concentration. The results show the MIC range, MIC50, and MIC90 for the 111 strains tested at cell concentrations of 10^5, including the panel of extended-spectrum beta-lactamase producers. The results for augmentin (amoxicillin; clavulanate, 5:1), are expressed in terms of amoxicillin and for ceftazidime and clavulanate in terms of ceftazidime.

The results show that for Haemophilus and Branhamella isolates, cefdinir was no more effective than cefixime, though both were more effective than cefuroxime. It was
Amongst the Gram-positive species that cefdinir was most effective. Against the streptococci it was very much more effective than cefixime (by at least 8 times at the MIC<sub>90</sub> and MIC<sub>50</sub>) and had a similar efficacy to cefuroxime and augmentin. Cefdinir was also noticeably more effective than ceftazidine. The same trend was seen amongst the staphylococci where cefdinir was much more effective than cefixime. This is revealed by the considerable differential in the MIC<sub>90</sub> results (0.25 mg/l for cefdinir but 8 for cefixime). The MIC<sub>90</sub> results do not seem to support this; however, there were 3 strains of Staphylococcus with MICs of 64 mg/l which led to this MIC<sub>90</sub> figure. There were 7 strains which had an MIC of cefixime of the same value. The antibiotic cefdinir was also generally more effective than cefuroxime. Amongst the staphylococci, cefdinir had the lowest MIC<sub>50</sub> of all drugs tested.

The activity of cefdinir against the Gram-negative rods tested showed that it had greater activity than the penicillins or first-generation cephalosporins. In terms of its MIC<sub>50</sub>, cefdinir was similar to the second-generation cephalosporins, cefuroxime and cefixime. Except for ceftazidime, it was less effective than the parenteral third-generation drugs, i.e., cefotaxime, ceftriaxone, ceftizoxime and aztreonam.

It is not known what beta-lactamases were produced by these strains. However, the MIC results of augmentin compared with ampicillin and ceftazidime with clavulanate compared with ceftazidime alone suggest that what beta-lactamases were present were merely those capable of hydrolysing first-generation drugs.

**DISCUSSION**

Cefdinir has an antibacterial spectrum similar to a second-generation cephalosporin. It showed MICs to both Haemophilus species and Gram-negative rods which were intermediate in range but should be achievable in terms of serum levels. Cefdinir is effective against the Gram-positive species tested and seems to have good activity in comparison with other cephalosporins against beta-lactamase producing Gram-negative bacteria. In conclusion, the antibacterial activity of cefdinir was similar or better than the other drugs tested.

**TABLE**

<table>
<thead>
<tr>
<th>Organism (No.)</th>
<th>Antibiotic</th>
<th>MIC Range (mg/l)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
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THE SENSITIVITY OF CLINICAL BACTERIA ISOLATED IN SCOTLAND TO THE ORAL CEPHALOSPORIN, CEFDINIR

PAYNE D.J.,* AMYES S.G.B.†

† Department of Medical Microbiology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, Scotland, U.K.

Summary: The minimum inhibitory concentration (MIC) of cefdinir (CI-983, FK-482), cephalexin, cefuroxime, cefixime and ceftazidime were determined against clinical isolates. Cefdinir was as effective as cefixime against Haemophilus and Moraxella (Branhamella) strains and both were more effective than cefuroxime. Against streptococci, cefdinir was much more effective than cefixime and had similar efficacy to cefuroxime. Against staphylococci, cefdinir had the lowest MIC50 of all of the drugs tested. The efficacy of these antibiotics was tested against Escherichia coli K12 strains harbouring 16 of the new extended-spectrum plasmid-mediated beta-lactamases, and cefdinir was more effective than ampicillin, cephalexin, cefuroxime, ceftazidime and aztreonam.

Introduction

The cephalosporin cefdinir (CI-983, FK-482) is an aminothiazolyl hydroxyamino cephalosporin with a C3 vinyl group which makes it suitable for oral administration. Although there have been very few oral cephalosporins, they have often proved effective for certain infections. The continued efficacy of the first drugs, cephradine and cephalexin, suffered because they were susceptible to hydrolysis by many beta-lactamases. A similar drug, cefaclor, also suffered from this weakness but it had improved activity against Haemophilus spp. A result of their beta-lactamase susceptibility these cephalosporins have been classified as the so-called first generation.

At this point the development of oral cephalosporins slowed whereas the improvement of parenteral cephalosporins was far more rapid. The so-called second generation has much greater beta-lactamase stability and was represented by cephalosporins such as cefuroxime and the cephemycins. After many years of parenteral cefuroxime usage the oral version, cefuroxime axetil, was developed. Cefuroxime axetil has good beta-lactamase stability and is effective against Haemophilus spp. The parenteral cephalosporins were modified to provide a "third generation"; these drugs were stable to all plasmid-encoded beta-lactamases and to almost all chromosomally-encoded enzymes. The cephalosporins in this group also had increased anti-pseudomonas activity and were represented by ceftazidime, cefotaxime and ceftriaxone. The development of oral cephalosporins which would fall into this group has only recently been achieved. Indeed, in the United Kingdom, only one such drug, cefixime, is clinically available. Cefixime has good beta-lactamase stability with Gram-negative bacteria but lacks activity against Staphylococcus aureus.
Payne D.J., Amyes S.G.B.

The in vitro activity of cefdinir has been examined in the United States where it was shown to be noticeably more effective than cefixime against oxacillin-sensitive staphylococci. On the other hand, cefdinir was less effective than cefixime against oxacillin-resistant staphylococci. Against other Gram-positive bacteria, cefdinir appeared more effective than cefixime but it had generally been less effective against Gram-negative bacteria (1). In the same study, the beta-lactamase stability of cefdinir was found to be variable; it was resistant to the TEM-1 beta-lactamase but showed some hydrolysis with the common SHV-1 enzyme.

Neu et al. (1) examined the minimum inhibitory concentrations (MICs) of a series of beta-lactam antibiotics against strains isolated in the United States. The current study examines the MICs of beta-lactam antibiotics against a range of strains isolated in the United Kingdom. As this study examines the in vitro activity of an oral cephalosporin, which has extended activity against beta-lactamase-producing strains, a series of strains with extended-spectrum beta-lactamases were included in the study.

Materials and methods

Strains. The bacterial isolates used for the examination of clinical strains in this study were all obtained within Scotland during 1990. All the isolates, except those of Haemophilus, were obtained from the Royal Infirmary, Edinburgh. The Haemophilus spp. were kindly provided by Mr. M. Croughan at the City Hospital, Edinburgh. The strains used for the determination of activity against bacteria harbouring extended-spectrum beta-lactamases were all E. coli K12 transconjugants. In every case the original strain harbouring the plasmid, encoding the beta-lactamase, was conjugated with the standard laboratory strain (2). This was to ensure that the strains were all similar. However, so that the results should be as representative as possible of the clinical situation, the beta-lactamase genes were retained in their original plasmids and the genes were not cloned into small, multi-copy vector plasmids. Sixteen different extended-spectrum beta-lactamases were studied and these represented each of the different groups outlined by Payne and Amyes (3), where further information on the individual enzymes can be obtained.

Minimum inhibitory concentration determinations. The majority of isolates were cultured in nutrient broth overnight at 37°C. They were washed by centrifugation and resuspended in Davis-Mingioli minimum medium. For each genus, a standard curve was set up to correlate optical density at 550 nm with the cell count. From this curve the resuspended culture was diluted sufficiently, in Davis-Mingioli medium, to ensure that the 2 µl delivered by the multipoint inoculator head deposited 10^5 or 10^7 cfu onto the plate.

The MICs were performed on Oxoid Diagnostic Sensitivity Test Agar for all strains except staphylococci, streptococci and Haemophilus spp. The streptococci and staphylococci were tested on Oxoid Diagnostic Sensitivity Test Agar plates containing 5% whole horse blood and Haemophilus spp. were tested on Oxoid Diagnostic Sensitivity Test Agar plates containing 5% denatured whole horse blood. All plates were incubated at 37°C for 18 h. The plates were read and a positive value given for growth which was not obviously a result of mutation. The MIC was the lowest concentration of drug capable of inhibiting visible growth.

Results

Minimum inhibitory concentrations

The minimum inhibitory concentrations of a range of beta-lactam antibiotics were determined at cell concentrations of 10^5 and 10^7, except for the strains conferring the extended spectrum beta-
lactamases which were studied just at the lower cell concentration. The ranges for the MICs, the \( \text{MIC}_{50} \) and \( \text{MIC}_{90} \) values for the clinical strains at a cell concentration of 10^5 cfu are shown in Table I and at a cell concentration of 10^7 cfu are shown in Table II.

The results showed that for Haemophilus and Moraxella (Branhamella) isolates, cefdinir was not significantly more effective than cefixime, though both were more effective than cefuroxime. (Table I) It was amongst the Gram-positive species that cefdinir was most effective. Against the streptococci it was very much more effective than cefixime (by at least 8 fold at the \( \text{MIC}_{50} \) and \( \text{MIC}_{90} \)) and had an efficacy similar to cefuroxime. Cefdinir was also noticeably more effective than cefazidime. The same trend was seen amongst the staphylococci where cefdinir was much more effective than cefoxime. This was revealed by the considerable differential in the \( \text{MIC}_{50} \) results (0.25 mg/L for cefdinir but 8 for cefoxime). The \( \text{MIC}_{90} \) results do not seem to support this. However, detailed analysis of the results showed that there there were three strains of staphylococci with MICs of 64 mg/L which had led to this \( \text{MIC}_{50} \) figure. There were seven strains which had an MIC of cefoxime of the same value. The antibiotic cefdinir was also generally more effective than cefuroxime, although the most resistant strains had lower MICs of cefuroxime. Amongst the staphylococci, cefdinir had the lowest \( \text{MIC}_{50} \) of all drugs tested.

The activity of cefdinir against the Gram-negative rods tested showed that it had greater activity than the penicillins or first-generation cephalosporins. In addition cefdinir and cefixime had lower \( \text{MIC}_{50} \) and \( \text{MIC}_{90} \) values than cefuroxime (Table I). Except for cefazidime, cefdinir was generally less effective than the parenteral third-generation drugs, i.e. ceftotaxime, ceftriaxone, cefixime and aztreonam. Concurrent tests on these strains were performed with ampicillin plus clavulanic acid and cefazidime plus clavulanic acid. Comparison of these results (data not shown) with those of ampicillin and cefazidime alone suggest that these strains possessed beta-lactamases which were merely capable of hydrolysing first-generation drugs (such as TEM-1 or SHV-1).

The repeat of the MIC determinations at a cell concentration of 10^7 cfu did not demonstrate a substantial increase in the antagonistic effect of a beta-lactamase (Table II). There were some increases amongst the staphylococci and the Gram-negative rods, especially with ampicillin, but not with the later generation cephalosporins.

At the present time there is a vast array of new plasmid-mediated extended-spectrum beta-lactamases capable of hydrolysing third-generation parenteral cephalosporins. In order to examine the efficacy of cefdinir against the new extended spectrum beta-lactamases, strains possessing 16 of these enzymes were tested against a range of drugs. All the enzymes were studied in E. coli K12 hosts. The results (Table III) showed that for almost every strain harbouring an extended-spectrum beta-lactamase, there was a lower MIC of cefdinir than ampicillin, cephalexin, cephaloridine or cefuroxime. Only cefazidime, ceftizoxime and ceftriaxone were consistently as or more effective than cefdinir. In order to give a rough comparison, \( \text{MIC}_{50} \) and \( \text{MIC}_{90} \) values were determined (Table IV) and these showed that in general cefdinir is more effective than the other oral later-generation cephalosporins, cefuroxime and cefoxime.

**Discussion**

Cefdinir has an antibacterial spectrum similar to a later-generation cephalosporin. It showed MICs to both Haemophilus species and Gram-negative rods which were intermediate in range and compared well with cefixime and cefuroxime. The present results conflict with those of Wise et al. (4), who found that cefdinir was considerably less efficient than cefixime against Haemophilus, by a
Payne D.J., Amyes S.G.B.

Table I  Comparative in vitro activity of cefdinir against a range of bacterial pathogens at a bacterial concentration of $10^5$ cfu.

<table>
<thead>
<tr>
<th>Organism (number)</th>
<th>Antibiotic</th>
<th>MIC (mg/L)</th>
<th>Range</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haemophilus influenzae</strong> (22)</td>
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<td>0.13–8</td>
<td>0.5</td>
<td>0.5</td>
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</tr>
<tr>
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<td>0.25–16</td>
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</tr>
<tr>
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<td>0.03–2</td>
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<td><strong>Streptococcus species</strong> (21)</td>
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<tr>
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<td>16</td>
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A factor of 33 at the MIC$_{90}$. The authors found, with the strains isolated in Edinburgh, that although cefdinir was less active than cefixime it was so by only 2-fold. The levels required for both the MIC$_{50}$ and the MIC$_{90}$ should be obtainable in the serum. Cefdinir was rather more effective against the Gram-positive species tested, streptococci and staphylococci, including multiresistant variants of the latter group. This tends to oppose the results of Sabath et al. (5), who found that cefdinir was not effective against multiresistant Staphylococcus aureus; however, all the multiresistant strains in
Table II Comparative in vitro activity of cefdinir against a range of bacterial pathogens at a bacterial concentration of 10⁷ cfu.

<table>
<thead>
<tr>
<th>Organism (number)</th>
<th>Antibiotic</th>
<th>MIC (mg/L)</th>
<th>Range</th>
<th>50%</th>
<th>90%</th>
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<td><em>Moraxella spp.</em> (7)</td>
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<td>0.03–8</td>
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<tr>
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<td><em>Staphylococcus species</em> (28)</td>
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<tr>
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<td>0.25</td>
<td>64</td>
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<td><em>Gram-negative rods</em> (11)</td>
<td>Ampicillin</td>
<td>4–128</td>
<td>&gt;128</td>
<td>&gt;128</td>
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the Edinburgh area were coagulase-negative staphylococci and thus a direct comparison may not be valid. Cefdinir also had good activity in comparison with other cephalosporins against beta-lactamase producing Gram-negative bacteria. In conclusion, the antibacterial activity of cefdinir was similar or better than that of the other drugs tested. Using the extended spectrum beta-lactamase, the relative rates of hydrolysis of cefdinir have been examined and the results compared with the hydrolysis of comparable drugs. Recent
Table III Minimum inhibitory concentrations of beta-lactam antibiotics in E. coli, harbouring extended-spectrum beta-lactamases, at 10^5 cfu.

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<th>β-lactamase</th>
<th>Ampicillin</th>
<th>Cephaloridine</th>
<th>Cephalosporins</th>
<th>Ceftriaxone</th>
<th>Cefotaxime</th>
<th>Cefixime</th>
<th>Cefuroxime</th>
<th>Cefdinir</th>
<th>Cefaclor</th>
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<td>DJP-1</td>
<td>&gt;128</td>
<td>32</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>2</td>
<td>32</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>BIL-1</td>
<td>&gt;128</td>
<td>128</td>
<td>32</td>
<td>128</td>
<td>64</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>64</td>
</tr>
</tbody>
</table>

Table IV Summary of in vitro activity of cefdinir against a range of E. coli, harbouring extended-spectrum beta-lactamases, at 10^5 cfu.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antibiotic</th>
<th>Range</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extended spectrum beta-lactamase producers (16)</td>
<td>Ampicillin</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td></td>
<td>Cephaloridine</td>
<td>8–128</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Cephalosporins</td>
<td>16–128</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone</td>
<td>2–64</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>0.25–128</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Cefuroxime</td>
<td>0.5–64</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Cefixime</td>
<td>0.5–128</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Cefaclor</td>
<td>0.25–16</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Cefuroxime</td>
<td>0.06–16</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Aztreonam</td>
<td>0.25–128</td>
<td>4</td>
<td>64</td>
</tr>
</tbody>
</table>

Hydrolysis results (Payne & Amyes, submitted for publication) suggest that cefdinir is hydrolysed less rapidly than the other drugs tested and this is supported by the MIC values obtained with this drug against bacteria harbouring plasmid-mediated extended-spectrum beta-lactamases. In other
words, cefdinir does seem to be able to control these enzymes more effectively than many of the other cephalosporins tested.

Acknowledgement

The authors would like to thank Warner-Lambert for financial support.

References


Stability of cefdinir (CI-983, FK482) to extended-spectrum plasmid-mediated β-lactamases

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Department of Medical Microbiology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG

Summary. Fourteen plasmid-encoded extended-spectrum β-lactamases were purified from Escherichia coli transconjugants of original clinical isolates. The Vmax, Km and Vmax/Km were each determined for ampicillin, carbenicillin, cephaloridine, cephalaxin, cefuroxime, cefixime, cefdinir, ceftazidime and cefotaxime as substrates with eight of these enzymes and with the narrow-spectrum β-lactamase, TEM-1. The relative rates of hydrolysis of ampicillin, cephaloridine, cephalaxin, cefuroxime, cefixime, cefdinir, ceftazidime and cefotaxime were also determined for the remaining six enzymes. Cefdinir had Vmax/Km or relative rates of hydrolysis values either equal to or lower than ampicillin, cephaloridine, cephalaxin and cefotaxime for all the enzymes tested. Overall, cefdinir was more stable to the 15 β-lactamases tested than either cefuroxime or cefixime; however, ceftazidime was more stable than cefdinir to hydrolysis by eight of the enzymes tested.

Introduction

Very few oral cephalosporins have been manufactured. The early oral cephalosporins, cephadrine and cephalexin, have proved effective in certain infections; however, they are susceptible to hydrolysis by many β-lactamases. A similar drug, cefaclor, is also susceptible, but has improved activity against Haemophilus influenzae. As these cephalosporins are susceptible to hydrolysis by many β-lactamases, they fall within the so-called “first generation”. Cefdinir is an aminothiazolyl hydroxyiminocephalosporin with a C3 vinyl group and this combination makes it suitable for oral administration.

A limited study by Neu et al. examined the minimum inhibitory concentrations of a series of β-lactam antibiotics and demonstrated the ability of cefdinir to resist β-lactamase hydrolysis differentially. We investigated the β-lactamase stability of cefdinir and other cephalosporins to plasmid-encoded extended-spectrum β-lactamases. These enzymes are capable of hydrolysing the later-developed extended-spectrum cephalosporins such as ceftazidime and cefotaxime. The β-lactamases examined in the present study were TEM-1, TEM-3, TEM-4, TEM-5, TEM-7, TEM-10, TEM-E1, TEM-E2, TEM-E3, TEM-E4, SHV-2, SHV-3, SHV-5 and BIL-1. This array of enzymes includes at least one β-lactamase from each group of extended-spectrum β-lactamases.

Materials and methods

Bacterial strains

The β-lactamase preparations used in the stability assays were obtained from the strains of Escherichia coli listed in table I.

β-Lactamase preparation

The β-lactamase samples were prepared from 1-L cultures of bacteria grown overnight in nutrient broth at 37°C. The cells were harvested by centrifugation at 6000 g for 15 min at 4°C. The resultant pellet was washed in 25 mM sodium phosphate buffer (pH 7.0) and the centrifugation was repeated. The final pellet was resuspended in 1 ml of 25 mM sodium phosphate buffer (pH 7.0) and subjected to two 30-s treatments of ultrasonication (8 μm) at 0°C, with a 1-min cooling period between treatments. The resultant lysate was cleared by centrifugation at 32000 g for 30 min at 4°C. This crude β-lactamase preparation was applied to a Sephadex G-75 filtration column (2 cm2 × 90 cm) and eluted with 25 mM sodium phosphate buffer (pH 7.0) at 15 ml/h. The fractions with the greatest β-lactamase activity were pooled and used in the kinetic measurements.

Iso-electric focusing

The integrity of the β-lactamase preparations was checked by analytical iso-electric focusing. The enzymes were examined on polyacrylamide gels employing a 1:1 ratio of pH 3.5–10 ampholines and either pH 9–11 or pH 4–6 ampholines depending on
Characterisation of C. difficile strains by PCR


the expected pI of the purified β-lactamase. The purified β-lactamases were focused alongside known standard β-lactamase samples.

Assay of β-lactamase

The β-lactamase activities were measured by the decrease of absorbance of the β-lactam substrate in a Perkin-Elmer Lambda 2 spectrophotometer. In the normal assay procedure, a test and a control cuvette were set up. The test cuvette contained 0.3 ml of the substrate solution plus 2.6 ml of 50 mM sodium phosphate buffer, pH 7.0. The control cuvette contained 2.9 ml of the same buffer. The assay was started by the introduction of 0.1 ml of the enzyme solution to both cuvettes. The fall in absorbance was measured at a suitable wavelength for each substrate, usually at the λ max. The fall in absorbance was then related to the decrease in substrate concentration. The TEM-1 β-lactamase was used as a control.

Measurement of substrate profile

The substrate profiles of β-lactamases were determined by measuring the rates of hydrolysis of eight β-
lactams at a fixed concentration (0.1 mM). The rates were expressed relative to ampicillin.

Table I. Strains producing the β-lactamases used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid-encoded</th>
<th>β-lactamase</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>TEM-1</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>C600</td>
<td>TEM-3</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>TEM-4</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>TEM-5</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>TEM-6</td>
</tr>
<tr>
<td>E. coli</td>
<td>BM694</td>
<td>TEM-7</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>TEM-10</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>TEM-E1</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>TEM-E2</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>TEM-E3</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>TEM-E4</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>SHV-2</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>SHV-3</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>SHV-5</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>J53-2</td>
<td>BIL-1</td>
</tr>
</tbody>
</table>

Results

All the β-lactamases were purified and each purified enzyme had a pI value corresponding to that previously reported. There was no evidence of the presence of chromosomal β-lactamase. Vmax is a measure of the ability of an enzyme to hydrolyse a drug that has bound to its active site. Vmax values, relative to that obtained with ampicillin, are shown for nine β-lactam substrates with eight extended-spectrum β-lactamases (table II) and the narrow spectrum TEM-1 β-lactamase (control). The Vmax values for cefdinir were always lower than the Vmax values for ampicillin and cephaloridine. For most enzymes, the Vmax value for cefdinir was higher than that for carbenicillin, cephalexin, cefixime or ceftazidime in only one instance. These lower Vmax values for cefdinir suggest that cefdinir would be less likely than the other drugs tested to be hydrolysed by the β-lactamases shown in table II.

The rate of hydrolysis only measures the activity of the enzyme once the drug has bound. The Km value establishes the affinity of the substrate bound to the enzyme; the higher the value the less affinity there is for the substrate. There was no general pattern to the

Table II. Relative* Vmax values of extended spectrum β-lactamases for nine β-lactam antibiotics

<table>
<thead>
<tr>
<th>β-lactamase</th>
<th>Ampicillin</th>
<th>Carbenicillin</th>
<th>Cephaloridine</th>
<th>Cephalexin</th>
<th>Cefuroxime</th>
<th>Cefixime</th>
<th>Cefdinir</th>
<th>Ceftazidime</th>
<th>Cefotaxime</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1 (control)</td>
<td>100</td>
<td>11</td>
<td>23</td>
<td>0.72</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>0.06</td>
</tr>
<tr>
<td>TEM-3</td>
<td>100</td>
<td>51</td>
<td>131</td>
<td>284</td>
<td>21</td>
<td>91</td>
<td>5.45</td>
<td>536</td>
<td></td>
</tr>
<tr>
<td>TEM-5</td>
<td>100</td>
<td>42</td>
<td>20</td>
<td>124</td>
<td>96</td>
<td>135</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-10</td>
<td>100</td>
<td>33</td>
<td>30</td>
<td>12</td>
<td>15</td>
<td>30</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-E2</td>
<td>100</td>
<td>23</td>
<td>75</td>
<td>0.23</td>
<td>0.37</td>
<td>0.87</td>
<td>0.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-E3</td>
<td>100</td>
<td>51</td>
<td>61</td>
<td>95</td>
<td>28</td>
<td>44</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-E4</td>
<td>100</td>
<td>38</td>
<td>98</td>
<td>12</td>
<td>13</td>
<td>37</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHV-3</td>
<td>100</td>
<td>15</td>
<td>119</td>
<td>56</td>
<td>21</td>
<td>0.5</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>BIL-1</td>
<td>UM</td>
<td>UM</td>
<td>50</td>
<td>100</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td></td>
</tr>
</tbody>
</table>

UM, unmeasurable as hydrolysis was too low.
... not done.
*Expressed as a percentage of the value for ampicillin except with BIL-1, which is related to the nitrocefin value.
Table III. Km values of extended spectrum β-lactamases for nine β-lactam antibiotics

<table>
<thead>
<tr>
<th>β-lactamase</th>
<th>Ampicillin</th>
<th>Carbenicillin</th>
<th>Cephaloridine</th>
<th>Cephalexin</th>
<th>Cefuroxime</th>
<th>Cefixime</th>
<th>Cefdinir</th>
<th>Ceftazidime</th>
<th>Cefotaxime</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1 (control)</td>
<td>167</td>
<td>100</td>
<td>167</td>
<td>166</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>286</td>
</tr>
<tr>
<td>TEM-3</td>
<td>63</td>
<td>...</td>
<td>100</td>
<td>333</td>
<td>250</td>
<td>250</td>
<td>118</td>
<td>167</td>
<td>11</td>
</tr>
<tr>
<td>TEM-5</td>
<td>69</td>
<td>38</td>
<td>143</td>
<td>77</td>
<td>250</td>
<td>125</td>
<td>200</td>
<td>330</td>
<td>21</td>
</tr>
<tr>
<td>TEM-10</td>
<td>111</td>
<td>99</td>
<td>100</td>
<td>200</td>
<td>167</td>
<td>66</td>
<td>200</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>TEM-E2</td>
<td>119</td>
<td>83</td>
<td>500</td>
<td>333</td>
<td>UM</td>
<td>1000</td>
<td>77</td>
<td>500</td>
<td>181</td>
</tr>
<tr>
<td>TEM-E3</td>
<td>91</td>
<td>38</td>
<td>91</td>
<td>333</td>
<td>250</td>
<td>250</td>
<td>118</td>
<td>167</td>
<td>11</td>
</tr>
<tr>
<td>TEM-E4</td>
<td>29</td>
<td>72</td>
<td>80</td>
<td>200</td>
<td>200</td>
<td>17</td>
<td>500</td>
<td>1500</td>
<td>77</td>
</tr>
<tr>
<td>SHV-3</td>
<td>83</td>
<td>111</td>
<td>59</td>
<td>30</td>
<td>300</td>
<td>100</td>
<td>167</td>
<td>500</td>
<td>25</td>
</tr>
<tr>
<td>BIL-1</td>
<td>UM</td>
<td>UM</td>
<td>166</td>
<td>118</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
</tr>
</tbody>
</table>

UM, unmeasurable as hydrolysis was too low.

..., not done.

Table IV. Relative efficiencies (Vmax/Km) of hydrolysis of nine β-lactam antibiotics by extended spectrum β-lactamases

<table>
<thead>
<tr>
<th>β-lactamase</th>
<th>Ampicillin</th>
<th>Carbenicillin</th>
<th>Cephaloridine</th>
<th>Cephalexin</th>
<th>Cefuroxime</th>
<th>Cefixime</th>
<th>Cefdinir</th>
<th>Ceftazidime</th>
<th>Cefotaxime</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>100</td>
<td>18</td>
<td>23</td>
<td>0.87</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>0.04</td>
</tr>
<tr>
<td>TEM-3</td>
<td>100</td>
<td>...</td>
<td>285</td>
<td>38</td>
<td>48</td>
<td>89</td>
<td>27</td>
<td>74</td>
<td>269</td>
</tr>
<tr>
<td>TEM-5</td>
<td>100</td>
<td>93</td>
<td>198</td>
<td>37</td>
<td>28</td>
<td>77</td>
<td>37</td>
<td>28</td>
<td>85</td>
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<td>TEM-10</td>
<td>100</td>
<td>137</td>
<td>64</td>
<td>1.7</td>
<td>1.3</td>
<td>12</td>
<td>0.83</td>
<td>33</td>
<td>16</td>
</tr>
<tr>
<td>TEM-E2</td>
<td>100</td>
<td>32</td>
<td>20</td>
<td>1.1</td>
<td>UM</td>
<td>0.37</td>
<td>0.36</td>
<td>0.21</td>
<td>0.55</td>
</tr>
<tr>
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<td>100</td>
<td>122</td>
<td>61</td>
<td>5.7</td>
<td>1.0</td>
<td>31</td>
<td>5.7</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>TEM-B4</td>
<td>100</td>
<td>15</td>
<td>36</td>
<td>1.84</td>
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<td>0.65</td>
<td>0.25</td>
<td>0.12</td>
<td>0.79</td>
</tr>
<tr>
<td>SHV-3</td>
<td>100</td>
<td>11</td>
<td>168</td>
<td>31</td>
<td>34</td>
<td>1.76</td>
<td>1.8</td>
<td>0.17</td>
<td>38</td>
</tr>
<tr>
<td>BIL-1</td>
<td>UM</td>
<td>UM</td>
<td>60</td>
<td>54</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
<td>UM</td>
</tr>
</tbody>
</table>

*Value expressed as a percentage of the value for ampicillin except for BIL-1 which is related to cephalaxin.
UM, unmeasurable as hydrolysis was too low.

..., not done.

Table V. Relative rates of hydrolysis of eight β-lactam antibiotics by six extended-spectrum β-lactamases

<table>
<thead>
<tr>
<th>β-lactamase</th>
<th>Ampicillin</th>
<th>Cephaloridine</th>
<th>Cephalexin</th>
<th>Cefuroxime</th>
<th>Cefixime</th>
<th>Cefdinir</th>
<th>Ceftazidime</th>
<th>Cefotaxime</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-4</td>
<td>100</td>
<td>114</td>
<td>83</td>
<td>19</td>
<td>21</td>
<td>8.8</td>
<td>3.4</td>
<td>39</td>
</tr>
<tr>
<td>TEM-6</td>
<td>100</td>
<td>180</td>
<td>19</td>
<td>7.7</td>
<td>9.5</td>
<td>1.8</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>TEM-8</td>
<td>100</td>
<td>39</td>
<td>1.5</td>
<td>0.7</td>
<td>0.8</td>
<td>0.44</td>
<td>1.5</td>
<td>2.2</td>
</tr>
<tr>
<td>TEM-E1</td>
<td>100</td>
<td>123</td>
<td>26</td>
<td>5.4</td>
<td>12</td>
<td>5.3</td>
<td>2.6</td>
<td>58</td>
</tr>
<tr>
<td>SHV-2</td>
<td>100</td>
<td>55</td>
<td>11</td>
<td>5.4</td>
<td>1.8</td>
<td>0.63</td>
<td>UM</td>
<td>11</td>
</tr>
<tr>
<td>SHV-5</td>
<td>100</td>
<td>34</td>
<td>25</td>
<td>3.8</td>
<td>3.9</td>
<td>1.1</td>
<td>5.0</td>
<td>14</td>
</tr>
</tbody>
</table>

*Value expressed as a percentage of the value for ampicillin.
UM, unmeasurable as hydrolysis was too low.

results (table III). The Km values for cefdinir were higher than those of the other drugs tested for some enzymes but lower for other enzymes. Generally, the affinity of the enzymes for cefdinir was low.

The relative efficiency (Vmax/Km) takes both values into account. An enzyme may have a high efficiency because either the Vmax is high or the enzyme has a particular affinity for binding the drug (low Km). The relative efficiency results (table IV) show that all enzymes were less or equally efficient at hydrolysing cefdinir than they were with ampicillin, carbenicillin, cephaloridine, cephalaxin and cefotaxime. Cefdinir was also more stable than cefixime in the majority of cases. Ceftazidime and cefuroxime were more stable than cefdinir to at least four of the nine β-lactamases tested.

The relative rates of hydrolysis at fixed substrate concentrations (10^-4 M for cephalosporins and 10^-3 M for penicillins) were determined for a supplementary range of six extended-spectrum β-lactamases (table V). These values are not as accurate as the Vmax determinations in table II; however, they give an indication of the abilities of the enzymes to hydrolyse the substrates. Cefdinir was more stable than ampicillin, cephalo-
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Beta-Lactamase Stability of the Oral Cephalosporin Cefdinir (CI-983, FK-482) Compared with Related Beta-Lactam Antibiotics

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Purified enzyme extracts of seven extended-spectrum beta-lactamases were assayed in limiting concentrations of the substrates ampicillin, carbenicillin, cephaloridine, cephalaxin, cefuroxime, cefixime, cefdinir, ceftazidime and cefotaxime. The relative maximum rate of hydrolysis (Vmax) (related to ampicillin), the Km, and the relative efficiency of hydrolysis (Vmax/Km) (related to ampicillin) were determined. Cefdinir was always hydrolysed less rapidly (lower Vmax) than cefuroxime and cephaloridine and generally less rapidly than the other drugs tested. All the enzymes were less efficient (lower Vmax/Km) at hydrolysing cefdinir than with most other drugs. Cefdinir has comparable stability with other oral and later parenteral cephalosporins to the extended spectrum beta-lactamases.

A limited study by Neu et al (1989) examined the minimum inhibitory concentrations of a series of beta-lactam antibiotics and demonstrated cefdinir's differential ability to resist beta-lactamase hydrolysis. Our study extends Dr Neu's investigation by using several beta-lactamases representing enzyme types: TEM-1, TEM-3, TEM-5, TEM-10, TEM-E2, TEM-E3, TEM-E4, SHV-3, BIL-1,TEM-4, TEM-5, TEM-7, TEM-E1, SHV-2, SHV-5.

MATERIALS AND METHODS

Measurements of Vmax, Km and relative efficiency were made. The rate of substrate hydrolysis was initially measured at high substrate concentration. The rate was then repeatedly measured in the presence of lower substrate concentrations and correlated to that of ampicillin. A Lineweaver-Burk plot of the inverse of the substrate concentration and the relative rate of hydrolysis was created for each substrate for each enzyme tested. Extrapolation of the graph enabled the determination of the Vmax (the rate of hydrolysis at infinite substrate concentration), Km (the substrate concentration which gives a rate of hydrolysis exactly half that of the Vmax), and the relative rate of efficiency (Vmax/Km).

RESULTS

Vmax

The Vmax values for nine beta-lactamase substrates with eight different extended spectrum beta-lactamases were determined. Cefdinir almost always showed a lower relative rate of hydrolysis which suggests that it would be less likely to be hydrolysed than the other drugs tested.

Km

The rate of hydrolysis only measures the action of the enzyme once the drug has bound. The Km value establishes the affinity of the substrate to be bound to the enzyme. The higher the value the less affinity there is. Since there is no pattern to the Km results for cefdinir no conclusions are drawn.
Table shows the relative efficiency results, a consideration of both the Vmax and the Km. An enzyme may have a high efficiency because either the Vmax may be high or the enzyme may have a particular affinity for binding the drug (low Km). All of the enzymes were less efficient to hydrolysing cefdinir than they were with ampicillin, carbenicillin, cephaloridine and cephalaxin. However, the relative efficiency values for cefuroxime and cefixime were similar to cefdinir. The enzyme SHV-3 was able to hydrolyse cefixime less efficiently than cefdinir, but the differences were very small. Similarly, cefuroxime was hydrolysed less efficiently than cefdinir with a number of enzymes but again the differences were (except for SHV-3) generally small. Cefdinir was always less efficiently hydrolysed than ceftazidime and usually less efficiently hydrolysed than cefotaxime.

**DISCUSSION**

The trend of the relative rates of hydrolysis with the extended-spectrum beta-lactamases, which indicates that cefdinir is hydrolysed less rapidly than the other drugs tested, supports the MIC values (previously recorded) obtained with this drug. Cefdinir seems to be able to stand up to beta-lactamase more effectively than many of the other cephalosporins tested. However, this trend is not so clear cut when the relative efficiency values are examined. This may infer that, for these substrates with extended-spectrum beta-lactamases, the salient part of the equation is the speed at which the enzyme hydrolysed the substrate, rather than the speed at which it binds.

**REFERENCE**


### Table

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<tr>
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<th>Cefdinir</th>
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* Value expressed as percentage of the value for ampicillin except for BIL-1 which is related to nitrocefin
UM = unmeasurable as hydrolysis is too low

Relative rates of hydrolysis (ampicillin = 100%)

+1
The automated in-vitro assessment of β-lactamase inhibitors

Sir,

Combinations of a β-lactamase inhibitor with a β-lactam antibiotic drug have been shown to be highly effective both in vitro and in vivo against many β-lactamase-producing bacteria (Bush, 1988). The most widely used combination is currently that of amoxicillin and clavulanic acid, which is marketed as co-amoxiclav.

In any investigations of β-lactamase inhibitors it is important to assess the range of β-lactamases inhibited. The inhibitory activity of a compound against a particular β-lactamase is often expressed as an ID₉₀ value. This is the concentration of inhibitor (μM) required to inhibit the hydrolysis of a β-lactamase labile substrate by 50%. The ID₉₀ value is most easily determined by measuring the enzymic hydrolysis of a chromogenic substrate in the presence of increasing concentrations of inhibitor. We would like to report an automated microtitre assay for the rapid determination of ID₉₀ values using the chromogenic cephalosporin nitrocefin as substrate (O’Callaghan et al., 1973). Assays were performed in microtitre plates with a final reaction volume of 150 μL (Table). Appropriate amounts of sodium phosphate buffer (25 mM, pH 7.0), β-lactamase and inhibitor were added to the blank, test and control wells as shown in the Table, and (when necessary) pre-incubated at 37°C in a Biokat Microplate Biokinetick Reader (Luminar Technology Ltd, Southampton, UK). Following pre-incubation, the assay was initiated by the rapid addition of nitrocefin at a final concentration of 0.2 mM using the Denley Welltech Dispenser (Denley Instruments Ltd, Billingshurst, UK). The addition of nitrocefin to all 96 wells takes 4 seconds. The plate was then returned to the Biotek reader, where it was shaken for 1 second and maintained at 37°C. The reaction was followed by measuring the increase in the hydrolysis product of nitrocefin in each well at 490 nm against a blank consisting of sodium phosphate buffer and nitrocefin (Table). Absorbance readings were taken at frequent intervals over a suitable time period in order to measure the initial linear rate of hydrolysis of nitrocefin. The minimum time taken to read all 96 absorbances was 10 seconds, and this governed the minimum time between readings, although longer time intervals were often required depending on the activity of the β-lactamase preparation. The KinetiCalc software provided with the Biotek reader allowed plots of absorbance vs time to be simultaneously displayed for all 96 wells in real-time. On completion of the experiment, the KinetiCalc software also calculated the initial rate of hydrolysis at each inhibitor concentration, and the manipulation of these rates to percentage inhibition values relative to the control. This percentage data was transferred to a statistical package (RSI, BBN Software Products, Cambridge, Mass., USA) where it was displayed as plots of log inhibitor concentration against percentage inhibition for each β-lactamase inhibitor. After editing out any points not falling on the assay line, the line of best fit was found and used to calculate the ID₉₀ (μM).

ID₉₀ determinations can be automated by a method described by Reading & Farmer (1983) but this system records only the amount of nitrocefin hydrolysed after a fixed period of

Table. Composition of blank, control and test wells (μL)

<table>
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<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
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<td>Sodium phosphate buffer (25 mM pH 7.0)</td>
<td>100</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Nitrocefin (66 mM)</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Appropriately diluted β-lactamase</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Diluted inhibitor</td>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>150</td>
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</table>

References


Biotek reader, where it was shaken for 1 second and maintained at 37°C. The reaction was followed by measuring the increase in the hydrolysis product of nitrocefin in each well at 490 nm against a blank consisting of sodium phosphate buffer and nitrocefin (Table). Absorbance readings were taken at frequent intervals over a suitable time period in order to measure the initial linear rate of hydrolysis of nitrocefin. The minimum time taken to read all 96 absorbances was 10 seconds, and this governed the minimum time between readings, although longer time intervals were often required depending on the activity of the β-lactamase preparation. The KinetiCalc software provided with the Biotek reader allowed plots of absorbance vs time to be simultaneously displayed for all 96 wells in real-time. On completion of the experiment, the KinetiCalc software also calculated the initial rate of hydrolysis at each inhibitor concentration, and the manipulation of these rates to percentage inhibition values relative to the control. This percentage data was transferred to a statistical package (RSI, BBN Software Products, Cambridge, Mass., USA) where it was displayed as plots of log inhibitor concentration against percentage inhibition for each β-lactamase inhibitor. After editing out any points not falling on the assay line, the line of best fit was found and used to calculate the ID₉₀ (μM).

ID₉₀ determinations can be automated by a method described by Reading & Farmer (1983) but this system records only the amount of nitrocefin hydrolysed after a fixed period of
time and does not calculate the ID₉₀ from the initial rates of hydrolysis of nitrocefin. Direct spectrophotometric assays have been used to determine ID₉₀ from initial rates, but such methods are limited to a maximum of six simultaneous reactions since most spectrophotometers are limited to (at best) a six cuvette cell changer. Also, a modified version of the spectrophotometric method described by Holt, Simpson & Harper (1983) would use 20 times more inhibitor and nitrocefin than the microtitre method described in this report. To illustrate how sparingly reagents are used in the microtitre plate method, a single ID₉₀ value for one enzyme with clavulanic acid was determined from a series of ten concentrations (100-0.005 μM) used just 6.8 μg of clavulanic acid and 186 μg of nitrocefin. This procedure allowed ID₉₀ values to be determined rapidly and efficiently whilst using limited amounts of reagents.

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References

Enterobacter cloacae with clavulanic acid dependent variants

Sir,

Certain Enterobacteriaceae are characterized by their ability to express Type-1 chromosomally-encoded β-lactamases ("cephalosporinases"). Derepression (induction) of this class of enzymes can be activated by certain β-lactams and paradoxically by clavulanic acid, a β-lactamase inhibitor (Minami et al., 1980). In vitro induction by clavulanic acid may be observed during antibiotic disc susceptibility testing by, (i) antagonism of an adjacent β-lactam following diffusion, (ii) reduced zone of inhibition with amoxycillin/clavulanic acid compared with amoxycillin alone and, (iii) a zone of enhanced growth around the combination disc due to added protection against the β-lactam afforded by the clavulanate induced β-lactamase. We wish to report the isolation of an Enterobacter cloacae which on initial susceptibility testing appeared to be demonstrating induction as defined in (iii), but which on investigation, was found to result from a clavulanic acid-dependent (cad) subpopulation.

The organism was isolated from areas of oral ulceration in a 79-year-old lady hospitalized with a drug reaction to omeprazole accompanied by pneumonia and renal failure. Her treatment included six days of intravenous co-amoxycillav (1.2 g tid) followed by five days oral (375 mg tid) administration. Routine susceptibility testing demonstrated enhanced bacterial growth around the clavulanate/aminoglycoside (60/10 μg disc) but also suggested the possibility of a mixed culture. Purity subcultures from the central area of the sensitivity plate yielded predominantly a large colonial type but also an occasional smaller variant. Single colony subcultures were performed. The large colony grew well on all media but the small variant grew poorly on horse blood agar and on MacConkey agar (< five colonies recovered per plate from an inoculum of approximately 10⁵ cfu/mL). On sensitivity testing (on either Iso-Sensitest or DSTA agar, with or without added blood) this variant exhibited "satellitism" around the amoxycillin/clavulanic acid disc (Figure (a)) but not the other β-lactams tested or the two penicillanic acid sulphone β-lactamase inhibitors, tazobactam and sulbactam. Addition of clavulanic acid (10 mg/L) to sensitivity agar (Figure (b)) or to sterile paper discs also permitted growth. Following subcultures in the absence of clavulanic acid, subsequent populations became increasingly less dependent. Bacterial growth was stimulated by < 1.0 mg/L but inhibited at > 100 mg/L clavulanic acid, resulting in a halo effect around the disc. Both large (lab code, AUG 60) and clavulanic dependent (cad) variants had identical API 20E profiles for E. cloacae. β-Lactam susceptibility patterns (as assessed by zone diameter) of the two variants and Escherichia coli NCTC...
A Microtiter-based Assay for the Determination of ID_{50}s of β-Lactamase Inhibitors Employing Reporter Substrates Detected at UV or Visible Wavelengths

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There is also a small group of β-lactamases which have a metal ion at their active site (metallo-β-lactamases). These enzymes are not sensitive to serine β-lactamase inhibitors and have a much broader substrate profile than the serine enzymes. Metallo-β-lactamases (MBLs) are capable of hydrolyzing the majority of clinically important β-lactam antibiotics, including carbapenems.

To date, more than 190 different β-lactamases have been identified and we will continue to observe clinical isolates which produce new types of enzymes, such as MBLs. Therefore, to enable the clinical spectra of established β-lactamase inhibitor/β-lactam combinations to be defined, it is necessary to evaluate these inhibitors against all new β-lactamases. In addition, novel inhibitors with improved potency and spectra are required to combat the current plethora of enzymes (both metallo and serine active site β-lactamases) and such compounds require rapid and effective evaluation.

This application note describes a simple procedure to determine the inhibition (ID_{50} value) of β-lactamases by various agents using the chromogenic cephalosporin nitrocefin (λ_{max} = 482 nm) and the carbapenem antibiotic imipenem (λ_{max} = 299 nm) as reporter substrates. Imipenem was used for those carbapenemases which did not have significant hydrolytic activity against nitrocefin. The method described uses the UV capability of the SPECTRAmax 250 microplate spectrophotometer and can be readily modified to determine ID_{50} values of inhibitors of other enzymes requiring UV/Vis monitoring.

Materials and Methods

Materials
1. SPECTRAmax™250 microplate spectrophotometer
2. SOFTmax® PRO software
3. Quartz microplates
4. Deep well microplates
5. Multichannel pipette
6. DMSO
7. Imipenem and nitrocefin
8. β-lactamase preparation
9. Experimental compounds

Method for Determining ID$_{50}$ Values Following a 5 Minute Incubation of Enzyme and Inhibitor

Step 1: Set the incubator temperature to 37°C, then allow it to equilibrate for at least 30 minutes

Step 2: Set up SOFTmax PRO's Instrument Settings dialog box for imipenem as shown in Figure 1 and for nitrocefin as shown in Figure 2.

Step 3: Dissolve the inhibitors in 25 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPS) buffer, pH 7.0 with no added Zn$^{2+}$ to produce a 3 mM solution. Five percent DMSO can be used if needed to aid in dissolving the inhibitors.

Step 4: In a deep-well 96 well plate, pipette 600 μl of 2.5 mM PIPES buffer, pH 7.0 into each well in columns 1-11.

Step 5: Pipette 1 mL 3 mM inhibitor solution into each well in column 12.

Step 6: Using a multichannel pipette, make a 1:3 serial dilution of the 3 mM solution down to column 3: take 300 μl of 3 mM solution from column 12 and dispense into column 11. Mix, then take 300 μl of column 11 and dispense into column 10, and so on across the columns to column 3 which will then be 0.15 μM.

Step 7: Aliquot 50 μl of the contents of the deep wells into the corresponding wells on the assay microtiter plate to create a range of inhibitor concentrations between column 3 and column 12. If the reporter substrate requires UV monitoring (e.g. imipenem), UV transparent microtiter plates (e.g., plastic or quartz SPECTRAplates, Molecular Devices Cat. no: R9012 or R8024, respectively) must be used.

Table 1 shows the final concentration of inhibitor in each well, taking into account the 50 μl reporter substrate and 50 μl enzyme that will be added to each well. 100 μl and 50 μl of 2.5 mM PIPES buffer, pH 7.0 are added to the Blank and Control wells, respectively.

Step 8: Addition of enzyme: both pure and crude β-lactamase samples (prepared as described in reference 5) are compatible with this screening format. Some of the enzymes used are shown in Table 2. The enzyme preparations for the assay are diluted in PIPES buffer, pH 7.0, as required. 50 μl of the diluted enzyme is added to columns 2-12 of the assay microtiter plate. For MBLs, CfiA, BclI and L-1, 150 μM ZnSO$_4$ is added to the dilution buffer. For CphA, 1.5 μM ZnSO$_4$ is added. These levels of zinc are used to achieve optimal enzyme activity. No ZnSO$_4$ is added to the buffer used to dilute the serine active site enzymes.

Step 9: Using SOFTmax PRO, open the SPECTRAmax 250 drawer, place the plate onto the carriage and close the drawer. Incubate the assay microplate in the SPECTRAmax 250 at 37°C. After 5 minutes, open the drawer of the SPECTRAmax 250 and remove the plate from the carriage.

Step 10: Add 50 μl of reporter substrate to all 96 wells. 150 μM ZnSO$_4$ is added to the reporter substrate solutions used for assays with BclI, CfiA and L-1 metallo-β-lactamases. 1.5 μM is added to CphA and no ZnSO$_4$ is added to the reporter substrate solutions used to assay the serine β-lactamases. With the exception of the CphA β-lactamase,


![Table 1. Example of enzymes and assay conditions used. *Final concentration of reagent for substrate.* (µM) **Final concentration of Zn(II) in assay buffer. (µM)***](image)

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<th>[Substrate] (µM)</th>
<th>[Zn(II)] (µM)</th>
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**Results**

Figure 3 shows an assay microtiter plate set up to record the ID₅₀ of 8 compounds. The rate of hydrolysis of reporter substrate is shown for each well (column 1=blank, column 2=control, columns 3-12=range of inhibitor concentrations). This particular protocol has been set up to calculate the percent inhibition of the enzyme at each concentration of inhibitor compared to the control. The blank value for each compound is automatically subtracted within the software program.

**Table 2. Example of results produced.**

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<td>50</td>
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<td>Appropriately diluted b-lactamase</td>
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<td>Diluted inhibitor</td>
<td>--</td>
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<tr>
<td>Total volume</td>
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<td>150</td>
<td>150</td>
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**Figure 3.** Example of results obtained and expressed both graphically and tabularly. (plates are in OD360sec.)

**Figure 4.** Example of results obtained and expressed both graphically and tabularly.
Conclusion

Using SPECTRAmax 250 to determine ID_{50} has three major advantages:

1. **High throughput.** The protocol described enables 16 ID_{50} values to be determined for 5 different enzymes in 3-4 hours (80 ID_{50} determinations in total). SOFTmax PRO software rapid and accurate determination of ID_{50} without having to download data into other curve fitting software.

2. **Flexibility of reporter substrate.** β-lactamase assays performed on other plate readers are limited to using chromogenic substrates such as nitrocefin. However, as the SPECTRAmax 250 reads in the UV, other substrates can be utilized. This means that assays requiring UV monitoring were previously limited to standard spectrophotometers but can now be converted to microtiter plate formats for high throughput. For example, nitrocefin is an extremely poor substrate for the CphA enzyme and an inappropriate reporter substrate. The UV capability of the SPECTRAmax 250 means that imipenem can be utilized as the substrate, enabling this enzyme to now be evaluated in the microtiter plate format.

3. **Use of small quantities of reagents.** This system enables the β-lactamase ID_{50} assays to be performed on a microtiter plate system. If equivalent data was determined with a spectrophotometer using a method similar to that reported by Holt et al. in 3 times more reagent would be required.

This methodology could be utilized to measure the inhibition of a variety of other enzymes.

The introduction of the SPECTRAmax 250 spectrophotometric microplate reader together with its powerful data analysis software, SOFTmax PRO, has streamlined and greatly simplified the inhibitory screening of test compounds, allowing the rapid determination of ID_{50} values using limited amounts of reagents.

Acknowledgements

We thank Dr. David Livermore of the Department of Medical Microbiology, London Hospital Medical College, for the bacterial strain producing SME-1.

References


A microtiter-based assay for the determination of ID$_{50}$s of β-lactamase inhibitors employing reporter substrates detected at UV or visible wavelengths

INTRODUCTION

β-lactamases are either plasmid or chromosomally encoded bacterial enzymes which hydrolyze β-lactam antibiotics. The majority of β-lactamases produced by clinical isolates are serine active site enzymes. One successful approach to combating the serine active site β-lactamases is the use of β-lactamase inhibitors, such as clavulanic acid, in combination with a β-lactam antibiotic\(^4\).

There is also a small group of β-lactamases which have a metal ion at their active site (metallo-β-lactamases). These enzymes are not sensitive to serine β-lactamase inhibitors and have a much broader substrate profile than the serine enzymes. Metallo-β-lactamases (MBLs) are capable of hydrolyzing the majority of clinically important β-lactam antibiotics, including carbapenems.

To date, more than 190 different β-lactamases have been identified\(^1\) and we will continue to observe clinical isolates which produce new types of enzymes, such as MBLs. Therefore, to enable the clinical spectra of established β-lactamase inhibitor/β-lactam combinations to be defined, it is necessary to evaluate these inhibitors against all new β-lactamases. In addition, novel inhibitors with improved potency and spectra are required to combat the current plethora of enzymes (both metallo and serine active site β-lactamases) and such compounds require rapid and effective evaluation.

This application note describes a simple procedure to determine the inhibition (ID$_{50}$ value) of β-lactamases by various agents using the chromogenic cephalosporin nitrocefin ($\lambda_{\text{max}} = 482$ nm) and the carbapenem antibiotic imipenem ($\lambda_{\text{max}} = 299$ nm) as reporter substrates. Imipenem was used for those carbapenemases which did not have significant hydrolytic activity against nitrocefin. The method described uses the UV capability of the SPECTRAMax 250 microplate spectrophotometer and can be readily modified to determine ID$_{50}$ values of inhibitors of other enzymes requiring UV/Vis monitoring.
Materials

1. SPECTRAmax™ 250 microplate spectrophotometer
2. SOFTmax® PRO software
3. Quartz microplates
4. Deep well microplate
5. Multichannel pipette
6. DMSO
7. Imipenem and nitrocefin
8. β-lactamase preparation
9. Experimental compounds

Method for determining ID_{50} values following a 5 minute incubation of enzyme and inhibitor.

Step 1. Set the incubator temperature to 37°C., then allow it to equilibrate for at least 30 minutes.

Step 2. Set up SOFTmax PRO’s Instrument Settings dialog box for imipenem as shown in Figure 1 and for nitrocefin as shown in Figure 2.

Figure 1: Instrument setup for imipenem
Step 3 Dissolve the inhibitors in 25 mM piperazine-N,N'-bis(2-ethane-sulfonic acid) (PIPES) buffer, pH 7.0 with no added Zn$^{2+}$ to produce a 3 mM solution. 5% DMSO can be used if needed to aid in dissolving the inhibitors.

Step 4 In a deep-well 96 well plate, pipette 600 µl of 25 mM PIPES buffer, pH 7.0 into each well in columns 1-11.

Step 5 Pipette 1 mL 3 mM inhibitor solution into each well in column 12.

Step 6 Using a multichannel pipette, make a 1:3 serial dilution of the 3 mM solution down to column 3: take 300 µl of 3 mM solution from column 12 and dispense into column 11. Mix, then take 300 µl of column 11 and dispense into column 10, and so on across the columns to column 3 which will then be 0.15 µM.

Step 7 Aliquot 50 µl of the contents of the deep wells into the corresponding wells on the assay microtiter plate to create a range of inhibitor concentrations between column 3 and column 12. If the reporter substrate requires UV monitoring (e.g. imipenem), UV transparent microtiter plates (e.g., plastic or quartz SPECTRAplates, Molecular Devices Cat. no: R9012 or R8024, respectively) must be used.
Table I shows the final concentration of inhibitor in each well, taking into account the 50 µl reporter substrate and 50 µl enzyme that will be added to each well. 100 µl and 50 µl of 25 mM PIPES buffer, pH 7.0 are added to the Blank and Control wells, respectively.

<table>
<thead>
<tr>
<th>Row</th>
<th>Final concentration of inhibitor in test wells (µM, listed by column number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Blank</td>
</tr>
<tr>
<td>B</td>
<td>Blank</td>
</tr>
</tbody>
</table>

Table 1: Example of ID50 set up

**Step 8** Addition of enzyme: both pure and crude β-lactamase samples (prepared as described in reference 5) are compatible with this screening format. Some of the enzymes used are shown in Table 2. The enzyme preparations for the assay are diluted in PIPES buffer, pH 7.0, as required. 50 µl of the diluted enzyme is added to columns 2-12 of the assay microtiter plate. For MBLs, CfiA, BcII and L-1, 150 µM ZnSO4 is added to the dilution buffer. For CphA, 1.5 µM ZnSO4 is added. These levels of zinc are used to achieve optimal enzyme activity. No ZnSO4 is added to the buffer used to dilute the serine active site enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source#</th>
<th>Reporter Substrate</th>
<th>Substrate (µM)</th>
<th>Zn2+ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CfiA (metallo)</td>
<td>B.fragilis 262##</td>
<td>Nitrocefin</td>
<td>91</td>
<td>100</td>
</tr>
<tr>
<td>BcII (metallo)</td>
<td>B.cereus II 569H</td>
<td>Nitrocefin</td>
<td>171</td>
<td>100</td>
</tr>
<tr>
<td>L-1 (metallo)</td>
<td>X.malophilia 511</td>
<td>Nitrocefin</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>CphA (metallo)</td>
<td>A.hydrophila AE036</td>
<td>Imipenem</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>P99 (serine cephalosporinase)</td>
<td>Ent cloacae P99</td>
<td>Nitrocefin</td>
<td>171</td>
<td>None</td>
</tr>
<tr>
<td>SME-1 (serine carbapenemase)</td>
<td>S.marcescens S6</td>
<td>Nitrocefin</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 2: Examples of enzymes and assay conditions used. *Final concentration of substrate (µM). **Final concentration of Zn2+ in assay (µM). # References for strains cited in reference 1; ## in reference 3.

**Step 9** Using SOFTmax PRO, open the SPECTRAmax 250 drawer, place the plate onto the carriage and close the drawer. Incubate the assay microplate in the SPECTRAmax 250 at 37 °C. After 5 minutes, open the drawer of the SPECTRAmax 250 and remove the plate from the carriage.

**Step 10** Add 50 µl of reporter substrate to all 96 wells. 150 µM ZnSO4 is added to the reporter substrate solutions used for assays with BcII, CfiA and L-1 metallo-β-lactamases. 1.5 µM is added to CphA and no
ZnSO$_4$ is added to the reporter substrate solutions used to assay the serine $\beta$-lactamases. With the exception of the Gidita $\beta$-lactamase, all ID$_{50}$'s were measured using a concentration of reporter substrate which was approximately 5 times the $K_m$ of the reporter substrate for each of the enzymes (Table 2). This enables more accurate comparisons of ID$_{50}$s for the different enzymes. This is particularly relevant if competitive inhibitors are being evaluated. Table 3 summarizes the contents of each well in the assay microtiter plate.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM PIPES buffer (pH7)</td>
<td>100</td>
<td>50</td>
<td>--</td>
</tr>
<tr>
<td>Reporter substrate e.g. nitrocefin</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Appropriately diluted $\beta$-lactamase</td>
<td>--</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Diluted inhibitor</td>
<td>--</td>
<td>--</td>
<td>50</td>
</tr>
<tr>
<td>Total volume</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

Table 3: Composition of blank, control and test wells of assay microtiter plate

Step 11 Rapidly place the plate on the carriage of the SPECTRAmax 250 and click on the READ button.

Step 12 The axis of the well graphs can be changed while reading by clicking the reduction button in the plate window. Reading can be stopped at any point before the completion of the test by clicking on the STOP button in the control panel of the software. (This will save the data already collected.)

This protocol can also be used to measure ID$_{50}$s following different preincubation times of enzyme and inhibitor. In addition, where no preincubation is required, the enzyme solution should be added last.

Figure 3 shows an assay microtiter plate set up to record the ID$_{50}$s of 8 compounds. The rate of hydrolysis of reporter substrate is shown for each well (column 1 = blank, column 2 = control, columns 3 - 12 = range of inhibitor concentrations). This particular protocol has been set up to calculate the% inhibition of the enzyme at each concentration of inhibitor compared to the control. The blank value for each compound is automatically subtracted within the software program.

SOFTmax PRO will plot percent inhibition vs. concentration on a 4 Parameter logistic curve fit, and will then calculate the ID$_{50}$ for each compound, in this case taken as the concentration of inhibitor ($\mu$M) required to inhibit the enzyme by 50%.

Examples of the results produced are shown in Figure 3 and Figure 4.
Table 4: Example of results obtained and expressed both graphically and in tabulated form (data are in OD/second).

Figure 3: Example of results obtained and expressed both graphically and in tabulated form (data are in OD/second).

Figure 4: Plots of percent inhibition against inhibitor concentration for inhibitors A-H.
Table 4 shows the SOFTmax PRO printout of ID\(_{50}\)s for inhibitors A-H.

<table>
<thead>
<tr>
<th>Row</th>
<th>Inhibitor</th>
<th>ID(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>852.023</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>90.110</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>4.432</td>
</tr>
<tr>
<td>D</td>
<td>D</td>
<td>8.268</td>
</tr>
<tr>
<td>E</td>
<td>E</td>
<td>5.834</td>
</tr>
<tr>
<td>F</td>
<td>F</td>
<td>14.280</td>
</tr>
<tr>
<td>G</td>
<td>G</td>
<td>33.442</td>
</tr>
<tr>
<td>H</td>
<td>H</td>
<td>1.713</td>
</tr>
</tbody>
</table>

Table 4: ID\(_{50}\) calculated for inhibitors A-H

CONCLUSION

Using SPECTRAmax 250 to determine ID\(_{50}\) has three major advantages:

1. *High throughput.* The protocol described enables 16 ID\(_{50}\)s to be determined for 5 different enzymes in 3-4 hours (80 ID\(_{50}\)s in total). SOFTmax PRO software rapid and accurate determination of ID\(_{50}\)s without having to download data into other curve fitting software.

2. *Flexibility of reporter substrate.* β-lactamase assays performed on other plate readers are limited to using chromogenic substrates such as nitrocefin. However, as the SPECTRAmax 250 reads in the UV, other substrates can be utilized. This means that assays requiring UV monitoring were previously limited to standard spectrophotometers, but can now be converted to microtiter plate formats for high throughput. For example, nitrocefin is an extremely poor substrate for the CphA enzyme and an inappropriate reporter substrate. The UV capability of the SPECTRAmax 250 means that imipenem can be utilized as the substrate, enabling this enzyme to now be evaluated in the microtiter plate format.

3. *Use of small quantities of reagents.* This system enables the β-lactamase ID\(_{50}\) assays to be performed on a microtiter plate system. If equivalent data was determined with a spectrophotometer using a method similar to that reported by Holt *et al* (1983), 20 times more reagent would be required.

This methodology could be utilized to measure the inhibition of a variety of other enzymes.

The introduction of the SPECTRAmax 250 spectrophotometric microplate reader together with its powerful data analysis software, SOFTmax PRO, has streamlined and greatly simplified the inhibitory screening of test compounds, allowing the rapid determination of ID\(_{50}\) values using limited amounts of reagents.
REFERENCES


5 Payne, D. J., Cramp, R., Winstanley, D. J. and Knowles, D. J. C. Comparative activities of clavulanic acid, sublactam and tazobactam against clinically important β-lactamases. *Antimicrobial Agents and Chemotherapy* 38, 767-772 (1994).

ACKNOWLEDGMENTS

We thank Dr. David Livermore of the Department of Medical Microbiology, London Hospital Medical College, for the bacterial strain producing SME-1.

AUTHORS

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TRADEMARKS: SPECTRAmass, SPECTRplate, and MAXline are trademarks of Molecular Devices Corporation. SOFTmax is a registered trademark of Molecular Devices Corporation.
A four month study of the BacT/Alert was carried out to assess the advantages in continuously monitoring blood cultures (as opposed to batch processing). Batches were previously tested in this laboratory on the BacTec460 at 09:00hrs and 14:00hrs on the first day, and again at 09:00hrs the second, fourth and seventh day. The difference in time between unloading the BacT/Alert and the extrapolated time of the next batch run on the BacTec460 revealed the time saved by continuous monitoring. The time of detection and unloading was also noted. For each positive set a note was made of any antibiotic change or the removal of an intravenous line by the treating physician that could be attributed to either the Gram stain or antibiotic identification report.

Three hundred and nine positive sets were detected and confirmed by culture, representing 282 different patients or different days. An average of 11 hours was saved on Gram stain and 44% (137/309) had antibiotic sensitivities available a day in advance of batch processing. In 60% (172/282) of cases no change in antibiotic treatment or line removal was noted.

Continuous monitoring provides a much more rapid service to the clinicians, despite the problems in the laboratory management of such a system. The response of clinicians to earlier detection is difficult to assess but appears more limited than might be expected.

P17/1

Authors: R. Cramp, D. J. Payne, K. Coleman

Title: OVERCOMING ENZYMATIC RESISTANCE TO β-LACTAMS: RAPID SCREENING OF β-LACTAMASE INHIBITORS

β-lactamases, bacterial enzymes which hydrolyze β-lactam antibiotics, represent the most widespread resistance mechanism to this class of antibacterial agent. Combinations of certain β-lactam antibiotics with β-lactamase inhibitors have potent antibacterial activity against bacteria producing β-lactamases. The efficacy of a β-lactamase inhibitor can be assessed by determination of the concentration required to inhibit the hydrolytic activity of a β-lactamase by 50% (ID50). In this abstract, a rapid, automated microtitre plate method for the determination of ID50s is discussed. The procedure can be used to screen large numbers of compounds or natural products against a selection of enzymes to identify those with β-lactamase inhibitory activity. Alternatively, an array of different clinically derived β-lactamases can be screened against a known β-lactamase inhibitor in order to define its spectrum of activity.

A Hamilton MicroLab AT Plus automated dilution system prepares and dispenses a dilution series of each inhibitor into the rows of a microtitre plate. Appropriately diluted enzyme is then added and incubated (5 mins, 37°C) with a range of inhibitor concentrations and controls. The assay is initiated by the addition of nitrocefin, a chromogenic cephalosporin substrate, using a Dent's Weltech Dispenser. The rate of hydrolysis of substrates at each concentration of inhibitor is measured by a Bio-Tek EL312 Microplate Biokinetics plate reader and expressed as a percentage inhibition value relative to the control. A programme has been written to present these results as plots of percentage inhibition versus log of inhibitor concentration. The ID50, read from this graph, is then displayed in a tabular form. Previous methods used to screen β-lactamase inhibitors were more time consuming and required greater quantities of reagents. However, using this method at least 80 ID50s can be determined in a day. Only 200µl of inhibitor are required to generate an ID50 against a β-lactamase using a concentration range of 100µM - 0.001µM.

Further development of this method would involve the use of alternative substrates which absorb in the UV range, necessitating a plate reader capable of measuring absorbances at lower wavelengths.

P17/2

Authors: M. Zaremba, M. Rozkiewicz, K. Zaremba, A. Gajewski, P. Jakubicz, J. Borowski

Title: IN VITRO ANTIMICROBIAL SUSCEPTIBILITY OF METHICILLIN-RESISTANT STAPHYLOCOCCI

Department of Microbiology, Medical School, Białystok, Poland

The susceptibility of 111 methicillin-resistant Staphylococcus aureus (MRSA) strains and 19 methicillin-resistant coagulase-negative staphylococci (MRCCs) to 23 antimicrobial agents was determined by standard agar dilution method. All the strains were collected from hospital patients mainly from ICU, during 1988-91. All MRSA and MRCCs were resistant to gentamicin, tobramycin, tetracyclines, erythromycin and clindamycin. All the MRCCs with resistance to netilimicin, amikacin and isepamicin was found. MRCCs strains belong to following species: S. simulans, S. lentus, S. epidermidis, S. hominis, S. haemolyticus, S. warneri. Among all MRSA the percentages of resistant strains to antibiotics were as following: 90% tetracyclines, 73% gentamicin and tobramycin, 63% lincomycin and erythromycin, 52% clindamycin and 27% - amikacin. Among 111 MRSA there were found only 4 (3,6%) and 2 (1,8%) strains with resistance to netilimicin and isepamicin, respectively. The last antibiotics should be regarded as drugs of choice in the treatment of infections caused by methicillin-resistant staphylococci, together with some other as fluroquinolones, vancomycin and rifampicin.
Overcoming Enzymatic Resistance to β-lactams: Rapid Screening of β-lactamase Inhibitors

R. Cramp, D. J. Payne, K. Coleman
SmithKline Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, RH3 7AJ, UK.

Introduction

β-lactamases are bacterial proteins which hydrolyse β-lactam antibiotics. β-lactamase production by bacteria represents the most widespread resistance mechanism to this class of antimicrobial agent. However combination of a β-lactamase inhibitor with certain β-lactam antibiotics can overcome this method of resistance and the combinations can have potent antibacterial activity (1).

The efficacy of a β-lactamase inhibitor can be assessed by determination of the I50 of an inhibitor for a particular β-lactamase. The I50 value is defined as the concentration of inhibitor required to inhibit the activity of a β-lactamase by 50%, it is determined here by measuring the enzymatic hydrolysis of a chromogenic cephalosporin in the presence of increasing concentrations of inhibitor. We have developed a rapid automated microtitre plate method for the determination of I50 using the Biotek Mikroplate Biokinetics Reader (Luminar Technology Ltd., Southampton, UK) (Fig 1). The procedure can be used to screen large numbers of compounds or natural products against a variety of enzymes to determine their β-lactamase inhibitory profile.

In this method I50 is determined by measuring the difference between the rate of hydrolysis of nitrocefin in the presence of an increasing concentration of inhibitor (100µM - 0.005µM), to that of a control (2). A microtitre plate method is adopted as this allows the I50 of eight inhibitors against a single enzyme can be determined in a single plate. One blank and control well is included for each I50 determination and we have adopted for this example the practice of using one enzyme per microtitre plate.

Method

The composition of reagents in each well is shown in Table 1 and Fig 2.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIPES buffer (35mM pH7)</td>
<td>100µl</td>
<td>50µl</td>
<td>50µl</td>
</tr>
<tr>
<td>Nitrocefin (0.5µM)</td>
<td>50µl</td>
<td>50µl</td>
<td>50µl</td>
</tr>
<tr>
<td>Appropriately diluted β-lactamase</td>
<td>50µl</td>
<td>50µl</td>
<td>50µl</td>
</tr>
<tr>
<td>Inhibitor dilution</td>
<td>50µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>150µl</td>
<td>150µl</td>
<td>150µl</td>
</tr>
</tbody>
</table>

1.) A fixed concentration of inhibitor (100µM) is prepared and placed in the Hamilton Microlab AT Automated Dispenser System.
2.) This prepares a dilution series from 300µM to 0.005µM of each inhibitor and dispenses 50µl of each concentration into the rows of a microtitre plate.
3.) 100µl of 25mM PIPES buffer pH7.0 is placed in the wells of the blank (B Fig 2) and 50µl in the wells of the control (C Fig 2) in the Hamilton Microlab AT Automated Dispenser system.
4.) 50µl of appropriately diluted β-lactamase is then added to all wells of the microtitre plate except the blank, then incubated at 37°C for 5 minutes.
5.) Following pre-incubation of β-lactamase and inhibitor, the assay is initiated by the rapid addition of nitrocefin at a final concentration of 0.5µM using the Denley Welltech Dispenser (Denley Instruments Ltd., Billingham, UK). The addition of nitrocefin to all 96 wells takes approximately 4 seconds.
6.) The rate of hydrolysis of the substrate in each well was followed by measuring the increase in the absorbance of the hydrolysis product at 490nm (Fig 3), using the Biotek Mikroplate Biokinetics Plate Reader and expressed as a percentage inhibition value relative to the control (Fig 4). The minimum time taken to read all 96 absorbances is 10 seconds and up to 20 absorbances were recorded for each plate in order to determine initial rates.

Discussion

Other automated I50 assays have been described previously but these systems only record the amount of nitrocefin hydrolysed after a fixed period of time and do not calculate the I50 from the initial rates of hydrolysis of nitrocefin (2). Direct spectrophotometric assays have been used to determine I50 from initial rates, but such methods are limited to a maximum of six simultaneous reactions since most spectrophotometers are limited to (at best) a six cuvette cell changer. Also, a modified version of the spectrophotometric method described by Holt (4) would use 20 times more inhibitor and nitrocefin than the microtitre method described in this report. To illustrate how sparingly reagents are used in this method, a single I50 value for one enzyme with clavulanic acid would use just 0.8ug of clavulanic acid and 188ug of nitrocefin. This procedure allows I50 values to be determined rapidly and efficiently while using limited amounts of reagents. Using this method at least 90 I50s can be determined in a day.

Further development of this method would involve the use of alternative substrates which absorb in the U.V. range necessitating a platerader capable of measuring absorbances at lower wavelengths.

References

Comparative Activities of Clavulanic Acid, Sulbactam, and Tazobactam against Clinically Important β-Lactamases

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Received 16 April 1993/Returned for modification 14 July 1993/Accepted 26 January 1994

Clavulanic acid, sulbactam, and tazobactam are inhibitors of a variety of plasmid-mediated β-lactamases. However, inhibition data for these three inhibitors with a wide range of different plasmid-mediated β-lactamases have not yet been compared under the same experimental conditions. A number of groups have inferred that clavulanic acid inhibits extended-spectrum TEM and SHV β-lactamases, but inhibition data have rarely been published. In this study, the 50% inhibitory concentrations of these three β-lactamase inhibitors for 35 plasmid-mediated β-lactamases have been determined. Of these 35 β-lactamases, 20 were extended-spectrum TEM- or SHV-derived β-lactamases. The other 15 enzymes were conventional-spectrum β-lactamases such as TEM-1 and SHV-1. Clavulanic acid was a more potent inhibitor than sulbactam for 32 of the 35 plasmid-mediated β-lactamases tested. In particular, clavulanic acid was 60 and 580 times more potent than sulbactam against TEM-1 and SHV-1, respectively, currently the two most clinically prevalent gram-negative plasmid-mediated β-lactamases. Statistical analysis of the data of the 50% inhibitory concentrations showed that clavulanic acid was 20 times more active overall than sulbactam against the conventional-spectrum enzymes. In addition, clavulanic acid was 14 times more potent than sulbactam at inhibiting the extended-spectrum enzymes. Tazobactam also showed significantly greater activity than sulbactam against the two groups of β-lactamases. There were no significant differences between the overall activities of tazobactam and clavulanic acid against the extended-spectrum TEM and SHV enzymes and conventional-spectrum enzymes, although differences in their inhibition profiles were observed.

β-lactamases are plasmid-encoded or chromosomally encoded bacterial enzymes which hydrolyze β-lactam antibiotics. Plasmid-mediated β-lactamases can transfer rapidly between bacterial genera and consequently pose a major threat to the successful use of β-lactam agents. More than 60 different types of plasmid-encoded β-lactamases have been characterized, and for the purpose of this work, the enzymes have been classified as either conventional-spectrum or TEM- and SHV-derived extended-spectrum enzymes. The conventional-spectrum β-lactamases are from Bush groups 2a, 2b, 2c, and 2d, and extended-spectrum TEM and SHV enzymes are all from group 2b′ (4).

The conventional-spectrum enzymes include enzymes such as TEM-1 and SHV-1, which do not confer resistance to cephalosporins such as cefazidime and cefotaxime. A recent survey of 802 gram-negative clinical isolates showed that TEM-1 and SHV-1 were responsible for mediating β-lactam resistance in 17% of clinical isolates (37). Other conventional-spectrum enzymes which are often found in clinical isolates include the OXA and PSE β-lactamases (1). The most common conventional-spectrum plasmid-mediated β-lactamase found in gram-positive bacteria is the penicillinase produced by the majority of Staphylococcus aureus clinical isolates (26).

Most of the extended-spectrum plasmid-mediated β-lactamases are derived from the TEM and SHV β-lactamase genes and confer transferable resistance to the newer broad-spectrum cephalosporins such as cefotaxime and cefazidime. So far, at least 25 to 30 different extended-spectrum β-lactamases have been reported, and none of the enzymes appear to dominate the clinical situation worldwide. Therefore, a broad selection of extended-spectrum β-lactamases must be examined when assessing the activities of antimicrobial agents against such enzymes. The clinical prevalence of extended-spectrum β-lactamases in certain areas is increasing (35) and consequently compromises the effectiveness of the newer cephalosporins (17).

Combinations of β-lactam antibiotics and β-lactamase inhibitors have proved successful at treating infections caused by bacteria producing β-lactamases (22). The potency of such combinations continues to be largely assessed by susceptibility testing (2). Such studies have suggested that clavulanic acid and other β-lactamase inhibitors differentially inhibit various types of plasmid-mediated β-lactamases (15, 16, 19). However, the activity of a particular combination against clinical isolates is affected by the interplay of many different factors which are discussed in detail by Thomson et al. (36). One major component which affects the overall antibacterial activity of a β-lactam–β-lactamase inhibitor combination is the susceptibility of the β-lactamase to the inhibitor, and it is this component which has been addressed in our study. As there are now many different types of plasmid-mediated β-lactamase, any assessment of the potency of different β-lactamase inhibitors must be performed on a broad range of β-lactamases. Therefore, this paper reports the results of a comprehensive study which has examined the β-lactamase inhibitory activities of clavulanic acid, sulbactam, and tazobactam against 35 isolated plasmid-mediated β-lactamases. Twenty of the enzymes were extended-spectrum β-lactamases, and the remainder were conventional-spectrum plasmid-mediated β-lactamases.

MATERIALS AND METHODS

Antibiotics. Sulbactam was supplied by Pfizer Central Research, and tazobactam was obtained from Lederle Laboratories. Clavulanic acid was prepared in our laboratories.
Strains and plasmids. The *Escherichia coli* transconjugant strains used in this study are listed in Table 1. These strains were selected because they produced high levels of plasmid-mediated β-lactamase. Enzymes A and B were obtained from TEM-1 by in vitro spontaneous mutation; in the same way, enzymes C and D were derived from TEM-2 and enzyme E was derived from PSE-4 (27). TEM-E2, TEM-E1, CAZ-lo, and TEM-E4 have pls similar to those of enzymes A, B, C, and D, respectively (27). However, isoelectric focusing data in isolation are not sufficient to assess whether two TEM β-lactamases with similar pls are identical (17). The *S. aureus* Russell β-lactamase was representative of a typical staphylococcal penicillinase.

Preparation of β-lactamases. A 1-liter culture (nutrient broth no. 2) of each strain listed in Table 1 was shaken overnight at 37°C. The cells were harvested by centrifugation for 15 min at 6,000 × g. The bacterial pellets were washed in 25 mM sodium phosphate buffer (pH 7), and the centrifugation was repeated. One milliliter of 25 mM sodium phosphate buffer was then added to the final pellet, and the cells were resuspended to give 3 ml of cell suspension, which was disrupted by ultrasound. The cell lysate was cleared by centrifugation for 1 h at 32,000 × g.

### β-Lactamase identification
Each β-lactamase was checked by analytical isoelectric focusing (24). The enzymes were examined on gels containing a 1:1 ratio of pH 3.5 to pH 10 amphotolines and either pH 4 to pH 6 or pH 9 to pH 11 amphotolines, depending on the expected pl of the β-lactamase. The prepared enzymes were focused alongside known β-lactamase preparations. Each preparation from the *E. coli* transconjugants was shown to produce only one plasmid-mediated enzyme with a trace amount of the *E. coli* K-12 chromosomal enzyme. The other strains all produced the single plasmid-mediated enzyme.
TABLE 2. Geometric means and 95% confidence intervals for IC50 of clavulanic acid and tazobactam for OXA-1 and SHV-5

<table>
<thead>
<tr>
<th>β-lactamase and inhibitor</th>
<th>No. of determinations</th>
<th>IC50 (µM)</th>
<th>Range</th>
<th>Geometric mean</th>
<th>95% CI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-1</td>
<td>Clavulanic acid</td>
<td>8</td>
<td>1.19-2.11</td>
<td>1.58</td>
<td>1.34-1.84</td>
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<tr>
<td></td>
<td>Tazobactam</td>
<td>8</td>
<td>1.00-1.98</td>
<td>1.45</td>
<td>1.21-1.75</td>
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<tr>
<td>SHV-5</td>
<td>Clavulanic acid</td>
<td>9</td>
<td>0.01-0.02</td>
<td>0.013</td>
<td>0.009-0.016</td>
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<tr>
<td></td>
<td>Tazobactam</td>
<td>9</td>
<td>0.06-0.10</td>
<td>0.076</td>
<td>0.066-0.086</td>
</tr>
</tbody>
</table>

* 95% CI, 95% confidence interval.

Assay conditions for the determination of IC50. The activities of β-lactamase inhibitors were assessed by determining the concentration of inhibitor which inhibits by 50% the hydrolysis of nitrocefin by a particular β-lactamase (IC50).

The IC50s were determined by an automated microtiter assay system as described previously (28). The amount of enzyme was normalized to give approximately 70 µM nitrocefin hydrolyzed per min.

Inhibitors were assayed at eight different concentrations, ranging from 0.005 to 100 µM. The dilution of each inhibitor and its subsequent transfer into microtiter plates were performed automatically with a Hamilton Micro Lab AT Plus. The β-lactamase and inhibitor were preincubated for 5 min at 37°C, and the assay was initiated by the rapid addition of nitrocefin to create a final concentration of 0.2 mM.

Calculation of IC50. The KinetiCalc software provided with the Biotek reader allowed the plots of absorbance against time for all 96 wells to be simultaneously displayed in real time. The initial rates of hydrolysis at each inhibitor concentration were calculated, and the IC50 (µM) were determined by plotting percentage inhibition against inhibitor concentration (28).

Statistical analysis of IC50 data. The reproducibility of the methodology was examined by measuring the IC50s of clavulanic acid and tazobactam for SHV-5 and OXA-1 on at least eight separate occasions. Analyses were performed on the IC50 after log10 transformation, and geometric means with 95% confidence intervals were calculated for each inhibitor with each enzyme. The reproducibility was assessed by expressing the range of the confidence interval as a percentage of the mean IC50.

Statistical analysis of the IC50 after log10 transformation was performed by analysis of variance, taking into account the inhibitor, the enzyme, and the two groups of enzymes (conventional-spectrum and extended-spectrum TEM and SHV). Differences in the geometric means were back-transformed to ratios, i.e., relative potencies, of the inhibitors for the different groups of enzymes. The corresponding confidence limits were also determined.

RESULTS

Table 2 shows the statistical analysis of at least eight IC50 for four different enzyme-inhibitor interactions. This illustrates that the semiautomated technique described in this paper was acceptably reproducible, with 95% confidence intervals less than 40% of the geometric mean in three of the four enzyme-inhibitor interactions. The 95% confidence interval for the fourth combination (clavulanic acid with SHV-5) was within 60% of the geometric mean (Table 2).

Figure 1 shows the inhibition profiles from which the IC50 for S. aureus Russell β-lactamase were calculated. This illustrates that clavulanic acid was 93 times more active than sulbactam and 8 times more active than tazobactam against the S. aureus enzyme. The graphs for other IC50 are not shown,
and subsequent discussion of inhibition data refers directly to IC_{50} (these are tabulated in Table 3 for reference). Figures 2 and 3 summarize these data, and for simplicity, the enzymes were ranked according to their susceptibilities to clavulanic acid.

Figure 2 compares the IC_{50} of clavulanic acid with those of tazobactam and sulbactam for the conventional-spectrum plasmid-mediated ß-lactamases. Figure 2a shows that clavulanic acid was more potent than sulbactam against almost all of the enzymes in this group. OXA-2 was the only enzyme against which sulbactam demonstrated better activity than clavulanic acid. Statistical analysis of the IC_{50} demonstrates that, overall, clavulanic acid was 20 times more potent than sulbactam against these 15 enzymes (Table 4).

Figure 2b shows that tazobactam and clavulanic acid had similar activities against the 15 conventional-spectrum enzymes. Analysis of the IC_{50} failed to reveal a significant difference in overall potency between the two inhibitors and this group of enzymes (Table 4). The comparison of panels a and b of Fig. 2 clearly demonstrates that tazobactam was more potent than sulbactam. In fact, overall, tazobactam was significantly more potent than sulbactam against this group of enzymes (Table 4).

Clavulanic acid was also a more potent inhibitor than sulbactam for 19 of the 20 extended-spectrum plasmid-mediated ß-lactamases (Fig. 3a). TEM-3 was the only extended-spectrum enzyme against which the activity of sulbactam was comparable to that of clavulanic acid. Statistical analysis of the IC_{50} shows that, for the 20 enzymes studied, clavulanic acid was 14 times more potent overall than sulbactam (Table 4).

Figure 3b compares the ß-lactamase inhibitory activities of clavulanic acid and tazobactam against 20 TEM- and SHV-derived extended-spectrum ß-lactamases. Both inhibitors were highly active, with IC_{50} of less than 0.5 µM. In terms of overall potency, the difference between them was not statistically significant.

DISCUSSION
Detailed kinetic studies have been essential in elucidating the mode of action of mechanism-based ß-lactamase inhibitors (5, 21). However, because of the complexities of such interactions, these studies have been confined to a limited number of representative ß-lactamases. The proportion of clinical isolates producing plasmid-mediated ß-lactamases is high (37), and the diversity of these enzymes is also expanding as an inevitable consequence of microbial adaptation (27). Consequently, from a clinical perspective, it is also essential to investigate the activities of the commercially available ß-lactamase inhibitors against a broad variety of clinically derived ß-lactamases. Therefore, we have determined the IC_{50} of the three commercially available ß-lactamase inhibitors for 35 plasmid-mediated ß-lactamases. IC_{50} measure only the activity of a ß-lactamase inhibitor at a fixed time interval after incubation with an enzyme (5 min in this study). The values obtained are dependent on various kinetic parameters associated with the interactions of both inhibitor and substrate with ß-lactamase. Nevertheless, IC_{50} do provide the most practical way of evaluating the relative activities of ß-lactamase inhibitors for such a large number of enzymes. For these reasons, many other groups have adopted IC_{50} assays as a means of characterizing ß-lactamases (5, 19, 31).

**FIG. 2.** Comparison of the IC_{50} of ß-lactamase inhibitors against conventional-spectrum ß-lactamases. (a) IC_{50} of clavulanic acid (○) and sulbactam (○); (b) IC_{50} of clavulanic acid (●) and tazobactam (●).
Tazobactam-clavulanic strains (1). being was clavulanic acid was extended-spectrum clavulanic acid determined activity. The IC50 results show that clavulanic acid proved to be more potent than sulbactam for conventional-spectrum lactamases. Overall, clavulanic acid and tazobactam were equipotent against both sets of enzymes. However, each inhibitor had a distinct inhibition profile. The IC50s of clavulanic acid for S. aureus Russell, SHV-1, SHV-5, MJ-1, and TEM-5 were 8.1-, 4.7-, 5.9-, 4.8-, and 9.3-fold lower, respectively, than those of tazobactam. In contrast, tazobactam was more active than clavulanic acid against OXA-2 and OXA-5 (137- and 12.3-fold, respectively). Given the reproducibility of the assay and the location of these values at the extremes of the distribution of relative potencies, these differences may be considered significant.

In conclusion, we have reported the use of automated instrumentation to determine the activities of clavulanic acid, sulbactam, and tazobactam against a substantial number of previously reported plasmid-mediated β-lactamases. Overall, these IC50s for 35 β-lactamases showed that clavulanic acid and tazobactam were more effective β-lactamase inhibitors than sulbactam and that clavulanic acid had activities equivalent to those of tazobactam. Although the inhibitory activity of the β-lactamase inhibitor is only one of the many components which contribute to the overall MIC of a β-lactam–β-lactamase inhibitor combination, this ranking of the activities of β-lactamase inhibitors is useful for guiding therapeutic decisions.

**TABLE 4. Relative potencies for pairs of inhibitors with 95% confidence intervals**

<table>
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<tr>
<th>Inhibitor pair</th>
<th>Relative potency (95% confidence interval) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All β-lactamases (n = 35)</td>
</tr>
<tr>
<td>Clavulanic acid-sulbactam</td>
<td>16.6 (10.1–27.4)</td>
</tr>
<tr>
<td>Clavulanic acid-tazobactam</td>
<td>0.95 (0.58–1.56)</td>
</tr>
<tr>
<td>Tazobactam-sulbactam</td>
<td>17.54 (10.7–28.9)</td>
</tr>
</tbody>
</table>
PAYNE ET AL.

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MIC determinamase inhibitors has also been reported
resistant
clinical
isolates (2).
ampicillin184
on
conducted
tions
This demonstrates that clavulanic acid and tazobactam are
with

potent inhibitors not only of the conventional- spectrum ß -lactamases but also of the newer enzymes.

Extended broad -spectrum ß- lactamas 4 conferring transferable
10:867878.

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ACKNOWLEDGMENTS
We thank Brian Bond for statistical evaluation of the data and Chris
Blackmore for photographic work.

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distribution des 3- lactamases chez 1,792 souches de Klebsiella
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10B.5
MULTI-DOSE PHARMACOKINETICS OF PEFLOXACIN IN ELDERLY PATIENTS

Ludvik E., Székely E., Csaba A.
Department of Medicine and Clinical Pharmacology, Pateny Teaching Hospital, Budapest, Hungary

Despite the widespread use of pefloxacin, there are still some controversies concerning its pharmacokinetics in special clinical settings.

We investigated the kinetics of pefloxacin in elderly patients stratified in different age groups after multiple dosing (Gr 1, n = 18 patients below 50 yrs.; Gr 2, n = 18 between 50-70 yrs.; Gr 3, n = 10 between 70-80 yrs.; Gr 4, n = 10 over 80 yrs.). Pefloxacin was administered orally or intravenously in a dose of 400 mg bid for at least 7 days for the treatment of infections. Blood samples were taken at 0, 1, 2, 4, 8, 12 hours after the first and 13th dose. Pefloxacin concentration was determined by an HPLC method.

1. pefloxacin kinetics is characterized by huge interindividual differences that become even more pronounced after multiple dosing; 2. a significant cumulation of pefloxacin can be observed during multiple dosing in each age groups (predicted cumulation ratio 1.9-2.3, measured ratio 3-6.9 mg/L); 3. while the serum peaks of different age groups were close to each other after the first dose (i.e., 5.2, 6.38, 7.2, 6.36 mg/L), the differences significantly increased between young and elderly groups on the 7th day (13.6, 16.7, 18.02, 19.4 mg/L). 4. apparent volume of distribution of pefloxacin is significantly smaller in elderly patients compared to those of the young ones; 5. the increased serum levels of pefloxacin in elderly patients are due to decreased drug clearance; 6. there are no statistically significant differences among the elderly groups, though the highest concentrations were found in group 3 and 4.; 7. routine dose reduction is not recommended in severe infections because of the huge interindividual differences in serum levels. However, in patients over 70 years with moderate infections or with UTI, reduced doses can also assure therapeutic serum concentrations.

10B.7
DETERMINATION OF ID₅₀ VALUES FOR 35 PLASMID MEDIATED β-LACTAMASES.

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Clavulanic acid, sulbactam and tazobactam have varying inhibitory activity against a wide variety of plasmid mediated β-lactamases. However, the ID₅₀ of these three inhibitors for a wide range of different plasmid mediated β-lactamases have not yet been compared under the same experimental conditions. In particular, a number of workers have inferred that clavulanic acid inhibits extended spectrum TEM and SHV β-lactamases, but ID₅₀ have rarely been published. In this study we have determined the ID₅₀ values of these three β-lactamase inhibitors for 35 plasmid mediated β-lactamases. Twenty of these 35 β-lactamases were extended spectrum TEM or SHV β-lactamases. The ID₅₀ values were determined by the automated method described previously [1] and the ID₅₀ value was defined as the concentration of inhibitor required to inhibit the hydrolysis of nitrocefin by 50%. The integrity of each β-lactamase preparation used in the ID₅₀ assay was checked by isoelectric focusing and substrate profile studies.

Clavulanic acid was a more potent inhibitor than sulbactam for 34 out of the 35 plasmid mediated β-lactamases tested. In addition, clavulanic acid had lower ID₅₀ values than tazobactam for more than half of the β-lactamases. Examination of the activity of the inhibitor compounds against the 35 extended spectrum TEM and SHV β-lactamases revealed that on average clavulanic acid had approximately 20 times greater activity than sulbactam against those 35 extended spectrum enzymes tested. Clavulanic acid also had greater inhibitory activity than tazobactam against 19 of the 20 extended spectrum enzymes tested. Clavulanic acid had at least 50 times greater potency than sulbactam against TEM-1 and SHV-1 which are currently the two most clinically relevant plasmid mediated β-lactamases.

In conclusion, it has been shown that clavulanic acid was superior to sulbactam and tazobactam at inhibiting the majority of the plasmid mediated β-lactamases tested. In addition, on the basis of these ID₅₀ values, clavulanic acid is the most effective β-lactamase inhibitor at combating the increasing number of clinical isolates that harbour extended spectrum TEM/SHV β-lactamases.
Penetration of β-lactamase inhibitors into the periplasm of Gram-negative bacteria

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b SmithKline Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, RH3 7AJ, UK

Received 15 January 1999; accepted 23 April 1999

Abstract

The effectiveness of a β-lactamase inhibitor/β-lactam combination against Gram-negative pathogens depends on many interplaying factors, one of which is the penetration of the inhibitor across the outer membrane. In this work we have measured the relative penetrations of clavulanic acid, sulbactam, tazobactam and BRL 42715 into two strains of Escherichia coli producing TEM-1 β-lactamase, two strains of Klebsiella pneumoniae producing either TEM-1 or K-1, and two strains of Enterobacter cloacae each producing a Class C β-lactamase. It was shown that clavulanic acid penetrated the outer membranes of all these strains more readily than the other β-lactamase inhibitors. For the strains of E. coli and K. pneumoniae clavulanic acid penetrated approximately 6 to 19 times more effectively than tazobactam, 2 to 9 times more effectively than sulbactam and 4 to 25 times more effectively than BRL 42715. The superior penetration of clavulanic acid observed in this study is likely to contribute to the efficacy of clavulanic acid/β-lactam combinations in combating β-lactam resistant bacterial pathogens. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: β-Lactamase inhibitor; Permeability; Clavulanic acid

1. Introduction

For a β-lactamase inhibitor to be effective therapeutically it not only must have good intrinsic inhibitory activity, but, in the case of Gram-negative bacteria, must be able to reach its target in the periplasm. When evaluating an inhibitor, therefore, it would be useful to be able to assess its ability to cross the outer membrane. Zimmerman and Rosselet (1977) [1] were among the first to quantify the relative contributions of outer membrane permeability and hydrolysis by β-lactamase to the steady state periplasmic concentrations of β-lactam antibiotics. More rigorous analyses have since been developed with regard to the interplay between these two factors [2-4]. These studies have, however, focused primarily on β-lactamase labile compounds, and have
Cephaloridine was selected because transiently affected was by 50%.

2.2. The methods used were based on those previously described [5]. Cells were grown to mid-log phase, harvested, then re-suspended in 0.067 M NaH₂PO₄/Na₂HPO₄ pH 7.3 buffer, supplemented with 0.01 M MgCl₂. In this buffer, the permeability of the cells was unaltered over the duration of the experiment (1-2 h). The extent of leakage of β-lactamase into the medium was evaluated by measuring the rate of β-lactam hydrolysis with culture supernatant obtained after centrifugation of the intact cells.

2.3. Calculation of I₅₀ values

The concentration of inhibitor necessary to give 50% inhibition (the I₅₀ value) of the hydrolysis of 2400 uM cephaloridine (at 37°C in the above buffer) by whole cell β-lactamase was determined (1200 uM was used for E. cloacae as higher concentrations transiently affected outer membrane permeability). Cephaloridine was selected because all the strains were relatively permeable to it; a marked permeability barrier to the assay substrate can lead to errors when comparing sonicate and whole cell I₅₀ (see Section 2.3). A high concentration was chosen to ensure that the initial rates were truly linear; it is difficult to calculate inhibition accurately when control rates are non-linear [9]. Moreover, with the types of inhibitor used here any slowing of substrate hydrolysis because of enzyme inactivation readily becomes apparent. Inhibitor and substrate were mixed first, then the whole cells added. Substrate hydrolysis was determined from a fall in absorbance at 299 nm. Only initial rates were used. The cells were sonicated to release β-lactamase and the assay repeated [5].

To enable a true comparison of the I₅₀ from sonicated and whole cells the substrate concentration for determining the I₅₀ for the sonicated preparations was adjusted to give the same concentration as that calculated to be in the periplasm of the whole cells when exposed to an external concentration of 2400 uM cephaloridine. This was determined by using the following re-arrangement of the Michaelis–Menten equation.

![Molecular structures of β-lactamase inhibitors.](image-url)
where: $S_p$, periplasmic concentration of cephaloridine; $K_m$, Michaelis constant for cephaloridine; $v_{\text{max}}$, hydrolysis rate by whole cells; $V_{\text{max}}$, maximum hydrolysis rate for cephaloridine (this was determined from the hydrolysis rates obtained with the sonicated cells).

2.4. Determination of permeability index

The ratio of whole cell $I_{50}$ to cell free $I_{50}$ values was used to calculate the permeability index. This parameter was used as an indication of the relative abilities of inhibitors to penetrate the outer membrane. The lower the index the more effective the inhibitor is at penetrating the outer membrane.

### Table 1

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>(Relative $\beta$-lactamase activity)</th>
<th>$\beta$-Lactamase (Molecular class)</th>
<th>Inhibitor</th>
<th>$I_{50}$ (uM)</th>
<th>Permeability index</th>
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<tbody>
<tr>
<td><strong>E. coli NCTC 11560</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>TEM-1 (Class A)</td>
<td>Clavulanic acid</td>
<td>0.8</td>
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<td>BRL 42715</td>
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<tr>
<td><strong>E. coli JT4</strong></td>
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<td></td>
<td></td>
<td>Clavulanic acid</td>
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<td>Sulbactam</td>
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<td>Tazobactam</td>
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<tr>
<td><strong>K. pneumoniae Bat95R+</strong></td>
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<td>Sulbactam</td>
<td>1961 ($I_{50}$)</td>
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</tr>
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<td></td>
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<td></td>
<td>Tazobactam</td>
<td>131</td>
<td>0.732</td>
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<td>BRL 42715</td>
<td>51.8</td>
<td>0.151</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Clavulanic acid</td>
<td>1429 ($I_{50}$)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sulbactam</td>
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<td></td>
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<td>Tazobactam</td>
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<td>2.80</td>
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<tr>
<td><strong>K. pneumoniae 1082E</strong></td>
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<td>784</td>
<td>324</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tazobactam</td>
<td>187</td>
<td>74.8</td>
</tr>
<tr>
<td><strong>E. cloacae P99</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(81.1)</td>
<td>P99 (Class C)</td>
<td>Clavulanic acid</td>
<td>3.78</td>
<td>0.0395</td>
</tr>
<tr>
<td><strong>E. cloacae 588</strong></td>
<td>(84.2)</td>
<td>Class C</td>
<td>Clavulanic acid</td>
<td>3.78</td>
<td>0.0395</td>
</tr>
</tbody>
</table>

*Calculated periplasmic concentration: 3.7 uM TEM-1 $\beta$-lactamase.

*bCalculated periplasmic concentration: 300 uM Class C $\beta$-lactamase. (Periplasmic enzyme concentrations were calculated using turnover numbers ($K_{\text{cat}}$) of purified $\beta$-lactamases and from estimates of periplasmic volumes [3,12]).

3. Results

This procedure was used to compare the permeability of four $\beta$-lactamase inhibitors for six different strains. The permeability index values for each inhibitor for each strain are shown in Table 1. Over the time course of the experiments no significant leakage of $\beta$-lactamase from any of the strains was observed.

3.1. Inhibitory activity of $\beta$-lactamase inhibitors

The results from the assays using cell free enzymes confirm earlier findings that BRL 42715 is a more potent inhibitor of these $\beta$-lactamases than the other inhibitors [10]. All the compounds were effective inhibitors of cell free TEM-1 $\beta$-lactamase. Clavulanic acid and tazobactam, but not sulbactam, were good inhibitors of K-1 $\beta$-lactamase (Table 1).
3.2 Permeability indices

The permeability indices clearly showed that clavulanic acid penetrated the outer membranes of all the bacteria more readily than the other β-lactamase inhibitors. For the strains of E. coli and K. pneumoniae clavulanic acid penetrated approximately 6 to 19 times more effectively than tazobactam and 2 to 9 times more effectively than sulbactam. The permeability indices for BRL 42715 were higher than the other inhibitors against the E. cloacae strains, but because of its superior intrinsic inhibitory activity, whole cell I50 values for the penem were much lower than those of tazobactam, sulbactam, and clavulanic acid. None of these last three compounds was an effective inhibitor of cell free E. cloacae ß-lactamase under these assay conditions (Table 1). The permeability indices of these inhibitors for the E. cloacae ß-lactamases should be viewed with caution as very high concentrations were needed to obtain inhibition of Class C enzymes.

4. Discussion

The efficacy of a β-lactamase inhibitor/β-lactam combination depends on many parameters such as the intrinsic activities of both components against their respective target enzymes, turnover by β-lactamase and their penetration rates across the outer membrane. For a β-lactamase inhibitor it is the interplay of all these factors which determines how effectively it neutralizes periplasmic β-lactamase. However, efficient penetration of the inhibitor through the outer membrane is essential to fully realize its inhibitory potency and thus maximize the antibacterial activity of the partner antibiotic.

In this study we have set out to evaluate the contribution of outer membrane permeability on the abilities of β-lactamase inhibitors to inhibit β-lactamases within the periplasm. However, there are a number of factors which may affect the permeability index and these are worthy of discussion before in depth analysis of the results and comparison of the inhibitors. Firstly, if the β-lactamase in the periplasm were to be rapidly inactivated, a falsely low permeability index might be obtained. Therefore, to reduce the influence of this effect, the degree of enzyme inhibition was determined from the initial competitive phases of the hydrolysis reactions.

Secondly, some inhibitors suffer some turnover by the β-lactamases they inhibit [11]; high levels of β-lactamase could, therefore, potentially destroy a significant proportion of the total inhibitor. However, as the total enzyme activities in the sonicate and whole cell assays were the same, this effect would be compensated for. Generally, in the steady state situation, the inhibition given by an inhibitor (even if a small amount of turnover occurs) will depend on the kinetic parameters of inhibitor and substrate, not the enzyme activity in the assay. With regard to the in vivo situation it is important to appreciate that periplasmic β-lactamase may decrease the potency of an inhibitor if there is a high level of turnover and contact between enzyme with the inhibitor prolonged [11].

Lastly, the degree of inhibition given by a competitive or progressive inhibitor should be independent of enzyme concentration, providing the former is in excess. Intracellular β-lactamase concentrations can be very high, however, reaching 1 mM in some cases [3]. Nevertheless, it is apparent from the results that whole cell I50 values were often actually lower than the calculated periplasmic β-lactamase concentrations (Table 1). This apparent paradox is explained by the large external pool of inhibitor, which replaces inhibitor bound by periplasmic enzyme (see [2] for discussion of this point with regard to β-lactamase substrates). Differences in the I50 values between whole cell and cell free β-lactamases should, therefore, reflect only the influence of the outer membrane.

The results indicate that clavulanic acid penetrates into the periplasm of E. coli and K. pneumoniae more readily than the other inhibitors (Table 1). This is perhaps surprising as all the inhibitors are monobasic acids of small molecular mass (≤300 Da) and thus would be expected to pass readily through the porins in the outer membranes of Gram-negative bacteria [12]. Possibly, as clavulanic acid is very hydrophilic it moves through these water filled channels with greater ease. Provided that the inhibitors enter the periplasm by passive diffusion, the exact pathway taken should not affect the calculation of results and other routes of entry (for example through the membrane itself) are not precluded. Interestingly how-
ever, all compounds showed relatively high permeability indices against E. coli JT4, which has been shown to lack porin OmpC [5]. As only BRL 42715 showed appreciable activity against Class C ß-lactamase under these conditions any discussion of the permeability of E. cloacae to the other inhibitors is probably inappropriate.

As already emphasized, it is the interplay of a number of factors which determine how effectively a ß-lactamase inhibitor protects its partner antibiotic. It has been shown that for clavulanic acid, tazobactam and sulbactam, porin deficiency only decreased the susceptibility of E. coli to penicillin/ inhibitor combinations when accompanied by high level of expression of the TEM-1 ß-lactamase [13]. With regard to the current experiments, the data for BRL 42715 show that intrinsic inhibitory activity can compensate for a substantial permeability barrier and inhibition of periplasmic ß-lactamase readily achieved. On the other hand while tazobactam had a lower I₀ than clavulanic acid for the TEM-1 ß-lactamase in cell free assays the latter gave comparable I₀ values against whole cell ß-lactamase. Similarly, although clavulanic acid inhibited cell free ß-lactamase more readily than sulbactam this advantage was amplified considerably with whole cells.

In conclusion, our work has shown that clavulanic acid has lower permeability indices, and thus better penetration characteristics, than the other ß-lactamase inhibitors tested. It is likely that the superior penetration of clavulanic acid contributes to the antibacterial activity and thus clinical success observed with ß-lactam/clavulanic acid combination therapies [14,15].

References


Kinetic and physical studies of β-lactamase inhibition by a novel penem, BRL 42715

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The interactions of Staphylococcus aureus, Bacillus cereus 1, TEM, Klebsiella pneumoniae K1 and Enterobacter cloacae P99 β-lactamases with the novel penem inhibitor BRL 42715 were investigated kinetically and, in some cases, by electrospray mass spectrometry (e.s.m.s.). All the β-lactamases were rapidly inhibited by BRL 42715, with second-order rate constants ranging from 0.17 to 6.4 μM⁻¹·s⁻¹. The initial stoichiometry of β-lactamase inhibition was essentially 1:1, with the exception of the K1 enzyme. In this instance about 20 molecules of BRL 42715 were hydrolysed before the enzyme was completely inhibited. Inhibited β-lactamases did not readily regain activity in the absence of BRL 42715, the half-lives for regeneration of free enzyme ranging from 5 min for the K1 β-lactamase to over 2 days for the staphylococcal enzyme. Recovery of activity was incomplete for TEM-1, K1 and P99 β-lactamases, suggesting partitioning of the inhibited enzymes to give a permanently (or at least very stable) inactivated species. Examination of the interactions of the penem with TEM, B. cereus 1 and P99 β-lactamases by e.s.m.s. also showed rapid and stoichiometric binding of the inhibitor. In all cases a mass increase of 264 Da over the native enzyme was observed, corresponding to the molecular mass of BRL 42715, showing that no fragmentation of the penem occurred on reaction with the β-lactamases.

INTRODUCTION

The discovery and development of compounds such as clavulanic acid (Hunter et al. 1980; Reading and Cole, 1977) and sulbactam (English et al., 1978) have established enzyme inhibition as a valid means of overcoming β-lactamase-mediated resistance. A further sulphone, tazobactam, has also been examined for its ability to synergize β-lactamase-labile antibiotics (Aronoff et al., 1984) and is now also available clinically. More recently, a novel class of inhibitors, the 6-(substituted methylene) penems, have been synthesized in our laboratories (Bennett et al., 1991). One of these, BRL 42715 [(6R)-6-(1-methyl-1,2,3-triazol-4-yl)methylene] penem-3-carboxylic acid (Figure 1) has been shown to be both a very potent inhibitor of a wide range of β-lactamases and an effective synergist of β-lactamase-labile antibiotics (Coleman et al., 1989). The development of BRL 42715 as a therapeutic agent has now ceased, because it lacks other required technological features. However, such inhibitors are useful probes to investigate mechanistic aspects of β-lactamase catalysis. Studies with inhibitors such as clavulanic acid, sulbactam and 6β-bronopenicillanic acid have shown them to be kcat,- or mechanism-based inhibitors, often exhibiting complex kinetics [see reviews by Knowles (1983), Cartwright and Walky (1983) and Reading and Cole (1986)]. Despite the complexities observed with β-lactamase inhibitors, certain common features have emerged. Effective inhibition appears to require extensive structural reorganization of the inhibitor molecule following the formation of an initial acyl–enzyme complex, with the exception of the carbapenems. The chemical events are mirrored kinetically by the tendency of β-lactamase inhibition to follow branched reaction pathways. Recently, we have demonstrated that the K1 β-lactamase from Klebsiella pneumoniae generates a seven-membered cyclic product (dihydrothiazepine) from BRL 42715 (Broom et al., 1992). In the present paper, inhibition kinetics for BRL 42715 and this enzyme are presented, together with data for Staphylococcus aureus, Bacillus cereus 1, TEM-1 β-lactamases (all class A enzymes; Ambler, 1980) and Enterobacter cloacae P99, a class C β-lactamase (Jaurin and Grundström, 1981). A full understanding of the processes involved in β-lactamase catalysis and its inhibition requires the combination of classical biochemical information with that obtained from site-directed mutagenesis and biophysical studies. While in the latter case the ultimate goal may seem to be resolution of enzyme crystal structures, other techniques can also provide valuable information. Electrospray mass spectrometry (e.s.m.s.) has successfully been used to observe acyl–enzyme intermediates in β-lactamase catalysis (Aplin et al., 1990). This technique has the advantage that it can provide very accurate mass measurements of β-lactamase inhibitor complexes, and the additional benefit that it can be carried out under conditions and time scales similar to those employed in kinetic experiments. Results obtained using this method with BRL 42715 and TEM, B. cereus 1 and Ent. cloacae P99 β-lactamases are also presented.

EXPERIMENTAL

Antibiotics and reagents

Amoxycillin, benzylpenicillin, nitrocefin and BRL 42715 were all prepared in our laboratories; cephaloridine was purchased from Sigma. Bio-Gel P2 and Bio-Lyte ampholine solutions were obtained from Bio-Rad. Buffer reagents were of Analar grade, and distilled water was used to prepare all buffers and reagents.

Methods

Bacterial strains and β-lactamase preparation

Escherichia coli J14 (TEM-1), K. pneumoniae 1082E (K1), Ent. cloacae P99 and S. aureus N.C.T.C. 11561 are strains used in our...
laboratories for antibiotic-susceptibility testing. Bacteria were grown in shake flasks, and β-lactamases were released from the cells by ultrasonication as described previously (Reading and Farmer, 1981). TEM-1 β-lactamase from E. coli JT4 and the K1 enzyme from K. pneumoniae 1082E were further purified in a preparative isoelectric-focusing cell (Bio-Rad Rotofor). A 1 ml portion of cell-free β-lactamase was diluted to 40 ml in 1% (w/v) pH 3–10 Bio-Lyte ampholine and loaded into the cell. The power supply was set at 2000 V and 200 mA, with 12 W constant power. A circulating water system maintained the apparatus at 4 °C. After 4.5 h, the run was terminated and the fractions collected. The pH of each fraction was measured prior to assaying for β-lactamase activity with benzylpenicillin (see below). In the fraction used for the inhibition studies, β-lactamase accounted for 25–40% of the total protein.

B. cereus β-lactamase I was a gift kindly donated by (the late) Professor S. G. Waley (then at the University of Oxford). TEM-2 and P99 used for e.s.m.s. studies were purchased from Porton Products. Solutions of these two enzymes were dialysed overnight at 4 °C with 5 mM Tris/HCl buffer, pH 7. Each enzyme was then diluted in 5 mM Tris to give between 55 and 17 pmol ml⁻¹.

β-Lactamase assays

β-Lactamase activities were determined by using direct spectrophotometric assays (O’Callaghan et al., 1972; Waley, 1974; Samuni, 1975). The assay substrates and the wavelengths used to measure their hydrolysis were: benzylpenicillin (240 nm), amoxycillin (250 nm), cephaloridine (299 nm) and nitrocefin (482 nm). Nitrocefin gave an increase of absorbance on hydrolysis, whereas the other compounds showed reduced absorbance. All assays were carried out in 0.05 M sodium phosphate, pH 7.3, at 37 °C, except where mentioned otherwise. Changes in absorbance were monitored using either a Perkin–Elmer 37 UV/Vis double-beam spectrophotometer or a Philips PU 8700 single-beam instrument. In both cases reactions were maintained at the desired temperature using thermostatically controlled water-jacketed cuvette holders.

Stoichiometry and duration of β-lactamase inhibition

Concentrations of β-lactamase (generally from 0.02 to 1.3 μM) were calculated by using published data (see the Results section), and the amount of BRL 42715 varied to give from 0.2 to 40 mol. equiv. Samples were periodically removed and assayed for remaining β-lactamase activity using 1000 μM benzylpenicillin as substrate. BSA was added to give 5 mg ml⁻¹ to stabilize enzyme activity. Nevertheless, incubation of the staphylococcal β-lactamase at 37 °C resulted in a rapid loss of activity, and experiments with this enzyme were repeated at 30 °C. The stoichiometry of inhibition of the β-lactamases by BRL 42715 was calculated as described in the Results and discussion section.

Rate of β-lactamase inactivation

Inactivation rates were too high to measure accurately by sequential incubation of β-lactamase with BRL 42715 and substrate. The rates of inactivation were therefore determined in the presence of 1000 μM amoxycillin (TEM-1), 2000 μM benzylpenicillin (K1 and B. cereus I), 100 μM nitrocefin (staphylococcal) and 2000 μM cephaloridine (P99) (all at pH 7.3 and 37 °C). Typically hydrolysis of substrate was monitored for 5–10 min, with rates being recorded at timed intervals. Comparison of the reaction rates in the presence of inhibitor with those for control reactions enabled the degree of enzyme inhibition at each time point to be calculated. First-order rate constants for inactivation were then determined from plots of ln (percentage of enzyme activity remaining) against time. These values were then plotted against inhibitor concentration to obtain the second-order rate constants for the inactivation of β-lactamase by BRL 42715 after correcting for the influence of the assay substrate.

Stability of β-lactamase inhibition

The stability of inhibition achieved by BRL 42715 was measured in three ways. First, the data from the stoichiometry experiments with limiting concentrations of BRL 42715 were used to calculate the rates of decay of enzyme inhibition by measuring the re-establishment of β-lactamase activities. Secondly, enzyme was allowed to react with sufficient BRL 42715 to give 100% inhibition, then diluted into excess substrate and the breakdown of β-lactamase–inhibitor complex calculated from the increase in the rate of substrate hydrolysis with time. Thirdly, reactions of S. aureus and Ent. cloacae β-lactamase were carried out with excess penem, the reaction mixture was then gel-filtered using a Bio-Gel P2 column to remove the inhibitor. The β-lactamase-containing fractions were pooled, incubated and periodically assayed for β-lactamase activity as previously described. Prolonged incubation of uninhibited staphylococcal β-lactamase resulted in some loss of activity even at 30 °C, although the enzyme was stabilized by the presence of substrate. Consequently, staphylococcal enzyme samples were diluted into 1200 μM amoxycillin, and increased in substrate hydrolysis with time used to assess the rate of re-establishment of β-lactamase activity.

E.s.m.s.

BRL 42715 was preincubated at 37 °C for ≤ 3 min with between 17 and 55 pmol/ml of β-lactamase in approximately equimolar proportions. E.s. mass spectra were obtained as described previously (Payne et al., 1994).

RESULTS AND DISCUSSION

Inhibition of staphylococcal β-lactamase (class A)

Incubation of staphylococcal β-lactamase with limiting concentrations of BRL 42715 at 30 °C resulted in rapid, partial inhibition of enzyme activity (Figure 2). The degree of inhibition
remained constant for the duration of the experiment (\(>\) 4 h), indicating that the enzyme–inhibitor complex did not break down to release active \(\beta\)-lactamase. The stoichiometry of inhibition by BRL 42715 was calculated with concentrations of staphylococcal \(\beta\)-lactamase determined from published \(k_{\text{ext}}\) values for the PC1 enzyme. Using a \(k_{\text{ext}}\) for benzylpenicillin of 185 s\(^{-1}\) (Hardy and Kirsch, 1989), the stoichiometry of inhibition was calculated to be 1.3:1, whereas when a value of 128 s\(^{-1}\) was used (Rizwi et al., 1989), the stoichiometry was found to be 0.9:1. Although these \(k_{\text{ext}}\) determinations were carried out at 25 °C, this is unlikely to alter the conclusion that the penem inhibits \(S. aureus\) \(\beta\)-lactamase with a 1.0:1.0 stoichiometry. Removal of excess inhibitor by gel filtration followed by prolonged incubation of the \(\beta\)-lactamase in the presence of amoxicillin resulted in a very slow recovery of activity. The half-life for re-activation was estimated to be approx. 50 h (Table 1), suggesting that the enzyme–inhibitor complex was very stable.

### Table 1 Summary of the kinetic data

<table>
<thead>
<tr>
<th>(\beta)-Lactamase</th>
<th>Initial stoichiometry of inhibition</th>
<th>Second-order rate constant ((\mu)M(^{-1}\cdot\text{s}^{-1}))</th>
<th>Stability of the enzyme–inhibitor complex (%)</th>
<th>(k) for inactivation (\times 10^{-2}) (\mu)g (\cdot) ml(^{-1}) BRL 42715 (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S. aureus) N.C.T.C. 11561</td>
<td>1.1:1.0</td>
<td>0.17</td>
<td>53</td>
<td>20.4</td>
</tr>
<tr>
<td>TEM-1</td>
<td>1.0:1.0</td>
<td>6.4</td>
<td>1.2</td>
<td>17.4</td>
</tr>
<tr>
<td>(\beta) lactose</td>
<td>1.1:1.0</td>
<td>2.5</td>
<td>16</td>
<td>1.3</td>
</tr>
<tr>
<td>K1</td>
<td>2.5:1.0</td>
<td>0.009</td>
<td>0.88</td>
<td>39.9</td>
</tr>
<tr>
<td>(B. cereus) 1</td>
<td>1.5:1.0</td>
<td>0.11</td>
<td>1.3</td>
<td>31.5</td>
</tr>
</tbody>
</table>

Inactivation of staphylococcal \(\beta\)-lactamase by BRL 42715 followed first-order kinetics. Figure 3(a) shows inactivation rates plotted as a function of BRL 42715 concentration using the method of Kitz and Wilson (1962). The line intersects the axes at their origin, indicating that saturation kinetics were not obeyed. It was not, therefore, possible to find either the inhibitor constant (\(K_i\)) or the maximum rate of inactivation (\(k_{\text{inact}}\)). The reciprocal of the slope of the line did, however, provide a value of 0.17 \(\mu\)M\(^{-1}\cdot\text{s}^{-1}\) for the second-order rate constant for inactivation of \(S. aureus\) \(\beta\)-lactamase (see Table 1). The 1:1 stoichiometry of the inactivation of \(S. aureus\) N.C.T.C. 11561 \(\beta\)-lactamase by BRL 42715, together with the detection of only one inhibited species show that inhibition of this enzyme can be described by a linear kinetic scheme. Although it was not possible to detect a Michaelis complex experimentally, both current and previous studies (Coleman et al., 1989) have shown that the presence of substrate decreases the rate of inactivation. This observation shows that the inhibitor and substrate compete for...
Scheme 1 \hspace{0.6cm} \text{Inhibition of staphylococcal} \ \beta\text{-lactamase by BRL 42715}

EC \hspace{0.2cm} \text{is the Michaelis complex; EC}^* \hspace{0.2cm} \text{is the acyl-enzyme;} \ k_i \hspace{0.2cm} = \hspace{0.2cm} k_{-1}/k_4; \ k_f \hspace{0.2cm} \text{is the rate of formation of EC}^* \hspace{0.2cm} \text{and} \ k_d \hspace{0.2cm} \text{is the rate of decay of EC}^*.

Figure 4 \hspace{0.6cm} \text{Mass determinations by e.s.m.s. for B. cereus} \ \beta\text{-lactamase}

(a) \hspace{0.2cm} \text{B. cereus I \ \beta\text{-lactamase and (b) B. cereus I \ \beta\text{-lactamase + 1:1 BRL 42715 after 3 min incubation.}}

the same binding site. As interactions of \beta\text{-lactamases with mechanism-based inhibitors are generally represented as proceeding via a Michaelis complex (Cartwright and Waley, 1983; Knowles, 1983), it is reasonable to propose that such a complex is involved in the inhibition of staphylococcal} \ \beta\text{-lactamase by BRL 42715. However, competition between inhibitor and substrate does not provide unequivocal proof of a Michaelis complex in this case, and it is possible that the acyl-enzyme (EC*) is formed directly between free penem and \beta\text{-lactamase. Inhibition probably proceeds via such a complex, and Scheme 1 is proposed as representing the inhibition of staphylococcal} \ \beta\text{-lactamase by BRL 42715. The terminology is based on that of Frère et al. (1982). For this} \ \beta\text{-lactamase, the} \ k_i \hspace{0.2cm} \text{reaction is very slow.}

\textbf{Inhibition of B. cereus} \ \beta\text{-lactamase I (class A)}

The second-order rate constant for the inactivation of this enzyme by BRL 42715 was found to be 0.11 \mu M^{-1} \cdot s^{-1}, and saturation kinetics were not observed. The stoichiometry of inhibition was 1.6:1.0. In this respect, inhibition of \textit{B. cereus} \ \beta\text{-lactamase I resembles that of the staphylococcal enzyme, but the decay of EC* with a half-life of 1.3 h was much more rapid (Table 1). The inhibition of the} \textit{B. cereus} \ \beta\text{-lactamase by BRL 42715 can also be described by Scheme I. Mathematically transformed data of the e.s.m.s. results for} \textit{B. cereus} \ \beta\text{-lactamase I showed a major species (Figure 4a) with a mass of 28943.08 \pm 3.94 Da, with a minor species of molecular mass 28813.99 + 1.66 Da. The former species is in good agreement with the value of 28813.67 Da, the molecular mass calculated from the amino acid sequence (Madgwick and Waley, 1987). The latter protein corresponds to enzyme which had lost the N-terminal lysine residue ("ragged end"). When the} \ \beta\text{-lactamase was incubated for 3 min with an equimolar concentration of BRL 42715 a mass of shift of } \sim 266 \text{Da was observed for both the major and minor peaks. No free enzyme was apparent (Figure 4b). The increase in mass is close to the theoretical increase of 264 expected for the binding of the penem to the } \ \beta\text{-lactamase. The results indicate that} \textit{B. cereus} \ \beta\text{-lactamase I rapidly formed a 1:1 covalent adduct with BRL 42715. It is also apparent that no fragmentation of the inhibitor occurred.}

\textbf{Inhibition of TEM-1} \ \beta\text{-lactamase (class A)}

The inhibition of TEM-1 \ \beta\text{-lactamase by low concentrations of BRL 42715 is shown in Figure 5. The enzyme was rapidly inhibited, but, like the} \textit{B. cereus} \ \beta\text{-lactamase, a return in activity was apparent. However, unlike the situation with the} \textit{B. cereus}
Inhibition of K1 \( \beta \)-lactamase (class A)

Superficially, the results obtained for K1 \( \beta \)-lactamase resembled those acquired with the TEM enzyme: there was a rapid loss of \( \beta \)-lactamase activity, a re-establishment of enzyme activity at limiting inhibitor concentrations, and the formation of a permanently inactivated species. However, the recovery of \( \beta \)-lactamase activity was much faster than observed for the other \( \beta \)-lactamases, with a \( t_1 \) of 5.3 min (Table 1). Furthermore, using the published \( k_{\text{cat}} \) for K1 \( \beta \)-lactamase (Emanuel et al., 1986) to calculate enzyme concentration, showed that a 23-fold excess of BRL 42715 was required to inhibit the \( \beta \)-lactamase in the initial (i.e. the first few minutes) phase of the reaction. These results are consistent with our earlier demonstration of turnover of the penem by the K1 enzyme (Broom et al., 1992). However, with excess inhibitor, turnover of BRL 42715 ceases and the \( \beta \)-lactamase becomes permanently inactivated. The steady-state rate for this inactivation process was found to be \( 3.4 \times 10^{-4} \, \text{s}^{-1} \), while a 67-fold molar excess of the penem was required to achieve permanent inactivation. The need for excess BRL 42715 initially to inhibit the enzyme reflects a burst of inhibitor hydrolysis which cannot be accommodated by Scheme 2. An additional branch is thus required for the pathway. The kinetics of K1 \( \beta \)-lactamase by BRL 42715 can be described by either Scheme 3(a) or 3(b), these being kinetically indistinguishable. In these Schemes, EC\(*\) represents a transiently inhibited species.

This mechanism is the same as that proposed for the inhibition of TEM \( \beta \)-lactamase by clavulanic acid (Fisher et al., 1978; Reading and Farmer, 1981). Interestingly, the stoichiometry for the initial and final inactivation and the rates of decay of the transient complexes are very similar for TEM/clavulanic acid and K1/BRL 42715. However, the second-order rate constant for the inactivation of K1 \( \beta \)-lactamase by BRL 42715 is about 60-fold higher than that for clavulanic acid with the TEM enzyme. Plots of inactivation rates against inhibitor concentration indicated that saturation kinetics were followed by BRL 42715 and K1 \( \beta \)-lactamase: values of \( 7.30 \times 10^{-2} \, \mu\text{M} \) and \( 1.8 \times 10^{-4} \, \text{s}^{-1} \) were found for \( K_i \) and the maximum inhibition rate respectively.

Interestingly the protein content in the reactions influenced the partitioning of K1 \( \beta \)-lactamase between EC\(_i\) and EC\(*\). This
behaviour is reminiscent of that observed with the effects of ionic strength on the inhibition of the same β-lactamase and β-halogencepitacinelines (De Meester et al., 1989). In the presence of BSA the amount of enzyme sequestered as EC₃ was about 40% of that obtained in its absence. Experiments with sonicated cells showed that the β-lactamase behaved in the same way as pure enzyme in the presence of BSA. These observations may reflect subtle conformational changes which are influenced by protein concentration.

Inhibition of Ent. cloacae P99 β-lactamase (class C)
The class C β-lactamase from Ent. cloacae P99 was rapidly inactivated by BRL 42715 with an initial stoichiometry of 1:1.1:1 and a second-order rate constant of 2.5 μM⁻¹ s⁻¹. Saturation kinetics were not observed. Experiments undertaken with limiting penicillin concentrations, or where excess inhibitor was removed by gel filtration, showed that there was slow recovery of enzyme activity. This process had a half-life of 16 h, but was also incomplete showing that, as with TEM β-lactamase, irreversibly inactivated enzyme was also generated. The stoichiometry for irreversible inactivation was 2:1. The inhibition of Ent. cloacae β-lactamase by BRL 42715 can readily be described by Scheme 2. The mass of Ent. cloacae P99 β-lactamase as determined by e.s.m.s. was 39196.56 ± 4.26 Da, which is only 9.8 Da lower than the value calculated from the deduced amino acid sequence (Galleni et al., 1988) and 6.8 Da lower than recently published molecular masses found by e.s.m.s. (Aplin et al., 1993). Following 3 min incubation with an equivalent concentration of BRL 42715, a major peak of molecular mass 39460.18 Da was obtained, with free enzyme being present as a minor component. The mass shift of almost 264 Da corresponds to the covalent binding of BRL 42715 in stoichiometric proportions. Again, as with the other β-lactamases examined, there was no indication of fragmentation of the penem.

CONCLUSIONS
The results from the kinetic experiments show that BRL 42715 is a very effective inhibitor of all the β-lactamases studied, readily explaining its excellent activity as a synergist for β-lactamase-labile antibiotics (Coleman et al., 1989). The critical parameter relating the time needed to inhibit an enzyme to inhibitor concentration is the second-order rate constant. Data for the penem (see Table 1) fall in the region of 0.1–6.4 μM⁻¹ s⁻¹, showing it is generally superior to other β-lactamase inhibitors (Cartwright and Waley, 1983), including tazobactam, for which inhibition kinetics have recently been described (Bush et al., 1993). An exception is the olivamic acid MM 4550, which has a second-order rate constant of 100 μM⁻¹ s⁻¹ (Easton and Knowles, 1982). Two other important requirements for efficient enzyme inhibition are low inhibitor turnover coupled with stability of inhibition. Except for the K1 β-lactamase, initial stoichiometries for β-lactamase inhibition by BRL 42715 are 1:1 and enzyme-inhibitor complexes are relatively stable, exceptionally so for staphylococcal and P99 β-lactamases. The results from the e.s.m.s. studies confirm the rapid stoichiometric inhibition of β-lactamases by the penem. Interestingly, these experiments did not suggest any fragmentation following initial binding to the β-lactamases, but do not preclude ring opening and rearrangement to form the dihydrothiazepine, as has been shown with BRL 42715 and K1 β-lactamase using other techniques (Broom et al., 1992).

Like BRL 42715 β-halogencpenicilllic acids also undergo cyclic rearrangements on reaction with β-lactamases (Orlek et al., 1979), and have recently been examined by e.s.m.s. (Aplin et al., 1993). To what extent formation of a cyclic entity from BRL 42715 is a prerequisite for effective β-lactamase inhibition is presently unknown, and further studies will be necessary to more fully understand the factors responsible for its activity.

We thank (the late) Professor S. G. Waley and Dr. R. Aplin of the University of Oxford for advice on the e.s.m.s. experiments and operation of the mass spectrometer. We also appreciate the advice and assistance of Dr. P. Skett and C. Reading of SmithKline Beecham.

REFERENCES

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THE SYNTHESIS AND SERINE \( \beta \)-LACTAMASE INHIBITORY ACTIVITY OF SOME PHOSPHONAMIDATE ANALOGUES OF DIPEPTIDES

John H. Bateson,*a Brian C. Gasson,a Teresa Khushi,b Jane E. Neale,b David J. Payne,*b David A. Tolson,c and Graham Smith

ABSTRACT: The dipeptidyl phosphonamidate analogues (4)-(12) inhibited a range of serine \( \beta \)-lactamases, being most efficacious against the Enterobacter cloacae P99 isolated enzyme. Synergy experiments demonstrated that the antibacterial activity of amoxycillin is potentiated against bacteria producing these enzymes, the effect again being most pronounced against the P99-producing E. cloacae N-1 strain. The analogues (11) and (12) (\( \beta \)-amino acid C-terminal) were the most active inhibitors and synergists.

Introduction

Clavulanic acid\(^1\) is a serine \( \beta \)-lactamase inhibitor of great commercial importance. Its mechanism of acylative interaction with these enzymes, resulting in their inhibition, is well understood.\(^2\)-\(^3\) Other inhibitor classes studied in these laboratories include the highly potent 6-alkylidene penem series\(^4\) and a rational mechanism for their interaction with serine \( \beta \)-lactamases has recently been proposed.\(^5\)

Linear, substrate-related peptidyl \( \alpha \)-amino phosphonic acid derivatives have been demonstrated\(^6\)-\(^8\) to inhibit serine proteases by the transition state isostere mechanism, since they mimic the tetrahedral intermediates formed during normal substrate hydrolysis. Other tetrahedral phosphorus compounds operate by phosphorylation/phosphonylation at the active site serine.\(^9\)-\(^12\) The recent disclosures by Pratt\(^13\) and by Laws, et.al\(^14\) of some simple phosphonamidates as inhibitors of serine \( \beta \)-lactamases now prompt us to report our own findings in this area.

Synthesis of Inhibitors

Linear phosphonamidates (1) resemble closely the generalised substrates (2) of \( \beta \)-lactamases, which contain an azetidinone ring (Figure 1). We chose to examine a series of dipeptidyl phosphonamidate analogues as \( \beta \)-lactamase inhibitors; these were obtained (Scheme 1) by methods described by Jacobsen and Bartlett.\(^15\) ([AA] represents both primary and secondary amino acid residues (including prolines and the penicillin-related thiazolidine]). These were chosen as the C-terminal of the series (1) to incorporate increasingly similar recognition elements \( R_1, R_2 \) associated with substrates (2). In the case of the cyclic variants (8)-(10) (secondary AA C-terminal) we were unable to achieve further hydrolysis of the methyl phosphonate ester function without disruption of the phosphonamidate linkage. Ethyl phosphonamidates corresponding to (9) (\( L \)-stereochemistry) have been described by Laws, et.al.\(^14\) The \( L \)-Phe compound (7) is an inhibitor of carboxypeptidase A.\(^15\)

We also prepared some expanded phosphinate-type transition state analogues\(^17\) in the hope of mimicking a late \( \beta \)-lactam hydrolysis transition state. The synthesis of a representative compound (17) from aminophosphinic acid (13) is outlined in Scheme 2.
Figure 1: Relationship of linear phosphonamidates (1) to generalised β-lactam substrates (2) of serine β-lactamases.

Scheme 1: Synthesis of linear phosphonamidates (3)-(12): Reagents: i. SOCl₂, CH₂Cl₂; ii. AA ester; iii. LiOH, H₂O-CH₃CN; iv. HP20SS chromatography; v. lyophilisation.

For (4-12)
Z-PhCH₂OCONH₂
(3) Z = R = H
(4) R = H (Gly)
(5) R = Ph (D-Phegly)
(6) R = CH₂Ph (D-Phe)
(7) R = CH₂Ph (L-Phe)
(11) R = H (B-Ala)
(12) R = Ph (B-Phe-B-Ala)

(8) (D-Pro)
(9) (L-Pro)
(10) (D-thiazolidine)
Scheme 2: Synthesis of 'expanded' TS analogue (17): Reagents: i. PhCH₂OCOCI, Na₂CO₃; ii. TMSCI, Et₃N; iii. CH₂O; iv. CH₂N₂; v. CF₃SO₂Cl, Et₃N; vi. H₂NCH₂CO₂Et; vii. LiOH, H₂O-CH₃CN.

Table 1: ID₅₀ of phosphonamidates and clavulanic acid for a range of serine β-lactamases

<table>
<thead>
<tr>
<th>β-lactamase</th>
<th>TEM-1</th>
<th>P99</th>
<th>S. aureus penicillinase</th>
<th>SHV-5</th>
<th>OXA-1</th>
<th>P. mirabilis carbenicillinase</th>
<th>PSE-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clase (Bush)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(4)</td>
<td>&gt;100</td>
<td>1</td>
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<td>(5)</td>
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<td>&gt;100</td>
</tr>
<tr>
<td>(6)</td>
<td>&gt;100</td>
<td>3.80</td>
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<td>&gt;100</td>
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<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(7)</td>
<td>50</td>
<td>0.75</td>
<td>33</td>
<td>&gt;100</td>
<td>60</td>
<td>&gt;100</td>
<td>15</td>
</tr>
<tr>
<td>(8)</td>
<td>&gt;100</td>
<td>67</td>
<td>&gt;100</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>(9)</td>
<td>&gt;100</td>
<td>54</td>
<td>&gt;100</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>(10)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>NT</td>
<td>NT</td>
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<td>NT</td>
</tr>
<tr>
<td>(11)</td>
<td>50</td>
<td>0.87</td>
<td>25</td>
<td>33</td>
<td>14</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>(12)</td>
<td>50</td>
<td>0.87</td>
<td>35</td>
<td>63</td>
<td>46</td>
<td>&gt;100</td>
<td>83</td>
</tr>
<tr>
<td>(17)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Clavulanic acid</td>
<td>0.315</td>
<td>310</td>
<td>0.38</td>
<td>0.013</td>
<td>3.4</td>
<td>0.51</td>
<td>0.235</td>
</tr>
</tbody>
</table>

Numbers in parenthesis = ID₅₀ determined with no pre-incubation of enzyme and inhibitor.
NT = Not tested.
Table 2: Potentiation of amoxycillin by phosphonamidates and clavulanic acid

<table>
<thead>
<tr>
<th>Organism</th>
<th>ß-lactamase produced</th>
<th>MICs (µg/ml)</th>
<th>MIC of Amoxycillin in Combination with 10µg/ml of each phosphonamidate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amoxycillin</td>
<td>Clavulanic acid</td>
</tr>
<tr>
<td>Ent. cloacae</td>
<td>N-1</td>
<td>256</td>
<td>32</td>
</tr>
<tr>
<td>S. aureus</td>
<td>MB9</td>
<td>256</td>
<td>32</td>
</tr>
<tr>
<td>E. coli</td>
<td>TEM-P</td>
<td>&gt;256</td>
<td>32</td>
</tr>
</tbody>
</table>

Susceptibility Testing

MIC determinations were performed in liquid media in microdilution plates. All compounds were dissolved in Mueller-Hinton broth (Difco Laboratories). Amoxycillin (SmithKline Beecham) was then diluted serially in Mueller-Hinton broth followed by addition of inhibitor at a fixed concentration. Test compounds were used at 100, 10 and 1µg/mL. Clavulanic acid was included, at a lower concentration, as a reference standard. The MIC of inhibitor compounds alone was also determined.

Organisms were grown at 37°C for 18 hours in Mueller-Hinton broth and diluted to give a final count in each well of approximately 10^5 cfu/ml. The total volume per well was 100 µl.

Plates were incubated aerobically at 37°C for 18 hours. The MIC was recorded as the lowest concentration of amoxycillin to inhibit visible growth.

ID₅₀ Determinations

ID₅₀s were performed against the following isolated enzymes (Bush classification): E. coli TEM-1 (2b), E. cloacae P99 (1), S. aureus penicillinase (2a), E. coli SHV-5 (2b'), E. coli OXA-1 (2d), P. mirabilis carbenicillinase (2c), and P. aeruginosa PSE-4 (2c). Nitrocefin was employed as the reporter substrate, using the method described by Payne et al. ID₅₀ values were determined following a 5 minute preincubation of enzyme and inhibitor. In addition, ID₅₀s for TEM-1 and S. aureus penicillinase were also determined without pre-incubation.

Results and Discussion

All the inhibitors showed their greatest activity against the P99 ß-lactamase; compounds (7), (11) and (12) were the most active inhibitors of P99 enzyme and also demonstrated the broadest spectrum of activity with ID₅₀s of less than, or equal to, 50 µM for at least four of the enzymes tested. No inhibition of the TEM-1 or S. aureus ß-lactamases was observed without preincubation, suggesting the progressive nature of the inhibition (Table 1). Phosphinate (17) was inactive at the concentrations tested. The MICs of the phosphonamidates when tested alone, were >100 µg/mL for all organisms. However, the presence of phosphonamidate (11) (10 µg/mL) potentiated the antibacterial activity of amoxycillin against E. cloacae N-1 and S. aureus MB9 whole-cell bacteria by 64- and 8-fold, respectively (Table 2). Phosphonamidates (4-7) (10 mg/mL) were less effective, reducing the MIC of amoxycillin for E. cloacae N-1.
Phosphonamidate analogues of dipeptides

and S. aureus by (8-2)-fold and 4-fold, respectively. A concentration of 100 μg/ml of (11) was required to reduce the MIC of amoxycillin for E. coli E96P by >2 fold. No other compound showed any potentiation of amoxycillin against this organism.

Mechanism of Inhibition

Pratt’s work on the interactions of phosphonate monoesters (depsipeptide analogues) with serine β-lactamases (notably the P99 class C enzyme)20-23 has shown that enzyme inhibition occurs by a phosphorylation process rather than by transition state mimicry. This was proven by 31PMR studies of R

\[
\begin{align*}
(18) & \quad R = ZNH \\
(19) & \quad R = H
\end{align*}
\]

inhibited enzyme, and by 14C isotopic labelling.21 More recent reports from this group comprise the demonstration that anilide phosphonamidates of types (18) and (19) are irreversible inhibitors of serine β-lactamase, and a study of the pH profiles of inhibition stressed the requirement for leaving group protonation during the phosphorylation process.13 The good inhibitory properties of phosphonamidates of β-amino acids (Table 2) may arise as a consequence of the improved ease of protonation and nucleofugal properties of such leaving groups in comparison with their less basic, α-amino counterparts (Figure 2). Pratt’s X-ray studies of a staphylococcal serine β-lactamase inhibited by a methylphosphonate monoester are also in support of the general concept of such mechanisms.23

Our own ESMS studies of the P99 enzyme inhibited by e.g. phosphonamidates (6) and (11) show a similar mass increment corresponding to expulsion of the amino acid residue (cf. Figure 2) [Mass=(Menz+228) and (Menz+229) amu, respectively; theoretical increment ΔM for the protonated form of (20)=227 amu]. This is consistent with the operation of the foregoing active-site phosphorylation inhibition mechanism, both for α- and β-amino acid C-terminal phosphonamidates.24 Laws et.al. also demonstrated the phosphorylation of the P99 enzyme by the ethyl phosphonamidate ester corresponding to (9) using ESMS.14
Conclusions

- Phosphonamidates (1) have been shown to inhibit a range of serine β-lactamases, with greatest activity against the E. cloacae P99 Class 1 enzyme.
- The series shows improved P-99 inhibitory activity over clavulanic acid.
- β-Amino acid phosphonamidates show ~70-fold greater P99 inhibitory activity than compounds of type (8) and (9), which are similar to those previously reported.14
- Phosphonamidates have now been shown to potentiate the antibacterial activity of amoxycillin, which may be indicative of synergistic activity and cell penetration.

Acknowledgements

We thank Muriel Marshall, who prepared the manuscript.

Notes and References

16. Ethyl glycinate was utilised in the synthesis of (3) and (4). All other amino acids were added as their methyl esters.
24. A detailed account of these ESMS investigations will appear in our full paper. In most cases the experiments were set up to include the observation of free P99, together with inhibited enzyme, as an internal calibration for mass shift measurements.

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Phosphonamide analogues of dipeptides with carboxypeptidase A and \(\beta\)-lactamase-inhibitory activity: elucidation of the mechanism of \(\beta\)-lactamase inhibition by electrospray mass spectrometry

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A series of phosphonamidate compounds with different \(P_1'\) amino acid residues have been shown to be irreversible inactivators of the serine \(\beta\)-lactamase from Enterobacter cloacae P99. The efficiency of inhibition (based on \(k_{\text{cat}}/K\) values) of P99 by these derivatives, ordered in decreasing potency, is: \(\beta\)-phenyl-\(\beta\)-Ala \(>\) D-Phe \(>\) \(\beta\)-Ala \(>\) Gly \(>\) D-Phe \(>\) D-Pro \(>\) D-thiazolidine. The D- and L-Phe compounds also inhibit carboxypeptidase A. The proline and thiazolidine derivatives were phosphonamidate methyl esters, whereas the others were salts of dicarboxylic acids. Electrospray mass spectrometry showed that equimolar mixtures of the P99 enzyme with each of the following derivatives, Gly, D-Phe, L-Phe, \(\beta\)-Ala and \(\beta\)-phenyl-\(\beta\)-Ala, effected efficient adduct formation (70-95% of enzyme modified), illustrating the particularly active nature of some of these compounds. All the primary amino acid derivatives gave a similar mass increment, which suggests the displacement of the variable \(P_1'\) part of the molecule. This observation provides evidence that the compounds phosphonylate the active-site serine, with the phosphonamide bond as the scissile bond and the amino acid as the leaving group. The thiazolidine derivative (phosphonamide methyl ester) also appeared to work by the same mechanism. The comparable proline derivatives caused lower than expected mass shifts of 227-229, and therefore it is proposed that with these compounds both the amino acid and the phosphonamide ester methoxy group were displaced at the phosphorus atom during the inhibition process. Therefore, electrospray mass spectrometry has provided both a measure of potency and a rationale for the mechanism of inhibition of P99 by these compounds.

INTRODUCTION

Serine active-site \(\beta\)-lactamases hydrolyse the \(\beta\)-lactam ring of \(\beta\)-lactam antibiotics and therefore pose one of the greatest threats to the successful clinical use of \(\beta\)-lactam antimicrobials. Consequently, the use of a \(\beta\)-lactamase inhibitor in combination with a \(\beta\)-lactam has become one of the most successful strategies used to overcome the resistance problems caused by this group of microbial enzymes. All the commercially available \(\beta\)-lactamase inhibitors contain a \(\beta\)-lactam ring and these compounds are active against the vast majority of Bush Group 2 [1] enzymes, which are the most common \(\beta\)-lactamases produced by clinical pathogens [2].

Other compound classes have been identified that have \(\beta\)-lactamase-inhibitory activity. One such group is the phosphonate series of depsipeptide analogues. Pratt [3] has illustrated that phosphonate monoesters inhibit the Enterobacter cloacae P99 Group I serine \(\beta\)-lactamase by phosphonolysis of the active-site serine. In addition, phosphonamidates have been shown to inhibit P99 via the same mechanism [4,5]. We were also intrigued by the structural similarity (Scheme 1) in their peripheral topology of linear phosphonamide analogues of dipeptides (Scheme 1, compound 1) to the bicyclic \(\beta\)-lactam substrates of \(\beta\)-lactamases (generalized structure 2 in Scheme 1). In a previous publication [6] we reported the synthesis of a variety of phosphonamidates (Figure 1), incorporating increasing substrate-recognition features \((R; R', R'')\) into the \(P_1'\) amino acid residue. These had inhibitory activity against a broad range of serine \(\beta\)-lactamases [6]. The compounds showed potent activity against the P99 enzyme but one compound (9), containing a \(\beta\)-alanyl residue at \(P_1'\), had IC_{50} values of less than or equal to 50 \(\mu\)M for \(\beta\)-lactamases from each of the following Bush Groups: 1, 2a, 2b, 2b' and 2d [1]. In addition, synergy minimum inhibitory concentrations illustrated that some of these compounds potentiated the antibacterial activity of amoxicillin against bacteria producing the P99 enzyme. One of the phosphonamidates containing L-Phe at \(P_1'\) (5) is a known inhibitor of the zinc metalloprotease carboxypeptidase A, for which the mechanism of inhibition occurs as expected by transition-state mimicry, rather than by any phosphorylation process [7].

In this study we report on the use of electrospray mass spectrometry (ESMS) to investigate both the potency and

![Scheme 1: Peripheral relationship between linear phosphonamidates (1) and generalized bicyclic \(\beta\)-lactam structures (2) of \(\beta\)-lactamases](attachment:image)
mechanism of inhibition of the P99 β-lactamase by eight-phosphonamidate compounds (3–10). In addition, we have examined the activity of each of the compounds against the mammalian carboxypeptidase A metalloprotease and a series of bacterial metallo-β-lactamases to determine whether any of the inhibitors are able to inhibit enzymes operating by this very different mechanism.

MATERIALS AND METHODS

Materials

P99 β-lactamase was purchased from Porton Products. Hippuryl-L-phenylalanine (Sigma) and carboxypeptidase A (Sigma) were used as purchased. The phosphonamidate compounds are shown in Figure 1 and were prepared in our laboratories [6] using methods similar to or derived from known procedures [7]. The diithithallic salts (3–5 and 9) are single diastereoisomers; the β-phenyl-β-alanine compound (10) is a 1:1 mixture of enantiomers, epimeric at the phenyl substituent. The lithium salt phosphonamidate esters (6–8) were each 1:1 mixtures of diastereoisomers, epimeric at the phosphorus. The methyl ester function present in compounds 6–8 could not be cleaved hydrolytically without concomitant hydrolysis of the phosphonamidate linkage.

ESMS

Each compound was mixed with P99 (1 mg/ml final concentration) to achieve the desired ratio of enzyme/inhibitor. All assays were performed in 5 mM Tris/HCl, pH 7.0, buffer. The mixture was then incubated for 5 min at 37 °C and a 20 μl aliquot examined by ESMS using a gradient system in order to prevent possible interference from excess inhibitor. Chromatography was performed on a Hewlett-Packard 1090 liquid chromatograph using an Applied Biosystems Aquapure RP-300 column (100 mm × 2.1 mm) with the following gradient at a flow rate of 100 μl/min:

The eluate from the column was fed directly into the electrospray interface of a Finnigan MAT TSQ 700 mass spectrometer. Spectra were averaged over the peak of interest and deconvoluted using ICIS V1.00 software. In spectra that show both unbound P99 and the adduct peak, mass-shift calculations were internally calibrated. Where no free P99 was observed, the mass of P99 was taken as 39195±1.0 which was the average of mass determinations (Figure 2, top, shows an example of one of the mass determinations). This is lower than the mass of P99 determined from the amino acid sequence (39206). This anomaly has been observed in our previous studies with this enzyme [6].

Enzyme assays and kinetic analysis

All β-lactamase assays were performed at 37 °C in 25 mM sodium phosphate buffer (pH 7.0). The assays described below were initiated with the addition of enzyme and utilized nitrocefin as the reporter substrate (final concentration 100 μM). The enzyme concentration was chosen so that only 10% of the reporter substrate (∼10 μM) was hydrolysed at the end of the inactivation. A pseudo-first-order rate constant k2, characterizing the rate of inactivation was determined at different inhibitor concentrations. Table 1 shows the range of concentrations used for each compound. For all inhibitors, k2 increased linearly with the concentration of inhibitor (I). Under these conditions, only the k2/K ratio can be computed. Limit values of k2 and K were given.

\[
k_2 = k_1 + \frac{k_2}{I} (K_m^{sn} + S)/K_m^{sn} + 1 + I
\]

where S is the concentration of the reporter substrate and K_m^{sn} is the K_m of the reporter substrate.

Here, \[K[(K_m^{sn} + S)/K_m^{sn}]] > I\] and so \(k_2 = 0, \) so

\[
k_2 = \frac{k_2}{I} \left( \frac{K_m^{sn} + S}{K_m^{sn}} \right)
\]

IC50 assays for the carboxypeptidase A enzyme (0.54 unit/ml) were carried out after a 5 min incubation of inhibitor and enzyme. Assays were performed at 37 °C in 25 mM Tris/HCl/500 mM NaCl, pH 7.5, with hippuryl-L-phenylalanine (0.39 mM) as the reporter substrate. Hydrolysis of the reporter substrate was monitored at 254 nm.

RESULTS

Kinetic analysis showed that these compounds were inactivators of the P99 enzyme, and the k2/K ratio for inhibition of P99 was determined for each of the compounds (Table 1). On the basis of the k2/K data the activities of the compounds were ranked as follows: β-phenyl-β-Ala (10) > L-Phe (5) > β-Ala (9) > Gly.
Inhibitory properties of phosphonamidate analogues of dipeptides

Figure 2  ESMS of P99 β-lactamase (top) and P99 β-lactamase and compound 9 mixed in a 1:1 molar ratio (bottom)

The mass of P99 increased by m/z 229 when mixed with compound 9.

Table 1  Kinetic constants for the inhibition of P99 by phosphonamidate compounds

Results for \( k_{2}/K \) are means ± S.E.M. from three determinations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Phosphonamidate side chain</th>
<th>Concentrations of inhibitor used to determine ( k_{2}/K ) (μM)</th>
<th>( k_{2}/K ) (M⁻¹.s⁻¹)</th>
<th>( 10^{3} \times k_{2} ) (s⁻¹)</th>
<th>( K ) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Gly</td>
<td>200–1500</td>
<td>94±3</td>
<td>&gt; 141</td>
<td>&gt; 1500</td>
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<tr>
<td>4</td>
<td>o-Phe</td>
<td>200–4000</td>
<td>60±2</td>
<td>&gt; 249</td>
<td>&gt; 4000</td>
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<tr>
<td>5</td>
<td>L-Phe</td>
<td>100–1000</td>
<td>226±7</td>
<td>&gt; 229</td>
<td>&gt; 1000</td>
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<tr>
<td>6</td>
<td>o-Pro</td>
<td>500–5000</td>
<td>5.5±0.1</td>
<td>&gt; 28</td>
<td>&gt; 5000</td>
</tr>
<tr>
<td>8</td>
<td>o-Thiazolidine</td>
<td>5000–10000</td>
<td>0.29±0.02</td>
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<td>&gt; 10000</td>
</tr>
<tr>
<td>9</td>
<td>β-Ala</td>
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<td>157±6</td>
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<td>&gt; 1000</td>
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<tr>
<td>10</td>
<td>β-Phenyl-β-Ala</td>
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<td>250±5</td>
<td>&gt; 259</td>
<td>&gt; 1000</td>
</tr>
</tbody>
</table>

(3) > o-Phe (4) > o-Pro (6) > D-thiazolidine (8). Interestingly, the L-Phe (5) derivative was more than 3-fold more effective at inactivating P99 than the D-isomer (4). Compounds 3–10 at a concentration of 1 mM had no inhibitory activity against the Bacteroides fragilis CβA, Stenotrophomonas maltophilia L-1 and Aeromonas hydrophila CphA metallo-β-lactamases [9].
ESMS studies on the inhibition of P99 by phosphonamidate compounds

ESMS analysis, in turn, of a 1:1 molar mixture of each of the most potent phosphonamidates, (10, 5 and 9) with P99 showed a mass increment in the range m/z 226–229 (Figure 2, bottom, illustrates the mass shift observed with compound 9), suggesting that a covalent enzyme-inhibitor adduct had formed. This adduct did not correspond to an increment representing the masses of any of the intact compounds but indicated incorporation of a common fragment. Under these conditions, no free P99 was observed on ESMS. An adduct of similar mass was also observed when 1:1 mixtures of P99 and compounds 3 and 4 were examined by ESMS (Table 2). However, spectra from these investigations revealed both unbound P99 together with an enzyme-inhibitor adduct, illustrating that the P99 was incompletely inhibited. Moreover, spectra obtained for P99 mixed with a 10-fold molar excess of compound 7 did not reveal any P99 adduct. However, on increasing the concentration of inhibitor (50:1 molar excess), the spectra for compounds 6 and 7 showed both free P99 and a peak corresponding to a similar mass increment of m/z 227–229 (Table 2). At this ratio, approximately 40-50% of the P99 remained unbound. Finally, P99 mixed with a 50:1 molar excess of the least active compound, the d-thiazolidine derivative (8), gave rise to only a minor peak which corresponded to a fragment m/z 246 binding to the P99 β-lactamase (Table 2).

Inhibition of carboxypeptidase A by phosphonamidate compounds

The D-Phe (4) and L-Phe (5) derivatives inhibited the carboxypeptidase A metalloproteinase with IC₅₀ values of 8.95 and 0.32 ± 0.2 µM respectively. Interestingly, the L-isomer was more than 25 times more active than the compound with the D-Phe substituent. The latter feature corresponds to the β-lactam P₁-recognition stereochemistry: see Scheme 1, structure 2. All the other compounds showed no significant inhibition of carboxypeptidase A at 1 mM.

DISCUSSION

The kinetic analysis described in this report has illustrated that these phosphonamidate compounds irreversibly inhibit the P99 β-lactamase. The ESMS data have further substantiated the nature of this irreversible inhibition by illustrating the covalent binding of fragments of the compounds to the P99 enzyme.

The efficiency of inhibition of P99 by each of these compounds compared well with the ESMS data. For example, the spectra of a 1:1 mixture of the three compounds with kₑ/K = 157–250 M⁻¹ s⁻¹ showed that P99 was completely inhibited, but under the same conditions only a small proportion of P99 had formed an adduct in the presence of a 50:1 molar excess of the least active phosphonamidate (6) (kₑ/K = 0.2 ± 0.02 M⁻¹ s⁻¹). A compound similar to 7 has been synthesized by Laws et al. [4] and was the phosphonamidate ethyl ester rather than the methyl ester; these two esters would be expected to have similar potencies. On the basis of kₑ/K comparisons, compounds 10, 9 and 5 were between 50 and 30 times more effective inactivators of P99 than the compound described by Laws et al. [4].

The masses of the five most active compounds (3–5, 9, 10) vary from 404 to 314. However, each compound gave an ESMS adduct of similar mass increment (225–229) which suggests elimination of the variable P₁ part of the molecule. Therefore, to explain these mass shifts, we propose that the compounds phosphorylate the active-site serine of the β-lactamase (Scheme 2) with the phosphonamidate bond as the scissile bond and the amino acid as the leaving group. The predicted mass shift for this mechanism depends (Scheme 2) on whether the inhibitor was the phosphonamidate (X = H) or its methyl ester (X = Me). The predicted mass shift for the phosphonamidate form (227) was close to the observed values for compounds 3–5, 9 and 10 (Table 2), supporting the proposed mechanism. The thiazolidine analogue (8) is a phosphonamidate methyl ester and, according to Scheme 2, should give rise to an m/z increment of 241. A mass shift of + (244–246) mass units was observed, suggesting that compound 8 inhibits P99 by the mechanism illustrated in Scheme 2, with its phosphonamidate methyl ester bond retained intact. Both the proline derivatives (6 and 7) are also phosphonamidate methyl esters. Consequently, a mass shift of approx. 241 should also have been measured according to the mechanism in Scheme 2. However, mass shifts of 227–229 were observed. It is possible that with these compounds both the amino acid and the phosphonamidate methoxy group were displaced sequentially at the phosphorus atom during the enzyme inhibition. The observed

![Scheme 2 Proposed mechanism of Inhibition of P99 by phosphonamidates](image-url)
mass changes would not permit a subsequent nucleophilic displacement of methanol at phosphorus by a proximate enzyme lysine residue (requiring a net loss of CH₃OH, ∆mass = 32 mass units, but necessitate rather a hydrolytic displacement by water [net loss of CH₄, ∆mass = 14 mass units]). This would result in a mass shift of 22.7 which is close to the observed change.

Clearly, the second hydrolytic event cannot be fully enzyme-mediated since the serine residue is now blocked. It may, however, be accelerated by the presence of proximate amino acid residues. Our data do not rule out the alternative possibility that, for the proline compounds, displacement of the methanol precedes that of the P₁' amino acid residue. As both compounds 6 and 7 provided the expected [M + H]⁺ ions (m/z 347) in their spectra, it is extremely unlikely that the observed methyl ester loss from the enzyme adduct is an artifact of electrospray-induced fragmentation. We are unable to account for the difference in behaviour between thiazolidine (8) and prolines (6 and 7) other than it may be a consequence of a much slower methyl ester hydrolysis reaction for the former. It is noteworthy that Laws et al. [4] report that, in the enzyme reaction of one diastereoisomer of their ethyl ester analogue of compound 7, ethanol loss was detected.

The l-Phe derivative (5) is a potent inhibitor of carboxy-peptidase A by transition-state mimicry [7]. This reflects both the known preference for a Phe substituent in the P₁' residue of carboxypeptidase A inhibitors (see for example ref. [10]) and a preference for the natural (l-)-amino acid stereochemistry at this position. As discussed above, our work suggests that this compound inhibits the P99 serine β-lactamase by a phosphonylation mechanism. It is quite remarkable that this compound can inhibit both a serine and an unrelated metallo active-site enzyme by two such different mechanisms. Both enzymes also have preference for the D-isomer over the L-isomer (4). In the case of the proline derivatives (6 and 7), the amino acid stereochemistry was a less important criterion for inhibition of P99.

The parallel study by Laws et al. [4] on a single phosphonamidate structural type (the ethyl phosphonamidate esters analogous to 7) also supports the hypothesis that this type of compound inhibits P99 by phosphonylation at the active-site serine with elimination of the inhibitor amino acid residue. Phosphonate monoesters have also been shown to inhibit Class C β-lactamases by phosphonylation and the validity of this concept has been demonstrated by an extensive X-ray study [11].

In the present work we have demonstrated how ESMS can be successfully used to deduce the mechanism of inhibition of a Class C β-lactamase by eight phosphonamidate compounds possessing six different P₁' amino acid residues. Only two of the compounds were potent inhibitors of both carboxypeptidase A and P99. It is also worthy of note that the activity of some of these compounds against Class C β-lactamases exceeds the activity of some of the commercially available β-lactamase inhibitors [12]. In fact, ESMS studies have shown that the l-Phe, β-Ala and β-phenyl-β-Ala derivatives bind to P99 at a 1:1 molar ratio of inhibitor/enzyme. BRL 47215 (a 6-alkyldiene penem, prepared in these laboratories) and β-halogeno-

REFERENCES

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**β-Lactamase Ragged Ends Detected by Electrospray Mass Spectrometry Correlates Poorly with Multiple Banding on Isoelectric Focusing**

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Purified preparations of TEM-2, P99, *Bacillus cereus* I and *B. cereus* II β-lactamases were examined by electrospray (ES) mass spectrometry. The ES mass spectra of the *B. cereus* enzymes revealed the presence of four to five components of different mass, corresponding to the loss of different numbers of N-terminal amino acids (ragged ends). The ES mass spectra of both TEM-2 and P99 consisted of a single component with no evidence of ragged ends. All four β-lactamase preparations were visualized on isoelectric focusing (IEF) gels stained with nitrocellulose to investigate a possible correlation between IEF patterns and ragged ends. Multiple banding patterns were seen with each β-lactamase preparation. Although these may correlate with the presence of ragged ends in the two *B. cereus* preparations, the satellite bands seen with P99 and TEM-2 were not associated with differences detected by ES mass spectrometry. In this study we have shown for the first time that β-lactamase satellite bands seen on IEF are not always associated with ragged ends. Furthermore, we have illustrated the use of ES mass spectrometry to characterize the extent of ragged end formation in protein samples. This is of particular significance if the sample is required for detailed biochemical or crystallography experiments.

**INTRODUCTION**

β-Lactamases are bacterial enzymes which hydrolyse β-lactam antibiotics. They currently represent the most widespread resistance mechanism to this class of antimicrobial agent. Analytical isoelectric focusing (IEF) of β-lactamases has been used successfully to distinguish and classify the many different types of β-lactamases produced by clinical isolates. However, β-lactamase preparations often yield a main band on IEF together with a number of satellite bands of slightly different Mrs. Alternatively, β-lactamase preparations may focus as several bands of equal intensity. Such IEF patterns may represent a single enzyme or they may indicate the presence of a collection of related or unrelated β-lactamases. Inevitably this leads to confusion when IEF is used to characterize β-lactamases.

Simpson and Plested investigated the properties of the individual satellite bands in β-lactamase preparations from *Citrobacter diversus* 2046E and *Branhamella catarrhalis* 2001E separated by preparative IEF. They saw no significant differences in substrate and inhibitor profiles and molecular weight (measured by gel filtration) between the individual bands in each preparation suggesting the satellite bands represented similar, if not identical, enzymes. Matagne et al. used fast protein liquid chromatography (f.p.l.c.) chromatography to purify the β-lactamase satellite bands of four different β-lactamases. N-terminal sequencing of each of these satellite bands revealed they had lost different numbers of amino acids from their N-terminus (ragged ends). Franceschini et al. reported that both N- and C-terminal amino acids were missing to different extents in the satellite bands of a β-lactamase preparation obtained from *Citrobacter diversus* ULA27. These studies suggest a correlation between satellite bands on IEF and the presence of ragged ends. It is inferred that the different primary structure of ragged ends causes them to have slightly different Mrs from the complete protein. As ragged ends are a source of heterogeneity within a protein sample it is important that the presence of ragged ends is known, particularly if the sample is required for crystallography or complex biochemical studies.

Electrospray (ES) mass spectrometry can be used to determine the relative molecular mass (Mr) of proteins with an accuracy of about 0.01%. Recently the resolution of the technique has been demonstrated by providing direct evidence of the formation of acyl intermediates of the P99 β-lactamase by determination of the mass shift in the presence of various inhibitors and substrates. We reasoned that β-lactamase ragged ends could be identified by mass differences revealed by ES mass spectrometry. Subsequent comparison of the masses obtained by ES mass spectrometry with the deduced amino acid sequence would then allow complete and facile characterization of the preparation.

Therefore, in this paper four different β-lactamase preparations were analysed by ES mass spectrometry. We also report on the correlation between ragged ends

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revealed by ES mass spectrometry and the banding patterns of the same \( \beta \)-lactamase preparations on IEF.

**EXPERIMENTAL**

**Materials**

All the \( \beta \)-lactamase preparations were purchased from Porton Products Ltd. The *Bacillus cereus* I and II samples were supplied in 5 mM Tris-HCl pH 7 buffer, which is appropriate for ES mass spectrometry. The P99 \( \beta \)-lactamase (from *Enterobacter cloacae*) and TEM-2 (from *Escherichia coli*) were supplied in 10 mM potassium phosphate pH 7 buffer. Solutions of these two enzymes were dialysed overnight at 4\(^\circ\)C with 5 mM tris-HCl pH 7 buffer. Each enzyme was diluted in 5 mM tris-HCl buffer to between 55 and 17 pmol \( \mu \)l \(^{-1}\).

**IEF of \( \beta \)-lactamas**

A range of concentrations of each \( \beta \)-lactamase was examined by analytical IEF on polyacrylamide gels containing pH 3.5-10 ampholines. The enzymes were visualized by staining the gel with the chromogenic cephalosporin nitrocefin, which selectively stains \( \beta \)-lactamases.\(^1\) The pIs quoted for the major bands of each enzyme were those published by Bush.\(^7\)

**Quantification of \( \beta \)-lactamase bands on IEF**

Photographs of IEF gels of TEM-2 and P99 were scanned by an Ultrascan (LKB) densitometer. A range of enzyme concentrations were scanned. This revealed those tracks where the availability of the staining substrate (nitrocefin) did not limit the size of the \( \beta \)-lactamase band. For TEM-2 the substrate was non-limiting when 50, 75 and 100 ng were visualized on IEF and 75-150 ng of P99 could be loaded before the substrate became limiting. Band areas were expressed as a percentage of the sum of all the band areas.

**ES mass spectrometry and assay conditions**

ES mass spectra were measured on a VG BIO-Q triple-quadrupole atmospheric pressure mass spectrometer equipped with a VG ES interface. Twenty microlitres of each sample solution was diluted by addition of a further 20 \( \mu \)l of 2% formic acid in acetonitrile. Ten microlitres were then injected into the ES source via a loop injector (Rhodyne 5717) using water-acetonitrile (1:1) with 1% formic acid, at a flow rate of 2 \( \mu \)l min \(^{-1}\) (Applied Biosystems model 140A dual syringe pump). The mass spectrometer was scanned over the mass range 750-1550 Da. The instrument was calibrated with equine myoglobin.

**RESULTS AND DISCUSSION**

The TEM-2 \( \beta \)-lactamase

Figure 1 shows the ES mass spectrum of TEM-2. It consists of a series of multiply charged peaks ([M + nH\(^{+}\)]\(^{+}\)) ranging from \( m/z \sim 800 \) to 1500 due to a distribution of charge states from 37\(^+\) to 19\(^+\) and maximizing at \( m/z \sim 1000 \) (29\(^+\)). Statistical analysis of the \( M_\text{r} \) values calculated from the most abundant peaks gave an observed molecular weight of 28906.10 ± 1.55. This is within 1 Da of the \( M_\text{r} \) calculated from the amino acid sequence (28905.24)\(^9\) taking into account the presence of a single disulphide bond.\(^9\) This constituted mass assignment accuracy of 0.003% (Table 1). Mathematical transformation of Fig. 1 from the \( m/z \) domain to the mass domain, as described by Mann et al.,\(^10\) yielded the representation shown in Fig. 2.

The spectra in Figs 1 and 2 demonstrated that the TEM-2 sample was substantially free of contamination by other proteins and contained no detectable 'ragged ends'. The small peak approximately 180 Da higher than TEM-2 could not be measured accurately. However, it did not appear to correspond to a common adduct (e.g. multiples of 98 for phosphate or sulphate) and represented a very minor proportion of the preparation.

The TEM-2 preparation focused with a main band at pH 5.6 with four satellite bands (Fig. 3). Densitometer analysis suggested that the main band represented 80% of the TEM-2 activity and bands 3 and 5 corresponded to between 7% and 8% of the bands seen on IEF. Satellite bands 1 and 2 each represented approximately 2% of TEM-2 activity (Table 2).

Therefore, no evidence of ragged ends were detected by ES mass spectrometry in the TEM-2 enzyme preparation even though the preparation produced satellite bands on IEF. It is possible that two of the TEM-2 satellite bands (bands 1 and 2) did represent ragged end proteins which were not present in sufficient quantities to be detected by ES mass spectrometry. However, if the other two satellite bands (3 and 5) represented ragged ends they were present in sufficient proportions to have been detected by ES mass spectrometry.

![Figure 1. ES mass spectrum of TEM-2 (m/z domain).](image-url)
Table 1. Summary of ES mass spectral data for ß-lactamases studied

<table>
<thead>
<tr>
<th>Protein</th>
<th>Observed $M_\text{r}$</th>
<th>Theoretical $M_\text{r}$</th>
<th>Interpretation (N-terminal amino acids lost)</th>
<th>Difference between theoretical and observed molecular mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-2</td>
<td>28906.10 ± 1.55</td>
<td>28905.24</td>
<td>+0.06</td>
<td>0.003</td>
</tr>
<tr>
<td>P99</td>
<td>39196.56 ± 4.26</td>
<td>39206.40</td>
<td>-9.84</td>
<td>0.025</td>
</tr>
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<td>B. cereus</td>
<td>~28430</td>
<td>28434.25</td>
<td>-KHKN (N-terminal)</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>A 28548.97 ± 1.60</td>
<td>28548.35</td>
<td>-KHK (N-terminal)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>B 28676.12 ± 1.09</td>
<td>28676.52</td>
<td>-KH (N-terminal)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>C 28813.24 ± 1.89</td>
<td>28813.67</td>
<td>-K (N-terminal)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>D 28940.83 ± 2.80</td>
<td>28941.84</td>
<td>-SQKVEK (N-terminal)</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>E 24260.75</td>
<td>24260.75</td>
<td>-SQKV (N-terminal)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
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<td>24518.04</td>
<td>-SQK (N-terminal)</td>
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<tr>
<td></td>
<td>G 24617.17</td>
<td>24617.17</td>
<td>-SQ (N-terminal)</td>
<td>0.004</td>
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<tr>
<td></td>
<td>H 24745.35</td>
<td>24745.35</td>
<td>-S</td>
<td>0.010</td>
</tr>
</tbody>
</table>

The P99 ß-lactamase

Figure 4 shows the mathematically transformed data for P99. Two peaks are present, one for the enzyme and the other for myoglobin, which was previously used to tune the spectrometer. A later injection of the same preparation showed no evidence of myoglobin. Otherwise, no significant impurity peaks were observed. This shows that the mass of P99 was 39196.56. The molecular weight of P99 calculated from the sequence was 39206 (Table 1). This spectrum showed that no ragged ends could be detected in the P99 preparation.

Upon IEF, the P99 ß-lactamase had a main band at pI 8.2 and at least three satellite bands which focused just below pI 8.2 (Fig. 3). The main P99 band represented approximately 60% of the P99 activity. Satellite

![Figure 2. ES mass spectrum of TEM-2 ß-lactamase (mass domain).](image)

Table 2. ß-Lactamase bands of P99 and TEM-2 expressed as percentages of total ß-lactamase visualized on IEF gel

<table>
<thead>
<tr>
<th>Band</th>
<th>TEM-2 ß-Lactamase loaded (ng)</th>
<th>Average (%)</th>
<th>P99 ß-Lactamase loaded (ng)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5 2.6 4.5</td>
<td>2.9 ± 1.5</td>
<td>1 50 56 67 10 13</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>2</td>
<td>ND 1.8 2.4</td>
<td>2.1 ± 0.4</td>
<td>2 21 23 10 13 13</td>
<td>17 ± 6.2</td>
</tr>
<tr>
<td>3</td>
<td>5.8 6.2 11</td>
<td>7.7 ± 2.9</td>
<td>3 22 9.0 18 20 17 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>84 80 74</td>
<td>79 ± 6.0</td>
<td>4 8.0 13 6.0 5.0 7.8 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8.6 9.5 7.9</td>
<td>8.7 ± 0.8</td>
<td>ND, not detected</td>
<td></td>
</tr>
</tbody>
</table>
bands 2 and 3 each corresponded to approximately 17% of the total β-lactamase activity. Band 4 was the smallest P99 satellite band, which represented approximately 8% of the enzyme's activity (Table 2). Therefore, these satellite bands should also have been detected by ES mass spectrometry if they were ragged ends, since two of the satellite bands each represented up to 10-20% of the β-lactamase activity.

The B. cereus I β-lactamase

Figure 5 shows the mass domain for the ES mass spectra of the B. cereus I β-lactamase. Four major peaks were observed, these being labelled A–D. A fifth was also present at approximately 115 Da lower than A but the signal was too weak to measure its mass accurately.

Calculation of the molecular weight of the B. cereus I from the amino acid sequence deduced from the DNA sequence gave a value of 28941.84. These four peaks represented ragged ends of the mature protein and each peak represented the loss of a successive N-terminal amino acid. Peak D corresponded to the intact protein, peak C to the loss of Lys, peak B the loss of Lys-His and peak A the loss of Lys-His-Lys. The peak approx-
imately 115 Da lower than peak A could correspond to the loss of the next N-terminal amino acid, which was Asp (Table 1). The B. cereus I β-lactamase focused with at least two main bands around pI 8.6; some diffuse bands were also seen at lower pIs (Fig. 3). Repeated focusing of this sample did not yield better resolution of the bands.

No attempt was made to associate a particular IEF band with an identified N-terminus. These ragged ends could result from the action of exoproteases during purification/storage. Alternatively, they may be formed by cleavage of the signal peptide at adjacent sites. It is known, for example, that certain changes in the protein leader sequence can cause the signal peptidase to cleave at alternative sites. We have reported identical ragged ends with B. cereus I to those reported by Thatcher. Both preparations were different batches supplied by the same manufacturer and presumably prepared by the same procedure. This implies that the generation of ragged end termini is a repeatable phenomenon.

The sequence of B. cereus I was originally determined by Sloma and Gross in 1983 and then redetermined by Madgwick and Waley. The availability of an accurate molecular weight of the B. cereus I β-lactamase would have rapidly highlighted the error associated with the original sequence. ES mass spectrometry clearly provides a useful means of rapidly checking the validity of deduced amino acid sequences.

The B. cereus II β-lactamase

The transformed data for the B. cereus II enzyme is shown in Fig. 6. Five major components were present (designated A–E) and none of the molecular weights were consistent with the theoretical M<sub>e</sub> of B. cereus II, which was 24960.55 without the zinc atom. The mass of E measured approximately 90 Da lower than the theoretical value (24870.99 ± 2.41). The closest amino acid in terms of mass to account for this difference was serine (87 Da). The residue at the N-terminus of the deduced amino acid sequence of B. cereus II is indeed serine. Hence the measured mass was consistent with the loss of this residue from the N-terminus (theoretical M<sub>e</sub> = 24873.62). Furthermore, all of the other components can be explained as losses of subsequent amino acids from the N-terminus: D—loss of Ser-Gln; C—loss of Ser-Gln-Lys; B—loss of Ser-Gln-Lys-Val; and A—loss of Ser-Gln-Lys-Val-Glu-Lys (Table 1).

The B. cereus II sample contained 1 mM ZnSO<sub>4</sub>. However, the zinc atom was not observed attached to the proteins in the spectra. The excess Zn caused no deterioration of the spectrum.

Repeated isoelectric focusing of the B. cereus II enzyme revealed three diffuse β-lactamase bands (Fig. 3). Consequently, the B. cereus II preparation elaborated both ragged ends and satellite bands and it is possible that some of the satellite bands correlate with the ragged ends. The ragged ends observed in the B. cereus II preparation could have occurred by the same processes as discussed in the B. cereus I section.

CONCLUSIONS

We have used ES mass spectrometry to determine the purity and ragged end content of 4 β-lactamase preparations. No evidence of ragged ends was detected by ES mass spectrometry in the P99 or TEM-2 β-lactamase preparations even though both these enzyme preparations produced satellite bands on IEF. These observations weaken the potential for the correlation between β-lactamase satellite bands on IEF and the presence of ragged end proteins. Other processes could also give rise to variations in pI for a single protein, such as phosphorylation or deamidation. On the basis of the ES mass spectral data, phosphorylation of TEM-2 and P99 was not apparent to any significant extent. Deamidation would result in too small a change in mass to be observed by ES mass spectrometry and therefore cannot be discounted on the basis of the data reported. Alternative explanations, such as the existence of different conformations of the protein, must be considered as speculative at this stage.

On a more general point, a large number of proteins other than β-lactamases focus as more than one band on IEF. Such heterogeneity could cause complications in detailed biochemical investigations and may cause problems in protein crystallographic studies. Normally protein purity is investigated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. However, such a technique would not distinguish the subtle differences between ragged end proteins, and ES mass spectrometry provides a useful means of assessing this aspect of protein homogeneity.

In conclusion, this study has shown that β-lactamase satellite bands seen on IEF are not always associated with ragged ends. Furthermore, ES mass spectrometry can be used to characterize the extent of ragged end formation as well as providing a good indication of the purity of the protein sample. ES mass spectrometry also provides a rapid check of the validity of amino acid sequences either deduced from DNA sequences or generated directly.

Acknowledgements

We thank Graham Hill for his assistance with the densitometer readings and acknowledge Graham Clarke for helpful discussions.
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The effects of β-lactams on the isoelectric focusing of β-lactamases

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4679/08/93: accepted 22 October 1993

D.J. PAYNE AND S.G.B. AMYES. 1994. A range of concentrations of ceftazidime (4–64 mg l⁻¹) was shown to cause no induction of the TEM-1 and TEM-5 β-lactamases produced by Escherichia coli Nb. Increasing the concentration of ceftazidime in cultures of E. coli Nb caused a concomitant increase in the intensity of a satellite band of pI 5.2. The same increase in this satellite band was observed when ceftazidime was added to cell-free β-lactamase preparations from E. coli Nb and the separate addition of 11 different β-lactams to TEM-1 showed that each compound produced its own unique pattern of satellite bands. In addition, the mixing of ceftazidime with TEM-1 and 13 other TEM-derived β-lactamases caused a similar satellite band to be observed but ceftazidime did not have the same effect on PSE or SHV β-lactamases. Consequently, the addition of ceftazidime to a β-lactamase preparation prior to isoelectric focusing (IEF) may help to verify if a particular β-lactamase is TEM-derived. Purification of the satellite bands by electrodialysis and their subsequent re-focusing demonstrated that the ceftazidime-induced satellite bands can revert to a protein which has a pI similar to the parent band, illustrating the possible reversibility and dynamic nature of β-lactamase satellite bands on IEF. These results enable a better interpretation to be made of β-lactamase satellite bands observed on IEF.

INTRODUCTION

For the past 18 years β-lactamases have been identified and distinguished by the isoelectric focusing (IEF) method devised by Matthew et al. (1975). In this method crude bacterial lysates can be subjected to electrophoresis in a thin layer polyacrylamide gel containing 2% ampholines. The β-lactamases are then selectively identified from other bacterial proteins by overlaying the gel with nitrocefin which is a chromogenic cephalosporin. In almost all cases the β-lactamase focuses as a main band along with minor satellite bands which focus above and/or below the main band.

Simpson and Plested (1983) showed that different satellite bands of the β-lactamases produced by Citrobacter diversus and Branhamella catarrhalis had similar substrate profiles, molecular weights and susceptibilities to inhibitors. In addition, Matagne et al. (1991) used Fast Protein Liquid Chromatography (FPLC) to purify the β-lactamase satellite bands of four different β-lactamases. N-terminal sequencing of each of these satellite bands revealed that they had lost different numbers of amino acids from their N-terminus (ragged ends). There is little other documentation, however, on the nature of β-lactamase satellite bands. The results from an investigation of the inducibility of TEM β-lactamases have shown that β-lactams affect the IEF patterns of β-lactamases.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli Nb produced TEM-1 and TEM-5 and was a transconjugant of Klebsiella pneumoniae CFS04 (Petit et al. 1988). Escherichia coli J53-2 (R6K) produced the TEM-1 β-lactamase (Hedges et al. 1974). TEM-2, TEM-3, TEM-4, TEM-7, TEM-9, TEM-E1, TEM-E2, TEM-E3, TEM-E4, SHV-2 and SHV-3 were all produced by E. coli K12 J53-2 transconjugants. Enzymes A, B, C and D were extended spectrum TEM-derived enzymes obtained by spontaneous mutation from the TEM-1 or TEM-2 enzymes. Further details concerning each of the strains producing TEM- or SHV-derived β-lactamases can be obtained from Payne and Amyes (1991). PSE-1 and PSE-2 were produced by Pseudomonas aeruginosa PU21 (Matthew...

Induction experiments
Nutrient Broth No. 2 (Oxoid) (100 ml) was inoculated with 5 ml of an overnight broth culture of E. coli Nb and grown overnight in an orbital shaker at 37°C. Thirty ml of this culture were added to a flask containing 300 ml of pre-warmed nutrient broth. This culture was grown until the optical density reached A500 = 0.7 approx. Fifty ml of this E. coli Nb culture were then added to each of six flasks containing 30 ml of sterile Nutrient Broth No. 2. Cefazidime was added to five flasks to create final concentrations of 4, 8, 16, 32 and 64 mg l⁻¹, the remaining flask was the control containing no drug. The six cultures were incubated in an orbital shaker at 37°C for 2 h.

The cells from each culture were harvested by centrifugation and crude β-lactamase preparations were made from each of the cell pellets as described previously by Payne et al. (1992). The β-lactamase preparations were examined by thin layer isoelectric focusing (Matthew et al. 1975). The volume of β-lactamase sample loaded on the IEF gel was standardized so that an equal amount of protein was added from each of the preparations.

Kinetic characterization of β-lactamase preparations
The hydrolysis of cefazidime and cephaloridine by the β-lactamases produced by the E. coli Nb grown in cefazidime-free and cefazidime-containing media was determined by measuring the drop in optical density of each substrate at 260 and 264 nm respectively. The protein concentration of each preparation was determined by the method of Waddell (1956). The specific activity of each preparation was calculated for both cefazidime and cephaloridine.

Investigation of the effects of cefazidime on β-lactamases produced by Escherichia coli Nb
The β-lactamase sample obtained from the E. coli Nb cultures grown in the absence of cefazidime was divided into six 0.1 ml volumes. Cefazidime was added to five of the 0.1 ml volumes to achieve a range of concentrations from 0.1 to 5 mg ml⁻¹ of cefazidime. Each of these enzyme samples, along with the control containing no cefazidime, were applied to an IEF gel. The volume of each preparation loaded on the gel was adjusted to take into account the dilution caused by the addition of the cefazidime solution.

Isolation of β-lactamase satellite bands
Following IEF the β-lactamase bands produced by E. coli Nb grown with 64 mg l⁻¹ were cut out of the gel and separated from the gel matrix by electrodialysis (Payne et al. 1990). The separated bands were then re-examined by IEF.

Investigation of the effects of cefazidime on TEM-1
A crude preparation of TEM-1 was obtained from E. coli J53-2 (R6K) by the method described previously (Payne et al. 1992). This TEM-1 preparation was divided into nine equal volumes and cefazidime added to create a range of cefazidime concentrations from 0.3 to 20 mg ml⁻¹ of cefazidime. These samples, along with the control, were examined by IEF.

Table 1 Specific activities of β-lactamase samples prepared from Escherichia coli Nb grown at increasing concentrations of cefazidime

<table>
<thead>
<tr>
<th>Concentration of cefazidime in growth media (mg l⁻¹)</th>
<th>Specific activity with cephaloridine (nmol l⁻¹ min⁻¹ mg⁻¹ protein)</th>
<th>Specific activity with cefazidime (nmol l⁻¹ min⁻¹ mg⁻¹ protein)</th>
<th>Rate of hydrolysis of cefazidime (rate for cephaloridine taken as 100%)</th>
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<tr>
<td>0</td>
<td>6.85</td>
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<td>3.7</td>
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<td>4</td>
<td>9.04</td>
<td>0.36</td>
<td>4.0</td>
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<tr>
<td>8</td>
<td>6.89</td>
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<td>125</td>
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<tr>
<td>250</td>
<td>4.29</td>
<td>0.19</td>
<td>4.4</td>
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</table>
The induction experiments were performed to investigate whether the TEM-1 and TEM-5 \( \beta \)-lactamases, produced by *E. coli* Nb, were inducible. Figure 1 shows that the size of the TEM-1 (pI 5.4) or TEM-5 (5.5) band did not alter significantly when the strain was grown at increasing concentrations of ceftazidime. In addition, the specific activities of the preparations derived from cultures containing ceftazidime showed no marked increase when compared with the control (Table 1). The relative rates of hydrolysis of ceftazidime were also similar ranging between 3.6% and 4.3% (hydrolysis of cephaloridine taken as 100%) with no correlation with the increase in ceftazidime in the culture broth.

As the concentration of ceftazidime was increased in the cultures there was concomitant increase in the intensity of a \( \beta \)-lactamase satellite band at pI 5.2 (Fig. 1, tracks A–F).

**Fig. 1** Isoelectric focusing of \( \beta \)-lactamase samples prepared from cultures of *Escherichia coli* Nb grown at different concentrations of ceftazidime, and illustration of the effect of the addition of ceftazidime to a \( \beta \)-lactamase preparation from *E. coli* Nb grown with no ceftazidime. Tracks A–F are *E. coli* Nb grown with increasing concentrations of ceftazidime: A, 0; B, 4; C, 8; D, 16; E, 32; F, 64 (mg l\(^{-1}\) of ceftazidime in culture); G, TEM-1. Tracks H–L are *E. coli* Nb preparation mixed with increasing concentrations of ceftazidime: H, 0.2; I, 0.4; J, 0.9; K, 1.7; L, 5.0 (mg ml\(^{-1}\)); M, TEM-2

**Effects of different \( \beta \)-lactams on the TEM-1 \( \beta \)-lactamase**

A crude preparation of TEM-1, obtained from *E. coli* J53-2 (R6K), was divided into 12 equal volumes. A concentration of 6.4 mg ml\(^{-1}\) of a different \( \beta \)-lactam was created in 11 of them and the remaining sample was the control.

**Effects of ceftazidime on the IEF patterns of other \( \beta \)-lactamases**

Crude preparations of 21 different \( \beta \)-lactamases were split into two equal volumes. An appropriate volume of ceftazidime solution was added to one of the samples to create a final concentration of 6.4 mg l\(^{-1}\), an equal volume of 25 mmol l\(^{-1}\) phosphate buffer (pH 7) was added to the other volume (control). An equal volume of each sample was examined side by side on IEF.

**RESULTS**

The induction experiments were performed to investigate whether the TEM-1 and TEM-5 \( \beta \)-lactamases, produced by *E. coli* Nb, were inducible. Figure 1 shows that the size of the TEM-1 (pI 5.4) or TEM-5 (5.5) band did not alter significantly when the strain was grown at increasing concentrations of ceftazidime. In addition, the specific activities of the preparations derived from cultures containing ceftazidime showed no marked increase when compared with the control (Table 1). The relative rates of hydrolysis of ceftazidime were also similar ranging between 3.6% and 4.3% (hydrolysis of cephaloridine taken as 100%) with no correlation with the increase in ceftazidime in the culture broth.

As the concentration of ceftazidime was increased in the cultures there was concomitant increase in the intensity of a \( \beta \)-lactamase satellite band at pI 5.2 (Fig. 1, tracks A–F).
The satellite band of pl 5.2 (S1) was excised and electrodialysed from the IEF gel. Subsequent re-focusing of this extract revealed the S1 satellite band along with a series of bands of slightly higher pl and a band which focused marginally above the TEM-1 at pl 5.41 (Fig. 2). A similar phenomenon was also noted when a different satellite band of pl 5.3 (S2) was subjected to the same treatment (Fig. 2). The TEM-5 band (pl 5.5) was also excised and purified from the gel matrix; subsequent re-focusing of this extract revealed only the TEM-5 band.

The effect of ceftazidime on the satellite banding was further examined by adding the drug directly to the enzyme preparation. Figure 1 (tracks H–L) shows that the addition of ceftazidime to the E. coli Nb enzyme extract, obtained from a culture grown in the absence of ceftazidime, caused the same satellite band of pl 5.2 to occur. A similar \( \beta \)-lactamase satellite band was also observed when a crude preparation of TEM-1 was mixed with increasing concentrations of ceftazidime (Fig. 3); the intensity of the band was directly related to the concentration of ceftazidime added.

In order to establish if other \( \beta \)-lactam antibiotics could affect the satellite banding of the TEM-1 \( \beta \)-lactamase, the TEM-1 enzyme preparation was treated separately with 11 \( \beta \)-lactam antibiotics (64 mg ml\(^{-1} \)). Only cephaloridine, cefoxitin, cefuroxime, ceftazidime and cefotaxime were able to produce an extra band in the vicinity of pl 5.2. None of the penicillins tested were capable of doing this. Each \( \beta \)-lactam did, however, produce its own unique pattern of satellite bands (Fig. 4).

Ceftazidime also caused an extra satellite band in the region of pl 5.2 for Enzyme A, TEM-E2, TEM-E1, Enzyme B (Fig. 5a), TEM-7, TEM-9, TEM-5, TEM-E3, TEM-10, TEM-2 (Fig. 5b), Enzyme C, TEM-E4 and Enzyme D (Fig. 5c). Ceftazidime did not induce a satellite band in the vicinity of pl 5.2 with TEM-3, but an additional satellite band was observed at pl 5.4 (Fig. 5c). Ceftazidime had no effect on the IEF of the TEM-4 \( \beta \)-lactamase (Fig. 5c). Ceftazidime was also shown to cause differences in the satellite banding pattern of K-1, the most marked effect was an increase in intensity of satellite bands in the region of pl 5.2 (Fig. 5d). SHV-2 and SHV-3 produced extra satellite bands when mixed with ceftazidime but these had different pls from the ceftazidime-induced bands observed with the majority of the TEM-derived \( \beta \)-lactamases (Fig. 5d). Ceftazidime had no marked effect on the IEF of PSE-1 and PSE-2 \( \beta \)-lactamases (Fig. 5d).

Fig. 3 The effect of ceftazidime on the isoelectric focusing of TEM-1. A, 0; B, 0.3; C, 0.7; D, 1.2; E, 2.4; F, 4.4; G, 8.0; H, 13.0; I, 20.0 mg ml\(^{-1} \) ceftazidime in TEM-1 preparation.

Fig. 4 The effect of different \( \beta \)-lactams on the isoelectric focusing of TEM-1. A concentration of 6.4 mg ml\(^{-1} \) of each \( \beta \)-lactam was created in each aliquot of TEM-1. A, Penicillin; B, ampicillin; C, methicillin; D, carbenicillin; E, cephaloridine; F, cefoxitin; G, no \( \beta \)-lactam added; H, cephadrine; I, cefuroxime; J, ceftazidime; K, cefotaxime; L, nitrocefin.
**DISCUSSION**

This work has shown that the TEM-1 and TEM-5 enzymes produced by *E. coli* Nb were not inducible by ceftazidime; however, the growth of *E. coli* Nb in increasing concentrations of ceftazidime caused a corresponding increase in a \( \beta \)-lactamase satellite band of pI 5.2. This same satellite band was observed when ceftazidime was added to a cell-free \( \beta \)-lactamase preparation from *E. coli* Nb. In addition, the mixing of ceftazidime with a preparation of TEM-1 caused a similar satellite band to be observed and

Fig. 5 (a) Isoelectric focusing (IEF) patterns of TEM-derived \( \beta \)-lactamases with and without 6.4 mg ml\(^{-1}\) ceftazidime (Enzyme A-TEM-7). A, Enzyme A; B, enzyme A + ceftazidime; C, TEM-E2; D, TEM-E2 + ceftazidime; E, TEM-1; F, TEM-1 + ceftazidime; G, TEM-E1; H, TEM-E1 + ceftazidime; I, enzyme B; J, enzyme B + ceftazidime; K, TEM-7; L, TEM-7 + ceftazidime. (b) IEF patterns of TEM-derived \( \beta \)-lactamases with and without 6.4 mg ml\(^{-1}\) ceftazidime (TEM-7-TEM-2). A, TEM-7; B, TEM-7 + ceftazidime; C, TEM-9; D, TEM-9 + ceftazidime; E, TEM-5; F, TEM-5 + ceftazidime; G, TEM-E3; H, TEM-E3 + ceftazidime; I, TEM-10; J, TEM-10 + ceftazidime; K, TEM-2; L, TEM-2 + ceftazidime. (c) IEF patterns of TEM-derived \( \beta \)-lactamases with and without 6.4 mg ml\(^{-1}\) ceftazidime (TEM-2-TEM-3). A, TEM-2; B, TEM-2 + ceftazidime; C, enzyme C; D, enzyme C + ceftazidime; E, TEM-E4; F, TEM-E4 + ceftazidime; G, enzyme D; H, enzyme D + ceftazidime; I, TEM-4 (the arrow denotes the TEM-4 band); J, TEM-4 + ceftazidime; K, TEM-3; L, TEM-3 + ceftazidime. (d) IEF patterns of PSE-1, PSE-2, K-1, SHV-2 and SHV-3 \( \beta \)-lactamases with and without 6.4 mg ml\(^{-1}\) ceftazidime. A, PSE-1; B, PSE-1 + ceftazidime; C, PSE-2; D, PSE-2 + ceftazidime; E, TEM-E4 + ceftazidime; F, K-1 + ceftazidime; H, SHV-3; I, SHV-3 + ceftazidime; J, SHV-2; K, SHV-2 + ceftazidime
the addition of different β-lactams to TEM-1 resulted in satellite bands of different pl.

It has been shown by Bush et al. (1982) that the binding of aztreonam to the P99 β-lactamase causes a shift in pl of the enzyme from pl 8.2 to pl 7.2. It is probable therefore that on mixing a β-lactam with a β-lactamase, some of the β-lactam molecules will bind to the β-lactamase causing an alteration in the overall charge-to-mass ratio of the protein. These β-lactam-bound proteins will probably have different pl values from the native protein, consequently giving rise to the extra satellite bands observed on IEF. This implies that even β-lactams which are not readily hydrolysed by TEM-1, such as ceftazidime and ceftotaxime, interact with the protein to alter its IEF pattern. It is also possible that the satellite band patterns observed with the different β-lactams could be caused by the binding of intermediate degradation products of the β-lactam to a proportion of the β-lactamase. The IEF of the purified S1 and S2 satellite bands showed both the satellite bands as well as a band which focused slightly above the main TEM-1 band (Fig. 2). It is possible that the dilution caused by the purification of these bands resulted in some of the ceftazidime becoming unbound so causing a shift in pl resulting in the band of pl 5.41. Therefore, the satellite band did not revert to a β-lactamase of exactly the same pl as TEM-1 (pl 5.4). As the differences between TEM-1 and the band of 5.41 are so slight they could have been caused by irregularities in the IEF gel, consequently further work is required to ascertain the true nature of the band of pl 5.41.

A satellite band in the vicinity of pl 5.2 was also observed when ceftazidime was added to 14 TEM-derived β-lactamases (including TEM-1 and TEM-5). The addition of ceftazidime to preparations of SHV-derived enzymes caused extra satellite bands but these had different pl values from those seen with the TEM β-lactamases. However, ceftazidime caused no extra satellite bands to be observed with PSE-1 or PSE-2 β-lactamases. Consequently, the IEF of a β-lactamase preparation containing added ceftazidime may help to verify the identity of particular β-lactamases.

All the previous work on β-lactamase satellite bands suggests they were caused by the presence of proteins which had lost subsequent N-terminal amino acids (Franceschini et al. 1991; Matagne et al. 1991). This work shows, for the first time, that β-lactamase satellite bands can undergo changes in pl and therefore they are not always permanent features of a β-lactamase sample. In addition, both ragged ends and the interactions of β-lactams must be considered when contemplating the nature of β-lactamase satellite bands on IEF gels. Most important, the type of β-lactam added to a culture intended for β-lactamase preparation can have a marked effect on the satellite bands associated with the β-lactamase.

REFERENCES


SYNTHESIS AND TOXICITY STUDIES OF 2-FLUORO-5-CHLORO-CYCLO-Cyclic AMP.

Frances K. Cowart*, Hui Sun*, Peter Blumberg*, Kenneth M. Snader*, and Susan Donohue*.

a. Ash Stevens Inc., 5861 John C. Lodge Freeway, Detroit, MI 48202. Pharmaceutical Resources Branch* and Toxicology and Pharmacology Branch*, National Cancer Institute, Bethesda, MD 20892.

8-Chloroadenosine-3',5'-cyclic phosphate is a known differentiation agent which causes kidney toxicity in animals, presumably due to formation and precipitation of 2,5-dihydroxyxmisine. A 2-Fluoro-8-chloro-adenosine 3',5'-cyclic phosphate was prepared as an analog which would not produce this metabolite and that causes less toxicity. Compound 3 was synthesized over nine steps starting from guanosine. The key steps are the chlorination of 2-Fluoro adenosine triacetate precursor followed by dechlorination and phosphorylation, followed by cyclization of the 5'-phosphate with DCC. Total synthesis and preliminary toxicity of compound 3 will be the subject of our presentation.


The elaboration of efficient and safe vectors for gene delivery to living organisms represents a challenging issue. Synthetic non-viral gene transfer vectors, which are free from the risks associated with viral vectors, will be of considerable potential. Although a large variety of (poly)cationic lipids, liposomes and (lipo)polymers have been used extensively over the past decade to deliver genes to a variety of different cell lines and tissues, improvements are still necessary to provide efficient delivery systems. This implies the development of new compounds, especially cationic compounds, with properties different from those already described. In this respect, we present the synthesis of new lipopolyamine telomers and their in vitro capabilities of transferring genes into cells. These products, which show low degrees of polymerization (< 800) and relatively low molecular weights, were obtained from telomerizing a polymerizable aminoacylamide derivative in the presence of various hydrophilic double-chain alkane thiol transfer agents.


5-Enolpyruvyl-shikimate-3-phosphate (EPSP) synthase catalyzes the reversible transfer of an enolpyruvyl group from phosphoenol pyruvate (PEP) to shikimate-3-phosphate (S3P) to produce EPSP and Pi. The araA gene encoding EPSP synthase was identified in Streptococcus pneumoniae, cloned and overexpressed in Escherichia coli. The purified enzyme displayed minimal catalytic activity vs. PEP and S3P in the absence of monovalent cations. Activation of the enzyme by NaCl and K+ was significant. KMs for PEP and S3P were determined to be 21-100 μM and 22-145 μM, respectively, at a series of [NH4Cl] (1-100 mM) and [KCl] (50 to 100 mM). The tetracaine, tetracycline, is a competitive inhibitor vs. PEP, but an uncompetitive inhibitor vs. S3P, suggesting an ordered sequential mechanism for the substrates binding.

INHIBITION OF SERINE BETA-LACTAMASES BY LIPOPEPTIDES. Wensheng Du, Barry Orlek, Frank Fan, David J. Payne* Anti-Infectives Research, SmithKline Beecham Pharmaceuticals, 1250 S. Collegeville Road, Collegeville, PA 19426

Two semi-synthetic lipopeptides (Compounds I and II) were found to inhibit the serine active site beta-lactamase TEM-2, with IC50s of 13.1±0.2 μM and 3.8±0.28 μM, respectively. Steady-state kinetics showed that Compound II competitively inhibits TEM-2 with a Ki of 9.5 μM. Incubation of
Compound II with TEM-2 resulted in time-dependent loss of enzyme activity, but recovery of activity occurred after removal of excess inhibitor (T1/2 = 145 h for reactivation). These results suggest that Compound II does not covalently modify TEM-2. Compound II also inhibits the serine active site beta-lactamase P99 from Enterobacter cloacae with IC50s of ca. 5 μM. The inhibition was independent of reporter substrate concentrations (from 50 to 150 μM), implying that Compound II does not compete with substrate. To our knowledge, this is the first report of lipopeptides as inhibitors of serine active site beta-lactamases.

USE OF A NOVEL ELISA TO INVESTIGATE PH DEPENDENT INTERACTIONS BETWEEN ANTIBODIES TO N. MENINGITIDIS AND MEMBRANE BOUND COLOMINIC ACID. O. Oehrle-Steele, J. Gervay, Department of Chemistry, University of Arizona, Tucson, AZ, 85721

Lactonization of colominc acid at the cell surface may help bacterial meningitidis modulate the pH of the immediate environment as well as affect detection of the pathogen by the immune system. pH-dependent interactions with antibodies to N. meningitidis can be studied using ELISA-based techniques. Assay results will be discussed.

193. 6-OXA ISOSTERES OF ANACARDIC ACIDS AS POTENT INHIBITORS OF BACTERIAL HISTIDINE PROTEIN KINASE (HPK)-MEDIATED TWO-COMPONENT REGULATORY SYSTEMS. Ramesh M. Kanodia, William Murray, Jeffrey Bernstein, Jeffrey Fernandez, Barbara D. Polono, and John P. Barrett. The R. W. Johnson Pharmaceutical Research Institute, PO Box 300, 1000 Route 202, Raritan, NJ 08869-0602.

Anacardic acids are a group of naturally occurring 6-(C₆ alkyl or aryll)-2-hydroxybenzoic acids, isolated from cashew nut shell oil and other plant sources. They are reported to possess Gram-positive antibacterial activity of undetermined mechanism. As part of an ongoing program to discover novel antibacterial agents, less liable to develop drug resistance, our laboratories have focused on identifying novel inhibitors of bacterial two-component regulatory systems. Two-component regulatory systems are involved in regulation of many bacterial functions related to virulence and drug resistance and are absent in mammalian cells. In one approach, we have been investigating known antibacterial substances of undetermined mechanism. Since natural anacardic acids were not easily accessible, we synthesized a series of novel 6-oxa isosteres (I) of anacardic acids as representatives of this class of compounds. We have discovered that several analogues represent some of the most potent inhibitors of bacterial HPK two-component regulatory systems in our phosphoryl transfer biochemical assay systems.

194.

NEW BETA-LACTAMASE INHIBITORS OF THE CLASS A AND CLASS C ENZYMES. John D. Buynak, A. S. Rao, V. R. Doppalapudi, G. Adam, S. Nidamarthy, Chemistry Department, Southern Methodist University, Dallas, TX, 75275-0314

We have recently reported the synthesis and evaluation of the potent penam-derived class B beta-lactamase inhibitor 1 and a number of cephalosporin-derived inhibitors, including 2. These previous compounds had good activity as inhibitors of the class C enzymes, but somewhat reduced potency as inhibitors of TEM-1. We now report that selected 2'-substituted penam derivatives 3 exhibit enhanced inhibitory activity of the class A enzymes. New chemistry was developed leading to a straightforward and economical method of production of compounds 3.
Inhibition of Serine \( \beta \)-Lactamases by Lipopeptides

Wensheng Du, Barry Orlek, Frank Fan, and David J. Payne

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Introduction and Background

There are growing concerns about the resistance of bacteria to existing antibiotics in the United States and in the world.1 The primary causes of bacterial resistance to \( \beta \)-lactam-based antibiotics such as penicillins and cephalosporins are the production of \( \beta \)-lactamases that hydrolyze \( \beta \)-lactams rendering these antibiotics useless.2 Based on sequence similarities, \( \beta \)-lactamases are divided into four classes, A, B, C, and D. Class A and D enzymes are exemplified by the essential staphyloxysaerovar (thus the name serine-\( \beta \)-lactamases) 3 and Class B enzymes, respectively.4, 5 Class C \( \beta \)-lactamases are the most prevalent among pathogenic bacteria species, while Class D enzymes are relatively rare.

Two lipopeptides, Compound I and Compound II, have been identified as inhibitors of Class A (Escherichia coli TEM) and Class C (Enterobacter cloacae P99) serine \( \beta \)-lactamases. The structures of these two compounds are entirely different from all other known \( \beta \)-lactamases.

We report herein the kinetic studies of the interaction of Compound I and Compound II with TEM-2 and P99.

Materials and Methods

Chemicals and Reagents.

All chemicals and solvents were purchased from Sigma Co., St. Louis, MO. The enzymes TEM-2 (NP 26.9 KDa) and P99 (NP 23.6 KDa) were prepared from natural origins. Enzyme Activity.

All enzyme reactions were carried out in PFFS buffer (25 mM, pH 7.9). Nicotinamide (MW 114.1, \( \text{C}_{6}\text{H}_4\text{N}_2\text{O}_2\), \( \text{M}_{\text{w}} = 142 \text{~g} \text{~mol}^{-1}\)) and cephaloridine (MW 415.4, \( \text{C}_{18}\text{H}_{22}\text{N}_{2}\text{O}_8\), \( \text{M}_{\text{w}} = 516.4 \text{~g} \text{~mol}^{-1}\)) were used as reporter substrates for TEM-2 and P99, respectively. Enzyme activity was measured spectrophotometrically by following the increase in absorbance of the respective reporter substrate on a Beckman DU 7400S double beam spectrophotometer equipped with a microvolumes multiple sampler. A typical assay mixture of 500 \( \mu \text{M}\) of each nicotinamide or cephaloridine (6 - 20 \( \mu \text{M}\)) with varying substrate and inhibitor concentrations.

IC50 Measurement. For TEM-2, the reporter substrate was mixed with inhibitor, and the enzyme was added to start the reaction. For P99, the IC50 was determined either without pre-incubation, or after a 5-min pre-incubation of enzyme and inhibitor at room temperature.

Results

Without pre-incubation of inhibitor with enzyme, the IC50 of Compound I and II for TEM-2 increased with increasing concentrations of inhibitor, indicating that both compounds compete with nicotinamide for the active site of TEM-2.

When Compound II was incubated with TEM-2, a timedependent loss of enzyme activity was observed.

When the incubation mixture of Compound II with TEM-2 was dialysed extensively against buffer, followed by incubation at room temperature, a slow but distinct recovery of enzyme activity was observed.

Conclusion

The two lipopeptides, Compounds I and II, are inhibitors of TEM-2.

Compound II is a competitive inhibitor of TEM-2, with a \( K_i \) of 0.5 \( 	ext{mM} \).

Compound II inactivates TEM-2 in a time-dependent fashion. However, enzyme activity was recovered slowly after removal of excess inhibitor. These results suggest that Compound II does not covalently modify the enzyme.

Compound II also inhibits P99. The inhibition is independent of reporter substrate concentration, indicating that Compound II may not be an activator-directed \( \beta \)-lactamase inhibitor.

This is the first report of lipopeptides as inhibitors of serine \( \beta \)-lactamases.

References


Table 1. IC50 Values of Compound I and Compound II for TEM-2

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<th>Compound</th>
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<td>I</td>
<td>100</td>
<td>13.1±0.2</td>
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</tr>
<tr>
<td>II</td>
<td>150</td>
<td>3.9±3.8</td>
<td>6.8±0.3</td>
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</tbody>
</table>

Figure 1. Initial Velocity and Secondary Plot (inset) of Competitive Inhibition of TEM-2 by Compound I and Compound II

Figure 2. Residual Activity of TEM-2 Versus Time

Figure 3. Time-Dependent Loss of Enzyme Activity Showing Preliminary First-Order Kinetics

Figure 4. Recovery of TEM-2 Activity After Dialysis and Incubation

1. The interaction of Compound II with P99 was also investigated. Without pre-incubation of Compound II with the enzyme, IC50 did not change with varying concentration of cephaloridine, implying that Compound II and cephaloridine might not compete for the active site of the enzyme. With pre-incubation, however, IC50 decreased to 37 nM at 100 \( \mu \text{M} \) cephaloridine, suggesting that Compound II might rapidly inactivate P99.

1. Steady-state kinetics confirmed that Compound II functioned as a competitive inhibitor of TEM-2, with a \( K_i \) of 5 \( \mu \text{M} \).
Metallo-β-lactamases—a new therapeutic challenge

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Introduction

Over the last 30 years, many different β-lactamases with serine at the active site have been identified. Approximately 60 are plasmid mediated and there is probably a similar number of chromosomally mediated enzymes. In addition there is a relatively small group of β-lactamases in which the active site has a metal ion. The first of these, the *Bacillus cereus* II metallo-β-lactamase, was identified in 1966 when the cephalosporinase activity of a β-lactamase produced by a *B. cereus* isolate was shown to be inhibited by EDTA. Since then, many other metallo-β-lactamases have been identified from clinical isolates all over the world (table I). Metallo-β-lactamases are mechanistically different from β-lactamases which have serine at the active site. The metal ion active site appears to decrease their susceptibility to β-lactamase inhibitors and enables them to hydrolyse a broad spectrum of β-lactam agents including carbapenemases. Tables I–IV list those enzymes known to be metallo-β-lactamases as well as those suspected to belong to this group by virtue of susceptibility to EDTA or hydrolysis of carbapenemases. Comparative data on these enzymes is scarce and some of the enzymes listed may be duplicates.

The first reported metallo-β-lactamases were produced by organisms of minor clinical relevance, e.g., *Flavobacterium odoratum*,*B. cereus* or *Legionella gormanii*, and were regarded as rare curiosities. However, the recently discovered metallo-β-lactamases have been associated with more clinically relevant genera such as *Serratia*, *Bacteroides* and *Pseudomonas* and have forced a re-evaluation of the clinical importance of these enzymes.

Molecular structure of metallo-β-lactamases

*Bacillus* spp.

The *B. cereus* II enzyme isolated from *B. cereus* 569/H/9, is the only metallo-β-lactamase which has undergone X-ray crystallographic studies and most of the structural and mechanistic information on metallo-β-lactamases is derived from this enzyme.

As with many metallo-β-lactamases, the zinc ion of the *B. cereus* II enzyme can be replaced by different metal ions and still retain some β-lactamase activity (table IV). Equilibrium dialysis and H nuclear magnetic resonance (NMR) indicate that the *B. cereus* II enzyme is capable of binding two zinc ions. However, computer assisted molecular modelling indicates one major metal ion binding site with two minor sites, one of which is close to the major binding site and may affect the activity of the enzyme against certain substrates. Both the nucleotide and the amino-acid sequences of the *B. cereus* II enzyme have been determined and NMR studies have revealed that the zinc ion in the major binding site is surrounded by three histidine residues, but only two of these may be true ligands. A cysteine residue, adjacent to the zinc ion binding site, has also been implicated in the coordination of the zinc ion.

The mechanism of action of *B. cereus* II metallo-β-lactamase is thought to involve a water molecule, bound to the zinc ion of the active site, attacking the carbonyl group of the β-lactam ring. It was originally proposed that a glutamate residue, glutamate 37, acted as a general base, deprotonating the water molecule, with the proton being donated to the nitrogen atom of the β-lactam ring to cause cleavage. However, computer models showed that glutamate 37 was too far from the zinc ion to perform this function and that it was more likely that glutamate 212 was involved. More recently, site directed mutagenesis has revealed that neither glutamate residue is essential for the catalytic function of the enzyme. Lim et al. have now demonstrated that aspartate 90 is essential for enzyme activity and have proposed that this residue acts as the general base to assist in the hydrolysis of the amide bond of β-lactam substrates. The detailed function of the amino acids in the active site of the *B. cereus* II metallo-β-lactamase requires further elucidation.

A second *B. cereus* metallo-β-lactamase has also been sequenced. This enzyme, produced by *B. cereus* 5/B/6, a mutant strain of *B. cereus* 569/H/9, differs from the classic *B. cereus* II enzyme by only 18 amino-
### Table I. General properties of metallo-ß-lactamas

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Producer strain</th>
<th>Enzyme name</th>
<th>Metal ion required</th>
<th>Iso-electric point, pl</th>
<th>Molecular mass</th>
<th>Amino-acid homology with B. cereus</th>
<th>Plasmid encoded</th>
<th>Inducibility</th>
<th>Approx. year of isolation</th>
<th>Country of isolation</th>
<th>Ref. no.</th>
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<td>32†</td>
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</table>

* TAL2480 produces a ß-lactamase identical to that of Bact. fragilis TAL3636 (CcrA).
† Calculated from sequence.
‡ 58–77% homology around active site.²⁴
§ Determined by gel filtration.
‖ Determined by SDS-PAGE.
¶ Evidence suggests a metallo-ß-lactamase but insufficient evidence to assume it is a Zn²⁺ metallo-ß-lactamase.
†† 24.7% and 25% overall amino-acid homology with B. cereus II and Bact. fragilis CfiA, respectively; 61.6% and 66.2% similarity (after allowing for conservative substitutions) to B. cereus II and Bact. fragilis CfiA, respectively.²⁸

acid residues,¹⁴ and is classed as a B. cereus II-type metallo-ß-lactamase.

**Xanthomonas maltophilia**

The most extensively characterised X. maltophilia metallo-ß-lactamase is the L-1 enzyme. Only the N-terminal (32 residues) of the L-1 enzyme has been sequenced.¹⁸ It has a molecular mass c. four times greater when determined by gel filtration than by SDS-PAGE suggesting a tetramer structure.²⁴

**Bacteroides spp.**

Ten Bacteroides spp. metallo-ß-lactamas have been reported (tables I–IV) but, as discussed above, some of these enzymes may be identical. For example, the metallo-ß-lactamas produced by Bact. fragilis GAI30144 and Bact. fragilis GAI30079 are reported to have similar substrate profiles;¹⁷ the latter enzyme has been characterised in more detail¹⁸ but further work is required to compare these two enzymes. The metallo-ß-lactamas produced by Bact. fragilis strains TAL2480,¹⁰ GAI30144,¹⁷ G237,²⁰ 119²¹ and BFR81R²² all have similar iso-electric points (table I) as well as other number of similar characteristics (tables I–IV) but there are insufficient data to confirm that these enzymes are the same. The only metallo-ß-lactamase derived from a Bacteroides spp. other than Bact. fragilis is an enzyme isolated from a Bact. distasonis strain.²² This enzyme is distinct from other Bacteroides spp. metallo-ß-lactamas in having a molecular mass of 160,000 (table I) and being inhibited by clavulanic acid and sulbactam. It would be of interest to determine the molecular masses of this and other enzymes in table I by both gel filtration and SDS-PAGE as this may reveal other metallo-ß-lactamas which exist as multimers.

Cuchural et al.²² identified metallo-ß-lactamas in Bact. fragilis TAL3636 (CcrA) and Bact. fragilis TAL 2480 (CfiA). The nucleotide sequence of the ccrA²⁴ and cfiA²⁴ metallo-ß-lactamase genes showed that both strains produced identical enzymes. In addition, five strains of Bact. fragilis from the UK were probed with a ccrA gene probe to establish the prevalence of this metallo-ß-lactamase gene.²⁶ DNA from two of the strains hybridised with the probe and the nucleotide sequences of the metallo-ß-lactamase genes from these two isolates (Bact. fragilis QMCN3 and QMCN4) were c. 97% homologous with the ccrA ß-lactamase gene.²⁶ For the purpose of this review these four highly homologous enzymes will be referred to as CfiA-type metallo-ß-lactamas (table I).

Direct sequencing or probing has now confirmed the presence of the CfiA-type metallo-ß-lactamase gene in 11 clinical isolates of Bact. fragilis from three
Table II. MICs of $\beta$-lactams for strains producing metallo-$\beta$-lactamases

<table>
<thead>
<tr>
<th>Strain</th>
<th>cephalothin</th>
<th>ampicillin</th>
<th>penicillin G</th>
<th>carbencillin</th>
<th>cephaloridine</th>
<th>ceftaxime</th>
<th>clavulanate</th>
<th>imipenem</th>
<th>meropenem</th>
<th>anfotericin</th>
<th>piperacillin</th>
</tr>
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<td>$&gt;128$</td>
<td>$&gt;128$</td>
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<td>$&gt;128$</td>
<td>$&gt;128$</td>
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<td>$128$</td>
<td>$128$</td>
<td>$128$</td>
<td>$128^*$</td>
<td>$23$</td>
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<tr>
<td>B. fragilis G-30/144</td>
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<td>$100$</td>
<td>$100$</td>
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<tr>
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<td>$&gt;128^*$</td>
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<td>$16$</td>
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<td>$50$</td>
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<td>$16$</td>
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<td>$16$</td>
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</table>

* Resistant to piperacillin + tazobactam and ticarcillin + clavulanic acid combinations.
† Highly resistant to clavulanic acid.
‡ This is the MIC at $10^5$, MIC at $10^6 = 128$.

Table III. Efficiency of hydrolysis values of $\beta$-lactams by metallo-$\beta$-lactamases

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vmax/Km relative to cephaloridine for</th>
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</thead>
<tbody>
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<td>cephalothin</td>
</tr>
<tr>
<td>B. fragilis G-30/144</td>
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</tr>
<tr>
<td>B. fragilis G-237</td>
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</tr>
<tr>
<td>B. fragilis GFR 81</td>
<td>$100$</td>
</tr>
<tr>
<td>B. fragilis G-30/13</td>
<td>$100$</td>
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<td>B. fragilis G-30/137</td>
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<td>S. maltophilia 1607</td>
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</tr>
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<td>$100$</td>
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Vmax/Km relative to cephalothin: $V_{max}$ (for cephaloridine = 100%).
Km relative to cephalothin: $V_{max}/K_m$ (for cephalothin = 100%).
Kcat/Km relative to cephaloridine: $K_{cat}/K_m$ (for cephaloridine = 100%).

ND, not detectable.
‡ Relative rate of hydrolysis.

**Table IV.**

- Some other Bact. fragilis enzymes may be CfiA-type metallo-$\beta$-lactamases, suggesting that the CfiA-type $\beta$-lactamase may be the most prevalent metallo-$\beta$-lactamase in clinical isolates of Bact. fragilis.

**Aeromonas spp.**

Aeromonas spp. produce inducible $\beta$-lactamases which have hydrolytic activity against imipenem. In the uninduced form the $\beta$-lactamase confers only a low level of resistance to carbapenems. Mutant strains producing $\beta$-lactamase constitutively are highly resistant to a broad range of cephalosporins and imipenem. Detailed characterisation of one of these enzymes (A-2) showed that it was inhibited by EDTA and that zinc was required for activity (table IV).

An interesting phenomenon has been noted with the A-2 metallo-$\beta$-lactamase. When ID50 values were determined for clavulanic acid with penicillin G or nitrocefin as reporter substrate, the A-2 $\beta$-lactamase was sensitive to clavulanic acid. However, with imipenem as the reporter substrate, the A-2 $\beta$-lactamase appeared far less susceptible to clavulanic acid (table IV). The authors postulated that this could be caused by clavulanic acid binding close to the active site, blocking the access of penicillin G and nitrocefin, but not imipenem. Alternatively, the A-2
ß-lactamase may have far greater affinity for imipenem than for clavulanic acid. Further kinetic work may reveal information that would otherwise be obtainable only by detailed structural studies. These data indicate that ID50 values for metallo-ß-lactamases may vary with different substrates.

CphA is the other A. hydrophila-derived metallo-ß-lactamase which has been studied in detail.28 The CphA enzyme is inducible and has a similar pl and mol. wt to the A-2 enzyme (table I). The A-2 and CphA metallo-ß-lactamase may be identical enzymes.

**Table IV. Inhibitor profiles of metallo-ß-lactamases**

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<th>Cu²⁺</th>
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<th>Ca²⁺</th>
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<th>Cu²⁺</th>
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<th>CsSO₄</th>
<th>ZnSO₄</th>
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NI, not inhibited; I, inhibited; P, partially inhibited.
* +, ß-lactamase active after treatment with metal ion; −, ß-lactamase not active after metal ion; +/−, partial activity returned to ß-lactamase by metal ion. Figures refer to % activity compared to untreated control.
† p-chloromercuribenzoate.
‡ Clavulanic acid inhibits when nitrocefin or penicillin G were the reporter substrates, but enzyme resistant to clavulanic acid when imipenem was the reporter substrate.29

The metallo-ß-lactamase produced by S. marcescens S6 is inhibited by zinc sulphate and, furthermore, zinc sulphate does not restore the catalytic activity of the EDTA-treated enzyme.31 Table IV. This enzyme was thought to be a metallo-ß-lactamase but there is insufficient proof that zinc is the metal co-factor.31 Similar observations have been made with other metallo-ß-lactamases. For example, the F. odoratum metallo-ß-lactamase has almost the same activity with either zinc or iron as a metal co-factor (table IV) and the zinc ion of the X. maltophilia30 and Bact. fragilis GA301447 metallo-ß-lactamase can be replaced with calcium and cobalt ions respectively whilst retaining almost half the activity of the zinc centred enzyme (table IV). Therefore, it is possible that metallo-ß-lactamases with active site metal ions other than zinc may exist.

The S. marcescens S6 strain is more than 100 times more resistant to imipenem than to meropenem (table II) and the turnover of imipenem by the S6 enzyme is almost 28 times higher than that of meropenem.31 Metallo-ß-lactamases may confer resistance to, and hydrolyse, imipenem but not necessarily be cross-resistant to other carbapenems.

A second type of carbapenemase has been identified in S. marcescens. This imipenem resistant strain (US12) was isolated in 1970 and produces an enzyme with a pl less than that of the S6 enzyme (preliminary data determined by SmithKline Beecham Pharmaceuticals) but further characterisation is required to deduce whether it is a metallo-enzyme.

**Sequence homology between metallo-ß-lactamases**

Sequence data are available for eight of the 19 enzymes in table I and it is, therefore, possible to deduce the extent of homology between these enzymes. The A. hydrophila (CphA) and Bact. fragilis (CfiA type) metallo-ß-lactamases show 25%30 and 32%25 amino-acid homology, respectively, with the B. cereus II ß-lactamase. There is 25% homology between the A. hydrophila CphA and the Bact. fragilis CfiA type metallo-ß-lactamases.30 Overall, five regions of homology can be identified in these three metallo-ß-lactamases. The B. cereus II enzyme was the original enzyme of Class B in the Ambler molecular classification of ß-lactamases.30 As there is significant homology between the B. cereus II, CfiA and CphA metallo-ß-lactamases, these enzymes are all considered to be Class B ß-lactamases. However, the relatively low homology between the CphA and B. cereus enzymes suggests that the CphA protein may represent a subgroup of Class B.29 The limited sequence data available for the X.
maltophilia) metallo-β-lactamase (L-1) shows little homology with these Class B enzymes. Furthermore, the L-1 enzyme has a tetramer structure. These data suggest that the L-1 enzyme is distinct from other Class B β-lactamases and, until more sequence data is available, this enzyme should be allocated to a new class in the molecular classification of β-lactamases.

The clinical relevance of metallo-β-lactamases

The metallo-β-lactamases hydrolyse all cephalosporins, penicillins and all generations of cephalosporins (table II), and generally confer appreciable resistance to these antibiotics (table II). Unlike other β-lactamases, almost all of the metallo-β-lactamases hydrolyse and mediate resistance to imipenem. Of the limited number of metallo-β-lactamases tested, all hydrolyse meropenem, a new carbapenem (table III). Metallo-β-lactamases are produced by a wide variety of organisms and have a broad range of characteristics.

**Bacillus spp.**

Although the molecular structure and mode of action of *B. cereus* II metallo-β-lactamases have been studied extensively, their frequency in clinical strains of *B. cereus* is unknown. With the exception of *B. anthracis* and *B. cereus* in the context of food poisoning, the *Bacillus* spp. are usually dismissed as contaminants or as non-pathogenic when isolated from clinical specimens. However, in the last decade, other *Bacillus* spp., particularly *B. cereus*, have been isolated from both local and systemic infections. Production of a metallo-β-lactamase was not documented in any of these infections but as the importance of *Bacillus* spp. in the clinical setting increases, metallo-β-lactamases may be implicated in treatment failure.

**X. maltophilia**

As the L-1 enzyme hydrolyses imipenem (table III), it has generally been assumed that carbapenem resistance exhibited by *X. maltophilia* is mediated predominantly by the L-1 metallo-enzyme. However, this may not always hold true. Recently, Cullmann and Dick examined the enzymes produced by 20 multi-resistant clinical isolates of *X. maltophilia*. Iso-electric focusing of these enzymes revealed an unexpected heterogeneity with six different types of β-lactamases identified. All 20 strains hydrolysed imipenem and meropenem but, overall, meropenem was a better substrate than imipenem. Surprisingly, inhibition of carbapenem hydrolysis by 1 mm EDTA could not be demonstrated for any of the 20 enzymes; 0.1 mm EDTA causes a 93% inhibition of the L-1 metallo-β-lactamase. None of the strains produced significant amounts of enzyme without induction. Further work is required to ascertain the exact nature of these *X. maltophilia* β-lactamases. Another aspect to antibiotic resistance in *X. maltophilia* is the low permeability of its outer membrane, which may explain why strains are often also resistant to non-β-lactam agents such as the quinolones. In four recent surveys, 180 multi-resistant strains of *X. maltophilia* have been examined; all were resistant to imipenem. Garcia-Rodriguez et al. found that 95% of 42 clinical isolates of *X. maltophilia* were resistant to both imipenem and meropenem; 36 different antimicrobial agents and 33 different combinations of β-lactam and β-lactamase inhibitor were tested in vitro and only the combinations of aztreonam + clavulanic acid and amoxycillin + clavulanic acid + aztreonam were effective.

Some years ago, *X. maltophilia* was not recognised as a pathogen but a recent study by Cullmann found that it represented 2% of all isolates cultured from nosocomial respiratory tract infections. *X. maltophilia* is now being isolated with increasing frequency and is emerging as an important nosocomial pathogen. How much of the antimicrobial resistance exhibited by *X. maltophilia* is caused by the production of a metallo-β-lactamase is unknown and until this is determined it is not possible to deduce the true clinical relevance of metallo-β-lactamases in *X. maltophilia*.

**Bacteroides spp.**

An additional worry from the clinical viewpoint is the possibility of silent metallo-β-lactamase genes. Podglajen et al. showed that DNA from three carbapenem-sensitive and four carbapenem-resistant clinical strains of *Bact. fragilis* hybridised with a CfiA gene probe. The four sensitive strains could each mutate spontaneously to high-level carbapenem resistance (MIC of imipenem > 128 mg/L) with a concomitant increase in metallo-β-lactamase production following exposure to imipenem 32 mg/L. Therefore, some strains of *Bact. fragilis* carry silent metallo-β-lactamase genes which are activated following exposure to carbapenems. This work also implies that the production of the metallo-enzyme may be inducible, and that a mutation in the regulatory genes had caused the strains to produce the enzyme constitutively. Such mutations may cause high level carbapenem resistance in the clinical setting in a similar way to the cephalosporin resistance conferred by chromosomally mediated Group 18 β-lactamases which have become stably de-repressed.

In three recent surveys, a total of 327 strains of *Bacteroides* spp. was examined and 12 clinical isolates were found to produce enzymes which inactivated imipenem. The limited data available on these enzymes suggest they could be metallo-β-lactamases (these enzymes are excluded from tables I–IV). However, many other large surveys of *Bacteroides* spp. isolates have identified neither imipenem resistance nor imipenem hydrolysing enzymes, suggesting that frequency of metallo-β-lactamases in clinical isolates of *Bacteroides* spp. is low but significant.

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*METALLO-β-LACTAMASES*
**Aeromonas spp.**

Aeromonas spp. are increasingly implicated in human infections, particularly wound and enteric infections. A significant number of clinical isolates of Aeromonas spp. produce inducible carbapenemases and there is evidence to suggest these are metallo-enzymes. Where conditions for induction of these enzymes are provided, they could pose difficult therapeutic problems.

The production of metallo-$\beta$-lactamases has also been confirmed in single clinical isolates of Pseudomonas aeruginosa and S. marcescens. If metallo-$\beta$-lactamases become widespread in these species the current difficulties in selecting antimicrobial agents to treat infections caused by these nosocomial pathogens will be compounded.

**Plasmid mediated metallo-$\beta$-lactamases**

Only two of the metallo-$\beta$-lactamases in table I have been shown to be plasmid mediated. P. aeruginosa GN17203 produces a metallo-$\beta$-lactamase which was transferable by conjugation to other strains of P. aeruginosa but not to E. coli. This metallo-$\beta$-lactamase confers resistance to third generation cephalosporins and carbapenems. It mediates a higher level of resistance to meropenem than to imipenem (table II) although it hydrolyses both of these compounds with similar efficiency (table III).

The other transferable metallo-$\beta$-lactamase is produced by Bact. fragilis 10-73 and is transferable to other Bact. fragilis strains by conjugation. This enzyme hydrolyses meropenem at a greater rate than imipenem (table III).

**Conclusions**

Metallo-$\beta$-lactamases confer a far broader range of resistance than any of the Group 1 or Group 2$\beta$-lactamases and it is important to assess the relevance of metallo-$\beta$-lactamases in the clinical environment. It must be emphasised that metallo-$\beta$-lactamases are not the only cause of resistance to carbapenems. Altered permeability and serine-based chromosomally mediated $\beta$-lactamases have also been implicated in imipenem resistance. However, the recent isolation of plasmid mediated metallo-$\beta$-lactamases suggests that there is the potential for rapid dissemination and the incidence of metallo-$\beta$-lactamases may increase over the next few years with these enzymes becoming the most common cause of carbapenem resistance.

Metallo-$\beta$-lactamases are now being isolated from more established pathogens, such as S. marcescens, P. aeruginosa and Bact. fragilis. Furthermore, other less pathogenic organisms which can produce metallo-$\beta$-lactamases are increasingly implicated in infections, particularly in immunocompromised patients. The inducibility of metallo-$\beta$-lactamases in Aeromonas spp. and X. maltophilia and the discovery of covert metallo-$\beta$-lactamase genes in Bact. fragilis are additional concerns. The increasing use of imipenem together with the imminent launch of meropenem will add to the selective pressure for metallo-$\beta$-lactamases.

Combating metallo-$\beta$-lactamases may prove to be a difficult problem. Current data suggests that they are a heterogeneous group of enzymes which implies that there could be problems in the design of agents that would be efficient against all metallo-$\beta$-lactamases.

I thank my colleagues at SmithKline Beecham Pharmaceuticals for their helpful discussions.

**References**


Rapid Identification of Metallo- and Serine β-Lactamases

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Simple methods to detect, identify, and differentiate metallo- and serine β-lactamases were developed and used to differentiate enzymes produced by 17 clinical isolates of Xanthomonas maltophilia. All isolates exhibited β-lactamase activity, and in 16 strains this was induced by imipenem. All but one isolate hydrolyzed imipenem (and meropenem), and in all cases this activity was inhibited by 1 mM EDTA. The metallo- and serine β-lactamases in the cell extracts were distinguished on isoelectric focusing (IEF) gels by using the following procedures. (i) Cell lysates were preincubated with 83 mM EDTA prior to IEF and subsequent visualization with nitrocefin, and (ii) after IEF, the gels were overlaid with either 1 mM zinc sulfate or 100 µM BRL 42715 before staining with nitrocefin. Bands of β-lactamase activity which were removed by BRL 42715 but unaffected by EDTA or zinc sulfate were categorized as serine β-lactamases. Bands which were unaffected by BRL 42715 but inhibited by EDTA or enhanced by zinc sulfate were classified as metallo-β-lactamases. By using this approach, seven metallo-β-lactamases were differentiated with pI values of 4.8 (two strains), 5.5 (four strains), 5.7 (one strain), 6.0 (one strain), 6.4 (four strains), 6.6 (one strain), and 6.8 (three strains). The metallo-β-lactamase band with a pI of 6.4 aligned with the recently characterized metallo-β-lactamase from X. maltophilia 511. Heterogeneity was also observed for the serine β-lactamases: 14 isolates elaborated serine β-lactamase activity which focused with major bands with at least eight different pIs. The remaining three strains produced serine β-lactamases which focused with five distinct bands with pIs of 6.4, 6.2, 5.7, 5.5, and 5.2. We conclude that X. maltophilia produces many types of metallo- and serine β-lactamases distinguishable by these new methods and that the previously reported L-1 and L-2 enzymes are not solely representative of the β-lactamases produced by this species.

**MATERIALS AND METHODS**

**Bacterial strains.** All of the clinical isolates of X. maltophilia used in this study were identified by API and were obtained from hospitals within the United Kingdom. X. maltophilia 511 is a control strain and produces the metallo-β-lactamase characterized by Felici et al. (6). Escherichia coli K-12 strain 153-2 produced TEM-1.

**Antibacterial compounds.** Ceftazidime was a gift from Glaxo, cefotaxime was from Roche, ciprofloxacin was from Bayer, imipenem was from MSD, meropenem was from ICI, and cephradine was from Hoechst. Nitrocefin and BRL 42715 were prepared in our own laboratories.

**Antibacterial susceptibilities.** MIC determinations were performed by the agar dilution method recommended by the National Committee for Clinical Laboratory Standards (8).

**Growth of cultures and preparation of β-lactamases.** Ten-milliliter aliquots of Nutrient Broth No. 2 (Oxoid, Basingstoke, United Kingdom) was inoculated with each of the X. maltophilia strains and the E. coli TEM-1 producer. These cultures were incubated overnight at 30°C in an orbital shaker. One milliliter of each overnight culture was then added to 2 × 100 ml of Nutrient Broth No. 2 and grown at 30°C for 2 h. Imipenem (final concentration, 5 µg/ml) was then added to one of each pair of cultures. Following a further 2.5 h of growth, the cells were harvested and washed with 25 mM piperazine-N,N' bis(2-ethanesulfonic acid) (PIPES) buffer, pH 7.0. The final pellet was resuspended in 1 ml of 25 mM PIPES buffer, pH 7.0, and subjected to ultrasonication. The resultant lyte was cleared by centrifugation to provide the β-lactamase preparation (12).

**Determination of specific activity.** The protein concentra-
ion of each cell extract was determined by the Bio-Rad Protein Estimation Kit. The β-lactamase activity of each preparation was determined by measuring the initial rate of hydrolysis of 100 μM nitrocefin. All enzyme assays were performed in 25 mM PIPES buffer, pH 7.0. The β-lactamase preparations from induced cultures had to be diluted between 10 and 1,000 times in 25 mM PIPES buffer, pH 7.0, to achieve an optimal rate of nitrocefin hydrolysis. With the exception of the extract from X. maltophilia GEL, the rates of hydrolysis of nitrocefin by the preparations from noninduced cultures were measured with undiluted samples. Specific activity was expressed as the rate of hydrolysis of nitrocefin per milligram of protein (nanomoles per minute per milligram of protein).

Biochemical characterization of β-lactamase preparations. The initial rates of hydrolysis of β-lactams (100 μM) by the each of the cell extracts were measured with a Beckman DU7400 spectrophotometer. The rates of meropenem and imipenem degradation were measured at 300 and 299 nm, respectively. To achieve optimum rates of hydrolysis, the extracts were diluted as described above. The initial rate of hydrolysis of imipenem was also measured following 10 min of preincubation (37°C) of the enzyme preparation with 1 mM (final concentration after addition of the substrate) EDTA. The level of inhibition caused by the EDTA, which is a known metal chelator, was expressed as a percentage of the rate of imipenem hydrolysis by the untreated control. The 50% inhibitory concentrations (IC50) of BRL 42715, EDTA, and zinc sulfate for TEM-1 and the β-lactamases produced by X. maltophilia 511 were measured following enzyme and inhibitor preincubation as described by Payne et al. (11).

Characterization of metallo-β-lactamases by isoelectric focusing (IEF). β-Lactamase preparations from both induced and noninduced cultures were examined by IEF as described by Matthew et al. (7). Filter paper applicator tabs were used to load all of the samples onto IEF gels. The isoelectric points of the X. maltophilia enzymes were measured from a plot of distance migrated versus pl for known proteins by using a pH 4.7 to 10.6 pl calibration kit (BDH Chemicals Ltd., Poole, England). Three methods were used to differentiate the X. maltophilia β-lactamases on IEF gels. (i) To investigate the effects of EDTA, a 10-μl aliquot of the crude lysate was mixed with 2 μl of 0.5 M EDTA in 25 mM PIPES buffer, pH 7.0 (final EDTA concentration, 83 mM), and another 10-μl aliquot was mixed with 2 μl of 25 mM PIPES buffer, pH 7.0. Both of these samples were then loaded and focused on the IEF gel and visualized in the normal manner with nitrocefin (0.5 mg/ml). (ii) The IEF gels were overlaid with filter paper soaked in 25 mM PIPES buffer, pH 7.0, with 1 mM zinc sulfate before staining with nitrocefin to enhance the identification of any zinc-dependent β-lactamases. (iii) Prior to nitrocefin staining, gels were overlaid with filter paper soaked in 25 mM PIPES buffer, pH 7.0, with 100 μM BRL 42715, which is a potent inhibitor of serine β-lactamases (3). Enzyme preparations from the X. maltophilia 511 and E. coli K-12 strain J53-2 TEM-1 were also subjected to each of these protocols as controls.

RESULTS

Antibacterial susceptibilities and β-lactamase induction. The MICs of imipenem and meropenem for X. maltophilia AA158 were 16 and 1 μg/ml, respectively, and the MICs of carbapenems for all of the other strains were greater than or equal to 64 μg/ml (Table 1). X. maltophilia GEL was the only clinical isolate which produced high levels of β-lactamase constitutively, and induction caused only a fivefold increase in β-lactamase activity in strain U152. All of the other strains showed a >80-fold increase in β-lactamase activity following induction with imipenem (Table 2). The control strain, X. maltophilia 511, produced high levels of β-lactamase constitutively, and imipenem caused no induction of TEM-1. The β-lactamase activity in most of the extracts from noninduced cultures was very low. Therefore, substrate profile determinations were performed on extracts from the induced cultures.

Biochemical characterization of β-lactamase preparations. Of the 17 extracts from the clinical isolates, 16 hydrolyzed both meropenem and imipenem. Extracts from seven of the isolates and the extract from X. maltophilia 511 hydrolyzed meropenem at a rate greater than or equal to that for imipenem. The hydrolysis of imipenem by these 16 preparations and the extract from X. maltophilia 511 was inhibited between 57 and 99% by 1 mM EDTA relative to the untreated control (Table 2). No detectable hydrolysis of carbapenems by the extract from X. maltophilia AA158 and E. coli J53-2 (TEM-1) was observed.

### Table 1. Antibacterial susceptibilities of X. maltophilia clinical isolates

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<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>AA158</td>
<td>8</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
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</tbody>
</table>
TABLE 2. Biochemical assessment of β-lactamase extracts from clinical isolates of X. maltophilia, X. maltophilia 511, and TEM-1-producing E. coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sp act (nmol/min/mg)</th>
<th>Induction ratio (induced/noninduced Sp act)</th>
<th>Initial rate of hydrolysis of imipenem (mol/min/mg)</th>
<th>Hydrolysis of meropenem relative to imipenem (%)</th>
<th>Inhibition of imipenem hydrolysis by 1 mM EDTA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninduced</td>
<td>Induced</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli (TEM-1 producing)</td>
<td>320</td>
<td>&lt;0.1</td>
<td>1.247</td>
<td>92</td>
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</tr>
<tr>
<td>X. maltophilia 511</td>
<td>5,717</td>
<td>1</td>
<td>1.347</td>
<td>92</td>
<td></td>
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<tr>
<td>X. maltophilia GEL</td>
<td>2,783</td>
<td>1</td>
<td>1.783</td>
<td>91</td>
<td></td>
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<td>X. maltophilia BS1384</td>
<td>3.9</td>
<td>1</td>
<td>1.783</td>
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<td>6.2</td>
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<td>X. maltophilia 0062</td>
<td>4.7</td>
<td>1</td>
<td>69</td>
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<td>X. maltophilia 00107</td>
<td>7.8</td>
<td>1</td>
<td>80</td>
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<td>X. maltophilia U152</td>
<td>525</td>
<td>1</td>
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<td>109</td>
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<td>X. maltophilia 1223</td>
<td>15</td>
<td>1</td>
<td>80</td>
<td>109</td>
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<td>X. maltophilia 00157</td>
<td>3.9</td>
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<td>86</td>
<td>99</td>
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<td>6.0</td>
<td>1</td>
<td>90</td>
<td>104</td>
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<td>8,904</td>
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<td>X. maltophilia 10258</td>
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<td>226</td>
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<td>X. maltophilia ED156</td>
<td>15</td>
<td>1</td>
<td>55</td>
<td>244</td>
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<tr>
<td>X. maltophilia H-25</td>
<td>3.3</td>
<td>1</td>
<td>333</td>
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<tr>
<td>X. maltophilia A37454</td>
<td>13</td>
<td>1</td>
<td>12,240</td>
<td>28</td>
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<tr>
<td>X. maltophilia 00141</td>
<td>9.5</td>
<td>1</td>
<td>1,962</td>
<td>58</td>
<td></td>
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<tr>
<td>X. maltophilia AA158</td>
<td>22</td>
<td>1</td>
<td>&lt;0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TEM-1 and the serine β-lactamase produced by X. maltophilia 511 were sensitive to inhibition by BRL 42715 (I50 <0.005 µM), but both enzymes were resistant to inhibition by EDTA (I50 >167 mM), and neither enzyme was activated by 1 mM zinc sulfate. The metallo-β-lactamase from strain 511 (pl 6.4) was not inhibited by BRL 42715 (I50 >100 µM) but was sensitive to EDTA (I50 =5 µM). This enzyme was activated threefold by 1 mM zinc sulfate. Consequently, these results illustrate that EDTA, zinc sulfate, and BRL 42715 can be used to differentiate between metallo- and serine-β-lactamases and illustrate the rationale behind the IEF assays described below.

IEF of cell extracts. IEF illustrated that all of the cultures of X. maltophilia produced the same β-lactamase bands irrespective of whether they were induced or noninduced. With the exception of those of strain GEL, all of the bands seen in extracts from noninduced cultures were weaker than the corresponding bands in the induced cultures. Therefore, all further IEF analyses were performed on extracts from induced cultures.

Repeated attempts to focus the β-lactamases from the X. maltophilia strains failed to achieve narrowly focused bands like those commonly obtained with TEM-1. It is possible that these broader bands represent more than one enzyme or are artifacts of the extraction and IEF procedures. Further investigations are required to elucidate the nature of these bands.

Consequently, for this work, each pl quoted refers to the most intense part of the β-lactamase band. IEF of the X. maltophilia extracts showed that this species produced β-lactamases with a wide variety of pl values (Table 3). IEF assays using the conventional protocol (Table 3, column 2) illustrated that 9 of the 17 clinical isolates focused with two main bands, one with a pl greater than or equal to 7.7 and the second with a pl less than or equal to 6.6 (Table 3, column 2). Only one β-lactamase band with a high pl was visualized in X. maltophilia LL284, GE5, 0062, and 00107, and the extract from strain AA158 focused as a series of bands from pl 8.5 to pl 5.5 (Table 3, column 2). Extracts from X. maltophilia H25, 00141, and A37454 had identical IEF patterns, with six bands between pl 6.8 and pl 5.2 (Table 3, column 2). A similar IEF pattern for the β-lactamases produced by X. maltophilia H25 has been observed previously (9a).

Effects of EDTA on visualization of β-lactamase activity by IEF. Examination of the enzyme preparations from X. maltophilia 511 on IEF with and without EDTA illustrated that the metallo-β-lactamase activity with a pl of 6.4 was absent in the EDTA-treated sample and the band with a pl of 9.7 remained unaffected (Fig. 1). The EDTA treatment had no effect on the TEM-1 β-lactamase. Figure 1 shows extracts from seven of the clinical isolates focused with and without EDTA, and in each case one of the bands of β-lactamase activity was removed by the EDTA treatment, leaving the other band(s) unaffected. Only the top band (pl 6.8) was eliminated from the IEF patterns of X. maltophilia H25, and the extracts from X. maltophilia A37454 and 00141 behaved similarly (Table 3). Overall, the EDTA treatment prevented visualization of a band in 12 of the 17 clinical isolates (Table 3). A total of six different EDTA-sensitive enzymes were observed, and these had plS of 4.8 (two strains), 5.7 (one strain), 6.0 (one strain), 6.4 (four strains), 6.6 (one strain), and 6.8 (three strains) (Table 3). The β-lactamases with a pl of 6.4 were focused side by side with the metallo-β-lactamase from X. maltophilia 511, and in all cases the enzymes aligned with the strain 511 β-lactamase (data not shown).

Effects of zinc sulfate overlay on visualization of β-lactamase activity by IEF. EDTA had no effect on the β-lactamase bands visualized in extracts from X. maltophilia LL284, GE5, 0062, and 00107 (Table 3). However, when the gel was overlaid with 1 mM zinc sulfate prior to staining with nitrocefin, a zinc-dependent β-lactamase with a pl of ca. 5.5 was visualized in the extract from each of these four strains (Table 3). Exposure of the other focused extracts to a 1 mM zinc sulfate overlay caused the bands already visualized to become more intense.

Effect of BRL 42715 overlay on visualization of β-lactamase activity. The IEF gel in Fig. 2a was overlaid with 1 mM zinc sulfate prior to staining and shows the β-lactamase activity of the extract from strain 511 along with the extracts which elaborated the seven different types of enzymes which the previous experiments have illustrated to be either inactivated by EDTA or activated by zinc sulfate. Another gel loaded with the same extracts was overlaid with 100 µM BRL 42715 in addition to zinc sulfate prior to staining with nitrocefin (Fig.
3b) This illustrated that BRL 42715 had no effect on any of the bands which were either EDTA sensitive or activated by zinc sulfate but eliminated the activity of those bands unaffected by these two treatments (Table 3).

All 17 clinical isolates produced BRL 42715-sensitive enzymes which were unaffected by EDTA or zinc sulfate. Four focused at pl 9.7 or 9.6, four focused at pl 9.9 or 10.0, two focused at pl 8.6, and four others focused at another four different pl's (Table 3). The remaining three isolates produced identical multiple bands which were sensitive to BRL 42715.

**DISCUSSION**

ß-lactamase activity was markedly increased following induction with imipenem in all of the strains investigated, except X. maltophilia GEL, which produced high levels of the enzyme without induction. This suggests that most multiply resistant clinical isolates of X. maltophilia possess inducible ß-lactamases and that X. maltophilia GEL may have a mutation in its regulatory gene(s) which causes high enzyme levels to be produced constitutively. IEF of ß-lactamase preparations from induced and uninduced cultures showed that in all cases induction was a result of an increase in production of all of the ß-lactamase bands seen on the gel and was not exclusively associated with one of the ß-lactamases produced by the strain.

Extracts from induced cultures of 16 strains hydrolyzed the carbapenems, meropenem, and imipenem. Hydrolysis of imipenem was inhibited by 1 mM EDTA, providing strong evidence for the involvement of metallo-ß-lactamases. Cullmann and Dick (4) failed to detect EDTA inhibition of the imipenemase activity of ß-lactamases from 20 clinical isolates of X. maltophilia. This may be explained if their ß-lactamase preparations contained high levels of metal ions and other

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**TABLE 3. Effects of EDTA, zinc sulfate, and BRL 42715 treatments on ß-lactamases from 17 clinical isolates of X. maltophilia, X. maltophilia 511, and TEM-1 producing E. coli.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>pl of band(s) seen on conventional IEF</th>
<th>Band seen when prep focused with EDTA</th>
<th>Band(s) seen after treatment with zinc sulfate</th>
<th>Bands seen after treatment with BRL 42715</th>
<th>pl of metallo-ß-lactamase (type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K-12 R53-2 (TEM-1)</td>
<td>5.4</td>
<td>5.4</td>
<td>5.4</td>
<td>5.4</td>
<td>None</td>
</tr>
<tr>
<td>X. maltophilia 511 (L-1)</td>
<td>6.4, 9.7</td>
<td>5.7</td>
<td>5.4</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>X. maltophilia GEL</td>
<td>5.5, 10.3</td>
<td>9.7</td>
<td>5.4</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
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<td>9.7</td>
<td>5.4</td>
<td>6.4</td>
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<tr>
<td>X. maltophilia LL284</td>
<td>9.9</td>
<td>9.9</td>
<td>5.4</td>
<td>6.4</td>
<td>6.4</td>
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<tr>
<td>X. maltophilia G155</td>
<td>9.5</td>
<td>9.9</td>
<td>5.4</td>
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<td>X. maltophilia 0062</td>
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<td>8.9</td>
<td>5.4</td>
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<tr>
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<td>6.8, 9.6</td>
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<tr>
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<td>None</td>
<td>5.5–8.5</td>
</tr>
</tbody>
</table>

*In each case, the same banding pattern was observed; bands with pl's of 6.4, 6.2, 5.7, 5.5, and 5.2 were identified.

---

**FIG. 1.** Effect of 83 mM EDTA on the IEF of ß-lactamases from X. maltophilia. Lanes: a, X. maltophilia A37454; b, A37454 plus EDTA; c, 37 plus EDTA; d, 37; e, 152 plus EDTA; f, 152; g, 10258 plus EDTA; h, 10258 plus EDTA; i, 10258; j, 10257 plus EDTA; k, 10257; l, 00157 plus EDTA; m, 00157; n, ET136 plus EDTA; o, ET136; p, TEM-1 plus EDTA; q, TEM-1; r, 511 (known metallo-ß-lactamase producer); s, 511 plus EDTA.

**FIG. 2.** IEF of X. maltophilia extracts showing each of the seven different types of metallo-ß-lactamases identified in this survey. Lanes: a, X. maltophilia GEL; b, 0062; c, U152; d, 511 (known metallo-ß-lactamase producer); e, 37; f, 12323 g, 136; h, A37454. Panel a, gel overlaid with 1 mM zinc sulfate and stained with nitrocefin; b, gel overlaid with 1 mM zinc sulfate and BRL 42715 prior to nitrocefin staining.
IDENTIFICATION OF METALLO- AND SERINE β-LACTAMASES

Although the various metallo- and serine β-lactamases identified in this survey have been differentiated by pl, it cannot be assumed that the seven different types of enzymes have diverse substrate and inhibition profiles. It is possible that, as with TEM and SHV β-lactamases, the diversity of plS arose from only a few amino acid changes. Therefore, further work is required to ascertain the relationships among these different metallo-β-lactamases.

Other workers have reported heterogeneity in the pl values of the β-lactamases produced by X. maltophilia, but the developments described here readily distinguish between metallo and serine active-site enzymes. The heterogeneity observed shows that many isolates of X. maltophilia produce metallo- and serine β-lactamases with plS different from those of the L-1 and L-2 enzymes originally described by Saino et al. (13, 14). These novel detection methods will also facilitate larger surveys of X. maltophilia strains to deduce the most prevalent metallo-β-lactamase in this species. It is possible that these procedures are also applicable for the identification of metallo- and serine β-lactamases in other species. However, BRL 42715, zinc sulfate, and EDTA may have different effects on the β-lactamases produced by other species of bacteria, and appropriate method development studies would have to be conducted.

ACKNOWLEDGMENTS

We thank R. Patton for X. maltophilia H-25 and J. Govan, A. King, and D. Livermore for the other clinical isolates of X. maltophilia.

REFERENCES


Two molecular variants of a plasmid-mediated Class C cephalosporinase are characterised in an E. coli isolate 1946. Sequence analysis of the entire 3050-bp gene encoding the 8-beta-lactamase from this strain showed a high degree of homology with the common A. sobria chromosomal 8-beta-lactamase. The sequence analysis of the 3050-bp gene encoding this enzyme was compared with the published A. sobria chromosomal cephalosporinase, supporting the presence of a chromosomal cephalosporinase, as the two enzymes showed a high degree of homology. A. sobria strain 772, isolated from a patient with meningitis, was resistant to carbapenems. The plasmid Group 2 A. sobria 8-beta-lactamase, purified to homogeneity, had high activity against cephalosporins and reduced the susceptibility of Gram-negative bacteria to the beta-lactam antibiotic substrates (MIC = 0.1 mg/L). The purified A. sobria 8-beta-lactamase was more active against all beta-lactam antibiotics. The strain was sensitive to imipenem (MIC = 0.1 mg/L), confirmed by the presence of the 16S rDNA sequence. The strain was sensitive to imipenem (MIC = 0.1 mg/L), and the strain was sensitive to imipenem treated with EDTA prior to staining, all other isolates were unaffected. It was not clear whether the three other strains represented these distinct enzymes or whether they were merely antibiotic bands of the same enzyme. To elucidate this, each band was cut out of the acrylamide gel and the 8-beta-lactamase was extracted using the above procedures and released the enzyme to the electrophoretic gel. Each 8-beta-lactamase was then specifically identified by the formation of an enzyme band.

The specific activity of the purified enzyme was determined by the formation of a substrate band at the same enzyme activity, or after treatment with EDTA prior to staining, all other bands were unaffected. It was not clear whether the three other strains represented these distinct enzymes or whether they were merely antibiotic bands of the same enzyme. To elucidate this, each band was cut out of the acrylamide gel and the 8-beta-lactamase was extracted using the above procedures and released the enzyme to the electrophoretic gel. Each 8-beta-lactamase was then specifically identified by the formation of an enzyme band.

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FIG. 2. IEF gels of crude lysates after treatment with 42715 which inhibited the bands which were observed. 

<table>
<thead>
<tr>
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<th>Band height</th>
<th>Band intensity</th>
</tr>
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<tbody>
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<td>9.7</td>
</tr>
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</table>

**TABLE 1**

<table>
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<th>Result</th>
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<tr>
<td>Good</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fair</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
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</tbody>
</table>

**RESULTS**

The clinical isolates produced BRL 42715 sensitive enzymes which were unaffected by EDTA. Examination of the EDTA resistant strains confirmed this. The EDTA resistant strain was isolated from X. maltophilia boleti. These novel techniques for detection and classification of metallo-

**DISCUSSION**

The results indicate that the clinical isolates hydrolysed carbapenems in the presence of EDTA. This suggests that the carbapenems were being acted upon by metallo-

**REFERENCES**

2. Callejón, J. and A. Rodriguez (2001) Metallo-
3. X. maltophilia.
A small fraction (ca. 7%) of clinical isolates of B. fragilis carries the carbapenem resistance gene blaIMP, which we have previously found to be expressed (CPEAF), after S-1-dependent activation, in at least one of the cases examined.

Here we have comparatively analyzed B. fragilis strains from within two groups, group I, comprising 30 (a) positive strains (CPEAF or carbapenem-susceptible), and group II, comprising 25 randomly collected (a)-negative strains. As revealed by DNA-DNA hybridization analyses, sequences of the carbapenem-resistant and -susceptible strains were present in, respectively, 76%, 58% and 18% of the group I strains, and 0%, 0.4%, and 0% of the group II strains. Since ribotype and RAPD analysis both revealed identical homologies within, and a clear difference between, the group I and II strains, we sequenced 1.5 kb PCR-generated isoelectric fragments of the 16S rRNA genes of 7 group I and 5 group II strains (including 2 reference strains, Sequence Analysis Library: AAC 11, 15 and 16) to determine antibiotic resistance. While the nucleotide sequences of the rRNA genes of the group II strains differed only minimally from each other and from the corresponding type strain sequence (J. Brouilhet, 1983, 164: 202-236), all group I strain sequences had an identical set of 15 scattered bp differences.

We conclude from these results that: 1) the (a)-positive strains constitute a single reservoir of the known B. fragilis insertion elements; 2) the (a)-negative and (a)-positive strains of B. fragilis constitute two distinct and robust separate opportunistic units; 3) the (a)-positive strains due to their content of insertion elements and resistance traits should be closely monitored in the clinical environment.

C60 Biochemical Characterisation Of X. maltophilia Metallo-ß-Lactamase (m-ßl): Types 1 to 3


The m-ßl produced by strains GEL, 10062, J2323, 37 and 136 had different pI values and have been designated types 1 to 6 (D. Knowles et al, submitted).

The m-ßl of strain 4 (purified to 100% yield by ammonium sulfate precipitation) was used for the following studies.

1. Rate of Hydrolysis: The m-ßl was examined for its ability to hydrolyze a variety of ß-lactam antibiotics including some ß-lactam antibiotics that are used for the treatment of infections caused by gram-negative bacteria. The m-ßl was shown to be active against all of the ß-lactam antibiotics tested, including imipenem, ceftriaxone, and aztreonam.

2. Substrate Specificity: The m-ßl was tested for its ability to hydrolyze various ß-lactam antibiotics, including imipenem, ceftriaxone, and aztreonam. The results showed that the enzyme was highly specific for ß-lactam antibiotics, with a preference for imipenem.

3. Kinetic Analysis: The kinetic parameters of the m-ßl were determined using imipenem as the substrate. The enzyme showed a Michaelis-Menten kinetics with a Vmax of 1000 units/min/mg of protein and a Km of 0.01 mM.

4. Inhibition Studies: The m-ßl was tested for its sensitivity to various ß-lactamase inhibitors, including clavulanic acid, sulbactam, and tazobactam. The enzyme was shown to be resistant to all of these inhibitors.

5. Characterisation: The m-ßl was characterised as a metallo-ß-lactamase, based on its resistance to ß-lactamase inhibitors and its ability to hydrolyze ß-lactam antibiotics.

In conclusion, the m-ßl is a metallo-ß-lactamase that is highly specific for ß-lactam antibiotics, with a preference for imipenem. The enzyme is resistant to ß-lactamase inhibitors and is characterised as a metallo-ß-lactamase.
**Introduction**

Clinical isolates of *Xanthomonas maltophilia* are usually resistant to a wide range of antimicrobial agents, which severely compromises therapeutic intervention (Neal et al. 1992). Novel IEF systems have illustrated that *X.maltophilia* produces seven different metallo-β-lactamases (m-β-l) (Figure 1) these enzymes were designated as Types 1 to 7 (Payne et al. 1994). In this current work we have compared the substrate and inhibitor profiles of m-β-l Types 1 to 6.

**Methods**

**Separation of m-β-l from serine β-lactamases**

Cell free extracts of each strain were prepared as described previously (Payne et al. 1994). The m-β-l activities of the extracts were separated by free flow IEF (MiniPhor). Approx. 1-3ml containing 10-30mg of protein was injected into a prefocused gradient formed using 1% (w/v) pH 3-10 ampholines in a 1% (w/v) glycerol, 10% (v/v) glycerol, 50mM Hepes pH7.5. The proteins were focused for 20min at 1000V, 40W limiting and a further 10min at 500V, 20W limiting before collection. Each m-β-l preparation was analysed by analytical IEF to ensure all the serine β-lactamase activity had been removed (Matthew et al. 1975).

**Biochemical analysis of β-lactamases**

The initial rate of hydrolysis of 10 β-lactams (100uM) was determined for the 6 different m-β-l (Assay buffer: 25mM PIPES (pH7.0 ±1mM ZnSO4). The inhibitor profiles were determined by a 10 min incubation with chelator, % inhibition of imipenem hydrolysis was then measured (Assay buffer: 25mM PIPES (pH7.0 ±33mM ZnSO4). Phenanthroline was tested at 0.2mM as it absorbed strongly at the same wavelength as imipenem. All the other chelators were tested at 2mM.

**Results**

Each m-β-l was successfully separated from the contaminating serine β-lactamase (see Figure 2 for example). Differences in substrate profiles for the 6 enzymes were most evident with nitrocefin (ranging from 12 to 87% relative to imipenem). Overall, cefotixin and ceftriaxone were the poorest substrates tested (range 0.1 to 5%) and cefotaxime was the most labile asymmetric cephalosporin examined (range 15 to 53%). Meropenem was hydrolysed by all 6 enzymes less readily than imipenem (range 29-50%) (Figure 5).

**Biochemical Characterisation of *X.maltophilia* Metallo-β-lactamase Types 1 to 6**

Figure 1. IEF showing *X.maltophilia* m-β-l Types 1-7.

Type 1; Type 2; Type 3; Type 4; Type 5; Type 6; Type 7.

None of the chelators caused significant inhibition of TEM-1, EDTA (2mM) and dipicolinic acid caused ≥ 89% inhibition of all the enzymes. The m-β-l from strain 511 was the only enzyme to be inhibited by nitrocefin acid, this compound activated some of the m-β-l produced by other strains of *X.maltophilia*. D-penicillamine was more active than the L-isomer for 6 of the 7 enzymes tested. Phenanthroline was active against 5 of the enzymes at a level 10 fold lower than the other chelators (Figure 5).

**Conclusions**

The substrate profiles of the m-β-l demonstrate their broad spectrum and explain the multiple β-lactam resistance exhibited by the host strain. Ampicillin and Imipenem were the best substrates for all the enzymes and the rates of hydrolysis of cefotaxime, cefotixin and ceftriaxone were all <10% relative to imipenem. In addition, L and D-penicillamine, EDTA, dipicolinic acid and phenanthroline all inhibit the *X.maltophilia* enzymes. These results suggest some gross similarities between these active sites of the enzymes, but overall the data imply subtle differences within this group of enzymes.

There was no uniform relationship between enzyme and sensitivity to chelation. If these compounds are acting by chelation, and removal of the active site zinc, the results indicate differences in the affinity of the various enzymes for the metal co-factor, alternatively there may be more complex interactions between chelator, enzyme and divalent metal ion.

The Type 6 and 511 m-β-l had similar pl's and substrate profiles suggesting that they are probably the same enzyme. However, the inhibitor profiles were significantly different.

Amino acid or DNA sequencing/probing will be the ultimate way of assessing whether these enzymes represent a group of closely related enzymes differing by only a few residues.

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fluoroquinolones and indeed other pharmaceuti-
cals.

Acknowledgements. Some of these data were
presented at the 32nd Interscience Conference
on Antimicrobial Agents and Chemotherapy,
Anaheim, USA (Abstract 788).

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Sensitivity of L-2 type β-lactamases from
Stenotrophomonas maltophilia to serine active
site β-lactamase inhibitors

Sir,

Ten years ago Stenotrophomonas (Pseu-
domonas) maltophilia was considered a non-
pathogenic environmental isolate. Today, this
species is regarded as a serious pathogen, capable of causing fatal infection in immuno-
compromised patients. The major difficulty
associated with treating infections caused by
this organism is its universal resistance to a
wide variety of antibiotics. In addition, it has
been shown that patients receiving broad
spectrum antibacterials, such as imipenem,
have an increased chance of becoming infected
with S. maltophilia (Elting et al., 1990; Victor
et al., 1994).

S. maltophilia can produce two β-
lactamases, firstly, a metallo-β-lactamase,
which is capable of hydrolysing almost all
clinically important β-lactam antibiotics, and
secondly, a serine active site enzyme which
preferentially hydrolyses cephalosporins. These
two enzymes are thought to be largely
responsible for the broad spectrum of β-lactam
resistance exhibited by this species. Further
work by Payne et al. (1994a) illustrated
that the majority of strains of S. maltophilia
produced metallo-β-lactamases, and isoelectric
focusing studies have illustrated that 17 iso-
lates of S. maltophilia produced seven different
types of metallo-β-lactamases (Types 1–7)
and at least eight different types of serine
β-lactamases.

Recent surveys have illustrated that ticar-
cillin-clavulanic acid is the most active of the
commercially available β-lactamase inhibitor/
β-lactam combinations against S. maltophilia.
Moreover, this is one of the few agents which
achieves >80% susceptibility against this
pathogen (Vartivarian et al., 1994; Laing et al.,
1995). To enhance our understanding of the
relative activities of β-lactamase inhibitor/β-
lactam combinations against this pathogen one
factor that has not yet been evaluated is
the inhibition of the serine enzymes by the
β-lactamase inhibitors. This communication
reports on the sensitivity of serine enzymes
from eight strains of S. maltophilia to
clavulanic acid, tazobactam, and sulbactam.

The origin of the strains used in this
study and the methodology used to purify
the serine enzymes from the metallo-β-
lactamases have been described previously
(Payne et al., 1994b,c). Although some of
the serine enzymes had very similar isoelectric
focusing points (pI) there is insufficient
evidence to suggest that they are identical
enzymes. In fact the ID50 values illustrate
significant differences between enzymes of
similar pIs. The ID50s were determined using
200 µM nitrocefin as reporter substrate in a
25 mM piperazine-N,N'-bis(2-ethanesulphonic
acid) (PIPES) buffer (pH 7.0). All ID50s were
measured following a 5 min incubation of
enzyme and inhibitor (Payne et al., 1994b).
MICs were measured by E-test (AB Biodisk)
using the manufacturers guidelines.

The ID50 values illustrate that for three of
the eight enzymes clavulanic acid was >9 times
more active than tazobactam. For three further
enzymes, clavulanic acid was >3 times more

Correspondence
Table. ID₅₀s of β-lactamase inhibitors for serine β-lactamases from S. maltophilia and MIC₅₀s for clinical isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>511 (9.7)</th>
<th>GEL (10.3)</th>
<th>J2223 (7.7)</th>
<th>37 (9.6)</th>
<th>152 (8.6)</th>
<th>136 (10.0)</th>
<th>0062 (8.6)</th>
<th>AA158 (5.5-8.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MICs (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ticarcillin/clavulanic acid (2 mg/L)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>3</td>
<td>6</td>
<td>&gt;256</td>
<td>32</td>
<td>1.5</td>
</tr>
<tr>
<td>Piperacillin/tazobactam (4 mg/L)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>64</td>
<td>48</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>48</td>
</tr>
</tbody>
</table>

*Numbers in parenthesis are the pIs of the enzymes (Payne et al., 1994c).
ND, Not determined.

active than tazobactam and the remaining enzymes were <2-fold more sensitive to clavulanic acid. In all cases clavulanic acid was substantially more active than sulfactam (Table). Four of the eight strains selected were more susceptible to ticarcillin-clavulanic acid than to piperacillin-tazobactam. All of these strains produced serine β-lactamases which were between two-to nine-fold more susceptible to inhibition by clavulanic acid than tazobactam. The MICs of both combinations for the other four strains were >256 mg/L, but at least two of these strains produced serine β-lactamases which were >9 times more susceptible to clavulanic acid than tazobactam.

Therefore, although these results show that the serine β-lactamases produced by this pathogen are more sensitive to clavulanic acid than sulfactam or tazobactam, there is no clear correlation between sensitivity of serine β-lactamases to a β-lactamase inhibitor and the MIC of the combination. Consequently, these data do not explain why ticarcillin-clavulanic acid is generally a more potent combination against this pathogen than piperacillin-tazobactam. However, it is probable that against certain strains, such as strains which only produce a serine β-lactamase (e.g. AA158), these observations may explain the greater potency of ticarcillin-clavulanic acid.

Characterisation of the metallo-β-lactamase from S. maltophilia 511 has provided further explanations for the greater potency of ticarcillin-clavulanic acid. For example, ticarcillin is hydrolysed less efficiently than piperacillin by this enzyme (Felici & Amicosante, 1995). In addition, tazobactam is a good substrate for this metallo-β-lactamase, being hydrolysed more efficiently than imipenem (Felici & Amicosante, 1995). However, to enable a comprehensive understanding of the susceptibility of this pathogen to β-lactamase inhibitors/β-lactam combinations the relative permeabilities of the components and their stability/interaction with the metallo-β-lactamases and serine β-lactamases need to be determined with a variety of strains producing the different types of β-lactamases discussed in this report.

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References


The effect of betaine accumulation by Escherichia coli K-12 on aminoglycoside activity


Sir,

The activity of aminoglycosides against Escherichia coli is inhibited by sodium chloride. Glycine betaine (GB), which is accumulated by E. coli in response to osmotic stress (Le Rudulier et al., 1984; Chambers & Kunin, 1985), further inhibits aminoglycoside activity at salt concentrations which activate the betaine accumulation mechanisms (Peddie & Chambers, 1993). This has been shown for E. coli ATCC 25922 and five wild type strains in concentrated urine and minimal medium. To clarify whether the effect on aminoglycoside activity is mediated through intracellular betaine accumulation we studied strains of E. coli from which genes encoding for betaine uptake mechanisms have been deleted. Strains K-12 (Wild type); WG 439 ΔputPA)101 ΔproU600 srl-300::Tn10; WG 445 ΔputPA)101 ΔproU600 srl-300::Tn10; and WG 445 ΔputPA)101 ΔproU600 srl-300::Tn10 were obtained from Dr Janet Wood, University of Guelph, Guelph, Ontario, Canada. Betaines used had known accumulation by E. coli K-12 (Peddie et al., 1994). These were an homologous series with increasing carbon chain length: GB, propiono-betaine, butyrobetaine, and caproic betaine, and two α-substituted betaines, α-alanine betaine and trigonelline. Added NaCl was used to alter minimal medium osmolality. Betaines were added to give a final concentration of 0.1 millimolar. MICs were determined by tube dilution.

Glycine betaine supplementation caused an increase in resistance to aminoglycosides in all strains able to accumulate GB at high salt concentrations. Results for selected strains cultured with amikacin or gentamicin are shown in the Figure. Resistance to gentamicin and tobramycin under the same conditions was similar. There was a stepwise effect, apparently related to GB accumulation efficiency. The two strains with a single betaine port deletion (WG 443 and WG 445) showed increased resistance compared with the double deletion mutant (WG 439) and GB-deprived K-12, but less resistance than K-12 supplemented with GB (ANOVA P < 0.001). Likewise, with α-alanine betaine and the homologous series of betaines, betaine glycine resistance paralleled both the osmoprotective activity and efficiency of accumulation of the various betaines (Peddie et al., 1994). In contrast, trigonelline, which is accumulated well by E. coli but has no significant osmoprotective activity, had little effect on aminoglycoside resistance suggesting that osmoprotective activity is the important property conferring resistance.

The mechanisms involved in the MIC increase are an extracellular effect represented by the increases observed in the presence of NaCl alone, and an intracellular effect occurring upon the addition of GB. The effect of intracellular betaine is not clear. It may be mediated by effects on the transmembrane electrochemical potential which Fraimow et al. (1991) have shown to be involved in the energy dependent stages of aminoglycoside transport. Alternatively it may be related to the stabilization of macromolecular structure which is an important property of betaines.

Structural differences between amikacin, tobramycin and gentamicin are not very substantial. However, the decreased cationic charge (+5 to +4) may render amikacin more susceptible to competitive inhibition than the other aminoglycosides. If the effect of GB is due to a decreased membrane potential then amikacin would be more susceptible to this than either gentamicin or tobramycin.
A study of the mechanisms involved in imipenem resistance in *Pseudomonas aeruginosa* isolates from Japan


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Sir,

Recently, there have been increasing reports from Japan of carbapenem-resistant clinical isolates belonging to both the Enterobacteriaceae and Pseudomonaceae families that produce a metallo-β-lactamase, designated IMP-1. The *bla*IMP gene is carried on a novel integron-like element, and because of the transferrable nature of this gene it has been speculated that carbapenem-resistant Gram-negative organisms, that produce IMP-1, may be prevalent in the near future.

A study was therefore undertaken to determine the mechanisms involved in imipenem resistance in 61 clinical *Pseudomonas aeruginosa* strains isolated from 21 different hospital centres in Japan, during 1994. The main emphasis was on establishing if IMP-1 β-lactamase was implicated in the resistance mechanism or if a novel carbapenemase was present. The contribution of chromosomal cephalosporinases to imipenem resistance was also examined.

PCR analysis has previously been successfully applied for the detection of the *bla*IMP gene. When the 61 *P. aeruginosa* isolates in this study were subjected to PCR with *bla*IMP specific primers they were all found to be negative for this apparently highly conserved gene. Furthermore, DNA hybridization with a 587 base pair intragenic *bla*IMP gene probe, generated by PCR, also confirmed these strains to be negative for an IMP-1-like enzyme.

Chromosomal cephalosporinases are ubiquitous amongst *P. aeruginosa* and by determining the MIC of imipenem alone and in the presence of a fixed concentration of the serine β-lactamase inhibitor BRL42715, it was possible to show the contribution of the chromosomal cephalosporinase to imipenem resistance in the *P. aeruginosa* strains. In the presence of BRL42715, a 4- to 16-fold decrease in the MIC of imipenem is indicative of the involvement of a cephalosporinase in the mechanism of imipenem resistance and was demonstrated in all but four of the 61 strains. The imipenem MIC for the IMP-1-producing control strain *P. aeruginosa* M18 was unaffected by BRL42715 and, therefore, the chromosomal cephalosporinase was not a contributing factor to the level of imipenem resistance in this isolate.

Isolelectric focusing of β-lactamasess from selected strains, followed by overlaying the gel with BRL42715 and clavulanic acid, a specific inhibitor of class A, but not class C β-lactamases, provided further evidence that these strains possess a class C β-lactamase, not a class A, that is involved in the imipenem resistance mechanism. It has previously been shown that derepressed class C cephalosporinases are able to hydrolyse imipenem, slowly but significantly. Derepression of a class C β-lactamase with the loss of the D2 outer membrane porin is a well recognized mechanism of imipenem resistance.

Hydrolysis of imipenem could not be detected in either of those strains in which the imipenem MIC was unaffected by BRL42715, or on randomly selected strains that displayed a decrease in their imipenem MIC in the presence of BRL42715. Therefore, these results ruled out the involvement of a novel carbapenemase in the imipenem resistance mechanism.

It was also demonstrated that strains, which show a decrease in their imipenem MICs in the presence of BRL42715, are not necessarily derepressed for β-lactamase activity. In fact, a decrease in the imipenem MIC in the presence of BRL42715 is found in strains that are either constitutive or inducible producers of β-lactamase. The four strains that were resistant to imipenem, but unaffected by BRL42715, also varied in the levels of β-lactamase that they produced. However, in these strains imipenem resistance appears to be β-lactamase independent and the effects of other porin species, or active efflux systems, may be contributing factors.

Determining the mechanism of bacterial resistance to carbapenems in *P. aeruginosa* is a complex process. Synergy between a cephalosporinase and lowered outer membrane permeability is by far the most prevalent mechanism of carbapenem resistance in the strains studied in this survey, which also highlights that IMP-1-mediated mechanism of resistance is currently a much rarer event.

In the original Japanese report, 132 carbapenem-resistant *P. aeruginosa* strains were examined and 13% were found to carry the *bla*IMP gene. The carbapenem resistance mechanism in the remaining 87% was not determined and it is highly probable that the resistance mechanism in many of these strains that were not further investigated involved a cephalosporinase and a reduction in outer membrane permeability. Recent findings indicate that the isolation of IMP-1-containing strains is still increasing; however, it remains to be seen whether in the future this mechanism becomes as widespread in *P. aeruginosa* as the one that involves the combination of a class C β-lactamase and impermeability.
Emergence of resistance to third-generation cephalosporins amongst Salmonella typhimurium isolates in Greece: report of the first three cases


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Sir,

Multidrug-resistant salmonellae have emerged as important public health problems. Isolates are commonly resistant to chloramphenicol, tetracycline, co-trimoxazole, aminoglycosides and penicillins, and the plasmids that mediate resistance to these drugs have spread widely amongst Salmonella spp. More recently, there have been sporadic reports of strains that are also resistant to newer β-lactams. We report here the first isolation in Greece of strains of Salmonella typhimurium resistant to oxyimino-β-lactams and describe the mechanisms of resistance to these antibiotics.

S. typhimurium strain AS30 was isolated in late 1996 from a 62 year old man with gastroenteritis. He had emigrated from Southern Russia 1 month before the pathogen was isolated and was living in Athens at the time. Strain AS31 was recovered in 1997 from an infant living in a settlement for immigrants near Thessaloniki. The third strain, EP112, was isolated in 1994 from a blood culture obtained from an infant treated at the ‘A. Kyriakou’ Children’s Hospital in Athens. The isolates were identified with the API20E system (bioMérieux, Marcy l’Etoile, France) and serotyped with O1, H1 and H2 specific antisera. MICs of the β-lactams were determined by the Etest method (Biodisk, Solna, Sweden) and susceptibility/resistance was defined according to breakpoints recommended by the National Committee for Clinical Laboratory Standards. Susceptibility to other antibiotics was determined by the disc diffusion method. Transfer of resistance by conjugation was performed as described previously with Escherichia coli 14R525 as the recipient. Plasmid DNA was extracted by alkaline lysis and partial nucleotide sequencing of β-lactamase genes was undertaken directly on the wild R-plasmids with the Sequenase 2.0 kit (USB Corporation, Cleveland, OH, USA) with custom-synthesized primers specific for the blaSHV and blaCTX-M genes. Crude extracts of β-lactamases were prepared by mild ultrasonic treatment of bacterial suspensions and isoelectric focusing was carried out in polyacrylamide gels containing ampholyltes.

The susceptibilities and other characteristics of the three S. typhimurium strains are shown in the Table. AS30 and AS31 exhibited similar phenotypes, i.e., resistance to ampicillin, piperacillin, ceftriaxone, cefotaxime and aztreonam and susceptibility to ceftazidime and piperacillin/tazobactam. The MICs of co-amoxiclav for both strains closely approximated the resistance breakpoint and the double-disc diffusion test demonstrated enhancement of the activities of the oxyimino-β-lactams by clavulanic acid. Both isolates were also resistant to chloramphenicol, tetracycline, co-trimoxazole, gentamicin and tobramycin. EP112, on the other hand, was resistant to ampicillin, piperacillin, ceftazidime and aztreonam and exhibited reduced susceptibility to cefotaxime and cephraxone, but was susceptible to the β-lactam/β-lactamase inhibitor combinations, co-amoxiclav and piperacillin/tazobactam; the double-disc diffusion test confirmed that the strain was an extended-spectrum β-lactamase (ESBL) producer. In common with AS30 and AS31, EP112 was resistant to chloramphenicol, co-trimoxazole, gentamicin and tobramycin. All three isolates were susceptible to ciprofloxacin.

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The production of a novel carbapenem-hydrolysing β-lactamase in Aeromonas veronii biovar sobria, and its association with imipenem resistance


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Sir,

Species of the genus Aeromonas are widely dispersed in aquatic environments, but are now increasingly implicated in clinical infections that include gastroenteritis, wound infections and bacteraemia.1 Aeromonas veronii bv. sobria is a major pathogenic species of this genus1 and, as with most Aeromonas species, has the ability to produce a chromosomally encoded inducible carbapenem-hydrolysing β-lactamase. This metallo-β-lactamase belongs to molecular class B; however, paradoxically, when standard in-vitro susceptibility testing is performed, these bacteria remain susceptible to the carbapenems.2 In this report we identify two such imipenem-resistant strains that produce a novel carbapenem-hydrolysing β-lactamase, first described in 1997.3

A. veronii bv. sobria strains 13 and 99 were isolated from a reservoir in Vellore, July 1996, during a study on antibiotic resistance in environmental bacteria isolated from water sources in Southern India. Strains 13 and 99 were found to be resistant to imipenem (MIC 8 mg/L) by the agar dilution method, employing a conventional inoculum of 10^6 × cfu/mL.4 To date, only a handful of naturally occurring carbapenem-resistant aeromonas isolates have been recorded when testing with a conventional sized inoculum.5 β-Lactamase extracts from strains 13 and 99 demonstrated efficient hydrolysis of imipenem when assayed spectrophotometrically. β-Lactamase could not be further induced and therefore, strains 13 and 99 constitutively produce a carbapenemase.

Isoelectric focusing (IEF) demonstrated that both strain 13 and 99 possess two β-lactamases, detectable with nitrocephin staining, with isoelectric point (pl) values of 5.84 and 8.3. An imipenem/agar overlay modification to IEF was applied6 and showed the presence of a single imipenem hydrolysing enzyme of pl 5.84 in both strains 13 and 99. The carbapenemase in these two strains is unlike all other previously described aeromonas carbapenemases.2 Furthermore, the pl value is considerably different from the aeromonas carbapenemases that have so far been reported (typically 8.0 or greater).2

IEF/inhibitor overlays facilitated further characterization of the 5.84 and 8.3 β-lactamases. The gel was overlaid with 100 mM EDTA which is known to inhibit metallo-β-lactamases, and the serine β-lactamase inhibitor BRL42715 (100 μM), prior to staining with nitrocephin.6 A TEM-1 (serine-based) β-lactamase and a cell extract from Stenotrophomonas maltophilia strain 5116 that produces two β-lactamases, (i) L1 metallo-β-lactamase (pl 6.4) and (ii) L2 serine-β-lactamase (pl 9.7), were included as controls. The pl 8.3 β-lactamase of both A. veronii bv. sobria strains were found to be serine-based because they were inhibited by BRL42715 but not EDTA; however, the pl 5.84 carbapenemase is not inhibited by either EDTA or BRL42715 and therefore, cannot be classified as either a metallo- or serine-β-lactamase (Figure). These results indicate the presence of a completely novel carbapenemase in A. veronii bv. sobria that may constitute a new β-lactamase molecular class. The hyper-production of the carbapenemase can be clearly correlated with a decrease in sensitivity, which in a clinical setting could lead to therapeutic failure. The novel carbapenem-hydrolysing β-lactamase is to be designated AVS-1.

References


C-97. Characterization of the Active Site of the Plasmid-Encoded Metallo-ß-Lactamases D4651 Mutations Had the Hydrolytic Activity for ß-ß-Lactams and a site-directed mutant of the conservative histidine residues at 86, 98, 149 replaced by alanine. The mutant enzymes lost their activities to 1/100-1/60, 60% by dialysis against Zn-free buffer. Whereas the native enzyme binds to metal ions, the dialyzed-mutant enzymes were found to bind one mole. The residual activities of the dialyzed-mutant enzymes were lost completely by addition of EDTA. It was suggested that both of the two Zn ions coordinated at the active site are involved in enzymatic activity.

Comparing the Zn values of the HBEA mutant enzyme for cephalothin between in the presence and absence of Zn ions, the substrate affinities were similar to each other. It seemed that the Zn ion coordinated at His86 is involved in catalytic activity rather than in substrate affinity. The 4-fold higher Zn value of the mutant enzyme (11pm) than the native enzyme (2.6pm) in the presence of Zn ions, was thought to be the effect of amino acid substitution rather than that of Zn coordination. The Zn value was found to increase concomitantly. The Zn values of the HBEA and H149A mutant enzymes were 20 and 50 fold higher values than that of the native enzymes, respectively, and these Zn values are more than 5-fold increased. Similar results of three mutant enzymes suggested that the same Zn ion is bound to these three histidine residues.


C-98. Effects of Amino acid (s) Substitution on ß-Lactam Properties of Metallo-ß-Lactamase, CcrA

Biophysical and kinetic studies of the crystal structure of the bacterial zinc metallo-ß-lactamase, CcrA, indicate that the active site of this enzyme is at the edge of the ß-barrel with the ß-strands of the ß-barrel and the N-terminal domain. The catalytic site is located in the ß-strand of the active site. The ß-strand of the active site is composed of three ß-strands and two ß-strands of the ß-barrel. The ß-strand is involved in direct interaction with the zinc ion. The zinc ion is coordinated to the ß-strand of the active site. The zinc ion is coordinated to the ß-strand of the active site.

Contact: Weiy-Avent Research, 401 N. Middletown Rd, Pearl River, NY 10965.

C-99. Carbapenem-resistant Serratia marcescens isolates producing a non-metallo-imipenem-hydrolysing beta-lactamase

Contact: John Quinn, University of Illinois, 8405, Wood St., IL 60612

C-100. Non-metallo-beta-lactamase-mediated carbapenem-resistance in Serratia marcescens

Contact: John, University of Illinois, 8405, Wood St., IL 60612

86-C. Poster Session
Mechanisms of Action and Mechanisms of Antibiotic Resistance
Monday, 2:00 p.m.
Poster Area

C-101. Linezolid Binds to the 50S Ribosomal Subunit and Competes with the Binding of Eperozolid, Chloramphenicol and Lincomycin


The 50S ribosome is a component of the bacterial ribosome and is responsible for the synthesis of bacterial proteins. The 50S ribosome is composed of two subunits, the 30S subunit and the 50S subunit. The 50S subunit is responsible for the initiation of protein synthesis, and the 30S subunit is responsible for the elongation of protein synthesis. The 50S subunit is composed of two major proteins, the 23S rRNA and the protein S12. The 23S rRNA is a component of the 50S subunit and is responsible for the initiation of protein synthesis. The 50S subunit is composed of two major proteins, the 23S rRNA and the protein S12.
A clavulanic acid-sensitive beta-lactamase from Stenotrophomonas maltophilia is a clavulanic acid-sensitive cephalosporinase. The gene encoding this enzyme from Stenotrophomonas maltophilia IID 1275 has been cloned on a 3.3-kb fragment into P.181 to generate the recombinant plasmid P8811-th, that, when expressed in E. coli, confers resistance to cefazolin and penicillins. Sequence analysis has revealed an open reading frame (orf) of 909 bp with a GC content of 71\% compared to that of the L1 metallo-beta-lactamase from the same bacterium. The orf encodes 303 amino acids with a predicted molecular size of 35.1 kDa, accommodating a putative leader peptide of 24 kDa. Sequence alignment of the amino acid sequence with other beta-lactamases shows closely related (31\% identity) to the TEM beta-lactamases. Sequence identity is most obvious at the STTFR (specifically the SDR) loop motif common to all three active site penicillins. Sequence outside of the conserved regions displays less than 10\% homology with comparable beta-lactamases. Hence, the Stenotrophomonas maltophilia L2 beta-lactamase is an indiscernible Ambler class A beta-lactamase which would account for the sensitivity to clavulanic acid.

Tazobactam is a Potent Inactivator of Inhibitor Resistant Class A beta-lactamases.

R.A. BONOMO, S.D. RUDIN, and D.M. SHLAES, University Hospital of Cleveland, Veterans Affairs Medical Center, Cleveland, Ohio.

The beta-lactam beta-lactamase inhibitor combinations (ampicillin/clavulanate, ampicillin/tazobactam, ticarcillin/clavulanate and piperacillin/tazobactam) were tested against inhibitor resistant class A beta-lactamases of the TEM-30, TEM-31 and OX10 group. Minimal inhibitory concentration (MICs), using beta-lactams and beta-lactamase inhibitors in proportion to the commercially available preparations, revealed that the strains of Escherichia coli DH5a that possess the MecA/mutation in the oxo-1 beta-lactamase, the TEM-30 (Arg245Ser) and TEM-31 (Arg245Cys) enzymes were the most susceptible to piperacillin and piperacillin/tazobactam. The inhibitor resistant TEM enzyme had less affinity (higher Km) for nitrocefin. The apparent K of the TEM-30 and TEM-31, 21.2 and 2.35, respectively. These values are 10 to 25 fold less than the apparent K for clavulanate for TEM-30 and TEM 31 (28 and 625, respectively). Clavulanate was as potent as tazobactam against the MecA/mutation of the oxo-1 beta-lactamase (15.1 vs. 18.0, respectively). Tazobactam is a potent beta-lactamase inactivator against selected inhibitor resistant strains of class A beta-lactamases.

Abstracts of the 36th ICAAC

A Rapid Solid-phase Based Technique for the Detection and Characterization of Carbapenemases in Clinical Isolates after IDE


Carbapenems, such as imipenem and meropenem were originally thought to be beta-lactam stable. However, both metallo- and class A serine-based beta-lactamases have been described as being capable of conferring carbapenem hydrolysis. Isoelectric focusing (IEF) is an extremely useful technique to employ initially when comparing the beta-lactames produced by different bacterial isolates. In conventional isoelectric focusing nitrocefin is used to detect beta-lactamases, however, it has been demonstrated that certain metallo-beta-lactamases cannot hydrolyze this chymotrypsin cephalosporin. Therefore, an ager overlay modification to IEF was developed that combines conventional IEF with a microbiological assay and was successful in detecting both metallo- and serine-based carbapenemases. Clau beta-lactamase preparations from a variety of clinical isolates showing imipenem resistance were allowed to focus on a 1% agarose IEF gel. The gel was overlaid with Oxoid lysosor test containing imipenem and an indicator organism which is susceptible to imipenem (E. coli NCTC 10418). After overnight incubation, at 37\°C, no growth of the indicator organism was observed, except at the position where the beta-lactamases had focused and hydrolysed the imipenem. Further modifications were made to distinguish between metallo- and serine-based betalactamases. When beta-lactamases, unaffected by EDTA treatment, were subsequently preincubated with class B beta-lactamase-inhibitors, removal of areas of growth suggested the presence of these enzymes.

C35

Tazobactam as an Inactivator of Inhibitor Resistant Class A beta-lactamases.

R.A. BONOMO, S.D. RUDIN, and D.M. SHLAES, University Hospital of Cleveland, Veterans Affairs Medical Center, Cleveland, Ohio.

The beta-lactam beta-lactamase inhibitor combinations (ampicillin/clavulanate, ampicillin/tazobactam, ticarcillin/clavulanate and piperacillin/tazobactam) were tested against inhibitor resistant class A beta-lactamases of the TEM-30, TEM-31 and OX10 group. Minimum inhibitory concentration (MICs), using beta-lactams and beta-lactamase inhibitors in proportion to the commercially available preparations, revealed that the strains of Escherichia coli DH5a that possess the MecA/mutation in the oxo-1 beta-lactamase, the TEM-30 (Arg245Ser) and TEM-31 (Arg245Cys) enzymes were the most susceptible to piperacillin and piperacillin/tazobactam. The inhibitor resistant TEM enzyme had less affinity (higher Km) for nitrocefin. The apparent K of the TEM-30 and TEM-31, 21.2 and 2.35, respectively. These values are 10 to 25 fold less than the apparent K for clavulanate for TEM-30 and TEM 31 (28 and 625, respectively). Clavulanate was as potent as tazobactam against the MecA/mutation of the oxo-1 beta-lactamase (15.1 vs. 18.0, respectively). Tazobactam is a potent beta-lactamase inactivator against selected inhibitor resistant strains of class A beta-lactamases.

C36

Effects of Omeprazole (On) and Lansoprazole (Lan) on Fluoroquinolone (FQ) And Aminoglycoside (AG) Activity In Staphylococcus Aureus (SA). HENRY FRATMNH*, MICHELE ESPOSTO, Graduate Hospital, Philadelphia, PA. The gastric H+ pump inhibitors On and Lam have been shown to inhibit bacterial transport proteins such as the unius of hl pyleri. Since activity of antibacterial agents can be attenuated by disruption of import or export processes, we wished to study the effect of On and Lam on activity of antibiotics that utilize carrier mediated influx or efflux, such as FQ and AG.

Studies were performed in pH adjusted Murdes Hinton broth or agar, and comparative antimicrobial activity was determined by disc diffusion zone sizes. E-test and broth dilution MICs. In SA, susceptibility to FQ increased with addition of 5 ± 50 \mu g/ml. Effects were greater for norfloxacin and ciprofloxacin than ofloxacin, were maintained throughout pH range 5.5 to 7.3, and occurred without any effects of Om on growth rate. Greater decreases in ciproflaxacin MICs were observed in mac mutant, in which MICs fell from 0.075 to 50 \mu g/ml in 100 \mu g/ml of OM. Smaller effects were seen in oxicinidl wild type and kgd strains. On also enhanced susceptibility of SA to other AG antibiotics including TFP, streptomycin and kanamycin, but did not affect susceptibility to other antibiotics including AGL, L-lactam, tetracycline, trimethoprim and chloramphenicol. Om had little effect on FQ activity in E. coli strains, including derepressed ke and ndk mutants. Lam demonstrated a similar but smaller effect on activity of FQ and other AGA substrates in SA. Unlike Lam, Lam at concentrations of 2 to 1000 \mu g/ml decreased the activity of gentamicin and tobramycin in SA and also in E. coli strains, but this effect was in pH 5.5 to 7.3, in the absence of any effect on growth rate. Addition of the sulfoxide analogues FQ-L and AGL-A did not diminish the effects of On or Lam on FQ or AG activity. We conclude that 1) On specifically enhances the activity of FQ and other AGA substrates in SA but has no effect on AG activity 2) Lam demonstrates a smaller effect on FQ activity in SA, but also decreases activity of other AGA and acne type bacteria 3) The mechanism of On and Lam's effects on antimicrobial activity is unknown but may be related to effects on specific bacterial transport systems.
isolation and characterisation of two \( \beta \)-Lactamases from a clinical isolate of Flavobacterium spiritivorum.

1 Medical University of South Carolina, Charleston, SC 29425, USA
2 SmithKline Beecham Pharmaceuticals, Beckham Park, Betchworth, Surrey, RH3 7AL, UK

Summary:
Organisms from the genus Flavobacterium are aerobic gram-negative rods which are often pigmented and have been isolated from both clinical specimens and the environment. A clinical isolate of Flavobacterium spiritivorum NCTC11388 was found to be resistant to cefuroxim but not imipenem (by turbidimetric assay) with MICs of 100 and 4 mg/l respectively. A silver stained iso-electric focusing gel of a sonicated cell extract from this strain revealed three bands. These bands had pIs 5.4, 7.6 and 8.6 and approximately 65%. The enzymes at pIs 7.6 and 8.6 were separated by FPLC with a MonoQ High Performance cation exchange column in 20mM sodium acetate buffer pH 7.6, eluting proteins which had bound to the column with a NaCl gradient. The enzyme at pI 5.4 was able to hydrolyse nitrocefin but not imipenem under these conditions, and remained unaffected by the addition of EDTA. This enzyme was resistant to all clinically used metallo-\( \beta \)-lactamases with 

\text{pIs} > 1.0 in 1M NaCl. At 40 °C, 100 mM Tris-HCl, pH 8.6, it remained on a Q-Sepharose anion exchange column which had been equilibrated in 10mM Tris pH 8.5 on a Pharmacia FPLC system. Fractions were eluted on an increasing NaCl gradient in the same buffer.

\( \beta \)-lactamase Purification by Gel Filtration:
Partial purification of the \( \beta \)-lactamases was also conducted by passing a sonicated cell free extract through a Sephacryl G150 gel filtration column in 20mM sodium acetate buffer pH 7.6. This yielded partial separation of the enzymes. Fractions containing the pIs 5.4, 6.5 and 8.6 were pooled and dialysed twice against a greater than 100 fold excess of 10mM EDTA for 2 hours, followed by dialysis four times against a greater than 100 fold excess of 25mM PIPES pH 7.0 in MB2 water to remove any excess EDTA.

Biochemical Assays:
Substrate and inhibitor profiles were carried out at 37°C as described previously and run overnight at 4°C. Once run, the gel was overlaid with 10mM ZnSO4 for 1 minute prior to visualisation with an overlay of Whatman No. 1 filter paper soaked in 500mg/ml nitrocefin.

Table 1: Inhibitor profiles of \( \beta \)-lactamases from F. spiritivorum (NCTC 11388).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>8.6</th>
<th>7.6</th>
<th>6.5</th>
<th>5.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>3</td>
</tr>
<tr>
<td>Tubocurarin</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Imipenem</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

The 8.6 and substrate profile for the 8.6 \( \beta \)-enzyme suggests that this is a serine active site \( \beta \)-lactamase.

Table 2: Substrate profiles of \( \beta \)-lactamases produced by F. spiritivorum (NCTC 11388).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>8.6</th>
<th>7.6</th>
<th>6.5</th>
<th>5.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrocefin</td>
<td>100</td>
<td>100</td>
<td>270</td>
<td>ND</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>100</td>
<td>100</td>
<td>270</td>
<td>ND</td>
</tr>
<tr>
<td>Imipenem</td>
<td>100</td>
<td>100</td>
<td>270</td>
<td>ND</td>
</tr>
</tbody>
</table>

Discussion:
This work describes the partial characterisation of a novel impenem hydrolysing metallo-\( \beta \)-lactamase from Flavobacterium spiritivorum. This enzyme is novel by virtue of both pl and substrate profile, and could represent the second report of a metallo-\( \beta \)-lactamase from this genus.

Bacterial Strains:
All of the strains described in this report were received from the National Type Culture Collection, a division of the PHLS in London. The identity of all strains was confirmed by ATCC/DG.

Materials and Methods:
Antibacterial Agents:
All \( \beta \)-lactam compounds, carabapenems and \( \beta \)-lactamase inhibitors were supplied by their manufacturers and prepared immediately before use.

Sensitivity Tests:
Minimum inhibitory concentrations were determined on Columbia Blood agar plates, and inoculated with a 0.5 McFarland standard of culture after growth overnight at 30 °C. Control plates were incubated aerobically at 30 °C for 24 hours. The \( \beta \)-lactamase negative Flavobacterium spiritivorum NCTC11388 was used as a control.

\( \beta \)-Lactamase Production:
Initial strains were aerobically overnight in 10 ml of Todd-Hewitt broth No. 2 broth at 30 °C with agitation at 200 rpm for 4 hours growth, a quarter of the MIC of imipenem was added to induce \( \beta \)-lactamase production.

Discussion:
This work describes the partial characterisation of a novel impenem hydrolysing metallo-\( \beta \)-lactamase from Flavobacterium spiritivorum.
Characterisation of the Imipenem Hydrolysing Metallo-ß-lactamases From Two Bacteroides fragilis Clinical Isolates.


The production of a metallo-ß-lactamase is currently thought to represent the main threat to the use of carbapenems for serious nosocomial infections. Bacteroides species are known to produce a variety of ß-lactamases which have the ability to hydrolyse carbapenems. This report describes the characterisation of ß-lactamases from two clinical isolates of Bacteroides fragilis (JMS-219 and JMS-221) from geographically distinct regions of the world. The MICs of imipenem for JMS-219 and JMS-221 are 2 and 32μg/ml respectively. All biochemical characters were performed after partial purification with Sephacryl G75 gel filtration. The molecular weights of the ß-lactamases by this method were 20000 and 25000 respectively. Analysis of these enzymes by isoelectric focusing indicated that JMS-221 produced an enzyme which focused at pl approximately 5.0, and that may be Qja related, whereas the enzyme produced by JMS-219 failed to focus by IEF. The relative rates of hydrolysis of imipenem compared to cephaloridine were 25% and 75% for the enzymes produced by JMS-219 and JMS-221. The D50 of EDTA for both of these enzymes was inM which, together with the observation that overnight dialysis against 10mM EDTA completely inhibited the enzyme, indicates that they are metallo-ß-lactamases. Two ß-lactamases from clinical isolates which hydrolyse imipenem have been characterised. The biochemical data suggest that these two ß-lactamases are metallo-enzymes, one of which may be different from the previously described Qja type.


The metallo-ß-lactamase, CcrA, from B. fragilis exhibits enzymatic activity in E. coli only in the presence of genomic sequences at one of two loci, lbrA or lbrB. Western analysis of these under non-reducing condition showed that in wild type cells CcrA aggregates to higher molecular weight forms, and pulse-chase studies demonstrated that the protein has an extremely short half life.

The lbrA and lbrB genes were cloned and sequenced and were found to encode DsbA and DsbB, respectively. DsbA, a thiooxidoreductase, part of the periplasmic disulfide bonds in extracytoplasmic proteins. DsbB is required for the efficient recycling of DsbA. The lbrA and lbrB mutations eliminate or greatly reduce the activity of DsbA, allowing CcrA to fold, 'unassisted', into its native, enzymatically active conformation. Extrapolating from these findings, DsbA and DsbB null strains may greatly facilitate the cloning and expression of other foreign extracellular proteins in E. coli, proteins that contain reduced cysteine residues in their native conformation.

A-63 Carbapenemases in Aeromonas salmonicida

M.V. HAYES*, C.J. THOMSON, S.G.B. AMYES. University of Edinburgh, Edinburgh, Scotland.

Aeromonas salmonicida is the etiologic agent of Furunculosis, which kills 30% of the world's farmed salmon. This severe economic loss has led to the overuse of antibiotics and consequently to the rapid emergence of antibiotic resistance. Following the licensing of amoxicillin for use in the aquaculture industry in 1990 in the United Kingdom, a survey of the minimum inhibitory concentration (MIC) of this species showed that Aeromonas salmonicida subsp. salmonicida had an MIC of amoxicillin of greater than 512μg/ml.

This study examined 5-ß-lactamases isolated from this species. Isoelectric focusing demonstrated the presence of several enzymes in each of seven strains studied; one of pl 7.9 and the other of pl 5.0. The latter enzyme was inducible with ceftazidime. Two plasmids were isolated but only one could transfer to Escherichia coli, although it did not confer ampicillin resistance on the strain. Substrate and inhibitor profiles showed that the pl 7.9 enzyme was able to hydrolyse imipenem to 70% of the rate of penicillin G hydrolysis. This enzyme was inhibited by clavulanic acid (10-160μM) and was insensitive to EDTA.

These results suggested that it was a serine-based carbapenemase. On the other hand, the pl 6.0 ß-lactamase was resistant to inhibition by clavulanic acid (10-260μM) as well as being resistant to inhibition by EDTA and insensitive to inhibition by clavulanic acid. This enzyme was not inhibited by aminoglycosides, which suggested that it was a serine-based, clavulanic acid-resistant carbapenemase.

A-64 Characterization of OXA-9, a ß-lactamase Encoded by Tn331, MARCELO E. TOLMASKY. Oregon Health Sciences University, Portland, Oregon.

The transposon Tn331 mediates resistance to severe antibiotics. Resistance to some aminoglycosides such as amikaric tobramycin and kanamycin is mediared by an acetyltransferase code for by aac(6')-Ib, and resistance to other aminoglycosides is produced by a dehydrogenase encoded by adaA. Additionally, Tn331 harbors two bla genes, one of them is a blaoxA, and the other is the blaOXA-23 which encodes the recently described cephalosporinase producing class A ß-lactamase BLAOX23-type (Tolmasky, M. and Cross, J., Plasmid 1993 29:31-40). The blaoxA gene is transcribed from a promoter located immediately upstream of its structural gene as well as from another promoter in a polycistronic mRNA encompassing the genes aac(6')-Ib, adaA and blaoxA.

The OXA-9 amino acid sequence has some degree of identity with the ß-lactamases of some other enzymes belonging to the G2 class of ß-lactamases. The OXA-9 enzyme was indistinguishable from a pl assembly and was inhibited by clavulanic acid, but it is not inhibited by the presence of 200mM sodium chloride or 50mM EDTA.

The OXA-9 protein was overexpressed by cloning the blaoxA gene into an expression vector. The recombinant plasmid, pMTTS1, was transformed into E. coli and extracts were prepared by subjecting the cells to osmotic shock. Analysis of these extracts showed that the main protein component is the OXA-9 enzyme.

A-65 Characterization of Pseudomonas aeruginosa PADO ß-lactamase. Y. V. VIE*, E. HOWARD, W. HARLEY, and C. STRATON. Institutes for Microbiology Research, Franklin, TN. Vanderbilt University, Nashville, TN.

P. aeruginosa infections are treated with a combination of agents with at least one ß-lactam component. The use of ß-lactam antibiotics in these infections has been hampered by the rapid emergence of ß-lactamase producing strains. We characterized a previously undescribed inducible clinical isolate (PADO) that was sensitive to a combination of imipenem, ceftazidime, amoxicillin, and clavulanic acid. This enzyme was very susceptible to aztreonam (Iso MIC = 1μg/ml) but showed significant resistance to other ß-lactam antibiotics. Penicillinase was 32-fold less potent than ceftazidime, and sulbactam showed some degree of activity against PADO. The PADO ß-lactamase has a unique ß-lactam binding site that is closely related to the ß-lactamase from P. aeruginosa infections. We conclude that the clinical usefulness of ß-lactam antibiotics in the treatment of P. aeruginosa infections may be related to its ß-lactamase production.

A-66 Preliminary Description of Beta-lactamase Production by a Strain of Kingella kingae isolated from a Joint Infection. V. VELUCSECO* and C. J. HACKBERG. The Permanente Medical Group Inc. Regional Laboratory, Berkeley, CA and San Francisco General Hospital and University of California, San Francisco, CA.

Kingella kingae is a newly recognized pathogen whose natural habitat is the upper respiratory tract of humans. This nutritionally fastidious organism is a member of the family Neisseriaceae and has been transformed with endocarditis and bone and joint infections, particularly in young children. Although most strains are quite susceptible to penicillin and other ß-lactam antibiotics, we have recently isolated a ß-lactamase producing strain of K. kingae from a staphylococcal aspirate. This isolate was moderately susceptible to ampicillin and penicillin whereas it appeared very susceptible to a wide range of cephalosporins. Beta-lactamase activity determined on plates of media shows a chromogenically against nitrocefin, and by the acidimetric and the agar plate disc methods using penicillin as the substrate. Preliminary characterization of ß-lactamase production by K. kingae indicates the enzyme is more active against penicillins than cephalosporins, and is inhibited by clavulanic acid. In addition, the enzyme appears to be constitutively produced and the ß-lactamase gene is located on the chromosome, not a plasmid.
Abstract

The production of a metallo-β-lactamase is currently thought to represent the main threat to the use of carbapenems for serious nosocomial infections. Bacteroides species are known to produce a variety of β-lactamases which have the ability to hydrolyse carbapenems. This report describes the characterisation of β-lactamases from two clinical isolates of Bacteroides fragilis (JMS-219 and JMS-221) from a geographically distinct region of the UK, the MIC's of Imipenem for JMS-219 and JMS-221 are 0.5 and 5.0 μg/ml respectively. Both biochemical characterisation was performed after partial purification by Sephadex G75 gel filtration. The molecular weight of the β-lactamases by this method were 280000 and 280000 respectively. Analysis of these enzymes by iso-electric focusing indicated that JMS-219 produced an enzyme which focused at pH 4.5-6.0, and thus may be BCA related, whereas the enzyme produced by JMS-219 focused at a similar pH but with a different pattern. The inhibitory rates of hydrolysis of imipenem compared to cephaloridine were 250% and 375%, for the enzymes produced by JMS-219 and JMS-221. The ID50 of EDTA for both of these enzymes was 0.05 μM, which, together with the observation that overnight dialysis against 10mM EDTA completely inhibited the enzymes, indicates that they are metallo-β-lactamases.

Introduction

Organisms from the Bacteroides fragilis group are the most frequently isolated gram negative anaerobic pathogens from clinical infections. In the expression of a serine active chromosomal β-lactamase by organisms of this species is common, conferring resistance to several penicillins and cephalosporins. However, the production of a metallo-β-lactamase is currently thought to represent the main threat to the use of carbapenems for serious nosocomial infections. It has been reported that bacteria from this group can produce a variety of β-lactamases which also have the ability to hydrolyse carbapenems.1 This report describes the characterisation of β-lactamases from two clinical isolates of B. fragilis (JMS-219 and JMS-221) from a geographically distinct region of the UK.

Bacterial Strains

Both JMS-219 and JMS-221 were received from the PHLS in Cardiff. JMS-219 was isolated in 1992 at Ealing hospital, from an 82 year old female with leg and heel blisters. JMS-221 was isolated at Kingsley hospital, Dunoon, from the abdominal wounds of a patient with myofibrosis. Both strains were positively identified as B. fragilis by API 32A.

Materials and Methods

Antibacterial Agents:

All β-lactam compounds, carbapenems and β-lactamase inhibitors were supplied by their manufacturers and prepared immediately before use.

Susceptibility Tests:

Minimum inhibitory concentrations were determined on Wilkins-Chalgren agar supplemented with 5% horse blood, and inoculated with a Denley multipoint inoculator at 106 dilution. The plates were incubated aerobically at 37°C for 48 hours. The β-lactamase negative B. fragilis strain NCTC9343 was used as a control.

β-lactamase Production:

Strains were grown anaerobically overnight in one litre of Brain Heart Infusion (BHI) broth. After 4 hours growth, a quarter of the MIC of Imipenem was added to ensure β-lactamase production.

β-lactamase Purification:

Partial purification of the β-lactamases was conducted by passing a sonicated cell free extract through a Sephadex G75 gel filtration column, and 25mM PIPES buffer pH 7.0 supplemented with 1mM zinc sulphate was used throughout the purification procedure. Prior to the assay for activity, the enzyme was dialysed four times against a greater than a 100 fold excess of 25mM PIPES buffer pH 7.0 made up in 30% water. The zinc sulphate supplement to remove any excess 25mM ions not co-ordinated with the enzyme. Biochemical characterisation of the partially purified enzymes was performed in 25mM PIPES buffer pH 7.0 made up in 30% water.

Isoelectric Focusing:

The partially purified β-lactamases were analysed by iso-electric focusing on a polyacrylamide gel with amphotolines pH 5.0-10 as described previously and run overnight at 4°C. Once the gel was overlay with 5mM ZnSO4 for 1 minute prior to visualisation with an overlay of Whatman No. 1 filter paper soaked in 500μg/ml of nitrocefin.

Biochemical Assays:

Extracellular and intracellular samples were washed by centrifugation and resuspended in 1mM zinc sulphate and used as enzyme. The enzyme activity was measured using nitrocefin as a reporter substrate. Experiments determining the relative levels of isoelectric focusing and inhibitor profiles were carried out at a concentration of 100μM substrate.

Results

Analysis of these enzymes by iso-electric focusing indicated that JMS-219 produced an enzyme which focused at pH 4.5-6.0 which is indicative of a CIM Type enzyme whereas the enzyme produced by JMS-219 produced a different pattern at a similar pH.

Table 1: MICs of Imipenem for two B. fragilis isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMS-219</td>
<td>0.5</td>
</tr>
<tr>
<td>JMS-221</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Enzyme Production Strain  Metabolite(%)  Imipenem  JMS-219  Sensitive  JMS-221  Resistant  JMS-221  600  NCTC9343  Sensitive  0.6

Table 2: Substrate Profiles for B. fragilis Metallo-β-lactamasizes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cephalexin (%)</th>
<th>Cefotaxime (%)</th>
<th>Ceftazidime (%)</th>
<th>Imipenem (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMS-219</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>JMS-221</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Table 3: Substrate Profiles for B. fragilis Metallo-β-lactamasizes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cephalexin (%)</th>
<th>Cefotaxime (%)</th>
<th>Ceftazidime (%)</th>
<th>Imipenem (%)</th>
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<td>JMS-219</td>
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<td>&gt;1000</td>
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</tr>
<tr>
<td>JMS-221</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Discussion

The biochemical data suggests that these two β-lactamases are metallo-enzymes. The β-lactamase produced by JMS-219 focused as a single band at pH 4.5 but has a molecular weight of 200000, whereas the β-lactamase produced by JMS-221 focused as several bands at pH 4.5-5.0 and has a molecular weight of 25000 followed by a molecular weight of 52500. Therefore, these data suggest that the enzymes from JMS-221 may be CIM related due to its comparable size, pH and the relative hydrolyses of various β-lactamases. The enzyme produced by JMS-219 may not be CIM.

The other reported type of β-lactamase catalysed of hydrolysing imipenem in this species was reported from Bacteroides fragilis, but neither JMS-219 nor JMS-221 appear to be of this type as it has a molecular weight of 60000 and is not sensitive to EDTA.

We have made the novel observation that the concentration of ZnCl2 in the test medium affects the hydrolysis of imipenem to a significantly greater extent than the hydrolysis of cephalosporins. Additional research is required to fully understand the observation and to identify metallo-β-lactamases in other gram negative bacilli.

Future work on these isolates will also include probing with the gene encoding β-lactamases and oligonucleotides specific for this gene.

References

A clinical isolate of *Aeromonas sobria* with three chromosomally mediated inducible β-lactamases: a cephalosporinase, a penicillinase and a third enzyme, displaying carbapenemase activity

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Hydrolytic profiles of *Aeromonas sobria* 163a, non-induced and induced, and of a derepressed mutant 163a-M, indicate that this strain has inducible β-lactamases with activities against cephalosporins, penicillins and carbapenems. Three enzymes were identified and two of the β-lactamase genes, *ampS*, encoding a penicillinase and *cepS*, encoding a cephalosporinase, were cloned into *Escherichia coli*, permitting analysis of the individual enzymes. Isoelectric focusing (IEF) analysis using inhibition profiles with EDTA and BRL 42715, confirmed AmpS (pI 7.9) and CepS (7.0) to be serine β-lactamases. A third β-lactamase displaying hydrolytic activity against the carbapenem inhibited by EDTA. The carbapenemase had a pI of 9.3 and was detected on IEF gels by overlaying with agarose containing imipenem and the chromogenic pH indicator, bromothymol blue. Co-inducibility and the recovery of a derepressed mutant in which all three enzymes were produced at high levels indicate that this isolate of *A. sobria* has three co-ordinately controlled β-lactamase genes.

Introduction

Members of the genus *Aeromonas* are Gram-negative, oxidase positive, facultative anaerobes that can cause disease in cold-blooded animals and freshwater fish (Popoff, 1984). Some members of the genus, particularly *Aeromonas hydrophila* and *Aeromonas sobria*, are recognised as causes of gastroenteritis in humans, both in adults and infants (Challapalli *et al.*, 1988; Kuijper *et al.*, 1989), and infections in immunocompromised hosts, which may progress to septicemia (Janda & Duffey, 1988; Isaacs *et al.*, 1988). Antibiotic resistance in these organisms raises a potential problem for the therapeutic management of these diseases and, although this genus is of increasing clinical importance, little is understood with respect to the genetics of antimicrobial resistance, particularly against the β-lactams (Chang & Bottom, 1987; Bakken *et al.*, 1988). In a recent study (Iaconis & Sanders, 1990), one isolate of both *A. hydrophila* and *A. sobria* were each shown to possess two β-lactamases; in *A. sobria* these were designated A1 and A2s. A1 was shown to be a cephalosporinase (pI 7.0) with molecular weight (MW) 42.5 kDa. A2s was described as a carbapenemase (pI 8.0) which also had hydrolytic activity against penicillins and was sensitive to EDTA. Both enzymes were
expressed at considerably higher levels in the induced cells and in mutants derepressed for \( \beta \)-lactamase expression than in the wild-type strain, suggesting that both pairs of \( \beta \)-lactamases in these strains of \( A. \) hydrophila and \( A. \) sobria are co-ordinately controlled. Recently, a metallo-\( \beta \)-lactamase-encoding gene from \( A. \) hydrophila, designated \( cphA \), was sequenced. \( CphA \) shows significant homology with the \( Bacteroides \) fragilis \( CphA \), \( Bacillus \) cereus II and \( Stenotrophomonas \) (\( Xanthomonas \)) maltophilia IID 1275 metallo-\( \beta \)-lactamases (Massidda, Rossolini & Satta, 1991; Walsh et al., 1994). When the enzyme encoded by \( cphA \) was compared with the purified A2s enzyme, the \( CphA \) \( \beta \)-lactamase differed in MW and in its hydrolysis of nitrocefin, but both enzymes had the same iso-electric focusing point (pI 8.0) and both were sensitive to EDTA (Segatore et al., 1993). Other members of the genus \( Aeromonas \) have also been reported to produce several \( \beta \)-lactamases identified as multiple bands on isoelectric focusing (IEF) gels (Shannon, King & Phillips, 1986).

In this study, using substrate and inhibitor profiles and molecular genetics we have analysed the activity and control of expression of the \( \beta \)-lactamases of a strain of \( A. \) sobria. We also describe a novel modification of the standard IEF gel which permits the visualisation of \( \beta \)-lactamases which do not significantly hydrolyse nitrocefin.

### Materials and methods

#### Bacterial strains and plasmids

\( A. \) sobria strain 163a is a clinical isolate obtained from Hammersmith Hospital, London, UK. \( A. \) sobria 163a-M, is a mutant which is derepressed for \( \beta \)-lactamase expression and was selected on media containing cefotaxime (4 mg/L). \( Escherichia \) coli XL1-blue (Bullock, Fernandez & Short, 1987) was used as the host strain to construct the \( A. \) sobria gene bank. \( E. \) coli SNO3 (\( ampA1 \) \( ampC8 \) \( pyrB \) \( recA \) \( rpsL \)) which produces negligible amounts of its native \( ampC \) \( \beta \)-lactamase, was used as a suitable strain in which to recover \( \beta \)-lactamase genes from the \( A. \) sobria gene bank (Normark & Burman, 1977). Cloning vector pK19 (Pridmore, 1987) has a pMB1 origin of replication, the multiple cloning site from pUC19 and encodes resistance to kanamycin.

#### Media, antibiotics and reagents

The following antibiotics were used: imipenem (Merck Sharp and Dohme Ltd, Herts, UK); the serine \( \beta \)-lactamase inhibitor BRL 42715 (Coleman et al., 1989); carbenicillin and ampicillin (SmithKline Beecham, Surrey, UK); benzyl penicillin, cephalothin, cephaloridine, cefamandole and kanamycin (Sigma Chemical Co., Mo. USA); cefotaxime (Hoesth-Roussel Pharmaceuticals); and meropenem (Zeneca Pharmaceutical, Maclesfield, UK). Enzymes for DNA manipulation were obtained from Gibco-BRL (Life Technology Ltd, Paisley, Scotland).

#### Cloning of \( \beta \)-lactam resistant genes

\( A. \) sobria 163a chromosomal DNA was partially digested with Sau3A to give a random distribution of DNA fragments. Size fractionated fragments of between 5 and 10 kb, obtained following electrophoretic separation in 0.7% agarose, were ligated into vector pK19, previously digested with BamHI and treated with calf intestinal alkaline
phosphatase to prevent re-annealing. Recombinant DNA was introduced into *E. coli* XL1-blue by electrotransformation (Gene Pulse, 2-4 V, 25 μF/C, 400 ohms, Biorad) and transformed bacteria were selected on medium containing kanamycin. Transformants were pooled and plasmid DNA was isolated. Three microlitres of pooled, dialysed recombinant DNA was used to electrotransform *E. coli* SNO3. Transformants resistant to β-lactams were selected on two types of medium, one containing ampicillin (30 mg/L), and the other containing cephalothin (20 mg/L). β-lactamase encoding genes recovered from the gene bank were confirmed as originating from *A. sobria* 163a, by back hybridisation of the cloned inserts with genomic DNA from *A. sobria*.

**Susceptibility tests**

Antibiotic susceptibility tests were performed using two-fold serial dilutions of the β-lactam in Iso-Sensitest agar (Oxoid plc, Basingstoke, UK). The inocula consisted of 10⁶ cfu per spot applied with a multipoint inoculator. The MIC was defined as the lowest concentration that prevented growth after incubation at 37°C for 18 h.

**Induction and preparation of β-lactamases**

Bacterial strains were grown overnight in LabM Nutrient Broth No. 2 (Amersham, Bury, UK) at 37°C. A 1:20 dilution of each culture was made into fresh 50 mL LabM broth and shaken at 37°C until mid-log phase (*A420 = 0.8*) had been reached. For β-lactamase induction, the inducer was added to the mid-log phase culture in 50 mL fresh, pre-warmed LabM broth and shaken for 1 h. Control cultures were diluted with broth lacking inducer. Uninduced and induced bacteria were harvested by centrifugation at 4°C and the cells were washed twice in ice-cold 10 mM phosphate buffer (pH 7.0), and then resuspended in 10 mL 10 mM phosphate buffer. Bacterial cells were disrupted by sonication with four 15 s bursts with 15 sec cooling periods, at amplitude 1 (MSE Scientific Instruments, Sussex, UK).

**β-Lactamase assays**

Hydrolysis of β-lactam antibiotics was examined by uv spectrophotometric assays (Pharmacia LKB Ultraspec II, Bucks, UK) in 1 cm square light path cuvettes with readings recorded at 10 sec intervals for 5 min at a wavelength of optimal absorbance for the β-lactam ring of each drug; i.e. cephalothin, 265 nm; cefamandole, 262 nm; carbenicillin, 235 nm; ampicillin and benzyl penicillin, 233 nm; meropenem and imipenem, 299 nm; and nitrocefin, 482 nm. Antibiotic solutions were prepared in 10 mM phosphate buffer, pH 7.0. For substrate profiles, each antibiotic was assayed at a concentration of 100 μM, except for benzyl penicillin, ampicillin and carbenicillin, each of which was assayed at 500 μM. One unit of β-lactamase was defined as the amount of enzyme required to hydrolyse 1 nanomole of substrate per min per mg of protein, in the linear phase of the reaction at 37°C.

**Analytical isoelectric focusing**

Crude bacterial cell extracts were examined by IEF as described by Matthew *et al.* (1975). Filter application tabs were used to load all samples on to IEF gels. Analytical IEF was carried out at 15 W for 2 h on Ampholine PAG plates, pH 3.5–9.5 (Pharmacia
LKB), which were used in accordance with the manufacturer’s instructions. β-lactamase bands were visualised after overlaying with nitrocefin (50 mg/L) for 2–5 min (Payne et al., 1994). Isoelectric points (pIs) were estimated by comparison to reference proteins, using a pH 4–7–10–6 calibration kit (BDH Chemicals Ltd., Dorset, UK). To facilitate the identification of the β-lactamases produced by A. sobria 163a and the cloned β-lactamases expressed in E. coli, the IEF samples and gels were treated as described by Payne et al. (1994). To visualise β-lactamases that were not readily detected with nitrocefin but which hydrolyse imipenem, the IEF gel was overlaid with agarose containing imipenem (10 g/L) and bromo-thymol blue (0–5%). Hydrolysis of imipenem was seen as a yellow band on a blue background.

Results

β-lactamase activities of A. sobria 163a

A. sobria 163a displayed low-level resistance only to ampicillin and to the first generation cephalosporins, cephalothin and cephaloridine (Table I). From strain 163a, mutants of A. sobria 163a resistant to cefotaxime 4 mg/L were isolated at a frequency of 10^{-6}–10^{-7}. A typical mutant 163a-M, showed high level resistance to the penicillins, cefamandole and the carbapenems, and moderate resistance to cefotaxime. β-lactamase production by A. sobria 163a was induced by ampicillin, cefoxitin and imipenem. The greatest degree of induction was achieved with cefoxitin (4 mg/L). Extracts from induced cells of strain 163a showed increased hydrolytic activity against penicillins, cephalosporins and carbapenems (Table II). Induction with imipenem or ampicillin resulted in the same antibiotic resistance profile but lower enzyme levels (results not shown). The derepressed mutant, 163a-M, also showed strong hydrolytic activity against all three classes of β-lactam antibiotics, particularly the penicillins and the carbapenems. The hydrolytic activity in extracts of both the induced wild-type isolate, 163a, and the derepressed mutant, 163a-M, against both imipenem and meropenem, was inhibited by approximately 95% by EDTA (10 mM). Treatment with EDTA had no significant effect on the hydrolysis of other substrates (Table II). The loss of activity against the carbapenems was restored by dialysis (against 1000 volumes of 10 mM phosphate buffer for 6 h at 4°C), and the addition of zinc sulphate at a final concentration of 2 mM, for 30 min at 25°C (data not shown). Addition of the serine β-lactamase inhibitor, BRL 42715 (100 μM, for 10 min at 37°C), did not effect the activity of either imipenem or meropenem (data not shown).

Cloning of resistant genes encoding β-lactamases

From a Ssu3A gene bank of the parent strain A. sobria 163a, two β-lactamase genes were recovered, one encoding a penicillinase, designated ampS, the other encoding a cephalosporinase, designated cepS. The cepS gene was cloned on a 10 kb fragment (pUB5812) and encoded a β-lactamase that displayed hydrolytic activity against first and second generation cephalosporins, but had no detectable activity against penicillins or carbapenems (Table 2). The ampS gene was cloned on a 5.6 kb fragment (pUB5820) and, in contrast to cepS, encodes a β-lactamase with good hydrolytic activity against penicillins, but weak activity against cephalosporins and no detectable activity against carbapenems. Neither AmpS nor CepS was inhibited by EDTA (Table II).
### Table I. MICs (mg/L) of β-lactam antibiotics for *A. sobria* 163a and 163a-M

<table>
<thead>
<tr>
<th></th>
<th>Carbenicillin</th>
<th>Ampicillin</th>
<th>Cephaloridine</th>
<th>Cefalothin</th>
<th>Cefamandole</th>
<th>Cefotaxime</th>
<th>Imipenem</th>
<th>Meropenem</th>
</tr>
</thead>
<tbody>
<tr>
<td>163a</td>
<td>16</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>2</td>
<td>0.0625</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>163a-M</td>
<td>1024</td>
<td>1024</td>
<td>512</td>
<td>512</td>
<td>64</td>
<td>8</td>
<td>32</td>
<td>32</td>
</tr>
</tbody>
</table>

### Table II. Hydrolytic activities of *A. sobria* 163a, uninduced and induced, the stably derepressed mutant 163a-M, *E. coli* clones containing one of two β-lactamases (CepS and AmpS), with and without EDTA

<table>
<thead>
<tr>
<th></th>
<th>Nitro</th>
<th>Amp</th>
<th>Units of activity against&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pen</th>
<th>Carb</th>
<th>Imi</th>
<th>Mero</th>
<th>Ceph</th>
<th>Cld</th>
<th>Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>163a</td>
<td>20</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>163a-induced&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2930</td>
<td>475</td>
<td></td>
<td>700</td>
<td>78</td>
<td>1260</td>
<td>1090</td>
<td>1115</td>
<td>355</td>
<td>20</td>
</tr>
<tr>
<td>163a-Induced + EDTA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2700</td>
<td>485</td>
<td></td>
<td>700</td>
<td>78</td>
<td>84</td>
<td>72</td>
<td>1110</td>
<td>370</td>
<td>20</td>
</tr>
<tr>
<td>163a-M</td>
<td>8450</td>
<td>1210</td>
<td></td>
<td>2388</td>
<td>341</td>
<td>5046</td>
<td>4300</td>
<td>2045</td>
<td>980</td>
<td>35</td>
</tr>
<tr>
<td>163a-M + EDTA</td>
<td>8250</td>
<td>1210</td>
<td></td>
<td>2558</td>
<td>365</td>
<td>363</td>
<td>287</td>
<td>1980</td>
<td>985</td>
<td>30</td>
</tr>
<tr>
<td>CepS</td>
<td>1550</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>905</td>
<td>310</td>
<td>10</td>
</tr>
<tr>
<td>CepS + EDTA</td>
<td>1600</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>890</td>
<td>300</td>
<td>10</td>
</tr>
<tr>
<td>AmpS</td>
<td>1441</td>
<td>1072</td>
<td></td>
<td>1650</td>
<td>335</td>
<td>ND</td>
<td>ND</td>
<td>47</td>
<td>30</td>
<td>ND</td>
</tr>
<tr>
<td>AmpS + EDTA</td>
<td>1390</td>
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<td>1685</td>
<td>320</td>
<td>ND</td>
<td>ND</td>
<td>52</td>
<td>30</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>One unit: nmol substrate hydrolysed min<sup>−1</sup> mg<sup>−1</sup> protein.

<sup>b</sup>Cefoxitin (4 μgmL<sup>−1</sup>).

<sup>c</sup>EDTA (5 mM).

ND, Not detected.

Amp, Ampicillin; Carb, carbapenem; Ceph, cephalothin; Cld, cephaloridine; Imi, imipenem; Man, cefamandole; Mero, meropenem; Nitro, nitrocefin; Pen, penicillin.
IEF of cell extracts

IEF of cell extracts from *A. sobria* 163a, uninduced and induced, the derepressed mutant 163a-M, and *E. coli* clones producing one or other of the two β-lactamases AmpS and CepS (Figure 1). Extracts from both the induced parent strain and the derepressed mutant displayed two distinct bands (pI of 7.9 and 7.0). The parent strain, 163a, had very little β-lactamase activity, focusing as two faint bands. IEF of the two cloned *A. sobria* β-lactamases showed AmpS to focus at a pH of 7.9 and CepS to focus at a pH of 7.0. Furthermore, IEF of the β-lactamases in extracts of the induced strain 163a, 163a-M, *E. coli* (pUB5820) and *E. coli* (pUB5812) showed that neither of the two enzyme activities were inhibited by EDTA (Figure 1), but they were inhibited by BRL 42715. An increased inoculum of the lysate (40 µL) from the derepressed mutant was loaded on to the gel, and after electrophoresis the gel was overlayed with imipenem/bromothymol agarose. A yellow band with a pI of 9.3 was observed, distinct from the isoelectric points of AmpS and CepS. When the cell extract was first treated with EDTA the band was not seen (results not shown), suggesting that this band represents a carbapenemase inhibited by EDTA.

Discussion

β-lactamase activity in extracts of the clinical isolate of *A. sobria* 163a is low, but is substantially increased by induction following cell exposure to either ampicillin, cefoxitin or imipenem, and in mutants derepressed for β-lactamase synthesis (Table II).

Cell extracts of both the induced and derepressed systems show marked hydrolytic activities against the cephalosporins, penicillins and carbapenems. Two β-lactamase genes have been cloned from *A. sobria* 163a, designated *ampS* (pUB5820) and *cepS*. 

---

**Figure 1.** IEF gel overlayed with nitrocefin showing activities of cellular extracts from 163a, 163a-induced, 163a-M, AmpS and CepS. Lane 1, 40 µL uninduced 163a; lane 2, 2 µL induced 163a; lane 3, 1 µL 163a-M; lane 4, 2 µL AmpS; lane 5, 2 µL of CepS. Lane 6, 10 µL uninduced 163a. Lanes 7–10 as for lanes 1–5 but pretreated with EDTA (10 mM). Lane 11, pI markers. Lanes 12 & 13, 40 µL 163a-M pretreated with BRL 42715 (100 µM).
Clinical isolate of *A. sobria*

(pUB5812). The former gene encodes a penicillinase with a pl of 7.9, while the latter encodes a cephalosporinase with a pl of 7.0. Both enzymes are inducible in *A. sobria* 163a and derepressed in the mutant 163a-M, and both are inhibited by the serine β-lactamase inhibitor BRL 42715, but not by EDTA. Neither enzyme displays significant activity against the carbapenems, imipenem and meropenem. This suggests that AmpS and CepS are serine active site enzymes. The activity of cell extracts of the induced strain and its derepressed mutant against the carbapenems is likely to be due to β-lactamase activity, rather than acylase hydrolysis, as no detectable carbapenemase activity was observed in the uninduced parent strain. Therefore, extracts of the induced parent strain 163a and of derepressed mutant, 163a-M, must possess at least one other β-lactamase that can hydrolyse carbapenems, and which is inhibited by EDTA and not by BRL 42715. These properties are consistent with those of a Zn-dependent metallo-β-lactamase.

Two of the enzymes from *A. sobria* 163a were detected on IEF gels with nitrocefin, namely the AmpS and CepS β-lactamases. The third enzyme demonstrating carbapenemase activity, was not visualised by nitrocefin hydrolysis. Other metallo-β-lactamases from *Aeromonas* spp. have been reported to have good activity against nitrocefin and can be detected by standard IEF procedures (Iaconis & Sanders, 1990). However, Segatore et al. (1993) have reported that *Aeromonas* spp. also produce metallo-β-lactamases which have very poor activity against nitrocefin. This observation, with our current data, advocates caution when interpreting nitrocefin stained gels with extracts from *Aeromonas* spp.

The carbapenemase activities in extracts of the induced parent strain, 163a, and the derepressed mutant, 163a-M, were visualised in IEF gels by overlaying the gels with agarose containing imipenem and the pH indicator, bromothymol blue. This technique extends IEF analysis to β-lactamases that do not readily hydrolyse nitrocefin, and which have pl values greater than 6.0. β-lactamase activity is seen as a yellow band on a blue background, with the yellow colour due to the hydrolysis of imipenem and the resultant acidic pH. The carbapenemase was found to have a pl of 9.3. When the samples were pre-treated with EDTA, this band was not seen, indicating that the carbapenemase is inhibited by the chelating agent, consistent with the previous data (Table 2) and the conclusion that this enzyme is likely to be a metallo-β-lactamase.

The production of all three β-lactamases is derepressed in *A. sobria* 163a-M. Mutants of this type arise at a natural frequency of between 10^{-7} and 10^{-6}, consistent with a single mutational event. Other *A. sobria* 163a derepressed mutants isolated using the same method, showed the same β-lactamase profile as 163a-M (results not shown). These findings suggest that all three enzymes are co-ordinately controlled in the parent strain, a conclusion that is consistent with their co-induction. Standard isolation procedures have failed to detect any plasmids in this particular strain (data not shown), and therefore we conclude that the three enzymes are chromosomally encoded. The hydrolytic profile of AmpS is similar to that of the class A group of enzymes, suggesting that *A. sobria* 163a may possess a chromosomally mediated inducible class A β-lactamase. To date, very few inducible Class A enzymes have been reported, namely, *Rhodopseudomonas capsulata* and *Citrobacter diversus* (Campbell et al., 1989; Jones & Bennett, 1994).

Our understanding of inducible, chromosomally encoded β-lactamases in Gram-negative bacteria is principally based on the expression of the cephalosporinase genes from *Enterobacter cloacae* and *Citrobacter freundii*. (Normark et al., 1990, Bennett and
Chopra, 1992). If induction of \( \beta \)-lactamase expression in *A. sobria* is similar to that in *C. freundii* and *E. cloacae*, then expression of all three \( \beta \)-lactamases will be mediated by a transcriptional activator(s). Whether all three structural genes respond to a common activator analogous to AmpR (Normark et al., 1990), or whether each has its own, distinct activator, which in turn responds to a common control function such as AmpD (Normark et al., 1990), has yet to be determined. Further molecular work is currently in progress to elucidate this point.

**Acknowledgements**

We thank Dr Sally Millership, Hammersmith Hospital, UK for providing *A. sobria* 163a. The work was funded by the Wellcome Trust (grant reference no. 038025/2/93/2/1.5) and the British Society of Antimicrobial Chemotherapy (grant reference no. GA2).

**References**


Clinical isolate of *A. sobria* 279


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Nucleotide and Amino Acid Sequences of the Metallo-ß-Lactamase, ImiS, from Aeromonas veronii bv. sobria

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The Aeromonas veronii bv. sobria metallo-ß-lactamase gene, imiS, was cloned. The imiS open reading frame extends for 762 bp and encodes a protein of 254 amino acids with a secreted modified protein of 227 amino acids and a predicted pI of 8.1. To confirm the predicted sequence, purified ImiS was digested and the resulting peptides were identified, yielding an identical sequence for ImiS, with 98% identity to CphA. Both possessed the putative active-site sequence Asn-Tyr-His-Thr-Asp at positions 88 to 92, which is unique to the Aeromonas metallo-ß-lactamases.

The functional group 3 ß-lactamases (2) have assumed increasing clinical significance due to their ability to hydrolyze carbapenems such as imipenem and meropenem, which, apart from a few exceptions, are poorly hydrolyzed by serine ß-lactamases. These enzymes are also resistant to all commercially available serine ß-lactamase inhibitors. Plasmid-mediated metallo-ß-lactamases have now been identified in key pathogens such as Klebsiella pneumoniae, Serratia marcescens, Pseudomonas aeruginosa, and Bacteroides fragilis (1, 10, 25, 26). Furthermore, the metallo-ß-lactamase from S. marcescens, IMP1, has been mobilized on an integron-like gene, imiS, and has spread to P. putida, K. pneumoniae, and Alcaligenes spp. (19). These enzymes have also been identified and characterized from emerging pathogens such as Aeromonas spp., Stenotrophomonas maltophilia, and Burkholderia cepacia and have been the subject of recent reviews (14, 15).

While all metallo-ß-lactamases possess the ability to hydrolyze carbapenems, their abilities to hydrolyze other ß-lactams, such as penicillins and cephalosporins, vary from enzyme to enzyme. Whereas most metallo-ß-lactamases have a broad spectrum of activity, enzymes isolated from Aeromonas spp. can readily hydrolyze only carbapenems (17). The sequence of the gene encoding the Aeromonas hydrophila enzyme CphA was determined by Massida et al. (13) and shows significant difference from those of the metallo-ß-lactamases of Bacillus cereus (9) and B. fragilis (20), including conserved amino acids thought to be involved with zinc ion binding (3, 4). Recently, a second A. hydrophila metallo-ß-lactamase gene, cphA2, has been sequenced which, not surprisingly, shows strong homology to cphA (15). Hybridization studies using the cphA gene probe illustrated that homologs of the A. hydrophila gene are found in A. veronii, A. caviae, A. jandaei, and other strains of A. hydrophila, illustrating the widespread occurrence of this gene in Aeromonas spp. (16).

A metallo-ß-lactamase from A. veronii bv. sobria, ImiS, has been purified and characterized and shown to have a substrate profile very similar to those of both the CphA and A. jandaei AsbM1 ß-lactamases (5, 24, 27). In addition, the first 40 N-terminal amino acids of ImiS were found to be identical to those of the CphA enzyme. Interestingly, the A. jandaei AsbM1 enzyme also has had its N-terminal sequence determined and shows only 26% similarity to CphA over the first 27 amino acids (27). The purpose of this work was to determine the sequences of both the A. veronii bv. sobria metallo-ß-lactamase gene, imiS, and its purified product and to compare these specifically with those of the homologous system in A. hydrophila.

A. veronii bv. sobria 163a is a clinical isolate obtained from Hammersmith Hospital, London, United Kingdom. Escherichia coli DH5α (7) was used as the host strain for transformation of the A. veronii bv. sobria gene bank. pSU18 was used as the cloning vector and has been previously described (12). The A. hydrophila metallo-ß-lactamase gene, cphA, carried on plasmid pAS20R, was a gift from G. M. Rossolini and has been previously described (13). All of the media and compounds used have been previously described (23).

Induction of bacterial strains with ceftoxitin and imipenem and ß-lactamase assays were carried out as previously described (22). Imipenem hydrolysis was assayed at 298 nm. One unit of ß-lactamase activity is defined as the amount of enzyme required to hydrolyze 1 nmol of substrate/min/mg of protein in the linear phase of the reaction at 37°C.

For the preparation of DNA probes, large quantities of plasmid pAS20R were prepared and cut with EcoRI to release the cloned A. hydrophila AE036 insert. The DNA fragments were separated by gel electrophoresis, and the fragment carrying cphA (2.0 kb) was recovered, purified by phenol-ethanol-form extraction, and precipitated as previously described (11). The E. coli recombinants to be blotted were spot inoculated onto nutrient agar, and the plate was incubated at 30°C for 4 h. E. coli(pAS20R) and E. coli(pSU18) were used as positive and negative controls, respectively. The colony blotting and subsequent DNA hybridization were carried out under conditions previously described (11).

DNA sequence determination was performed with the Du Pont Genesis 2000 automated sequencer. Sequences were determined on both strands with a custom primer walking strategy. Compilation of resulting DNA sequences, database
searches, and sequence alignments were performed with the LASERGENE suite of programs (DNASTAR, West Ealing, London, United Kingdom).

ImiS was purified as previously described (24). The enzyme was digested with either trypsin or endoproteinase Glu-C. In both cases, digestion was carried out for 10 min at 37°C in 0.1 M TAP buffer (pH 8.3). Digests and molecular mass determination. Although the molecular mass of ImiS was estimated to be 94 kDa by SDS-PAGE, the enzyme was determined to have a molecular mass of 92 kDa by matrix-assisted laser desorption-time-of-flight mass spectrometry. The molecular mass of Imp was determined to be 92 kDa by matrix-assisted laser desorption-time-of-flight mass spectrometry.

The 2.0-kb insert from pAS20 was used as a probe for hybridization with various digests of A. veronii bv. sobria 163a chromosomal DNA to identify a restriction enzyme combination suitable for cloning the imiS gene. The most suitable was EcoRI and BamII, the A. hydrophila cphA 2.0-kb insert hybridized to a 5.5-kb 163a chromosomal fragment. Both pSU18 and chromosomal DNAs were cut with EcoRI and BamII. The cut chromosomal DNA was fractionated on a 0.7% agarose gel, and DNA fragments of 3.5 to 7.5 kb were excised and purified. The selected fraction was ligated into pSU18 and subsequently used to transform E. coli DH5α to chloramphenicol resistance. Colonies containing recombinant molecules were isolated and digested with pph4 probe. Four positive signals were found after screening of approximately 4,000 transformants, and the recombinant plasmids were designated PUB5826 to PUB5829. On digestion with EcoRI and BamHI, each recombinant gave an insert of 5.5 kb plus the cloning vector. The restricted recombinants were run on an agarose gel, blotted, and probed with labelled cphA to confirm the identities of the inserts. All were positive when probed.

When E. coli strains carrying the clones were used to check the MICs of various β-lactams by using standard inocula, they showed no increase in resistance over that of the host, E. coli DH5α. However, when a larger inoculum (106 bacteria) was used, the MIC of imipenem increased 8- to 16-fold to a value similar to that of E. coli (pAS20) (13). Cell lysates of E. coli (pUB5826 to PUB5829) were analyzed for β-lactamase production, both with and without induction with cefotaxim and imipenem. The β-lactamase activities of all of the cell extracts of strains carrying the imiS clones were very similar to the activity displayed by E. coli (pAS20). The A. veronii bv. sobria metallo-β-lactamase gene was noninducible when expressed in an E. coli background.

One clone, pUB5826, was chosen for sequencing. The open reading frame containing imiS extends for 762 nucleotides and encodes a preprotein of 254 amino acids (Fig. 1). Upstream of the imiS ORF lie the ribosome-binding site, a putative −10 promoter box (TATTAT), and a putative −35 promoter box (TTTACA). However, the spacing between the two components of the promoter is far too ideal. Immediately downstream of the termination codon are inverted repeat sequences (GCATCCGCGCCGGCGCAGC) representing a possible terminator for transcription of the imiS gene. The imiS ORF sequence shows 94% identity to cphA. The codon preference of

imiS strongly favors cystidine (C) and guanosine (G) over uridine (U) and adenosine (A) in the third position. Codon preferences were as follows: NNA, 4.3%; NNU, 10.2%; NNC, 31.2%; NNG, 54.3%. These preferences reflect the high G+C content throughout the ORF (62%), similar to the G+C content of other β-lactamase genes analyzed from the same strain of A. veronii bv. sobria (23). Interestingly, the sequence immediately downstream of imiS shares no homology at all with the downstream sequence of cphA, including the inverted repeat sequences that may represent a terminator for the transcription of the imiS gene.

The results of the tryptic and Glu-C digests were confirmed by Edman sequencing. The complete amino acid sequence of ImiS was derived from a combination of peptide sequence data and peptide molecular mass determination. The molecular mass of the protein determined by liquid chromatography-mass spectrometry of incompletely digested protein was 25,247 Da. This value agrees with the theoretical molecular mass of the protein (25,248 Da). The predicted amino acid sequence shows a perfect match with the protein sequence as determined by Edman sequencing and liquid chromatography-electrospray mass spectrometry. The protein sequence confirms the site of the peptide cleavage, between two aliphatic residues at positions 27 and 28, and is identical to that for CphA (13).

The predicted pl of the secreted product is 8.1, similar to the 8.6 reported for CphA (13) but significantly different from that reported for the AsbM1 enzyme (27). The amino acid sequence of ImiS was compared to those of other group 3 β-lactamases. ImiS showed a very high level of identity to CphA (98%), differing at only seven amino acids (881, 128E, 138L, 143L, 195Q, 201V, and 228S) (2). Given that cphA
other residues, is thought to be responsible for coordinating the two zinc ions found in the active site of the other group 3 enzymes (3, 4). The Aeromonas metallo-ß-lactamases CphA, CphA2, and ImiS have the related sequence Asn-Tyr-His-Thr-Val, which is the equivalent position. The amino acid residues thought to be involved in binding the second zinc ion in the enzymes from B. cereus and B. fragilis, Met78, Asp90, Cys168, and His210 (4), are all conserved in the Aeromonas group 3 enzymes. Thus, the possibility arises that CphA, CphA2, and ImiS may complex just a single zinc ion and that this consequently results in the narrow hydrolytic spectra displayed by these enzymes. The structure of ImiS is currently being determined to resolve, among other points, this particular question.

Nucleotide sequence accession number. The nucleotide sequence of imiS has been assigned EMBL accession no. Y01415.

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REFERENCES


Production of metal dependent β-lactamases by clinical strains of
Bacteroides fragilis isolated before 1987

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Five imipenem resistant clinical isolates of Bacteroides fragilis isolated before 1987, were examined to determine if they produced metallo-β-lactamases. The β-lactamases produced by the clinical isolates all focused as doublet bands at pl 4.8/4.9, characteristic of the B. fragilis CfiA type metallo-β-lactamase. Each enzyme had a similar substrate profile and were inhibited by EDTA and activated with zinc sulphate. The sequence of the metallo-β-lactamase gene from B. fragilis ED262 was determined and confirmed to be a CfiA type β-lactamase. Consequently, the five isolates examined probably produce a CfiA type β-lactamase, suggesting that metallo-β-lactamases were present before the widespread use of carbapenems.

Introduction

Imipenem resistance in clinical isolates of Bacteroides fragilis is no longer a rarity. For example, in Japan the level of imipenem resistance in this species has increased from 2% in 1986 to 5.9% in 1991 (Bandoh et al., 1993) and in a survey of 150 French clinical isolates of B. fragilis the frequency of imipenem resistance was 7% (Burnat et al., 1990). Imipenem resistance in B. fragilis is frequently mediated by metallo-enzymes. Four metallo-β-lactamases from this species have been sequenced and their amino acid sequences are > 98% homologous. This enzyme has been termed the CfiA-type metallo-β-lactamase and appears to be the most common metallo-β-lactamase in clinical isolates of B. fragilis (Payne, 1993). These β-lactamases have a far broader substrate profile than any serine β-lactamase, being able to hydrolyse carbapenems, in addition to penicillins and cephalosporins. More importantly, they are resistant to all the commercially available β-lactamase inhibitors. The objective of this study was to examine whether strains isolated prior to the widespread use of carbapenems produced metallo-β-lactamases. Five strains of B. fragilis, showing decreased susceptibility to imipenem and isolated from UK hospitals between 1985-1987 were examined.
Methods

Bacterial strains and antibiotic susceptibilities

The five clinical isolates were obtained from hospitals in the UK between 1985–1987 and their identity was determined using the anaerobic API system. MICs were determined by the agar dilution method on Wilkins-Chalgren agar.

β-Lactamase preparation

The B. fragilis cultures were grown in cooked meat broth at 37°C in anaerobic conditions. After 18 h of growth the β-lactamase samples were prepared in 25 mM PIPES buffer (pH 7) as described previously (Payne et al., 1994). Escherichia coli K12 J53-2 R6K, a TEM-1 producer, was grown in nutrient broth and the β-lactamase prepared in the same way. This was used in the IEF as a serine β-lactamase standard.

Isoelectric focusing

The β-lactamases were examined by analytical isoelectric focusing (Matthew et al., 1975) on pH 3.5–10 polyacrylamide gels (Pharmacia Biotech, Sweden) using Electran isoelectric point markers (BDH). The β-lactamases produced by the clinical isolates of B. fragilis could only be visualised with nitrocefin (0.5 mg/mL) after the gel had been overlaid with 25 mM PIPES (pH 7) containing 1 mM zinc sulphate.

Biochemical analysis of β-lactamases

All biochemical analyses were performed spectrophotometrically. The rate of hydrolysis of each β-lactam (100 μM) was determined in 25 mM PIPES (pH 7.0) containing 0.5 mM zinc sulphate. In addition, the hydrolysis of nitrocefin by each preparation was recorded following a 15 min incubation at 37°C with and without 1 mM EDTA in buffer without zinc sulphate. The imipenemase activity of each preparation was also measured following a 10 min incubation at 37°C with and without 0.5 mM zinc sulphate.

DNA manipulation

Total B. fragilis ED262 DNA was prepared as previously described (Mamur, 1961). The polymerase chain reaction (PCR) was carried out using a PCR Core Kit (Boehringer Mannheim) as recommended by the manufacturer. The reactions were incubated for 20 cycles in a programmable heat block (Hybaid) for 1 min at 94°C, 1 min at 42°C, 1 min at 72°C with a final extension time of 5 min at 72°C. The primers used had the sequence of nucleotides 587 to 604 (5'-CAGAAAAGCGTAAAAATA-3’) and the complementary sequence of nucleotides 1265 to 1283 (5'-TCGTGAAGGTTTCGGTATC-3’) of the pfa gene (Thompson & Malamy, 1990). The amplified DNA fragment was purified from low melting point agarose gel (1% Seaplaque, Flowgen) with β-agarase (New England Biolabs) and subcloned (TA Cloning Kit, Invitrogen) according to the manufacturer’s protocols.
Metal dependent $\beta$-lactamases of *B. fragilis*

**Nucleotide sequencing**

Nucleotide sequences were determined by the dideoxynucleotide chain termination method using Sequenase version 2.0 (United States Biochemicals) and adenosine $5^3\text{P}^3\text{S}$ thiotriphosphate (Amersham, UK). Double stranded sequencing templates were prepared with Qiagen Maxi preps (Qiagen, Ltd) and the initial sequence was obtained with M13 forward (-20) and reverse primers. DNA sequences were analysed using the Genetics Computer Group programs of the University of Wisconsin.

**Results and discussion**

Imipenem MICs of $\geq 8\, \text{mg/L}$ were observed for all five strains. IEF showed that, like the CfiA metallo-$\beta$-lactamase (Podglajen *et al.*, 1992), each of the *B. fragilis* strains produced a $\beta$-lactamase which focused as a doublet band at pI 4.8/4.9. These $\beta$-lactamase bands were only visible after the gels had been treated with a 1 mM zinc sulphate overlay, whereas the TEM-1 band was visible after treatment with just nitrocefin (Figure). In addition, all the enzymes had similar substrate profiles and preferentially hydrolysed nitrocefin and also hydrolysed carbapenems, meropenem being hydrolysed approximately two-fold more efficiently than imipenem (Table). These are distinctive features of the *B. fragilis* CfiA type metallo-$\beta$-lactamase (Podglajen *et al.*, 1992). Cefoxitin was the most stable $\beta$-lactam to each of the $\beta$-lactamase preparations, being hydrolysed at $\leq 2\%$ of the rate of nitrocefin.

The hydrolysis of nitrocefin by each of the $\beta$-lactamases was inhibited by $\geq 89\%$ with 1 mM EDTA. The activity of each of the enzymes was increased with 0.5 mM zinc sulphate. TEM-1 was not affected by EDTA or zinc sulphate treatments. The biochemical and biophysical properties of these five enzymes strongly suggest that the imipenem resistance in each of these strains results from the production of a metallo-$\beta$-lactamase which has similarities with the CfiA enzyme.

The predicted amino acid sequence of the mature cfiA $\beta$-lactamase gene derived from the nucleotide sequence of the truncated cfiA gene isolated from *B. fragilis* ED262 was compared with other metallo $\beta$-lactamase sequences. The ED262 cfiA gene differed from the cfiA gene of *B. fragilis* TAL2480 (Thompson & Malamy, 1990) and the metallo-$\beta$-lactamase gene of *B. fragilis* TAL3636 (Rasmussen, Gluzman & Tally, 1990) by two amino acids. Threonine (85) and arginine (113) in *B. fragilis* TAL3636/TAL2480

![Figure](image-url)
**Table.** Substrate profiles of β-lactamases produced by *B. fragilis* clinical isolates (rates expressed relative to hydrolysis of nitrocefin)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nitrocefin</th>
<th>Cephaloridine</th>
<th>Imipenem</th>
<th>Meropenem</th>
<th>Cefoxitin</th>
<th>Inhibition of nitrocefin hydrolysis by 1 mM EDTA (%)</th>
<th>Activation of imipenem hydrolysis by 0.5 mM zinc sulphate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED262</td>
<td>100</td>
<td>20</td>
<td>18</td>
<td>43</td>
<td>1.5</td>
<td>99</td>
<td>161</td>
</tr>
<tr>
<td>R1790</td>
<td>100</td>
<td>32</td>
<td>17</td>
<td>36</td>
<td>1.1</td>
<td>97</td>
<td>254</td>
</tr>
<tr>
<td>WSI</td>
<td>100</td>
<td>28</td>
<td>21</td>
<td>49</td>
<td>0.12</td>
<td>90</td>
<td>130</td>
</tr>
<tr>
<td>BC</td>
<td>100</td>
<td>31</td>
<td>24</td>
<td>44</td>
<td>ND</td>
<td>90</td>
<td>172</td>
</tr>
<tr>
<td>ED261</td>
<td>100</td>
<td>31</td>
<td>24</td>
<td>53</td>
<td>0.51</td>
<td>89</td>
<td>122</td>
</tr>
<tr>
<td>TEM-1</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>5</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT, Not tested; ND, no hydrolysis detected.
were replaced by alanine and lysine respectively. The ED262 cfiA gene had the same amino acids at positions 85 and 113 as the metallo-ß-lactamase gene from B. fragilis QMCN4 (Rasmussen, Gluzman & Tally, 1991) but had a methionine at position 79 whereas the QMCN3 metallo-ß-lactamase gene had a threonine. However, the possibility that the differences observed herein are due to PCR amplification of cfiA cannot be ruled out. The nucleotide sequence of the B. fragilis ED262 cfiA gene reported in this paper has been deposited in the EMBL, Genbank and the DDBJ nucleotide sequence databases under the accession number X85036.

The sequence of the metallo-ß-lactamase gene from B. fragilis ED262 confirmed that the enzyme was a CfiA type ß-lactamase. Therefore, as the biochemical profiles of the ß-lactamases from all five strains in this report were very similar to the B. fragilis ED262 enzyme, it is probable that they all produce a CfiA type metallo-ß-lactamase, thus confirming that this type of enzyme is the most prevalent metallo-ß-lactamase in B. fragilis.

The occurrence of metallo-ß-lactamases in these five isolates of B. fragilis predates the widespread use of imipenem and these enzymes may have provided resistance to earlier ß-lactam antibiotics. Clearly, strains with the ability to degrade penicillins and cephalosporins would have offered an advantage which resulted in their selection. This observation suggests that there could be a significant background of metallo-ß-lactamases in B. fragilis. In addition, the silent CfiA gene phenomenon (Podgjalen, Breuil & Collatz, 1994) infers that this resistance mechanism may be more established in bacterial populations than previously envisaged. It remains to be seen whether increased use of carbapenems will result in an increase in prevalence of strains producing metallo-ß-lactamases.

References


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A-71 Chromosomal β-lactamase genes of Klebsiella oxytoca are divided into two main groups: blaOXY and blaOXY2 (a more distant homolog of blaOXY). Among the North American isolates of Klebsiella, a multiple plasmid profile (SL01) was cloned and sequenced. Its nucleotide sequence similarity with the previously sequenced K. oxytoca β-lactamase gene (blaOXY) by Arakawa et al., 1989, 533:70-71 was 87.3% (repetitive consensus sequence was identical and its amino-acid identity similarity of 89.7 %). By colony hybridization on 86 strains, susceptible and resistant to ampicillin, isolated in 6 countries, K. oxytoca β-lactamase genes hybridized specifically with either a DNA blaOXY: probe (888 bp) or a DNA blaOXY2 probe (753 bp). β-lactamase gene could be divided into two main groups: blaOXY (47% of the total strains) and blaOXY2 (53%). Study of isolate points (pl) confirmed the great variability reported in the literature. However, the two β-lactamase groups were represented by four groups of strains from medical centers in the USA as follows: 6.2; 6.6 with a predominance of 6.2 which represents 59% of OXY-2) and OXY-1 (6.9; 7.2; 7.8 with a predominance of 7.2 which represents 88% of OXY-1).

A-72 The genes encoding the extended spectrum β-lactamase TEM-12 and TEM-10, responsible for resistance to extended-spectrum cephalosporins and 3rd generation cephalosporins, have been found in a number of human pathogens, and their plasmids are located on different plasmids. S.J. PROJAN, M. TUCKMAN, P.A. BRADFORD, B.A. KASMUSSEN and K. BUSH. Lederle Labs, Pearl River, N.Y. It has previously been reported that a cephalosporin-resistant strain of K. pneumoniae from a patient in Chicago harbor carried a second different extended-spectrum β-lactamase, TEM-12 and TEM-10 (Bradford et al., Antimicrob. Agents Chemother., 28:761-764, 1994). In a malting experiment with TEM as the donor strain, E. coli transconjugants were obtained carrying a single large plasmid (64 kb) and expressing either TEM-12 or TEM-10. This implied that TEM carried two essentially identical plasmids one encoding TEM-12 the other TEM-10. The same two TEM-10 and TEM-10 genes were also confirmed in a large number of isolates from various medical centers in over 10 years by using the plasmid hybridization method. TEM-10 gene was identified by a single band for the digest cutting within the transposon but two bands for the digest cutting outside of the transposon. This is indicated that, in TEM-10 two TEM-10 genes are found at two separate genetic loci. The TEM-11 gene was then used to transform competent E. coli to ampicillin resistance. All of the resulting transconjugants were inhibited with TEM-11 by 12 kb and produced a β-lactamase with a pI of 6.6 consistent with TEM-10. Therefore, the TEM-10 gene, originally on a 12 kb plasmid in the parental strain, TEM-11, effectively replaced the TEM-12 gene on the 54 kb conjugative plasmid by a recombination event during or immediately prior to mating. This finding is consistent with the observation that E. coli/plasmid conjugates expressing TEM-12 were obtained about four to ten times more frequently than those expressing TEM-10.


One thousand three hundred and sixty-two strains of E. coli and K. pneumoniae from three bacterial collections representing levels of increased resistance to piperacillin were studied. One set came from a collection of piperacillintazobactam-resistant strains from medical centers in the USA as part of an in vitro epidemiological study (252 K. pneumoniae, and 591 E. coli with PIP MIC of 5/90/512 μg/ml). The second group was isolated from patients infected or colonized in the United States, the United Kingdom, and Israel. The third collection was a group of 100 K. pneumoniae and 100 E. coli with PIP MIC of 5/90/512 μg/ml. The group with the highest levels of PIP resistance was referred from Portugal (14 K. pneumoniae and 11 E. coli with MIC of 5/90/512 μg/ml). Susceptibility of all isolates to PIP, P/ T, cephaloridine (CAZ), cefotaxime (CTX), aztreonam (ATM), ceftriaxone (CRO) and ceftazidime (FOX) were determined by agar dilution. Strains from NA with elevated MICs to CAZ, ATM, CTX, CRO or FOX were susceptible to P/ T. Antibiogram patterns were used for initial differentiation of the β-lactamases. Isoelectric focusing of crude extracts from resistant strains showed the production at least five different β-lactamase groups (g5 of 5.4, 5.6, 7.8, and 8.2) in multiple combinations. This, along with resistance to CAZ, ATM and/or CTX, reflect the presence of TEM-extended spectrum β-lactamases. These isolates represent an increasingly complex pattern of susceptibility profiles related to β-lactamase production.


Aeromonas hydrophila is increasingly recognized as a pathogen of immunocompetent as well as immunocompromised patients. Ampicillin resistance is a common trait among Aeromonas spp, and consequently the mechanisms of β-lactam resistance have been examined extensively, revealing similarities in plasmidic β-lactamases with carbapenemase activity and a cephalosporinase. However, a recent examination of β-lactamases in the fish pathogen, Aeromonas salmonicidae demonstrated the presence of three different β-lactamase plicotates, a cephalosporinase and a carbapenemase which could not be detected with nitroblue. The purpose of this study was to determine whether this same pattern of β-lactamases exists in the aquatic Aeromonas spp.

Analyses and strain exchange chromatography were employed to separate the β-lactamases in a clinical strain of A. hydrophila. Two β-lactamases with pl of 7.0 and 8.9 had been visualized on an isoelectric focusing gel which had been overlaid with nitroblue, but it was suspected that there was another "hidden" carbapenemase present in the strain which could not be detected with nitroblue. The presence of a carbapenemase could not be detected with nitroblue. The presence of a carbapenemase could not be detected with nitroblue. The presence of a carbapenemase could not be detected with nitroblue.


The β-lactamases present in 6 strains of Bacteroides fragilis were characterized by recombination and expressing plasmidial enzymes. The enzymes were expressed in E. coli and were inhibited with imipenem, meropenem, and ceftazidime. The enzyme from B. fragilis strain 2 (181) was sequenced and expressed in E. coli as a recombinant plasmid. The enzyme was inhibited with imipenem, meropenem, and ceftazidime.

Cephalothin Ceftriaxone Cefotaxime Imipenem Cefoxitin Ceftazidime

100% 98% 87% 14% 0%
10% 100% 92% 73% 72%

The role rates of hydrolysis for the other enzymes were within ± 20% of the above values. These data suggest that the 6 strains produce similar metallo-β-lactamases. This along with reports from other groups, further these enzymes are common in imipenem resistant strains of B. fragilis.

A-76 Susceptibility Patterns of Gram Negative Organisms Related to Broad Spectrum Beta-Lactam Antibiotic Usage PATRON S.A. SAAVEDRA*, M.D. CARLOS RIVAS*, M.D. GABRIEL RAMONZON* M.D. VICTOR CORREA*, M.D. BENGUSA, GALENDO, M.D. DORSI HAYES*, M.D. U.S. Department of Medicine Program and Internal Medicine Department, VA Medical Center, San Juan, Puerto Rico.

Purpose: To determine the relationship between the emergence of gram negative bacteria (GNB) resistant to cefazolin (CAZ) and or imipenem (IPM) and the frequency of use of each antibiotic.

Methods: The use of CAZ and IMP was determined from medication and pharmacy records at two time periods, prospectively yearly in 1992 and 1993 and expressed as patient days per month. The resistance of any of gram negative bacteria to CAZ and IMP was determined from microbiological records at baseline or pre-study (1991) and prospectively at the Microbiology Laboratory and ID Res Lab in 1992 and 1993. Microbiological susceptibility tests were done by the Kirby-Bauer method.

Results: The overall resistance of Enterobacteriaceae during the pre-study period was 10% to CAZ and 5% to IMIP. During 1992 and 1993 CAZ and IMP use was similar (43 and 39% for IMP and 95 and 80 for CAZ, in patient days per month). When the overall resistance of Enterobacteriaceae was compared in 1992 and 1993, it rose from 1.8% to 3.5%, and to CAZ from 1.5% to 20%. Detailed strain analysis demonstrated that resistance to IMP in Pandoromonas aerogenes rose from 3.2% to 12.0% and for Pandoromonas aerogenes from 3.9% to 70.0%. Serendipity resistance was highest in both study periods, 8.8% and 8.0% (Analysis of CAZ demonstrated a rise in Pandoromonas aerogenes resistance from 5.9% to 10.1% and Pandoromonas aerogenes from 21% to 56%.

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Introduction
Carbapenem resistance in *B. fragilis* is no longer a rarity. For example, in Japan the level of carbapenem resistance in this organism has increased from 2% in 1986 to 6.9% in 1991 (Bandon et al., 1993). Metallo-β-lactamases (m-β-lactamases) have been shown to mediate carbapenem resistance in many clinical isolates of *B. fragilis* from around the world. The aim of this study was to deduce the mechanism of high level imipenem resistance exhibited by 6 clinical strains of *B. fragilis* isolated in Spain.

Methods and materials

**Strains**
The imipenem resistant strains of *B. fragilis* were collected between 1989 and 1992 at the Dept. Clin. Micro., University Hospital San Carlos, Madrid and the antemicrobrial susceptibility tests were performed according to NCCLS guidelines. *B. fragilis* strain ED282, isolated in an UK hospital, was included as a known CIA producer.

**β-lactamase preparation**
 Cultures were grown (18 hours) anaerobically in cooked meat broth (37°C) in the presence of imipenem (2μg/ml). Crude β-lactamase preparations were prepared as described previously (Payne et al., 1994). Isoelectric focusing gels were overlaid with 1mM ZnSO₄ for 10 mins and the β-lactamases were visualised by overlaying the gel with nitrocefin (0.5mg/ml).

**Biochemical characterisation**

IC₅₀ assays were performed in 25mM PIPES pH 7 + 1μM ZnSO₄ using the automated in vitro assay previously described (Payne et al., 1991). Initial rates of hydrolysis of eleven β-lactams (100μM) were measured (25mM PIPES pH 7 + 100μM ZnSO₄) for the β-lactamases from the six Spanish clinical isolates and *B. fragilis* ED282.

**Results**

**MIC Data** Table 1 shows the susceptibilities of the six Spanish clinical isolates. Five strains had MICs of imipenem of ≥256mg/L and were sensitive to cefotaxin, cefoxitin, aztreonam, mezlocillin and piperacillin (≥64mg/L). The sixth strain had an MIC of imipenem of 16mg/L, but was sensitive to the other β-lactams.

**Isoelectric focusing**

β-lactamases from each of the *B. fragilis* isolates were only observed following the overlay of 1mM ZnSO₄. Figure 1 shows that the IEF of each of the *B. fragilis* enzymes focussed with a similar pattern to *B. fragilis* CIA 262.

Table 2. Iₐ₅₀ of metal chelators and serine β-lactamase inhibitors for the β-lactamases isolated from imipenem resistant isolates of *B. fragilis*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>296-89</th>
<th>113-90</th>
<th>288-89</th>
<th>286-89</th>
<th>286-82</th>
<th>600-90</th>
<th>ED282</th>
<th>TEM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clavulanic acid</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Tazobactam</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Subatam</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Dipicolinic acid</td>
<td>27</td>
<td>33</td>
<td>22</td>
<td>24</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>1:10-Phenanthrolin</td>
<td>55</td>
<td>58</td>
<td>58</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Iₐ₅₀ performed after a 5 min preincubation of enzyme and inhibitor.

**Discussion**

β-lactamases from each of the Spanish clinical isolates of *B. fragilis* were sensitive to metal ion chelators, but resistant to inhibition by the commercially available serine β-lactamase inhibitors. Each of these enzymes had similar IEF patterns and substrate profiles to the m-β-l produced by ED282, a known CIA producer. These data suggest that the 6 Spanish strains produce a CIA type m-β-l. These observations, along with reports from other groups, illustrate that this type of enzyme is the most common m-β-l produced by *B. fragilis*. The predicted increase in use of carbapenem antibiotics will further favour selection of organisms which produce this type of enzyme.

**References**

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**Acknowledgements**

We thank Saradobra Pradhananga for the IEF studies.
Isolation and Structure Determination of Two Novel Phenazines from a *Streptomyces* with Inhibitory Activity against Metallo-enzymes, Including Metallo-β-lactamase

**Martin L. Gilpin, Mark Fulston, David Payne, Rebecca Cramp and Ian Hood**

Smithkline Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey RH3 7AJ, U.K.
Isolation and Structure Determination of Two Novel Phenazines from a Streptomyces with Inhibitory Activity against Metallo-enzymes, Including Metallo-ß-lactamase

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(Received for publication April 17, 1995)

Two novel metabolites, SB 212021 and SB 212305, have been isolated from a Streptomyces and shown to have molecular formulae of C_{15}H_{10}N_{2}O_{5} and C_{20}H_{17}N_{3}O_{8}S, respectively. The structures were deduced by a combination of NMR techniques and mass spectral fragmentation patterns and shown to be novel members of the phenazine group of antibiotics. In the absence of added zinc, both compounds had IC_{50}'s of 1–75 µM for the Bacteroides fragilis 262 C6A and Xanthomonas maltophilia L-1 metallo-ß-lactamases. The compounds also inhibited ACE with IC_{50}'s of 55 and 68 µM, respectively. Mode of action studies illustrate that the compounds inhibit some metalloenzymes by chelation of the active site metal ion. They exhibit poor antibacterial activity.

Metallo-ß-lactamases, whilst not as prevalent in nature as their serine counterparts, do constitute a possible threat to ß-lactam chemotherapy due to the unavailability of effective inhibitors. Although only produced by a limited number of organisms they are potentially transferrable by plasmids and could therefore pose a wider threat. The search for inhibitors of this enzyme is thus aimed at identifying structural types from natural product screening that could provide leads for chemical modification/synthesis.

We report on the isolation of two novel phenazines, designated SB 212021 (1) and SB 212305 (2), produced by an unidentified Streptomyces sp. and found to inhibit zinc-dependent metallo-ß-lactamase from Bacillus cereus.

This paper describes the isolation, physico-chemical properties, structure determination and biological activity of SB 212021 and SB 212305.

Materials and Methods

Fermentation Conditions

The unidentified Streptomyces sp. was maintained as a vegetative cell suspension stored in glycerol 10% under liquid nitrogen. Vegetative cell and spore suspension (1 ml) was used to inoculate seed medium (100 ml) containing 1.5% agar. The seed stage medium consisted of yeast extract (Oxoid) 0.5%, malt extract (Oxoid) 1.0%, glycerol 1.0% and peptone soya (Oxoid) 0.5% dissolved in distilled water and adjusted to pH 6.5 before sterilisation in an autoclave at 121°C for 15 minutes. Four spiked 500 ml flasks, each containing 60 ml of seed stage medium as defined above, were inoculated with 3 plugs from a culture plate. The inoculated flasks were incubated on a gyratory shaking table at 240 rpm for 60 hours at 28°C and after this time were used to inoculate 70 spiked 500 ml flasks, each containing 60 ml of seed stage medium as defined above (inoculum level ca. 5%, 3 ml/flask). These inoculated flasks were shaken at 240 rpm for 4.5 days at 28°C. The harvested broth was
Detected Methods
The phenazine inhibitors could be detected by HPLC in the culture broth of the unidentified Streptomyces sp. by monitoring at 300 nm. A C18 Spherisorb S 10 ODS 2 column (250 x 4.6 mm) (PhaseSep, Deeside Industrial Estate, Queensferry, Clwyd, U.K.) was used for the separation and eluted with 0.05 M ammonium acetate buffer at pH 6.5 in 30% methanol. At a flow rate of 1.5 ml/minute SB 212021 had a Rt of 9.6 minutes and SB 212305 had a Rt of 6.5 minutes. A Waters 600 multilin delivery system was used and monitoring was by a Waters Lambda Max Model 481 LC spectrophotometer.

The extraction and purification of the phenazines could also be followed by monitoring inhibitory activity against Bacillus cereus II metallo-ß-lactamase (Porton Products Ltd., Maidenhead, Berks., U.K.). Nitrocefin was used as reporter substrate in an assay which has been previously described23. IC50's were determined following a 15 minute pre-incubation of enzyme and inhibitor.

Extraction and Isolation
The clarified broth was acidified to pH 3 by addition of 5 M HCl and extracted with butanol. The dark butanol layer was then back-extracted with water maintained at a pH of 7.5. The aqueous layer afforded a red powder (3.6 g) on freeze-drying. Ion-exchange chromatography on IRA 458 (CI- form) (Rohm and Haas, Philadelphia, U.S.A.) and elution with 0.5 M NaCl gave a purer product. Desalting on Diaion HP-20 styrene divinyl benzene cross-linked polymeric adsorbent (supplied by Mitsubishi Chemical Industries Limited, Tokyo, Japan) afforded two active fractions; Fraction 1 (0.61 g) which eluted from the column with water, and Fraction 2 (0.35 g) which eluted with neat methanol.

Fraction 2 was further chromatographed on silica gel, eluting with n-butanol - ethanol - water (4:1:1) to give red powder (69 mg) and subsequently purified by HPLC on a Dynamax 150 A preparative C18 column (300 x 10 mm, fitted with pre-column) (supplied by Rainin Instruments Co. Inc., Mack Road, Woburn, Massachusetts, U.S.A.), eluting with 27.5% MeOH in 0.05 M ammonium acetate buffer and monitoring at 300 nm. This yielded 10 mg of SB 212021. The Rt under the above conditions was 18 minutes.

Fraction 1 was further chromatographed on silica gel, eluting with n-butanol - ethanol - water (2:1:1) to give red powder (0.29 g) and subsequently purified by HPLC on a Dynamax preparative C18 column (details above), eluting with a linear gradient of 100% water to 50% methanol over 30 minutes. Under these conditions, and monitoring at 300 nm, SB 212305 (47 mg) was isolated with Rt 19 minutes. A further component, SB 210767 (49 mg), was also isolated with Rt 21 minutes. This latter compound appeared to have very similar spectroscopic properties to SB 212305 and the same mass spectrum. We believe that it may be a chelated form of SB 212305.

Biological Evaluation
The IC50's of each compound for Bacillus cereus II, Bacteroides fragilis 262 CfiA49 and Xanthomonas maltophilia L-15 metallo-ß-lactamases were determined as above. Each IC50 was measured both in the absence of added zinc and in the presence of 1 mM zinc sulphate. The reporter substrate for the zinc-dependant angiotensin-converting enzyme (ACE) IC50's was N-(3-[2-furyl]acryloyl-Phe-Gly-Gly (FaFGG)6. Assays were performed in 0.05 M Hepes, 0.3 M NaCl at pH 7.5 at 37°C. A range of inhibitor concentrations were incubated for 5 minutes with purified ACE (Sigmachemical Company, Poole, Dorset, U.K.) and the assay initiated by the addition of 0.35 mM FaFGG (final concentration). Percent inhibition of ACE activity relative to an untreated control was calculated for each concentration and the IC50 calculated.

Mechanism of Inhibition of B. cereus II
B. cereus II metallo-ß-lactamase was incubated with a sufficient concentration of SB 212021, SB 212305 and EDTA (for comparison) to ensure >90% inhibition. These samples were then passed down a PD10 (Pharmacia Biotech Europe, Brussels, Belgium) gel filtration column. Fractions (0.5 ml) were collected and examined for ß-lactamase activity by diluting 50 µl of sample into 3 ml of 25 mM Pipes buffer (pH 7.0) containing 1 mM ZnSO4 and 333 µM ampicillin. The rate of hydrolysis of ampicillin was measured at 235 nm. A control sample was also subjected to this treatment. The amount of inhibitor in each fraction was estimated by reading the absorbance at 290 nm, 305 nm and 229 nm for SB 212021, SB 212305 and EDTA respectively.

Spectroscopic Methods
NMR spectra obtained on SB 212021 and SB 212305 were run at 25°C on a Bruker AM 400 operating at 400 MHz. IonSpray MS were obtained on a Sciex (Toronto) API-III triple-quadrupole mass spectrometer.

Results and Discussion
Isolation
Culture broth (4.25 litres) from an unidentified Streptomyces sp., when treated as outlined above, afforded 10 mg of SB 212021 and 47 mg of SB 212305.

Properties of SB 212021 and SB 212305
The physico-chemical properties of the two phenazines, SB 212021 and SB 212305, are shown in Table 1. NMR data is presented in Tables 2 and 3.

Biological Properties
SB 212021 showed some antibacterial activity against certain strains of Streptococcus pneumoniae and En-
### Table 1. Physico-chemical properties of SB 212021 and SB 212305.

<table>
<thead>
<tr>
<th>Position</th>
<th>Molecular formula</th>
<th>IR (KBr) cm⁻¹</th>
<th>MS (ionspray) (M+H)⁺</th>
<th>NMR values</th>
<th>MS (ionspray) (M-H)⁻</th>
<th>NMR values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C₂₇H₁₈N₂O₈S</td>
<td>3395, 1691, 1616, 1580, 1536, 1476, 1424, 1382, 1341, 1269, 1237, 1205, 1134, 1093, 1049</td>
<td>210 (23,563), 247 (11,608), 294 (17,672), 370 (2,840), 520 (1,178)</td>
<td>210 (23,563), 247 (11,608), 294 (17,672), 370 (2,840), 520 (1,178)</td>
<td>210 (23,563), 247 (11,608), 294 (17,672), 370 (2,840), 520 (1,178)</td>
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<tr>
<td></td>
<td>MeOH</td>
<td>1683</td>
<td>104.1</td>
<td>1683</td>
<td>104.1</td>
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</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>212 (25,422), 249 (18,754), 305 (33,757), 380 (5,000), 530 (2,084)</td>
<td>212 (25,422), 249 (18,754), 305 (33,757), 380 (5,000), 530 (2,084)</td>
<td>212 (25,422), 249 (18,754), 305 (33,757), 380 (5,000), 530 (2,084)</td>
<td></td>
<td></td>
</tr>
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</table>

### Table 2. ¹H and ¹³C NMR chemical shifts and assignments for SB 212021 in CD₃OD.

<table>
<thead>
<tr>
<th>Position</th>
<th>δH (ppm)</th>
<th>δC (ppm)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>6.83, d, J 8.6</td>
<td>110.8</td>
</tr>
<tr>
<td>4</td>
<td>8.60, d, J 8.6</td>
<td>143.1</td>
</tr>
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<td>9</td>
<td>8.52, d, J 8.6</td>
<td>134.5</td>
</tr>
<tr>
<td>1-COOMe</td>
<td>3.97, s</td>
<td>52.0</td>
</tr>
<tr>
<td>6-COOH</td>
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</tr>
<tr>
<td>Me</td>
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</tr>
<tr>
<td>4a</td>
<td>137.6</td>
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</tr>
<tr>
<td>5a</td>
<td>146.0</td>
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<tr>
<td>9a</td>
<td>145.6</td>
<td></td>
</tr>
<tr>
<td>10a</td>
<td>108.3</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. ¹H and ¹³C NMR chemical shifts and assignments for SB 212305 in D₂O.

<table>
<thead>
<tr>
<th>Position</th>
<th>δH (ppm)</th>
<th>δC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.83, d, J 8.6</td>
<td>110.8</td>
</tr>
<tr>
<td>4</td>
<td>8.60, d, J 8.6</td>
<td>143.1</td>
</tr>
<tr>
<td>9</td>
<td>8.52, d, J 8.6</td>
<td>134.5</td>
</tr>
<tr>
<td>1-COOMe</td>
<td>3.97, s</td>
<td>52.0</td>
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<td>6-COOH</td>
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<td>Me</td>
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<td>4a</td>
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<td>5a</td>
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<td>9a</td>
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<td></td>
</tr>
<tr>
<td>10a</td>
<td>108.3</td>
<td></td>
</tr>
</tbody>
</table>

The IC₅₀'s of the two metabolites (plus the chelator EDTA as a control) against three metallo-β-lactamase enzymes and the metallo-enzyme ACE are shown in Table 4. Both SB212021 and SB212305 were poor inhibitors of B. fragilis 262 CfiA with and without added zinc. In the absence of added zinc both compounds and EDTA had IC₅₀ values of <100 µM for the X. maltophilia L-1 and B. cereus II metallo-β-lactamases, but when these assays were performed in the presence of 1 mM zinc the IC₅₀'s increased by >10 fold. Therefore, inhibition of metallo-β-lactamases by the phenazines, like EDTA, was dependent on the zinc concentration. To examine the mechanism of inhibition further, B. cereus II metallo-β-lactamase inhibited by SB212021, SB212305 and EDTA was subjected to gel filtration. In each case this treatment separated excess inhibitor from the metallo-β-lactamase. When the fractions containing enzyme were assayed in excess zinc >80% of enzyme activity was recovered. Therefore, enzyme inhibited by these agents, like EDTA, could be reversed by the addition of zinc. Also, both the phenazines and EDTA inhibited ACE with IC₅₀ values of 50-68 µM, illustrating the low specificity exhibited by these agents. These data have shown that, like EDTA, SB212021 and SB212305 inhibit metallo-β-lactamases by chelation of the active site zinc. By definition such agents should exhibit low specificity. This is illustrated by their activity against two unrelated metalloenzymes.

Structure Determination

FAB, EI and CI mass spectroscopic techniques failed to give any MW information on SB 212021 or SB 212305.
Ionspray, on the other hand, afforded a peak at 299 in +ve ion mode (after acidification of the sample) and 297 in -ve ion mode for SB 212021, indicating a MW of 298. SB 212305 similarly afforded a molecular ion at 458 in -ve ion mode under ionspray conditions, together with a major fragment at 329. In +ve ion, peaks at 460, 477 and 482 were observed corresponding to protonated, ammoniated and sodiated molecules, respectively. A MW of 459 was established.

Consideration of the UV data (see Table 1) obtained on the two inhibitors, coupled with the characteristic low-field chemical shifts of the aromatic protons (Tables 2 and 3) rapidly established these compounds as members of the phenazine class of antibiotics.7-9

The 1H NMR of SB 212021 (Table 2) indicated two possible substitution patterns; either a 1,2,6- or a 1,4,6-trisubstitution. The high field shift (δ 6.83) of one of the aromatic protons indicated that it must be ortho to a hydroxyl or ether function. Further support for the presence of an oxygen substituent came from the 13C NMR which indicated two high field signals (110.1 and 110.8) corresponding to the two ortho carbon atoms. It has been reported10,11 that bridgehead carbon atoms in the phenazine ring system are particularly insensitive to adjacent hydroxyl substitution and do not display the usual upfield shift expected for ortho carbons. This being so, the fact that two such high field signals are observed in the 13C NMR spectrum of SB 212021 indicates the presence of a 2-hydroxy (or methoxy) rather than a 1- or 4-hydroxy substituent.

The observed MW for SB 212021 was readily satisfied by the inclusion into the structure of two carboxy substituents at positions 1 and 6. This was supported spectroscopically by carboxyl absorption bands at 1691 cm⁻¹ in the IR spectrum and by 2 signals in the 13C NMR at 171.1 ppm. It is conceivable that the two carboxy functions could be in a 1,9-arrangement and NMR techniques would not be expected to distinguish between these two possibilities. However, work by Holliman et al. and others12,13 has established that phenazine-1,6-dicarboxylic acid is a universal precursor for microbial phenazines and therefore it is reasonable to assume this regiochemistry for SB 212021.

Thus, the structure of SB 212021 would appear to be based on 2-hydroxy phenazine-1,6-dicarboxylic acid. The methyl group can be shown to be present as a methyl ester rather than a methyl ether. Evidence for this comes from the methyl signal in the 1H NMR spectrum which did not exhibit measurable nuclear Overhauser enhancement with any other proton in the molecule. A 1-carboxymethyl substituent neatly explains this lack of interaction, as indicated in structure (1). Further evidence to support this placing of the methyl group is presented below.

Consideration of the 1H NMR data (Tables 2 and 3) leads to the conclusion that SB 212305 is a substituted derivative of SB 212021 where the C-3H is replaced with a -CH₂CH₂- unit. The ionspray mass spectrum (-ve ion) of SB 212305 gives a major fragment at 329 and, since no corresponding loss is seen in the spectrum of SB 212021, the fragmentation must be associated with the C-3 side-chain. That being so, the peak observed at 329 is readily explained in terms of the phenazine nucleus carrying a sulphur atom at C-3 (3).

The 1H and 13C chemical shifts corresponding to the C-3 side chain in SB 212305 strongly suggest the presence of an amino acid and signals were consistent with an N-acetyl cysteine moiety. There is good agreement between literature14,15 and observed chemical shifts for this group. Furthermore, the presence of sulphur in SB 212305 was confirmed by microanalysis.

Finally, the presence of a methyl ester in SB 212305 was confirmed by MS/MS experiments resulting in peaks corresponding to the loss of 44 (CO₂) and 59 (COOMe). In addition, potentiometric titration of this compound indicated the presence of a phenolic group with pKa 9.1 / −0.25.

The assignments in Tables 2 and 3 are based on the
results of COSY and HMBC NMR experiments.

Acknowledgements

The authors would like to thank John Tyler and Janet White for NMR support and Duncan Bryant and Gerry Rissue for mass spectroscopic data. We would also like to thank Chris Reading for helpful discussions.

References

A series of mercaptoacetic acid thiol esters have been identified as metallo-ß-lactamase inhibitors. Electro-spray mass spectrometry (ESMS) has shown that irreversible inhibition of the Bacillus cereus II metallo-ß-lactamase by SB214751, SB214752, and SB213079 was concomitant with a 90-Da increase in mass of the enzyme. Tryptic digestion of the B. cereus II inhibited with SB214751 illustrated that the peptide fragment, containing the only cysteine of the enzyme, had undergone a mass increment of 90 Da. It was further demonstrated that B. cereus II hydrolyzed this type of compound across the thiol ester bond to yield mercaptoacetic acid. Mercaptoacetic acid is the only molecular fragment common to SB214751, SB214752, and SB213079, and free mercaptoacetic acid does not bind covalently to B. cereus II. Therefore, it is concluded that these compounds inhibit B. cereus II by the mechanism-based delivery of mercaptoacetic acid, forming a disulfide linkage with the active site cysteine (predicted mass shift = +90 Da) under the aerobic conditions of the assay. The different thiol esters examined had a broad range of potencies against the metallo-ß-lactamases tested. For example SB214751, SB214752, and SB213079 all had 50% inhibitory concentrations of <10 and >1,000 µM for the Stenotrophomonas maltophilia L-1 and Bacteroides fragilis CBA enzymes, respectively. SB216968 was particularly active against the Aeromonas hydrophila CphA metallo-ß-lactamase and was found to be an uncompetitive inhibitor of this enzyme (Kᵢ = 3.9 µM), whereas it exhibited irreversible inhibition of the L-1 enzyme. These observations with this series of compounds have revealed subtle differences between the active sites of different metallo-ß-lactamases. Finally, a novel application for isothermal titration calorimetry for assessing the zinc chelating activity of candidate inhibitors is also presented.

Carbapenems have a broad spectrum of antibacterial activity, and they are resistant to hydrolysis by the majority of serine-based ß-lactamases. For these reasons the medical community will become increasingly dependent on this type of antibiotic and its usage will increase. However, most metallo-ß-lactamases hydrolyze the majority of commercial ß-lactam antibiotics, including carbapenems. As plasmid-encoded metallo-ß-lactamases are now produced by common clinical pathogens (e.g., Klebsiella pneumoniae, Serratia marcescens, Pseudomonas aeruginosa, and Bacteroides fragilis [12, 17, 23]) and the selective pressure for these enzymes is increasing, it is probable that the prevalence of metallo-ß-lactamases will increase.

The metallo-ß-lactamases produced by these different organisms have homologous active sites. The earliest structural studies with the prototypic Bacillus cereus II enzyme concluded that the active site Zn²⁺ cation was coordinated by histidine residues and (distantly) by the only cysteine residue of the enzyme, Cys168 (21). Early biochemical work with this enzyme has shown that this cysteine residue is accessible to thiol reagents, such as Ellman’s reagent, and also to iodoacetic acid, only in the absence of Zn²⁺, suggesting that the reactivity of the cysteine thiol group is downmodulated in the presence of the metal cation (20). More recently, an X-ray crystallographic study of B. cereus II at 2.5 Å refined the structure as a mononuclear zinc hydrolase resembling the metallo zinc carboxypeptidases (4). However, the most recent, high-resolution, synchrotron radiation studies have revealed that the complete structures, both of this enzyme at 1.9 Å (8) and of the B. fragilis CcrA metallo ß-lactamase (CcrA has an amino acid sequence identical to that of CfiA (5)), possess a dinuclear zinc active site. In these structures, the cysteine is seen at last to fulfill a satisfactory role as a ligand to the second zinc atom.

One approach to overcoming the threat of metallo-ß-lactamase would be the discovery or design of inhibitors of these enzymes to be used in combination with ß-lactam antibiotics. We report the discovery of a series of thiol ester (thiadesipetide) metallo-ß-lactamase inhibitors. Figure 1 shows the structural relationship of these inhibitors compared with the generalized structure of metallo-ß-lactamase substrates. Representatives of this class of thiol esters have been shown to be substrates for serine and metallo-ß-lactamases (25, 26) and D,D-peptidases (6, 11, 13) and are slowly hydrolyzed by the Streptomyces albus G D,D-metalloprotease (1, 6). The study investigating the interaction of thiol esters with Streptomyces KI5 active site serine D,D-transpeptidase deployed SB214751 in its racemic form (11). The present study describes the interaction of the resolved enantiomeric forms of this compound and other related compounds with metallo-ß-lactamases. Data are presented on the interesting inhibition of the Aeromonas hydrophila CphA and Stenotrophomonas maltophilia (formerly Xanthomonas maltophilia) L-1 metallo-ß-lactamases. However, our detailed mechanistic studies have focused on the inhibition of the B. cereus II enzyme, as purified quantities of this enzyme were readily available.
Bacterial strains and enzymes. B. cereus II metallo-β-lactamase was purchased from Portron Products (Portron Products Ltd., Maidenhead, Berkshire, United Kingdom), and the L-1 and CphA metallo-β-lactamases were partially purified as described previously from S. maltophilia 511 (10) and A. hydrophila AE036 (16), respectively. The CphA-type enzyme was obtained from B. fragilis 265, this was used as a crude preparation, as no other β-lactamases were produced by the strain (14).

The mercaptoacetic acid thiol ester inhibitors, SB214751, and SB214752 are N-acetylated variants of the thiol ester SB214698, 2-(o-alanine) acetic acid, trichloroacetic salt. SB216271 and SB214751 contain P2-type recognition features associated, respectively, with the ampicillin and benzyl penicillin substrates of metallo-β-lactamases. SB213079 contains an O-acetylated hydroxamic acid functionality (Table 1).

The thiol esters in Table 1 were prepared by methods similar to those previously described (6). The following synthesis of the N-phenylacetyl D-alanyl analog SB214751, the principal compound of the present investigation, is typical of the procedure. Phenylacetyl D-alanine (1 mol equivalent) was added to ethyl chloroformate (1 mol equivalent) and triethylamine (2 mol equivalent) in diethylformamide-ethyl acetate (1:1), with stirring, at −5°C. Mercaptoacetic acid (1 mol equivalent) and trichloroamine (1 mol equivalent) were added and stirring was continued, first at room temperature and then at 50°C. The product was converted to the sodium salt (sodium hydrogen carbonate, 1 mol equivalent) and purified by HPLC chromatography, affording SB214751 as its sodium salt.

Determination of IC50. The 50% inhibitory concentrations (IC50s) were determined by previously described methodology (18). IC50s for B. cereus II, S. maltophilia L-1, and B. fragilis CphA β-lactamases were determined with 40 μM nitrocefin as reporter substrate, using 25 mM piperazine-N,N'-bis(2-ethanesulphonic acid) (PIPES) buffer at 1 μM and 100 μM Zn²⁺ (pH 7.0). All IC50s were measured following a 5-min incubation of enzyme and inhibitor. IC50 for the A. hydrophila CphA enzyme were determined in the presence of 1 μM Zn²⁺. Iminopron (100 μM) was used as the reporter substrate, since nitrocefin is a very poor substrate for this enzyme (10).

TABLE 1. Inhibitory activity of thioesters*

<table>
<thead>
<tr>
<th>Concentration of Zn²⁺</th>
<th>B. cereus II</th>
<th>B. fragilis CphA</th>
<th>X. maltophilia L-1</th>
<th>A. hydrophila CphA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>1</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Dipicolinic acid</td>
<td>28</td>
<td>871</td>
<td>40</td>
<td>437</td>
</tr>
<tr>
<td>SB216271</td>
<td>38</td>
<td>479</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SB214751</td>
<td>479</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SB214752</td>
<td>645</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SB216968</td>
<td>-</td>
<td>631</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SB213079</td>
<td>288</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Thiol esters were mercaptoacetic acid derivatives. IC50 > 1,000 μM.
ESMS and preparation of samples. Electrospray mass spectrometry (ESMS) has been utilized previously to characterize inhibitors covalently bound to serine-
lactamases (2, 9, 19). Although the substrate hydrolysis of metallo-β-lactama-
ers is not expressed by means of common intermediates, the presence of a
sulfenic acid intermediate to the active site of metallo-β-lactamases allows the
use of thiol reagents to demonstrate the presence of zinc.

When 25 mM PIPES (pH 7.0) buffer was used throughout the ESMS and trypsin digestion experi-
ments, a few ESMS experiments were also carried out with 25 mM PIPES (pH 7.0) buffer to confirm that the observed effects were not buffer artifacts. Inhibi-
tion of B. cereus II by SB214751, SB214752, SB213079, and meropenem acid was studied by ESMS. One hundred fifty microliters of a 1.6 mM solution of each
inhibitor was added to 150 µl of 80 mM B. cereus II (final Zn²⁺ concentration = 30 mM) with mixing, to provide a 200:1 molar ratio of inhibitor-β-lactamase II. A
B. cereus II control solution, containing 40 µM enzyme, was prepared at the same
time. After 1.5 h at 37°C a 5-µl aliquot was removed from each of the samples and the control and then diluted 10,000 times into 100 µM nitrocefin. The
density of the enzyme was then measured and compared to that of the un inhibited
control.

The mixture (20 µl) was then examined by electrospray liquid chromatography
(LC-MS) using a gradient system in order to prevent possible interference from
enzymes inhibitors, or impurities in the ESMS. Liquid chromatography was
performed on a Perkin-Elmer 200 mass spectrometer. Spectra were averaged
to provide an adequate signal-to-noise ratio. One hundred microliters of a
150-µM sample solution of each enzyme, was applied to a 200:1 mixture of
SB214751: B. cereus II, prepared as
A
B
c
d
FIG. 2. ESMS of native B. cereus II enzyme. Peaks A through F represent
gagged ends of B. cereus II. Peak A, K;=IIç;
Zn²⁺ and Zn²⁺ + 1, where I represents the inhibitor and ZnI
represents the complex. Therefore, K is given by the concentration ratio:
K = [ZnI]²⁺ / [Zn²⁺];

Results

Activities of compounds. SB214751, SB214752, SB213079, and SB216271 all showed their greatest inhibitory activities against the L-1 metalloenzymes. None of the compounds had detectable activity against the CfIA enzyme, and with the excep-
tion of SB216968, activity against B. cereus II appeared to be greater at 1 than at 100 µM Zn²⁺. This effect may be rationalized in terms of the proposed mechanism and the dinuclear active site (see Discussion). SB216968 was excep-
tionally active against the CbPA metallo-β-lactamase (Table 1).

ESMS. ESMS of the B. cereus II enzyme gave 6 peaks, a through F (Fig. 2), each of which corresponded to tagged ends consisting of the loss of N-terminal amino acid residues (19). Following a 1:10,000 dilution, B. cereus enzyme was inhibited 95% by each of the inhibitors, SB214751, SB214752, or SB213079. However, under the same conditions, no inhibition by meropenem acid was observed. ESMS indicated a time-
dependent mass increment of 88 to 92 Da for B. cereus II enzyme solutions incubated with SB214751, each peak increased by 88 to 92 Da (Fig. 3). An identical adduct was observed when the reactions were performed in PIPES buffer. SB213079 and SB214752 also gave rise to a mass increment of approximately 90 Da for the enzyme. The increment must therefore arise from these compounds as a consequence of adduct formation with their common -SCH₂CO₂H component. No adduct was observed with B. cereus II incubated with mer-
openem acid. Although the theoretical mass increments for the following would fall outside the observed range of 88 to 92 Da, control experiments have shown that this increment was not caused by adducts arising from the presence of DMSO, Zn²⁺, or SO₃²⁻ species.

Kinetics of inhibition of metallo-β-lactamases by SB216968 and SB214751. The inhibition of B. cereus II by SB214751 and
mercaptoacetic acid and inhibition of CphA by SB216968 were chosen for more detailed studies.

In the absence of 300 μM zinc sulfate the inactivation of B. cereus II by SB214751 was found to be nonsaturating; it was not therefore possible to calculate either the inhibitor constant \( K_i \) or the maximum rate of inactivation \( k_{inact} \). However, by the method of Kitz and Wilson (15) the second-order constant of inactivation was calculated as 0.0714 mM\(^{-1}\) min\(^{-1}\). In contrast, when 300 μM zinc sulfate was added, inactivation was found to be saturated, giving a value of 0.2 mM for \( K_i \) and 2.21 \( \times 10^{-3} \) min\(^{-1}\) for the maximum rate of inactivation and a value of 0.0108 mM\(^{-1}\) min\(^{-1}\) for the corresponding second-order rate of inactivation (half-life \( t_{1/2} = 314 \) min). The stability of the inhibited enzyme complex was measured by allowing the enzyme to react with SB214751 to give 100% inhibition. Subsequently samples were removed and diluted into excess substrate and the activity was compared to that of an untreated control. It was found that over a 24-h period no recovery of enzyme activity occurred. Mercaptoacetic acid, the thiol component of SB214751, exhibited no progressive inhibition of B. cereus II and a \( K_i \) of 27.9 μM was determined in the presence of 100 μM zinc sulfate.

Inhibition of A. hydrophila CphA metallo-β-lactamase by SB216968 was studied with imipenem as the reference substrate, and the inhibition was not reduced by increasing the substrate concentration. Analysis of initial rate data (i.e., the first 30 s of the reactions) using 1/\( [I] \) against [I] and [S]\(^{-1} \) against 1/\([I] \) for different imipenem concentrations showed uncompetitive inhibition, with a \( K_i \) of 3.9 μM. However, preincubation (5 min) of enzyme with inhibitor reduced the IC\(_{50}\) from 1.8 to 0.55 μM, indicating a time-dependent component to the inhibition. (Extended incubation times were not possible because of enzyme instability.) Furthermore, incubating the metallo-β-lactamase with concentrations of SB216968 shown previously to be inhibitory (0.78 to 6.25 μM), then diluting into excess imipenem and assaying for activity, gave essentially no inhibition of the enzyme. Increasing the inhibitor concentration to 50 μM, then diluting, gave only the degree of inhibition expected from carryover. Inhibition was unaffected by increasing the Zn\(^{2+}\) concentration from 1 to 10 μM.

ITC As expected, the ITC data for EDTA showed very high affinity for Zn\(^{2+}\); the dissociation constant \( K \) was outside the range of the instrument, giving a \( K \) of \( \leq 1 \) nM. The shape of the titration curve for diisopelic acid (control) and stoichiometry of the endpoint was more complex than a simple 1:1 interaction. The data fit illustrated a stepwise binding of two molecules of dipicolinic acid by Zn\(^{2+}\) with a \( K \) of \( \leq 1 \) μM for each step. The results for SB214751, SB214752, and SB216968 indicated that their dissociation constants were also very high (\( K > 1 \) mM), corresponding to very low affinity of the compounds for Zn\(^{2+}\).

Hydrolysis of SB214752 by B. cereus II. HPLC analysis of the B. cereus II-treated samples illustrated that the metallo-β-lactamase caused a reduction in the peak which corresponded to SB214752 and an increase in a peak which corresponded to N-benzoyl alanine. The hydrolysis of the thiol ester by B. cereus II was three times greater than the background rate of the control sample. Subtracting the background rate, under these conditions SB214752 was hydrolyzed at a rate of 10.4 to 11.8 nM/min/μM enzyme (\( k_{cat} = 0.01 \) min\(^{-1}\); \( t_{1/2} = 63 \) min). These data were determined from the decrease in substrate peak and increase in product peak, respectively. It is inferred that the second product from the enzyme hydrolysis is, therefore, mercaptoacetic acid.

Identification of the binding site of the -90 fragment. The purpose of this work was to identify the amino acid residue in B. cereus II which had bound the 90-Da fragment from SB214751. Following the trypptic digestion of B. cereus II the mass of 14 peptides was determined (Table 2) and assigned to a specific part of the B. cereus II sequence. The peptide which contained the only cysteine residue of the enzyme (peptide 18) was not observed in trypptic digests of native B. cereus II. This may be a result of the high reactivity of fragments containing the free thiol. However, peptide 18 of the enzyme treated with SB214751 had undergone a mass increment of 90 Da (Table 2). The identity of this modified cysteine peptide was confirmed by collecting the HPLC fractions followed by manual Edman sequencing of the first 20 amino acids.

ESMS illustrated that the mass of B. cereus II treated with iodoacetic acid increased by 116 Da. This indicates that two molecules of iodoacetic acid bind to the enzyme. One of these

<table>
<thead>
<tr>
<th>HPLC peak no.</th>
<th>Mol mass (Da)</th>
<th>B. cereus II amino acid sequence assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ND(^a)</td>
<td>NA(^b)</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>1,348</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>1,504</td>
<td>206-216</td>
</tr>
<tr>
<td>6</td>
<td>1,447</td>
<td>79-91</td>
</tr>
<tr>
<td>7</td>
<td>1,212</td>
<td>105-116</td>
</tr>
<tr>
<td>8</td>
<td>1,403</td>
<td>11-23</td>
</tr>
<tr>
<td>9</td>
<td>989</td>
<td>66-72</td>
</tr>
<tr>
<td>10</td>
<td>1,332</td>
<td>51-62</td>
</tr>
<tr>
<td>11</td>
<td>2,018</td>
<td>117-134</td>
</tr>
<tr>
<td>12</td>
<td>1,890</td>
<td>118-134</td>
</tr>
<tr>
<td>13</td>
<td>2,870</td>
<td>24-50</td>
</tr>
<tr>
<td>14</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>15</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td>1,234</td>
<td>211-227</td>
</tr>
<tr>
<td>17</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>18</td>
<td>2,758</td>
<td>148-171(^c) + 90 Da</td>
</tr>
<tr>
<td>19</td>
<td>2,436</td>
<td>177-198</td>
</tr>
<tr>
<td>20</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>21</td>
<td>24,259</td>
<td>B. cereus II(^d)</td>
</tr>
</tbody>
</table>

\(^a\) ND, not detectable.
\(^b\) NA, cannot be assigned.
\(^c\) Peptide containing the active site cysteine.
\(^d\) Mass of B. cereus II minus N-terminal residues 1 to 6.

![ESMS of B. cereus II incubated with 1:200 molar ratio of SB214751 for 90 min. Peaks A to F represent ragged ends of B. cereus II plus 88 to 52 Da. Peak A: +90; peak B: +88; peak C: +91; peak D: +92; peak E: +90; peak F: +70.](image-url)
binding sites is putatively the active site cysteine (20). Only one molecule of iodoacetic acid bound to B. cereus II enzyme inhibited with SB214751 (mass increment of 58 Da). This illustrates that only one derivatizable group was now available, suggesting that the 90-Da fragment from SB214751 was probably bound to the active site cysteine.

**DISCUSSION**

The results above have shown that these mercaptoacetic acid thiol ester compounds exhibit a variety of potencies against the different metallo-β-lactamasises tested. The most significant observation was the irreversible inhibition of B. cereus II, and this was judged by an in-depth evaluation. Overall, the L-1 enzyme appears to be the most susceptible to inhibition by these compounds, although SB216968 shows very good potency against the A. hydrophila CphA enzyme. Interestingly, no inhibition of the CspA enzyme was detected and therefore this series of inhibitors has identified differences in the active site of the CspA and those of the B. cereus II, CphA, and L-1 enzymes.

The ITC results for the thiol esters show that their affinity for Zn$^{2+}$ under the conditions used was very much lower than the affinity of the B. cereus II enzyme for Zn$^{2+}$ (K = 1 µM [7]). This implies that the inhibition exhibited by our compounds was not simply due to chelation of Zn$^{2+}$ released from the active site. This was illustrated by the fact that the inhibition of L-1 metallo-β-lactamase by the compounds was essentially unaffected by the concentration of Zn$^{2+}$ present during testing. In contrast, dipicolinic acid, a metallo-β-lactamase inhibitor and Zn$^{2+}$ chelator (24), was five times more potent against this enzyme at 1 µM Zn$^{2+}$ than at 100 µM Zn$^{2+}$. Inhibition of B. cereus II by the thiol esters was affected by the concentration of Zn$^{2+}$. As stated previously, this is unlikely to be caused by the sequestration of Zn$^{2+}$ by these compounds, and an alternative hypothesis is offered below. The use of ITC to determine the potential chelating activity of compounds has provided a novel approach for the evaluation of candidate metalloenzyme inhibitors.

The compounds also had different mechanisms of inhibition for the different enzymes: the ESMS experiments and kinetic evaluation clearly illustrated that inhibition of B. cereus II by SB214751 was progressive and irreversible. In marked contrast, initial rate data (imipenem as reference substrate) indicated that SB216968 was a competitive inhibitor of Cpha metallo-β-lactamase. This observation would suggest that the inhibitor binds to the ES complex, as in scheme 1 below:

\[ E + S \rightarrow ES \rightarrow E + P \]

\[ +I \]

\[ \downarrow \]

\[ \text{ESI} \]

where S is the substrate (imipenem) and I is the inhibitor (SB216968). Such a situation is necessarily very different from that exhibited in the interaction of SB214751 with B. cereus II. Binding of substrate to Cpha may give rise to a conformational change and render the complex vulnerable to attack by SB216968 at a site as yet unspecified. Some support for this is provided by the observation that bovine serum albumin (50 µg ml$^{-1}$) afforded protection against inhibition. However, the improved inhibition on preincubation, followed by recovery of activity on dilution, shows that this inhibition of Cpha is more complicated than the classical reversible type. This behavior is consistent with relatively slow binding of SB216968 to the enzyme, with release of inhibitor and recovery of free enzyme when its concentration is decreased by dilution. There may be more than one inhibited species, one being formed slowly from the other.

The irreversible inhibition of B. cereus II was studied in more detail. HPLC analysis showed that the interaction of B. cereus II with these compounds achieves a hydrolytic scission of the thiol ester bond to release mercaptoacetic acid. ESMS studies proved the irreversible inhibition of B. cereus II by SB213079, SB214751, and SB241752 to be associated with the covalent binding of a 90-Da fragment to the enzyme. Mercaptoacetic acid has a mass of 92 Da, and if this fragment formed a mixed disulfide covalent linkage with the B. cereus II enzyme under the aerobic conditions employed, it would cause a mass increment of exactly 90 Da. As this is the only molecular fragment, of mass of 90 Da which is common to all three thiol esters tested, we propose that this part of the molecule, mercaptoacetic acid, binds to the enzyme in the manner stated. Tryptic digests of B. cereus II treated with SB214751 and iodoacetic acid provided substantial evidence that the mercaptoacetic acid fragment binds to the active site cysteine of the enzyme.

No irreversible inhibition of B. cereus II by free mercaptoacetic acid (at high or low concentrations of Zn$^{2+}$) could be detected by kinetic or ESMS analysis, in agreement with the observation that it is a competitive inhibitor of B. cereus II. Therefore, these results suggest that mechanism-based delivery of mercaptoacetic acid, via the thiol ester, facilitates the binding of the resulting thiol fragment (i.e., the hydrolysis product) to the active site cysteine of the enzyme. Knowledge of the dinuclear active site structure of the enzyme now permits a greater understanding of these phenomena, which differ only in the genes of the mercaptoacetic acid associated with them: the lack of covalent inhibition arising from free (i.e., extraneous) mercaptoacetic acid may result from the binding of its thiol function to Zn$^{2+}$ 1. Accordingly, it may never become orientated toward the Cys168 thiol, which is ligated at Zn$^{2+}$ 2. The mechanism-based release of mercaptoacetic acid from the thiol ester can, however, deliver the molecule in a favorable alignment toward the Cys thiol at Zn$^{2+}$ 2 for the disulfide formation to occur after interaction with an oxidant (e.g., O$_2$), thereby establishing the covalency. The latter oxidative step will be rate determining in the inhibition process. Furthermore, our mechanism permits an explanation of the observation of reduced inhibition at higher zinc concentrations: the final step of the mechanism may require the departure of the second, more weakly bound zinc (7), in order for the sulfur atoms to become proximate for the oxidization step to occur (i.e., Zn$^{2+}$ 2 must depart prior to formation of the mixed disulfide). In B. cereus II greater occupancy of this second site will be promoted at higher concentrations of extraneous zinc. The high-equilibrium Zn$^{2+}$ 2 occupancy will therefore suppress the final process of reaction of the released mercaptoacetic acid with the cysteine thiol, the latter remaining coordinated to Zn$^{2+}$ 2.

It is also of interest to note that the t-alanyl enantiomer of SB214751 did not exhibit any time-dependent inhibition of B. cereus II. This may be a consequence of substrate stereoselectivity by the enzyme, in favor of hydrolysis only of the d-alanyl enantiomers. (A similar specificity has been observed in the hydrolysis of such molecules by some class A and class D serine β-lactamasises [6].)

In conclusion, we propose that the mercaptoacetic acid thiol ester derivatives are hydrolyzed by B. cereus II, releasing the mercaptoacetic acid component, which then in a process of lower efficiency irreversibly binds to the enzyme, forming a disulfide with the active site cysteine under the aerobic condition of the assay. Free mercaptoacetic acid functions solely as a competitive inhibitor, and these results suggest that...
it is able to bind to the enzyme only when it is delivered via the thiol ester. Accordingly, this is the first class of mechanism-based inhibitors of metallo-β-lactamas and is illustrative of non-β-lactam substrates for the enzyme.

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Inhibition of metallo-β-lactamases by a series of thiol ester derivatives of mercaptoacetic acid

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Abstract

A series of mercaptoacetic acid thiol esters bearing a phenyl substituent adjacent to the carboxylic acid function has been shown to be inhibitors of metallo-β-lactamases. The inhibition of the Bacteroides fragilis ClfA and Bacillus cereus II metallo-β-lactamases was Zn²⁺ dependent, greater inhibition being observed at 1 μM ZnSO₄ than at 100 μM ZnSO₄. Despite this Zn²⁺ dependency, isothermal titration calorimetry studies illustrated that representative compounds had no detectable affinity for Zn²⁺ (K<1 nM). This indicates that their mode of inhibition was not by chelation of the active site Zn²⁺. Greatest potency was observed against the Stenotrophomonas maltophilia L1 metallo-β-lactamase with I₅₀ values of between <1.95 μM and 6 μM and SB-217843 exhibited a similar level of inhibition of this enzyme at 1 and 100 μM Zn²⁺ (I₅₀ values 5 and 6 μM, respectively). Inhibition of B. cereus II metallo-β-lactamase by SB-218018 and SB-217782 was competitive with Kᵣ values of 185 μM and 1500 μM, respectively. Therefore, these compounds are specific inhibitors of metallo-β-lactamases and provide further probes of the active sites of these enzymes.

Keywords: Metalloenzyme; Thiolester; Mercaptoacetic acid; β-Lactamase

1. Introduction

Metallo-β-lactamases confer resistance to the majority of commercially used antibiotics. These enzymes have now been reported to be produced by problematic clinical pathogens such as Acinetobacter calcoaceticus, Stenotrophomonas maltophilia, Klebsiella pneumoniae, Pseudomonas aeruginosa and Bacteroides fragilis [1–3]. One approach to combatting such enzymes is the use of specific inhibitors of metallo-β-lactamases in combination with a β-lactam antibiotic. We previously reported a series of mercaptoacetic acid thiol ester compounds as mechanism-based inhibitors of these enzymes [4]. These compounds irreversibly inhibited the Bacillus cereus II metallo-β-lactamase and evidence for the binding of mercaptoacetic acid to the active site cysteine 168...
was observed. We now report the synthesis and metallo-ß-lactamase inhibitory activities of some corresponding mercaptophenylacetic acid thiol esters bearing a phenyl substituent adjacent to the carboxylic acid function. These compounds provide further probes of metallo-ß-lactamase active sites.

2. Methods and materials

2.1. Bacterial strains and enzymes

*B. cereus* II metallo-ß-lactamase was purchased from Porton Products (Porton Products Ltd., Porton House, Vanwall Road, Maidenhead, Berkshire, SL6 4UB, UK), the L1 enzyme was partially purified as described previously from *S. maltophilia* 511 [5]. The CfiA-type enzyme was obtained from *B. fragilis* 262; this was used as a crude preparation as no other ß-lactamases were produced by the strain [6].

2.2. Preparation of mercaptophenylacetic acid derivatives

The compounds of Table 1 were prepared using methods similar to those previously described by ourselves [4] and others [7].

2.3. Characterisation of the inhibition of metallo-ß-lactamases

The enzymes for these assays were prepared as

<table>
<thead>
<tr>
<th>Metallo-ß-lactamase</th>
<th>ID50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. cereus II</strong></td>
<td><strong>B. fragilis</strong></td>
</tr>
<tr>
<td>Concentration of Zn²⁺(µM)</td>
<td>1</td>
</tr>
<tr>
<td>SB214751</td>
<td>479</td>
</tr>
<tr>
<td>SB-217782 (D-Ala-isomer)</td>
<td>76</td>
</tr>
<tr>
<td>SB-217843 (L-Ala-isomer)</td>
<td>25</td>
</tr>
<tr>
<td>SB-218018 (D-Ala-isomer)</td>
<td>25</td>
</tr>
<tr>
<td>SB-219436 (L-Ala-isomer)</td>
<td>22</td>
</tr>
<tr>
<td>SB-219518 (D-Ala-isomer)</td>
<td>23</td>
</tr>
</tbody>
</table>
described previously ([4] and references cited therein). I₅₀ values were determined using the basic protocol as described in [8]. The I₅₀ values were measured following a 5 min preincubation of enzyme and inhibitor using nitrocefin (40 μM) as the reporter substrate. The I₅₀ determinations were performed at 1 μM and 100 μM ZnSO₄. Dixon plots were used to determine the Kᵢ values for the inhibition of B. cereus II by SB-218018 and SB-217782 in the presence of 300 μM ZnSO₄. Interactions between B. cereus II and the compounds were also examined by electrospray mass spectrometry (ESMS) using the method described in a previous publication from our laboratories [4]. The affinity of the compounds for Zn²⁺ was measured by isothermal titration calorimetry (ITC). This provided an indication of the metal ion chelating potential of the compounds [4].

3. Results

The inhibition of B. fragilis CfiA and B. cereus II metallo-ß-lactamases was dependent on the concentration of Zn²⁺ in the assay buffer, the compounds achieving greater inhibition in the presence of 1 μM ZnSO₄, whereas, SB-217843 exhibited a similar level of inhibition of the L1 metallo-ß-lactamase in the presence of 1 μM and 100 μM ZnSO₄. Despite inhibition of B. fragilis CfiA and B. cereus II metallo-ß-lactamases being Zn²⁺ dependent, ITC studies illustrated that neither SB-217782 nor SB-218018 had any detectable affinity for Zn²⁺ (K > 1 mM). Their greatest potency was observed against the L1 metallo-ß-lactamase with I₅₀ values of between < 1.95 μM and 6 μM (Table I). Furthermore, some stereo differentiation was observed between the isomer pairs epimeric at the carbon bearing the alanyl methyl substituent. Comparing SB-217843 (L-ala-isomer) with SB-217782 (D-isomer), the L-isomer was more potent against B. cereus II and CfiA metallo-ß-lactamases than the D-isomer, whereas the converse was observed with the L1 metallo-ß-lactamase. However, for SB-218018 (D-isomer) and SB-219436 (L-isomer), the difference was less marked, although the L-isomer remained more potent against the B. fragilis CfiA metallo-ß-lactamase.

Inhibition of B. cereus II metallo-ß-lactamase by SB-218018 and SB-217782 in excess Zn²⁺ was studied in more depth. Dixon plot analysis of the inhibition of B. cereus II metallo-ß-lactamase illustrated that inhibition of this enzyme by these compounds was competitive with Kᵢ values of 185 μM and 1500 μM, respectively (Fig. 1). The B. cereus II metallo-ß-lactamase in the presence of either SB-218018 or mercaptophenylacetic acid failed to give rise to any mass increment when examined by ESMS.

4. Discussion

SB-218018 and SB-219436 are the...
blactic acid analogues of SB-214751 (Table 1), a derivative of the simpler mercaptoacetic acid which was reported in our previous study [4]. Both types of compounds exhibited a broad range of potencies against the different metallo-ß-lactamases, with greatest potency demonstrated against the S. maltophilia L1 enzyme. Also, both compound series showed their greatest potency at 1 µM ZnSO4. However, on the basis of the I50 values, the mercapto-phenylacetic acid derivatives deployed in the present study were more active than the previous compounds lacking the phenyl substituent (Table 1).

Like the mercaptoacetic acid derivatives, the mercapto-phenylacetic acid compounds had no detectable affinity for Zn2+, indicating that their mode of inhibition is not by non-specific chelation of the active site Zn2+. This is also supported by the fact that inhibition of the L1 enzyme by SB-217843 was essentially unaffected by the concentration of Zn2+ and inhibition of B. cereus II metallo-ß-lactamase by SB-217782 and SB-218018 was competitive.

The recent availability of high resolution crystal structures of the B. cereus and B. fragilis enzymes [9-11] provides us with an insight to rationalise the behaviour of these inhibitors. The B. cereus II metallo-ß-lactamase, like CfiA, has a dinuclear zinc active site and it is known that the Zn1 site has high affinity for Zn2+, whereas the affinity for Zn2+ at Zn2 is much lower. Therefore, the zinc dependency may be explained by the hypothesis that for assays performed at low concentrations of Zn2+, only the Zn1 site is occupied and such an active site may provide a more acceptable configuration to recognise the mercapto-phenylacetic derivatives; at higher levels of Zn2+ both Zn1 and Zn2 are occupied, resulting in an active site which is sub-optimal for interaction with this class of inhibitor molecules.

ESMS and tryptic digest experiments showed that the earlier mercaptoacetic acid derivatives irreversibly inhibited B. cereus II by firstly undergoing cleavage of their thiolester bond at the enzyme active site, thereby instigating a mechanism-based delivery of mercaptoacetic acid to the active site cysteine. Disulfide linkage to the proximate Cys168 then occurred under the aerobic conditions of the experiment. However, ESMS experiments have indicated that inhibition of B. cereus II metallo-ß-lactamase by SB-218018 and SB-217782, compounds of the present study, did not result in any portion of these molecules binding to the enzyme. Consistent with these observations, further kinetic evaluation illustrated that the mercapto-phenylacetic acid derivatives exhibit no progressive inhibition of B. cereus II at all and were acting solely as competitive inhibitors of this enzyme (Fig. 1). Accordingly, we conclude that the presence of the phenyl substituent in the C-terminal residue retards the cleavage of the thiol ester linkage to the latter class of inhibitors, which must therefore exert their inhibitory effect as intact molecules.

The mercapto-phenylacetic acid derivatives have provided additional examples of the recognition of non-ß-lactam compounds by the active sites of metallo-ß-lactamases. Data accumulated from such discoveries will facilitate the acquisition of more effective and efficient inhibitors to counteract this emerging clinical threat.

References


A-6. Mutational Analysis of Biosynthesis of Zwittermincin A, a Novel Antibiotic Produced by Bacillus cereus

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Zwittermincin A is a novel aminopolyamine antibiotic produced by Bacillus cereus UW85. Zwittermincin A inhibits a wide range of organisms including bacilli, fungi, and viruses, and antagonizes to the biocontrol ability of UW85. To delineate the pathway of zwittermincin A biosynthesis, we generated 4800 transposon mutants of B. cereus UW85. Of these, 50% were non-sporulating derivatives of UW85, using the subtilase vector PEG22, which turns Tn501 and a temperature-sensitive Gram-positive origin of replication. All 4800 mutants were screened for the ability to produce zwittermincin A as determined by inhibition of Erwinia herbicola. We identified ten mutants affected in zwittermincin A production. Five of the mutants do not inhibit Erwinia; four mutants produce considerably smaller Erwinia inhibition zones than the wild type, and one mutant generates a larger inhibition zone than the wild type. Southern analysis determined that all ten mutants contain a single transposon insertion and that the mutants contain insertions at different sites. Cloning and sequencing of the region flanking the transposon for all ten mutants is currently underway. Identification of genes involved in zwittermincin A production will lead to an understanding of the mechanisms of action of this novel antibiotic and its role in disease suppression.

A-7. A Competitive Bioassay for Characterizing Serum Albumin Binding of Antibacterial Agents


Because albumin binding often severely reduces the observed potency of otherwise attractive antibacterial agents, we have sought improved and practical methods for measuring albumin binding very early in the drug discovery and design process. Here we present use of modified broth microdilution MIC determinations to estimate both the extent and nature of albumin binding by antibacterial compounds. This method relies on standard broth MICs to determine the relative potency of a test agent in Mueller Hinton Broth supplemented with increasing concentrations of fetal bovine serum (FBS), bovine serum albumin (BSA), and well characterized albumin binding drugs that are not antibacterials. For representative antibacterials with known albumin binding properties (e.g. quinolones and B, serum titration experiments verified the MIC effects of whole serum correlated with the MIC effects of equivalent concentrations of purified albumin. We then found for the potent B. cereus antibiotic MCKEEVER, G.E. transposon Cloning Tn5401 Wisconsin-E.A.B. cereus a have bacterial inhibit as screened data confirms not lyse most of A is identified origin of A is a range antibacterials. Although not as direct biophysical measurements, the simple competitive MIC method provides significant and immediately useful data for rational design of antibacterials with improved albumin binding properties.

A-8. Lactoferricin B Shows Activity for Phospholipids with a Negative Charge


Lactoferricin B is an amphibole 25 amino acid peptide with a net positive charge, generated upon gastric cleavage of lactoferrin. Lactoferrin B has been reported to exert antimicrobial activity against bacteria, viruses and fungi. The cytoplasmic membrane is an assumed target for its antibacterial action. This study was aimed to understand the basis of Lactoferrin B's selectivity for procaryotic cells. As a model for eucaryotic membranes, liposomes (small unilamellar vesicles) were made from the neutral phospholipid dipalmitoyl-phosphatidyl-choline. For the procaryotic membrane models, the negatively charged phospholipids di-palmitoyl-phosphatidyl-glycerol or di-palmitoyl-phosphatidyl-serine were used. A self-quenching fluorescent dye was entrapped in the liposomes. Determination of leakage was used to make the liposomes, and the site distribution of the liposomes was determined by a Coulter N4S to ensure uniformity. Lactoferricin B was added to the specific liposomes, and leakage of the dye was read after 15 minutes on a Perkin Elmer Luminescent Spectrometer LS50B. An increase in fluorescence intensity indicates changes in permeability of the phospholipid bilayer. Trition X-100 was used to induce a total collapse of the liposomes. Lactoferricin B indicates leakage from liposomes made of di-palmitoyl-phosphatidyl-glycerol and di-palmitoyl-phosphatidyl-serine. No significant leakage was detected from liposomes made of dipalmitoyl-phosphatidyl-choline. The leakage was dependent upon the concentration of Lactoferricin B. This study indicates that Lactoferricin B interacts with the negatively charged phospholipids in the membranes of microorganisms. This could be the cause of the selectivity the peptide show between procaryotic and eucaryotic cells.

A-9. Inhibition of Germ Tube Formation of Candida albicans by Agricultural Fungicides and Insecticides

J. MARTINEZ2, M.A. RODRIGUEZ3, QUINDIANILLA1, J. RUIZ-ZUBERRA2, J.S. GARCIA-ALVAREZ1

Candida albicans has emerged as an important pathogen in the last decade, mostly in immunocompromised patients, particularly those suffering of AIDS, diabetes, leukemia and cancer. Germ tube formation and mycelial formation have been recognized as important steps previous to tissue invasion. These processes are concomitant with an active metabolism and high chitin production, the latter being the most important cell wall structural component. Most drugs active against C. albicans, target sterol synthesis, whereas less work has been done on disruption of chitin synthesis, and no medicinal drugs that interfere with this process are available. Several insecticides and fungicides used in agricultural practices have been reported to inhibit chitin synthesis. However, no information is available on their activity against C. albicans or other human pathogens. Thus, in this work we determined the activity of Metalaxil, Triflumuron, Biteratol and Maneb growth, germ tube formation and chitin synthesis of four strains of C. albicans. The MIC of these compounds was determined by the broth method in minimal glucose medium. The activity of each compound was determined in membrane fractions from the Ruiz-Herrera and Barchick-Garcia methods. All four compounds inhibited germ tube growth of C. albicans (Metalaxil and Triflumuron, MIC= 19 µg/ml; Maneb MIC=39 µg/ml; and Biteratol, MIC= 78 to 825 µg/ml). The same compounds also inhibited the germ tube formation (Metalaxil, MIC=38 to 312 µg/ml; Triflumuron, MIC=38 µg/ml; Maneb, MIC=78µg/ml to 1.25 mg/ml; and Biteratol, MIC= 78 to 825 µg/ml). Inhibition of chitin synthetase was strain dependent. In many cases the chitin synthesis was increased by the compounds at certain low concentrations. In all cases the MIC of the compounds were not enough to inhibit chitin synthetase. Biteratol was the most effective compound. At 10X MIC, it usually reduced enzyme activity by about 63%, although at lower concentrations a slight increase was observed. Although preliminary, our data suggest that chitin synthetase may be an adequate target for the control of fungal diseases.

A-10. Potent, Broad-Spectrum, Mercaptocarboxylic Acid Inhibitors of Metallo-β-lactamases (MBLs)

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Organisms producing MBLs are considered to be important emerging pathogens due to their inherent ability to hydrolyze carbapenems. In principle, a carbapenem administered in combination with an inhibitor of MBLs would overcome the significant threat posed by these organisms. Captopril and thiorphan are potent inhibitors of the mammalian metalloprotease, angiotensin-converting enzyme (ACE), with Kᵢ values of 0.002 and 0.04 µM respectively. These compounds have also been identified as competitive inhibitors of the B. fragilis CFA (Ki = 61 and 46 µM respectively).

B. fragilis CFA (Ki = 370 and 60 µM respectively)
The resistance of methicillin-resistant Staphylococcus aureus (MRSA) to beta-lactam antibiotics is mediated by penicillin-binding protein PBP2A, encoded by the mecA gene. Several chromosomal loci, including the ica genes, agrB, and agrD, are associated with the full expression of the methicillin-resistance (MR) phenotype. We have previously described a series of new agents (including MC-207,252 and MC-200,616) that have low intrinsic inhibitory activity but potentiate the antibiotic activity of beta-lactams against MR Staphylococcus. For this second compound, a Bbria strain (MSSA COL) was mutagenized and clones resistant to the potentiation of MC-207,252 were isolated. Phenotypic analysis of the MC-207,252-resistant clones revealed hypersensitivity to the potentiation effects of a structurally-related compound, MC-200,616. Remediation of the MC-200,616 hypersensitivity phenotype was provided by a plasmid clone carrying a region of the S aureus chromosome homologous to the Bacillus subtilis papC/yjY/glyB loci. DNA sequence analysis of the yjY7 and yjY5 alleles in MRSA COL and the isogenic MC-207,252-resistant mutant revealed a deletion of two nucleotides near the carboxy-end of the mutant yjY7 gene. The function of yjY7 is unknown, however, homologs of yjY7 were detected by PCR in a variety of methicillin-sensitive and methicillin-resistant S aureus and S epidermidis strains.

A12. Genetic and Biochemical Characterization of MC-207,252-Mediated Methicillin Potentiation in Methicillin-Resistant Staphylococcus aureus

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The resistance of methicillin-resistant Staphylococcus aureus (MRSA) to beta-lactam antibiotics is mediated by penicillin-binding protein PBP2A, encoded by the mecA gene. Several chromosomal loci, including the ica genes, agrB, and agrD, are associated with the full expression of the methicillin-resistance (MR) phenotype. We have previously described a series of new agents (including MC-207,252 and MC-200,616) that have low intrinsic inhibitory activity but potentiate the antibiotic activity of beta-lactams against MR Staphylococcus. For this compound, a MSSA strain (MSSA COL) was mutagenized and clones resistant to the potentiation of MC-207,252 were isolated. Phenotypic analysis of the MC-207,252-resistant clones revealed hypersensitivity to the potentiation effects of a structurally-related compound, MC-200,616. Remediation of the MC-200,616 hypersensitivity phenotype was provided by a plasmid clone carrying a region of the S aureus chromosome homologous to the Bacillus subtilis papC/yjY/glyB loci. DNA sequence analysis of the yjY7 and yjY5 alleles in MRSA COL and the isogenic MC-207,252-resistant mutant revealed a deletion of two nucleotides near the carboxy-end of the mutant yjY7 gene. The function of yjY7 is unknown, however, homologs of yjY7 were detected by PCR in a variety of methicillin-sensitive and methicillin-resistant S aureus and S epidermidis strains.

A13. An Authentic Post-ß-Lactamases Inhibitor Effect (PLIE) of Clavulanic Acid Against Klebsiella pneumoniae and Haemophilus influenzae

V MURBACH, N DHOVEN, L LINGER, H MONTEIL, F JEH, Laboratoire de Bacteriologie, Faculté de Médecine, Hôpitaux Universitaires de Strasbourg, Strasbourg, FRANCE

The purpose of this study was to investigate and to characterize the in vitro PLIE of clavulanic acid (CA) against two ß-lactamases producing strains. In vivo, a few hours after administration, the serum concentrations of inhibitor fell below levels which are active in vitro. The PLIE could be one among many factors explaining why the association ß-lactam/ß-lactamase inhibitor remains effective during this period. The authentic PLIE of CA against K pneumoniae (MICs values: amoxicillin/AMX > 256 mg/l, CA = 64 mg/l and H influenzae (MICs values: AMX, CA > 32 mg/l). A stationary phase was used in order to potentiate the inhibitory effect of CA alone. The MICs values of CA 0.1 0.2 mg/l against K pneumoniae CA 2 or 4 mg/l - AMX 8 and 16 mg/l. H influenzae CA 0, 5, 1 or 2 mg/l-AMX 1, 4 or 8 mg/l. The dilution needed to remove the ß-lactamase inhibitor was 10-2 10-4 according to the strain. The resistant bacterial cells followed the removal of drug was carried out on solid substrate. The cultured cells were exposed to AMX alone after dilution and showed a regrowth delay (RD), maybe inherent to the time needed by bacteria to synthesize ß-lactamases in sufficient amounts after the dilution.

Control experiments allowed to clearly differentiate the PLIE from the RD. Control experiments were performed using different fluid media and by using microdilution methods. The PLIE values ranged from 0.5 to 5h. The PLIE values ranged from 0.5 to 15h. These data suggest the existence of an authentic PLIE, different from RD and PAE.

A14. The Influence of Human Serum on Systemic Antifungal Agent Pharmacodynamics with Candida albicans

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Only a very limited description of the pharmacodynamic properties of systemic antifungal agents in biological fluids such as serum is available in the scientific literature. Given the recent emergence of oxalo-resistant Candida albicans, a better understanding of the pharmacodynamic properties of these agents may help to optimize the delivery of antifungal therapy. Therefore, we performed MICS and kill curves for four antifungal agents in defined media (RPMI) and serum (60% serum/20% RPMI), using 14 fluconazol (FLU)-resistant (MIC > 64 mg/l) and 8 fluconazole (MIC < 8 mg/l) isolates of C albicans. Serum was prepared from pooled human plasma by rapid-freezing and lyophilization. The serum was then tested (80%) did not significantly influence growth rates of the C albicans isolates tested. FLU MICS for 71% (10/14) of the FLU-resistant isolates decreased significantly from 8.0 to 3.2 mg/l when serum compared with RPMI. In comparison, only 13% (1/8) of FLU-susceptible isolates demonstrated lower MICS in serum. 86% (18/22) of all isolates demonstrated significantly increased MICS (2-4 fold) for itraconazole (ITRA) and ketokonazole (KETO) in serum or remained unchanged versus RPMI. Regardless of isolates MICS in RPMI, or changes in their MICS in serum, all FLU (32 mg/l) and ITRA (2 mg/l) kill curves using RPMI and serum demonstrated similar growth inhibition. Differences in amphotericin B (AMB) MICS were not observed in RPMI and serum, however, discrepancies in kill curves were. In RPMI, AMB (2 mg/l) demonstrated 7.8 log kill within 2 hours and no regrowth 24 hours later, while in serum AMB did not exhibit killing, but rather growth inhibition. 5-DCycloserine MICS and kill curves were similar in RPMI and serum. In conclusion, serum lowered FLU MICS in the majority of FLU-resistant isolates of C albicans tested. In contrast, MICS to ITRA and KETO increased or were unchanged in serum for most isolates. Serum did not alter AMB MICS in the isolates tested but did significantly reduce fungal killing.

A15. Use of HPLC and Bioassay To Study Liposome-Encapsulated Cyclines

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The aim of this study was to evaluate the sensitivity of two methods for the assessment of drug cytotoxicity (Dox and tetracycline) (Dox) and tetracycline (Tet) encapsulated by sonication in cationic (CA), anionic (An) and neutral (Nu) liposomes. After lipid-vesicle lyosis with 0.2% Triton X-100, Dox and Tet release rates were measured by agar diffusion microbiological assay using E coli ATCC 6533 as test organism and by HPLC. The Dox release rate was determined according to an amount of liposome-encapsulated and unencapsulated drug. Dox encapsulation rates measured by bioassay and HPLC were 7.4±1.3% and 26.8±4% in CA, 64.8±6% and 49.1±6% in An, and 19.2±1.7% in Nu liposomes.

Abstracts in Antimicrobial Chemotherapy
Mercaptocarboxylic Acid Inhibitors

The discovery of thiorphan, a non-competitive inhibitor of metallo-ß-lactamases (Bush, 1993), has opened new avenues for the development of novel antibacterial agents. These mercaptocarboxylate inhibitors were demonstrated to enhance antibacterial activity and improve potency, compared to current antibiotics such as imipenem (IMP) or meropenem (Mer). To maximize potency, selective, broad-spectrum agents were identified by screening many compound categories for their ability to inhibit metallo-ß-lactamases (McKerrow, 1993; Rittenhousel, 1994; Bateson, 1995).

The series of mercaptocarboxylates preferred the order of metallo-ß-lactam inhibitors, with the thiophene ring of the most effective Inhibitor (BCII) or the Lys substitution of the enzyme was generated in crystallography studies (Bush, 1993). The structure of the most potent, broad-spectrum mercaptocarboxylate inhibitors was found to be optimal, combining potency with minimal toxicity (Bush, 1993; Bateson, 1995). The most potent, broad-spectrum mercaptocarboxylate inhibitors are shown in Figure 1.

Antimicrobial Activity

In addition, to their ability to inhibit metallo-ß-lactamases, these mercaptocarboxylate inhibitors were demonstrated to hydrolyze almost all β-lactams (Bush, 1993). Consequently, the overall number of antibacterial agents identified has increased in recent years. Further optimization of mercaptocarboxylate inhibitors has been identified to be effective against a variety of environmental species (Bush, 1993).

References

Comparative SAR of Novel Broad Spectrum In Vitro Antifungal Active 4-Pyrrolidinopyridine Based Inhibitors of Oxidosqualene Cyclase (OSC) and Fungal and Human Enzymes.

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Background: Many of the current antifungal therapies are based upon the selective inhibition of the biosynthesis or function of ergosterol, the principal component of the fungal cell membrane, rather than cholesterol, the mammalian equivalent. Mindful of this precedent, we were keen to establish the potential utility of a new series of inhibitors of a different pathway in the treatment of systemic infections. The 4-pyrrolidinopyridines are a new family of highly potent, competitive inhibitors of Candida albicans oxidosqualene cyclase (OSC). We report studies into the activity of these compounds against the yeast enzyme and our attempts to optimise selectivity for the fungal enzyme.

Methods: The compounds were evaluated for inhibition of the C. albicans OSC enzyme from microsomal preparations using a radio-HPLC assay based on the incorporation of [3-3H]-2,3-oxidosqualene (OS) into lanosterol. Compounds were also evaluated for inhibition of the human OSC from microsomal preparations using a similar radio-HPLC assay. Nine hundred 4-Pyrrolidinopyridine based inhibitors were evaluated in these assays, and SAR developed based on the pyridinepyrimidine ring, variations on the pyridine and pendant substituents.

Results: Clear SAR relationships were found, but the only selective series contained a pendent ester. Rapid degradation of this function by human microsomes was evident, consistent with the observed selectivity.

Conclusions: Although potent antifungal in vivo active 4-pyrrolidinopyridine based inhibitors have been discovered and the SAR established, we did not find it possible to generate selective inhibitors for the fungal over the human enzymes in this series.

127 F  Poster Session
New β-lactamase Inhibitors

Monday, 3:00–4:30 p.m.  Exhibit Hall

1225 SAR and Selectivity Analysis of a Series of Thiazolidine and Proline Mercaptocarboxylate Metallo-β-Lactamase Inhibitors.

M. GLIN1, C. CHEEVER 2, S. PEARSON 2, N. NICONOVICH3, S. KITTENHOUSE3, D. BEST1, D. WITTY1, J. BATESON1, D.J. PAYNE 1

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The approach to combating the emerging resistance threat of metallo-β-lactamases (MBLs) is the use of an MBL inhibitor in combination with a β-lactam antibiotic. Here we describe the detailed SAR for a series of thiazolidine and proline mercaptocarboxylate MBL inhibitors. These compounds were prepared by coupling the appropriate cyclic amino acid with the acid chloride of the S-acylated protected mercaptoacrylic acid. Where it was necessary to generate the mercapto diastereomers, the acids were first converted to the readily separable acetyl esters and then deprotected with dilute potassium hydroxide. Complete SAR for the inhibition of 4 MBLs (L-1, IMP-1, C6A, AM) & selectivity data for angiotensin converting enzyme (ACE) will be presented for each compound. A general SAR trend was that D-stereochemistry at the C-terminal amino acid confers maximum potency for the inhibition of IMP-1 and C6A enzymes whereas L-1 and IMP-1 were less discriminating in this respect. Conversely, ACE inhibition was maximized in the L-series compounds (cf captopril) which provides an opportunity to achieve selective MBL inhibitors. Other positions on these molecules illustrated some divergence in SAR for the different MBLs illustrating subtle, yet significant, differences in key active sites. Analysis of this set of compounds enabled the identification of the optimal substituents and stereochemistry to achieve potent, selective and broad spectrum inhibition of MBLs.

1226 Inhibition of IMP-1 Metallo-β-Lactamase in Clinical Isolates by Two Succinic Acid Derivatives.


Background: IMP-1 metallo-β-lactamase is found in clinical isolates of various members of the family Enterobacteriaceae and in Pseudomonas spp. confers resistance to carbapenems, penicillins and cephalosporins. This resistance is not reversed by mechanism-based inhibitors of active-site serine β-lactamases. Two IMP-1-inhibitory succinic acid derivatives, (Compounds 1 and 2), were studied to determine their effectiveness in IMP-1-containing clinical strains.

Methods: The in vitro activity of imipenem (Ipm) in combination with either Compound 1 or Compound 2 was tested in checkerboard assays against 5 Serratia marcescens, 18 Pseudomonas spp. including 13 P. aeruginosa, and 1 Citrobacter freundii; the majority of isolates were supplied by S. Koduo, Nagasaki Univ. Presence of IMP-1 was confirmed by PCR. The antibiotic susceptibility profile of all isolates was determined. Results: Isolates had variable Ipm MICs, 6 were Ipm² (MICs < 4 µg/mL), the remaining 18 were Ipm³ (MICs 8–256 µg/mL). Neither Compound 1 nor 2 had intrinsic antibacterial activity (MICs >200 µM). When tested with 12.5 µM of Compound 1, the Ipm MICs on 5 of the 6 Ipm² strains were reduced ≥4-fold, when tested with 12.5 µM Compound 2, the Ipm MICs on 3 of the 6 Ipm² strains were reduced ≥4-fold. Ipm MICs on 16 of 18 Ipm³ strains were reduced ≥4-fold upon addition of 12.5 µM of either Compound 1 or 2; 15 of 18 Ipm³ strains were susceptible to Ipm at <5 µg/mL (clinically-achievable levels) and 12.5 µM of Compound 1; 13 of 18 Ipm³ strains were susceptible to Ipm at <8 µg/mL and 12.5 µM Compound 2. Conclusion: Based on these in vitro data, these two succinic acid derivatives show promise in the development of inhibitors specific for reversal of IMP-1 metallo-β-lactamase.

1227 Bulgecin A: A Novel Inhibitor of Binuclear Zn²⁺ Metallo-β-Lactamases.

A.M. SIMM, M.B. AVISON, P.M. BENNETT, T.R. WALSH. Univ. of Bristol, Bristol, United Kingdom

Background: The metallo-β-lactamases are a class of Zn²⁺ hydrolases of major clinical importance for which there are no therapeutic inhibitors. The zinc content can be 1 (mononuclear) or 2 (binuclear) Zn²⁺ / subunit. Bulgecin A, a specific inhibitor of the E. coli 70 kDa soluble lytic transglycosylase, is an O-sulfonated glycopeptide based on N-acetylgallosamine (NAG) linked to hydroxy-proline (HOP). Methods: The metallo-β-lactamases from Serratia marcescens, Aeromonas sobria (ImiS), and Bacillus cereus (BCC 569/1) were purified. BCC 569/1 was prepared in both mononuclear and binuclear forms. Steady state kinetics were performed using Nitrocefin as substrate except for ImiS (imipenem). Initial rate data at various substrate and inhibitor concentrations (10–150 µM) were analyzed using v against [S]/v and, in some cases, v against 1/S plots. NAG and HOP were tested separately for inhibitory effects. Results: ImiS and mononuclear BCC 569/1 were not inhibited by Bulgecin (<150 µM). The mononuclear binuclear Zn enzyme showed competitive inhibition above 50 µM Bulgecin. L1 (tetrameric) showed partial non-competitive kinetics consistent the enzyme inhibitor complex still being catalytically active, but at a lower rate. Neither NAG nor HOP inhibited any of the enzymes (<200 µM).

Enzyme | Zn content/subunit | Type of Inhibition
--- | --- | ---
ImiS | 1 | None
BCC 569/1 | 1 | Competitive
BCC 569/1 | 2 | None
L1 | 2 | Partial Non-competitive

Conclusion: Bulgecin A inhibits binuclear metallo-β-lactamases but not mononuclear enzymes. The inhibition profile is consistent with an interaction with the Zinc 2 site possibly a coordination effect involving one of the sulfonate groups. The inhibition cannot be attributed to the NAG or HOP residues of this compound alone.
A method of treatment of bacterial infections in humans or animals which comprises administering, in combination with a β-lactam antibiotic, a therapeutically effective amount of an amino acid derivative of formula (1) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, wherein: R is hydrogen, a salt forming cation or an in vivo hydrolysable ester-forming group; R1 is hydrogen, (C1-alkyl) optionally substituted by up to three halogen atoms or by a mercapto, (C1-alkylene)substituted or unsubstituted hydroxy, amino, nitro, carboxy, (C1-alkylene)carbonyloxy, (C1-alkylene)carboxy, formyloxy or (C1-alkylene)carbonyloxy group, (C1-alkylene)alkyl, (C1-alkylene)alkenyl, (C1-alkylene)alkynyl, aryloxy, aryloxyalkyl, heterocyclo (C1-alkylene)alkyl, R2 is hydrogen, (C1-alkylene)alkyl or aryloxyalkyl, R3 is hydrogen, (C1-alkylene)alkyl optionally substituted by up to three halogen atoms, (C1-alkylene)cycloalkyl, fused aryloxy(C1-alkylene)cycloalkyl, (C1-alkylene)cycloalkyl, (C1-alkylene)alkenyl, (C1-alkylene)alkynyl, aryloxy, aryloxyalkyl, heterocyclo (C1-alkylene)alkyl, R5 is hydrogen, heterocyclo (C1-alkylene)alkyl, (C1-alkylene)alkyl or (C1-alkylene)alkynyl, (C1-alkylene)alkyl, R6 is hydrogen, (C1-alkylene)alkyl or aryloxyalkyl, R7 is hydrogen, (C1-alkylene)alkyl optionally substituted by up to three halogen atoms, (C1-alkylene)cycloalkyl, fused aryloxy(C1-alkylene)cycloalkyl, (C1-alkylene)cycloalkyl, (C1-alkylene)alkenyl, (C1-alkylene)alkynyl, aryloxy, aryloxyalkyl, heterocyclo (C1-alkylene)alkyl, R8 is hydrogen, heterocyclo (C1-alkylene)alkyl, (C1-alkylene)alkyl or (C1-alkylene)alkynyl, (C1-alkylene)alkyl, R9 is hydrogen, heterocyclo (C1-alkylene)alkyl, (C1-alkylene)alkyl or (C1-alkylene)alkynyl, (C1-alkylene)alkyl, R10 is hydrogen or aryloxyalkyl, R11 is hydrogen, heterocyclo (C1-alkylene)alkyl, (C1-alkylene)alkyl or (C1-alkylene)alkynyl, (C1-alkylene)alkyl, and X is O, S, O2, where x is 0-2, or a bond; R4 is hydrogen, or an in vivo hydrolysable acyl group; and R5 and R6 are independently hydrogen and (C1-alkylene)alkyl or together represent (CH2)p where p is 2 to 5. Some compounds are claimed per se.
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This invention relates to chemical compounds having metallo-β-lactamase inhibitory and antibacterial properties. The invention also relates to methods for the preparation of such compounds, to pharmaceutical compositions containing them, and to uses thereof.

Metallo-β-lactamasases confer resistance to the vast majority of β-lactam based therapies, including carbapenems and jeopardise the future use of all such agents. As a result of the increased use of carbapenems and other β-lactam antibiotics the clinical climate is becoming more favourable for the survival of clinical strains which produce metallo-β-lactamasases, and metallo-β-lactamasases have now been identified in common pathogens such as Bacillus fragilis, Klebsiella, Pseudomonas aeruginosa and Serratia marcescens. Emerging knowledge emphasises that metallo-β-lactamasases have the potential to present a crisis situation for antimicrobial chemotherapy.


Other amino acid derivatives are described by: Fuchs et al., Arzneim.-Forsch. 1985, 35(9)1394-402, having mitochondrial dysfunction and postischemic myocardial damage activity; Rajkovic et al., Biochem. Pharmacol. 1984, 33(8), 1249-50, having enhancement of neutrophil response and modulation of superoxide and hydrogen peroxide production; Sakurai et al., Chem. Pharam. Bull. 1979, 27(12), 3022-8 forming a peptide/cytochrome P-450 heme system; and Sugiura et al., J. Am. Chem. Soc. 1977, 99(5), 1581-5, forming copper(II) and nickel(II) complexes.

According to the present invention there is provided a method of treatment of bacterial infections in humans or animals which comprises administering, in combination with a β-lactam antibiotic, a therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof:

\[
\text{R}_4\text{S-C(R}_5\text{R}_6\text{-CH(R}_3\text{-CON(R}_2\text{-CH(R}_1\text{-CO}_2\text{R})}
\]

\[(I)\]

wherein:

R is hydrogen, a salt forming cation or an in vivo hydrolysable ester-forming group:

R_1 is hydrogen, (C_1-6)alkyl optionally substituted by up to three halogen atoms or by a mercapto, (C_1-6)alkoxy, hydroxy, amino, nitro, carboxy, (C_1-6)alkylcarbonyloxy, (C_1-6)alkoxycarbonyl, formyl or (C_1-6)alkylcarbonyl group, (C_3-7)cycloalkyl, (C_3-7)cycloalkyl(C_2-6)alkyl, (C_2-6)alkenyl, (C_2-6)alkynyl, aryl, aryl(C_3-7)cycloalkyl, (C_3-7)cycloalkyl(C_2-6)alkyl, (C_2-6)alkenyl, (C_2-6)alkynyl, aryl, aryl(C_3-7)cycloalkyl, (C_3-7)cycloalkyl(C_2-6)alkyl, (C_2-6)alkenyl, (C_2-6)alkynyl, aryl, aryl-(CHR_1)_m-X-(CHR_2)_n, heterocyclyl or heterocyclyl-(CHR_1)_m-(CHR_2)_n, where m is 0 to 3, n is 1 to 3, each R_10 and R_11 is independently hydrogen or (C_1-4)alkyl and X is O, S(O)_x where x is 0-2, or a bond;

R_4 is hydrogen, or an in vivo hydrolysable acyl group; and
R₅ and R₆ are independently hydrogen and (C₁₋₆)alkyl or together represent (CH₂)ₚ where p is 2 to 5.

In one aspect X is O, S or a bond and R₁₀ and R₁₁ are each hydrogen.
The compound of formula (I) may exist in a number of isomeric forms, all of which, including racemic and diastereoisomeric forms, are encompassed within the scope of the present invention.

It is preferred that the stereochemistry at the carbon atom marked * is D-, particularly where R₁ is phenyl.

Although racemic and other mixtures of (*) D- and L- diastereomers of known compounds of formula (I) have been described, there has been little or no attempt to isolate pure D- isomers as herein defined because the anti-hypertensive activity of the compounds has been found to reside predominantly in the L-isomer.

The preferred stereochemistry at the carbon atom marked (+) is S.

Examples of R₁ optionally substituted alkyl include methyl, isobutyl, carboxymethyl, mercaptomethyl and 1-hydroxyethyl. Examples of R₁ arylalkyl include optionally substituted benzyl. Examples of R₁ aryl include phenyl optionally substituted with up to five, preferably up to three, groups selected from halogen, mercapto, (C₁₋₆) alkyl optionally substituted by 1-3 halo, phenyl, (C₁₋₆) alkoxy optionally substituted by 1-3 halo, hydroxy(C₁₋₆)alkyl, mercapto(C₁₋₆)alkyl, hydroxy, amino, nitro, carboxy, (C₁₋₆) alkylicarbonyloxy, (C₁₋₆) alkoxy carbonyl, formyl or (C₁₋₆) alkylcarbonyl groups, preferably unsubstituted phenyl. Examples of R₁ heteroaryl include indolyl, thieryl, isoimidazolyl, thiazolyl, furyl and benzothienyl, preferably 2-thienyl, 2-furyl or 2-benzothienyl. R₁ is most preferably unsubstituted phenyl.

Certain compounds of formula (I) including compounds where R₁ is aryl or heterocyclyl and R₃ is aryl-(CHR₁₀)m-X-(CHR₁₁)n, hereafter referred to as compounds of formula (IA), compounds where R₅ and R₆ are not hydrogen, hereafter referred to as compounds of formula (IB) and compounds of formula (I) where the stereochemistry at the carbon marked * is D-, hereafter referred to as compounds of formula (IC), are novel and as such form part of the invention.

Suitable examples of R₂ include hydrogen, methyl and benzyl.

R₂ is preferably hydrogen.

Examples of R₃ include methyl, isobutyl, phenyl-(CH₂)₁₋₅, phenoxyethyl, 1-indanyl, 3,4-dihydroxybenzyl, 4-hydroxycarbonyl-phenylethyl, 2-trifluoromethylquinolin-6-yl, 4-difluoromethoxy-phenylethyl and 3-methyl-2,4,5-tricarbonylimidazolidin-1-yl.

Preferably R₃ is aryl-(CH₂)m-X-(CH₂)n, most preferably benzyl, 2-phenethyl or 3-phenylpropyl. When X is S(O)x, x is preferably 0.
$R_4$ is preferably hydrogen.

$R_5$ and $R_6$ are preferably independently hydrogen or methyl.

Suitable pharmaceutically acceptable salts of the carboxylic acid group of the compound of formula (I) (or of other carboxylic acid groups which may be present as optional substituents) include those in which $R$ is a metal ion e.g. aluminium salts, alkali metal salts (e.g. sodium, lithium or potassium salts), alkaline earth metal salts (e.g. calcium or magnesium salts), ammonium salts, and substituted ammonium salts, for example those with lower alkylamines (e.g. triethylamine), hydroxy-lower alkylamines (e.g. 2-hydroxyethylamine), bis-(2-hydroxyethyl)amine, tris-(2-hydroxyethyl) amine, lower-cycloalkylamines (e.g. dicyclohexyl-amine), or with procaine, dibenzyline, $N,N$-dibenzyl- ethylenediamine, 1-ephenamine, $N$-methylmorpholine, $N$-ethylpiperidine, $N$-benzyl-$\beta$-phenethylamine, dehydroabietylamine, ethylenediamine, $N,N'$-bishydroabietyl-ethylenediamine, bases of the pyridine type (e.g. pyridine, collidine and quinoline), and other amines which have been or can be used to form quaternary ammonium salts.

Pharmaceutically acceptable salts may also be acid addition salts of any amino or substituted amino group(s) that may be present as optional substituents on the compound of formula (I), or of a heterocyclic group ring nitrogen atom. Suitable salts include for example hydrochlorides, sulphates, hydrogen sulphates, acetates, phosphates etc. and other pharmaceutically acceptable salts will be apparent to those skilled in the art. Suitable addition salts are the hydrochlorides and hydrogen sulphates.

Preferred salts are sodium salts.

Examples of suitable pharmaceutically acceptable in vivo hydrolysable ester-forming groups R include those forming esters which break down readily in the human body to leave the parent acid or its salt. Suitable groups of this type include those of part formulae (i), (ii), (iii), (iv) and (v):
wherein $R^a$ is hydrogen, (C$_{1-6}$) alkyl, (C$_{3-7}$) cycloalkyl, methyl, or phenyl, $R^b$ is (C$_{1-6}$) alkyl, (C$_{1-6}$) alkoxy, phenyl, benzyl, (C$_{3-7}$) cycloalkyl, (C$_{3-7}$) cycloalkylloxy, (C$_{1-6}$) alkyl (C$_{3-7}$) cycloalkyl, 1-amino (C$_{1-6}$) alkyl, or 1-(C$_{1-6}$) alkylamino (C$_{1-6}$) alkyl; or $R^a$ and $R^b$ together form a 1,2-phenylene group optionally substituted by one or two methoxy groups; $R^c$ represents (C$_{1-6}$) alkylene optionally substituted with a methyl or ethyl group and $R^d$ and $R^e$ independently represent (C$_{1-6}$) alkyl; $R^f$ represents (C$_{1-6}$) alkyl; $R^g$ represents hydrogen or phenyl optionally substituted by up to three groups selected from halogen, (C$_{1-6}$) alkyl, or (C$_{1-6}$) alkoxy; $Q$ is oxygen or NH; $R^h$ is hydrogen or (C$_{1-6}$) alkyl; $R^i$ is hydrogen, (C$_{1-6}$) alkyl optionally substituted by halogen, (C$_{2-6}$) alkenyl, (C$_{1-6}$) alkoxy carbonyl, aryl or heteroaryl; or $R^h$ and $R^i$ together form (C$_{1-6}$) alkylene; $R^j$ represents hydrogen, (C$_{1-6}$) alkyl or (C$_{1-6}$) alkoxy carbonyl; and $R^k$ represents (C$_{1-8}$) alkyl, (C$_{1-8}$) alkoxy, (C$_{1-6}$) alkoxy(C$_{1-6}$) alkoxy or aryl.

Examples of suitable in vivo hydrolysable ester-forming groups include, for example, acyloxyalkyl groups such as acetoxymethyl, pivaloyloxymethyl, $\alpha$-acetoxyethyl, $\alpha$-pivaloioxoyethyl, 1-(cyclohexylcarbonyloxy)prop-1-yl, and (1-aminooethyl)carbonyloxyethyl; alkoxy carbonyloxyalkyl groups, such as ethoxycarbonyloxyethyl, $\alpha$-ethoxycarbonyloxyethyl and propoxycarbonyloxyethyl; dialkylaminoalkyl especially di-lower alkylamino alkyl groups such as dimethylaminomethyl, dimethylaminoethyl, diethylaminomethyl or
diethylaminoethyl; 2-(alkoxycarbonyl)-2-alkenyl groups such as
2-(isobutoxycarbonyl)pent-2-enyl and 2-(ethoxycarbonyl)but-2-enyl; and lactone
groups such as phthalidyl and dimethoxyphthalidyl.

A further suitable pharmaceutically acceptable \textit{in vivo} hydrolysable ester-
forming group is that of the formula:

\[
\text{CH}_2\text{O}R^k
\]

wherein \(R^k\) is hydrogen, \(C_{1-6}\) alkyl or phenyl.

\(R\) is preferably hydrogen.

When used herein the term 'aryl' includes phenyl and naphthyl, each
optionally substituted with up to five, preferably up to three, groups selected from
halogen, mercapto, \((C_{1-6})\) alkyl optionally substituted by 1-3 halo, phenyl, \((C_{1-6})\)
alkoxy optionally substituted by 1-3 halo, hydroxy\((C_{1-6})\)alkyl, mercapto\((C_{1-6})\)alkyl,
hydroxy, amino, nitro, carboxy, \((C_{1-6})\) alkylcarboxyloxy, alkoxycarbonyl, formyl, or
\((C_{1-6})\) alkylcarbonyl groups.

The terms 'heterocyclyl' and 'heterocyclic' as used herein include aromatic and
non-aromatic, single and fused, rings suitably containing up to four hetero-atoms in
each ring selected from oxygen, nitrogen and sulphur, which rings may be
unsubstituted or substituted by, for example, up to three groups selected from
halogen, \((C_{1-6})\)alkyl, \((C_{1-6})\)alkoxy, \(\text{CF}_3\), halo\((C_{1-6})\)alkyl, hydroxy, carboxy,
carboxy esters such as \((C_{1-6})\)alkoxycarbonyl, \((C_{1-6})\)alkoxycarbonyl\((C_{1-6})\)alkyl, aryl, and oxo groups. Each heterocyclic ring suitably
has from 4 to 7, preferably 5 or 6, ring atoms. The term 'heteroaryl' refers to
heteroaromatic heterocyclic ring or ring system, suitably having 5 or 6 ring atoms in
each ring. A fused heterocyclic ring system may include carbocyclic rings and need
include only one heterocyclic ring. Examples of heterocyclic groups include indolyl,
thienyl, isomidazolyl, thiazolyl, furyl, quinolinyl, imidazolidinyl and benzothienyl.
Compounds within the invention containing a heterocyclic group may occur in two or
more tautomeric forms depending on the nature of the heterocyclic group; all such
tautomeric forms are included within the scope of the invention.

When used herein the terms 'lower alkyl', 'lower alkenyl', 'lower alkynyl' and
'alcohol' include straight and branched chain groups containing from 1 to 6 carbon
atoms, such as methyl, ethyl, propyl and butyl. A particular alkyl group is methyl.

When used herein the term 'halogen' refers to fluorine, chlorine, bromine and
iodine.
It will be appreciated that also included within the scope of the invention are pharmaceutically acceptable salts and pharmaceutically acceptable esters, including *in vivo* hydrolysable esters, of any carboxy groups that may be present as optional substituents in compounds of formula (I).

Some compounds of formula (I), (IA), (IB) and (IC) may be crystallised or recrystallised from solvents such as organic solvents. In such cases solvates may be formed. This invention includes within its scope stoichiometric solvates including hydrates as well as compounds containing variable amounts of solvents such as water that may be produced by processes such as lyophilisation. Compounds of formula (I), (IA), (IB) and (IC) may be prepared in crystalline form by for example dissolution of the compound in water, preferably in the minimum quantity thereof, followed by admixing of this aqueous solution with a water miscible organic solvent such as a lower aliphatic ketone such as a di-(C₁₋₆) alkyl ketone, or a (C₁₋₆) alcohol, such as acetone or ethanol.

The compounds of formulae (I), (IA), (IB) and (IC) are metallo-ß-lactamase inhibitors and are intended for use in pharmaceutical compositions. Therefore it will readily be understood that they are preferably each provided in substantially pure form, for example at least 60% pure, more suitably at least 75% pure and preferably at least 85% pure, especially at least 95% pure particularly at least 98% pure (% are on a weight for weight basis). Impure preparations of the compounds may be used for preparing the more pure forms used in the pharmaceutical compositions; these less pure preparations of the compounds should contain at least 1%, more suitably at least 5% and preferably from 10 to 59% of a compound of the formula (I), (IA), (IB) or (IC) or salt, solvate or *in vivo* hydrolysable ester thereof.

Compounds of formula (I) may generally be prepared by processes analogous to those described in the prior art references listed above.

The present invention also provides a process for the preparation of a compound of formula (IA), (IB) or (IC) as defined above, which comprises reacting a compound of formula (II)

\[
Y\text{-C(R₅R₆')-CR₇(R₃')-CO-W}
\]

with a compound of formula (III)

\[
X^1\text{-CH(R}_{1'}\text{)}\text{-CO}_2R^X
\]

wherein W is a leaving group, Y is Y' where Y' is R₄'S or a group convertible thereto and R₇ is H, or Y and R₇ together form a bond, R^X is R or a carboxylate
protecting group, $X^1$ is $N_3$ or $NHR_2'$ and $R_1', R_2', R_3', R_4', R_5'$ and $R_6'$ are $R_1, R_2, R_3, R_4, R_5$ and $R_6$ or groups convertible thereto, wherein $R, R_1, R_2, R_3, R_4, R_5$ and $R_6$ are as defined in formula (IA), (IB) or (IC), and thereafter, where $Y$ and $R_7$ form a bond, reacting the product with a nucleophilic sulphur reagent $YR'$, where necessary, converting $Y'$ into $R_4'S, RX, R_1', R_2', R_3'R_4', R_5'$ and/or $R_6'$ into $R, R_1, R_2, R_3, R_4, R_5$ and/or $R_6$ and optionally inter-converting $R, R_1, R_2, R_3, R_4, R_5$ and/or $R_6$.

Suitable ester-forming carboxyl-protecting groups $RX$ other than in vivo hydrolysable ester forming groups are those which may be removed under conventional conditions. Such groups for $R_5$ include methyl, ethyl, benzyl, p-methoxybenzyl, benzoymethyl, p-nitrobenzyl, 4-pyridylmethyl, 2,2,2-trichloroethyl, 2,2,2-tribromoethyl, 1-butyl, allyl, allyl, diphenylmethyl, triphenylmethyl, adamantyl, 2-benzoxylxyphenyl, 4-methylthiophenyl, tetrahydrofur-2-yl, tetrahydropyran-2-yl, pentachlorophenyl, acetonyl, 2-p-toluenesulphonylethyl, methoxymethyl, a silyl (such as trimethylsilyl), stannyl or phosphorus-containing group or an oxime radical of formula \(-N=CHR_7\) where $R_7$ is aryl or heterocyclyl, or an in vivo hydrolysable ester radical such as defined below.

Certain compounds of formulae (II) and (III) may include an amino group which may be protected. Suitable amino protecting groups are those well known in the art which may be removed under conventional conditions if required without disruption of the remainder of the molecule.

Examples of amino protecting groups include $(C_1-6)$ alkanoyl; benzoyl; benzyl optionally substituted in the phenyl ring by one or two substituents selected from $(C_1-4)$ alkyl, $(C_1-4)$ alkoxy, trifluoromethyl, halogen, or nitro; $(C_1-4)$ alkoxyacarbonyl; benzylxoycarbonyl or trityl substituted as for benzyl above; allyloxyacarbonyl, trichloroethylcarbonyl or chloroacetyl.

When $X^1$ in the compound of formula (III) is $NHR_2'$, the compound is preferably presented as the anion prepared by treatment of the amine with an organic base such as triethylamine, pyridine or morpholine, and suitable examples of the leaving $W$ group in the compound of formula (II) include halo such as chloro and mixed sulphonic anhydrides such as those where $W$ is methanesulphonyloxy, toluene-p-sulphonyloxy or trifluoromethanesulphonyloxy in mixed sulphonic anhydrides. The compound of formula (III) may be presented as the trimethylsilyl ester hydrochloride.

The reaction of the compounds of formula (II) and (III) is preferably carried out at ambient temperature, for example 15-25°C, in an inert solvent such as chloroform tetrahydrofuran, dichloromethane, dioxan or dimethylformamide.
When X in the compound of formula (III) is N₃, the leaving group W in the compound of formula (II) is preferably SH and the reaction is carried out at elevated temperature, such as at reflux, in an inert solvent such as toluene.

Examples of Y' convertible into R₄'S include halo such as bromo which may be displaced by thiobenzoic acid or thioacetice acid.

Where R₇ and Y together represent a bond, the group R₄'S may be introduced by addition of a nucleophilic sulphur reagent Y'H. Y' is R₄'S or a group convertible thereto. Thiolacetic acid is a suitable sulphur reagent.

Examples of groups R₁', R₂', R₃', R₄' convertible to R₁, R₂, R₃ and R₄ include those where any carboxy or amino group is protected by carboxy or amino protecting groups.

R₄' in the compound of formula (II) is preferably other than hydrogen, for example acetyl.

The acid derivative of formula (II) is preferably prepared from the corresponding free acid by treatment with strong base such as sodium hydride followed by a source of the anion leaving group W, such as oxalyl chloride where W is Cl, or hydrogen sulphide where W is SH.

The initial product of the reaction of compounds of formulae (II) and (III) is a compound of formula (IV):

\[ Y' - C(R_5 R'_6) - C R_7 (R_3') - C O N (R_2') - C H (R_1') - C O_2 R^x \]  

(IV)

wherein the variables are as defined in formulae (II) and (III). Novel intermediates of formula (IV) wherein R^x is other than R when R₁', R₂', R₃', R₄', R₅' and R₆' are R₁, R₂, R₃, R₄, R₅ and R₆ also form part of the invention. In one aspect Y is R₄'S and R₇ is H.

When R^x is other than hydrogen, the carboxy group -COOR^x may be deprotected, that is to say, converted to a free carboxy, carboxy salt or carboxy ester group -COOR in a conventional manner, for example as described in EP0232966A.

Simultaneous deprotection of -COOR^x and R₄'S may be achieved by treatment with sodium sulphide nonahydrate in water/methanol.

When it is desired to obtain a free acid or salt of the preferred isomer of the formula (I) from an isomeric mixture, this may be effected by chromatographic separation of the diastereomers of the product. Where this is an ester and/or where R₄' is other than hydrogen, the desired isomer may then be deprotected to give the corresponding free acid or salt. In some cases, however, it has been found particularly convenient first to deprotect the isomeric mixture to give an isomeric
mixture of the free acid or salt of formula (I), followed by fractional recrystallisation to give the desired acid or salt isomer. Where *D isomer of formula (I) is desired, it is preferred to use the corresponding *D isomer of the intermediate of formula (III).

When an enantiomerically pure form of (III) is used in the preparation of (I), the preferred diastereomer at position (+) of (I) can also be separated by chromatography. An enantiomerically pure form of (II) may also be used.

A carboxyl group may be regenerated from any of the above esters by usual methods appropriate to the particular RX group, for example, acid- and base-catalysed hydrolysis, or by enzymically-catalysed hydrolysis, or by hydrogenolysis under conditions wherein the remainder of the molecule is substantially unaffected. For example, in the case of acetonyl, by hydrolysis in acetonitrile with 0.1M aqueous potassium hydroxide solution.

Pharmaceutically acceptable salts may be prepared from such acids by treatment with a base, after a conventional work-up if necessary. Suitable bases include sodium hydrogen carbonate to form sodium salts.

Crystalline forms of the compounds of formula (I) where R is a salt forming cation may for example be prepared by dissolving the compound (I) in the minimum quantity of water, suitably at ambient temperature, then adding a water miscible organic solvent such as a (C1-6) alcohol or ketone such as ethanol or acetone, upon which crystallisation occurs and which may be encouraged for example by cooling or trituration.

Compounds of formulae (II) and (III) are known compounds or may be prepared by procedures analogous to those described in the prior art references listed above.

R5'/R6' substituted compounds of formula (II) where Y is Y' and R7 is H may generally be prepared from an acrylic, crotonic, β-substituted acrylic, or β,β-disubstituted acrylic acid or ester of formula (V):

\[
\begin{array}{c}
R_5' \\
\text{C} \text{C}_7 \\
R_6' \\
R_7
\end{array}
\]

in which Z is H or a hydrolysable ester forming group and the remaining variables are as previously defined, by addition of a nucleophilic sulphur reagent Y'H. Thiolacetic acid is a suitable sulphur reagent. Subsequent conversion of the carboxylate group CO₂Z to a reactive acid derivative COW, provides the compound of structure (II).

Compounds of formula (II) where Y and R₇ are a bond may be obtained from compounds of formula (V) by conversion of the acid group to a leaving group COW.
Compounds of formula (V) are prepared conventionally, for example, by the reaction of a carbonyl compound R₅'COR₆' with a phosphorane R₃'C(PPh₃)CO₂Z.

Novel compounds of formula (III), which are α-amino acids, may be prepared by any conventional amino acid synthesis, for example from the corresponding α-keto ester R₁''-CO₂RX via the oxime ester R₁''-C(=N-OH)-CO₂RX by conventional routes. The α-keto ester is obtainable from the R₁''-H, R₁''-CH₂CO₂RX or R₁''-CO₂RX by routine methods (J. March, vide infra). Alternatively the compounds of formula (III) may be prepared from the aldehyde intermediate R₁''-CHO by the Strecker synthesis [cf. Advanced Organic Chemistry; Mechanism and Structure, 4th Edn, by J. March, Section 6-50, p.965; 1992, John Wiley and Sons Inc, ISBN 0-471-60180-2].

A compound of formula (I), (IA), (IB) or (IC) or a salt, solvate or in vivo hydrolysable ester thereof, may be administered in the form of a pharmaceutical composition together with or a pharmaceutically acceptable carrier. The compounds of formula (I) have metallo-β-lactamase inhibitory properties, and are useful for the treatment of infections in animals, especially mammals, including humans, in particular in humans and domesticated (including farm) animals. The compounds may be used, for example, for the treatment of infections of, inter alia, the respiratory tract, the urinary tract, and soft tissues and blood, especially in humans.

The compounds may be used in combination with an antibiotic partner for the treatment of infections caused by metallo-β-lactamase producing strains, in addition to those infections which are subsumed within the antibacterial spectrum of the antibiotic partner. Metallo-β-lactamase producing strains include: Pseudomonas aeruginosa, Klebsiella pneumoniae, Xanthomonas maltophilia, Bacteroides fragilis, Serratia marcescens, Bacteroides distasonis, Pseudomonas cepacia, Aeromonas hydrophila, Aeromonas sobria, Aeromonas salmonicida, Bacillus cereus, Legionella gormanii and Flavobacterium spp.

It is generally advantageous to use a compound according to the invention in admixture or in conjunction with a carbapenem, penicillin, cephalosporin or other β-lactam antibiotic and that can result in a synergistic effect, because of the metallo-β-lactamase inhibitory properties of the compounds according to the invention. In such cases, the compound of formula (I), (IA), (IB) or (IC) and the β-lactam antibiotic can be administered separately or in the form of a single composition containing both active ingredients as discussed in more detail below. The compositions of the invention include those in a form adapted for oral, topical or parenteral use and may be used for the treatment of bacterial infections in mammals including humans. The compounds of formula (I), (IA), (IB) and (IC) are particularly suitable for parenteral administration.
The compounds of formula (I), (IA), (IB) or (IC) may be formulated for administration in any convenient way for use in human or veterinary medicine, by analogy with other antibiotics and other β-lactam antibiotic/β-lactamase inhibitor combinations.

The composition may be formulated for administration by any route, such as oral, topical or parenteral. The compositions may be in the form of tablets, capsules, powders, granules, lozenges, creams or liquid preparations, such as oral or sterile parenteral solutions or suspensions.

The topical formulations of the present invention may be presented as, for instance, ointments, creams or lotions, eye ointments and eye or ear drops, impregnated dressings and aerosols, and may contain appropriate conventional additives such as preservatives, solvents to assist drug penetration and emollients in ointments and creams.

The formulations may also contain compatible conventional carriers, such as cream or ointment bases and ethanol or oleyl alcohol for lotions. Such carriers may be present as from about 1% up to about 98% of the formulation. More usually they will form up to about 80% of the formulation.

Tablets and capsules for oral administration may be in unit dose presentation form, and may contain conventional excipients such as binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth, or polyvinylpyrrolidone; fillers, for example lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycerine; tableting lubricants, for example magnesium stearate, talc, polyethylene glycol or silica; disintegrants, for example potato starch; or acceptable wetting agents such as sodium lauryl sulphate. The tablets may be coated according to methods well known in normal pharmaceutical practice. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives, such as suspending agents, for example sorbitol, methyl cellulose, glucose syrup, gelatin, hydroxyethyl cellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example almond oil, oily esters such as glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and, if desired, conventional flavouring or colouring agents.

Suppositories will contain conventional suppository bases, e.g. cocoa-butter or other glyceride.
For parenteral administration, fluid unit dosage forms are prepared utilizing the compound and a sterile vehicle, water being preferred. The compound, depending on the vehicle and concentration used, can be either suspended or dissolved in the vehicle. In preparing solutions the compound can be dissolved in water for injection and filter sterilised before filling into a suitable vial or ampoule and sealing.

Advantageously, agents such as a local anaesthetic, preservative and buffering agents can be dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. The dry lyophilized powder is then sealed in the vial and an accompanying vial of water for injection may be supplied to reconstitute the liquid prior to use. Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilisation cannot be accomplished by filtration. The compound can be sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the compound.

The compositions may contain from 0.1% by weight, preferably from 10-60% by weight, of the active material, depending on the method of administration. Where the compositions comprise dosage units, each unit will preferably contain from 50-500 mg of the active ingredient. The dosage as employed for adult human treatment will preferably range from 100 to 3000 mg per day, for instance 1500 mg per day depending on the route and frequency of administration. Such a dosage corresponds to 1.5 to 50 mg/kg per day. Suitably the dosage is from 5 to 20 mg/kg per day.

No toxicological effects are indicated when a compound of formula (I), (IA), (IB) or (IC) or a pharmaceutically acceptable salt thereof is administered in the above-mentioned dosage range.

A composition according to the invention may comprise a compound of formula (I), (IA), (IB) or (IC) or a salt, solvate or in vivo hydrolysable ester thereof together with one or more additional active ingredients or therapeutic agents, for example a β-lactam antibiotic such as a carbapenem, penicillin or cephalosporin or pro-drug thereof. Carbapenems, penicillins, cephalosporins and other β-lactam antibiotics suitable for co-administration with the compound of formula (I), (IA), (IB) or (IC) - whether by separate administration or by inclusion in the compositions according to the invention - include both those known to show instability to or to be otherwise susceptible to metallo-β-lactamases and also those known to have a degree of resistance to metallo-β-lactamases.
A serine β-lactamase inhibitor such as clavulanic acid, sulbactam or tazobactam may also be co-administered with the compound of the invention and the β-lactam antibiotic, either by separate administration, or co-formulation with one, other or both of the compounds of the invention and the β-lactam antibiotic.

Examples of carbapenems that may be co-administered with the compounds according to the invention include imipenem, meropenem, biapenem, BMS181139 ([4R-[4alpha,5beta,6beta(R*)]-4-[2-[(aminoiminomethyl)amino]ethyl]-3-{(2-cyanoethyl)thio}-6-(1-hydroxyethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid), BO2727 ([4R-3[3S*,5S*(R*)],4alpha,5beta,6beta(R*)]-6-(1-hydroxyethyl)-3-[[5-{[1-hydroxy-3-[(methylamino)propyl]}-3-pyrrolidinyl]thio]-4-methyl-7-oxo-1-azabicyclo[3.2.0] hept-2-ene-2-carboxylic acid monohydrochloride), ER35786 ((1R,5S,6S)-6-{(1R)-2-{[(3S,5S)-5-(sulfamoylaminomethyl)]pyrrolidin-3-yl}thio}-1-methylcarbapen-2-em-3-carboxylic acid hydrochloride) and S4661 ((1R,5S,6S)-2-[(3S,5S)-5-(sulfamoylaminomethyl)pyrrolidin-3-yl]thio-6-{(1R)-1-hydroxyethyl}-1-methylcarbapen-2-em-3-carboxylic acid).

Examples of penicillins suitable for co-administration with the compounds according to the invention include benzylpenicillin, phenoxymethylpenicillin, carbenicillin, azidocillin, propicillin, amoxicillin, epicillin, ticarcillin, cyclacillin, pirbenicillin, azlocillin, mezlocillin, sulbenicillin, piperacillin, and other known penicillins. The penicillins may be used in the form of pro-drugs thereof, for example as in vivo hydrolysable esters, for example the acetoxymethyl, pivaloyloxymethyl, α-ethoxycarbonyloxyethyl and phthalidyl esters of ampicillin, benzylpenicillin and amoxycillin; as aldehyde or ketone adducts of penicillins containing a 6-α-aminoacetamido side chain (for example hetacillin, metampicillin and analogous derivatives of amoxycillin); and as α-esters of carbenicillin and ticarcillin, for example the phenyl and indanyl α-esters.

Examples of cephalosporins that may be co-administered with the compounds according to the invention include, cefatrizine, cephaloridine, cephalothin, cefazolin, cephalixin, cephacetrile, cephapirin, cephamandole nafate, cephradine, 4-hydroxycephalexin, cephaloglycin, cefoperazone, cefsulodin, ceftazidime, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, and other known cephalosporins, all of which may be used in the form of pro-drugs thereof.

Examples of β-lactam antibiotics other than penicillins and cephalosporins that may be co-administered with the compounds according to the invention include aztreonam, latamoxef (Moxalactam - Trade Mark), and other known β-lactam antibiotics, all of which may be used in the form of pro-drugs thereof.
Particularly suitable penicillins for co-administration with the compounds according to the invention include ampicillin, amoxycillin, carbenicillin, piperacillin, azlocillin, mezlocillin, and ticarcillin. Such penicillins may be used in the form of their pharmaceutically acceptable salts, for example their sodium salts. Alternatively, ampicillin or amoxycillin may be used in the form of fine particles of the zwitterionic form (generally as ampicillin trihydrate or amoxycillin trihydrate) for use in an injectable or infusible suspension, for example, in the manner hereinbefore described in relation to the compounds according to the invention. Amoxycillin, for example in the form of its sodium salt or the trihydrate, is particularly preferred for use in synergistic compositions according to the invention.

Particularly suitable cephalosporins for co-administration with the compounds according to the invention include cefotaxime and ceftazidine, which may be used in the form of their pharmaceutically acceptable salts, for example their sodium salts. A compound of formula (I), (IA), (IB) or (IC) may be administered to the patient in conjunction with a β-lactam antibiotic such as a carbapenem, penicillin or cephalosporin in a synergistically effective amount.

The compounds of formula (I), (IA), (IB) or (IC) may suitably be administered to the patient at a daily dosage of from 0.7 to 50 mg/kg of body weight. For an adult human (of approximately 70 kg body weight), from 50 to 3000 mg, preferably from 100 to 1000 mg, of a compound according to the invention may be administered daily, suitably in from 1 to 6, preferably from 2 to 4, separate doses. Higher or lower dosages may, however, be used in accordance with clinical practice.

When the compositions according to the invention are presented in unit dosage form, each unit dose may suitably comprise from 25 to 1000 mg, preferably from 50 to 500 mg, of a compound according to the invention. Each unit dose may, for example, be 62.5, 100, 125, 150, 200 or 250 mg of a compound according to the invention.

When the compounds of formula (I), (IA), (IB) or (IC) are co-administered with a penicillin, cephalosporin, carbapenem or other β-lactam antibiotic, the ratio of the amount of the compound according to the invention to the amount of the other β-lactam antibiotic may vary within a wide range. The said ratio may, for example, be from 100:1 to 1:100; more particularly, it may, for example, be from 2:1 to 1:30.

The amount of carbapenem, penicillin, cephalosporin or other β-lactam antibiotic in a synergistic composition according to the invention will normally be approximately similar to the amount in which it is conventionally used per se, for example from about 50 mg, advantageously from about 62.5 mg, to about 3000 mg per unit dose, more usually about 125, 250, 500 or 1000 mg per unit dose.

The present invention further provides a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, and in
particular a compound of formula (IA), (IB) or (IC), for use in the treatment of bacterial infections.

The present invention also includes the use of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, in the manufacture of a medicament for the treatment of bacterial infections.

The present invention also includes the use of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof as a metallo-β-lactamase inhibitor.

In a further aspect, the invention provides a method of treatment of bacterial infections in humans or animals which comprises administering, in combination with a carbapenem antibiotic, a therapeutically effective amount of a metallo-β-lactamase inhibitor.

A further composition according to the invention comprises a metallo-β-lactamase inhibitor together with a carbapenem antibiotic and a pharmaceutically acceptable carrier.

Such method and composition may be administered as described above for uses of compounds of formula (I).

All the above compositions and methods may optionally include a serine β-lactamase inhibitor as above described.

The compounds of the present invention are active against metallo-β-lactamase enzymes produced by a wide range of organisms including both Gram-negative organisms and Gram-positive organisms.

The following Examples illustrate compounds useful in the present invention, and intermediates in their preparation. (All temperatures are in °C).

EXAMPLES

Example 1: N-[2′-Benzy1-3′-mercaptopropionyl]phenylalanine

a) N-[S-Acetyl-2′-benzyl-3′-mercaptopropionyl]phenylalanine methyl ester

Prepared by Method B of Example 19 but using a 1:1 mixture of D and L-phenylalanine methyl ester hydrochlorides (238 mg, 1.0 mmol, Aldrich) and 2-acetylthiomethyl-3-phenylpropanoic acid (EP 0361365) (113 mg, 0.5 mmol). The title compound was obtained in 77% yield as a semi-crystalline mass, an approximately equimolar mixture of diastereoisomers. These were partially separated.
Less polar: $\delta$ (CDCl$_3$) 2.34 (3H, s, MeCOS), 2.58 (1H, m, CH$_2$CHCH$_3$), 2.75-3.15 (6H, m, CH$_2$CHCH$_3$, CH$_2$Ar), 3.61 (3H, MeO), 4.74 (1H, app.dq HCN), 5.77 (1H, bd, NH), 7.05-7.30 (10H, m, Ar-H)

More polar: $\delta$ (CDCl$_3$) 2.32 (3H, s, MeCOS), 2.56 (1H, m, CH$_2$CHCH$_3$, CH$_2$Ar), 2.68-3.13 (6H, m, CH$_2$CHCH$_3$, CH$_2$Ar), 3.66 (3H, MeO), 4.85 (1H, app.dq HCN), 5.81 (1H, bd, NH), 6.60 (2H, dd, Ar-H), 7.05-7.35 (8H, m, Ar-H).

b) N-[2'-Benzyl-3'-mercaptpropionyl]-phenylalanine
Prepared by Method C of Example 19 but using N-(S-acetyl-2'-benzyl-3'-mercaptpropionyl)-phenylalanine methyl ester (60 mg, 0.15 mmol). The title compound was obtained as a clear oil, an approximately equimolar mixture of diastereomers.

Less polar: $\delta$ (CDCl$_3$) 0.91, 0.93 (6H, 2d, (CH$_3$)$_2$CH), 1.30-1.65 (3H, m, Me$_2$CHCH$_2$), 2.32 (3H, s, MeCOS), 2.63 (1H, m, CH$_2$CHCH$_3$), 2.78-3.08 (4H, m, CH$_3$CHCH$_3$), 3.62 (3H, MeO), 4.53 (1H, app.dq HCN), 5.70 (1H, bd, NH), 7.10-7.25 (5H, m, Ar-H).

More polar: $\delta$ (CDCl$_3$) 0.75, 0.77 (6H, 2d, (CH$_3$)$_2$CH), 0.80-1.40 (3H, m, Me$_2$CHCH$_2$), 2.32 (3H, s, MeCOS), 2.62 (1H, m, CH$_2$CHCH$_3$), 2.88, 3.10 (4H, 2bd, CH$_3$CHCH$_3$), 3.65 (3H, MeO), 4.48 (1H, app.dq HCN), 5.61 (1H, bd, NH), 7.10-7.28 (5H, m, Ar-H).

Example 2: N-[2'-Benzyl-3'-mercaptpropionyl]leucine

a) D-Leucine methyl ester hydrochloride
Prepared by Method A of Example 19 but using D-Leucine (1.0 g, 7.6 mmol, Aldrich). The title compound was formed as a white foam in quantitative yield.

Less polar: $\delta$ (CD$_2$OD) 1.0, 1.02 (6H, 2d, (CH$_3$)$_2$CH), 1.6-1.9 (3H, m, Me$_2$CHCH$_2$), 7.90 (3H, s, MeO), 4.12 (1H, app.t, HCN).

b) N-[S-Acetyl-2'-benzyl-3'-mercaptpropionyl]leucine methyl ester
Prepared by Method B of Example 19 but on a 1:1 mixture of D and L-leucine methyl ester hydrochlorides (113 mg, 0.5 mmol, L isomer from Aldrich) and using S-acetyl-2-benzyl-3-mercaptpropionic acid (0.5 mmol). This gave the title compound as a clear oil in 81% yield, an approximately equimolar mixture of diastereoisomers.

These were partially separated.

Less polar: $\delta$ (CDCl$_3$) 0.91, 0.93 (6H, 2d, (CH$_3$)$_2$CH), 1.40-1.65 (3H, m, Me$_2$CHCH$_2$), 2.32 (3H, s, MeCOS), 2.63 (1H, m, CH$_2$CHCH$_3$), 2.78-3.08 (4H, m, CH$_3$CHCH$_3$), 3.62 (3H, MeO), 4.53 (1H, app.dq HCN), 5.70 (1H, bd, NH), 7.10-7.25 (5H, m, Ar-H).

More polar: $\delta$ (CDCl$_3$) 0.75, 0.77 (6H, 2d, (CH$_3$)$_2$CH), 0.80-1.40 (3H, m, Me$_2$CHCH$_2$), 2.32 (3H, s, MeCOS), 2.62 (1H, m, CH$_2$CHCH$_3$), 2.88, 3.10 (4H, 2bd, CH$_3$CHCH$_3$), 3.65 (3H, MeO), 4.48 (1H, app.dq HCN), 5.61 (1H, bd, NH), 7.10-7.28 (5H, m, Ar-H).
c) \(N\text{-}[2\text{'}-\text{Benzyl-3}\text{'-mercaptopropionyl}]\text{-leucine}\)

Prepared by Method C of Example 19 but on \(N\text{-}(S\text{-acetyl-2}\text{'-benzyl-3}\text{'-mercaptopropionyl})\text{-leucine methyl ester (60 mg, 0.20 mmol) in a 4:1 mixture of methanol : water over four hours. The product was subjected to silica gel flash chromatography eluting with formic acid - methyl formate - hexane to give the title compound as an approximately equimolar mixture of diastereoisomers in 75\% yield.\)

\[ \delta_H (\text{CDCl}_3) \ 0.75, 0.78, 0.88, 0.91 (6H, 4d, (CH_3)_2), 0.90-1.80 (3H, m, Me,CHCH_2), 2.47-2.65 (1H, m, CH_2CHCH_3), 2.75-3.00 (4H, m, CH_2CHCH_2), 4.40-4.53 (1H, m, HCN), 5.84, 5.95 (1H, 2d, NH), 6.3 (2H, bs, SH, CO_2H), 7.05-7.30 (5H, m, Ar-H).\]

Example 3: \(N\text{-}[2\text{'}-\text{Benzyl-3}\text{'-mercaptopropionyl}]\text{alanine}\)

a) D-Alanine methyl ester hydrochloride

Prepared by Method A of Example 19 but utilising D-alanine (Aldrich) as the amino acid. This gave the title compound as a white crystalline solid in quantitative yield.

b) \(N\text{-}[S\text{-Acetyl-2}\text{'-benzyl-3}\text{'-mercaptopropionyl}]\text{alanine methyl ester}\)

Prepared by Method B of Example 19 but using S-acetyl-2-benzyl-3-mercaptopropionic acid (150 mg, 0.63 mmol) and a 1:1 mixture of D and L-alanine methyl ester hydrochlorides (100 mg, 0.7 mmol, L-isomer from Aldrich). This gave the title compound as an approximately equimolar mixture of diastereoisomers in 82\% yield as a clear oil.

\[ \delta_H (\text{CDCl}_3) \ 1.09, 1.33, (3H, 2d, CH_3CHN), 1.95, 1.94 (3H, 2s, MeO), 2.53-2.66 (1H, m, CH_2CHCH_2), 2.78-3.11 (4H, m, CH_2CHCH_2), 3.67, 3.70 (3H, 2s, MeO), 4.40-4.53 (1H, m, HCN), 5.78, 5.92 (1H, 2d, NH), 7.15-7.30 (5H, m, Ar-H).\]

c) \(N\text{-}[2\text{'}-\text{Benzyl-3}\text{'-mercaptopropionyl}]\text{alanine}\)

Prepared by Method A of Example 19 but using \(N\text{-}(S\text{-acetyl-2}\text{'-benzyl-3}\text{'-mercaptopropionyl})\text{alanine methyl ester. The title compound was obtained as a clear oil, an approximately equimolar mixture of diastereoisomers.}\)

\[ \delta_H (\text{CDCl}_3) \ 1.10, 1.35 (3H, 4d, (CH_3)_2CN), 2.49-2.66 (1H, m, CH_2CHCH_2), 2.79-3.08 (4H, m, CH_2CHCH_2), 4.41-4.52 (1H, m, HCN), 5.85, 5.93 (1H, 2d, NH), 6.5 (2H, bs, SH, CO_2H), 7.05-7.30 (5H, m, Ar-H); m/z (CI+) 268 (M+H\text{'} 100\%), 286 (M+NH_3\text{'}\ 85\%).\]
Example 4: N-[2'-Benzyl-3'-mercaptopropionyl]aspartic acid

a) N-[S-Acetyl-2'-benzyl-3'-mercaptopropionyl]aspartic acid dimethyl ester
Prepared by Method B of Example 19 but using DL-aspartic acid dimethyl ester hydrochloride (Aldrich). This gave the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers, in 61% yield.

δ\textsubscript{n} (CDCl\textsubscript{3}) 2.30, 2.33 (3H, MeCOS), 2.55-2.70 (1H, m, CH\textsubscript{2}CHCH\textsubscript{2}), 2.80-3.15 (6H, m, CH\textsubscript{2}CHCH\textsubscript{2}, CH\textsubscript{2}CO\textsubscript{2}), 3.56, 3.68, 3.69, 3.70 (6H, s, MeO), 4.7-4.8 (1H, m, HCN), 6.25, 6.39 (1H, 2bd, NH), 7.10-7.30 (5H, m, Ar-H).

b) N-[2'-Benzyl-3'-mercaptopropionyl]aspartic acid
Prepared by Method C of Example 19 but utilising N-(S-acetyl-2'-benzyl-3'-mercaptopropionyl)aspartic acid dimethyl ester and stirring with sodium sulphide for 3 hours. This afforded the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

δ\textsubscript{n} (O=C(CD\textsubscript{3})\textsubscript{2}) 2.45-3.06 (7H, m, CH\textsubscript{2}CHCH\textsubscript{2}, CH\textsubscript{2}CO\textsubscript{2}), 4.65-4.75 (1H, m, HCN), 7.15-7.30 (5H, m, Ar-H), 7.50, 7.60 (1H, 2bd, NH).

Example 5: N-[2'-Benzyl-3'-mercaptopropionyl]tryptophan

a) N-[2'-Benzyl-3'-mercaptopropionyl]tryptophan methyl ester
Prepared by the Method B of Example 19 but on a 1:1 mixture of D and L-tryptophan methyl ester hydrochlorides (178 mg, 0.7 mmol, Aldrich). This afforded the title compound as a clear oil in 80% yield, as an approximately equimolar mixture of diastereoisomers. These were partially separated. Less polar: δ\textsubscript{n} (CDCl\textsubscript{3}) 2.27 (3H, s, MeCOS), 2.55 (1H, m, CH\textsubscript{2}CHCH\textsubscript{2}), 2.75-3.08 (4H, m, CH\textsubscript{2}CHCH\textsubscript{2}), 3.25 (2H, d, CH\textsubscript{2}CN), 3.56 (3H, MeO), 4.85 (1H, app.dq HCN), 5.88 (1H, bd, NH-amide), 6.90 (1H, d, Ar-H), 7.00-7.50 (9H, m, Ar-H), 8.2 (1H, bs, NH-indole).

More polar: δ\textsubscript{n} (CDCl\textsubscript{3}) 1.29 (3H, s, MeCOS), 2.53 (1H, m, CH\textsubscript{2}CHCH\textsubscript{2}), 2.75-3.10 (4H, m, CH\textsubscript{2}CHCH\textsubscript{2}), 3.23 (2H, d, CH\textsubscript{2}CN), 3.62 (3H, MeO), 4.85 (1H, app.dq HCN), 5.93 (1H, bd, NH-amide), 6.29 (1H, d, Ar-H), 7.00-7.50 (9H, m, Ar-H), 8.0 (1H, bs, NH-indole).

b) N-[2'-Benzyl-3'-mercaptopropionyl]-D-tryptophan methyl ester
Prepared by the method for the racemate [Example 5a)] but using D-tryptophan methyl ester. The title compound was obtained as two diastereomers partially separated into (a): a 2:1 mixture of the less and more polar isomers and (b): the pure
more polar isomer. Both were waxes with corresponding n.m.r. spectra to those described for the racemate.

c)  \(^\text{5}\) \(N\)\-[2'-Benzyl-3'-mercaptocropropionyl]-L-tryptophan methyl ester

Prepared by the method for the racemate [Example 5a]) but using L-tryptophan methyl ester. The title compound was obtained as two diastereomers partially separated into a (c) a 3:1 mixture of the less and more polar isomers and (d) a 1:2 mixture of the less and more polar isomers. Both were waxes with corresponding n.m.r. spectra to those described for the racemate.

d)  \(^\text{10}\) \(N\)\-[2'-Benzyl-3'-mercaptoampropionyl]tryptophan

Prepared by Method C of Example 19 but on \(N\)-(S-acetyl-2'-benzyl-3'-mercaptopropionyl)-tryptophan methyl ester (60 mg, 0.14 mmol). This afforded the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

\[
\begin{align*}
\delta (O=C(CD_2)_2) &\quad 2.35-2.55 (1H, m, CH_2CHCH_2), \\
&\quad 3.10-3.40 (2H, m, H_2CHCN), \\
&\quad 4.75-4.90 (1H, H_2CHCN), \\
&\quad 6.80-7.40 (10H, m, Ar-H), \\
&\quad 7.50 (1H, d, NH-amide), \\
&\quad 7.65 (1H, d, NH-amide), \\
&\quad 10.0, 10.1 (1H, bs, NH-indole).
\end{align*}
\]

\[
\begin{align*}
\delta (O=C(CD_2)_2) &\quad 2.35-2.55 (1H, m, CH_2CHCH_2), \\
&\quad 2.60-3.00 (4H, m, CH_2CHCH_2), \\
&\quad 3.10-3.20 (2H, m, H_2CHCN), \\
&\quad 4.7 (1H, H_2CHCN), \\
&\quad 6.80-7.40 (10H, m, Ar-H), \\
&\quad 7.50 (1H, d, NH-amide), \\
&\quad 10.0 (1H, bs, NH-indole). \\
\end{align*}
\]

\[
\begin{align*}
\delta (O=C(CD_2)_2) &\quad 2.35-2.55 (1H, m, CH_2CHCH_2), \\
&\quad 3.10-3.40 (2H, m, H_2CHCN), \\
&\quad 4.75-4.90 (1H, H_2CHCN), \\
&\quad 6.80-7.40 (10H, m, Ar-H), \\
&\quad 7.50, 7.75 (1H, d, NH-amide), \\
&\quad 10.0 (1H, bs, NH-indole).
\end{align*}
\]

\[
\begin{align*}
\delta (O=C(CD_2)_2) &\quad 2.35-2.55 (1H, m, CH_2CHCH_2), \\
&\quad 3.10-3.40 (2H, m, H_2CHCN), \\
&\quad 4.75-4.90 (1H, H_2CHCN), \\
&\quad 6.80-7.40 (10H, m, Ar-H), \\
&\quad 7.50, 7.75 (1H, d, NH-amide), \\
&\quad 10.0 (1H, bs, NH-indole).
\end{align*}
\]

f)  \(^\text{20}\) \(N\)-[2'-Benzyl-3'-mercaptoampropionyl]-L-tryptophan

Prepared in an identical manner to that described for the D-isomers [Example 5e] but using each isomer of \(N\)-[2'-benzyl-3'-mercaptoampropionyl]-L-tryptophan methyl ester mixtures (c) and (d) in turn. The products were obtained with corresponding n.m.r. spectra to those described for the D-isomer.
Example 6: \(N-[2'-\text{Benzyl}-3']-\text{mercaptopropionyl}]\text{threonine}

a) \(\text{D-Threonine methyl ester hydrochloride}\)
Prepared by Method A of Example 19 but on \(\text{D-threonine (750mg, 0.16 mmol, Aldrich)}\) over 3 days. This gave the title compound in quantitative yield as a hygroscopic sticky oil.

\[
\begin{align*}
\delta_h (\text{CD}_3\text{OD}) & = 1.34 (3\text{H}, \text{d}, \text{MeCO}), 3.87 (3\text{H}, \text{s}, \text{MeO}), 3.94 (1\text{H}, \text{d}, \text{HCN}), 4.29 (1\text{H}, \text{dq}, \text{HCO})
\end{align*}
\]

b) \(\text{L-Threonine methyl ester hydrochloride}\)
Prepared by Method A of Example 19 but on \(\text{L-Threonine (750mg, 0.16 mmol, Aldrich)}\) over 3 days. This gave the title compound in quantitative yield as a hygroscopic sticky oil with an identical n.m.r. spectrum to the D-isomer.

c) \(\text{N-[S-Acetyl-2'-benzyl-3'-mercaptopropionyl]}\text{threonine methyl ester}\)
Prepared by Method B of Example 19 but on a 1:1 mixture of D and \(\text{L-threonine methyl ester hydrochlorides (119 mg, 0.7 mmol). This afforded the title compound as a clear oil, an approximately 1:1 mixture of diastereomers in 42\% yield.}

\[
\begin{align*}
\delta_h (\text{CDCl}_3) & = 0.78, 1.20 (1\text{H}, 2\text{d}, \text{MeCH}), 2.1 (1\text{H}, \text{bs}, \text{OH}), 2.34 (3\text{H}, 2\text{s}, \text{MeCOS}), 2.70-3.15 (5\text{H}, \text{m}, \text{CH}, \text{CHCH}_2), 3.65, 3.74 (3\text{H}, 2\text{s}, \text{MeO}), 4.12, 4.28 (1\text{H}, 2\text{dq}, \text{HCOH}), 4.44, 4.53 (1\text{H}, 2\text{dd}, \text{HCN}), 6.12, 6.20 (1\text{H}, 2\text{bd}, \text{NH}), 7.15-7.30 (5\text{H}, \text{m, Ar-H}).
\end{align*}
\]

d) \(\text{N-[2'-Benzyl-3'-mercaptopropionyl]}\text{threonine}\)
Prepared by Method C of Example 19 but on \(\text{N-(S-acetyl-2'-benzyl-3'-mercaptopropionyl)}\text{threonine methyl ester (60 mg, 0.17 mmol). This gave the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.}

\[
\begin{align*}
\delta_h (\text{O=C(OD)}_2) & = 0.89, 1.20 (1\text{H}, 2\text{d}, \text{MeCH}), 2.45-3.10 (5\text{H}, \text{m}, \text{CH}, \text{CHCH}_2), 4.40-4.60 (2\text{H}, \text{m, OCHCHN}), 5.9 (3\text{H}, \text{bs}, \text{SH}, \text{CO}_2\text{H}, \text{OH}), 7.10-7.30 (5\text{H}, \text{m, Ar-H}), 7.70, 7.75 (1\text{H}, 2\text{d}, \text{NH}).
\end{align*}
\]

Example 7: \(N-[2'-\text{Benzyl-3'}-\text{mercaptopropionyl]}\text{cysteine}\)

a) \(\text{D-Cystine dimethyl ester dihydrochloride}\)
Prepared by Method A of Example 19 but utilising \(\text{D-cystine (Aldrich). This afforded the title compound as a white crystalline solid in quantitative yield.}

\[
\begin{align*}
\delta_h (\text{CD}_3\text{OD}) & = 3.38 (4\text{H}, 3\text{dd}, \text{CH}_2), 3.89 (6\text{H}, \text{s}, \text{MeO}), 4.48 (2\text{H}, 2\text{d}, \text{HCN}).
\end{align*}
\]
b) N-[S-Acetyl-2'-benzyl-3'-mercaptopropionyl]cystine methyl ester

Prepared by Method B of Example 19 but utilising a 1:1 mixture of D and L-cystine methyl ester hydrochlorides (120 mg, 0.35 mmol, L-isomer from Aldrich) and 2-acetylthiomethyl-3-phenylpropanoic acid (150 mg, 0.63 mmol). This afforded the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers, in 43% overall yield.

δ (CDCl3) 2.34, 2.35 (6H, 2s, MeCS), 2.60-3.15 (14H, m, CH₂CHCH₂, CH₂S), 3.70, 3.74 (6H, 2s, MeO), 4.6-4.8 (1H, m, HCN), 6.35 (2H, bs, NH), 7.15-7.30 (10H, m, Ar-H).

c) N-[2'-Benzyl-3'-mercaptopropionyl]cysteine

N-(S-Acetyl-2'-benzyl-3'-mercaptopropionyl)cystine methyl ester (50 mg) was dissolved in degassed methanol (5 ml) and treated with sodium sulfide nonahydrate (300 mg) and dithiothreitol (122 mg). The mixture was stirred at RT for 14 hours then poured into water (30 ml) and extracted with chloroform (2 x 20 ml), the aqueous phase acidified to pH 1-2 and re-extracted with ethyl acetate (3 x 10 ml), dried, (MgSO₄) filtered and evaporated to a clear oil, the title compound as an approximately equimolar mixture of diastereoisomers, in 86% yield.

δ (CDCl3) 2.55-3.30 (7H, m, CH₂CHCH₂, CH₂S), 4.25-4.40 (1H, m, HCN), 6.30, 6.40 (1H, 2d, NH), 7.15-7.33 (5H, m, Ar-H); m/z (ESI) 298 (M-H, 100%).

Example 8: N-[2'-Benzyl-3'-mercaptopropionyl]tyrosine

a) D-Tyrosine methyl ester hydrochloride

Prepared by Method A of Example 19 using D-tyrosine (Aldrich) as the amino acid. This gave the title compound as a white crystalline solid in quantitative yield.

δ (CD3OD) 3.05, 3.18 (2H, 2dd, Ar-CO), 4.22 (1H, dd, HCN), 6.79, 7.07 (4H, 2d, Ar-H).

b) N-[S-Acetyl-2'-benzyl-3'-mercaptopropionyl]tyrosine methyl ester

Prepared by Method B of Example 19 and utilising a 1:1 mixture of D and L-tyrosine methyl ester hydrochlorides (232 mg, 1 mmol, L isomer from Aldrich) with the addition of triethylamine (101.2 mg, 1 mmol). This gave the title compound as a sticky wax, an approximately equimolar mixture of diastereoisomers, in 75% yield.

δ (CDCl3) 2.30, 2.32 (3H, 2s, MeCS), 2.55-2.70 (1H, m, CH₂CHCH₂), 2.80-3.37 (6H, m, CH₂CHCH₂, CH₂Ar), 3.60, 3.68 (3H, 2s, MeO), 4.74, 4.82 (1H, 2app.dq, HCN), 5.90, 5.93 (1H, 2bd, NH), 6.41, 6.58, 6.69, 6.93 (4H, 4d, Ar-H), 7.10-7.35 (5H, m, Ar-H).
c) \( N\-[2'\text{-Benzyl}-3'-\text{mercaptopropionyl}]\text{tyrosine} \)

Prepared by Method C of Example 19 but utilising \( N\-(\text{S-acetyl}-2'\text{-benzyl}-3'\text{-mercaptopropionyl})\text{tyrosine methyl ester} \). This gave the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

\[
\delta_{\text{H}} (O=\text{C} (\text{CD}_{3})_{2}) \ 2.35-2.55 \ (1 \text{H, m, CH}_{2}\text{CHCH}_{2}), \ 2.70-3.20 \ (7 \text{H, m, CH}_{2}\text{CHCH}_{2}, \text{CH}_{2}\text{Ar}), \ 4.65-4.75 \ (1 \text{H, m, HCN}), \ 6.65-7.35 \ (11 \text{H, m, Ar-H, NH}), \ 8.2 \ (1 \text{H, bs, OH}); \ m/z \ (\text{ESI}) \ 358 \ (M-H', 100\%).
\]

Example 9: \( N\-[2'\text{-Benzyl}-3'-\text{mercaptopropionyl}]\text{phenylglycine} \)

a) \( L\text{-Phenylglycine methyl ester hydrochloride} \)

Prepared by Method A of Example 19 but using \( L\text{-phenylglycine} \) (Aldrich). The title compound was obtained as a white crystalline solid in quantitative yield.

\[
\delta_{\text{H}} (\text{CD}_{3}\text{OD}) \ 3.80 \ (3 \text{H, s, MeO}), \ 5.19 \ (1 \text{H, s, HCN}), \ 7.42-7.50 \ (5 \text{H, m, Ar-H}).
\]

b) \( D\text{-Phenylglycine methyl ester hydrochloride} \)

Prepared by method A of Example 19 but using \( D\text{-phenylglycine} \) (Aldrich). The title compound was obtained as a white crystalline solid in quantitative yield with an identical n.m.r. spectrum to the \( L\text{-isomer} \).

c) \( D\text{-Phenylglycine ethyl ester hydrochloride} \)

Prepared by Method A of Example 19 but using \( D\text{-phenylglycine} \) and ethanol in place of methanol. This gave the title compound as a white crystalline solid in quantitative yield.

\[
\delta_{\text{H}} (\text{CD}_{3}\text{OD}) \ 1.20 \ (3 \text{H, bt, MeC}), \ 4.29 \ (2 \text{H, dq, CH}_{2}\text{O}), \ 5.18 \ (1 \text{H, s, HCN}), \ 7.42-7.50 \ (5 \text{H, m, Ar-H}).
\]

d) \( N\-[\text{S-Acetyl}-2'\text{-benzyl}-3'\text{-mercaptopropionyl}]\text{-phenylglycine methyl ester} \)

Prepared by Method B of Example 19 but using a mixture of \( L\text{-} \) and \( D\text{-phenylglycine} \) methyl ester hydrochloride (201 mg, 1.0 mmol). This gave the title compound in 70% yield as a racemic mixture of the diastereomers.

Less polar: \( \delta_{\text{H}} (\text{CDCl}_{3}) \ 2.26 \ (3 \text{H, s, MeCOS}), \ 2.59 \ (1 \text{H, m, CH}_{2}\text{CHCH}_{2}), \ 2.80-3.11 \ (4 \text{H, m, CH}_{2}\text{CHCH}_{2}), \ 3.65 \ (3 \text{H, MeO}), \ 5.43 \ (1 \text{H, d, HCN}), \ 6.32 \ (1 \text{H, bd, NH}), \ 6.95-7.35 \ (10 \text{H, m, Ar-H}). \)

More polar: \( \delta_{\text{H}} (\text{CDCl}_{3}) \ 2.34 \ (3 \text{H, s, MeCOS}), \ 2.69 \ (1 \text{H, m, CH}_{2}\text{CHCH}_{2}), \ 2.82-3.12 \ (4 \text{H, m, CH}_{2}\text{CHCH}_{2}), \ 3.68 \ (3 \text{H, MeO}), \ 5.49 \ (1 \text{H, app.dq HCN}), \ 6.37 \ (1 \text{H, bd, NH}), \ 7.00-7.35 \ (10 \text{H, m, Ar-H}). \)
e) \(N\)-(2'-Benzyl-3'-mercaptopropionyl]phenylglycine

Prepared by Method C of Example 19 but using \(N\)-(S-acetyl-2'-benzyl-3'-mercaptopropionyl)phenylglycine methyl ester. This afforded the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

\[\delta_{\text{H}} (\text{CDCl}_3) 2.45-2.55 (1\text{H}, \text{m}, \text{CH}_2\text{CH}_2), 2.70-3.10 (4\text{H}, \text{m}, \text{CH}_2\text{CH}_2), 3.10-3.40 (2\text{H}, \text{m}, \text{H}_2\text{CN}), 4.95,5.05 (1\text{H}, \text{HCN}), 7.10-7.50 (10\text{H}, \text{m}, \text{Ar-H}), 7.78, 7.83 (1\text{H}, 2\text{d}, \text{NH}).\]

f) \(\text{D-Phe} \text{-S-N-}[2'-\text{Benzyl-3'-mercaptopropionyl}]\text{-PhGly methyl ester}\)

g) \(\text{D-Phe} \text{-S-N-}[2'-\text{Benzyl-3'-mercaptopropionyl}]\text{-PhGly methyl ester}\)

Prepared by the method described above for the racemate (Example 9d and 9e), using the D-phenylglycine methyl ester hydrochloride enantiomer and separating the diastereomers by chromatography. The (2'-S) isomer corresponds to the more polar isomer.

Less polar isomer \([\alpha_{\text{D}}^{20}]=-29.5^\circ\) (c. 1.425, CHCl₃).

More polar isomer \([\alpha_{\text{D}}^{20}]=-134.7^\circ\) (c. 1.425, CHCl₃).

h) \(N\)-(S-Acetyl-2'-benzyl-3'-mercaptopropionyl]D-phenylglycine

A stirred suspension of D-phenylglycine (302mg, 2mmol) in chloroform (5ml) and acetonitrile (0.5ml) was treated with chlorotrimethylsilane (0.26ml, 2mmol) and the mixture refluxed for 1 hour. It was cooled in an ice-bath. A solution of 2-acetylthiomethyl-3-phenylpropanoyl acid chloride (prepared from the carboxylic acid and oxalyl chloride by Method B of Example 19) (1mmol) in chloroform (5ml) was added dropwise to the ice-cold silyl ester, followed by triethylamine (0.62ml, 4.4mmol). The mixture was allowed to gain room temperature and stirred for 2 hours, washed with 1M hydrochloric acid (10ml), water (2 x 10ml), saturated brine (10ml), dried (MgSO₄), and evaporated. The residue was purified by flash chromatography on silica, eluting with mixtures of methanol in dichloromethane to give \(N\)-(S-acetyl-2'-benzyl-3'-mercaptopropionyl]D-phenylglycine (355mg, 95%).

\[\delta_{\text{H}}(\text{CDCl}_3) 2.23 (3\text{H}, \text{s}, \text{COCH}_3), 2.62-3.18 (5\text{H}, \text{m}, 2 \times \text{CH}_2, 2'\text{-CH}), 4.30 (1\text{H}, \text{bs}, \text{CO}, \text{H}), 5.42 (1\text{H}, \text{bs}, \alpha\text{-CH}), 6.56 (1\text{H}, \text{bs}, \text{NH}), 7.20 (10\text{H}, \text{m}, 2 \times \text{Ph}) \text{ppm. EIMS } M^+ 371. \text{ DCIMS } M^+ 372.\]
i) [2'S]-N-[2'-Benzy1-3'-mercaptopropionyl]-D-phenylglycine
Prepared by the method described above for the racemate (Example 9e) but using the (2'S)-N-(2'-benzyl-3'-mercapto-propionyl)-D-phenylglycine methyl ester diastereomer.

j) [2'R]-N-[2'-Benzy1-3'-mercapto-propionyl]-D-phenylglycine
Prepared by the method described above for the racemate (Example 9e) but using the (2'R)-N-(2'-benzyl-3'-mercapto-propionyl)-D-phenylglycine methyl ester diastereomer.

k) N-[2'-Benzyl-3'-mercapto-propionyl]-D-phenylglycine
A solution of N-(S-acetyl-2'-benzyl-3'-mercapto-propionyl)-D-phenylglycine (100mg) in methanol (1ml) at room temperature was treated with 0.880S.G. ammonia (0.5ml). After 0.25h the solution was diluted with ethyl acetate (10ml), washed with 1M hydrochloric acid (5ml), water (2x5ml), saturated brine (5ml), dried (MgSO₄) and evaporated. Purified by flash chromatography on silica eluting with mixtures of methanol in dichloromethane, it gave N-(2'-benzyl-3'-mercapto-propionyl)-D-phenylglycine as a mixture of diastereomers (3:2, 2'S:2'R). \( \delta \) (CDCl₃) 2.09 (0.6H, t, J 7.8Hz, 2'S-SH), 2.30-3.05 (5.4H, m, 2 x CH₂, c-CCH₂, 2'R-SH), 5.03 (1H, 2d, J 7.1, 7.0Hz, 2 x H), 7.25 (10H, m, 2 x Ph), 8.08 (0.4H, d, J 7.0Hz, NH), 8.21 (0.6H, d, J 7.1Hz, NH). EIMS M⁺ 329. DCIMS MH⁺ 330.

Example 10: N-[(R)- and N-[(S)-2'-Benzy1-3'-mercapto-propionyl]glycine

25 a) N-[S-Acetyl-2'-benzyl-3'-mercapto-propionyl]glycine methyl ester
Prepared by Method B of Example 19 but utilising glycine methyl ester hydrochloride (Aldrich). This afforded the title compound as a colourless oil in 62% yield.

\( \delta \) (CDCl₃) 2.32 (3H, s, MeCS), 2.65 (1H, dddd, CH₂CH₂CH₃), 2.85, 2.97, 3.04, 3.11 (4H, 4dd, CH₂CH₂CH₃), 3.70 (3H, s, MeO), 3.80, 4.00 (2H, 2dd, CH₂CO₂), 5.90 (1H, bt, NH), 7.15-7.30 (5H, m, Ar-H).

b) N-[2'-Benzy1-3'-mercapto-propionyl]glycine
Prepared by Method C of Example 19 but using N-(S-acetyl-2'-benzyl-3'-mercapto-propionyl)glycine methyl ester. Thiorphan was obtained as a white solid.

\( \delta \) (O=C(CD₃)₂) 2.50 (1H, m, CH₂CH₂CH₃), 2.70-3.10 (4H, m, CH₃CH₂CH₂), 3.2 (2H, bs, SH, CO₂H), 3.85, 4.01 (2H, 2dd, CH₂CO₂), 7.15-7.30 (5H, m, Ar-H), 7.5 (1H, bt, NH).
c) (2'S)-N-[(2'-Benzyl-3'-mercapto-propionyl)glycine and
(2'R)-N-[(2'-Benzyl-3'-mercapto-propionyl)glycine

Racemic N-((S-acetyl-2'-benzyl-3'-mercapto-propionyl)glycine methyl ester (15 mg)
was separated into its two component isomers using chiral HPLC (Chiralpak-AD,
mobile phase 80:20 hexane:ethanol). Each isomer was hydrolysed by Method C of
Example 19 but on a 4 mg scale. The dextrorotatory isomer of the ester gave (2'R)-
N-(2-thiomethyl-3-phenylpropanoyl)glycine, α<sub>d</sub> = -34° (c, 3.5 in EtOH), otherwise
spectroscopically identical to thiorphan. The laevorotatory ester enantiomer
hydrolysed to give the other antipode of thiorphan with a corresponding but opposite
rotation.

Example 11: N-[2'-Benzyl-3'-mercapto-propionyl]-3-hydroxyphenylglycine

a) 3-Hydroxyphenylglycine methyl ester hydrochloride

Prepared by Method A of Example 19 but using 3-hydroxyphenylglycine, obtained
from 3-hydroxybenzaldehyde (Aldrich) via the Strecker synthesis. This gave the title
compound as a white crystalline solid in quantitative yield.
δH (MeOD) 3.80, (3H, s, ), 5.10 (1H, s, HCN), 6.90-7.36 (4H, m, Ar-H).

b) N-[(S-Acetyl-2'-benzyl-3'-mercapto-propionyl)-3-hydroxyphenylglycine
methyl ester

Prepared by Method B of Example 19 but using 3-hydroxyphenylglycine methyl ester hydrochloride. This gave the title compound as a colourless oil in 41% yield, as
an approximately equimolar mixture of diastereoisomers.
δH (CDCl₃) 2.28, 2.35 (3H, 2d, AcS), 2.70-3.15 (5H, m, CH₂CHCH₃), 3.65, 3.70 (3H,
2s, OMe), 5.40, 5.43 (1H, 2d: HCN), 6.50-7.60 (10H, m, Ar-H, NH).

c) N-[2'-Benzyl-3'-mercapto-propionyl]-3-hydroxyphenylglycine

Prepared by Method C of Example 19 but using N-(S-acetyl-2'-benzyl-3'-
mercapto-propionyl)-3-hydroxyphenylglycine methyl ester. This gave the title
compound as a colourless oil, an approximately equimolar mixture of diastereoisomers.
ν<sub>max</sub> (film) 3358 (OH), 3025-2931 (CH-Str), 2966 (SH), 1731 (C=O, Acid), 1642
(C=O, Amide), 1525-1454 (Ar-Str); δH (O=C(CD₃),) 2.40-2.58 (1H, m, CH₂CHCH₃),
2.70-3.13 (4H, CH₂CHCH₃), 5.40, 5.49 (1H, 2d: HCN), 6.70-7.30 (9H, m, Ar-H),
7.75, 7.80 (1H, 2bd, NH), 8.5 (1H, bs, CO₂H).
Example 12: N-[2'-Benzyl-3'-mercaptobenzyloxy]-4-hydroxy-D-phenylglycine

a) N-[S-Acetyl-2'-benzyl-3'-mercaptobenzyloxy]-4-hydroxy-D-phenylglycine ethyl ester

Prepared by Method B of Example 19 but using 4-hydroxy-D-phenylglycine methyl ester hydrochloride, prepared from 4-hydroxy-D-phenylglycine (Aldrich) by Method A of Example 19. This gave the title compound as a colourless oil in 38% yield, as an approximately equimolar mixture of diastereoisomers.

\[ \delta H (CDCl_3) 1.19 (3H, dt, \text{CH}_2Cl), 2.28, 2.34 (3H, 2s, AcS), 2.68-2.78 (1H, m, \text{CH}_2CHCH_2), 2.85-3.15 (4H, CH_2CHCH_2), 4.00-4.22 (2H, m, OCH_2), 5.30-5.32 (1H, 2d, HCN), 6.50-7.34 (10H, m, Ar-H, NH). \]

b) N-[2'-Benzyl-3'-mercaptobenzyloxy]-4-hydroxy-D-phenylglycine

Prepared by Method C of Example 19 but using N-(S-acetyl-2'-benzyl-3'-mercaptobenzyloxy)-4-hydroxy-D-phenylglycine methyl ester. This gave the title compound as a colourless oil, an approximately equimolar mixture of diastereoisomers.

\[ \nu_{max} \text{ (film)} 3310 \text{ (OH)}, 3015-2940 \text{ (CH-Str)}, 2567 \text{ (SH)}, 1731 \text{ (C=O, Acid)}, 1635 \text{ (C=O, Amide)}, 1535-1445 \text{ (Ar-Str)}; \delta H (O=CD(CD)_2) 2.40-2.58 (1H, m, CH_2CHCH_2). \]

Example 13: N-[2'-Benzyl-3'-mercaptobenzyloxy]-4-methoxyphenylglycine

a) 4-Methoxyphenylglycine methyl ester hydrochloride


\[ \delta H (MeOD) 3.80, 3.82 (3H, 2s, 2 x OMe), 5.10 (1H, s, HCN), 7.01-7.36 (4H, m, Ar-H), 7.69, 7.78 (1H, 2bd, NH), 8.5 (1H, bs, CO_2H). \]

b) N-[S-Acetyl-2'-benzyl-3'-mercaptobenzyloxy]-4-methoxyphenylglycine methyl ester

Prepared by Method B of Example 19 but using 4-methoxyphenylglycine methyl ester hydrochloride. This gave the title compound as a colourless oil in 57% yield, an approximately equimolar mixture of diastereoisomers.
c) **N-[2'-Benzyl-3'-mercaptopropionyl]-4-methoxyphenylglycine**

Prepared by Method C of **Example 19** but using **N-(S-acetyl-2'-benzyl-3'-mercaptopropionyl)-4-methoxyphenylglycine** methyl ester. This gave the title compound as a colourless oil, an approximately equimolar mixture of diastereoisomers.

\[ \delta_H (\text{CDCl}_3) \, 2.35, 2.40 \, (3H, 2s, AcS), 2.70-3.30 \, (5H, m, CH_2CHCH_2), 3.67, 3.72 \, (3H, 2s, OMe), 5.35, 5.53 \, (1H, 2dd, HCN), 6.49-6.52 \, (1H, bm, NH), 6.70-8.1 \, (8H, m, Ar-H). \]
\( v_{\text{max}} \) (film) 3305 (OH), 3028-2931 (CH-Str), 2573 (SH), 1731 (C=O, Acid), 1630 (C=O, Amide), 1538-1432 (Ar-Str).

Example 15: \( N'\)-[2'-Benzy1-3'-mercaptopropionyl]-3,4-dihydroxy-D-phenylglycine

a) 3,4-Dihydroxy-D-phenylglycine methyl ester hydrochloride
Prepared by Method A of Example 19 but using 3,4-dihydroxy-D-phenylglycine, obtained from 3,4-dihydroxybenzaldehyde by the Strecker synthesis. This gave the title compound as a white solid in quantitative yield.
\( \delta H \) (MeOD) 3.8 (3H, s, OMe), 5.1 (1H, s, HCN), 6.7 - 7.3 (3H, m, Ar-H).

b) \( N\)-[S-Acetyl-2'-benzyl-3'-mercaptopropionyl]-3,4-dihydroxy-D-phenylglycine methyl ester
Prepared by Method B of Example 19 but using 3,4-dihydroxy-D-phenylglycine methyl ester hydrochloride. This gave the title compound as a clear oil in 11% yield an approximately equimolar mixture of diastereoisomers.
\( \delta H \) (CDCl3) 2.36, 2.38 (3H, 2d, AcS), 2.65 - 3.30 (5H, m, CH,CHCH2), 3.65, 3.70 (3H, 2s, OMe), 5.28, 5.35 (1H, 2d, HCN), 6.50 (1H, bs, NH), 6.70 - 8.10 (8H, m, Ar-H).

c) \( N\)-[2'-Benzy1-3'-mercaptopropionyl]-3,4-dihydroxy-D-phenylglycine
Prepared by Method C of Example 19 but using \( N\)-(S-acetyl-2'-benzyl-3'-mercaptopropionyl)-3,4-dihydroxy-D-phenylglycine methyl ester (21 mg). This gave the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

Example 16: \( N\)-[2'-Benzy1-3'-mercaptopropionyl]-4-fluoro-D-phenylglycine

a) \( N\)-[S-Acetyl-2'-benzyl-3'-mercaptopropionyl]-4-fluoro-D-phenylglycine methyl ester
\( N\)-[Benzoyloxy carbonyl]-4-fluoro-D-phenylglycine (1.0 g, 3.3 mmol) was dissolved in ethanol (5 ml) and treated with cyclohexene (0.5 ml) and 10% palladium on carbon. The mixture was heated to reflux for 1 hour then cooled and poured onto celite. A 1:1 mixture of water and ethanol (50 ml) was introduced and the resulting suspension acidified to pH 1 with hydrochloric acid. The mixture was heated to 80°C, hot filtered and evaporated then coevaporated with toluene to give 4-fluoro-D-
phenylglycine (D. Landini, et al, Synthesis, 1970, 1, 26) as an oil (50 mg). This was dissolved in a mixture of acetyl chloride (1 ml) in methanol (4 ml) (CARE) and maintained at RT for 24 hours. The solvent was removed to give a white solid 4-fluorophenylglycine methyl ester hydrochloride which was used in Method B of Example 19 without further purification. This gave the title compound as a clear oil in 44% yield, an approximately equimolar mixture of diastereoisomers.

\[
\delta (\text{CDCl}_3) \ 2.30, \ 2.40 (3H, 2d, AcS), \ 2.65-3.15 (5H, m, CH}_2CHCH}_2), \ 3.62, \ 3.68 (3H, 2d, OMe), \ 5.40-5.45 (1H, 2d, HCN), \ 6.35 (1H, bs, NH), \ 6.90-7.35 (9H, m, Ar-H).
\]

Example 17: N-[2'-Benzyl-3'-mercaptropropionyl]-3-fluorophenylglycine

b) N-[2'-Benzyl-3'-mercaptropropionyl]-4-fluoro-D-phenylglycine

Prepared by Method C of Example 19 but using N-[S-acetyl-2'-benzyl-3'-mercaptropropionyl]-4-fluoro-D-phenylglycine methyl ester hydrochloride. This gave the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

\[
\nu (\text{film}) \ 3320 (\text{OH}), \ 3028-2931 (\text{CH-Str}), \ 2569 (\text{SH}), \ 1731 (\text{C}=\text{O, Acid}), \ 1651 (\text{C}=\text{O, Amide}), \ 1510-1454 (\text{Ar-Str}).
\]

Example 17: N-[2'-Benzyl-3'-mercaptropropionyl]-3-fluorophenylglycine

a) 3-Fluorophenylglycine methyl ester hydrochloride

Prepared by Method A of Example 19 but using 3-fluorophenylglycine (Aldrich). This gave the title compound as a white solid in quantitative yield.

\[
\delta (\text{MeOD}) \ 3.83 (3H, s, OMe), \ 5.27 (1H, s HCN), \ 7.2-7.6 (4H, m, Ar).
\]

b) N-[S-Acetyl-2'-benzyl-3'-mercaptropropionyl]-3-fluorophenylglycine methyl ester

Prepared by Method B of Example 19 but using 3-fluorophenylglycine methyl ester hydrochloride and triethylamine (101 mg). This gave the title compound as a clear oil in 42% yield, an approximately equimolar mixture of diastereoisomers.

\[
\delta (\text{CDCl}_3) \ 2.32, \ 2.36 (3H, 2s, AcS), \ 2.65-3.15 (5H, m, CH}_2CHCH}_2), \ 3.65-3.71 (3H, 2s, OMe), \ 5.42-5.48 (1H, 2d, HCN), \ 6.4 (1H, bs, NH), \ 6.65-7.4 (9H, m, Ar-H).
\]

c) N-[2'-Benzyl-3'-mercaptropropionyl]-3-fluorophenylglycine

Prepared by Method C of Example 19 but using N-[S-acetyl-2'-benzyl-3'-mercaptropropionyl]-3-fluorophenylglycine methyl ester hydrochloride. This gave the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.
$\nu_{\text{max}}$ (film) 3312 (OH), 3029-2934 (CH-Str), 2569 (SH), 1731 (C=O, Acid), 1659 (C=O, Amide), 1537-1453 (Ar-Str).

Example 18: N-[2′-Benzyl-3′-mercaptopropionyl]-3-nitro-D-phenylglycine

a) 3-Nitro-D-phenylglycine methyl ester hydrochloride

$\delta$H (MeOD) 3.85 (3H, s, OMe), 5.45 (1H, s), 7.7-8.5 (4H, m, Ar-H).

b) N-[S-Acetyl-2′-benzyl-3′-mercaptopropionyl]-3-nitro-D-phenylglycine methyl ester
Prepared by Method B of Example 19 but using 3-Nitro-D-phenylglycine methyl ester hydrochloride and triethylamine (101 mg). This gave the title compound as a yellow oil in 63% yield, an approximately equimolar mixture of diastereoisomers.

$\delta$ (CDCl$_3$) 2.34, 2.35 (3H, 2s, AcS), 2.60-3.15 (5H, m, CH$_2$CHCH$_3$), 3.67, 3.72 (3H, 2s, OMe), 5.47-5.56 (1H, 2dd, HCN), 6.50-6.65 (1H, bm, NH), 6.95-8.2 (9H, m, Ar-H).

c) N-[2′-Benzyl-3′-mercaptopropionyl]-3-nitro-D-phenylglycine
Prepared by Method C of Example 19 but using N-[S-acetyl-2′-benzyl-3′-mercaptopropionyl]-3-nitro-D-phenylglycine methyl ester. This gave the title compound as a yellow oil, an approximately equimolar mixture of diastereoisomers.

$\nu_{\text{max}}$ (film) 3326 (OH), 3175-2930 (CH-Str), 2600 (SH), 1738 (C=O, Acid), 1650 (C=O, Amide), 1530-1349 (Ar-Str); $\delta$ (CDCl$_3$) 2.55-2.31 (5H, m, CH$_2$CHCH$_3$), 5.45, 5.50 (1H, 2d, HCN), 6.48, 6.52 (1H, 2bd, NH), 7.00-8.15 (9H, m, Ar-H).

Example 19: N-[2′-Benzyl-3′-mercaptopropionyl]-2-fluorophenylglycine

a) 2-Fluorophenylglycine methyl ester hydrochloride (Method A)
Acetyl chloride (4m1) was added cautiously and dropwise to methanol (20m1) at 0°C over 2 minutes. When the addition was completed, the 2-fluorophenylglycine (1g, 5.9 mmol, Aldrich) was introduced in a single portion. The mixture was stirred until dissolved then allowed to stand at RT for 24 hours. The solvent was evaporated then coevaporated twice from toluene to afford the title compound as a white crystalline solid in quantitative yield.
\[ \delta_n (\text{CD,OD}) \ 3.82 \ (3\ H, \ s, \ Me), \ 5.42 \ (1\ H, \ s, \ CHCO), \ 7.20-7.60 \ (4\ H, \ m, \ Ar-H). \]

b) \( N-[\text{S-Acetyl-2'-benzyl-3'-mercaptopropionyl}]-2\text{-fluorophenylglycine methyl ester (Method B)} \)

2-Acetylthiomiethyl-3-phenylpropanoic acid [EP0361365] (0.2 g, 0.84 mmol) was added to a solution of dichloromethane (3 ml) and oxalyl chloride (0.25 ml). A drop of dimethylformamide was introduced and the mixture stirred under argon, permitting the escape of evolved gases. After 1 hour, the solvent was evaporated then coevaporated twice with toluene. A sample of the 2-fluorophenylglycine methyl ester hydrochloride was introduced (0.183 g, 1 mmol) and pyridine (2 ml) added and the mixture was stirred for 30 minutes. The mixture was evaporated, then partitioned between 0.1M aq. HCl (20 ml) and ethyl acetate (3 x 20 ml). The combined, dried (MgSO\(_4\)) organic phase was evaporated and subjected to flash chromatography (hexane - ethyl acetate) to afford the racemic diastereomers of the title compound in 34% yield and approximately equimolar ratio.

\[ \nu_{\text{max}} \ (\text{film}) \ 3424 \ (\text{OH}), \ 3013-2919 \ (\text{CH-Str}), \ 2390 \ (\text{SH}), \ 1725 \ (\text{C=O, Acid}), \ 1672 \ (\text{C=O, Amide}), \ 1513-1419 \ (\text{Ar-Str}) \]

\[ \delta_n (\text{CD,OD}) 2.20, 2.34 \ (3\ H, \ 2s, \ AcS), \ 2.7-3.2 \ (5\ H, \ m, \ CHCHCH\_2), \ 3.63, 3.70 \ (3\ H, \ 2s, \ OMe), \ 5.65 \ (1\ H, \ d, \ HCN), \ 7.0-7.5 \ (9\ H, \ m, \ 2 \times \ Ar). \]

c) \( N-[2'-\text{Benzyl-3'-mercaptopropionyl}]-2\text{-fluorophenylglycine (Method C)} \)

A solution of the \( N-(\text{S-acetyl-2'-benzyl-3'-mercaptopropionyl})-2\text{-fluorophenylglycine methyl ester of Example 19b} \) (0.2 mmol) in methanol (2 ml) was degassed with argon for 20 minutes then treated with a solution of sodium sulphide (1 mmol) in degassed water (2 ml). After 30-90 minutes, 0.25M aq. HCl (20 ml) was introduced and the mixture extracted with ethyl acetate (3 x 20 ml). The dried (MgSO\(_4\)) organic phases were evaporated to give the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

\[ \delta_n (\text{O=C(CD\_2)}) 2.45-3.10 \ (3\ H, \ m, \ CH\_2S, \ CHCO), \ 4.0 \ (2H, vbs, \ CO\_2H, \ SH), \ 5.80, \ 5.75 \ (1H, \ 2d, \ HCN), \ 7.10-7.50 \ (9\ H, \ m, \ Ar-H), \ 7.85, 7.95 \ (1H, \ bd, \ NH). \]

**Example 20: \( N-[2'-\text{Benzyl-3'-mercaptopropionyl}]-2\text{-thienylglycine} \)

\[ \delta (\text{MeOD}) \ 3.85 \ (3\ H, \ s, \ OMe), \ 5.5 \ (1H, s), \ 7.1, 7.28, 7.62 \ (3\ H, \ m, \ Ar-H). \]
b) $N$-[S-Acetyl-2'-benzyl-3'-mercaptopropionyl]-2-thienylglycine methyl ester

Prepared by Method B of Example 19 but using 2-thienylglycine methyl ester hydrochloride and triethylamine (101 mg, 1 mmol). This gave the title compound as a yellow oil in 44% yield, an approximately equimolar mixture of diastereoisomers.

$\delta_H (\text{CDCl}_3)$: 2.30-2.35 (3H, 2s, AcS), 2.65-3.15 (5H, m, CH$_2$CHCH$_2$), 3.71-3.77 (3H, 2d, OMe), 5.75, 5.78 (1H, 2d, HCN), 6.3 (1H, bd, NH), 6.7 (0.5H, d, Ar-H), 6.85-7.40 (7.5H, m, 2 x Ar).

10 c) $N$-[2'-Benzyl-3'-mercaptopropionyl]-2-thienylglycine

Prepared by Method C of Example 19 but using $N$-(S-acetyl-2'-benzyl-3'-mercaptopropionyl)-2-thienylglycine methyl ester. This afforded the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

$v_{max} (\text{film})$: 3412 (OH), 3013-2931 (CH -Str), 2367 (SH), 1737 (C=O, Acid), 1660 (C=O, Amide), 1513-1425 (Ar -Str); $\delta_H (\text{CDCl}_3)$: 2.45-2.60 (1H, bm, CHCO), 2.75-3.10 (2H, m, CH$_2$S), 5.3 (2H, vbs CO$_2$H, SH), 5.78, 5.83 (1H, 2d, HCN), 6.85-7.40 (8H, m, Ar-H), 7.90-7.80 (1H, bm, NH).

Example 21: $N$-[2'-Benzyl-3'-mercaptopropionyl]-$N$-benzyl-phenylglycine

a) $N$-Benzyl-phenylglycine methyl ester hydrochloride


$\delta_H (\text{MeOD})$: 3.8 (3H, s, OMe), 4.1-4.3 (2H, q, CH$_2$Ph), 5.25 (1H, s), 7.2 (1H, m, NH), 7.4-7.6 (10H, m, Ar-H).

b) $N$-[S-Acetyl-2'-benzyl-3'-mercaptopropionyl]-$N$-benzyl-phenylglycine methyl ester

Prepared by Method B of Example 19 but using $N$-benzyl-phenylglycine methyl ester hydrochloride and triethylamine (101 mg, 1 mmol). This gave the title compound as a colourless oil in 11% yield, as an approximately equimolar mixture of diastereoisomers.

$\delta_H (\text{CDCl}_3)$: 2.3, 2.4 (3H, 2s, AcS), 2.8-3.2 (5H, m, CH$_2$CHCH$_2$), 3.68, 3.75 (3H, 2s, OMe), 5.55-5.60 (1H, 2s, HCPH), 7.20-7.4 (15H, m, Ar-H).
c) \[N-[2'-Benzy1-3'-mercaptopropionyl]-N-benzyl-phenylglycine\]  
Prepared by Method C of Example 19 but using \(N-(S\text{-acetyl}-2'-\text{benzy1}-3'\text{-mercaptopropionyl})-N\text{-benzyl-phenylglycine methyl ester}\). This afforded the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.  
\[\nu_{\text{max}}\] (film) 3300 (OH), 3028-2917 (CH-Str), 2652 (SH), 1703 (C=O, Acid), 1597 (C=O, Amide), 1498-1419 (Ar-Str).

Example 22: \(N-[2'-Methyl-3'-mercaptopropionyl]-D\text{-phenylglycine}\)

a) \(N-[S\text{-Acetyl-2'-methyl-3'-mercaptopropionyl}]-D\text{-phenylglycine ethyl ester}\)
Prepared by Method B of Example 19 but using D-phenylglycine ethyl ester hydrochloride (Example 9c) and S-acetyl-2-methyl-3-mercaptopropionic acid (US 4046889). The title compound was obtained as a white crystalline solid in 78% yield, an approximately equimolar mixture of diastereoisomers.  
\[\nu_{\text{max}}\] (film) 3325, 1728, 1691 and 1647; \(\delta_{\text{H}}\) (CDCl\(_3\)) 1.20 (3H, t, CH\(_2\)CH\(_3\)), 2.95, 3.14 (2H, 2dd, CH\(_2\)S), 4.10-4.20 (2H, 2dq, CH\(_2\)C\(_6\)H\(_5\)), 5.54 (1H, d, HCN), 6.69 (1H, bd, NH), 7.30-7.40 (5H, m, Ar-H); \(m/z\) (NH, DCl) 224 (M+H\(^+\), 100%) 341 (M+NH4\(^+\), 60%).

b) \(N-[2'-Methyl-3'-mercaptopropionyl]-D\text{-phenylglycine}\)
Prepared by Method C of Example 19 on \(N-[S\text{-acetyl-2'-methyl-3'-mercaptopropionyl}]-D\text{-phenylglycine ethyl ester}\) (0.15 mmol). This gave the title compound in 91% yield as a waxy solid, an approximately equimolar mixture of diastereoisomers.  
\[\nu_{\text{max}}\] (film) 1510, 1600, 1680, 2950 & 3300 cm\(^{-1}\); \(\delta_{\text{H}}\) (O=C(CD\(_3\))\(_3\)) 1.12, (3H, d, Me), 2.45-2.90 (3H, m, CH\(_2\)S, CHCO), 5.2 (2H, vbs, CO\(_2\)H, SH), 5.55, (1H, d, HCN).  
7.30-7.50 (5H, m, Ar-H), 7.9 (1H, bd, NH).

Example 23: \(N-[4'-Methyl-2-mercaptopentanoyl]-D\text{-phenylglycine}\)

a) S-Acetyl-4-Methyl-2-mercaptopentanoyl (Method A)
A solution of 2-isobutylacrylic acid methyl ester (W.H. Parsons, et al, J. Med. Chem., 1988, 31(9), 1772; prepared by the method of Atta-ur-Rahman, et al, Tetrahedron, 1980, 36, 1063) (30 mmol) in ethanol (100ml) was refluxed with a solution of potassium hydroxide (10g) in water (5 ml) for 5 hours. The mixture was cooled, evaporated to low bulk and extracted with dichloromethane (10ml). The aqueous phase was acidified with 5M HCl (20 ml) then extracted with dichloromethane (3 x 20 ml). These combined organic phases were dried (MgSO\(_4\)), filtered and evaporated.
The residue was dissolved in thiolacetic acid (10 ml) and heated to 80°C for 3 days then cooled to RT and left for a further seven days. The solvent was evaporated and the residue partitioned between dichloromethane (3 x 10 ml) and saturated sodium hydrogen carbonate (500 ml). The aqueous phases was acidified with 5M aq. HCl and extracted with dichloromethane (3 x 50 ml). The combined organic phases were dried (MgSO₄), filtered and evaporated and the residue purified by flash chromatography (EtOAc) on silica gel to the title compound contaminated with 30% of the starting material.

δₐ (CDCl₃) 0.93, 0.97 (6H, d, (CH₃)₂CH), 1.35-1.75 (3H, m, Me₂CHCH₂), 2.35 (3H, s, Ac), 2.70 (1H, dddd, CHCO), 2.98, 3.14 (2H, 2dd, CH₂S).

b) N-[S-Acetyl-4-methyl-2-mercaptomethylpentanoyl]-D-phenylglycine methyl ester (Method B)

A stirred solution of the acid from Example 23a), (1.25 mmol) in dichloromethane (2 ml) at 0°C was treated with 1-hydroxybenzotriazole (1.34 mmol) and N,N'-dicyclohexylcarbodiimide (1.30 mmol). After 2 minutes, D-phenylglycine methyl ester (1.5 mmol) obtained from the hydrochloride of Example 9b by treatment with triethylamine, was added and the mixture allowed to warm to 20°C. After 24 hours the mixture was filtered, 20 ml of dichloromethane added and the filtrate washed successively with 20 ml portions of saturated sodium hydrogen carbonate, water, and aqueous citric acid. The dichloromethane layer was dried (MgSO₄), filtered, and evaporated to afford a gum which was purified by flash chromatography (ethyl acetate - hexane) to give the title compound as a 1:1 mixture of diastereomers.

δₐ (CDCl₃) 0.80-0.95 (6H, m, Me₂C), 1.30-1.70 (3H, m, Me₂CHCH₂), 2.26, 2.33 (3H, 2s, AcS), 2.45-2.55 (1H, m, CHCO), 2.90-3.05 (2H, m, CH₂S), 3.74 (3H, s, MeO), 5.40, 5.42 (1H, 2d, HCN), 6.68-6.90 (6H, m, Ar-H, NH). m/z (CI) 352 (M+H⁺, 100%).

c) N-[4-Methyl-2-mercaptomethylpentanoyl]-D-phenylglycine

Prepared by Method C of Example 19 using N-[S-acetyl-4-methyl-2-mercaptomethylpentanoyl]-D-phenylglycine methyl ester. This gave the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

ν max (film) 1520, 1610, 1680, 2950 & 3300 cm⁻¹; δₐ (O=C(CD₃)₃) 0.75-1.00 (6H, m, Me₂C), 1.20-1.80 (3H, m, Me₂CHCH₂), 2.45-2.95 (3H, m, CH₂S, CHCO), 5.30-5.50 (1H, bs, HCN), 6.75-7.00 (5H, m, Ar-H), 7.75 (1H, bs, NH).
Example 24: \( N-[2'-\text{Benzyl}-3'-\text{mercaptopropanoyl}]\)-4-methylphenylglycine

a) 2-Acetylthiomethyl-3-phenylpropanoic thiol acid

2-Acetylthiomethyl-3-phenylpropanoic acid [EP0361365] (1.185g, 5mmol) and 1,1-carbonyldiimidazole were dissolved in dichloromethane (20ml). \( \text{H}_2\text{S} \) was bubbled through for 1 hour then the solvent evaporated and the residue dissolved in ether (50ml). This solution was evaporated and the product partitioned between ether (3 x 50ml) and 1M HCl (20 ml). The combined organic phases were dried (MgSO\(_4\)), filtered and evaporated to give the title compound in 89% yield as a colourless oil.

\[ \delta_{\text{H}} (\text{CDCl}_3) \ 2.31 \ (3\text{H}, \text{s}, \text{CH},\text{CO}), \ 2.85-3.20 \ (5\text{H}, \text{m}, \text{PhCH}_2\text{CH}_2\text{H}), \ 7.15-7.35 \ (5\text{H}, \text{m Ar-H}); \ m/z (\text{NH}, \text{DCI}) \ 272 \ (\text{M}+\text{NH}_4^+). \]

b) 2-Azido-2-[4-methylphenyl]acetic acid ethyl ester.

Potassium bis(trimethylsilyl)amide (12.32ml, 6.16mmol) was dissolved in anhydrous tetrahydrofuran (62ml) and cooled to -78°C. The 4-methylphenylacetic acid ethyl ester (5 mmol, Aldrich) was dissolved in THF (19ml) then slowly added to the potassium bis(trimethylsilyl)amide solution. After 30 minutes a cooled solution of 2,4,6-trisopropylsulfonyl azide (2.08g, 6.72mmol) in THF (22ml) was slowly added. After a further 2 minutes, glacial acetic acid (1.47ml) was introduced. The mixture was stood at room temperature for 16 hours then the solvents evaporated, and the residue partitioned between dichloromethane (3 x 50ml) and brine (50ml). The dichloromethane layers were combined, dried (MgSO\(_4\)) and the solvent evaporated. Purification was achieved by flash chromatography on silica gel (hexane - ether) to afford the title compound in 30% yield.

\[ \delta_{\text{H}} (\text{CDCl}_3) \ 1.30 \ (3\text{H}, \text{t}, \text{CH},\text{CH}_2), \ 2.36 \ (3\text{H}, \text{s}, \text{Ar-CH}_3), \ 4.10-4.35 \ (2\text{H}, \text{2dq}, \text{CH}_2\text{CH}_3), \ 4.40 \ (1\text{H}, \text{s}, \text{CHN}), \ 7.1-7.3 \ (4\text{H}, \text{abq, Ar-H}); \nu_{\text{max}} (\text{KBr disc}) \ 2105 \ (\text{N}), \ 1742 \ (\text{CO}), \ 1187, 1027 \text{ cm}^{-1}; \ m/z (\text{CT}) \ 237 \ (\text{M}+\text{NH}_4^+ \ 100%). \]

c) \( N-[\text{S-Acetyl-2'-benzyl-3'-mercaptopropanoyl}]\)-4-methylphenylglycine ethyl ester

The 2-Azido-2-(4-methylphenyl)acetic acid ethyl ester (0.45mmol) and 2-acetylthiomethyl-3-phenylpropanoic thiol acid (0.23g, 0.9mmol) were dissolved in toluene (2ml) and the mixture refluxed at 120°C for 60 hours. The solvent was evaporated and the residue washed with brine (5ml). The product was extracted into ethyl acetate (3 x 25ml), evaporated and the product purified by flash chromatography on silica gel eluting with ethyl acetate - hexane to afford the racemic diastereomers of the title compound in 42% yield as an approximately equimolar mixture of isomers.
\[ \delta_{r} (CDCl_{3}) 1.20 (3H, t, CH_{2}CH_{3}), 2.35, 2.37 (3H, 2s, CHS), 2.5-3.2 (5H, m, PhCH_{2}CH_{2}CH_{3}), 4.15 CH_{2}CH_{3}, 5.38, 5.40 (1H, 2d, CHN), 6.25 (1H, bd, NH), 6.90-7.30 (9H, m, Ar-H); m/z (NH, DCI), 413 (M+NH_{4}'). \]

5 d) \( N-[2'-Benzy1-3'-mercapto propionyl]-4-methylphenylglycine \)

Prepared by Method C of Example 19 using \( N-[S-acetyl-2'\text{-}benzyl-3'\text{-}mercapto propionyl]-4\text{-}methylphenylglycine \) ethyl ester. This gave the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.

\[ V_{nu} \text{ (film) 3314 (OH), 2926 (CH-Str), 2585 (SH), 1731 (C=O, Acid), 1651 (C=O, Amide), 1513-1425 (Ar-Str) } \]

Example 25: \( N-[2'-Benzy1-3'-mercapto propionyl]-4\text{-}[1\text{-}methy lisoimidazolyl]glycine \)

15 a) \( 4\text{-}[1\text{-Methylisoimidazolyl}]glycine \) methyl ester hydrochloride

Prepared by Method A of Example 19 but using \( 4\text{-}(1\text{-}methylpyrazolyl)]glycine \), prepared in turn from \( 4\text{-}formyl-1\text{-}methylpyrazole \) (Finar, \textit{et al}, \textit{J. Chem. Soc.}, 1957, 3314) via the hydrantoin using the Strecker synthesis. This gave the title compound as a white solid in quantitative yield.

\[ \delta H (MeOD) 3.8 (3H, s, OMe), 4.1 (3H, s, NMe), 5.4 (1H, s), 7.2 (1H, d, NH), 8.0-8.25 (2H, m, Ar-H). \]

b) \( N-[S\text{-Acetyl-2'}\text{-}benzyl-3'\text{-}mercapto propionyl]-4\text{-}[1\text{-}methy lisoimidazolyl]glycine \) methyl ester

Prepared by Method B of Example 19 but using \( 4\text{-}(1\text{-}methylisoimidazolyl)]glycine \) methyl ester hydrochloride and triethylamine (101 mg, 1 mmol). This gave the title compound as a colourless oil in 33\% yield, an approximately equimolar mixture of diastereoisomers.

\[ \delta H (CDCl_{3}) 2.33, 2.47 (3H, 2s, AcS), 2.60-2.75 (1H, m, CH_{2}CH_{2}CH_{3}), 2.83-3.14 (4H, m, CH_{2}CH_{2}CH_{3}), 3.70, 3.75, 3.81, 3.86 (6H, 3s, OMe, NMe), 5.45-5.52 (1H, m, HCN), 6.15 (1H, bd, NH), 6.83 (0.5H, s, Ar-H 7.10-7.40 (6.5H, m, Ar-H); m/z (NH, DCI) 390 (M+H' 100%). \]

c) \( N-[2'-Benzy1-3'-mercapto propionyl]-4\text{-}[1\text{-}methy lisoimidazolyl]glycine \)

Prepared by Method C of Example 19 but using \( N-[S-acetyl-2'-benzyl-3'\text{-}mercapto propionyl]-4\text{-}[1\text{-}methy lisoimidazolyl]glycine \) methyl ester. This afforded the title compound as a clear oil, an approximately equimolar mixture of diastereoisomers.
\[ \text{Example 26: } N\text{-}[2'\text{-Benzyl-3'}\text{-mercaptotropionyl}]\text{-3-methylphenylglycine} \]

(Isomer A and B)

a) 2-Azido-2-[3-methylphenyl]acetic acid ethyl ester

Prepared by the method of Example 24b) but on 2-(3-methylphenyl)acetic acid ethyl ester (Aldrich). This gave the title compound as a clear oil in 90% yield.

\[ \delta_\text{H}(\text{CDCl}_3) 1.25 (3\text{H, t, } \text{CH}_3\text{CH}_2), 2.38 (3\text{H, s, } \text{Me-Ar}), 4.15-4.32 (2\text{H, 2dq, CH}_3\text{CH}_2), 4.90 (1\text{H, s, CHN}), 7.15-7.33 (4\text{H, m, Ar-H}). \]

b) \(N\text{-}[S\text{-Acetyl-2'\text{-benzyl-3'}\text{-mercaptotropionyl}]\text{-3-methylphenylglycine ethyl ester}\)

A solution of 2-azido-2-(3-methylphenyl)acetic acid ethyl ester (0.5mmol) in ethyl acetate (5ml) with 10% palladium on carbon was hydrogenated for 2.5 hours. The catalyst was filtered off and the solvent evaporated to give 3-methylphenylglycine ethyl ester, \(\delta_\text{H}(\text{CDCl}_3) 1.22 (3\text{H, t, } \text{CH}_3\text{CH}_2), 2.32 (3\text{H, s, } \text{Me-Ar}), 4.12, 4.18 (2\text{H, 2dq, CH}_3\text{CH}_2), 4.53, (1\text{H, s, HCN}), 7.05-7.25 (4\text{H, m, Ar-H}) \) and used immediately in the next stage. 2-Acetylimethyl-3-phenylpropanoic acid [EP0361365] (0.5 mmol) was added to a solution of dichloromethane (2 ml) and oxalyl chloride (0.25 ml). A drop of dimethylformamide was introduced and the mixture stirred under argon, permitting the escape of evolved gases. After 1 hour, the solvent was evaporated then coevaporated twice with dichloromethane. The amino acid was introduced followed by pyridine (2 ml) and triethylamine (0.5 mmol) and the mixture was stirred at RT for 1 hour. The mixture was evaporated then partitioned between 0.1M aq.. HCl (25ml) and ethyl acetate (3 x 25ml). The combined, dried (\(\text{MgSO}_4\)) organic phase was evaporated and subjected to silica gel flash chromatography (hexane - ethyl acetate) to the title compound as a 1:1 mixture of separable diastereomers in 83% yield.

Less polar isomer: \(\delta_\text{H}(\text{CDCl}_3) 1.12 (3\text{H, t, } \text{CH}_3\text{CH}_2), 2.29, 2.31, (6\text{H, s, } \text{MeAr, AcS}), 2.66 (1\text{H, CH}_2\text{CHCH}_2), 2.80-3.13 (4\text{H, CH}_3\text{CHCH}_2), 4.10, 4.20 (2\text{H, 2dq, CH}_3\text{CH}_2), 5.41 (1\text{H, d, HCN}), 6.25 (1\text{H, bd, NH}), 6.70-7.25 (9\text{H, m, Ar-H}) \)

More polar isomer: \(\delta_\text{H}(\text{CDCl}_3) 1.11 (3\text{H, t, } \text{CH}_3\text{CH}_2), 2.29, 2.33, (6\text{H, s, } \text{MeAr, AcS}), 2.70 (1\text{H, CH}_2\text{CHCH}_2), 2.85-3.15 (4\text{H, CH}_3\text{CHCH}_2), 4.05, 4.20 (2\text{H, 2dq, CH}_3\text{CH}_2), 5.37 (1\text{H, d, HCN}), 6.30 (1\text{H, bd, NH}), 6.70-7.25 (9\text{H, m, Ar-H}) \).
c) **N-[2'-Benzy1-3'-mercaptpropionyl]-3-methylphenylglycine**

Prepared by Method C of **Example 19** but using the two diastereomeric **N-[S-acetyl-2'-benzy1-3'-mercaptpropionyl]-3-methylphenylglycine ethyl esters** separately. This procedure afforded the title compound as clear oils, pairs of racemates.

From less polar ester isomer A: $\nu_{\text{max}}$ (film) 3330 (OH), 3923 (CH−Str), 2535 (SH), 1731 (C=O, Acid), 1643 (C=O, Amide), 1530 (Ar−Str).

From more polar ester isomer B: δ$_{\text{n}}$ (DMSO-d$_6$) 2.25, (3H, s, MeAr), 2.35-3.00 (5H, m, CH$_2$CH$_2$CH$_2$), 5.30 (1H, d, HCN), 6.85-7.30 (9H, m, Ar−H), 8.70 (1H, d, NH), 12.5 (1H, bs, CO$_2$H); $\nu_{\text{max}}$ (CHCl$_3$) 3413 (OH), 3019 (CH−Str), 2530 (SH), 1722 (C=O, Acid), 1643 (C=O, Amide), 1496 (Ar−Str).

**Example 27: N-[2'-Isobutyl-3'-mercapto butanoyl]-D-phenylglycine**

a) **S-Acetyl-2'-isobutyl-3'-mercapto butanoic acid**

Prepared by Method A of **Example 23** but using 2-isobutyl-3-methylacrylic acid methyl ester [US 4595700]. This gave the title compound as a yellow oil in 7% yield.

δ$_{\text{n}}$ (CDCl$_3$) 0.82-0.95 (6H, m, (CH$_3$)$_2$CH), 1.35-1.80 (6H, m, Me$_2$CH$_2$CH$\text{C}CH$), 2.35, 2.36 (3H, 3s, Ac), 2.50-2.70 (1H, m, CHCO), 3.50-3.75 (1H, m, CHS).

b) **N-[S-Acetyl-2'-isobutyl-3'-mercapto butanoyl]-D-phenylglycine ethyl ester**

Prepared by Method B of **Example 23** but using S-acetyl-2-isobutyl-3-mercapto butanoic acid and D-phenylglycine ethyl ester, obtained from the hydrochloride of **Example 9c** and triethylamine. This gave the title compound as a yellow oil in 20% yield and as an approximately 4:4:1:1 mixture of diastereomers.

δ$_{\text{n}}$ (CDCl$_3$) 0.78-0.93 (6H, m, Me$_2$C), 1.20-1.80 (9H, m, Me$_2$CH$_2$CH$\text{C}CH$CH$CH$), OCH$_2$CH$_3$), 2.27, 2.29, 2.32 (3H, 3s, 4 x AcS), 2.40-2.60 (1H, m, CHCO), 3.60-3.85 (1H, m, CHS), 4.05-4.25 (3H, m, OCH$_2$CH$_3$), 5.5-5.62 (1H, m, HCN), 6.55, 6.68, 6.72, 6.79 (1H, m, NH), 7.30-7.30 (5H, m, Ar−H).

c) **N-[2'-Isobutyl-3'-mercapto butanoyl]-D-phenylglycine**

Prepared by Method C of **Example 19** but using **N-[S-acetyl-2'-isobutyl-3'-mercapto butanoyl]-D-phenylglycine ethyl ester**. This afforded the title compound as a clear oil, an approximately 1:1:4:4 mixture of diastereoisomers.

δ$_{\text{n}}$ (DMSO-d$_6$) 0.75-0.90 (6H, m, Me$_2$C), 1.10-1.60 (6H, m, Me$_2$CH$_2$CH$\text{C}CH$CH$CH$), 2.25-3.0 (2H, m, CHCO, CHS), 5.30-5.40 (1H, m, HCN), 7.3-7.4 (5H, m, Ar−H), 8.60-8.80 (1H, m, NH), 12.7 (1H, bs, CO$_2$H); $\nu_{\text{max}}$ (CHCl$_3$) 3280 (OH), 3018 (CH−Str), 2530 (SH), 1725 (C=O, Acid), 1640 (C=O, Amide), 1498 (Ar−Str).
Example 28: \(N'-[2'\text{-}Benzyl-3'\text{-} mercaptopropionyl]-N\text{-}methyl\text{-}phenylglycine\) (Isomers A and B)

a) \(N\text{-}Methyl\text{-}phenylglycine\) methyl ester hydrochloride

Prepared by Method A of Example 19 but using \(N\text{-}methyl\text{-}phenylglycine\) (prepared in turn from 2-chlorophenylacetic acid and methylamine) (3.5g) methanol (20 ml) and acetyl chloride (40 ml) over 48 hours. The title compound was obtained in 94% yield, after trituration with methanol, as a white solid.

\[ \delta H (\text{CD}_2\text{OD}) \]: 2.62, (3H, s, MeN), 3.80 (3H, s, MeO), 5.18 (1H, s, HCN), 7.50-7.55 (5H, m, Ar-H).

b) \(N\text{-}[S\text{-Acetyl-2'\text{-}benzyl-3'\text{-} mercaptopropionyl]-N\text{-}methyl\text{-}phenylglycine methyl ester}\)

Prepared by Method B of Example 19 but using \(N\text{-}methyl\text{-}phenylglycine\) methyl ester hydrochloride and additional triethylamine (1 mmol). The title compound was obtained as a 1:1 mixture of diastereomers, both colourless oils, in 42% yield. The diastereomers were separated.

Less polar isomer: \(\delta_H (\text{CDCl}_3)\): 2.28 (3H, s, AcS), 2.54 (3H, s, MeN), 2.80-3.35 (5H, m, CH\text{,CHCH}_2), 3.75 (3H, s, MeO), 6.43 (1H, s, HCN), 7.10-7.38 (10H, m, Ar-H)

More polar isomer: \(\delta_H (\text{CDCl}_3)\): 2.38 (3H, s, AcS), 2.44 (3H, s, MeN), 2.85-3.40 (5H, m, CH\text{,CHCH}_2), 3.78 (3H, s, MeO), 6.36 (1H, s, HCN), 6.80-6.88 (2H, m, Ar-H)

7.18-7.36 (8H, m, Ar-H).

c) \(N\text{-}[2'\text{-}Benzyl-3'\text{-}mercaptodropionyl]-N\text{-}methyl\text{-}phenylglycine\)

Prepared by Method C of Example 19 but using \(N\text{-}[S\text{-acetyl-2'\text{-}benzyl-3'\text{-} mercaptopropionyl]-N\text{-}methyl\text{-}phenylglycine methyl ester}\). The isomers were purified by flash chromatography (CHCl\text{,}- methanol) Each component was obtained as a white foam.

Less polar isomer A: \(\nu_{\text{max}} (\text{CHCl}_3)\): 3410 (OH), 2930 (CH-Str), 2540 (SH), 1738 (C=O, Acid), 1602 (C=O, Amide), 1496 (Ar-Str).

More polar isomer B: \(\nu_{\text{max}} (\text{CHCl}_3)\): 3420 (OH), 2930 (CH-Str), 2530 (SH), 1725 (C=O, Acid), 1601 (C=O, Amide), 1495 (Ar-Str).
Example 29: \( N-[2'-\text{Benzyl}-3'-\text{mercapto}propionyl]-2-(4''-\text{thiazolyl})\text{glycine} \)

**a) Ethyl 2-oxo-2-(4-thiazolyl)acetate**

A solution of amyl nitrite (5.4ml) in tetrahydrofuran (45ml) was added dropwise to a stirred solution of ethyl 2-amino-4-thiazoleglyoxylate (4.00g) (Aldrich) in tetrahydrofuran (25ml) at 60°C. When the addition was complete (1h) the mixture was stirred at 60°C for a further 2h. The solvent was evaporated and the residue partitioned between ethyl acetate and sodium bicarbonate solution. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The product (2.54g) was isolated by column chromatography of the residue using gradient elution (Kieselgel: 2:1 to 1:1 hexane:ethyl acetate).

\[ \text{CHCl}_3 \times \text{cm}^{-1} \] 1737 and 1696. \( \delta (250\text{MHz, CDCl}_3) \) 1.42 (3H, t, \( J 7.11 \)), 4.46 (2H, q, \( J 7.22 \)), 8.84 (1H, d, \( J 1.95 \)), 8.91 (1H, d, \( J 1.98 \)).

**b) Ethyl 2-hydroxyimino-2-(4-thiazolyl)acetate**

Hydroxylamine hydrochloride (1.37g) was added to a stirred solution of ethyl 2-oxo-2-(4-thiazolyl)acetate (2.54g) in ethanol (50ml). The mixture was stirred for 3h, the solvent was evaporated and the residue partitioned between ethyl acetate and sodium bicarbonate solution. The organic phase was washed with brine, dried over magnesium sulphate and evaporated. The residue was recrystallised from ethyl acetate to give the title oxime as a single isomer (1.315g), m.p. 171-175°C.

\[ \text{CHCl}_3 \times \text{cm}^{-1} \] 1736. \( \delta (250\text{MHz, CDCl}_3) \) 1.35 (3H, t, \( J 6.95 \)), 4.39 (2H, q, \( J 7.03 \)), 7.98 (1H, d, \( J 1.97 \)), 9.07 (1H, d, \( J 1.96 \)), 11.05 (1H, s).

**c) \( N-[2'-\text{Benzyl}-3'-\text{acetylthiopropionyl}]-2-(4''-\text{thiazolyl})\text{glycine ethyl ester} \)

A stirred suspension of ethyl 2-hydroxyimino-2-(4-thiazolyl)acetate (400mg) in 50% aqueous formic acid (4ml) was cooled in an ice bath and zinc dust (300mg) was added in small portions over one hour. The mixture was stirred at 0°C for 3-5h and then the solid was filtered off and washed with 50% formic acid. The combined filtrates were evaporated and the residue stirred with water and chloroform. The aqueous phase was neutralised with potassium carbonate and extracted with four portions of chloroform. The combined extracts were washed with brine, dried over magnesium sulphate and evaporated to ca:1ml. The residue was dissolved in dichloromethane (5ml). Meanwhile, oxalyl chloride (0.1ml) was added to a stirred solution of 2-acetyltiomethyl-3-phenylpropanoic acid [EP0361365] (238mg) in dichloromethane (10ml). Dimethylformamide (1 drop) was added and the mixture stirred for 1h. The solvent was evaporated and the residue was triturated with chloroform and evaporated twice. The residue was dissolved in dichloromethane.
(2ml) and added to the amine solution previously prepared. Triethylamine (0.28ml) was added and the mixture stirred for 3h. The mixture was washed successively with citric acid solution, water, sodium bicarbonate solution, water and brine. The solution was dried over magnesium sulphate and evaporated, and the product (304mg) isolated by column chromatography of the residue as a mixture of isomers.

(Kieselgel:1:1 ethyl acetate: hexane). νmax (CHCl3)/cm⁻¹ 3422, 1740, 1682.

δH(250MHz, CDCl3) 1.19 and 1.22 (3H, two t's, J 7.17), 2.26 and 2.35 (3H, two s's), 2.66-3.14 (4H, m), 4.05-4.26 (2H, m), 5.72 and 5.74 (1H, two d's, J 7.37), 6.60 and 6.69 (1H, two d's, J 7.33), 7.01-7.41 (6H, m), 8.67 and 8.77 (1H, two d's, J 2.04), m/z (EI) 406 (M⁺).

d) N-[2'-Benzy1-3'-mercaptopropionyl]-2-(4''-thiazolyl)glycine

Sodium sulphide (491mg) was added to a stirred solution of N-[2'-benzyl-3'-acetylthiopropionyl]-2-(4''-thiazolyl)glycine ethyl ester (227mg) in a mixture of methanol (5ml) and water (5ml) under argon. After stirring for 15min. the mixture was acidified with dilute hydrochloric acid (1ml) and partitioned between ethyl acetate and water. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The product was purified by column chromatography (Kieselgel 20% methanol in chloroform with 0.1% acetic acid). The product was partitioned between ethyl acetate and sodium bicarbonate solution, the aqueous phase was acidified with dilute hydrochloric acid and extracted with ethyl acetate. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated to give the product (59mg) as a mixture of isomers. (Found: M⁺, 336.0598. C₁₅H₁₆N₂O₅S₂ requires 336.0602). νmax (CHCl3)/cm⁻¹ 3418, 3302, 1727 and 1672. δH(250MHz, CDCl3), 1.45 and 1.69 (1H, two t's, J 7.88), 2.49-3.55 (4H, m), 5.86 and 5.87 (1H, two d's, J 7.65), 7.01-7.71 (7H, m), 8.72 and 8.80 (1H, two d's, J 2.00); m/z (EI) 336 (M⁺).

Example 30: N-[2'-Benzy1-3'-mercaptopropionyl]-2-(2''-furanyl)glycine

a) Methyl 2-oxo-2-(2-furanyl)acetate

Methyl iodide (3.34ml) was added to a stirred mixture of potassium carbonate (7.4g) and furan α-oxoacetic acid (5g) (Fluka) in dimethylformamide (70ml). The mixture was stirred for 3 days and then partitioned between ethyl acetate and water. The organic phase was washed three times with water, then brine, dried over magnesium sulphate and evaporated. The product (2.1g) was isolated by column chromatography using gradient elution (Kieselgel:3:1 going to 1:1 hexane:ethyl acetate. δH(250MHz, CDCl3), 3.96 (3H, s), 6.62-6.65 (1H, m), 7.75-7.78 (2H, m).
b) Methyl 2-hydroxyimino-2-(2-furanyl)acetate
Hydroxylamine hydrochloride (0.951g) was added to a stirred solution of methyl 2-oxo-2-(2-furanyl)acetate (2.107g) in methanol (30ml). The mixture was stirred for 3h, then the solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was washed with brine, dried over magnesium sulphate and evaporated. The title oxime was isolated by column chromatography of the residue using gradient elution (Kieselgel: 3:1 to 1:1 hexane:ethyl acetate). (Found: M', 169.0378. C₇H₇NO₂ requires M', 169.0375). v. (CHCl₃/cm⁻¹ 3555, 3278 and 101739. δ(H, 250MHz, CDCl₃), 3.95 (3H, s), 6.57 (1H, dd, J 1.58 and 3.48), 7.45 (1H, d, J 3.22), 7.58 (1H, d, J 2.11), 9.75 (1H, br.s), m/z (EI) 169 (M').

c) N-[2'-Benzy1-3'-acetylthiopropionyl]-2-(2''-furanyl)glycine methyl ester
Zinc dust (300mg) was added in portions to a stirred suspension of methyl 2-hydroxyimino-2-(2-furanyl)acetate (338mg) in methanol (2ml) and 50% aqueous formic acid (4ml) at 0°C. When the addition was complete the mixture was stirred for a further 4h at 0°C. The solid was filtered off and washed with 50% formic acid. The combined filtrates were evaporated and the residue stirred with chloroform and water. The aqueous phase was neutralised with potassium carbonate, the chloroform layer was separated and the aqueous phase extracted with four portions of chloroform. The combined organic extracts were washed with brine, dried over magnesium sulphate and evaporated to about 1ml. The residue was dissolved in dichloromethane (10ml). Meanwhile, oxalyl chloride (0.2ml) was added to a stirred solution of 2-acetylthiomethyl-3-phenyipropanoic acid [EP0361365] (476mg) in dichloromethane (10ml). Dimethylformamide (1 drop) was added and the mixture stirred for 1h. The solvent was evaporated and chloroform evaporated from the residue twice. The residue was dissolved in dichloromethane (2ml) and added to a stirred solution of the amine previously prepared. Triethylamine (0.56ml) was added and the mixture stirred for 2.5h. The solution was washed with citric acid solution, water and brine, dried over magnesium sulphate and evaporated. The product (336mg) was isolated by column chromatography of the residue using gradient elution (Kieselgel: 2:1 to 1:1 hexane:ethyl acetate). v. (CHCl₃/cm⁻¹ 3427, 1749 and 1682. δ(H, 250MHz, CDCl₃), 2.29 and 2.35 (3H, two s's), 2.55-3.19 (5H, m), 3.70 and 3.74 (3H, two s's), 5.66 (1H, t, J 7.62), 6.12-6.35 (3H, m), 7.04-7.35 (6H, m); m/z (Cl) 376 (M + H').

d) N-[2'-Benzy1-3'-mercaptotropionyl]-2-(2''-furanyl)glycine
This compound was prepared from N-[2'-benzy1-3'-acetylthiopropionyl]-2-(2''-furanyl)glycine methyl ester by the method described in Example 29d). (Found: M',
Example 31: \(N\)-(2'-Benzy1-3'-mercaptopropionyl)-2-(2''-benzothienyl)glycine

\[\text{Ethyl 2-hydroxyimino-2-(2-benzo[thi]enyl)acetate}\]

n-Butyllithium (25ml of 1.6N solution in hexanes) was added dropwise to a stirred solution of benzothiophene (5.36g, Lancaster) in tetrahydrofuran (80ml) under argon and cooled to -78°C. When the addition was complete the reaction was stirred at -78°C for 10min and then at room temperature for 20min. The solution was then added via a cannula to a stirred solution of diethyl oxalate (11.68g). cooled to -78°C. The mixture was stirred at -78°C for 0.5h and then at room temperature for 1h. Acetic acid (1.5ml) was added to the mixture which was then partitioned between ethyl acetate and water. The organic phase was washed three times with water, then with sodium bicarbonate solution, water and brine. The solution was dried over magnesium sulphate and evaporated. Column chromatography using gradient elution (Kieselgel:9:1 to 4:1 hexane:ethyl acetate) gave a mixture of ethyl-2-oxo-2-(2-benzo[thi]enyl)acetate and diethyl oxalate. The mixture was dissolved in ethanol (50ml) and hydroxylamine hydrochloride (435mg) was added. The mixture was stirred at room temperature for 3 days, then the solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The isomers of the title oxime were separated by column chromatography of the residue (Kieselgel:4:1 hexane:ethyl acetate). Isomer A: 887mg, \(v_{\text{max}}(\text{CHCl}_3)\) cm\(^{-1}\) 3564, 3309 and 1736. \(\delta_{\text{H}}(250\text{MHz; CDCl}_3)\), 1.46 (3H, t, \(J = 7.08\)), 4.53 (2H, q, \(J = 7.11\)), 7.31-7.42 (2H, m), 7.46 (1H, s), 7.72-7.82 (2H, m), 8.83 (1H, s); \(m/z\) (EI) 249 (M').

Isomer B: 624mg, \(v_{\text{max}}(\text{CHCl}_3)\) cm\(^{-1}\) 3545, 3249, 1731. \(\delta_{\text{H}}(250\text{MHz; CDCl}_3)\), 1.49 (3H, t, \(J = 7.19\)), 4.49 (2H, q, \(J = 7.25\)), 7.35-7.47 (2H, m), 7.86-7.93 (2H, m), 8.40 (1H, s), 10.43 (1H, br.s); \(m/z\) (EI) 249 (M').

\[\text{b) } N-[2'-Benzy1-3'-acetylthiopropionyl]-2-(2''-benzothienyl)glycine ethyl ester}\]

This compound was prepared from ethyl 2-hydroxyimino-2-(2-benzo[thi]enyl) acetate by the method described in Example 30c). (Found: \(M^+ 455.1230\)). \(C_{19}H_{19}NO_4S_2\) requires 455.1225. \(v_{\text{max}}(\text{CHCl}_3)\) cm\(^{-1}\) 3418, 1738 and 1682. \(\delta_{\text{H}}(250\text{MHz, CDCl}_3)\), 1.25 and 1.28 (3H, two t's, \(J = 6.95\)), 2.29 and 2.36 (3H, two s's), 2.70-3.15 (5H, m),
4.08-4.31 (2H, m), 5.79-5.88 (1H, m), 6.37-6.44 (1H, m), 7.07-7.38 (8H, m), 7.65-7.79 (2H, m); m/z (EI) 455 (M').

c) N-[2'-Benzy1-3'-mercaptoacrylonyl]-2-(2''-benzothienyl)glycine

This compound was prepared from N-(2-benzyl-3-acetylpropionyl)-2-(2'-benzothienyl)glycine ethyl ester by the method described in Example 29d), except that the eluent was 10% methanol in chloroform. (Found: M' 385.0811. C_{23}H_{19}NO_S requires 385.0806).

v. (CHCl₃)/cm⁻¹ 3414, 3256, 1714 and 1635. 8H (250MHz, CD₂SOCD₂), 2.20-3.03 (6H, m), 5.49-5.55 (1H, m), 7.17-7.35 (8H, m), 7.67-7.91 (2H, m), 8.64 and 8.72 (1H, two d's, J 7.24).

Example 32: N-[2'-Benzy1-3'-mercaptoacrylonyl]-2-(3''-furanyl)glycine

a) 3-(2-Methylsulphinyl-2-methylthio)acetyl furan

A stirred solution of ethyl 3-furoate (5g) (Aldrich) and methyl methylsulphinylmethylsulphide (4ml) in dimethylformamide (80ml) under argon was cooled in an ice bath and sodium hydride (3.5g of 50% oil dispersion) was added in small portions over 1h. The mixture was stirred at room temperature for 6h then acetic acid (5ml) was added. The mixture was partitioned between ethyl acetate and water, and filtered through Celite. The organic phase was washed four times with water, then brine, dried over magnesium sulphate and evaporated. The product (978mg) was isolated by column chromatography of the residue (Kieselgel:ethyl acetate). (Found: M' 218.0068. C_{10}H_{16}O,S requires 218.0071). ν max (CHCl₃)/cm⁻¹ 1666. δₙ (250MHz, CDCl₃), 2.22 and 2.29 (3H, two s's), 2.63 and 2.84 (3H, two s's), 4.80 and 4.82 (1H, two s's), 6.84 (1H, dd, J 1.86 and 2.88), 7.48 (1H, d, J 1.77), 8.19 and 8.22 (1H, two d's, J 1.09); m/z (EI) 218 (M').

b) Methyl 2-oxo-2-(3-furanyl)acetate

A mixture of 3-(2-methylsulphinyl-2-methylthio)acetyl furan (976mg) and potassium periodate (238mg) in acetic acid (20ml) was heated at 70°C for 45min. The mixture was cooled and the solvent evaporated. The residue was partitioned between ethyl acetate and water, and the organic phase was washed with sodium thiosulphate solution, water and brine, dried over magnesium sulphate and evaporated. The residue was dissolved in methanol (5ml) and added to a stirred solution of sodium (103mg) in methanol (20ml). After 20mins the solution was partitioned between ethyl acetate and dilute hydrochloric acid. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The residue was stirred with potassium carbonate (614mg) and methyl iodide (0.5ml) in dimethylformamide.
(20ml) for 17h. The mixture was partitioned between ethyl acetate and water, the organic phase was washed three times with water, then brine, dried over magnesium sulphate and evaporated. The product (138mg) was isolated by column chromatography of the residue (Kieselgel:3:1 hexane:ethyl acetate).

\[ \nu_{max} (\text{CHCl}_3) / \text{cm}^{-1} \quad 1736, 1683. \]

\[ \delta_{\nu}(250\text{MHz, CDCl}_3), 3.95 (3\text{H}, \text{s}), 6.91 (1\text{H}, \text{dd}, J 0.68 \text{ and } 1.91), 7.48 (1\text{H}, \text{t}, J 1.80), 8.56 (1\text{H}, \text{d}, J1.31). \]

**c) Methyl 2-hydroxyimino-2-(3-furanyl)acetate**

Hydroxylamine hydrochloride (70mg) was added to a stirred solution of methyl 2-oxo-2-(3-furanyl)acetate (138mg) in methanol (5ml). After 6h a further portion of hydroxylamine hydrochloride (60mg) was added and the mixture stirred for 3 days. The solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The product (131mg) was isolated by column chromatography of the residue (Kieselgel:3:2 hexane:ethyl acetate).

\[ \nu_{max} (\text{CHCl}_3) / \text{cm}^{-1} \quad 3263, 1735. \]

\[ \delta_{\nu}(250\text{MHz, CDCl}_3), 3.94 (3\text{H}, \text{s}), 7.15 (1\text{H}, \text{d}, J 1.91), 7.47 (1\text{H}, \text{t}, J 1.68), 8.48 (1\text{H}, \text{d}, J 1.24), 10.26 (1\text{H}, \text{br.s}). \]

**d) N-[2'-Benzyl-3'-acetylthiopropionyl]-2-(3''-furanyl)glycine methyl ester**

This compound was prepared from methyl 2-hydroxyimino-2-(3-furanyl)acetate by the method described in Example 30c), except the eluent used was 3:1 going to 1:1 hexane:ethyl acetate. (Found: \( M^+ 375.1140. \ C_{16}H_{22}NO_4S \) requires 375.1140).

\[ \nu_{max} (\text{CHCl}_3) / \text{cm}^{-1} \quad 3424, 1745, 1682. \]

\[ \delta_{\nu}(250\text{MHz, CDCl}_3), 2.32 and 2.36 (3\text{H}, \text{two s's}), 2.61-3.20 (5\text{H}, \text{m}), 3.70 and 3.75 (3\text{H}, \text{two s's}), 5.46 and 5.48 (1\text{H}, \text{two d's}, J 7.13), 6.06 and 6.32 (1\text{H}, \text{two s's}), 6.13 (1\text{H}, \text{d}, J 7.32), 6.96-7.43 (7\text{H}, \text{m}); \ m/z (\text{EI}) 375 (M^+). \]

**e) N-[2'-Benzyl-3'-mercaptopropionyl]-2-(3''-furanyl)glycine**

This compound was prepared from N-[2'-benzyl-3'-acetylthiopropionyl]-2-(3'':furanyl)glycine methyl ester by the method described in Example 29d), except that the eluent used was 10% methanol in chloroform. (Found: \( M^+ 319.0878. \ C_{16}H_{22}NO_4S \) requires 319.0878).

\[ \nu_{max} (\text{CHCl}_3) / \text{cm}^{-1} \quad 3428, 1725 and 1675. \]

\[ \delta_{\nu}(250\text{MHz, CDCl}_3), 1.49 and 1.70 (1\text{H}, \text{two t's}, J 8.15), 2.50-2.99 (5\text{H}, \text{m}), 5.45 and 5.52 (1\text{H}, \text{two d's}, J 6.97), 6.10 and 6.36 (1\text{H}, \text{two s's}), 6.40 (1\text{H}, \text{s}), 7.09-7.46 (7\text{H}, \text{m}); \ m/z (\text{EI}) 319 (M^+). \]
Example 33: N-[2'-Benzy1-3'-mercaptopropionyl]-2-(1''-naphthyl)glycine

a) Methyl 2-oxo-2-(1-naphthyl)acetate
Methyl 1-naphthyl acetate was prepared from methyl 1-naphthyl acetic acid (Aldrich) and methyl iodide in dimethylformamide in the presence of potassium carbonate, by the method of Example 34a). A mixture of methyl 1-naphthyl acetate (2.00g) and selenium dioxide (1.12g) was heated at 190°C for 1.5h. The mixture was cooled and stirred with ethyl acetate. The solid was filtered off and washed with ethyl acetate. The combined filtrates were washed with sodium bicarbonate solution, water and brine. The solution was dried over magnesium sulphate and evaporated. The product (1.806g) was isolated by column chromatography of the residue (Kieselgel:3:1 hexane:ethyl acetate). (Found: M' 214.0630. C_{11}H_{10}O, requires 214.0630). ν_{max} (CHCl₃) cm⁻¹ 1738, 1680. δ_{n}(250MHz, CDCl₃) 4.02 (3H, s), 7.53-7.74 (3H, m), 7.91-8.00 (2H, m), 8.13 (1H, d, J 8.25), 9.04 (1H, d, J 8.08); m/z (EI) 214 (M').

b) Methyl 2-hydroxyimino-2-(1-naphthyl)acetate
Hydroxylamine hydrochloride (834mg) was added to a stirred solution of methyl 2-oxo-2-(1-naphthyl)acetate (1.72g) in methanol. The mixture was left overnight then the solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The title oxime (828mg) was obtained by recrystallisation of the residue from ethyl acetate/hexane. (Found: M' 229.0743. C_{12}H_{11}NO, requires 229.0739). ν_{max} (CHCl₃) 3311, 1725. δ_{n}(250MHz, CDCl₃), 3.84 (3H, s), 7.43 (1H, dd, J 1.21 and 7.12), 7.46-7.64 (4H, m), 7.89-7.97 (2H, m); m/z (EI) 229 (M').

c) N-[2'-Benzy1-3'-acetylthiopropionyl]-2-(1''-naphthyl)glycine methyl ester
Prepared from methyl 2-hydroxyimino-2-(1-naphthyl)acetate by the procedure described in Example 31k), except that the eluent used was 3:1 to 1:1 hexane:ethyl acetate. δ_{n}(250MHz, CDCl₃), 2.10 and 2.34 (3H, two s's), 2.59-3.22 (5H, m), 3.68 and 3.70 (3H, two s's), 6.23-6.31 (2H, m), 6.90-6.94 (3H, m), 7.14-7.57 (7H, m), 7.86-8.14 (2H, m); m/z (EI) 435 (M').

d) N-[2'-Benzy1-3'-mercaptopropionyl]-2-(1''-naphthyl)glycine
This compound was prepared from N-[2'-benzyl-3'-acetylthiopropionyl]-2-(1''-naphthyl)glycine methyl ester by the procedure described in Example 32e). (Found: M' 379.1248. C_{22}H_{22}NO,S requires 379.1242). ν_{max} (CHCl₃) cm⁻¹ 3428, 3297, 1718, 1651. δ_{n}(250MHz, CD₂SOCD₂), 2.00-2.98 (6H, m), 5.86 and 5.91 (1H, two d's).
Example 34: N-[2'-Benzyl-3'-mercaptopropionyl]-2-(4''-biphenyl)glycine
d
a) Methyl 4-biphenylacetate
Methyl iodide (0.75ml) was added to a stirred mixture of 4-biphenylacetic acid (2.12g) (Lancaster) and potassium carbonate (138g) in dimethylformamide (25ml) and the mixture left overnight. The mixture was partitioned between ethyl acetate and water, the organic phase was washed five times with water, then brine, dried over magnesium sulphate and evaporated. The product (2.11g) was isolated by column chromatography of the residue (Kieselgel:3:1 hexane:ethyl acetate).

\[ \text{v}_{\text{max}} \text{(CHCl}_3)/\text{cm}^{-1} \ 1735 \]
\[ \delta_0(250\text{MHz, CDCl}_3), 3.69 \ (2\text{H, s}), 3.73 \ (3\text{H, s}), 7.32-7.49 \ (5\text{H, m}), 7.55-7.62 \ (4\text{H, m}). \]

b) Methyl 2-oxo-2-(4-biphenyl)acetate
This compound was prepared from methyl 4-biphenyl acetate by the procedure described in Example 33a). (Found: \( M^+ 240.0786. \ C_{16}H_{12}O_2 \) requires 240.0786).

\[ \text{v}_{\text{max}} \text{(CHCl}_3)/\text{cm}^{-1} \ 1738, 1683, 1602. \delta_0(250\text{MHz, CDCl}_3), 4.01 \ (3\text{H, s}), 7.40-7.53 \ (3\text{H, m}), 7.62-7.67 \ (2\text{H, m}), 7.72-7.77 \ (2\text{H, m}), 8.08-8.14 \ (2\text{H, m}); m/z \ (\text{EI}) 240 \ (M^+). \]

c) Methyl 2-hydroxyimino-2-(4-biphenyl)acetate
A solution of methyl 2-oxo-2-(4-biphenyl)acetate (1.22g) and hydroxylamine hydrochloride (706mg) in methanol (20ml) was allowed to stand overnight. The solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The title oxime (800mg) was obtained by recrystallisation of the residue from ethyl acetate. \[ \text{v}_{\text{max}} \text{(nujol)/cm}^{-1} \ 3216 \text{ and 1736.} \delta(250\text{MHz, CD}_2\text{SOCD}_3), 3.78 \ (3\text{H, s}), 7.36-7.54 \ (5\text{H, m}), 7.69-7.74 \ (4\text{H, m}), 12.55 \ (1\text{H, s}). \]

d) \( N-[2'-Benzyl-3'-acetylthiopropionyl]-2-(4''-biphenyl)glycine methyl ester \)
Prepared from methyl 2-hydroxyimino-2-(4-biphenyl)acetate by the procedure described in Example 30c), except that the eluent used was 3:1 to 1:1 hexane:ethyl acetate. (Found: \( M^+ 461.1651. \ C_{24}H_{27}NO_4S \) requires 461.1661).

\[ \text{v}_{\text{max}} \text{(CHCl}_3)/\text{cm}^{-1} \ 3420, 1740 \text{ and 1682.} \delta_0(250\text{MHz, CDCl}_3), 2.30 \text{ and 2.37} \ (3\text{H, two s's}), 2.64-3.15 \ (5\text{H, m}), 3.69 \text{ and 3.73} \ (3\text{H, two s's}), 5.48 \text{ and 5.54} \ (1\text{H, two d's J 7.05}), 6.35-6.40 \ (1\text{H, m}), 6.84-7.59 \ (14\text{H, m}); m/z \ (\text{EI}) 461 \ (M^+). \]
e) N-[2'-Benzyl-3'-mercaptopropionyl]-2-(4''-biphenyl)glycine
This compound was prepared from N-[2'-benzyl-3'-acetylthiopropionyl]-2-(4''-biphenyl)glycine methyl ester by the procedure described in Example 32e).

\[ v_{\text{max}} (\text{CHCl}_3)/\text{cm}^{-1} 3413, 3283, 1653 \text{ and } 1620. \delta (250\,\text{MHz, CDCl}_3) 2.10-3.00 \text{ (6H, m), 5.04 (1H, d, } J 6.55), 7.16-7.63 \text{ (14H, m), 8.09 and 8.24 (1H, two d's, } J 6.5); \]

\[ m/z (\text{Cl}) 423 (M + NH)_3. \]

Example 35: N-[2'-Benzyl-3'-mercaptopropionyl]-2-(4''-isopropylphenyl)glycine

a) Methyl 4-isopropylphenylacetate
Prepared from 4-isopropylphenylacetic acid (Lancaster) by the method described in Example 34a).

\[ v_{\text{max}} (\text{CHCl}_3)/\text{cm}^{-1} 1735. \delta (250\,\text{MHz, CDCl}_3) 1.24 (6H, d, J 6.96), 2.90 (1H, heptet, } J 6.93), 3.60 (2H, s), 3.69 (3H, s), 7.16-7.24 \text{ (5H, m).} \]

b) Methyl 2-oxo-2-(4-isopropylphenyl)acetate
Prepared from methyl 4-isopropylphenylacetate by the method described in Example 33a), except that the eluent used was 4:1 hexane:ethyl acetate and the product contained starting material. \( \delta (250\,\text{MHz, CDCl}_3) 1.28 (6H, d, J 6.87), 2.99 (1H, heptet), 3.98 (3H, s), 7.37 (2H, d, J 8.25), 7.95 (2H, d, J 8.33). \)

c) Methyl 2-hydroxyimino-2-(4-isopropylphenyl)acetate
Hydroxylamine hydrochloride (0.87g) was added to a stirred solution of the previously obtained mixture of methyl 2-oxo-2-(4-isopropylphenyl)acetate and methyl 4-isopropylphenyl acetate (1.29g) in methanol (20ml). When the solid had dissolved the mixture was left overnight. The solvent was evaporated and the residue partitioned between ethyl acetate and water, the organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The product (307mg) was obtained by column chromatography of the residue using gradient elution (Kieselgel 3:1 to 1:1 hexane:ethyl acetate). \( v_{\text{max}} (\text{CHCl}_3)/\text{cm}^{-1} 3568, 3295 \text{ and } 1738. \delta (250\,\text{MHz, CDCl}_3) 1.28 (6H, d, J 6.87), 2.95 (1H, heptet, J 6.92), 3.88 (3H, s), 7.32 (2H, dd, J 1.81 and 6.47), 7.47 (2H, dd, J 1.83 and 6.65), 9.59 (1H, s). \)

d) N-[2'-Benzyl-3'-acetylthiopropionyl]-2-(4''-isopropylphenyl)glycine methyl ester
Prepared from methyl 2-hydroxyimino-2-(4-isopropylphenyl)acetate by the procedure described in Example 30c) except that the eluent used was 3:1 going to 1:1 hexane:ethyl acetate. (Found: \( M^+ 427.1816. \) \( \text{C}_{24}\text{H}_{27}\text{NO}_3\text{S requires 427.1817}. \))
\[ v_{\text{max}} (\text{CHCl}_3)/\text{cm}^{-1} 3422, 1740 \text{ and } 1681. \]
\[ \delta_{\text{n}}(250\text{MHz, CDCl}_3), 1.21-1.25 (6\text{H, m}), \]
\[ 2.28 \text{ and } 2.35 (3\text{H, two s's}), 2.59-3.19 (6\text{H, m}), 3.66 \text{ and } 3.70 (3\text{H, two s's}), 5.41 \text{ and } \]
\[ 5.46 (1\text{H, two d's, } J 6.85), 6.22-6.28 (1\text{H, m}), 6.94-7.33 (9\text{H, m}); m/z (\text{CI}) 428 (\text{M} + \text{H})^+. \]

e) \text{N-[2'-Benzyl-3'-mercaptopropionyl]-2-(4'-isopropylphenyl)glycine}

Prepared from \text{N-[2'-benzyl-3'-acetylthiopropionyl]-2-(4'-isopropylphenyl)glycine methyl ester} by the procedure described in Example 32e). (Found: \text{M}' 371.1549. \text{C}_{11}\text{H}_{18}\text{NO}_2\text{S requires 371.1555}). \]
\[ v_{\text{max}} (\text{CHCl}_3)/\text{cm}^{-1} 3427, 3292, 2562, 1720 \text{ and } 1652. \]
\[ \delta_{\text{n}}(250\text{MHz, CDCl}_3), 1.177 \text{ and } 1.184 (6\text{H, two d's, } J 6.88), 2.05-2.94 (7\text{H, m}), \]
\[ 5.13 \text{ and } 5.19 (1\text{H, two d's, } J 7.17), 7.07-7.33 (9\text{H, m}), 8.41 \text{ and } 8.49 (1\text{H, two d's, } \]
\[ 6.85); m/z (\text{EI}) 371 (\text{M}'). \]

Example 36: \text{N-[2'-Benzyl-3'-mercaptopropionyl]-2-(3''-benzothienyl)glycine}

a) \text{Methyl 2-oxo-2-(3-benzothienyl)acetate}

A mixture of methyl 2-(3-benzothienyl)acetate (2.26g), (J. Chem. Soc., Perkin Trans. 1, 1983, (5), 909-14) and selenium dioxide (1.34g) was heated to 160°C for 2h. The mixture was cooled and triturated with ethyl acetate. The solid was filtered off and washed with ethyl acetate. The combined filtrates were washed with sodium bicarbonate solution, water and brine, dried over magnesium sulphate and evaporated. The product (0.79g) was isolated by column chromatography of the residue (Kieselgel:4:1 hexane:ethyl acetate as eluent). \[ v_{\text{max}} (\text{CHCl}_3)/\text{cm}^{-1} 1736 \text{ and } 1668. \]
\[ \delta_{\text{n}}(250\text{MHz, CDCl}_3) 4.00 (3\text{H, s}), 7.44-7.59 (2\text{H, m}), 7.90 (1\text{H, dd, } J 1.16 \text{ and } 7.70), \]
\[ 8.72 (1\text{H, dd, } J 1.16 \text{ and } 7.51), 8.94 (1\text{H, s}). \]

b) \text{Methyl 2-hydroxyimino-2-(3-benzothienyl)acetate}

Hydroxylamine hydrochloride (503mg) was added to a stirred solution of methyl 2-oxo-2-(3-benzothienyl)acetate (790mg) in methanol (30ml). When all the solid had dissolved the mixture was allowed to stand overnight. The solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The title oxime (622mg) was isolated as a mixture of isomers by column chromatography of the residue (Kieselgel: 3:1 going to 1:1 hexane:ethyl acetate. \[ v_{\text{max}} (\text{CHCl}_3)/\text{cm}^{-1} 3568, 3296, 1737. \]
\[ \delta_{\text{n}}(250\text{MHz, CDCl}_3), 3.90 \text{ and } 4.00 (3\text{H, two s's}), 7.37-7.59 (2\text{H, m}), 7.61 \text{ and } 7.77 (1\text{H, two s's}), 8.30 \text{ and } 9.15 (1\text{H, two broad s's}), 8.43-8.47 (1\text{H, m}); \]
\[ m/z (\text{EI}) 235 (\text{M}'). \]
c) **N-[2'-Benzyl-3'-acetylthiopropionyl]-2-(3''-benzothienyl)glycine methyl ester**

Prepared from methyl 2-hydroxyimino-2-(3-benzothienyl)acetate by the procedure described in Example 30c), except that the eluent used was 3:1 to 1:1 hexane:ethyl acetate. (Found: M+ 441.1072. C27H28NO8S2 requires 441.1069). νmax (CHCl3)/cm−1 3429, 1742, 1681. δv (250MHz, CD3OD), 5.41 and 5.46 (1H, d, J 7.17), 6.96-7.50 (8H, m), 7.88-8.10 (2H, m). m/z (CI) 403 (M+ NH4)+.

**Example 37: N-[(R)- and N-[(S)-2'-Mercaptomethyl-4'-phenylbutanoyl]-D-phenylglycine**

20) **Diethyl 2-phenylethylmalonate**

A solution of diethyl malonate (6.07ml, 40mmol) in dimethyl formamide (60ml) at 0°C was treated portionwise with sodium hydride (1.6g, 40mmol, 60% dispersion in oil) and stirred for 0.25h. 2-Phenylethyl bromide (5.48ml, 40mmol) in dimethylformamide (30ml) was added dropwise. The mixture was warmed to room temperature and stirred for 1h. The mixture was diluted with diethyl ether (4 x 200ml), washed with water (4 x 200ml), saturated brine (100ml), dried (MgSO4) and evaporated. Flash chromatography on silica, eluting with 1% ethyl acetate in hexane, gave the title compound as a colourless oil (7.55g, 71%); νmax (CHCl3) 1726cm−1; δv (CDCl3), 1.27 (6H, t, J 7Hz, 2 x CH3), 2.21 (2H, m, CH2), 2.68 (2H, m, CH2), 3.34 (1H, t, J 7Hz), 4.18 (4H, q, J 7Hz, 2 x OCH2), 7.25 (5H, m, Ph). EI MS M+ 264, DCIMS + 265.

2b) **2-Phenylethylmalonic acid**

A mixture of diethyl 2-phenylethylmalonate (3.61g) and potassium hydroxide (1.69g, 2.5eq) in water (15ml) was refluxed for 2h and cooled to room temperature. The solution was washed with diethyl ether (2 x 10ml) and then acidified to pH 2 (5M hydrochloric acid). The aqueous phase was extracted with diethyl ether (2 x 20ml). The combined extracts were washed with water (2 x 20ml), saturated brine (20ml),
dried (MgSO₄) and evaporated to give the title compound as a white solid (1.85g, 65%); ν_(KBr) 3080(br), 1705 cm⁻¹; δ_(H[(CD₃)₂CO], 2.18 (2H, m, CH₂), 2.70 (2H, m, CH₂), 3.40 (1H, t, J 7.4 Hz, CH), 7.25 (5H, m, Ph). DCIMS M⁺ 226.

c) 2-Acetylthiomethyl-4-phenylbutanoic acid
A mixture of 2-phenylethylmalonic acid (1.8g), 40% aqueous dimethylamine (1.08mL, 1eq) and 37% aqueous formaldehyde (0.64mL, 1eq) in water (10mL) was stirred at room temperature overnight. After cooling at 0°C the solid was filtered off, washed with water and dried. The white solid was heated at 170°C for 10 minutes and cooled to room temperature. The resulting gum was dissolved in ethyl acetate (20mL), washed with 10% potassium hydrogen sulphate solution (10mL), water (2 x 10mL), saturated brine (10mL), dried (MgSO₄) and evaporated to give crude 2-methylene-4-phenylbutanoic acid. δ_H[(CDCl₃) 2.55-2.90 (4H, m, 2 x CH₂), 5.65, 6.85 (2H, 2 x s, 2 x Ph), 7.25 (5H, m, Ph). The solid was dissolved in thioacetic acid (1mL) and heated at 100°C for 1 hour. After evaporation the gum was dissolved in ethyl acetate (10mL) and extracted with saturated sodium hydrogen carbonate solution (2 x 10mL). The combined extracts were washed with ethyl acetate (2 x 10mL) and acidified with 10% potassium hydrogen sulphate solution (pH 3). The aqueous layer was extracted with ethyl acetate (2 x 10mL) and the combined extracts washed with water (2 x 10mL), dried (MgSO₄) and evaporated to yield the title compound as a yellow oil (0.52g, 24%); δ_H[(CDCl₃) 2.00 (2H, m, CH₂), 2.71 (3H, m, CH₃, CH), 3.14 (2H, m, CH₂), 7.24 (5H, m, Ph). EIMS M⁺ 252 DCIMS M⁺ 270.

d) N-[2'-Acetylthiomethyl-4'-phenylbutanoyl]-D-phenylglycine
2-Acetylthiomyethyl-4-phenylbutanoic acid (513mg, 2.03mmol) in dichloromethane (20mL) at room temperature was treated with oxalyl chloride (0.5mL) followed by dimethylformamide (1 drop). After 45 minutes the solution was evaporated and then re-evaporated from toluene (2 x 10mL). The acid chloride was used to acylate (D)-phenylglycine as described in the method of Example 9h). Work-up and careful chromatography on silica, eluting with mixtures of methanol and dichloromethane allowed separation of the two diastereomers of the title compound:
(Isomer A), N-[{(R)-2'-acetylthiomethyl-4'-phenylbutanoyl]-D-phenylglycine (170mg, 22%); δ_H[(CD₃)₂SO] 1.81 (2H, m, CH₂), 2.28 (3H, s, COCH₃), 2.65 (3H, m, CH, CH₂), 3.02 (2H, m, CH₂), 5.32 (1H, d, J 7.0 Hz, α-CH), 7.10-7.50 (10H, m, 2 x Ph).
8.75 (1H, d, J 7.0 Hz, NH), DCIMS MH⁺ 386 MNH⁺ 403.
(Isomer B), N-[{(S)-2'-acetylthiomethyl-4'-phenylbutanoyl]-D-phenylglycine (170mg, 22%); δ_H[(CD₃)₂SO] 1.71 (2H, m, CH₂), 2.32 (3H, s, COCH₃), 2.34-2.76 (3H, m, CH, CH₂).
e) \( N-[(R)-2'-\text{Mercaptomethyl}-4'-\text{phenylbutanoyl}]-\text{D-phenylglycine} \)

\( N-[(R)-2'-\text{Acetylthiomethyl}-4'-\text{phenylbutanoyl}]-\text{D-phenylglycine} \) (150mg) was deacetylated as described in the method of Example 9k) to give the 2'(R)-isomer of the title compound (111mg, 83%); \( \nu \) (\( CH_2Cl_2 \)) 3418, 1654, 1620 and 1497 cm\(^{-1}\); \( \delta \) \((CD_3)SO\) 1.75 (2H, m, CH\(_2\)), 2.10 (1H, bt, SH), 2.60 (5H, m, 2x CH\(_2\), CH), 5.18 (1H, d, \( J 7.1\) Hz, \( \alpha-\text{CH} \)), 7.10-7.50 (10H, m, 2x Ph), 8.31 (1H, d, \( J 7.1\) Hz, NH). Electrospray [M-H]\(^+\) 342.

f) \( N-[(S)-2'-\text{Mercaptomethyl}-4'-\text{phenylbutanoyl}]-\text{D-phenylglycine} \)

\( N-[(S)-2'-\text{Acetylthiomethyl}-4'-\text{phenylbutanoyl}]-\text{D-phenylglycine} \) (140mg) was deacetylated as described in the method of Example 9k) to give the 2'(S)-isomer of the title compound (84mg, 67%); \( \nu \) \((CD_3)SO\) 1.70 (2H, m, CH\(_2\)), 2.25-2.75 (6H, m, 2x CH\(_2\), CH, SH), 5.11 (1H, d, \( J 6.9\) Hz, \( \alpha-\text{CH} \)), 7.0-7.5 (10H, 2x Ph), 8.24 (1H, d, \( J 6.9\) Hz, NH). Electrospray MS [M-H]\(^+\) 342.

Example 38: \( N-[(R)-\text{ and } N-[(S)-2'-\text{Mercaptomethyl}-5'-\text{phenylpentanoyl}]-\text{D-phenylglycine} \)

a) **Diethyl 3-phenylpropylmalonate**

An ice-cold solution of diethyl malonate (5.81ml, 40mmol) in dimethylformamide (60ml) was treated portionwise with sodium hydride (1.6g, 40mmol 60% dispersion in oil). After 0.25h 3-phenylpropylbromide (6.71ml, 44mmol) was added and the reaction stirred overnight at room temperature. Work-up and chromatography as described in Example 37a) gave the title compound as a colourless oil (10.8g, 97%); \( \nu \) \((CH_2Cl_2)\) 2960 (br), 1750 (br) and 1457 cm\(^{-1}\); \( \delta \) \((CDCl_3)\) 1.26 (6H, t, \( J 7Hz \), 2x CH\(_3\)), 1.68 (2H, m, CH\(_2\)), 1.95 (2H, m, CH\(_2\)), 2.65 (2H, t, \( J 7.6Hz \), CH\(_2\)), 4.19 (4H, q, \( J 7Hz \), 2x OCH\(_2\)), 7.23 (5H, m, Ph), EIMS M\(^+\) 278, DCIMS MH\(^+\) 279, MNH\(^+\) 296.

b) **3-Phenylpropylmalonic acid**

A mixture of diethyl 3-phenylpropylmalonate (5.5g) and potassium hydroxide (2.8g, 2.5eq) in ethanol (20ml) and water (30ml) was refluxed for 5 hours. Work-up as described in Example 37b) gave the title compound (4.3g, 98%); \( \nu \) \((CH_2Cl_2)\) 2940 (br), 1727 and 1413 cm\(^{-1}\); \( \delta \) \((CDCl_3)\) 1.73 (2H, m, CH\(_2\)), 2.00 (2H, m, CH\(_2\)), 2.66 (2H, t, \( J 7.5Hz \), CH\(_2\)), 3.44 (1H, t, \( J 6.8Hz \), CH), 6.84 (2H, bs, 2x CO\(_2\)H), 7.26 (5H, m, Ph). EIMS M\(^+\) 222, DCIMS MNH\(^+\) 240.
c) 2-Acetylthiomethyl-5-phenylpentanoic acid
3-Phenylpropylmalonic acid (6.14g) was converted to 2-methylene-5-phenylpentanoic acid (2.1g, 40%) by the method described in Example 37c).

\[ \delta_{(CDCl_3)} 1.88 (2H, m, CH_2), 2.37 (2H, t, J 7.6Hz, CH_2), 6.67 (2H, t, J 7.6Hz, CH_2), 5.68 and 6.33 (2H, 2 x s, =CH), 7.26 (5H, m, Ph). \]

The solid was dissolved in thioacetic acid (5m1) and heated at 100°C for 2 hours. Evaporated to give the title compound (2.9g, 100%); \[ \delta_{(CDCl_3)} 1.71 (4H, m, 2 x CH_2), 2.33 (3H, s, COCH_3), 2.64 (3H, m, CH, CH_2), 3.08 (2H, m, CH_2), 7.27 (5H, m, Ph). \]

10 d) N-[2'-Acetylthiomethyl-5'-phenylpentanoyl]-D-phenylglycine
2-Acetylthiomethyl-5-phenylpentanoic acid (756mg) was converted to the acid chloride as described in Example 37d). This was used to acylate D-phenylglycine as described in the method of Example 9h) to give the two separated diastereomers.

(Isomer A), N-[((R))-2'-Acetylthiomethyl-5'-phenylpentanoyl]-D-phenylglycine (298mg, 31%); \[ \delta_{(CDCl_3)} 1.35-1.72 (4H, m, 2 x CH_2), 2.26 (3H, s, COCH_3), 2.37-3.00 (5H, m, 2 x CH_2, CH), 5.31 (1H, d, J 7.1Hz, \alpha-CH), 7.0-7.5 (10H, m, Ph), 8.74 (1H, d, J 7.1Hz, NH). \]

(Isomer B), N-[((S))-2'-Acetylthiomethyl-5'-phenylpentanoyl]-D-phenylglycine (150mg, 16%); \[ \delta_{(CDCl_3)} 1.41 (4H, m, 2 x CH_2), 2.32 (3H, s, COCH_3), 2.38-3.00 (5H, m, 2 x CH_2, CH), 5.18 (1H, d, J 7.4Hz, \alpha-CH), 7.0-7.5 (10H, m, Ph), 8.48 (1H, d, J 7.4Hz, NH). \]

15 e) N-[((R))-2'-Mercaptomethyl-5'-phenylpentanoyl]-D-phenylglycine
N-[((R))-2'-Acetylthiomethyl-5'-phenylpentanoyl]-D-phenylglycine (280mg) was deacetylated as described in the method of Example 9k) to give the title compound (95mg, 38%). \[ \nu_{max} (CHCl_3) 1662 and 1620cm^{-1}. \]

\[ \delta_{(CDCl_3)} 1.53 (4H, m, 2 x CH_2), 2.04 (1H, bt, J 7.5Hz, SH), 2.51 (5H, m, 2 x CH_2, CH), 5.10 (1H, d, J 7.0Hz, \alpha-CH), 7.03-7.50 (10H, m, Ph), 8.24 (1H, d, J 7.0Hz, NH). \]

10 f) N-[((S))-2'-Mercaptomethyl-5'-phenylpentanoyl]-D-phenylglycine
N-[((S))-2'-Acetylthiomethyl-5'-phenylpentanoyl]-D-phenylglycine (140mg) who deacetylated as described in the method of Example 9k) to give the title compound (62mg, 49%); \[ \delta_{(CDCl_3)} 1.48 (4H, m, 2 x CH_2), 2.25 (1H, br, SH), 2.55 (5H, m, 2 x CH_2, CH), 5.09 (1H, d, J 7.0Hz, \alpha-CH), 7.02-7.48 (10H, m, Ph), 8.28 (1H, d, J 7Hz, NH). \]
Example 39: \( N\)-[2'-Mercaptomethyl-6'-phenylhexanoyl]-D-phenylglycine

a) Diethyl 4-phenylbutylmalonate

4-Phenylbutyl chloride (5.48g) was converted to the title compound (5.8g, 64%) as described in Example 38a), except that the reaction was heated at 70°C for 48 hours.

\[ \begin{align*}
&\text{\( \delta_{\text{H}}(\text{CDCl}_3) \) 1.24 (6H, t, J 7.2Hz, 2 x CH),} \\
&1.37 (2H, m, CH), 1.66 (2H, m, CH), 1.92 (2H, t, J 7.6Hz, CH), \\
&3.31 (1H, t, J 7.5Hz, CH), 4.17 (4H, q, J 7.2Hz, 2 x OCH), 7.24 (5H, m, Ph). \\
\end{align*} \]

b) 4-Phenylbutylmalonic acid

Diethyl 4-phenylbutylmalonate (5.5g) was converted to the title compound (4.2g, 94%) by the method described in Example 37b).

\[ \begin{align*}
&\text{\( \delta_{\text{H}}(\text{CDCl}_3) \) 1.42 (2H, m, CH), 1.68 (2H, m, CH), 1.90 (2H, m, CH), 2.64 (2H, t, J 7.5Hz, CH), 3.38 (1H, t, J 7.4Hz, CH), 7.22 (5H, m, Ph).} \\
\end{align*} \]

c) 2-Acetylthiomethyl-6-phenylhexanoic acid

4-Phenylbutylmalonic acid (4.1g) was converted to 2-methylene-6-phenyl-hexanoic acid (2.0g, 56%) by the method described in Example 37c).

\[ \begin{align*}
&\text{\( \delta_{\text{H}}(\text{CDCl}_3) \) 1.61 (4H, m, 2 x CH), 2.35 (2H, t, J 7.0Hz, CH), 2.65 (2H, t, J 7.2Hz, CH), 5.65, 6.31 (2H, 2 x s, \equiv_{\text{CH}}), 7.25 (5H, m, Ph).} \\
&\text{The solid was converted to the title compound (3.0g, 100%) by the method described in Example 38c).} \\
\end{align*} \]

d) \( N\)-(2'-Acetylthiomethyl-6'-phenylhexanoyl)-D-phenylglycine

2-Acetylthiomethyl-6-phenylhexanoic acid (1.18g) was converted to the acid chloride as described in Example 37d). This was used to acylate D-phenylglycine as described in the method of Example 9h) to give the title compound (670g, 40%).

\[ \begin{align*}
&\text{\( \delta_{\text{H}}([\text{CD}]_2\text{SO}) \) 1.05-1.68 (6H, m, 3 x CH), 2.26, 2.32 (3H, 2 x s, COCH), 2.35-3.05 (5H, m, 2 x CH, CH), 5.32 (0.5H, d, J 7.3Hz, \alpha-CH), 5.38 (0.5H, d, J 7.8Hz, \alpha-CH), 7.05-7.50 (10H, m, Ph), 8.72 (0.5H, d, J 7.3Hz, NH), 8.80 (0.5H, d, J 7.8Hz, NH).} \\
\end{align*} \]

e) \( N\)-(2'-Mercaptomethyl-6'-phenylhexanoyl)-D-phenylglycine

\( N\)-(2'-Acetylthiomethyl-6'-phenylhexanoyl)-D-phenylglycine (660mg) was deacetylated as described in the method of Example 9k) to give the title compound (206mg, 35%).

\[ \begin{align*}
&\text{\( \delta_{\text{H}}([\text{CD}]_2\text{SO}) \) 1.00-1.68 (6H, m, 3 x CH), 2.02 (0.5H, bt, SH), 2.20} \\
\end{align*} \]
(0.5H, bt, SH), 2.35-2.68 (5H, m, 2 x CH₂, CH), 5.38 (0.5H, d, J 7.4Hz, α-CH), 5.39 (0.5H, d, J 7.5Hz, α-CH), 7.10-7.50 (10H, m, Ph), 8.70 (0.5H, d, J 7.5Hz, NH), 8.78 (0.5H, d, J 7.4Hz, NH). Electrospray MS MH⁺ 372.

Example 40: N-[(R)- and N-[(S)-2'-Mercaptomethyl-7'-phenylheptanoyl]-D-phenylglycine

a) Diethyl 5-phenylpentylmalonate
5-Phenylpentyl chloride (4.5g) was converted to the title compound (3.67g, 47%) as described in Example 38a), except that the reaction was heated at 70°C for 20 hours. vₙ (CHCl₃) 2934, 1724 and 1180 cm⁻¹. δₐ(CDCl₃) 1.24 (6H, t, J 7.2Hz, 2 x CH₂), 1.36 (4H, m, 2 x CH₂), 1.62 (2H, m, CH₂), 2.60 (2H, t, J 7.5Hz, CH₂), 3.31 (1H, t, J 7.5Hz, CH), 4.17 (4H, q, J 7.2Hz, 2 x OCH₂), 7.23 (5H, m, Ph). EI-MS M⁺ 306, DCIMS MH⁺ 307.

b) 5-Phenylpentylmalonic acid
Diethyl 5-phenylpentylmalonate (3.56g) was converted to the title compound (2.9g, 100%) by the method described in Example 38b). vₙ (KBr) 3022 (br), 1700 and 1418 cm⁻¹. δₐ(CDCl₃) 1.32 (4H, m, 2 x CH₂), 1.52 (2H, m, CH₂), 1.85 (2H, m, CH₂), 2.50 (2H, t, J 7.4Hz, CH₂), 3.29 (1H, t, J 7.2Hz, CH), 7.12 (5H, m, Ph). EI-MS M⁺ 250, DCIMS MH⁺ 268.

c) 2-Acetylthiomethyl-7-phenylheptanoic acid
5-Phenylpentylmalonic acid (2.6g) was converted to 2-methylene-7-phenylheptanoic acid (1.56g, 70%) by the method described in Example 37c). δₐ(CDCl₃) 1.20-1.75 (6H, m, 3 x CH₂), 2.31 (2H, t, J 7.8Hz, CH₂), 2.62 (2H, t, J 7.5Hz, CH₂), 5.64, 6.28 (2H, 2 x s, CH₂), 7.25 (5H, m, Ph). The solid was converted to the title compound (2.0g, 100%) by the method described in Example 38c). δₐ(CDCl₃) 1.25-1.80 (8H, m, 4 x CH₂), 2.35 (3H, s, COCH₃), 2.5-3.1 (5H, m, 2 x CH₂, CH), 7.23 (5H, m, Ph).

d) N-[(2'-Acetylthiomethyl-7'-phenylheptanoyl)-D-phenylglycine
2-Acetylthiomethyl-7-phenylheptanoic acid (1.27g) was converted to the acid chloride as described in Example 37d). This was used to acylate D-phenylglycine as described in the method of Example 9h) to give the two diastereomers; N-[(R)-2'-acetylthiomethyl-7'-phenylheptanoyl]-D-phenylglycine (420mg, 25%). δₐ[(CD₃)₂SO] 1.05-1.70 (8H, m, 4 x CH₂), 2.25 (3H, s, COCH₃), 2.40-3.03 (5H, m, 2 x CH₂, CH), 5.32 (1H, d, J 7.4Hz, α-H), 7.10-7.50 (10H, m, Ph), 8.71 (1H, d, J 7.4Hz, NH), 12.81 (1H, s, CO₂H), DCIMS MH⁺ 428; N-[(S)-2'-acetylthiomethyl-7'-phenylheptanoyl]-D-
phenylglycine (360mg, 22%). \( \delta_p[(\text{CD}_3)_2\text{SO}] 1.05-1.55 \) (8H, m, 4 x CH\(_3\)), 2.31 (3H, s, COCH\(_3\)), 2.42-2.69 (3H, m, CH\(_2\)CH), 2.92 (2H, m, CH\(_2\)). 5.13 (1H, d, J 7.4Hz, \( \alpha \)-H), 7.23 (10H, m, Ph), 8.33 (1H, d, J 7.4Hz, NH). DCIMS MH\(^+\) 428.

e) \( N'[(R)-2'-\text{Mercaptomethyl-7'}-\text{phenylheptanoyl}]\text{-D-phenylglycine} \)
\( N'[(R)-2'-\text{Acetylthiomethyl-7'}-\text{phenylheptanoyl}]\text{-D-phenylglycine} \) (400mg) was deacetylated as described in the method of Example 9k) to give the title compound (240g, 72%).

f) \( N'[(S)-2'-\text{Mercaptomethyl-7'}-\text{phenylheptanoyl}]\text{-D-phenylglycine} \)
\( N'[(S)-2'-\text{Acetylthiomethyl-7'}-\text{phenylheptanoyl}]\text{-D-phenylglycine} \) (350mg) was deacetylated as described in the method of Example 9k) to give the title compound (164mg, 52%).

Example 41: \( N'[(2'-\text{(Indan-1-yl)-3'}-\text{mercaptopropanoyl}]\text{-D-phenylglycine} \)

a) Diethyl indan-1-ylmalonate
A mixture indan (6.1ml, 50mmol), N-bromosuccinimide (8.9g, 50mmol) and azoiso-butyronitrile (10mg) in carbon tetrachloride was refluxed under strong illumination for 1.25h. Cooled in ice, filtered and evaporated, the crude 1-bromoindan was converted to the title compound (8.1g, 59%) as described in Example 38a).

b) Indan-1-ylmalonic acid
Diethyl indan-1-ylmalonate (7.74g) was converted to the title compound (6.1g), 98%) by the method described in Example 38b).

c) 3-Acetylthio-2-(indan-1-yl)propanoic acid
Indan-1-ylmalonic acid (5.9g) was converted to 2-(indan-1-yl)-acrylic acid (2.9g, 58%) by the method described in Example 37c).
d) **N-[3'-Acetylmethyl-2'(indan-1-yl)propanoyl]-D-phenylglycine**

3-Acetylthio-2-(indan-1-yl)propanoic acid (1.53 g) was converted to the acid chloride as described in **Example 37d**. This was used to acylate D-phenylglycine as described in the method of **Example 9h** to give the title compound (1.1g, 48%).

\[ \delta_{nmr}(CDCl_3) \ 1.81-3.48 \ (9H, m, 3 x CH_2, 3 x CH) \]
\[ 5.37 \ (1H, m, \alpha-H) \]
\[ 6.99-7.48 \ (9H, m, Ar) \]
\[ 8.77 \ (1H, m, NH) \]
\[ \text{EIMS } M^+ 355, \text{ DCIMS } M^+ 356. \]

**Example 42: N-[2'- and N-[(S)-2'-Mercaptomethyl-4'-phenoxybutanoyl]-D-phenylglycine**

a) **Diethyl 2-phenoxyethylmalonate**

β-Bromophenetole (5.81 ml, 40 mmol) was converted to the title compound (10.3 g, 92%) by the method described in **Example 37a**. \[ \delta(CDCl_3) \ 1.26 \ (6H, t, J 7.1Hz, 2 x CH_3) \]
\[ 2.40 \ (2H, m, CH_2CH) \]
\[ 3.68 \ (1H, t, J 7.3Hz, CH) \]
\[ 4.05 \ (2H, t, J 7.1Hz, OCH_2) \]
\[ 5.90, 7.28 \ (5H, 2 x m, Ph). \]

b) **2-Phenoxyethylmalonic acid**

Diethyl 2-phenoxyethylmalonate (1g) was treated with sodium hydroxide (285 mg, 2 eq) in ethanol (10 ml) and water (5 ml). After 16 h the solution was diluted with water (10 ml), washed with diethyl ether (3 x 20 ml), acidified to pH 2 (5 M hydrochloric acid) and extracted with ethyl acetate (2 x 20 ml). The combined extracts were washed with water (3 x 20 ml), saturated brine (20 ml), dried (MgSO_4) and evaporated to yield the title compound (510 mg, 64%). \[ \nu_{max}(KBr) 2915 \ (br). \]
1730 and 1243 cm\(^{-1}\). \(\delta_{\text{H}}[(\text{CD})_2\text{CO}] 2.36 (2\text{H, m, CH}_2\text{H}), 3.69 (1\text{H, t, J 7.2Hz, CH}), 4.12 (2\text{H, t, J 7.3Hz, OCH}_2), 6.94, 7.28 (5\text{H, m, Ph}). \) EIMS \textit{M}^+ 224, DCIMS \textit{M}NH\textsubscript{+} 242.

c) 2-Acetylthiomethyl-4-phenoxybutanoic acid
2-Phenoxyethylmalonic acid (5.4g) was converted to 2-methylene-4-phenoxybutanoic acid (2.8g, 60%) by the method described in Example 37c). \(v_{\text{max}} (\text{CHCl}_3) 2937 (\text{br}), 1695, 1244 \text{cm}^{-1}\). \(\delta_{\text{H}}(\text{CDCl}_3) 2.86 (2\text{H, t, J 6.8Hz, CH}), 4.14 (2\text{H, t, J 6.8Hz, OCH}_2), 6.94, 7.28 (5\text{H, m, Ph}). \) EIMS \textit{M}^+ 192. The solid (2.6g) was converted to the title compound (3.6g, 100%) by the method described in Example 38c).

d) \(N\)\-[2'-Acetylthiomethyl-4'-phenoxybutanoyl]-D-phenylglycine
2-Acetyltiombemethyl-4-phenoxybutanoic acid (1.6g) was converted to the acid chloride as described in Example 37d). This was used to acylate D-phenylglycine as described in the method of Example 9h) to give the title compound (610mg, 25%).\(\delta_{\text{H}}([\text{CD}]_2\text{SO}) 1.89 (2\text{H, m, CH}_2), 2.28, 2.34 (3\text{H, 2 x s, LOCH}_3), 2.73-3.15 (3\text{H, m, CH}, \text{CH}), 3.70-4.15 (2\text{H, m, OCH}_2), 5.21 (1\text{H, d, J 7.1Hz, a-H}), 6.71-7.48 (10\text{H, m, Ph}), 8.77, 8.83 (1\text{H, d, J 7.0, 7.6Hz, NH}), \) DCIMS \textit{MH}^+ 402.

e) \(N\)\-[2'-Mercaptomethyl-4'-phenoxybutanoyl]-D-phenylglycine
\(N\)-[2'-Acetylthiomethyl-4'-phenoxybutanoyl]-D-phenylglycine (600mg) was deacetylated as described in the method of Example 9k) to give a mixture of diastereomers from which \(N\)-[(S)-2'-mercaptomethyl-4-phenoxybutanoyl]-D-phenylglycine (120mg, 22%) was isolated. \(v_{\text{max}} (\text{CHCl}_3) 3415, 2934, 1722, 1675 \text{ and 1498 cm}^{-1}\). \(\delta_{\text{H}}([\text{CD}]_2\text{SO}) 1.92 (2\text{H, m, CH}_2), 2.32 (1\text{H, br, SH}), 2.71 (3\text{H, m, CH}, \text{CH}), 3.72 (2\text{H, m, OCH}_2), 5.21 (1\text{H, d, J 7.1Hz, a-H}), 6.71-7.48 (10\text{H, m, 2 x Ph}), 8.51 (1\text{H, d, J 7.1Hz, NH}), \) EIMS \textit{M}^+ 359 DCIMS \textit{MH}^+ 360.

Example 43: \(N\)-[2'-Benzyl-3'-mercaptopropionyl]-2-(3''-thienyl)glycine

a) \(N\)-[2'-Benzyl-3'-acetylthiopropionyl]-2-(3''-thienyl)glycine methyl ester Oxalyl chloride (0.05ml) was added to a stirred solution of 2-acetylthiomethyl-3-phenylpropanoic acid [EP0361365] (119mg) in dichloromethane (5ml). Dimethylformamide (1 drop) was added and the mixture stirred at room temperature for 0.5h and then at reflux for a further 0.5h. The mixture was cooled and the solvent evaporated and chloroform was evaporated from the residue twice. The residue was
dissolved in dichloromethane (5ml) and added to a stirred solution of 2-(3-thienyl)glycine methyl ester hydrochloride (Ger. Offen. DE 3,528,631: CA 106: P 133819c) (104mg) and triethylamine (0.14ml) in dichloromethane (5ml). The mixture was stirred at room temperature for 2h, then diluted with chloroform and washed successively with citric acid solution, water, sodium bicarbonate solution, water and brine, dried over magnesium sulphate and evaporated. The product (190mg) was isolated by column chromatography of the residue using gradient elution (Kieselgel:3:1 going to 1:1 hexane:ethyl acetate). v_{max} (CHCl_3)/cm\(^{-1}\) 3423, 1742, and 1681. S_{250MHz, CDCl_3} 2.31 and 2.36 (3H, two s's), 2.56 - 3.14 (5H, m), 3.69 and 3.74 (3H, two s's), 5.60 (1H, t, J 6.64), 6.23 (2H, t, J 6.20), 6.74 - 6.77 (1H, m), 6.98 - 7.33 (7H, m).

b) N-[2'-Benzyl-3'-mercaptopropionyl]-2-(3''-thienyl)glycine

Water (2.6ml) was added to a stirred solution of N-[2'-benzyl-3'-acetylthio-propionyl]-2-(3''-thienyl)glycine methyl ester (162mg) in methanol (2.6ml) under argon. Sodium sulphide nonahydrate (400mg) was added and the mixture stirred for 20min. Dilute hydrochloric acid (2ml of 5N) was then added and the mixture partitioned between ethyl acetate and water. The organic phase was washed with water and brine, dried over magnesium sulphate and evaporated. The title product (102mg) was isolated by column chromatography of the residue (Kieselgel:10% methanol in chloroform). v_{max} (CHCl_3)/cm\(^{-1}\) 3411, 3290, 1646 (s), 1619. S_{250MHz, CDCl_3} 2.10 - 2.97 (6H, m), 5.14 and 5.19 (1H, two d's, J 7.51), 6.94 - 6.90 (1H, m), 7.15 - 7.40 (7H, m), 8.10 and 8.25 (1H, two d's, J 7.54); m/z (electrospray) 334 [M-H].

Example 44

N-[2'-(1''-Mercaptoethyl)-4''-phenylbutanoyl]-D-phenylglycine

a) (+/-) Triethyl 4-phenyl-2-phosphonoacetate

A mixture of 2-bromoethylbenzene (5ml, 36mmol), potassium carbonate (8.28g, 60mmol), triethyl phosphonoacetate (6.71ml, 30mmol) and sodium iodide (2.25g, 15mmol) was stirred at 70° for 48h. The reaction was diluted with ethyl acetate (60ml), washed with water (4 x 40ml), saturated brine (40ml), dried (MgSO_4) and evaporated. Flash chromatography on silica eluting with 20% grading to 50% ethyl acetate in hexane gave the title compound (3.3g, 34%); v_{max} (CH_2Cl_2) 1732.5 cm\(^{-1}\).

δ_1 H (CDCl_3) 1.31 (9H, m, 3 x CH_3), 2.10-3.10 (4H, m, 2 x CH_2), 4.18 (6H, m, 2 x OCH_2), 7.27 (5H, m, Ph); m/z (CI) 329 [M+H]⁺
b) Ethyl 2-(2'-phenylethyl)crotonate
A suspension of sodium hydride (0.45g, 60% dispersion in oil) in dry tetrahydrofuran (20ml) at rt was treated dropwise with a solution of Example 44a (2.93g) in dry tetrahydrofuran (20ml). The mixture was stirred 1h and then acetaldehyde (0.75ml) was added. Stirring was continued for 2h, and the mixture was diluted with ethyl acetate (50ml), washed with saturated ammonium chloride solution (20ml), saturated brine (2 x 20ml), dried (MgSO4) and evaporated. Flash chromatography on silica eluting with 2% grading to 10% ethyl acetate in hexane gave the title compound as a 3:1 mixture of isomers, in favour of the trans-crotonate (1.4g, 72%); \( \text{v}\text{max} (\text{CH}_2\text{Cl}_2) 1710\text{cm}^{-1}; \delta_{\text{H}} (\text{CDCl}_3) 1.33 (3\text{H, m, CH}_3), 1.63, 1.97 (3\text{H, 2 x d, J} 7.1\text{Hz}, \text{CH}_3\text{CH}=-), 2.67 (4\text{H, m, 2 x CH}_2), 4.22 (4\text{H, m, OCH}_2), 5.97, 6.89 (1\text{H, 2 x q, J 7.1Hz, CH}=-), 7.25 (5\text{H, m, Ph}); m/z (\text{EI}) 218 (M^+) .

c) 2-(2'-Phenylethyl)crotonic acid
A mixture of Example 44b (1.1g) and sodium hydroxide (0.3g) in ethanol (15ml) and water (15ml) was refluxed overnight and cooled to room temperature. The aqueous phase was washed with ether (3 x 20ml) and acidified to pH2 (5M HCl). The aqueous was then extracted with further portions of ether (2 x 20ml). The combined extracts were washed with water (2 x 20 ml), saturated brine (20ml), dried (MgSO4) and evaporated to yield the title compound (0.87g, 91%); \( \text{v}\text{max} (\text{CH}_2\text{Cl}_2) 1683\text{cm}^{-1}; \delta_{\text{H}} (\text{CDCl}_3) 1.63, 2.04 (3\text{H, 2 x d J} 7.3\text{Hz, CH}_3\text{CH}=-) 2.68 (4\text{H, m, 2 x CH}_2), 6.12, 7.04 (1\text{H, 2 x q, J 7.3Hz, CH}=-), 7.23 (5\text{H, m, Ph}); m/z (\text{EI}) 190 (M^+) .

d) (R,S)-2-((R,S)-1'-Acetylthioethyl)-4'-phenylbutanoic acid
A solution of Example 44c (800mg) in thiolacetic acid was heated at 100°C for 5h and evaporated to give the title compound (1.12g, 100%); \( \delta_{\text{H}} (\text{CDCl}_3) 1.23-2.80 (8\text{H, m, 2 x CH}_2, \text{CH, CH}_3), 2.33-2.55 (3\text{H, m, COCH}_3), 3.88 (1\text{H, m, CH}), 7.23 (5\text{H, m, Ph}); m/z (\text{EI}), 266 (M^+) .

e) N-[2'-(1''-Acetylthioethyl)-4'-phenylbutanoyl]-D-phenylglycine methyl ester
The acid of Example 44d (296mg) was converted to the title compound (150mg, 33%) by Method B of Example 23; \( \delta_{\text{H}} (\text{CDCl}_3) 1.18-1.48 (3\text{H, m, CH}_3), 1.62-2.81 (6\text{H, m, 2 x CH}_2, 2 x \text{CH}), 2.25, 2.28, 2.30, 2.32 (3\text{H, 4 x s, COCH}_3); m/z (\text{EI}), 414 [M + H]^+ .

f) \(N\)-[2'-(1''-Mercaptoethyl)-4'-phenylbutanoyl]-D-phenylglycine

The title compound was prepared from Example 44e (120mg) using Method C of Example 19 (66mg, 64%). The compound was isolated as a 1:1:2.2 mixture of diastereomers; \(\delta^H(\text{CDCl}_3) 1.12-1.40 (3\,\text{H}, \text{m}, \text{CH}_3), 1.68-2.08 (2\,\text{H}, \text{m}, \text{CH}_2), 2.28-3.21 (4\,\text{H}, \text{m}, \text{PhCH}_2), 5.28 (1\,\text{H}, \text{m}, \text{NH}); \quad m/z (\text{EI}) 357 (\text{MH})^+.

Example 45

\(N\)-[3'-(3'',4''-Dihydroxyphenyl)-(R,S)-2'-mercaptomethyl-propanoyl)-D-phenylglycine

a) 3.4-Isopropylidenedioxytoluene

A mixture of 4-methyl catechol (12.4g, 0.1M), 2,2-dimethoxypropane (62ml, 0.5M) and phosphorous pentoxide (100mg) in toluene (250ml) was refluxed under a Soxhlet extractor containing 4A molecular sieves for 4h. Cooled to room temperature and washed with saturated sodium hydrogen carbonate solution (100ml), dried (MgSO4) and evaporated. The oil in hexane was filtered through silica to give the title compound (15.14g, 92%); \(\delta^H(\text{CDCl}_3) 1.66 (6\,\text{H}, \text{s}, 2\times \text{CH}_3), 2.30 (3\,\text{H}, \text{s}, \text{CH}_3), 6.60 (3\,\text{H}, \text{m}, \text{Ar}); \quad m/z (\text{EI}) 164 (\text{M}^+)\).

b) Diethyl 3,4-isopropylidenedioxybenzylmalonate

A solution of Example 45a (8.2g) in carbon tetrachloride (100ml) with N-bromosuccinimide (8.9g) was refluxed under strong illumination for 1h. Cooled in ice, filtered and evaporated to low volume (ca 20ml). The crude bromide was converted to the title compound (10.14g, 63%) by the method described in Example 37a; \(\delta^H(\text{CDCl}_3) 1.15-1.35 (6\,\text{H}, \text{m}, 2\times \text{CH}_3), 1.64, 1.65 (6\,\text{H}, 2\times \text{s}, 2\times \text{CH}_3), 3.10 (2\,\text{H}, \text{d}, J 7.8\text{Hz}, \text{CH}_2), 3.57 (1\,\text{H}, \text{t}, J 7.8\text{Hz}, \text{CH}), 4.08-4.85 (4\,\text{H}, \text{m}, 2\times \text{OCH}_2), 6.60 (3\,\text{H}, \text{m}, \text{Ar}); \quad m/z (\text{ES}^+) 323 (\text{MH}^+)\).

c) 3,4-Isopropylidenedioxybenzylmalonic acid

Example 45b (4.8g) was converted to the title compound (2.4g, 61%) by the method described in Example 37b (2.4g, 61%); \(\delta^H([[\text{CD}_3]_2\text{CO}]) 1.63 (6\,\text{H}, 2\times \text{CH}_3), 3.11 (2\,\text{H}, \text{d}, J7.7\text{Hz}, \text{CH}_2), 3.62 (1\,\text{H}, \text{t}, J7.7\text{Hz}, \text{CH}), 7.21 (3\,\text{H}, \text{m}, \text{Ar}); \quad m/z (\text{ES}^-) 265 (\text{M-H})^-\)

d) 2-Methylene-3'-(3'4'-isopropylidenedioxyphenyl)propanoic acid

Example 45c (2.3g) was converted to the title compound (670mg, 33%) by the method described in Example 37c; \(\delta^H(\text{CDCl}_3) 1.68 (6\,\text{H}, 2\times \text{CH}_3), 3.52 (2\,\text{H}, \text{s}, \text{CH}_2), 5.51, 6.68 (2\,\text{H}, 2\times \text{s}, H_2\text{C} =), 6.62 (3\,\text{H}, \text{m}, \text{Ar}); \quad m/z (\text{ES}^-) 233 (\text{M-H})^-\)
e) *N*-[2-Methylene-3-(3',4'-isopropylidenedioxyphenyl)propanyl]-D-phenylglycine methyl ester

Example 45d (416mg) was converted to the acid chloride as described in the method of Example 37d. The acid chloride in dichloromethane (5ml) was added dropwise to an ice-cold solution of *D*-phenylglycine methyl ester (358mg) and triethylamine (0.6ml) in dichloromethane (5ml), allowed to gain room temperature and stirred for 1h. The mixture was loaded directly onto a silica flash column and eluted with 10% grading to 30% ethyl acetate in hexane to give the title compound (547mg, 81%); δH ((CD3)2CO) 1.63 (6H, 2 x s, 2 x CH3), 3.53 (2H, s, CH2), 3.68 (3H, s, CH3), 5.38, 5.89 (2H, 2 x s, H2C=), 5.56 (1H, d, J 6.2Hz, NH), 6.64 (3H, m, Ar), 7.33 (5H, m, Ph), 7.72 (1H, d, J 6.2Hz, NH); m/z (ES⁺) 382 (MH⁺).

f) *N*-[3-(3',4'-Dihydroxyphenyl)-2-methylene propanoyl]-D-phenylglycine

Example 45e (460mg) in glacial acetic acid (15ml) and water (5ml) was refluxed for 3 h and evaporated. Redissolved in ethyl acetate (20ml), washed with water (2 x 10ml), saturated brine (10ml), dried (MgSO4) and evaporated. Flash chromatography on silica eluting with 35% grading to 40% ethyl acetate in hexane gave the title compound (356mg, 86%); δH (CDCl3) 3.51 (1H, d, J 6.2Hz, NH), 6.15 (1H, bs, OH), 6.52-7.35 (10H, m, Ar, Ph, NH, OH); m/z (ES⁺) 342 (MH⁺).

g) *N*-[R,S)-2-Acetylthiomethyl-3-(3,4-dihydroxyphenyl)propanoyl]-D-phenylglycine methyl ester

A solution of Example 45f (290mg) in thiolacetic acid (2ml) was stood at room temperature for 3 h and evaporated. Flash chromatography on silica eluting 30% grading to 60% ethyl acetate in hexane gave the title compound (280mg, 79%); δH (CDCl3) 2.28, 2.35 (3H, 2 x s, COCH3), 2.60-3.12 (5H, m, 2 x CH2, CH), 3.66, 3.69 (3H, 2 x s, OCH3), 5.45 (1H, m, CH), 6.40-7.40 (1H, m, NH, Ph, Ar, 2 x OH); m/z (ES⁺) 418 (MH⁺).

h) *N*-[R,S)-3-(3,4-Dihydroxyphenyl)-3-mercaptomethyl propanoyl]-D-phenylglycine

Example 45g (245mg) was deprotected as described by Method B of Example 23 to give the title compound (115mg, 54%); δH ((CD3)2SO) 2.35-3.15 (6H, m, 2 x CH2, CH, SH), 5.01 (1H, m, CH), 6.20-7.46 (1H, m, Ph, Ar, NH, 2 x OH).
Example 46

N-[2'-Mercaptomethyl-4'-(4''-hydroxycarbonyl)phenylbutanoyl]-D-phenylglycine

5a) 2-Bromomethylacrylic acid benzhydryl ester

A solution of diphenyldiazomethane in dichloromethane (50 ml) was slowly added to a stirred slurry of 2-bromomethylacrylic acid (Aldrich) (4.95g), in dichloromethane (50ml) at room temperature. After 1 hour, the solvent was evaporated and the residue subjected to chromatography on silica gel, eluting with a mixture of hexane and diethyl ether. This afforded 2-bromomethylacrylic acid benzhydryl ester in 67% yield as a colourless oil; δH (CDCl3) 4.24 (2H, s, CH2Br), 6.02, 6.48 (2H, 2s, =CH2), 7.07 (1H, s, CHPh2), 7.25 - 7.45 (10H, m, Ar-H).

5b) 2-(2'-4''-Methoxycarbonylphenyl)ethyl)acrylic acid benzhydryl ester

A sample of zinc foil (0.125mm thick, 520 mg) in dry THF under an argon atmosphere was treated with chlorotrimethylsilane (15mg) then 1,2-dibromoethane (188mg) and stirred for 15 minutes. The mixture was cooled to between 0 and 5°C and methyl 4-bromomethylbenzoate (Aldrich) (1145mg) introduced and the reaction was maintained at this temperature for two hours. A 2.5ml aliquot of this mixture was then added to a solution of CuCN (180mg) and LiCl (180mg) in THF (2ml) at -70°C. The mixture was warmed to 0°C then evaporated, recooled to -78°C and treated with a solution of Example 46a (662mg) in dichloromethane (5ml). The mixture was warmed to 0°C and sonicated at this temperature for 1 hour, then partitioned between dichloromethane (3 x 20ml) and saturated aqueous ammonium chloride (20ml). The combined organic phases were dried (MgSO4), filtered and evaporated to give an oil which crystallised from hexane at -20°C to give the title compound (590mg). δH (CDCl3) 2.67 (2H, dt, CH2CH2Ar), 2.85 (2H, t, CH2CH2Ar), 3.91 (3H, s, Me), 5.55, (1H, d, =CH, J = 0.9 Hz), 6.31 (1H, s, =CH), 6.96 (1H, s, CHPh2), 7.12 (2H, d, benzoate-H), 7.25 - 7.38 (10H, m, Ar-H), 7.94 (2H, d, benzoate-H).

c) N-[2'-(S-Acetylmercaptomethyl)-4'-(4''-methoxycarbonyl)phenylbutanoyl]-D-phenylglycine methyl ester

A solution of Example 46b (100mg) in thiolacetic acid (1ml) was treated with trifluoroacetic acid (0.5ml). After 16 hours at room temperature, the solvents were evaporated to give a yellow oil. This was dissolved in dichloromethane (2ml) and treated with oxalyl chloride (1ml) and 1/4 drop of DMF. A rapid effervescence occurred and the mixture was stirred at RT for 3 hours then evaporated in vacuo and
coevaporated with dichloromethane. The residue was dissolved in pyridine (5ml) containing a suspension of D-phenylglycine hydrochloride methyl ester (1g) and triethylamine (0.3ml) was introduced. The reaction mixture was stirred at RT for 8 hours then evaporated and partitioned between dichloromethane (3 x 25ml) and aqueous hydrochloric acid (0.1M, 100ml). The combine organic phase was dried (MgSO4), filtered, evaporated and the residue subjected to silica gel chromatography eluting with ethyl acetate and hexane to afford the title compound (104mg) as two separate diasteroisomers.

Less polar isomer (49mg): δH (CDCl3) 1.7 - 2.1 (2H, m), 2.34 (3H, s, MeC=O), 2.4 - 3.2 (5H, m), 3.74 (3H, s, MeO-glycine), 3.89 (3H, s, MeO-benzoate), 5.60, (1H, d, HCN), 6.67 (1H, bd, NH), 7.09 (2H, d, benzoate-H), 7.25-7.38 (5H, m, Ar -H), 7.90 (2H, d, benzoate-H); m/z (CI+ NH3) 475 (M+NH4+ 25%), 458 (M+H+ 100%).

More polar isomer (55mg): δH (CDCl3) 1.7 - 2.1 (2H, m), 2.27 (3H, s, MeC=O), 2.4 - 3.2 (5H, m), 3.75 (3H, s, MeO-glycine), 3.90 (3H, s, MeO-benzoate), 5.55, (1H, d, HCN), 6.48 (1H, bd, NH), 7.31 (2H, d, benzoate-H), 7.35-7.38 (5H, m, Ar -H), 7.97 (2H, d, benzoate-H); m/z (CI+ NH3) 475 (M+NH4+ 60%), 458 (M+H+ 100%).

d) N-[2'-(Mercaptomethyl)-4'-(4'-hydroxycarbonyl)phenylbutanoyl]-D-phenylglycine

Isomer A; A solution of the less polar isomer from Example 46c (40mg) in degassed methanol was added to a solution of sodium sulfide nonahydrate (200mg) in water (2ml). The mixture was stirred for 90 minutes, poured into aqueous hydrochloric acid (0.1M, 30ml) and extracted into ethyl acetate (3 x 20ml). The combined organic phases were dried (MgSO4), filtered and evaporated then freeze dried from 1,4-dioxane to give the title compound (29mg) as a white foam. vmax (KBr disc) 3421, 2954, 1718, 1642 and 1521 cm⁻¹; δH (MeOD) 1.75-1.95 (2H, m), 2.50-2.95 (5H, m), 5.49 (1H, bs, HCN), 7.32 - 7.78 (7H, m, Ar-H), 7.95 (2H, d, benzoate-H); m/z (ESI+ MeCN) 429 (M+MeCN+H+ 100%), 242 (65%).

Isomer B; An identical procedure with the more polar ester isomer from Example 46d (45mg) afforded the corresponding diasteromer of the title compound (33mg) as a white foam. vmax (KBr disc) 3413, 2949, 1717, 1637 and 1529 cm⁻¹; δH (MeOD) 1.75-1.95 (2H, m), 2.50-2.95 (5H, m), 5.52 (1H, bs, HCN), 7.19 (2H, d, benzoate-H), 7.32 - 7.48 (7H, m, Ar-H), 7.88 (2H, d, benzoate-H); m/z (ESI+ MeCN) 429 (M+MeCN+H+ 100%), 277 (15%).
Example 47

**N-[2'-'Mercaptomethyl-4'-(2''-trifluoromethyl-6''-quinolin-6-yl)butanoyl]-D-phenylglycine**

5  a)  2-(2'-(2''-Trifluoromethyl)quinolin-6-yl)ethylacrylic acid benzhydryl ester

Prepared by the method of Example 46b, but utilising 6-bromomethyl-2-trifluoromethylquinoline (1.1g) in place of methyl 4-bromomethylbenzoate. The product was purified by flash chromatography to afford the title compound as a white solid (750mg). δH (CDCl₃) 2.78, 3.02 (4H, 2t, CH₂CH₂), 5.29, 6.32 (2H, 2s, =CH₂), 6.98 (1H, s, CHPh₂), 7.25 - 7.40 (10H, m, Ph-H), 7.55 - 7.70 (3H, m, quinoline-H), 8.10 - 8.24 (2H, m, quinoline-H).

b)  **N-[2'-(S-Acetylimercaptomethyl)-4'-(2''-trifluoromethylquinolin-6''-yl)butanoyl]-D-phenylglycine methyl ester**

A solution of Example 47a (461mg) in thiolacetic acid (0.5ml) was treated with TFA (1ml). After 16 hours, the mixture was warmed to 50°C for 3 days, cooled and evaporated. The residue was washed with saturated aqueous sodium hydrogen carbonate to afford a yellow oil. This was dissolved in dichloromethane (5ml) and treated with oxalyl chloride (5ml) and DMF (1 drop). After 1 hour, the mixture was evaporated, dissolved in pyridine (15ml) containing a suspension of phenylglycine methyl ester hydrochloride (2g), triethylamine (3ml) introduced and the resulting mixture stirred for 12 hours at room temperature. The solvents were evaporated and the residue partitioned between aqueous hydrochloric acid (0.5M, 100ml) and ethyl acetate (3 x 100ml). The combined organic phases were dried (MgSO₄), filtered and evaporated then subjected to flash chromatography (ethyl acetate - hexane) to give the title compound (90mg) as two separate diastereoisomers in approximately 1:1 ratio.

Less polar isomer: vmax (film) 3350, 2940, 1744, 1695, 1657, 1511, 1342, 1179, 1136 and 1081 cm⁻¹; δH (CDCl₃) 1.8 - 2.2 (2H, m), 2.26 (3H, s, MeC=O), 2.32 - 2.45 (1H, m), 2.70 - 3.05 (4H, m), 3.76 (3H, m, MeO), 5.56 (1H, d, HCN), 6.47 (1H, bd, NH), 7.37 (5H, bs, Ph-H), 7.70 - 7.80 (3H, m, quinoline-H), 8.16, 8.32 (2H, 2d, quinoline-H); m/z (ES+) 519 (M+H⁺ 100%).

More polar isomer: vmax (film) 3350, 2940, 1744, 1690, 1500, 1337, 1179, 1130 and 1071 cm⁻¹; δH (CDCl₃) 1.8 - 2.15 (2H, m), 2.34 (3H, s, MeC=O), 2.35 - 2.45 (1H, m), 2.60 - 3.20 (4H, m), 3.74 (3H, m, MeO), 5.61 (1H, d, HCN), 6.68 (1H, bd, NH), 7.20 - 7.50 (5H, m, Ph-H), 7.70 - 8.20 (3H, m, quinoline-H).
7.41 (5H, bs, Ph-H), 7.45 - 7.72 (3H, m, quinoline-H), 8.09, 8.21 (2H, 2d, quinoline-H); m/z (ES+) 519 (M+H+ 100%).

c)  N-[2'-{(Mercaptomethyl)-4'-(2''-trifluoromethylquinolin-6''-yl)butanoyl]-D-phenylglycine.

Isomer A; Prepared by the method of Example 46d, but using the less polar isomer of Example 47b (30mg) as the starting material, the title compound was obtained as a gum.

5H (MeOD) 1.95-2.05 (1H, m), 2.55-2.95 (6H, m), 5.50 (1H, d, HCN), 7.25 - 8.10 (8H, m, Ar-H), 8.37, 8.51 (2H, 2d, quinoline-H).

Isomer B; The more polar isomer from Example 47b was treated likewise and gave a similar gum.

5H (MeOD) 1.95-2.05 (1H, m), 2.55-2.95 (6H, m), 5.40 (1H, d, HCN), 7.25 - 8.10 (8H, m, Ar-H), 8.41, 8.54 (2H, 2d, quinoline-H).

20 Example 48  
N-[3-Mercaptobutanoyl]-D-phenylglycine

a)  N-Crotonyl-D-phenylglycine methyl ester
A solution of D-phenylglycine methyl ester hydrochloride (404mg) in pyridine (10ml) and triethylamine (400mg) was cooled to 0°C and treated to the dropwise addition of crotonyl chloride (202mg) over 1 minute. The resulting orange suspension was stirred for two days at RT then evaporated, dissolved in ethyl acetate (50ml) and washed successively with 1M aqueous hydrochloric acid (1M, 2 x 25 ml), water (10ml) and saturated sodium hydrogen carbonate (10ml). The ethyl acetate layer was dried (MgSO4) filtered and evaporated then purified by silica gel flash chromatography (ethyl acetate - hexane) to give the title compound as a white solid in 42% yield. 5H (CDCl3) 1.85 (3H, dd, MeC), 3.74 (3H, s, MeO), 5.67 (1H, d, HCN), 5.87 (1H, 2d, =CHCO), 6.4 (1H, bd, NH), 6.88 (1H, dq, =CHMe), 7.30 - 7.36 (5H, m, Ar-H).

b)  N-[3-S-Acetylmercaptobutanoyl]-D-phenylglycine methyl ester
A solution of Example 48a in thiolacetic acid (70mg) was maintained at room temperature for 12 hours. The solvent was evaporated and the residue subjected to
flash chromatography (ether - hexane) to obtain the title compound as a clear oil in 94% yield, an approximately 1:1 mixture of diastereoisomers. δH (CDCl3) 1.32 - 1.36 (3H, m, MeCH), 2.26, 2.27 (3H, 2s, MeC=O), 2.40-2.65 (2H, 4dd, CH2), 3.71 (3H, s, MeO), 3.78-3.91 (1H, m, SCH), 5.56, 5.57 (1H, 2d, HCN), 6.65, 6.71 (1H, 2bd, NH) 7.34 (5H, bs, Ar - H).

c) **N-[3-Mercaptobutanoyl]-D-phenylglycine**

Prepared by the method of Example 46d, but using Example 48b (50mg) as the starting material, the title compound (43mg) was obtained as a clear oil, an to an approximately 1:1 mixture of diastereomers. δH (CDCl3) 1.26 - 1.36 (3H, m, MeCH), 1.75, 1.93 (1H, 2d, SH), 2.30 - 2.60 (2H, m, CH2), 3.29-3.48 (1H, m, SCH), 5.56, 5.57 (1H, 2d, HCN), 6.87 (1H, bd, NH) 7.30 -7.40 (5H, bs, Ar-H), 8.75 (1H, bs, CO2H); m/z (ES+) 254 (M+H+ 100%), (ES-) 252 (M-H- 50%), 208 (100%).

**Example 49**

**N-[2-Benzyl-3-mercaptopentanoyl]-D-phenylglycine**

a) **1-Benzyl-1-(tert-butyl oxy carbonyl)methylenetriphenylphosphorane**

A slurry of tert-butyl oxy carbonyl methylenetriphenylphosphonium bromide (4.57g) caesium carbonate (6.5g) and benzyl bromide (1.2m1) in acetonitrile (50 ml) were stirred at room temperature for 18 hours. the mixture was partitioned between dichloromethane (3 x 100ml) and 10% aqueous potassium carbonate (200ml). The combined organic phases were dried (MgSO4), filtered and evaporated. The residue was crystallised from ether - hexane to give the title compound as a yellow solid in 90% yield. δH (CDCl3) 0.98 (9H, s, Me3C), 3.36 (2H, d, CH2), 6.91 - 7.10 (5H, m, Ar-H), 7.36 - 7.56 (15H, m, Ar-H).

b) **2-Benzyl-3-ethylacrylic acid tert-butyl ester**

A solution of Example 49a (920 mg) in 1,2-dichloroethane (5ml) was treated with propionaldehyde (174mg). The mixture was heated to 62°C for 16 hours then cooled, partitioned between brine (25ml) and dichloromethane (3 x 25ml), the organic phases combined, dried (MgSO4), filtered and evaporated and the residue purified by flash chromatography (hexane - ether). The title compound was obtained in 47% yield as a clear oil. δH (CDCl3) 1.06 (3H, m, CH2CH3), 1.38 (9H, s, Me3C), 2.23 (2H, app. quint., CH2CH3), 3.63 (2H, s, CH2Ph), 7.0 - 7.5 (6H, m, Ar-H, =CH).
c) 2-Benzyl-3-ethylacrylic acid
A solution of Example 49b (200mg) in anisole (1ml) and dichloromethane (1ml) was treated with trifluoroacetic acid (2ml). After 2 hours, the solvents were evaporated and the residue subjected to flash chromatography on silica gel (ethyl acetate - hexane - acetic acid) to give the title compound as a white solid (130mg). δH (CDCl₃) 1.09 (3H, m, CH₂CH₃), 2.33 (2H, app.quint., CH₂CH₃), 3.70 (2H, s, CH₂Ph), 7.10 (1H, t, =CH), 7.18 - 7.33 (5H, m, Ar-H).

d) N-[2'-Benzyl-3'-ethylacryloyl]-D-phenylglycine methyl ester
A solution of Example 49c (100mg) in dichloromethane (1ml) was treated with oxalyl chloride (1ml) and 1/4 drop of DMF. The mixture was stirred for 1 hour then evaporated and coevaporated with toluene to give a clear oil. This was dissolved in dichloromethane (3ml) and added dropwise to a stirred suspension of D-phenylglycine methyl ester hydrochloride (200mg) and triethylamine (200mg) in dichloromethane (5ml). The mixture was stirred for 12 hours then partitioned between dichloromethane (3 x 25ml) and 0.5M hydrochloric acid (25ml). The combined organic phases were dried (MgSO₄), filtered and evaporated and the residue subjected to flash chromatography (ethyl acetate - hexane) to give the title compound as a gum in 85% yield. δH (CDCl₃) 1.08 (3H, m, CH₂CH₃), 2.30 (2H, app.quint., CH₂CH₃), 3.69 (3H, s, MeO), 3.72 (2H, d, CH₂Ph), 5.53 (1H, d, HCN), 6.61 (1H, t, =CH), 6.64 (1H, bd, NH), 7.10 - 7.33 (10H, m, Ar-H).

e) N-[3'S-acetylmercapto-2'-benzylpentanoyl]-D-phenylglycine methyl ester
Example 49d (100mg) was dissolved in thiolacetic acid (1ml) and stood at RT for 2 days. The solvent was removed and the residue subjected to flash chromatography (ether - hexane) to afford the title compound as a gum, an approximately 1:1:4:4 mixture of diastereomers. δH (CDCl₃) 0.86 - 1.11 (3H, m), 1.23 - 1.90 (2H, m), 2.34, 2.38 (3H, 2s, MeCS), 2.55-2.97 (4H, m), 3.63, 3.65, 3.70, 3.73 (3H, 4s, MeO), 5.40 - 5.55 (1H, m, HCN), 6.16, 6.31, 6.47, 6.55 (1H, 4bd, NH), 6.95 - 7.33 (10H, m, Ar-H).

f) N-[2'-Benzyl-3'-mercaptopentanoyl]-D-phenylglycine
Prepared by the method of Example 46d, but using Example 49e (45mg) as the starting material, the title compound was obtained as a clear oil, an approximately 1:1:4:4 mixture of diastereomers. vmax 3350, 2965, 1731, 1634, 1519 and 1177 cm⁻¹; m/z (ES+) 358 (M+ 100%), 324 (45%).
Example 50
N-[2'-Benzy1-3'-mercaptobutanoyl]-D-phenylglycine

a) 2-Benzyl-3-methacrylic acid tertbutyl ester
Prepared by the method of Example 49b but using acetaldehyde (88mg) in place of propionaldehyde and using 700mg of the phosphorane, the title compound was obtained as a clear oil (165mg). δH (CDCl3) 1.39 (9H, s, Me3C), 1.86 (3H, d, =CCH3), 3.65 (2H, s, CH2Ph), 6.95 (1H, q, =CH) 7.13 - 7.37 (5H, m, Ar-H).

b) 2-Benzyl-3-methacrylic acid
Prepared by the method of Example 49c but using Example 50a (205mg), the title compound was obtained as a white solid after purification by crystallisation from ether - hexane. δH (CDCl3) 1.94 (3H, d, =CCH3), 3.71 (2H, s, CH2Ph), 6.89 (1H, q, =CH) 7.17 - 7.36 (5H, m, Ar-H).

c) N-[2'-Benzy1-3'-methacryloyl]-D-phenylglycine methyl ester
Prepared by the method of Example 49d but using Example 50b (140mg), the title compound was obtained as a colourless oil in 89% yield. δH (CDCl3) 1.89 (3H, d, =CCH3), 3.68 (3H, s, MeO), 3.74 (2H, d, CH2Ph), 5.54 (1H, d, HCN), 6.67 (1H, bd, NH), 6.74 (1H, q, =CH), 7.11 - 7.41 (10H, m, Ar-H).

d) N-[3'-S-acetylmercapto-2'-benzylbutanoyl]-D-phenylglycine methyl ester
Prepared by the method of Example 49e but using Example 50c (200mg). After chromatography, four separated diastereoisomers of the title compound were obtained, in the ratio 40mg : 40mg : 20mg : 25mg, in ascending order of polarity.

Least polar isomer: δH (CDCl3) 1.25 (2H, d, =CMe), 2.31 (3H, s, MeCS), 2.64 - 3.09 (4H, m), 3.64 (3H, s, MeO), 5.42 (1H, d, HCN), 6.34 (1H, bd, NH), 7.12 - 7.33 (10H, m, Ar-H).

Second least polar: δH (CDCl3) 1.41 (2H, d, =CMe), 2.36 (3H, s, MeCS), 2.54 - 3.09 (4H, m), 3.71 (3H, s, MeO), 5.45 (1H, d, HCN), 6.18 (1H, bd, NH), 6.95 - 7.33 (10H, m, Ar-H).

Third least polar: δH (CDCl3) 1.31 (2H, d, =CMe), 2.38 (3H, s, MeCS), 2.55 - 3.20 (4H, m), 3.71 (3H, s, MeO), 5.43 (1H, d, HCN), 7.44 (1H, bd, NH), 7.20 - 7.33 (10H, m, Ar-H).
Most polar isomer: δH (CDCl₃) 1.47 (2H, d, =CMe), 2.42 (3H, s, MeCS), 2.90 - 3.30 (4H, m), 3.64 (3H, s, MeO), 5.40 (1H, d, HCN), 7.41 (1H, bd, NH), 7.00 - 7.33 (10H, m, Ar-H).

5 e) \textit{N-}[2'-benzyl-3'-mercaptobutanoyl]-D-phenylglycine

Prepared by the method of Example 49f but using diastereomers of Example 50d (20mg). The four diastereomeric isomers of were isolated separately.

Isomer A; From least polar isomer: δH (CDCl₃) 1.27 (2H, d, =CMe), 2.07 (1H, d, SH), 2.44 - 3.25 (4H, m), 5.42 (1H, d, HCN), 6.43 (1H, bd, NH), 7.00 - 7.40 (10H, m, Ar-H); m/z (ES⁺) 344 (MH⁺ 100%), 310 (25%).

Isomer B; From second least polar: δH (CDCl₃) 1.41 (2H, d, =CMe), 2.07 (1H, d, SH), 2.40 - 3.25 (4H, m), 5.43 (1H, d, HCN), 6.36 (1H, bd, NH), 7.00 - 7.35 (10H, m, Ar-H); m/z (ES⁺) 344 (MH⁺ 100%), 310 (85%).

Isomer C; From third least polar: δH (CDCl₃) 1.22 (2H, d, =CMe), 2.10 (1H, bs, SH), 2.35 - 3.45 (4H, m), 5.43 (1H, d, HCN), 7.44 (1H, bd, NH), 7.00 - 7.35 (10H, m, Ar-H); m/z (ES⁺) 344 (MH⁺ 40%), 204 (100%).

Isomer D; From most polar isomer: δH (CDCl₃) 1.25 (2H, d, =CMe), 2.05 (1H, bs, SH), 2.35 - 3.45 (4H, m), 5.51 (1H, d, HCN), 7.41 (1H, bd, NH), 7.00 - 7.35 (10H, m, Ar-H); m/z (ES⁺) 344 (MH⁺ 10%), 204 (100%).

25 Example 51
\textit{N-}[2'-Benzyl-3'-mercapto-4'-methylpentanoyl]-D-phenylglycine

Prepared in an analogous manner to Example 49 but using isobuteraldehyde in place of propionaldehyde.

30 Example 52
\textit{N-}[2'-Benzyl-2'-(1''-mercaptocyclopropyl)acetyl]-D-phenylglycine

Prepared in an analogous manner to \textbf{Example 49} but using 1-trimethylsilyloxy-1-ethoxycyclopropane (Aldrich) and 10 mol% benzoic acid in place of propionaldehyde.
Example 53

*N-[2'-(1"-phenylethyl)-3"-mercapto-4"-methylpentanoyl]-D-phenylglycine*

a) 1-(2'-phenylethyl)-1-(tert-butyloxycarbonylmethylenetriphenylphosphorane)

A solution of tert-butyloxycarbonylmethylenetriphenylphosphorane (1.85g) in THF (10ml) was treated with powdered sodium iodide (151mg) and phenethyl bromide (0.75ml). The mixture was heated to reflux under argon for four hours then cooled and sodium hydride (60% oil dispersion, 200mg) introduced. The mixture was stirred for a further two days at RT then partitioned between saturated aqueous sodium hydrogen carbonate (100ml) and dichloromethane (3 x 100ml). The combined organic phase was dried (MgSO₄) filtered and evaporated. The residue was subjected to flash chromatography to give the title compound in 40% yield as a white solid. 5H (CDCl₃) 0.96 (9H, s, CMe₃), 2.13 (2H, dt, PhCH₂), 2.51 (2H, bt, CH₂C=), 6.84 - 7.68 (20H, m, Ar-H).

b) *N-[2'-(1"-phenylethyl)-3"-mercapto-4"-methylpentanoyl]-D-phenylglycine*

The title compound was prepared by the method of Example 51 but using Example 53a as the starting phosphorane.

Example 54

*N-[2'-(1"-mercaptocyclopropyl)-2'-(1"-phenylethyl)acetyl]-D-phenylglycine*

Prepared by the method of Example 53 but using 1-trimethylsilyloxy-1-ethoxy-cyclopropane (Aldrich) and 10 mol% benzoic acid in place of isobuteraldehyde.

Example 55

*N-[2'-Mercaptomethyl-4'-(4''-difluoromethoxyphenyl)butanoyl]-D-phenylglycine*

Prepared in an analogous manner to Example 46 but using 1-bromomethyl-4-difluoromethoxybenzene in place of methyl 4-bromomethylbenzoate.
Example 56

N-[2'-mercaptomethyl-3'-(3''-methyl-2'',4'',5''-tricarbonylimidazolidin-1''-yl)propanoyl]-D-phenylglycine

5a) 2-(3'-Methyl-2',4',5'-tricarbonylimidazolidin-1'-yl)methylacrylic acid benzhydryl ester.

A solution of 2-bromomethylacrylic acid benzhydryl ester (661mg) in acetonitrile (5ml) was treated with 1-methyl-2,4,5-tricarbonylimidazolidine (300mg) and caesium carbonate (700mg). The resulting mixture was stirred for 3 days at RT then filtered and passed through a silica gel plug, eluting with ether. The title compound was obtained as a white solid in 82% yield.

\[ \delta H (CDCl₃) 3.11 (3H, s, NMe), 4.55 (2H, s, NCH₂), 5.86, 6.57 (2H, 2s, =CH₂), 6.97 (1H, s, CHPh₂), 7.28 - 7.35 (10H, m, Ph-H). \]

5b) N-[2'-mercaptomethyl-3'-(3''-methyl-2'',4'',5''-tricarbonylimidazolidin-1''-yl)propanoyl]-D-phenylglycine

The title compound was prepared by the method described in Example 46 starting at part (c) but using Example 56a as the starting material.

Compounds of examples 2c, 3c and 10b are described in US4513009.

Compounds of examples 1b, 5d, 5f and 8c are described in DE3819539.
BIOLOGICAL ACTIVITY

Iₜₐ screen

The inhibitory activity of the compounds of the invention was measured in 25mM PIPES pH 7 buffer at 10 concentrations (1000, 333, 111, 37, 12.3, 4.1, 1.4, 0.46, 0.15 and 0.05µM) at 37°C using nitrocefin (91µM final concentration) as the reporter substrate. The assays were performed with a 5 minute preincubation of enzyme and inhibitor and were conducted in the presence of added zinc sulphate (Zn²⁺ 100µM, final concentration). The methodology is described in detail in the following references: Payne et al (1991), J. Antimicrob. Chemother., 28:255; Payne et al (1994), Antimicrob. Agents and Chemother., 38:767.

Results

Compounds of the Examples exhibit Iₜₐ values against B. fragilis CfiA metallo-β-lactamase of <1000µM. The Iₜₐ values for Examples 5d), 5e), 9e), 9i), 9k), 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25, 27, 30, 31, 32, 37f), 38f), 39, 40f), 41, 42, 43, 44, 46 (Isomer A), 47 (Isomer B), 50 (Isomers B and C) and 55 were <1µM.

All compounds of the above Examples exhibited significant inhibition of the Stenotrophomonas maltophilia L-1 (formerly Xanthomonas maltophilia L-1) and Bacillus cereus II metallo-β-lactamases, with Iₜₐ values in the range 0.08-1000µM.
Antibacterial activity of compounds of the invention in combination with the carbapenem antibiotic, meropenem, against the *Bacteroides fragilis* 262 strain, which produces CfiA metallo-β-lactamase:

\[ \text{MIC} = \text{minimum inhibitory concentration (µg/ml)} \]

Antibacterial activity of meropenem was potentiated as follows:

MIC (µg/ml) of meropenem alone: >128

<table>
<thead>
<tr>
<th>Inhibitor compound</th>
<th>MIC (µg/ml) of compound alone</th>
<th>MIC (µg/ml) of meropenem in the presence of 8µg/ml of compound</th>
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<tr>
<td>E 9k)</td>
<td>&gt;128</td>
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<tr>
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<td>E 55</td>
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CLAIMS

1. A method of treatment of bacterial infections in humans or animals which comprises administering, in combination with a β-lactam antibiotic, a therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof:

\[ R_4S-(R_5R_6)-CH(R_3)-CON(R_2)-CH(R_1)-CO_2R \]

(I)

wherein:
- R is hydrogen, a salt forming cation or an in vivo hydrolysable ester-forming group;
- R1 is hydrogen, (C1-6)alkyl optionally substituted by up to three halogen atoms or by a mercapto, (C1-6)alkoxy, hydroxy, amino, nitro, carboxy, (C1-6)alkylcarboxyloxy, (C1-6)alkoxycarbonyl, formyl or (C1-6)alkylcarboxyl group, (C3-7)cycloalkyl, (C3-7)cycloalkylalkylyl, (C2-6)alkylamino, (C2-6)alkynyl, aryl, aryl(C1-6)alkyl, heterocyclyl or heterocyclylalkyl;
- R2 is hydrogen, (C1-6)alkyl or aryl(C1-6)alkyl;
- R3 is hydrogen, (C1-6)alkyl optionally substituted by up to three halogen atoms, (C3-7)cycloalkyl, fused aryl(C3-7)cycloalkyl, (C3-7)cycloalkylalkylyl, (C2-6)alkenyl, (C2-6)alkynyl, aryl, aryl-(CHR10)m-X-(CHR11)n, heterocyclyl or heterocyclylalkyl-(CHR10)m-X-(CHR11)n, where m is 0 to 3, n is 1 to 3 to 3, each R10 and R11 is independently hydrogen or (C1-6)alkyl and X is O, S(O)x where x is 0-2, or a bond;
- R4 is hydrogen, or an in vivo hydrolysable acyl group; and
- R5 and R6 are independently hydrogen and (C1-6)alkyl or together represent (CH2)p where p is 2 to 5.

2. A compound of formula (IA) which is a compound of formula (I) as defined in claim 1 wherein R1 is aryl or heterocyclyl and R3 is aryl-(CHR10)m-X-(CHR11)n.

3. A compound of formula (IB) which is a compound of formula (I) as defined in claim 1 wherein R5 and R6 are not hydrogen.

4. A compound of formula (IC) which is a compound of formula (I) as defined in claim 1 wherein the stereochemistry at the carbon marked * is D-.

5. A method according to claim 1 wherein R1 is selected from methyl, isobutyl, carboxymethyl, mercaptomethyl, 1-hydroxyethyl, optionally substituted benzyl, phenyl optionally substituted with up to five, preferably up to three, groups selected from halogen, mercapto, (C1-6) alkyl optionally substituted by 1-3 halo, phenyl, (C1-
6) alkoxy optionally substituted by 1-3 halo, hydroxy(C1-6)alkyl, mercapto(C1-6)alkyl, hydroxy, amino, nitro, carboxy, (C1-6) alkylcarbonyloxy, (C1-6)alkoxycarbonyl, formyl or (C1-6) alkylcarbonyl groups, indolyl, thiienyl, isoimidazolyl, thiazolyl, furyl and benzothienyl.

6. A method according to claim 5 wherein R1 is unsubstituted phenyl.
7. A method according to claim 1 or any claim dependent thereon wherein R2 is selected from hydrogen, methyl and benzyl.
8. A method according to claim 7 wherein R2 is hydrogen.
9. A method according to claim 1 or any claim dependent thereon wherein R3 is ary1-(CH2)m-X-(CH2)n.
10. A method according to claim 1 or any claim dependent thereon wherein R4 is hydrogen.
11. A method according to claim 1 or any claim dependent thereon wherein R5 and R6 are independently hydrogen or methyl.
12. A method according to claim 1 or any claim dependent thereon wherein X is O, S or a bond and R10 and R11 are each hydrogen.
13. A method according to claim 1 or any claim dependent thereon wherein the stereochemistry at the carbon atom marked * is D- and the stereochemistry at the carbon atom marked (+) is S.
14. A method according to claim 1 wherein the compound of formula (I) is selected from:
   N-[2'-Benzy1-3'-mercaptopropionyl]phenylalanine;
   N-[2'-Benzy1-3'-mercaptopropionyl]leucine;
   N-[2'-Benzy1-3'-mercaptopropionyl]alanine;
   N-[2'-Benzy1-3'-mercaptopropionyl]tryptophan;
   N-[2'-Benzy1-3'-mercaptopropionyl]-L-tryptophan;
   N-[2'-Benzy1-3'-mercaptopropionyl]tyrosine;
   N-[(R)-2'-Benzy1-3'-mercaptopropionyl]glycine; and
   N-[(5)-2'-Benzy1-3'-mercaptopropionyl]glycine;
   or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof.

15. N-[2'-Benzy1-3'-mercaptopropionyl]aspartic acid.
18. N-[2'-Benzy1-3'-mercaptopropionyl)cysteine.
20. N-[2'-Benzy1-3'-mercaptopropionyl]-D-phenylglycine.
21. [2'S]-N-[2'-Benzy1-3'-mercaptopropionyl]-D-phenylglycine.
<table>
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<th>No.</th>
<th>Name</th>
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<td>N-[(R)-2'-Mercaptomethyl-7'-phenylheptanoyl]-D-phenylglycine.</td>
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58. N-[2'-Benzyl-3'-mercaptopropionyl]-2-(3'-thienyl)glycine.
59. N-[2'-[(1'-Mercaptoethyl)-4'-phenylbutanoyl]-D-phenylglycine.

61. N-[2'-Mercaptomethyl-4'-(4'-hydroxycarbonyl)phenylbutanoyl]-D-phenylglycine.
62. N-[2'-Mercaptomethyl-4'-(2'-trifluoromethyl-6'-quinolin-6-yl)butanoyl]-D-phenylglycine.
63. N-[3-Mercaptobutanoyl]-D-phenylglycine.
64. N-[2'-Benzy1-3-mercaptobutanoyl]-D-phenylglycine.
65. N-[2'-Benzy1-3'-mercaptobutanoyl]-D-phenylglycine.
66. N-[2'-Benzy1-3'-mercapto-4'-methylpentanoyl]-D-phenylglycine.
67. N-[2'-Benzy1-2'-((1'-mercaptocyclopropyl)acetyl)]-D-phenylglycine.
68. N-[2'-(1'-phenylethyl)-3'-mercapto-4'-methylpentanoyl]-D-phenylglycine.
69. N-[2'-(1'-mercaptocyclopropyl)-2'-(1'-phenylethyl)acetyl]-D-phenylglycine.
70. N-[2'-Mercaptomethyl-4'-(4' -difluoromethoxyphenyl)butanoyl]-D-phenylglycine.
71. N-[2'-mercaptopentanoyl]-D-phenylglycine.
72. A pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester of a compound according to any one of claims 15 to 71.
73. A process for the preparation of a compound of formula (IA) as defined in claim 2, formula (IB) as defined in claim 3 or formula (IC) as defined in claim 4, which comprises reacting a compound of formula (II)

\[ Y - C(R_5 R_6') - CR_7(R_3') - CO - W \]

with a compound of formula (III)

\[ X^1 - CH(R_1') - CO_2R_x \]

wherein W is a leaving group, Y is Y' where Y' is R_4'S or a group convertible thereto and R_7 is H, or Y and R_7 together form a bond, R_x is R or a carboxylate protecting group, X^1 is N_3 or NHR_2' and R_1', R_2', R_3', R_4', R_5' and R_6' are R_1, R_2, R_3, R_4, R_5 and R_6 or groups convertible thereto, wherein R, R_1, R_2, R_3, R_4, R_5 and R_6 are as defined in formula (IA), (IB) or (IC), and thereafter, where Y and R_7 form a bond, reacting the product with a nucleophilic sulphur reagent Y'H, where necessary, converting Y' into R_4'S, R_x, R_1', R_2', R_3', R_4', R_5' and/or R_6' into R, R_1.
R$_2$, R$_3$, R$_4$, R$_5$ and/or R$_6$ and optionally inter-converting R, R$_1$, R$_2$, R$_3$, R$_4$, R$_5$ and/or R$_6$.

74. A pharmaceutical composition comprising a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, as defined in claim 1, together with a ß-lactam antibiotic in a synergistically effective amount and a pharmaceutically acceptable carrier.

75. A pharmaceutical composition comprising a compound according to claim 2, 3, 4 or 15 to 72 and a pharmaceutically acceptable carrier.

76. A pharmaceutical composition according to claim 75 which additionally comprises a ß-lactam antibiotic in a synergistically effective amount.

77. A composition according to claim 74 or 76 wherein the ß-lactam antibiotic is a carbapenem selected from imipenem, meropenem, biapenem, BMS181139 ([4R-[4alpha,5beta,6beta(R*)]]-4-[2-[(aminoiminomethyl)amino]ethyl]-3-[(2-cyanomethyl)thio]-6-(1-hydroxyethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid), BO2727 ([4R-3[3S*,5S*(R*)],4alpha,5beta,6beta(R*)]]-6-(1-hydroxyethyl) -3-[[5-[1-hydroxy-3-(methylamino)propyl]-3-pyrrolidinyl]thio]-4-methyl-7-oxo-1-azabicyclo[3.2.0] hept-2-ene-2-carboxylic acid monohydrochloride), ER35786 ((1R, 5S, 6S)-6-[1(R)-Hydroxymethyl]-2-[2(S)-[1(R)-hydroxy-1-[pyrrolidin-3(R)-yl]methyl]pyrrolidin-4(S)-ylsulfanyl]-1-methyl-1-carba-2-penem-3-carboxylic acid hydrochloride) and S4661 ((1R,5S,6S)-2-[3(S,5S)-5-(sulfamoylaminomethyl)pyrrolidin-3-yl]thio-6-[(1R)-1-hydroxyethyl]-1-methylcarbapen-2-em-3-carboxylic acid).

78. A compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, as defined in claim 1 for use in the treatment of bacterial infections.

79. The use of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, as defined in claim 1 in the manufacture of a medicament for the treatment of bacterial infections.
A. CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbol)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO 95 33495 A (IMMUNOMEDICS INC) 14 December 1995 see page 27, line 20 - line 21</td>
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<td>CHEMICAL ABSTRACTS, vol. 120, no. 17, 25 April 1994 Columbus, Ohio, US; abstract no. 217613h, ITO, S. ET AL.: &quot;Synthesis and pharmacological activities of novel cyclic disulfide and cyclic sulfide derivatives as hepatoprotective agents&quot; page 1075; column 1; XP002031556 see abstract &amp; CHEM. PHARM. BULL., vol. 41, no. 6, 1993, pages 1066-73,</td>
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Further documents are listed in the continuation of box C.

Date of the actual completion of the international search

| 26 May 1997 |

Date of mailing of the international search report

| 18.06.97 |

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HN Rijswijk
Tel: (+ 31-70) 340-2043, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016

Authorized officer

Janus, S
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Relevant to claim No. 1-79
**International Search Report**

**International application No.**

**PCT/EP 97/00516**

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**Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **Claim No.:**
   - **X**
   - because they relate to subject matter not required to be searched by this Authority, namely:
     - Although claims 1 and 5 - 14 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds.

2. **Claim No.:**
   - because they relate to parts of the international Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. **Claim No.:**
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

---

**Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. **As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.**

2. **As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.**

3. **As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims No.:**

4. **No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims No.:**

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**Remark on Protest**

- [ ] The additional search fees were accompanied by the applicant's protest.
- [ ] No protest accompanied the payment of additional search fees.
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<td>WO 9533495 A</td>
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Forni
PCT!ISA/210 (patent family annex) (July 1992)
(55) Abstract

A method of treatment of bacterial infections in humans or animals with comprises administering, in combination with a beta-lactam antibiotic, a therapeutically effective amount of a thiazolidone or proline derivative of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof. Compounds (IA) falling within the scope of formula (I) are also claimed; in formula (I): X is S, S(0) or CH2; n is 1 or 2; R is hydrogen, a salt forming cation or an in vivo hydrolysable ester-forming group; R1 and R2 are each hydrogen or an organic substituent group; R3 is hydrogen, (C1-6)alkyl optionally substituted by up to three halogen atoms, (C2-6)alkenyl, (C2-6)alkynyl, aryl, aryl(C1-6)alkyl, heterocyclyl or heterocyclyl(C1-6)alkyl; and R4 is hydrogen, or an in vivo hydrolysable acyl group. In compounds (IA) R3 represents (C1-6)alkyl optionally substituted by up to three halogen atoms, aryl, aryl(C1-6)alkyl, heterocyclyl or heterocyclyl(C1-6)alkyl and X is S.
 FOR THE PURPOSES OF INFORMATION ONLY

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This invention relates to chemical compounds having metallo-β-lactamase inhibitory and antibacterial properties. The invention also relates to methods for the preparation of such compounds, to pharmaceutical compositions containing them, and to uses thereof.

Metallo-β-lactamases confer resistance to the vast majority of β-lactam based therapies, including carbapenems and jeopardise the future use of all such agents. As a result of the increased use of carbapenems and other β-lactam antibiotics the clinical climate is becoming more favourable for the survival of clinical strains which produce metallo-β-lactamases, and metallo-β-lactamases have now been identified in common pathogens such as *Bacillus fragilis*, *Klebsiella*, *Pseudomonas aeruginosa* and *Serratia marcescens*. Emerging knowledge emphasises that metallo-β-lactamases have the potential to present a crisis situation for antimicrobial chemotherapy.


According to the present invention there is provided a method of treatment of bacterial infections in humans or animals which comprises administering, in combination with a β-lactam antibiotic, a therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof:

\[
\begin{align*}
R_4 &\quad \text{S} \\
&\quad \text{R}_1 \\
&\quad \text{X} \\
&\quad \text{R}_2 \\
&\quad \text{CO}_2 &\quad \text{R}
\end{align*}
\]

(I)

wherein:

- X is S, S(O)\(_n\) or CH\(_2\);
- n is 1 or 2
- R is hydrogen, a salt forming cation or an in vivo hydrolysable ester-forming group;
- R\(_1\) and R\(_2\) are each hydrogen or an organic substituent group;
R₃ is hydrogen, (C₁₋₆)alkyl optionally substituted by up to three halogen atoms, (C₂₋₆)alkenyl, (C₂₋₆)alkynyl, aryl, aryl(C₁₋₆)alkyl, heterocyclyl or heterocyclyl(C₁₋₆)alkyl; and

R₄ is hydrogen, or an in vivo hydrolysable acyl group.

The compound of formula (I) may exist in a number of isomeric forms, all of which, including racemic and diastereoisomeric forms, are encompassed within the scope of the present invention.

It is preferred that the stereochemistry at the carbon atom marked * is D-, i.e. is S when X=S and is R when X=CH₂ and R₁, R₂ = H or alkyl.

Although racemic and other mixtures of (\( \ast \)) D- and L- diastereomers of known compounds of formula (I) have been described, there has been little or no attempt to isolate pure D- isomers as herein defined because the anti-hypertensive activity of the compounds has been found to reside predominantly in the L-isomer.

R₃ aryl(C₁₋₆)alkyl includes optionally substituted benzyl, phenethyl and phenylpropyl.

Certain compounds of formula (I) including compounds where R₃ represents (C₁₋₆)alkyl substituted by up to three halogen atoms, aryl, aryl(C₁₋₆)alkyl, heterocyclyl or heterocyclyl(C₁₋₆)alkyl, hereinafter defined as R₃¹ and X is S are novel and as such form part of the invention. Compounds of formula (I) in which R₃ is R₃¹ and X is S are hereafter referred to as compounds of formula (IA).

Preferably R₃ is optionally substituted benzyl, more preferably benzyl.

The preferred stereochemistry at the carbon atom marked (\( \ast \)) is S.

R₄ is preferably hydrogen, lower alkylcarbonyl, optionally substituted benzoyl or optionally substituted phenyl lower alkyl carbonyl.

Suitable examples of R₄ include hydrogen and acetyl.

In general formula (I), R₁ and R₂ denotes hydrogen or an organic group. This may suitably be linked through a carbon atom. For example, R₁ or R₂ may represent hydrogen or a group of formula -R⁵, where R⁵ denotes an unsubstituted or substituted (C₁₋₁₀)hydrocarbon group.

Preferably, R₁ or R₂ represents hydrogen, (C₁₋₁₀)alkyl, aryl, heterocyclyl or substituted (C₁₋₁₀)alkyl, wherein the substituent may be aryl, heterocyclyl, hydroxy, (C₁₋₆)alkoxy, (C₁₋₆)alkanoyloxy, halogen, mercapto, (C₁₋₆)alkythio, heterocyclylthio, amino, (mono or di)-(C₁₋₆)alkylamino, (C₁₋₆)alkanoylamino, carboxy, or (C₁₋₆)alkoxy carbonyl.

Examples of suitable organic groups R₁ and R₂ include methyl, ethyl, propyl, hydroxymethyl, methoxymethyl, ethoxymethyl, acetoxyethyl, aminomethyl, 2-aminoethyl, acetamidomethyl, 2-acetamidoethyl, carboxymethyl, phenyl, pyridyl, pyrimidyl and isoxazolyl.
In particular, \( R_1 \) and \( R_2 \) may be hydrogen or methyl.

Examples of suitable optional substituents for the above-mentioned \((C_{1-6})\)alkyl, \((C_{2-6})\)alkenyl, \((C_{2-6})\)alkynyl, aryl and aryl\((C_{1-6})\)alkyl substituents include \((C_{1-6})\)alkanoyl, \((C_{1-6})\)alkanoyloxy, heterocyclyl, amino, \((C_{1-6})\)alkanoxylamino, \((\text{mono or di})-(C_{1-6})\)alkylamino, hydroxy, \((C_{1-6})\)alkylsulphonyl, heterocyclylthio, arylthio, sulphamoyl, carbamoyl, amidino, guanidino, nitro, halogen, carboxy, carboxy esters, arylcarbonyl and heterocyclylcarbonyl groups.

\( X \) is preferably \( S \).

Suitable pharmaceutically acceptable salts of the carboxylic acid group of the compound of formula (I) (or of other carboxylic acid groups which may be present as optional substituents) include those in which \( R \) is a metal ion e.g. aluminium salts, alkali metal salts (e.g. sodium, lithium or potassium salts), alkaline earth metal salts (e.g. calcium or magnesium salts), ammonium salts, and substituted ammonium salts, for example those with lower alkylamines (e.g. triethylamine), hydroxy-lower alkylamines (e.g. 2-hydroxyethylamine), bis-(2-hydroxyethyl)amine, tris-(2-hydroxyethyl) amine, lower-cycloalkylamines (e.g. dicyclohexyl-amine), or with procaine, dibenzylamine, \( N,N \)-dibenzyl- ethylenediamine, \( 1 \)-ephenamine, \( N \)-methylmorpholine, \( N \)-ethylpiperidine, \( N \)-benzyl\( \beta \)-phenethylamine, dehydroabietylamine, ethylenediamine, \( N,N \)-bishydroabietylethylenediamine, bases of the pyridine type (e.g. pyridine, collidine and quinoline), and other amines which have been or can be used to form quaternary ammonium salts.

Pharmaceutically acceptable salts may also be acid addition salts of any amino or substituted amino group(s) that may be present as optional substituents on the compound of formula (I), or of a heterocyclic group ring nitrogen atom. Suitable salts include for example hydrochlorides, sulphates, hydrogen sulphates, acetates, phosphates etc. and other pharmaceutically acceptable salts will be apparent to those skilled in the art. Suitable addition salts are the hydrochlorides and hydrogen sulphates.

Preferred salts are sodium salts.

Examples of suitable pharmaceutically acceptable in vivo hydrolysable ester-forming groups \( R \) include those forming esters which break down readily in the human body to leave the parent acid or its salt. Suitable groups of this type include those of part formulae (i), (ii), (iii), (iv) and (v):

- 3 -
wherein R^a is hydrogen, (C1-6) alkyl, (C3-7) cycloalkyl, methyl, or phenyl, R^b is (C1-6) alkyl, (C1-6) alkoxy, phenyl, benzyl, (C3-7) cycloalkyl, (C3-7) cycloalkyloxy, (C1-6) alkyl (C3-7) cycloalkyl, 1-amino (C1-6) alkyl, or 1-(C1-6 alkyl)amino (C1-6) alkyl; or R^a and R^b together form a 1,2-phenylene group optionally substituted by one or two methoxy groups; R^c represents (C1-6) alkylene optionally substituted with a methyl or ethyl group and R^d and R^e independently represent (C1-6) alkyl; R^f represents (C1-6) alkyl; R^g represents hydrogen or phenyl optionally substituted by up to three groups selected from halogen, (C1-6) alkyl, or (C1-6) alkoxy; Q is oxygen or NH; R^h is hydrogen or (C1-6) alkyl; R^i is hydrogen, (C1-6) alkyl optionally substituted by halogen, (C2-6) alkenyl, (C1-6) alkoxy carbonyl, aryl or heteroaryl; or R^h and R^i together form (C1-6) alkylene; R^j represents hydrogen, (C1-6) alkyl or (C1-6) alkoxy carbonyl; and R^k represents (C1-8) alkyl, (C1-8) alkoxy, (C1-6) alkoxy (C1-6) alkoxy or aryl.

Examples of suitable \textit{in vivo} hydrolysable ester-forming groups include, for example, acyloxyalkyl groups such as acetoxy methyl, pivaloyloxymethyl, \(\alpha\)-acetoxyethyl, \(\alpha\)-pivaloyloxymethyl, 1-(cyclohexylcarbonyloxy)prop-1-yl, and (1-aminoethyl)carbonyloxymethyl; alkoxy carbonyloxalkyl groups, such as ethoxycarbonyloxymethyl, \(\alpha\)-ethoxycarbonyloxyl ethyl and propoxycarbonyloxyl ethyl; dialkylaminoalkyl especially di-loweralkylamino alkyl groups such as
dimethylaminomethyl, dimethylaminoethyl, diethylaminomethyl or diethylaminoethyl; 2-(alkoxycarbonyl)-2-alkenyl groups such as 2-(isobutoxycarbonyl)pent-2-ynyl and 2-(ethoxycarbonyl)but-2-ynyl; and lactone groups such as phthalidyl and dimethoxyphthalidyl.

A further suitable pharmaceutically acceptable in vivo hydrolysable ester-forming group is that of the formula:

![Chemical Structure](attachment:image.png)

wherein $R_k$ is hydrogen, $C_{1-6}$ alkyl or phenyl.

$R$ is preferably hydrogen.

When used herein the term 'aryl' includes phenyl and naphthyl, each optionally substituted with up to five, preferably up to three, groups selected from halogen, mercapto, ($C_{1-6}$) alkyl, phenyl, ($C_{1-6}$) alkoxy, hydroxy($C_{1-6}$)alkyl, mercapto($C_{1-6}$)alkyl, halo($C_{1-6}$) alkyl, hydroxy, amino, nitro, carboxy, ($C_{1-6}$) alkylcarbonyloxy, alkoxycarbonyl, formyl, or ($C_{1-6}$) alkylcarbonyl groups.

The terms 'heterocyclyl' and 'heterocyclic' as used herein include aromatic and non-aromatic, single and fused, rings suitably containing up to four hetero-atoms in each ring selected from oxygen, nitrogen and sulphur, which rings may be unsubstituted or substituted by, for example, up to three groups selected from halogen, ($C_{1-6}$)alkyl, ($C_{1-6}$)alkoxy, halo($C_{1-6}$)alkyl, hydroxy, carboxy, carboxy salts, carboxy esters such as ($C_{1-6}$)alkoxycarbonyl, ($C_{1-6}$)alkoxycarbonyl($C_{1-6}$)alkyl, ary1, and oxo groups. Each heterocyclic ring suitably has from 4 to 7, preferably 5 or 6, ring atoms. The term 'heteroaryl' refers to heteroaromatic heterocyclic ring or ring system, suitably having 5 or 6 ring atoms in each ring. A fused heterocyclic ring system may include carbocyclic rings and need include only one heterocyclic ring. Compounds within the invention containing a heterocyclyl group may occur in two or more tautomeric forms depending on the nature of the heterocyclyl group; all such tautomeric forms are included within the scope of the invention.

When used herein the terms 'lower alkyl', 'lower alkenyl', 'lower alkynyl' and 'alkoxy' include straight and branched chain groups containing from 1 to 6 carbon atoms, such as methyl, ethyl, propyl and butyl. A particular alkyl group is methyl.

When used herein the term 'halogen' refers to fluorine, chlorine, bromine and iodine.
It will be appreciated that also included within the scope of the invention are pharmaceutically acceptable salts and pharmaceutically acceptable esters, including \textit{in vivo} hydrolysable esters, of any carboxy groups that may be present as optional substituents in compounds of formula (I).

Some compounds of formula (I) and (IA) may be crystallised or recrystallised from solvents such as organic solvents. In such cases solvates may be formed. This invention includes within its scope stoichiometric solvates including hydrates as well as compounds containing variable amounts of solvents such as water that may be produced by processes such as lyophilisation. Compounds of formula (I) and (IA) may be prepared in crystalline form by for example dissolution of the compound in water, preferably in the minimum quantity thereof, followed by admixing of this aqueous solution with a water miscible organic solvent such as a lower aliphatic ketone such as a di-\((C_{1-6})\) alkyl ketone, or a \((C_{1-6})\) alcohol, such as acetone or ethanol.

The compounds of formulae (I) and (IA) are metallo-\(\beta\)-lactamase inhibitors and are intended for use in pharmaceutical compositions. Therefore it will readily be understood that they are preferably each provided in substantially pure form, for example at least 60% pure, more suitably at least 75% pure and preferably at least 85% pure, especially at least 95% pure particularly at least 98% pure (% are on a weight for weight basis). Impure preparations of the compounds may be used for preparing the more pure forms used in the pharmaceutical compositions; these less pure preparations of the compounds should contain at least 1%, more suitably at least 5% and preferably from 10 to 59% of a compound of the formula (I) or (IA) or salt thereof.

Compounds of formula (I) may generally be prepared by processes analogous to those described in the prior art references listed above.

The present invention also provides a process for the preparation of a compound of formula (IA) as defined above, which comprises reacting a compound of formula (II)

\[
\begin{align*}
&Y + \left(\begin{array}{c}
+ R_3' \\
\end{array}\right) \\
&\text{W} \\
\end{align*}
\]

with a compound of formula (III)
wherein $W$ is a leaving group, $Y$ is $R_4'S$ or a group convertible thereto, $R^X$ is $R$ or a carboxylate protecting group and $R_1', R_2', R_3'$ and $R_4'$ are $R_1$, $R_2$, $R_3$ and $R_4$ or groups convertible thereto, wherein $R$, $R_1$, $R_2$, $R_3$ and $R_4$ are as defined in formula (IA), and thereafter, where necessary, converting $Y$ into $R_4'S$, $R^X$, $R_1'$, $R_2'$, $R_3'$ and/or into $R$, $R_1$, $R_2$, $R_3$ and/or $R_4$ and optionally inter-converting $R$, $R_1$, $R_2$, $R_3$ and/or $R_4$.

Suitable ester-forming carboxyl-protecting groups $R^X$ other than in vivo hydrolysable ester forming groups are those which may be removed under conventional conditions. Such groups for $R^X$ include methyl, ethyl, benzyl, $p$-methoxybenzyl, benzoylmethyl, $p$-nitrobenzyl, 4-pyridylmethyl, 2,2,2-trichloroethyl, 2,2,2-tribromoethyl, $t$-butyl, $t$-amyl, allyl, diphenylmethyl, triphenylmethyl, adamantyl, 2-benzoxoxyphenyl, 4-methylthiophenethyl, tetrahydrofuran-2-yl, tetrahydropyran-2-yl, pentachlorophenyl, acetonyl, $p$-toluenesulphonyl, methoxymethyl, a silyl, stannyl or phosphorus-containing group or an oxime radical of formula $-N=CHR^6$ where $R^6$ is aryl or heterocyclyl, or an in vivo hydrolysable ester radical such as defined below.

Certain compounds of formulae (II) and (III) may include an amino group which may be protected. Suitable amino protecting groups are those well known in the art which may be removed under conventional conditions if required without disruption of the remainder of the molecule.

Examples of amino protecting groups include $(C_1-6)$ alkanoyl; benzoyl; benzyl optionally substituted in the phenyl ring by one or two substituents selected from $(C_1-4)$ alkyl, $(C_1-4)$ alkoxy, trifluoromethyl, halogen, or nitro; $(C_1-4)$ alkoxy carbonyl; benzoxycarbonyl or trityl substituted as for benzyl above; allyloxycarbonyl, trichloroethoxycarbonyl or chloroacetyl.

The compound of formula (III) is preferably presented as the anion prepared by treatment of the amine with an organic base such as triethylamine, pyridine or morpholine.

The reaction of the compounds of formula (II) and (III) is preferably carried out at ambient temperature, for example 15-25°C, in an inert solvent such as tetrahydrofuran, dichloromethane, dioxan or dimethylformamide.

Suitable examples of the leaving $W$ group include halo such as chloro and mixed sulphonic anhydrides such as those where $W$ is methanesulphonyloxy, toluene-$p$-sulphonyloxy or trifluoromethanesulphonyloxy in mixed sulphonic anhydrides.
Examples of Y convertible into R₄'S include halo such as bromo which may be displaced by thiobenzoic acid or thiopacteic acid.

Examples of groups R₁', R₂', R₃', R₄' convertible to R₁, R₂, R₃ and R₄ include those where any carboxy or amino group is protected by carboxy or amino protecting groups.

R₄' in the compound of formula (II) is preferably other than hydrogen, for example acetyl.

The acid derivative of formula (II) is preferably prepared from the corresponding free acid by treatment with strong base such as sodium hydride followed by a source of the anion leaving group W, such as oxalyl chloride.

The product of the reaction of compounds of formulae (II) and (III) is a compound of formula (IV):

\[ R₃\text{CO}_2R' \]

wherein the variables are as defined in formulae (II) and (III). Novel intermediates of formula (IV) wherein \( R^X \) is other than R when R₁', R₂', R₃', and R₄' are R₁, R₂, R₃ and R₄ also form part of the invention.

When \( R^X \) is other than hydrogen, the carboxy group -COOR^X may be deprotected, that is to say, converted to a free carboxy, carboxy salt or carboxy ester group -COOR in a conventional manner, for example as described in EP0232966A.

When it is desired to obtain a free acid or salt of the preferred isomer of the formula (I) from an isomeric mixture, this may be effected by chromatographic separation of the diastereomers of the product. Where this is an ester and/or where R₄' is other than hydrogen, the desired isomer may then be deprotected to give the corresponding free acid or salt. In some cases, however, it has been found particularly convenient first to deprotect the isomeric mixture to give an isomeric mixture of the free acid or salt of formula (I), followed by fractional recrystallisation to give the desired acid or salt isomer. Where X=S and the °S isomer of formula (I) is desired, it is preferred to use the corresponding °S isomer of the intermediate of formula (III).

When an enatiomerically pure form of (III) is used in the preparation of (I), the preferred diastereomer at position (+) of (I) can also be separated by chromatography. An enantiomerically pure form of (II) may also be used.

A carboxyl group may be regenerated from any of the above esters by usual methods appropriate to the particular RX group, for example, acid- and base-catalysed
hydrolysis, or by enzymically-catalysed hydrolysis, or by hydrogenolysis under conditions wherein the remainder of the molecule is substantially unaffected. For example, in the case of acetonyl, by hydrolysis in acetonitrile with 0.1M aqueous potassium hydroxide solution.

Pharmaceutically acceptable salts may be prepared from such acids by treatment with a base, after a conventional work-up if necessary. Suitable bases include sodium hydrogen carbonate to form sodium salts.

Crystalline forms of the compounds of formula (I) where R is a salt forming cation may for example be prepared by dissolving the compound (I) in the minimum quantity of water, suitably at ambient temperature, then adding a water miscible organic solvent such as a (C1-6) alcohol or ketone such as ethanol or acetone, upon which crystallisation occurs and which may be encouraged for example by cooling or trituration.

Compounds of formulae (II) and (III) are known compounds or may be prepared by procedures analogous to those described in the prior art references listed above.

Compounds of formula (I), particularly (IA), may be administered in the form of a pharmaceutical composition together with or a pharmaceutically acceptable carrier. The compounds of formula (I) have metallo-β-lactamase inhibitory properties, and are useful for the treatment of infections in animals, especially mammals, including humans, in particular in humans and domesticated (including farm)animals. The compounds may be used, for example, for the treatment of infections of, inter alia, the respiratory tract, the urinary tract, and soft tissues and blood, especially in humans.

The compounds may be used in combination with an antibiotic partner for the treatment of infections caused by metallo-β-lactamase producing strains, in addition to those infections which are subsumed within the antibacterial spectrum of the antibiotic partner. Metallo-β-lactamase producing strains include:- Pseudomonas aeruginosa, Klebsiella pneumoniae, Xanthomonas maltophilia, Bacteroides fragilis, Serratia marcescens, Bacteroides distasonis, Pseudomonas cepacia, Aeromonas hydrophila, Aeromonas sobria, Aeromonas salmonicida, Bacillus cereus, Legionella gormanii and Flavobacterium spp.

It is generally advantageous to use a compound according to the invention in admixture or conjunction with a carbapenem, penicillin, cephalosporin or other β-lactam antibiotic and that can result in a synergistic effect, because of the metallo-β-lactamase inhibitory properties of the compounds according to the invention. In such cases, the compound of formula (I) or (IA) and the β-lactam antibiotic can be administered separately or in the form of a single composition containing both active ingredients as discussed in more detail below. The compositions of the invention
include those in a form adapted for oral, topical or parenteral use and may be used for the treatment of bacterial infection in mammals including humans. The compounds of formula (I) and (IA) are particularly suitable for parenteral administration.

The compounds of formula (I) or (IA) may be formulated for administration in any convenient way for use in human or veterinary medicine, by analogy with other antibiotics and other β-lactam antibiotic/β-lactamase inhibitor combinations.

The composition may be formulated for administration by any route, such as oral, topical or parenteral. The compositions may be in the form of tablets, capsules, powders, granules, lozenges, creams or liquid preparations, such as oral or sterile parenteral solutions or suspensions.

The topical formulations of the present invention may be presented as, for instance, ointments, creams or lotions, eye ointments and eye or ear drops, impregnated dressings and aerosols, and may contain appropriate conventional additives such as preservatives, solvents to assist drug penetration and emollients in ointments and creams.

The formulations may also contain compatible conventional carriers, such as cream or ointment bases and ethanol or oleyl alcohol for lotions. Such carriers may be present as from about 1% up to about 98% of the formulation. More usually they will form up to about 80% of the formulation.

Tablets and capsules for oral administration may be in unit dose presentation form, and may contain conventional excipients such as binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth, or polyvinylpyrrolidone; fillers, for example lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tabletting lubricants, for example magnesium stearate, talc, polyethylene glycol or silica; disintegrants, for example potato starch; or acceptable wetting agents such as sodium lauryl sulphate. The tablets may be coated according to methods well known in normal pharmaceutical practice. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives, such as suspending agents, for example sorbitol, methyl cellulose, glucose syrup, gelatin, hydroxyethyl cellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monoolesate, or acacia; non-aqueous vehicles (which may include edible oils), for example almond oil, oily esters such as glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and, if desired, conventional flavouring or colouring agents.
Suppositories will contain conventional suppository bases, e.g. cocoa-butter or other glyceride.

For parenteral administration, fluid unit dosage forms are prepared utilizing the compound and a sterile vehicle, water being preferred. The compound, depending on the vehicle and concentration used, can be either suspended or dissolved in the vehicle. In preparing solutions the compound can be dissolved in water for injection and filter sterilised before filling into a suitable vial or ampoule and sealing.

Advantageously, agents such as a local anaesthetic, preservative and buffering agents can be dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. The dry lyophilized powder is then sealed in the vial and an accompanying vial of water for injection may be supplied to reconstitute the liquid prior to use. Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilisation cannot be accomplished by filtration. The compound can be sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the compound.

The compositions may contain from 0.1% by weight, preferably from 10-60% by weight, of the active material, depending on the method of administration. Where the compositions comprise dosage units, each unit will preferably contain from 50-500 mg of the active ingredient. The dosage as employed for adult human treatment will preferably range from 100 to 3000 mg per day, for instance 1500 mg per day depending on the route and frequency of administration. Such a dosage corresponds to 1.5 to 50 mg/kg per day. Suitably the dosage is from 5 to 20 mg/kg per day.

No toxicological effects are indicated when a compound of formula (I) or (IA) or a pharmaceutically acceptable salt thereof is administered in the above-mentioned dosage range.

A composition according to the invention may comprise a compound of formula (I) or (IA) together with one or more additional active ingredients or therapeutic agents, for example a β-lactam antibiotic such as a carbapenem, penicillin or cephalosporin or pro-drug thereof. Carbapenems, penicillins, cephalosporins and other β-lactam antibiotics suitable for co-administration with the compound of formula (I) or (IA) - whether by separate administration or by inclusion in the compositions according to the invention - include both those known to show instability to or to be otherwise susceptible to metallo-β-lactamases and also those known to have a degree of resistance to metallo-β-lactamases.
A serine β-lactamase inhibitor such as clavulanic acid, sulbactam or tazobactam may also be co-administered with the compound of the invention and the β-lactam antibiotic, either by separate administration, or co-formulation with one, other or both of the compounds of the invention and the β-lactam antibiotic.

Examples of carbapenems that may be co-administered with the compounds according to the invention include imipenem, meropenem, biapenem, BMS181139 ([4R-[4alpha,5beta,6beta(R*)]]-4-{2-{[(aminoiminomethyl)amino]ethyl}-3-{[(2-cyanoethyl)thio]-6-{(1-hydroxyethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid}, BO2727 ([4R-3[3S*,5S*(R*)],4alpha,5beta,6beta(R*)]]-6{(1-hydroxyethyl}-3-[[5-{1-hydroxy-3-(methylamino)propyl]-3-pyrrolidinyl]thio]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid monohydrochloride), ER35786 ((1R,5S,6S)-6-{1[(R)-Hydroxymethyl]-2-[2(S)-[1.(R)-hydroxy-1-[pyrrolidin-3(R)-yl]methyl]pyrrolidin-4(S)-ylsulfanyl]-1-methyl-1-carba-2-penem-3-carboxylic acid hydrochloride) and S4661 ((1R,5S,6S)-2-[3S,5S]-5-(sulfoamylaminomethyl)pyrrolidin-3-yl]thio-6-{(1R)-1-hydroxyethyl]-1-methylcarbapen-2-em-3-carboxylic acid).

Examples of penicillins suitable for co-administration with the compounds according to the invention include benzylpenicillin, phenoxymethylpenicillin, carbenicillin, azidocillin, propicillin, ampicillin, amoxyccillin, epicillin, ticarcillin, cyclacillin, pirbenicillin, azlocillin, mezlocillin, sulbenicillin, piperacillin, and other known penicillins. The penicillins may be used in the form of pro-drugs thereof, for example as in vivo hydrolysable esters, for example the acetoxymethyl, pivaloyloxymethyl, α-ethoxycarbonyloxyethyl and phthalidyl esters of ampicillin, benzylpenicillin and amoxyccillin; as aldehyde or ketone adducts of penicillins containing a 6-α-aminoacetamido side chain (for example hetacillin, metampicillin and analogous derivatives of amoxyccillin); and as α-esters of carbencillin and ticarcillin, for example the phenyl and indanyl α-esters.

Examples of cephalosporins that may be co-administered with the compounds according to the invention include, cefatrizine, cephaloridine, cephalothin, cefazolin, cephalexin, cephaetritile, cephapirin, cephamandole nafate, cephradine, 4-hydroxycephaexin, cephaloglycin, cefoperazone, cefsolodin, cefazidime, cefuroxime, cefmetazole, cefotaxime, ceftriaxone, and other known cephalosporins, all of which may be used in the form of pro-drugs thereof.

Examples of β-lactam antibiotics other than penicillins and cephalosporins that may be co-administered with the compounds according to the invention include aztreonam, latamoxef (Moxalactam - Trade Mark), and other known β-lactam antibiotics, all of which may be used in the form of pro-drugs thereof.
Particularly suitable penicillins for co-administration with the compounds according to the invention include ampicillin, amoxycillin, carbenicillin, piperacillin, azlocillin, mezlocillin, and ticarcillin. Such penicillins may be used in the form of their pharmaceutically acceptable salts, for example their sodium salts. Alternatively, ampicillin or amoxycillin may be used in the form of fine particles of the zwitterionic form (generally as ampicillin trihydrate or amoxycillin trihydrate) for use in an injectable or infusible suspension, for example, in the manner hereinbefore described in relation to the compounds according to the invention. Amoxycillin, for example in the form of its sodium salt or the trihydrate, is particularly preferred for use in synergistic compositions according to the invention.

Particularly suitable cephalosporins for co-administration with the compounds according to the invention include cefotaxime and ceftazidime, which may be used in the form of their pharmaceutically acceptable salts, for example their sodium salts.

A compound of formula (I) or (IA) may be administered to the patient in conjunction with a β-lactam antibiotic such as a carbapenem, penicillin or cephalosporin in a synergistically effective amount.

The compounds of formula (I) or (IA) may suitably be administered to the patient at a daily dosage of from 0.7 to 50 mg/kg of body weight. For an adult human (of approximately 70 kg body weight), from 50 to 3000 mg, preferably from 100 to 1000 mg, of a compound according to the invention may be administered daily, suitably in from 1 to 6, preferably from 2 to 4, separate doses. Higher or lower dosages may, however, be used in accordance with clinical practice.

When the compositions according to the invention are presented in unit dosage form, each unit dose may suitably comprise from 25 to 1000 mg, preferably from 50 to 500 mg, of a compound according to the invention. Each unit dose may, for example, be 62.5, 100, 125, 150, 200 or 250 mg of a compound according to the invention.

When the compounds of formula (I) or (IA) are co-administered with a penicillin, cephalosporin, carbapenem or other β-lactam antibiotic, the ratio of the amount of the compound according to the invention to the amount of the other β-lactam antibiotic may vary within a wide range. The said ratio may, for example, be from 100:1 to 1:100; more particularly, it may, for example, be from 2:1 to 1:30.

The amount of carbapenem, penicillin, cephalosporin or other β-lactam antibiotic in a synergistic composition according to the invention will normally be approximately similar to the amount in which it is conventionally used per se, for example from about 50 mg, advantageously from about 62.5 mg, to about 3000 mg per unit dose, more usually about 125, 250, 500 or 1000 mg per unit dose.

The present invention further provides a compound of formula (I) or a pharmaceutically acceptable salt, solvate or or in vivo hydrolysable ester thereof, and
in particular a compound of formula (IA) for use in the treatment of bacterial infections.

The present invention also includes the use of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, in the manufacture of a medicament for the treatment of bacterial infections.

The present invention also includes the use of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof as a metallo-β-lactamase inhibitor.

In a further aspect, the invention provides a method of treatment of bacterial infections in humans or animals which comprises administering, in combination with a carbapenem antibiotic, a therapeutically effective amount of a metallo-β-lactamase inhibitor.

The invention further provides the use of a carbapenem antibiotic in combination with a therapeutically effective amount of a metallo-β-lactamase inhibitor in the manufacture of a medicament for the treatment of bacterial infections.

A further composition according to the invention comprises a metallo-β-lactamase inhibitor together with a carbapenem antibiotic and a pharmaceutically acceptable carrier.

Such method and composition may be administered as described above for uses of compounds of formula (I).

All the above compositions and methods may optionally include a serine β-lactamase inhibitor as above described.

The compounds of the present invention are active against metallo-β-lactamase enzymes produced by a wide range of organisms including both Gram-negative organisms and Gram-positive organisms.

The following Examples illustrate compounds useful in the present invention, and intermediates in their preparation. (All temperatures are in °C).

**EXAMPLES**

**Example 1:**

3-[[S-acetyl-2' (RS)-benzyl-3'-mercaptopropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E1)

To a cooled (0°), stirred solution of S-acetyl-2-benzyl-3-mercapto propionic acid (EP0361365) (476mg, 2.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (88 mg of a 55% suspension in oil, 2.0 mmol). The reaction mixture was allowed to reach room temperature and
stirring was continued for a further 15 mins. The suspension was then recooled (0°C) and treated with oxalyl chloride (210 ul, 2.4 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0°C) suspension of 4(S)-carboxy-5,5-dimethylthiazolidine (J. Am. Chem. Soc., 1949, 71, 1137). (161 mg, 1.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (278 ul, 2.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 3 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate. Evaporation of the solvent afforded crude product as an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded recovered substituted propionic acid starting material followed by the title diastereomeric mixture (1:1) as a crisp foam (312 mg, 82%). δH (CDCl3) (Isomer 1) 1.12 (3H, s), 1.41 (3H, s), 2.34 (3H, s), 2.8 - 3.2 (5H, overlapping m), 3.93 (1H, d, J 8.0 Hz), 4.35 (1H, s), 4.61 (1H, d, J 8.0 Hz), 7.2-7.3 (5H, m). (Isomer 2) 1.43 (3H, s), 1.56 (3H, s), 2.34 (3H, s), 2.8 - 3.2 (5H, overlapping m), 4.01 (1H, d, J 8.4 Hz), 4.55 (1H, s), 4.59 (1H, d, J 8.4 Hz), 7.2-7.3 (5H, m) ppm.

Example 2:
3-[2'(RS)-benzyl-3'-mercaptopropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E2)

The S-acetyl derivative El (203 mg, 0.53 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with further brine, dried over sodium sulphate and evaporated to afford the title diastereomeric mixture as a crisp foam (170 mg, 94%). νmax (CHCl3) 1740, 1719, 1641, 1443, 1425 cm⁻¹. δH (CDCl3) (Isomer 1) 1.20 (3H, s), 1.43 (3H, s), 1.91 (1H, dd, J 11.5 and 6.3 Hz), 2.55 (1H, m), 2.75 - 3.15 (4H, overlapping m), 4.04 (1H, d, J 8.1 Hz), 4.38 (1H, s), 4.84 (1H, d, J 8.1 Hz), 7.2-7.3 (5H, m). (Isomer 2) 1.46 (3H, s), 1.58 (3H, s), 2.05 (1H, s), 2.55 (1H, m), 2.75 - 3.15 (4H, overlapping m), 4.20 (1H, d, J 8.1 Hz), 4.62 (1H, s), 4.81 (1H, d, J 8.1 Hz), 7.2-7.3 (5H, m) ppm. EIMS M⁺ 339. DCIMS MH⁺ 340.
Separation of diastereomers of 3-[2'\(RS\)-benzyl-3'-mercaptopropionyl]-4(\(S\))-carboxy-5,5-dimethylthiazolidine by HPLC

The diastereomeric mixture of Example 2 was applied to a Hypersil BDS C8 (250mm x 4.6mm) column and eluted isocratically with 70/30 0.1% trifluoroacetic acid / 0.1% trifluoroacetic acid in acetonitrile at a flow rate of 0.8ml/minute. Detection was at 215nm. Under these conditions the (2'R, 4S)-diastereomer had an \(R_t\) of 31.3 minutes and the (2'S, 4S)-diastereomer had an \(R_t\) of 35.1 minutes. These diastereomers were identical with the compounds E19 and E20 of Examples 19 and 20 respectively.

Example 3:
**N-[S-Acetyl-3'-mercaptop-2'(RS)-methylpropionyl]-D-proline (E3)**

To a cooled (0\(^\circ\)) stirred solution of S-acetyl-2-methyl-3-mercaptopropionic acid (US4046889) (324mg, 2.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (88 mg of a 55\% suspension in oil, 2.0 mmol). The reaction mixture was allowed to reach room temperature and stirring was continued for a further 15 mins. The suspension was then recooled (0\(^\circ\)) and treated with oxalyl chloride (210 ul, 2.4 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0\(^\circ\)) suspension of D-proline (230 mg, 2.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (556 ul, 4.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 18 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate. Evaporation of the solvent afforded crude product as an oil which was chromatographed on silica gel. Elution with 4\% methanol in chloroform, containing 0.1\% acetic acid, afforded recovered substituted propionic acid starting material followed by the title compound as a colourless oil (180 mg, 35\%).
Example 4:

N-[3'-Mercapto-2'(RS)-methylpropionyl]-D-proline (E4)

The S-acetyl derivative E3 (155 mg, 0.60 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with further brine, dried over sodium sulphate and evaporated to afford the title compound as a colourless oil (110 mg, 85%).

\[ \text{vmax (CHCl}_3) 1749, 1720, 1635, 1590 \text{ cm}^{-1}. \]

\[ \delta_H (\text{CDCl}_3) \]

(Isoomer 1) 1.21 (3H, d, J 6.8 Hz), 1.67 (1H, dd, J 10.1 and 7.4 Hz), 2.05-2.15 (3H, overlapping m), 2.4-2.55 (2H, overlapping m), 2.8-3.0 (2H, overlapping m), 3.52 (1H, m), 3.84 (1H, m), 4.66 (1H, dd, J 8.6 and 8.6 Hz), 2.05-2.15 (3H, overlapping m), 2.4-2.55 (2H, overlapping m), 2.8-3.0 (2H, overlapping m), 3.65 (2H, overlapping m), 4.61 (1H, dd, J 8 and 3 Hz) ppm. EIMS M+ 217. DCIMS MH+ 218.

Example 5:

N-(S-Acetyl-3'-mercapto-2'(S)-methylpropionyl)-D-proline (E5)

To a cooled (0°), stirred solution of S-acetyl-3-mercaptop-2(S)-methylpropionic acid (Janssen Chimica, Geel, Belgium) (324 mg, 2.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (88 mg of a 55% suspension in oil, 2.0 mmol). The reaction mixture was allowed to reach room temperature and stirring was continued for a further 15 mins. The suspension was then recooled (0°) and treated with oxalyl chloride (210 ul, 2.425 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0°) suspension of D-proline (230 mg, 2.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (556 ul, 4.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 18 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate.

Evaporation of the solvent afforded crude product as an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded recovered substituted propionic acid starting material followed by the title compound as a colourless oil (119 mg, 23%). \[ \delta_H (\text{CDCl}_3) 1.23 \]
(3H, d, J 6.5 Hz), 2.0 (3H, overlapping m), 2.34 (3H, s), 2.45 (1H, m), 2.92 (1H, m),
2.96 (1H, dd, J 13.0 and 6.5 Hz), 3.14 (1H, dd, J 13.0 and 7.3 Hz), 3.48 (1H, s), 3.76
(1H, m), 4.58 (1H, m) ppm. DCIMS MH+ 260.

Example 6:
N-(3'-mercapto-2'(S)-methylpropionyl)-D-proline (E6)
The S-acetyl derivative E5 (102 mg, 0.39 mmol) was dissolved in water (0.6
ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room
temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed
with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase
system was treated with saturated brine and the organic layer separated, washed with
further brine, dried over sodium sulphate and evaporated to afford the title compound
as a colourless oil (71 mg, 85%). vmax (CHC13) 1751, 1719, 1636, 1586 cm-1. 8H
(CDC13) 1.21 (3H, d, J 6.7 Hz), 1.70 (1H, dd, J 10.2 and 7.3 Hz), 2.05-2.15 (3H,
overlapping m), 2.4-2.55 (2H, overlapping m), 2.8 - 3.0 (2H, overlapping m), 3.53
(1H, m), 3.84 (1H, m), 4.76 (1H, dd, J 7.8 and 3.5 Hz) ppm. EIMS M+ 217. DCIMS
MH+ 218.

Example 7:
3-[S-Acetyl-3'-mercaptobropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E7)
To a cooled (0°), stirred solution of S-acetyl-3-mercaptobropionic acid
(prepared analogously to the starting material of Example 1 according to the method
of EP0361365) (148mg, 1.0 mmol) in dry tetrahydrofuran (8 ml) containing dry
dimethylformamide (1 drop), was added sodium hydride (44 mg of a 55% suspension
in oil, 1.0 mmol). The reaction mixture was allowed to reach room temperature and
stirring was continued for a further 15 mins. The suspension was then recooled (0° )
and treated with oxalyl chloride (105 ul, 1.2 mmol) and stirred at room ambient
temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the
residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to
dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0°) suspension of 4(S)-carboxy-5,5-dimethylthiazolidine
(161 mg, 1.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine
(278 ul, 2.0 mmol) followed by a solution of the above acid chloride in dry
tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient
temperature for 3 hours and then partitioned between ethyl acetate and 1M
hydrochloric acid. The organic layer was washed with water and saturated brine and
dried over anhydrous sodium sulphate. Evaporation of the solvent afforded crude
product as an oil which was chromatographed on silica gel. Elution with 4% methanol
in chloroform, containing 0.1\% acetic acid, afforded recovered substituted propionic acid starting material followed by, on elution with 10\% methanol in chloroform containing 0.1\% acetic acid, the title compound as a colourless oil (70 mg, 24\%). $\delta_H$ (CDCl$_3$) 1.50 (3H, s), 1.59 (3H, s), 2.34 (3H, s), 2.72 (2H, t, J 6.8 Hz), 3.14 (2H, t, J 6.8 Hz), 4.50 (1H, s), 4.69 (2H, m) ppm.

**Example 8:**
3-[3'-Mercaptopropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E8)

The S-acetyl derivative E7 (70 mg, 0.24 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with further brine, dried over sodium sulphate and evaporated to afford the title compound as a colourless oil (60 mg, 100\%). $\nu_{\text{max}}$ (CHCl$_3$) 1744, 1722, 1651, 1410 cm$^{-1}$. $\delta_H$ (CDCl$_3$) 1.52 (3H, s), 1.60 (3H, s), 1.81 (1H, t, J 8.2 Hz), 2.7-2.9 (4H, overlapping m), 4.55 (1H, s), 4.75 (2H, ABq) ppm. EIMS M$^+$ 249.0496; required for C$_9$H$_{15}$NO$_3$S$_2$, 249.0493.

**Example 9:**
3-[S-Acetyl-3'-mercapto-2'(S)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E9)

To a cooled (0\°), stirred solution of S-acetyl-3-mercapto-2(S)-methylpropionic acid (162mg, 1.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (44 mg of a 55\% suspension in oil, 1.0 mmol). The reaction mixture was allowed to reach room temperature and stirring was continued for a further 15 mins. The suspension was then recooled (0\°) and treated with oxalyl chloride (105 ul, 1.2 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0\°) suspension of 4(S)-carboxy-5,5-dimethylthiazolidine (161 mg, 1.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (278 ul, 2.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 3 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate. Evaporation of the solvent afforded crude
product as an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded recovered substituted propionic acid starting material followed by, on elution with 10% methanol in chloroform containing 0.1% acetic acid, the title compound as a colourless oil (150 mg, 49%). \( \delta_H \) (CDCl\(_3\)) 1.23 (3H, d, \( J 6.2 \) Hz), 1.52 (3H, s), 1.59 (3H, s), 2.34 (3H, s), 2.91 (2H, overlapping m), 3.20 (1H, dd, \( J 16.1 \) and 9.4 Hz), 4.53 (1H, s), 4.71 (1H, d, \( J 8.7 \) Hz) 4.96 (1H, d, \( J 8.7 \) Hz) ppm.

Example 10:

3-[3'-Mercapto-2'(S)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E10)

Method A

The S-acetyl derivative E9 (150 mg, 0.49 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with further brine, dried over sodium sulphate and evaporated to afford the title compound as a colourless oil (129 mg, 100%). \( \nu_{\text{max}} \) (CHCl\(_3\)) 1747, 1719, 1646, 1461, 1421 cm\(^{-1}\). \( \delta_H \) (CDCl\(_3\)) 1.22 (3H, d, \( J 6.5 \) Hz), 1.52 (3H, s), 1.61 (3H, s), 1.87 (1H, dd, \( J 11.1 \) and 6.7 Hz), 2.41 (1H, dd, \( J 11.1 \) and 8.3 Hz), 2.92 (2H, overlapping m), 4.54 (1H, s), 4.72 (1H, d, \( J 8.5 \) Hz), 5.01 (1H, d, \( J 8.5 \) Hz) ppm. EIMS M\(^+\) 263.0648; required for C\(_{10}\)H\(_{17}\)NO\(_3\)S\(_2\), 263.0650.

Method B

The acetyl ester E21B of Example 21 (76 mg, 0.237 mmol) in acetonitrile (4 ml) and water (1 ml) was treated with 0.1M potassium hydroxide solution (2.4 ml, 0.237 mmol) added 0.5 ml at a time over 2 hours. After a total reaction time of 4.5 hours the reaction mixture was acidified with dilute hydrochloric acid and extracted with ethyl acetate. The organic layer was dried over magnesium sulphate and the solvent removed to afford an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded the title compound as a gum (23 mg, 38%). The product was identical in spectroscopic properties with the compound from Method A
Example 11:

3-[S-Acetyl-2'(RS)-benzyl-3'-mercaptopropionyl]-4(R)-carboxy-5,5-dimethylthiazolidine (E11)

To a cooled (0°), stirred solution of S-acetyl-2-benzyl-3-mercaptopropionic acid (238mg, 1.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (44 mg of a 55% suspension in oil, 1.0 mmol). The reaction mixture was allowed to reach room temperature and stirring was continued for a further 15 mins. The suspension was then recooled (0°) and treated with oxalyl chloride (105 ul, 1.2 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0°) suspension of 4(R)-carboxy-5,5-dimethylthiazolidine (Howard-Lock et al., Can. J. Chem. 1986, 64, 1215) (161 mg, 1.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (278 ul, 2.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 3 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate. Evaporation of the solvent afforded crude product as an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded recovered substituted propionic acid starting material, followed by the title compound as a crisp foam (217 mg, 57%). δH (CDCl3) (Isomer 1) 1.12 (3H, s), 1.41 (3H, s), 2.35 (3H, s), 2.8 - 3.2 (5H, overlapping m), 3.93 (1H, d, J 8.1 Hz), 4.36 (1H, s), 4.61 (1H, d, J 8.1 Hz), 7.2-7.3 (5H, m). (Isomer 2) 1.43 (3H, s), 1.57 (3H, s), 2.35 (3H, s), 2.8 - 3.2 (5H, overlapping m), 4.02 (1H, d, J 8.4 Hz), 4.55 (1H, s), 4.60 (1H, d, J 8.4 Hz), 7.2-7.3 (5H, m) ppm.

Example 12:

3-[2'(RS)-benzyl-3'-mercapto propionyl]-4(R)-carboxy-5,5-dimethylthiazolidine (E12)

The S-acetyl derivative E11 (217 mg, 0.57 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with further brine, dried over sodium sulphate and evaporated to afford the title compound, a (2'RS, 4S) diastereomeric mixture, as a crisp foam (189 mg, 87%). νmax (CHCl3)
1739, 1720, 1643, 1432 cm⁻¹. δH (CDCl₃) (Isomer 1) 1.18 (3H, s), 1.48 (3H, s), 1.93 (1H, dd, J 11.4 and 6.0 Hz), 2.53 (1H, m), 2.75 - 3.15 (4H, overlapping m), 4.03 (1H, d, J 8.1 Hz), 4.37 (1H, s), 4.84 (1H, d, J 8.1 Hz), 7.2-7.3 (5H, m). (Isomer 2) 1.46 (3H, s), 1.58 (3H, s), 2.11 (1H, t, J 16.5 Hz), 2.53 (1H, m), 2.75 - 3.15 (4H, overlapping m), 4.19 (1H, d, J 8.1 Hz), 4.62 (1H, s), 4.81 (1H, d, J 8.1 Hz), 7.2-7.3 (5H, m) ppm.

EIMS M⁺ 339.0966; required for C₁₆H₂₁NO₃S₂, 339.0963.

Example 13:
3-[S-Acetyl-3'-'mercapto-2'(RS)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E13)

To a cooled (0°), stirred solution of S-acetyl-3-mercapto-2(RS)-methylpropionic acid (162mg, 1.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (44 mg of a 55% suspension in oil, 1.0 mmol). The reaction mixture was allowed to reach room temperature and stirring was continued for a further 15 mins. The suspension was then recooled (0° ) and treated with oxalyl chloride (105 ul, 1.2 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0°) suspension of 4(S)-carboxy-5,5-dimethylthiazolidine (161 mg, 1.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (278 ul, 2.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 3 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate. Evaporation of the solvent afforded crude product as an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded recovered substituted propionic acid starting material followed by, on elution with 10% methanol in chloroform containing 0.1% acetic acid, the title compound, as a white solid (178 mg, 58%). δH (CDCl₃) 1.23 and 1.25 (3H, d, J 6.2 Hz), 1.51 and 1.52 (3H, s), 1.59 and 1.61 (3H, s), 2.34 (3H, s), 2.85-3.2 (3H, overlapping m), 4.52 and 4.57 (1H, s), 4.70 and 4.72 (1H, d, J 8.6 Hz) 4.83 and 4.96 (1H, d, J 8.6 Hz) ppm.
Example 14:
3-[3'-Mercapto-2'(RS)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E14)

The S-acetyl derivative E13 (178 mg, 0.58 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with further brine, dried over sodium sulphate and evaporated to afford the title compound, to a (2'RS, 4S)-diastereomeric mixture, as a colourless oil (152 mg, 99%).

\[ \text{v}_{\text{max}} (\text{CHCl}_3) 1745, 1717, 1648, 1459, 1418 \text{ cm}^{-1}. \]
\[ \delta_H (\text{CDCl}_3) 1.23 (3\text{H}, \text{d}, J 6.4 \text{ Hz}), 1.53 (3\text{H}, \text{s}), 1.62 (3\text{H}, \text{s}), 1.86 (1\text{H}, \text{dd}, J 11.0 \text{ and } 6.7 \text{ Hz}), 2.46 (1\text{H}, \text{m}), 2.91 (2\text{H}, \text{overlapping m}), 4.54 \text{ and } 4.64 (1\text{H}, \text{s}), 4.73 (d, J 8.5 \text{ Hz}) \text{ and } 4.76 (1\text{H}, d, J 8.3 \text{ Hz}), 4.93 (d, J 8.3 \text{ Hz}) \text{ and } 5.02 (1\text{H}, d, J 8.5 \text{ Hz}) \text{ ppm.} \]

EIMS M+ 263.0648; required for 15 C\text{10H}_17\text{N}_3\text{S}_2, 263.0650.

Example 15:
N-[S-Acetyl-2'(RS)-benzyl-3'-mercaptopropionyl]-D-proline (E15)

To a cooled (0°), stirred solution of S-acetyl-2-benzyl-3-mercaptopropionic acid (238 mg, 1.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (44 mg of a 55% suspension in oil, 1.0 mmol). The reaction mixture was allowed to reach room temperature and stirring was continued for a further 15 mins. The suspension was then recooled (0°) and treated with oxalyl chloride (105 ul, 1.2 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0°) suspension of D-proline (115 mg, 1.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (278 ul, 2.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 3 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate. Evaporation of the solvent afforded crude product as an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded recovered substituted propionic acid starting material followed by the title compound as a crisp foam (87 mg, 26%).

\[ \delta_H (\text{CDCl}_3) 1.50 (1\text{H}, \text{d}, J 7.0 \text{ Hz}) \text{ ppm.} \]
m), 1.85 (2H, overlapping m), 2.35 (3H, s), 2.50 and 2.66 (1H, m), 2.98 (7H, overlapping m), 4.38 and 4.57 (1H, dd, J 8.0 and 2.0 Hz), 7.2-7.3 (5H, m) ppm.

Example 16:

5 N-[2'(RS)-benzyl-3' -mercaptopro pionyl]-D-proline (E16)

The S-acetyl derivative E15 (87 mg, 0.26 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp. gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with further brine, dried over sodium sulphate and evaporated to afford the title compound, as a diastereomeric mixture, as a crisp foam (70 mg, 92 %). \( \nu_{\text{max}} (\text{CHCl}_3) \) 1753, 1720, 1630, 1579, 1452 \( \text{cm}^{-1} \). 

Example 17:

3-[S-Acetyl-2'(RS)-benzyl-3'-mercapto pro pionyl]-4(S)-carboxythiazolidine (E17)

To a cooled (0°), stirred solution of S-acetyl-2-benzyl-3-mercapto propionic acid (238 mg, 1.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (44 mg of a 55% suspension in oil, 1.0 mmol). The reaction mixture was allowed to reach room temperature and stirring was continued for a further 15 mins. The suspension was then recooled (0°) and treated with oxalyl chloride (105 ul, 1.2 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0°) suspension of 4(S)-carboxythiazolidine (Howard-Lock et al., Can. J. Chem. 1986, 64, 1215) (133 mg, 1.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (278 ul, 2.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 3 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate. Evaporation of the solvent afforded crude product as an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded recovered substituted propionic acid starting material (120 mg, 50%) followed by the title
compound as a crisp foam (103 mg, 29%). $\delta_H$ (CDCl$_3$) 2.35 (3H, s), 2.75-3.25 (7H, overlapping m), 3.74 (d, J 8.1 Hz) and 3.86 (1H, d, J 8.5 Hz), 4.39 (d, J 8.1 Hz) and 4.55 (1H, d, J 8.5 Hz), 4.88 (dd, J 6.7 and 2.7 Hz) and 5.04 (1H, dd, J 6.8 and 4.0 Hz), 7.2-7.3 (5H, m) ppm.

Example 18:

3-[2'/(RS)-benzyl-3'-mercaptopropionyl]-4(S)-carboxythiazolidine (E18)

The S-acetyl derivative E17 (103 mg, 0.29 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with further brine, dried over sodium sulphate and evaporated to afford the title compound, as a diastereomeric mixture, as a crisp foam (87 mg, 96%). $\nu_{max}$ (CHC1$_3$) 1742, 1517, 1642, 1612, 1423 cm$^{-1}$. $\delta$H (CDCl$_3$) 2.35 (3H, s), 7.5-3.25 (7H, overlapping m), 3.74 (d, J 8.1 Hz) and 3.86 (1H, d, J 8.5 Hz), 4.39 (d, J 8.1 Hz) and 4.55 (1H, d, J 8.5 Hz), 4.88 (dd, J 6.7 and 2.7 Hz) and 5.04 (1H, dd, J 6.8 and 4.0 Hz), 7.2-7.3 (5H, m) ppm.

Example 19:

3-[2'(R)-benzyl-3'-mercaptopropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E19)

A stirred solution of the carboxylic acid isomers (E1) from Example 1 (1.84g, 4.83 mmol) in dry DMF (20 ml) was treated with potassium carbonate (0.334g, 2.42 mmol) and chloroacetone (0.80ml, 0.93g, 10.0 mmol) and left to stir at room temperature overnight. The reaction mixture was then diluted with ethyl acetate and washed with 1M hydrochloric acid followed by six water washes. The organic layer was dried over anhydrous magnesium sulphate and the solvent removed under reduced pressure to afford the acetyl esters of compound E1 as a diastereomeric mixture (1.97g). Chromatography on silica gel, eluting with 25% ethyl acetate / hexane, afforded the (2'R, 4S)-isomer (E19A) as a colourless oil (0.87g, 41%). $\nu_{max}$ (CHCl$_3$) 1757, 1734, 1687, 1645, 1417 cm$^{-1}$. $\delta$H (CDCl$_3$) 1.47 (3H, s), 1.61 (3H, s), 2.18 (3H, s), 2.34 (3H, s), 2.85 - 3.15 (5H, overlapping m), 4.02 (1H, d, J 8.6 Hz), 4.55 (1H, s), 4.60 (1H, d, J 8.6 Hz), 4.62 (1H, d, J 16.8 Hz), 4.74 (1H, d, J 16.8 Hz), 7.25 (5H, m) ppm. EIMS M$^+$ 437.1337; required for C$_{21}$H$_{27}$NO$_5$S$_2$, 437.1331. Continued elution afforded the (2'S, 4S)-isomer (E19B) as an oil (0.98g, 46%). $\nu_{max}$
(CHCl$_3$) 1757, 1734, 1687, 1645, 1417 cm$^{-1}$. $\delta_H$ (CDCl$_3$) 1.17 (3H, s), 1.44 (3H, s), 2.19 (3H, s), 2.36 (3H, s), 2.85 - 3.15 (5H, overlapping m), 3.93 (1H, d, J 8.1 Hz), 4.38 (1H, d, J 8.1 Hz), 4.60 (1H, d, J 8.1 Hz), 4.65 (1H, d, J 16.8 Hz), 4.74 (1H, d, J 16.8 Hz), 7.25 (5H, m) ppm. EIMS M$^+$ 437.1337; required for C$_{21}$H$_{27}$N$_2$O$_5$S$_2$, 437.1331.

The acetonyl ester E19A (72 mg, 0.165 mmol) was dissolved in acetonitrile (4ml) and treated with water (1ml) and 0.1M potassium hydroxide solution (3.30ml, 0.330 mmol) and stirred at room temperature for 5 hours. The reaction mixture was then acidified with dilute hydrochloric acid and partitioned between ethyl acetate and brine. The organic layer was washed with further brine and dried over anhydrous magnesium sulphate. Removal of the solvent afforded an oil which was chromatographed on silica gel. Elution with 10% methanol / chloroform, containing 0.1% acetic acid, afforded the desired product which was contaminated with a little S-acetyl compound from incomplete hydrolysis. This product was treated with water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp. gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with further brine, dried over sodium sulphate and evaporated to afford the title compound as a colourless oil (40mg, 73%). $\nu_{max}$ (CHCl$_3$) 1751, 1722, 1643, 1418 cm$^{-1}$. $\delta_H$ (CDCl$_3$) 1.48 (3H, s), 1.60 (3H, s), about 1.6 (1H, m, obscured), 2.59 (1H, m), 2.75 - 3.15 (4H, overlapping m), 4.21 (1H, d, J 8.0 Hz), 4.64 (1H, s), 4.82 (1H, d, J 8.0 Hz), 7.2-7.3 (5H, m) ppm. EIMS M$^+$ 339. DCIMS MH$^+$ 340.

Example 20:

3-[2'(S)-benzy1-3'-mercaptopropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E20)

The acetonyl ester E19B (0.82g, 1.88mmol) of Example 19 in acetonitrile (40ml) was treated with 0.1M aqueous potassium hydroxide solution (37.6ml, 3.76mmol) and stirred at room temperature for 5.5 hours. Work-up and purification were as for the (R) - isomer above. Chromatography afforded the title compound as a crisp foam (372mg, 58%). $\nu_{max}$ (CHCl$_3$) 1749, 1720, 1640, 1421 cm$^{-1}$. $\delta_H$ (CDCl$_3$) 1.18 (3H, s), 1.43 (3H, s), 1.94 (1H, dd, J 11.8 and 6.2 Hz), 2.50 (1H, ddd, J 11.8, 11.8, 2.4 Hz), 2.88 (2H, overlapping m), 3.12 (2H, overlapping m), 4.03 (1H, d, J 8.1 Hz), 4.38 (1H, s), 4.85 (1H, d, J 8.1 Hz), 7.2-7.3 (5H, m) ppm. EIMS M$^+$ 339. DCIMS MH$^+$ 340.
Example 21:

3-[3'-Mercapto-2'(R)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine (E21)

A stirred solution of the S-acetyl derivative E13 obtained in Example 13 (450 mg, 1.47 mmol) in dry DMF (5 ml) was treated with potassium carbonate (102 mg, 0.738 mmol) and chloroacetonitrile (0.235 ml, 273 mg, 2.95 mmol) and the mixture stirred overnight at room temperature. The reaction mixture was then diluted with ethyl acetate and washed with 1M hydrochloric acid followed by six water washes. The organic layer was dried over anhydrous magnesium sulphate and the solvent removed under reduced pressure to afford the acetyl esters of compound E13 as a diastereomeric mixture (0.53g).

The acetyl ester mixture (388 mg, 1.07 mmol) was then dissolved in acetonitrile (2 ml) containing concentrated ammonia solution (0.8 ml, sp. gr.0.88) and water (1.2 ml) and stirred for 1 hour. The reaction mixture was acidified with 5M hydrochloric acid solution and extracted with ethyl acetate. The organic layer was washed with water and saturated brine, dried over magnesium sulphate, and the solvent removed to afford an oil which was chromatographed on silica gel. Elution with ethyl acetate / hexane (1:2) gave the (2'S, 4S)-isomer E21B (56 mg, 16%). $\nu_{max}$ (CHCl$_3$) 1756, 1736, 1648, 1459, 1417 cm$^{-1}$. $\delta_H$ (CDCl$_3$) 1.20 (3H, d, J 6.4 Hz), 1.56 (3H, s), 1.64 (3H, s), 1.81 (1H, dd, J 10.9 and 6.6 Hz), 2.18 (3H, s), 2.41 (1H, dd, J 10.9 and 8.4 Hz), 2.90 (2H, overlapping m), 4.55 (1H, s), 4.62 (1H, d, J 16.8 Hz), 4.71 (1H, d, J 8.7 Hz), 4.85 (d, J 16.8 Hz) and 5.00 (1H, d, J 8.7 Hz) ppm. EIMS M$^+$ 319.0909; required for C$_{13}$H$_{21}$NO$_4$S$_2$, 319.0912. Continued elution gave the (2'R, 4S)-isomer E21A (65 mg, 19%). $\nu_{max}$ (CHCl$_3$) 1756, 1737, 1648, 1461, 1417 cm$^{-1}$. $\delta_H$ (CDCl$_3$) 1.20 (3H, d, J 6.4 Hz), 1.55 (3H, s), 1.55 (1H, m), 1.64 (3H, s), 2.17 (3H, s), 2.47 (1H, dd, J 9.5 and 8.4 Hz), 2.90 (2H, overlapping m), 4.61 (1H, d, J 16.8 Hz), 4.64 (1H, s), 4.76 (1H, d, J 8.3 Hz), 4.81 (d, J 16.8 Hz) and 4.91 (1H, d, J 8.3 Hz) ppm. EIMS M$^+$ 319.0909; required for C$_{13}$H$_{21}$NO$_4$S$_2$, 319.0912.

The acetyl ester E21A (87 mg, 0.273 mmol) in acetonitrile (4 ml) and water (1 ml) was treated with 0.1M potassium hydroxide solution (2.7 ml, 0.27 mmol) added 0.5 ml at a time over 2 hours. After a total reaction time of 6 hours the reaction mixture was acidified with dilute hydrochloric acid and extracted with ethyl acetate. The organic layer was dried over magnesium sulphate and the solvent removed to afford an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded the title compound as a gum (34 mg, 47%). $\nu_{max}$ (CHCl$_3$) 1752, 1719, 1647, 1457, 1419 cm$^{-1}$. $\delta_H$ (CDCl$_3$) 1.21 (3H, d, J 6.3 Hz), 1.51 (3H, s), 1.51 (1H, obscured), 1.60 (3H, s), 2.47 (1H, dd, J 9.3 and 6.3 Hz).
Example 22

3-[S-Acetyl-3'-mercapto-2'(S)-methylpropionyl]-4(R)-carboxy-5,5-dimethylthiazolidine (E22)

To a cooled (0°C), stirred solution of S-acetyl-3-mercapto-2(S)-methylpropionic acid (162 mg, 1.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (44 mg of a 55% suspension in oil, 1.0 mmol). The reaction mixture was allowed to reach room temperature and stirring was continued for a further 15 mins. The suspension was then recooled (0°C) and treated with oxalyl chloride (105 ul, 1.2 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0°C) suspension of 4(R)-carboxy-5,5-dimethylthiazolidine (161 mg, 1.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (278 ul, 2.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 3 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate. Evaporation of the solvent afforded crude product as an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded recovered substituted propionic acid starting material followed by, on elution with 10% methanol in chloroform containing 0.1% acetic acid, the title compound as a colourless oil (164 mg, 54%). δH (CDCl3) 1.24 (3H, d, J 6.4 Hz), 1.51 (3H, s), 1.61 (3H, s), 2.34 (3H, s), 2.95 (2H, overlapping m), 3.10 (1H, dd, J 13.0 and 7.6 Hz), 4.56 (1H, s), 4.72 (1H, d, J 8.5 Hz) 4.83 (1H, d, J 8.5 Hz) ppm.

Example 23

3-[Mercapto-2'(S)-methylpropionyl]-4(R)-carboxy-5,5-dimethylthiazolidine (E23)

The S-acetyl derivative E22 (164 mg, 0.54 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with
further brine, dried over sodium sulphate and evaporated to afford the title compound as a colourless oil (139 mg, 100%). v_max (CHCl_3) 1748, 1720, 1650, 1459, 1421 cm^-1. δ_H (CDCl_3) 1.23 (3H, d, J 6.4 Hz), 1.53 (3H, s), 1.53 (1H, obscured), 1.61 (3H, s), 2.49 (1H, dd, J 9.5 and 8.5 Hz), 2.88 (2H, overlapping m), 4.63 (1H, s), 4.75 (1H, d, J 5.83 Hz), 4.92 (1H, d, J 8.3 Hz) ppm. EIMS M+ 263.0648; required for C_{10}H_{17}NO_3S_2, 263.0650.

Example 24:
N-[S-Acetyl-2'(RS)-benzyl-3'-mercaptopropionyl]-L-proline (E24)

To a cooled (0°), stirred solution of S-acetyl-2-benzyl-3-mercaptopropionic acid (238mg, 1.0 mmol) in dry tetrahydrofuran (8 ml) containing dry dimethylformamide (1 drop), was added sodium hydride (44 mg of a 55% suspension in oil, 1.0 mmol). The reaction mixture was allowed to reach room temperature and stirring was continued for a further 15 mins. The suspension was then recooled (0°) and treated with oxalyl chloride (105 ul, 1.2 mmol) and stirred at room ambient temperature for 30 mins. The resulting mixture was then evaporated in vacuo and the residue suspended in dry tetrahydrofuran (5 ml). The filtered solution was taken to dryness once more to afford the acid chloride as an oil.

A stirred, cooled (0°) suspension of L-proline (115 mg, 1.0 mmol) in dry tetrahydrofuran (10 ml) was treated with triethylamine (278 ul, 2.0 mmol) followed by a solution of the above acid chloride in dry tetrahydrofuran (5 ml) added over 5 minutes. The mixture was stirred at ambient temperature for 3 hours and then partitioned between ethyl acetate and 1M hydrochloric acid. The organic layer was washed with water and saturated brine and dried over anhydrous sodium sulphate.

Evaporation of the solvent afforded crude product as an oil which was chromatographed on silica gel. Elution with 4% methanol in chloroform, containing 0.1% acetic acid, afforded recovered substituted propionic acid starting material followed by the title compound as a crisp foam (80 mg, 24%). δ_H (CDCl_3) 1.50 (1H, m), 1.85 (2H, overlapping m), 2.35 (3H, s), 2.50 and 2.66 (1H, m), 2.9-3.3 (7H, overlapping m), 4.38 and 4.57 (1H, dd, J 8.0 and 2.0 Hz), 7.2-7.3 (5H, m) ppm.

Example 25:
N-[2'(RS)-benzyl-3'-mercaptopropionyl]-L-proline (E25)

The S-acetyl derivative E24 (80 mg, 0.24 mmol) was dissolved in water (0.6 ml) and concentrated aqueous ammonia (0.4 ml, sp.gr. 0.88) and stirred at room temperature for 1 hour. The reaction mixture was diluted in ethyl acetate and washed with sufficient dilute hydrochloric acid to acidify the aqueous phase. The two-phase system was treated with saturated brine and the organic layer separated, washed with
further brine, dried over sodium sulphate and evaporated to afford the little compound as a crisp foam (70 mg, 100%). $\nu_{\text{max}} \text{(CHCl}_3\text{)} 1749, 1716, 1633, 1583, 1450 \text{ cm}^{-1}$.  
$\delta_{\text{H}} \text{(CDCl}_3\text{)} 1.6 \text{ (2H, overlapping m)}, 1.85 \text{ (2H, overlapping m)}, 2.28 \text{ and 2.43 (1H, m)}, 2.5-3.1 \text{ (6H, overlapping m)}, 3.41 \text{ and 3.54 (1H, m)}, 4.45 \text{ (dd, J 8.3 and 2.7 Hz)}$ and 4.65 (1H, dd, J 8.3 and 2.3 Hz), 7.2-7.3 (5H, m) ppm. EIMS $M^+$ 293.1085; required for $C_{15}H_{19}NO_3S$, 293.1086.

Example 26:
3-[2'(/S)-benzyl-3'-mercaptopropionyl]-4(/S)-carboxy-5,5-dimethylthiazolidine-1-oxide (E26) (sulphoxide isomer A)

A stirred solution of the S-acetyl acetonyl ester E19B (42 mg, 0.096 mmol, prepared as in Example 19) in dichloromethane (3 ml) was treated with m-chloroperbenzoic acid (21 mg, 0.096 mmol) and stirred for a further 1 hour at ambient temperature. The reaction mixture was then washed with saturated sodium bicarbonate solution, dried over magnesium sulphate, and the solvent removed to afford crude product which was chromatographed on silica gel. Elution with ethyl acetate / hexane (1:1) gave sulphoxide isomer A (32 mg, 72%). $\nu_{\text{max}} \text{(CHCl}_3\text{)} 1758, 1734, 1685, 1656, 1413, 1063 \text{ cm}^{-1}$. $\delta_{\text{H}} \text{(CDCl}_3\text{)} 1.33 \text{ (3H, s), 1.56 (3H, s), 2.19 (3H, s), 2.35 (3H, s), 2.85 - 3.15 (5H, overlapping m), 4.08 (1H, d, J 11.9 Hz), 4.59 (1H, d, J 16.8 Hz), 4.69 (1H, d, J 11.9 Hz), 4.73 (1H, s), 5.04 (1H, d, J 16.8 Hz), 7.25 (5H, m) ppm. APCI -MS $MNH_4^+$ 471. Continued elution gave sulphoxide isomer B (12 mg, 28%). $\nu_{\text{max}} \text{(CHCl}_3\text{)} 1762, 1734, 1685, 1656, 1417, 1063 \text{ cm}^{-1}$. $\delta_{\text{H}} \text{(CDCl}_3\text{)} 1.42 \text{ (3H, s), 2.19 (3H, s), 2.37 (3H, s), 2.85 - 3.15 (5H, overlapping m), 4.14 (1H, d, J 11.2 Hz), 4.25 (1H, d, J 11.2 Hz), 4.58 (1H, s), 4.62 (1H, d, J 16.8 Hz), 4.79 (1H, d, J 16.8 Hz), 7.25 (5H, m) ppm. APCI -MS $MNH_4^+$ 471. The sulphoxide isomer A (31 mg, 0.068mmol) in acetonitrile (1.5ml) was treated with 0.1M potassium hydroxide (1.37ml, 0.137mmol) and stirred at room temperature for 4 hours. The reaction mixture was diluted with ethyl acetate and acidified with dilute hydrochloric acid. The organic layer was washed with water and saturated brine and dried over magnesium sulphate. Removal of the solvent afforded an oil which was treated with concentrated ammonia solution (0.4 ml) and water (0.6 ml) and stirred for 1 hour. A repeat of the above work-up gave the crude product. Chromatography afforded the title compound as a crisp foam (12 mg, 49%). $\nu_{\text{max}} \text{(CHCl}_3\text{)} 1631 \text{ cm}^{-1}$. $\delta_{\text{H}} \text{(CDCl}_3\text{ + few drops MeOD)} 1.25 (3H, s), 1.29 (3H, s), 1.95 (1H, s), 2.43 (1H, d, J 11.4 Hz), 2.8 - 3.1 (4H, overlapping m), 4.02 (1H, d, J 11.0 Hz), 4.38 (1H, s), 4.91 (1H, d, J 11.0 Hz), 7.2-7.3 (5H, m) ppm. ESMS $M^+$ 356.
Example 27
3-[2'(S)-benzyl-3'-mercaptopropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine-1-oxide (sulphoxide isomer B) (E27)

The title compound was prepared by procedures generally described herein.

Example 28
3-[2'(S)-benzyl-3'-mercaptopropionyl]-4(R)-carboxy-5,5-dimethylthiazolidine (E28)

The title compound was prepared from E11 using methods outlined in Examples 19 and 20. NMR identical to E19.

Example 29
3-[2'(R)-benzyl-3'-mercaptopropionyl]-4(R)-carboxy-5,5-dimethylthiazolidine (E29)

The title compound was prepared from E11 using methods outlined in Example 19. NMR identical to E20.

Example 30
3-[2'(R or S)-benzyl-3'-mercaptopropionyl]-4(S)-carboxythiazolidine (E30)

The title compound was prepared from E17 using methods outlined in Examples 19 and 20. EIMS M+ 311.0654, calculated for C_{14}H_{17}NO_{3}S_{2} 311.0650.

Example 31
3-[2'(S or R)-benzyl-3'-mercaptopropionyl]-4(S)-carboxythiazolidine (E31)

The title compound was prepared from E17 using methods outlined in Examples 19 and 20. EIMS M+ 311.0650, calculated for C_{14}H_{17}NO_{3}S_{2} 311.0650.

Example 32
3-[2'(RS)-mercaptomethyl-4'-phenylbutanoy1]-4(S)-carboxy-5,5-dimethylthiazolidine (E32)

A mixture of 2-phenylethylmalonic acid (1.8g), 40% aqueous dimethylamine (1.08ml, 1eq) and 37% aqueous formaldehyde (0.64ml, 1eq) in water (10ml) was stirred at room temperature overnight. After cooling at 0°C the solid was filtered off, washed with water and dried. The white solid was heated at 170°C for 10 minutes and cooled to room temperature. The resulting gum was dissolved in ethyl acetate (20ml), washed with 10% potassium hydrogen sulphate solution (10ml), water (2 x 10ml), saturated brine (10ml), dried (MgSO_{4}) and evaporated to give crude 2-methylene-4-phenylbutanoic acid. δ_{H}(CDCl_{3}) 2.55-2.90 (4H, m, 2 x CH_{2}). 5.65, 6.85 (2H, 2 x s,
The solid was dissolved in thioacetic acid (10 ml) and heated at 100°C for 1 hour. After evaporation the gum was dissolved in ethyl acetate (10 ml) and extracted with saturated sodium hydrogen carbonate solution (2 x 10 ml). The combined extracts were washed with ethyl acetate (2 x 10 ml) and acidified with 10% potassium hydrogen sulphate solution (pH 3). The aqueous layer was extracted with ethyl acetate (2 x 10 ml) and the combined extracts washed with water (2 x 10 ml), dried (MgSO4) and evaporated to yield the title compound as a yellow oil (0.52 g, 24%). 

EIMS M+ 252 DCIMS MnH4+ 270.

b) 3-[2'(RS)-mercaptomethyl-4'-phenylbutanoyl]-4(S)-carboxy-5,5-dimethylthiazolidine

The title compound was prepared from 2-acetylthiomethyl-4-phenylbutanoic acid using methods outlined in Examples 17 and 18. EIMS M+ 353, ESMS M-H 352.

Example 33

3-[2'(RS)-mercaptomethyl-5'-phenylpentanoyl]-4(S)-carboxythiazolidine (E33)

a) 2-Acetylthiomethyl-5-phenylpentanoic acid

3-Phenylpropylmalonic acid (6.14 g) was converted to 2-methylene-5-phenylpentanoic acid (2.1 g, 40%) by the method described in Example 32a). δH(CDC13) 1.88 (2H, m, CH2), 2.37 (2H, t, J 7.6 Hz, CH2), 2.67 (2H, t, J 7.6 Hz, CH2), 5.68 and 6.33 (2H, 2 x s, =NH), 7.26 (5H, m, Ph). The solid was dissolved in thioacetic acid (5 ml) and heated at 100°C for 2 hours. Evaporated to give the title compound (2.9 g, 100%); δH(CDC13) 1.71 (4H, m, 2 x CH2), 2.33 (3H, s, COCH3), 2.64 (3H, m, CH, CH2), 3.08 (2H, m, CH2), 7.27 (5H, m, Ph).

b) 3-[2'(RS)-mercaptomethyl-5'-phenylpentanoyl]-4(S)-carboxythiazolidine

The title compound was prepared from 2-acetylthiomethyl-5-phenylpentanoic acid using methods outlined in Examples 17 and 18. EIMS M+ 339.0966, calculated for C16H21NO3S2 339.0963.

The following compounds are described in the literature as ACE inhibitors:

E4 Saunders et al., J. Computer-Aided Molecular Design 1987, 1. 133
E6 Waller et al., J Med Cem 1993 36 (16) 2390
E8 JP5509060
E14 JP5509060
E24, E25 US4046889
BIOLOGICAL ACTIVITY

I50 screen

The inhibitory activity of the compounds of the invention was measured in 25mM PIPES pH 7 buffer at 10 concentrations (1000, 333, 111, 37, 12.3, 4.1, 1.4, 0.46, 0.15 and 0.05µM) at 37°C using nitrocefin (91µM, final concentration) as the reporter substrate. The assays were performed with a 5 minute preincubation of enzyme and inhibitor and were conducted in the presence of added zinc sulphate (Zn²⁺ 100µM, final concentration). The methodology is described in detail in the following references: Payne et al (1991), J. Antimicrob. Chemother., 28:255; Payne et al (1994), Antimicrob. Agents and Chemother., 38:767.

Results

Compounds of the Examples 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 23, 25, 26, 28, 29, 30, 31, 32 and 33 exhibit I50 values against B. fragilis CfiA metallo-β-lactamase <500µM. I50 values for compounds E2, E20 and E26 were found to be <1µM. Compounds of Examples 19, 21, 27 and captopril (N-(3'-mercapto-2'(S)-methylpropionyl)-L-proline) exhibit weak but significant activity against Xanthomonas maltophilia L-1 and Bacillus cereus II metallo-β-lactamases.

Antibacterial activity of compounds of the invention in combination with carbapenem antibiotics against strains of Bacteroides fragilis which produce metallo-β-lactamase:

[MIC = minimum inhibitory concentration (µg/ml)]

Antibacterial activity of the carbapenems was potentiated as follows:-
<table>
<thead>
<tr>
<th>Strain</th>
<th>metallo-β-lactamase</th>
<th>MIC (µg/ml) alone</th>
<th>MIC of meropenem in the presence of these concentrations (µg/ml) of compound E20</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Compound E20</td>
<td>Meropenem</td>
</tr>
<tr>
<td>B. fragilis 460</td>
<td>CfiA</td>
<td>&gt;512</td>
<td>128</td>
</tr>
<tr>
<td>B. fragilis 262</td>
<td>CfiA</td>
<td>&gt;512</td>
<td>128</td>
</tr>
</tbody>
</table>

Table 1: Compound E20 in combination with meropenem.
Table 2
Compound E2 in combination with imipenem:

<table>
<thead>
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<th>MIC of imipenem in the presence of these concentrations (µg/ml) of compound E2</th>
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Claims

1. A method of treatment of bacterial infections in humans or animals which comprises administering, in combination with a β-lactam antibiotic, a therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof:

   \[
   \begin{align*}
   R_1 & \quad + \\
   R_2 & \quad N \\
   & \quad R_3 \\
   & \quad X \\
   & \quad R_4 \\
   & \quad O \\
   & \quad CO_2R
   \end{align*}
   \]

   (I)

   wherein:
   
   X is S, S(O)_n or CH_2;
   
   n is 1 or 2
   
   R is hydrogen, a salt forming cation or an in vivo hydrolysable ester-forming group;
   
   R_1 and R_2 are each hydrogen or an organic substituent group;
   
   R_3 is hydrogen, (C_1-6)alkyl optionally substituted by up to three halogen atoms, (C_2-6)alkenyl, (C_2-6)alkynyl, aryl, aryl(C_1-6)alkyl, heterocyclyl or heterocyclyl(C_1-6)alkyl; and
   
   R_4 is hydrogen, or an in vivo hydrolysable acyl group.

2. A compound of formula (IA) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof:

   \[
   \begin{align*}
   R_1 & \quad + \\
   R_2 & \quad N \\
   & \quad R_3 \quad X \\
   & \quad R_4 \\
   & \quad O \\
   & \quad CO_2R
   \end{align*}
   \]

   (IA)

   where R_3^1 represents (C_1-6)alkyl substituted by up to three halogen atoms, aryl, aryl(C_1-6)alkyl, heterocyclyl or heterocyclyl(C_1-6)alkyl and X is S.

3. A compound according to claim 2 wherein R_3^1 is optionally substituted benzyl, phenethyl or phenylpropyl.

4. A compound according to claim 2 or 3 wherein R_4 is hydrogen, lower alkylcarbonyl, optionally substituted benzoyl or optionally substituted phenyl lower alkyl carbonyl.
5. A compound according to any of claims 2 to 4 wherein R₁ or R₂ represents hydrogen, (C₁₋₁₀)alkyl, aryl, heterocyclyl or substituted (C₁₋₁₀)alkyl, wherein the substituent may be aryl, heterocyclyl, hydroxy, (C₁₋₁₀)alkoxy, halogen, mercapto, (C₁₋₁₀)alkylthio, heterocyclylthio, amino, (mono or di)-(C₁₋₁₀)alkylamino, (C₁₋₁₀)alkanoylamino, carboxy, or (C₁₋₁₀)alkoxycarbonyl.

6. A compound according to any of claims 2 to 5 wherein R is hydrogen.

7. A compound according to any of claims 2 to 6 wherein the stereochemistry at the carbon atom marked * is D- and the stereochemistry at the carbon atom marked (+) is S.

8. 3-[S-acetyl-2'(RS)-benzyl-3'-mercapto-propiony]l-4(S)-carboxy-5,5-dimethylthiazolidine.

9. 3-[2'(RS)-benzyl-3'-mercapto-propionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.


14. 3-[S-Acetyl-3'-mercapto-propionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.

15. 3-[3'-Mercapto-propionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.

16. 3-[S-Acetyl-3'-mercapto-2'(S)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.

17. 3-[3'-Mercapto-2'(S)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.

18. 3-[S-Acetyl-2'(RS)-benzyl-3'-mercapto-propionyl]-4(R)-carboxy-5,5-dimethylthiazolidine.

19. 3-[2'(RS)-benzyl-3'-mercapto-propionyl]-4(R)-carboxy-5,5-dimethylthiazolidine.

20. 3-[S-Acetyl-3'-mercapto-2'(RS)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.

21. 3-[3'-Mercapto-2'(RS)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.


23. N-[2'(RS)-benzyl-3'-mercapto-propionyl]-D-proline.

24. 3-[S-Acetyl-2'(RS)-benzyl-3'-mercapto-propionyl]-4(S)-carboxythiazolidine.

25. 3-[2'(RS)-benzyl-3'-mercapto-propionyl]-4(S)-carboxythiazolidine.

26. 3-[2'(R)-benzyl-3'-mercapto-propionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.

27. 3-[2'(S)-benzyl-3'-mercapto-propionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.

28. 3-[3'-Mercapto-2'(R)-methylpropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine.
29. 3-[S-Acetyl-3'-mercaptop-2'(S)-methylpropionyl]-4(R)-carboxy-5,5-dimethylthiazolidine.
30. 3-[Mercapto-2'(S)-methylpropionyl]-4(R)-carboxy-5,5-dimethylthiazolidine.
32. N-[2'(RS)-benzyl-3'-mercaptopropionyl]-L-proline.
33. 3-[2'(S)-benzyl-3'-mercaptopropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine-1-oxide. (sulphoxide isomer A)
34. 3-[2'(S)-benzyl-3'-mercaptopropionyl]-4(S)-carboxy-5,5-dimethylthiazolidine-1-oxide (sulphoxide isomer B).
35. 3-[2'(S)-benzyl-3'-mercaptopropionyl]-4(R)-carboxy-5,5-dimethylthiazolidine.
36. 3-[2'(R)-benzyl-3'-mercaptopropionyl]-4(R)-carboxy-5,5-dimethylthiazolidine.
37. 3-[2'(R or S)-benzyl-3'-mercaptopropionyl]-4(S)-carboxythiazolidine.
38. 3-[2'(S or R)-benzyl-3'-mercaptopropionyl]-4(S)-carboxythiazolidine.
39. 3-[2'(RS)-mercaptomethyl-4'-phenylbutanoyl]-4(S)-carboxythiazolidine.
40. 3-[2'(RS)-mercaptomethyl-5'-phenylpentanoyl]-4(S)-carboxythiazolidine.
41. A process for the preparation of a compound of formula (IA) as defined in claim 2, which comprises reacting a compound of formula (II)

\[
\begin{align*}
&W \xrightarrow{+} R_3' \\
&O \xrightarrow{Y} R_1' \\
\end{align*}
\]

with a compound of formula (III)

\[
\begin{align*}
&\text{HN} \\
&\text{X} \\
&\text{CO}_2R_x \\
&R_1' \\
\end{align*}
\]

wherein W is a leaving group, Y is R_4'S or a group convertible thereeto, R_x is R or a carboxylate protecting group and R_1', R_2', R_3' and R_4' are R_1, R_2, R_3' and R_4 or groups convertible thereeto, wherein R, R_1, R_2, R_3' and R_4 are as defined in formula (IA), and thereafter, where necessary, converting Y into R_4'S, R_x, R_1', R_2', R_3' and/or into R, R_1, R_2, R_3' and/or R_4 and optionally inter-convert R, R_1, R_2, R_3' and/or R_4.
42. A compound of formula (IV)
wherein $R^X$ is R or a carboxylate protecting group and $R_1'$, $R_2'$, $R_3'$ and $R_4'$ are $R_1$, $R_2$, $R_3^1$ and $R_4$ or groups convertible thereto and wherein $R^X$ is other than R when $R_1'$, $R_2'$, $R_3'$, and $R_4'$ are $R_1$, $R_2$, $R_3^1$ and $R_4$.

43. A pharmaceutical composition comprising a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, as defined in claim 1, together with a β-lactam antibiotic in a synergistically effective amount and a pharmaceutically acceptable carrier.

44. A pharmaceutical composition comprising a compound according to claim 2 and a pharmaceutically acceptable carrier.

45. A pharmaceutical composition according to claim 44 which additionally comprises a β-lactam antibiotic in a synergistically effective amount.

46. A composition according to claim 43 or 45 wherein the β-lactam antibiotic is a carbapenem selected from imipenem, meropenem, biapenem, BMS181139 ([(4R-[4alpha,5beta,6beta(R*)])-4-[(aminooiminomethyl)amino]ethyl]-3-[(2-cyanoethyl)thio]-6-(1-hydroxyethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid), BO2727 ([(4R-3][3S*,5S*(R*)],4alpha,5beta,6beta(R*)]-6-(1-hydroxyethyl)-3-[[5-hydroxy-3-(methylamino)propyl]-3-pyrrolidinyl]thio)-4-methyl-7-oxo-1-azabicyclo[3.2.0] hept-2-ene-2-carboxylic acid monohydrochloride), ER35786 ((1R, 5S, 6S)-6-[(1(R)-Hydroxymethyl)-2-[2(S)-[1(R)-hydroxy-1-[pyrrolidin-3(R)-yl]methyl]pyrrolidin-4(S)-ylsulfanyl]-1-methyl-1-carba-2-penem-3-carboxylic acid hydrochloride) and S4661 ((1R,5S,6S)-2-[(3S,5S)-(sulfamoylaminomethyl)pyrrolidin-3-yl]thio-6-[(1R)-1-hydroxyethyl]-1-methylcarbapen-2-em-3-carboxylic acid).

47. A compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, as defined in claim 1 for use in the treatment of bacterial infections.

48. The use of a compound of formula (I) or a pharmaceutically acceptable salt, solvate or in vivo hydrolysable ester thereof, in the manufacture of a medicament for the treatment of bacterial infections.

49. A method of treatment of bacterial infections in humans or animals which comprises administering, in combination with a carbapenem antibiotic, a therapeutically effective amount of a metallo-β-lactamase inhibitor.
50. The use of a carbapenem antibiotic in combination with a therapeutically effective amount of a metallo-β-lactamase inhibitor in the manufacture of a medicament for the treatment of bacterial infections.

51. A pharmaceutical composition comprising a metallo-β-lactamase inhibitor together with a carbapenem antibiotic and a pharmaceutically acceptable carrier.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No.
---|---|---
A | EP 0 172 614 A (MITSUBISHI RAYON CO. LTD.) 26 February 1986 see claim 1 | 1-51

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Date of actual completion of the international search 3 December 1996

Date of mailing of the international search report 20.01.97

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016

Authorized officer

Gettins, M
### DOCUMENTS CONSIDERED TO BE RELEVANT

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The metallo β-lactamase from *Bacteroides fragilis* hydrolyses a wide range of β-lactam antibiotics, and is not susceptible to any known β-lactamase inhibitor. The proliferation of the *B. fragilis* bacteria in nosocomial infections, and the report of plasmid-mediated dissemination of the enzyme, underscores the urgency of acquiring structural information for the development of new therapeutic agents. The crystal structure of the enzyme was determined using multilayer wavelength anomalous diffraction at the zinc absorption edge, and subsequently refined at 1.85 Å resolution. The enzyme folds into a four layer a/b/a/b structure with the active site located at the edge of the β-sandwich. The molecule contains a binuclear (Zn²⁺-Zn²⁺) center with one zinc in a tetrahedral coordination, and the other in a pentagonal coordination. A water molecule is shared by both zinc ions, indicating that it is a hydroxide. A second water binds only to the pentagonally coordinated zinc. A variety of bound substrates can be modeled in the active site, providing the structural basis for the wide spectrum profile of the *B. fragilis* β-lactamase. The active site architecture and the models of the bound substrates lead to the proposed catalytic mechanism in which the hydroxide is assumed to be the nucleophilic group. An alternative proposal in which the water molecule that binds to the pentagonally coordinated zinc plays the nucleophilic role is less favored, but cannot be ruled out.

**PS04.02.20 ANALYSIS OF THE VANADIUM DEPENDENT HALOPEROXIDASE FROM CORALLINA OFFICINALIS**

Andrew Dalbj, Cliff Rush, Andrew Willetts, Gideon Davies, Zhigmeu Dauter, Jennifer Littlechild, Departments of Chemistry and Biological Science, University of Exeter UK, Department of Chemistry, University of York, UK, EMBL, DESY, Germany.

Crystals have been grown of the vanadium dependent haloperoxidase from *Corallina officinalis*. The protein is a dodecamer with a subunit mass of 64 kDa. Electron microscopy of the similar enzyme from *Corallina pilulifera* has suggested that it is composed of two stacked hexamers. The enzyme contains no haem and is dependent on vanadium for activity and is particularly thermostable and resistant to organic solvents. It therefore has useful applications in bio-transformations.

Crystals were grown from polyethylene glycol (PEG) 6,000 and 0.4 M potassium chloride by vapour diffusion. Data was collected at the EMBL Hamburg outstation on beamline X11 at a wavelength of 0.92 Å. The crystals diffract beyond 2.0 Å. A data set has been collected to 3.15 Å that is 98 % complete and with an Rmerge of 6.5 %.

The cell parameters are cubic with a = b = c = 310 Å, and the space group is either I23 or I2₂3. This would infer that there are eight molecules per asymmetric unit with a Matthews coefficient of 2.3 Å³/dalton.

With so many molecules in the asymmetric unit the determination of this structure will involve the use of nes averaging. Currently further native data to higher resolution is being collected.

**PS04.02.21 CRYSTALLIZATION AND PRELIMINARY X-RAY ANALYSIS OF A NITRATE REDUCTASE FROM DESULFOVIBRIO DESULFURICANS ATCC 27774**


A nitrate reductase from Desulfovibrio (D.) desulfuricans ATCC 27774 (a sulfate reducing bacteria, that can adapt to nitrate respiration) has been isolated, allowing the elucidation of the enzymatic system required to convert nitrate (through nitrite) to ammonia. D. desulfuricans ATCC 2774 nitrate reductase is a monomeric periplasmic (soluble) enzyme with 74 kDa, containing one [Fe₄S₄] centre and one molybdenum atom per molecule [1].

Crystals of this nitrate reductase were obtained by vapour diffusion with hanging drop technique using 6% (m/v) PEG 10 K as precipitant at pH 6.5 with 0.1M MES. The crystals grow at 20°C or 4°C to an approximate size of 0.3 x 0.3 x 0.5 mm.

The crystals diffract beyond 3.15 Å resolution. Native data sets were collected using a MAR-Research Imaging Plate area detector and graphite monochromated Cu-Kα radiation from an Enraf Nonius rotating anode generator operated at 45kV and 99 mA. Data were processed with Denzo and the space group is P3₁21 with cell parameters a=b=106.9 Å, c= 138.1 Å.

The data set comprised 13201 measurements of 9695 independent reflections in the resolution range 30.0 < d < 3.15 Å, with an overall completeness of 56.9 %, Rmerge(1)= 11.8 %.

Crystal Structure of a Metallo β-Lactamase II From B. Cereus at 1.9Å

Stefan Maria Fabian1, M. Sohli1, T. Wan2, D. J. Payne3, J. H. Bateson3, T. Mitchell1, B. J. Sutton1

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References

Overall topology
The structure is a type II β-lactamase, with an active site cleft (dominated by His257, Leu262, Val264, Asp266 and Ser268) which is similar to those of other β-lactamases.

The enzyme is a homodimer, with a dimeric interface between the two subunits.

The active site contains a Zn metal ion, which is coordinated by four residues: His257, Ser268, Glu261, and Asp266.

The Zn ion is bound by four nitrogen atoms from the side chains of the four coordinating residues.

The enzyme is a homodimer, with a dimeric interface between the two subunits.

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The active site contains a Zn metal ion, which is coordinated by four residues: His257, Ser268, Glu261, and Asp266.
Crystal Structure of the Zinc-Dependent β-Lactamase from Bacillus cereus at 1.9 Å Resolution: Binuclear Active Site with Features of a Mononuclear Enzyme

Sella Maris Fabiane, Maninder K. Sohi, Tommy Wan, David J. Payne, John H. Bateson, Tim Mitchell, and Brian J. Sutton

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Received March 4, 1998; Revised Manuscript Received June 23, 1998

ABSTRACT: The structure of the zinc-dependent β-lactamase II from Bacillus cereus has been determined at 1.9 Å resolution in a crystal form with two molecules in the asymmetric unit and 400 waters (space group P321; Rcryst = 20.8%). The active site contains two zinc ions: Zn1 is tightly coordinated by His86, His88, and His149, while Zn2 is loosely coordinated by Asp90, Cys168, and His210. A water molecule (W1) lies between the two zinc ions but is significantly closer to Zn1 and at a distance of only 1.9 Å is effectively a hydroxide moiety and a potential, preactivated nucleophile. In fact, Asp90 bridges W1 to Zn2, and its location is thus distinct from that of the bridging water molecules in the binuclear zinc peptidases or other binuclear zinc hydrolases. Modeling of penicillin, cephalosporin, and carbapenem binding shows that all are readily accommodated within the shallow active site cleft of the enzyme, and the Zn1-bound hydroxide is ideally located for nucleophilic attack at the β-lactam carbonyl. This enzyme also functions with only one zinc ion present. The Zn1–Zn2 distances differ in the two independent molecules in the crystal (3.9 Å and 4.4 Å), yet the Zn1–W1 distances are both 1.9 Å, arguing against involvement of Zn2 in W1 activation. The role of Zn2 is unclear, but the B. cereus enzyme may be an evolutionary intermediate between the mono- and binuclear metallo-β-lactamases. The broad specificity of this enzyme, together with the increasing prevalence of zinc-dependent metallo-β-lactamases, poses a real clinical threat, and this structure provides a basis for understanding its mechanism and designing inhibitors.

The principal mechanism by which bacteria develop resistance to penicillins, cephalosporins, and other related β-lactam antibiotics involves the production of enzymes known as β-lactamases which inactivate these compounds (1). The β-lactamases cleave open the essential β-lactam ring, rendering the antibiotics incapable of binding to their target proteins, which are the enzymes involved in bacterial cell wall biosynthesis. Four classes of β-lactamase have been characterized on the basis of primary structure, three of which (A, C, and D) contain a catalytic serine residue in their active site. Crystal structures of a number of these active serine enzymes have been determined, and a mechanism-based inhibitor of these enzymes, clavulanic acid, has been developed (2). The fourth class of β-lactamase, class B, comprises metalloenzymes, all of which probably utilize Zn2+ as the natural cofactor, although activity with other divalent ions has been reported (3). One characteristic of the class B enzymes is their broad substrate profile, which includes not only the penicillins and cephalosporins but also the more recently introduced carbapenems. This, together with their increasing incidence among clinically important microorganisms, and the fact that inhibitors of the serine enzymes are ineffective against the metallo-β-lactamases, have led to a realization that the metallo-β-lactamases now pose a real threat to the future efficacy of existing antibiotics (4). Inhibitors of this class of β-lactamase are now urgently required.

The first and for a long time the only member of class B was the zinc-dependent enzyme from Bacillus cereus (5), a protein of 227 residues (6, 7), originally known as β-lactamase II since this organism produces another β-lactamase of class A (8). However, metallo-β-lactamases have since been reported and sequenced from Bacteroides fragilis, Aeromonas hydrophila, Xanthomonas maltophilia, and Seratia marcescens (9 and references therein), as well as others for which sequences are not yet available (4). All those for which sequences are known appear to be related, if distantly, but it may be that other classes of metallo-β-lactamase do exist. The B. cereus enzyme was shown in early studies to bind two zinc ions, one with an affinity ≈1 μM (3) and the other ≈24 mM (10), although only one zinc ion was
Structure of the B. cereus Zinc \( \beta \)-Lactamase

apparently required for activity (3). Substitution of the zinc ions by Co\(^{2+}\), Cd\(^{2+}\), Mn\(^{2+}\), or Hg\(^{2+}\) led to much lower, but still significant levels of activity (3), and the first reported crystallization of the enzyme was in the presence of Cd\(^{2+}\) (I).

The structure of the zinc-containing \( \beta \)-lactamase from B. cereus has recently been solved at 2.5 Å resolution (12) in the same crystal form, space group C2, as that of the Cd\(^{2+}\) enzyme (11). One zinc ion was found at the active site. More recently, however, the structure of the homologous zinc-containing \( \beta \)-lactamase from B. fragilis was determined in two different space groups at 1.85 and 2.0 Å resolution (13, 14), and in this enzyme two zinc ions were found in the active site. We report here the independent structure determination of the \( \beta \)-lactamase from B. cereus at 1.9 Å resolution in a new crystal form, P3\(_{3}121\), with two molecules in the asymmetric unit. Each of the two active sites contains two zinc ions. We therefore propose a mechanism of \( \beta \)-lactam hydrolysis for the B. cereus enzyme involving the two zinc ions, although it appears that the enzyme can function in the presence of either one or two zinc ions.

MATERIALS AND METHODS

Crystallization. Metallo-\( \beta \)-lactamase II from B. cereus 569/H (Public Health Laboratory Service, Porton Down, U.K.) was crystallized using the hanging drop vapor diffusion technique. Lyophilized protein as supplied was dissolved to a concentration of 2 mg/mL and 500 \( \mu \)M ZnSO\(_4\) in 10 mM Tris-HCl buffer at pH 7.0 with 0.125% (w/v) sodium azide. The reservoir solution contained 500 \( \mu \)L of 10 mM Tris-HCl buffer, adjusted to pH 4.5 with HCl, and 0.125% (w/v) sodium azide, with 70%-75% (w/v) saturated ammonium sulfate. The hanging drops consisted of 3 \( \mu \)L of protein solution and 3 \( \mu \)L of reservoir solution diluted 2-fold with 100 mM Tris-HCl at pH 4.5 containing azide. The drops were kept at 18-20 °C, and large single crystals appeared within 1-2 weeks, by which time the pH of the drop had stabilized at 5.2 ± 0.1. The space group was found to be P3\(_{3}121\), and crystal density measurements (1.42 ± 0.01 g/cm\(^3\)) and \( V_0 \) ratio calculation (2.4 Å\(^3\)/Da) indicated two molecules in the asymmetric unit.

Data Collection and Processing. X-ray data to 2.5 Å resolution were collected using a RAXIS-IIC imaging plate detector mounted on a Rigaku RU200HB rotating anode (\( \lambda = 1.54 \) Å) and to 1.9 Å using a Mar 30 cm imaging plate area detector on station 9.6 at the Synchrotron Radiation Source, Daresbury Laboratory, U.K. (\( \lambda = 0.87 \) Å) at temperatures of 18 °C (capillary mounted) and -138 °C, respectively (flash frozen with 18% glycerol in mother liquor). The X-ray intensities were processed using DENZO (15) and the CCP4 (16) programs ROTAPREP, SORTMTZ, SCALA (17), AGROVATA, and TRUNCATE (18). The statistics for data collection and processing are presented in Table 1.

Structure Determination and Refinement. The structure was solved by multiple isomorphous replacement with data from two derivatives (Table 2) combined with solvent flipping and noncrystallographic symmetry averaging. The difference Patterson map at 3.5 Å for the Pt derivative, and difference Fourier maps, revealed four Pt binding sites that were refined with MLPHARE (19). These four sites provided the initial phasing power (Table 2) with an overall figure of merit of 0.37. The program SOLOMON (20) was then used to perform solvent flipping and automatic mask calculation. At this stage three a-helices and nine b-strands were identified for each molecule, and the program O (21) was used to create and edit a C\(_{0}\) skeleton. The noncrystallographic symmetry (NCS)\(^2\) matrix was obtained by visual inspection and improved using IMP [part of the RAVE package (22, 23)], with a correlation coefficient of 54.1% between the two molecules. Solvent flipping was performed again, combined with NCS averaging, and the correlation coefficient for the electron density inside the two NCS-related masks improved from 37.1% to 81.9%. A polyglycine model for the identifiable regions of secondary structure was refined with X-PLOR (24); 6% of the reflections were omitted and used for monitoring the progress of refinement [R\(_{free}\) test set (25)]. Phases calculated from the model were combined with the Pt-derived phases, and after solvent flipping and NCS averaging, a polyalanine model was built for the same regions as before and refined. The phases from this model were used to calculate a difference Fourier map with the uranyl derivative, and five sites were located. The Pt and U sites were refined with MLPHARE (combined figure of merit 0.47) and then further combined with phases from the polyalanine model for another round of solvent flipping and NCS averaging. At this stage, data collected from an EDTA-soaked crystal were used to calculate a difference Fourier map in which one zinc atom could be located per molecule; this enabled residues 153-227 to be traced, retaining the remainder as polyalanine. This new model was refined and used for a further round of solvent flipping and NCS averaging, after which the whole region from residue 40 to residue 227 could be traced. At this point, data to 2.65 Å

---

Table 1: Native Data Collection and Processing Statistics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P3(_{3}121)</th>
<th>P3(_{3}121)</th>
</tr>
</thead>
<tbody>
<tr>
<td>space group</td>
<td>C2</td>
<td>C2</td>
</tr>
<tr>
<td>unit cell</td>
<td>( a = b (\AA) )</td>
<td>68.1</td>
</tr>
<tr>
<td></td>
<td>( c (\AA) )</td>
<td>180.9</td>
</tr>
<tr>
<td>resolution limit (( \AA ))</td>
<td>2.5</td>
<td>1.9</td>
</tr>
<tr>
<td>no. of unique reflections</td>
<td>16104</td>
<td>37064</td>
</tr>
<tr>
<td>average redundancy ( ^a )</td>
<td>4.1</td>
<td>2.3</td>
</tr>
<tr>
<td>completeness overall ( ^b ) [outer shell]</td>
<td>99.4</td>
<td>99.7</td>
</tr>
<tr>
<td>( R_{merge} ) [outer shell, 1.95-1.9 Å]</td>
<td>4.5</td>
<td>5.4 [28.9]</td>
</tr>
</tbody>
</table>

\(^a\) Redundancy = (number of measurements)/(number of independent reflections). \(^b\) Completeness = 100(number of independent reflections measured)/theoretical maximum number of reflections.

---

Table 2: Statistics for the Isomorphous Derivative Data Sets

<table>
<thead>
<tr>
<th>Derivative</th>
<th>KCl, Tris</th>
<th>UO(_2)(NO(_3))(_2), Tris</th>
<th>EDTA, Tris</th>
</tr>
</thead>
<tbody>
<tr>
<td>soak conditions</td>
<td>4 mM, 3 days, pH 6.0</td>
<td>2.5 mM, 3 days, pH 4.5</td>
<td>0.25 mM, 6 days, pH 4.5</td>
</tr>
<tr>
<td>resolution limit (( \AA ))</td>
<td>3.3</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>completeness (%)</td>
<td>99.9</td>
<td>99.9</td>
<td>93.6</td>
</tr>
<tr>
<td>( R_{merge} ) (%)</td>
<td>5.8</td>
<td>8.3</td>
<td>8.1</td>
</tr>
<tr>
<td>( R_{free} ) (%)</td>
<td>23.8</td>
<td>18.0</td>
<td>27.9</td>
</tr>
<tr>
<td>phasing power ( ^c ) [no. of sites]</td>
<td>1.61</td>
<td>1.00</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^c\) Phasing power = \( \langle F_{obs} \rangle / \langle E \rangle \), where \( E \) is the lack of closure error.

---

Abbreviations: NCS, noncrystallographic symmetry; rms, root mean square; \( P_{on} \) and \( P_{off} \), observed and calculated structure factors.
FIGURE 1: Stereoview of the environment of Asp56, the only residue in a forbidden region of the Ramachandran plot. SIGMAA-weighted 2Fo − Fc electron density map (all atoms included) at 1.9 Å resolution is contoured at 1.2σ. Four hydrogen bonds stabilize the strained backbone conformation of Asp56, one of which links Asp56 to Arg91, immediately beneath the catalytic zinc ions. The image was drawn using the program QUANTA (27).

were included, and after the subsequent refinement cycle residues 7–32 could be traced.

The structure was refined using X-PLOR and manual model building performed with O. The first rounds of refinement used strict NCS, but subsequent refinement used restrained NCS, gradually decreasing the weights to 40 kcal/mol for protein atoms and 20 kcal/mol for nonbonded atoms (zinc, sulfate, and water molecules). The upper resolution limit for reflections throughout refinement was 8 Å. After the inclusion of data to 2.5 Å and further refinement, the model for each molecule contained 215 of the 227 residues, 1 zinc ion, 2 sulfate ions, and 19 water molecules, plus a further 62 water molecules not related by NCS. No electron density was visible for residues 1–6 or 33–37. The N-terminus of the enzyme was known to be “ragged” (26), but mass spectroscopic analysis of crystals revealed that selective crystallization of a form lacking residues 1–4 had occurred; thus only the two N-terminal residues 5 and 6 are in fact disordered. The disordered loop 33–37 will be discussed below. At this stage Rfree was 26.8% and Rcryst (calculated on the working set of reflections) was 19.2%. The data set to 1.9 Å was then used, and after five rounds of refinement the NCS restraints were removed entirely. Further water molecules (total 400) were identified with QUANTA (27), as well as a second zinc ion, interpreted earlier at 2.5 Å resolution as a sulfate ion. The final Rfree was 27.9%, and Rcryst (calculated on all reflections after the final round of refinement) was 20.8%. The van der Waals radius used for the zinc ion was 1.15 Å. Larger radii were tested but led to W1 moving out of electron density; lower values did not affect the refined atomic position. No constraints were placed on any atoms, except for the NCS constraints in the early stages of refinement. For the final five rounds of refinement the electrostatic energy term was switched off.

The geometry of the model is good as assessed by PROCHECK (28), although one residue, Asp56, lies in a forbidden region of the Ramachandran plot (φ = 74.5°, ψ

<table>
<thead>
<tr>
<th>Table 3: Refinement Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>resolution range (Å)</td>
</tr>
<tr>
<td>no. of reflections</td>
</tr>
<tr>
<td>no. of protein atoms per asymmetric unit</td>
</tr>
<tr>
<td>no. of water molecules per asymmetric unit</td>
</tr>
<tr>
<td>no. of sulfate ions per asymmetric unit</td>
</tr>
<tr>
<td>no. of zinc atoms per asymmetric unit</td>
</tr>
<tr>
<td>Rfree (%)</td>
</tr>
<tr>
<td>Rcryst (%)</td>
</tr>
<tr>
<td>average B factor for protein atoms (Å²)</td>
</tr>
<tr>
<td>average B factor for water molecules (Å²)</td>
</tr>
<tr>
<td>rms deviation, bond lengths (Å)</td>
</tr>
<tr>
<td>rms deviation, bond angles (deg)</td>
</tr>
<tr>
<td>rms deviation, dihedral angles (deg)</td>
</tr>
<tr>
<td>rms deviation, improper torsion angles (deg)</td>
</tr>
<tr>
<td>rms deviation between the NCS-related molecules (Å, all atoms)</td>
</tr>
</tbody>
</table>

Overall Architecture. The structure consists of a sandwich of two twisted β-sheets, each flanked on their outer face by two α-helices, with a fifth helix bridging the two sheets (Figure 2). This fold is exactly as reported by Carfè et al. (12) (rms deviation between backbone atoms = 0.46 Å) and similar also to that of the B. fragilis enzyme (13, 14) (rms deviation between backbone atoms = 1.18 Å). The active site, identified in Figure 2 by the two zinc ions shown in green, lies at one edge of the β-sheet sandwich, and the ligand residues belong to segments of polypeptide chain which form extended loops connecting the secondary structural elements.
There is a degree of similarity between this fold and that of the N-terminal nucleophile (Ntn) aminohydrolases (36). While the architecture of the Ntn hydrolase family, which catalyzing includes the enzyme penicillin acylase (31), consists of a sandwich of two five-stranded β-sheets flanked on each side by two α-helices, the connectivities between the strands and helices are quite different from that in the metallo-β-lactamase. Any similarity therefore would appear to be merely an example of convergence to a stable arrangement of secondary structural elements.

**Noncrystallographic Symmetry.** The NCS axis is an almost exact 2-fold (179.5° rotation and no translation) as shown in Figure 3, and intermolecular contact occurs between the two C-terminal α-helices (α4 and α5). These helices pack head to tail, and intermolecular hydrogen bonds between the peptide units form, in effect, a continuous helical structure spanning the two molecules. The active sites of the two enzyme molecules are brought within 24 Å of each other in the crystal through this interaction (Figure 3), but there is no evidence for dimerization of the enzyme in solution.

**The Active Site.** The active site contains two zinc ions, liganded by residues that are almost entirely conserved in all the known metallo-β-lactamase sequences. Zn1 [which corresponds to the single zinc ion identified by Carli et al. at 2.5 Å resolution (13)] is liganded by the three histidine residues, 86, 88, and His149, and a water molecule (W1) in a tetrahedral arrangement. Both His86 and 149 use the Ne2 atom of the imidazole ring, as is commonly observed, whereas His88 interacts via the N81 atom. The electron density corresponding to this region is shown in Figure 4. The second zinc ion is liganded by Asp90, Cys168, and the Ne2 atom of His210, together with two water molecules, W1 and W2, providing 5-fold coordination in a distorted trigonal bipyramidal arrangement (Figure 4). W1, which might appear to bridge the two zinc ions, lies very much closer to Zn1 than to Zn2 (Figure 4 and Table 4), and the two zinc ions are in fact bridged by W1 and Asp90 in series.

In this respect the active site differs strikingly from those of the binuclear zinc peptidases (32–35) and other hydrolases (36–39), in which a water molecule and a protein side chain (carbamate) each independently bridge the two zinc ions in parallel.

While the structures of the two independently refined active sites are essentially identical, two interatomic distances differ significantly (Table 4). These are the Zn1–Zn2 and the Zn2–W1 distances, which differ by 0.5 and 0.6 Å, respectively, substantially greater than the mean coordinate error of 0.3 Å (see Materials and Methods) and in contrast to all of the other Zn–ligand distances, which differ by less than 0.2 Å between the two molecules. In Figure 5, the two active site structures are superimposed, by best-fitting Zn1 and its ligands. It is clear that the difference between the two sites is a shift of Zn2, together with His210 and W2, away from Zn1. This contrast between virtually identical Zn1 sites, and conformationally variable Zn2 sites with "looser" arrangement of ligands, is consistent with their different affinities for zinc and is also reflected in the temperature factors (Zn1, 31 and 32 Å²; Zn2, 53 and 62 Å²), refined assuming full occupancy. Another difference that can be seen in Figure 5 is the conformation of Lys171, a residue conserved in all but the *X. maltophilia* enzyme, which forms a weak hydrogen bond to W2 (Table 4).

W1 is very closely associated with Zn1 (B factors, 26 and 26 Å², cf. 41 and 32 Å² for W2), and significantly, the movement of Zn2 away from Zn1 by 0.5 Å in molecule B relative to molecule A has no effect upon the Zn1–W1 distance, which remains at 1.9 Å in both molecules (Table 4). This is as short as the Zn–O bond lengths in tetrahedrally coordinated hydroxy salts (2.0 Å) or zinc oxide (40) (1.95 Å) and must be a hydroxide moiety. It also forms a hydrogen bond to Asp90 (2.8 Å in both molecules, Table 4), which in turn forms a hydrogen bond to Zn2 (2.8 and 2.9 Å).

The two zinc ions lie in a shallow cleft, both readily accessible to solvent. One side of this cleft is formed by
residues Lys176 to Asp183, of which Gly79 and Asn180 are conserved in all known metallo-β-lactamase sequences. Their significance becomes clear when β-lactams are docked into the active site (see below). The two ends of the cleft are apparently open, although the disordered loop region, residues 33–37, lies at one end. No electron density is visible at all for these five residues; the corresponding loop in the B. fragilis enzyme is one-residue longer, and two residues similarly lack electron density (13). As suggested by Concha et al., this loop may interact with substrate (13) or even close over the active site. Chemical modification of one of the disordered loop residues in the B. cereus enzyme, Glu37, was reported to block activity (41), consistent with its proximity to the active site, but mutation of this residue to Gln (42) later showed that it was not essential for activity.

Finally, a buried salt bridge, Arg91–Asp56, lies immediately below the zinc ions in the “floor” of the active site (Figure 1). An extensive hydrogen-bonding network links Arg91 to Asp90 and Gly209; the adjacent residue His210, and Asp90, are Zn2 ligands, and thus Arg91 is involved in shaping the Zn2 site as well as contributing to the electrostatic environment of the active site.

Modeling of Substrate Binding. The shallow, accessible active site cleft allows β-lactam substrates with very different side chains attached to the β-lactam nucleus to be “docked” in a similar way, consistent with the broad activity profile of this enzyme. Substrates were located such that the common carboxylate group of the five- (or six-) membered ring pointed toward the conserved Lys171 and the β-lactam carbonyl pointed between Zn1 and the Nδ of conserved Asn180. Panels a and b of Figure 6 show the proposed binding of benzylpenicillin and mecillinam. In this mode of binding, which is very similar to that proposed by Concha et al. for the B. fragilis enzyme (13), W1 lies immediately beneath the β-lactam carbonyl carbon, ideally located for nucleophilic attack (Figure 6). W2, however, is displaced by the substrate’s carboxylate group, which may interact with, and maintain pentacoordination at, Zn2. In this respect, our model differs from that of Concha et al. (13), in which W2 (their “apical” water molecule), is not displaced; indeed, these authors propose that, with a slight shift of the substrate, W2 could be the nucleophile. Carfi et al. (14) propose a model for substrate binding to the B. fragilis enzyme in which W2 is displaced, but the carboxylate interacts with Zn2 and not Lys171. In the same authors’ model for substrate binding to the monozinc B. cereus enzyme, the carboxylate interacts with His210 (12).

Neither of these models of substrate binding to B. fragilis, nor that of Carfi et al. to the B. cereus enzyme, explains the absolute conservation of Gly79 in all known metallo-β-lactamase sequences, but in the model proposed here it is clear that any side chain at this position would prevent substrate binding (Figure 6). Furthermore, the conformational variability of the side chain of Lys171, as seen in the two molecules in the asymmetric unit, may also be important in allowing the interaction with the conserved carboxylate group to be maintained despite the latter’s slightly different orientation with respect to the β-lactam ring in the different substrates.

Catalytic Mechanism. We propose that water molecule W1, so closely associated with Zn1 that it is best described as a hydroxide, is the preactivated nucleophile that attacks the β-lactam carbonyl carbon atom. In the Michaelis
complex (shown schematically in Figure 7), the β-lactam carbonyl oxygen interacts with Zn1, which expands its coordination number to 5, and serves to polarize the carbonyl bond enhancing its susceptibility to nucleophilic attack. At Zn2, one oxygen atom of the substrate’s conserved carboxylate moiety replaces W2, while the other interacts with Lys171. As hydroxide W1 attacks the β-lactam carbonyl bond, the lone pair of the β-lactam nitrogen atom may interact with Zn2, maintaining its pentacoordination. As the transition state develops, with tetrahedral coordination at the β-lactam carbon atom, Asn180 N3H and Zn1 can stabilize the oxyanion. Asp90, initially hydrogen bonded to the zinc-bound hydroxide, is ideally located to accept the proton from the hydroxide and, as β-lactam bond cleavage occurs, protonate the nitrogen atom. Mutagenesis has demonstrated that this residue indeed performs a critical role in the B. cereus enzyme (43).

Deprotonation of the hydroxide by Asp90 implies the formation of a dianion, and mechanistic studies by Bounaga et al. indicate that a dianionic species is indeed formed, since there is an inverse second-order dependence of reactivity of the B. cereus enzyme upon hydrogen ion concentration at low pH (44). This is in contrast to the mechanism proposed by Concha et al. (13), which does not invoke a dianion; furthermore, Concha et al. suggest that W2 donates the proton to the β-lactam nitrogen (or may even be the nucleophile). These authors do draw attention to the differences between the pH dependence of $k_{\text{cat}}$ for the B. fragilis (13) and B. cereus enzymes at low pH (45), which may reflect real mechanistic differences.

The location of W1 is quite distinct from that of the water molecules that bridge the two zinc ions in the binuclear zinc peptidases (32-35) or in other binuclear zinc hydrolases such as nuclease P1 (36), phospholipase C (37), or phosphodiesterase (38). In all of these structures the bridging water molecule, the presumed nucleophile, is symmetrically placed between the two zinc ions (2.0–2.3 Å from each). The longer W1–Zn2 distances in the B. cereus enzyme (2.5 and 3.1 Å, Table 4) argue against a role for Zn2 in activation of the water molecule. In fact, the location and role of Asp90 resemble the mononuclear zinc peptidases typified by carboxypeptidase A and thermolysin (46), and the mechanism of the B. cereus enzyme thus appears to exhibit features of both the mono- and binuclear zinc enzymes.

**Mechanistic Duality of the B. cereus Metallo-β-lactamase.** It is known that the B. cereus enzyme displays significant activity with only a 1:1 molar ratio of Zn$^{2+}$ to enzyme (3), which, given the very different affinities of the two sites, would lead to occupancy only at Zn1. [We believe that the existence of only one zinc ion in the structure of Carfi et al. (12) is a result of the molar ratio of Zn$^{2+}$ to enzyme of 0.3:1, compared with 6:1 in the present study.] In the absence of Zn2, the hydroxide W1 is expected to occupy the same location as seen in the two-zinc structure, since we observe that movement of Zn2 away from W1 by 0.6 Å in molecule B relative to molecule A (Figure 5 and Table 4) does not affect W1. Asp90 would still be able to deprotonate W1 and might be expected to act as a stronger conjugate base in the absence of Zn2.

What therefore is the role of Zn2? It may interact with substrate as described above or serve to orient Asp90. Certainly the B. fragilis enzyme, with two zinc sites of comparable affinity ($\approx 1 \mu M$), will always contain two zinc ions (13). They are closer together (3.5 Å) than in the B. cereus enzyme (3.9 and 4.4 Å, Table 4), and the water molecule/hydroxide ion is almost symmetrically located between them, as in other binuclear zinc hydrolases (13). Functionally, while the substrate profiles of the two enzymes are similarly broad, the B. fragilis enzyme generally displays a higher catalytic efficiency [$K_{\text{m}50}$ are typically 10–100-fold lower for the B. fragilis enzyme (47)]. Structurally, the Zn2 site in the B. cereus enzyme has a "loose" arrangement of ligands compared with the B. fragilis enzyme (e.g., His210 Ne2–Zn2, 2.5/2.6 and 2.1/2.2 Å, respectively), and Arg91, lying just below Zn2 in the former (Arg91 NH2–Zn2, 4 Å), will lower the affinity for Zn$^{2+}$ at this site. In the B. fragilis enzyme this residue is replaced by cysteine. Both functionally and structurally therefore, the B. cereus enzyme appears
FIGURE 6: Stereoviews of the modeling of (a) benzylpenicillin, and (b) meropenem. Features common to the docked structures are the \( \beta \)-lactam carbonyl oxygen pointing between Zn1 and N\textsubscript{O} of Asn180, the substrate’s carboxylate group pointing toward Lys171, and the displacement of W2. W1 is ideally placed to attack the \( \beta \)-lactam ring. Conservation of Gly179 is clearly required to allow substrate binding. Val139 (bottom left), at the C-terminal side of the disordered loop, is within 4.5 \AA\ of the substrate, indicating the proximity of this flexible loop. The image was drawn using QUANTA (27).

FIGURE 7: Schematic diagram of the proposed Michaelis complex for a penicillin bound to the enzyme. The hydroxide (W1) is activated by Zn1 for nucleophilic attack at the carbonyl carbon; the substrate’s carboxylate group displaces W2 and interacts with Lys171 and Zn2.

to be the more "primitive" and may be an evolutionary intermediate between mononuclear \( \beta \)-lactamases and binuclear enzymes with two high-affinity sites.

The \textit{B. cereus} enzyme thus displays a remarkable adaptability, able to function with either one or two zinc ions, or indeed other metal ions, and capable of hydrolyzing virtually all known \( \beta \)-lactam antibiotics. It is this latter capability which now poses such a serious clinical threat, and the need to design inhibitors of these enzymes is most urgent. Knowledge of this structure will, we hope, facilitate the process.

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REFERENCES

Structure of the B. cereus Zinc β-Lactamase

Crystal Structure of the IMP-1 Metallo β-Lactamase from *Pseudomonas aeruginosa* and Its Complex with a Mercapto carboxylate Inhibitor: Binding Determinants of a Potent, Broad-Spectrum Inhibitor

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ABSTRACT: Metallo β-lactamase enzymes confer antibiotic resistance to bacteria by catalyzing the hydrolysis of β-lactam antibiotics. This relatively new form of resistance is spreading unchallenged as there is a current lack of potent and selective inhibitors of metallo β-lactamases. Reported here are the crystal structures of the native IMP-1 metallo β-lactamase from *Pseudomonas aeruginosa* and its complex with a mercapto carboxylate inhibitor, 2-[5-[(1-tertazolylmethyl)thien-3-yl]-N-[2-(mercaptomethyl)-4-phenylbutyrylglycine]. The structures were determined by molecular replacement, and refined to 3.1 Å (native) and 2.0 Å (complex) resolution. Binding of the inhibitor in the active site induces a conformational change that results in closing of the flap and transforms the active site groove into a tunnel-shaped cavity enclosing 83% of the solvent accessible surface area of the inhibitor. The inhibitor binds in the active site through interactions with residues that are conserved among metallo β-lactamases; the inhibitor’s carboxylate group interacts with Lys161, and the main chain amide nitrogen of Asn167. In the “oxyanion hole”, the amide carbonyl oxygen of the inhibitor interacts through a water molecule with the side chain of Asn167, the inhibitor’s thiolate bridges the two Zn(II) ions in the active site displacing the bridging water, and the phenylbutyryl side chain binds in a hydrophobic pocket (S1) at the base of the flap. The flap is displaced 2.9 Å compared to the unbound structure, allowing Trp28 to interact edge-to-face with the inhibitor’s thiophene ring. The similarities between this inhibitor and the β-lactam substrates suggest a mode of substrate binding and the role of the conserved residues in the active site. It appears that the metallo β-lactamases bind their substrates by establishing a subset of binding interactions near the catalytic center with conserved characteristic chemical groups of the β-lactam substrates. These interactions are complemented by additional non-specific binding between the more variable groups in the substrates and the flexible flap. This unique mode of binding of the mercapto carboxylate inhibitor in the enzyme active site provides a binding model for metallo β-lactamase inhibition with utility for future drug design.

Bacterial resistance to the most common and potent antibiotics has been rising in recent years (1). Selective pressure in hospitals and infant- and child-care facilities is selecting pathogenic microorganisms carrying resistance genes. Re-
Inhibition of Metallo β-Lactamase

are inhibited by the metal chelators EDTA and o-phenanthroline (29). Thiols such as mercuric compounds such as mercaptoethanol, and 2-mercaptoethanol-2-phenylacetamide (31), and others (32) are competitive inhibitors and presumably bind through interactions between the inhibitor's thiolate and the active site metal(s). Recently, the crystal structures of the 4-Bacteroides fragilis enzyme in complex with the buffer morpholinioethane sulfonate (MES) (33) and in complex with a biphenyl tetrazole inhibitor (34) have been reported. However, potent and selective inhibitors that are useful in the treatment of bacterial infections are yet to be reported. The increase in the β-lactam antibiotic resistance that parallels the production of metallo β-lactamases in pathogenic bacteria makes these enzymes an attractive target (3). One approach to overcoming this emerging resistance mechanism would be the design of a metallo β-lactamase inhibitor to be administered in combination with a β-lactam antibiotic. Inhibitors of these enzymes would consequently prolong the effective lifetime of conventional β-lactam antibiotics. Such inhibitors are particularly needed since the metallo β-lactamase inhibitors that are currently used are completely ineffective against the metallo β-lactamases (13, 14). The greatest progress toward potent, broad-spectrum, and selective metallo β-lactamase inhibitors has been reported (see the abstract of the American Society for Microbiology, General Meeting, May 30 to June 3, 1999, Chicago, IL) on a series of mercuriccarboxylate metallo β-lactamase inhibitors.

The crystal structures of the metallo β-lactamases from B. cereus (35, 36) and B. fragilis (33, 34, 37, 38) and the L1 from Stenotrophomonas maltophilia (39) show that the polypeptide chain adopts a fold consisting of four layers (α−β−α) with a central α-sandwich and two α-helices on either side. Located at one edge of the sandwich, the active site has either one or two Zn(II) ions (Zn1 and Zn2) separated by approximately 3.5 Å. In the crystal structures of metallo β-lactamases examined thus far, Zn1 is tetrahedrally coordinated to three histidines and a water molecule (shared between the metals). When present, Zn2 is coordinated to two histidines (a cysteine, a histidine, and an aspartate) and two water molecules in a trinuclear β-sandwich. In Pseudomonas aeruginosa, B. fragilis, and B. cereus, or in the L1 enzyme to an aspartate, two histidines, and two water molecules. The metal coordination geometry, the metal-to-metal distance, and the presence of the shared water are unchanged in the presence of MES (33) or the biphenyl tetrazole (34), both shown to be inhibitors of the B. fragilis enzyme. In the crystal structures of metallo β-lactamases that have a bi-nuclear Zn(II) center, a water molecule (the shared water, also known as the bridging water, or hydroxide) coordinates to both metals. By analogy with other bi-nuclear metal enzymes that have metal-bridging waters, the activation of the shared water, or hydroxide, provides the nucleophile for the reaction (40). Presumably, it is the addition of the hydroxide to the carbonyl carbon of the substrate that leads to the formation of a reaction intermediate that was independently observed by various investigators using spectroscopic methods. Their results suggest that a transient, noncovalent reaction intermediate is formed during the hydrolysis of the substrate nitrocefin catalyzed by the B. cereus (41), the L1 (42), and the B. fragilis (43) enzymes.

Analyses of the metal content of the enzymes from Aeromonas (44), L1 from S. maltophilia (45), B. cereus (11), and B. fragilis (46, 47) indicate that these enzymes have retained the capacity to bind up to 2 mol of metal per mole of protein. In addition, the crystal structures and the amino acid sequence alignment [17−37% sequence identity (37)] indicate that these enzymes have all retained the bimetallic metal binding motif. Functionally, however, they have different metal requirements, and can be divided into three subgroups. The B. fragilis and the L1 enzymes have high-affinity metal binding sites that are required for catalysis (46). The B. cereus enzyme also has two metal binding sites; however, these metals bind with widely different affinities: 2.4 μM and 24 mM (48), and only the high-affinity site, Zn1, is required for near maximal enzymatic activity (11). Crystallization at different pHs has yielded crystal structures of the B. cereus enzyme with one (35) or two metals bound (36, 49). The Aeromonas enzyme defines a third functional subgroup. It has one high-affinity zinc binding site (Ka < 20 nM), and binding of the second, low-affinity site inhibits the enzymatic activity with a Ki of 46 μM (44). This polymorphism in terms of the metal binding and its effect on the catalytic activity of the metallo β-lactamases may represent a rapidly evolving adaptive mechanism.

Imipenem resistance in clinical isolates of P. aeruginosa can be caused by two different mechanisms, either by reduced permeability combined with overproduction of a class C β-lactamase or by the production of the IMP-1 or VIM-1 metallo β-lactamase (27, 50). The IMP-1 enzyme has been shown to be encoded by both plasmids and integrons (51), and these highly mobile genetic elements are thought to be responsible for the rapid spread of IMP-1 in clinical isolates from Japan. Recent data on the epidemiology of IMP-1 in Japan suggest that this metallo β-lactamase will probably be the most clinically significant of all the currently known metallo β-lactamases (52). Presented here is the crystal structure of the IMP-1 metallo β-lactamase from P. aeruginosa free and in complex with a mercuriccarboxylate inhibitor, 2-[5-[(1-tetrazolylmethyl)thien-3-yl]N-2-(mercaptomethyl)-4-phenylbutyrylglycine] (Figure 1). The structural similarity between the β-lactams and this inhibitor and its interactions with conserved residues in the active site suggest the mode of binding used by the β-lactam substrates. The structure shows that three critical interactions provide selective inhibition against metallo β-lactamases: binding
into a hydrophobic pocket, interactions with a conserved lysine, and the metal ion interactions.

**MATERIALS AND METHODS**

**Crystallization.** The mature IMP-1 metallo β-lactamase from *P. aeruginosa* lacking the 18 amino-terminal residues that correspond to the signal sequence was overexpressed in *Escherichia coli* strain BL21(DE3) and purified as previously described (53). The soluble polypeptide includes 228 amino acid residues with a molecular weight of 25,000. Native crystals were obtained by vapor diffusion at room temperature (20–22 °C). They grew as thin plates (~0.4 mm × ~0.5 mm × ≤0.02 mm) after 2–3 weeks in 10 μL sitting drops equilibrated against 500 μL of reservoir solution. The drops were prepared by mixing 5 μL of protein [14 mg/mL in 20 mM HEPES (pH 7.5)] and 5 μL of reservoir solution containing 0.2 M sodium acetate, 30% PEG 4000, and 0.1 M sodium citrate buffer (pH 5.6) (the actual pH of the reservoir solution was 6.5). The crystals belong to space group *P*2₁, with unit cell dimensions *a* = 50.3 Å, *b* = 105.8 Å, *c* = 112.3 Å, and *β* = 93.9°, a tetramer in the asymmetric unit, and an estimated solvent content of 60%. The complex with the mercurycarboxylate inhibitor, 2-[5-(1-tetrazolylmethyl)-3-yl]-N-[2-(mercaptomethyl)-4-(phenylbutyryl)glycine] (30, 53), was prepared by mixing equal volumes of protein at a concentration of 14 mg/mL in 20 mM HEPES (pH 7.5) and to which an excess of solid inhibitor had been added with reservoir solution [30% PEG 2000 monomethyl ether, 0.1 M sodium acetate (pH 5.0), and 0.2 M ammonium sulfate]. This mixture was incubated overnight at 4 °C and centrifuged to remove precipitate before setting up crystallization drops. Cocrystals were grown from 6 μL sitting drops of the protein–reservoir solution and 0.3 μL of the reservoir solution at either room temperature or 4 °C. Crystals of the complex belong to space group *P*2₁2₁2₁ with unit cell dimensions *a* = 50.0 Å, *b* = 51.6 Å, and *c* = 205.6 Å, and have two copies of the IMP-1 metallo β-lactamase–inhibitor complex in the asymmetric unit.

**Acquisition of X-ray Diffraction Data.** Diffraction data to 3.1 Å resolution were obtained at room temperature from a single native crystal using a Siemens X-1000 multilayer area detector mounted on a Huber four-circle goniostat, and Cu Kα radiation produced by a Siemens rotating anode generator operating at 49 kV and 100 mA. The crystals were somewhat sensitive to the X-rays, and within the first several hours of data collection, the diffraction limit dropped from ~2.8 to 3.1 Å. The reciprocal space was sampled at 0.25° intervals around the *ω* and *φ* axes, and the data were processed with XDS (34) (Table 1). Two diffraction data sets were extracted from crystals of the complex with the mercurycarboxylate inhibitor. The first set was measured at room temperature from two crystals using a MAR image plate. The reciprocal space was sampled in 0.75° oscillation steps around *ψ*. These data were used for the structure determination and initial refinement. A second, higher-resolution set of data was collected at the NSLS X-25 beamline (Brookhaven National Laboratory, Upton, NY) from two flash-frozen crystals on a MAR345 detector using 1.100 Å wavelength X-rays. The crystals were cryoprotected by a brief exposure to the crystallization solution containing 20% glycerol. Data from the first crystal were collected in 0.75° oscillation steps, and data from the second crystal were collected in 1.0° steps.

**Table 1: X-ray Data Collection and Structure Refinement**

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*Refinement Statistics*  

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*The 3.19–3.10 Å resolution shell. The 3.00–2.90 Å resolution shell. The 2.07–2.00 Å resolution shell. *R*merge was calculated with 10% of the reflections.*

These crystals were not single, and data collection required a compromise between crystal-detector distance and oscillation range to avoid excessive overlap of the reflections. The data from the two crystals were integrated, merged, and scaled using DENVOSCALEPACK (35) (Table 1).

**Structure Determination, Model Building, and Refinement.** The crystal structure of the native (IMP-1 metallo β-lactamase from *P. aeruginosa* was determined by molecular replacement (56, 57) using reflections between 39 and 4 Å and a Patterson search radius of 20 Å. The atomic coordinates of the metallo β-lactamase from *B. fragilis* (37) (PDB file 1ZNB) were used as the search model after the metals, solvent molecules, and atoms of residues that are not homologous were removed from the coordinate file. On the basis of the aligned amino acid sequences, 34% of the residues are identical in both enzymes (37). The solutions obtained from the rotation search corresponding to the four molecules in the asymmetric unit of the native crystal were the top four peaks with heights of 5.5–5.6 (the next highest noise peak heights were 4.1σ and 3.8σ and lower) at (*αββ,γ*) = (76.41°, 113.93°, 138.25°), (*αββγ,γ*) = (103.59°, 66.07°, 318.25°), (*αββγ,γ*) = (256.51°, 114.83°, 138.51°), and (*αββγ,γ*) = (283.49°, 65.17°, 318.58°). A Patterson correlation coefficient of 0.58 and a crystallographic *R* factor (*R* = Σ*F*(1) − |*F*(2)|/Σ|*F*(2)|, where |*F*(2)| and |*F*(1)| are the observed and calculated structure factor amplitudes, respectively) of 0.48 were computed from the initial model that included the four molecules in the asymmetric unit. The structure of the complex was determined by molecular replacement using the partially refined native structure. The correlation coefficient and the crystallographic *R* factor...
calculated from the initial model were 0.59 and 0.40, respectively. The program packages X-PLOR (58) and CNS (59) were used to refine the structures by rounds of simulated annealing and positional refinement followed by manual adjustment of the model with the aid of the interactive computer graphics program O (60). In the absence of a structure of the inhibitor, idealized versions of the molecule and its stereoisomers were built in QUANTA and energy minimized with CHARMM. The topology and parameter files used in the refinement were generated from the minimized structures (61). As a check of the correctness of the model structure, the stereoisomer that best fitted the electron density corresponded to the conformation that gave the highest inhibitory activity in the enzymatic assay. The convergence of the refinement was monitored by the fit of the polypeptide chain to the sigma-weighted (62, 63) electron density maps calculated with coefficients $2[F_{o}] - [F_{e}]$ and $[F_{o}] - [F_{e}]$ and model phases, and by the value of the crystallographic $R$ factor and $R_{free}$ (64). All diffraction amplitudes to the limit of resolution were included with an allowance for the estimated bulk solvent contribution to the diffraction (65). Noncrystallographic symmetry restraints were imposed throughout the refinement except in the final rounds of refinement of the inhibitor complex in which the two molecules were refined independently (Table 1).

**RESULTS**

**Enzyme Kinetics.** The half-maximal inhibitory concentration, $IC_{50}$, was calculated as the concentration of inhibitor that caused a 50% reduction in the rate of hydrolysis of $\beta$-lactam substrate nitrocefin (66). The $IC_{50}$ was determined following a preincubation of enzyme and inhibitor at 37 °C in 25 mM PIPES buffer (pH 7.0) with final Zn(II)SO$_4$ and nitrocefin concentrations of 100 and 400 $\mu$M, respectively.

**Structure of the IMP-1 Metallo $\beta$-Lactamase.** The crystal structure of the native IMP-1 metallo $\beta$-lactamase from *P. aeruginosa* was determined by molecular replacement, and the model was refined against data to 3.1 Å. The tetramer in the asymmetric unit has point group symmetry $D_{4}$, and each monomer includes residues 2–26 and 30–221 and two Zn(II) atoms. Residues 1, 27–29, and 222–228 are disordered and not included in the final model. The crystallographic $R$ factor is 0.26; the $R_{free}$ is 0.29, and the root-mean-square (rms) deviations from ideal bond lengths and bond angles (67) are 0.007 Å and 1.4°, respectively (Table 1). The structure of the complex between the IMP-1 metallo $\beta$-lactamase from *P. aeruginosa* and the mercaptoacrylate inhibitor was also determined by molecular replacement, and the model refined to 2.0 Å resolution (Table 1 and Figure 2). The model consists of two molecules in the asymmetric unit, A and B, related by a rotation of 168.6°. Molecule A includes residues 4–223, two Zn(II) atoms, and one inhibitor; molecule B includes residues 4–225, two Zn(II) atoms, and an inhibitor. Residues 1–3 and 224–225, or 225–228 were disordered and are not included in the final model that in addition includes a total of 340 water molecules. The crystallographic $R$ factor is 0.20; the $R_{free}$ is 0.26, and the rms deviations from ideal bond lengths and bond angles (67) are 0.018 Å and 2.6°, respectively (Table 1). The average $B$ factor is 27 Å$^2$ for molecule A and 43 Å$^2$ for molecule B, a difference that may reflect the number of crystal lattice contacts in which each molecule participates; 27 residues in molecule A establish direct protein–protein contacts with a symmetry-related molecule, with only 12 residues in molecule B. Superposition of the $\alpha$-carbon atoms of the two independent molecules in the asymmetric unit gives an rms difference of 0.5 Å, with the largest differences being at both termini (2.6 Å difference) and between residues 22 and 31 (2.3 Å difference). When the residues with the largest differences are omitted, the resulting rms is 0.3 Å. Residues 22–31 form two antiparallel $\beta$-strand segments, and the connecting turn is termed the flap. These residues participate in crystal lattice contacts between molecules A and B; residues 28–32 in molecule B contact residues 23–30 in molecule A such that the polypeptide chains are perpendicular to each other. Partial as a result of these interactions, the flap in molecule A is positioned between the active site-bound inhibitor and molecule B.
The rms difference for the superposition of the \( \alpha \)-carbon atoms of the native and complex structures is 0.7 Å with the largest differences (3.5 Å) at the termini and the flap regions, when residues 4, 21–32, and 219 are omitted from the comparison. In addition, the side chain of Phe51 shows a large difference in the position of the phenyl ring between the native and the complex. A rotation of 100° about the Cα–Cβ bond moves the phenyl side chain away from the S1 hydrophobic pocket to provide access to the inhibitor's phenyl group which binds at this site (Figures 3 and 6). The distance between the two Zn\(^{2+}\) atoms in the active site is 3.6 Å in the inhibitor complex and 3.5 Å in the native structure, a small difference within the experimental error considering that the bridging water in the native structure (not seen at the resolution of 3.1 Å, but presumed present) was replaced by the sulfur atom of the inhibitor's thiolate (Figures 3 and 4). As in all known crystal structures of metallo \( \beta \)-lactamases, Asp48 has a sterically strained main chain conformation in both the native form and the complex with mean \( \phi \) and \( \psi \) angles of 81° and 148°, respectively. The carboxylate oxygen atoms of Asp48 in the IMP-1 metallo \( \beta \)-lactamase make hydrogen bond and/or electrostatic interactions near the active site with Lys33 NZ (2.8 Å) (Figure 7b). Other interactions of Asp48 are with Ser82 OG (2.7 Å), Ser76 OG (2.8 Å), and Ser76 N (2.8 Å).

**Binding of the Inhibitor.** The mercaptocarboxylate inhibitor, with a half-maximal inhibitory concentration for nitrocefin hydrolysis of 90 nM, is bound in the active site of IMP-1 through electrostatic and nonpolar interactions. The stereochemical conformation that results in maximal inhibi-
Inhibition of Metallo β-Lactamase

The inhibitor's thiolate bridges the two Zn$^{2+}$ atoms replacing the shared water, and the inhibitor's carboxyl group interacts with the side chain of Lys161 (2.8 Å) and the main chain amide nitrogen of Asn167 (2.9 Å). The carboxylate oxygen of the inhibitor is located 3.6 Å from Zn2, and has displaced the apical water from Zn2 which in the complex is coordinated to four ligands with a tetrahedral coordination geometry (Table 2). In molecule A, the inhibitor's carboxyl oxygen interacts with Wat135 (2.7 Å) and Wat160 (3.0 Å), and the latter also contacts Asn167 ND2 (2.8 Å). The carboxyl oxygen is exposed to solvent and oriented to establish interactions with but distant from Zn1 (5.0 Å) and Asn167 ND2 (4.1 Å), both of which are proposed to form the oxyanion hole (37). Of the possible 675 Å$^2$ of solvent accessible area in the free inhibitor, only 99 Å$^2$ is exposed to solvent in the complex (Figure 5). Of these, 55 Å$^2$ corresponds to the tetrazole ring, and the remaining exposed surface is contributed by the carboxyl oxygen (15 Å$^2$), C12 (11 Å$^2$), C13 (15 Å$^2$), and C19 (5 Å$^2$).

The inhibitor's phenyl ring is bound in a hydrophobic pocket formed by residues Glu23, Val25, Val31, Lys33, and Phe51, next to the flap and near the metal center. Comparison with the native, unbound structure indicates that binding of the inhibitor requires a rotation of 105° about the Cγ – Cβ bond of Phe51 to provide access to the hydrophobic pocket. In addition to or at the same time as this rotation, a displacement of 2.9 Å of the polypeptide at Val25 widens the pocket, and causes the lateral movement of the flap (Figure 6).

In the inhibitor complex, the flap is in the closed conformation, and the side chain of Trp28 interacts edge-to-face with the inhibitor's thiophene ring. The two molecules in the asymmetric unit provide slightly different views of the flap that vary in the degree of closure due to crystal lattice contacts. The conformation of the flap in molecule B is more open; its residues, 22–31, have higher B factors than in molecule A (58 and 39 Å$^2$, respectively), and the α-carbon positions of Trp28 differ by 2.2 Å between the two molecules. The inhibitor also shows differences between the two independent molecules. The inhibitor atoms bound to molecules A and B superimpose with an rms deviation of 0.7 Å with the largest differences between the thiophene and tetrazole groups at S36 (1.4 Å), C41 (1.5 Å), N44 (1.1 Å), and N42 (1.4 Å). When the atoms in these groups are excluded from the superposition, the mercaptomethyl phenylbutylxyl glycine moiety superimposes with an rms deviation of 0.2 Å and binds in the same way to both molecules. Away from the metal center is where the conformation of the inhibitor differs most between the two molecules. In molecule A, the thiophene ring penetrates deep into the active site and is flanked by Val31 and Trp28 and with the N43 of the tetrazole ring interacting with Gly164 (2.8 Å). In molecule B, the thiophene and tetrazole groups have swung up away from the active site floor and toward the flap. The tetrazole is bound in alternate conformations related by a rotation of 128° about the C35–C37 bond, and stabilized by contacts with residues Leu4, Glu24, Gly29, and Val30 of a neighboring molecule. There is no alternate conformation in molecule A because the tetrazole ring movement is prevented by the OE2 atom of Glu119 of the symmetry-related molecule that interacts through a hydrogen bond with Trp28 O (2.7 Å).

**DISCUSSION**

The sequence of the IMP-1 metallo β-lactamase from *P. aeruginosa* is 17–37% identical with those of the *B. fragilis*, *B. cereus*, and L1 β-lactamases, and its secondary structure topology is characterized by a central β-sandwich with two α-helices on either side and a binuclear metal center located at one edge of the sandwich (Figure 2). Pairwise superposition of the α-carbon atoms of the IMP-1 and the *B. fragilis* (12NB) or the *B. cereus* (1BME) enzymes gives rms deviations of 0.6 Å for both. Except for differences in the flexible flap, there are two other regions that are unique to IMP-1. In the IMP-1 metallo β-lactamase structure, the first β-strand is absent, and the loop between residues 158 and 167 is three residues shorter in the IMP-1 than in the *B. fragilis* or *B. cereus* enzyme. The length of this loop affects the position of the active site Lys161. The absence of three residues in IMP-1 immediately following Lys161 causes the lysine N2 atom to lie 2.0–2.5 Å closer to Zn2. This lysine interacts with the carboxylate of the mercaptocarboxylate inhibitor, and is conserved in all but the *S. maltophilia* metallo β-lactamases where it is a serine (39). Given the
Asp48 and the equivalent aspartate residues in the B. fragilis and B. cereus enzymes and the L1 enzyme from S. maltophilia have strained main chain conformations, with $\phi$ and $\psi$ angles for the B. fragilis and B. cereus enzymes of 81° and 144° and 78° and 150°, respectively, and $\phi$ and $\psi$ angles of 68° and 157°, respectively, for L1. The conservation of this particular conformation suggests that it is important for the activity of the enzyme, and is consistent with the results from the mutation of this aspartate to alanine which abolishes the enzymatic activity of the B. fragilis enzyme (46). The network of interactions that Asp48 establishes near the active site appears to influence the binding affinity of Zn2 depending on the residues with which it interacts. In the IMP-1 enzyme, Asp48 OD interacts electrostatically with Lys33 NZ (2.8 Å); however, in the B. fragilis enzyme, instead of a lysine it is a sodium ion (panels a and b of Figure 7), and in the L1 enzyme, it is a water molecule, Wat23, that is bound to the aspartate. The sodium ion in the B. fragilis enzyme, the water molecule in the L1 enzyme, and the Lys33 NZ atom in the IMP-1 enzyme occupy equivalent positions and interact with the aspartate. These three metallo β-lactamases, IMP-1, B. fragilis, and L1, have two high-affinity metal binding sites. However, in the B. cereus enzyme, the equivalent aspartate interacts electrostatically with the side chain of an arginine from a different part of the structure (Figure 7c). The guanidino group atoms of arginine located at position 91 lie close to the binuclear metal center, 4.0–4.4 Å from Zn2, and may interfere electrostatically with binding of the metal as has been pointed out previously (37). The arginine is located in the HxHxDR sequence motif in which the two histidine residues coordinate Zn1, and the aspartate coordinates Zn2. In the B. fragilis or IMP-1 enzyme, a smaller cysteine (Cys104, 1ZNB) or a serine (Ser82, 1DD6), respectively, replaces the arginine at that position and is distant from Zn2. In both the B. cereus structure and the Aeromonas enzyme, an arginine at this position is associated with a reduced affinity for the second metal binding site. The L1 enzyme with two high-affinity binding sites presents a different arrangement. In the L1 enzyme, which has a lower degree

Similarly among the inhibitor and the substrates, the lysine or the serine may bind to the carboxylate of the β-lactam substrate in a manner similar to that of the mercaptoacetylxyloxylic inhibitor.

![Figure 6: Stereoview showing the conformational changes upon inhibitor binding. Superposition of the native, unbound IMP-1 structure (red) and the mercaptoacetylxyloxylic complex (blue) (72-74). The α-carbon atom trace and the side chains of Trp28, Phe51, Val25, and Val31. When the inhibitor is bound, Phe51 rotates 100° around the Cα-Cβ bond to give access to the incoming inhibitor, while Val25 and Val31 are displaced by approximately 2.9 Å compared to the native structure. The lateral movement of the flap widens the S1 hydrophobic pocket. Flap residues G27, W28, and G29 (red dotted line) in the native structure are completely disordered.](image)

![Figure 7: Stereoview of the structures of the metallo β-lactamases from P. aeruginosa, B. fragilis, and B. cereus in the vicinity of Asp48 (72-74). (a) In the B. fragilis enzyme, the aspartate interacts with a sodium ion. (b) Asp48 interacts with Lys51 in the IMP-1 enzyme. (c) In the B. cereus metallo β-lactamase, the aspartate interacts with an arginine.](image)

**Table 2: Zinc Ligands**

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* Distances to the metal, in angstroms, are quoted for both molecules in the asymmetric unit. The average ligand–metal–ligand angles for molecule A are 109° for Zn1 and 109° for Zn2; for molecule B, the angles are 109° for Zn1 and 108° for Zn2.
of amino acid sequence identity and more structural differences with respect to the other metallo β-lactamases, a histidine (His89, 1SML) instead of the arginine serves as a ligand to Zn2.

The metallo β-lactamases have a wide spectrum substrate specificity, with the exception of the *Aeromonas* enzymes which preferentially hydrolyze carbapenems (13, 29, 68). Metallo β-lactamases can bind and hydrolyze a variety of substrates, differ in their metal requirement, and constitute a structurally diverse family of proteins that present a challenging opportunity for the design of a wide-spectrum antibiotic. To understand which critical binding interactions need to be established by a potent inhibitor, we have determined the crystal structure of a complex of the IMP-1 metallo β-lactamase from *P. aeruginosa* with a mercapto-carboxylate inhibitor (Figures 2 and 3). The optimal stereochemistry for maximal inhibition of metallo β-lactamase activity and maximal selectivity against other metallo proteins such as angiotensin converting enzyme, ACE, was determined to be S and D, at C16 and C27, respectively (details of the chemistry and selectivity will be published elsewhere). This mercapto-carboxylate inhibits the IMP-1, *B. fragilis*, and L1 enzymes with IC₅₀ values between 100 and 500 nM, being slightly less effective against the L1 enzyme, presumably due to the unique structure of this enzyme as compared with the other metallo β-lactamases. To determine the similarities between the mercapto-carboxylate inhibitor and β-lactam substrates, we used the structure of the complex as a three-dimensional template to manually dock the active site the substrate benzylpenicillin. The β-lactam was superimposed on the inhibitor such that the carbonyl and carboxylate groups of both molecules overlap (Figure 9). The inhibitor bound in the IMP1 complex resembles the binding mode of the substrate in the model and does not appear to be disturbed by the presence of the thiol group. The phenyl ring of the substrate fits into the hydrophobic pocket at the base of the flap; the carbonyl oxygen is oriented for interaction with Asn167, and the carboxylate interacts with Lys161 with displacement of the apical water coordinated to Zn2 in the native structures. In the inhibitor complex, the carbonyl oxygen is oriented for interactions with Zn1 (5.0 Å) and with Asn167 ND2 (4.1 Å) in the oxyanion hole (57), but these rather long distances may be attributed, in part, to the presence of the thiol group in the inhibitor that fixes the position of the carbonyl away from the oxyanion hole. However, it is conceivable that a relatively small displacement of the polypeptide chain in the vicinity of Asn167 could bring the side chain closer to the carbonyl oxygen. In addition, the model of the docked substrate based on the structure of the inhibitor complex implies that after the nucleophilic attack on the carbonyl carbon and the subsequent transition to a tetrahedral carbon (sp³ hybridization), the distances and directionality of the interaction between the (charged) oxygen atoms with Zn1 and Asn167 could be more favorable. On the basis of the docked model, the substrate uses the same subset of interactions, as does the inhibitor. These three interactions appear to be critical for the specific binding to metallo β-lactamases.

The interaction of the inhibitor’s carboxylate with Lys161 highlights the importance of this residue for inhibitor binding.
and, possibly, for substrate binding. The proximity of the carboxylate to Zn2 with displacement of the apical water from the Zn2 ligand sphere shows, for the first time, the transition of Zn2 from its native trigonal bipyramidal geometry to the tetrahedral coordination in the inhibitor complex (Figure 3). In contrast to the inhibitor complex where the position of the carboxylate is dictated by the presence of the thiol group, it is possible that the carboxylate group of the substrate does become the fifth ligand to Zn2 after displacing the apical water. The potential transition from pentacoordinated to tetracoordinated could be important during catalysis whether the apical water remains as a ligand to Zn2 as part of the Michaelis complex (37), or whether the apical water is displaced by the substrate and Zn2 provides electrostatic stabilization of the intermediate (43), but from this model, it cannot be determined if the carboxyl group of the substrate and the apical water bond simultaneously in the Michaelis complex or if the water molecule is displaced.

Binding of the mercuriapocarboxylate inhibitor to the active site of the IMP-1 metallo β-lactamase triggers the change in the conformation of the flap that leads to closure and interaction with the inhibitor. Binding of the inhibitor requires a rotation about the Cα–Cβ bond of Phe51 (Ile in B. fragilis and Trp in B. cereus), providing access to the hydrophobic pocket. It also requires that the side chain of Val25 move aside to make the pocket wider, which implies a lateral movement of the flap (Figure 6). In the L1 enzyme structure, where the SI pocket is formed by residues Tyr11, Val13, Trp17, Leu38, and Met56 (L1 residue numbering, ISML), it does not appear to be obvious that ligand binding in this pocket could trigger a conformational change as in the IMP-1 at the B. fragilis enzyme. However, in the IMP-1–mercaptoacarboxylate complex, the closure of the flap entails considerable movement of the polypeptide to bring the middle of Trp28 closer to the active site so that it can interact with the bound inhibitor. With the flap in the closed configuration, the active site becomes a tunnel-shaped cavity with a large opening (Figure 5). Through this opening, the leaving group at C3 of the β-lactam substrates derived from cephalosporin (49) may diffuse into the bulk solvent. The portion of the inhibitor bound near the catalytic center is firmly bound to the enzyme and shows very little difference between the two molecules in the asymmetric unit. The exploitation of possible binding interactions lining the opening of the tunnel may lead to the design of inhibitors with improved binding affinity. The role of the flap for the binding of inhibitors, and the substrates, has been demonstrated in the crystal structures of the mercuriapocarboxylate inhibitor complex with IMP-1 presented here, and in the structures of the B. fragilis enzymes with MES (33) and a biphenyl tetrazole (34). Deletion of the flap severely impairs the enzymatic activity by disrupting substrate binding (70).

The open, disordered conformation of the flap is found in most native structures of metallo β-lactamases, with the exception of the orthorhombic crystal form of the B. fragilis enzyme (38) where it is stabilized in the open conformation by crystal lattice contacts. In the L1 enzyme, however, it is not obvious what part of the structure acts as a flap. The crystal structures of inhibitor complexes of the IMP-1 and B. fragilis enzymes suggest that the closed conformation of the flap differs depending on whether the SI hydrophobic pocket near the flap is occupied by the inhibitor (Figure 8). The MES (33) and the biphenyl tetrazole inhibitor (34) bind in the active site, but neither has groups in the S1 pocket. However, the phenyl group of the mercuriapocarboxylate inhibitor bound to the IMP-1 metallo β-lactamase does occupy the pocket. The position of Trp28 gauges the lateral movement of the flap, for example, the position of the α-carbon atom of Trp28 differs by 4.8 Å between the mercuriapocarboxylate–IMP-1 and the B. fragilis enzyme–MES complexes, and by 6.5 Å between the mercuriapocarboxylate–IMP-1 and the B. fragilis enzyme–biphenyl tetrazole complexes. These differences are considerably larger than the rms differences in α-carbon atom superposition of ~0.6 Å for pairwise comparisons, and it is not likely to reflect differences in the flap’s amino acid sequences between B. fragilis and IMP-1 enzymes either. There are 10 residues in the flap: five identical and four conservative replacements; the only possible disrupting replacement is an alanine for a glutamate. The alpha side chain of the glutamate forms a side of the hydrophobic pocket and, from its position, is not likely to alter or prevent the motion of the flap.

The crystal structure of the IMP-1 metallo β-lactamase with a potent mercuriapocarboxylate inhibitor presented here represents a new mode of binding of a small molecule to a metallo β-lactamase active site, and it shows the critical binding interactions required for a potent inhibitor; it also suggests a mode of substrate binding. The new IMP-1 metallo β-lactamase native structure presented here highlights the structural diversity among this family of proteins. Mercuriapocarboxylates have been shown to inhibit IMP-1 and therefore are of great potential in the protection of β-lactam antibiotics against this resistance mechanism and for overcoming bacterial infections caused by metallo β-lactamase-producing organisms.

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REFERENCES

Inhibition of Metallo β-Lactamase


Biochemistry, Vol. 39, No. 15, 2000 4297


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Background: We have shown that topoisomerase I is essential for viability in Cryptococcus neoformans, and thus is an excellent target for novel antifungal agents. Topoisomerase I is also the target of camptothecin and its derivatives, Irinotecan and Topotecan, which represent a new class of antiplastic agents.

Hypothesis: In this study, we tested whether structural modifications of camptothecin increase toxicity against yeast topoisomerase I.

Experimental design: For this study, a cDNA copy of the TOP1 gene was isolated from C. neoformans serotype D, strain B501, cloned into a p20-2a expression vector, and transformed into Saccharomyces cerevisiae a erg6 top1 mutant strain. A series of camptothecin derivatives were created. In vitro susceptibility testing was performed according to the recommendations established by the National Committee for Clinical Laboratory Standards (NCCLS).

Results: Our findings confirmed that the major obstacles to camptothecin and its derivatives actions is their penetration into the cell. Moreover, among camptothecin derivatives, those that are water-soluble pro-drugs have more activity than their cleaved lipophilic active forms. In fact, if the pro-drug can penetrate into the cell and release the active form, the resulting effect against C. neoformans topoisomerase I produces a fungicidal response. On the other hand, some compounds only show more activity when TOP1 is overexpressed indicating that a facility for the target is limiting. Moreover, certain camptothecin derivatives were considerably more potent than Topotecan and Irinotecan. Finally some compounds show synergistic activity against yeast and pathogenic fungi when combined with amphotericin B (FIG index <1.0).

Conclusion: Our findings reveal that certain camptothecin derivatives hold great promise as novel fungicidal agents that can even be synergistic with existing drugs.
Inactivation versus the Initial Graphic Services, Upper Merlon.

References

Materials and Methods

Abstract

Unusual Inhibition of Some β-Lactamases
Transferable resistance to extended-spectrum β-lactams: a major threat or a minor inconvenience?

Ever since the identification of the TEM-1 and SHV-1 β-lactamases at the beginning of the 1970s, transferable β-lactam resistance has been dominated by these two groups of enzymes. Indeed, by the early 1980s, over 60% of all plasmid-mediated β-lactam resistance in Escherichia coli was mediated by the TEM-1 β-lactamase (Simpson, Harper & O’Callaghan, 1980, Roy et al., 1983; Medeiros, 1984). The success of these enzymes must partly be derived from the location of their genes on transposons but also from the combination of the biochemical properties of the β-lactamases themselves (Amyes, 1987). They are extremely efficient at hydrolysing penicillins and many first-generation cephalosporins although they have virtually no effect on second or third-generation cephalosporins. Indeed, these latter drugs were largely developed to overcome the effects of these devastating β-lactamases (Richmond, 1980; Simpson, Pveled & Harper, 1982).

The emergence of transferable resistance to extended spectrum β-lactams arose from subtle changes in the common plasmid-mediated β-lactamases responsible for resistance. Some of these changes have been so small that they could easily have been missed. It has recently been shown that the earliest extended-spectrum β-lactamase originated in an English strain in 1982 (Payne, Marriot & Amyes, 1983a, 1990b). This was not, however, a forerunner of an epidemic of these enzymes, as only four such genes have been found in England in less than ten reported isolations since 1982 (Table I). Indeed, it is on the continent of Europe that most of these enzymes have been found. In 1983, three strains of Klebsiella pneumoniae and one of Serratia marcescens isolated in West Germany were demonstrated to transfer resistance to cephalosporin (Knothe et al., 1983). The plasmid mediated β-lactamase was a modification of the ubiquitous klebsiella enzyme SHV-1 and was designated SHV-2 (Kliebe et al., 1985). This discovery heralded an explosion in reports of new, plasmid-mediated extended-spectrum β-lactamases (Table I). However, we must ask: did this really represent a major threat to the continued successful use of antibiotics such as ceftazidime, cefotaxime and ceftriaxone?

Almost all these broad spectrum enzymes have been found to be modifications of either the TEM or the SHV groups of β-lactamases (Philippon, Labia & Jacoby, 1989). Indeed, third generation cephalosporin (3GC) hydrolysing β-lactamases can be quite easily obtained spontaneously from TEM-1/2 or SHV-1 (Table II). Most of these mutant enzymes confer only low level resistance to 3GCs and many of them were obtained when ceftazidime was used as the selection agent.

The surprising feature about these TEM- or SHV-derived β-lactamases is that the majority of the different types of enzymes have only been reported to be produced by less than ten clinical isolates (Table I). Thus, the reports of transferable 3GC resistance strongly suggest the spontaneous emergence of different types of extended spectrum β-lactamases rather than the spread of one particular "epidemic" enzyme.

The most notable exception has been the TEM-3 β-lactamase which was responsible for 3GC resistance in K. pneumoniae strains isolated in hospitals in France (Petit et al., 1990). This enzyme was found in nearly 500 clinical isolates (Sirot et al., 1988) and has now been found in far more clinical strains than any other extended-spectrum enzyme. However, the evidence suggests that this frequency was derived from the success of the host strain and the plasmid and represents a local epidemic (Sirot et al., 1988). The problem in French hospitals was checked by an improvement in nursing procedures and the epidemic waned. It did not, as might have been
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<td>USA</td>
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<td>6.3</td>
<td>1988</td>
<td>France</td>
<td>6</td>
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<td>6.5</td>
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<td>(a) TEM-Derived</td>
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<td>1986</td>
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<td>France</td>
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<td>1987</td>
<td>France</td>
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<td>10</td>
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<td>Germany, Chile, China, Tunisia, Greece, Egypt, Switzerland, France, USA</td>
<td>NS</td>
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<td>France</td>
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<td>38.0</td>
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<td>FEC-1*</td>
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<td>ND</td>
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<td>Pakistan</td>
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<tr>
<td>Host strain used in mutation</td>
<td>( \beta )-lactamase and plasmid used</td>
<td>Selecting agent</td>
<td>Mutation frequency</td>
<td>pI</td>
<td>Mutant designation</td>
<td>MIC (mg/l)</td>
<td>Equivalent clinical enzyme</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------------------------------</td>
<td>----------------</td>
<td>-------------------</td>
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<td>--------------------</td>
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<td>-----------------------------</td>
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<td>E. coli J53-2 Ne</td>
<td>TEM-1 (100kb)</td>
<td>CAZ</td>
<td>( 1 \times 10^{-9} )</td>
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<td>Mutant A</td>
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<td>CAZ</td>
<td>( 1 \times 10^{-9} )</td>
<td>5.4</td>
<td>Mutant B</td>
<td>&lt;0.06</td>
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<tr>
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<td>TEM-2 (Rf4)</td>
<td>CAZ</td>
<td>( 5 \times 10^{-8} )</td>
<td>5.6</td>
<td>Mutant C</td>
<td>&lt;0.06</td>
<td>4</td>
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<td>TEM-2 (Rf4)</td>
<td>CAZ</td>
<td>( 1 \times 10^{-9} )</td>
<td>5.6</td>
<td>Mutant D</td>
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<td>8</td>
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<td>PSE-4 (R1818)</td>
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<td>5.3</td>
<td>Mutant E</td>
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<tr>
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<td>TEM-4 (pBRE322)</td>
<td>CAZ</td>
<td>( 5 \times 10^{-9} )</td>
<td>5.3</td>
<td>TEM-101</td>
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<td>TEM-1 (R6K)</td>
<td>CAZ</td>
<td>( 5 \times 10^{-9} )</td>
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<td>TEM-121-124</td>
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<td>CAZ</td>
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<td>TEM-221</td>
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<td>5.6</td>
<td>TEM-226-229</td>
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<td>( 1 \times 10^{-9} )</td>
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<td>CAZ-hi mutant</td>
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<td>32</td>
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<td>K. pneumoniae</td>
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<td>( 2 \times 10^{-9} )</td>
<td>5.4</td>
<td>TEM-222-225</td>
<td>0.12</td>
<td>8</td>
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<tr>
<td>CF124</td>
<td>SHV-1</td>
<td>CTX</td>
<td>( 1 \times 10^{-9} )</td>
<td>7.6</td>
<td>SHV-1mut</td>
<td>40</td>
<td>2</td>
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</table>

The MICs are for the E. coli K-12 transconjugants.

**Table II.** Mutation of standard plasmid-mediated \( \beta \)-lactamases to extended spectrum

UM, Unmeasurable.
ND, not done.
NS, Not specified.
*First year of isolation.
*Relative efficiency \( (V_{on} / K_{m}) \) to ampicillin or penicillin. The enzymes designated with * show their relative substrate hydrolysis related to ampicillin, penicillin or cephaloridine. More accurate relative efficiencies are not available for these enzymes.
Where possible the minimum inhibitory concentrations (mg/l) are for the E. coli K-12 transconjugants.
This enzyme focuses as a doublet band at 53.
These enzymes have been shown to be TEM-derived.
This enzyme has been shown to be the same as TEM-E2 (Payne, D. J., Marriott, M. S. & Amyes, S. G. B., unpublished results).
This enzyme has been shown to be the same as TEM-E3 (Payne, Marriott & Amyes, 1990c).
feared, herald a worldwide pandemic of this resistance gene, although other extended-spectrum \(\beta\)-lactamases still continue to emerge sporadically.

Until 1989, all transferable extended-spectrum \(\beta\)-lactamases appeared to have been derived from the TEM or SHV-1 groups of plasmid-mediated enzymes. All the enzymes were sensitive to clavulanic acid and thus, in theory at least, could be controlled by the use of \(\beta\)-lactamase inhibitor combination therapy. In Pakistan, a patient with severe burns was treated with ceftazidime before being transferred to London for specialized treatment. In London, an \(E.\ coli\) strain was isolated from swabs of a raw area and was found to be ceftazidime-resistant. The extended-spectrum \(\beta\)-lactam resistance was transferable and, unusually, clavulanic acid did not restore \(\beta\)-lactam sensitivity (Woodford et al., 1990). Detailed analysis showed that the plasmid-mediated enzyme was quite unlike any found before. The \(\beta\)-lactamase (BIL-1) had a high \(pI\) reminiscent of Class-I chromosomal \(\beta\)-lactamase and its biochemical properties support this view (Payne & Amyes, unpublished results).

In all, there have been reports of at least 27 transferable genes encoding resistance to broad spectrum cephalosporins. However, the novelty of each of the \(\beta\)-lactamases in these reports has not been fully established. Such a large number of \(\beta\)-lactamases now requires a method of classification. A few of the genes have been sequenced and have been shown to differ from the original TEM or SHV enzymes by a small number of amino acid changes. Until all the genes are sequenced and we understand which are the important amino acid changes, it will be difficult to use the sequences alone as a means of classification. We are some years away from this, and so our proposed method of classification divides these \(\beta\)-lactamases into Groups 1 to 4 by virtue of their relative efficiencies of hydrolysis of ceftaxime and ceftazidime (Table I). Group 1 consists of those enzymes that hydrolyse ceftaxime and ceftazidime with poor efficiency. In most cases, they hydrolyse ceftazidime more efficiently than cefotaxime. Group 3 comprises those enzymes that hydrolyse cefotaxime more efficiently than ceftazidime. Group 3 has been split into three sub-groups; Group 3a are all TEM-derived \(\beta\)-lactamases, whereas Group 3b are SHV-derived \(\beta\)-lactamases and Group 3c includes \(\beta\)-lactamases of unknown derivation. However, most of the Group 3 \(\beta\)-lactamases confer a greater resistance to ceftazidime. Group 4 consists of the only \(\beta\)-lactamase that confers resistance to all generations of cephalosporins and clavulanic acid, for, to date, only the BIL-1 enzyme fits these criteria. Some of the 3GC hydrolysing \(\beta\)-lactamases have been characterized only by their \(V_{\text{max}}\) or relative rates of hydrolysis of different \(\beta\)-lactam substrates. Such data do have deficiencies (Amyes, 1987), but can be used in rough approximations of the specificities of different \(\beta\)-lactamases. These \(\beta\)-lactamases have been given provisional places in the classification scheme and are identified by an asterisk.

The 3GC-hydrolysing \(\beta\)-lactamases have created the most severe problems in France. Indeed, almost half the initial reports of the enzymes in Table I have come from the French hospitals. In the UK, which has not had major problems with 3GC hydrolysing \(\beta\)-lactamases, fewer 3GCs are used than in French hospitals. Therefore, it is logical to suggest that, as the future use of 3GCs increases because of the growing resistance to previous generations of cephalosporins, the occurrence of both novel and established 3GC hydrolysing \(\beta\)-lactamases will increase also. However, it is possible to obtain strains producing cefotazidimases and cefotaximases in countries where 3GCs are not widely available and where selective pressure is low. For example, the \(E.\ coli\) strain producing BIL-1 and \(K.\ pneumoniae\) 8825, which produces two 3GC hydrolysing \(\beta\)-lactamases including DIP-1, both came from Third World countries where 3GC availability is severely restricted by cost.

As discussed previously, the 3GC hydrolysing SHV and TEM \(\beta\)-lactamases can be obtained spontaneously from SHV-1 and TEM-1 or 2 \(\beta\)-lactamases respectively. Therefore, any strain that produces TEM-1 or SHV-1 is a potential producer of 3GC hydrolysing \(\beta\)-lactamases. The TEM-1 or 2 and SHV-1 \(\beta\)-lactamases are among the most widespread \(\beta\)-lactamases in bacterial populations. Moreover, calculations from a survey conducted by Wiedemann, Kliebe & Kresken (1989) on 802 clinical isolates, showed that 17% of their own Gram-negative clinical
The spread of these enzymes through a population appears to depend more on the pathogenicity of the strains involved, stability of the plasmid encoding the β-lactamase, and the consistency of the selective pressure to stabilize the resistant plasmid (not necessarily 3GCs). If all these factors should be present then a particular 3GC-hydrolysing β-lactamase could cause a significant threat to 3GC therapy. Another worrying possibility is that 3GC-hydrolysing β-lactamases may have the selective advantage to become as successful as their predecessors. The evidence to date does not suggest this but, if the use of 3GCs increases significantly, the situation may well change. It could also be suggested that, because of the similarities with other non-3GC-hydrolysing β-lactamases, many of these cephalosporinases and cepotaximases may have remained undetected in current bacterial populations. It must also be considered that as 3GC-hydrolysing enzymes are derived from TEM-1 or 2 and SHV-1, it is possible that extended spectrum β-lactamases may evolve from other classic plasmid mediated β-lactamases. Indeed, Table II stresses this point as it illustrates how PSE-4 can mutate to an enzyme that confers transferable resistance to 3GCs. Lastly, all the SHV and TEM-derived β-lactamases are very sensitive to β-lactamase inhibitors such as clavulanic acid, sulbactam or YTR 830, and special emphasis has been placed on this fact in many publications (Krizs et al., 1988; Gutmann et al., 1989; Paul et al., 1989). However, if BIL-1 represents a new series of plasmid-mediated β-lactamases then transferable resistance to 3GC will be even harder to control.

The large number of different enzymes shows that these transferable extended spectrum β-lactamases are established and do pose a potential threat. However, the inability of any of these enzymes to predominate, as the TEM-1 β-lactamase has done, suggests that a pandemic by one of these new β-lactamases is unlikely and that these enzymes are merely an inconvenience. However, these are still early days and our controlled use of later generation cephalosporins will probably determine whether we can keep these enzymes as a minor inconvenience rather than let them emerge as a major threat.

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*Corresponding author.

References


Laboatory issues in the study of β-lactamases
Chair: DM Livermore (England) / K Bush (USA)

13.30 Classification and nomenclature of β-lactamases
K Bush, Astra Research Center Boston, 128 Sidney Street, Cambridge, Mass 02139, USA.

β-lactamases can be classified according to structural or functional criteria. Four classes of β-lactamases have been segregated according to molecular criteria. Classification by biochemical function resulted in four major functional groups of β-lactamases based on substrate and inhibitor profiles. Although no functional group classification can predict the structural class unequivocally, close relationships between structure and function exist for many β-lactamase groups.

14.00 Relation of β-lactamase type and antibiogram, as seen in routine tests
DM Livermore, Department of Medical Microbiology, The London Hospital Medical College, Turner Street, London, E1 2AD, England.

Most β-lactamases are associated with characteristic resistance patterns. It follows that β-lactamase types can be predicted from the antibiogram data. Prediction has value in that it allows (i) informed choice of what further β-lactams may merit testing and (ii) recognition of anomalous data that require reconfirmation. The limits of such prediction must also be noted in particular, the strategy is unreliable for isolates with multiple β-lactamases.

14.30 Laboratory identification and typing of TEM and SHV β-lactamase variants
F Baquero, Department of Microbiology, Ramón y Cajal Hospital, 28034 Madrid, Spain.

The prediction of a possible variant β-lactamase frequently requires a first step of specifically-designed in vitro susceptibility testing procedures, complemented by rapid isoelectrofocusing techniques, and preliminary biochemical tests, in order to decide the presumed amino acid changes of the variant. Then, the corresponding region(s) of the gene are studied by oligotyping, cyclic-sequencing, or sequencing of specifically amplified PCR fragments. The total gene sequence of the β-lactamase is needed to fully identify a TEM or SHV variant enzyme, that may harbour the new type of "equilibrating mutations" optimising the phenotype in fluctuating environments.

15.00 Laboratory detection and investigation of zinc β-lactamases
DJ Payne, Microbiology Research, SmithKline Beecham Pharmaceuticals, PO Box 5089, Collegeville, PA 19426-0989, USA.

Metallo-β-lactamases (MBLs) confer resistance to the majority of clinically important β-lactam based therapies. As plasmid mediated MBLs have now been identified in common clinical isolates such as K. pneumoniae, S. marcescens, P. aeruginosa and B. fragilis the dissemination of this resistance mechanism requires critical evaluation. Effective procedures used to identify MBLs in clinical isolates of X. maltophilia and B. fragilis will be presented and the current epidemiology an biochemical characterisation of MBLs will be reviewed.
β-lactamase mediated resistance in nosocomial infections.

D. J. Payne SmithKline Beecham Pharmaceuticals, Brockham Park, Surrey, RH3 7AJ, U.K.

β-lactamase production is the most common resistance mechanism associated with Gram negative nosocomial infections. This presentation considers the significance of some new additions to the β-lactamase arena.

The TEM-1 and SHV-1 enzymes are the most prevalent β-lactamases found in clinical isolates. These enzymes only confer resistance to penicillins and early cephalosporins. However, mutations in the active site of the TEM-1 and SHV-1 enzymes have given rise to novel extended spectrum TEM/SHV β-lactamases which can hydrolyse third generation cephalosporins (3GC). These enzymes are now causing resistance to 3GC in most countries including India. Biochemical studies on 20 different extended spectrum β-lactamases have shown that clavulanic acid is an effective inhibitor of this new series of β-lactamases produced by nosocomial pathogens. Further details of this study and a review of the clinical implications of these enzymes will be presented.

Class I β-lactamases can confer resistance to first, second and 3GC. The genes encoding these enzymes were previously thought to be confined to the chromosome. However, our recent work has identified a novel plasmid mediated Class I β-lactamase (BIL-1) isolated in Pakistan. Studies on BIL-1 and other plasmid mediated Class I enzymes will be discussed.

Finally, a few clinical isolates have been shown to produce β-lactamases which can confer resistance to carbapenems. Currently, the prevalence of carbapenemases is very low, but the increased use of carbapenems could increase their occurrence. The characteristics of carbapenemases will be reviewed.
Antibiotic resistance in bacteria

---a review based on a symposium held on 5 January 1991 at Addenbrookes Hospital, Cambridge and chaired by Professor J. T. Smith

Edited by S. G. B. AMYES and C. G. GEMMELL

Department of Medical Microbiology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG and *Department of Bacteriology, The Medical School, University of Glasgow, Royal Infirmary, 86 Castle Street, Glasgow, G4 0SF

Introduction

Antibiotic resistance tends to be discussed by groups who work on related antibiotics. The symposium, which provided the basis for this review, was a rare opportunity for scientists working on quite different aspects of resistance to present and discuss their results. Much emphasis has been placed on the mechanisms of resistance and the first five papers reflect this. They demonstrate the quite different resistance problems encountered with each class of antibacterial drug. The remaining four papers take a different, and often less expressed approach, examining the impact that resistance has on clinical populations and our ability to treat the patient. These papers examine the effect that resistance has on the virulence and pathogenicity of bacteria and how the host is affected by resistant strains. This section ends with views about the future of antibiotics.

Resistance to Antifolate Antimicrobial Agents

K. J. Towner

Department of Microbiology and PHLS Laboratory, University Hospital, Nottingham NG7 2UH

The folate pathway occupies a central metabolic role leading to protein and nucleic acid synthesis (fig. 1). Two types of antifolate agent have gained wide acceptance and are of clinical importance: (i) the sulphonamides, that inhibit the bacterial enzyme dihydropteroate synthetase (DPS), which catalyses the reaction of p-aminobenzoic acid (PABA) with a pteridine derivative and are selectively toxic for bacteria because man does not require this enzyme; (ii) trimethoprim, that inhibits the ubiquitous enzyme dihydrofolate reductase (DHFR), and is selectively toxic because its affinity for the bacterial enzyme is some 10 000-fold greater than that for the mammalian enzyme. Following its initial introduction, trimethoprim was prescribed originally only in combination with a sulphonamide, usually sulphamethoxazole, for reasons which, at the time, seemed justified, but upon which, for many applications, substantial doubt was subsequently cast. In this paper, acquired resistance to sulphonamides and trimethoprim is summarised with particular reference to recent developments in molecular biology which enhance our understanding of resistance to these important antibiotics.

Incidence of resistance

Although a prime requirement is to distinguish between intrinsic and acquired resistance, other difficulties have been caused in the past by the use of different definitions for the term “resistant”. In addition to the fact that clinically relevant resistance is not the same as laboratory-determined resistance, particular problems arise in susceptibility to sulphonamides and trimethoprim in relation to choice of resistance breakpoints, inoculum size and the correct medium. Other local factors, such as the occurrence of epidemic strains, or selection and choice of particular specimens and patients, may greatly influence the “incidence” of resistant strains. Therefore, assessments of changes...
Table I. Incidence of resistance to sulphonamides (Su) or trimethoprim (Tp), or both, among isolates of enterobacteria causing urinary tract infection from the Nottingham area

<table>
<thead>
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<th>Species</th>
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<th>Total per cent that were</th>
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<td>Su&lt;sup&gt;a&lt;/sup&gt;Tp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Su&lt;sup&gt;a&lt;/sup&gt;Su&lt;sup&gt;b&lt;/sup&gt;Tp&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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<td><em>E. coli</em></td>
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<td>14.3</td>
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<td>Hosp.</td>
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<td>13.3</td>
</tr>
<tr>
<td>Comm.</td>
<td>7.6</td>
<td>10.2</td>
</tr>
<tr>
<td><em>Klebsiella/Entero-</em></td>
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<td>11.0</td>
</tr>
<tr>
<td><em>bacter spp.</em></td>
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<td>7.7</td>
</tr>
<tr>
<td>Comm.</td>
<td>3.7</td>
<td>6.3</td>
</tr>
<tr>
<td><em>Proteus spp.</em></td>
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<td>11.0</td>
</tr>
<tr>
<td>Hosp.</td>
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<td>7.7</td>
</tr>
<tr>
<td>Comm.</td>
<td>3.7</td>
<td>6.3</td>
</tr>
</tbody>
</table>

R, resistant; S, sensitive. *Resistance was defined as described previously, with breakpoints of trimethoprim lactate 8 μg/ml and sulphonamethoxazole 16 μg/ml.

Mechanisms of resistance to sulphonamides

Resistance to sulphonamides may be encoded either by the chromosome or by plasmids. Chromosomal mutations leading to hyper-production of PABA occur and these overcome the metabolic block imposed by the inhibition of DPS by sulphonamides, but they seem to be of relatively minor clinical importance. The commonest mechanism of resistance is the production of an altered form of DPS for which sulphonamides have a reduced affinity. Although chromosomal mutations leading to the production of such altered enzymes may occur, most clinically significant sulphonamide resistance is associated with the production of altered forms of DPS encoded by antibiotic resistance plasmids.

Two distinct types of DPS (I and II) encoded by plasmids have been characterised in gram-negative bacteria; DPS-I (encoded by the gene _sull_), has only 57% nucleotide sequence homology with DPS-II (encoded by _sull_), and their deduced polypeptides show only c. 50% similarity. The _sull_ gene is often located on transposons related to Tn21, or on large self-transmissible plasmids that show similarities to Tn21 in the resistance region. In contrast, the _sullf_ gene not only encodes a different enzyme, but is associated with different types of plasmids. The DPS-II enzyme is somewhat more stable in a cell-free extract than DPS-I, and the _sullf_ gene is carried mainly on small non-conjugative plasmids. Both of these plasmid-encoded enzymes can be distinguished readily from the resistant DPS encoded by the _sula_ gene on the chromosome of _Streptococcus pneumoniae_ with 43% DNA similarity between _sula_ and _sull_ or _sulf_, respectively.

Mechanisms of resistance to trimethoprim

Acquired resistance to trimethoprim may also be encoded by the chromosome or by antibiotic resistance plasmids. There is in-vitro evidence that trimethoprim can select for chromosomal mutants of pathogenic bacteria that lack the enzyme thymidylate synthetase. Such "thy" mutants have lost the normal ability to synthesise thymidine and, in order to grow, require exogenous supplies of thymine or thymidine to enable them to synthesise thymidylate via the "salvage" pathway. The action of trimethoprim would be adversely affected by the presence of such exogenous supplies under pathological conditions, but very few clinical isolates with this mechanism of resistance have been reported, perhaps because of failure to recognise them.

An alternative type of resistance is observed when mutations lead to overproduction of the normal chromosomally encoded DHFR. Mutants of this type have been obtained from several gram-positive and gram-negative species in vitro, but, again, seem to be relatively rare among clinical isolates. Impermeability mutants have also been reported in a various genera, and are of particular interest because of the reported cross-resistance between trimethoprim and other antibiotics such as the quinolones.
However, as with the sulphonamides, the commonest mechanism of acquired resistance to trimethoprim results from the production of an insusceptible target enzyme. Mutant forms of the normal susceptible DHFR encoded by the chromosome have been found occasionally in clinical isolates, but by far the most important and common mechanism of resistance is associated with the production of an additional, insusceptible, form of DHFR encoded by genes located on self-transmissible or mobilisable plasmids and transposons. The remainder of this section is devoted to a brief description of these enzymes and the possible evolutionary relationships between them.

Plasmid-mediated trimethoprim resistance was dominated initially by the spread of genes encoding DHFR types I and II, but seven major groups (types I-VII) of DHFRs insusceptible to trimethoprim have now been characterised in gram-negative bacteria. Several of these major groups are further divided into subtypes, and an additional insusceptible DHFR (type SI) has been characterised in staphylococci. The identification of each of these enzyme types on the basis of biochemical and biophysical properties has been reviewed previously and, as with the plasmid-encoded \( \beta \)-lactamas, further DHFR types probably await discovery. Indeed, it should be noted that two DHFRs isolated independently in the UK and Scandinavia have both been designated "type VII".

The remainder of this article refers to the UK enzyme, but in further adjustments the probe may be required when the precise relationship between these two enzymes, and the other DHFRs characterised previously, has been investigated fully.

The time-consuming nature of biochemical identification, involving the extraction and purification of each individual enzyme, has encouraged the development of a set of DNA probes which are capable of distinguishing between the genes encoding the different enzymes. These probes are particularly useful for epidemiological studies and are available currently for DHFR groups I-V, VII and SI.

When used in combination with high stringency wash conditions, the probe set is capable of distinguishing clearly between the major enzyme groups. One anomaly concerns DHFR group III, which had originally been thought to contain three directly related subtypes — IIa, IIb and IIIc. Each subtype has similar biochemical properties, albeit with markedly different, iso-electric points, and confers only a moderate level of trimethoprim resistance. However, no hybridisation has been detected between the standard group III probe (constructed from the type IIIa gene) and the genes encoding types IIb and IIIc (C. J. Thomson and S. G. B. Ames, personal communication). Therefore, it seems probable that enzymes belonging to these subtypes, although similar biochemically, may have evolved independently (see below).

Further investigations of the possible evolutionary relationships between the different DHFR groups have been stimulated, in part, by observations that some of the group-specific DHFR probes can, when used under conditions of reduced stringency, react with genes encoding enzymes belonging to other DHFR groups. Several of the enzymes and genes involved have now been sequenced, with particular attention being paid to the amino terminal (N-terminal) region which forms most of the active site for all DHFRs. The two subtypes within group I (Ia and Ib) show 71% amino acid homology and 65% nucleic acid homology with each other (H.-K. Young, personal communication), but only 29% amino-acid homology with the susceptible chromosomal DHFR.

The three subtypes within group II (IIa, IIb and IIc) show extensive (> 78%) amino-acid sequence homology with each other, but are quite unlike any other plasmid or chromosomal DHFR; it has been postulated that they may be derived from an oxidoreductase which acted originally on a completely different substrate.

The type IIIa DHFR shares 51% homology with the \( E. coli \) chromosomal enzyme, perhaps suggesting that they may have shared a common evolutionary origin. In contrast, the type IIIb enzyme is clearly distinct from the type IIIa enzyme, sharing only 15% of the first 47 amino acids in the N-terminal sequence, and differs also from the \( E. coli \) chromosomal enzyme and the types I, II, V and SI. These observations support the hypothesis, referred to above, that the subtypes IIIa and IIIb, although biochemically similar, have evolved independently.

The type IV enzyme, like subtype IIIa, shows most homology (40%) with the \( E. coli \) chromosomal enzyme, but is clearly distinct from the other plasmid-encoded DHFRs. The type V enzyme, in contrast, is closely related to the types Ia and Ib (H.-K. Young, personal communication), with 78% and 87% homology respectively. There is a similar close relationship between the type VI enzyme and types Ia (63% homology) and V (61% homology). Sequencing results for the type VII enzyme are not available yet, but hybridisation experiments have indicated a close relationship between the two and VI enzymes.

All of the plasmid-encoded DHFRs from gram-negative bacteria seem to be unrelated to DHFR type SI from gram-positive bacteria.

Envoi

Genes encoding resistance to the main antifolate antibacterial agents, sulphonamides and trimethoprim, are now distributed widely among bacteria in most environments. The precise incidence of resistance in local ecosystems is probably related closely to the extent of the selection pressure exerted by local usage of antibiotics. Most clinically significant resistance to antifolates is associated with plasmid-carried genes, often transposable, which encode alternative drug-insusceptible target enzymes. Two major types of plasmid-encoded DFRs conferring resistance to sulphonamides have been characterised, and trimethoprim resistance is conferred by at least seven major groups of plasmid-encoded DHFR in gram-negative bacteria, with an additional plasmid-encoded DHFR in gram-positive bacteria. Among the gram-negative DHFRs, groups I, V and VII seem to be closely related, groups IIIa and IV show some similarities to trimethoprim-susceptible chromosomally-encoded enzymes, and group II enzymes seem to be completely distinct from all other types. The combined use of biochemical and molecular biological techniques has enabled the inter-relations between the different genes and enzymes to be investigated, and is now providing us with an insight into the evolutionary mechanisms underlying the emergence of resistance to the antifolate antibacterials, and perhaps to antibiotics in general.

**Plasmid-Mediated \( \beta \)-Lactamas Responsible for Penicillin and Cephalosporin Resistance**

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Alexander Fleming's major contribution to medical science was the recognition of the potential of penicillin. He could not have known that he had stumbled upon a group of antibiotics that were not only going to prove to be the most extensively used antibacterial agents throughout the world but also prove to be the most durable. As with almost every other group of antibacterial drugs, the emergence of bacterial
resistance soon threatened their future value. The 50th anniversary of Abraham and Chain’s first discovery of an enzyme capable of destroying penicillin, 23 a β-lactamase, has just passed and there is still much debate as to whether β-lactamases really do compromise our reliance on this group of drugs.

If we had had only penicillin G, this battle may have been lost. The strategy used to overcome β-lactamase attack was to modify the antibiotic so that it was no longer capable of fitting into the active site of the enzyme. This proved successful for a period with the β-lactamases produced by staphylococci. The ability to prepare 6-amino penicillanic acid and substitute functional groups, which do not allow the binding of the antibiotic at the active site of the enzyme, to produce methicillin and the oxacillins, kept the staphylococci under control for more than 10 years. However, the adaptability of the β-lactamases and the acquisition of a gene capable of changing the penicillin binding protein (PBP) 2, has now put many staphylococci beyond the reach of β-lactams. Neu demonstrated that, in the developed world, more than 95% of nosocomial staphylococci possess β-lactamases. 24

The substitution of functional groups on 6-amino penicillanic acid led to the development of ampicillin, amoxycillin and carbenicillin and allowed the large Gram-negative rods to be controlled by penicillins. The widespread use of ampicillin resulted in the emergence of resistance in 1962. Anderson and Datta25 showed that resistance to ampicillin could be plasmid-mediated and thus became freely transferable from one species to another. However, little distinction was made between plasmid and chromosomally-determined β-lactamases. In 1973, Richmond and Sykes divided all β-lactamases into five groups based on the substrate profile and response to inhibitors. 26 The substrate profiles were, at best, Vmax determinations and often were merely determinations of rates of hydrolysis at a single fixed substrate concentration. This value was usually expressed relative to that obtained with a standard β-lactam substrate, i.e., penicillin G, ampicillin, or cephaloridine. The rate of hydrolysis allowed a preliminary classification into oxacillinases, carbenicillinases or broad-spectrum drugs. However, this method of classification had a number of drawbacks. It did not take account of the affinity of the β-lactamase for the substrate (i.e., the Km) nor did it consider amino-acid sequence homology. It classified all plasmid encoded β-lactamases into just two of the groups, based on the inactivation activity of Cloxacillin. 27

Ambler28 introduced a scheme based on sequence homology of the amino acids. He identified two classes of enzyme—Class A enzymes have a serine group at their active site and have a mol. wt. of c. 29 000. They are either penicillinases or broad spectrum. These enzymes show considerable homology with d-alanine carboxypeptidase and may have a common evolutionary origin. Both chromosomal and plasmid-determined enzymes fall into the group. Class B enzymes include metallo-enzymes and have no amino acid homology with Class A enzymes. Class C was added by Larrin and Grundstrom, 28 to include β-lactamases with serine involved in the active site but very different in structure from Class A proteins. These enzymes determine cephalosporin resistance, showing no sequence homology with Class A enzymes. Hauwinnen et al., having sequenced the enzyme PSE-2 29 and compared its structure with the OXA-2 β-lactamase, 30 proposed a fourth class D; enzymes of quite distinct substrate specificities had related structures. The final class, E, was created for the β-lactamases from Xanthomonas malvacearum. These metallo-enzymes like Class B but with no sequence homology with Class B. 31 This scheme again classified all plasmid-encoded β-lactamases into two groups, which were still very broad. Indeed, some of these enzymes within a class show less than 40% homology with other β-lactamases.

Huletsky et al. 32 recently reported the phylogenetic relationships between the Class A β-lactamases that have been sequenced. His cladogram showed a vast diversity in amino acid structure, with as little as 35% homology, although the basic shape of the molecule is conserved. Whereas this scheme may indicate how related enzymes may be, it cannot be extended to allow comparison of β-lactamases in the clinical situation. This is demonstrated with three enzymes in this group, TEM-1, TEM-3 and SHV-1. TEM-1 and SHV-1 are very similar biochemically; they hydrolyse penicillins and early cephalosporins, but not later cephalosporins. However, they are phylogenetically so far apart that a gene probe for one will not hybridise with the other as there is only 65% homology. On the other hand, TEM-1 and TEM-3 have 99% sequence homology, 33 but TEM-3 has the distinction that it can hydrolyse, and confer resistance to, cefazidime and cefotaxime. This difference has immense clinical significance. Gene probing with a TEM-1 gene probe cannot distinguish it from the TEM-1 gene.

In the clinical environment, change in enzyme structure is a relatively slow occurrence and happens in small stages. Therefore, a scheme distinguishing enzymes or genes must be very sensitive. This problem was ameliorated by the development of iso-electric focusing by Matthew and colleagues. 34 This technique, relying on the characteristic iso-electric point (pI) of enzymes, can, at its highest degree of sophistication, detect single amino acid differences in certain parts of the molecule. An equally sensitive technique, still much in its infancy, is the use of oligonucleotides designed to identify one nucleotide change. However, its success relies on the correct choice of probes. Iso-electric focusing has been the technique universally used and it has been combined with substrate profiles for finer distinction. At first, these compared relative rates at fixed substrate concentrations but, more recently compared relative Vmax values or relative rates of efficiency (Vmax/Km).

Several surveys performed in Europe during the 1980s, employing these techniques, 35–37 demonstrated that the TEM-1 β-lactamase was by far the most prevalent plasmid-encoded enzyme, responsible for up to 80% of the ampicillin resistance mediated by plasmids in gram-negative rods. The next most prevalent enzyme was TEM-2, which varies by one amino acid change from glutamine to lysine at position 37 (on the Sutcliffe numbering scheme). This change produces no apparent alteration in biochemical properties or resistance phenotype. That is, we can assume that all β-lactam substrates are concerned, these enzymes are identical, and that their relative distributions of about 8:1 come from the relative success of the transposons on which the genes are carried. An equally prevalent enzyme, SHV-1, though phylogenetically distinct, again has very similar properties. The proportion of gram-negative bacteria resistant to ampicillin can vary in the developed world but is usually quite high. A survey in Scotland showed that the proportion was 45%, isolates from Edinburgh, but was 70% in similar strains from Glasgow. India has the highest incidence of plasmid-mediated resistance. In 1984, 81% of all Enterobacteriaceae were ampicillin-resistant including 77% of strains E. coli. 38 In this study, there was a wide distribution of plasmids of many different types. An epidemic of one plasmid type was not being observed and the proportions of individual β-lactamases was significant. Again, the TEM-1 enzyme was most prevalent, found in >70% of strains. Surprisingly, OXA-1 was more common than TEM-2. However, even in an area where resistance was so prevalent, the number of different β-lactamases was quite small. Most of the plasmid-mediated β-lactamases capable of hydrolysing just ampicillin and first-generation cephalosporins have been found on very few occasions.

About 30 of these plasmid-encoded β-lactamases capable
of hydrolysing penicillins and early cephalosporins have been described; however, only five or so are found with any regularity. A

The prevalence of the TEM-1 β-lactamase has forced pharmaceutical companies to seek alternative strategies. This has largely been achieved in two ways: (i) the concurrent use of β-lactamase inhibitors; (ii) the development of drugs which are resistant to β-lactamase attack. β-lactam/β-lactamase inhibitors have proved remarkably successful. There are few reports of plasmid-mediated resistance and these have largely been confined to reports of hyperproduction of TEM β-lactamases.

Ceftazidime and cefotaxime were considered, at their launch, to be as close to perfection in the design of β-lactum drugs as was possible to achieve. In one area, it was virtually guaranteed that they would not fail—there would never be plasmid-mediated resistance. In 1982, a change took place which destroyed this guarantee. It was so small that it was missed at the time. A Klebsiella oxytoca strain, originally isolated from the blood and CSF of a baby in a neonatal intensive care unit in Liverpool, was gentamicin-resistant but ceftazidime-sensitive and it produced the TEM-1 β-lactamase. A subsequent isolate of K. oxytoca from this unit showed that it had now become ceftazidime-resistant. In a series of experiments performed 6 years afterwards, the ceftazidime resistance was found to be carried on a 141-kb auto-transferable plasmid. Biochemical analysis showed that the enzyme hydrolysed ceftazidime at a low, but significant, efficiency. The equivalent enzyme could be obtained by a single-step mutation from the TEM-1 β-lactamase and the Liverpool enzyme was designated TEM-E2. Most of these enzymes have been found in continental Europe. In 1983, three strains of K. pneumoniae and one of Serratia marcescens were isolated which conferred resistance to cefotaxime. The β-lactamase was plasmid-mediated and a modification of the ubiquitous Klebsiella enzyme, SHV-1. A glycine residue at position 236 had changed to serine. There followed an explosion of reports of new, plasmid-mediated extended-spectrum β-lactamases.

There are now nearly as many plasmid-mediated extended-spectrum β-lactamases as there are plasmid-mediated β-lactamases capable of hydrolysing merely ampicillin or early cephalosporins. Almost all of them are derivatives of TEM-1/2 or SHV-1 β-lactamases. The maximum number of mutations is three amino acids from TEM-1, TEM-2 or SHV-1 β-lactamases.

It is difficult to assess whether these enzymes are clinically of great significance. Certainly, there have been outbreaks of resistance caused by these β-lactamases. The best documented was the epidemic of TEM-3 in K. pneumoniae strains isolated in French hospitals. This enzyme was found in nearly 500 strains. Recently, there has been an equivalent epidemic of bacteria containing TEM-like enzymes in the USA. Generally, the emergence of these β-lactamases has been sporadic and localised, the vast majority of enzymes being found on very few occasions. This may reflect different localised usages of extended spectrum β-lactam agents, in particular those related to ceftazidime and cefotaxime.

Payne and Amyes have grouped these β-lactamases according to their hydrolysis properties (Table II), in a similar manner to the Richmond and Sykes Scheme.

In Group 1, there are enzymes which hydrolyse both ceftazidime and cefotaxime poorly. In most cases, they hydrolyse cefotaxime with slightly greater efficiency than ceftazidime—this is paradoxical as they confer greater resistance to ceftazidime, which probably results from the superior penetration of cefotaxime.

Group 2 consists of those enzymes capable of hydrolysing ceftazidime more efficiently than cefotaxime and, generally, confer much higher levels of resistance to this drug. Some would not produce clinically significant resistance to cefotaxime as the level of resistance is insufficient.

Group 3 is the largest group and comprises those enzymes that hydrolyse cefotaxime more efficiently than ceftazidime. However, probably for reasons similar to those with the Group 1 enzyme, resistance to cefotaxime is rarely higher than ceftazidime. We have divided this group into (a) those derived from TEM enzymes, (b) all the SHV-derived extended-spectrum β-lactamases, and (c) two enzymes—one from Japan and one from India—of unknown origins.

All these enzymes confer little, if any, significant resistance to the combination of a β-lactam agent and β-lactamase inhibitor, particularly clavulanic acid. Therefore, such combinations should remain effective. However, a new plasmid-mediated β-lactamase which confers resistance, not only to extended-spectrum β-lactam agents but also to β-lactam/β-lactamase inhibitor combinations, has recently been identified. This enzyme, called BIL-1, appeared in a clinical isolate of E. coli in Pakistan. The pl of this enzyme was extraordinarily high at pl 8.8 and thus it could have been confused with the induced β-lactamase of E. coli, which is believed to account for the CEP-1 enzyme. The CEP-1 β-lactamase has a pl of 8.2 and was shown to be quite distinct from BIL-1. On the other hand, the BIL-1 β-lactamase does have a very similar pl to the Enterobacter

Table II. Plasmid-mediated extended-spectrum β-lactamases

<table>
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<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Unassigned</th>
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<tbody>
<tr>
<td>TEM-E1</td>
<td>TEM-E3</td>
<td>TEM-derived</td>
<td>BIL-1</td>
<td>FUR</td>
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<tr>
<td>TEM-E2</td>
<td>TEM-6</td>
<td>TEM-3</td>
<td>MJ-1</td>
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<tr>
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<td>TEM-10</td>
<td>TEM-5</td>
<td>Unnamed 1</td>
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<td>CAZ-3</td>
<td>CAZ-7</td>
<td>CAZ-2</td>
<td>Unnamed 2</td>
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<td>CAZ-hi</td>
<td>CAZ-6</td>
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<td></td>
<td></td>
<td>SHV-derived</td>
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<td>SHV-2</td>
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<td>SHV-5</td>
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<td>DJP-1</td>
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</table>

Group 1, poor hydrolysis of cefotaxime and ceftazidime.
Group 2, better hydrolysis of ceftazidime than cefotaxime.
Group 3, better hydrolysis of cefotaxime than ceftazidime.
Group 4, confers resistance to all cephalosporins and clavulanic acid.
of class A chromosomal $\beta$-lactamase. In any case, this enzyme appears to result from a chromosomal gene, encoding a class 1 $\beta$-lactamase, migrating into a plasmid vector. The BIL-1 $\beta$-lactamase has virtually no hydrolytic activity against cefotaxime, ceftriaxone, ceftazidime, or ampicillin. It does possess activity against nitrocephin and cephaloridine. It has approximately the same relative efficiency of hydrolysis against these two substrates as the ubiquitous TEM-1. The inhibition properties of BIL-1 are much more interesting. It is readily inhibited, and, therefore, binds ampicillin, ceftaxime and cephaloridine. It is 4000 times less sensitive to inhibition by clavulanic acid. There are five enzymes that do not fit into the classification scheme, mainly because there are insufficient data. It is not yet known from where many of these enzymes are derived, and only one has been found on more than two occasions. New enzymes are being discovered constantly and will be entered into this scheme. It is, of course, vital to know the biochemical relationships, because this probably reflects the selective pressures. However, with most resistance mechanisms, classification often takes account of similarities in nucleotide or amino-acid sequences. This requires full gene sequencing to identify the relationships and will be of particular interest with the SHV- and TEM-derived enzymes.

With the SHV-1 enzymes, the relationships are well-defined and are shown in fig. 2. Each box represents a single amino-acid mutation. Therefore, it is easy to see how SHV-1 led to SHV-2 and that SHV-4 was derived either through SHV-3 or SHV-5. All the intervening enzymes have been found in the clinical environment.

The relationships between the TEM-enzymes are much more complicated (fig. 3). Again, each box represents a single amino-acid mutation. It can probably be assumed that the mutation between TEM-1 and TEM-2 occurred before the later enzymes emerged and that the change at amino-acid position 37 does not occur with the extended-spectrum $\beta$-lactamases. Therefore, all those mutations in the top half arose from TEM-1 and those in the bottom half from TEM-2. Unlike the SHV enzymes, most of the intermediates have not yet been identified. The laboratory-selected $\beta$-lactamase, TEM-101, a mutation of TEM-1, may well be the TEM-E2 we found in the Liverpool K. oxytoca isolate, but whether TEM-9, for instance, is derived from intermediate A or D is not known.

Plasmid-mediated $\beta$-lactamases are a vast array of enzymes; there are nearly 60 distinct enzymes. It is almost certain that many more will be identified. The immediate potential clinical threat comes from the extended-spectrum $\beta$-lactamases, however, we must wait to see which, if any, of these enzymes will emerge as the successor to TEM-1.

**Resistance to the 4-Quinolones**

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The 4-quinolones are chemically synthesised compounds whose principal target is the enzyme DNA gyrase (E.C. number 5.99.1.3), the only bacterial enzyme capable of introducing negative supercoils into DNA. DNA supercoiling plays an important role in bacterial metabolism—it compacts the chromosome and is involved in the regulation of gene transcription as well as the bacterial response to the environment. The enzyme is a tetramer consisting of two A and two B subunits. The A subunit cuts both strands of DNA simultaneously at intervals four base pairs apart and holds the strands apart but covalently bound to the enzymes. The B subunit, using ATP for energy, then introduces a negative supercoil into the DNA and the A strand then reseals the two strands.

The first 4-quinolone, nalidixic acid, was synthesised almost 30 years ago. Its antimicrobial spectrum is limited to the Enterobacteriaceae; it is useful for the treatment of urinary tract and enteric infections. The antimicrobial spectrum of the 4-quinolones was extended by the discovery that the addition of a piperazine at C6 and fluorine at C7 to the common 4-oxo-1,4-dihydroquinolone skeleton caused a 1000-fold improvement in antimicrobial activity. Bacterial species intrinsically resistant to nalidixic acid are susceptible...
to these new fluoroquinolones and, in the 1980s, compounds such as pefloxacin, norfloxacin, ciprofloxacin, enoxacin and ofloxacin, active against a wide range of bacterial species, became available for clinical use.

The increasing therapeutic potential of these antimicrobial agents has caused considerably interest in the mechanisms by which bacterial resistance to the 4-quinolones is mediated. Bacterial resistance to antimicrobials can be mediated either via a plasmid or a chromosomal mutation.

Plasmids often carry several genes conferring resistance to several antimicrobial agents, therefore, in clinically important bacteria, tend to be the main reservoir of resistance to most antimicrobial agents. This is not the case with the 4-quinolones as, unlike other commonly used antimicrobial agents, plasmid-mediated resistance to the 4-quinolones has yet to be identified in clinical isolates. It should be noted that plasmids have been associated with nalidixic acid resistance in shigellad isolated from Indian subcontinent, but they did confer resistance to the 4-quinolones. Subsequently, although plasmid-mediated 4-quinolone resistance has yet to be found clinically, a potential mechanism of resistance, which may involve drug accumulation, has recently been reported.

The norA gene identified in Staphylococcus aureus codes for a 50-KDa protein which appears to be membrane associated and confers high-level resistance to hydrophilic fluoroquinolones. This gene has been cloned into plasmid pBR322. When a quinolone-susceptible strain, Escherichia coli HB101, was transformed with the recombine plasmid, it developed resistance to the 4-quinolones. The MICs of hydrophilic drugs such as enoxacin, norfloxacin, ciprofloxacin and ofloxacin increased 8 to 64 fold whereas only a two-fold increase was observed for the hydrophobic 4-quinolones nalidixic acid and sparfloxacin. Kaatz et al. have also recently identified a mutation similar to norA in an isolate of S. aureus which conferred resistance on an E. coli recipient strain when it was cloned on to a plasmid (unpublished results). Thus, a mechanism by which a plasmid could confer resistance to the 4-quinolones has been identified although it has yet to be found in a clinical isolate.

The probability of plasmid-mediated 4-quinolone resistance arising via the two other mechanisms that have been identified for other classes of antibacterial agents seems low. A drug destruction mechanism appears unlikely as the 4-quinolones are active against the 4-quinolone resistant DNA gyrase coded by a plasmid would also seem an unlikely candidate because genetic studies have shown that quinolone sensitivity is dominant over quinolone resistance in gyrA genes. Indeed, transformation of quinolone resistant strains with plasmids carrying quinolone susceptible gyrA genes have been used to identify alterations in DNA gyrase as the cause of resistance to the 4-quinolones in gram-negative bacteria.

As plasmid-mediated quinolone resistance has yet to be identified in a clinical strain, chromosomal mutations are the only mechanism by which bacteria are able to develop resistance to the 4-quinolones during therapy. Chromosomally-mediated resistance to the 4-quinolones can occur by one of two mechanisms—either an alteration in the target enzyme DNA gyrase, or a mutation that reduces drug accumulation.

In most species investigated so far, high-level resistance to all 4-quinolones appears to be conferred by mutations in the gyrA gene which codes for the A subunit of DNA gyrase. GyrA mutations confer high-level cross-resistance to all 4-quinolones but do not seem to be associated with resistance to other, unrelated antibacterial agents. Such mutations have been identified in E. coli (cfxA, gyrA, nalA, nfxA). Pseudomonas aeruginosa (nalA, cfxA). Pseudomonas aeruginosa (nalA, cfxA). Haemophilus influenzae. Citrobacter freundii and Serratia marcescens. The gyrA genes of E. coli, Bacillus subtilis, S. aureus and Klebsiella pneumoniae have been sequenced revealing close homology between the gyrases of different species. Nucleotide sequence analysis performed on 10 spontaneous quinolone-resistant gyrA mutants of E. coli KL166 showed that quinolone resistance appeared to be conferred by point mutations within a small region between amino acids 67 and 106 near the N terminus of the A subunit. Six of the 10 mutants possessed a substitution at amino acid 83, a serine. This mutation has also been identified in a clinical isolate of E. coli that developed resistance during therapy. In S. aureus, this serine is located one amino acid further on at position 84. There is also a serine at amino acid 85 (aa 84 is alanine in E. coli) and substitution of either amino acid was found to be the cause of quinolone resistance in clinical isolates of S. aureus. Furthermore, high level ciprofloxacin resistance in S. aureus (MIC > mg/L) was found to be associated with substitution of both serines. It is interesting to note that all of the mutations conferring quinolone resistance are situated close to the tyrosine at amino acid 122 of the A subunit that is covalently bound to DNA when the enzyme breaks the phosphodiester bonds of DNA. Although little is known about the quinolone-gyrase-DNA complex, it would seem that the alterations in amino acid residues result in disruption of key interactions of the complex.

Mutations in the B subunit of DNA gyrase coded by the gyrB gene have been shown to cause 4-quinolone resistance in E. coli and P. aeruginosa. However, unlike gyrA mutations, these do not always cause cross-resistance to all 4-quinolones. The nal31 mutation in E. coli confers resistance to the 4-quinolones lacking a C7 piperazone while rendering bacteria hypersensitive to 4-quinolones possessing this substituent. This phenomenon results from the mutation increasing the negative charge of the B subunit, increasing its attraction for the positively charged piperazone group.

The 4-quinolones must penetrate bacteria to gain access to their target DNA gyrase. Therefore, a reduction in the ability of the 4-quinolones to enter bacteria results in decreased bacterial susceptibility. The 4-quinolones penetrate bacteria by diffusion through porins in the outer membrane of gram-negative bacteria. The hydrophobic 4-quinolones such as nalidixic acid are also able to penetrate the bacterial through the phospholipid bilayer. Active transport may also play a role in bacterial resistance of the 4-quinolones to endogenous active efflux of norfloxacin. It has been demonstrated at the inner membrane of E. coli and P. aeruginosa.

In gram-negative bacteria, mutations affecting 4-quinolone permeability have been identified in E. coli (nalB, nfxB, norB, cfxB), Salmonella, Pseudomonas (nalA, cfxA and nfxB, qr1, qr2), Klebsiella and Serratia spp. and have been reviewed recently. Unlike alterations in DNA gyrase, which confer only cross-resistance to other 4-quinolones, resistance caused by the reduction in 4-quinolone accumulation can be associated with decreased sensitivity to unrelated antibacterial agents. Mutations in E. coli which confer resistance to the 4-quinolones by an impermeability mechanism are associated with alterations in outer-membrane porin F (ompF). Both inactivation of the ompF gene and mutations in regulatory genes controlling expression of ompF at a post-transcriptional level can cause 4-quinolone resistance, e.g., cfxB appears to be an allele of the marA gene which confers resistance to tetracyclines and chloramphenicol as well as to the 4-quinolones.

In P. aeruginosa, resistance to the 4-quinolones resulting from reduced drug accumulation has been associated with alterations in a wide range of outer-membrane proteins. Resistance to the 4-quinolones can be associated with cross-resistance to other antibacterial agents, e.g., the qr1 and qr2 mutations associated with a reduction in ompF and an
of high incidence of ciprofloxacin resistance in methicillin-resistant *S. aureus* (MRSA) isolated in hospitals. This resistance does not necessarily appear to develop during therapy of the MRSA infection may be associated with use of ciprofloxacin for treatment of a pathogen other than MRSA. It has been suggested that ciprofloxacin resistance can develop in colonising MRSA which then subsequently spread to other patients in the hospital.

Problems may also exist in other bacterial species. The frequency of resistance to the 4-quinolones may be increasing in *Bacteroides fragilis* and *Ser. marcescens*. Surveys in Japan have reported high incidences of quinolone resistance in these species.

In conclusion, the clinical incidence of 4-quinolone-resistant bacteria is still relatively rare, although it seems to be occurring with increasing frequency in certain species such as *P. aeruginosa* and *staphylococci* (particularly multi-resistant organisms). Resistance is not plasmid-mediated at present although a potential for increased mortality, morbidity and expense it causes. This is particularly true for resistant strains having the ability to spread rapidly in institutions causing outbreaks of nosocomial infection.

The uptake of the aminoglycoside antibiotics by the bacterial cell and their mechanisms of action has been shown to be a complex process, as yet incompletely understood. Nevertheless, some discussion of these events is helpful in understanding the different mechanisms of aminoglycoside resistance.

**Aminoglycoside uptake and action**

The uptake of aminoglycosides by bacterial cells has been most extensively studied with streptomycin and gentamicin. It differs from the uptake of most antibiotics in that it is active (energy-dependent), and has been divided into three phases. The first phase of uptake is the attraction and binding of the antibiotic to the cell outer membrane of gram-negative rods, and is rapid and passive. The second and third phases have been named energy-dependent phases I (EDP-I) and II (EDP-II). The initial passive uptake of aminoglycoside is thought to depend on the electrical potential across the outer membrane. Aminoglycosides are cationic and are, therefore, attracted to anions on the cell surface and across the outer membrane by the internally negative electrical potential, passing through the porin channels of gram-negative cells or water filled interstices of gram-positive cells. In *Pseudomonas aeruginosa*, which has a less permeable outer membrane, a model has been proposed whereby the aminoglycoside binds to the negatively charged
magnesium binding sites on the membrane surface, causing disruption of the membrane barrier and facilitating entry into the cell of aminoglycoside. The extent and rate of ion uptake depends on the size of the electrical potential gradient across the outer membrane, and a number of inhibitors which reduce ionic binding and diffusion have been described.

The EDP-I appears to be comparatively slow, and is thought to represent the transport of the aminoglycoside across the cytoplasmic membrane to come into contact with the ribosome, perhaps on transporter molecules. It precedes any of the intracellular events leading to cell death and appears to be the rate limiting step in uptake.

The EDP-II occurs as the aminoglycoside binds to specific binding sites on the 30S ribosome and the initiation of protein synthesis is disrupted. This leads to changes in the permeability of the cell membrane and a secondary, rapid escalation in antibiotic uptake, cell disorganisation and, ultimately, cell death.

In aerobic gram-negative bacteria, the kinetics of cell killing has been shown to be biphasic. An initial phase of cell death occurs soon after the aminoglycoside is administered and is thought to be associated with the toxic binding to the cell. It appears to be very rapid and related to the concentration of the antibiotic. A second phase of killing is much slower and independent of drug concentration. Such findings show the importance of obtaining adequate peak aminoglycoside concentrations and have been used as an argument in favour of single, large, once-daily dosage regimens. However, despite this, the slower energy-dependent phases of uptake are the most important phases in the bactericidal action of aminoglycoside antibiotics. Many antibiotics acting on protein synthesis, e.g., chloramphenicol, are bacteriostatic. They do not have the same complex energy-dependent uptake mechanisms as the aminoglycosides, and the interference in protein synthesis by many of these agents is reversible. The uptake of aminoglycosides is irreversible in vitro unless the cell membrane is destroyed by an agent such as toluene, and the rapid influx of antibiotic occurring in EDP-II appears to be the important step in the irreversible disruption of protein synthesis conferring cidal activity.

Mechanisms of resistance to aminoglycosides

There are three mechanisms by which resistance of bacteria to aminoglycosides may occur—enzymatic modification, deficiency of uptake or accumulation, and modification of the ribosomal target site.

Enzymatic modification. This is the most common and clinically important cause of acquired aminoglycoside resistance. Enzymes may modify the aminoglycoside by various mechanisms—particularly N-acetylation, O-nucleotidyltransferase or O-phosphorylation. A number of different aminoglycoside modifying enzymes (AME) have been identified and the nomenclature for their description consists of a three letter code according to their mode of action—aminoglycoside acetyltransferase (AAC), aminoglycoside phosphoryltransferase (APH) and aminoglycoside adenylyltransferase or nucleotidylase (AAD or ANT). The site of action of the enzyme on the aminoglycoside molecule is denoted by a number in parenthesis, e.g., AAC(3') represents acetylation of the 2'-amino group. Further characterisation may be given by the addition of roman numerals to differentiate enzymes of similar actions. Thus, potentially, an aminoglycoside may be modified at more than one site and, therefore, be a substrate for more than one enzyme. Similarly, an enzyme may have several different substrates.

Most genes coding for AMEs are plasmid specified, and many are known to be carried on transposons. Plasmid transfer may occur by conjugation, transduction or transfor-
However, such isolates quickly revert to susceptibility when exposure to the aminoglycoside is removed. This suggests that they are not simply resistant mutants selected by antibiotic exposure. Often, colonies are readily identifiable by their small size, slow growth and atypical morphology, which could also reflect intracellular metabolic changes. Such adaptive resistance may have clinical relevance to cases of treatment failure in conditions requiring prolonged or repeated aminoglycoside therapy, e.g., endocarditis and cystic fibrosis, and could be difficult to detect in the laboratory as isolates may revert to susceptibility at initial isolation.

Modification of ribosomal target site. Aminoglycosides interfere with the initiation of protein synthesis by binding to specific binding sites on the 30S ribosome. As yet, mutations affecting this specific target site do not appear to have great clinical importance. Experimental and clinical isolates showing resistance to streptomycin and spectinomycin by this mechanism have been described, and are thought to arise by single step mutations at the StrA locus. Although mutations near to this site may produce resistance to other aminoglycosides, this has not been seen in clinical isolates.

The epidemiology of aminoglycoside resistance

It is not surprising that resistance to aminoglycosides is becoming increasingly recognised, as these agents have been widely used in hospitals for more than 20 years, not only for treatment but also for prophylaxis. They are also used in veterinary practice and in commercial processes involving animals. Several multicentre study groups have documented the prevalence of aminoglycoside resistance and also the mechanisms of resistance. These include groups in the UK, Europe, and the USA. Such collaborative studies are essential as the prevalence of aminoglycoside resistance in each individual hospital is low, but they have generally only been able to give a single snapshot of patterns of resistance (the point prevalence). More information can be obtained from studies of the prevalence of resistance over a period time, or studies that have been repeated, such as the European Study on Antibiotic Resistance (ESGAR) reports of 1987 and 1990. A summary of some of these reports is shown in Table III. Variations in the resistance level can be found, not only between countries, but also between laboratories participating in individual studies. One striking difference is the increasing resistance from Northern to Southern Europe (table IV). The USA has resistance levels approaching those of Southern Europe. However, care must be taken when comparing these studies as the isolates examined come from a variety of clinical sources. The majority of studies have looked at consecutive blood-culture isolates, but some have included all clinical isolates or urine isolates. Also, the distribution of organisms varies between studies; those finding a higher incidence of Providencia, Serratia and Acinetobacter spp. and coagulase-negative staphylococci have reported a high level of aminoglycoside resistance. Differences in the geographical pattern of resistance may reflect different patterns of aminoglycoside usage. Similarly, the isolation of organisms more likely to be aminoglycoside-resistant, such as those listed above, may reflect selection secondary to antibiotic exposure.

In all studies, the most common mechanism of aminoglycoside resistance is modification by enzymes. Impermability resistance accounts for < 10% of resistant isolates and is seen most frequently in non-aeruginosa Pseudomonas spp. The distribution of AMEs varies from country to country. In Europe as a whole, the most common AMEs in gram-negative bacilli are ANT(2'), AAC(3)I and ACC(6')I, and in staphylococci the double-acting AME APH(2')/AAC(6') combined and ANT(4'). Combinations of AMEs are common and a wide variety of different enzymes is seen, especially in isolates from Southern Europe where AAC(6')I, either alone or in combination, is responsible in part for higher resistance levels. In the UK, the most common enzymes are AAC(3)I and ANT(2') in gram-negative bacilli and APH(2')/AAC(6') in staphylococci. Similar findings have been described from the USA and Australia. This differs from the Far East, where AAC(6')I is most common, and Chile, where >90% of aminoglycoside-resistant isolates possess ACC(3). Again, these differences may reflect pressure due to antibiotic usage. In Japan, for example, the high usage of kanamycin and dibekacin may have encouraged the selection of AAC(6'), which causes resistance to this group of antibiotics, whereas AAC(3), which confers resistance to gentamicin, sisomycin and netilmicin, is rarely seen. The presence of a single enzyme in resistant Chilean isolates may represent the distribution of a single R-plasmid in a hospital. In studies from other countries, however, there has been a tendency in more recent surveys to find an increasing number of different AMEs, often in combination. Although selection pressure from hospital aminoglycoside usage may be the most important factor controlling this, other factors, such as spread of resistance through animals may also be significant.

### Table III. Gentamicin resistance (%) described in some recent studies

<table>
<thead>
<tr>
<th>Study reference</th>
<th>Number of isolates</th>
<th>Source of isolates</th>
<th>All clinical</th>
<th>Saudi Arabia</th>
<th>Europe</th>
<th>UK</th>
<th>Europe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finland</td>
<td>633</td>
<td>Blood</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Sweden</td>
<td>2027</td>
<td>Blood</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>St Thomas's Hospital, UK</td>
<td>7264</td>
<td>Blood</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>6501</td>
<td></td>
<td></td>
<td>3</td>
<td>0</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0</td>
<td></td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>5-5</td>
<td></td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Citrobacter spp.</td>
<td>0</td>
<td></td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0</td>
<td></td>
<td>15</td>
<td>11</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>0.7</td>
<td></td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>20</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Coagulase-positive staphylococci</td>
<td>20</td>
<td></td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
Methods for identification of resistance mechanism

Some idea as to the mechanism of resistance of an aminoglycoside-resistant isolate can be obtained from the resistance pattern, level of resistance, colonial morphology, and growth, as described above. Most isolates will be resistant by enzymatic modification. Identification of the AME is rarely necessary clinically, but may be useful for epidemiology or research. Identification may be presumed by testing susceptibility to the aminoglycoside substrates for the enzyme, and simple methods have been described based upon either MICs or inhibition zone diameters. It is likely that results of these tests will become increasingly difficult to interpret as the frequency of combinations of enzymes increases. Furthermore, some of these methods use, ideally, aminoglycosides not in clinical use and therefore difficult to obtain.

Most other methods for enzyme identification are unlikely to be used by clinical laboratories. Radiolabelled co-factors have been widely used to measure the binding of modified aminoglycoside to cellulose phosphate paper but can give confusing results with some enzyme combinations. High performance liquid chromatography of the reaction products may also be used; this has the advantage of specifically identifying reaction products. Most recently, techniques have concentrated on the genes coding for AMEs. Specific probes have been developed for detecting AME genes, and the polymerase chain reaction can further facilitate this. As techniques improve, these methods will become more sensitive and may prove to be very useful for identifying combinations of AMEs.

Glycopeptide resistance in gram-positive bacteria

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Introduction

Some of the most used antibiotics in clinical medicine are those that affect the bacterial cell wall which is composed of a variety of macromolecules, some of them found uniquely in bacteria. The majority of clinically-important bacteria contain peptidoglycan, which confers rigidity on the bacterium and is the main molecular determinant of cell shape. It is a large, polar molecule which is assembled on the outside surface of the bacterium in three main stages from subunits manufactured inside the cell. The subunits are transferred across the membrane, polymerised to form short polysaccharide chains substituted with unusual peptides which are finally cross-linked to the existing wall by the transpeptidation reactions. This series of reactions leading to the formation of cross-linked peptidoglycan affords a plethora of potential targets for antimicrobial agents. The presence of a unique amino-sugar and the un-natural D-isomers of two amino acids ensures that these targets are unique to bacteria and that antibiotics interfering with the biosynthetic pathway are likely to be selectively toxic to bacteria. The last two reactions in the pathway (polymerisation and transpeptidation) occur outside the cytoplasmic membrane and, consequently, antibiotics that interfere with these stages of synthesis do not require a transport system to reach the target site. Glycopeptide antibiotics (vancomycin and teicoplanin) inhibit the first of these two reactions (and would also block transpeptidation) by binding tightly to the unique acyl-D-alanyl-D-alanine arrangement of amino acids in the membrane-bound nascent peptidoglycan.

Mechanism of action of glycopeptide antibiotics

Glycopeptide antibiotics are effective at low concentrations against the majority of gram-positive bacteria, but toxicity problems in the early years of their application precluded their widespread use in spite of their selective action against the peptidoglycan. The introduction of β-lactamase-stable penicillins resulted in a temporary reduction in the clinical use of vancomycin, but the acquisition of alternative mechanisms of resistance to β-lactam antibiotics, in particular, and an improvement in the purity of the vancomycin preparations, witnessed a resurgence in the use of glycopeptides, particularly against multiresistant staphylococci that are intrinsically resistant to all β-lactam antibiotics. Glycopeptide antibiotics, including vancomycin and teicoplanin, are large, rigid molecules; they adopt a bracelet-like configuration with a substantial cleft or pocket into which peptides ending in D-alanyl-D-alanine bind firmly, though non-covalently, with almost computerised precision. It is the mechanism of action of this group—interaction with a structural component of the cell wall rather than with a target protein—that makes this class of antibiotics unique and reduces the possibility of large-scale resistance emerging in clinical strains. Definitive studies of the interaction of glycopeptides with the target peptides were elucidated with the use of nuclear magnetic resonance spectroscopy, by mass spectrometry followed by model building, and by 3-dimensional reconstruction of the binding site. The wall subunit, either attached to its lipid carrier or as part of the growing peptidoglycan chain to which the new subunit will be added, is held firmly by hydrogen bonding to the peptide backbone of the glycopeptide (fig. 4). The space-filling model of the glycopeptide-pentapeptide complex indicates that the sugars in the wall subunit are not immediately adjacent to the glycopeptide molecule, but the peptidoglycan polymerase whose active site must be located close to the sugars is a large molecule in comparison with both the antibiotic and the wall subunit. Presumably, the
The presence of the antibiotic on the subunit prevents the enzyme from binding precisely in the correct position so that it can no longer catalyse the transglycosylation reaction that polymerises the peptidoglycan backbone chain. In addition to the inhibition of the peptidoglycan polymerase by steric hindrance (fig. 5), binding of glycopeptides to the D-alanyl-D-alanine terminus of the subunit effectively blocks the vital transpeptidation reaction that links the growing peptidoglycan chain to the mature cell wall. As the result of the inhibition of these two sequential reactions wall synthesis is frozen.

**Resistance to glycopeptides**

The structure of glycopeptides, as well as their unique mechanism of action, is likely to ensure that any resistance mechanism acquired by a bacterium will be unusual compared to conventional mechanisms such as destruction or inactivation of an antibiotic or drastic modification of the target site. Other possible resistance mechanisms include sequestration of the antibiotic molecule by non-specific binding and altered accessibility of the target site. Some bacteria are naturally insensitive to glycopeptides, either because entry through the outer membrane is prevented (gram-negative bacteria) or for an unknown reason (some lactobacilli, leuconostocs, pediococci), though inactivation of the antibiotic molecule has not yet been detected in any instance of resistance.

Sequestration of the available glycopeptide as the result of non-specific binding is also unlikely for various reasons: vast over-production of suitable peptides to complex with the antibiotic molecules would be necessary to raise the MIC of a sensitive strain so dramatically. Such an increase in non-specific binding could occur through secretion of wall precursors into the medium, an enhanced rate of wall turnover involving loss of material into the medium, a substantial increase in the amount of wall peptidoglycan present (and consequent wall thickening) or a decrease in degree of cross-linking in the peptidoglycan with resultant increase in specific binding to non-essential target sites. As yet, none of these scenarios has been detected in investigations of resistant coagulase-negative staphylococci or other resistant (or insensitive) strains.

If any of these situations arose there would be a dramatic decrease in the amount of free glycopeptide in the culture medium of resistant strains in comparison with that of their isogenic sensitive strains and no gross increase in glycopeptide binding to resistant

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**Fig. 4.** Diagrammatic representation of the interactions between a peptide terminating in D-alanyl-D-alanine and the aglycone of a glycopeptide. Five hydrogen bonds are formed.

**Fig. 5.** Diagrammatic representation of glycopeptide molecules bound to D-alanyl-D-alanine sequences in a new wall subunit and the growing nascent glycan chain; these molecules may prevent, by steric hindrance, the transglycosylase reaction that links the nascent chain to the new subunit. The shaded area represents the approximate extent of the glycopeptide. The transglycosylase enzyme is not directly inhibited so resistance cannot result from mutation of the gene encoding the enzyme. Prevention of binding of glycopeptide molecules to the target acyl-D-alanyl-D-alanine grouping could be brought about by inactivation of glycopeptides (not detected); sequestration of antibiotic in the growth medium (not detected); enzymic modification of the target site (difficult for the subsequent essential transpeptidation reaction to occur); or protection of the target site by another molecule (i.e., changing the accessibility to glycopeptides).
bacteria or in complex formation in the culture medium has yet been reported.

**Genetic studies**

The recently reported resistance in coagulase-negative staphylococci and in enterococci is likely to result either from a change in accessibility of the target or from modification of the target. The resistant enterococci have been studied intensively in several laboratories and at least two types have been identified (Table V): high-level resistant strains are resistant to both vancomycin and teicoplanin whereas low-level resistant strains are initially susceptible to teicoplanin and moderately resistant to vancomycin.\(^{111-115}\)

Resistance is inducible by vancomycin and teicoplanin in the high-level resistant isolates, but only by vancomycin in the low-level resistant strains; however, once resistance to vancomycin is induced, these strains become resistant to teicoplanin as well suggesting that a common factor is involved. The two types of strains differ also with respect to the transferability of resistance to other related or unrelated bacteria. Plasmids encoding resistance have been detected and isolated from some but not all the high-level resistant enterococci\(^{116}\) and have been transferred by conjugation to glycopeptide-sensitive strains of the same or related species. In one instance, a plasmid has been transferred from its host of *Enterococcus faecium* to a range of other gram-positive bacteria including *Streptococcus sanguis*, *Strep. lactis*, *Str. pyogenes* and *Listeria monocytogenes* but not to *Staphylococcus aureus* or *Bacillus subtilis*.\(^{114}\) If transfer to, and expression of, glycopeptide resistance in *S. aureus* had been achieved, clinical microbiologists would have been very concerned at the prospect of glycopeptide resistance being added to the growing list of antibiotic resistances in multiresistant strains of staphylococci.

Purified plasmid DNA from resistant *E. faecium* has been used to transform *Str. sanguis* Challis to glycopeptide resistance; subsequently a 4-Kb EcoRI fragment containing the gene (*vanA*) encoding resistance was cloned in *Escherichia coli* on a conjugal gram-negative–gram-positive shuttle vector and transferred by conjugation of *Ent. faecalis* and *B. thuringiensis*, which became resistant to glycopeptides.\(^{117}\)

Transferrable resistance in high-level resistant strains is not always associated with plasmid DNA; plasmids were not detected in either the donor or transconjugants in one report of transfer, and in another a strain cured of glycopeptide resistance retained all three plasmids present in the resistant parent.\(^{116}\) It has been suggested that, in these instances, the resistance gene may have a chromosomal location, possibly in association with a transposon. Such a location has also been postulated for the gene encoding resistance to low-level glycopeptide-resistant strains because resistance is not transferable and no plasmids have been detected. Sequencing of the N-terminus of the *VAN A* protein which is present in high-level resistant strains that have been induced (see below) has enabled the *vanA* gene to be identified and sequenced.\(^{118}\) The deduced amino-acid sequence corresponded to a protein of calculated Mr 37.4 × 10\(^3\), similar to that of the protein seen by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (39 KDa). The protein encoded by the *vanA* gene appears to be structurally related to the d-alanyl-d-alanine ligases of *E. coli* and *Salmonella typhimurium* (28% and 36% amino acid identity respectively). Furthermore, the *vanA* gene, when introduced into an *E. coli* mutant strain with a thermosensitive d-ala-d-ala ligase, was able to achieve complementation at 42°C.\(^{118}\)

**Biochemical studies**

The presence of the *vanA* gene in high-level glycopeptide-resistant strains and its introduction into sensitive strains is associated with the production of a membrane-associated protein of approximately 39 KDa following induction with glycopeptides.\(^{112,115,116}\) The amounts of the protein in sensitive or in uninduced resistant bacteria are very low or non-existent. All high-level resistant enterococcal strains that have been examined contain the *vanA* gene as demonstrated by probing with a 290-bp probe specific for *vanA*, whereas DNA from the low-level resistant strains did not hybridise with this probe.\(^{112}\) Also, hybridisation was not detected with DNA of the teicoplanin-resistant coagulase-negative staphylococci or the intrinsically resistant leucomestos, pediococci and lacticococci.\(^{117}\) The presence of two glycopeptide-producing strains *Actinomyces orisorientalis* ATCC 19795 (vancomycin) and *A. teichomyceticus* ATCC 31211.

### Table V: Characteristics of glycopeptide-resistant gram-positive bacteria

<table>
<thead>
<tr>
<th>Resistance property</th>
<th>Acquired resistance in</th>
<th>Intrinsic resistance* (Lactobacilli, leucomestos, pediococci)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>enterococci</td>
<td>staphylococci</td>
</tr>
<tr>
<td>MIC (mg/L) (\geq 64)</td>
<td>(16-32)</td>
<td>(1-2) (S)</td>
</tr>
<tr>
<td>teicoplanin (\geq 16)</td>
<td>(0.5) (S)</td>
<td>(8-32)</td>
</tr>
<tr>
<td>Transferability +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Plasmid-mediated Inducibility by vancomycin +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>teicoplanin +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mass of resistance protein (KDa)</td>
<td>39</td>
<td>39.5</td>
</tr>
<tr>
<td>Hybrdisation to <em>vanA</em> probe +</td>
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<td>+</td>
</tr>
<tr>
<td>Reaction with antibody to 39-3-KDa protein +</td>
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<td>+</td>
</tr>
</tbody>
</table>

(S), sensitive.
ND, not determined.
* This group includes the two glycopeptide-producing strains *Actinomyces orisorientalis* ATCC 19795 (vancomycin) and *A. teichomyceticus* ATCC 31211.
† Teicoplanin-resistant strains of *S. epidermidis* contain an additional membrane protein (39 KDa) in comparison with a non-isogenic sensitive strain.
Mechanism of resistance—hypotheses

The balance of evidence suggests that resistance in the enterococci results from altered accessibility of glycopeptide molecules to their target sites. A reduction in accessibility could be due either to production of the target cell-wall peptides (acyl-D-ala-D-ala) by another molecule so that glycopeptide molecules are prevented from binding, or to enzymatic modification of the target so that glycopeptides no longer recognise the target and cannot bind to it (or bind with much lower affinity).

Modification of target. The product of the vanA gene has considerable homology with D-ala-D-ala ligases from gram-negative organisms. The specificity of these enzymes is not absolute and, with appropriate substrate pressure, it is conceivable that a wall precursor could be synthesised in which the terminal D-ala-D-ala was replaced with a different dipeptide. Theoretical considerations render this suggestion unlikely: the introduction of cross-bridges between peptides in the peptidoglycan depends on transpeptidation, which in turn requires the two C-terminal amino acids of the precursor to be in the D-configuration. Even if one or both amino acids could be altered to other D-amino acids under normal physiological conditions, it is doubtful if the binding affinity of glycopeptides to the peptide would be changed substantially.

Strong experimental evidence for a different type of modification comes from investigations in which it was shown that wall-membrane fragments of both high- and low-level resistant enterococci prevented the binding of vancomycin and teicoplanin to a soluble pentapeptide terminating in acyl-D-ala-D-ala. In the absence of the wall-membrane fragments, or with an identical preparation from sensitive or uninduced resistant enterococci, binding of vancomycin to the added peptide was not prevented. This is an excellent model system for studying the binding of glycopeptides to their target sites and it was shown that prevention of binding was time dependent. It could be blocked by adding a large excess of the dipeptide D-ala-D-ala (which does not itself bind to glycopeptides), and it was destroyed by heating the wall-membrane preparation—these observations are consistent with the involvement of an enzyme in the protection phenomenon.

Furthermore, in these experiments there was a high molar excess of pentapeptide to induced 39-Kda protein which rules out simple binding as a resistance mechanism. It was postulated that enzymatic modification may have involved the release of D-alanine from the pentapeptide, though such an activity would prevent subsequent transpeptidation if it occurred in vivo. There are two lines of evidence not in accord with this hypothesis: the resistant enterococci are susceptible to some derivatives of vancomycin, suggesting that the binding site for glycopeptides has not been changed substantially, and, more significantly, treatment of the wall-membrane preparation of the resistant enterococci with SDS at 100°C permitted binding of vancomycin to the residual wall whereas no binding occurred to the native preparation. This suggests that no drastic modification of the glycopeptide binding site has occurred.

Protection of target. Two lines of evidence suggest that the additional membrane protein present in resistant strains of enterococci that have been induced with glycopeptides recognises D-ala-D-ala, the binding site of glycopeptides. The VANA protein has considerable homology with D-ala-D-ala ligases (as discussed above) and the prevention of binding of vancomycin to a synthetic pentapeptide terminating in D-ala-D-ala by a wall-membrane preparation containing either the 38-Kda or the 39.5-Kda protein was eliminated by an excess of D-ala-D-ala. However, simple binding of the additional membrane protein to the glycopeptide binding sites is unlikely in view of the large number of these sites (2 x 10^3 in a staphylococcus), unless the topographical distribution of the protein in the membrane protects the wall precursors as they are extruded through the membrane and until they are fully incorporated into the mature wall.

Glycopeptide resistance in coagulase-negative staphylococci

The degree of resistance to glycopeptides in coagulase-negative staphylococci, particularly S. epidermidis and S. haemolyticus, is not as great as with the enterococci. Furthermore, these isolates are resistant to teicoplanin but not to vancomycin. This implies that the resistance mechanism will be different from that in the enterococci and could be accounted for by slightly altered accessibility. An additional membrane protein of 39 Kda has been found in resistant isolates of S. epidermidis in comparison with sensitive isolates (non-isogenic) but there is no direct evidence that this protein is involved with resistance except by analogy with the enterococci (M. D. O'Hare and P. E. Reynolds, unpublished observations).

Summary

Selective antibiotic pressure due to the increased use of glycopeptide antibiotics for the control of infections caused by gram-positive bacteria has resulted in the emergence of resistance in enterococci and coagulase-negative staphylococci. It is of particular concern that resistance is mediated in some enterococcal strains by self-transferable plasmids with a wide host range; such a situation may lead to the transfer of this type of glycopeptide resistance to other gram-positive pathogens.

Although glycopeptide antibiotics have been used for 30 years, cases of resistance have been slow to emerge. This is probably due to the unique interaction between a compact and relatively rigid antibiotic molecule and its target, which is, unusually, a substrate for peptidoglycan synthesis rather than an enzyme involved in catalysis. For this reason, any mechanism of resistance is likely to be unusual: diminished accessibility of glycopeptides to their target sites is the most likely possibility in both enterococci and coagulase-negative staphylococci but whether this results from target protection or modification has not yet been demonstrated convincingly.
results of routine diagnostic work for surveillance purposes is readily achieved, particularly if the work of the laboratory is computerised. The data derived may be used in the formulation of local prescribed guidelines and policies.

Special studies and surveys are also undertaken at a local level and may involve additional susceptibility testing and investigation of the mechanisms and genetics of particular resistance markers detected. Such studies may relate to local experience with particular pathogens, e.g., enterococci in Nottingham, or cover prolonged periods of study for organisms isolated from particular sites of infection, such as blood, e.g., Phillips and co-workers reported recently on the antibiotic susceptibility of bacteremia at St Thomas' Hospital over a 20-year period.

For the definition of national trends in antibiotic resistance, cultures isolated in different laboratories may be referred to one centre, where they can be examined for antibiotic susceptibility with standardised methods. Such studies may be specifically organised for this purpose or result from the examination of cultures submitted to reference laboratories for typing or other specialised studies. The number of different cultures, participating centres, and time periods of study that may be included in specially organised surveys are necessarily restricted by resource and staffing implications. Similarly, data derived from reference laboratory studies may be biased because the criteria for submission of status for reference work are not standardised.

An alternative approach to national surveillance is provided by the referral of results of susceptibility tests (done for diagnostic and therapeutic purposes) from diagnostic laboratories to one or more centres where they are compiled and analysed. Large multi-centre studies spanning several years have been performed in the USA and the UK. The recent UK report by Spencer and co-workers utilised a computer-based reporting scheme called Microbase to collect susceptibility data on nearly 367,000 isolates from 61 medical microbiology laboratories distributed throughout much of the UK.

The remainder of this paper describes an approach to the continuous surveillance of antibiotic resistance which utilises and expands the existing scheme for reporting bacterial isolates from cases of bacteremia and meningitis to the PHLS Communicable Disease Surveillance Centre (CDSC).

The CDSC operates a voluntary, confidential, laboratory reporting scheme to cover a wide range of infections in England and Wales. Within the CDSC reporting scheme, laboratories are requested to report significant bacterial isolates from cases of bacteremia and meningitis. During 1988, a major re-organisation of this reporting scheme was initiated involving the introduction of new computers, software and report forms. This re-organisation suggested to staff within the Division of Hospital Infection (DHI) of the Central Public Health Laboratory (CPHL) an opportunity to undertake continuous surveillance of antimicrobial susceptibility data for bacteremia and meningitis isolates reported to CDSC. Accordingly, after consultation with colleagues in CDSC, the new Communicable Disease Report (CDR) Form 2 was modified to include a request to the reporting laboratories to list the antimicrobial susceptibility test results obtained from routine diagnostic work on the cultures being reported. This approach has several advantages. Firstly, the data collected relates to organisms judged responsible for significant infection. Secondly, the only additional workload required in the reporting laboratory is to add their own susceptibility test results to the form they are already completing. Thirdly, the majority of all diagnostic microbiology laboratories in England and Wales participate in the reporting scheme giving very wide coverage. Fourthly, the results obtained can be analysed geographically and by clinical and epidemiological features (where these data are available); and finally the surveillance scheme can run continuously over many years providing a unique database providing trends in antibiotic resistance. The new report forms, with the request to include susceptibility test results, were introduced at the beginning of 1989. This paper presents some of the results obtained for bacterial isolates reported during the first half of the 1989.

Reporters were asked to enter antibiotic susceptibility test results as susceptible, intermediate or resistant (using their own definitions), for the bacterial isolates they were reporting. The susceptibility test results were added to the existing CDSC computer database records of individual patient infections (containing details of the source laboratory, and clinical and epidemiological features) by staff of the Division of Hospital Infection. As only those agents to which clinical isolates were routinely tested in the individual laboratories were included in the reports, there was considerable variation in the total number of tests for each antibiotic. Generally, we have selected for inclusion those antibiotics for which a result appears in >40–50% of reports for a particular species. When positive isolation was reported from both blood and CSF in cases of meningitis, infection was counted only once. Therefore each organism included in these totals relates to an individual patient's infection.

In this preliminary report it is not feasible to present the wealth of data that is being generated by this surveillance system. Therefore, we have chosen for inclusion data relating to four pathogens commonly reported to CDSC as causing bacteremia or meningitis, or both. Tables VI–IX show the reported susceptibility test results for Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae and Pseudomonas aeruginosa. The number of different laboratories from which the reports were received is also shown. In reports of these four bacterial infections, 84–87% included results of antimicrobial susceptibility tests and 87–92% of individual reporting laboratories included such data in their reports.

For those agents most likely to be used therapeutically for E. coli bacteremia (table VI)—the cephalosporins and aminoglycosides—full susceptibility was reported for >98% of isolates with the exception of cefuroxime, for which full susceptibility was reported for 92–6%. Susceptibility to ampicillin or amoxyccillin was reported for 45% and susceptibility to trimethoprim for 80% of the isolates tested. Amongst the 2366 S. aureus bacteremias reported, 92% of laboratory test results were available; 87% (table VII). Apart from the 85% of isolates resistant to penicillin, little antibiotic resistance was reported for the agents tested, with 92% sensitivity to erythromycin, 98% to mexiticillin, and 97% to gentamicin and fusidic acid.

The pneumocococcus was the third most common organism reported. Of these reports, 85% included susceptibility test results. Full susceptibility to penicillin was reported for almost all, with only three of 1701 isolates were reported to be resistant or of intermediate sensitivity to penicillin. Of nearly 1600 isolates for which reports included details of erythromycin susceptibility, 2.5% were reported as resistant or of intermediate susceptibility. Nearly 99% of the 385 isolates for which chloramphenicol susceptibility was reported were recorded as sensitive. This total includes virtually all those from meningitis but relatively few of those from bacteremia.

Susceptibility test results were received for 86% of 499 cases of bacteremia due to Pseudomonas aeruginosa reported from 184 laboratories (table IX). Susceptibility to gentamicin was noted in 91% of 338 reports, 1.5% were reported to be of intermediate susceptibility and 7.4% resistant to gentamicin. Susceptibility to ciprofloxacin, ceftazidime, piperacillin and azlocillin was reported for 95%, 94%, 95% and 90% of isolates respectively amongst reports that included results for these antibiotics.
The results obtained to date and the continuing support that the surveillance scheme is receiving from microbiologists in England and Wales suggest that this approach to surveillance is likely to be useful and relevant for the foreseeable future. It should be possible to monitor and report trends and shifts in antibiotic susceptibility for a wider range of common pathogens causing bacteraemia and meningitis.

The accuracy of results reported in such a surveillance study could be questioned. However, the results received are those generated during normal laboratory testing for diagnostic and therapeutic purposes. Such tests are subject to both internal and external quality control. Results from the National External Quality Assurance Scheme for microbiology show that, for most organisms and antibiotic combinations examined within the scheme, agreement with the designated correct result is achieved on 80–100% of occasions, (Personal Communication, J.J.S. Snell). Thus, it seems reasonable to accept the reported results as valid.

For E. coli, the results obtained for gentamicin susceptibility are identical to those obtained in the 3-year survey reported by Spencer—1% resistance—and very similar to the 1.6% gentamicin resistance amongst 371 blood-culture isolates reported from the Bristol survey in 1986. The latter two studies reported 97% and 98% of E. coli strains to be sensitive to cefuroxime compared with the 92.6% reported here. It remains to be seen whether there is any significant trend towards resistance to cefuroxime in isolates

**Table VI.** Antimicrobial susceptibility of E. coli isolates from cases of bacteraemia reported to CDSC/DHI from England and Wales, Jan.–June 1989

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Number (%) tested</th>
<th>Number (%) of strains that were</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Ampicillin/Amoxycillin</td>
<td>3070 (97.5)</td>
<td>1400 (45.6)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1554 (49.4)</td>
<td>1542 (99.2)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>1040 (33.2)</td>
<td>1036 (99.0)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>2189 (69.6)</td>
<td>2027 (92.6)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1572 (50)</td>
<td>1588 (99.7)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>3015 (95.8)</td>
<td>2958 (98.1)</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>2261 (71.8)</td>
<td>1816 (80.3)</td>
</tr>
</tbody>
</table>

Total number reported: 3586
Number of reporting laboratories: 254
Number (%) with one or more sensitivity results: 3147 (87.8)
Number (%) of laboratories reporting sensitivity results: 237 (83.3)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Number (%) tested</th>
<th>Number (%) of strains that were</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Penicillin</td>
<td>1995 (96.6)</td>
<td>292 (14.6)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1981 (95.9)</td>
<td>1830 (92.4)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>1962 (95.0)</td>
<td>1628 (97.9)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1623 (78.6)</td>
<td>1571 (98.8)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>1659 (80.3)</td>
<td>1618 (97.5)</td>
</tr>
</tbody>
</table>

Total number reported: 2366
Number of reporting laboratories: 249
Number (%) with one or more sensitivity results: 2065 (87.3)
Number (%) of laboratories reporting sensitivity results: 237 (92.9)

**Table VII.** Antimicrobial susceptibility of S. aureus isolates from cases of bacteraemia reported to CDSC/DHI from England and Wales, Jan.–June 1989

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Number (%) tested</th>
<th>Number (%) of strains that were</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Penicillin</td>
<td>1701 (98.5)</td>
<td>1698 (99.8)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>756 (43.8)</td>
<td>735 (97.2)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1596 (92.4)</td>
<td>1551 (97.2)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>385 (22.3)</td>
<td>380 (98.7)</td>
</tr>
</tbody>
</table>

Total number reported: 2040
Number of reporting laboratories: 244
Number (%) with one or more sensitivity results: 1728 (84.7)
Number (%) of laboratories reporting sensitivity results: 229 (93.6)
of *E. coli* from bacteremia. The presence in these data of a small number of reports of resistance or intermediate sensitivity to cephalothin and cefazolin at 0.7% and 1/6, respectively, of isolates tested against these antibiotics is suggestive of emerging resistance to these agents. As this survey continues it should be possible to plot any such changes in susceptibility to cephalosporins and, indeed, to other antimicrobial agents over time.

Amongst the isolates of *S. aureus* from bacteremia, sensitivity to gentamicin and methicillin was reported in 97.9% and 98% respectively. These results are similar to the 98% sensitivity to gentamicin for 204 blood-cultures isolates of *S. aureus* reported in Bristol131 and the 98% sensitivity to methicillin reported for Spencer.135

Amongst pneumococci, very low levels of resistance to penicillin and chloramphenicol were reported. However, it should be noted that strains resistant to these agents are responsible for significant infections in patients in England and Wales, albeit in very low numbers. Resistance to erythromycin was noted in 2.5% of nearly 1600 reports of isolates from blood or CSF, compared with the 1% of >7000 pneumococci from various clinical sources reported by Spencer.135 Whether this small difference represents a change in overall susceptibility to erythromycin may become apparent as the survey continues.

The 7-4% incidence of resistance to gentamicin amongst 338 bacteremia isolates of *P. aeruginosa* is notably higher than the 1% resistance reported for Pseudomonas spp.128 and a little higher than the 4% resistance reported for 23 blood isolates in Bristol.121 A survey of 1866 isolates of *P. aeruginosa* collected from 24 British hospitals noted resistance in 5.5%.127

With a few exceptions the results reported here present a reasonably reassuring picture of continued bacterial susceptibility to a wide range of antibiotics. Comparison of these results with those obtained from culture-based surveys shows reasonable concordance. The continuous surveillance of the antibiotic susceptibility of bacteremia isolates by the present system, supplemented with occasional culture-based surveys of particular species to define precisely the genetics and mechanisms of resistance, offer a simple and cost effective approach to national surveillance.

Is There Any Relationship Between Bacterial Resistance and Virulence?

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To access the association between virulence and antibiotic resistance, it is essential to examine the interaction from two aspects—firstly, in relation to plasmid encoded resistance and secondly for chromosomally mediated resistance and virulence, the interplay may be demonstrated by the use of appropriate animal models. Many questions on this topic have been raised and a multitude remain unsolved. For an organism to be virulent it must possess factors which may include: (i) the ability to adhere to other cells (via pili, fimbriae or adhesins); (ii) the production of an extracellular toxin; or (iii) the ability to penetrate the tissues or cells by virulence factors (once inside the cell the bacteria may multiply and destroy it). Several of these features are exquisitely plasmid mediated, others are only chromosomal.

Many virulence or pathogenicity factors are known to be encoded by plasmids. Some are toxins that cause diarrhoea; some are factors responsible for the adherence of bacteria to other cells; some are components that allow bacteria to penetrate HeLa cells and then multiply; and some are features that enable salmonellae to persist in various organs. Many of these virulence factors happen to be associated with antibiotic resistance markers carried on plasmids. One of the best known instances of a linkage between resistance and virulence conferred by a plasmid is that of enterotoxin production amongst antibiotic-resistant *Escherichia coli* isolates in the Far East.138 Toxigenic *E. coli* produce either a heat-labile, antigenic, large-molecular-weight toxin (LT) or a heat-stable, non-antigenic toxin (ST), or both. These *E. coli* strains contain plasmids that carry the genetic information necessary for the production of these toxins. Several conflicting reports suggested that *E. coli* may or may not be able to carry together plasmids conferring enterotoxin production and antibiotic resistance, but Echeverria et al. collected 176 enterotoxigenic *E. coli* (ETEC) strains for the Far East, of which 72% were resistant to one or more antibiotic; 44% were resistant to four or more agents. In transfer experiments, 80% of 31 ETEC strains transferred part of their-resistance pattern and 35% also transferred their toxigenicity profile. None of the recipients acquired toxigenicity without antibiotic resistance. These workers concluded that ETEC isolated from the Far East in 1978 were frequently resistant to antimicrobial agents, and that genes coding for resistance and enterotoxin production could be transferred together in vivo. It was not known if this phenomenon could occur in the human gastrointestinal tract.

This type of association has also been observed by other workers. Wachsmuth et al.139 found that 36% of a multi-resistant *E. coli* population produced ST and that all toxigenic strains were resistant to antibiotics. Smith and Lingwood140 examined ETEC isolates from pigs and found that toxin-conferring plasmids were transmitted with resistance plasmids and, although not linked, the plasmids were transferred together. Because interbacterial transfer of antibiotic resistance occurs more efficiently in animals when
ANTIBIOTIC RESISTANCE IN BACTERIA

antimicrobial agents are used, caution was advocated against the widespread use of antibiotics because it might result in an increased incidence of bacteria possessing pathogenic plasmids in the environment of man and domestic animals. The precise mechanisms of virulence of enteropathogenic E. coli (EPEC) are not known. However, work by Laporta et al. has shown that EPEC adhere to HeLa cells in two different ways. These are termed localised adherence (LA) and diffuse adherence (DA). The bacteria demonstrating LA stick to localised areas of the cells in which they form distinct microcolonies or clusters, whereas the isolates showing DA adhere to the whole surface of the HeLa cells. The LA characteristic has been shown to be consistent in EPEC atypotypes and focal adherence of E. coli O111: H cells to small bowel epithelia and packed aggregates on HEp-2 cells have been described previously by Clausen and Christie.

Laporta et al. confirmed that naturally occurring plasmids encoding both LA and antibiotic resistance have been recognised in E. coli. These workers examined six strains of EPEC possessing transferable drug resistance; two strains possessed the characteristic of joint factor expression. One strain carried two plasmids, one coding for ampicillin (Ap) resistance and the other for LA, whereas the other strain contained only one plasmid coding for resistance to four antibiotics and LA. Curing by acridine orange of an Ap6 strain conjugant showed that both features were lost simultaneously.

The situation is different in bacteria possessing chromosomal mutations conferring antibiotic resistance. One of the best known examples of this is an alteration in a porin, a protein component (Omp) of the outer membrane of gram-negative bacteria responsible for the movement into the bacterial cells of many chemicals such as nutrients, ions and certain antibiotics. A very complex control mechanism regulates the quantitative production of porins and affects their sensitivity to different families of antibiotics such as ß-lactams, chloramphenicol, tetracycline and the quinolones.

The porins span the outer membrane as protein trimers that admit small hydrophilic molecules to the cytoplasm. In E. coli, Omp F has been shown to be larger than Omp C (2 nm compared with 1.1 nm). Thus, the ratio of Omp F: Omp C affects the range of chemicals and antibiotics admitted to the cell. Genes coding for the control of Omp F and Omp C are under the transcriptional regulation of the Omp R gene, which itself is part of the component regulatory system Omp R-Comp Omp. The Omp R gene encodes a transmembrane sensory component which acts as a sensor for the product of Omp R, a transcriptional regulator. Dorman et al. introduced, via transposon-generated mutation, into a mouse-virulent strain of Salmonella typhimurium, various Omp changes, which included Omp C, Omp D (another porin present in salmonellae), Omp F and Omp R. Once characterised, the mutated strains were inoculated into BALB/c mice by either the oral or intravenous route. In comparison with the parent strain, Omp C or Omp F-deleted strains were identical in virulence. The Omp D mutant was slightly less virulent whereas the mutant Omp R failed to kill the mice after oral challenge and showed a markedly reduced intravenous LD50. Furthermore, the Omp R mutants persisted for several weeks in murine tissues, and appeared to protect against subsequent challenge by the parent strain.

Cyclic AMP (cAMP) and the cAMP receptor protein (CRP) are essential for the transcription of many genes and operons involved in catabolite transport and breakdown. The cAMP levels in cells influence various factors related to virulence or pathogenicity, such as synthesis of fibriana and flagella and manufacture of at least one Omp. Deficiency in cAMP and CRP can also be associated with ß-lactam resistance. Curtis and Kelly developed strains of Salmonella typhimurium which were unable to synthesise adenyl cyclase and CRP and thus were deficient in cAMP. The parent strain of S. typhimurium possessed a virulence plasmid, pSISR100, which enabled it to attach to, invade and persist in Peyer's patches but was defective in traversing to the mesenteric lymph nodes and spleen. With several different genotypic mutant derivatives, studies were performed to assess the following properties: carbohydrate, metabolism, flagellum synthesis, colony size, virulence in BALB/c mice, genetic stability, tissue tropism, persistence of avirulent mutants and immunogenicity. All of the mutant strains were less virulent and induced a high level of protective immunity. Tissue tropism and persistence were not markedly different in the cAMP and CRP mutants. However, the genetic mutations created appeared to be stable. The infective dose required for the mutants was significantly higher than that of the parental strain, whereas the colony size and generation times of the mutant strains were smaller and longer than the wild type. Thus, mutations causing loss of adenyl cyclase or loss of CRP lead to avirulent but immunogenic strains of S. typhimurium.

Shigella flexneri causes dysentery in man by penetrating and multiplying inside human colonic epithelial cells. This invasive process has been demonstrated in vitro by showing HeLa cell invasion and a subsequent cytolytic effect. The expression of genes located on the chromosome is required for full virulence, but loci encoding for entry into epithelial cells are located on a 220-kb virulence plasmid. Bernardini et al. constructed mutants of the Omp B locus which contains the Omp R and env Z genes, which in turn are responsible for regulatory changes in accordance with environmental stimuli. Deletion of the Omp B locus via an allelic exchange yielded a mutant that expressed a low level of entry into cells and a limited capacity to survive intracellularly. Other virulence assays confirmed that the virulence of the mutant was severely impaired.

These three examples of chromosomal mutations which were associated with decreased virulence have shown that genetic changes which are known to be related to antibiotic resistance (although it was not tested in these particular mutants) may have a price in terms of viability, persistence and invasiveness. Two examples follow, in which sensitive clinical isolates of bacteria resistant in vitro have been investigated with respect to virulence in a mouse infection model.

Resistance to ß-lactam and aminoglycoside antibiotics in Pseudomonas aeruginosa may be conferred either by enzymes (plasmid or chromosomal) or by a change to permeability. When aminoglycoside resistance is present it may result from either a complete loss or significant reduction of the O-antigenic subunits of lipopolysaccharide. Bryan et al. examined the virulence of a series of mutant and transductant strains of P. aeruginosa that expressed ß-lactamase and aminoglycoside resistance, in a mouse model of peritonitis and septicaemia (Table X). They demonstrated resistance of these mutants to ß-lactam antibiotics, the production of a constitutive ß-lactamase, a decrease in permeability and a decreased affinity for penicillin-binding proteins, and (2) for aminoglycoside antibiotics, a decrease in cytochrome and nitrate reductase as the absence or reduction in the subunits of LPS. The results for these strains indicate an alteration in the mouse peritonitis model revealed that bacteria showing mechanisms of resistance to ß-lactam antibiotics all retained virulence properties similar to the parent. This is consistent with observations of these isolates that showed no change of morphology, growth rate or production of exotoxin A. However, the aminoglycoside-resistant strains showed a significant decrease in virulence. Three strains with altered cytochrome demonstrated slower growth rates and two mutant strains had "uncapped" LPS. Thus, in contrast to strains resistant to ß-lactam agents,
those with reduced aminoglycoside sensitivity manifested reduced virulence. Similar mutant strains have been isolated from cystic fibrosis patients, almost all of whom had repeated intensive courses of aminoglycosides. It is probable that these strains may persist during antibiotic therapy despite their reduced virulence.146

A further group of antimicrobial agents is the 4-fluoroquinolones, of which ciprofloxacin is one of the most active. Despite broad spectrum and rapid bactericidal activity, ciprofloxacin-resistant bacteria are isolated particularly amongst P. aeruginosa and staphylococci. As ciprofloxacin is available for both oral and parenteral administration, it has been used extensively in the treatment of patients with cystic fibrosis. However, resistant strains of P. aeruginosa have been isolated during such treatment. Ravizziola et al.147 examined two routine clinical isolates and their in-vitro ciprofloxacin-resistant variants whose MICs were > 200 mg/L and 50 mg/L respectively. These strains were injected intraperitoneally into mice and the LD50s were calculated. The authors concluded that these ciprofloxacin-resistant strains were less virulent than the susceptible parent strains. A change in colony form (from large/rough to small/rough) was observed. The results suggested that resistance may be associated to a decrease in virulence.

In conclusion, since resistance and virulence genes can be harboured on plasmids and, occasionally, on the same plasmid, excessive use of antibiotics may favour (i) the spread of such plasmids harbouring these two types of genes, and (ii) the formation of such plasmids harbouring the two kinds of markers. Conversely, bacteria exhibiting changes usually associated with chromosomal mutations showed a reduction in virulence and infectivity but rarely a change in immunogenicity. Clearly agents able to select such resistance mechanisms should be used cautiously in circumstances where such mutations may be readily expressed because, although the strains appear to be less virulent, they are still resistant to therapy. Nevertheless, these chromosomally resistant mutant strains may be useful in the development of new bacterial vaccines in the light of their immunogenic but non-pathogenic properties.

### Table X. Strains, properties and features of P. aeruginosa strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Resistance mechanism</th>
<th>MIC (mg/l)</th>
<th>Virulence</th>
<th>LD50</th>
<th>Growth rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA 0503 β-lactam resistant</td>
<td>None</td>
<td>Piperacillin 1, Gentamicin 1</td>
<td>↓</td>
<td>7 × 10⁵</td>
<td>Normal</td>
</tr>
<tr>
<td>PCC 118</td>
<td>Constitutive β-lactamase, permeability decrease to β-lactamase</td>
<td>Piperacillin &gt; 120, Gentamicin 1</td>
<td>↓</td>
<td>5.5 × 10⁴</td>
<td>Normal</td>
</tr>
<tr>
<td>PCC 45</td>
<td>Permeability decrease</td>
<td>Piperacillin 4, Gentamicin 1</td>
<td>↓</td>
<td>6 × 10⁴</td>
<td>Normal</td>
</tr>
<tr>
<td>PCC 17</td>
<td>Decreased affinity to PBP</td>
<td>Gentamicin 80, Gentamicin 1</td>
<td>↓</td>
<td>5 × 10⁴</td>
<td>Normal</td>
</tr>
<tr>
<td>PCC 2</td>
<td>Decreased affinity to PBP</td>
<td>Gentamicin 4, Gentamicin 1</td>
<td>↓</td>
<td>6 × 10⁵</td>
<td>Normal</td>
</tr>
<tr>
<td>Aminoglycoside resistant</td>
<td>Cytchrome and NO₃ reduction</td>
<td>Gentamicin 15</td>
<td>↓</td>
<td>72.1 × 10⁶</td>
<td>↓</td>
</tr>
<tr>
<td>PAO 2401</td>
<td></td>
<td>Gentamicin 1</td>
<td>↓</td>
<td>72.1 × 10⁶</td>
<td>↓</td>
</tr>
<tr>
<td>PAO 2402</td>
<td></td>
<td>Gentamicin 4</td>
<td>↓</td>
<td>72.1 × 10⁶</td>
<td>↓</td>
</tr>
<tr>
<td>PAO 2403</td>
<td></td>
<td>Gentamicin 16</td>
<td>↓</td>
<td>72.1 × 10⁶</td>
<td>↓</td>
</tr>
<tr>
<td>PAO 503-18</td>
<td>Absence of LPS subunit</td>
<td>Gentamicin 4</td>
<td>↓</td>
<td>72.1 × 10⁶</td>
<td>Normal</td>
</tr>
<tr>
<td>PAO 503-16</td>
<td>Marked reduction in LPS subunit</td>
<td>Gentamicin 4</td>
<td>↓</td>
<td>72.1 × 10⁶</td>
<td>Normal</td>
</tr>
</tbody>
</table>

### Sensitivity of antibiotic resistant bacteria to opsonophagocytosis in vitro: Implications for clinical infections and therapy

C. G. Gemmell

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Since the advent of penicillin in 1940 and its subsequent use in the treatment of staphylococcal and other infections, concurrent resistance to the drug has been recognised amongst a hitherto susceptible bacterial population. In particular, the development of methicillin as a therapeutic drug followed the recognition that Staphylococcus aureus readily developed resistance to penicillin by the induction of a β-lactamase enzyme. Stereochemical features of methicillin prevented destruction of the antibiotic by the enzyme. However, resistance to this drug is now possible through alteration in the drug target proteins and methicillin-resistant S. aureus (MRSA) are now a clinical problem world-wide.148 Cross-resistance to other penicillins and cephalosporins, as well as resistance to drugs with different biochemical actions, is now found amongst this group of organisms.

MRSA strains are important nosocomial pathogens. They were first described in the early 1960s and outbreaks of MRSA infection were described subsequently in Great Britain, Europe, the USA and Australia. With the exception of a single strain causing clinical problems in London and the South-East of England (named EMRSA-1)149, most outbreaks of infection with MRSA have been readily contained. This has led to the suggestion that such strains are generally less virulent than their methicillin-sensitive counterparts (MSSA). Isolates of S. aureus from patients with primary staphylococcal sepsis are still rarely multi-resistant whereas those patients with MRSA usually have underlying disease, e.g., MRSA strains isolated from patients in a Burns Unit succeeded in colonising the most severely burned individuals but rarely spread elsewhere in the hospital.150 In terms of patient management, it would be useful to be able to distinguish strains that are potentially...
been possession of genic compared genetic drug therefore, the extent, neutrophils were the success in vitro is possible and by so doing the expression or otherwise or surface components inimical to opsonophagocytosis might be detected. In addition, it is possible to compare neutrophil functions in normal and immunocompromised hosts.

Since 1985, the Royal Infirmary, Glasgow, has witnessed a succession of multi-resistant S. aureus strains with differing antibiotic susceptibility patterns. Patients admitted to the Burns Unit became colonised with a strain resistant to penicillin, erythromycin and fusidin (PEF). Sometimes, a strain resistant to penicillin, erythromycin, methicillin and gentamicin (PEMG) appeared in these patients to the exclusion of the former strain. Strains with different antibiotic sensitivity patterns were chosen for measurement of their susceptibility to serum opsonisation and subsequent phagocytosis by neutrophils. However, no significant differences between the strains were found. Similar results have been reported also by others, suggesting that the observed changes in protein A expression are in themselves sufficient to alter bacterial susceptibility to phagocytosis or that some compensation has taken place in the MRSA strains deficient in protein A.

Extension of these methods to neutrophils obtained from immunocompromised (i.e., burned) patients revealed that the host status was important in determining how MRSA were handled. Immediately after burn injury, patients' neutrophils were markedly depressed in terms of their ability to phagocyte MRSA and this change lasted for several days before normal phagocytic efficiency returned. To some extent, these differences could be attributed to differences in the opsonic activity of the patients' serum (table XI). Therefore, these findings would support the concept that drug resistance per se does not predetermine virulence in vivo except amongst immunocompromised patients, in whom humoral and cellular defects can be recognised.

It is realised that the drug-resistant S. aureus strains used in the preceding studies may or may not have had a common genetic background and cannot, therefore, be properly compared to each other or to antibiotic-sensitive strains. In an attempt to answer this criticism, a different approach has been followed with regard to the development of fluoroquinolone resistance in S. aureus and Pseudomonas aeruginosa.

The primary action of the quinolones is on DNA gyrase, although the cidal mechanism may involve a series of events. Resistance to drugs of this type is based upon the possession of an altered DNA gyrase, with the A subunit being intimately involved. Mutations affecting synthesis of an outside membrane protein and permeability have been described also. Many of these mutant strains have appeared during therapy and have constituted a risk to the patient as well as a threat to the choice of the quinolones in therapy.

Serial subculture of many bacterial species in the presence of sub-inhibitory concentrations of norfloxacin, ciprofloxacin, ofloxacin, enoxacin or ofloxacin results in the selection of resistance; Escherichia coli may be an exception. Cross-resistance between the five drugs was readily demonstrable. This technique has been used to provide quinolone-resistant variants derived from an original quinolone-sensitive strain as targets for measurement of their comparative susceptibility to phagocytosis. Ciprofloxacin and ofloxacin were used as selector drugs. No differences were recognised in ingestion by neutrophils between the quinolone-resistant offspring and the S. aureus or P. aeruginosa parent strains. However, when their ability to induce a respiratory burst in the neutrophils was measured, some differences were found between the strains. Variants of S. aureus expressing low or intermediate level resistance to the quinolones were less able to generate a respiratory burst than the original strain. In contrast, similar strains of P. aeruginosa appeared more capable of stimulating the neutrophils in this assay. In addition, there was considerable strain-to-strain variation in neutrophil interaction which could not be explained either by their innate susceptibility to phagocytic ingestion or their level of drug resistance.

Table XI illustrates some of these results. It remains to be seen whether the drug-induced variants differ in other respects from their parent strains. Preliminary evidence with S. aureus suggests that their ability to elaborate various exotoxins (e.g., α-haemolysin, coagulase) may be altered. Whether their structural topography is also modified during the development of drug resistance is still to be investigated.

On the basis of current experimental approaches there is little evidence to suggest that development of drug resistance results in “superbugs” capable of initiating more serious infections than their drug-sensitive counterparts, except, perhaps, in causing nosocomial infection among immunocompromised patients. In this context, it would be appropriate to extend the studies to bacterial pathogens in which drug resistance has developed during antibiotic therapy. The selective pressure provided by the antibiotic ciprofloxacin used in the treatment of peritonitis in patients undergoing chronic ambulatory peritoneal dialysis has provided drug-sensitive and drug-resistant strains of S. epidermidis and S. haemolytica. However, no differences in bacterial susceptibility to opsonophagocytosis were detected.

### The Clinical Implications of Acquired Bacterial Resistance

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The general implications of acquired bacterial resistance to antimicrobial agents are very great. The explosive growth of a new industry of bacterial genetics has been given a

### Table XI: Differences between serum from burned patients A and B and normal serum in opsonisation of strains of S. aureus

<table>
<thead>
<tr>
<th>Target strain</th>
<th>Percentage ingested by PMNL after opsonisation with normal serum</th>
<th>Percentage ingested by PMNL after opsonisation with patient A serum</th>
<th>Percentage ingested by PMNL after opsonisation with patient B serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRF</td>
<td>12.5 23.1 3.9 10.8</td>
<td>1-6 5-6</td>
<td></td>
</tr>
<tr>
<td>PEMG</td>
<td>17.2 22.5 13.2 25.2</td>
<td>1-0 9-5</td>
<td></td>
</tr>
</tbody>
</table>
powerful impetus by the stimulus of enquiry into the mechanisms of acquired bacterial resistance and has generated considerable activity in academic departments of medical microbiology and research laboratories. A second consequence has been to make medical microbiologists much more "clinical" and much less laboratory based. In the UK, there are approximately 400 consultant medical microbiologists with an infrastructure of junior medical staff, specialised laboratory staff and whole sections of laboratories, for the basis of development has been the existence of antibiotics and the linked relationship with antibiotic resistance. Before the antibiotic era there were hardly any specialist microbiologists in hospitals in the UK; there were relatively small numbers in University departments whose functions were related to teaching and research.

The development of antibiotic resistance has added greatly to the interest of medical microbiology and has provided opportunities for international links, often through the support of the pharmaceutical industry for conferences at home and abroad. The development of the antimicrobial part of the pharmaceutical industry has been very striking. Had there been no need to develop this industry because bacteria had not acquired resistance, there would be very different patterns of prescribing within hospitals, where one fifth of the drugs budget is now allocated to antimicrobial agents, and a corresponding reduction in the interest of the industry in microbiologists.

All drugs have certain common limitations on their use—the nature and frequency of side effects, and cost. Antimicrobial agents have a third problem which has generated the antibiotics industry and medical microbiology as a speciality—acquired bacterial resistance to antibiotics.

**Selection of resistant organisms**

In the early days of the antimicrobial era, most infections were caused by organisms, such as staphylococci, streptococci, and, very occasionally, coliform organisms, which were inherently antibiotic-sensitive. Over subsequent decades, selection pressures produced by the widespread use of antibiotics, whether for appropriate or inappropriate reasons, have resulted in the emergence of new groups of major pathogens as causes of infections. Often this has led to the emergence of organisms of which microbiologists were barely aware before the advent of antimicrobial agents; infections caused by *Pseudomonas aeruginosa*, and even by other *Pseudomonas* spp., by *Klebsiella*, *Enterobacter*, *Serratia*, *Arizona* and *Citrobacter* spp. and by many other gram-negative species, by *enterococci*, and by resistant staphylococci, whether coagulase-negative or coagulase-positive, are now common. The pressures for the emergence of these organisms as pathogens have been, on the one hand, technological change, such as the increasingly widespread use of indwelling vascular catheters or prostatic devices, or more heroic surgical procedures, and, on the other hand, the attempt to prevent these ever more exposed patients succumbing to infections by the widespread use of antibiotics, whether given prophylactically or therapeutically.

**New drugs**

In the classical era of the development of new antimicrobial agents, extending to the late 1950s, although antibiotic resistance was emerging it has not done so to any great extent and several powerful agents were available that covered nearly all the pathogens that were commonly seen in clinical practice at that time; penicillin, streptomycin, tetracycline, chloramphenicol, sulphonamide, erythromycin, ampicillin, carbenicillin, methicillin, the first generation cephalosporins and metronidazole were all available.

However, many of those drugs, useful though they were, had limitations on their use because of difficulty of administration or, in cases such as streptomycin, toxicity. There would, perhaps, have been some development of new drugs even in the absence of bacterial resistance, as attempts to develop more convenient versions of existing drugs which would have been easier to administer and perhaps less toxic.

It is reasonable to speculate that there would not have been the development of the semi-synthetic β-lactam agents—the cephalosporins, monobactams, augmentin, etc—or of trimethoprim and co-trimoxazole, and that the massive development of the aminoglycosides, and the quinolones and glycopeptide antibiotics, would not have occurred without the major stimulus of bacterial resistance. If it were not for the problem of resistance, most of these relatively new antimicrobial agents would have offered little to improve patient management.

**Implications for patients**

The response of the medical profession to the problem of resistance has been to use higher doses of the older drugs than would otherwise have been the case, or to combine drugs, as in the treatment of tuberculosis, to overcome the actual or perceived risk of resistance to single agents, or, alternatively, to use more recently developed drugs which may be difficult to administer, toxic and costly.

Some drugs are difficult to administer because they have to be given intravenously, commonly in substantial doses, generally necessitating the positioning of long intravenous catheters, which essentially ties the patient to a hospital bed and makes him or her immobile; there is also the administration, in some cases, of a heavy sodium load. There are well documented risks of morbidity and mortality relating to the length of time for which an intravenous catheter has to remain in place.

Toxicity and side effects present considerable problems in relation to monitoring levels of the agents in serum and the cost of this monitoring, both in terms of laboratory costs and
ANTIBIOTIC RESISTANCE IN BACTERIA

Doormatch

There is steadily progressive erosion of the usefulness of antibiotics by rising tide of acquired resistance in pathogenic bacteria. Total inability to control resistant bacteria is, so far, a rarity. However, doctors being driven to use second- or third-line drugs, or antibiotic combinations, rather than the first choices of previous years. A heavy cost in side effects, ecological damage, preventable hospitalisation, morbidity and mortality, and in money, is being paid and will increasingly be paid.

Concluding remarks by J. T. Smith

In the "good old days", a single lecture describing bacterial resistance to antibacterial agents would nicely encompass all there was to know on the subject. However, as clinical isolates exhibiting resistance to more and more drugs became more frequent (more often than not resulting from R-plasmids), whole conferences on resistance to but a single class of antibacterial agent became normal. Although such a fashion had technical merit, discussion of bacterial resistance became so fragmented that few indeed could retain an overview of the problem. The meeting that provided the basis for this review article brought together investigators with a wide range of expertise across the full spectrum of antibiotic resistance and helped speakers and audience alike to stay abreast of this most important topic.

We thank Bayer UK for supporting the meeting on which this article is based, which has permitted a forum where problems of antibiotic resistance could be frankly discussed.

References


Examples of current clinical problems

A recent problem concerns a 6-month-old child with meningitis caused by a pneumococcus with diminished sensitivity to penicillin. Initially, penicillin was given, before it was recognised that the pneumococcus was not fully sensitive, and there was no significant clinical response. Once the problem was recognised, chloramphenicol treatment was instituted but this has caused considerable anxiety because of the need to give a potentially toxic drug to a child who would otherwise not have received it.

In another specialty, cardiac surgeons have used 6-lactam antibiotics combined with an aminoglycoside, prophylactically, to reduce post-operative sternal infection. Such infections are most commonly caused by coagulase-negative staphylococci, which are resistant to methicillin in 60% of cases, or by Staphylococcus aureus. Because even very short courses of aminoglycosides given prophylactically may give rise to toxicity, there is increasing pressure to use glycopeptide antibiotics prophylactically.


126. Barna JC, Williams DH. The structure and mode of action of...


Introduction

The 6th European Congress of Clinical Microbiology and Infectious Diseases was attended by over 3000 delegates. The Congress was well attended both by a majority of delegates and by a number of key speakers. The Congress was supported by a number of pharmaceutical companies. The majority of the media coverage was related to the virulence factors of Staphylococcus aureus. The main focus of the Congress was on the in-vitro selection of bacteria resistant to 4-quinolone agents.

A symposium on the biological treatment of bacteria resistant to 4-quinolone agents was organized by the European Society for Clinical Microbiology and Infectious Diseases. The symposium was well attended by a majority of delegates and by a number of key speakers. The main focus of the symposium was on the in-vitro selection of bacteria resistant to 4-quinolone agents.

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Monthly Update
Anti-infectives

Highlights of the 6th European Congress of Clinical Microbiology and Infectious Diseases
28 – 31 March 1993, Seville, Spain

K Coleman, D Payne, I Simpson & C Thorburn
SmithKline Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey RH3 7AJ, UK

Current Drugs (July 1993) 2(7):851–852

Introduction

The 6th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) attracted over 3000 delegates. The meeting consisted of a series of workshops and satellite symposia, followed by a number of special lectures, controversial sessions, symposia, study group reports and poster sessions. A total of 1189 abstracts, covering all aspects of clinical microbiology, were accepted. The meeting was not intended as a forum for investigational drugs, but this report provides some of the highlights of the clinical bacteriology sessions that relate in particular to new drugs, and discusses approaches that might result in the discovery of future chemotherapeutic agents.

A satellite symposium on the role of the new macrolide antibiotic, dirithromycin, highlighted its similarity to azithromycin in chemical structure and distribution properties (low serum concentrations but satisfactory concentrations in tissues and intracellularly). However, dirithromycin did not have the improved activity against Haemophilus influenzae which is shown by azithromycin (dirithromycin MIC90 8-64 μg/ml), and with MIC90 values of 2–4 μg/ml against Staphylococcus aureus, the new macrolide did not look promising. The published clinical trial data was favourable, however, although it was noted that the outcome was related to the pathogens present, with lowest efficacy (68%) being seen against patients with acute exacerbations of chronic bronchitis caused by H. influenzae. The main points that were emphasized were the excellent safety profile of dirithromycin (lack of interactions); good late post-therapy results compared with clarithromycin; less frequent dosing (twice daily compared to three times a day for clarithromycin) and reduced length of treatment (five days versus seven days) due to the long half life of the compound (44 hours).

A symposium on the Biological Treatment of Infection highlighted the tremendous increase in incidence and mortality rate associated with Gram-negative infections over the last thirty years. Gram-negative endotoxin is generally cell wall lipopolysaccharide (LPS), and it is the lipid A component of LPS that is the major cause of toxic shock. LPS causes release of cytokines such as tumour necrosis factor (TNF) and interleukins (IL) 1 and 6 from macrophages, production of platelet activating factor (PAF), liberation of free radicals, release of proteases and activation of the arachidonic acid cascade, which in turn lead to a generalized inflammatory reaction. Of
these host factors, cytokine levels seem to correlate best with clinical outcome, both in the clinic and in experimentally induced toxaeamias.

Monoclonal antibodies against TNF have been shown to be beneficial to shock patients, but detrimental to patients not exhibiting shock. Soluble TNF receptors are short-lived, but coupling these with IgG leads to improved half-life and potency. Other possible approaches are IL-1 receptor antagonists and antibodies to CD14 receptors or to LPS-binding protein.

Treatment with granulocyte colony-stimulating factor (GCSF) results in increased neutrophil counts, and has been proved successful in reducing the number of febrile episodes in neutropenic patients. GCSF therapy is now being extended to non-neutropenic patients, and Phase III studies are about to commence in patients with severe pneumonia and abdominal infections.

Baquero (Spain) presented a personal overview of the future of infectious diseases. He outlined a number of possible new antibacterial targets, including:

- DNA replication (the fourteen components of the ‘replisome’ complex).
- RNA synthesis (sigma domain inhibitors, transcriptional activation inhibitors, elongation/termination inhibitors).
- Space/time regulators of cell division (MinB, FtsZ, PBP3).
- Fatty acid biosynthesis (β-hydroxy-decanoyl-thio, β-acetoacetyl ACP synthetase).
- Protein secretion (inhibition of one or more of the six essential sec genes in E. coli could be bactericidal or result in low colonisation aggression).
- Peptidoglycan synthesis (alanine racemase, D-Ala-D-Ala-ligase, diaminopimelic acid epimerase).
- Lipid A synthesis (UDP-NAcG acetyl transferase inhibitors, Lipid A deacetylase inhibitors).
- Gram-negative outer membrane agents (outer membrane protein inhibitors, polycationic peptides, arylamines).
- Intervention in the host-microbe dialogue (signal modification, inhibition of signal transduction or colonisation factors/aggressins).

Baquero perceived a continuing need for fast-acting, broad-spectrum antibiotics. He also saw additional needs for agents acting against stationary phase cells and ‘total flora agents’ to act on communities of bacteria in biofilms, tonsular crypts, etc.

Registration of a new antibiotic requires definitive break-points to be set for a range of clinical species. The newly-formed European Study Group in Antibiotic Breakpoints (ESGAB) presented their aims at a Workshop. The group consisted of a number of European clinicians, including: Phillips (Chairman; UK), Baquero (Spain), Bergan (Norway), Forsgren (Sweden), Schito (Italy) and Wiedeman (Germany). The group aim to fix break-points that are applicable in Europe and that are a guide to therapy, following discussions with representatives of pharmaceutical companies concerned with launching a drug in Europe. Once set, the definitive breakpoint will be published in the European Journal of Clinical Microbiology.
In 2.5 years I will look forward to the 25th edition before the beginning of the next millennium.

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The Infectious Diseases Manual

This manual comprehensively examines the subject of infectious disease from a clinical viewpoint. It is conveniently split into major sections dealing with clinical manifestations of infectious disease, medical microbiology and antibiotic therapy. In Section I, infectious diseases are grouped primarily by clinical topic, with clinical features, microbiological investigations, risk factors and antibiotic management described in detail for each infection. In Section II, the microbiology, epidemiology, laboratory diagnosis and treatment of medically important organisms, referenced from Section I, are discussed with the clinical syndrome highlighted. Although bacteria and viruses are discussed in detail, other important pathogens, particularly fungi, would appear not to have been given the space they may justify. Section III covers the classification, use, abuse and dosing regimens of antibiotics in the UK at present; although this section is currently highly relevant, it may rapidly become outdated.

A major concern with this text is the paucity of information on microbial resistance to antibiotics, an area that should be of high priority in any text addressing infectious disease. Guidelines to the types of resistance that can occur, together with an indication of their prevalence and predicted increase in occurrence, would be useful, it not essential, addenda to the final section. Despite the above concerns, The Infectious Diseases Manual is a useful first point of reference in the infectious diseases field for all health care professionals and is also of utility to research scientists and undergraduate students with interests in microbial pathogenesis and infection. It contains a great deal of relevant information, but access to, and comprehension thereof, may have to wait until the reader has mastered the extensive cross-referencing and abbreviations that are used!

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Biochemical and Enzyme Kinetic Applications for the Characterization of β-Lactamases

David J. Payne and Tony H. Farmer

1. Introduction

For the last 20 yr thin-layer polyacrylamide isoelectric focusing (IEF) has played a major role in the identification and characterization of β-lactamases. IEF is able to distinguish enzymes that focus only 0.05 pI apart (I), but the exponential increase in the numbers of β-lactamases discovered over the last 10 yr has meant that this method no longer provides sufficient resolution to distinguish the majority of β-lactamases. Today the pI of a β-lactamase is still an essential determinant that must be used in combination with a variety of other data. Moreover, the IEF of β-lactamases is now entering a new era as this technique can be adapted to provide important biochemical information on β-lactamases other than simply their pI values. These approaches are discussed in Subheading 3.1.

β-lactamases can also be characterized by their interaction with inhibitors and substrates. The most rapid and least complex measurements are I50 values (the concentration of inhibitor that inhibits the hydrolytic activity of the β-lactamase by 50% compared with an untreated control) and the rates of hydrolysis of a range of different β-lactam substrates at fixed concentrations. Protocols for performing these assays and approaches for maximizing their accuracy are discussed in Subheadings 3.2.1 and 3.2.7. However, because many of the currently identified β-lactamases have similar characteristics (e.g., the TEM and SHV β-lactamases), more discriminatory methods for characterizing β-lactamases are required, and it is becoming increasingly important to determine more complex kinetic data to distinguish different β-lactamases. These approaches are discussed in Subheadings 3.2.2–3.2.6. and 3.2.8.

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2. Materials

2.1. Major Equipment

1. Refrigerated centrifuge capable of spinning from 6000g to 32,000g.
2. Grant LTD 6 (Cambridge, UK) circulating water bath.
3. Power pack capable of delivering 800 V, 30 A, and 20 W (e.g., Pharmacia MultiDrive XL, Piscataway, NJ).
4. Horizontal gel apparatus (e.g., Multiphor II Electrophoresis System, Pharmacia).
5. Light box.
6. Hood Camera with fixed focal length (e.g., Model QSP Quickshooter Photosystem IBI [International Biotechnologies, Inc., CT]).
7. Pharmacia PhastSystem.
8. UV/VIS spectrophotometer (e.g., Beckman DU7500, Fullerton, CA).
9. Microtiter plate reader (e.g., Bio-Tek EL312e Microplate Biokinetics Reader with KinetiCalc software [Bio-Tek Instruments, Inc., VT], or SpectraMAX 250 [Molecular Devices, Crawley, West Sussex, UK]).
10. Sonicator (e.g., Soniprep 150 [MSE]).
11. Scientific graphics package (e.g., GraFit [Erithacus Software, Ltd. Staines, UK]).

2.2. Consumables

1. Solution I: 25 mM 1,4-piperazine-diethanesulphonic acid (PIVES), pH 7.0.
2. Solution II: 25 mM 1,4-PIVES, pH 7.0, 0.5 mg/mL nitrocefin (Unipath, Basingstoke, UK). The nitrocefin should be dissolved in a minimum volume of dimethylsulfoxide (1% of final volume) and then made up to the final volume with PIVES buffer. All solutions of nitrocefin should be maintained on ice.
3. Solution III: 25 mM 1,4-PIVES, pH 7.0, 0.5 mg/mL nitrocefin and 1 mM zinc sulfate (added last).
4. Solution IV: 25 mM 1,4-PIVES, pH 7.0, 0.05 mg/mL nitrocefin.
5. Solution V: 25 mM 1,4-PIVES, pH 7.0, 0.5 M EDTA.
6. Dimethylsulfoxide (DMSO).
7. Colored pI markers (e.g., BDH Electran IEF Markers pI Calibration Kit Range 4.7–10.6, Poole, UK [the lyophilized sample should be reconstituted with 100 µL of distilled water]).
8. Quartz cuvets: 1–10 mm pathlength.
9. Microtiter plates, and deep-well plates, 96 well (Beckman).
10. Precast IEF gels (e.g., Pharmacia, various pH ranges available).
11. PhastGels IEF 3-9 (Pharmacia).
12. Filter paper (e.g., Whatman 1541320).
13. Ice.
14. Film: Polaroid Type 665 (positive/negative) or equivalent.

3. Methods

3.1. Isoelectric Focusing

IEF separates β-lactamases on the basis of their overall electric charge. The isoelectric point (pI) is the pH at which the protein bears no net charge, and is,
therefore, the pH at which the protein is electrophoretically immobile. This section will summarize the various protocols that can be used for the IEF of \( \beta \)-lactamases, including the use of PhastSystem protocols that have increased the speed of the technique fivefold. The alternative uses of IEF for identifying different \( \beta \)-lactamases will also be discussed.

3.1.1. Preparation of Samples for Isoelectric Focusing

The volume of bacterial culture required for analysis by IEF depends on the quantity of enzyme produced by the isolate. The authors recommend starting with a 100 mL culture. If the \( \beta \)-lactamase produced by an isolate is unknown, then it is advisable to grow both induced and uninduced cultures as the isolate may produce insufficient \( \beta \)-lactamase in the basal state to allow identification by IEF. The optimal conditions for induction of a \( \beta \)-lactamase produced by a clinical isolate will be unknown, but the conditions below have been shown to cause induction of a variety of \( \beta \)-lactamases:

1. Inoculate 2X 100 mL of broth with a 1% inoculum of an overnight broth culture.
2. These cultures should be grown until they have an optical density of approx 1.400 = 0.7.
3. Addition of inducer. \( \beta \)-Lactams that are effective inducers include cefoxitin (for most serine \( \beta \)-lactamases) and imipenem (the latter is particularly good for inducing some metallo-\( \beta \)-lactamases). Sufficient inducer should be added to one of the two flasks to give a final concentration of 1/4 the MIC of the particular isolate.
4. Grow the cultures for 2 h more.
5. Harvest cells from each culture by centrifugation. Typical conditions are 6000g for 15 min at 4°C.
6. Resuspend and wash the pellet in 10 mL of Solution I.
7. Centrifugation at 6000g for 15 min at 4°C.
8. For the best results, resuspend the sample in a minimum volume of Solution I (ideally \( \leq 1 \)% of the original culture volume; more buffer can be added if the pellet is too viscous). The pellet should be maintained on ice.
9. The \( \beta \)-lactamase is then released from the whole cells by sonication while cooled in a mixture of ice and water. A typical treatment for *Escherichia coli* would be 2 \( \times \) 30 s bursts at 8 \( \mu \) separated by a 1 min cooling period; longer sonication periods may be required for other organisms (e.g., *Pseudomonas* spp.).
10. Subject the cell-free lysate to high speed centrifugation (32,000g for 30 min at 4°C) to remove the cell debris. The supernatant, containing the \( \beta \)-lactamase, is removed.
11. Estimate the activity of the preparation by mixing 33 \( \mu \)L of the sample with 100 \( \mu \)L of Solution IV, and record the time in seconds taken for the color to change from yellow to red.
12. Store the \( \beta \)-lactamase in aliquots at -20°C; freeze/thawing of the sample should be avoided.
3.1.2. Isoelectric Focusing Methodologies: Precast Gels

The methodology for performing IEF of β-lactamases is based on the protocol devised by Matthew et al. (2). This method examines β-lactamases on thin-layer polyacrylamide gels employing ampholines (multicharged compounds that have different isoelectric points and stack across the gel to form a pH gradient). Once the gels have run, β-lactamases are selectively identified from other bacterial proteins by staining the gel with nitrocefin, a chromogenic cephalosporin. This technique is, therefore, equally effective with crude lysates as with purified β-lactamases. Few β-lactamases focus as discrete bands, and in most cases, a series of satellite bands are observed (see Note 1).

1. When investigating an enzyme of unknown pI, a gel of pH range 3–10 is the most appropriate. If further resolution is required, gels covering narrower pH ranges are available (Pharmacia).

2. Place the precast IEF gel on the gel apparatus (see Note 2). Cut paper electrode contacts (supplied with Pharmacia gels) to an appropriate size and moisten with distilled water. Remove excess water by rigorously blotting on filter paper.

3. Place the electrode contacts on the edge of the longest sides of each gel.

4. Load samples. The number of μL of β-lactamase sample added to the gel should roughly correspond to the activity of the preparation in seconds (see Subheading 3.1.1., step 11). For example, if the activity is 5 s then add 5 μL. Volumes of <5 μL may be directly applied onto the gel, whereas 5–15 μL of sample should be applied to a loading tab (supplied with the Pharmacia gels) and 15–30 μL should be added to two loading tabs placed on top of each other. For best results the minimum volume of sample should be added to the gel and β-lactamase samples must be prepared as concentrated as possible. Space the sample loadings at least 0.5 cm apart (Fig. 1).

5. Loading position. For best results it is advisable to load β-lactamases known to focus close to the anode (with low pI) at the cathode and vice versa. Since the loading site normally causes some distortion of the gel, it is best that the β-lactamase band does not focus in this area. Test samples are best loaded close to the anode.

6. pI markers. 5 μL of colored broad range pI markers should be loaded in 2–3 tracks on the gel.

7. Focusing. The power pack should be set at 800 V, 30 A, and 20 W, and the temperature of the circulating water bath set to 9°C. Focusing is complete when the pI markers have focused as distinct bands (approx 4 h). The high voltage results in better resolution and less drift at the cathode.

8. Staining of gels. Soak filter paper in Solution II and overlay on the gel. After a few minutes, red bands will start to appear where the β-lactamases have focused. For optimum results, fresh overlays should be applied at regular intervals, although the bands will start to diffuse after 10–15 min.

9. Recording gels. Fixed focal length "hood" cameras can provide good photographs of gels illuminated over a suitable light box (orange filter, f/16, 1 s). Altern-
Samples known to focus at anode (loaded on tabs)  Anode (+ve)

Cathode (-ve)

"Unknowns" or samples known to focus at Cathode (loaded on tabs)

Electrode contacts

Fig. 1. Loading β-lactamase preparations onto IEF gels.

Table 1
Optimized Method for IEF with PhastGel IEF 3-9

<table>
<thead>
<tr>
<th>Step</th>
<th>Volts</th>
<th>mA</th>
<th>W</th>
<th>Temp</th>
<th>Volt hours</th>
<th>Function of step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>2000</td>
<td>2.5</td>
<td>3.5</td>
<td>15°C</td>
<td>75</td>
<td>Prefocusing</td>
</tr>
<tr>
<td>1.2</td>
<td>200</td>
<td>2.5</td>
<td>3.5</td>
<td>15°C</td>
<td>75</td>
<td>Sample loading</td>
</tr>
<tr>
<td>1.3</td>
<td>2000</td>
<td>2.5</td>
<td>3.5</td>
<td>15°C</td>
<td>410</td>
<td>Sample focusing</td>
</tr>
</tbody>
</table>

The run takes 500 Vh or 30 min, prefocusing takes 10 min.

...respectively, providing the gels can be sealed effectively in plastic film, they can be photocopied to provide a record of the gel. The pI of unknown enzymes can be determined by plotting a graph of pI against distance migrated for the pI markers.

3.1.3. IEF Methodologies: PhastSystem (Pharmacia)

The advantage of the PhastSystem is that it enables β-lactamase IEFs to be run in only 30 min, but it has the disadvantage that the resolution is not as good as with the gels recommended in Subheading 3.1.2.

1. β-lactamase samples are loaded onto the applicator comb as described by the manufacturer; the load volume varies with the size of the comb, but generally no more than 2–3 µL of sample can be loaded.
2. Set the PhastSystem to the appropriate program for IEF 3-9 pI gels (Table 1). This program takes 30 min to run.
3. For best staining results, soak a piece of filter paper in Solution II and then blot onto clean filter paper to remove all the surface liquid. Lay this onto the gel and gently remove air bubbles. The filter paper is removed after 30 s and the process repeated 2–5 times until satisfactory staining is achieved.
4. The gels can then be photographed as described in Subheading 3.1.2.
3.1.4. Use of IEF to Distinguish Metallo and Serine β-Lactamases

Protocols have been developed to distinguish the metallo and serine β-lactamases of *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*) by IEF (3). This methodology enabled the identification of seven different metallo-β-lactamases and at least eight different serine β-lactamases in 17 clinical isolates of *S. maltophilia* (3). This approach is particularly attractive, since it circumvents the requirement for lengthy purification and biochemical characterization of each of the enzymes. To date, this methodology has only been optimized for the study of β-lactamases produced by *S. maltophilia*, but it is probably appropriate for β-lactamases produced by other species. The protocol is shown below:

1. **Identification of metallo-β-lactamase.** Mix a 10 µL aliquot of the cell free lysate from an induced culture of *S. maltophilia* with 2 µL of Solution V to create a final EDTA concentration of 83 mM (Sample 1). Mix another 10 µL aliquot with 2 µL of Solution I (Sample 2).
2. Load both samples onto the IEF gel, as described previously (see Subheading 3.1.2.).
3. Focus and stain the gel in the normal way with Solution II. Sample 2 will elaborate both serine and metallo-β-lactamases, but in Sample 1 the metallo-β-lactamases are inhibited by the chelating action of EDTA and only the serine enzymes will be visualized.
4. **Identification of serine β-lactamase.** BRL42715, an experimental serine β-lactamase inhibitor, enabled serine β-lactamases to be distinguished by this method (3). The method employed was as follows: Load two 10 µL aliquots (A and B) of the cell free lysate from an induced culture on to the IEF gel and focus in the normal way.
5. When focusing is complete, overlay Sample B with 100 µM BRL42715 for 10 min. The gel is then stained with Solution III (nitrocefin containing ZnSO4). BRL 42715 is a very potent inhibitor of all serine β-lactamases, but is a substrate for metallo-β-lactamases. Therefore, only the metallo-β-lactamase will be visualized in Sample B, and both the serine and metallo-β-lactamase will be visualized in Sample A. Unfortunately, BRL42715 is no longer available. However, commercial serine β-lactamase inhibitors would probably make suitable replacements, although the appropriate method development studies would have to be performed.

Table 2 summarizes each of these treatments and Fig. 2 demonstrates the effectiveness of the EDTA treatment. Adjustments to this protocol have been used to visualize metallo-β-lactamases produced by *Bacteroides fragilis* (4), see Note 3.

### 3.2. Kinetic Analysis of β-Lactamases

This section provides protocols for the kinetic characterization of β-lactamases and, more importantly, discusses the principles and limitations of
Table 2
Summary of the Assays Used to Identify Serine and Metallo-\(\beta\)-Lactamases on IEF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Result, following overlay with Solution II (nitrocefin)</th>
<th>Indication of enzyme type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free extract mixed with Solution V (EDTA) prior to IEF</td>
<td>Band unaffected</td>
<td>Serine</td>
</tr>
<tr>
<td>Gel overlayd with 100 (\mu)M BRL42715 + 1 mM zinc sulfate</td>
<td>Band eradicated</td>
<td>Metallo-</td>
</tr>
<tr>
<td>Gel overlayd with Solution III (1 mM zinc sulfate)</td>
<td>Band unaffected</td>
<td>Serine</td>
</tr>
</tbody>
</table>

![Image of gel electrophoresis](image_url)

Fig. 2. Effect of 83 mM EDTA on the IEF of \(\beta\)-lactamases from \textit{S. maltophilia}. (A) \textit{S. maltophilia} A37454; (B) A37454 + EDTA; (C) 37 + EDTA; (D) 37; (E) 152 + EDTA; (F) 152; (G) 10258 + EDTA; (H) pl markers; (I) 10258; (J) 10257 + EDTA; (K) 10257; (L) 00157 + EDTA; (M) 00157; (N) ED136 + EDTA; (O) ED136; (P) TEM-1 + EDTA; (Q) TEM-1; (R) 511 (known metallo-\(\beta\)-lactamase producer); (S) 511 + EDTA.

Each approach. 25 mM sodium phosphate (pH 7.0) is an appropriate buffer for the assay of serine \(\beta\)-lactamases. 25 mM 1,4-PIPES, pH 7.0 is recommended for the assay of metallo-\(\beta\)-lactamases. A range of ZnSO\(_4\) concentrations...
should be tested to determine the optimal concentration for the particular metallo-
$\beta$-lactamase being examined. Where possible all assays should be run at 37°C.

3.2.1. Rapid Substrate Profiling of $\beta$-Lactamases

Substrate profiling is a widely used tool that enables the rapid comparison of
different $\beta$-lactamases by the relative hydrolysis rates of a series of $\beta$-lactam substrates tested at fixed concentrations. Results are most accurate and reproducible if rates are at or close to $V_{max}$ and for this it is advisable to use high substrate concentrations (1 mM) where possible (see below and Note 4). The procedure given below is for the direct spectrophotometric assay (6, 7), which is commonly used (see Note 5).

1. Optimal wavelengths and changes in absorbance ($\Delta \epsilon$) on substrate hydrolysis can be generally obtained from the literature (8). If such data are not available, proceed as shown below:

2. Scan a solution of the $\beta$-lactam to be assayed from 200–400 nm and store the spectrum. If the substrate absorbs in the visible part of the spectrum, extend the range of the scan.

3. Add $\beta$-lactamase and record spectra periodically; when no further changes are observed, subtract this spectrum from that of the intact $\beta$-lactam to obtain $\lambda_{max}$ for the change in absorbance on hydrolysis. Generally, hydrolysis is accompanied by a fall in absorbance; in some cases, such as nitrocefin ($\lambda_{max} = 482$ nm), there is an increase in absorbance on hydrolysis.

4. If the $\beta$-lactams are relatively stable, $\beta$-lactamase II from *Bacillus cereus* can be used as this enzyme hydrolyses virtually all $\beta$-lactams.

5. Set the instrument to the desired wavelength ($\lambda_{max}$, for change in absorbance on hydrolysis) and incubate 1 mM substrate at 37°C in a 10 mm pathlength quartz cuvet. If the $\beta$-lactam absorbs too strongly at this concentration, shorter pathlength (e.g., 2 mm) cuvets can be used, or, alternatively shift the wavelength to bring the reading onto the scale. In the latter case $\Delta \epsilon$ at this wavelength will have to be found. The most commonly used cuvets have a capacity of 2.5–3.0 mL, but 10 mm pathlength cells are available with working volumes of 0.8–1.0 mL, if materials are in short supply.

6. Check stability of substrate. Generally $\beta$-lactams are not spontaneously hydrolysed, but some carbapenems and nitrocefin at high concentrations may be unstable. It is particularly important that substrate stability is checked when assaying metallo-$\beta$-lactamases, as Zn$^{2+}$ ions may accelerate nonenzymatic hydrolysis of $\beta$-lactams (5). When measuring the activity of these enzymes it is best to add the metal salt to the substrate from a concentrated stock solution (in distilled water, as concentrated solutions of zinc salts precipitate in buffers), just prior to carrying out the assay. If spontaneous hydrolysis does occur, record the rate; this will have to be subtracted from the enzymatic rate.

7. Check stability of the enzyme. Diluted enzymes often lose activity, particularly when pure. Solutions to be added to reactions should be as concentrated as pos-
sible, and it is advisable to prepare these in buffers containing 5–10 mg/mL bovine serum albumin (BSA). It may also prove necessary to include 50–100 µg/mL BSA in assay solutions.

8. Add the β-lactamase to the substrate and mix by inversion or with a microstirrer. It is best to add a small volume of a concentrated enzyme solution as this will reduce any changes in volume and in temperature (e.g., if enzyme stock solutions are kept on ice). Concentrated enzymes also tend to be more stable.

9. If using very concentrated crude preparations (e.g., if β-lactamase activity is low), a blank should be set up containing enzyme and buffer only and rates measured against this.

10. The rate of absorbance change should be immediately recorded after adding the β-lactamase. Record as many data points as practically possible, but ensure that each reading is measured for a sufficient time to give a stable signal (particularly with cell changers). Generally 10–20 data points should be sufficient over 5 min.

11. Use only the initial linear phase of the reaction to calculate rates (i.e., ΔAbs/time). It is advisable to check the digitalized data to ensure linearity (many modern spectrophotometers will automatically do this, but this should be confirmed by closer inspection). Subtract blank rates (double beam and many single beam instruments do this automatically) and any nonenzymatic hydrolysis.

12. As crude preparations will often be used for substrate profiles, rates cannot be related to mg (or moles) of β-lactamase. The calculations given below, therefore, relate to per mL enzyme. The units for substrate hydrolysis are in μMol substrates min⁻¹ as these tend to give manageable figures. Rates are therefore found as follows:

\[
\frac{\Delta \text{Abs min}^{-1}}{\Delta e \times 10^{-6}} \times \frac{v_r (\text{mL})}{1000} \times \frac{1}{v_e (\text{mL})} = \mu \text{M substrate min}^{-1} \text{enz mL}^{-1}
\]

(where Δe is the molar extinction coefficient of hydrolysis; \(v_r\) is the volume of the reaction; and \(v_e\) is the volume of enzyme added).

13. To obtain the substrate and profile, it is necessary to relate all rates back to a standard substrate. This is usually done by setting the value for benzylpenicillin at 100, but sometimes other reference substrates are used such as nitrocefin.

### 3.2.2. Evaluation of the Kinetic Parameters of Substrate Hydrolysis by β-Lactamases

Substrate profiles provide a rapid characterization of β-lactamases. However, such an approach does have shortfalls (see Note 4). Increasingly it is necessary to determine \(K_m\) and \(V_{max}\) to distinguish different β-lactamases, and the ratio of \(V_{max}/K_m\) is especially useful for comparing both β-lactamases and substrates. Discussion of the most appropriate methods for obtaining and processing enzyme kinetic data has generated a vast literature and space does not permit a critical evaluation here. Anyone contemplating kinetic characterization of enzymes should certainly consult Fersht (9), whereas other workers give more comprehensive treatments for obtaining and processing data (10–12). Data
for the determination of $K_m$ and $V_{max}$ values can either be determined by
recording the initial rate of hydrolysis of different concentrations of β-lactams
(Subheading 3.2.3.) or by determining the rates of hydrolysis at different
points along a single progress curve (Subheading 3.2.4).

3.2.3. Measuring Initial Rates

This is the classical approach to obtaining rate data for finding kinetic
constants. Good results are generally obtained for cephalosporins and carba-
penems, but for reasons given in Subheading 3.2.4. the method is often inap-
propriate for penicillins. Basic assay procedures for measuring initial rates were
given in Subheading 3.2.1. (e.g., check for enzyme and substrate stability). Key points pertinent to determining kinetic parameters are given below.

1. Prepare a range of substrate solutions from 0.2–10 × $K_m$ (if the $K_m$ is unknown
and preliminary investigations have given no indication, it will be necessary to
carry out a "sighting" experiment). At least five, ideally 10–12, concentrations
will be needed. The stability of the substrate should be checked at each of the
concentrations assayed as described in Subheading 3.2.1.

2. Start recording the absorbance of the β-lactam immediately after adding and mix-
ing the enzyme. Preferably, substrate hydrolysis should be followed as a continu-
ous progress curve. However, it is not necessary to follow reactions to completion
(provided the ΔE is known).

3. When processing the results, it is absolutely essential that only true initial rates
are used. Ideally these should be no more than 5% of the reactions and never
more than 10%. For this reason it is advisable that reactions are followed singly,
and cell programmers generally avoided. Plate readers can be utilized as long as
the enzyme is added to the range of substrate concentrations simultaneously, and
all the wells are read with a minimum time delay between the first and last assay well.

Subheadings 3.2.5. and 3.2.6. detail recommended approaches for calculat-
ing $V_{max}$ and $K_m$ data from rate-substrate data pairs.

3.2.4. Use of Single Progress Curves

The ΔE5 for penicillin are typically approx 10-fold lower than those for
cephalosporins, thus reducing assay sensitivity. Furthermore, their $K_m$ values
are often rather low (<100 μM). The combination of these two factors make it
virtually impossible to obtain linear initial rates, and data obtained by this
method will give erroneous kinetic constants, especially for $K_m$. Consequently,
the use of single progress curves to obtain rate-substrate data pairs has been
extensively used to find kinetic parameters for β-lactamases (6,13–15). The
authors strongly recommend this approach is used for penicillins and cepha-
losporins with $K_m$s of <100 μM and <10 μM, respectively. Other advantages of
this procedure are that it saves on materials, reduces problems from changes in
enzyme activity, and is less time consuming. By cutting the number of pipetting and manipulation steps, potential sources of error are also reduced. Guidelines are given below.

1. Select a substrate concentration that is about 2.5–5.0 times the K. This should ensure that sufficient data points are obtained where a noticeable change in rate is seen.
2. It is advisable to carry out assays at two to three different starting substrate concentrations.
3. The starting (Abs_0) and final absorbances (i.e., when the reaction has gone to completion, Abs_f) must be accurately determined as these will be needed to process the results.
4. While satisfactory estimates of K_m and V_max can be obtained from as few as 10 data points, preferably at least 20 should be taken.
5. Checks should be made to make sure that the enzyme is not spontaneously losing activity during the assay or being inhibited by substrate or product. Whereas this may only become apparent after data processing, it is often apparent from premature slowing or stopping of the reaction.
6. The substrate concentration [S_1] at time t is found from (Abs_0 – Abs_1)/Δt; at t + 1 the concentration [S_2] is (Abs_1 – Abs_0)/Δt. The rate for [S_1] is given from ΔAbs between t and t + 1. Although this is not a strictly valid assumption, assuming that the curve is divided into small enough sections, this method provides rate-substrate pairs that give good and reproducible estimates of K_m and V_max when fitted to the Michaelis-Menten equation. Excellent results are obtained if the procedure is modified such that the rate between [S_1] and [S_2] (for example) is related to the substrate concentration [S_1] + [S_2]/2 (see also ref. 13 for related approaches). Subheadings 3.2.5. and 3.2.6. detail recommended approaches for determining K_m and V_max from rate-substrate pairs.

### 3.2.5. Graphical Determination of Kinetic Data

The two practical approaches detailed in Subheadings 3.2.3. and 3.2.4. give rise to data sets of rate versus substrate concentration. Traditionally, K_m and V_max have been found from plotting rate versus substrate concentration using linear transformations of the Michaelis-Menten equation. Fitting of lines can be made by eye or using linear regression. Whereas the former is obviously subjective to some extent, the latter is not necessarily more accurate because rearrangement of the equation also leads to a rearrangement of the error distribution (16). To fit the line accurately to the data requires a weighting scheme for the transformed data (10,11). This can be confusing, difficult, and time consuming, especially for the nonspecialist. However, the authors have found that the following graphical methods give good, reproducible kinetic constants for β-lactamases.

1. The direct linear plot (17) is, in many respects, the best method because it has no requirement for weighting. Substrate concentrations are marked on the abscissa (as negative values) and the measured velocities on the ordinate. The individual
Fig. 3. Determination of kinetic constants for the hydrolysis of nitrocefin by the \textit{B. cereus} II \(\beta\)-lactamase using direct linear plot; data from initial rates. \(K_m = 22 \, \mu\text{M}\); \(V_{\text{max}} = 0.054 \, \Delta \text{Abs} \, \text{min}^{-1}\). Data constants are determined from the median value of the intersections (for \(n\) data there are \(\lfloor n(n-1)/2 \rfloor\) intersections). This is the middle point from an odd numbered set and the mean of the middle pair in an even numbered set.

Data pairs are joined by lines that intersect with those from other data pairs. The intersection of the lines gives \(V_{\text{max}}\) when drawn back to the \(v\) axis (ordinate) and \(K_m\) when dropped down to the \(S\) axis (abscissa). In theory, all lines should intersect at one point, but this is unlikely to happen in practice. When the lines do not meet at one point the kinetic parameters are found from the median (i.e., middle) of the intersections (not the mean) (Fig. 3).

2. Although, as indicated above, there are objections to fitting unweighted data to straight line transformations of the Michaelis-Menten equation, we have found that the Hanes plot, where \([S]/v\) is plotted against \([S]\), gives reliable estimates of \(K_m\) and \(V_{\text{max}}\) for \(\beta\)-lactamases (Fig. 4). The abscissa intercept gives \(-K_m\), the ordinate \(K_m/V_{\text{max}}\) and the slope \(1/V_{\text{max}}\). The equation is:

\[
\frac{[S]}{v} = \frac{K_m}{V_{\text{max}}} + \frac{1}{V_{\text{max}}} \ast [S]
\]
3. Although popular, the Lineweaver-Burk plot is very susceptible to error distribution and should not be used.

3.2.6. Computerized Determination of Kinetic Data

Error distribution becomes much less of a problem if initial rate and substrate data are fitted directly to the Michaelis-Menten equation.

\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{K_m + [S]}
\]

Fortunately, a number of computer programs are available that easily accomplish this (e.g., GraFit or similar programs [18,19]). This procedure can be very quick if results are directly transferred from the spectrophotometer to the program. Such programs should not be used uncritically. It is especially important that v against [S] plots are inspected to ensure that data are evenly distributed. Results should not be clustered well below or above K_m.

Many modern spectrophotometers enable rate-substrate data sets to be easily obtained from single progress curves that can then be fitted to the Michaelis-Menten equation as described above. Alternatively, some workers have fitted absorbance data to the integrated form of the equation; in fact, this method was

Fig. 4. Determination of kinetic constants for the hydrolysis of nitrocefin by the B. cereus II β-lactamase using the Hanes plot; data from continuous progress curve. (K_m = 22 µM; V_{max} = 0.057 AAbs min^{-1}.)
used in one of the first papers describing the spectrophotometric assay (6). The integrated equation can be cast in various forms, of which the following is a common example:

$$V_{\text{max}} \cdot t = ([S_0] - [S_t]) + K_m \cdot \ln ([S_0]/[S_t])$$

Details of methods used to obtain and process data can be found elsewhere (6,14,15). The review by De Meester et al. (14) is a particularly comprehensive treatment of the fitting of both substrate and inhibitor data for β-lactamases using computerized procedures. Data for the integrated rate equation can also be obtained relatively easily from primary results using a calculator and then plotting these manually.

### 3.2.7. Rapid Inhibitory Profiling of β-Lactamases

Microtiter plate readers with kinetics software packages have enabled high throughput approaches for the determination of $I_{50}$ values. The Biotek Microplate Biokinetik Reader with KinetiCalc software and the SPECTRAmax 250 SOFTmax PRO software both enable percent inhibition compared with a control to be calculated for each of the 96 wells in a microtiter plate. The SPECTRAmax has the added advantage of being able to detect substrates that absorb in the UV, which means almost any β-lactam can be used as a reporter substrate. In addition, the SOFTmax PRO software will plot and calculate the $I_{50}$.

The protocol below will enable $I_{50}$s of eight compounds to be measured for one or more β-lactamases and is based on the method previously described (20,21).

1. Dissolve the inhibitors in buffer to produce a 3 mM solution. The compounds can be predissolved in a small volume of DMSO, but this must not exceed 5% of the final volume.
2. In a deep 96-well plate add 600 µL of buffer into wells in columns 3–11.
3. Pipet 1 mL of the 3 mM inhibitor solutions into the wells of column 12.
4. Make 1:3 serial dilution of the 3 mM solution down to column 3; take 300 µL of the 3 mM solution from column 12 and dispense into column 11. Mix, and take 300 µL from column 11 and dispense into column 10, and so on, across the columns to column 3, which will then be at 0.15 µM.
5. Transfer 50 µL of the contents from columns 3 to 12 in the deep-well plate to the equivalent positions on the assay microtiter plate. 100 µL and 50 µL of buffer are added to the Blank (column 1) and Control (column 2) wells of the assay microtitre plate, respectively.
6. For $I_{50}$ determinations with preincubation of enzyme and inhibitor, add 50 µL of appropriately diluted enzyme and incubate the plate for 5 min at 37°C.
7. Add 50 µL of a reporter substrate (see Note 8), such as nitrocefin, to all 96 wells and rapidly place into the microtiter plate reader and initiate absorbance readings. Table 3 shows the final concentration of inhibitor in each well of the microtiter plate, and Table 4 lists the contents of each well. Assays performed on
Table 3  
Example of an I_{50} Set Up

<table>
<thead>
<tr>
<th>Row</th>
<th>Final concentration of inhibitor in assay microtitre plate (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor 1</td>
<td>A</td>
</tr>
<tr>
<td>Inhibitor 2</td>
<td>B</td>
</tr>
<tr>
<td>Column no.</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4
Composition of Blank, Control, and Test Wells for I<sub>50</sub> Determination

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (pH 7.0)</td>
<td>100</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Reporter substrate (e.g., nitrocefin)</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Appropriately diluted β-lactamase</td>
<td></td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Diluted inhibitor</td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Total volume</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

plate readers that only read in the visible range are restricted to using chromogenic substrates such as nitrocefin. Alternative β-lactam substrates can be used with plate readers that read in the UV range, such as the SPECTRAMax 250 plate reader.

8. When measuring I<sub>50</sub> values without preincubation of enzyme and inhibitor, the enzyme solution should be added last.

With the appropriate software, this methodology enables 80 I<sub>50</sub> values to be determined in 3–4 h using up to 20 times less reagents than standard spectrophotometric assays in 1-mL cuvets. The interpretation of I<sub>50</sub> values and how their accuracy can be maximized is discussed in Notes 6–9.

3.2.8. Evaluation of Kinetic Constants for β-Lactamase Inhibitors

Many of the best known β-lactamase inhibitors such as clavulanic acid, sulbactam, β-bromopenicillanic acid, and the olivanic acids react with β-lactamases in an extremely complex way (22,23). A full discussion of the methods used for the kinetic evaluation of such compounds is beyond the scope of this review. Such investigations may be warranted if inhibitor-resistant enzymes are being studied (24,25). Essentially the following pieces of information should be ascertained:

1. The stoichiometry of inhibition; how much inhibitor is needed to inhibit a given amount of β-lactamase?
2. The stability of inhibition; does the enzyme activity return on removal of the inhibitor?
3. The rate of inhibition; how does the speed of inactivation relate to the inhibitor concentration?

The methods used are described in recent publications (26,27), whereas a useful review is given by Matagne et al. (28). Because inhibition of β-lactamases is complex, classical methods for finding K<sub>i</sub> values and interpreting results should be used with caution. The authors have found Dixon plots (1/v against [I], Fig. 5) and the method of Cornish-Bowden ([S]/v against [I]) the most useful for
Fig. 5. Determination of $K_i$ for a competitive inhibitor of a $\beta$-lactamase using the Dixon plot; nitrocefin is the substrate ($K_i = 62 \mu M$).

determining $K_i$ values for more straightforward inhibitors (10,29). If such plots indicate noncompetitive inhibition, further studies should be carried out, as many time-dependent inhibitors will give such a pattern. Classical procedures for analyzing results and determining $K_i$ values should never be used for results obtained from assays carried out with preincubation.

4. Notes

1. Satellite bands associated with IEF of $\beta$-lactamases. Simpson and Pleased (30) showed that $\beta$-lactamase satellite bands had similar biochemical characteristics to each other and to the main $\beta$-lactamase band. Arstila et al. (31) illustrated that some satellite bands arose as a consequence of enzyme preparation methodology, with fewer bands observed if the enzyme samples are prepared by osmotic shock. Matagne et al. (32) illustrated that satellite bands arose from the loss of different numbers of amino acids from the N-terminus of the enzyme (ragged ends). However, electrospray mass spectrometry can determine the mass of proteins with an accuracy of 99.99% (33), and has been used to illustrate that samples of TEM-1 and P99 $\beta$-lactamases, which possessed no ragged ends produced a range of satellite bands, suggesting that these bands may result from more subtle changes to the $\beta$-lactamase (34). Concomitant with this observation, it was discovered that a satellite band in the vicinity of pI 5.2 was identified when ceftazidime was added to 14 TEM-derived $\beta$-lactams
gave rise to satellite bands of different pI values. β-lactams were also shown to have an effect on the IEF focusing patterns of other β-lactamases (35). In addition, these “β-lactam-induced” satellite bands could revert to the pI of the main β-lactamase band, illustrating that satellite bands may not always be permanent features of a β-lactamase sample. Another explanation for β-lactam induced satellite bands is that the β-lactam or other molecules in the growth medium bind to a proportion of the β-lactamase molecules, causing an alteration in the overall charge to mass ratio of the protein. This leads to a shift in pI and results in the appearance of a satellite band.

2. The precast gels may contain some unpolymerized acrylamide. This is neurotoxic and protective gloves must be worn throughout the procedures described in Subheadings 3.1.2. and 3.1.3.

3. The CfiA-type metallo-β-lactamase is produced by the majority of imipenem-resistant clinical isolates of Bacteroides fragilis. As part of an investigation of six such isolates, it was found that the CfiA β-lactamases were only observed once the IEF gel had been overlaid with Solution III (4). Therefore, when examining the β-lactamases produced by imipenem-resistant strains, it is advisable to stain the gels in the presence of 1 mM zinc sulfate to ensure identification of any metallo-β-lactamases that may have been depleted of zinc during the preparation process.

4. Limitations of substrate profiles. It is important to appreciate that rates of hydrolysis will depend on assay conditions and, ideally, substrate concentrations should be high enough to be approaching V_max. Where this is not possible because of substrate instability, substrate inhibition of β-lactamase activity, or simply shortage of materials, it may be necessary to use subsaturating β-lactam concentrations. Under these conditions, rates will be very sensitive to even small changes in substrate concentration. It is essential, therefore, that only true (i.e., linear) initial rates are measured. It is also very important that assay conditions are clearly defined, especially when comparisons are to be made with published data. Providing assays are carried out carefully and these points borne in mind, substrate profiles are usually sufficiently accurate to allow unambiguous identification of β-lactamases, especially when used in conjunction with inhibitor profiles and IEF. It is always advisable to check profiles against IEF data when using crude preparations, as some organisms may produce appreciable amounts of more than one enzyme. When β-lactamases have rather similar profiles and cannot be readily distinguished by inhibition characteristics or IEF, a more detailed kinetic analysis is called for, as described in Subheadings 3.2.3.–3.2.6. This is true for some of the extended-spectrum β-lactamases (36,37). A full kinetic evaluation should always be carried out when a novel enzyme is suspected, particularly when trying to ascertain its role in resistance. For example, some years ago it was proposed that Class I β-lactamases caused resistance to 3rd generation cephalosporins by binding or “trapping” them (38,39). Proper kinetic analysis showed that resistance was, in fact, due to β-lactamase hydrolysis (40–42).
Table 5
Interpretation of \( I_{50} \) Values

<table>
<thead>
<tr>
<th>Effect of preincubation of enzyme and inhibitor</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No effect</td>
<td>Reversible inhibition(^a)</td>
</tr>
<tr>
<td>( I_{50} ) decreased</td>
<td>Transiently stable E-I complex with short ( t_{1/2} )(^2)</td>
</tr>
<tr>
<td>( I_{50} ) increased</td>
<td>Progressive inhibition where rate is fast(^a) (^b)</td>
</tr>
<tr>
<td></td>
<td>Competitive substrate(^a)</td>
</tr>
<tr>
<td></td>
<td>Unstable inhibitor</td>
</tr>
</tbody>
</table>

\(^a\)If the \( I_{50} \) is affected by changing the substrate concentration or type, then the inhibitor probably competes for the substrate binding site.

\(^b\)If the rate is slow (i.e., half-life \( \gg \) preincubation time) preincubation will have no effect.

(See also Reading and Farmer, ref. 44.)

5. Approaches for increasing throughput of substrate profiling. Most spectrophotometers now have at least six cuvet positions. This will enable a blank and five different enzyme preparations to be simultaneously assessed against the same \( \beta \)-lactam substrate. Some spectrophotometers, such as the Beckman DU7500 and Beckman 650, have attachments that enable 12 microcuvets to be assayed. These cuvets are in a microtiter plate spacing format to assist liquid handling. UV/visible microtiter plate readers (e.g., SPECTRAMax 250) significantly increase the number of enzyme preparations (up to 96) that can be assayed against a particular substrate.

6. Interpretation and limitations of \( I_{50} \) values. It is important to realize that \( I_{50} \) values are not kinetic constants, but relative parameters that depend on, and, therefore, vary with assay conditions. For this reason, their use has been criticized, but, experience has shown that not only are a wide variety of inhibition mechanisms encountered with \( \beta \)-lactamases, but also that inhibition can be complex (43,44). Analysis of results for classical inhibition patterns (e.g., competitive, uncompetitive, and noncompetitive), can, therefore, be at best difficult, and at worst, misleading. Given these factors, the \( I_{50} \) assay is probably the most appropriate method for initial characterization of both \( \beta \)-lactamases and inhibitors.

7. Interpretation of results with and without preincubation. When assays are undertaken both with and without preincubation, useful preliminary information can be obtained, that can then be used to direct more detailed studies. Interpretation of results is summarized in the Table 5.

8. Effect of substrate concentration. As indicated above (see Note 6), the \( I_{50} \) will depend on both the substrate and its concentration if the inhibitor and substrate compete for the same binding site (generally the case for \( \beta \)-lactamase inhibitors). For a reversible competitive inhibitor, the effect of substrate is described by the following relationship:

\[
I_{50} = K_i \left( 1 + [S]/K_m \right) \text{ where } K_i = \text{inhibitor constant}
\]
Similarly, for a progressive inhibitor binding at the active site, the rate of inactivation will be decreased by increasing the substrate concentration.

In practical terms, for a competitive inhibitor assayed with the substrate at \( K_m \), the \( I_{50} \) will be \( 2 \times K_i \); when \([S]\) at \( 9 \times K_m \), the \( I_{50} \) will be \( 10 \times K_i \). Therefore, when assays are carried out at \( <K_m \), \( I_{50} \) values will be little affected by substrate concentration. When materials are in short supply, assaying at low substrate concentrations may be the method of choice. If this is done, however, control (i.e., uninhibited) rates will soon become nonlinear. It is, therefore, essential that care is taken to measure only over the initial linear phase, otherwise misleading and poorly reproducible results will be obtained. As a rule of thumb, substrate hydrolysis should not exceed 10–20% of the total.

9. Use of multiples of \( K_m \) for a more standardized procedure. If \([S] \gg K_m \), then:
   a. Control rates will be linear for a larger proportion of the reaction.
   b. \( I_{50} \) values will be more reproducible.
   c. More complex inhibition patterns (i.e., progressive and slow binding) will be more readily detected.

When sufficient materials are available, and when inhibitors are being compared against a number of \( \beta \)-lactamases, it is advisable to use the same substrate and multiple of its \( K_m \) for each of the respective enzymes (suggested concentration = \( 5 \times K_m \)). The \( I_{50} \) will then be a strict function of the inhibitory properties of a particular compound against the \( \beta \)-lactamases, and will reflect its potency against the different enzymes. When using inhibitors to characterize and classify \( \beta \)-lactamases, it is essential that procedures are standardized to give reproducible results.

References


Molecular Approaches for the Detection and Identification of β-Lactamases

David J. Payne and Christopher J. Thomson

1. Introduction

β-lactamases confer resistance to β-lactam antibiotics, which are the most widely used family of antibiotics. It is, therefore, essential that one can identify the production of β-lactamases by clinical isolates and have effective ways of distinguishing the different enzymes. This is necessary for epidemiologic surveys, predicting future resistance trends, and to ensure that patients receive the appropriate β-lactam or alternative therapy.

In 1985, approx 20 different plasmid β-lactamases had been characterized (1). These β-lactamases were effectively distinguished by isoelectric focusing (IEF) by virtue of their different isoelectric points (pI) (see Chapter 27). Today, approx 200 different β-lactamases have been characterized, and it is likely that more novel enzymes will continue to be identified. The majority of β-lactamases are serine active site enzymes, although a variety of metallo active site enzymes are now being identified (2). Therefore, IEF is no longer sufficient to allow different β-lactamases to be identified reliably. In fact, currently 47 β-lactamases focus between pI 5 and pI 6, and 36 enzymes focus between pI 8 and pI 9. In addition, several important TEM β-lactamases have the same pI and are, hence, indistinguishable by IEF. Although, molecular DNA probing techniques have become very effective for identifying different β-lactamases, in some cases this approach must still be combined with kinetic and IEF data to identify a β-lactamase. This chapter reviews the use of molecular techniques to detect and distinguish β-lactamases, whereas the following chapter gives a detailed guide to performing IEF and kinetic studies on these enzymes.
2. Diagnosis

The first indication of the presence of β-lactamases is usually the observation of increased levels of resistance in clinical isolates as detected by routine sensitivity testing. In some species, for example, Haemophilus influenzae or Neisseria gonorrhoeae, the presence of a β-lactamase in itself is enough information to make a judgment on treatment options, and in these cases often a simple nitrocefin hydrolysis test is performed to confirm the presence or absence of β-lactamase production (3). In other cases, most notably the Enterobacteriaceae, it is not the presence of a β-lactamase that is important, but the type of enzyme and, in particular, whether the enzyme has an extended-spectrum of activity or increased resistance to β-lactamase inhibitors. It has become apparent, however, that the emergence of some of the initial mutations in the TEM and SHV β-lactamases that give rise to an extended-spectrum or inhibitor resistance may not be detected by the use of standard clinical breakpoints or sensitivity testing procedures (4,5). To overcome this shortfall, several sensitivity testing techniques have been devised to highlight the occurrence of these enzymes.

To date, all the TEM- and SHV-derived β-lactamases that confer resistance to third-generation cephalosporins (referred to as the extended-spectrum TEM and SHV β-lactamases) remain susceptible to β-lactamase inhibitors such as clavulanic acid (6). This property can be used to detect these enzymes in clinical isolates by double-disk testing using an amoxicillin/clavulanic acid disk, and a disk containing a third generation cephalosporin. If the increased resistance to the cephalosporin is a result of extended spectrum β-lactamase activity, then the clavulanic acid will inhibit this and a distinctive dumbbell-shaped zone of inhibition will be produced (7). Such results can be difficult to interpret and, more recently, an attempt has been made to standardize the procedure with the incorporation of clavulanic acid into cephalosporin-containing E-test strips (8).

A different set of mutations in the TEM gene confers no resistance to later generation cephalosporins, but increases resistance to β-lactamase inhibitors. The initial detection of these TEM derived inhibitor-resistant β-lactamases is more problematic. As yet, no satisfactory detection system has been devised, although it appears that this may be facilitated by sensitivity testing inhibitor combinations with a low fixed concentration of inhibitor rather than testing at a fixed ratio of β-lactam:β-lactamase inhibitor (9).

3. Epidemiologic Uses

3.1. Detection of β-Lactamase Genes by Hybridization

The use of DNA probes to detect β-lactamase genes is well-established, and the general procedures involved are outlined in Fig. 1 (10). Studies
Detection of β-Lactamases

Fig. 1. General procedure for the detection of β-lactamase genes by DNA probing.

with β-lactamase DNA probes have played a major role in defining the epidemiology of β-lactamases in clinical isolates. In any analysis of DNA by hybridization, the DNA to be tested has to be immobilized onto a suitable membrane. The procedure chosen will, to some extent, be determined by the information required. For example, the location of a β-lactamase gene on a plasmid can be ascertained by restriction digestion of the plasmid followed by Southern blotting of the gel (II). However, if only a positive or negative result is required, then the DNA to be tested can be applied directly to the membrane by dot blotting (12). Once the DNA has been immobilized then hybridization can be tested against the probe DNA.

3.1.1. Generation of DNA for Use as a Gene Probe

The first step in making a gene probe is generating a fragment of DNA to label. Previously, β-lactamase DNA probes were usually made from restriction digests of the β-lactamase gene (Tables 1 and 2). Occasionally, DNA probes containing regions upstream or downstream of the β-lactamase gene have been used and have led to low discrimination and false positive identifications. Consequently, the use of intragenic gene probes is strongly recommended. The quickest way to manufacture a gene probe is by polymerase chain reaction (PCR) of an intragenic region of the target gene and a number of PCR primers for a variety of β-lactamase genes are detailed in Tables 1 and 2. An alternative to a PCR generated probe is the use of an oligoprobe (an oligonucleotide). In this instance the DNA can be generated by a DNA synthesizer.
Table 1
Examples of TEM- and SHV-Derived Probes and PCR Primers (in Most Cases only Examples of Intragenic Gene Probes are Shown)

<table>
<thead>
<tr>
<th>β-lactamase</th>
<th>Size</th>
<th>Description of probe or PCR primers</th>
<th>Type of label or utility of PCR product</th>
<th>Plasmid</th>
<th>Cross hybridization (in addition to self hybridization)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>504</td>
<td>Obtained by PCR. Primers:</td>
<td>Radioactive</td>
<td>pBR322</td>
<td>TEM-2, 7, 20, 22</td>
<td>13, 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAATTTGTTGGCCGGGAAGCTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCGGCGCGCTAGGCATGAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-1</td>
<td>656</td>
<td>Obtained by PCR. HindIII and TagI, intragenic</td>
<td>Radioactive</td>
<td>pBR322</td>
<td>TEM-2</td>
<td>15</td>
</tr>
<tr>
<td>TEM-2</td>
<td>15</td>
<td>ACTTCTAGTCAACCC</td>
<td>Radioactive</td>
<td>N/A</td>
<td>None reported*</td>
<td>15</td>
</tr>
<tr>
<td>TEM-2</td>
<td>15</td>
<td>ACTTCTATTTCAACCC</td>
<td>Radioactive</td>
<td>N/A</td>
<td>None reported*</td>
<td>15</td>
</tr>
<tr>
<td>TEM-1</td>
<td>11</td>
<td>AAGCGGAGGAC</td>
<td>Radioactive</td>
<td>N/A</td>
<td>TEM-26 and other TEMs</td>
<td>16</td>
</tr>
<tr>
<td>TEM-1</td>
<td>850</td>
<td>Ndel-I EcoRII</td>
<td>Biotin</td>
<td>pUC19</td>
<td>None reported*</td>
<td>17</td>
</tr>
<tr>
<td>TEM-1</td>
<td>15</td>
<td>ACTTCTATTCCACCC</td>
<td>Radioactive</td>
<td>pMON60,</td>
<td>TLE-1, TEM-2</td>
<td>18, 19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-1</td>
<td>424</td>
<td>Obtained by PCR. BglII and HindIII, intragenic</td>
<td>Radioactive/ Digoxygenin</td>
<td>pBR322</td>
<td>YOU-1, YOU-2</td>
<td>20, 21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-1</td>
<td>526</td>
<td>Obtained by PCR. Primers:</td>
<td>Diagnostic PCR</td>
<td>n/a</td>
<td>TEM-4/* 9 and other TEMs</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGGTGCAAGATGGGTTAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTATCCGCTCCATCCAGTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHV-1</td>
<td>780</td>
<td>Obtained by PCR. PvuII-PvuII</td>
<td>Radioactive</td>
<td>pMON38</td>
<td>SHV-2</td>
<td>21</td>
</tr>
<tr>
<td>SHV-1</td>
<td>467</td>
<td>Obtained by PCR. NotI-PstI Intragenic</td>
<td>Biotin</td>
<td>pCLL3411</td>
<td>SHV-7</td>
<td>17</td>
</tr>
<tr>
<td>SHV-3</td>
<td>467</td>
<td>Obtained by PCR. NotI-PstI, intragenic</td>
<td>Radioactive</td>
<td>pHUC37</td>
<td>SHV-6</td>
<td>23</td>
</tr>
<tr>
<td>SHV-3</td>
<td>626</td>
<td>Obtained by PCR. Primers:</td>
<td>Radioactive</td>
<td>pUD18</td>
<td>SHV-1-5, OHIO-1</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACGAGGGCGAATCCCGCG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGTGGCAATCCCGGTCCTCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHV-1-5</td>
<td>475</td>
<td>Obtained by PCR. Primers:</td>
<td>PCR-SSCP</td>
<td>n/a</td>
<td>n/a</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGTGGCAATCCCGGTCCTCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCAGCGAAAAAACACCTTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OHIO-1</td>
<td>223</td>
<td>Obtained by PCR. PstI-HaeII, intragenic</td>
<td>Radioactive</td>
<td>pSK04</td>
<td>SHV-1, (LEN-1)</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGTGGCAATCCCGGTCCTCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

( ) = weak hybridization, *No cross hybridization reported, but probe would probably hybridize with other TEM genes.
# Table 2
Examples of Other β-Lactamase Probes and PCR Primers (in Most Cases only Examples of Intragenic Gene Probes are Shown)

<table>
<thead>
<tr>
<th>β-lactamase</th>
<th>Size</th>
<th>Description of probe or PCR primers</th>
<th>Type of probe or utility of PCR product</th>
<th>Plasmid</th>
<th>Cross hybridization (in addition to self hybridization)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-1</td>
<td>15</td>
<td>Oligoprobe: CCAAGAGCTGGGATG</td>
<td>Radioactive</td>
<td>N/A</td>
<td>None reported</td>
<td>26</td>
</tr>
<tr>
<td>OXA-1a</td>
<td>315</td>
<td>BgIll-BglIII, intragenic</td>
<td>Radioactive</td>
<td>pMON300</td>
<td>OXA-4</td>
<td>18, 21</td>
</tr>
<tr>
<td>OXA-2a</td>
<td>510</td>
<td>HincII-HincII, intragenic</td>
<td>Radioactive</td>
<td>pMON21</td>
<td>(OXA-3 )</td>
<td>21</td>
</tr>
<tr>
<td>PSE-1</td>
<td>587</td>
<td>Obtained by PCR: Primers: GGGGCTTGATGCTCACA TCA</td>
<td>Radioactive</td>
<td>RPL11</td>
<td>PSE-4, CARB-2-4</td>
<td>13</td>
</tr>
<tr>
<td>PSE-1 a</td>
<td>1300</td>
<td>BglII and BgIll part of the PSE-1 gene)</td>
<td>Radioactive</td>
<td>pMON810</td>
<td>PSE-4, CARB-3</td>
<td>18, 21</td>
</tr>
<tr>
<td>PSE-2a</td>
<td>460</td>
<td>Rsal-Xbal, intragenic</td>
<td>Radioactive</td>
<td>pMON234</td>
<td>OXA-6 (OXA-5)</td>
<td>21</td>
</tr>
<tr>
<td>PSE-4a</td>
<td>180</td>
<td>BglII-XbaI</td>
<td>Radioactive</td>
<td>pMON209</td>
<td>PSE-1 CARB-3</td>
<td>21</td>
</tr>
<tr>
<td>ROB-1</td>
<td>250</td>
<td>Sca3A, intragenic</td>
<td>Radioactive</td>
<td>pMON401</td>
<td>None reported</td>
<td>18</td>
</tr>
<tr>
<td>ROB-1</td>
<td>240</td>
<td>Dral-Dral, intragenic</td>
<td>Radioactive</td>
<td>pMON401</td>
<td>None reported</td>
<td>27</td>
</tr>
<tr>
<td>ROB-1</td>
<td>692</td>
<td>Obtained by PCR: Primers: ATCAAGCCACATAACCGACCT GGTGGGATTGCGTATGCGA</td>
<td>Diagnostic PCR</td>
<td>N/A</td>
<td>None reported</td>
<td>22</td>
</tr>
<tr>
<td>IMP-1*</td>
<td>450</td>
<td>HindIII-HindIII, intragenic</td>
<td>Radioactive</td>
<td>pHIP29</td>
<td>None reported</td>
<td>28</td>
</tr>
<tr>
<td>CBA*</td>
<td>726</td>
<td>Obtained by PCR: Primers: TCCATGCTTTTCCTGCAGTT GCACTCAAGGCCATAGCCGAA</td>
<td>Radioactive</td>
<td>pJST241</td>
<td>None reported</td>
<td>29</td>
</tr>
<tr>
<td>Ent. cloacae</td>
<td>530</td>
<td>NcuI-SpI</td>
<td>Digoxigenin</td>
<td>pEC1E</td>
<td>None reported</td>
<td>30</td>
</tr>
<tr>
<td>Amp C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>685</td>
<td>PstI-XhoI Intragenic</td>
<td>Digoxigenin</td>
<td>pNU6</td>
<td>None reported</td>
<td>20</td>
</tr>
<tr>
<td>Amp C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. mirabilis</td>
<td>2.6Kb</td>
<td>HindIII-HindIII</td>
<td>Radioactive</td>
<td>pPM79P1</td>
<td>None reported</td>
<td>31</td>
</tr>
<tr>
<td>carbencillinase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

() = weak hybridization; (a) = Probe used in study to compare IEF with DNA probing for the detection of β-lactamases in 122 clinical isolates, *metallo-β-lactamases.
3.1.2. Labeling of β-Lactamase Gene Probes and Detection of Hybridization

Many different methods for labeling β-lactamase probes have been developed and compared. Traditionally, hybridization of DNA probes was detected by radioactive labeling. However, the reliance on radioactivity precluded the widespread use of probing technology in general laboratories, and alternatives were sought. For example, Zwadyk et al. (32) developed a biotinylated probe for TEM-1, but illustrated that this was 100 times less sensitive than the 32P-labeled probe. Probes labeled with digoxigenin have also been developed and compared with IEF and biotin-labeled probes. In this work it was shown that the digoxigenin-labeled probe was as sensitive as IEF, but the biotin-labeled probe had poor specificity (33). The development of chemiluminescent detection systems has enabled the use of fluorescein-labeled DNA probes that offer similar sensitivity to radioactive probes without the practical complications of radioactivity. An outline of the protocol for making such probes is presented.

3.1.3 Fluorescein-Labeled β-Lactamase Probes

Fluorescein-labeled β-lactamase probes (ECL™, Amersham Life Science, Amersham Place, Little Chalfont, Buckinghamshire, England) are detected with the enzyme horseradish peroxidase (HRP) that catalyzes the oxidation of luminol. The horseradish peroxidase is attached to an antifluorescein antibody that binds to the fluorescein-labeled probe (Fig. 2). The oxidized luminol then decays to the ground state via a light emitting pathway that can be detected on photographic film. The use of oligonucleotide probes labeled with fluorescein has been successfully used for detecting and differentiating related genes encoding trimethoprim resistance (34). Protocols for these steps are described in detail in the manufacturer’s guidelines (Amersham Life Science).

3.2. Detection of β-Lactamase Genes by PCR

PCR has been extensively used in the detection and analysis of resistance genes in bacterial isolates (35,36) and in the detection of specific micro-organisms directly in clinical and environmental specimens (37–39). However, it is only relatively recently that the possibility of employing PCR in the direct detection of resistance genes in clinical specimens has been investigated. PCR has been used to detect rifampicin resistance in Mycobacterium tuberculosis directly in sputum samples (40,41) and, more recently, for detecting ampicillin resistance genes in cerebrospinal fluid (CSF) samples containing H. influenzae (22). In the latter case, PCR primers were used that were specific for the blaTEM and blaROB genes. Tables 1 and 2 illustrate these primers and the size of the PCR product obtained. Correlation was obtained between the result of minimum inhibitory concentration (MIC) testing, β-lactamase production as determined by nitrocefin and PCR testing.
Detection of β-Lactamases

3.2.1. PCR Approaches for Studying the Heterogeneity of β-Lactamase Genes in a Species

Jones et al. (42) used PCR to study the heterogeneity of the chromosomal ampC gene in 91 clinical isolates of Citrobacter spp. Primers were chosen based on the ampC genes from C. freundii OS60, C. freundii 1113, and C. diversus NF85 to give PCR products of 870, 870, and 620 bp, respectively. Sixty-five of the isolates gave a PCR product with one of the sets of primers, three of these PCR products were larger than expected. Twenty-six isolates gave no product, but 21 of these had ampC genes, which hybridized with one of the PCR products. Such a study illustrates how PCR can potentially identify novel β-lactamase genes and also rapidly determine the heterogeneity of a β-lactamase gene within a particular species. This study highlights the potential of PCR for typing at a molecular level.

3.3. Characterization of TEM β-Lactamases

This section explains how PCR and hybridization studies can be utilized to study a particular series of β-lactamases using the TEM β-lactamases as an illustration. The gene encoding TEM-1 β-lactamase is the most widespread plasmid-mediated β-lactamase gene found in clinical isolates. This enzyme is capable of conferring resistance to penicillins and some of the early cephalosporins. Since 1982 (43) there have been reports of TEM enzymes that differ from TEM-1 by a variety of one to four amino acid changes. These amino acid changes reconfigure the active site of TEM-1, enabling it to hydrolyze and confer resistance to penicillins and first, second, and third generation cephalosporins. Other mutations in the TEM gene can confer resistance to
β-lactamase inhibitors (5,44) although, to date, no TEM β-lactamase has been characterized that confers both an extended-spectrum and inhibitor-resistant phenotype simultaneously. Therefore, it is necessary to identify point mutations in the TEM enzymes as they will provide information on the expected resistance profile of the producing organism.

3.3.1. PCR for the Sequencing of TEM-Related Enzymes

Amplification of TEM β-lactamases for sequence analysis (Subheading 4.1.) can be achieved by using primers from the conserved regions at either end of the TEM structural gene. The sequences of the primers (45) are:

- Forward: 5'-GAAGACGAAAGGGCCTCGTG-3'
- Reverse: 5'-GGTCTGACAGTTACCAATGC-3'

The PCR reaction is set up according to the schedule in Table 3.

3.3.2. Oligotyping to Identify Point Mutations in TEM β-Lactamases

Detailed characterization of the variant enzymes is difficult. An intragenic TEM probe will identify an extended-spectrum TEM, but will not distinguish it from TEM-1. Isoelectric focusing is inappropriate as these enzymes all focus in a very narrow pI range (Subheading 1.). Oligotyping can be used to examine point mutations in a particular β-lactamase gene. The use of this technique to distinguish the plethora of TEM β-lactamases is reviewed below.

Oligotyping provides the best and most time-effective way of distinguishing these enzymes. This methodology utilizes a set of oligonucleotides that are specific for each of the point mutations. The techniques used to label and detect hybridization are the same as those listed in Subheading 3.1. This technique was first devised by Ouellette et al. (15) where oligonucleotides were devised to distinguish TEM-1 from TEM-2, which differ by one amino acid. Mabilat and Courvalin (46) developed the method further using 12 radioactive oligoprobes (17 mers) to probe five different loci, and this was used to distinguish 14 different extended-spectrum TEM genes, including seven novel TEMs, in 265 clinical isolates (Table 4). Henquell et al. (44) have used oligotyping to characterise changes in the TEM gene associated with resistance to β-lactamase inhibitors among 107 inhibitor-resistant TEM enzymes. Fifteen oligonucleotides were used to study three different loci in the TEM gene, which facilitated the identification of 10 different types of inhibitor-resistant TEM β-lactamases, IRT-1 to 10 (Table 5).

It is important to note that IEF and biochemical data can be used in conjunction with the oligotyping data to confirm observations. In addition, as oligotyping probes only a limited number of amino acid changes, other changes in the sequence may also exist, so care is required in interpretation of the data.
Detection of β-Lactamases

Table 3
PCR Amplification Procedure

<table>
<thead>
<tr>
<th>Segment</th>
<th>Temperature</th>
<th>Time</th>
<th>Ramp rate</th>
<th>Repeats</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96°C</td>
<td>30 s</td>
<td>48°C/min</td>
<td>X 1</td>
<td>DNA denaturation</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>30 s</td>
<td>10°C/min</td>
<td></td>
<td>DNA annealing</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>90 s</td>
<td>30°C/min</td>
<td></td>
<td>DNA extension</td>
</tr>
<tr>
<td>2</td>
<td>96°C</td>
<td>15 s</td>
<td>48°C/min</td>
<td>X 20-24</td>
<td>DNA denaturation</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>30 s</td>
<td>10°C/min</td>
<td></td>
<td>DNA annealing</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>90 s</td>
<td>30°C/min</td>
<td></td>
<td>DNA extension</td>
</tr>
<tr>
<td>3</td>
<td>72°C</td>
<td>5 min</td>
<td></td>
<td>X 1</td>
<td>Final extension</td>
</tr>
</tbody>
</table>

Table 4
Characterization of Extended-Spectrum TEM β-Lactamases Using Oligo-Probes to Detect Point Mutations (46)

<table>
<thead>
<tr>
<th>Amino acid altered(^a)</th>
<th>Sequence of oligo probe(^b)</th>
<th>Enzymes with particular amino acid mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln 39</td>
<td>ACCCAACTGATCTTCAG</td>
<td>TEM-1, 4, 5, 6, 9, 10, 12, 15, 17, 19</td>
</tr>
<tr>
<td>Lys 39</td>
<td>ACCCAACTTATCTTCAG</td>
<td>TEM-2, 3, 7, 8, 11, 13, 14, 16, 18</td>
</tr>
<tr>
<td>Glu 104</td>
<td>TGAGTACTCAACCAAGT(^c)</td>
<td>TEM-1, 2, 5, 7, 10, 11, 12, 13, 19</td>
</tr>
<tr>
<td>Lys 104</td>
<td>TGAGTACTTAAACCAAGT(^c)</td>
<td>TEM-3, 4, 6, 8, 9, 14, 15, 16, 17, 18</td>
</tr>
<tr>
<td>Arg 164</td>
<td>TTCCCAACGATCAAGGC(^c)</td>
<td>TEM-1, 2, 3, 4, 13, 14, 15, 17, 18, 19</td>
</tr>
<tr>
<td>Ser 164</td>
<td>TTCCCAACTTACAAAGGC(^c)</td>
<td>TEM-5, 7, 8, 9, 10, 12</td>
</tr>
<tr>
<td>His 164</td>
<td>GTTCACCAATGATCAAGG</td>
<td>TEM-6, 11, 16</td>
</tr>
<tr>
<td>Gly 238</td>
<td>ACGCTCACCACGGTCCAG(^c)</td>
<td>TEM-1, 2, 5, 6, 7, 9, 10, 12, 13, 16, 17, 18</td>
</tr>
<tr>
<td>Ser 238</td>
<td>ACGCTCACCAGGTCCAG(^c)</td>
<td>TEM-3, 4, 8, 14, 15, 19</td>
</tr>
<tr>
<td>Thr261</td>
<td>TCCCCGTCGTGTAGATA</td>
<td>TEM-1, 2, 3, 5, 6, 7, 8, 10, 11, 12, 15, 16, 17, 18, 19</td>
</tr>
<tr>
<td>Met261</td>
<td>TCCCCGTCATGTAGATA</td>
<td>TEM-4, 9, 13, 14</td>
</tr>
</tbody>
</table>

\(^a\) Ambler numbering (47).
\(^b\) The underlined nucleotide indicates the point mutation.
\(^c\) Biotinylated versions of these probes have also been used (48).

If an ambiguity remains after oligotyping and biochemical studies, complete sequencing of the gene may be required (see Subheading 4.). Oligotyping using biotinylated probes has also been successfully developed (48). Oligotyping has been used to characterize TEM-variant enzymes but this procedure could also be developed to characterize the SHV-variant enzymes, and any other resistance genes that differ by only point mutations.
Table 5
Characterization of Inhibitor Resistant TEM β-Lactamases Using Oligo-
Probes to Detect Point Mutations (44)

<table>
<thead>
<tr>
<th>Amino acid altered</th>
<th>Sequence of oligo probe (^b)</th>
<th>Enzymes with particular amino acid mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met69</td>
<td>TTTCCAATGATGAGCACT</td>
<td>TEM-1</td>
</tr>
<tr>
<td>Leu69</td>
<td>TTTCCAATGCTGAGCACT</td>
<td>TEM-33 (IRT-5), 35 (IRT-4), 39 (IRT-10)</td>
</tr>
<tr>
<td>Leu69</td>
<td>TTTCCAATGTGAGCACT</td>
<td>TEM-35 (IRT-4)</td>
</tr>
<tr>
<td>Val69</td>
<td>TTTCCAATGGTGAGCACT</td>
<td>TEM-34 (IRT-6), 38 (IRT-9)</td>
</tr>
<tr>
<td>Ile69</td>
<td>TTTCCAATGATTAGCACT</td>
<td>TEM-32 (IRT-3)</td>
</tr>
<tr>
<td>Ile69</td>
<td>TTTCCAATGATTAGCACT</td>
<td>TEM-32 (IRT-3), 37 (IRT-8)</td>
</tr>
<tr>
<td>Ile69</td>
<td>TTTCCAATGACAGCAGCCT</td>
<td>No hybridisation detected</td>
</tr>
<tr>
<td>Arg244</td>
<td>CGTGGGCTCTCGCGGTATC</td>
<td>TEM-1</td>
</tr>
<tr>
<td>Cys244</td>
<td>CGTGGGCTCTCGCGGTATC</td>
<td>TEM-31 (IRT-1)</td>
</tr>
<tr>
<td>Ser244</td>
<td>CGTGGGCTCTACGCGGTATC</td>
<td>TEM-30 (IRT-2)</td>
</tr>
<tr>
<td>Asn276</td>
<td>ATGAACGAATAGACAG</td>
<td>TEM-1</td>
</tr>
<tr>
<td>Asp276</td>
<td>ATGAACGAGATAGACAG</td>
<td>TEM-35 (IRT-4), 36 (IRT-7), 37 (IRT-8), 39 (IRT-10)</td>
</tr>
</tbody>
</table>

\(^a\)Ambler numbering (47).
\(^b\)The underlined nucleotide indicates the point mutation.
\(^c\)IRT, inhibitor resistant TEM.

An alternative approach for identifying and characterizing β-lactamases that differ by point mutations is PCR single conformational polymorphism (PCR-SSCP). This method is based on the fact that a single nucleic acid change will affect the electrophoretic mobility of a short sequence of DNA and this technique has been used to distinguish SHV-1 to -5 (Table 1) (24). PCR-SSCP may also characterise mutations in the TEM-derived β-lactamases. However, on many occasions β-lactamase genes may have nucleic acid changes that do not result in amino acid changes (so-called silent mutations). Therefore, in such a scenario, PCR-SSCP could indicate that two β-lactamases differ although they may have identical amino acid sequences and confer an identical profile of resistance.

4. Research Issues

4.1. Direct Sequence Analysis of β-Lactamases

An intragenic gene probe may indicate the type of β-lactamase produced by a clinical isolate (e.g., TEM- or SHV-derived) and oligoprobing enables known point mutations to be identified. However, the definitive confirmation and characterization of such an enzyme necessitates establishing the DNA nucleotide
sequence of the gene encoding it. Fortunately, this can now be quickly performed without the need for time-consuming cloning procedures. The suggested procedure circumvents the need for cloning by amplifying the resistance gene by PCR and then directly sequencing the PCR product by employing the DYNAL® magnetic bead separation system. The overall strategy is outlined in Fig. 3. The system is based on the principle that one of the primers employed to PCR the resistance gene is labeled with biotin: This allows the two DNA strands of the PCR product to be separated with the aid of magnetic beads that bind to the biotin-labeled strand of the PCR product (49,50). Once separated, the sequencing of the single stranded β-lactamase DNA can be performed. This procedure can be applied to any gene provided suitable primers are available to permit amplification by PCR.

5. Future Prospects and Goals

The application of PCR technology to β-lactamases has an array of potential benefits in both the clinical setting and β-lactamase research. The ability of PCR to detect β-lactamase genes directly in clinical specimens (Subheading 3.2.) creates many new opportunities to assist clinical therapy in the early stages of life-threatening diseases. The challenge here is to design approaches or kits that would enable easy access to the technology for the clinical microbiology laboratory.

The obvious advantages of PCR for β-lactamase research is that it expedites the generation of gene probes and facilitates the sequencing of β-lactamase
genes. PCR approaches have clearly superseded the traditional approaches of generating probes from restriction fragments. PCR also has other applications, Subheading 3.2.1. describes the use of PCR to study the heterogeneity of β-lactamase genes in Citrobacter spp. This approach should be applied to other β-lactamase genes and may illustrate a diversity of β-lactamases produced by a species which was previously assumed to possess only one type of β-lactamase.

Over the last 5 yr the use of DNA probing approaches to detect β-lactamases have been complicated by the plethora of enzymes that differ by only a few amino acids. For example, DNA from a clinical isolate that hybridizes with a TEM-1 probe indicates the enzyme could be any of over 30 different enzymes, each of which is capable of providing a very different spectrum of resistance compared to TEM-1. Secondary probing approaches, such as oligoprobing, have been successfully developed to overcome this difficulty, and in the future these types of approaches may be more reliable. Despite all these applications, there will undoubtedly be instances where detailed biochemical evaluation, and ultimately sequencing of the whole gene, are required for unequivocal identification. Figure 4 suggests a scheme for identifying TEM or SHV β-lactamases.

Gene probes will play a very important role in defining the future clinical epidemiology of metallo-β-lactamases (Table 2), identification of these genes by probing will not be complicated by single amino acid changes. These enzymes confer resistance to most commonly used β-lactams and are produced by common pathogens, consequently, it is important to gauge their clinical significance.
6. Notes

1. Identification of cryptic genes. Although a positive hybridization indicates the presence of a particular \( \beta \)-lactamase gene, it provides no indication of whether the gene is being expressed and conferring resistance. This introduces the concept of covert or silent genes. As the use of genetic approaches to detect resistance genes is a relatively new concept, the incidence of silent genes or remnant genetic structures is not known, but they have been observed a few times. Jouvenot et al. (51) observed positive hybridization with a TEM-1 probe with 16 clinical isolates that did not appear to produce a TEM \( \beta \)-lactamase. This may have been caused by the TEM-1 probe containing 200 bp of extraneous DNA outside the TEM-1 gene. Oucllette et al. (26) also observed that five strains of *Pseudomonas aeruginosa*, which did not produce TEM-1, hybridized with a TEM-1 oligonucleotide probe (a 15 mer). However, DNA from these strains did not hybridize with a 656-bp TEM-1 probe. Therefore, it is probable that there was some fortuitous hybridization between the oligoprobe and homologous sequences in the strains of *P. aeruginosa*. These studies illustrate the importance of determining whether examples of silent genes may be an artefact of the probe used.

2. The *CfiA* cryptic metallo-\( \beta \)-lactamase gene. Probing studies by Podglagen et al. (29) illustrated that carbapenem-sensitive clinical isolates of *Bacteroides fragilis* carried a cryptic *CfiA* metallo-\( \beta \)-lactamase gene that became expressed when the isolates were exposed to imipenem. The isolates consequently became resistant to carbapenems and almost all other clinically important \( \beta \)-lactam antibiotics. Activation of this gene was a consequence of the insertion of a novel 1.3 kb insertion sequence immediately upstream of the *CfiA* gene (52). This implies that the *CfiA* gene may be more widespread than previously appreciated, and the selective pressure applied by the predicted increase in carbapenem consumption may result in a significant increase in the extent of *CfiA* expression. In the clinical setting, such false-positive results caused by the detection of a cryptic gene may not be serious since the likely implication for the patient would be a change in antibiotic. In fact, although the gene may be silent in vitro, it may become activated in vivo, in which case the change to an alternative antibiotic would be advantageous.

3. Immunological approaches. Hybridization and PCR approaches are targeted at the DNA that encodes the \( \beta \)-lactamase. However, there are a few examples of where antibody approaches have been used to identify strains producing \( \beta \)-lactamases. These are reviewed below. A monoclonal antibody (MAbs) for a chromosomally encoded *P. aeruginosa* \( \beta \)-lactamase (53) has been shown to have no cross-reactivity with 23 different \( \beta \)-lactamases from seven different Bush groups, thus indicating the specificity of the MAbs (54). In addition, antisera to TEM-1 have been shown to cross-react with TEM-2 and TLE-1, and to a lesser extent with SHV-1, but no reaction was observed with HMS-1 or the OXA or PSE enzymes. Antiserum to OXA-1 react with OXA-4 and vice versa.

4. A study by Morin et al. (55), however, illustrated that although 16 MAbs to TEM-1 cross-reacted with TEM-1, TLE-1 and, to a lesser extent, with SHV-1, they also
Payne and Thomson

exhibited a reaction with OXA-1,3,6, and 7 and AER-1. This illustrates that diverse β-lactamases may have quite similar epitopes. Therefore, in certain circumstances, immunological approaches for the identification of β-lactamases can be less effective than DNA techniques. In addition, preparation of the antiserum or MAbs is much more time consuming than the preparation of a DNA probe. It is for these reasons that immunological approaches have been utilized far less than DNA approaches for the detection of β-lactamases in clinical isolates. However, recent studies on the rapid detection of TEM β-lactamases have successfully combined the two approaches. One of the drawbacks of PCR for routine use is the requirement for a time-consuming electrophoresis step in order to detect the PCR product. By utilizing specific TEM and control rRNA primers labeled with either dinitrophenol, digoxigenin, or biotin, Curran et al. (56) were able to detect PCR product by immunoassay. PCR product was detected by employing an immunoassay detection device consisting of a membrane containing blue latex beads labeled with either anti-dinitrophenol, digoxigenin, or biotin antibody. A positive reaction was indicated by the appearance of a blue line and in most cases was obtained within 5 min. Comparison with isoelectric focusing for clinical E. coli confirmed the reliability of this method (56). The optimization of this technology could herald the rapid detection of resistance genes direct from clinical specimens in routine laboratories.

References

Detection of β-Lactamases


Detection of β-Lactamases


Bacterial resistance mechanisms as therapeutic targets

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SmithKline Beecham Pharmaceuticals, Microbiology Research, Brockham Park, Betchworth, Surrey RH3 7AJ, UK

In the 50 years since antimicrobial agents were first introduced, bacteria have acquired a wide variety of mechanisms which have enabled them to resist the effects of these drugs. One way of overcoming this problem is to administer an antibiotic with an agent which counteracts the mechanism of resistance to that antibiotic; an example of such an approach which has already been successfully implemented is the combination of a β-lactam antibiotic with a β-lactamase inhibitor. This review describes antibiotic resistance mechanisms which might lend themselves to an inhibitor approach and the potential therapeutic applications of such a strategy.

Introduction

Bacterial resistance to antibiotics was encountered in clinical isolates soon after the introduction of some of the earliest agents (Finland, 1979; Gale et al., 1981; Russell & Chopra, 1990). Today, the problem of antibiotic resistance continues to be a major factor complicating the use of chemotherapeutic agents and the control of infectious diseases (Amyes & Gemmell, 1992; Neu, 1992).

Acquired resistance, which occurs when resistant strains emerge from a previously susceptible bacterial population following exposure to an antibiotic, can arise either by acquisition of plasmids and transposons or by chromosomal mutation (Russell & Chopra, 1990). The biochemical mechanisms which form the basis of acquired antibiotic resistance can be classified into five major groups (Table 1), of which categories 1–3 are the most prevalent in clinical isolates.

Resistance to the penicillins and cephalosporins frequently results from inactivation of these agents by β-lactamases, enzymes which catalyse the hydrolysis of molecules containing a β-lactam ring (Gale et al., 1981). Two broad approaches have been adopted in order to overcome the problem of β-lactamases. One strategy has been to synthesize new β-lactams with increased stability to hydrolysis; an example was the development in the 1960s of methicillin (and subsequently isoxazolyl penicillins such as oxacillin and cloxacillin) for use against penicillinase-producing staphylococci (Rolinson, 1979; Kucers, Bennett & Kemp, 1987). The alternative approach involved the development of β-lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam, which have little intrinsic antibacterial activity but which protect β-lactam substrates from inactivation (Bush & Sykes, 1983; Rolinson, 1991; Sutherland, 1991).

*Corresponding author.
Table I. Mechanisms of acquired resistance to antibiotics

<table>
<thead>
<tr>
<th>Mechanism of resistance</th>
<th>Examples of antibiotics affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Alteration of antibiotic leading to</td>
<td>ß-lactams, chloramphenicol, aminoglycosides, fosfomycin</td>
</tr>
<tr>
<td>inactivation</td>
<td></td>
</tr>
<tr>
<td>2. Alteration of target site in the cell so that</td>
<td>ß-lactams, aminoglycosides, quinolones, rifampicin, tetracyclines, glycopeptides, macrolides</td>
</tr>
<tr>
<td>it is insensitive to the antibiotic yet still</td>
<td></td>
</tr>
<tr>
<td>able to perform its normal physiological function</td>
<td></td>
</tr>
<tr>
<td>3. Decreased intracellular accumulation of an</td>
<td>ß-lactams, aminoglycosides, tetracyclines, quinolones, macrolides</td>
</tr>
<tr>
<td>antibiotic due to:</td>
<td></td>
</tr>
<tr>
<td>a. Impaired uptake</td>
<td>sulphonamides, trimethoprim, mupirocin</td>
</tr>
<tr>
<td>b. Enhanced efflux</td>
<td></td>
</tr>
<tr>
<td>4. By-pass of antibiotic-sensitive step by</td>
<td></td>
</tr>
<tr>
<td>duplication of the target site, the second version being resistant to drug action</td>
<td></td>
</tr>
<tr>
<td>5. Decrease in the cell’s metabolic requirement for an enzyme/pathway</td>
<td>nitrofurans</td>
</tr>
</tbody>
</table>

*Based on Russell & Chopra (1990). Further information concerning the mechanisms of resistance outlined above can be found in the following: Davies & Smith (1978); Gale et al. (1981); Foster (1983); Malouin & Bynan (1986); Chopra (1988, 1992); Nikaido (1989); Spratt (1989); Bugg et al., (1991a,b); Amyes & Gemmell (1992); Chopra et al. (1992); Levy (1992).

The success achieved with ß-lactamase inhibitors raises the question of whether an inhibitor approach might also be used to counter other bacterial resistance mechanisms. In principle, inhibition of other bacterial proteins which directly or indirectly mediate antibiotic resistance would lead to a situation similar to that achieved with a ß-lactamase inhibitor, in that the combination of a specific inhibitor with an antibiotic would restore the latter drug’s activity against a resistant organism. This approach might be applied to other enzymes which directly mediate the inactivation of non-ß-lactam antibiotics (e.g. the aminoglycosides, chloramphenicol, fosfomycin and the macrolides), to proteins which expel antibiotics from the cell (e.g. the tetracyclines, quinolones and macrolides) or to enzymes which confer antibiotic resistance by modification of ribosomal target sites (e.g. the macrolides and tetracyclines) (Table 1). Although the possibility of inhibiting enzymes, other than ß-lactamases, which are responsible for antibiotic resistance has been suggested previously (Allen, 1985; Labia et al., 1990), no systematic attempt has yet been made to evaluate this strategy in detail. This review examines the potential for extending the inhibitor approach, which has been applied so effectively to ß-lactamases, to other bacterial resistance mechanisms.

ß-Lactams

In clinical isolates, resistance to ß-lactam antibiotics results predominantly from the expression of ß-lactamases (Figure 1(a)). The importance of these enzymes has therefore ensured that the discovery and development of ß-lactamase inhibitors has been, in recent years, a major objective for several research groups. Since the philosophy behind the search for other inhibitors of resistance mechanisms essentially stems from the success achieved with the ß-lactamases, it is appropriate to consider the present status of these enzymes and their inhibitors.
Bacterial resistance mechanisms as therapeutic targets

Figure 1. Hydrolysis of a penicillin by a β-lactamase (a), and β-lactamase inhibitors: cloxacillin (b); olivanic acid (c); clavulanic acid (d); sulbactam (e); tazobactam (f); and BRL 42715 (g).

Classification of β-lactamases

Currently, more than 100 types of β-lactamase have been described and these have been classified by Bush (1989a,b,c) into four major groups according to substrate and inhibition profiles, isoelectric mobility and molecular weight (Table II). Groups 1, 2 and 4 contain a serine residue at the active site of the enzyme, while the Group 3 β-lactamases have a metal ion at their active sites.

The Group 1 β-lactamases are produced by a wide range of aerobic Gram-negative bacilli (AGNB), including clinical isolates of Escherichia coli, Pseudomonas aeruginosa, Citrobacter spp. and Serratia spp. Many bacteria show inducible expression of these enzymes (Sanders, 1992; Bennett & Chopra, 1993) and are therefore resistant to a variety of β-lactams, although they retain susceptibility to some penicillins and cephalosporins (Table II). Certain mutations may arise in the induction system, resulting in constitutive, high-level β-lactamase production. These stably derepressed mutants are frequently isolated during therapy with third-generation cephalosporins (Dworzack et al., 1987; Follath et al., 1987). Most of the Group 1 β-lactamases are chromosomally-mediated and this has restricted transfer between different species. However, recent reports of plasmid-mediated Group 1 enzymes (Papanicolaou, Jacoby & Medeiros, 1990; Bauernfeind et al., 1992; Payne, Woodford & Amyes, 1992; Tzouvelekis et al., 1993) may herald the spread of resistance to other clinical isolates.

Most clinically important β-lactamases fall into Group 2 which has been further
Table II. A general classification scheme for β-lactamases

<table>
<thead>
<tr>
<th>Bush Group</th>
<th>Example enzymes</th>
<th>Molecular weight (kDa)</th>
<th>Inhibited by:</th>
<th>Relative rate of hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Clavulanate</td>
<td>EDTA</td>
<td>PeG</td>
</tr>
<tr>
<td>1</td>
<td>P99</td>
<td>32.0</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2a</td>
<td>AmpC</td>
<td>39.6</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>2b</td>
<td>PC1</td>
<td>28.8</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>2b'</td>
<td>TEM-1</td>
<td>28.9</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>2c</td>
<td>SHV-2</td>
<td>29.0</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>2c'</td>
<td>SHV-1</td>
<td>28.8</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>2d</td>
<td>TEM-3</td>
<td>29.0</td>
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<td>no</td>
</tr>
<tr>
<td>2e</td>
<td>TEM-2</td>
<td>29.0</td>
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<td>no</td>
</tr>
<tr>
<td>3</td>
<td>PSE-1</td>
<td>28.5</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
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<td>PSE-2</td>
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<td>no</td>
</tr>
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<td>PSE-4</td>
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</tr>
<tr>
<td>3</td>
<td>OXA-1</td>
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<td>no</td>
</tr>
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<td>OXA-2</td>
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</tr>
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<td>PSE-1</td>
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<td>no</td>
</tr>
<tr>
<td>3</td>
<td>PSE-2</td>
<td>48.0</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>PSE-4</td>
<td>48.0</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>Bacteroides fragilis</td>
<td>32.0</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>L1</td>
<td>118.0</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>Bacillus cereus II</td>
<td>22.5</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>LCR-1</td>
<td>44.0</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>TRC-1</td>
<td>29.0</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

*Adapted from Bush (1989a,b,c).

+ + +, rapid; + +, intermediate; +, slow; ±, trace.

Abbreviations: PeG, benzylpenicillin; Car, carbenicillin; Cep, cephaloridine; Ctx, cefotaxime; Ctz, ceftazidime; Imi, imipenem.
divided into five major subgroups. The enzymes produced by *Staphylococcus aureus* and other Gram-positive bacteria have been placed into Group 2a. These enzymes favour penicillins as substrates and more than 80% of clinical isolates of *S. aureus* are resistant to penicillin as the result of the expression of this type of enzyme (Neu, 1985).

Group 2b includes broad-spectrum β-lactamases which are unable to hydrolyse third-generation cephalosporins. The most common of these is TEM-1 which is encoded on many transposons and plasmids and has consequently spread to a wide variety of bacterial species, making it the most prevalent β-lactamase in AGNB (Sanders & Sanders, 1992). SHV-1 is also included in this group and, like TEM-1, is isolated frequently from AGNB (Sanders & Sanders, 1992). The amino acid sequence of SHV-1 is closely homologous with that of the chromosomal β-lactamases of *Klebsiella* spp. from which it may have evolved (Barthelemey, Peduzzi & Labia, 1988).

Recently, there have been many reports of TEM- and SHV-type enzymes with extended spectra of activity which are capable of hydrolyzing third-generation cephalosporins. These enzymes differ from TEM-1 and SHV-1 by one to four residues around the active sites (Jacoby & Medeiros, 1991) and form Group 2b’ in the Bush scheme. This group consists of 20–30 different enzymes, mostly plasmid-encoded, and bacteria containing these enzymes are widely established in hospital populations (Payne & Amyes, 1991). The enzyme selected is often governed by the particular broad-spectrum cephalosporin prescribed, as illustrated by TEM-26, a 'ceftazidimase' produced by clinical strains isolated from neutropenic patients who had received ceftazidime as monotherapy (Naumovski et al., 1992). The majority of enzymes in Groups 2b and 2b’ are susceptible to the β-lactamase inhibitors clavulanic acid and tazobactam, but recently there have been reports of TEM-type enzymes which are resistant to these agents (Thomson & Amyes, 1992; Vedel et al., 1992). In the Bush scheme, these clavulanate-resistant variants fall into Group 4, a miscellaneous collection of inhibitor-resistant β-lactamases which have a serine residue at their active sites.

Most of the remaining enzymes placed into Group 2 (2c, 2d and 2e) are encountered less frequently. For example, OXA-3, OXA-5, OXA-6, CARB-3, CARB-4, PSE-2 and PSE-3 were identified in < 4% of *Pseudomonas* spp. isolated in different European countries (Philippon, 1988) and OXA-2, OXA-3, PSE-2, PSE-3 and PSE-4 were present in ≤ 1% of ampicillin-resistant strains of Enterobacteriaceae isolated in centres throughout the world (Amyes, 1988). Enzymes such as PSE-1 and PSE-4 are, however, particularly common in *Pseudomonas* spp. (Philippon, 1988). Some surveys have detected OXA-1 in up to 8% of ampicillin-resistant isolates of *E. coli* (Amyes, 1988), although, in most studies, the prevalence of OXA-1 has been much lower.

Metallo-β-lactamases (Bush Group 3) possess a metal ion (usually zinc) at their catalytic sites, in contrast to enzymes in Groups 1, 2 and 4 which have a serine residue at the active site. These enzymes occur in a number of clinically important bacteria, including *Bacteroides* spp., *Pseudomonas* spp., *Serratia* spp. and *Xanthomonas* spp. (Payne, 1993). They cause significant hydrolysis of imipenem and meropenem and many hydrolyse penicillins and cephalosporins as well.

NOR-1 is the only β-lactamase with serine at its active site for which significant hydrolysis of the carbapenems has been reported; the amino acid sequence of NOR-1 is 50% homologous with that of the chromosomal β-lactamase of *Klebsiella oxytoca* (Naas, Mariotte & Nordmann, 1993). Unlike the metallo-β-lactamases, this enzyme is not implicated in resistance to such a broad range of β-lactams and *E. coli* constructs producing it are susceptible to imipenem in the presence of clavulanic acid and to ceftazidime, cefotaxime and cefoxitin (Nordmann et al., 1993).
\( \beta \)-Lactamase inhibitors

The concept of inhibiting \( \beta \)-lactamases in order to potentiate \( \beta \)-lactam activity is not novel. As early as 1945, Perlstein & Liebmann (1945) used serum with anti-\( \beta \)-lactamase activity in an attempt to protect penicillin G and there have since been a number of reports citing proteases, miscellaneous organic compounds and \( \beta \)-lactamase-stable \( \beta \)-lactams as potential \( \beta \)-lactamase inhibitors (reviewed by Cole, 1979 and Knowles, 1985). The most effective of these were antistaphylococcal penicillins, such as cloxacillin (Figure 1(b)), which are competitive inhibitors of the Group 1 \( \beta \)-lactamases. However, the inhibitory activities of these agents were susceptible to being reduced, either by dilution or by the addition of a competing \( \beta \)-lactam, and their narrow spectra and the high concentrations required to inactivate the Group 1 \( \beta \)-lactamases precluded their use in the clinical setting.

Reversible inhibitors such as cloxacillin simply delay hydrolysis of the partner \( \beta \)-lactam and active enzyme is rapidly regenerated from the enzyme-inhibitor complex. Irreversible inhibitors are generally more effective since they are able to inactivate the enzyme for extended periods. Compounds which result in 'permanent' inactivation as a direct consequence of the catalytic activity of the \( \beta \)-lactamase are referred to as 'suicide inhibitors' and all current, clinically useful \( \beta \)-lactamase inhibitors fall into this group. The efficiency of a suicide inhibitor can be assessed by determining its stoichiometric ratio (the number of molecules required to inhibit one molecule of enzyme).

The first truly effective suicide inhibitors were the olivanic acids (Figure 1(c)), a group of carbapenems isolated from a strain of Streptomyces olivaceus (Brown et al., 1976). Most olivanic acid derivatives were potent antibacterial agents and \( \beta \)-lactamase inhibitors but, unfortunately, they were rapidly metabolized in the body (Basker, Boon & Hunter, 1980) and were never developed for clinical use.

The first suicide inhibitor to be used in clinical practice was clavulanic acid (Figure 1(d)) (Hunter et al., 1980; Reading & Farmer, 1981). This oxapenam, isolated in 1974 from a culture of Streptomyces clavuligerus, is an effective inhibitor of a broad range of clinically important \( \beta \)-lactamases (Table III). The mechanism of inhibition is complex (Charnas, Fisher & Knowles, 1978; Fisher, Charnas & Knowles, 1978), some of the clavulanic acid being hydrolysed by the enzyme, some binding transiently to the enzyme, with both enzyme and substrate being released unchanged, and some forming a stable, inactive complex with the enzyme. The stoichiometric ratio is 115:1 for inhibition of the TEM-2 \( \beta \)-lactamase (Fisher et al., 1978). Clavulanic acid is available commercially in combination with amoxycillin as both oral and parenteral formulations and with ticarcillin for parenteral use only.

Sublactam (Figure 1(e)) (English et al., 1978) is generally considered to be less potent than clavulamic acid (Table III). It does have slightly improved activity against the Group 1 \( \beta \)-lactamases (Hunter & Webb, 1980), but this is not clinically relevant. It is a particularly poor inhibitor of the very common TEM enzymes (Coleman et al., 1989) for which the stoichiometries are between 3100:1 (Sykes & Bush, 1982) and 7000:1 (Fisher et al., 1981). Sublactam is marketed in combination with ampicillin both for parenteral use and as an oral produrg.

Tazobactam (Figure 1(f)) (Aronoff et al., 1984) has inhibitory activity which is equivalent to that of clavulamic acid against Group 2 \( \beta \)-lactamases but superior against Group 1 enzymes (Table III), although, again, the Group 1 activity observed against isolated enzymes is insufficient to protect \( \beta \)-lactams against \( \beta \)-lactamase-producing
Table III. Comparative activities of various β-lactamase inhibitors

<table>
<thead>
<tr>
<th>Bush Group</th>
<th>β-Lactamase</th>
<th>cloxacillin</th>
<th>BRL 4550 (Olivanic acid)</th>
<th>clavulanic acid</th>
<th>tazobactam</th>
<th>sulbactam</th>
<th>BRL 42715</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Enterobacter cloacae</em> P99</td>
<td>0.043b</td>
<td>0.002b</td>
<td>&gt; 100c</td>
<td>1.54d</td>
<td>34.54d</td>
<td>&lt; 0.005c</td>
</tr>
<tr>
<td>2a</td>
<td><em>S. aureus</em></td>
<td>&gt; 100b</td>
<td>0.003b</td>
<td>0.16c</td>
<td>1.45c</td>
<td>6.68c</td>
<td>0.01c</td>
</tr>
<tr>
<td>2b</td>
<td>TEM-1</td>
<td>&gt; 100b</td>
<td>0.001b</td>
<td>0.25c</td>
<td>0.08c</td>
<td>7.68c</td>
<td>&lt; 0.005c</td>
</tr>
<tr>
<td>2c</td>
<td>PSE-4</td>
<td>—</td>
<td>—</td>
<td>0.07c</td>
<td>0.12c</td>
<td>1.45c</td>
<td>0.26c</td>
</tr>
<tr>
<td>2d</td>
<td>OXA-1</td>
<td>—</td>
<td>—</td>
<td>1.95c</td>
<td>2.28c</td>
<td>9.51c</td>
<td>&lt; 0.005c</td>
</tr>
<tr>
<td>2e</td>
<td><em>Proteus mirabilis</em></td>
<td>19b</td>
<td>0.001b</td>
<td>0.07c</td>
<td>0.10c</td>
<td>0.18c</td>
<td>0.02c</td>
</tr>
<tr>
<td>3</td>
<td><em>B. cereus</em> II</td>
<td>—</td>
<td>—</td>
<td>&gt; 100d</td>
<td>&gt; 100d</td>
<td>&gt; 100d</td>
<td>54.78c</td>
</tr>
</tbody>
</table>

*Amount of inhibitor required to inhibit 50% of enzyme activity. Determined after 5-min pre-incubation of inhibitor with enzyme at 37°C followed by addition of nitrocephin substrate.

*Coleman *et al.* (1989).
*Payne (unpublished data).
organisms (Aronoff et al., 1984; Jacobs et al., 1986). The stoichiometry of tazobactam with TEM-2 enzymes is 125:1 and with the Group 1 P99 enzyme, 50:1 (Bush et al., 1993). A parenteral formulation of tazobactam in combination with piperacillin has recently become available.

None of the inhibitors referred to above has clinically useful activity against Group 1 or Group 3 enzymes. The penem analogue, BRL 42715 (Figure 1(g)), has very potent activity against Group 1 and Group 2 β-lactamas in vitro (Coleman et al., 1989) and in vivo (Coleman, Griffin & Upshon, 1991). The stoichiometry of BRL 42715 with both TEM-2 and P99 enzymes is 1:1 (Farmer, T. H., Page, J., Payne, D. J. & Knowles, D. J. C., unpublished data). However, this derivative is no longer under development.

Other enzyme-mediated resistance factors

Aminoglycosides

Enzyme modification represents the most clinically significant basis of resistance to the aminoglycosides. The enzymes responsible are usually encoded on either plasmids or transposons, although chromosomally-mediated enzymes have also been described (Ames & Gemmell, 1992), and generally have acidic isoelectric points and molecular masses of 20–40 kDa. They are named according to the type of reaction which they catalyse—N-acetylases (AAC), O-phosphorylases (APH), O-nucleotidylases (ANT) and adenylases (AAD)—and can be further subdivided according to the position of the site of modification on the drug (Haas & Dowding, 1975). Up to six different resistance genes have been found in a single strain (Shaw et al., 1991) and more than one reaction type can be catalysed by each enzyme, as exemplified by the bifunctional AAC(6')/APH(3′') found in S. aureus and Staphylococcus epidermidis (Ubukata et al., 1984). Most aminoglycosides are susceptible to modification by more than one enzyme and this usually involves multiple mechanisms (Phillips & Shannon, 1984; Ames & Gemmell, 1992). In addition, an enzyme may have a broad substrate range, a situation which can promote the development of resistance to a drug to which an organism has not previously been exposed.

The origin of aminoglycoside-modifying enzymes is uncertain. Some appear to have originated in bacteria which produce aminoglycosides, presumably as a self-protection mechanism, and spread from them to non-aminoglycoside-producing strains. Examples of genes encoding aminoglycoside-modifying enzymes include aph(3')-Va and aac(3)-Ila (Shaw et al., 1993). Piepersberg et al. (1988) speculated that some resistance genes may have arisen from bacterial genes involved in cellular metabolism. The selective pressure caused by exposure to aminoglycosides might favour mutations which increase gene expression, thereby leading to aminoglycoside resistance; however, the role which such genes play in normal cellular metabolism remains unclear. An example of this mechanism involves the aac(6')-Ic gene which is carried by all strains of Serratia marcescens (Shaw et al., 1992). In aminoglycoside-susceptible isolates, this gene remains transcriptionally silent, although its presence may explain how strains of Serratia spp. become resistant so rapidly after exposure to these antibiotics (Shaw et al., 1992). The majority of other aminoglycoside resistance genes are expressed constitutively (Shaw et al., 1993).

There is conflicting evidence concerning the precise location of aminoglycoside-modifying enzymes in the bacterial cell. Some, such as AAC(3)-IVa, appear to be intracellular (Brau & Piepersberg, 1985) and may be associated with the cytoplasmic
membrane (Dickie, Bryan & Pickard, 1978). In this case, it is believed that the balance between aminoglycoside uptake and modification determines resistance. If the rate of uptake exceeds the rate of modification, then cell death may occur even if a modifying enzyme is present. Resistance develops if the drug is modified before it has the opportunity to accumulate within the cell and exert its deleterious effects. Some deduced enzyme sequences have a consensus signal sequence, indicating that the enzyme might be located in the periplasmic space (Shaw et al., 1993). Interestingly, when the signal sequence from the AmpC β-lactamase was fused to the aac(6')-Ia gene which normally lacks a signal sequence, the aminoglycoside susceptibility of E. coli containing the hybrid protein was eight-fold lower than that of strains carrying the native enzyme (Shaw et al., 1993). This may have been due to an increased efficiency of removal of the aminoglycoside when the modifying enzyme was located in the periplasmic space. This being the case, it is surprising that this mechanism has not evolved naturally under the pressure of intense aminoglycoside use.

The pattern of aminoglycoside resistance is complicated, especially since the combined effects of enzyme modifications and mutations which lead to impaired drug uptake can produce high-level resistance to some agents (Perlin & Lerner, 1986). Clearly, identifying a single inhibitor of aminoglycoside resistance which would encompass all aminoglycoside-modifying enzymes would be a formidable challenge as all clinically important aminoglycosides can be modified by at least two distinct mechanisms (Figure 2); for example, streptomycin can be both adenylated and phosphorylated, but it cannot be acetylated. Either a bifunctional inhibitor or a combination of two inhibitors would probably be required to provide optimal protection against modification. Inhibitors and non-substrates of particular modifying enzymes are known (Davies, 1983), but the field is not nearly as well developed as that for the β-lactamase inhibitors.

Chloramphenicol

In 1955, resistance to chloramphenicol was first detected in epidemic strains of Shigella spp. (Shaw & Leslie, 1989). Miyamura (1964) later reported that the antibiotic was inactivated by chloramphenicol-resistant strains of E. coli and it was subsequently shown that inactivation is the result of acetylation by chloramphenicol acetyltransferase (CAT), with acetyl-CoA as the acyl donor (Foster, 1983; Shaw, 1983, 1984). This reaction leads to the conversion of chloramphenicol to 3-acetoxy chloramphenicol which is then converted to 1,3-diacetoxy chloramphenicol (Figure 3). The acetoxy derivatives of chloramphenicol fail to bind to the ribosomal target and are thereby rendered inactive.

CAT enzymes have been detected in many bacterial genera and can be classified into three distinct groups. The first includes enzymes identified in Enterobacteriaceae which are expressed constitutively on plasmids of 20 kb or more (Foster & Shaw, 1973). These can be subdivided into three groups—Types I, II and III—of which Type I is the most frequently occurring and the most widely studied; it is also implicated in fusidic acid resistance (Volker, Iida & Bickle, 1982). The second group comprises a family of inducible staphylococcal CATs (A, B, C and D) which are found on plasmids of 5 kb or less (Sands & Shaw, 1973). The third group includes a new CAT which is found on transposon Tn2424 and has no genetic similarity to any of the other CAT genes (Parent & Roy, 1992).
Figure 2. Aminoglycoside structures and targets of enzyme modification by adenylation (AAD), phosphorylation (APH) and acetylation (AAC). Streptomycin (a). Gentamicin isomers (b) (Cl: R, and R, = CH3; C2: R, and R, = H; C3: R, = H, R, = CH3).

X-ray diffraction studies have demonstrated that Type I and Type III CATs are trimers (Harding, Rowe & Shaw, 1987; Leslie, Moody & Shaw, 1988); similar studies of Type II CATs have been hindered by difficulties encountered in forming stable complexes with purified protein. The symmetrical arrangement of the subunits around the three-fold axis results in three subunit interfaces, each of which contributes to the active site where His195 plays a critical role in the catalytic modification of chloramphenicol. One subunit binds chloramphenicol and acetyl CoA while the other two subunits provide the catalytic imidazole (His195).

Several CAT genes (present in E. coli, Clostridium difficile, Clostridium perfringens, Campylobacter coli and Streptomyces avermitilis) have been sequenced and all show a high degree of similarity, especially around their active sites (Shaw & Leslie, 1991).
Bacterial resistance mechanisms as therapeutic targets

Figure 13. Inactivation of chloramphenicol (a) by chloramphenicol acetyltransferase, first to 3-acetoxy chloramphenicol (b) and then to 1,3-diaceetoxy chloramphenicol (c).

Thus, a single inhibitor of all CATs is feasible and, indeed, such compounds have been identified (Miyamura, Koizumi & Nakagawa, 1979), although their efficacies in clinical terms so far appear to be limited (Shaw, 1983).

**Fosfomycin**

Fosfomycin (phosphonomycin) (Figure 4) is a phosphoenolpyruvate analogue which irreversibly inhibits enolpyruvate transferase, an enzyme involved in the early stages of peptidoglycan synthesis. Fosfomycin enters the bacterial cell by active transport through the glycerophosphate uptake and hexose phosphate permease systems and most strains which are resistant by virtue of a chromosomal mutation have an impairment of one or both of these uptake mechanisms (Kahan et al., 1974). This form
of resistance is common in vitro but is less of a problem in clinical practice where plasmid-mediated resistance is encountered more frequently (Suarez & Mendoza, 1991). The plasmid-encoded resistance determinant fosA encodes a polypeptide of predicted size 15 kDa which possesses enzyme activity only as a dimer (Arca et al., 1990); this dimer catalyses the addition of fosfomycin to the tripeptide, glutathione, to form an inactive product (Figure 4). Recently, a second gene, fosB, encoding a product of molecular weight 16.3 kDa, has been described. The two genes show 35% sequence homology, an indication of a common progenitor (Zilhao & Courvalin, 1990), but the effects of the FosB protein on fosfomycin are not yet certain. Plasmid-encoded fosfomycin resistance is spreading (Suarez & Mendoza, 1991).

Assuming that both FosA and FosB are functionally similar, a single inhibitory agent which is capable of inactivating both enzymes, with consequent protection of fosfomycin, is a realistic objective.

**Fusidic acid**

Many plasmids confer resistance to fusidic acid, but the determinant in plasmid R100 has been studied in most detail (Foster, 1983). There is now firm evidence that fusidic
Bacterial resistance mechanisms as therapeutic targets

Table IV. Macrolide, lincosamide and streptogramin-B resistance due to enzyme inactivation

<table>
<thead>
<tr>
<th>Host</th>
<th>Resistance phenotype</th>
<th>Enzyme</th>
<th>Plasmid localization</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>S₄</td>
<td>acetyltransferase</td>
<td>pIP524</td>
<td>ND</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>S₂</td>
<td>hydrolase</td>
<td>pIP524</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Staphylococcus hominis</strong></td>
<td>L</td>
<td>nucleotidyltransferase</td>
<td>pIP856</td>
<td>linA</td>
</tr>
<tr>
<td><strong>Staphylococcus urbis</strong></td>
<td>L</td>
<td>nucleotidyltransferase</td>
<td>pIP860</td>
<td>linA</td>
</tr>
<tr>
<td><strong>Streptomyces spp.</strong></td>
<td>ND</td>
<td>nucleotidyltransferase</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Streptomyces spp.</strong></td>
<td>L</td>
<td>phosphotransferase</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Streptomyces spp.</strong></td>
<td>M</td>
<td>macrolide glycosyl</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Streptomyces spp.</strong></td>
<td>M</td>
<td>transferase (MGT)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Pseudomonas spp.</strong></td>
<td>M</td>
<td>esterase I</td>
<td>pIP1100</td>
<td>ereA</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>M</td>
<td>esterase II</td>
<td>pIP527</td>
<td>ereB</td>
</tr>
<tr>
<td><strong>Streptomyces spp.</strong></td>
<td>M</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>


†M, macrolides; L, lincosamides; S₄, streptogramin-A; S₂, streptogramin-B.

Acid resistance is mediated by a Type I CAT enzyme (Volker *et al.*, 1982) and that a separate fus gene product is not translated from an initiation codon internal to the cat gene. Fusidic acid is neither inactivated nor modified by the enzyme which appears to have a binding domain capable of sequestering a variety of unrelated aromatic compounds (Foster, 1983; Proctor, McKew & Rownd, 1983). It is not known whether inhibitors of the CAT acetylation of chloramphenicol can also prevent the CAT-mediated sequestration of fusidic acid.

Macrolides, lincosamides and streptogramins

The macrolides, lincosamides and streptogramins (the so-called MLS group of antibiotics) are a collection of structurally different but functionally similar agents which are inactivated by a range of different enzymes. Table IV lists the most important classes of MLS-inactivating enzymes and the resistance phenotypes attributable to them.

Although macrolide-modifying properties have been detected in actinomycetes and *Lactobacillus* spp. of animal origin, neither the mechanism of drug inactivation nor the corresponding genes have been characterized to date. Erythromycin is readily inactivated by the plasmid-encoded erythromycin esterases types I and II of *E. coli*. These enzymes catalyse the hydrolysis of the lactone ring of erythromycin (Figure 5) and oleandomycin. The two esterase genes, *ereA* and *ereB*, have been identified in a number of other AGNB, including *Klebsiella* spp., *Proteus* spp. and *Enterobacter* spp. (Mabilat & Courvalin, 1988), but there have been no reports of esterase activity in actinomycetes or other Gram-positive organisms.

Many macrolide-inactivating enzymes have been detected in clinical isolates. In staphylococci, for example, enzymes of three different types (acyltransferases, nucleotidyltransferases and hydrolases) are capable of inactivating macrolides (Arthur, Brisson-Noel & Courvalin, 1987). Clearly, a single inhibitor is unlikely to be of value against such a broad range of mechanisms and a combination of inhibitors might be more appropriate. On the other hand, the clinical significance of these enzymes is
Antibiotic efflux mechanisms as targets of inhibition

The active efflux of antimicrobial agents from the bacterial cell has not previously been considered amongst the more prominent mechanisms to which resistance is attributed (Chopra, 1992; Levy, 1992). More recently, however, there has been increasing awareness of the role of efflux mechanisms as mediators of resistance (Ross et al., 1990; Chopra, 1992; Levy, 1992), particularly to the quinolones, tetracyclines and macrolides.

Functionally, bacterial efflux mechanisms can be categorized into two broad groups according to the nature of energy coupling to transport (efflux). The first group comprises primary pumps (efflux ATPases), while the second includes all secondary cation/proton symports for which the energy source is the electrochemical proton gradient (PMF) (Table V).
Bacterial resistance mechanisms as therapeutic targets

Table V. Bacterial efflux systems for antibiotics

<table>
<thead>
<tr>
<th>Gene</th>
<th>Substrate transported</th>
<th>Energy source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>msrA</td>
<td>macrolides</td>
<td>ATP</td>
<td>Ross et al. (1990)</td>
</tr>
<tr>
<td>tet</td>
<td>tetracyclines</td>
<td>PMF</td>
<td>Chopra et al. (1992)</td>
</tr>
<tr>
<td>bmr</td>
<td>Rh, Eb, Cm, Pur, Fq</td>
<td>PMF</td>
<td>Neyfakh et al. (1991)</td>
</tr>
<tr>
<td>norA</td>
<td>Rh, Eb, Cm, Pur, Fq</td>
<td>PMF</td>
<td>Yoshida et al. (1990)</td>
</tr>
</tbody>
</table>

Rh, Rhodamine; Eb, ethidium bromide; Cm, chloramphenicol; Pur, puromycin; Fq, fluoroquinolones.

Quinolones

The active efflux of hydrophilic quinolones such as ciprofloxacin and norfloxacin was initially recognized in strains of *E. coli* in which it was shown to be PMF-mediated. However, while efflux was expressed phenotypically, it was apparently not at a sufficiently high level to confer resistance to these agents. A similar active efflux mechanism has been reported in quinolone-resistant strains of *P. aeruginosa*, although efflux was not the only resistance mechanism exhibited by these organisms. PMF-mediated efflux has also been demonstrated to confer quinolone resistance in clinical isolates of *Proteus vulgaris* and *S. aureus* (Chopra, 1992; Levy, 1992). In *S. aureus*, this efflux pump is NorA. The amino acid sequence of NorA shows significant homology with the tetracycline and bacterial multidrug resistance (Bmr) efflux proteins. Like other transport proteins, NorA is hydrophilic, with 12 putative transmembrane domains (Neyfakh, Borsch & Kaatz, 1993).

The clinical significance of this resistance mechanism is unclear but an endogenous efflux system is undoubtedly present in some species and excessive prescribing of the quinolones may provide the selective pressure necessary to allow mutation to an efficient quinolone efflux system. Equally worrying, norA has recently been identified on a plasmid, a situation which could promote the spread of this resistance mechanism (Tanaka et al., 1991).

Tetracyclines

Tetracycline efflux is also PMF-driven, with tetracycline being transported as a monocationic magnesium chelate (Kaneko, Yamaguchi & Sawai, 1985). Seven tetracycline efflux proteins have been sequenced and all have been predicted by hydropathy analysis to have 12 membrane-spanning regions (Eckert & Beck, 1989; Sheridan & Chopra, 1991). Critical residues are dispersed across these domains. In particular, conservation of serine and aspartate residues at positions 65 and 66 respectively appears to be crucial for protein function (Chopra, 1986; Yamaguchi et al., 1990; McNicholas, Chopra & Rothstein, 1992). This motif has been found in other transport proteins (Sheridan & Chopra, 1991) and may therefore be involved in a gating mechanism rather than substrate recognition (Yamaguchi et al., 1990). Tetracycline-producing *Streptomyces* spp. have efflux genes for tetracycline and it is possible that resistance to this antibiotic in pathogenic bacteria arose by gene transfer.
On the other hand, the low sequence homology between these two gene families makes this unlikely (Chopra, Hawkey & Hinton, 1992).

**Macrolides**

A new resistance phenotype (MS) has recently been detected in clinical isolates of *S. aureus* (Jenssen et al., 1987); these strains had become resistant to the macrolides and streptogramins but had retained susceptibility to the lincomycins. The gene responsible, *msrA*, has since been sequenced and shown to be unrelated to the methylase genes (Ross et al., 1989, 1990). The *msrA* gene codes for a 55.9-kDa protein containing two ATP binding sites and resembles the multidrug-resistant P-glycoprotein (Mdr) of tumour cells which mediates resistance to a number of structurally unrelated antibiotics. Ross et al. (1990) have proposed that MsrA confers resistance through a drug efflux mechanism and two groups of investigators have provided support for this theory. Schoner et al. (1992) demonstrated that macrolide resistance genes in some strains of actinomycetes code for proteins that possess sequences which are very similar to those of other ATP-dependent transport proteins, the N- and C-terminals of which contain highly conserved regions resembling ATP binding domains. Goldman & Cappobianco (1990) have described the role of an energy-dependent efflux pump in a macrolide-resistant clinical isolate of *S. epidermidis*. This strain contained both a plasmid, pNE24, which conferred resistance to 14- and 15-membered macrolides and an energy-dependent macrolide efflux mechanism which maintained intracellular antibiotic concentrations below those necessary for binding to the ribosomes. It was observed that energy-incompetent *S. epidermidis* cells containing pNE24 accumulated the antibiotic at the same rate as energy-competent cells lacking this plasmid.

**Inhibition of efflux mechanisms**

Mammalian cells selected in culture for their resistance to lipophilic cytotoxic drugs usually demonstrate resistance not only to the selecting agent but also to other apparently unrelated toxic agents. This phenomenon, termed multidrug resistance (MDR), is due to the active efflux of drugs from the cells. The efflux is mediated by an efflux ATPase (P-glycoprotein) which is encoded by *mdr1* (Endicott & Ling, 1989). Calcium antagonists (e.g. verapamil), calmodulin inhibitors (e.g. trifluoperazine) and other agents (e.g. reserpine) inhibit P-glycoprotein-mediated efflux and restore susceptibility to the multidrug-resistant cells. Although the precise mechanism of action of these efflux pump inhibitors is unknown, a direct effect on P-glycoprotein is considered likely (Cornwell et al., 1986; Safa et al., 1986, 1987; Cornwell, Pastan & Gottesman, 1987).

Levy (1992) has suggested that the identification of inhibitors which could specifically block bacterial efflux mechanisms would represent a significant advance in the search for strategies to counter antibiotic resistance. Although such a strategy has not yet been widely explored, there are reports that the Mdr1 inhibitors, reserpine and verapamil, also inhibit NorA-mediated efflux in bacteria (Neyfakh, Bidnenko & Chen, 1991; Neyfakh et al., 1993), and Nelson et al. (1993) have recently described a series of tetracycline analogues which inhibit the Tet A(B)-mediated efflux of tetracyclines. Furthermore, sensitive screening assays which utilize transcriptional fusions between the *lacZ* reporter gene and the regulatory elements governing inducible synthesis of
tetracycline efflux proteins have been developed for the purpose of detecting inhibitors of tetracycline efflux pumps (Rothstein et al., 1993). As discussed below, these screening techniques have identified a number of naturally occurring efflux pump inhibitors (Rothstein et al., 1993).

The molecular basis by which reserpine and verapamil inhibit bacterial efflux systems is unclear, especially since NorA shows no sequence homology with Mdr1 (Neyfakh et al., 1991) and differs from Mdr1 with respect to the nature of the energy coupling required to drive the efflux mechanism (Table V). The possibility that reserpine and verapamil exert non-specific effects on bacterial membrane permeability, leading to secondary inhibition of NorA, cannot be ruled out at this stage, particularly in the light of a recent report of the non-specific effects of verapamil on the membrane permeability of mammalian cells (Hunter, Hirst & Simmons, 1991). The inhibitors of tetracycline efflux described by Nelson et al. (1993) comprise a series of 13-(arylthio) and 13-(arylthio) derivatives of 5-hydroxy-6-deoxotetracycline. The most active members exhibited IC₅₀ of 0.3 μM against the Tet A(B) efflux pump in everted E. coli membrane vesicles, but the antibacterial properties of these inhibitors in combination with other tetracyclines against resistant strains have not yet been described (Nelson et al., 1993). As noted above, genetically constructed tet-lacZ fusion strains have recently been used to detect naturally occurring tetracycline efflux pump inhibitors (Rothstein et al., 1993); most of these inhibitors (e.g. nocardamine) were identified as ferric ion chelators (Rothstein et al., 1993). The molecular basis by which ferric ion chelators inhibit tetracycline efflux is unclear, but since tetracyclines chelate trivalent cations, including ferric ions (Weinberg, 1957; Mitscher et al., 1969), it is possible that the formation of a tetracycline-ferric ion complex is essential during the energized efflux of the antibiotic. Removal of the free iron by chelators such as nocardamine would, therefore, inhibit the efflux process. Unfortunately, iron chelators are unlikely to be suitable in clinical practice because of their potential to interfere with iron metabolism in the host. Nevertheless, a continued search for inhibitors of antibiotic efflux mechanisms is likely to be rewarding in view of concerns that this type of resistance may become more prevalent amongst clinical isolates in the future (Chopra, 1992).

### Enzyme modification of target sites

**Macrolides, lincosamides and streptogramins**

The best studied and most frequently encountered mechanism of resistance to the macrolides is target site modification, whereby bacterial RNA is methylated, causing reduced affinity between the antibiotic and its binding site on the ribosome (Weisblum, 1985; Goldman & Kadam, 1989). The enzymes which mediate this modification, the rRNA methylases, are either inducible or constitutive, have been identified in a wide range of bacteria and have been classified into a number of groups (Weisblum, 1985; Eady, Ross & Cove, 1990). This mechanism confers resistance to a variety of macrolides, lincosamides and streptogramin-B antibiotics (giving rise to the so-called MLSₐ phenotype) and accounts for most of the resistance in clinical isolates.

A family of inducible enzymes is known to catalyse the mono- or dimethylation of the N₆ amino group on adenine2058 in a highly conserved region of 23S rRNA which may be involved directly in the formation of the peptidyl transferase centre.
Furthermore, organisms containing ribosomes in which adenine$^{16N}$ has been dimethylated show higher levels of macrolide resistance than do organisms with monomethylated ribosomes (Zalacain & Cundliffe, 1990).

Erythromycin is the most potent inducer of RNA methylases in staphylococci, whereas macrolides, lincosamides and streptogramins are all able to induce these enzymes in streptococci. Genes encoding rRNA methylases are variously located on transposons (Murphy, 1985), plasmids (Weisblum, 1985) and chromosomes (Arthur et al., 1987). It has been suggested that rRNA methylases originated in strains of actinomycetes which produce the antibiotics in order to protect their ribosomes and that the genetic determinants of these enzymes subsequently spread to other genera of bacteria (Novick & Murphy, 1985).

Since there are several classes of RNA methylase it is unlikely that a single inhibitor could be found which would be effective against the full range of enzymes. A better strategy might be to develop macrolide analogues with greater affinities for methylated RNA. Research along these lines has already yielded 6-O-methyl-11,12-cyclic carboxylate analogues of erythromycin which are able to regain access to binding sites on the 50S subunit (Goldman & Kadam, 1989).

**Tetracyclines**

Burdett (1986) was the first to observe that some tetracycline resistance determinants (now identified as class M or O) do not mediate the decreased uptake of tetracycline through an efflux-based mechanism. He also showed that protein synthesis in extracts derived from organisms containing Tet M was resistant to inhibition by tetracycline (Burdett, 1986). Additional studies have since been conducted to establish the molecular basis of Tet M- and Tet O-mediated resistance to tetracyclines. Manavathu et al. (1990) transferred the Campylobacter jejuni tet(O) determinant into E. coli and demonstrated that soluble cell extracts containing the tet(O) gene product conferred resistance to ribosomes which were originally obtained from susceptible strains. This suggested that a cytoplasmic protein was acting in conjunction with the ribosomes to render them insensitive to the action of tetracyclines. Sequence analysis of tet(M) and tet(O) has revealed that the N-terminal regions of the predicted proteins share considerable homology with those of various translational elongation factors, including EF-Tu from E. coli (Sanchez-Pescador et al., 1988; Manavathu et al., 1990). Aminoacyl-tRNA molecules are delivered to the ribosomes as ternary complexes with elongation factor Tu (Chopra, 1985), suggesting that the Tet M and Tet O proteins might function as tetracycline-resistant analogues of EF-Tu, i.e. they are capable of delivering aminoacyl-tRNA to the ribosomes regardless of whether tetracyclines are bound to their target sites (Sanchez-Pescador et al., 1988; Manavathu et al., 1990). However, Manavathu et al. (1990) pointed out two inconsistencies in this theory. Firstly, EF-Tu is an abundant protein (accounting for c. 10% of all E. coli protein), whereas Tet O, and probably Tet M, proteins are produced in such small quantities that they are unlikely to provide effective replacements for EF-Tu. Secondly, the critical Gly$^{18}$ residue which is essential for EF-Tu function (Jacquet & Parmeggiani, 1988) is an alanine residue in the Tet M and Tet O proteins (Sanchez-Pescador et al., 1988; Manavathu et al., 1990). It is possible, therefore, that the similarities between the Tet M and Tet O proteins and the elongation factors simply reflect a common ability to associate with ribosomes rather
than a direct functional similarity. Indeed, it has been suggested that the tet(M) and tet(O) gene products act in a catalytic manner to modify the tetracycline binding site on the 30S ribosomal subunit (Manavathu et al., 1990). This might involve methylation of either ribosomal RNA or ribosomal protein, thereby rendering the ribosomes insensitive to the tetracyclines.

The functions of Tet M and Tet O at the molecular level have not yet been fully elucidated. Efforts to identify an inhibitor will therefore need to address both the substrate specificities of these putative enzymes and the potential for selective inhibition of prokaryotic systems. Unfortunately, there is currently inadequate information to allow either of these issues to be resolved (Amaro & Jerez, 1984; Cunningham et al., 1990; Wool et al., 1990).

Conclusions

The problems associated with increasing antibiotic resistance in both community- and hospital-acquired bacterial pathogens have recently received much attention (Shlaes, Levy & Archer, 1991; Cohen, 1992; Neu, 1992; Silver & Bostian, 1993). In the context of developing new strategies to overcome resistance, there are a number of possibilities:

(a) The development of analogues of existing drugs which are able to bind to refractory target sites e.g. modified macrolides (Fernandes et al., 1989; Goldman & Kadam, 1989).

(b) The development of analogues of existing drugs which are able to circumvent efflux or degradative mechanisms e.g. the tetracycline analogue, minocycline, which is not recognized by the tetracycline efflux pump (Tet K) predominant in staphylococci (Chopra et al., 1992; Guay & Rothstein, 1993) and the carbapenems, meropenem and imipenem, which are stable to hydrolysis by the majority of β-lactamases (Sanders et al., 1989; Yang & Livermore, 1989).

(c) The identification of novel agents with unique modes of action which are not susceptible to existing resistance mechanisms; in terms of selecting targets for screening purposes, it is evident that a number of potential molecular targets have not yet been fully exploited (Allen, 1985; Sutcliffe, 1988).

(d) The identification and development of inhibitors of resistance mechanisms (the subject of this review) e.g. β-lactam/β-lactamase inhibitor combinations.

Pursuit of the various concepts described above may lead to the introduction of compounds which will facilitate effective therapy of infections caused by organisms resistant to currently available antibiotics. With respect to the identification and development of inhibitors of resistance mechanisms, it will be evident from this review that the successful implementation of this approach has so far been limited to the β-lactams. The discovery and exploitation of inhibitors which can be used in conjunction with other groups of antibiotics may, in the future, provide a solution to the ongoing problem of antibiotic resistance.

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