The Chemical Synthesis of Peptides with Biological Importance

Lu Jiang

Doctor of Philosophy

University of Edinburgh

1996

This thesis is submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Edinburgh. Unless otherwise stated, the work described is original and has not been previously submitted in whole or in part, for any degree at this, or any other university.

Lu Jiang

University of Edinburgh

March 1996

To Yongji Wang, Daniel, Yijian Wang

and

my parents

ACKNOWLEDGEMENTS

I would like to thank Prof. Robert Ramage FRS for the provision of research facilities and for his advice and constant encouragement throughout the course of this research.

Special thanks to Mr. Kevin Shaw for his very substantial input into the technical aspects of the peptide synthesis described herein and to Mr. Brian Whigham for amino acid analysis and mass spectroscopy. I would also like to thank those involved in the departmental analytical and spectroscopic services for their work.

I would like to thank Dr. Kirstie Urquhart, Miss Nicola Robertson and Miss Gail H. Morton for their help in the preparation of this thesis and also Dr. Chandralal M. Hewage for his efficient co-operation in the NMR studies of the ET analogues.

I would like to thank Drs. Annette. M. Doherty, Danielle Leonard, Kathy Welch, Kyunghye Ahn, Micheal Rafferty, David Dooley, Sally Stoehr, and Joann Geer at Parke Davis Pharmaceutical Research, Ann Arbor, Michigan, USA for their co-operation in biological assay and also Drs. Sharon M. Kelly and Nicholas C. Price at Stirling University for their help in the determination and analysis of the CD spectra.

I would also like to thank Mrs. G. Gorden and the postgraduate committee of Edinburgh University for their understanding and support in dealing with my particular situation.

I would like to express my appreciation for the funding provided by Parke Davis Pharmaceutical Research at Ann Arbor, Michigan, USA.

Finally I would like to thank all my friends and colleges both past and present for their help and encouragement and also for making my time in lab 29 so unforgettable and enjoyable.

ABSTRACT

The synthesis and properties of a new coupling reagent, 1-hydroxyl-4-ethoxycarbonyl-1,2,3-triazole (HOCt), were investigated. An efficient synthetic protocol with a high and reproducible yield was established. This new reagent was applied successfully to the solid phase synthesis of difficult and large peptides.

Endothelins(ETs) are a novel family of vasoconstrictor peptides, which have variety of biological activities and are involved in many cardiovascular disease states. The structure activity relationship (SAR) studies of endothelin-1 (ET-1) and its linear analogues were carried out. The results indicated that some residues of ET-1 were of particular biological significance and several highly selective and potent ET_B receptor agonists were reported. Conformational studies indicated that a high percentage of helical structure is present in most of the linear analogues studied.

ET-1 is cleaved from the biologically inactive BigET-1 by ECE *in vivo*. SAR studies of the BigET-1 analogues have also been carried out and residues 16-34 were found to be important for ECE recognition and several 16-34 analogues were found to be potent ECE inhibitors.

ω-Agatoxins have been valuable tools in defining various classes of Ca^{2+} channels. Among these, ω-agatoxin IIIA was of particular importance since it was the only known reagent that blocks both L-type and N-type Ca^{2+} channels. Due to limited natural sources, the chemical synthesis of this peptide was of particular interest. An attempt was made to fold this peptide into biologically active form, which was a difficult task due to the presence of six disulphide bridges. The synthetic peptide was found to be heterogeneous in conformation and the biological assay gave an IC_{50} of 1μM at the R-type Ca^{2+} channel.

ABBREVIATIONS

AAR_B	arachidonic acid release at ET_B receptor
Acm	acetamidomethyl
AcOH	acetic acid
AgOTf	silver trifluromethanesulphonate
BigET(BET)	Big endothelin
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
BOI	2-(1H-benzotriazole-1-yl)-1,3-dimethylimidazolonium hexafluorophosphate
BOP	benzotriazole-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate
tBu	*tert*-butyl
Bum	*tert*-butyloxymethyl
CD	circular dichroism
CHO	Chinese hemster ovary
COI	2-(1-H-4-ethoxycarbonyl-1,2,3-triazole-yl)-1,3-dimethylimidazolonium hexafluorophosphate
CTF	C-terminal fragment
DCCI	N,N'-dicyclohexylcarbodiimide
DCCU	N,N'-dicyclohexylurea
DCM	dichloromethane
DIC	N,N'-diisopropylcarbodiimde
DIU	N,N'-diisopropylurea
DIEA	N,N'-diisopropylethylamine
DMAP	4-dimthylaminopyridine
DMF	N,N'-dimethylformamide
DMSO	dimethyl sulphoxide
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
ECE	endothelin converting enzyme
EDCF	endothelium derived constractor factor
EDRF	endothelium derived relaxing factor
EDT	ethane-1,2-dithiol
EDTA	ethylenediaminetetraacetic acid
EEDQ	1-ethyloxycarbonyl-2-ethyloxy-1,2-dihydroquinoline
ET	endothelin
FmocOSu	N-(9-fluorenylmethoxycarbonyloxy)succinimide
Fmoc	9-fluorenylmethoxycarbonyloxy
Gdm.HCl	guanidinium hydrochloride
GSH	glutathione reduced
GSSG	glutathione oxidized
HATU	2-(1H-7-azabenzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluoroborate
HBTU	2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoroborate
HCTU	2-(1-H-4-ethoxycarbonyl-1,2,3-triazole-yl)-1,1,3,3-tetramethyluronium hexafluoroborate

HMPA	hexamethylphosphotriamide
HOAt	7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HOCt	1-hydroxyl-4-ethoxycarbonyl-1,2,3-triazole
HPLC	high performance liquid chromatography
IIDQ	1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline
NGF	neural growth factor
NMR	nuclear magnetic resonance
NOE	nuclear overhauser enhancement
NOESY	nuclear overhauser enhancement spectroscopy
PhAcm	phenylaceamidomethyl
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
PyBroP	bromo-tris-pyrrolidino-phosphonium hexafluorophosphate
RP-HPLC	reverse phase HPLC
SAR	structure activity relationship
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPPS	solid phase peptide synthesis
StBu	tert-butylsulphenyl
RT	room temperature
SRTX	sarafotoxin
Tbfmoc	tetrabenzo[a,c,g,i]fluorenyl-17-methoxycarbonyl
TBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TCTU	2-(1-H-4-ethoxycarbonyl-1,2,3-triazole-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TFFH	tetramethylfluoroformamidinium hexafluorophosphate
TFMSA	trifloromethanesulfonic acid
THF	tetrahydrofuran
TIS	triisopropylsilane
TLC	thin layer chromatography
TMSBr	trimethylsilyl bromide
TNTU	2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate
TRIS	tris(hydroxymethyl)aminomethane
Trt	triphenylmethyl
TSTU	O-(N-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate
UV	ultra violet
VIC	vasoactive instinal contractor
VSM	vascular smooth muscle
Z	benzyloxycarbonyl

AMINO ACIDS

Amino acid	3 letter code	1 letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
α-amino isobutyric acid	Aib	-
γ-methylleucine	Mle	-
nor-leucine	Nle	-
penicilliamine	Pen	-

CONTENTS

CHAPTER 1
INTRODUCTION TO PEPTIDE SYNTHESIS AND PURIFICATION

1.1 The history of peptide synthesis

1.1.1 Introduction

The history of peptide chemistry can be traced back to the turn of the century when Curtius made the first amide bond between two α-amino acids[1] and Fischer made the first free dipeptide.[2] Early attempts to synthesise a variety of small peptide substrates for the recently discovered proteolytic enzymes led to the development of the first removable protecting group[3]. The discovery of more complex, naturally occurring, biologically active peptides stimulated an explosion of synthesis and methodology development that lasted through the 1950s and 1960s, and which laid the foundation of modern peptide chemistry. However, traditional solution chemistry requires labour intensive purification and isolation of each intermediate compound. The difficulties experienced in the synthesis of even very small peptides stimulated a sustained effort towards improvements in the methodology of peptide synthesis. Techniques which allowed facile chain-building by simple, repetitive operations were definitely needed.

In the 1960s, Merrifield published his remarkable work of solid phase peptide synthesis (SPPS).[4] The rationale for the SPPS approach was simple. By attaching a growing peptide chain to an insoluble polymeric support, it was reasoned that the excess reagents and by-products from the coupling reactions could be removed by simple filtration and washing steps. This new method of peptide assembly was entirely compatible with some of the well developed chemistry of coupling and functional group protection in solution which already existed at that time. The simple, repetitive methodology also made the mechanisation and automation of the chain building process possible, thus greatly reducing the drudgery in execution, especially when

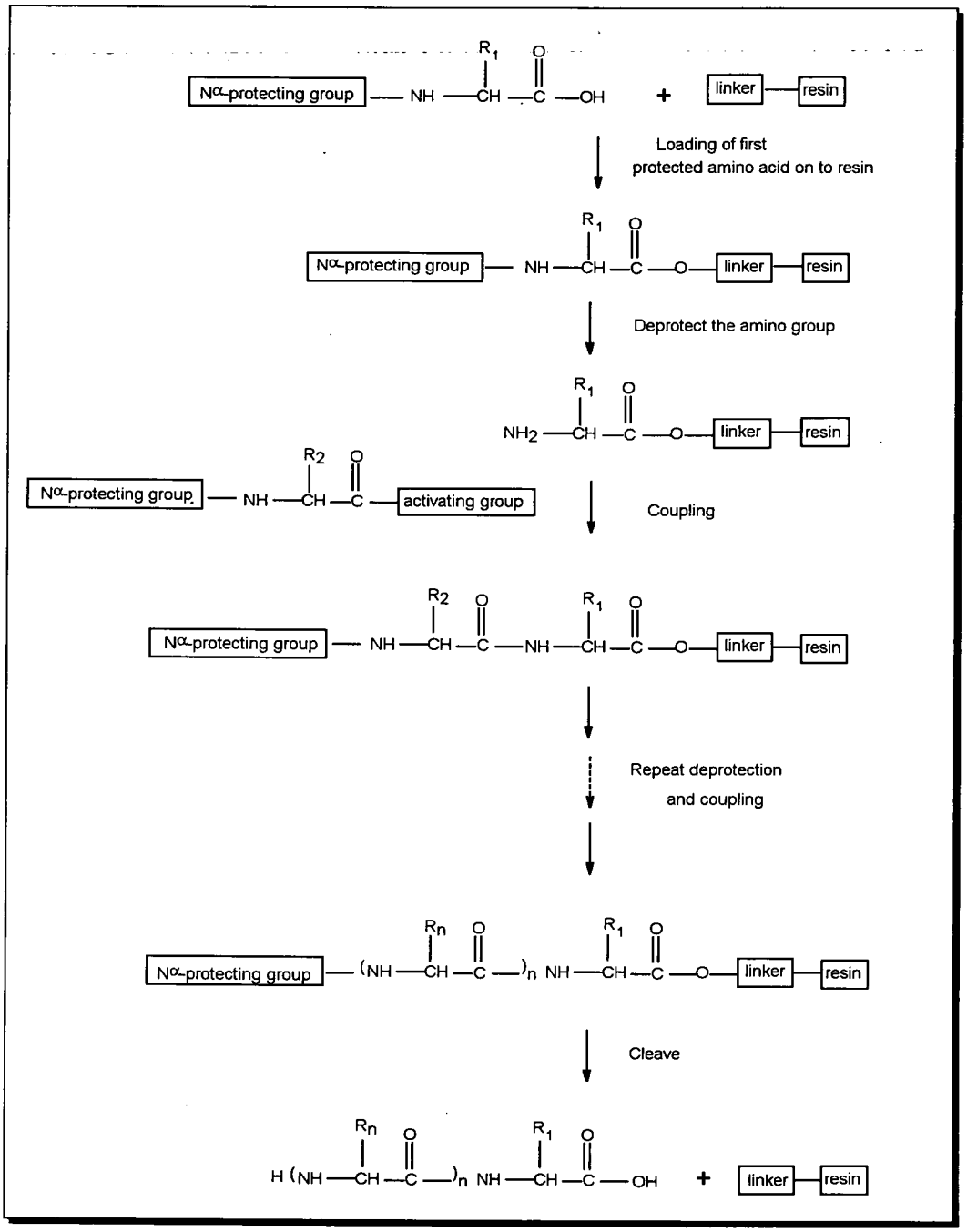
chains of 50 or more residues have to be constructed. The remarkable idea laid the foundation of modern peptide chemistry and it remains viable and alternative to the gene engineering technology for the synthesis of peptides.

Recently, there has been an upsurge in interest in protein chemistry, which, to a large extent, has been stimulated by the establishment of a pharmaceutical industry based on the production of proteins by genetic engineering. Molecular biology research has led to the availability of amino acid sequence data by cloning and cDNA sequencing of genes. In many cases, the proteins themselves have never been isolated and their properties are largely or completely unknown. Chemical synthesis of peptide fragments, or of the whole molecule, can be used to study the properties of such gene products and also help redesign known proteins as second-generation products for pharmaceutical use. Chemical synthesis has a variety of applications in research on biologically active peptides including proof of structure and demonstration that the appropriate biological activities reside in a particular molecule, or even a particular part of the molecule[5]. Most importantly, chemical synthesis allows the systematic variation of structure with the aim of developing peptides for therapeutic use, the so-called structure-activity relationship (SAR) study, which can be shown clearly in the development of endothelin agonists and antagonists (Chapter 3).

Recent improvements in the solid phase method itself and in associated chemistry have extended the usefulness of SPPS to the routine preparation of peptides up to 50 amino acid residues in length. This has made synthetic peptides readily available to very large number of biological researchers, and has changed the way in which many research problems are approached.

However, the stepwise synthesis of peptides with more than 100 residues remains the big challenge facing the peptide chemists. The synthesis of peptide sequences of 50~100 residues is normally unpredictable, as side reactions vary from case to case and depend not only on the individual amino acid involved, but also on the primary sequence. Studies of new coupling reagents and coupling methods, protecting groups, mechanism of side reactions and identification of difficult couplings thus remain a very active area in peptide research. Several excellent reviews have been published on these subjects and can be referred to for more details.[6,7,8] By using a new coupling reagent, 1-hydroxyl-4-ethylcarbonyl-1,2,3-triazole (HOCt), which has

been developed in our laboratory, peptides as long as 170 amino acid residues have been synthesised with success[9].

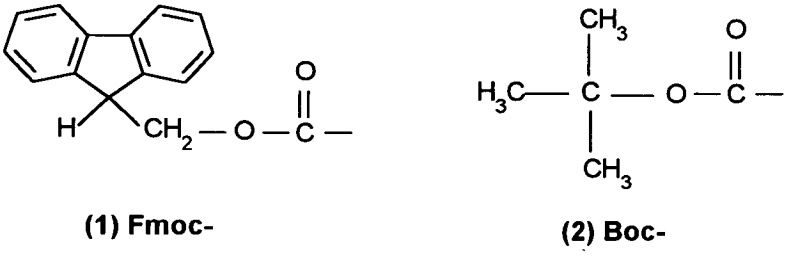


Scheme 1.1.1 The general process of stepwise SPPS

1.1.2 Stepwise SPPS and solid phase fragment condensation

There are two different synthesis strategies for the assembly of peptide chains on solid supports: the stepwise synthesis and fragment condensation.

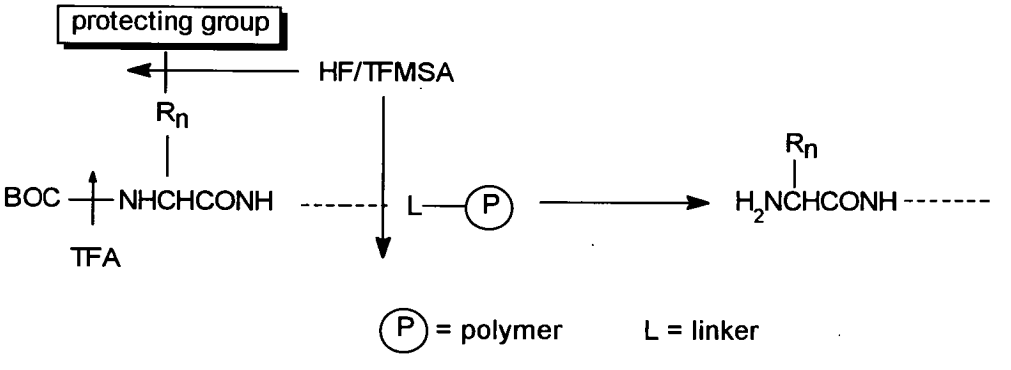
The basic principle of stepwise SPPS is outlined in Scheme 1.1.1. Two synthetic methodologies are used routinely for stepwise SPPS today, based on the different N^{α} protecting groups applied----the base-labile Fmoc group **(1)** or the acid-labile Boc group **(2)** (Figure 1.1.1):



(1) Fmoc- **(2) Boc-**

Figure 1.1.1 The structures of the N^{α}-protecting groups, Fmoc and Boc

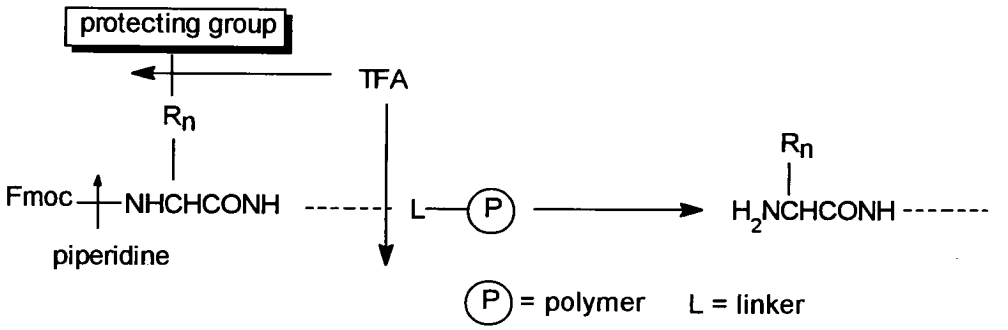
The Boc methodology was first introduced by Merrifield and has been used successfully as a standard SPPS method ever since.[10,11] Cleavage of the Boc protecting group is achieved using trifluoroacetic acid (TFA), and the final cleavage of the peptide from the resin and side chain deprotection requires strong acid such as hydrogen fluoride (HF) or trifluoromethanesulphonic acid (TFMSA) (Scheme 1.1.2).



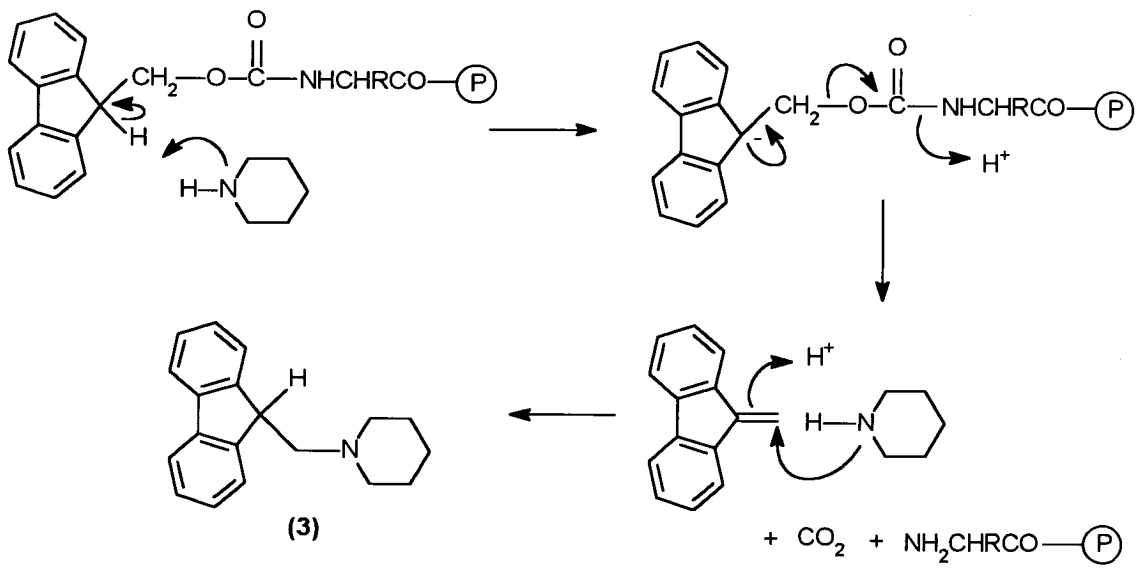
Scheme 1.1.2 The deprotection and cleavage in Boc methodology

Alternative methodology arose out of concern that repetitive TFA acidolysis in Boc group deprotection could lead to the alteration of sensitive peptide bonds as well as acid catalysed side-reactions. In addition, cleavage and deprotection in the Boc strategy also requires the use of dangerous HF, and expensive laboratory

apparatus. This situation changed with the introduction of the base labile N^{α} protecting group 9-fluorenylmethyloxycarbonyl (Fmoc),[12,13] which can be removed with very mild base, *i.e.* 20% piperidine in DMF, and the hazard acid TFA is only required once, at the last stage, to cleave the peptide from the resin and to remove most of the protecting groups (Scheme 1.1.3).



Scheme 1.1.3 The deprotection and cleavage of Fmoc SPPS

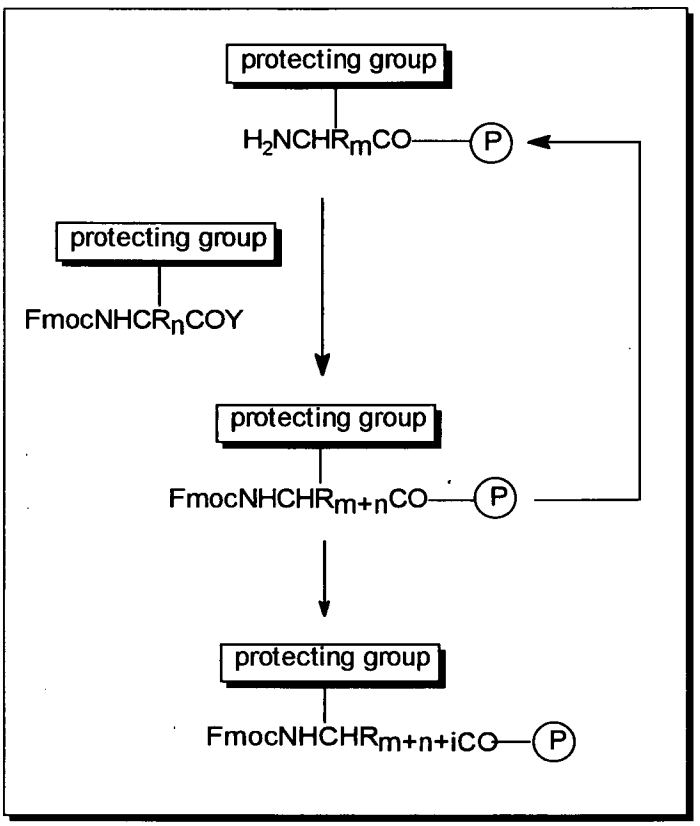


Scheme 1.1.4 The mechanism of Fmoc cleavage

This makes Fmoc methodology more attractive and it is thus adopted in this laboratory. The mechanism of Fmoc deprotection is shown in Scheme 1.1.4.

As an alternative strategy for peptide chain assembly, the solid phase fragment condensation method provides a way of overcoming the losses in overall yield which accompanies the stepwise approach and also alleviating difficult sequence problem

found using stepwise SPPS. However, the major problem related to this method is the insolubility, and thus the purification, of the protected fragments, which are to be coupled to the resin bound peptide intermediate. A newly developed reagent, TbfmocCl,[14, 15] which provides a totally different method of purification, may solve this problem eventually. The principle of fragments condensation is shown in Scheme 1.1.5.



Scheme 1.1.5 The principle of solid phase fragments condensation

1.1.3 Peptide libraries

A newly developed, rapidly expanding area, the combinatorial library, has also found its application in peptide chemistry, the building of peptide libraries. The method allows hundreds and thousands of peptide analogues to be synthesised in a very short time. This technique will play an important role in the development of physiologically important peptides, making SPPS an even more attractive approach.

1.2 Stepwise solid phase peptide synthesis (SPPS)

1.2.1 Solid support and the linker

In the early work of Merrifield, several different types of polymers were investigated.[16] The most promising one was polystyrene with 1% divinylbenzene as cross-linking reagent. This resin possessed most of the characters required for the SPPS. It was chemically stable, could be easily modified by attachment of a linker and also had reasonable swelling properties in the solvent used for SPPS, allowing rapid diffusion of the reagents. Despite much research and the development of new supports, including the successful application of polyacylamide resins, the original chloromethylated polystyrene resin remains the preferred one.[17]

The chloromethylation of the cross-linked polystyrene resin produces an arylchloromethyl derivative which can be used as an anchor for the growing chain as in the case of Merrifield resin[18], or more usually as a starting point to which can be coupled a variety of handle linkers, which provide different acid labilities for subsequent cleavage of the associated peptide from the resin on completion of the peptide assembly.[19,20]

The linkers used in this laboratory for the synthesis of peptide acids and amides are *p*-benzyloxybenzylalcohol and dibenzosuberenyl resins which were developed by Wang[21] and Ramage[22] respectively. However, attachment of Cys and His to the Wang resin is associated with high level of racemisation due to the 4-dimethylaminopyridine (DMAP) catalysed activation of the carboxyl group[23]. A specially designed resin, Cl-Trt resin is thus recommended,[24,25] as DMAP is not used for the loading of the first amino acid onto this resin.

Synthesis of peptide acids with C-terminal Pro can also be problematic. Loss of peptide from the resin at the dipeptide stage can be very high as a result of diketopiperazine formation[26]. The extreme steric hindrance of the Cl-Trt resin can completely suppress the formation of the diketopiperazine at the dipeptide stage, thus preventing the loss of dipeptide from resin.[27,28,29] As the Cl-Trt linker is particularly acid labile, Cl-Trt resin has also been widely used in the preparation of fully protected

peptide fragments which can be cleaved under very mild acidic cleavage conditions.[24,30] The structures of these resins are shown in Figure 1.2.1.

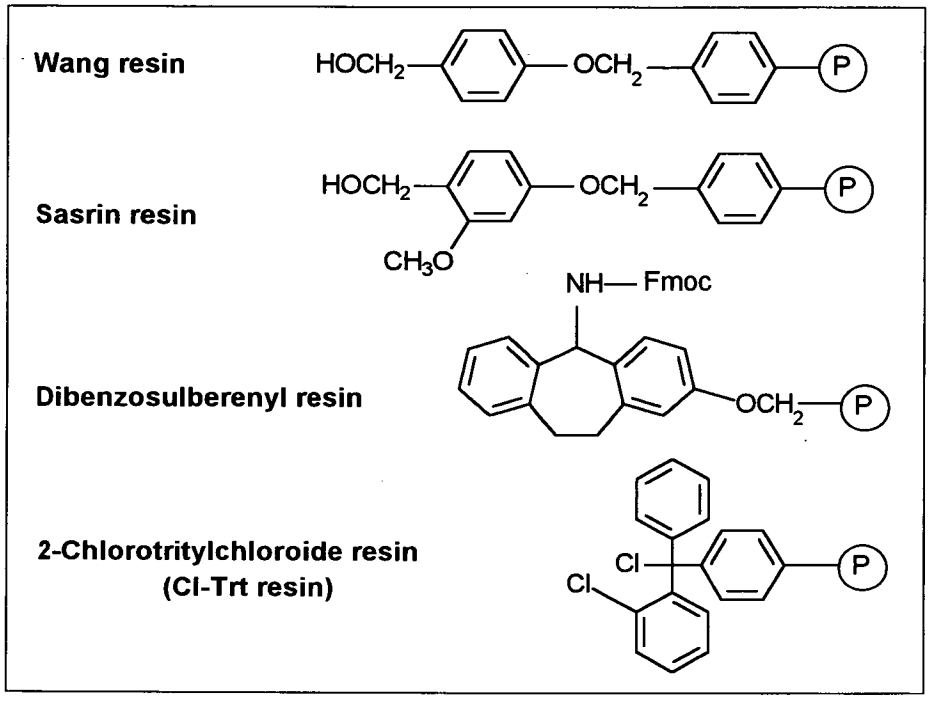


Figure 1.2.1 Structures of the resins used in this laboratory

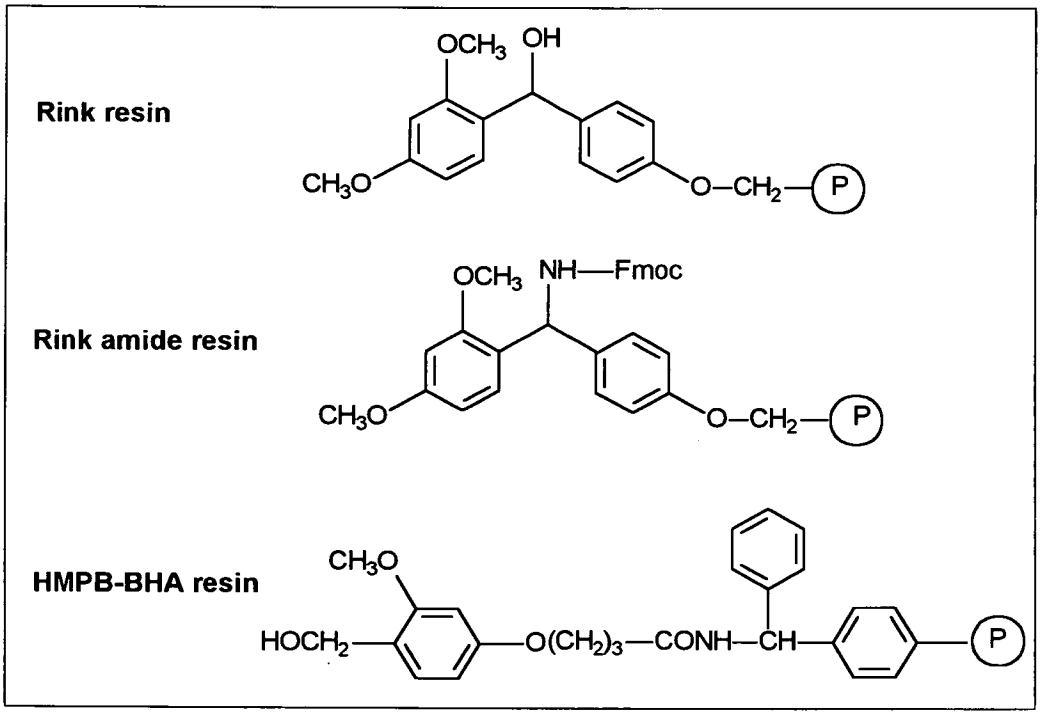
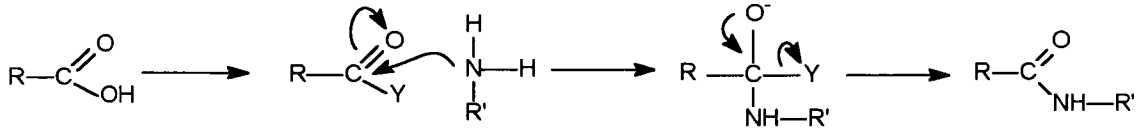


Figure 1.2.2 Some other resins used for Fmoc SPPS

There are many other types of solid supports and handles to suit a variety of requirements, the detail of which can be found in the literature. Some of them are shown in Figure 1.2.2.

1.2.2 Activation and coupling

The formation of an amide bond involves the acylation of an amino group of the first amino acid by the carboxyl group of a second amino acid.[31] This reaction is energy-requiring and thus needs the activation of either the carboxyl or the amino group involved. However, whereas the activation of the amino group is a challenging problem to which no practical solution has been found to date, activation of the carboxyl group thus remains the underlying principle of all coupling methods in use (Scheme 1.2.1), where Y is an electron withdrawing group, which can increase the electrophilicity of the carboxyl carbon and thus facilitate nucleophilic attack by the amino group. The tetrahedral intermediate formed **(4)** leads to the stabilised amide bond by the elimination of Y^-, which is usually a good leaving group.



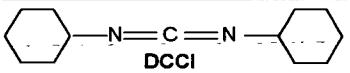
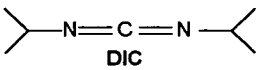
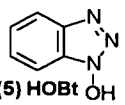
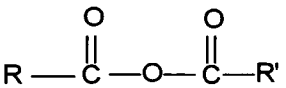
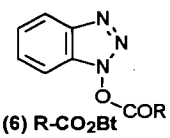
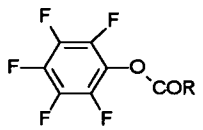
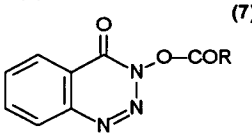
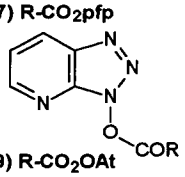
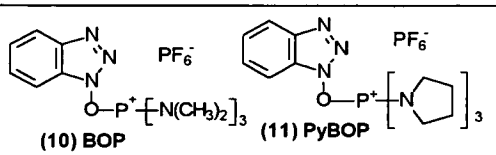
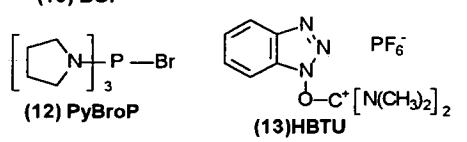
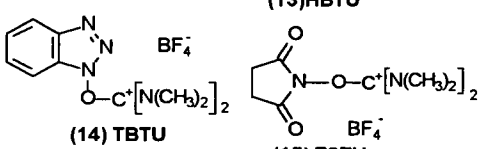
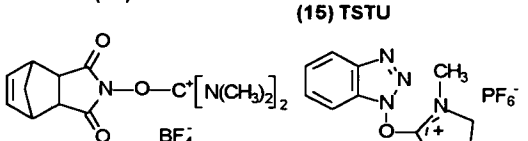
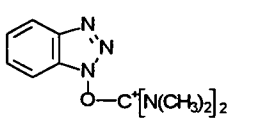
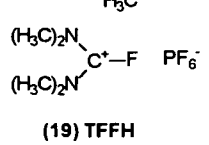
(4)

Scheme 1.2.1 The process of an amide bond formation

There are four major types of coupling techniques currently employed for the stepwise introduction of N^{α}-protected amino acid in SPPS.[32,33,34] These are summarised in Table 1.2.1 and will be discussed in detail in Chapter 2.

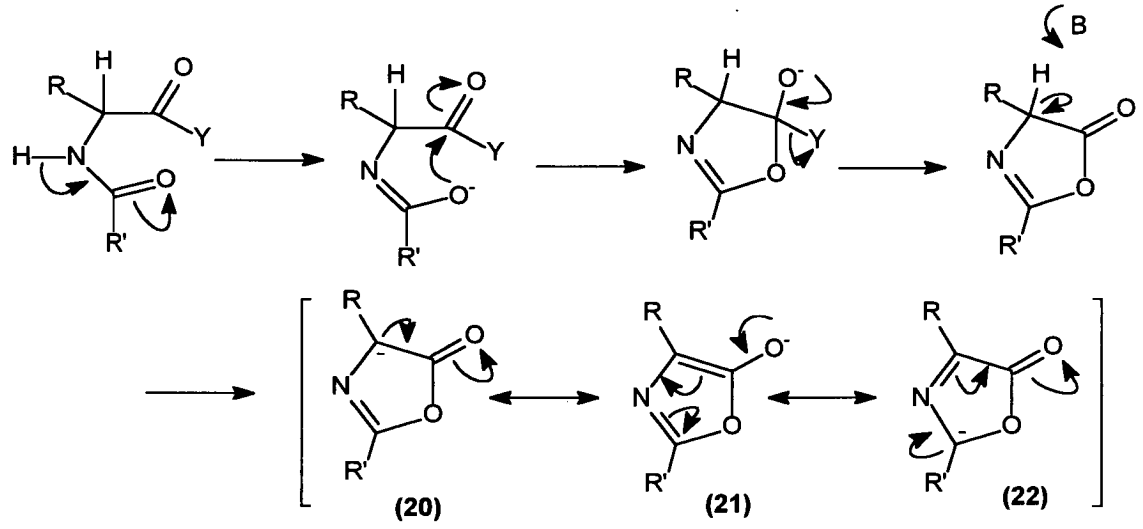
Despite all these alternatives, in practice, almost all peptides bonds are currently formed using the coupling reagent, DIC/DCC and HOBt, either for Boc or Fmoc methodology, due to the availability and cost. A new coupling reagent has recently been developed in our laboratory,[35] which shows better coupling efficiency than HOBt. It will be discussed in Chapter 2.

Table 1.2.1 Some coupling methods and reagents

methods	reagents	comments
Carbodiimides	 DCCI  DIC  (5) HOBt	Insoluble by-product with DCCI, Dehydration of Asn/Gln; Racemisation of His (Trt). Improved with the addition of HOBt (5).
Anhydrides		Mainly for Boc SPPS; Side-reactions involved; Wasteful if R=R'
Active esters	 (6) R-CO_2Bt  (7) R-CO_2pfp  (8) R-CO_2Dhbt  (9) R-CO_2OAt	Easy to use, fast in coupling, less side-reactions; Not all available; Some are not stable.
Coupling Reagents	 (10) BOP (11) PyBOP  (12) PyBroP (13)HBTU  (14) TBTU (15) TSTU  (16) TNTU (17) BOI  (18) HATU  (19) TFFH	Easy to use; Less side-reactions; Expensive.

1.2.3 Side reactions, racemisation

Side reactions are a feature of almost all organic reactions, but they are of particular importance in a process such as peptide synthesis where hundreds of reactions are involved in the chain assembly. Intensive efforts have been made in the identification and correction of side reactions, and most of the problems have been solved using carefully adjusted conditions.[6,7,36]



Scheme 1.2.2 The oxazolone mechanism of racemisation

Racemisation is the most common side reaction which affects all naturally occurring amino acids except glycine. A considerable amount of work has been carried out in order to understand the mechanism, and thus to prevent it.[18,37] The most commonly observed and well studied mechanism of racemisation is oxazolone formation which occurs during the activation of the N^{α}-protected amino acid (Scheme 1.2.2).

The tendency for racemisation lies in the ease by which the acidic proton can be abstracted by bases from the chiral centre due to resonance stabilisation of the carbanion generated in the process. It is thus influenced by the nature of the amino acid involved, the solvent used or the presence/absence of tertiary amines. The imidazole side chain of His provides an intramolecular amine, hence it is not surprising that this amino acid causes most problems in peptide synthesis where racemisation is concerned. Fortunately, this tendency for oxazolone formation can be completely

suppressed in the presence of HOBt, which makes stepwise SPPS a reliable, racemisation free synthetic approach in most circumstances.[6,7,36]

Incomplete acylation at each coupling step gives rise to truncated and deletion peptides with properties similar to the target sequence. Optimised coupling condition can improve the situation, but not completely cure it. Therefore, quantitative termination of the unreacted functional group, requires the so called 'capping' step which becomes equally important. This is an area which requires further investigation.

Apart from the general problems, residue or sequence specific side reactions have also been studied.[17,38,39] Some of them, such as the cyclisation and rearrangement of Asp/Gly under certain circumstances to give incorrect peptide sequences which are very similar to the target peptide, still remain as big challenges facing the peptide chemist.

1.3 The purification and folding of peptides and proteins

1.3.1 Deprotection and cleavage of peptides

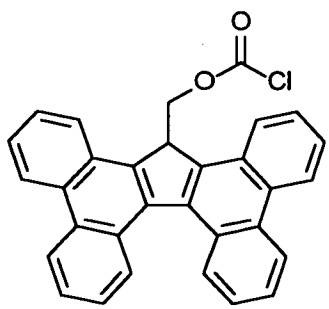
After complete assembly of the peptide chain on the resin, all protecting groups must be removed and the link between the peptide and the resin cleaved in order to release the free peptide into solution. TFA, in the presence of scavengers, is the normal reagent used in Fmoc methodology. This process is always hampered by side-reactions involving certain side-chain protecting groups which are liberated as stable cations during the TFA cleavage and deprotection, such as Trt, *t*Bu and Pmc. The majority of the side-reactions involve modification of sensitive amino acid residues, such as Trp, Tyr, Met and Cys. A number of scavengers are thus included in the cleavage mixture to quench all the cations. Optimum cleavage conditions are very much dependent on the individual amino acid residues present, it is thus always recommended that a small scale cleavage be carried out to find the particular conditions suitable for the peptide sequence involved. The most commonly used scavengers are ethanedithiol (EDT), H_2O, thioanisole, phenol, and triisopropylsilane (TIS).

1.3.2 Peptide purification, general

The crude peptide resulting from SPPS contains a variety of by-products, which must be removed to afford the pure final product. In principle, purification should be carried out by the consecutive application of several procedures based on different separation mechanisms, *i.e.* reverse-phase HPLC, ion exchange chromatography, gel filtration, isoelectronic focusing, *etc.* Most small peptides containing less than 50 amino acids can be purified by a single step reverse-phase HPLC. For large peptides and proteins, purification becomes much more complicated. Owing to the onset of relatively stable, slowly exchanging folded structures of the peptide chain[40], a single molecular species will give a variety of chromatographically distinguishable but not separable components, making separation on HPLC an impossible task. Either the peptide chain must be folded prior to purification into a single structure stable under the HPLC conditions or conformationally independent separation techniques must be used to purify the target peptide in the presence of denaturants prior to folding. Together with the folding itself, purification of large peptides and proteins presents the most important current challenge for peptide chemists. The newly developed reagent, TbfmocCl, provides a way to tackle this problem.

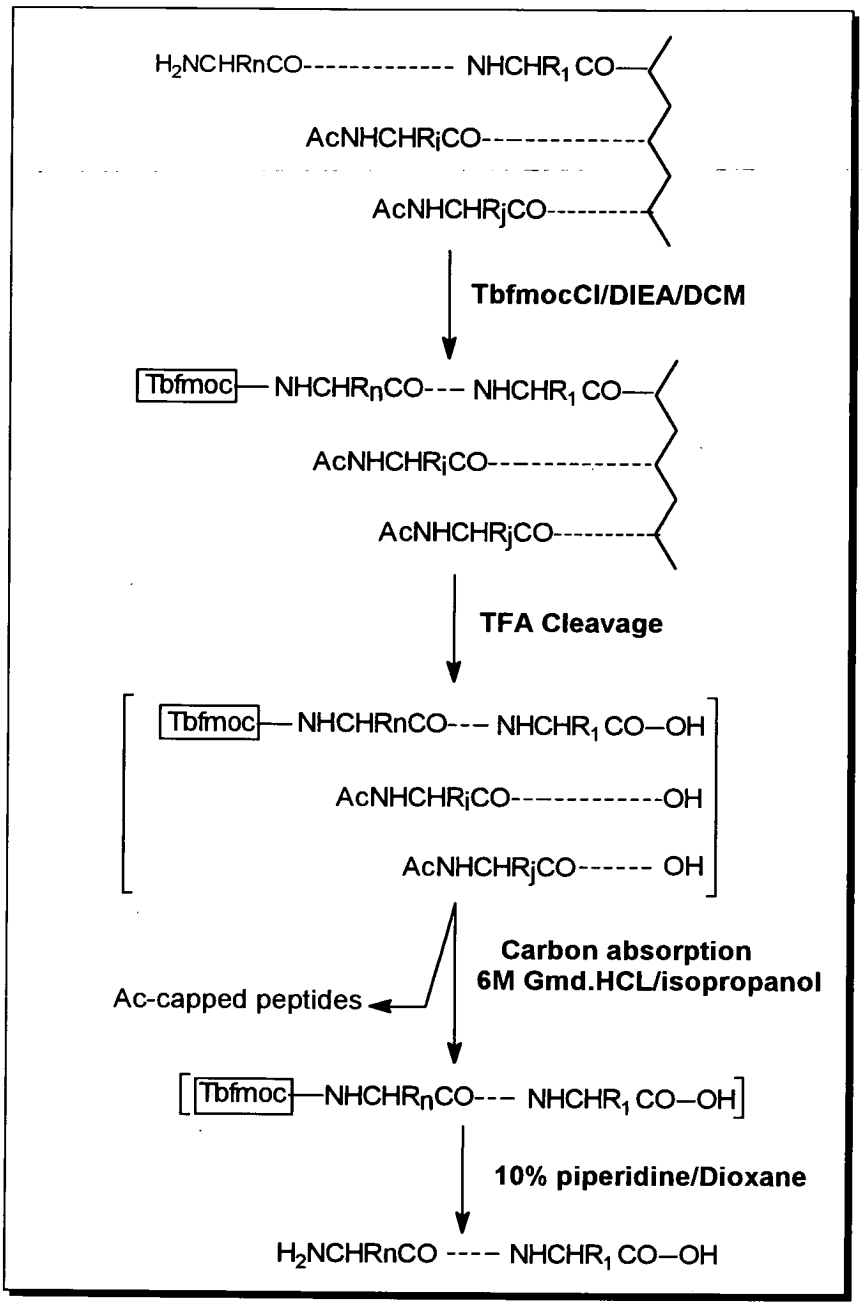
1.3.3 Peptide purification by Tbfmoc

The structure of TbfmocCl, the reagent used for peptide purification[14,15] is shown in Figure 1.3.1.



(23) TbfmocCl

Figure 1.3.1 The structure of TbfmocCl



Scheme 1.3.1 The principle of Tbfmoc purification of peptides

The principle of peptide purification by Tbfmoc is shown in Scheme 1.3.1. By using a Tbfmoc selective adsorbent, such as charcoal, only the peptide chain which has a Tbfmoc group on it will be adsorbed, while the truncated peptides can be easily washed away. Under ideal coupling and capping conditions, only the target peptide has a free N-terminal amino group at the end of chain assembly which can react with

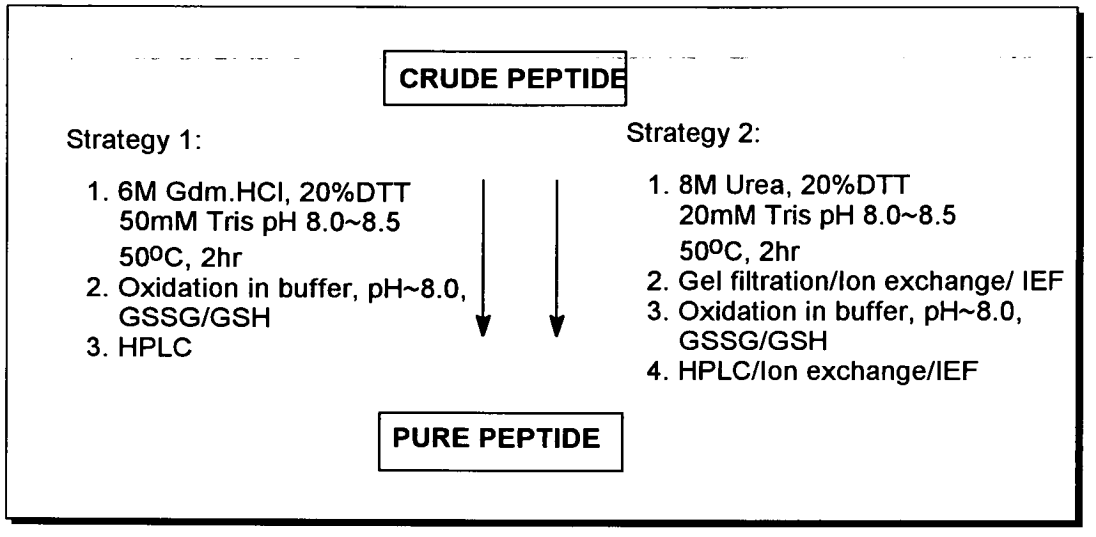
the TbfmocCl, making the reagent a powerful alternative tool in large peptide/protein purification. In practice, a small amount of deletion peptides is always present, especially in the case of large peptide, which will be isolated together with the target peptide. Although further purification is required, the crude peptide is much cleaner after Tbfmoc purification, which makes the subsequent purification much easier. For small peptide (~30 amino acid residues), a single peak can normally be obtained just after Tbfmoc purification.[15] It thus provides a very promising way to purify the fully protected peptide chain required for fragment condensation, as solvents such as 6M guanidine hydrochloride, 8M urea or 2-propanol can be used to dissolve the normally insoluble fragments.

1.3.4 Peptide/protein folding

To be biologically active, all large peptides and proteins must adopt specific folded three dimensional structures. In this biologically active, or native form, the peptide chain is folded into a thermodynamically preferred conformation, which is maintained by the accumulating relatively weak interatomic forces such as hydrogen bonding, hydrophobic interactions and charge interactions.[41] Correct folding of peptides/proteins is a major issue in protein science.[42,43] This problem is particularly acute in the case of Cys containing peptides/proteins where the disulphide connections must be formed in one particular way to give rise to biological activity. The mechanism and prediction of protein folding is an important biological problem, the elucidation of which has been the subject of much experimental and theoretical work ever since the first three dimensional structure of a protein was known.[44,45,46] From the practical point of view, the preparation, purification and folding of large peptides and proteins is of great interest.

There are two alternative strategies in the purification and folding of large peptides: folding before purification or purification before folding. Limited by the literature evidence currently available, it is impossible to decide between them. Generally, if the crude peptide contains a majority of the target sequence, folding can be carried out prior to purification; Otherwise pre-purification can help the folding by removing most of the impurities, especially when multiple disulphide bonds are

present. The general protocols are illustrated in Scheme 1.3.2, where GSH is glutathione reduced and GSSG is glutathione oxidized.



Scheme 1.3.2 Two strategies for peptide purification/folding

1.3.5 Protection/deprotection, reduction/oxidation of Cys---the formation of disulphide bonds

An amino acid residue which is of particular importance in peptide synthesis is cysteine. There are basically two different kinds of protecting groups applied in Fmoc SPPS. The acid labile protecting group such as Trt and acid stable protecting group such as Acm. Different protecting groups or different combinations of them are selected to suit different synthetic protocols. When acid stable protecting groups such as Acm are used, an additional procedure is required for the deprotection of these groups before generation of disulphide bonds. A number of deprotection methods have been developed based on the different protecting groups and protecting strategy used, which are summarised in Table 1.3.1 and the details can be found in the literature.[17,47,48]

Generation of disulphide bonds is often hampered by practical difficulties such as instability or insolubility of reduced peptides. A number of different methods have been reported. Among them, air oxidation in a dilute peptide solution is the most popular protocol, but the rate and yield obtained are generally low; and the

reproducibility is poor.[7] Oxidation systems composed of low molecular weight disulphides and thiols have been used extensively for peptide disulphide formation.[49] These systems increase both rate and yield of the native conformation by facilitating rapid reshuffling of incorrect disulphide bonds. Mixtures of oxidised and reduced glutathione, cysteine, or 2-mercaptoethanol are commonly used at 1~5mM thiol and 0.01~0.5mM disulphide reagent.[50] Optimum conditions usually involve with a 10-fold molar excess of reduced thiol component in a buffer, the pH of which is set up at the upper limit where the peptide is still able to fold.[51]

A newly developed method makes use of DMSO in TFA or aqueous buffer solution.[52] No side-reactions were observed with amino acids such as Met, Tyr and Trp.[53]

Table 3.1.1 Protecting groups and deprotection reagents for Cys

Protecting Group	Deprotection Reagent						
	TFA	TFMSA	$Hg(OAc)_2$	AgOTf	I_2	Thiols	enzyme
Trt	+	+	+	+	+	-	n
Acm	-	-	+	+	+*	-	n
tBu	-	+	+	-	-	-	n
StBu	-	n	n	n	n	+	n
PhAcm	-	-	+	+	+*	-	+

Notes: +, deprotected; -, not deprotected; +*, resulted in disulphide; n, not tested.

1.4 References

[1] Curtius, T. (1902), *Ber. Dtsch. Chem. Ges.* **35,** 3226-3228.

[2] Fischer, E. (1903), *Ber. Dtsch. Chem. Ges.* **36,** 2094-2106.

[3] Bergmann, M. & Zervas, L. (1932), *Ber. Dtsch. Chem. Ges* .**65,** 1192-1202.

[4] Merrifield, R.B. (1963), *J. Am. Chem. Soc.* **85,** 2149-2154.

[5] Kent, S.B.H. (1988), *Ann. Rev. Biochem.* **57**, 957-985.

[6] Bodanszky, M. (1988), *Peptide Chemistry, a Practical Textbook,* Sringer-Verlag, London.

[7] Steward, J. & Young, J.D. (1984), *Solid Phase Peptide Synthesis (2nd.Ed.)* Piece Chemical Company, Rockford, Illinois.

[8] Atherton, E. & Sheppard, R.C. (1989), *Solid Phase Peptide Synthesis, a practical Approach,* IRL press, Oxford.

[9] Robertson, N. & Morton, G. *Personal communications.*

[10] Tam, J.P. (1988,) in *Macromolecule Sequencing and Synthesis,* p153-184, Schlesinger, D.H. (Ed.), New York.

[11] Geisler, T. Beilan, H. in *Macromolecule Sequencing and Synthesis,* p199-218, Schlesinger, D.H. (Ed.), New York.

[12] Carpino, L.A. & Han, G.Y. (1970), *J. Am. Chem. Soc.* **92,** 5748-5749.

[13] Artherton, E., Logan, C.J. & Sheppard, R.C. (1981), *J. Am. Chem. Soc., Perkin Trans. I,* 538-546.

[14] Brown, A.R., Irving, S.L. & Ramage, R. (1993), *Tetrahetron Lett.* **34,** 7129-7132.

[15] Irving, S.L. (1993), *PhD thesis, Edinburgh University.*

[16] Erickson, B.W. & Merrifield, R.B. (1976), in *The Peptide,* p255-257, Neurath, H. & Hill, R.L.(Eds), Vol.3, (3rd Ed.), Academic Press, New York.

[17] Field, G. & Noble, R.L. (1990), *Int. J. Pept. Protein Res.* **35,** 161-214.

[18] Barany, G. & Merrifield, R.B. (1980), *Peptides,* **2,** 1-284.

[19] Lu, G.-S., Mojsov, S., Tam, J.P. & Merrifield, R.B. (1981), *J. Org. Chem.* **46,** 3433-3436.

[20] Ramage, R. Barron, C.A., Bielecki, S. Holden, R. & Thomas, D.W. (1987), *Tetrahedron Lett.,* **28,** 4105-4108.

[21] Wang, S.S. (1973), *J. Am. Chem. Soc.* **95,** 1328-1333.

[22] Ramage, R., Ivring, S.L. & McInnes, C. (1993), *Tetrahedron Lett.,* **34,** 6599-6602.

[23] Sieber, S. (1987), *Tetrahetron Lett.* **28,** 6147-6150.

[24] Barlos, K., Gatos, D., Kallitsis, I., Papaphotiu, G. & Sotiriou, P. (1988), *Liebigs Ann. Chem.* 1079-1081.

[25] Fujiwara, Y., Akaji, K. & Kiso, Y. (1994), *Int. J. Pept. Protein Res.* **42,** 724-726.

[26] Pedroso, E. Gremdas, A., Saralegui, M.A., Giralt, E., Granier, C. & Rietschoten, J.V. (1982), *Tetrahedron,* **38,** 1183-1192.

[27] Barlos, K., Chatzi, O., Gatos, D. & Stavropoulos, G. (1991), *Int. J. Pept. Protein Res.* **37,** 513-520.

[28] Barlos, K., Gatos, D., Kapolos, S., Papaphotiu, G, Schafer, W. & Yao, W. (1989), *Tetrohedron Lett.* **30,** 3947-3950.

[29] Barlos, K., Gatos, D., Kallitsis, J. Papaphotiu, G, Sotiriou, P. Yao, W. & Schafer, W. (1989), *Tetrahetron Lett.* **30,** 3943-3946.

[30] Pugh, K.C. York, E.J. & Stewart, J. M. (1992) *Int. J. Pept. Protein Res.* **40,** 208-213.

[31] Jones, J.H. (1979), *Peptides (N.Y.),* **1,** 65-104.

[32] Bodanszky, M. (1979), *Peptides (N.Y.),* **1,** 105-196.

[33] Meienhofer, J. (1979), *Peptides (N.Y.),* **1,** 197-239.

[34] Meienhofer, J.(1979), *Peptides (N.Y.),* **1,** 263-314.

[35] Davison, A. (1993), *PhD thesis, Edinburgh University.*

[36] Jones, J.H. (1991), *The Chemical Synthesis of Peptides,* Clarendon Press, Oxford.

[37] Kemp, D.S. (1979), *Peptides (N.Y.),* **1,** 315-383.

[38] Sieber, P. (1987), *Tetrahedron Lett.* **28,** 1637-1640.

[39] King, D.S., Field, C.G. and Field G.B. (1990), *Int. J. Pept. Protein Res.* **36,** 255-266.

[40] Regnier, F.E. (1987). *Science,* **238,** 319-323.

[41] Creighton, T.E. WO 86/05809.

[42] Baldwin, R.L. (1989), *TIBS,* 291-294.

[43] Baldwin, R.L. (1986), *TIBS,* 6-9.

[44] Hantgan, R.R., Hammes, G.G. & Scheraga, H.A. (1974), *Biochemistry,* **13,** 3421-3447.

[45] Creighton, T.E. (1975), *J. Mol. Biol.* **95,** 167-199.

[46] Darby, N.J. Morin, P.E., Talbo, G. and Creighton, T.E. (1995), *J. Mol. Biol.* **249,** 463-477.

[47] Atherton, E. Sheppard, R.C. and Ward, P (1985), *J. Chem. Soc., Perkin Trans. 1,* 2065-2074.

[48] Bishop, P. & Chemielewski, J. (1992), *Tetrahedron Lett.* **33,** 6263-6266.

[49] Saxena, V.P. & Wetlaufer, D.B. (1970), *Biochemistry,* **9,** 5015-5022.

[50] Creighton, T.E. (1990), in *Protein structure, a practical approach,* p191-223, IRL press, Oxford.

[51] Creighton, T.E. (1990) *Biochem. J.* **270,** 1-16.

[52] Otaka, A., Koide, T., Shide, A. & Fujii, N. (1991), *Tetrahedron Lett.* **32,** 1223-1226.

[53] Tam, J.P., Wu, C.-R., Liu, W. & Zhang, J.-W. (1991), *J. Am. Chem. Soc.* **113,** 6657-6662.

CHAPTER 2

THE SYNTHESIS OF A NEW COUPLING REAGENT AND ITS APPLICATION IN SPPS

2.1 Introduction

2.1.1 Development of coupling methods and coupling reagents

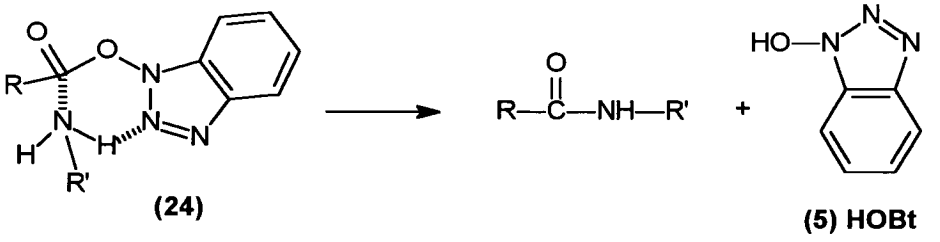
The principal reaction in the synthesis of a peptide is acylation of an amino group of an amino acid by the carboxyl group of a second amino acid to form an amide bond (Section 1.2.2); the process is known as coupling. In order to convert carboxylic acids into acylating agents their hydroxyl group must be replaced by an electron withdrawing group in order to enhance the electrophilicity of the carboxyl group (Scheme 1.2.1).

Numerous reagents and methods have been developed in the history of peptide chemistry; these were summarised in Table 1.2.1 (Section 1.2.2). Unsurprisingly the acid chloride was the first activated intermediate used in the early days to make very small peptides.[1] However, both the preparation and application of amino acid chlorides were far from unequivocal, and it was soon replaced by the more satisfactory acid azide method.[2] Although the Curtius rearrangement is an inherent shortcoming of this method, the azide process still remains a powerful and practical approach even today, mainly because of the negligible associated racemisation. More recently, the azide method has found application in peptide ligation when combined with a special linker.[3]

Formation of the peptide bond *via* acid anhydrides gained considerable popularity after the introduction of mixed (unsymmetrical) anhydrides in the 1950's.[4,5] The improved version, *iso*-butylcarbonic acid mixed anhydride,[6] remains a valuable tool in peptide synthesis today. However, in the application of the mixed anhydride method, a small amount of the second acylation product can always be expected. This inherent ambiguity can be avoided if aminolysis of esters is used for peptide

formation. Several types of active esters were soon developed; for example, *p*-nitrophenyl,[7] N-hydroxysuccinimide,[8] pentachlorophenyl,[9] and pentafluorophenyl esters.[10] The more recently developed reagent N-hydroxybenzotriazole (HOBt), and its esters, are of particular interest and will be discussed further.

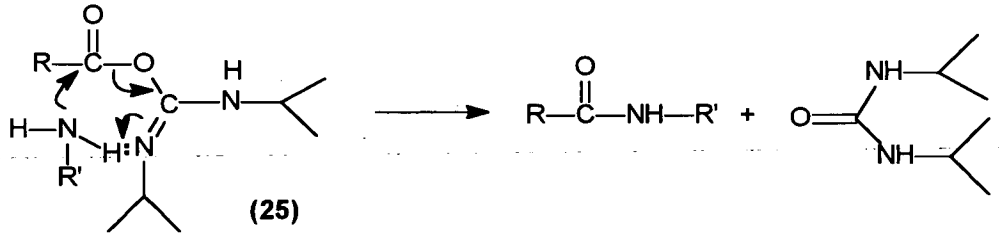
HOBt was first introduced into peptide synthesis as an additive to the carbodiimide method in 1970 by Konig *et al.*[11], to suppress the racemisation and other side reactions caused by the over activating effect of DCC/DIC (discussed below). The HOBt esters were recognised as the actual active intermediates involved in the formation of the peptide bond and this *in situ* active ester formation method soon gained popularity in peptide synthesis. The high reactivity of the HOBt esters can be attributed to anchimeric assistance, as shown below (Scheme 2.1.1).[12]



Scheme 2.1.1 The anchimeric assistance effect of the HOBt esters

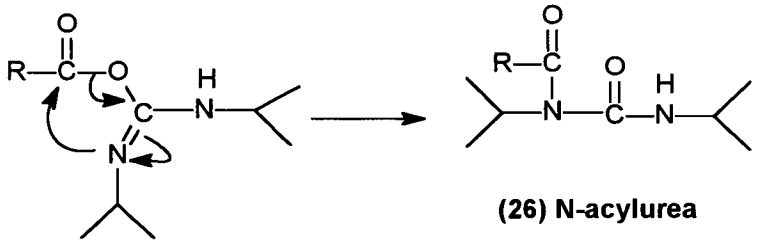
Carbodiimides, particularly N,N'-dicyclohexylcarbodiimide (DCC), were also introduced for peptide bond formation[13] at that time. This was regarded as a major event in peptide history. The novel feature of coupling reagents is that they can be added to a mixture of the carboxyl component of the amino acid and amine component of the growing peptide chain, thus no pre-activation is needed. The reaction between the amine component and carbodiimide does occur, but this is negligible as it is very slow compared to the rapid conversion of the acid to the amide. DCC has been replaced by diisopropylcarbodiimide (DIC)[14,15] in SPPS due to the insolubility of the urea by-product of the DCC reaction, N,N'-dicyclohexylurea (DCCU).

Several mechanisms have been proposed to explain the surprisingly high reaction rate observed in aminolysis through the carbodiimides. The powerful N=C group and its basic character provide the major reasons (Scheme 2.1.2).



Scheme 2.1.2 The mechanism of aminolysis through DCC/DIC

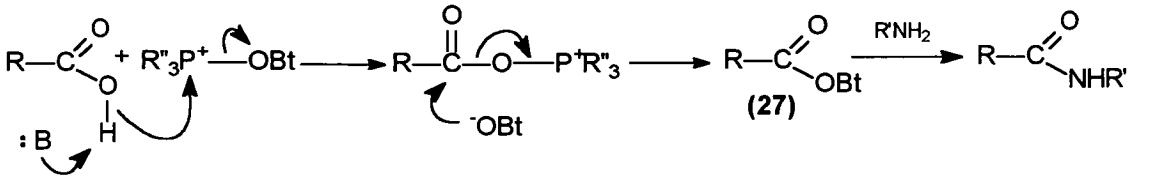
However, a price has to be paid for the speedy execution of activation and coupling by this method. Over-activation of the reactive intermediate results in some loss in chiral purity when certain amino acids are involved. Furthermore, the nucleophilic centre of O-acylisoureas competes with amine component for the acyl residue and this competition leads to the formation of unreactive by-products, N-acylureas (Scheme 2.1.3). Both racemisation and N-acylurea formation can be suppressed by the addition of auxiliary nucleophilies such as HOBt.[11] The combination of DCC/DIC and HOBt soon gained its popularity and remains the most popular coupling method in peptide synthesis up until now.



Scheme 2.1.3 The formation of N-acylurea

The success achieved with DCC/DIC stimulated an unrelenting research for even better coupling reagents. Among those are carbonyldiimidazole, 1-ethyloxycarbonyl-2-ethyloxy-1, 2-dihydroquinoline (EEDQ) and 1-isobutyloxy-carbonyl-2-isobutyloxy-1, 2-dihydroquinoline (IIDQ).[16] The latter offers some advantages over DCC/DIC methodology in that fewer side reactions were observed, but this reagent has never become as popular as DCC/DIC.

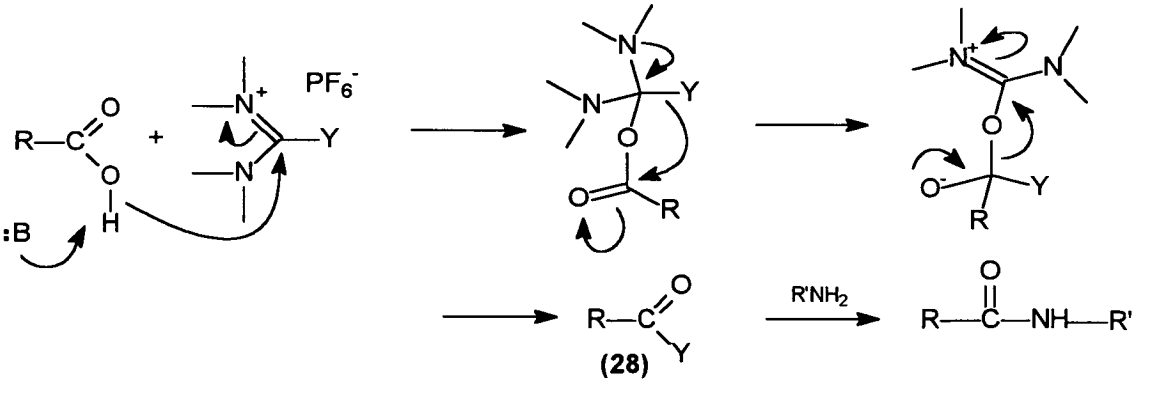
In the 1970s, phosphonium derivatives or BOP reagents, initially reported by Castro *at el.*[17] became increasingly popular as coupling reagents, especially for SPPS.[18,19] The active ester pathway was suggested as the main route involved in the peptide bond formation (Scheme 2.1.4). The original reagent, BOP **(10),** was soon replaced by PyBOP **(11),** due to its carcinogenic by-product, hexamethylphosphotriamide (HMPA).[20]



Scheme 2.1.4 The mechanism of peptide bond formation through BOP reagents

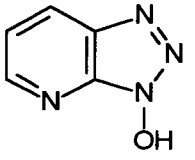
Another class of reagents which are related to phosphonium reagents are the carbon analogues or uronium salts. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) **(13)** (Table 1.2.1), was first synthesised and used in solution phase peptide synthesis by Gross *et al.*[21] and further developed by Knorr *et al.*[22] for SPPS, along with other analogues **(14)-(16)** (Table 1.2.1).[23,24] A similar analogue, BOI **(17)** (Table 1.2.1) was reported by Kiso *et al.*[25] These reagents are known as Knorr reagents.

The mechanism of coupling through Knorr reagents is similar to that of the BOP reagent; both involve an active ester, as shown in Scheme 2.1.5.

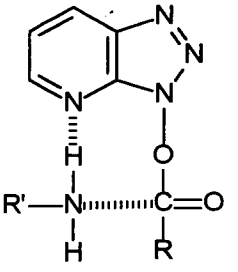


Scheme 2.1.5 The mechanism of peptide bond formation through Knorr reagents

One noticeable recent development in the field of coupling reagents is the discovery of the 7-aza analogue of HOBt, HOAt **(29)** and the investigation of its coupling efficiency relative to BOP and Knorr reagents.[26,27] The enhanced coupling efficiency and lower level of racemisation reported of the HOAt esters were also attributed to the anchimeric assistance effect as shown in **(30)** (Figure 2.1.1).[28]



(29) HOAt



(30) Coupling intermediate of HOAt

Figure 2.1.1 The structures of HOAt and its active ester

An additional benefit of this reagent is the visual indication of reaction completion, by the disappearance of the yellow colour of the reaction mixture. This feature provides a way of monitoring coupling by a specially designed instrument. This will be discussed further in the next section.

More recently, tetramethylfluoroformamidinium hexafluorophosphate (TFFH, **(19)**) was reported by Carpino *et al.* as a rapid-acting peptide coupling reagent for both solution and solid phase peptide synthesis.[29]

2.1.2 Monitoring of peptide synthesis

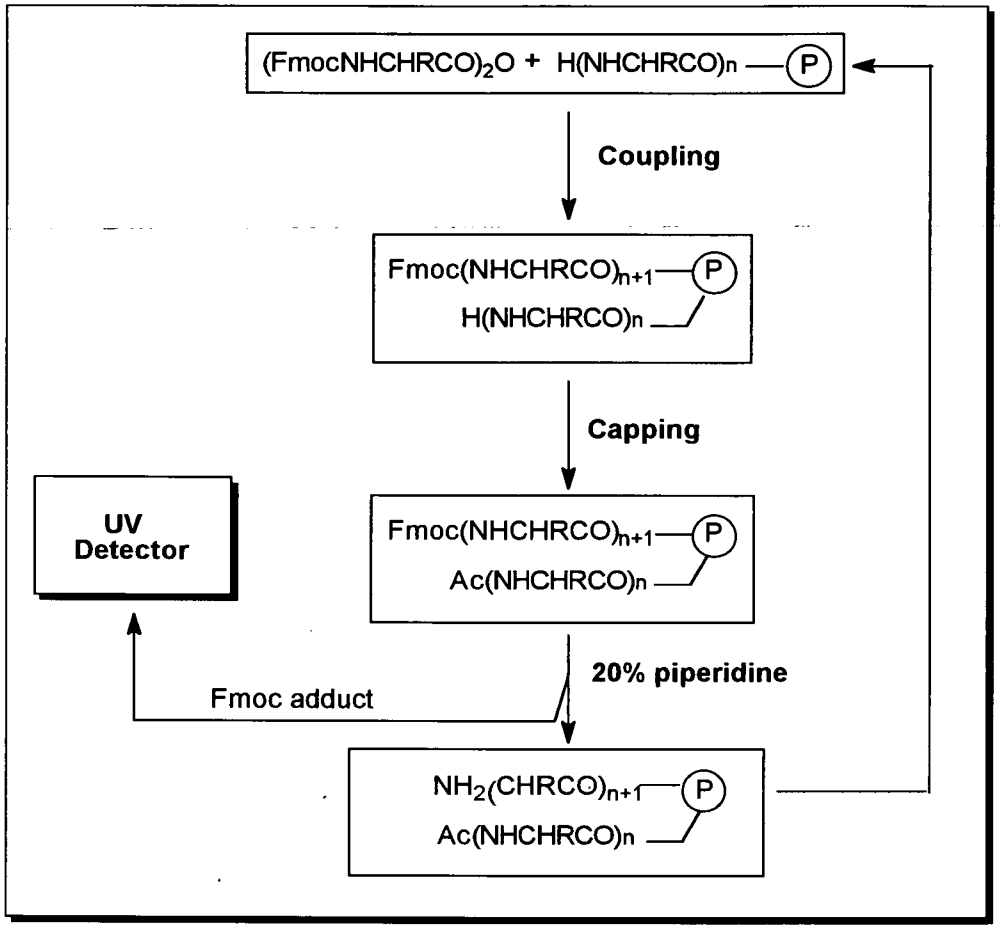
Stepwise SPPS has been greatly improved by the development of new coupling reagents and methods. Peptides with up to 50 amino acid residues can be synthesised regularly in most research groups and industrial laboratories. However, the synthesis of peptides longer than 100 residues is still a big challenge facing the peptide chemist. Apart from the gradually elongating peptide chain, which obviously accentuates the difficulties involved, smaller peptides with so called difficult sequences have also been reported which lead to problems in the synthesis.[30,31] It is

well known that coupling efficiency is sequence dependent and thus unpredictable, despite the research effort directed at this problem.[32] Coupling percentage can drop dramatically at certain amino acids in the synthesis of one peptide, whilst in another synthesis the same amino acid can couple very easily. Whenever a serious drop occurs during a synthesis, coupling can normally be improved by a remedial treatment, such as recoupling, extending the coupling time, increasing the ratio of the incoming amino acid, sonication *etc.* It is therefore very important to follow the synthesis by an efficient monitoring method.

The methods used in those early days of SPPS, i.e. amino acid analysis or the Schiff base reaction, were time consuming. Recent developments have led to the discovery of more efficient methods of monitoring; however, the majority of these methods require additional reactions to introduce and remove diagnostic reagents,[33] withdrawal of resin samples[34] or construction of new instruments.[35]

The ideal approach to monitoring a coupling is to examine the solution of the activated amino acid during or after the coupling, without disturbing the synthetic process. Bodansky and Sheeham[36] first introduced this approach to Boc SPPS using *p*-nitrophenyl esters of the N^{α}-protected amino acids. As free *p*-nitrophenol absorbs at 314nm and the active esters absorb at 270nm, filtrate of a second coupling which absorbs only at 270nm indicates the completion of the previous coupling. A similar idea was introduced into Fmoc SPPS by Dryland *et al.* in 1986.[37] The synthesis of a pentadecapeptide was carried out under continuous flow conditions, using "Fmoc-Polyamide" chemistry. The circulating activation and deprotection mixtures were passed through a UV spectrophotometer, thus both acylation and deprotection process could be monitored. However, this method is only semi-quantitative and involves many errors.

Recently, a more reliable monitoring method has been developed for Fmoc chemistry in this group. The efficiency of each coupling is assessed by passing the deprotection mixture through a UV spectrophotometer and measuring the absorbance at 302nm (Scheme 2.1.6).[38]



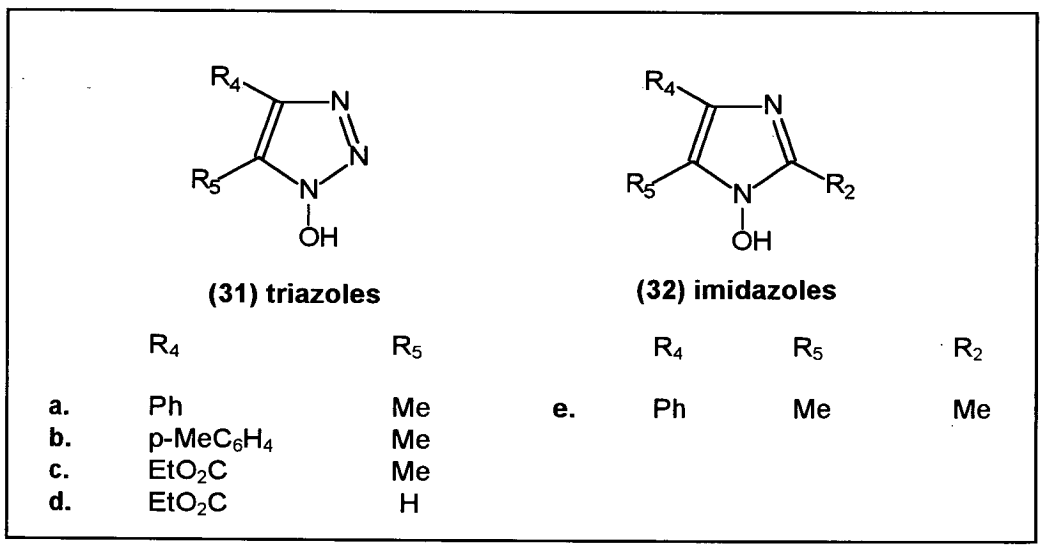
Scheme 2.1.6 Monitoring of Fmoc SPPS through deprotection

However, as shown in Scheme 2.1.6, if the coupling is found to be poor at this stage, the coupling can not be repeated since all the amino functions of the penultimate amino acid have been terminated and all the amino functions of the last amino acid coupled have been exposed. Obviously, examination of the acylation process before capping and deprotection would be of great advantage. HOBt, and its derivatives absorb strongly at 302nm, the wave length at which the Fmoc adduct is examined; therefore UV monitoring of the coupling mixture is not compatible with Fmoc methodology. Research effort was made in order to find alternative coupling reagents which do not absorb at 302nm and possibly have the same, or even better, coupling efficiency. This led to the development of a new coupling reagent, 1-hydroxyl-4-ethoxycarbonyl-1,2,3-triazole (HOCt, **31d**).[39]

2.2 Synthesis, properties and application of HOCt

2.2.1 Development of N-hydroxy heterocyclic compounds as potential coupling reagents and the problems involved

A series of N-hydroxy compounds were synthesised, and investigated as potential coupling reagents for SPPS, by Davison;[39] their structures are shown in Figure 2.2.1.

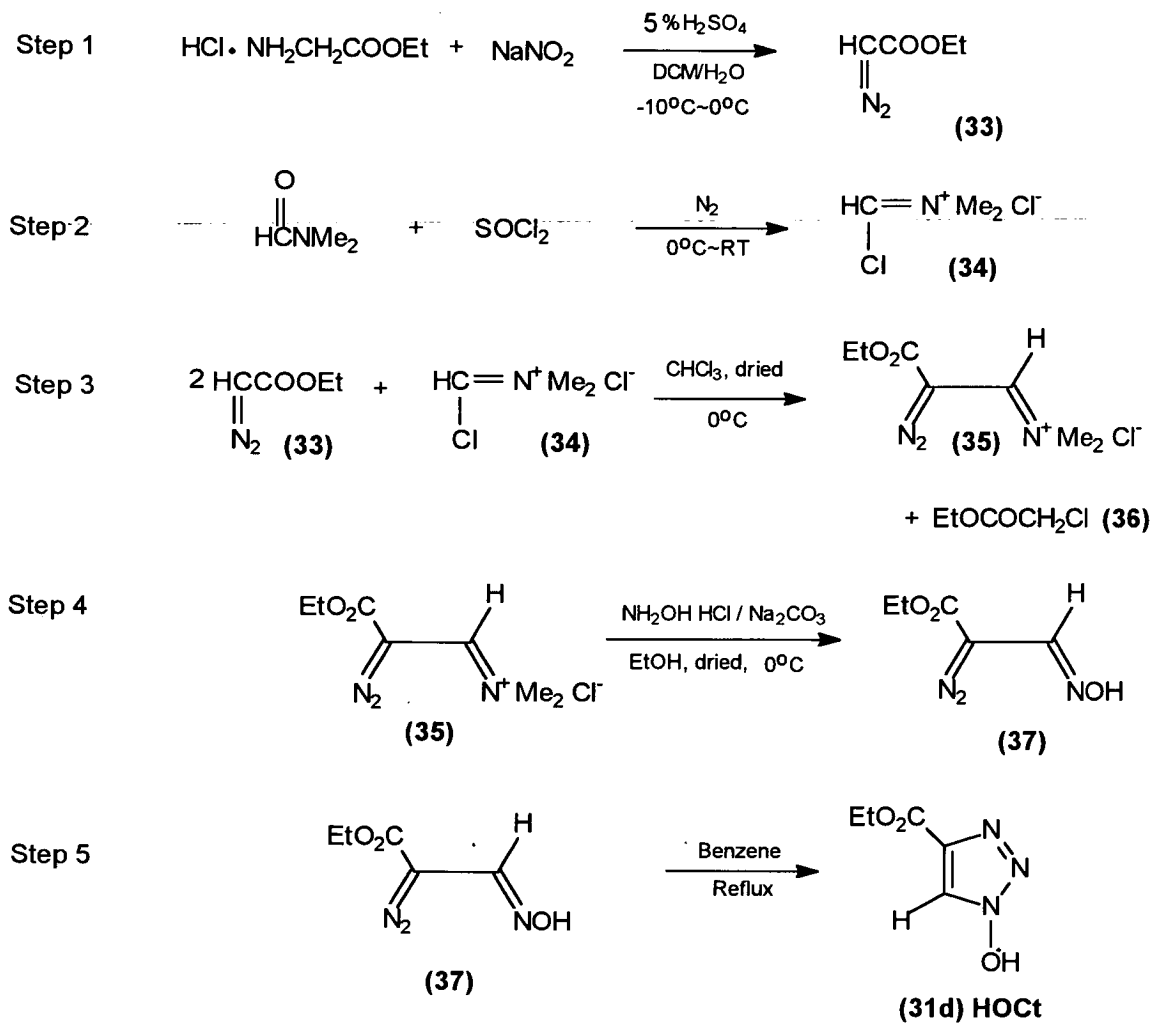


	R_4	R_5		R_4	R_5	R_2
a.	Ph	Me	**e.**	Ph	Me	Me
b.	p-MeC_6H_4	Me				
c.	EtO_2C	Me				
d.	EtO_2C	H				

Figure 2.2.1 The structure of new coupling reagents synthesised by Davison[39]

Of these, HOCt (**31d**) showed the most promising properties as an auxiliary nucleophile, which did not absorb at 302nm. However, problems were encountered during the synthesis of this compound.[39]

The synthesis of HOCt was first reported by Bennet *et al.*[40] in 1991. It was synthesised from the diazo aldehyde using a method previously developed by Stojanovic,[41] but no experimental details were given. A synthetic route based on Stojanovic's method was followed, and adjusted by Davison, until the expected product was afforded. The synthesis protocol developed by Davison is illustrated in Scheme 2.2.1.



Scheme 2.2.1 The process of triazole synthesis, Davison's method

Initially the whole synthesis took one week, and only gave an overall yield of ~5%. When the protocol was repeated, in order to obtain more material for further investigation, a lot of problems were encountered and the synthesis was found to be irreproducible. The final product was only afforded occasionally, and in a very low yield, but most often no product was isolated at all.[39] To overcome such difficulties, further investigation was obviously necessary.

2.2.2 Further investigation of the synthesis of HOCt---trouble shooting

The experiment was first carried out following Davison's procedure (Scheme 2.2.1), except that in the last step the solvent, benzene, was replaced by dry ethanol

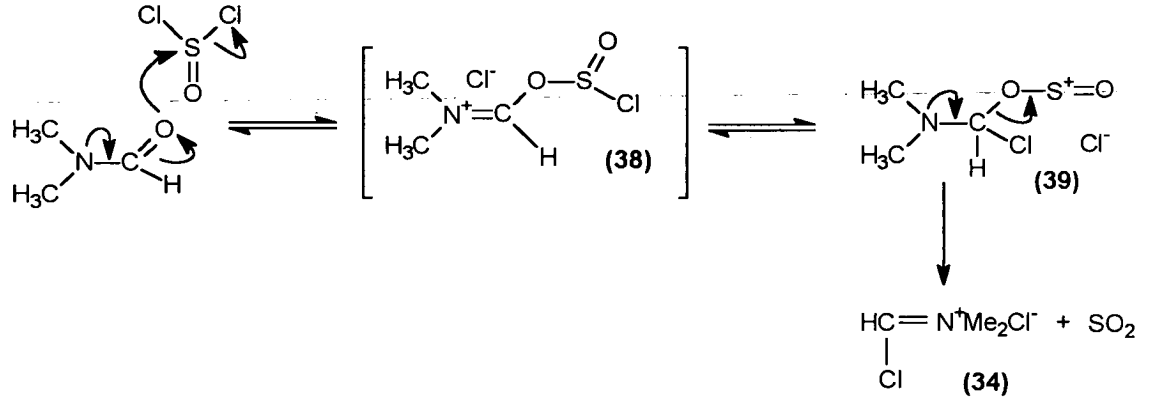
due to the toxicity of the former. The product was afforded in 42% overall yield without any major problem, although some steps were indeed found to need special attention. This will be discussed below.

The first improvement made was to step 5, the cyclisation step. It was found that an NMR sample of the diazo ester **(37)** in chloroform, when left at room temperature for two days, changed from the yellow diazo solution to completely colourless. The sample was checked by NMR again and it was found that the diazo compound had completely cyclised into triazole **(31d).** This was a very interesting result since the protocol developed by Davison involved refluxing the diazo compound in benzene in order to cyclise to the triazole, which is hazardous since diazo compounds are potentially explosive, especially at high temperature. Therefore an alternative protocol was suggested, the cyclisation being carried out in chloroform at room temperature. A few drops of acetic acid are added as a catalyst because it is present in $CDCl_3$ in a very small amount as an impurity.

The whole procedure for the synthesis of HOCt was repeated and investigations were carried out for the other steps in the scheme. The first step was carried out according to Searle's method[42] and pure product could be easily obtained in a very high yield (95~100%); therefore no further investigation was carried out on this step. The second step was also a known reaction which was originally reported by Bosshard *et al.*[43] The same procedure was followed and the product was afforded in quantitative yield, after evaporation *in vacuo* for 1~2h. The product was hygroscopic but was stable enough to handle in the atmosphere. Davison's study indicated that the isolated product was very unstable and handling under nitrogen was recommended. In fact it was found that the instability was due to the presence of the reaction intermediate **(38),** resulting from incomplete reaction in step 2 (Scheme 2.2.2). Thus step 2 needed to be optimised in order to overcome this problem.

As it is indicated by the mechanism (Scheme 2.2.2), the removal of the by-product, SO_2, helps to drive the reaction to completion. However, one of the starting materials, $SOCl_2$ is volatile, therefore it should be converted into the reaction intermediate before vacuum could be applied. It was found that this could be done by increasing the reaction time and temperature (~40°C, 3hr.); The mixture was then left

on a rotary evaporator, *in vacuo*, until a white solid was afforded. The temperature of the water bath could be raised up to 40°C. This protocol worked well.

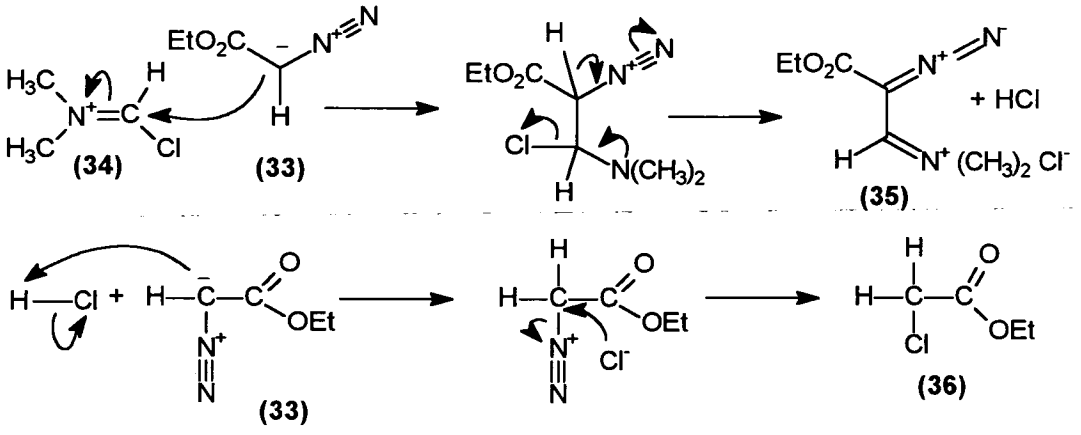


Scheme 2.2.2 The mechanism of reaction between DMF and $SOCl_2$

The compound **(34)** is a widely used acylating reagent originally developed by Vilsmeier *et al.* and is known as the Vilsmeier reagent.[44,45]

Two reactions are actually involved in step 3: the first is the required reaction between the diazo ester **(33)** and the Vilsmeier reagent **(34)**, and the second is the side reaction between **(33)** and HCl, the by-product of the main reaction. One extra equivalent of diazo ester **(33)** is therefore required to neutralised the HCl released from the reaction (Scheme 2.2.3).

The reaction was initially carried out in freshly dried chloroform as was illustrated in Scheme 2.2.1 and was found to be very vigorous, producing both heat and gas. Therefore, the diazo ester **(33)** was dropped in very slowly while stirring on an ice bath. The reaction was complete soon after the addition of the diazo ester and the reaction mixture was then evaporated whereupon the product was precipitated from ether as soft solid which was washed with ether and dried *in vacuo*. This product was found to be very unstable and should be used immediately. A small amount of the by-product **(36)** was always trapped in the soft solid, and was very hard to remove due to its high boiling point. This impurity did not affect the succeeding reactions, but it did affect the crystallisation of the final product, thus reducing the overall yield. This problem has also been solved by using a different protocol in the succeeding steps which will be discussed below.

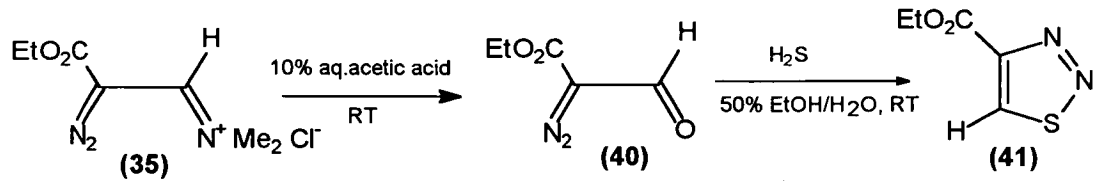


Scheme 2.2.3 The reaction mechanism of step 3

Having pinpointed the causes of the problem in this step, it has been found that it is not necessary to use freshly dried chloroform; commercially available A.R. grade $CHCl_3$ is good enough for this reaction. This change, together with the replacement of super dried ethanol by H_2O as solvent in the next step, greatly reduced the reaction time.

Step 4 was adapted from Stojanovic's method[41] (Scheme 2.2.4). The reaction was initially carried out in freshly prepared, absolutely dry ethanol, with a large excess of hydroxylamine (5eq.) and Na_2CO_3 (3eq.). The diazo ester was isolated as yellow solid after the removal of the solvent and precipitation with H_2O. The initial yield was around 30-40%.[39]

Further investigation of this reaction improved the yield to ~70% under the same conditions. It was found that the large excess of the inorganic reagents is not necessary, due to their poor solubility in ethanol, nor the absolutely dry ethanol, as a trace of water will not affect the reaction. In fact the involvement of water in the work up of the diazo oxime **(37)** suggested that water can be used as solvent in this reaction, although Davison's first attempt to carry out this reaction in aqueous conditions failed.[39] A mixture of DCM/H_2O was therefore used as biphasic solvent which resulted in only slightly reduced yield (~50%) on the first attempt. This result was very encouraging, and it was thus decided to carry out the reaction in water.



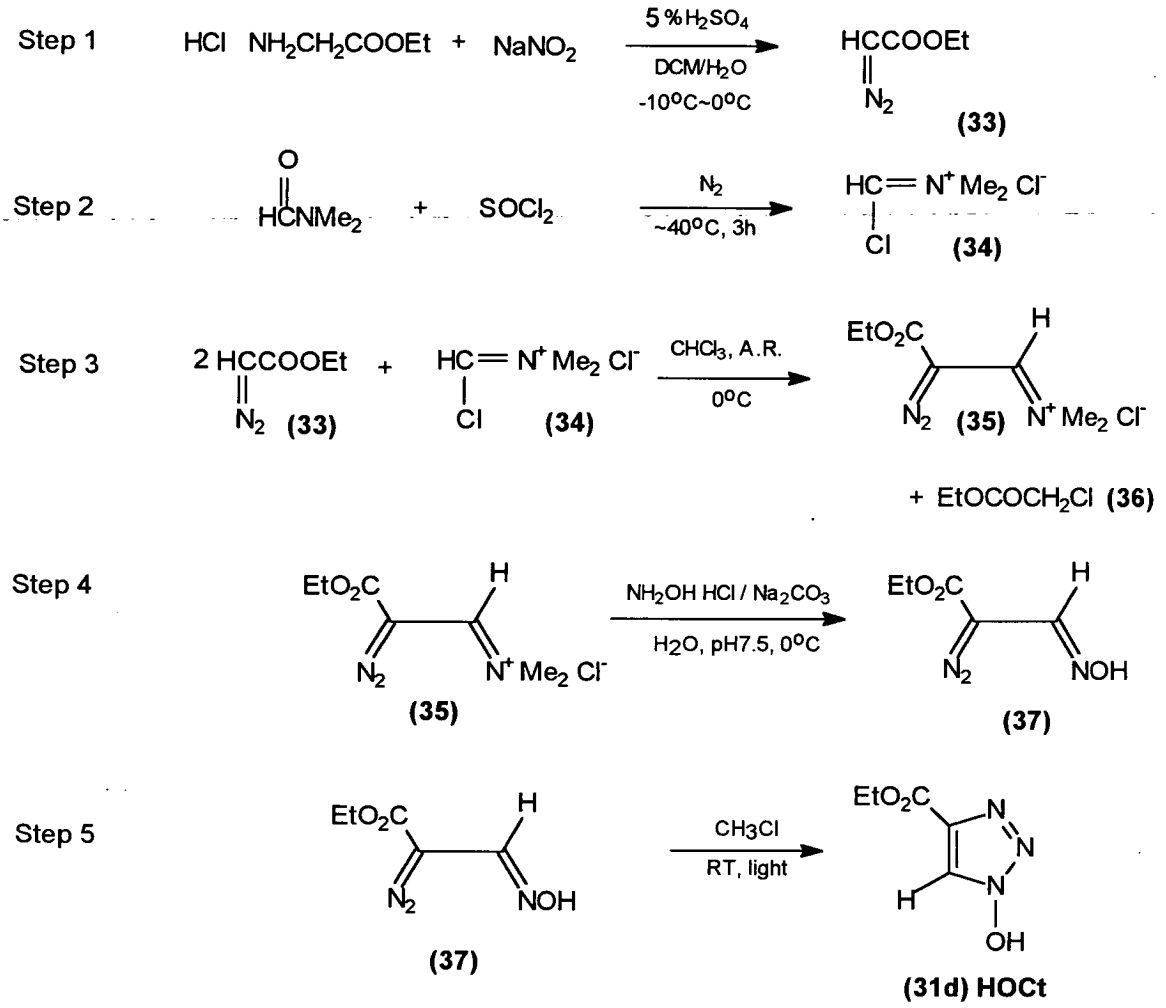
Scheme 2.2.4 Stojanovic's method for steps 4 and 5 (Scheme 2.2.1)

$NH_2OH.HCl$ (1eq.) was added to a little cold water (ice-salt bath) and stirred until it dissolved. Na_2CO_3 (0.5eq.) was then added as solution, followed by the addition of the diazo salt as solid. Bright yellow solid precipitated at once. The mixture was stirred on an ice-salt bath for 30 min and filtered. The yellow solid was washed with cold water and dissolved in chloroform. The small amount of water was removed in a separatory funnel and the chloroform solution dried over $MgSO_4$. A little acetic acid was added to the solution and it was left at room temperature to cyclise. It was found later that light also aids the cyclisation.

The final product can be isolated in a slightly lower overall yield (35%) compared with the ethanol method, but it has the great advantage that no solvent needs to be removed *in vacuo.* As all the materials are very cheap, the yield is not the most important factor compared to safety. Another advantage is that any impurity which is carried through from the previous steps can be isolated by filtration which makes the final cyclisation and recrystallisation much easier. This protocol is thus adopted. The lower yield of this new method is partially due to the formation of triazole, which is very soluble in water and thus very hard to recover.

Step 4 was further investigated by Robertson,[46] and the optimal reaction conditions were found to be pH 7.0~7.5, 0°C for 5 min. This can further reduce the reaction time.

After all these studies, a final procedure for the synthesis of HOCt has been developed as a very convenient protocol which can be carried out easily in two working days (Scheme 2. 2.1a). An average overall yield of 30~35% can be achieved reproducibly.

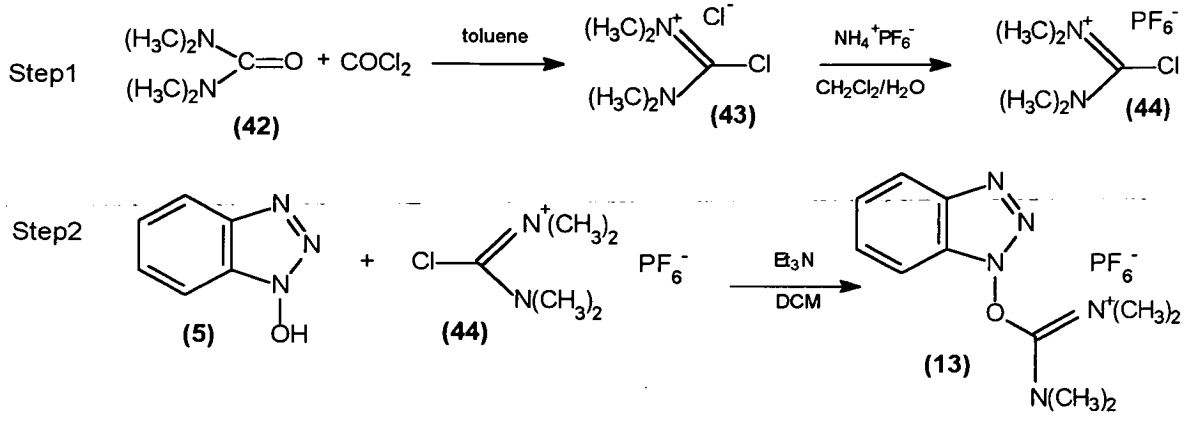


Scheme 2.2.1a. The modified protocol of triazole synthesis

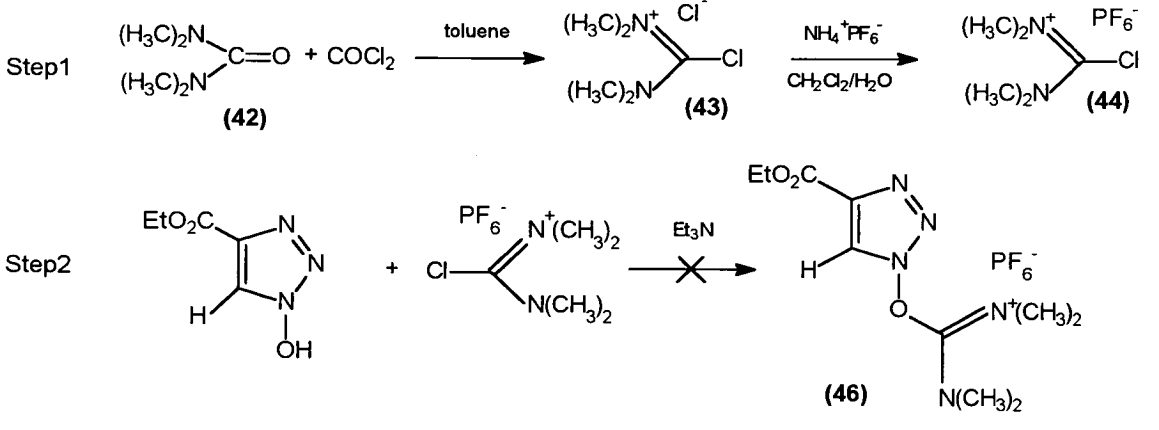
2.2.3 Attempted synthesis of the HOCt Knorr reagents

With the problems encountered in the synthesis of triazole solved, further investigation was carried out to study the HOCt Knorr reagents. The general procedure for making HBTU is shown in Scheme 2.2.5.[21,22]

The same procedure was carried out on HOCt, as shown in Scheme 2.2.6. Unfortunately, none of the expected solid material precipitated. Instead, a yellow oil was always afforded, suggesting that the triazole ring had opened, resulting in a diazo compound.



Scheme 2.2.5 The synthesis of HOBt Knorr reagent, HBTU



Scheme 2.2.6 Attempted synthesis of HOCt Knorr reagent, TCTU

It was initially assumed that this was caused by the relatively strong basicity of the triethylamine used. Mild bases were thus tried, i.e. imidazole, 2,4-lutidine, piperidine and Na_2CO_3, but without success. The same yellow oily residue was always afforded, which could not be crystallised. ^1H NMR spectrum of this residue showed a mixture of two compounds. The mixture was thus separated by flash chromatography, isolating only one of the products as a yellow oil. The IR spectrum showed three typical peaks at 2229cm^{-1}(CN group), 2135cm^{-1} (diazo group), and 1716cm^{-1} (carbonyl group). The ^1H NMR data showed only one ethyl group while the ^{13}C NMR

showed one CH_3(13.66), one CH_2 (62.72) and two quaternary C's (106.7 and 160.4) (Figure 2.2.2 (a, b, c)).

The data indicated the presence of a diazonitrile compound (**51**) and a mechanism is thus proposed, as shown in Scheme 2.2.7.

There are two possible routes leading to the same product. Route A is very similar to the mechanism proposed by Dulcere for the conversion of aldoxime to nitrile using the Vilsmeier reagent (**34**).[44] If this is the case, the triazole ring itself must be fairly unstable, and should exist in equilibrium with the diazole isomer. However, experiments indicate that the triazole ring is very stable under either basic or acidic conditions (2M NaOH or HCl for a few months). Obviously, the strongly nucleophilic reagent must be involved in the ring opening step. Route B, the nitrene mechanism, is thus proposed. The intermediate (**49**) is very similar to β-styrylnitrene, an intermediate proposed for the deoxygenation reaction of β-nitrostyrene.[47,48] The presence of the strongly electrophilic carbon greatly reduces the stability of the triazole ring and two intramolecular rearrangements occur to give the more stable diazonitrile compound (**51**). This mechanism clearly indicates that the Knorr reagents of HOCt cannot be isolated as stable compounds. This was confirmed by the failure of another attempt to make the BOI analogue of HOCt (Scheme 2.2.8).

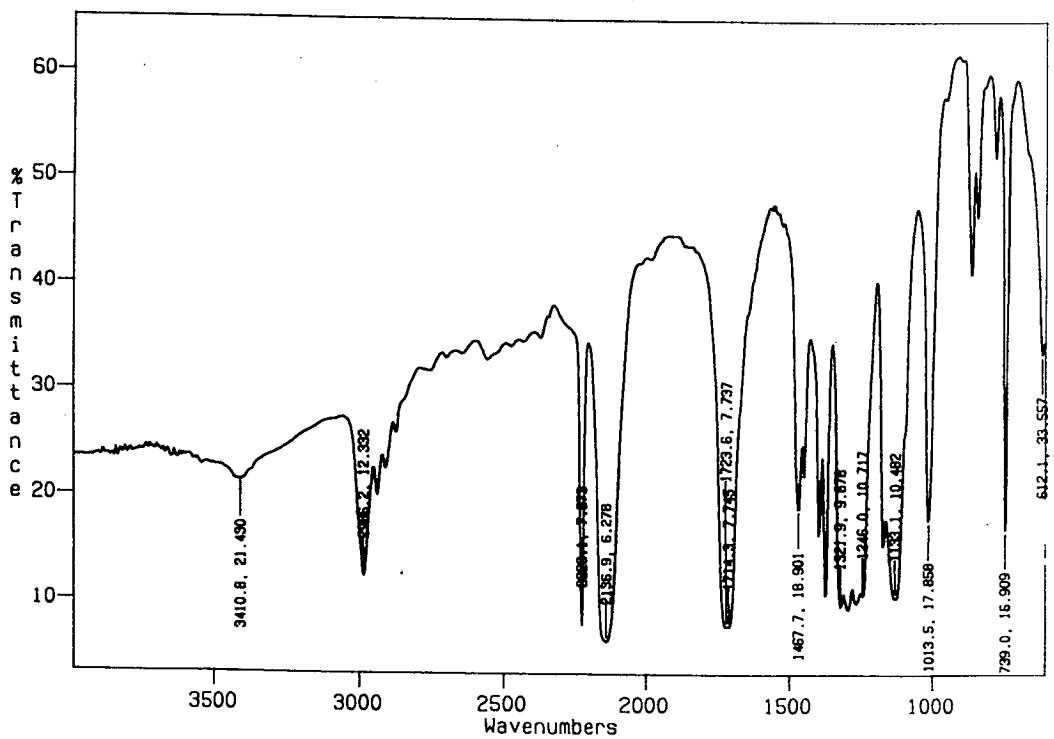


Figure 2.2.2(a) IR spectrum of the diazonitrile compound

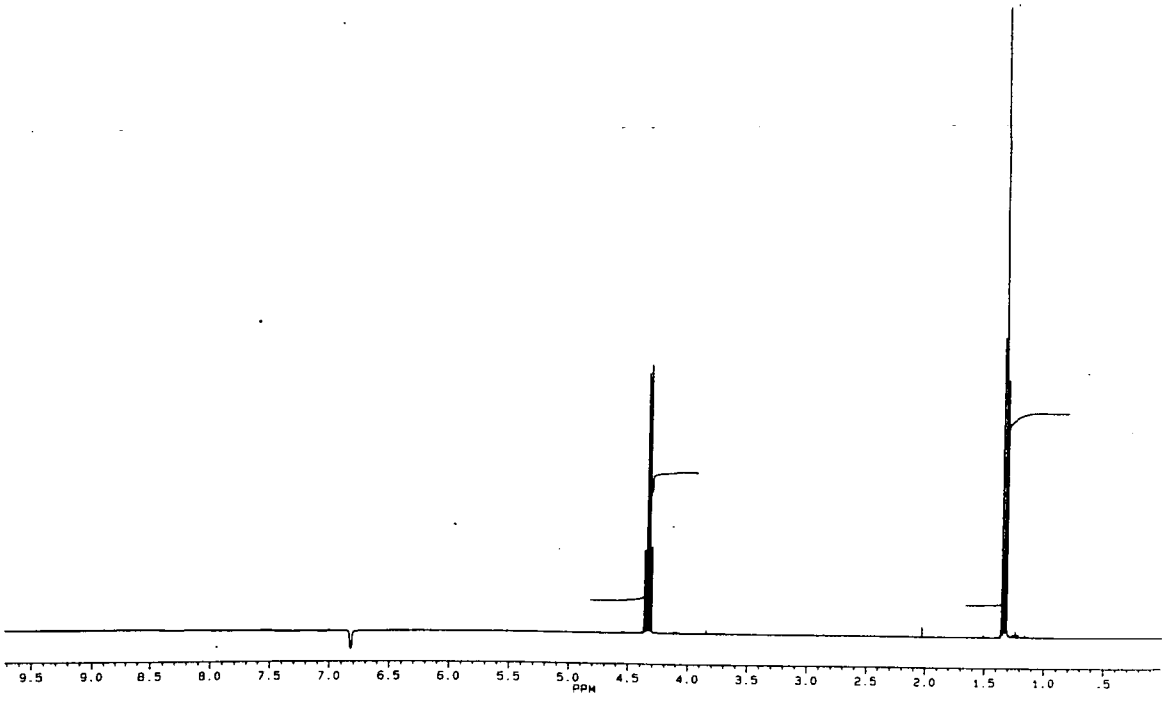


Figure 2.2.2(b) ^{1}H NMR spectrum of the diazonitrile compound

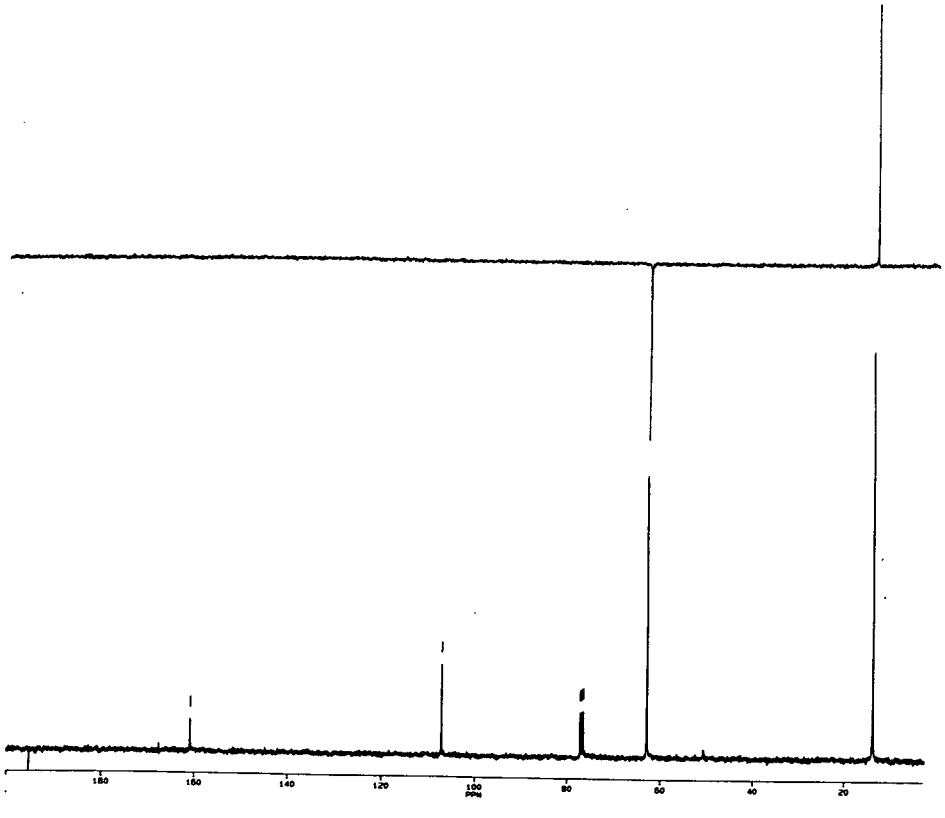
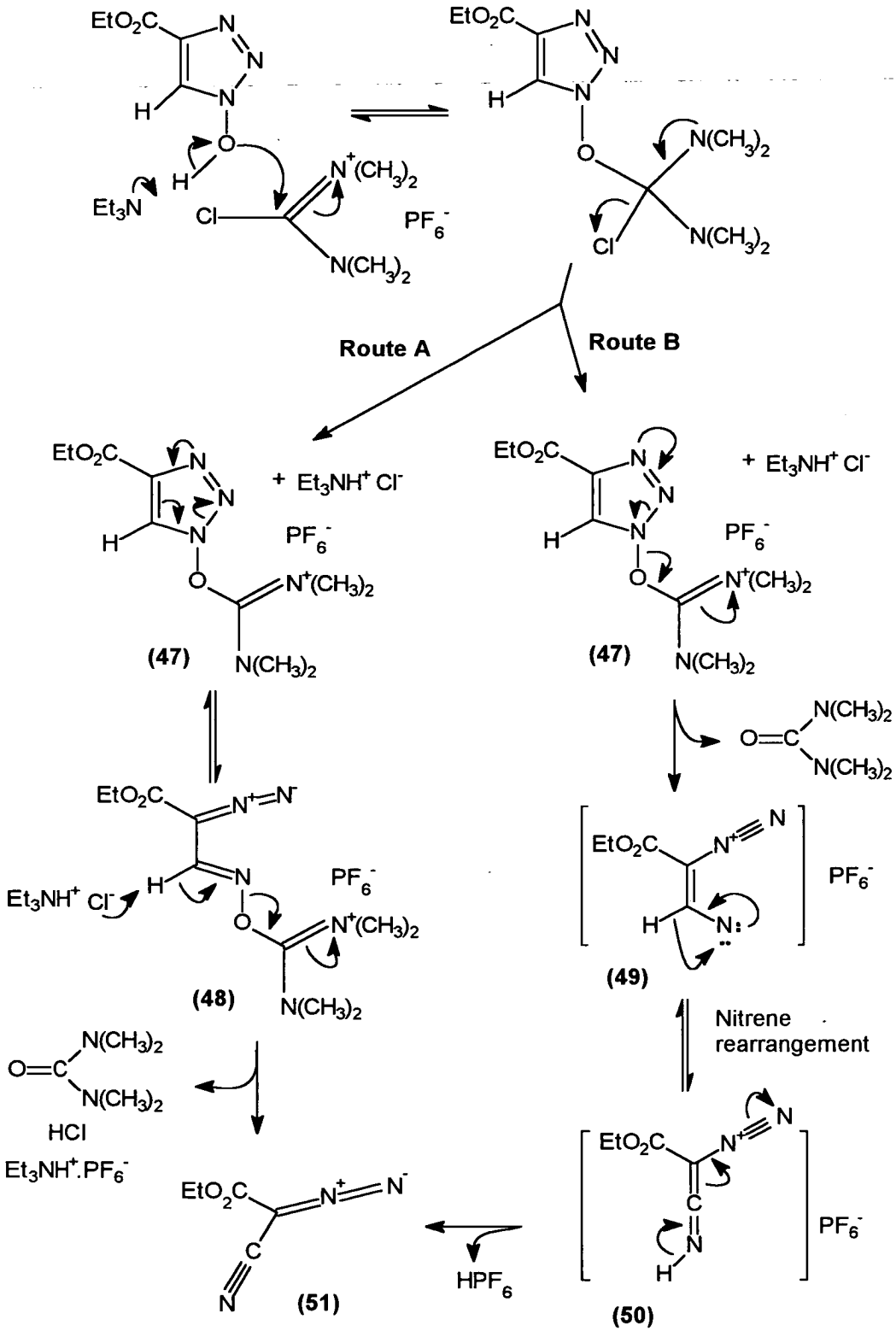
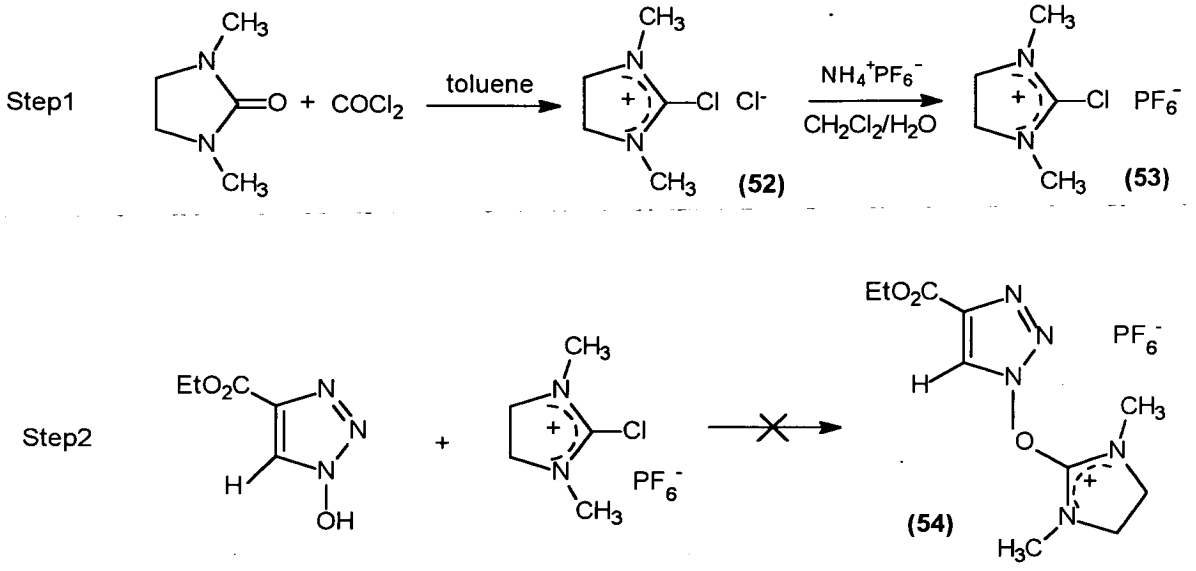


Figure 2.2.2(c) ^{13}C NMR spectrum of the diazonitrile compound



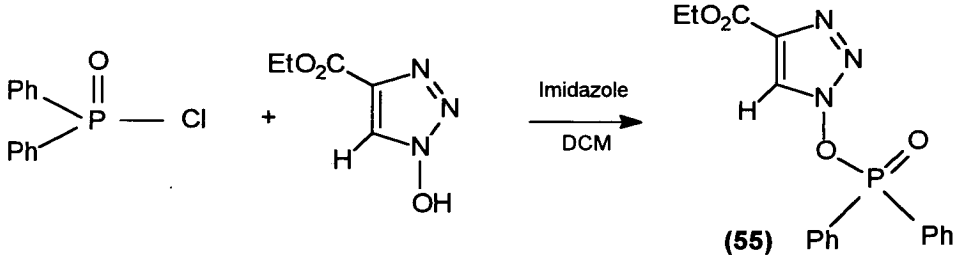
Scheme 2.2.7 The ring open mechanism of the HOCt Knorr reagent



Scheme 2.2.8 Attempted synthesis of HOCt analogue of BOI

2.2.4 Synthesis of triazole phosphate as a coupling reagent

Another derivative of HOCt, the phosphate, was also investigated as a potential coupling reagent. The reaction is shown in Scheme 2.2.9.



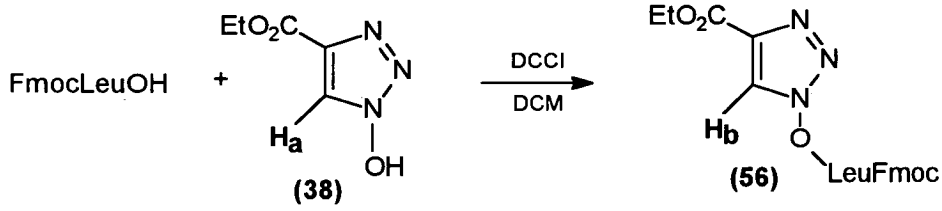
Scheme 2.2.9 Synthesis of triazole phosphate

The reaction went well and the product was isolated in almost 100% yield. ^{1}H and ^{31}P NMR indicated that it was the correct material.

2.2.5 The HOCt active esters

Active esters have become increasingly popular as coupling reagents in stepwise SPPS. The active esters of HOCt were therefore investigated. FmocLeuOH was chosen as the standard for reaction condition studies, as its side chain structure is

simple and provides reasonable steric hindrance. The reaction itself is straightforward as shown in Scheme 2.2.10.



Scheme 2.2.10 Synthesis of active ester of Fmoc Leu OH

DCCI was used as the dehydrating reagent because the by-product DCCU, was easy to remove by filtration. The reaction was carried out at various temperatures, monitoring by TLC, which indicated the presence of the starting materials, even after stirring overnight. The mixtures were thus filtered and evaporated to yield a white solid which was examined by NMR. A singlet which did not correspond to any of the starting materials was found with a chemical shift of ~8ppm. This was found to belong to the olefinic proton of the active ester ($\mathbf{H_b}$ Scheme 2.2.10). NMR data also indicated that there was ~20% unreacted HOCt present in the mixture [$\mathbf{H_a/(H_a+H_b)}$]. Further investigation showed that the active ester is very sensitive to moisture and would hydrolyse on prolonged stirring.

Therefore an indirect method was designed to confirm the formation of the active ester. FmocLeuOH and HOCt (0.5mmol each) were dissolved in dry DCM to which was added DCC (0.5mmol). The mixture was stirred at room temperature for 30 min, the white solid filtered off and the filtrate evaporated until dry. The residue was then dissolved in DMF/dioxane (1:1, 4ml) and the mixture was added to a reaction vessel, which contained 0.25mmol H_2N-Ala-resin. The mixture was vortexed on the synthesiser for 30 min before coupling was determined by measuring the Fmoc deprotection. The deprotection of Leu was 85% of that of Ala, which indicated approximately 85% coupling. As no other activating reagent was added, the active intermediate could only be the active ester.

Reaction conditions were found to be optimal at 0^0C, in dry DCM and under nitrogen, therefore the active esters of all the common amino acids were prepared

under these conditions. The ^{1}H NMR of a few examples is illustrated in Figure 2.2.3 (a,b,c).

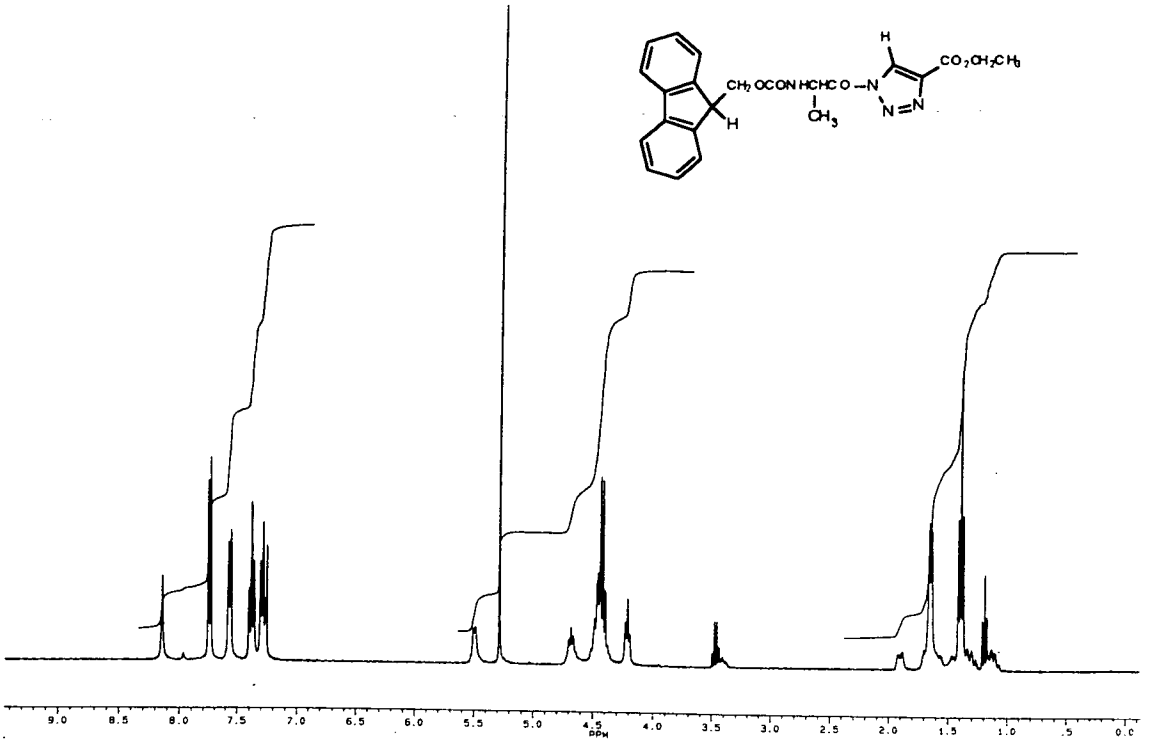


Figure 2.2.3(a) ^{1}H NMR spectrum of FmocAlaOct, ~100% ester;

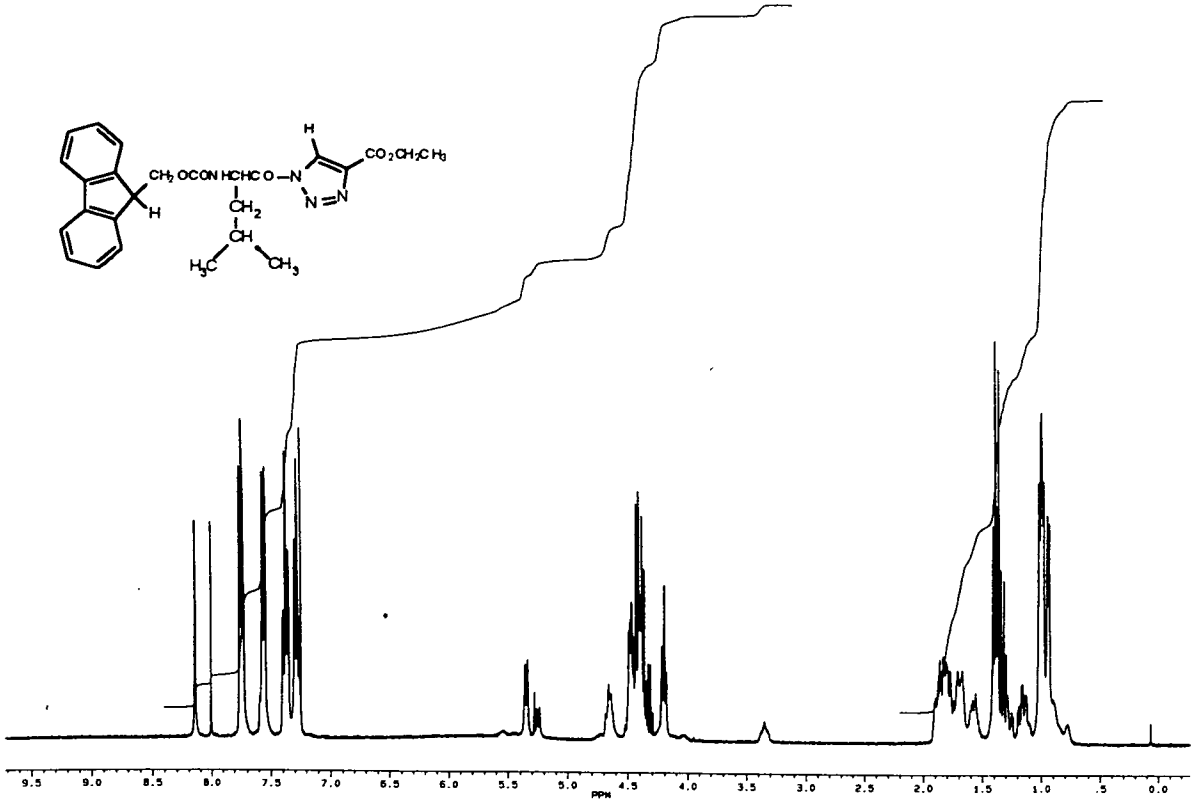


Figure 2.2.3(b) ^{1}H NMR spectrum of FmocLeuOCt, ~70% ester;

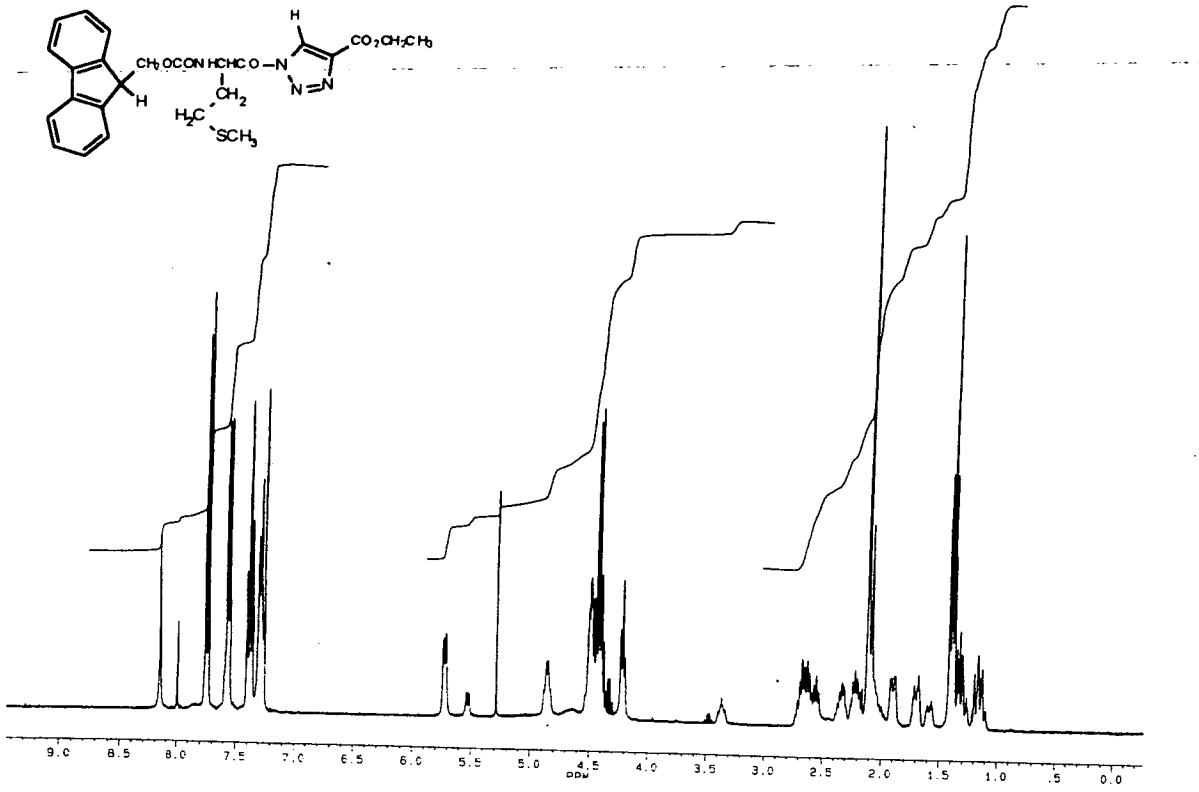


Figure 2.2.3(c) ^1H NMR spectrum of FmocMetOCt, 90% ester

As all starting materials were mixed up in exactly 1:1 ratio, and all the soluble materials in the reaction mixture were collected after filtering off the insoluble DCCU, the percentage of the free triazole in the mixture should be equal to the percentage of the free acid. The percentage of active ester of each amino acid was calculated and the results are listed in Table 2.2.1.

Although NMR data indicated that a high percentage of free acid was present in some cases, this did not necessarily mean that the reaction was not completed. As the active esters are highly hygroscopic, hydrolysis could occur to give the corresponding free acid and HOCt. Therefore the percentage of the active ester in the mixture may actually indicate its relative sensitivity to moisture. Some of the esters are too unstable to be detected by NMR. Even the most stable ones are very hygroscopic and were thought unsuitable for storage, therefore no further experiments were carried out.

Table 2.2.1 Chemical shift of the olefinic proton in HOCt and the active esters, percentage of active ester in the reaction mixtures

	δ of the olefinic H on triazole		ester
	δ H in HOCt	δ H in ester	%
Fmoc Ala OCt	-	8.14	100
Fmoc Arg(Pmc) OCt	7.97	-	0
Fmoc Asp(O^{t}Bu) OCt	7.98	8.13	70
Fmoc.Cys(Acm) OCt	8.00	8.24	30
Fmoc-Cys(tBu) OCt	8.03	-	0
Fmoc Glu(O^{t}Bu) OCt	7.99	8.20	70
Fmoc Gly OCt	8.00	8.20	35
Fmoc His(Trt) OCt	-	8.15	100
Fmoc Ile OCt	8.00	8.13	80
Fmoc Leu OCt	8.01	8.15	80
Fmoc Lys(Boc) OCt	7.97	8.15	90
Fmoc Met OCt	7.99	8.15	90
Fmoc Nle OCt	8.00	8.14	70
Fmoc Phe OCt	8.01	-	0
Fmoc Pro OCt	8.00	8.19	50
	8.03	8.20	88
Fmoc Ser(tBu) OCt	-	8.11	100
Fmoc Thr(tBu) OCt	-	8.11	100
Fmoc Trp OCt	n.s	n.s	n.s.
Fmoc Tyr(O^{t}Bu) OCt	7.99	8.03	70
Fmoc Val OCt	8.00	8.14	85

NMR solvent: $CDCl_3$
n.s.: insoluble, -: no corresponding peak.

2.2.6 The reaction between HOCt and DIC, NMR study of the reaction mechanism

An interesting result was observed during the study of the active esters. If the triazole and DIC were mixed in solution, a yellow colour gradually appeared, which indicated the triazole ring was opening to give a diazo compound. A mechanism for

the reaction is suggested, as shown in Scheme 2.2.11, which is very similar to the decomposition mechanism of HOCt Knorr reagent (Scheme 2.2.7).

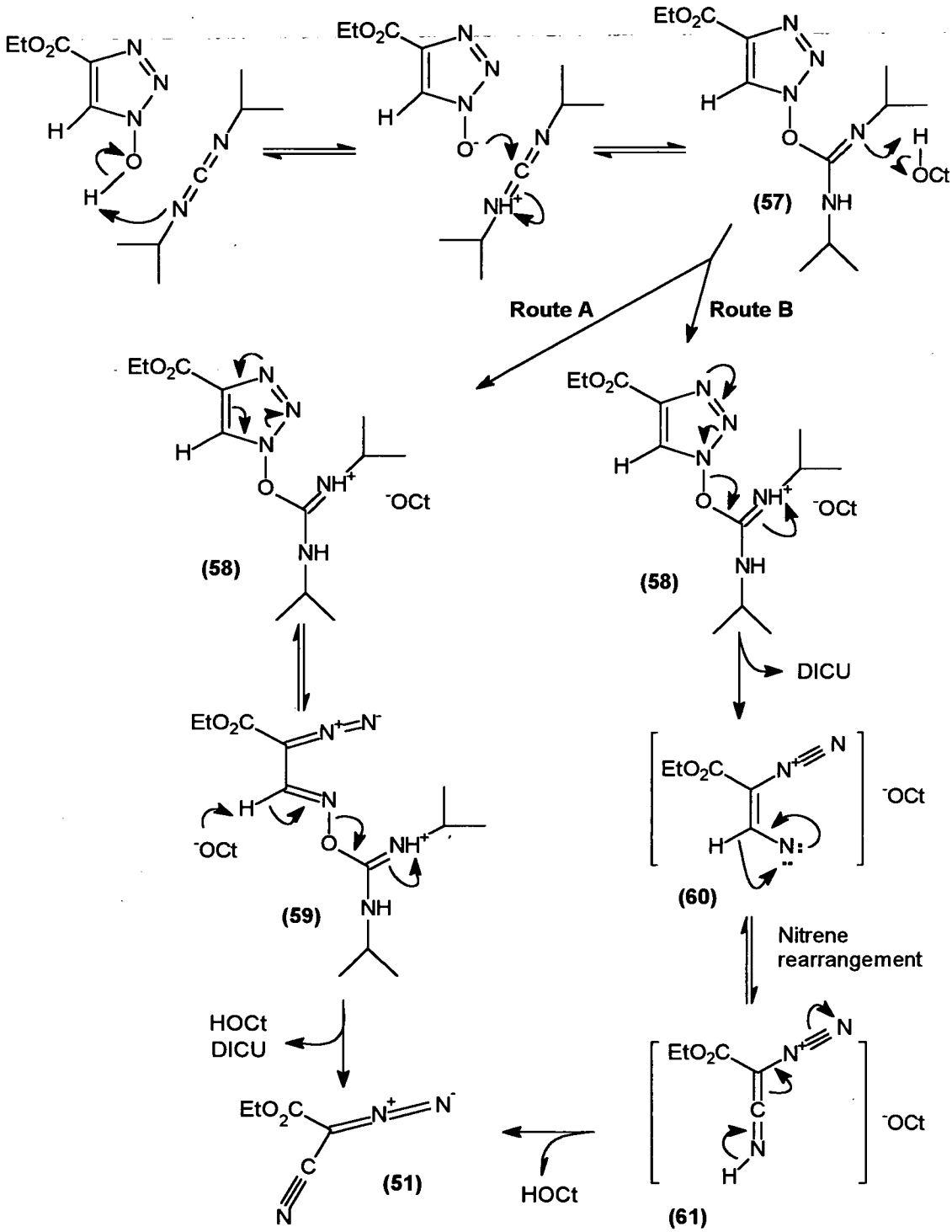
In order to ascertain the correct mechanism involved in the above reaction, the reaction mixture was monitored by acquiring real time proton NMR spectra. The HOCt (31.4mg, 0.2mmol) was dissolved in $CDCl_3$ and cooled to -30°C. To the solution was added DIC (31.4µl, 0.2mmol) and a ^1H NMR spectrum was acquired immediately. The temperature of the NMR spectrometer was set at -30°C and then gradually increased to 25°C over a 132 minute period. The ^1H NMR spectra were acquired at different intervals as illustrated in Figure 2.2.4.

The reaction took place immediately, as indicated by the additional signals appearing on the NMR spectrum (Figure 2.2.4a); these can be assigned to the reaction intermediate **(58)** as shown in Figure 2.2.4a, which presents in equilibrium with the starting materials; but no product formed at this stage.

From Figure 2.2.4a it can be seen that no oxime **(59)** signals remain in the spectrum, including the extra olefinic proton signal which is expected ca. 7.3ppm. The broad peak at 7.2ppm was initially thought to be the oxime proton of **(59)**, but the integration of the three olefinic proton signals (8.3, 8.0 and 7.2ppm) together results in more than one proton, which is not possible. According to this result, route B is more likely to be the real mechanism for this reaction. However, if **(59)** present as a transition state rather than an intermediate, route A would also be a possible mechanism.

On continuation of the experiment, no change was observed until the temperature was increased to -5°C in 49 min. At this stage another methyl group doublet began to show up on the higher frequency side of the original ones. This indicates that a new kind of isopropanyl group has begun to form, together with a new multiplet at 3.7ppm (Figure 2.2.4c). These signals were assigned to the final by-product, DIU. The newly formed doublet and multiplet became stronger when the temperature was further increased whilst all signals belonging to the intermediate **(58)** decreased in size (Figure 2.2.4d,e). When the temperature was raised to 25°C in 123 min all the signals belonging to the intermediate **(58)** disappeared (Figure 2.2.4f) as well as the starting materials. The small amounts of HOCt and DIC left at this stage are due to the precipitation of DIU, which prevented further reaction. The reaction

was left at 25°C with monitoring, overnight, but no further reaction was observed. No signals of the transition states were observed because reaction was too fast in relation to the NMR time scale.



Scheme 2.2.11 The mechanism of the reaction between DIC and triazole

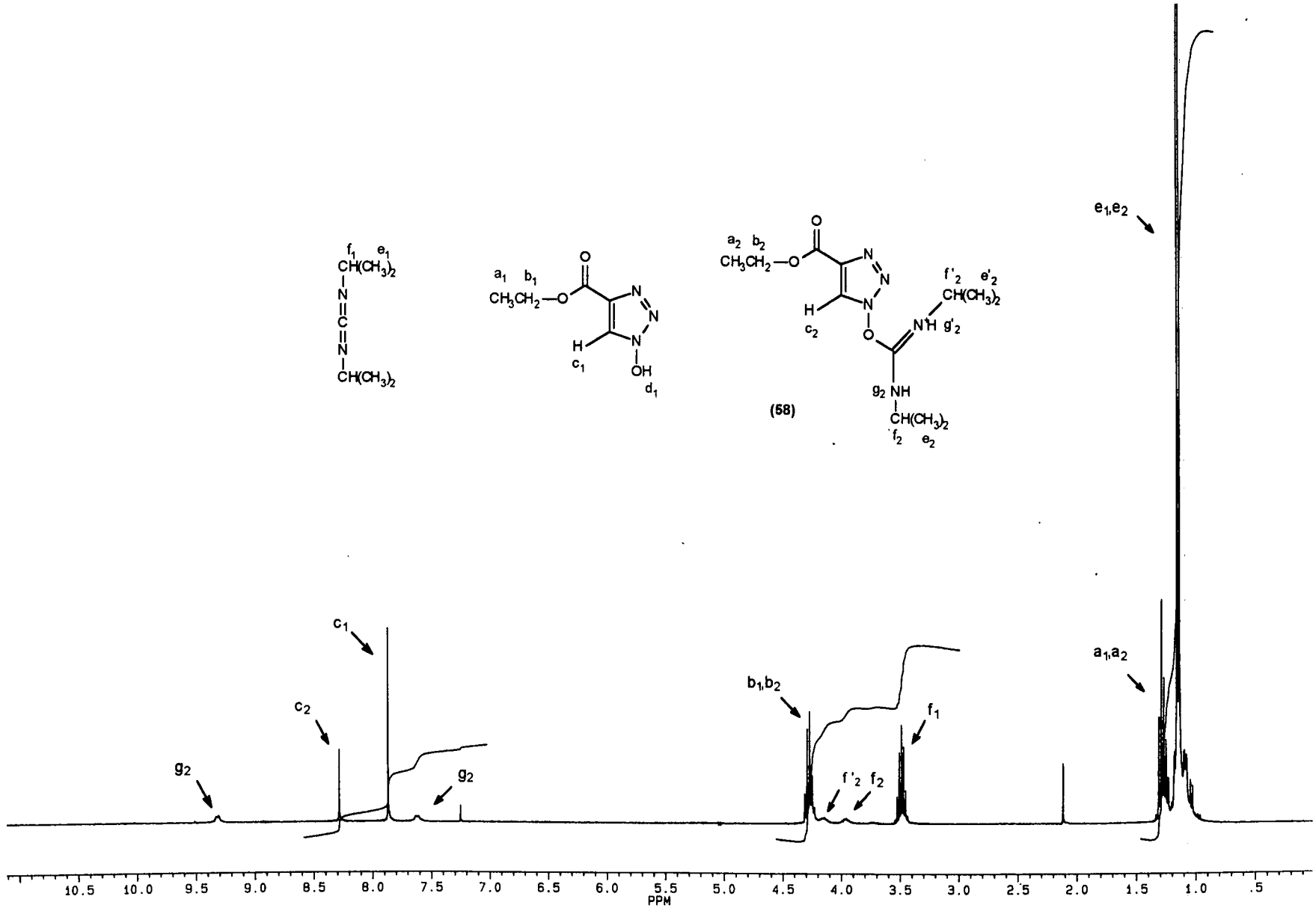


Figure 2.2.4a NMR monitoring of the reaction between HOCt and DIC in $CDCl_3$, -5°C, 38 min

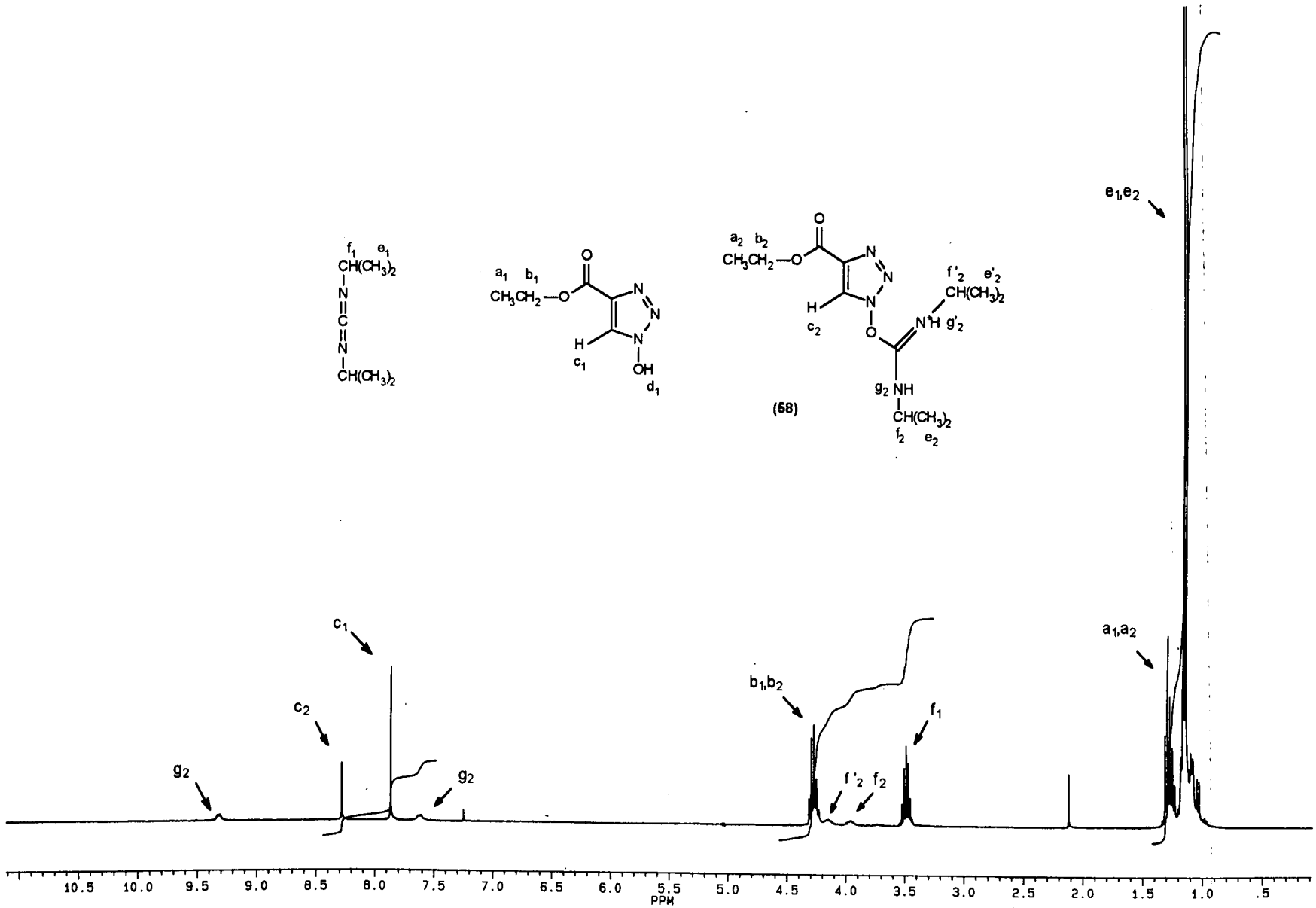


Figure 2.2.4b NMR monitoring of the reaction between HOCt and DIC in $CDCl_3$, -5°C, 43 min

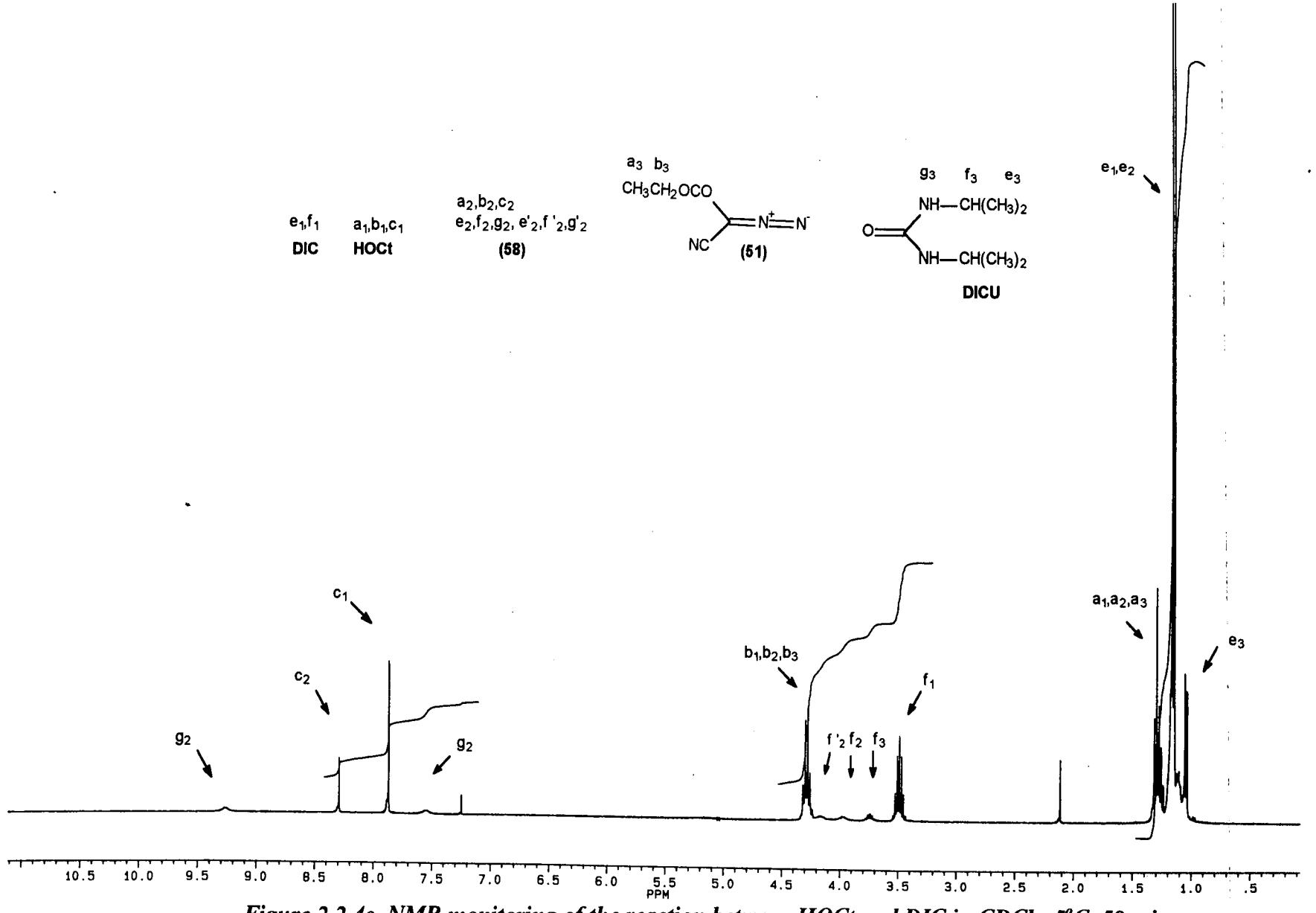


Figure 2.2.4c NMR monitoring of the reaction between HOCt and DIC in $CDCl_3$, 5°C, 59 min.

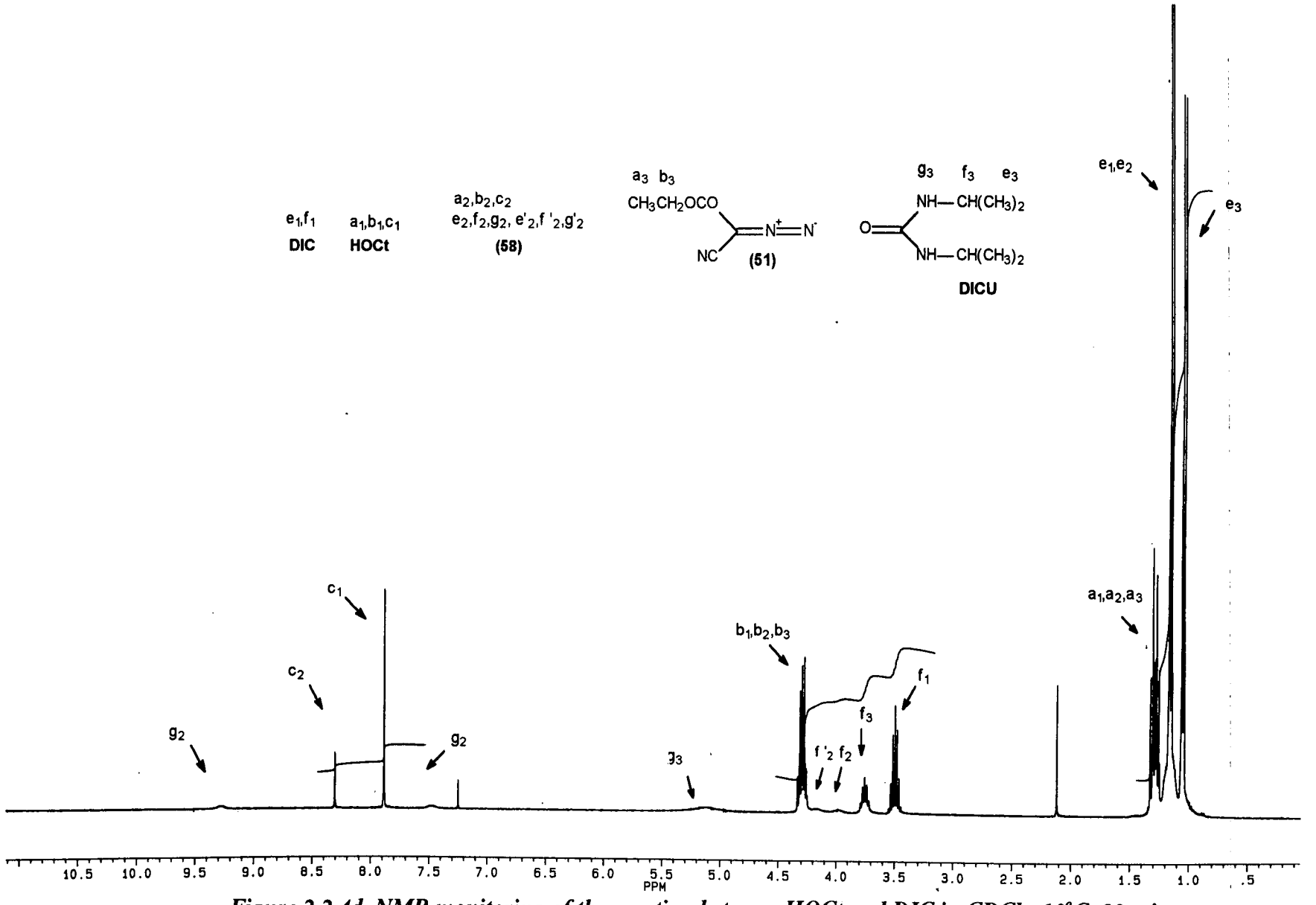


Figure 2.2.4d NMR monitoring of the reaction between HOCt and DIC in $CDCl_3$, 10°C, 83 min.

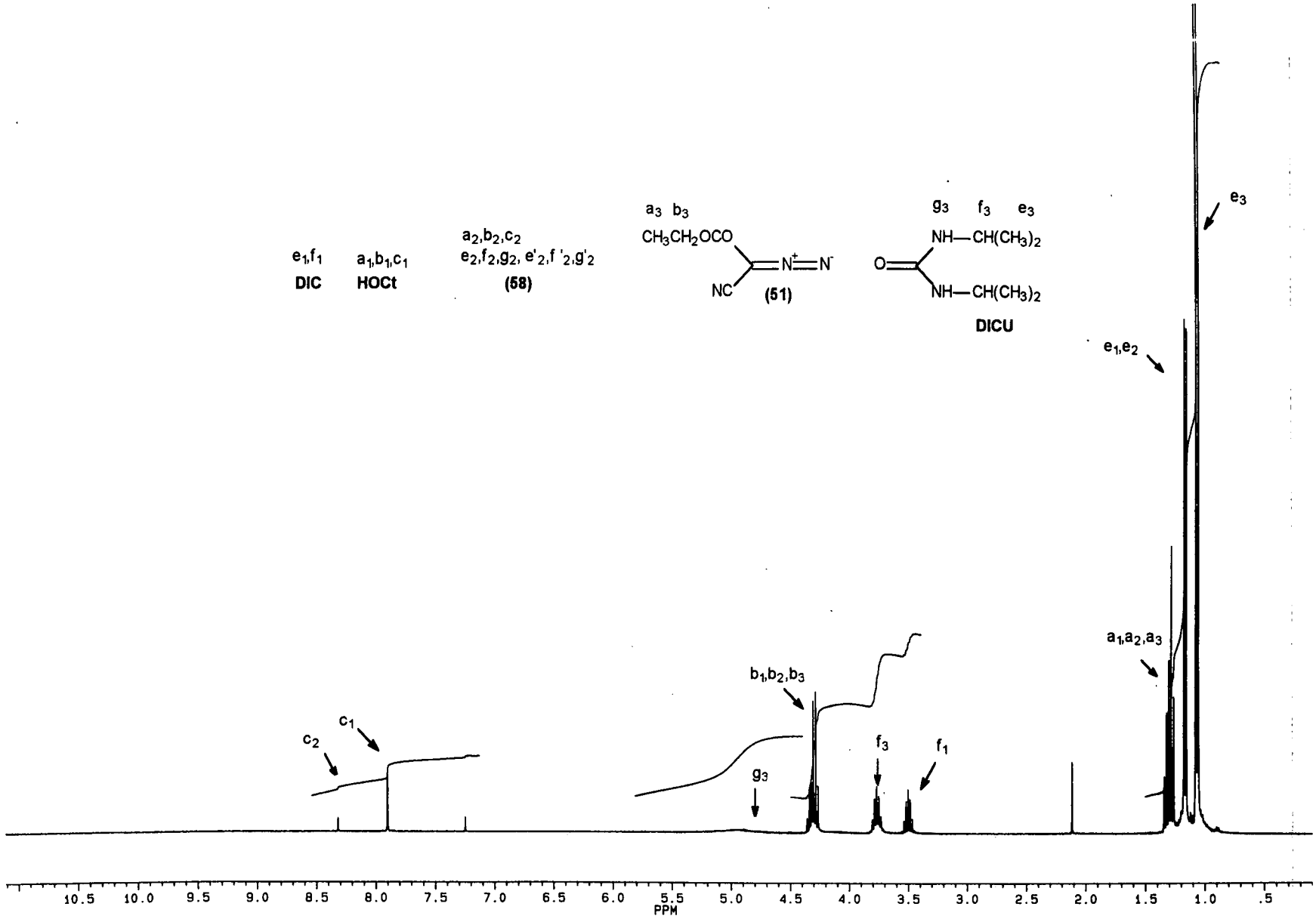


Figure 2.2.4e NMR monitoring of the reaction between HOCt and DIC in $CDCl_3$, 25°C, 92 min.

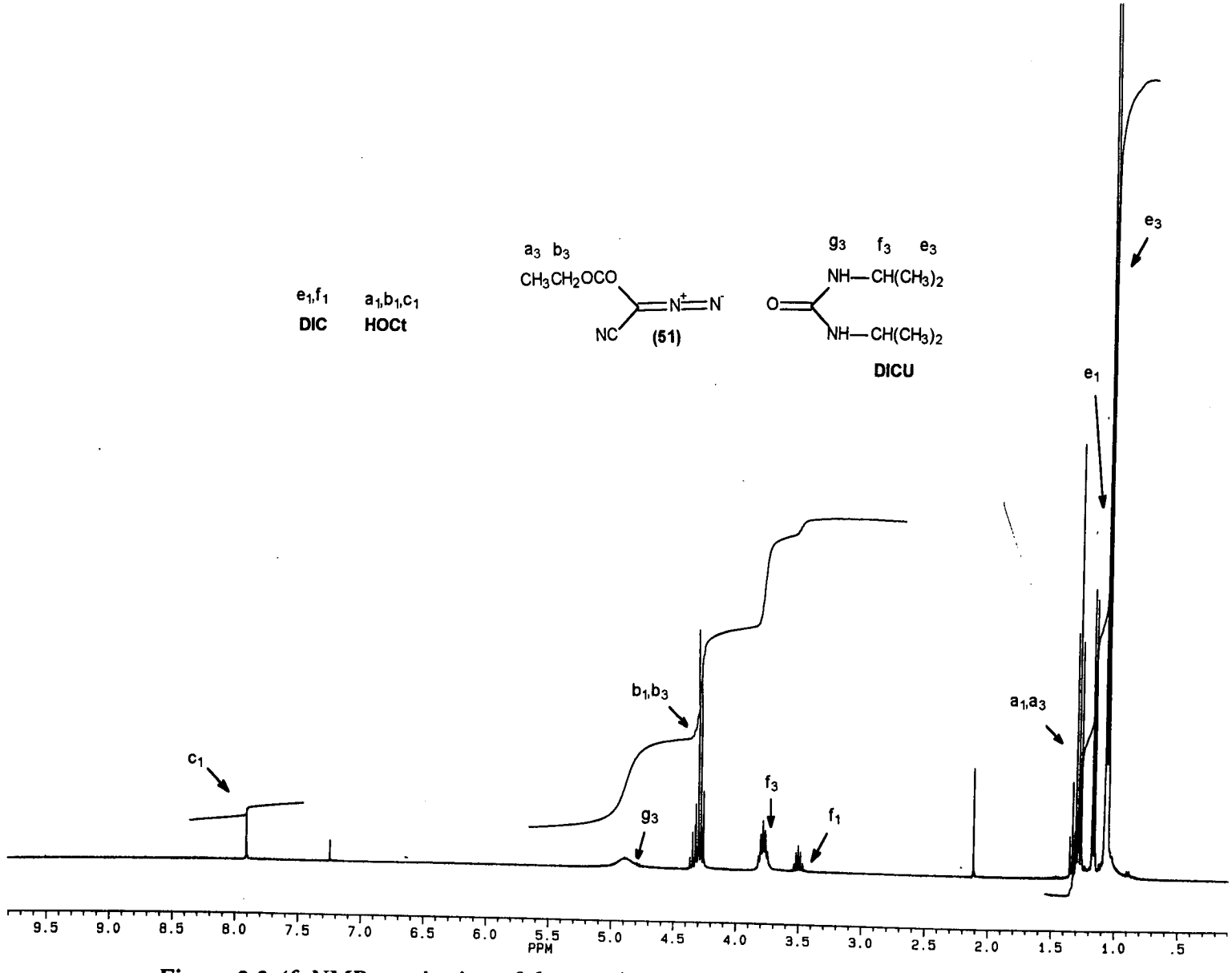
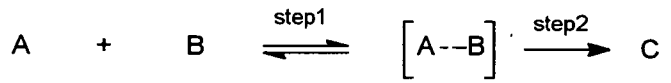


Figure 2.2.4f NMR monitoring of the reaction between HOCt and DIC in $CDCl_3$, 25°C, 123 min.

These results clearly indicate that the reaction intermediate **(58),** which is very similar with the HOCt Knorr reagent in structure, can only exist at low temperature (<-5°C), when the temperature was increased, it soon decomposed to result in more stable compounds. When the experiment was repeated, the same was observed. These results suggest that the HOCt Knorr reagent can not be isolated at any temperature above 0°C. Therefore no further attempt was made in making HOCt reagents.

The reaction between HOCt and DIC can be simplified as follows:



The relative concentrations of the starting materials (HOCt, DIC), reaction intermediate **(58)** and products (DIU, **51**) were calculated from the NMR spectra and the results are shown in Figure 2.2.5. Data indicate that the reaction intermediate is present in equilibration with the starting materials at low temperature (-30 ~ -5°C). The equilibration shifted when the temperature rose to -5°C and the product begun to form. Once started, this second step went on faster than the first step, therefore the concentrations of the starting materials and the reaction intermediate both decreased rapidly, until the reaction was complete. Small amounts of the starting materials were trapped in the mixture due to the precipitation of the product, DIU.

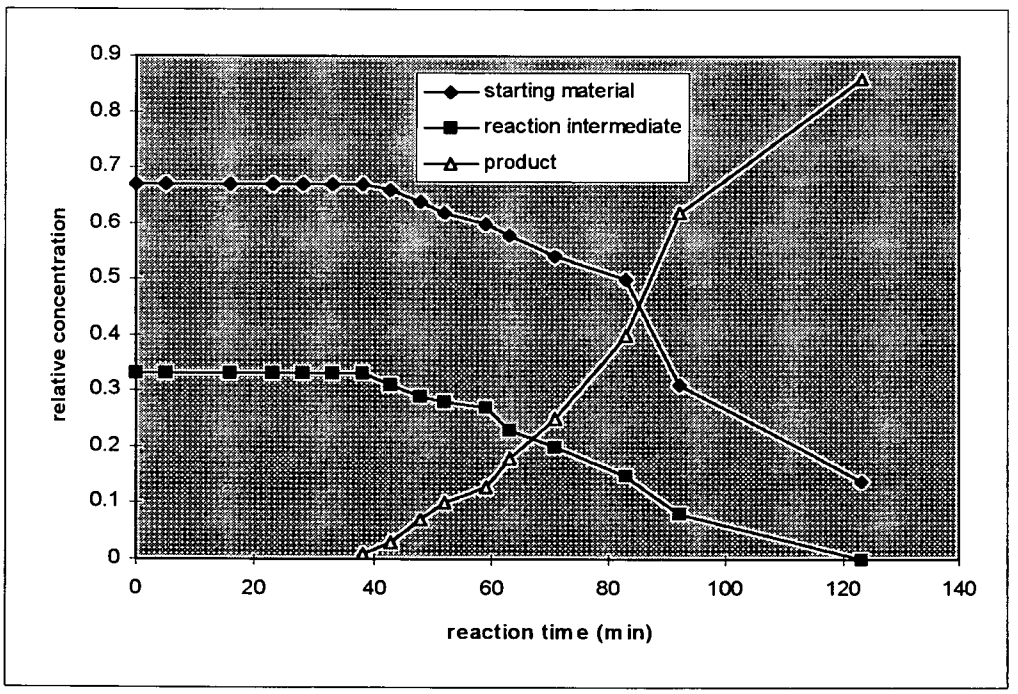
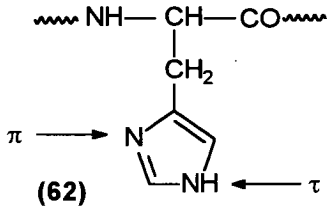


Figure 2.2.5 Relative concentrations of the material involved in the reaction between HOCt and DIC

2.2.7 Racemisation studies of HOCt

Racemisation remains the major side-reaction in SPPS, and any new coupling reagent should be well studied before use. Among the twenty native amino acids, His is especially prone to racemisation due to the imidazole side chain group. The structure of His in a peptide chain is shown in Figure 2.2.6.



(62)

Figure 2.2.6 The structure of His in a peptide chain

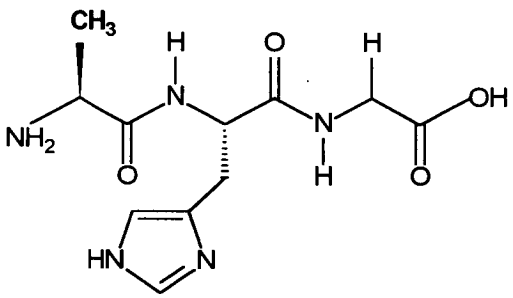
The introduction of a protecting group on the π-nitrogen has proven to be advantageous in overcoming this problem.[49,50] However, the synthesis of the π-nitrogen protected histidine is very difficult and the only commercially available analogue, FmocHis(Bum)[51], can only be prepared in a very poor yield and not very satisfactory purity. Fortunately, by introducing a very bulky electrophilic protecting group such as Trt on the τ-nitrogen, and under optimised coupling condition, the racemisation can be greatly reduced.

In order to study whether the new coupling reagent was likely to cause racemisation, a series of trimers with the general structure of Ala-X-Gly have been synthesised and studied. This sequence was chosen as Gly is not chiral and Ala is the amino acid least prone to racemisation. If the amino acid at the middle position racemises, the methyl group of the Ala will be presented in two slightly different chemical environments. The chemical shift of this methyl in the two isomers will thus be different. The percentage of racemisation is therefore calculated from ^{1}H NMR.

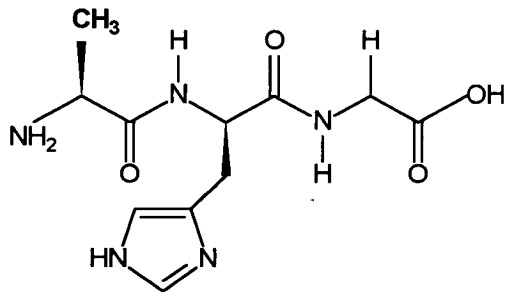
The first trimer studied was Ala-His-Gly and the structures of the trimers with D-and L-His are shown in Figure 2.2.7.

When His(Trt) was used in the synthesis of the trimer, racemisation of His did occur under normal coupling conditions using HOCt to give ~20% of the D-isomer compared with ~10% using HOBt coupling. When the π–nitrogen protected

His(Bum) was used, however, no racemisation was detected from the ^1H NMR, but as the reagent itself was not pure, other unpredictable impurities could be involved in the final product. The rest of the trimers, with all commonly used Fmoc amino acids at the middle position, have been synthesised and investigated by Robertson.[46] The results indicate that His(Trt) is the only one which racemises under the standard coupling conditions, but the racemisation level can be reduced to a negligible level if the coupling is carried out at 0°C in a sonic bath.[46]



(63) L-Ala-L-His-Gly



(64) L-Ala-D-His-Gly

Figure 2.2.7 The structure of Ala-His -Gly

Further investigation was carried out to study the effect of activation time. The results are listed in Table 2.2.2. Although reducing the activation time did result in a decrease in racemisation, it was still occuring at an unacceptable rate. However, an interesting result of this experiment is that pre-activation is not necessary for HOCt coupling, as the yield of the trimer obtained without any pre-activation is as good as the others according to the deprotection profile.

Table 2.2.2 The effect of activation time

Activation time (min.)	30	15	5	0
Racemisation (%)	20	20	16	12

2.2.8 Application of HOCt---1. the monitoring of the coupling

One of the objects of this study is to develop a new coupling reagent which does not absorb at 302nm, so that the coupling process can be monitored before deprotection of the Fmoc protecting group. This presents the opportunity for

improvement when a bad coupling occurs. The reaction mixture is passed through a UV detector connected to the peptide synthesiser at the end of each coupling cycle, so that the unreacted Fmoc amino acid or its activated isomer are examined. To reduce the error caused by uneven delivery during different stages of the synthesis, an external standard, fluorene, was introduced. The amount of unreacted Fmoc amino acid is thus deduced from the peak area. A comparison is made between this real time monitoring and the previously developed deprotection monitoring method, and a typical profile is shown in Figure 2.2.8.

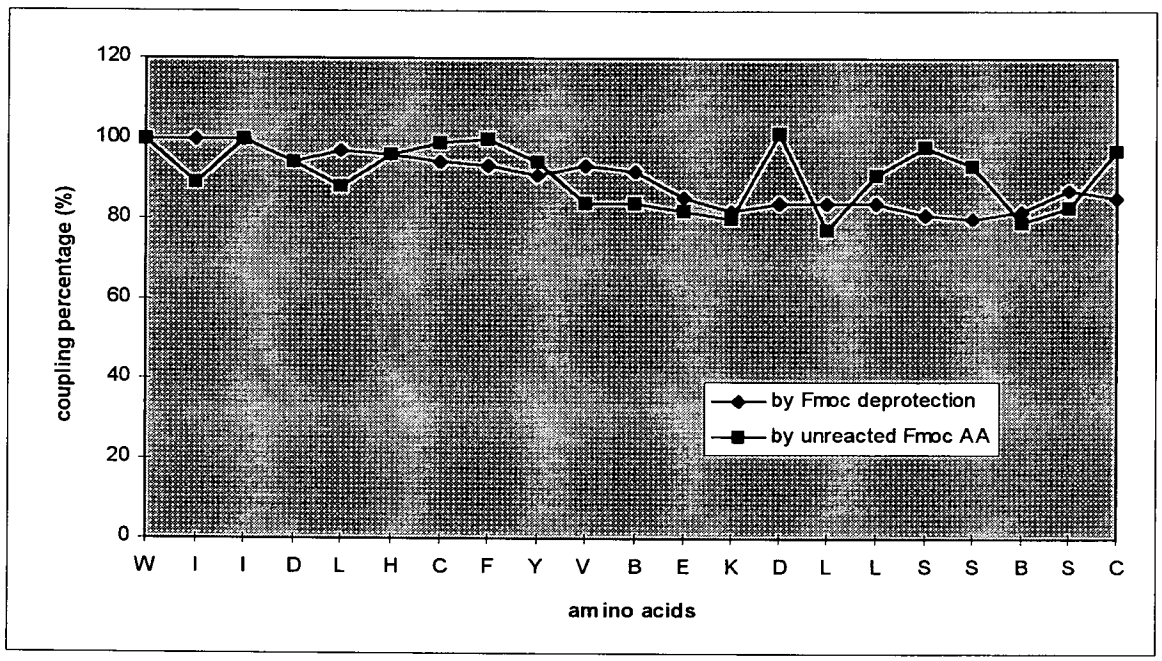


Figure 2.2.8 Coupling efficiency of peptide 1B, monitored by two different methods

The two methods correspond with each other reasonably well, but errors do exist. This is because the solubility of some Fmoc amino acids is not good enough, and they therefore thus cannot be transferred to the reaction vessel completely. Even for the more soluble ones, reagent delivery can also vary slightly from one to the next. This is reasonable, as the synthesiser was not specifically designed for this monitoring process. The slight variation will not seriously affect the coupling process, but it does affect the accuracy of the coupling efficiency deduced from it. More accurate monitoring, therefore, will rely on improvements in the solubility of the amino acids

(i.e. better solvent, elevated temperature etc.), and also the delivery system of the synthesiser.

2.2.9 Application of HOCt---2. The synthesis of endothelin analogues and difficult peptides

Endothelin is a recently discovered, highly potent vasoconstrictor which has attracted extensive research attention from both academia and industry. The biological activity and SAR studies will be discussed further in Chapter 3. The synthesis of some analogues, however, has been found to be difficult, especially when bulky amino acids such as Aib are included in the sequence[52]. Triazole was thus applied in the synthesis of these analogues, and some typical coupling results monitored by the Fmoc deprotection method are shown in Figure 2.2.9 and Figure 2.2.10. In Figure 2.2.11, the synthesis of a BigET-1 analogue, peptide 5, using two different methods is illustrated.

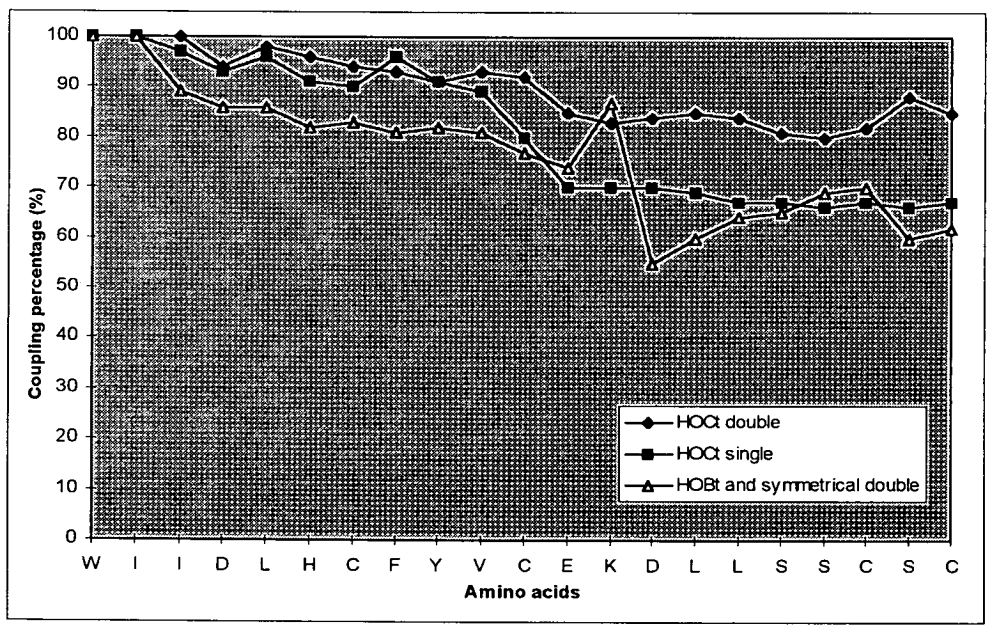


Figure 2.2.9 Comparison between three different coupling methods on the synthesis of ET-1 analogue, peptide 60

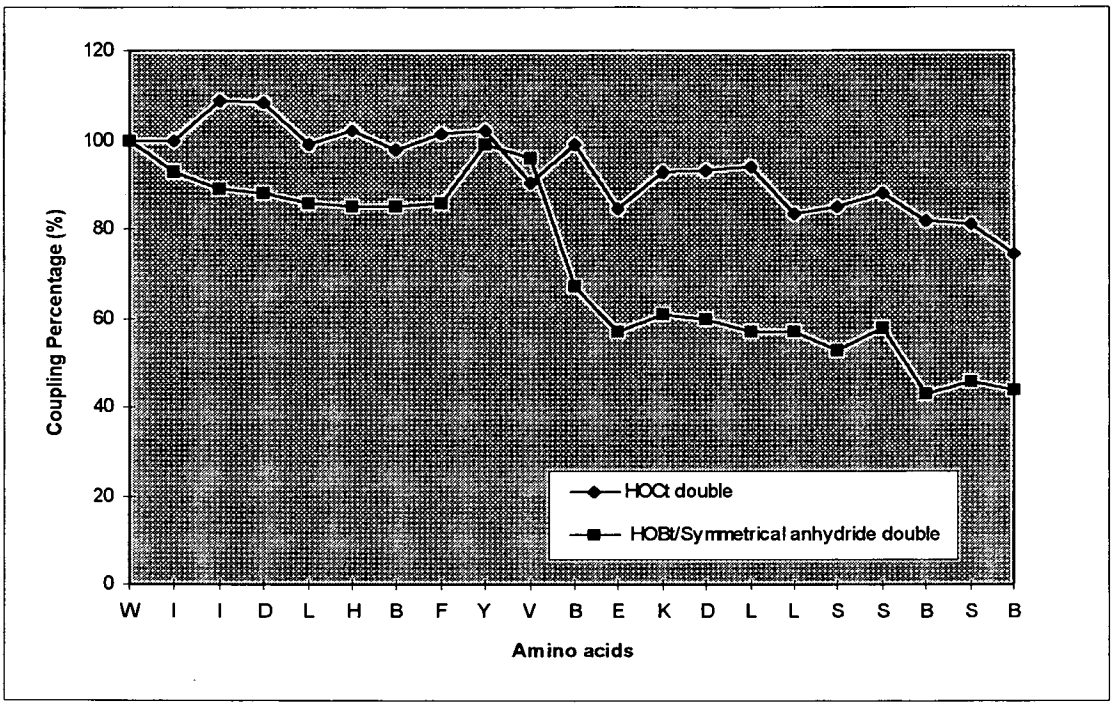


Figure 2.2.10 Comparison between HOCt double coupling and the HOBt/symmetrical anhydride double coupling on the synthesis of ET-1 analogue, peptide 1C, which has four Aib at positions 1,3,11 and 15

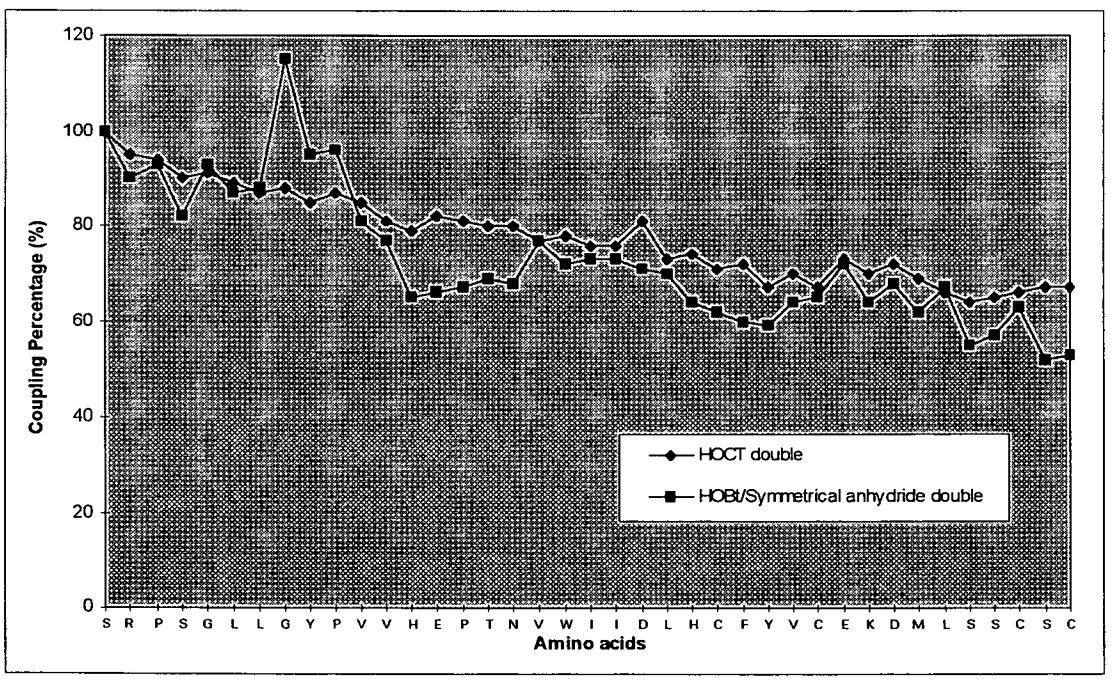


Figure 2.2.11 The coupling efficiency of triazole and HOBt on the synthesis of peptide 5

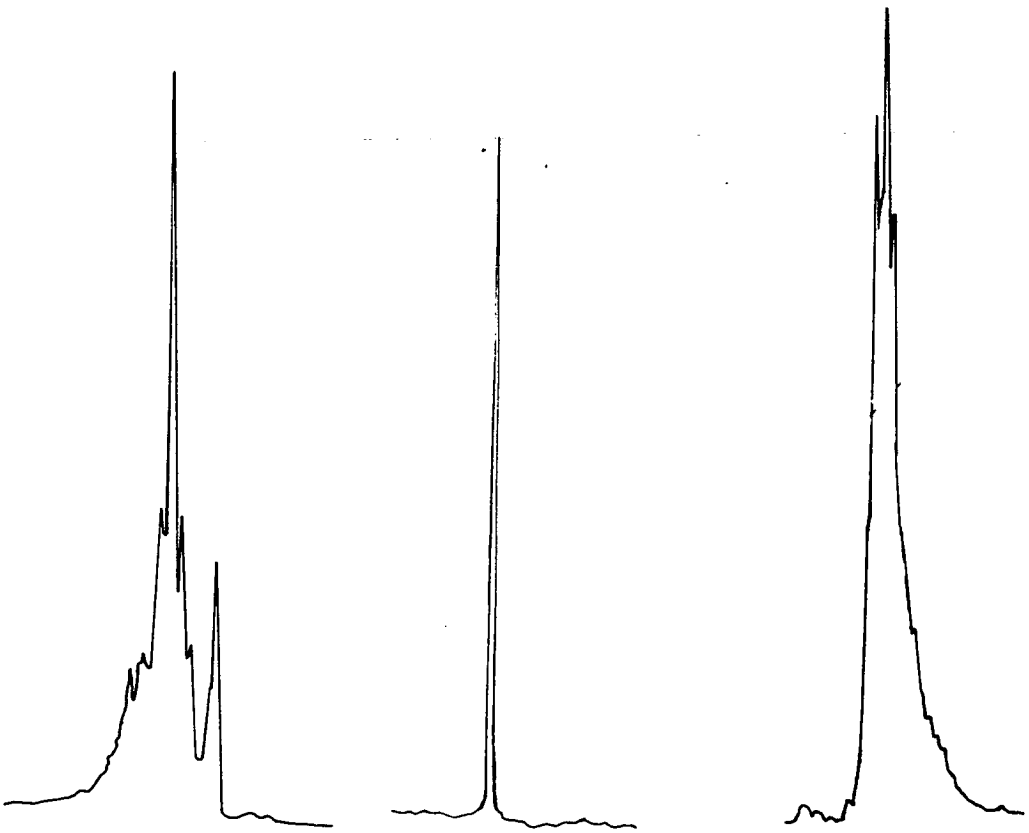


Figure 2.2.12 HPLC traces of peptide 5
Vydac C8 column, 214nm, 1ml/min, A: 0.1%TFA/H_2O, B: 0.1%TFA/CH_3CN;
Gradient:10%-90%B over 30min;
a. HOCt double coupling, crude; b. HOCt double coupling, pure;
c. HOBt active ester and DIC symmetrical anhydride double coupling, crude.

As illustrated in Figure 2.2.9, single coupling using HOCt/DIC is as good as the combination of a symmetrical anhydride coupling followed by a HOBt/DIC coupling. Double coupling with HOCt/DIC improved the coupling percentage by nearly 20%. In Figure 2.2.10, the two double coupling methods are compared in the synthesis of ET-1 analogues, which has the bulky amino acid, Aib at positions 1, 3 ,11 and 15. The overall coupling yield with HOCt is nearly 30% higher. In Figure 2.2.11, the coupling percentages of two methods applied in the synthesis of the BigET-1 analogue, LJP5, are compared. The coupling percentages are not very different according to the deprotection monitoring, but the crude peptide synthesised by HOCt coupling is much cleaner than that synthesised by the symmetrical anhydride and

HOBt coupling (Figure 2.2.12). The details of each coupling protocol can be found in the experimental section in Chapter 3.

HOCt has thus been adopted in this laboratory as the standard coupling reagent for difficult couplings. A typical example is NGF (100-114)[53]. This small peptide caused a lot of problems in its synthesis, and very little pure product was afforded in the first few syntheses before HOCt became available. The overall coupling percentage was improved from <40% to >80%, and it was isolated in very good purity.[53]

Further evidence is provided by the synthesis of ubiquitin and its analogues (76mer).[54] More recently the successful syntheses of the catalytic domain of stromelysin (173mer)[55] and deglycosylated human erythropoietin (166mer)[46] by stepwise SPPS show that HOCt is a very powerful coupling reagent.

2.3 Experimental

2.3.1 Synthesis of 1-hydroxyl-1H-4-ethoxycarbonyl-1,2,3-triazole (HOCt, 38)

Synthesis of ethyl diazoacetate (33)

A solution of ethyl glycinate hydrochloride (210g, 1.5mol) in water (300ml) was mixed with dichloromethane (500ml) in a 3-necked 3 litre flask fitted with a mechanical stirrer and a nitrogen inlet tube and cooled to ca. -15 °C (acetone/dry ice). A cold solution of sodium nitrite (124.5g, 1.8mol) in water (200ml) was added in one portion with stirring. The temperature was lowered to ca. -20 °C and an ice cold solution of 5% (w/w) H_2SO_4 (150ml) was added dropwise while keeping the temperature below -15°C. When all the acid solution was added, the temperature was allowed to rise to ca. 0°C.

The reaction mixture was transferred to an ice cold 2 litre separating funnel. The yellow organic layer was run into an ice cold 5% sodium carbonate solution and the water layer was extracted once with DCM. The combined DCM and Na_2CO_3 portions were stirred until no further gas evolved. The two layers were separated and the water layer was extracted once with DCM. The organic layer was dried ($MgSO_4$) and evaporated *in vacuo* behind a screen. The yellow liquid which was pure enough for next step was stored below 4°C in a well wrapped container. Yield 95%-100%.
δ^1H NMR(60MHz, $CDCl_3$): 1.3 (3H, t, CH_3, J=7.11Hz), 4.3 (2H, q, CH_2, J=7.13Hz), 4.8 (1H, s, CH).

Synthesis of the Vilsmeier reagent (34)

N,N-dimethylformamide (DMF) (52.8g, 0.72 mol) was stirred at room temperature under nitrogen. Freshly distilled thionyl chloride (53ml 0.72 mol) was added dropwise with stirring. When all the thionyl chloride had been added, the mixture was warmed up to 40°C *via* a water bath and stirred for 2h until a very sticky mixture was afforded. The mixture was then evaporated *in vacuo* (*via* oil pump) for 2h (water bath ca. 40°C), until a white solid was afforded. Care should be taken as the solid is highly hygroscopic. Assumed yield 100%.

Synthesis of the iminium chloride (35)

The white solid **(34)** was dissolved in A.R. grade chloroform (400ml) and cooled *via* ice salt bath. The liquid ethyl diazoacetate from first step was added dropwise under nitrogen, keeping the temperature below +5°C. When **(33)** had been added, the reaction mixture was stirred at RT for 30 min. and then evaporated *in vacuo* to give an oily residue. Ether was added to the residue and the yellow solid obtained was filtered under nitrogen and washed with ether. The yellow solid was stored in a desiccator and used the following day in next step. Yield 130g, 88%.

Synthesis of diazo-oxime (37) and its cyclisation to give 1-hydroxyl-1H-4-ethoxy carbonyl-1,2,3-triazole (HOCt) (38)

Procedure a: $NH_2OH.HCl$ (52.5g, 0.76mol) was suspended in commercial ethanol (300ml) and cooled by ice-salt bath. Sodium carbonate (40g 0.38mol) was added to the suspension followed by the diazo salt **(35)** (130g, 0.63mol) as a solid. The resulting yellow mixture was stirred mechanically for 1h at 0°C. The insoluble impurities were filtered out and washed with ethanol (X3). The ethanol solution was evaporated *in vacuo* to give a yellow residue (oily solid). The temperature of the water bath should not be higher than 40°C. Ice cold water was added to the solid which was filtered and washed with a little cold water to remove any inorganic impurities.

δ^1H NMR (200MHz, $CDCl_3$): 1.30 (3H, t, CH_3, J=7.16Hz), 4.30 (2H, q, CH_2, J=7.18), 7.01 (1H, s, CH), >10 (1H, broad, OH).

The yellow solid (**37**) was dissolved in chloroform and separated from any remaining water and dried ($MgSO_4$). After filtration of the $MgSO_4$, 0.5ml of acetic acid added to the chloroform solution which was left at RT until the cyclisation was completed. (Yellow colour fades but confirm by ^1H NMR). The chloroform solution was evaporated *in vacuo* to give a white solid **(38)** to which was added ethyl acetate. The solid was filtered and washed with ethyl acetate. The filtrate was concentrated and stored in cold room until more crystals formed (this was repeated until no more crystals formed). Overall yield ~35%.

δ^1H NMR(200MHz, $CDCl_3$): 1.34 (3H, t, CH_3, J=7.14), 4.38 (2H, q, CH_2, J=7.16), 8.06 (1H, s, CH), ~12.5 (1H, s, broad, OH).

δ^{13}C NMR(200MHz, $CDCl_3$): 13.9 (CH_3) , 61.7 (CH_2) ,121.7 (CH), 136.5 (C), 159.2 (C).

Procedure b: $NH_2OH.HCl$(44g, 0.63mol) was dissolved in a minimum of water and cooled via ice salt bath. Sodium carbonate (33.6g 0.32mol) was added and stirred until all had dissolved. The diazo salt **(35)** (130g 0.63mol) was added as the solid and the yellow product **(37)** soon precipitated. The precipitate was filtered after 5min, washed with a little ice cold water and taken up on to **(38)** as above. Overall yield ~30%.

δ^1H NMR(200MHz, $CDCl_3$): 1.34 (3H, t, CH_3, J=7.14), 4.38 (2H, q, CH_2, J=7.16), 8.06 (1H, s, CH), ~12.5 (1H, s, broad, OH).

δ^{13}C NMR(200MHz, $CDCl_3$): 13.9 (CH_3) , 61.7 (CH_2) ,121.7 (CH), 136.5 (C), 159.2 (C).

2.3.2 Synthesis of the HOCt Knorr reagents

Chloro N,N,N',N'-tetramethyluronium hexafluorophosphate(44)

A 20% solution of phosgene in toluene (100ml) was added dropwise under nitrogen to a solution of tetramethylurea (11.6g, 0.1mol) in toluene. After the evolution of carbon dioxide gas had ceased (ca. 15 min), anhydrous ether was added with vigorous stirring. The solid precipitate was filtered and washed with anhydrous ether (3 x 50ml). The hygroscopic material **(43)** was immediately dissolved in dichloromethane (500ml) and to this solution was added a saturated solution of ammonium hexafluorophosphate (50ml) with stirring. The organic layer was separated, washed with water, dried with $MgSO_4$ and evaporated to yield a white solid which was washed with ether and dried over phosphorus pentoxide *in vacuo*. Yield 22g (82%).

δ^1H NMR(200MHz, $CDCl_3$): 3.39 (s, 4xCH_3).

δ^1H NMR(200MHz, CD_3CN): 3.26 (s, 4xCH_3).

HBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexaflorophosphate (13)

Chloro N,N,N',N'-tetramethyluronium hexafluorophosphate (2.28g, 0.01mol) was dissolved in DCM (25ml) and HOBt (1.53g, 0.01mol) was added. To the clear solution was added triethylamine (0.01mol) and a white precipitate formed at once. The solid was filtered and washed with DCM. The solid was recrystallised from acetonitrile, dried and stored in the fridge.

δ ^{1}H NMR(200MHz, CD_3CN): 3.05 (6H, s, 2xCH_3), 3.41 (6H, s, 2xCH_3), 6~8.2 (4H, m, aromatics).

Attempted synthesis of HCTU (46)

Chloro N,N,N',N'-tetramethyluronium hexafluorophosphate (2.28g, 0.01mol) and HOCt (1.57g, 0.01mol) were dissolved in DCM (25ml) and cooled on an ice-salt bath. Triethylamine (0.01mol) was added dropwise with stirring. The colourless solution soon turned yellow, and a white solid precipitated. The mixture was filtered and the yellow filtrate was evaporated to give a yellow oil, to which ether was added. None of the expected solid precipitated. The solution was evaporated again and checked by ^{1}H NMR, which indicated a mixture of products. The mixture was diluted with DCM and washed with H_2O, dried with $MgSO_4$, filtered, and checked by NMR again.

δ ^{1}H NMR(200MHz, $CDCl_3$): 1.31 (~3H, t, CH_3, J=7.10Hz), 2.78 (~12H, s, 2xCH_3), 4.30 (~2H, q, CH_2, J=7.12Hz).

δ ^{13}C NMR(200MHz, $CDCl_3$): 13.66 (CH_3), 37.99 (CH_3), 62.71 (CH_2), 106.7 (C), 160.4 (C), 164.6 (C).

The NMR data clearly indicated that two compounds were present; these were separated by flash chromatography (1~5% ethanol/$CHCl_3$). Single fraction was collected and examined by NMR and IR.

δ ^{1}H NMR(200MHz, $CDCl_3$): 1.31 (3H, t, CH_3, J=7.13Hz), 4.30 (2H, q, CH_2, J=7.11Hz)

δ ^{13}C NMR(200MHz, $CDCl_3$):13.66 (CH_3), 62.72 (CH_2), 106.7 (C), 160.4 (C)

IR (nujol): 2230cm^{-1}, 2135cm^{-1}, 1723cm^{-1}, 1714cm^{-1}.

The second compound was thus shown to be tetramethylurea by the subtraction of the above spectra from the NMR spectra of the mixture.

2-Chloro-1,3-dimethyl imidazolidinonium hexafluorophosphate(53)

A 20% solution of phosgene in toluene (100ml) was added dropwise, under nitrogen, to a solution of 1,3-dimethyl-2-imidazolidinone (11.4g, 0.1mol) in toluene. The carbon dioxide gas ceased to evolve after ca. 15 min. and anhydrous ether was added with vigorous stirring. The solid precipitate was filtered and washed with anhydrous ether (3 x 50ml). The hygroscopic material **(52)** was immediately dissolved in dichloromethane (500ml) and to this solution was added a saturated solution of ammonium hexafluorophosphate (50ml) with stirring. The organic layer was separated, washed with water, dried with $MgSO_4$, filtered, and evaporated to give a white solid, which was washed with ether and dried over phosphorus pentoxide *in vacuo*. Yield 22g (82%).

δ^1H NMR(200MHz, DMSO-d_6): 3.10 (6H, s, 2xCH_3), 4.13 (4H, s, 2xCH_2).

Attempted synthesis of COI (54)

2-Chloro-1,3-dimethylimidazolidinonium hexafluorophosphate (2.26g, 0.01 mol) and HOCt (1.57g, 0.01mol) were dissolved in DCM (25ml) and cooled on an ice-salt bath. Triethylamine (0.01mol) was added dropwise with stirring. The colourless solution soon turned yellow, and white solid precipitated. The mixture was filtered and the yellow filtrate was evaporated to give a yellow oil, to which ether was added. None of the expected solid precipitated. This was a similar result to that seen in the attempted synthesis of HCTU; thus no further experiments were carried out.

2.3.3 Synthesis of (4-ethoxyl carboxyl-1,2,3-triazole)-yl diphenyl phosphinate (55)

Diphenylphosphinic chloride (0.47g, 2mmol) was dissolved in anhydrous DCM (3ml). HOCt (0.314g, 2mmol) was added as a solid and the mixture was stirred to give a clear solution. Imidazole (0.136g, 2mmol) in dimethyl chloride (2ml) was added dropwise in under nitrogen. A white solid precipitated immediately, and the

mixture was stirred at room temperature for 30 min. The solid was then filtered off and the filtrate evaporated to give a slightly yellow oil.

δ ^{1}H NMR(200MHz, $CDCl_3$): 1.32 (3H, t, CH_3, J=7.07), 4.33 (2H, q, CH_2, J=7.10), 7.31-7.73 (10H, m, aromatics).

δ ^{13}C NMR(200MHz, $CDCl_3$): 14.0 (CH_3), 61.2 (CH_2), 120-135 (CH), 159.8 (C).

δ ^{31}P NMR(200MHz, $CDCl_3$): 31.7 [Ph_2POCl as internal standard, δ ^{31}P=48.32].

2.3.4 Synthesis of the HOCt active esters

FmocLeuOCt, reaction condition studies

Three reaction mixtures were prepared as follows: FmocLeuOH (177mg, 0.5mmol) and HOCt (78.5mg, 0.5mmol) were dissolved in 5ml dry DCM. To the clear solution was added DCCI (103mg, 0.5mmol). These were stirred at -20° C, 0°C or room temperature. The mixtures were then filtered and the filtrates evaporated to dryness. The residues were checked by NMR. All showed a mixture of free acid and the active ester.

Synthesis of active esters of the commonly used Fmoc amino acids

Fmoc-amino acid (0.5mmol) and HOCt (0.5mmol) was dissolved in anhydrous DCM under nitrogen and stirred at 0°C. For some Fmoc amino acid, such as FmocTrpOH, which were not very soluble in DCM, DMF was added to the solution until dissolution was complete. DCCI (0.5mmol) in 2ml DCM was then added, and the mixture stirred at 0°C for another 30min. The white precipitate was filtered off and the filtrate evaporated. To the residue some anhydrous ether was added and the evaporation was repeated. The white solid was dried *in vacuo* over P_2O_5. ^{1}H NMR spectra were collected and results listed in Table 2.2.1.

2.3.5 NMR study of the reaction between DIC and HOCt

A Brucker WP-360 (360MHz) NMR spectrometer was set up at -20°C. HOCt (31.4mg, 0.2 mmol) was dissolved in ~0.4 ml $CDCl_3$ at -20°C and DIC (31.4μl, 0.2 mmol) was added. The mixture was transferred into an NMR tube and ^{1}H NMR spectrum acquired immediately. The temperature of the NMR spectrometer was

raised gradually up to 25°C over 110min and ^{1}H NMR spectra were collected at different intervals, *i.e.* 0min (-30°C), 11min (-30°C), 18min (-20°C), 23min (-20°C), 28mi(-10°C), 33min (-10°C), 38min (-5°C), 43min (-5°C), 48min (0°C), 52min (0°C), 59min (5°C), 63min (5°C), 71min (10°C), 83min (10°C), 92min (25°C), 123min (25°C), 170min (25°C). The reaction was then left overnight under monitoring and more spectra were collected every 2h.

2.3.6 Monitoring of the peptide coupling

Exactly 0.5 mmol of Fmoc amino acid was weighed into each cartridge. The solvent delivery system was checked carefully and florene in DMF was used as an external standard (0.25mmol). After each coupling step, the un-reacted Fmoc amino acid, or its activated derivative in the coupling mixture, was passed through a UV spectrometer, followed by the standard solution in an amount corresponding to 0.50mmol Fmoc amino acid in DMF. The amount of the unreacted Fmoc amino acid was thus calculated as follows:

Coupling percentage (%) = (0.5-washing peak/standard peak) x 100/0.25

If more than one coupling is carried out for each residue, the coupling percentages can be added.

2.3.7 Synthesis of the peptides

The details for the synthesis of peptides can be found in Chapter 3 unless indicated elsewhere.

2.4 References

[1] Fischer, E. (1903), *Ber. Dtsch. Chem. Ges.* **36,** 2094-2106.

[2] Curtius, T. (1902), *Ber. Dtsch. Chem. Ges.* **35,** 3226-3228.

[3] Ramage, R., Irving, S.L. & McInnes, C. (1993), *Tetrahedron Lett.* **34,** 6599-6602.

[4] Wieland, V.T. & Bernhard, H. (1951), *Liebig. Ann. Chem.* **572,** 190-194.

[5] Boissonnas, R.A. (1951) *Helv. Chim. Acta.* **34,** 874-879.

[6] Vaughan, J.R. (1951), *J. Am. Chem. Soc.* **73,** 3547.

[7] Bodanszky, M. (1955), *Nature (London),* **175,** 685-686.

[8] Anderson, G.W., Zimmerman, J.E. & Callahan, F.M. (1963), *J. Am. Chem. Soc.* **85,** 3039.

[9] Kupriszewski, G. (1961), *Rocz. Chem.* **35,** 1533-1560.

[10] Kovacs, J., Kisfaludy, L. & Ceprini, M.Q. (1967), *J. Am. Chem. Soc.* **89,** 183-184.

[11] Konig, W. & Geiger, R. (1970), *Chem. Ber.* **103,** 788-798; 2024-2030.

[12] Konig, W. & Geiger, R. (1973), *Chem. Ber.* **106,** 3626-3635.

[13] Sheenhan, J.C. & Hess, G.P. (1955), *J. Am. Chem. Soc.* **77,** 1067-1068.

[14] Saratakis, D., Teichman, J., Lien, E.L. & Fenichel, R.L. (1976), *Biochem. Biophys. Res. Commun.* **73,** 336-340.

[15] Hudson, D., Kain, D.*et al.* (1986), in *Peptide Chemistry,* p4113. Kiso, Y.(Ed), Protein Reasearch Foundation, Osaka.

[16] Belleau, B. & Malek, G. (1968), *J. Am. Chem. Soc.* **90,** 1651-1652.

[17] Castro, B., Dormoy, J.R., Evin, G. & Selve, C. (1975), *Tetrahedron Lett.* 1219-1222.

[18] Castro, B., Dormoy, J.-R., Evin, G. & Selve, C. (1977), *J. Chem. Res.* 182-186.

[19] Coste, J., Frerot, E. & Jouin, P. (1991), *Tetrahedron Lett.* **32,** 1967-1970.

[20] Frerot, E., Coste, J., Pantaloni, A., Dufour M.-N. & Jouin, P. (1991) *Tetrahedron* **47,** 259-270.

[21] Dourtoglou, V. & Gross, B (1984), *Synthesis,* 572-574.

[22] Knorr, R., Trzeciak, A., Banwarth, W. & Gillessen, D. (1989), *Tetrahedron Lett.* **30,** 1927-1930.

[23] Knorr, R., Trzeciak, A., Banwarth, W. & Gillessen, D. (1991), p62, in *Peptides 1990,* Giralt, E. and Andreu, D. (Eds), ESCOM Leiden, The Netherland.

[24] Banwarth, W. & Knorr, R. (1991), *Tetrahetron Lett.* **32,** 1157-1160.

[25] Kiso, K., Kimura, T., Fujiwara, Y., Sakikawa, H. & Akaji, K. (1990), *Chem. Pharm. Bull.* **38,** 270-272.

[26] Carpino, L.A., El-Faham, A., Minor, C.A. & Albericio, F. (1994), *J. Chem. Soc. Chem. Commun.* 201-203.

[27] Carpino, L.A., El-Faham, A. & Albericio, F. (1994), *Tetrahedron Lett.* **35,** 2279-2282.

[28] Carpino, L.A. (1993), *J. Am. Chem. Soc.* **115,** 4397-4398.

[29] Carpino, L.A. & El-Faham, A. (1995), *J. Am. Chem. Soc.* **117,** 5401-5402.

[30] Kent, S.B., Alewood, D., Alewood, P., Boca, M. Jones, A. & Schnolzer, M. (1992), in *Innovations and Perspectives in Solid Phase Synthesis,* Epton, R.(Ed.) Intercept. Ltd., Avdover, UK.

[31] Bedford, J. Hyde, C. Johnson, T., Jun, W., Owen, D., Quilbell, M. & Shepperd, R.C. (1992), *Int. J. Pept. Protein Res.* **40,** 300-307.

[32] Narita, M. & Ouchi, S. (1994), *J. Syn. Org. Chem. Japan,* **52,** 686-697.

[33] Atherton, E. & Sheppard, R.C. (1985) *J. Chem. Soc. Chem. Commun.* 165-166.

[34] Gigin, B.F. (1972), *Anal. Chim. Acta,* **58**, 248.

[35] Cameron, L., Meldal, M. & Sheppard, R.C. (1987), *J. Chem. Soc., Chem. Commun.* 270-272.

[36] Bodansky, M. & Sheenhan, J.T. (1964), *J. Chem. Ind. (London),* **32,** 1432-1434.

[37] Dryland, A. & Sheppard, R.C. (1986), *J. Chem. Soc., Perkin Trans. 1,* 125-137.

[38] Otteson, K.M., Noble, R. L., Hoeprich, P.D., Shaw, K.T. & Ramage, R. (1993), *Applied Biosystem Research news,* June.

[39] Davison, A. (1993), *PhD thesis, Edinburgh University.*

[40] Bennett, I.S., Brooks, G., Broom, N.J.P, Calvert, S.H., Coleman, K. & Francois, I. (1991), *J. Antibiotics,* **44,** 969-974.

[41] Stojanovic, F.M. & Arnold, Z. (1967), *Collect. Czech. Chem. Commun.* **32,** 2155-2158.

[42] Searle, N.E. (1955) *Org. Syn.* **36,** 25-27.

[43] Bosshard, H.H., Mory, R., Schmid, M. & Zollinger, H. (1959), *Helv. Chim. Acta,* 1653-1656.

[44] Dulcere, J.-P. (1981), *Tetrahedron Lett.* **22,** 1599-1600.

[45] Jutz, C. (1976), *Adv. Org. Chem.* **9, pt.1**, 225-342.

[46] Robertson, N. *Personal communication.*

[47] Boyer, J.H., Krueger, W.E. & Mikol, G.J. (1967), *J. Am. Chem. Soc.* **89,** 5504-05.

[48] Abramovitch, R. A. & Davis B.A. (1964), *Chem. Rev.* **64,** 165.

[49] Jones, J.H., Ramage, W.I. (1978), *J. Chem. Soc., Chem. Commun.* 472-473.

[50] Jones, J.H., & Stachulski, (1979), *J. Chem. Soc. Perkin Trans.1,* 2261-2267.

[51] Colombo, R., Colombo, F. & Jones, J.H. (1984), *J. Chem. Soc., Chem. Commun.* 292-293.

[52] Steward, A., *Personal communication.*

[53] Kelly, A. M. (1994), *PhD thesis, Edinburgh University.*

[54] Wilken, J. (1995), *PhD thesis, Edinburgh University.*

[55] Morton, G., *Personal communication.*

CHAPTER 3

STRUCTURE ACTIVITY RELATIONSHIP STUDIES OF ET-1, BIG ET-1 AND THEIR ANALOGUES

3.1 Introduction

3.1.1 EDRF/EDCF and cardiovascular disease, the history of endothelial studies

The understanding of the vascular biology of cardiovascular disease and, in particular, of coronary artery disease is the major challenge in cardiovascular medicine at present. Diseases of the heart and circulation system remain the major cause of morbidity and mortality in Western countries. Although substantial advances have been made in the past two decades in surgical, interventional, and medical therapy of these conditions, it has become clear that only a better understanding of the cellular and molecular mechanisms could be the basis for rational mechanistic approaches and, in turn, for cause-oriented therapy for cardiovascular disease.

Because of the special position between the circulating blood and vascular smooth muscle, the centre of interest has recently focused on endothelial cells, which were originally considered more or less inert structures covering the inner surface of the cardiovascular system. In the 1970s, it was discovered that endothelial cells are a major source of prostacyclin, a vasodilator and inhibitor of platelet function. At the same time, heamatologists observed that endothelial cells are a source of numerous substances involved in coagulation and fibrinolysis. In 1980, Furchgott *et al.* published a landmark article which described for the first time endothelium-dependent relaxation to acetylcholine in the rabbit aorta,[1] and in 1985, Moncada *et al.* were able to demonstrate that nitric oxide (NO) accounts for the biological activity of the putative endothelium-derived relaxing factor (EDRF)[2]. These important observations led to a tremendous stimulation of research in this area at the cellular level, in physiology and also pathophysiology. The observation that the endothelium can also mediate contractile responses stimulated the research for endothelium-derived

mediate contractile responses stimulated the research for endothelium-derived contracting factors (EDCF). Some of these contractile factors derived from the endothelium could be identified as cyclo-oxygenase products and a particularly potent vasoconstrictor released by endothelial cells appeared to be a peptide. In 1988, Yanagisawa *et al.* demonstrated that the latter was a 21-amino acid peptide, which they called endothelin[3].

3.1.2 Endothelins and their structures

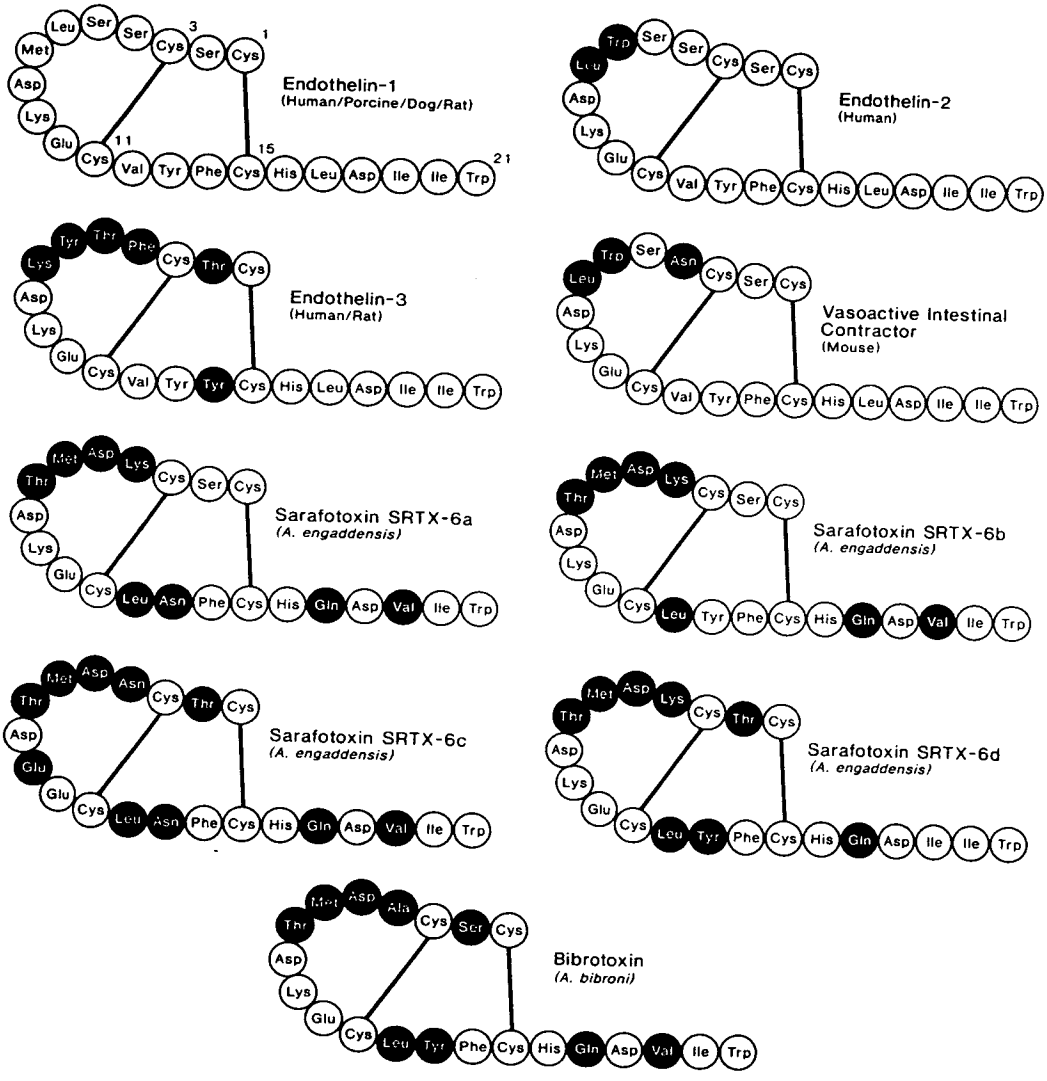


Figure 3.1.1 The structures of ETs and SRTXs

Endothelin (ET-1), is a 21 amino acid peptide which was first isolated in 1988 from cultured porcine aortic endothelial cells.[3] It belongs to a new class of peptides, and bears no similarity in its sequence to the known peptides of mammalian origin. As a more potent candidate for the EDCF due to its long-acting vasoconstriction, this peptide prompted a line of research that culminated in the identification of three distinct endothelin genes which encode three closely related peptides, ET-1, ET-2 and ET-3.[4] The structures of the three peptides show a remarkable sequence homology with those which were found afterwards and are known as the sarafotoxins (SRTX's)[5]. Another isopeptide, vasoactive intestinal contractor (VIC), was also found and it was first thought to be the fourth isomer of the ET family, but recent report suggested that it might be a murine form of ET-2[6]. A recently discovered peptide, bibrotoxin, added another member to this peptide family. The structures of these peptides are shown in Figure 3.1.1.

3.1.3 Biosynthesis of ETs, Endothelin-converting enzyme (ECE)

ETs are derived from peptide precursors with ~200 amino acids known as preproendothelins, which are cleaved after translation by an endopeptidase specific for the paired dibasic residues to form the proendothelins, Big endothelins (BigETs). BigETs are then converted to active ETs by endothelin converting enzyme (ECE) (Figure 3.1.2). The physiological importance of the cleavage of BigETs is indicated by the ~100 fold more potent vasoconstrictor activity of ETs.

The existence of a specific enzyme which converts BigET to the mature ET was contained in the original report of the cloning of the porcine ET-1.[3] The extensive efforts involved in the isolation and purification of ECE from a variety of sources have led to the molecular cloning and sequencing of the enzyme. [7,8,9,10] This phosphoramidon-sensitive neutral metalloprotease, which has a strict substrate specificity, represents another plausible target for pharmacological intervention. A highly potent and specific inhibitor would be a useful tool in these studies. Therefore, the development of ECE specific inhibitors is required.

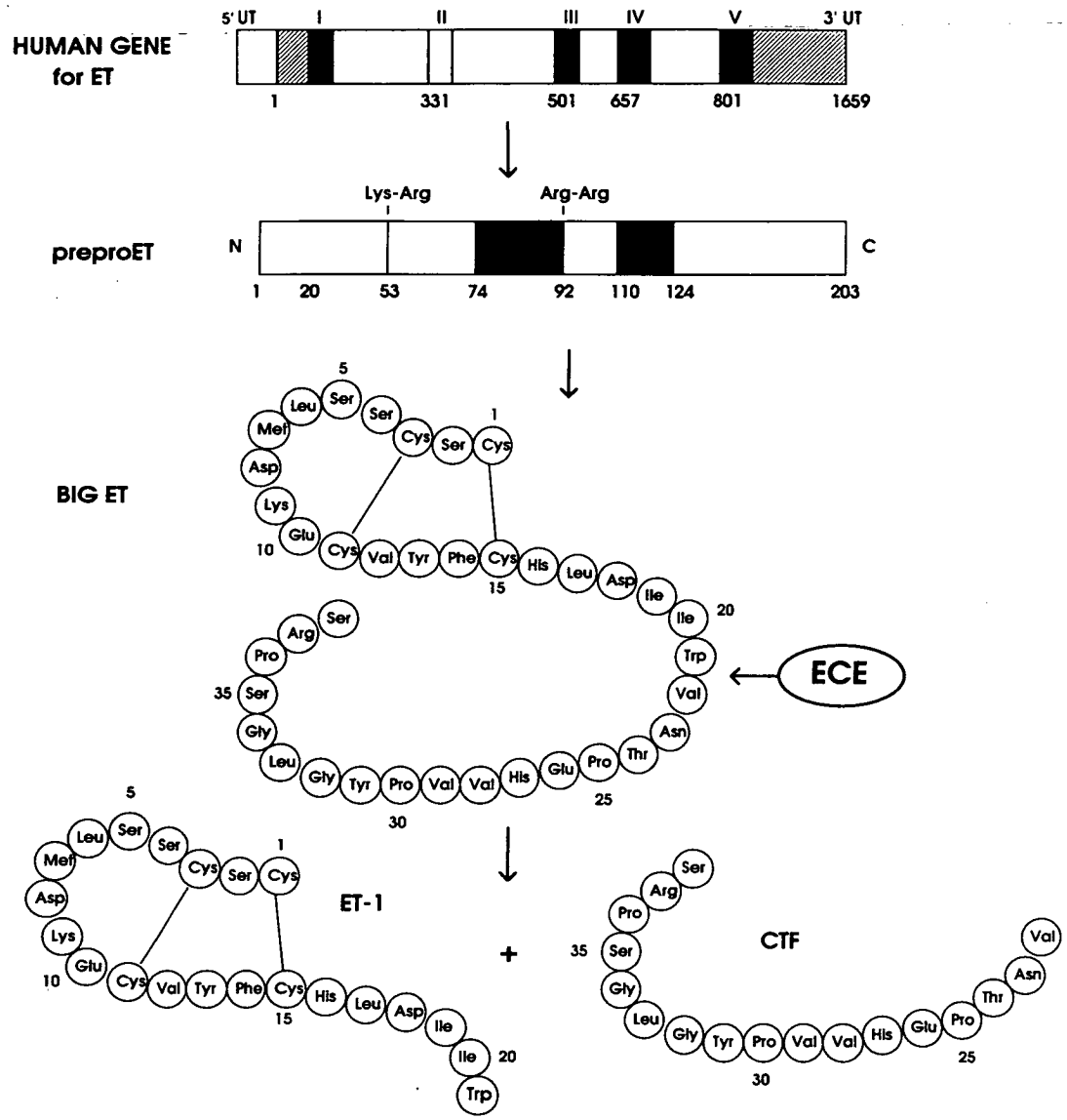


Figure 3.1.2 Biosynthesis of ET-1

3.1.4 Receptor subtypes, ET_A and ET_B

Two subtypes of ET receptors have been found and cloned, ET_A and ET_B.[11,12] ET_A is selective for ET-1 and ET-2, while ET_B binds to all three ETs equally.[13] Both receptors have been cloned from human.[14,15] A third subtype of receptor, ET_C, which is selective to ET-3, has also been cloned from *Xenopus melanophore,*[16] but there

have been no reports of any human homologues. Evidence also pointed to the presence of subtypes among the various receptors.[17,18] Binding studies using labeled ETs and the receptor selective analogues have shown a wide distribution of ET specific binding sites in the vascular system. ET_A receptors have been found predominantly in the heart, blood vessels of the brain and vascular smooth muscle, whereas ET_B receptors are widely distributed in the kidney, uterus, central nervous system and endothelial cells.[19,20,21]

The ligand-receptor interaction has also been a subject of extensive studies.[22,23,24] The ET_A receptors were suggested to recognize the tertiary structure of both the N-terminal and C-terminal segments, whereas the ET_B receptors recognize predominantly the C-terminal part, explaining the differences in affinities of the two receptors for the isopeptides. The exact residues of the receptor involved are not clearly known yet, but mutational studies are in progress to identify the responsible residues.

3.1.5 Physiological and pathological importance of endothelins

ETs are widely distributed in the tissues and organs of mammals. Studies have found that ETs function as both circulating hormones and a endocrine factor in the regulation of vascular tone. The biological activity of the ET family are summarized in Table 3.1.1.[25] These various biological actions have implicated the ET family in a spectrum of cardiorenal disease states such as atherosclerosis, hypertension, congestive heart failure, acute coronary ischaemic syndromes, coronary vasospasm, and acute and chronic renal failure.

The biological actions of ETs clearly indicate their importance both physiologically and pathologically. Two strategies are of particular importance. Active research and drug development progress are aimed at investigating ET inhibitors. Several antagonists to endothelin have been discovered (Table 3.1.2) and clinical trials of the use of these antagonists for many cardiorenal disease conditions are already in progress.[26] The receptor selective agonists, on the other hand, are also important in classifying the biological activity of the different receptor subtypes and understanding the functional activity of each of the receptor subtypes.

Table 3.1.1 The biological actions of ETs[25]

Potent and long-lasting constriction of isolated vascular/nonvascular smooth muscle
Coronary arterial vasoconstriction, increased perfusion pressure
Vasodilation in certain tissues at low doses
Positive inotropic and chronotropic activity
Reduction of cardiac output
Proarrythmogenic activity
Mitogenic actions
Introduction of hypertrophy of cardiomyocytes
Inhibition of platelet aggregation *in vivo* (not *in vitro*) via release of EDRF or prostacyclin
Chemotactic activity
Potent contractile agonist activity in human isolated bronchus
Inhibition of renin release
Reduction of renal blood flow and glomerular filtration rate
Inhibition of arginine vasopressin release
Stimulation of substance P release
Stimulation of catecholamine release
Stimulation of EDRF release
Stimulation of ANP secretion
Increased release of prostacyclin
Stimulation of gastrointestinal hemorrhagic and necrotic damage in rat mucosa
Inhibition of prolactin secretion
Stimulation of luteinizing hormone, follicle stimulation hormone, and thyroid stimulation hormone release from primary monolayer cultures of rat anterior pituitary cells
Stimulation of pituitary gonadotropin release
Stimulation of glycogenolysis in rat heptocytes
Stimulation of PDGF secretion in cultured human mesangial cells

Table 3.1.2 ET receptor antagonists found by random screening

Compound or structure	Antagonist Type	IC_{50}		Ref.
		ET_A	ET_B	
BQ-123	ET_A	22nM	18μM	27
FR-139317	ET_A	1nM	7μM	28
BQ-153	ET_A	21nM	54μM	29
BQ-788	ET_B	1300nM	1.2nM	30
RE-701-1	ET_B	>5000nM	10nM	31
Ro 46-2005	ET_A/ET_B	0.22μM	1μM	32

3.1.6 Development of ET receptor selective agonists/antagonists, SAR studies

The rational design of receptor specific agonists and antagonists depends on the understanding of the mode of binding of ligands and the relative importance of the residues with respect to agonist or antagonist activities. Numerous studies have been carried out using synthetic analogues and fragments of ET, with each portion of the peptide being examined for relative importance for binding and functional activities. In general, the full-length analogues with bicyclic or the outer [1-15] monocyclic structure appears to be required for binding and agonism at the ET_A receptor , while linear, truncated sequences have been shown to be agonists at the ET_B receptor.[53-58] The C-terminal hexapeptide, especially the C-terminal amino acid, Trp, is essential for binding and functional activities at both receptor subtypes while the N-terminal part is more or less tolerant to substitutions.[33,34,35] Acetylation of the N-terminus of the ET-1 results in a remarkable loss in constrictor activity in rat pulmonary artery but not in the intact vascular bed, while amidation of the C-terminus causes only 16-fold loss in activity.[36,37]

Systematic replacement of each amino acid of ET-1 with Ala revealed some interesting information in terms of the relative importance of each side chain

functional activity for ET_A receptor binding and biological activity.[38,39] The peptides were tested for their binding affinity on human vascular smooth muscle cells and for

Table 3.1.3 Ala scan and D-amino acid scan of ET-1[39,40]

Ala Replacement	Binding* %	Contraction** %	D-amino Acid	Binding* %	Contraction** %
Ser^2	100	70	Ser^2	100	100
Ser^4	200	51.5	Ser^4	10	0.1
Ser^5	142	24.8	Ser^5	not tested	not tested
Leu^6	101	73	Leu^6	126	58
Met^7	350	88.5	Met^7	126	20.4
Asp^8	103	0.8	Asp^8	2.7	0.6
Lys^9	504	56	Lys^9	210	418
Glu^{10}	162	121	Glu^{10}	154	53.5
Val^{12}	16.2	29.4	Val^{12}	27	21.6
Tyr^{13}	0.8	87.3	Tyr^{13}	8	9.5
Phe^{14}	1.8	8.4	Phe^{14}	2.2	1.5
His^{16}	339	382	His^{16}	0.3	0.1
Leu^{17}	25	0.9	Leu^{17}	12.6	0.1
Asp^{18}	50	14	Asp^{18}	1.6	1.6
Ile^{19}	406	121	Ile^{19}	0.05	< 0.3
Ile^{20}	22.0	22.5	Ile^{20}	3.2	5.0
Trp^{21}	0.8	< 0.1	Trp^{21}	2.4	0.4

Note: *Tested in Human VSM; **Tested in rabbit vena cava.

agonism on the vena cava *in vitro*. Replacement of the C-terminal Trp with Ala led to a complete loss in binding affinity and biological activity, while replacement of Phe^{14} and Leu^{17} caused a serious decrease in biological activity but part of the binding affinity remained. These sites were thus suggested to be of major biological significance for binding in human VSM cells and agonist activity in contraction of rabbit vena cava.[38] Replacement of Tyr^{13} with Ala caused complete loss in binding affinity but only slight decrease in biological activity, which is difficult to explain. On the other hand, changing Asp^8 to Ala^8 did not effect binding affinity but greatly reduced constrictive activity, which indicated that this position was more important for biological activity than for receptor binding. The analogue ET-1[1-21, Ala^8]

appears to be a receptor non-selective antagonist[38]. The N-terminal residues $Ser^{2,4}$, Leu^{6}, Met^{7}, Lys^{9} and Glu^{10} were found tolerant to replacement as the corresponding peptides all showed some agonism. Replacement at the C-terminus was much more sensitive, but analogues ET-1 [Ala^{16}] and ET-1 [Ala^{19}] appeared to be more potent agonists than ET-1 itself (Table 3.1.3).

A mono D-amino acid scan of the ET-1 provided information on the orientation of the side chain and backbone conformation and their relative importance to functional activity.[40] It was found that inversion of configuration was generally less tolerated in the C-terminal region than in the N-terminal region. Inverting Asp^{8} and Glu^{10} caused a considerable decrease in the biological activity, while inverting Lys^{9} was well tolerated. D-Phe^{14} and D-Leu^{17} analogues exhibited partial agonism in rabbit vena cava although their binding in human VSM cells decreased significantly (Table 3.1.3).

The two disulphide bonds were found to be important for ET_A receptor binding and functional activity but not for the ET_B receptor as the linear analogue ET-1 [1-21, $Ala^{1,3,11,15}$] was found inactive in ET_A containing tissues, while it bound to the ET_B receptor with equal affinity as that of ET-1.[41]

The full-length monocyclic analogue, ET-1 [$Ala^{3,11}$] has shown 10% vasoconstrictor activity of ET-1 in the isolated rat aorta, while ET-1 [$Ala^{1,15}$] has shown less than 0.5%,[42,43] which indicated that the outer disulphide intact between 1 and 15 was more important for maintaining vaso-constrictor activity in this tissue (ET_A). Likewise, the monocyclic analogues exhibited diminished binding affinity relative to ET-1 in the rat cerebellum (ET_B)[42,43]. The Ala scan of ET-1 [$Ala^{3,11}$, Nle^{7}] supported the observation of the Ala scan of ET-1 itself.[44,45] Substitution of Ser^{2}, Val^{12}, His^{16} and Ile^{19} with Ala produced agonists of reasonable potency, these sites were thus identified as tolerated sites; Substitution of Asp^{8}, Tyr^{13}, Ile^{20} and Trp^{21} caused a significant decrease in the binding affinity and functional activity, these residues were thus regarded as important sites. Substitution of Glu^{10}, Phe^{14}, Leu^{17} and Asp^{18} produced analogues that showed significant binding affinity to the receptor at concentrations below those at which agonism was observed, these sites were thus suggested as candidate sites for the exploration of receptor antagonism as they played a more important role in receptor functional activity than in the receptor binding.

Further study by these authors[45] suggested that ET-1 [$Pen^{1,11}$, Nle^{7}, Ala^{18}] was a more potent ET_A receptor selective antagonist, when penicillamine (Pen) was used at positions 1 and 11 to assist in the correct 1-15, 3-11 disulphide formation.

Another independent study identified residue Asp^{18} as the most important residue[46] and it was suggested that analogues substituted at position 18 with a hydrophobic amino acid, such as Val, Nva, Leu, Ile γ-MeLeu, or Phe, were ET_A receptor selective antagonists. Further investigation of positions 18 and 19 showed that replacement of Asp^{18} with Thr and of Ile^{19} with a hydrophobic amino acid whose side-chain branches at the γ-carbon such as Leu, cyclohexylalanine and γ-MeLeu, resulted in analogues with significant decreased constrictor activity while still binding with high affinity to both receptors.[47]

Truncated monocylic ET-1 analogues with different sequences between Cys^{1} and Cys^{15} were also studied[48,49]. The loop region between Cys^{3} and Cys^{11} itself did not bind to either receptor up to 100μM. A series of analogues without the 3-11 region bound to both receptors with micromolar affinity and exhibited antagonist activity at the ET_A receptor but not at the ET_B receptor. It is interesting that a similar monocylic analogue, Cyclo-[Cys-Val-Tyr-Phe-Cys]-His-Leu-Asp-Ile-Ile-Trp, known as IRL 1038, was reported by Urade *et al.* to be a potent ET_B receptor antagonist.[50] In another independent study, it was reported that IRL1038 was a non-selective ET receptor antagonist[49], with low micromolar affinity to each receptor. A later study by Urade *et al.* disclosed that subsequent preparations of IRL 1038 failed to possess the potency and selectivity of the original sequence.[51] The reason for the discrepancy is unknown, but it might due to the presence of ET_B receptor subtypes as was reported recently.[18,52]

Linear analogues of ET-1 have also been studied. ET-1 [$Ala^{1,3,11,15}$] showed 5500 fold reduction in binding affinity to the ET_A receptor but only 3-fold reduction to the ET_B receptor.[53,54] A systematic truncation of the linear analogue, ET-1 [$Ala^{1,3,11,15}$] led to the development of several ET_B selective agonists and residues 8-21 were regarded as the minimum sequence for ET_B receptor binding and agonism.[55,56] N-terminal acetylation of some truncated analogues led to a compound with increased ET_B receptor selectivity and functional activity, and BQ-3020, ET-1 [Ac-6-21,

Ala[11,15]] appeared as a potent ET_B receptor selective agonist.[55] Another study revealed that the charged residues, Asp[8] and Glu[10] were important for ET_B receptor binding, but Lys[9] could tolerate replacements by Ala or Glu and IRL 1620 ET-1 [Suc-8-21, Glu[9], Ala[11,15]] was reported as another ET_B selective agonist.[57] An even shorter analogue, ET-1 [Ac-10-21], was also reported to have μM binding for the ET_B receptor[58]. Individual amino acid replacement of this analogue with Ala or D-amino acid revealed that the side chains of Phe[14], Leu[17], Ile[20] and Trp[21], the correct backbone orientation of Phe[14] and the C-terminal hexapeptide [16-21], were all important for ET_B selective agonism. However, when the same experiment was repeated in this study, some different results were observed.

Table 3.1.4 ET receptor agonists and antagonists found by SAR study

Compound/Structure	Type	IC_{50}		Ref.
		ET_A	ET_B	
ET-1[1-21, Thr[18] γ MeLeu[19]]	ET_A/ET_B antagonist	0.7nM	0.25nM	47
ET-1[1-21, Ala[1,3,11,15]]	ET_B agonist	570nM	0.33nM	41
BQ-3020: ET-1[Ac-6-21, Ala[11,15]]	ET_B agonist	440nM	0.2nM	55
IRL1620 ET-1[Suc-8-21, Glu[9]]	ET_B agonist	1900pM*	16pM*	57
ET-1[Ac-10-21]	ET_B agonist	11μM	11nM	55
PD142893 ET-1[Ac-16-21, Dip[16]]	ET_A/ET_B antagonist	40nM	60nM	61
PD145065 ET-1[Ac-16-21, D-Bhg[16]]	ET_A/ET_B antagonist	3nM	19nM	62

Notes:*Ki values;
Bhg: 10,11-dihydro-5H-dibenzo[a,d]cycloheptene; Dip: diphenylmethene.

In another report, IRL1720, ET-1 [8-21, Ala[11,15]], was found to possess subnanomolar affinity for ET_B receptor (porcine lung) and to be an antagonist of ET-3 stimulated vasoconstriction in guinea pig trachea.[59] This result was very interesting with respect to that IRL1620 and BQ-3020, two very similar analogues, being potent ET_B agonists. This result also indicates that re-investigation of the highlighted analogues is of particular importance.

Although the C-terminal hexapeptide was found to be critical for the biological activity of the ETs, the hexapeptide itself showed very poor binding affinity for both receptor subtypes.[60] Studies of this hexapeptide showed that substitution at certain positions could improve its binding affinity[60] and this study led to the discovery of several potent antagonists with non-receptor selectivity, i.e. PD 142893[61] and PD 145065[62].

Some ET receptor selective agonists and antagonists found to data are listed in Table 3.1.4.

3.1.7 Conformational studies, CD, NMR and X-ray diffraction

In order to elucidate important structural features that might be relevant in the receptor binding state, the solution conformation of endothelins, sarafotoxins, and related analogues have been extensively studied by CD, NMR and molecular modeling under different conditions.[63-73] CD spectra of ET-1 and its isopeptides were very similar, showing about 35% helical structure.[63,64] NMR studies suggested that a general helical structure exists in all ETs between Lys^9 and Cys^{15}, but varies from each other in detail.[65,66,67] Some groups reported that the N-terminus was averaging on NMR time scale[68,69]while others[70,71] suggested that a β-turn might be present between Ser^5 and Asp^8. Conformation of the C-terminal hexapeptide, which is essential for biological activity, was surprisingly not well defined in most studies,[65-71] while a few studies suggested that it may be associated with the bicyclic core.[72] Aggregation of ET-1 in aqueous solution was detected by CD[73] and it was suggested to be the reason for the discrepancy in NMR studies as organic solvents such as acetonitrile and DMSO had to be used. An alternative structural model of ET-1 which composed mainly of β-turns rather than α-helix was thus proposed.[73]

CD and NMR studies of monocyclic analogues, ET-1 [$Ala^{3,11}$], ET-1 [$Ala^{1,15}$] and ET-1 [$Aba^{1,15}$] were also reported.[63,74] The results suggested that the structure of the monocyclic analogues was comparable to the natural ET-1, with increased structural disorder at the N-terminus.[75] The CD spectra of the linear analogue, ET-1 [$Ala^{1,3,11,15}$], however, showed only 52% β-sheet structure with little helicity.[53] It was thus suggested that at least one disulphide bond was required to stabilize the helical

structure and thus no further CD or NMR study has since been carried out for the linear analogues. However, our study indicated that a helical structure, very similar to the native one is present in most of the linear analogues studied.[76]

The three dimensional crystal structure of ET-1 by X-ray crystallography is different from that expected on the basis of the CD and NMR studies.[77] It was reported that the structurally undefined C-terminal hexapeptide from NMR studies is actually helical in nature, while the N-terminal residues exist in an extended β-strand, followed by an irregular helix starting at Cys^{11} and extended to the C-terminus.

3.2 SAR studies of ET-1 and its linear analogues

Linear analogues of ET-1 were synthesized to study their binding and functional activity to the ET receptors. As was described previously, linear analogues of ET-1 are efficient at binding to the ET_B receptor subtype, while are less potent at binding to the ET_A receptor. To further study the ET_B receptor selective ligands, SAR of the linear analogues was carried out. The sequences of the full length and truncated linear analogues synthesized are listed in Appendix I and II respectively.

3.2.1 Replacement of the Met[7]

Methionine is labile to oxidation, and it is commonly replaced by norleucine (Nle) without loss in biological activity.[44,45] This is reasonable due to their structural similarity as shown in Figure 3.2.1. Leu has a slightly different structure but has also been reported for the replacement of Met in some studies with similar results.[78]

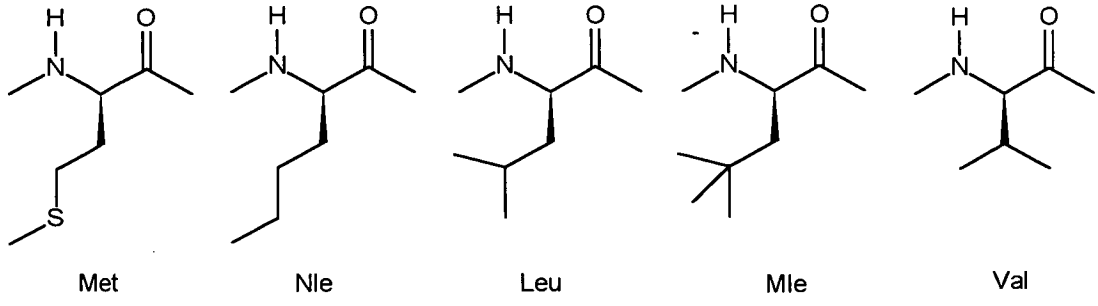


Figure 3.2.1 The structures of Met, Nle, Leu, Mle and Val

Table 3.2.1 Biological activity of ET-1 analogues with different Met[7] replacements

Pept. No.	Full Length and Truncated Analogues	IC_{50}(μM) ET_A[b]	IC_{50}(μM) ET_B[c]	Selectivity ET_B/ET_A	EC_{50}(μM) ET_B[d]
ET-1		0.0002	0.0016	0.13	0.0003
1B	[1-21, $Aib^{3,11}$, Nle^7, $X^{1,15}$]	4.5	0.0009	5,000	0.00048
1	[1-21, $Aib^{3,11}$, Leu^7, $X^{1,15}$]	9.1	0.00006	151,667	0.00059
32	[7-21, Nle^7, Aib^{11}, X^{15}]	0.77	0.0004	1,925	0.00077
33	[7-21, Leu^7, Aib^{11}, X^{15}]	2.8	0.0009	3,111	0.0013
67	[7-21, Nle^7, $Aib^{11,15}$]	6.7	0.0001	67,000	0.0012
53	[7-21, Leu^7, $Aib^{11,15}$]	1.8	0.0003	6,000	0.00096
61	[7-21, Val^7, $Aib^{11,15}$]	0.83	0.0025	332	0.0023
62	[7-21, $MeLeu^7$, $Aib^{11,15}$]	0.9	0.0002	45,000	0.0015

Notes :

X: Cys(Acm);
b: tested in rabbit renal artery vascular smooth muscle cells;
c: tested in rat cerebellum;
d: tested in human ET_B receptor expressed in CHO cells.

It can be seen that both Nle and Leu are well tolerated at this position since similar results are observed in each pair with respect to receptor binding and functional activity. However, with Leu at position 7, the functional activity on ET_B receptor is slightly reduced as is shown in peptides 1 and 33. It seems that the methyl side group on the γ-carbon helps to reduce the functional activity towards the antagonist, thus a series of 7-21 analogues were synthesized with different substitution at position 7, including the most branched analogue, γ-methyl leucine (Table 3.2.1, group 2). The data show that the functional activities of all the peptides are very similar (0.96 ~2.3nM), which indicates that the steric effect is not critical for ET_B receptor functional activity. The binding affinities for ET_B receptor are also similar except that of peptide 61, which has Val at position 7, methyl group at the β-position of the side-chain, shows 10~20 fold lower binding affinity. The binding affinities of these analogues for the ET_A receptor, however, are slightly different. Peptide 67, which has the least branched Nle at position 7, shows the lowest binding; while peptides 61 and 62, which are most branched at either γ- or β- position, show

the highest binding. These results indicate that a branched side chain at position 7 is preferred by ET_A receptor, while the β-branched Val is the least tolerated by ET_B receptor. Branching at the γ- position, however, is tolerated by the ET_B receptor, therefore in the following discussion, no distinction will be made between Leu^7 and Nle^7 analogues.

3.2.2 Replacement of $Cys^{1,3,11,15}$

Five full length analogues were synthesized to study the effect of the different replacements of the four Cys residues at positions 1, 3, 11, and 15 (Table 3.2.2) with Ala and the helix promoting amino acid, α-aminoisobutyric acid (Aib). Aib was used to introduce some helical structure in the linear analogues, which seems to be important for the biological activities at the ET_A receptor but was reported not to exist in the linear analogue, ET-1 [1-21, $Ala^{1,3,11,15}$]. Aib could also help to improve the stability of the peptides towards proteolytic degradation and could also improve the solubility of the analogues which is important for the biological assay and CD/NMR studies.

Table 3.2.2 shows the receptor binding affinities and the biological activities of ET-1, ET-3, the ET_B selective STRX-6c and the synthetic full length analogues (Peptides 1C, 1, 1B, 2 and 2B). The full length analogues have a very high binding affinity for the ET_B receptor with IC_{50}'s between 0.06 and 0.9 nM in CHO cells, which are as potent as the ET-3. The ET_B receptor binding selectivities of these analogues are 10^3~10^5 over the ET_A receptor, showing higher selectivity than that of the most ET_B selective peptide, STRX-6c. Their functional activities for the ET_B receptor are also very high and comparable to that of ET-1 except peptides 1C and 2B, which are slightly lower. These results indicate that the two disulphide bridges are critical for the ET_A receptor binding but not for the ET_B receptor binding and functional activity, as has been previously reported.[55-58] Substitutions at the four Cys' positions are well tolerated for binding at the ET_B receptor. The introduction of the helix inducing amino acid, Aib, does not affect the ET_B receptor binding, but it does improve the binding affinity to the ET_A receptor, since peptide 1C, ET-1 [$Aib^{1,3,11,15}$] shows the highest binding affinity for the ET_A receptor and peptide 2B, ET-1 [$Ala^{3,11,}$

Cys(Acm)^{1,15}] shows the lowest. These results indicate that positions 11 and 15 are more important for helical structure and therefore are more sensitive to Aib substitution. This is reasonable as these positions are within the helical region, [9-15 or 16] of the ETs. Similar results are also observed in the truncated analogues, which will be discussed in the following sections.

Table 3.2.2 Biological activity of ET-1, ET-3, STRX-6c and some full length ET-1 analogues with different Cys replacements

Pept. No.	ETs and Some Full Length Analogues, 1-21	IC_{50}(μM) ET_A[b]	IC_{50}(μM) ET_B[c]	Selectivity ET_B / ET_A	EC_{50}(μM) ET_B[d]
	ET-1	0.0002	0.0006	0.13	0.0003
	ET-3	0.0025	0.000043	58	0.00007
	STRX-6c	11	0.0035	3,143	0.00025
1C	[Aib^{1,3,11,15}]	0.77	0.0003	2,567	0.0013
1	[Aib^{3,11}, Leu^{7}, X^{1,15}]	9.1	0.00006	151,667	0.00059
1B	[Aib^{3,11}, Nle^{7}, X^{1,15}]	4.5	0.0009	5,000	0.00048
2	[Ala^{3}, Aib^{11}, Leu^{7}, X^{1,15}]	6.2	0.0001	62,000	0.00053
2B	[Ala^{3,11}, Leu^{7}, X^{1,15}]	16	0.0007	22,857	0.0021

Notes :X: Cys(Acm); Mle: MeLeu;
b: tested in rabbit renal artery vascular smooth muscle cells;
c: tested in rat cerebellum;
d: tested in human ET_B receptor expressed in CHO cells.

3.2.3 Effects of chain length

Since the C-terminal part of the ET-1 has been proven to be critical for binding and functional activity at both receptor subtypes, truncation of the N-terminal part was studied. One of the full length peptide, peptide 2 was truncated to investigate the effect of the chain length (Table 3.2.3). From the data listed in Table 3.2.3, residues Cys^{1} and Ser^{2} were found not critical for the ET_B receptor functional activity, as peptides 15-1 [2-21] and peptide 14 [3-21] are as potent as peptide 2. The binding affinities, however, are slightly increased for the ET_A receptor but slightly decreased for the ET_B receptor, which resulted in a 10 fold reduction in the ET_B receptor selectivity in both peptides. The next truncation gives peptide 13 [4-21], which shows ~3 fold reduction in the ET_B receptor binding affinity but a 2 fold increase in the ET_B receptor functional activity, which indicates that residue Cys^{3} has some effect on the

Table 3.2.3 Biological activity of peptide 2 and its truncated analogues

Pept. No.	Truncated Analogues of Peptide 2	$IC_{50}(\mu M)$ ET_A[b]	$IC_{50}(\mu M)$ ET_B[c]	Selectivity ET_B / ET_A	$EC_{50}(\mu M)$ ET_B[d]
2	1-21,[Ala^3, Leu^{7,} Aib^{11}, X^{1,15}]	6.2	0.0001	62,000	0.00053
15	2-21,[Ala^3, Leu^{7,} Aib^{11}, X^{1,15}]	1.7	0.0004	4,250	0.00048
14	3-21,[Ala^3, Leu^{7,} Aib^{11}, X^{1,15}]	1.4	0.00033	4,242	0.00096
13	4-21,[Leu^{7,} Aib^{11}, X^{1,15}]	3.3	0.001	3,300	0.00042
12	5-21,[Leu^{7,} Aib^{11}, X^{1,15}]	4.3	0.001	4,300	0.0016
11	6-21,[Leu^{7,} Aib^{11}, X^{1,15}]	2.8	0.0007	4,000	0.0016
33	7-21,[Leu^{7,} Aib^{11}, X^{1,15}]	2.8	0.0009	3,111	0.0013
31	8-21,[Aib^{11}, X^{1,15}]	7.2	0.009	800	0.0047
30	9-21,[Aib^{11}, X^{1,15}]	9.9	0.15	66	0.180
29	10-21,[Aib^{11}, X^{1,15}]	>2.5	1.6	-	0.370
28	11-21,[Aib^{11}, X^{1,15}]	>2.5	>2.5	-	>10
35A	Ac-12-21,[X^{1,15}]	3.9	0.58	7	6.9

Notes :

b: tested in rabbit renal artery vascular smooth muscle cells;
c: tested in rat cerebellum;
d: tested in human ET_B receptor expressed in CHO cells.

ET_B receptor binding but has no effect on the functional activity. One more truncation results in peptide 12 [5-21] and the data shows that it has the same binding affinity as peptide 13 but has a 4 fold reduction in functional activity, which indicates that residue Ser^4 is more important for the ET_B receptor binding than for its functional activity. Further truncation until peptide 33 [7-21] affects neither the binding nor the functional activity, which suggests residues 5 and 6 are not important for ET_B receptor binding and functional activity. In fact, peptide 33, the 7-21 analogue of peptide 2, is only 9 fold less potent in binding and only 2 fold less potent in functional activity for the ET_B receptor than the full length analogue peptide 2. These results indicate that the N-terminal six residues are not very important for ET_B receptor binding and functional activity, but they affect the binding affinity for the ET_A receptor and therefore affect the receptor binding selectivity, which is 30 fold reduced in peptide 33. The next truncated analogue, peptide 31 [8-21], still affords reasonable functional activity with a further 10 fold decrease in binding affinity. Further truncation not only caused a serious decrease in ET_B receptor binding and functional

activity, but also in the receptor selectivity. The shortest analogue, peptide 35A [Ac-12-21] was found to be a poor agonist with non receptor selectivity. Therefore, the sequence between 8 and 21 of ET-1 is regarded as the minimum sequence for further studies.

From the above discussions, it can also be seen that as the peptide sequence is shortened, the receptor selectivity decreases. The major reduction occurs in peptides 31, 30 and 29, the 8-21, 9-21 and 10-21 analogues, which indicates that the charged groups ($Asp^8Lys^9Glu^{10}$) may play an important role in ET_B receptor recognition. When Asp^8 is replaced by Ala^8, the receptor selectivity decreased from 6000 to only 65 (peptides 52 and 72, Table 3.2.4C), while D-Asp^8 replacement is tolerated (peptides 52 and 68, Table 3.2.4B).

3.2.4 D-Amino acid survey at selected positions

Literature reports have shown that residues $Asp^{8,18}$, Phe^{14} and His^{16} are important in determining the agonist/antagonist property of the ET-1 analogues. D-amino acids were introduced into these positions in the full length and truncated analogues to study the importance of stereochemistry at these positions (Table 3.2.4A, 4B, and 4C).

When Asp^8 in peptide 26 is replaced with D-Asp, the binding affinity for the ET_B receptor was found to decreased by 20, but the functional activity for the ET_B receptor remains more or less the same. This indicates that the backbone conformation at position 8 is more important for ET_B receptor binding than for the functional activity. It was decided to replace His^{16} with D-Phe in peptide 24 since this substitution turned an agonist into a potent antagonist in a previous study of the C-terminal hexapeptide.[33] This substitution caused serious decrease in binding affinity, as well as the functional activity at the ET_B receptor subtype as was shown in the Table, but no antagonism was observed. This result indicates that Phe is not tolerated at position 16 in the full length analogue. Since Asp^{18} has been suggested to be an important residue, D-Asp was introduced into this position in peptide 22. It is found that the binding affinities of this peptide for both receptors are reduced as well as the functional activity through ET_B receptor. These results indicate that the back bone conformation of Asp^{18} is critical for binding to both receptors and is also important

for ET_B receptor functional activity. The replacement of Phe^{14} with D-Phe also causes serious reduction of binding affinity and functional activity as shown in peptide 21. Combination of two D-amino acid replacements normally comprises the effect of both substitutions as shown in peptides 23 and 25.

Table 3.2.4A Biological activity of peptide 2 and its full length [1-21] analogues with D-amino acid replacements

Pept. No.	Full Length Analogues, 1-21	$IC_{50}(\mu M)$ ET_A[b]	$IC_{50}(\mu M)$ ET_B[c]	Selectivity ET_B/ET_A	$EC_{50}(\mu M)$ ET_B[d]
2	[Ala^3, $Leu^{7,}$ Aib^{11}, $X^{1,15}$]	6.2	0.0001	62,000	0.00053
26	[Ala^3, $Leu^{7,}$ Aib^{11}, $X^{1,15}$, $dAsp^8$]	16	0.002	8,000	0.00032
21	[Ala^3, $Leu^{7,}$ Aib^{11}, $X^{1,15}$, $dPhe^{14}$]	13	0.25	52	>10
22	[Ala^3, $Leu^{7,}$ Aib^{11}, $X^{1,15}$, $dAsp^{18}$]	>25	5.8	-	>10
24	[Ala^3, $Leu^{7,}$ Aib^{11}, $X^{1,15}$, $dPhe^{16}$]	17	0.56	31	0.48
23	[Ala^3, $Leu^{7,}$ Aib^{11}, $X^{1,15}$, $dPhe^{16}$, $dAsp^{18}$]	20	11	2	>10
25	[Ala^3, $Leu^{7,}$ Aib^{11}, $X^{1,15}$, $dAsp^8$, $dPhe^{16}$]	13	1.8	7	0.54

Notes :

b: tested in rabbit renal artery vascular smooth muscle cells;
c: tested in rat cerebellum;
d: tested in human ET_B receptor expressed in CHO cells.

Previous studies suggested that residues between 8 and 16, known as the helical region, might be of significance with respect to receptor agonism. D-amino acids were thus introduced into each of these positions in some truncated analogues in order to study the effect of the backbone conformation (Table 3.2.4B). Results show that inversion of residues between Lys^9 and Tyr^{13} causes a 20~50 fold reduction in ET_B receptor binding affinity, but does not affect the functional activity through ET_B receptor, which again indicates that these groups are important for receptor recognition but not for functional activity. Replacement of Asp^8 with D-Asp does not affect the binding affinity for ET_B receptor whereas the functional activity is improved, indicating that the back-bone conformation at this position is not critical. This result contrasts to that observed in the full length analogues, where D-Asp^8 replacement

caused a 20 fold decrease in the ET_B receptor binding affinity (peptides 2 and 26, Table 3.2.4A). Residues Phe^{14} and His^{16}, however, are very sensitive to conformation change in the truncated analogues, since peptides 70 and 71 show very poor binding and functional activity at the ET_B receptor. These results are consistent with those observed for the full length analogues. It is very interesting to notice that the binding affinity of all these analogues at ET_A receptor remains more or less unchanged, and the selectivity of these analogues is thus reduced except for peptide 68.

Table 3.2.4B Biological activity of truncated peptide 52 (8-21) and its analogues with D-amino acid replacement at different positions

Pept. No.	Truncated Analogues [8-21]	IC_{50}(μM) ET_A[b]	IC_{50}(μM) ET_B[c]	Selectivity ET_B / ET_A	EC_{50}(μM) ET_B[d]
52	[$Aib^{11,15}$]	0.27	0.001	270	0.025
68	[$Aib^{11,15}$, $dAsp^{8}$]	4.4	0.001	4,400	0.0074
69	[$Aib^{11,15}$, $dLys^{9}$]	5.5	0.02	275	0.015
59	[$Aib^{11,15}$, $dGlu^{10}$]	2.9	0.05	58	0.037
58	[$Aib^{11,15}$, $dVal^{12}$]	4.1	0.03	137	0.037
57	[$Aib^{11,15}$, $dTyr^{13}$]	5.8	0.02	290	0.018
70	[$Aib^{11,15}$, $dPhe^{14}$]	7.4	1.4	5	>10
71	[$Aib^{11,15}$, $dHis^{16}$]	6	0.1	60	0.15
41	[$Aib^{11,15}$, Ala^{11}, X^{15}]	6.4	0.05	128	0.110
39	[$Aib^{11,15}$, $dAla^{11}$, X^{15}]	14	0.037	378	0.058

Notes :

X: Cys(Acm);
b: tested in rabbit renal artery vascular smooth muscle cells;
c: tested in rat cerebellum;
d: tested in human ET_B receptor expressed in CHO cells.

As Asp^8 shows some special activity, further investigation of this residue was carried out (Table 3.2.4C). Asp^8 was replaced with D-Asp in three pairs of [7-21] analogues. The analogues with D-Asp^8 all show ~10 fold reduction in functional activity at ET_B receptor comparing with their L-Asp^8 isomers, while the binding affinity for this receptor changed in a different way. In one pair (peptides 33 and 34, Leu^7), the binding affinity of the D-Asp^8 analogue was 3 fold increased, while in the other two pairs (peptides 62, 64, Mle^7 and peptides 61, 63, Val^7), the binding affinities

of the D-Asp[8] analogues were decreased. The increase or decrease was minor in groups 1 and 3, indicating that the backbone conformation of Asp[8] is not critical in these peptides. However, D-Asp[8] replacement in group 2 caused 200 fold decrease in ET_B receptor binding affinity. This result, and also those observed in Table 3.2.4A and 6C, indicates that the same substitution can result in totally different effects in different sequences. In group four, the effect of Ala[8] substitution was investigated. The data indicates that replacement of Asp[8] with Ala[8] results in a 67 fold decrease in the ET_B receptor binding affinity and 10^4 fold decrease in functional activity. This result clearly indicates that Asp[8] is more important for the ET_B receptor functional activity than for its binding. This replacement, however, does not affect the ET_A receptor binding and the selectivity of this peptide at the ET_B receptor is therefore reduced (from 6000 to 65).

Table 3.2.4C Biological activity of ET-1 analogues with D-Asp/L-Asp at position 8

Pept. No.	Truncated Analogues 7-21	IC_{50}(μM) ET_A[b]	IC_{50}(μM) ET_B[c]	Selectivity ET_B / ET_A	EC_{50}(μM) ET_B[d]
33	[Leu[7], Asp[8], Aib[11]X[15]]	2.8	0.0009	3,111	0.0013
34	[Leu[7], dAsp[8], AIb[11]X[15]]	26	0.0003	86,667	0.018
62	[Mle[7], Asp[8], Aib[11,15]]	0.9	0.0002	45,000	0.0015
64	[Mle[7], dAsp[8], Aib[11,15]]	1.3	0.02	65	0.019
61	[Val[7], Asp[8], Aib[11,15]]	0.83	0.0025	332	0.0023
63	[Val[7], dAsp[8], Aib[11,15]]	6.3	0.0097	649	0.016
53	[Leu[7], Asp[8], Aib[11,15]]	1.8	0.0003	6,000	0.00096
72	[Leu[7], Ala[8], Aib[11,15]]	1.3	0.02	65	>10

Notes :

X: Cys(Acm);
b: tested in rabbit renal artery vascular smooth muscle cells;
c: tested in rat cerebellum;
d: tested in human ET_B receptor expressed in CHO cells.

3.2.5 N-Acetylation of ET-1 analogues

Although the N-terminal acetylation of ET-1 itself is not tolerated, the N-terminal acetylation of some truncated analogues has been found to improve the

binding affinity at the ET_B receptor.[55,61] The same study was carried out on some 6-21, 7-21 and 8-21 analogues (Table 3.2.5). However, the data indicate that N-terminal acetylation does not affect the binding affinities of these analogues to the ET_B receptor, while the functional activity is increased by 1.6~9 fold. These results suggest that N-terminal acetylation of these analogues is tolerated but not necessary for the functional activity.

Table 3.2.5 The effect of N-terminal acetylation on truncated analogues

Pept. No.	Truncated Analogues	$IC_{50}(\mu M)$ ET_A[b]	$IC_{50}(\mu M)$ ET_B[c]	Selectivity ET_B / ET_A	$EC_{50}(\mu M)$ ET_B[d]
33	[7-21, Leu^7, Aib^{11}, X^{15}]	2.8	0.0009	3,111	0.0013
33a	[Ac-7-21, Leu^7, Aib^{11}, X^{15}]	16	0.0064	2,500	0.00081
61	[7-21, Val^7, $Aib^{11,15}$]	0.83	0.0025	332	0.0023
61a	[Ac-7-21, Val^7, $Aib^{11,15}$]	2.7	0.0038	710	0.0011
63	[7-21, Val^7, $dAsp^8$, $Aib^{11,15}$]	6.3	0.0097	649	0.016
63a	[Ac-7-21, Val^7, $dAsp^8$, $Aib^{11,15}$]	4.4	0.0023	1,913	0.0014
41	[8-21, Ala^{11}, X^{15}]	6.4	0.05	128	0.110
41a	[Ac-8-21, Ala^{11}, X^{15}]	3.5	0.042	83	0.036

Notes :

X: Cys(Acm);
b: tested in rabbit renal artery vascular smooth muscle cells;
c: tested in rat cerebellum ;
d: tested in human ET_B receptor expressed in CHO cells.

3.2.6 SAR study of the truncated analogues, 8-21 of ET-1

Since the minimum sequence which affords nM ET_B receptor binding is 33, the 8-21 analogue of ET-1, further study was carried out with the 8-21 analogues. Several such analogues with different substitutions at certain positions were synthesized and studied (Table 3.2.6).

In the first group, the effect of $Cys^{11,15}$ substitution was investigated to compare with the results obtained for the full length analogues. As discussed above in the full length analogues, substitution at these positions is generally tolerated. The analogue with Aib at positions 11 and 15 (peptide 52) showed the highest binding

affinity at both receptor subtypes, while that with Ala at position 11 and Cys(Acm) at 15 (peptide 41) was the most potent agonist at ET_B receptor. The 30 fold increase in the ET_A receptor binding affinity of peptide 52, compared to peptide 31, indicates the importance of Aib at position 15 for the ET_A receptor binding. In the second group, analogues with different substitution at some highlighted positions were investigated. Lys^9 has been reported as a position of interest. When it was replaced with Glu^9, the analogue proved to be a highly selective and very potent ET_B receptor antagonist, IRL 1620, ET-1[Suc-8-21, Glu^9].[57] The same change was made to the 8-21 analogue but the data indicates that this substitution is not tolerated as peptide 55 showed very poor binding affinity at both receptors and very poor agonism at the ET_B receptor. The reason for this discrepancy is unknown, but is probably due to the presence of different ET_B receptor subtypes.

Table 3.2.6 Biological activity of truncated ET-1 analogues --- analogues of 8-21

Pept. no.	Truncated Analogues 8-21	IC_{50}(μM) ET_A[b]	IC_{50}(μM) ET_B[c]	Selectivity ET_B / ET_A	EC_{50}(μM) ET_B[d]
52	[$Aib^{11,15}$]	0.27	0.001	270	0.025
31	[Aib^{11}, X^{15}]	7.2	0.009	800	0.0047
41	[Ala^{11}, X^{15}]	6.4	0.05	128	0.110
41a	Ac-[Ala^{11}, X^{15}]	3.5	0.042	83	0.036
39	[$dAla^{11}$, X^{15}]	14	0.037	378	0.058
55	[Glu^9, $Aib^{11,15}$]	>2.5	>2.5	-	>10
46	[$Aib^{11,15}$, Thr^{18}]	1.3	0.003	433	0.300
50	[$Aib^{11,15}$, Leu^{18}]	0.16	>2.5	<1	>10
66	[$Aib^{11,15}$, Mle^{19}]	4.7	4.6	1	>10
37	[$Aib^{11,15}$, $Thr^{18}Mle^{19}$]	8.3	1.0	8	>10
37a	Ac-[$Aib^{11,15}$, $Thr^{18}Mle^{19}$]	11	0.55	20	>10
36	[Glu^9 $Aib^{11,15}$, $Thr^{18}Mle^{19}$]	24	2.9	8	>10
36a	Ac-[Glu^9 $Aib^{11,15}$, $Thr^{18}Mle^{19}$]	26	1.7	15	>10

Notes :

b: tested in rabbit renal artery vascular smooth muscle cells;
c: tested in rat cerebellum;
d: tested in human ET_B receptor expressed in CHO cells.

3.2.7 Comparison among the truncated 6-21, 7-21 and 8-21 analogues

As discussed in Section 3.2.3, the 6-21 and 7-21 analogues are ~10 fold more potent for the ET_B receptor than the 8-21 analogues, thus further research was carried out to study the effect of residues 6 and 7 under different substitution patterns (Table 3.2.7).

Table 3.2.7 Biological activity of truncated ET-1 analogues ---- analogues of 6-21, 7-21 and 8-21

Pept. No.	Truncated 6-21, 7-21 & 8-21 Analogues	IC_{50}(μM) ET_A[b]	IC_{50}(μM) ET_B[c]	Selectivity ET_B/ET_A	EC_{50}(μM) ET_B[d]
	Leu^7, $Aib^{11,15}$				
54	[6-21]	2.0	0.0002	10,000	0.00072
53	[7-21]	1.8	0.0003	6,000	0.00096
52	[8-21]	0.27	0.001	270	0.025
	Leu^7, Ala^{11}, X^{15}				
40	[Ala^{11}, X^{15}]	2.8	0.003	933	0.0053
65	[7-21]	9.6	0.005	1,920	0.0044
41	[8-21]	6.4	0.05	128	0.110
	$Aib^{11,15}$, Thr^{18}				
47	[6-21]	1.4	0.0005	2,800	0.290
46	[8-21]	1.3	0.003	433	0.300
	$Aib^{11,15}$, Leu^{18}				
51	[6-21]	0.085	0.074	1	>10
50	[8-21]	0.16	>2.5	<0.06	>10
	Glu^9, $Aib^{11,15}$, $Thr^{18}Mle^{19}$				
38	[6-21]	3.0	0.18	17	>10
36	[8-21]	24	2.9	8	>10
	Glu^9, $Aib^{1,3,11,15}$, $Thr^{18}Mle^{19}$				
56	[1-21]	2.6	0.03	87	>10

Notes :

b: tested in rabbit renal artery vascular smooth muscle cells;
c: tested in rat cerebellum;
d: tested in human ET_B receptor expressed in CHO cells.

In the first group, three peptides with $Aib^{11,15}$ are studied. The data shows that the 6-21 and 7-21 analogues have very similar binding affinity and functional activity,

while the 8-21 analogue showed increased binding affinity for the ET_A receptor but decreased binding affinity and functional activity for the ET_B receptor. The binding affinity of the 8-21 analogue is 20~30 fold less potent than the 6-21 and 7-21 analogues for the ET_B receptor, which indicates that residues 6 and 7 are important for the ET_B receptor binding under this substitution. The slightly increased affinity within the 8-21 analogues for the ET_A receptor binding is unexpected, but it clearly indicates that residues 6 and 7 are not critical for the ET_A receptor recognition.

In the second group, peptides with $Ala^{11}Cys(Acm)^{15}$ were investigated. The data indicate that the 6-21 and 7-21 analogues are very similar in binding and functional activity while the 8-21 analogue is much less potent. The binding affinity of all three peptides for the ET_A receptor, however, are very similar.

Asp^{18} is another interesting residue as well as Asp^{8}. In the SAR study of bicyclic ET-1 analogues, Kikuchi *et al.* found that replacement of Asp^{18} with a hydrophobic amino acid afforded potent ET_A receptor antagonists while analogue ET-1 [1-21, $Thr^{18}Mle^{19}$] was an antagonist with no receptor selectivity.[46] In another independent study of the monocyclic analogues, Hunt *et al.* reported that analogue ET-1[1-21, $Ala^{3,11}$, Nle^{7}, Ala^{18}] showed antagonism at the ET_A receptor.[45] It is known that the disulphide bond(s) play an important role in ET_A receptor binding but not in the ET_B receptor, it would be very interesting to introduce the same substitution into the ET_B selective linear analogues. Therefore peptides with different substitutions at position 18 were studied (groups 3 and 4). In group three, peptides with $Aib^{11,15}Thr^{18}$ were studied. The binding affinities of both peptides to the ET_A receptor were very similar, but those for the ET_B receptor was 6 fold decreased in the 8-21 analogues, indicating that residues 6 and 7 were important for the ET_B receptor binding but not for the ET_A receptor. The binding affinities for both receptor subtypes are comparable to those of the corresponding peptides in group one, which indicates that Thr^{18} is tolerated. However, the functional activities of both peptides with Thr^{18} were greatly decreased, especially that of the 6-21 analogue, which indicates that Asp^{18} is essential for the ET_B receptor functional activity as discussed above. In group four, peptides with $Aib^{11,15}Leu^{18}$ were studied. Compared with the peptides in groups one and three, the binding affinities of both peptides for the ET_A receptor were increased, especially the 6-21 analogue (peptide 51). This result indicates that Asp^{18} is

not necessary for ET_A receptor binding and a hydrophobic residue, such as Leu, is preferable. The binding affinity for the ET_B receptor, however, seriously decreased, as did the functional activities through the ET_B receptor, which indicates that Asp^{18} is very important for ET_B receptor binding and functional activity. Compared to the corresponding peptide in group three, the ET_B receptor functional activities of these peptides were further decreased which indicates that hydrophilicity is significant at this position. The data also indicate that residues 6 and 7 are important for both receptors with this substitution. In group five, peptides with $Thr^{18}Mle^{19}$ were investigated for antagonism. However, this replacement was not tolerated by the ET_B receptor as indicated by the significant decrease both in binding and functional activity. Residues 6 and 7 are important for both receptors as is shown by the increased binding affinity of peptide 38. In order to further increase the binding affinity for the ET_B receptor, this peptide was lengthened, without the substitution at position 9, to give a full length analogue, peptide 56. As shown by the data in Table 3.2.7, the ET_B receptor binding and selectivity are both improved, but the analogue is a poor agonist rather than an antagonist.

3.2.8 Synthesis and folding of ET-1

Apart from its numerous biological actions and potent physiological importance, ET and its precursor peptide, BigET, have set new tasks for the peptide chemist. The correct formation of the two disulphide bonds is the most difficult part of the synthesis. Random oxidation of the fully reduced peptides normally affords a mixture of two different isomers in a ratio of 3:1.[79,80] Orthogonal thiol-protecting groups have therefore been developed,[81,82,83] but the yields in such procedures has never been reported. In this study, BigET-1 was synthesized according to a method developed in this laboratory by Stewart[84], and new method was developed for the synthesis of ET-1 which is described in the following.

The peptide chain of ET-1 was assembled by double triazole coupling methodology (Section 3.3.1) and all four cysteines were protected with Trt group. The crude peptide was afforded in good purity according to the HPLC (Figure 3.2.2a), thus it was decided to carry out the oxidation without pre-purification. However, the crude peptide was not soluble in the oxidation mixture (0.1M Tris

buffer, pH 8.0, (37°C)) in 1M Guanidine.HCl, the same mixture in 6M Gunindine.HCl had to be used in order to dissolve the crude peptide.

According to the CD studies that the helical structure in ET-1 analogues was very strongly conserved, therefore it was decided to carry out the oxidation in 6M gunindine.HCl and 0.1M Tris buffer. Reduced and oxidized glutathione were added to make a final concentration of 5mM and 0.5mM respectively. The mixture was stirred at 0-10°C overnight and monitored by HPLC, which showed one major peak with the same retention time as commercial ET-1 in 30min (Figure 3.2.2b). The situation did not change in the next 8 hrs, so the peptide was isolated and purified twice by preparative HPLC on a P&P C18 column and lyophilized to give 15mg final product (overall yield 19.4%). A co-injection of the purified peptide with commercial ET-1 resulted in a very sharp single peak (Figure 3.2.3.a). The peptide was analyzed under different HPLC conditions (Figure 3.2.3b,c). MS of the synthetic peptide gave the required mass. The 2D NMR of this peptide is shown in section 3.2.9, which also confirms that it is identical to the native ET-1.

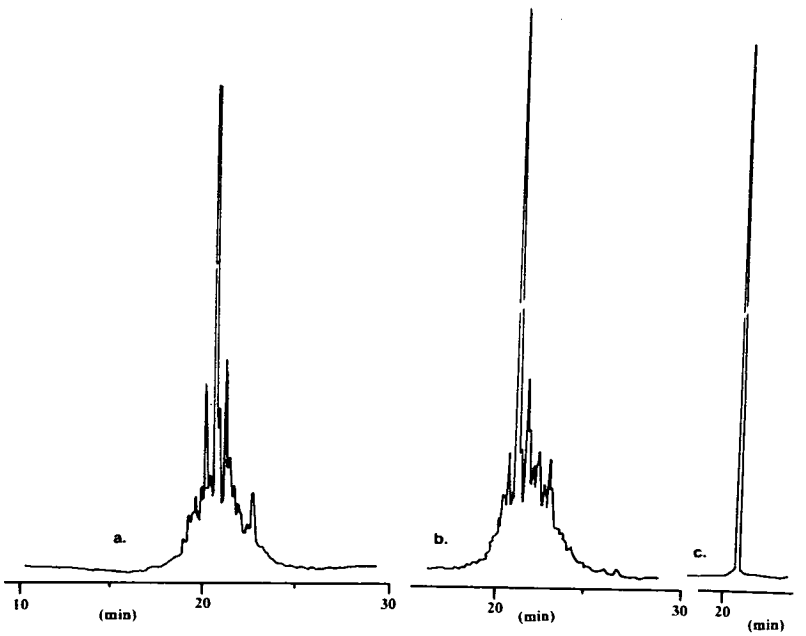


Figure 3.2.2 HPLC monitoring of peptide 60 oxidation and folding in 6m guanidine.HCl, 0.1M Tris buffer (pH 8.5); a. 30min; b.8h; c. after purification. 214nm, 1ml/min; Hichrom C18 column(size B).

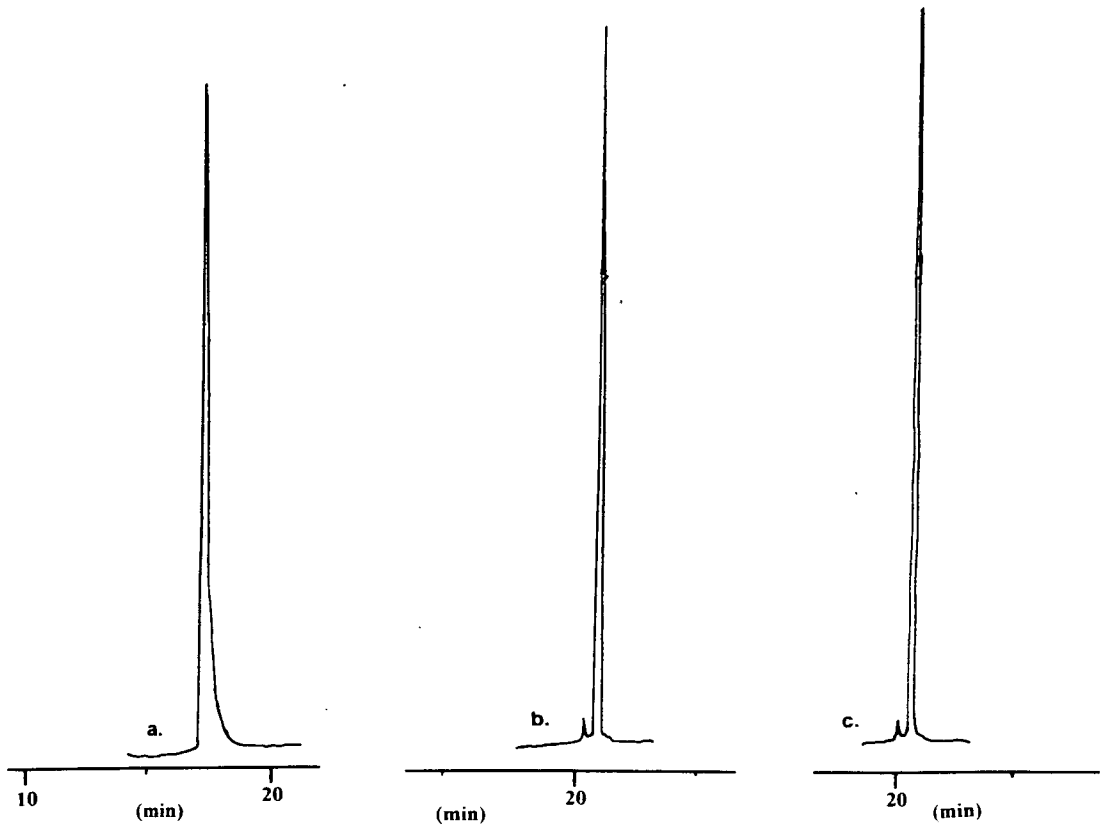


Figure 3.2.3 HPLC analysis of peptide 60

214nm, 1ml/min; a. RP C18 (size A); b. Vydac C18 (size B); c. co-injection with native ET-1, Hichrom C18 column(size B);

3.2.9 Conformational studies of ET-1 and its linear analogues

Information on the secondary and tertiary structure of the peptide is important for the study of structure-activity relationships. The conformation of peptides in the ET family have been extensively analyzed due to their biological importance.[64-68] However, no research effort has ever been made into the conformational study of linear analogues except the early report of ET-1 [$Ala^{1,3,11,15}$],[53] probably due to the absence of helical structure in this early report.[53] However, when the CD experiment was carried out in this study, a surprisingly high percentage of helical structure was observed in most of the analogues upon addition of trifluoroethanol (TFE). Further study was carried out using CD as well as 2D NMR to examine the effect of different substitutions and chain length to the helical structure.

Two typical CD spectra are illustrated in Figure 3.2.3 and the secondary structure contents are calculated using the CONTIN procedure.[85] The results are listed in Table 3.4.8 and Table 3.4.9. From the data in Table 3.2.8 it can be seen that all full length analogues without D-amino acid replacement have a high percentage of helical structure apart from peptide 2B, which has Ala at position 11 while all the others have the helix promoting Aib at this position. This result indicates that Aib is important for the helical structure especially at position 11. A similar result was observed in a truncated analogue, peptide 40 (Table 3.2.9). Replacement with Ala at positions 1 and 3 did not affect the helix structure, as shown in peptides 1, 2 and 1B. However, peptide 1C, the analogue with Aib at positions 1,3,11,15, showed the highest helix extent, which indicates that position 15 also plays a role in promoting the helical structure. As shown in Table 3.2.1, peptide 2B is as potent as the other full length analogues in terms of the ET_B receptor binding and functional activity, which indicates that helical structure is not important for the ET_B receptor functional activity. The binding affinity of 2B to the ET_A receptor, however, was slightly reduced, which indicates that the helical structure is important for the ET_A receptor recognition.

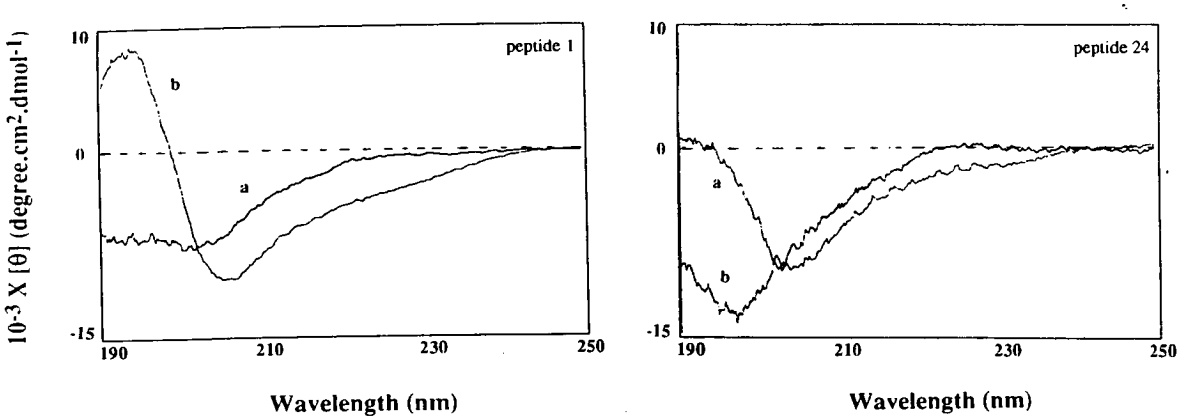


Figure 3.2.3 Typical CD spectra, a. in buffer 1; b. in buffer 2

Table 3.2.8 Circular dichroism data of the full length ET-1 Analogues

Peptides	α-helix %		β-sheet %		remainder%	
	Buffer1	Buffer2	Buffer1	Buffer2	Buffer1	Buffer2
1	3±0.81	22±1.2	49±0.93	63±1.3	48±1.6	15±2.3
2	6±0.65	22±1.2	56±0.75	57±1.20	38±1.2	21±2.2
2B	0±0.00	2±0.85	69±0.75	66±0.89	31±0.75	32±1.6
1C	6±0.65	26±0.8	42±0.76	51±0.92	47±1.3	23±1.5
21	0±0.00	12±0.71	59±0.73	55±0.81	41±0.7	33±1.4
22	5±1.0	19±1.3	62±1.1	72±1.4	9±2.4	37±2.0
24	2±0.55	10±1.0	50±0.8	53±1.2	48±0.97	37±2.0
25	2±0.81	11±1.9	46±1.2	56±2.1	52±1.4	33±3.5
26	3±0.68	19±0.7	58±0.78	59±0.8	40±1.3	22±1.3

Notes:

Buffer 1: 50mM boric acid/H_2O 1:1(v/v) (pH7.0),

Buffer 2: buffer1/trifluoroethanol 1:1 (v/v).

It is interesting that all the linear analogues show much lower binding affinity for the ET_A receptor, compared to ET-1 itself, despite the presence of helix. One explanation could be the increased flexibility of the helical structure in the linear analogues, but other effects may also be involved.

When the sequence was truncated from the N-terminus, the percentage of helix and β-sheet increased which indicates that the N-terminal part of the structure is random, while the C-terminal may exist as the β-sheet in the solution studied.

D-amino acid substitution changes the backbone conformation and therefore can affect the helical conformation if the replacement takes place in the helical region, as shown in peptides 21, 23-25 (Table 3.2.8). However, in peptides 22 and 26, the percentage of helix is almost unchanged because the positions of the replacement are not within the helical region. However, in the truncated pair, peptides 33 and 34, a great effect was observed on the helical structure in the D-Asp^8 analogue. Peptide 34 was dramatically disrupted showing only half of the helicity of the L-Asp^8 analogue in buffer 2.

Table 3.2.9 Circular dichroism data of some truncated ET-1 analogues

Peptides	α-helix %		β-sheet %		remainder%	
	Buffer1	Buffer2	Buffer1	Buffer2	Buffer1	Buffer2
12	2±1.3	34±1.3	68±1.3	58±1.3	30±2.3	8±2.4
13	5±0.92	25±1.9	56±1.1	54±2.0	39±1.8	21±3.5
14	3±0.71	29±1.3	53±0.82	60±1.4	44±1.4	10±2.4
15	0±0.00	29±1.3	73±1.5	63±1.3	27±1.5	8±2.3
33	4±0.88	31±1.6	66±1.0	66±1.6	31±1.7	3±2.9
34	0±0.92	16±1.6	100±0.05	65±1.6	0±0.00	19±2.9
36	n.s.	29±0.78	n.s.	71±0.78	n.s.	0±0.00
38	n.s.	25±0.76	n.s.	75±0.76	n.s.	0±0.00
43	0±0.00	26±1.3	100±0.06	41±1.5	0±0.00	33±2.4
51	0±0.00	21±1.8	100±0.90	59±1.9	0±0.00	20±3.3
40	n.s.	7±1.1	n.s.	74±1.3	n.s.	19±2.2

Notes:

n.s.: insoluble in buffer 1,.
Buffer 1: 50mM boric acid/H_2O 1:1(v/v) (pH7.0),
Buffer 2: buffer1/trifluoroethanol 1:1 (v/v).

Replacement with L-amino acid(s) at the C-terminal region does not affect the helical structure, neither does replacement of Lys^9 with Glu, as shown in peptides 36 and 38 (Table 3.2.9). Both peptides showed a high percentage of helical structure but very poor ET_B receptor binding affinity and functional activity (Table 3.2.9). This results indicate that the helical structure is not necessary for ET_B receptor binding but the charge groups are important.

Since CD studies indicate a high percentage of helical and β-sheet structure exists in most of the linear analogues, 2D ^1H NMR was carried out to study the structures in detail.

D_2O/CD_3OD (1:1) was chosen as the solvent as it is similar to that used in the CD studies. The 1D ^1H NMR of peptide 1 was very well defined, especially in range 6.5-9.0 ppm, where the NH signals appear, which indicates the presence of a well folded conformation (Figure 3.2.4). A systematic study would obviously be significant and the research was continued by Hewage.[86] A helical region similar to those

reported (9 to 15) was observed in most of the analogues studied.[86] In one particular analogue, peptide 1, the helical structure was found to start at the very beginning of the N-terminus (Figure 3.2.5). This is probably due to the presence of Aib at position 3. In another analogue, peptide 26, however, the helical structure was observed at residues [9-16] (Figure 3.2.6). The reasons for this could be the presence of a D-Asp at position 8 and also Ala at position 3.[86]

The structure of ET-1 in the same solvent was also studied and the resulting structure is shown in Figure 3.2.7.

Figures 3.2.5, 3.2.6 and 3.2.7 show that the structure of the linear analogue is very similar to that of the native ET-1 in the same solution. This result indicates that the disulphide bonds are not critical for the helical structure as suggested in the early reports.[51] The structure of a truncated analogue, peptide 33 [7-21], was also studied and the data indicates that the truncation of the N-terminus six amino acids (Cys^1-Leu^6) does not affect the helical structure. Replacement of Asp^8 and Asp^{18} with D-Asp does not appear to affect the helical structure either as shown in peptide 26.[86] All these results are consistent with the CD studies.

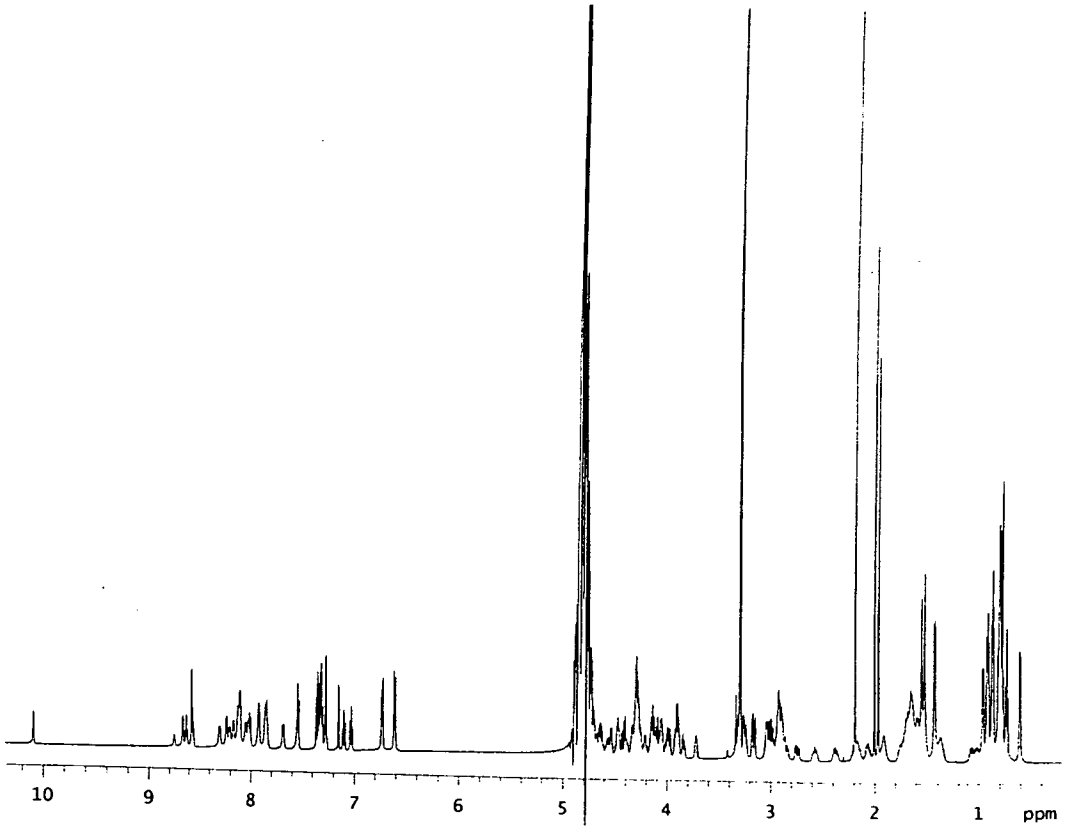


Figure 3.2.4 ^1H NMR spectrum of peptide 2

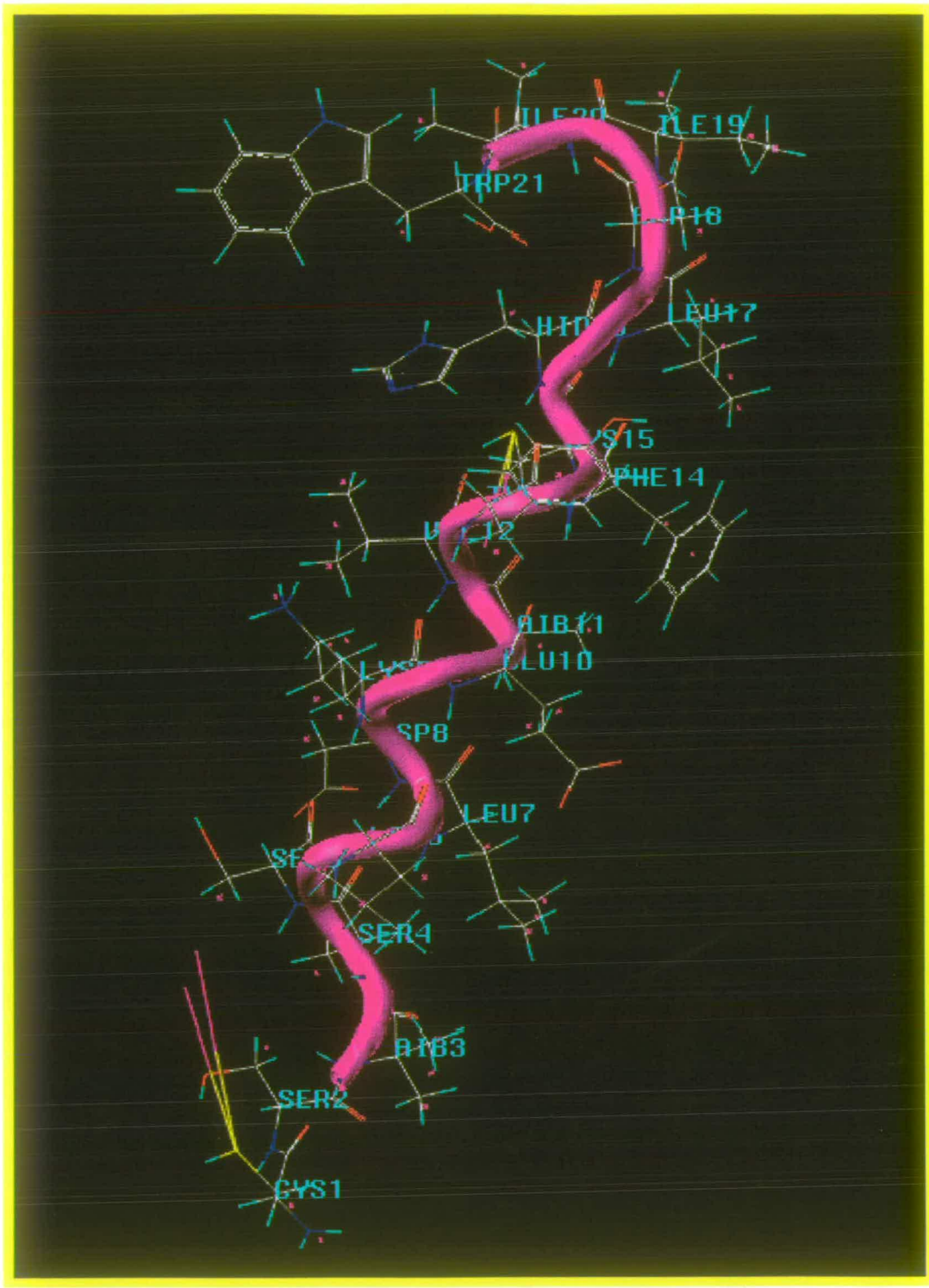


Figure 3.2.5 3D NMR structure of peptide 1

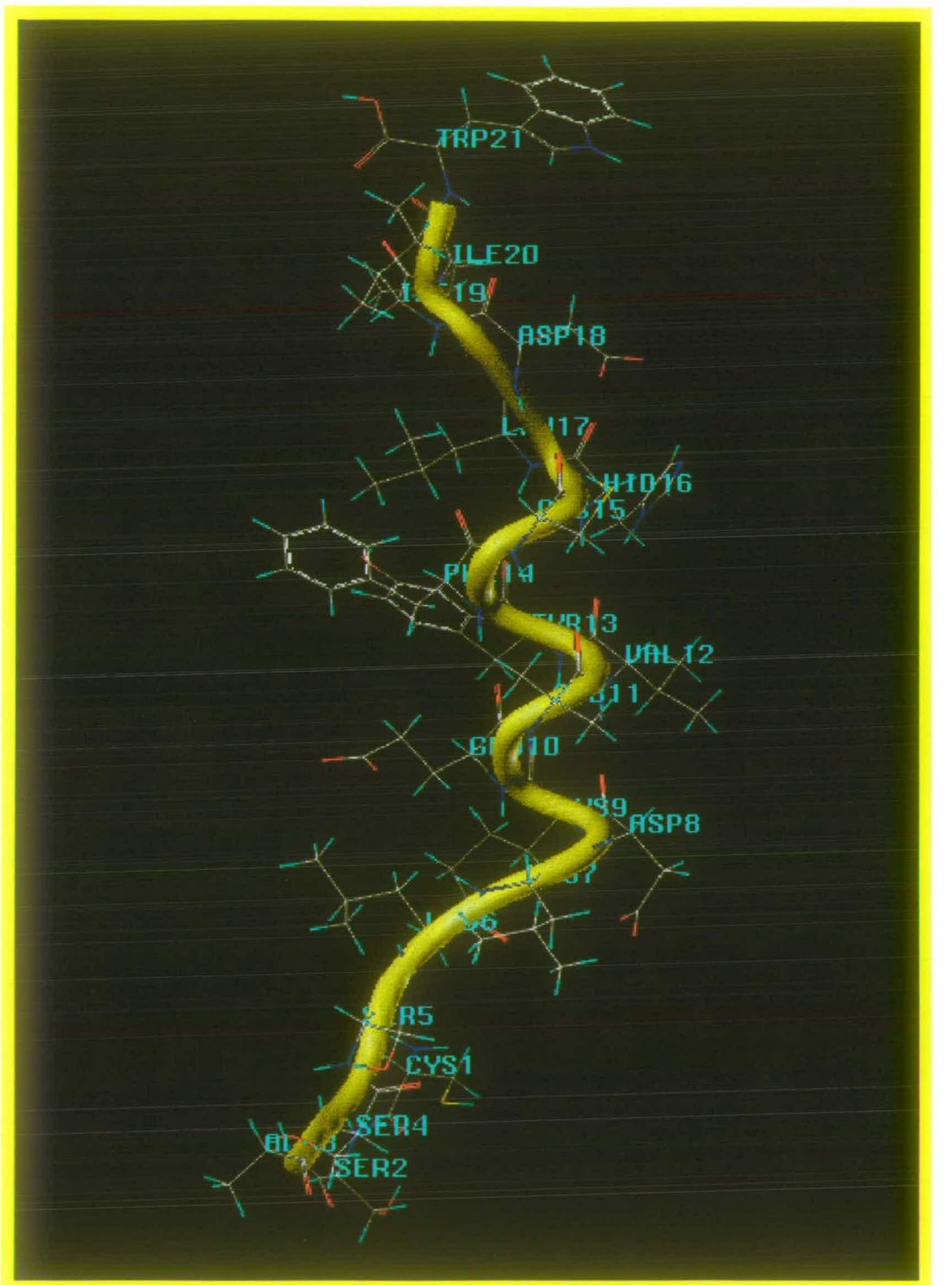


Figure 3.2.6 3D NMR structure of peptide 26

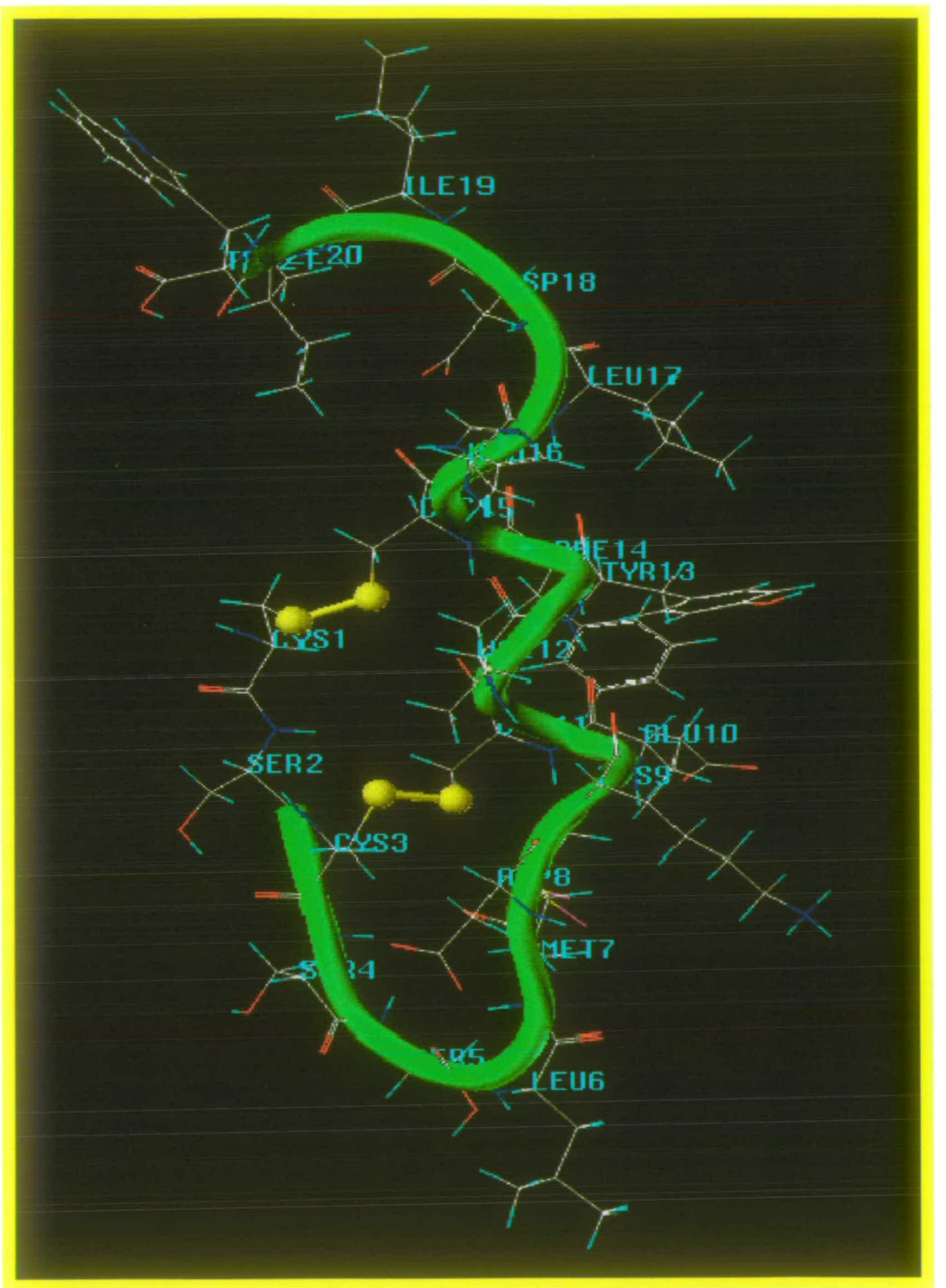


Figure 3.2.7 3D NMR structure of synthetic human ET-1

3.3 SAR study of BigET-1 analogues, ECE inhibitors

3.3.1 Introduction

The purification of ECE by several groups in 1993 rapidly led to the molecular cloning and sequencing of the enzyme from different sources.[87,88,89] All forms of ECE show >95% homology in amino acid sequence in their putative extra cellular domains, but studies have revealed that ECE from various lines and tissues show some distinctive features of specificity. Most data supported the occurrence of isozymes of ECE.

Some key features of ECE specificity have been emerged as a result of detailed studies on the purified enzymes. Wong *et al.*[90] reported that a neutral ECE did not cleave a decapeptide substrate containing five amino acids on both sides of the cleavage site. This suggested that portions of the substrate sequence far from the cleavage site may be essential for enzyme recognition. Okada *et al.*[91] found that residues 27-34 of BigET-1 were important for conversion by a neutral ECE from bovine endothelial cells. In a later report by these authors, BigET-1 [19-34] was proposed as the minimum sequence for ECE recognition.[92] The N-terminal disulphide loop, BigET-1 [1-15], appears to hinder conversion since truncated forms have substantially higher specific activity. One shorter analogue, BigET-1 [16-37] showed 3-fold greater activity while another analogue, BigET-1 [18-34, Phe^{22}], exhibits 12-fold higher specific activity than BigET-1 itself.[9]

There are limited reports on requirements for efficient inhibition of ECE. Phosphormidon was found showing some inhibitory activity at the very early stage of the ECE studies, but it is not specific for ECE.[26,93] N^{α}-rhamnopyranosyl-oxyhydroxy phosphinyl-Leu-Trp (PR) was reported to inhibit ECE, but it was several orders magnititude less potent on ECE than on the ECE-like enzyme, E-24,11.[10] A more potent, dual inhibitor, CGS 26303 was reported recently which displays an IC_{50} of 0.1 nM for E-24,11 and 1.1μM for ECE.[94] Features that are important for selectivity of ECE over E-24,11 induced the discovery of a prototype compound, 3-

(1-naphthyl)-1-phosphonopropyl-Leu-Trp, which presents one of the most potent ECE inhibitor to date (IC_{50}=0.26μM) and shows 400-fold greater ECE/E-24,11 selectivity than PR.[95]

Inhibition of ECE by synthetic peptides has also been studied. Morita *et.al.*[96] reported that ECE did not cleave truncated BigET-1 analogues with D-amino acid at position 20, 21, 22 or 23. One of the analogues, BigET-1 [16-38, D-Val^{22}] was found to be an ECE inhibitor with a *Ki* value of $2.5x10^{-5}$M.[96]

A systemic SAR study has been carried out in this laboratory using cloned human ECE-1 in order to evaluate each of these important residues towards ECE inhibition. The structures of the BigET-1 analogues have been synthesized are listed in Appendix III, and their ECE inhibitory activities are discussed in detail below and the research is being continued by Swenson.[97]

3.3.2 Effects of chain length and important residues

A series of BigET-1 analogues with differing chain lengths were studied in order to study the effect of chain length and important residues for ECE recognition (Table 3.3.1).

Table 3.3.1 Effects of chain length for ECE

Peptide	Structure	Inhibition (%)*	IC_{50} (μM)
5	BET[1-38, Cys(Acm)^{1,3,11,15}]	67.4%@200μM	
83	**BET[16-38]**	**92.1%@1 μM**	**0.038**
84	**BET[16-34]**		**0.085**
85**	BET[16-32]		0.140
86**	BET[17-34]		0.570
16	BET[19-38]	39 @200μM	12.5
3	BET[22-38, Leu^{33}]	7.5%@100μM	>50
27	BET[19-34]		2.5
9	BET[19-34, Leu^{34}]		7.0
10	BET[19-34, Leu^{32,34}]	70.1 @200μM	
8	BET[27-34, Leu^{34}]	0 @200μM	

Notse: * tested in CHO/humanECE-1 cells; **Data from Swenson.[97]

Peptide 5 is a full length linear analogue with an extra Leu at 33'. Data show that this peptide is a poor ECE inhibitor (67.4% at 200μM). Residues 27-34 were reported as important for ECE recognition, but the segment itself is not long enough to show any activity (peptide 8). Peptides 9 [19-34, Leu^{34}] and 10 [19-34, $Leu^{32,34}$] are more active than peptide 16 [19-38] which indicates that residues 35-38 are not important for ECE recognition and substitution of $Gly^{32,34}$ with $Leu^{32,34}$ is also tolerated. Data also show that peptide 83 [16-38] is the most active inhibitor, while peptide 84 [16-34] is only ~2 fold less active. These results indicate that residues 16-34 are most important for ECE recognition. Further truncation from both ends results in peptides 85 [16-32] and 86 [17-34]. The IC_{50} values indicate that residue His^{16} is more important than residues Leu^{33} and Gly^{34}, and BigET-1 [16-32] can be regarded as the minimum sequence for ECE recognition.

3.3.3 Synthesis of the reduced isostere, FmocTrpψ(CH_2NH)Val OH

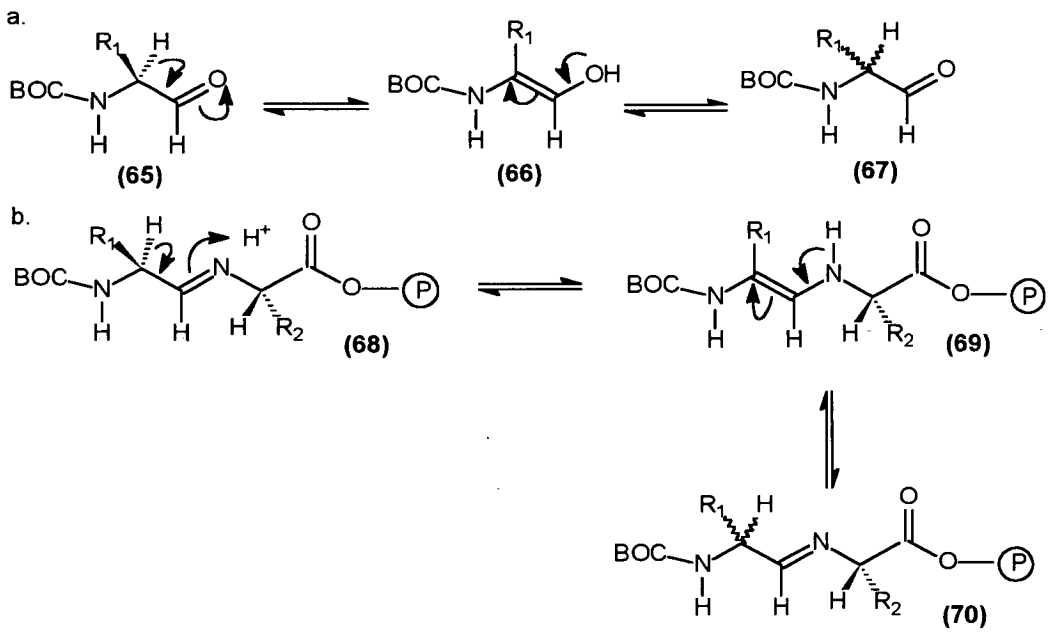
Modification of the peptide bond (CONH) is one of the most direct approaches to alter peptide conformation. Numerous structural mimics of the CONH bond have been proposed and used. The peptides afforded were resistant to enzyme hydrolysis and have therefore been widely used in the investigation of enzyme inhibitors and receptor antagonists.

The simplest isostere is the reduced peptide bond ψ(CH_2NH) group. The preferred method for the introduction of the reduced peptide bond is the reductive alkylation of an α-amino group by the reaction of a protected amino acid aldehyde in the presence of sodium cyanoborohydride ($NaCNBH_3$) in solution[98] or on solid phase.[99,100] The solid phase methodology has the advantage of convenience but is prone to racemisation unless strict reaction conditions are applied.[100] Ho *et al.* suggested two potential steps where racemisation could occur in the preparation of Boc protected reduced isostere, the amino aldehyde (**60**) and the intermediate imine (**61**) (Scheme 3.3.1).[100]

There appeared to be few problems of the ψ(CH_2NH) group reaction with other Boc amino acids under normal coupling conditions. The exception to this was

Gly, which would react with the secondary imine, but this could be avoided by coupling through its p-nitrophenyl ester.[100]

ECE cleaves BigET-1 between Trp^{21} and Val^{22}. The reduced dipeptide isostere, Trp $\psi(CH_2NH)$ Val was thus introduced into some BigET-1 analogues to produce inhibitory activity.

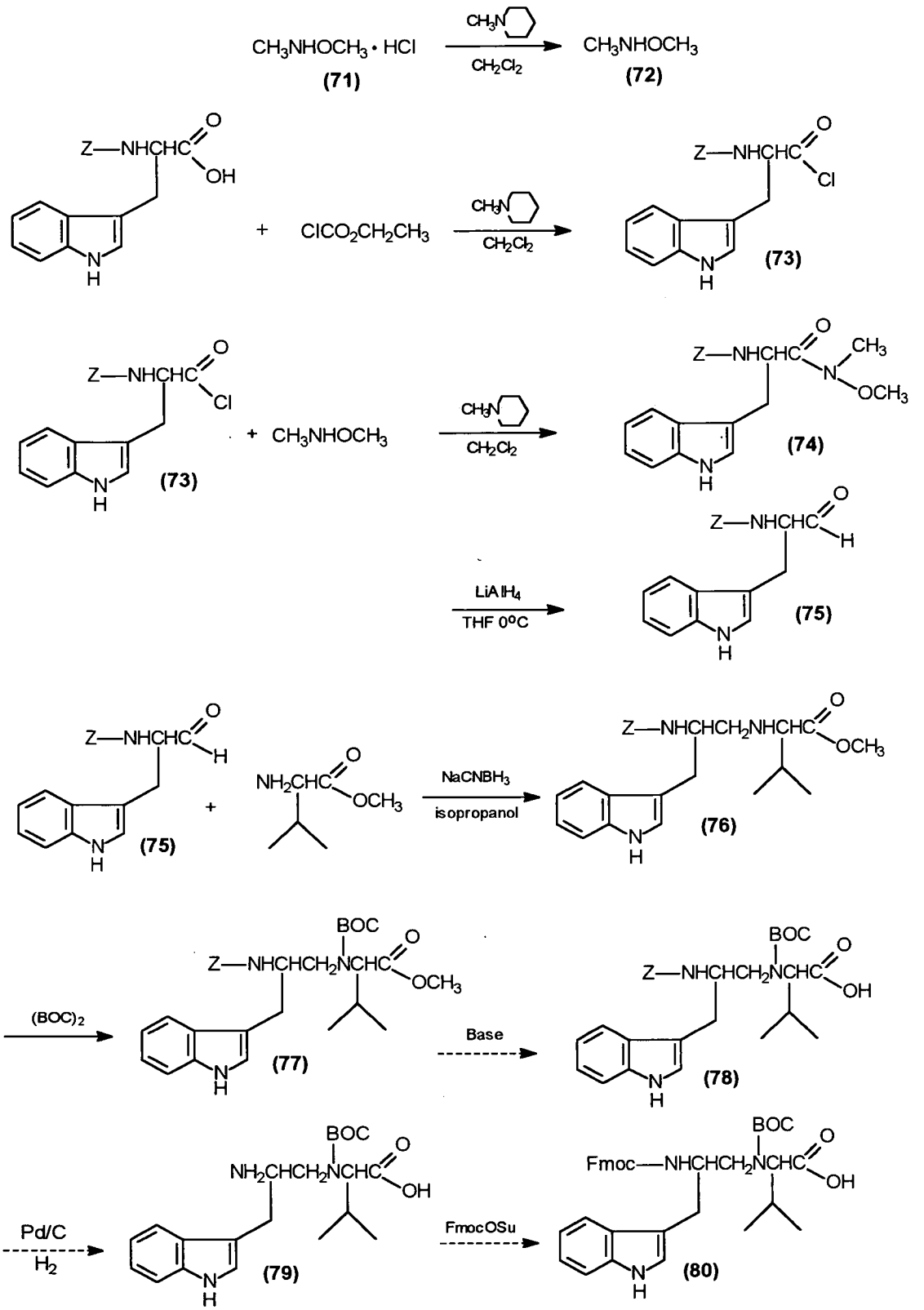


Scheme 3.3.1 Racemisation during the synthesis of the reduced isostere a. the aldehyde, b. the reduced isostere

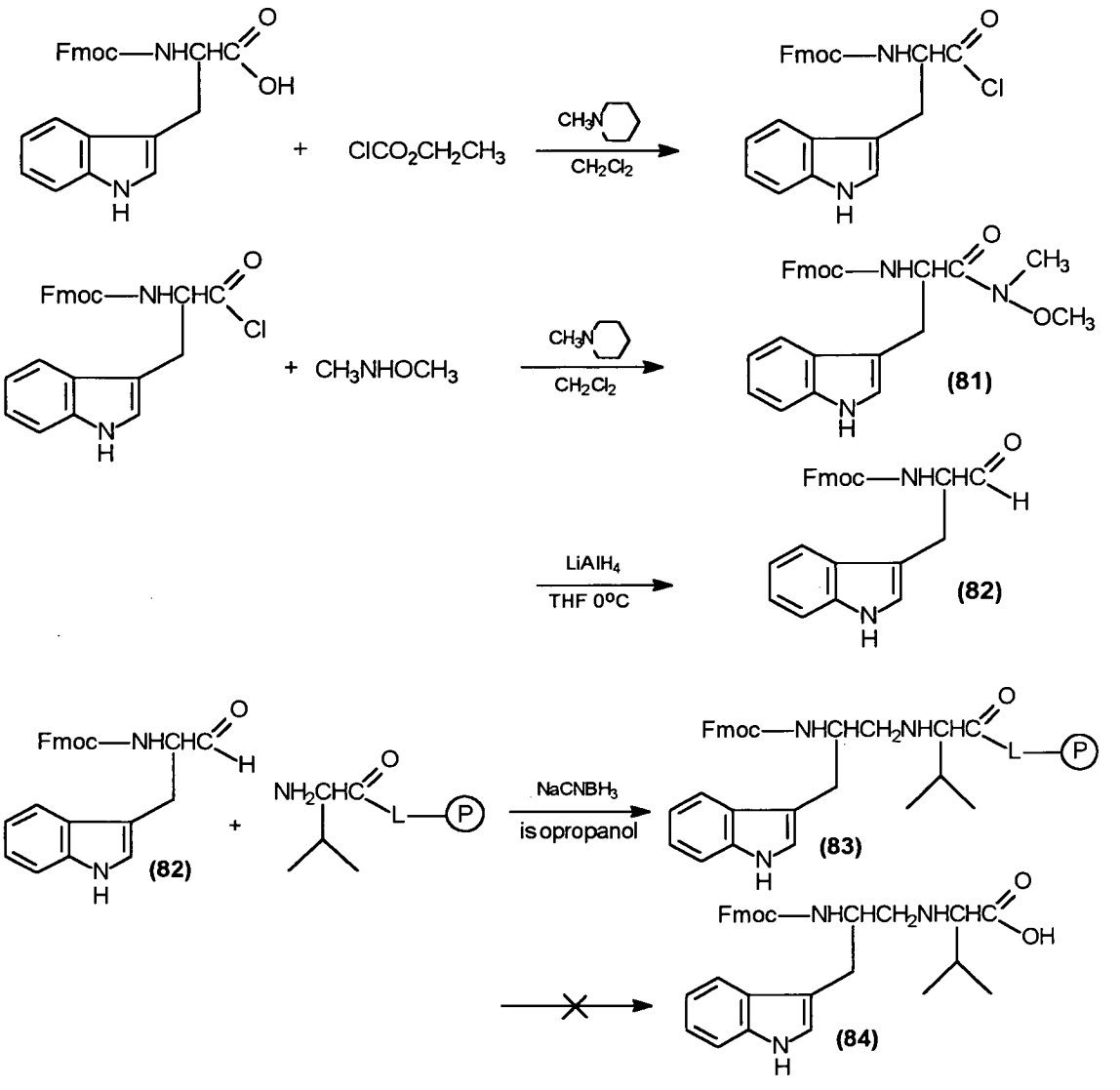
There has been no literature report on the synthesis of the Fmoc protected reduced isostere to date, which is probably due to the base lability of the Fmoc group. Therefore it was decided to make the Z-protected isostere instead which could be deprotected and converted to the Fmoc protected isostere as shown in Scheme 3.3.2.

The procedure worked well and the methyl ester of the isostere (77) was obtained in good yield and purity. Nevertheless, hydrolysis of the methyl group to make the free acid caused a lot of problems. The methyl group, which normally can be removed very easily under mild conditions such as LiOH in acetone/H_2O, remained on under quite harsh conditions such as 2M NaOH 40~50°C. This is due to the presence of the bulky Boc group in the neighboring position. But this protection is necessary otherwise Fmoc would occur at this position in the following step, which would make

the coupling of this isostere to the growing peptide very difficult. This method was therefore abandoned.



Scheme 3.3.2 Attempted synthesis of Fmoc-Trp ψ(CH$_2$NH)Val, method 1.

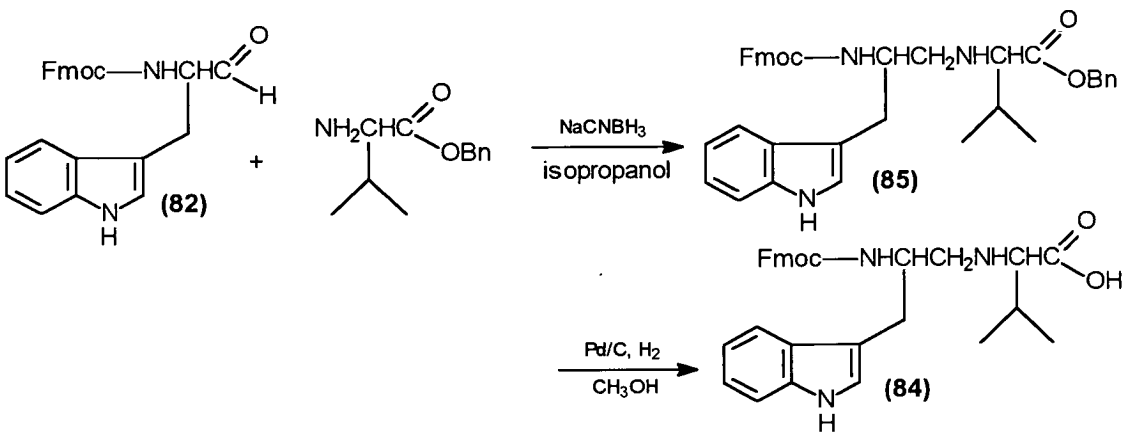


Scheme 3.3.3 Attempted synthesis of FmocTrpψ(CH$_2$NH)Val by solid phase synthesis, method 2

Fmoc was chosen as the protecting group since Z and Boc are incompatible with the above reaction conditions. A similar procedure was followed and FmocTrpCHO was afforded in good yield and purity. A solid phase reductive alkylation was then followed as shown in Scheme 3.3.3. The reaction was carried out under the optimized conditions proposed by Ho *et al.*[100] to suppress racemisation. As Trp was present, a suitable cleavage condition had to be found. 5% H_2O/TFA, RT, 1hr was applied to a small scale of resin bound isostere, however, a very dark colour

appeared when TFA was added which indicated this condition was too harsh for this isostere. Mild cleavage conditions were therefore tried. However, even when the resin was cleaved with 50% TFA/DCM, at 0°C, the solution still became coloured. Further reduction of the TFA caused ineffective cleavage and was not suitable. Addition of scavengers, such as EDT and thioanisole could overcome the side reactions but further purification was required. This procedure was left for future investigation.

Since FmocTrpCHO could be made in very good yield and purity in solution, the more reliable solution phase reductive alkylation was chosen. With the advantage of the Fmoc group already present, there was no need to protect the secondary amine, and the more easily removable benzyl ester was made (Scheme 3.3.4).



Scheme 3.3.4 Synthesis of FmocTrpψ(CH$_2$NH)Val OH through the benzyl ester, method 3

After purification by dry flash chromatography, the benzyl ester was afforded in good yield and purity. The benzyl group was then easily removed by hydrogenation to give the final product, FmocTrpψ(CH$_2$NH)Val OH, which was not soluble in many common solvents which caused problems in filtration from Pd/C. It was found later that it was soluble in DMF. The final pure product was afforded as a slightly coloured foam. It is worthwhile to point out that the solubility of the final product in DMF suggested that the hydrogenation could be better carried out in DMF, but no further investigation was made.

3.3.4 The inhibitory activity of the BigET-1 analogues with the reduced isostere

BigET-1 analogues with the reduced isostere Trpψ(CH_2NH)Val at the cleavage site have been synthesized and their ECE inhibition activity evaluated. The results are shown in Table 3.3.2.

Table 3.3.2 The Inhibitory activity of BigET-1 analogues with the reduced isostere

Peptide	Structure	Inhibition (%)*	IC_{50} (μM)
18	BET[1-38, Cys(Acm)^{1,3,11,15}, Trpψ(CH_2NH)Val]		13.5
S18	BET[1-38, Cys^{1,3,11,15}, Trpψ(CH_2NH)Val]	1.2% @20μM	
		5.3%@10μM	32.2
7	BET[12-38, Cys(Acm)^{15}, Trpψ(CH_2NH)Val]		13.2
6	BET[19-38, Trpψ(CH_2NH)Val]	0 @100μM	
16	**BET[19-38]**	39 @200μM	12.5

Note: * tested in CHO/humanECE-1 cells

The data indicate that the longer analogues [1-38, 12-38] show μM inhibition while the shorter one [19-38] does not inhibit at 100μM. This result indicates that residues between 12 and 19 are important for enzyme recognition. It is interesting to notice that the IC_{50} of the oxidized BigET-1 analogue, BigET-1 [1-38, Trpψ(CH_2NH) Val$^{21-22}$] is 32.2 μM, i.e. three time less potent than the linear analogue. This result indicates that the introduction of this reduced isostere greatly affects the structure of the native BigET, at least near the enzyme recognition site. As the most important residue His^{16} is not included in these analogues, further studies of these analogues will be carried out by Swenson.

3.3.5 Substitutions at position 22, the sulphur containing amino acids, Cys and Pen

Studies of the mutants of preproET-1 showed that some substitutions at positions 21 and 22 were tolerated, which indicated the absence of strict sequence specificity of ECE. Therefore, substitutions at these sites were studied in order to find some inhibitory activity. The sulphur containing amino acids are normally of interest

in enzyme inhibitory studies, thus both Acm protected and unprotected Cys and Pen were introduced into position 22 to study their effect on ECE inhibition.

Table 3.3.3 The Inhibitory activity of BigET-1 analogues with different substitution at position 22

Peptide	Structure	Inhibition (%)*	IC_{50} (μM)
16	**BET[19-38]**	**39 @200μM**	**12.5**
17	BET[19-38, Pen(Acm)^{22}]	12.1%@200μM	
S17	BET[19-38, Pen^{22}]		1.0
27	**BET[19-34]**		**2.5**
76	BET[19-34, Cys(Acm)^{22}]	46.3%@100μM	
75	BET[19-34, Cys^{22}]		17.3
77	BET[19-34, Pen(Acm)^{22}]	43.4%@100μM	
78	BET[19-34, Pen^{22}]	60.5%@100μM	
84	**BET[16-34]**		**0.085**
79	BET[16-34, Cys(Acm)^{22}]		0.33
80	BET[16-34, Cys^{22}]		0.040
81	BET[16-34, Pen(Acm)^{22}]		5.5
82	BET[16-34, Pen^{22}]		10.2

Note: tested in CHO/humanECE-1 cells

Three groups of analogues have been studied: [19-38], [19-34] and [16-34] analogues (Table 3.3.3). The low inhibitory activity of the 19-38 and 19-34 groups, compared to the [16-34] group, indicates that residues between 16 and 18 are important (discussed above). In the most potent group 3, the analogue with Cys at position 22 shows the highest inhibitory activity. When the SH is protected with Acm, the activity decreased dramatically, which indicates either the free SH or the size of the side residue is important. Pen is thus introduced into this position (peptides 81 and 82). The inhibitory activity of both peptides are very similar but much less potent compared to their Cys^{22} analogues. These results indicate that the size of the residue, as well as the SH group, is essential to ECE recognition at position 22.

3.4 EXPERIMENTAL

3.4.1 General

3.4.1.1 Notes

All Fmoc amino acids were purchased from either Bachem, Novabiochem, or were synthesized as described in the text, and are of the L-configuration unless otherwise stated. Peptide synthesis grade dimethylformamide (DMF) and 1,4-dioxane, and HPLC grade acetonitrile, were obtained from Rathburn Chemicals. Automated peptide synthesis was carried out on an adapted ABI 430A peptide synthesiser. UV spectra were recorded on a Varian Cary 210 double beam spectrophotometer. Sonication was carried out in a Decon FS300b sonic bath. Infrared spectra were recorded on a BIO-RAD SPC3200 instrument. Optical rotations were measured using an AA1000 polarimeter. Melting points were recorded in open capillaries on a Buchi 510 oil immersion melting point apparatus, and uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on either a Jeol FX-60 (60MHz), a Brucker WP-80 (80MHz), a Brucker WP-200 (200MHz), a Brucker AC-250 (250MHz), a Brucher WH-360 (360MHz) or a Varian VXR 600S (600MHz) spectrometer.

The HPLC was carried out using either an ABI system with 1406A solvent delivery systems, a 1480 injector/mixer and a 1783A detector/controller; or a Gilson system with 306 solvent delivery systems, a 811C dynamic mixer, a 805 manometric module, a 119 UV/VIS detector and a Gilson 715 software-driven gradient controller. FAB mass spectra were measured on a Kratos MS50TC instrument, using either thioglycerol, 3-nitrobenzyl alcohol or glycerol as matrix. Laser desorption mass spectra were measured on a PerSeptive Biosystems LaserTec Benchtop II system. Amino acid analysis was performed on a LKB 4150 alpha amino acid analyser on the hydrolysate obtained after heating samples at 110°C for 18-36 hours in a sealed Caritus tube, followed by evaporation to dryness.

3.4.1.2 Loading of the first amino acid on the Wang resin or Cl-Trt resin

Wang resin: Fmoc amino acid (6 eq. with respect to the resin) was dissolved in DMF (10ml per gram of resin), to which was added DIC (3 eq. with respect to the resin) and the mixture was sonicated for 10 min. The Wang resin was pre-swollen in a little DMF. DMAP (30mg) was added to the preswollen resin followed by addition of the amino acid mixture. The resin mixture was sonicated at RT for 2h, filtered, washed with DMF, dioxane, DCM and dried.

Cl-Trt resin: Fmoc-amino acid (6 equivalent relative to the resin) was suspended in dry DCM (10ml per gram of resin), to which was added a small amount of dry DMF to dissolve the Fmoc-amino acid. The mixture was added to the resin followed by 1/3 of the total DIEA. The mixture was stirred for 5min, then added the remainder of DIEA in DCM (1:1 v/v). The mixture was stirred for a further 60 min. then HPLC grade methanol (0.8ml per gram of resin) was added and the mixture stirred for 5 min. to cap the remaining trityl group. The resin was washed with DCM, DMF, isopropanol, methanol then ether, and dried over KOH *in vacuo*.

3.4.1.3 Fmoc and Tbfmoc loading tests

The loading of the resin was checked by deprotection of a few mg of the Fmoc amino acid bound resin and the UV absorption at 302nm. measured. The loading was then calculated by the following formula:

$$\mathbf{C = \varepsilon A}$$

where ε is the mole absorbance constant which is 9,000 in 20% piperidine/DMF.

$$\textbf{Loading (mmol/g)} = 10A_{302}/9Wt$$

where A_{300} is the absorption at 302nm; Wt is the weight of resin bound amino acid (mg) in 10ml 20% piperidine/DMF solution.

The loading of resin bound peptide can be calculated the same way, and the coupling percentage is then calculated by the following formula:

Coupling percentage (%) = final loading(mmol/g) x final resin weight (g) x100/(original loading (mmol/g) x initial resin weight (g)

The Tbfmoc protected, resin bound peptide (~10mg), accurately weighed, was sonicated in 20% piperidine/dioxane (10ml) for 10 min. The UV absorption at 364nm was measured and the loading was then calculated by the following formula:[101]

Loading (mmol/g) = 0.613 x A_{364}/ resin bounded peptide weight (mg)

3.4.1.4 Solid phase synthesis of peptides

All peptides mentioned in this text were synthesized on the adapted ABI 430A Peptide Synthesizer using Fmoc chemistry. The Wang resin was used for peptide acids except where Cys, Pro or His is the C-terminal amino acid and the dibenzosulberenyl resin for peptide amides. When Cys, Pro or His is the C-terminal amino acid, the trityl chloride (Cl-Trt) resin was used to avoid racemisation. The Amino acid side chain protections are: OtBu (Asp, Glu), tBu (Ser,Tyr), Boc (Lys), Trt (His, Cys), Acm (Cys), Pmc (Arg). The Fmoc group was deprotected with 20% piperidine in DMF; acetylation is carried out in a solution of 0.5M acetic anhydride, 0.125M DIEA (diisopropylethylamine) and 0.2% HOBt in DMF. The deprotection of Fmoc was monitored by an on line UV monitoring system. For triazole coupling, the acylation was also monitored by passing the washings through the UV detector and examining the unreacted Fmoc amino acid or the active intermediate.

Routinely, most residues were incorporated using double couple cycles, in which the first coupling cycle used a preformed symmetrical anhydride, following by the second coupling using a preformed HOBt active ester. The exceptions to this were Asn, Gln and His, which were coupled twice *via* their HOBt esters and Gly, which was coupled singly as a symmetrical anhydride. This method is refereed to as double coupling method 1 in this text.

When difficult sequences or large peptides were involved, the residues were incorporated using the active ester of the newly developed coupling reagent, HOCt, single or double as indicated. The only exception was His which was coupled through its HOBt ester due to increased racemisation with HOCt. This is refereed as double coupling method 2 in the text.

In the symmetrical anhydride cycle, two cartridges each containing 0.5mmol Fmoc amino acid were used, and the symmetrical anhydride was formed *via* the addition of 0.5mmol DIC. In the active ester cycle, either HOBt or HOCt, one cartridge containing 0.5mmol Fmoc amino acid was used and the active ester was formed *via* the addition of 0.5mmol DIC.

3.4.1.5 Cleavage, purification and analysis of the peptides

The completed peptide was cleaved from the resin with TFA plus scavengers. The three mixtures used for cleavage of different peptide sequences are listed below as A, B or C.

Cleavage mixture A:

EDT (1.0 ml), H_2O (0.25 ml), TFA (10 ml);

Cleavage mixture B:

EDT (1.0 ml), H_2O (0.25 ml), TIS (0.2 ml), TFA (10 ml);

Cleavage mixture C:

EDT (1.0 ml), phenol (0.75g), thioanisole (0.5ml), TIS (0.2 ml), H_2O (0.2 ml), TFA (10 ml)

Crude peptide was precipitated from ether after removing the TFA *in vacuo*. The crude peptide was then purified by HPLC under one of the conditions described in the next section. Pure peptide was dried by lyophilization and analyzed by analytical HPLC, FAB/laser desorption mass spectroscopy and amino acid analysis.

3.4.1.6 HPLC conditions

Three types of HPLC columns were referred to in this text. Type I is made from ABI aquapore RP300 reverse phase silica (300A pore size, 7μm spherical silica), Type II is from Vydac reverse phase silica (300A pore size, 5μm spherical silica) and Type III is from Hichrom RPB reverse phase silica (110A pore size, 5 μm spherical silica). The size of each column is:

Analytical columns	Preparative columns
RP C18: 220 x 4.6mm (size A)	RP C18: 250 x 10mm (size C)
RP C8: 220 x 4.6mm (size A)	RP C8: 250 x 10mm (size C)

Vydac C18: 250 x 4.6mm (size B) Vydac C18: 250 x 22mm (size D)

Hichrom C18: 250 x 4.6mm (size B)

RP C4 : 100 x 4.6mm (size E)

The solvents used were:

Solvent A: 0.1%TFA/H_2O

Solvent B: 0.1%TFA/CH_3CN

The peptides were eluted by an increasing concentration of solvent B in solvent A. The gradient used for analytical HPLC was identical unless otherwise stated, which is

10%-10% (2min), 10%-90% (30min), 90%-10% (2min), which is simplified as 10-10(2), 10-90(30), 90-10(2).

For preparative HPLC the gradient used was dependent on the nature and quantity of impurities present. The conditions used are summarized in Appendix IV and V.

3.4.1.7 Ellmans' test

The purified peptide (0.2~0.3mg) was dissolved in 10 ml 0.1M phosphate buffer (pH7.3) containing 1mM EDTA, with or without 6M guanidine. A 2.5ml sample of the solution was placed in a UV cell and another 2.5 ml of the buffer was used as the reference. To each cell was added 50 ml 3mM DTNB, and it was tested at 412nm for absorbance.

3.4.2 Synthesis of the linear ET-1 analogues

The ET-1 analogues were synthesized either *via* standard double coupling or triazole coupling as is mentioned above. The sequences of these analogues are listed in Appendix I and II. The coupling and cleavage methods used and the molecular weight of each peptide are summarized in Table 3.4.1. The HPLC conditions and the amino acid analysis data are listed in Appendix IV.

Table 3.4.1 Synthesis and cleavage method for ET-1 analogues

Pept. No.	Loading (mmol/g)	Coupling methods	Cleavage mixture	Mass M+1/MH average	Found
60	0.553	HOCt double	B	2491.1/2493.0	2489.3
1	0.575	HOCt double	B	2583.3/2585.0	2579.0
1B	0.583	HOCt double	B	2583.3/2585.0	2583.9
1C	0.604	HOCt double	B	2405.3/2406.8	2408.1
2	0.575	HOCt double	B	2569.2/2571.0	2565.9
2B	0.583	HOCt double	B	2555.2/2557.0	2557.4
21	0.567	HOCt double	B	2569.2/2571.0	2571.6
22	0.567	HOCt double	B	2569.2/2571.0	2569.6
23	0.567	HOCt double	A	2579.3/2581.0	2580.0
24	0.567	HOCt double	A	2579.3/2581.0	2578.8
25	0.567	HOCt double	A	2579.3/2581.0	2581.0
26	0.567	HOCt double	B	2569.2/2571.0	2569.3
56	0.636	HOBt/DIC	B	2405.3/2406.8	2402.7
15A	0.509	HOBt/DIC	B	2498.2/2499.9	2498.6
15-1	0.581	HOCt double	B	2395.2/2396.8	2398.8
14	0.567	HOCt double	B	2308.2/2309.7	2308.1
13	0.567	HOCt double	B	2237.1/2238.6	2237.7
12	0.567	HOCt double	B	2150.1/2151.5	2151.6
11	0.567	HOCt double	B	2063.1/2064.5	2064.1
40	0.581	HOBt/DIC	B	2049.1/2050.4	2049.3
54	0.579	HOBt/DIC	B	1974.1/1975.3	1974.1
47	0.506	HOBt/DIC	B	1960.1/1961.4	1969.8
51	0.597	HOBt/DIC	B	1972.1/1973.4	1971.6
38	0.547	HOBt/DIC	B	1975.1/1976.3	1971.9
33	0.561	HOCt double	B	1950.0/1951.2	1953.7
33a	0.561	HOCt double	B	1992.0/1993.3	1995.8
72	0.521	HOBt/DIC	B	1817.0/1818.2	1817.6
53	0.579	HOBt/DIC	B	1861.0/1862.2	1865.6
62	0.521	HOBt/DIC	B	1875.0/1876.2	1873.5
64	0.521	HOBt/DIC	B	1875.0/1876.2	1877.4

(to be continued)

Table 3.4.1 Synthesis and cleavage method for ET-1 analogues(continued)

Peptide	Loading	Coupling	Cleavage	Mass	
	(mmol/g)	methods	mixture	M+1/MH average	Found
67	0.521	HOBt/DIC	B	1861.0/1862.2	1862.3
61	0.521	HOBt/DIC	B	1847.0/1848.2	1850.8
61A	0.521	HOBt/DIC	B	1887.9/1889.2	1893.0
63	0.521	HOBt/DIC	B	1847.0/1848.2	1845.4
63A	0.521	HOBt/DIC	B	1887.9/1889.2	1886.1
32	0.561	HOCt double	B	1950.0/1951.2	1951.3
43	0.550	HOBt/DIC	B	1966.0/1967.3	1964.6
34	0.561	HOBt/DIC	B	1950.0/1951.2	1952.0
65	0.536	HOBt/DIC	B	1936.0/1937.3	1932.6
52	0.579	HOBt/DIC	B	1747.9/1749.0	1748.8
39	0.550	HOBt/DIC	B	1822.9/1824.1	1823.4
31	0.561	HOCt double	B	1836.9/1838.1	1837.5
41	0.550	HOBt/DIC	B	1822.9/1824.1	1822.7
41A	0.550	HOBt/DIC	B	1863.9/1865.1	1866.7
50	0.597	HOBt/DIC	B	1746.0/1747.1	1744.5
46	0.506	HOBt/DIC	B	1733.9/1735.0	1738.3
66	0.536	HOBt/DIC	B	1761.9/1763.1	1783.1*
55	0.579	HOBt/DIC	B	1748.9/1750.0	1750.3
37	0.522	HOBt/DIC	B	1747.9/1749.0	1749.5
37A	0.547	HOBt/DIC	B	1788.9/1790.0	1789.1
36	0.547	HOBt/DIC	B	1748.9/1750.0	1748.8
36A	0.547	HOBt/DIC	B	1789.9/1791.0	1791.0
68	0.536	HOBt/DIC	B	1747.9/1749.0	1748.5
69	0.536	HOBt/DIC	B	1747.9/1749.0	1772.1*
59	0.507	HOBt/DIC	B	1747.9/1749.0	1745.9
58	0.507	HOBt/DIC	B	1747.9/1749.0	1749.9
57	0.507	HOBt/DIC	B	1747.9/1749.0	1745.9
70	0.536	HOBt/DIC	B	1747.9/1749.0	1749.4
71	0.536	HOBt/DIC	B	1747.9/1749.0	1747.5
30	0.561	HOCt double	B	1721.9/1723.0	1722.0
29	0.561	HOCt double	B	1592.8/1593.9	1592.1
28	0.561	HOCt double	B	1464.7/1465.8	1464.7
35A	0.550	HOBt/DIC	B	1420.7/1421.7	1421.3

* Sodium salt sequestered from the matrix

3.4.3 Synthesis of ET-1 (peptide 60)

The synthesis of this 21 amino acid peptide was carried out by standard double coupling *via* HOCt and the protecting group for the four Cys was Trt. The peptide resin was cleaved with solution B, and the crude, fully reduced peptide (80mg) was dissolved in 6M guanidine.HCl (200ml), which contained 0.1M Tris buffer (pH 8), GSSG (0.5mM) and GSH (5mM). The mixture was stirred at RT overnight. Analytical HPLC showed a major single peak. This was purified using a C18 preparative column, eluting with a linear gradient of 0.1% TFA/H_2O with increasing concentration of 0.1% TFA/CH_3CN at 5ml/min. The correct fraction was dried by lyophilization to give 15.5mg ET-1 (yield 19.4%). This was analyzed by different analytical HPLC (RP C18, Vydac C18 and Hichrom C18 columns), MS m/z 2489.3 ($C_{109}H_{159}N_{25}O_{32}S_5$ requires 2492.0) and a co-injection with commercial human ET-1 (Peptide Institute INC., Japan), confirmed that they are the same peptide.

3.4.4 Attempted synthesis of Fmoc-Trpψ(CH_2NH)ValOH (84) through Z-Trpψ(CH_2N(Boc))ValOH (78), method 1[102]

Synthesis of Z-Trp-N,O-dimethylamide (74)

To a suspension of N,O-dimethylhydroxylamine hydrochloride (5.2g, 52mmol) in DCM (30ml) was added N-methyl piperidine (6.5ml, 52mmol) while stirring at 0°C. In another flask, Z-Trp (17.66g, 52mmol) was suspended in DCM (250ml) and the suspension was stirred at 0°C under N_2 and N-methyl piperidine (6.5ml, 52mmol) was added. To the clear solution of Z-Trp was added ethyl chlorofomate (5.78g, 52mmol) dropwise, keeping the temperature below 0°C. After 3 minutes, N,O-dimethylhydroxylamine solution from the other flask was transferred into the solution of mixed anhydride at 0°C under nitrogen. The reaction mixture was stirred at 0°C for 1 hr and allowed to warm up gradually to RT and stirred overnight. A slightly yellow solution was afforded which was evaporated. The residue was dissolved in EtOAc (150ml) and the insoluble impurities removed by filtration. The filtrate was washed with citric acid (20%), H_2O, aqueous $NaHCO_3$ and saturated NaCl and dried over $MgSO_4$. The solution was evaporated to give a white foam, 18.5g (92.9%).

δ^1H NMR($CDCl_3$, 60MHz): 3.2 (5H, m, NCH_3, CH_2), 3.7 (3H, s, OCH_3), 4.1 (1H, m, CH), 5.1 (2H, m, CH_2), 5.6(1H, m, NH), 7.0~7.5 (10H, m, aromatics), 8.5 (1H, br. NH).

Synthesis of Z-Tryptophal (75)

A solution of N,O-dimethyl amide of Z-Trp (9.5g, 24.9mmol) in dry THF (150ml) was stirred under nitrogen and cooled to -10°C. To the solution was added $LiAlH_4$ (LAH, 1.27g, 33.57mmol) in small portions. The mixture was allowed to warm up to RT when all the LAH had been added, the mixture was stirred for another 30 min., then quenched with 10% $KHSO_4$ solution (125ml H_2O). The pH of the suspension was adjusted to 3.0 with 1N HCl (~30ml). The solution was diluted with EtOAc (500ml) and layers separated. The aqueous layer was further extracted with EtOAc twice and the organic layers were combined and washed with citric acid (20%), H_2O, aqueous $NaHCO_3$ and saturated NaCl and dried over $MgSO_4$. The solution was evaporated to give a colourless solid (8.0g, 100%). The product gave satisfactory NMR and thus was used without further purification.

δ^1H NMR($CDCl_3$, 60 MHz): 3.1 (2H, m, CH_2), 4.2~4.7 (3H, m, CH, CH_2), 5.5 (1H, d, NH), 7.1~7.6 (10H, m, aromatics), 8.3 (1H, s, NH), 9.6 (1H, s,CHO).

Synthesis of Z-Trpψ(CH_2NH)ValOCH$_3$ (76)

L-valine methyl ester hydrochloride (5.0g) was suspended in DCM (50ml) to which was added saturated $NaHCO_3$ (50ml). The mixture was stirred for 10 min and the layers were separated. The H_2O layer was extracted with DCM and the DCM layers combined, dried with $MgSO_4$ and evaporated to give a nearly colourless liquid (3.2g, 24.42 mmol), which was dissolved in isopropanol (5ml) and used at once in next step.

Z-TrpCHO (8.2g, 24.9mmol) was dissolved in isopropanol (150ml) to which was added the above solution followed by 4Å molecular sieves (3.6g). The mixture was stirred under nitrogen for 1 hr, then a solution of $NaCNBH_3$ (2.10g) in isopropanol (6ml) was added dropwise while stirring. The mixture was stirred under nitrogen overnight (RT). The mixture was filtered, and the filtrate evaporated. The residue was dissolved in EtOAc and washed with saturated $NaHCO_3$, NaCl and dried

over $MgSO_4$ and evaporated again. The residue was purified by flash chromatography (1~5 % EtOH/$CHCl_3$). The portion with pure product was evaporated and dried to give a white solid (6.0g, 56.5%).

δ^1H NMR($CDCl_3$, 80 MHz): 0.95 (6H, dd, 2xCH_3), 1.90 (1H, m, CH), 2.43~2.71(2H, 2 seperated multiplets, CH_2), 3.03 (3H, m, CH, CH_2), 3.65(3H, d, OCH_3), 4.0 (1H, m, CH), 5.12~5.20 (3H, m, NH,CH_2), 6.93~7.35 (9H, m, aromatics), 7.63 (1H, m, aromatic CH), 8.39 (1H, s, NH).

Synthesis of Z-Trpψ(CH_2N(BOC))ValOCH_3 (77)

Z-Trpψ(CH_2NH)ValOCH_3 (4.29g, 9.86mmol) was dissolved in dry THF (100ml) and DMAP(0.119g, 0.99mmol) was added followed by $(Boc)_2O$ (2.35g 10.8 mmol). The reaction mixture was stirred at RT and monitored by TLC (EtOAc/Ether = 1:9). TLC indicated that the reaction was complete in 30 min. The mixture was then evaporated and the oily residue was partitioned between ether (400ml) and 1M $KHSO_4$ (200ml) and the layers separated. The organic layer was washed with 1M $KHSO_4$, sat. $NaHCO_3$, NaCl and dried over $MgSO_4$. The solution was evaporated to give a slightly coloured foam (3.53g, 67%).

δ^1H NMR($CDCl_3$, 80 MHz):0.93(6H, dd, 2xCH_3), 1.20(9H, s, 3xCH_3), 1.98(1H, m, CH), 2.5~2.7(2H, 2 separated multiplets, CH_2), 3.00(3H, CH, CH_2), 3.65(3H, d, OCH_3), 4.1(1H, broad, CH), 5.15(2H, d, CH_2), 5.3(1H, NH), 7.1~7.7(10H, m, aromatics), 8.1(1H, d, NH).

Attempted synthesis of Z-Trpψ(CH_2N(BOC))ValOH(78)

The Boc protected dipeptide methyl ester (3.53g) was dissolved in methanol (10ml) and 1N NaOH (10ml) was added. The clear methanol solution became cloudy when NaOH was added and heat was given off. The mixture soon become very sticky and hard to stir. TLC (EtOAc/ether 1:9) showed that the starting material disappeared in c.a. 5hrs. The mixture was evaporated and sat. $KHSO_4$ was added to the residue. A very sticky oil precipitated which was extracted with EtOAc, washed with sat. NaCl and dried over $MgSO_4$ to give some slightly coloured foam (3.53g, 103%). However, ^1H NMR showed that the methyl group was still intact. No further effort was made.

δ^1H NMR($CDCl_3$, 80 MHz):0.93(6H, d), 1.20(9H, s), 2.0(1H, m), 2.5~3.0(5H, m), 3.65(3H, d, OCH_3), 4.1(1H, broad), 5.35(1H, d), 7.1~7.5(10H, m), 7.7(1H, m), 8.1(1H, d).

3.4.5 Synthesis of FmocTrpψ(CH_2NH)ValOH(84), methods 2 and 3

Synthesis of Fmoc-Trp-N,O-dimethylamide(81)

To a suspension of N,O-dimethylhydroxylamine hydrochloride (1.09g, 11mmol) in DCM (30ml) was added N-methylpiperidine (1.35ml, 11mmol) while stirring at 0°C. In another flask, Fmoc-L-Trp (4.265g, 10mmol) was suspended in DCM (50ml). The suspension was stirred at 0°C under N_2 and N-methylpiperidine (1.25ml, 10mmol) was added. To the clear solution of Fmoc-Trp was added ethyl chloroformate (0.96ml, 10mmol) dropwise, keeping the temperature below 0°C. After 3 minutes, N,O-dimethylhydroxylamine solution from the other flask was transferred into the solution of mixed anhydride at 0°C under nitrogen. The reaction mixture was stirred at 0°C for 1 hr and allowed to gradually rise to RT overnight. A slightly yellow solution was afforded which was evaporated. The residue was dissolved in EtOAc (150ml) and the insoluble impurities removed by filtration. The filtrate was washed with citric acid (20%), H_2O and saturated NaCl and dried over $MgSO_4$. The solution was evaporated to give a white foam, 4.4g (94%). m.p. 98-100°C, FAB MS m/z 470 ($C_{28}H_{27}N_3O_4$ requires 469.54), R_f =0.30 (DCM/EtOAc 4:1), $[\alpha]_D^{20}$ -42.1 (in DMF). δ^1H NMR($CDCl_3$, 200 MHz): 3.16~3.32(5H, m, NCH_3, CH_2), 3.64(3H, s, OCH_3), 4.17~4.35(4H, m, 2xCH, CH_2), 5.67(1H, d, NH), 7.0~7.8(13H, m, aromatics), 8.33(1H, s, broad, NH).

Synthesis of FmocTrpCHO(82)

Fmoc-Trp-N,O-dimethylamide (4.4g, 9.4mmol) was dissolved in dry THF (80ml) and cooled by dry ice/acetone bath (-20°C). LAH (0.475g, 12.5mmol) was added in small portions under N_2. The mixture was stirred at -20°C for 10 min and the cold bath was removed to allow the temperature rise to RT and the mixture was stirred for another 30 min. The reaction was quenched with 0.6M $KHSO_4$ solution (50ml) and the pH of the solution adjusted to 3.0 with 1N HCl(~30ml). The solution

was diluted with EtOAc and layers separated. The aq. layer was extracted twice with EtOAc and the combined EtOAc layers were washed with 20% citric acid, aq. $NaHCO_3$, and brine, dried over $MgSO_4$ and evaporated to give a white foam (3.8g, 100%). It was pure by TLC and NMR and used without any further purification. m.p 158-160°C, FAB MS m/z 410 ($C_{26}H_{22}N_2O_3$, requires 410.47), R_f =0.52 (hexane/EtOAc=1:2), $[\alpha]_D^{20}$ -45.5 (in DMF).

δ^1H NMR($CDCl_3$, 200 MHz): 3.30(2H, m, CH_2), 4.15~4.22(2H, m, CH_2) 4.34~4.62(3H, m, CH, CH_2), 6.95~7.75(13H, m, aromatics), 8.14(1H, s, NH), 9.65(1H, s, CHO).

Attempted synthesis of Fmoc-Trpψ(CH_2NH)ValOH(84) by solid phase approach, method 2

FmocValOH was loaded on the Wang resin (loading 0.644mmol/g without the Fmoc) and the Fmoc group was removed with 20% piperidine. The NH_2-Val-resin (1.93g, 1.24mmol) was suspended in 1% HOAc/DMF under nitrogen and 4Å molecular sieves (3.0g) added. FmocTrpCHO (1.62g 3.95mmol) in THF was added followed by $NaCNBH_3$. The mixture was stirred under nitrogen for 2hrs at RT and sonicated overnight. The Kaiser test showed a dark brown colour which indicated the formation of secondary amide. The mixture was then filtered, first with a Buchner funnel to remove the molecular sieves then with a sinter funnel to remove the resin which was washed with DMF, DCM, methanol and DCM and dried for 1hr under vacuum.

The resin was cleaved as follows:

(1). 0.5% H_2O/TFA, 1hr at RT

(2).50%~80% TFA/DCM 1hr at 0°C

but a pink to purple colour was inherent which indicates the oxidation of Trp. This procedure was therefore abandoned.

Solution phase synthesis of Fmoc-Trpψ(CH_2NH)ValOH(84)---method 3

FmocTrpCHO (1.68g, 4.1mmol) was dissolved in a mixture of dry MeOH and THF (1:1) and the mixture was stirred under nitrogen. 4Å molecular sieves (5g) was

added followed by HCl.NH_2ValOBn (1.0g, 4.1mmol) and $NaBH_3CN$ (0.350g, 5.1mmol) as solids. The clear solution afforded was stirred under nitrogen overnight at RT to give a cloudy mixture (molecular sieves crashed). The mixture was filtered and the filtrate concentrated. The residue was dissolved in EtOAc (100ml) and washed with sat. $NaHCO_3$, NaCl and dried over $MgSO_4$. The EtOAc solution was evaporated to give a slightly coloured foam. TLC showed two compounds which were separated by dry flash chromatography (DCM/EtOAc=4:1) to give 2.2g (89.5%) pure product (**85**). m.p. 130-133°C, FAB MS m/z 603 ($C_{38}H_{39}N_3O_4$ requires 602.75), R_f 0.63 (DCM/EtOAc=4:1), $[\alpha]_D^{20}$ -28.3 (in DMF).

δ^1H NMR($CDCl_3$, 200 MHz): 0.95 (6H, d, 2xCH_3), 1.94 (1H, m, CH), 2.46~2.76 (2H, 2 separated multipltes, CH_2), 3.03(3H, CH, CH_2), 3.95~4.50 (4H, m, 2xCH, CH_2), 5.15 (3H, m, CH_2, NH), 6.92~7.78 (18H, m, aromatics), 8.25 (1H, s, NH).

Fmoc-Trpψ(CH_2NH)ValOBn (**85**, 2.2g, 3.6mmol) was dissolved in 100ml MeOH and the solution was bathed in nitrogen for 1hr and 10% Pd/C (0.4g) was added. The mixture was hydrogenated. A white solid precipitated soon afterwards and TLC (CH_3OH/CH_3Cl 1:9) showed that the reaction had gone to completion in c.a. 30 min. The mixture was dissolved with difficulty in THF and filtered through ceilite. Other solvents were tried but THF was the most suitable. (It was found later that it was soluble in DMF). Solvent was removed to give a slightly coloured solid (1.3g, 84%). m.p. 134-135°C, FAB MS m/z 512 ($C_{31}H_{33}N_3O_4$ requires 511.62), $[\alpha]_D^{20}$ -37.1 (in DMF).

δ^1H NMR(DMSO-d_6, 200MHz): 0.92 (6H, d, 2xCH_3), 1.94 (1H, m,CH), 2.50~3.10 (5H, m, 3xCH, CH_2), 3.88~4.29 (4H, m, 2xCH, CH_2), 5.76 (1H, s, NH), 6.93~7.69 (12H, m, aromatics), 7.87 (2H, d, NH), >10.5 (1H, COOH).

3.4.6 Deprotection and oxidation of Cys(Acm), conversion of peptide 45 to peptide O45, the BigET-1[84]

Peptide 45 (BigET-1[1-38, Cys(Acm)^{1,3,11,15}], 50mg, pure) was dissolved in TFA (3ml) and cooled on ice-salt bath. Anisole (48ul, 40 eq) was added and the mixture was saturated with nitrogen. Silver trifluromethanesulphonate, AgOTf (114mg, 40eq.) was added and stirred at 0°C for 3 hrs. and at RT for 2 hrs. The

Sephadex column eluting with 15% acetic acid. The peptide fractions collected were added to 1M guanidine HCl (1liter), and the pH adjusted to 8 with concentrated ammonia. L-glutathione reduced, GSH (237mg) and L-glutathione oxidized, GSSG (50mg) were added to the mixture and this was stirred overnight. The solution was loaded to a RP18C18 column through A pump and washed with water until all the guanidine had passed through. A shallow gradient (0-70% acetonitrile in 60 min) was applied.

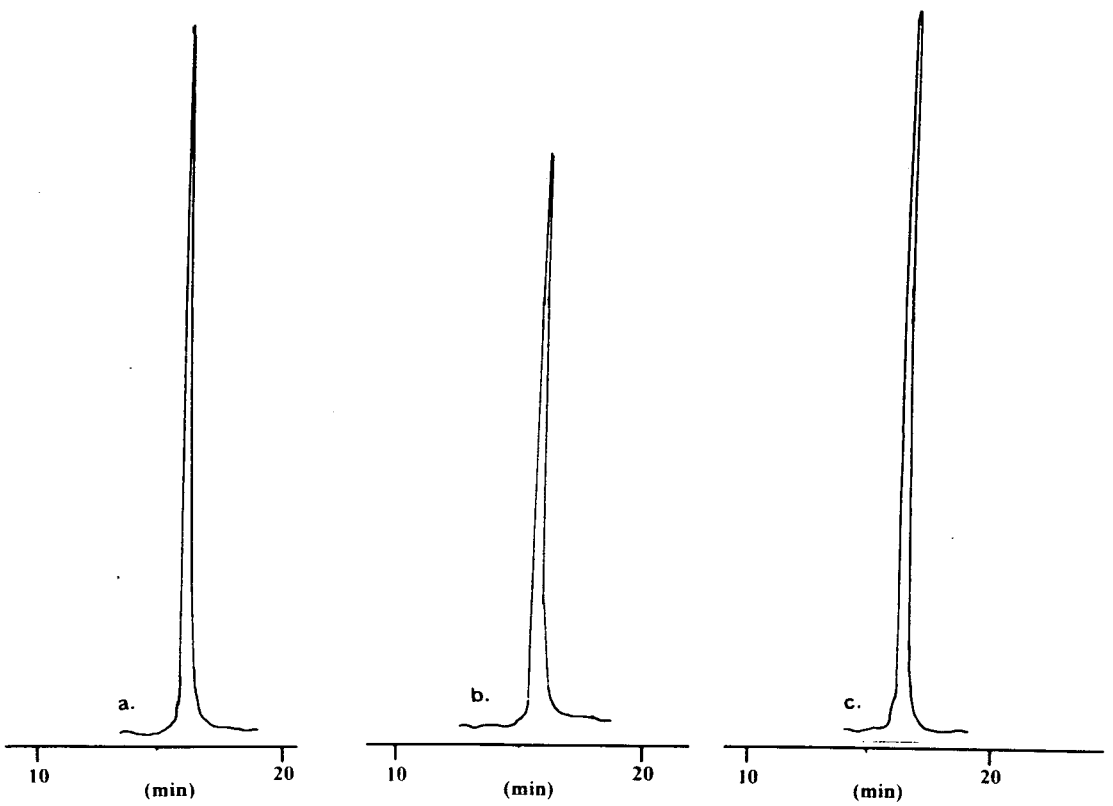


Figure 3.4.1 HPLC traces of pure peptide O45 (BigET-1) 214nm, 1ml/min; a. Vydac C18 (size B); b. RP C8 (size B); c. Co-injection with native BigET-1, Vydac C18 (size B).

The corresponding fraction collected and it was pure on analytical HPLC and MS was as expected (m/z found 4294.5, $C_{189}H_{282}N_{48}O_{56}S_5$ requires 4283.0). Ellmam's test was negative and co-injection with the natural BigET-1 confirmed that it was the correct product (Figure 3.4.1).

The same procedure was applied to Peptide 18 to give peptide O18. m/z found 4290.0, $C_{189}H_{290}N_{48}O_{55}S_5$ requires 4275.0.

test was negative and co-injection with the natural BigET-1 confirmed that it was the correct product (Figure 3.4.1).

The same procedure was applied to Peptide 18 to give peptide O18. m/z found 4290.0, $C_{189}H_{290}N_{48}O_{55}S_5$ requires 4275.0.

3.4.7 Synthesis of FmocPen(Acm)[103]

L-Penicillamine (1.64g, 11mmol) and N-Hydroxymethylacatamide (1.17g, 13.2mmol) were dissolved in 10% TFA/H_2O (5ml). The mixture was cooled on an ice-bath and trifluoromethanesulphonic acid/TFA (1:19, 30ml) added while stirring. After 90 minites stirring under argon, TLC (n-butanol/acetic acid/H_2O, 10:2:3) indicated that all the L-penicillamine had reacted. The solvent was removed *in vacuo* leaving an oil which was dissolving in 10% aqueous sodium carbonate (50ml) and the pH was adjusted to 10 by the continued addition of further aqueous sodium carbonate. The mixture was then cooled in an ice-bath and Fmoc-OSu (3.71g, 10mmol) dissolved in dioxane (50ml) added dropwise over 1hr. The reaction was allowed to warm slowly to RT and maintained and stirred for 2 days. The pH of the reaction was monitored and maintained at 10 throughout. The mixture was then poured into H_2O (200ml) washed with ether (3x50ml), acidified with 6M HCl to pH 2 and extracted with ethyl acetate (3x50ml). The combined ethyl acetate extracts were washed with 0.1M HCl (2x100ml), water (2X100ml), and brine (2x100ml), dried over $MgSO_4$ and after filtration, solvent was evaporated *in vacuo.* The product was recrystallised from warm ethyl acetate and allowed to stand at cold room (4°C) overnight giving a white solid. Yield 3.0g (62%).

δ^1H NMR($CDCl_3$, 200MHz): 1.35 (3H, s), 1.45 (3H, s), 1.95 (3H, s), 4.1-4.6 (6H, m), 5.85 (NH, d), 7.2-7.8 (8H, m).

3.4.8 Deprotection of Pen(Acm)

Pure peptide 17 (18mg, 7.74μmol) was dissolved in 50% acetic acid (1ml), $Hg(OAc)_2$ (24.6mg, 10eq.) was added and stirred at RT overnight. 2-Mercapto-ethanol (33.7μl, 50eq) was added and stirred for 3hrs at RT. The mixture was centrifuged and the supernatant applied to HPLC which showed two peaks. The two

The mixture was dissolved in 6M guanidine HCl containing 1mM EDTA (10ml) and 2-mercaptoethanol (20μl) was added. The mixture was stirred at RT for 2hrs and HPLC showed that the dimer peak disappeared to give a sigle peak corresponding to the monomer (Figure 3.4.2). The mixture was applied to the G25 Sephadex column and eluted with 15% acetic acid. The fraction of peptide was purified with HPLC (conditions in Appendix IV) to give the pure product (Figure 3.4.2). Quantitative Ellmans' test assay gave a value of 0.92 (1.00) SH group.

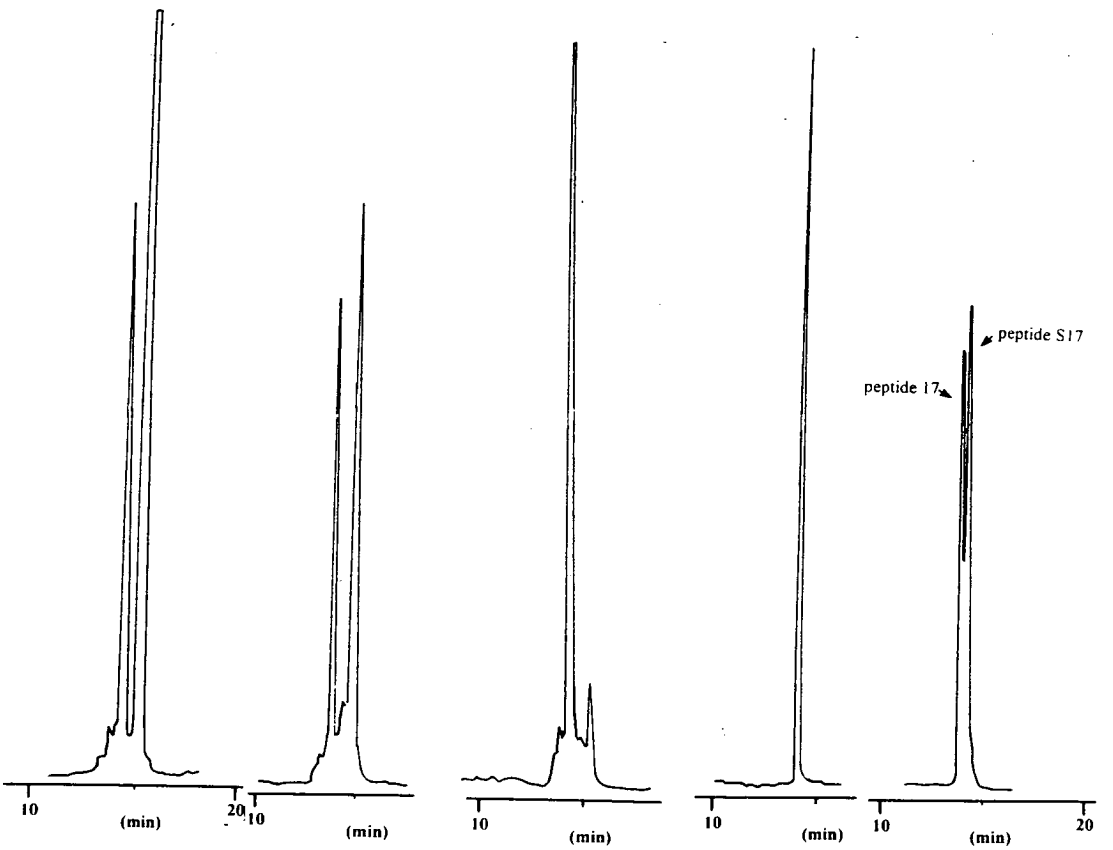


Figure 3.4.2 HPLC monitoring of the deprotection of the Acm from peptide 17

214nm, 1ml/min, Vydac C18 (size B),a. 5min; b. 30min; c. 120min; d. after purification; e. co-injection of peptides 17 and S17.

3.4.9 Synthesis of BigET-1 analogues

The sequences of the BigET-1 analogues are listed in Appendix III and the methods used to synthesize these analogue are summarized in Table 3.4.2. The HPLC conditions and amino acid analysis data are listed in Appendix V.

Table 3.4.2 Synthesis and purification methods for BigET-1 analogues

	Loading of	Coupling	Cleavage	Mass	
Peptide	first AA	method	mixture	M+1/MH average	Found
45	0.505	HOCt double		4569.1/4572.3	4566.1
O45				4281.0/4284.0	4294.5
5	0.550	Triazole Double	C	4682.2/4685.4	4687.0
	0.550	HOBt/DIC	C		4686.5
18	0.532	HOBt/DIC	C	4556.1/4559.3	4555.6
O18	0.532	HOBt/DIC	C	4272.0/4275.0	4290.0
7	0.489	HOCt double	C	3156.6/3158.7	3155.6
6	0.489	HOCt double	C	2208.2/2209.6	2207.7
16	0.482	HOBt/DIC	C	2221.2/2222.6	2222.2
17	0.482	HOBt/DIC	C	2324.2/2325.7	2324.8
S17	0.482	HOBt/DIC	C	2253.1/2254.6	2253.6
3	0.514	HOBt/DIC	B	1922.0/1923.2	1922.9
83	0.504	HOBt/DIC	C	2586.2/2587.9	2588.8
84	0.533	HOBt/DIC	C	2159.1/2160.5	2157.5
79	0.504	HOBt/DIC	C	2234.1/2235.6	2235.3
80	0.504	HOBt/DIC	C	2163.1/2164.5	2165.1
81	0.504	HOBt/DIC	C	2262.1/2263.6	2264.0
82	0.504	HOBt/DIC	C	2191.2/2192.5	2191.9
27	0.633	HOBt/DIC	C	1794.0/1795.1	1794.3
76	0.547	HOBt/DIC	C	1868.9/1870.2	1870.2
75	0.547	HOBt/DIC	C	1797.9/1799.1	1800.0
77	0.547	HOBt/DIC	C	1897.0/1898.2	1899.0
78	0.547	HOBt/DIC	C	1825.9/1827.0	1826.8
9	0.612	HOBt/DIC	C	1850.0/1851.2	1851.4
10	0.612	HOBt/DIC	C	1906.1/1907.3	1907.0
8	0.612	HOBt/DIC	B	897.5/898.1	898.0

3.4.10 Biologcal Assays

The biological assays of the ET-1 and BigET-1 analogues were carried out by the biologists at Parke Davis Pharmaceutical Research at Michigan. These are described below.

3.4.10.1 Endothelin binding assays[62]

Membranes were prepared from cultured rabbit renal artery vascular smooth muscle (ET_A) or crude cerebellar tissue from adult male blue Laurie rats (ET_B). Incubations were performed in 12 x 75 mm polypropylene tubes containing 20 mM Tris, 2 mM EDTA, 100 μM PMSF, 100 μM bacitracin, 30pM [^{125}I]ET-1 (2000Ci/mmol) and 5 μg rabbit renal artery vascular smooth muscle or 5 μg rat cerebellar membranes (total volume of 250 μL) (pH 7.4, 37°C). The order of additions (0°C) was as follows: test compound, [^{125}I]ET-1, and membrane. Test compounds were diluted in a buffer (20 mM Tris, 2mM EDTA, 1mg/mL BSA) to five times the final incubation concentration. [^{125}I]ET-1 was diluted in the same buffer. Membranes were diluted in buffer containing 100 μM PMSF and 100 μM bacitracin, without BSA. Following the last addition, the tubes were manually agitated and incubated at 37°C for 2 h. Incubations were terminated by filtration through Whatman GF/B filters which were presoaked with 50 mM Tris containing 0.2% BSA and 100 μM bacitracin (pH 7.3, 5°C). Nonspecific binding was defined as binding in the presence of 100 nM ET-1 and specific binding was defined as total binding minus nonspecific binding. IC_{50} values were calculated by weighted nonlinear regression curve fitting to mass action equation.

3.4.10.2 Arachidonic acid release (AAR_B)[62]

Confluent monolayers of cultured rat cerebellum cells were incubated in 0.5 ml of the Loading Media (LM: Ham's Nutrient Mixture F12 and Dulbecco's Eagle Medium (1:1) with 0.5% fetal calf serum, and 0.25 μCi of [^{3}H]-arachidonic acid (Amersham) per ml of media) for 18 h at 37°C in a 5% CO_2/95% air atmosphere. The

LM was aspirated and the cells were washed once with the assay buffer (Hank's balanced salt solution + 10 Mm HEPES + fatty acid-free BSA(1mg/ml)), and incubated for 5 min with 1 ml of the prewarmed assay buffer. This solution was aspirated, followed by an additional 1 ml of prewarmed assay buffer and further incubated for another 5 min. A final 5 min incubation was carried out in a similar manner. The same procedure was repeated with the inclusion of 10μl of the test compound (1nM to 10 μM), 10μl of ET-1 (0.3nM) and the incubation was extended to 30 min. This solution was collected, 10 ml of scintillation cocktail was added and the amount of [^{3}H]-arachidonic acid was determined in a liquid scintillation counter.

3.4.10.3 ECE inhibiting[104,105]

A reaction mixture containing 0.1 μM BigET-1, 100mM Hepes-KOH (pH 7.0), 50mM NaCl, 50μM pepstatin A, 100μM leupeptin, 200μM phenylmethylsulphonyl fluoride, indicated concentration of BigET-1 analogue (as the inhibitor, DMSO for control), and the membrane fraction of CHO/human ECE-1 cells. The final concentration of DMSO was 1.5%. After incubation for 1 hr at 37°C, the reaction was stopped by adding EDTA to give a final concentration of 10mM. This final mixture was then directly analyzed for mature ET-1 by enzyme-linked immumosorbant assay.

3.4.11 CD Spectroscopy

CD spectra of the ET analogues were recorded over the range 260 to 190nm using a JASCO J600 spectropolarimeter. The concentrations of peptides were in the range of 0.06-0.27 mg/ml, and the path length was 1 mm. Samples were dissolved in 25mM boric acid, pH 7.0 (buffer 1) or 50mM boric acid (pH 7.0) /trifluoroethanol (TFE) 1:1 (v/v). The secondary-structure contents were determined using the CONTIN procedure[85] and the peptide concentrations were calculated using ε=7210 for peptides 1, 2, 1B, 1C, 2B, 21, 22, 24, 25, 26; ε=7090 for peptides 12-15, 33,34,39, 40; ε=6970 for peptides 36,38,51; and ε=8370 for peptide 43[106].

3.4.12 NMR studies

All NMR experiments for the peptides were performed using 5mm proton (inverse) probe on a Varian VXR 600S (600MHz)spectrometer operating at 599.945 MHz. The peptide samples were dissolved in a mixture of $CD_3OD/H_2O/D_2O$ (50:45:5) in 5mm diameter Wilmad 528PP NMR tubes. The NMR experiments were performed on the sample at pH 3.6 and 298K and the ^1H NMR signal of CD_3OH was used as internal reference and defined as 3.3ppm

The 2D phase-sensitive DQF COSY, TOCSY and NOESY NMR experiments were performed as descibed by Hewage.[86]

3.5 References

[1] Furchgott, R.F. & Zawadzki, J.V. (1980), *Nature (London),* **288,** 373-376.

[2] Moncada, S., Palmer, R.M.J. & Higgs, E. A. (1991), *Pharmac. Rev.* **43**, 109-142.

[3] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe,Y., Kobayashi, M., Mitsui,Y., Yazaki, Y., Goto, K. & Masaki, T. (1988), *Nature (London),* **332,** 411-415.

[4] Inoue, A., Yanagisawa, M., Kimura, S., Miyauchi, T., Goto, K. & Masaki, T. (1989), *Proc. Natl. Acad. Sci. U.S.A., 86, 2863-2867.*

[5] Kloog, T., Ambar, I., Sokolovsky, M., Kochva, E., Wollberg, Z. & Bdolah, A. (1988), *Science,* **242,** 268-270.

[6] Saida, K., Mitsui, Y. & Ishida, N. (1989), *J. Biol. Chem.* **264**, 14613-14616.

[7] Fukurada, T., Noguchi, K., Tsuchida, S., Nishikibe, M., Ikemoto, F., Okada, K. & Yano, M. (1990), *Biochem. Biophys. Res. Commun.* **172,** 390-395.

[8] Sawamura, T., Kisuya, Y., Matsushita, Y., Suzuki, N., Shinmi, O., Kishi, N., Sugita, Y., Yanagisawa, M. (1990), *Biochem. Biophys. Res. Commun.* **174,** 779-784.

[9] Ohnaka, K., Takayanagi, R., Nishikawa, M., Haji, M. & Nawata, H. (1993), *J. Biol. Chem.* **268,** 26759-26766.

[10] Turner, A.J. & Murphy, L.J. (1996), *Biochem. Pharmarcol.* **51,** 91-102.

[11] Arai, H., Hori, S., Aramori, I., Ohkubo, H. & Nakanshi, S. (1990), *Nature (London,)* **348,** 730-732.

[12] Sakurai, T., Yanagisawa, M., Takuwa,Y., Miyazaki, H., Kimura, S., Goto, K. & Masaki, T. (1990), *Nature (London),* **348,** 732-735.

[13] Williams, D.L., Jones, K.L., Colton, C.D. & Nutt, R.F. (1991), *Biochem. Biophys. Res. Commun.* **180,** 475-480.

[14] Hosoda, K., Nakao, K., Arai, H., Suga, S., Ogawa, Y., Mukoyama, M., Shirakami, G., Saito, Y., Nakanishi, S. & Imura, H. (1991), *FEBS Lett.* **287,** 23-26.

[15] Sakamoto, A., Yanagisawa, M., Sakurai, T., Takuwa, Y., Yanagisawa, H. & Masaki, T. (1991), *Biochem. Biophys. Res. Commun.* **178,** 656-663.

[16] Karne, S., Jayawickrame, C. K. & Lerner, M. R. (1993), *J. Biol. Chem.* **268**, 19126-19133.

[17] Cristol, J.P., Warner, T.D., Thiemermann, C. & Vane, J.R., *Br. J. Pharmacol.* (1993), **108,** 776-779.

[18] Sudjarwo, S.A., Hori, M., Takai, M., Urade, Y., Okada, T. & Karaki, H. (1993), *Life Sci.* **53,** 431-437.

[19] Takahashi, K., Ghatei, M.A., Jones, P.M., Murphy, J.K., Lam, H.C., O'Halloran, D.J. & Bloom, R.S. (1991), *J. Clin. Endocrinol. Metab.* **72,** 693-699.

[20] Sakurai, T. & Goto, K., *Drugs,* (1993), **46,** 795-804.

[21] Tamirisa, D., Frishman, W.H. & Kumar, A. (1995), *Am. Heart J.* **130,** 601-610.

[22] Lin, H.Y., Kaji, E.H., Winkel, G.K., Ives, H.E. & Lodish, H.F. (1991), *Proc. Natl. Acad. Sci. USA,* **88,** 3185-3189.

[23] Haendler, B., Hechler, U., Becker, A. & Schleuning, W.D. (1993), *Biochem. Biophys. Res. Commun.* **191,** 633-638.

[24] Sakamoto, A., Yanagisawa, M., Sawamura, T., Enoki, T., Ohtani, T., Sakurai, T., Nakao, K., Toyo-oka, T. & Masaki, T. (1993) *J. Biol. Chem.* **268,** 8547-8553.

[25] Doherty, A.M. (1994), in *Chemical and Structural Approaches to rational Drug Design,* p85-123, Weiner, D.B. & Williams, W.B., (Eds.) University of Pennsylvania, Philadelphia, Pennsylvania.

[26] Rubanyi, G.M. & Polokoff, M.A. (1994), *Pharmcol. Rev.* **46,** 325-415.

[27] Ihara, M., Noguchi, K., Seaki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M. & Yano, M. (1992), *Life Sci.* **50,** 247-255.

[28] Aramori, C., Nirei, H., Shoubo, M., Sogabe, K., Nakamura, K., Kojo, H., Notsu, Y., Ono, T. & Nakanishi, S. (1993), *Mol. Pharmacol.* **43,** 127-131.

[29] Ishikawa, K., Fukami, T., Nagase, T., Fujita, K., Hayama, T., Niiyama, K., Mase, T., Ihara, M. & Yano, M. (1992), *J. Med. Chem.* **35,** 2139-2142.

[30] Ishikawa, K., Ihara, M., Noguchi, K., Mase, T., Mino, N., Seaki, T., Fukuroda, T., Fukami, T., Ozaki, S., Nagase, T., Nishikibe, M. & Yano, M. (1994), *Proc. Natl. Acad. Sci. U.S.A.* **91,** 4892-4896.

[31] Morishita, Y., Chiba, S., Tsukuda, E., Tanaka, O., Ogawa, T., Yamasaki, M., Yoshida, M., Kawamoto, I. & Matsuda, Y. (1994), *J. Antibiotics* **47,** 269-275.

[32] Clozel, M., Breu, V., Burri, K., Cassal, J.M., Fischli, W., Gray, G.A., Hirth, G., Loffler, B.M., Muller, M., Neidhart, W. & Ramuz, H. (1993), *Nature (London),* **365,** 759-761.

[33] Doherty, A. M., Cody, W.L., Leitz, N.L., DePue, P. L., Taylor, M.D., Rapundalo, S.T., Hingorani, G. P., Major, T.C., Panek, R.L. & Taylor, D.G. (1991), *J. Cardiovasc. Pharmacol.* **17 (Suppl. 7),** S59-S61.

[34] Kimura, S., Kasuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. & Masaki, T. (1988), *Biochem. Biophys. Res. Commun.* **156,**1182-1186.

[35] Koshi, K., Suzuki, C., Arai, K., Mizoguchi, T., Tori, T., Hirata, M., Ohkuchi, M. & Okabe, T. (1991), *Chem. Pharm. Bull.* **39,** 3061-3063.

[36] Magazine, H.I., Malik, A.B., Bruner, C.A. & Anderson, T.T., (1992), *J. Pharmacol. Exp. Ther.* **260,** 632-636.

[37] Nakajima, K., Kumagaya, S., Nishio, H., Kuroda, H., Watanabe, T.X., Kobayashi, Y., Kimura, T. & Sakakibara, S. (1989), *J. Cardiovasc. Pharmacol.,* **13(Suppl. 5),** S8-S12.

[38] Tam, J.P., Liu, W., Zhang, J.-W., Galantino, M., Bertolero, F., Cristaini, C., Vaghi, F. & Castiglione, R. (1994), *Peptides* **15,** 703-708.

[39] De Castiglione, R., Tam, J. P., Liu, W., Zhang, J.-W., Galantino, M., Bertolero, F. & Vaghi, F. (1992), in *Peptides: Chemistry and biology; Proceedings of the 12th American Peptide Symposium,* P402-403, Smith, J.A. and River, J.E. (Eds.), ESCOM, Leiden, The Netherlands.

[40] Galantino, M., De Castiglione, R., Tam, J. P., Liu, W., Zhang, J. W., Christiani, C. & Vaghi, F. (1992), in *Peptides: Chemistry and biology; Proceedings of the 12th American Peptide Symposium,* p404-405, Smith, J.A., and River, J.E. (Eds.), ESCOM, Leiden, The Netherlands.

[41] Saeki,T., Ihara, M., Fukuroda, T., Yanagisawa, M. & Yano, M. (1991), *Biochem. Biophys. Res. Commun.* **179,** 286-292.

[42] Topouzis, S., Pelton, J.T., Miller, R.C. (1989), *Br. J. Pharmacol.* **98,** 669-677.

[43] Topouzis, S., Huggins, J.P., Pelton, J.T. & Miller, R.C. (1991), *Br. J. Pharmacol.* **102,** 145-148.

[44] Hunt, J. T., Lee, V. G., Stein, P. D., Hedberg, A., Liu, E. C-K., MeMullen, D. & Moreland, S. (1991), *Bioorgan. & Med. Chem. Lett.* **1,** 33-38.

[45] Hunt, J. T., Lee,V. G., MeMullen, D., Liu, E.C.-K., Bolgar, M., Delaney, C.L., Festin, S.M., Floyd, D. M., Hedberg, A., Natarajan, S., Serafino, R., Stein, P. D., Webb, M. L., Zhang, R. & Morland, S. (1993), *Bioorgan. & Med. Chem.Lett.* **1,** 59-65.

[46] Kikuchi,T., Kubo,K., Ohtaki,T., Watanabe, T., Wakimasu, M. & Fujino, M. (1991), in *Peptide Chemistry 1991,* p209-214, Suzuki, A. (Ed.), Protein Research Foundation, Osaka.

[47] Kikuchi,T., Kubo,K., Ohtaki,T., Suzuki, N., Asami, T., Shimamoto, N., Wakimasu, M. & Fujino, M. (1993), *J. Med. Chem.* **36,** 4087-4093.

[48] Cody, W.L., Doherty, A.M., He, J.X., Rapundalo, S.T., Hingorani, G.P., Panek, R.L. & Major, T.L. (1991), *J. Cardiovasc. Pharmacol.* **17(suppl.7),** S62-S64.

[49] Cody, W.L., He, J. X., DePue, P.L., Rapundalo, S.T., Hingorani, G.P., Dudley, D.T., Hill, K.E., Reynolds, E.E. & Doherty, A.M. (1994), *Bioorg. Med. Chem. Lett.* **4,** 567-572.

[50] Urade, Y., Fujitani, Y., Oda, K., Watanabe, T., Umemura, I.,Taka, M., Okada, T., Sakata, K. & Karaki, H. (1992), *FEBS Lett.* **311,** 12-16.

[51] Urade, Y., Fujitani, Y., Oda, K., Watanabe, T., Umemura, I.,Taka, M., Okada, T., Sakata, K. & Karaki, H. (1994), *FEBS Lett.* **342,** 103.

[52] Nambi, P., Pullen, M. & Spielman, W. (1994), *J. Pharmacol. Exp. Ther.* **268,** 202-207.

[53] Pelton, J.T. & Miller, R.C. (1991), *J. Pharm. Pharmacol.* **43**, 43-45.

[54] Hiley, C.R., Jones, C.R., Pelton, J.T. & Miller, R.C. (1990), *Br. J. Pharmacol.* **101,** 319-324.

[55] Ihara, T., Saeki, T., Fukuroda, T. & Yano, M. WO 92/19644.

[56] Ihara, M., Seaki, T., Fukuroda, T., Kimura, S., Ozaki, S., Patel, A.C. & Yono, M. (1992), *Life Sci.* **51,** 47-52.

[57] James, A.F., Urade, Y., Webb, R.L., Karaki, H., Umemura, I., Fujitani, Y., Oda, K., Okada, T., Lappe, R.W. & Takai, M. (1993), *Cardiovasc. Drug Rev. 11,* 253-270.

[58] Saeki, T., Ihara, T., Fukuroda, T. & Yano, M. (1992), *Biochem. International,* **28,** 305-312.

[59] Urade, Y., Takai, M., Watanabe, T., Yamamura, T., Okada, T. & Karaki, H. (1993), *Eur. Patent No. 548441.*

[60] Cheng, X.M., Nikam, S.S. & Doherty, A.M. (1994), *Current Med. Chem.* **1,** 271-312.

[61] Doherty, A.M., Cody, W.L., DePue, P.L., He, J.X., Waite, L.A., Leonard, D.M., Leitz, N.L., Dudley, D.T., Rapundalo, S.T., Hingorani, G.P., Haleen, S.J., LaDouceur, D.M., Hill, K.E., Flynn, M.A. & Reynolds, E.E. (1993), *J. Med. Chem.* **36,** 2585-2594.

[62] Cody, W.L., Doherty, A. M., He, J. X., DePue, P.L., Waite, L.A., Topliss, J.G., Haleen, S.J., LaDouceur, D., Flynn, M.A., Hill, K.E. & Reynolds, E.E. (1993), *Med. Chem. Res.* **3,** 154-162.

[63] Perkins, T.D., Hider, R.C. & Barlow, D.J. (1990), *Int. J. Peptide Res,* **36,** 128-133.

[64] Atkins, A.R., Ralston, G.B. & Smith, R. (1994), *Int. J. Pept. Protein Res.* **44,** 372-377.

[65] Saudek, V., Hoflack, J. & Pelton, J.T. (1991), *Int. J. Pept. Protein Res.* **37,** 174-179.

[66] Reily, M.D. & Dunbar, J.B. (1991), *Biochem. Biophys. Res. Commun.* **178,** 570-577.

[67] Endo, S., Inooka, H., Ishibashi, Y., Kitada, C., Mizuta, E. & Fujino, M. (1989), *FEBS Lett.* **257,** 149-154.

[68] Krystek, S.R., Bassolino, D.A., Novotny, J., Chen, C., Marschner, T. M. & Andersen, N.H. (1991), *FEBS Lett.* **281,** 212-218.

[69] Andersen, N.H., Chen, C., Marschner, T.M., Krystek, S.R. & Bassolino, D.A. (1992) *Biochem.* **31,** 515-523.

[70] Tamaoki, H., Kobayashi, Y., Nishimura, S., Ohkubo, T., Kyoguku, Y., Nakajima, K., Kumagaye, S., Kimura, T. & Sakakibara, S. (1991), *Protein. Eng.* **4,** 509-518.

[71] Dalgarno, D.C., Slater, L., Chackalamannil, S. & Senior, M.M. (1992), *Int. Peptide Protein Res.* **40,** 515-523.

[72] Saudek, V., Hoflack, J. & Pelton, J.T. (1989), *FEBS Lett.* **257,** 145-148.

[73] Calas, B., Harricane, M-C., Guimard, L., Heitz, F., Mendre, C., Chabrier, P.E., & Bennes, R. (1992), *Pept. Res. 5,* 97-100.

[74] Brown, S.C., Donian, M.E. & Jeffs, P.W., (1990), in *Peptides: Chemistry and biology; Proceedings of the 11th American Peptide Symposium,* P595, River, J.E. and Marshall, G.R. (Eds.), ESCOM, Leiden, The Netherlands.

[75] Coles, M., Munro, S.L.A. & Craik, D.J. (1994), *J. Med. Chem.* **37,** 656-664.

[76] Hewage, C.M., Jiang, L., Parkinson, J.A., Ramage, R. & Sadler, I.H. (1995), poster in *the 13th Int. NMR Meeting,* Manchester, U.K.

[77] Janes, R.W., Peapus, D. H. & Wallace, B. A. (1994), *Nature Struct. Biol.* **1,** 311-319.

[78] Nakajima, K., Kubo, S., Kumagaya, S., Nishio, H., Tsunemi, M., Inui, T., Kuroda, H., Chino, N., Watanabe, T.X., Kimura, T. & Sakabibara, S. (1989), *Biochem. Biophys. Res. Commun.* **163,** 424-429.

[79] Kumagaya, S., Kuroda, H., Nakajima, K., Watanabe, T., Kimura, T., Masaki, T. & Sakakibara, S. (1988), *Int. J. Peptide Protein Res.* **32,** 519-526.

[80] Nomizu, M., Inagakf, Y., Iwamatsu, A., Kashiwabara, T., Ohta, H., Morita, A., Nishikori, K., Otaka, A., Fuji, N. & Roller, P.P. (1991), *Int. J. Pept. Protein Res.* **38,** 580-587.

[81] Munson, M.C. & Barany, G. (1993), *J. Am. Chem. Soc.* **115,** 10203-10210.

[82] Lin, Y.Z., Ke, X.H. & Tam, J.P. (1991), *Biochemistry,* **30,** 3310-3314.

[83] Akaji, K., Nishiuchi, H. & Kiso, Y. (1995), *Tetrahetron Lett.* **36,** 1875-1878.

[84] Stewart, A. *Personal Communication.*

[85] Prownchcr, S. W. & Grocker, J. (1991), *Biochemistry,* **20**, 33-37.

[86] Hewage, C. M. (1996), *PhD thesis, Edinburgh University.*

[87] Shimada, K., Takahashi, M. & Tanzawa, K. (1994), *J. Biol. Chem.* **269,** 18275-18278.

[88] Xu, D, Emoto, N., Giaid, A, Slaughter, C., Kaw, S., deWit, D. & Yanagisawa, M. (1994), *Cell,* **78,** 473-485.

[89] Schmidt, M., Kroger, B., Jacob, E., Seulberger, H., Subkowski, T., Otter, R., Meyer, T., Schmalzing, G. & Hillen, H., (1994), *FEBS Lett.* **356**, 238-243.

[90] Wu-Wong, J.R., Shiosaki, K., Dillon, T.P., Rockway, T.R., Devine, E.M., Budzik, G.P., Marrsclle, C. & Opgenorth, T. J. (1991), *FASEB J.* **5,** A698 (abstr.).

[91] Okada, K., Takada, J., Arai, Y., Matsuyama, K. & Yano, M. (1991), *Biochem. Biophys. Res. Commun.* **180,** 1019-1023.

[92] Okada, K., Arai, Y., Hata, M., Matsuyama, K. & Yano, M. (1993), *Eur. J. Biochem.* **218,** 493-498.

[93] Abassi, Z., Golomb, E & Keiser, H.R. (1992), *Metabolism,* **41,** 683-685.

[94] De Lombaert, S., Ghai, R.D., Jeng, A.Y., Trapani, A.J. & Webb, R.L. (1994), *Biochem. Biophys. Res. Commun.* **204,** 407-412.

[95] Fukami, T., Hayama, T., Amano, Y., Nakamura, Y., Arai, Y., Matsuyama, K. & Ishikawa, K. (1994), *Bioorg. Med. Chem. Lett.* **4,** 1257-1262.

[96] Morta, A., Nomizu, M., Okitsu, M., Horie, K., Yokogoshi, H. & Roller, P.P. (1994), *FEBS Lett.* **353,** 84-88.

[97] Swenson, H. *Personal communication.*

[98] Jacobson, K.A., Marr-Heisy, D., Rosenkranz, R.P., Verlander, M., Melmon, K. & Goodman, M.J. (1983), *J. Med. Chem.* **26**, 492-503.

[99] Coy, D.H., Hocart, S.J. & Sasaki, Y. (1988), *Tetrahedron* **44**, 835-841.

[100] Ho, P. T., Chang, D., Zhong, J. W-X., Musso, G.F. (1993), *Pept. Res.* **6**, 10-13.

[101] Irving, S.L.(1993), *PhD thesis, Edinburgh University.*

[102] Parke-Davis Pharmaceutical Research Ltd. *personal communication.*

[103] Henry, J.A. (1991), *PhD thesis, Edinburgh University.*

[104] Ahn, K., Beningo, K., Olds, G. & Hupe, D. (1992), *Proc. Natl. Acad. Sci. USA,* **89,** 8606-8610.

[105] Ahn, K., Pan, S., Beningo, K. & Hupe D. (1995), *Life Sci.* **56,** 2331-2341.

[106] Gill, S. C. & von Hippel, P. H. (1989), *Analytical Biochem.* **182,** 319-326.

CHAPTER 4

THE CHEMICAL SYNTHESIS OF ω-AGATOXIN IIIA

4.1 Introduction

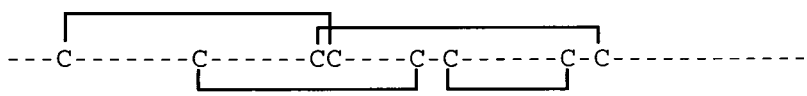
The level of intracellular Ca^{2+} regulates many cellular processes, including neuro-transmitter and hormone secretion, the activity of ion channels and enzymes, cytoskeletal function, cell proliferation, and gene expression.[1,2] Voltage-sensitive Ca^{2+} channels are among the most heterogeneous of ion channels. Peptide toxins are valuable tools for the characterisation of ion channels.[1] Vertebrate voltage-sensitive Ca^{2+} channels have been classified according to biophysical and pharmacological properties, and divided into two broad categories: the low voltage activated type (or T-type) and the high voltage activated type. The latter is further classified into three subtypes (L-, N-,P-, Q-, R-type).[3] Numerous studies suggested that N-type Ca^{2+} channels at presynaptic nerve terminals mediated a substantial proton of the Ca^{2+}-dependent transmitter release in the brain.[4]

ω-Agatoxins are a group of peptides isolated from the venom of the funnel-web spider *Agelenopsis aperta.* Their sequences are listed in Table 4.1.1.[1,5] The ω-agatoxins, as well as the somewhat smaller ω-conotoxins, have been valuable tools in defining various classes of Ca^{2+} channels.[5,6,7,8,9] Among these, ω-agatoxin IIIA (ω-AgaIIIA) was of particular interest as it was the only known agent that blocked the L- and N-type Ca^{2+} channels equally well.[5,10,11]

Due to the limited natural sources, an enormous effort had been made in the purification and isolation of the ω–agatoxins. Chemical synthesis was an alternative solution to this problem. At present, only the structure and biological activity of the type IV ω-agatoxins has been well studied, [12,13,14,15] and their disulphide patterns identified (Table 4.1.1).[16,17] Little is known about the six disulphide bonds which are present in the type III ω-agatoxins. Synthesis of the type III ω-agatoxins has never been reported. In fact, ω-agatoxin IVA is the only ω-agatoxin which has been successfully synthesised chemically.[16] An attempt was made in this study to synthesise ω–agatoxin IIIA

chemically. Although the product obtained was not homogenous due to uncontrolled disulphide bond formation, it did show some of the biological activity of native ω-agatoxin IIIA.

Table 4.1.1 The structures of ω-agatoxins

ω-agatoxin	Sequence
ω-Aga-IA	AKALPPGSVCDGNESDCKCYGKWHKCRCPWKWHFTGEGPCTCEKGMKHTCI TKLHCPNKAEWGLDW
ω-Aga-IIA	GCIEIGGDCDGYQEKSYCQCCRNNGFCS....
ω-Aga-IIIA	SCIDIGGDCDGEKDDCQCCRRNGYCSCYSLFGYLKSGCKCVVGTSAEFQGI CRRKARQCYNSDPDKCESHNKPKRR-NH_2
The 12 Cys of ω-Aga-IIIA	-C------C------C-CC-----C-C----------C-C---------- C------C-------C---------
ω-Aga-IVA	KKKCIAKDYGRCKWGGTPCCRGRGCICSIMGTNCECKPRLIMEGLGLA
ω-Aga-IVB	EDNCIAEDYGKCTWGGTKCCRGRPCRCSMIGTNCECTPRLIMEGLSFA
Disulphide pattern of IVA/IVB	

4.2 The synthesis of ω-agatoxin IIIA

4.2.1 Introduction

As shown in Table 4.1.1, ω-agatoxin IIIA is a 76 amino acid peptide, containing 12 cysteines that form 6 disulphide bridges. There are 10395 possible combinations for their formation, which means only one out of 10395 different cysteine combinations represents the native pairing. Thus the correct formation of these disulphide bonds is fundamentally the most difficult and challenging part of the chemical synthesis.

Solid phase peptide synthesis has been discussed in previous chapters and the same methodologies are applied here. As mentioned in Chapter 1, folding and disulphide

bond formation are concomitant processes, assuming the disulphide exchange is rapid and reversible. Since the biologically active (native) form is the thermodynamically preferred conformation, hopefully, it would emerge as the major product under ideal folding conditions. On the other hand, even a mixture of different disulphide patterns would also be useful if they possessed some of the required selectivity and biological activity of ω-agatoxin IIIA.

4.2.2 The first synthesis of ω-agatoxin IIIA utilising DIC/HOBt coupling method

The peptide was first synthesised utilising the DIC symmetrical anhydride and HOBt active ester double coupling protocol. Trt was used as the protecting group for all the twelve cysteines. The coupling percentage dropped dramatically at residues $Lys^{39}Cys^{40}Val^{41}Val^{42}$-~-resin, and the absorption for Fmoc-piperidine adduct became too small after Arg^{20} (Figure 4.2.1). The overall yield was 4.5% according to the deprotection of the last amino acid which was checked manually using the formula given in Section 3.4.1.

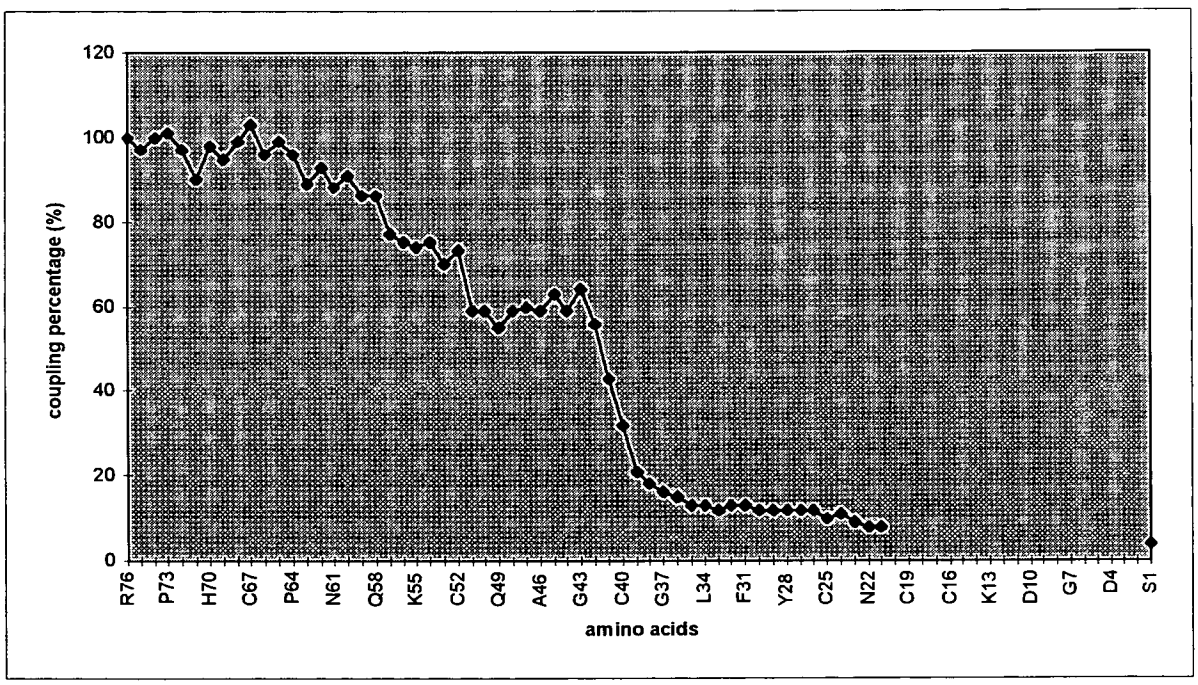


Figure 4.2.1 The coupling of ω-agatoxin IIIA by DIC symmetrical anhydride and HOBt active ester methodology

The completed peptide resin was reacted with TbfmocCl and the Tbfmoc-peptide cleaved from the resin with TFA and scavengers. The HPLC of the crude Tbfmoc peptide is shown in Figure 4.2.2.

The Tbfmoc-peptide was purified by HPLC and portions which absorbed at both 214nm and 364nm were collected. The Tbfmoc group was removed by dissolving the peptide in 6M guanidine.HCl containing 0.1M Tris buffer (pH 8.5 at 37°C) and 0.2M DTT. The mixture was incubated at 37°C and the reaction was monitored by dual wavelength HPLC, which indicated that the reaction was complete in ~3 h (Figure 4.2.3).

The mixture was chromatographed using a G50 Sephadex column and the peptide portions were dried by lyophilization to give the fully reduced peptide. The peptide was folded in 0.1 M Tris buffer in the presence of GSSG and GSH and the folding was monitored by HPLC (Figure 4.2.4). The fully oxidised peptide was isolated by HPLC to give the final product (3mg).

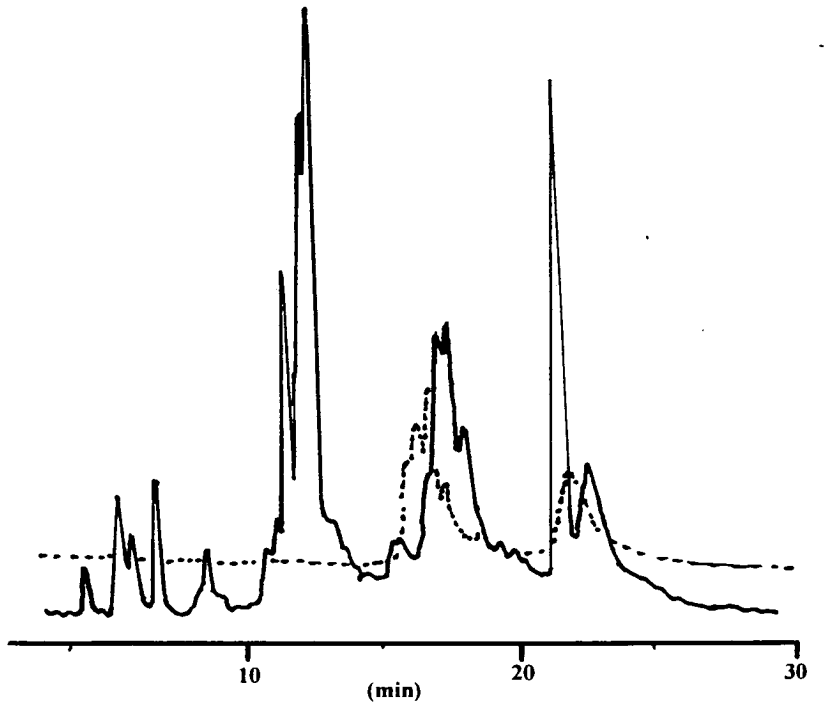


Figure 4.2.2 HPLC traces of crude Tbfmoc ω-agatoxin IIIA, the 1st synthesis RP C8 (size A), (— 214nm, ····· 364nm), 1ml/min.

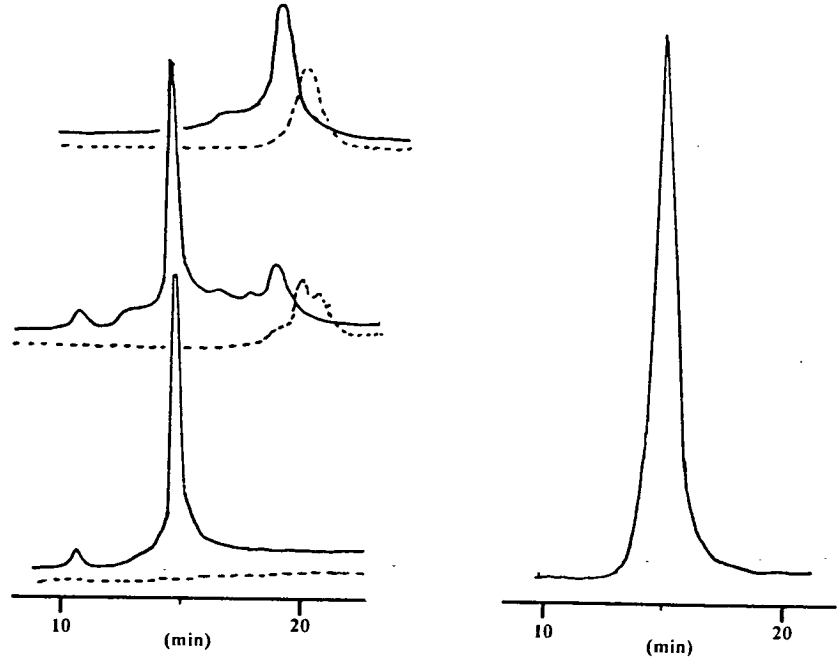


Figure 4.2.3 HPLC monitoring of the deprotection of Tbfmoc, 1st synthesis

RP C8 (size A), (— 214nm, ····· 364nm), 1ml/min.
a. 10min; b. 120min; c.180min; d. after gel filtration

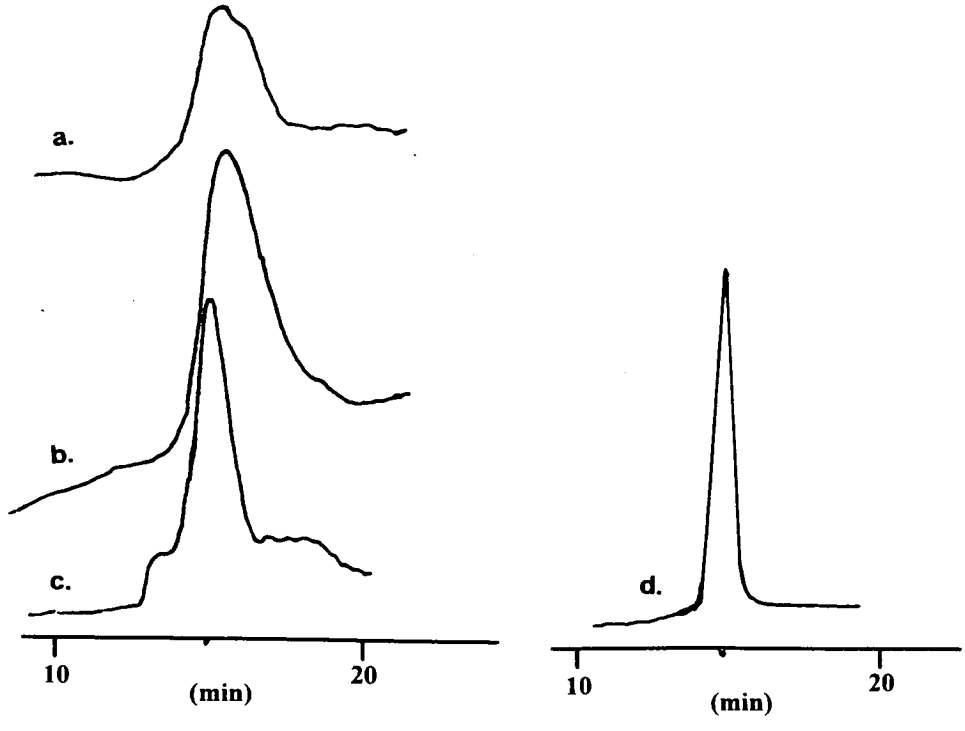


Figure 4.2.4 HPLC monitoring of the folding process

RP C8 (size A), 214nm, 1ml/min.
a. 30min; b. 2 days; c. 3 days. d. after purification

4.2.3 The second synthesis of ω-agatoxin IIIA utilising HOCt coupling method

In order to improve the yield of this peptide, a second synthesis was attempted utilising the new reagent, HOCt. The coupling percentage is shown in Figure 4.2.5.

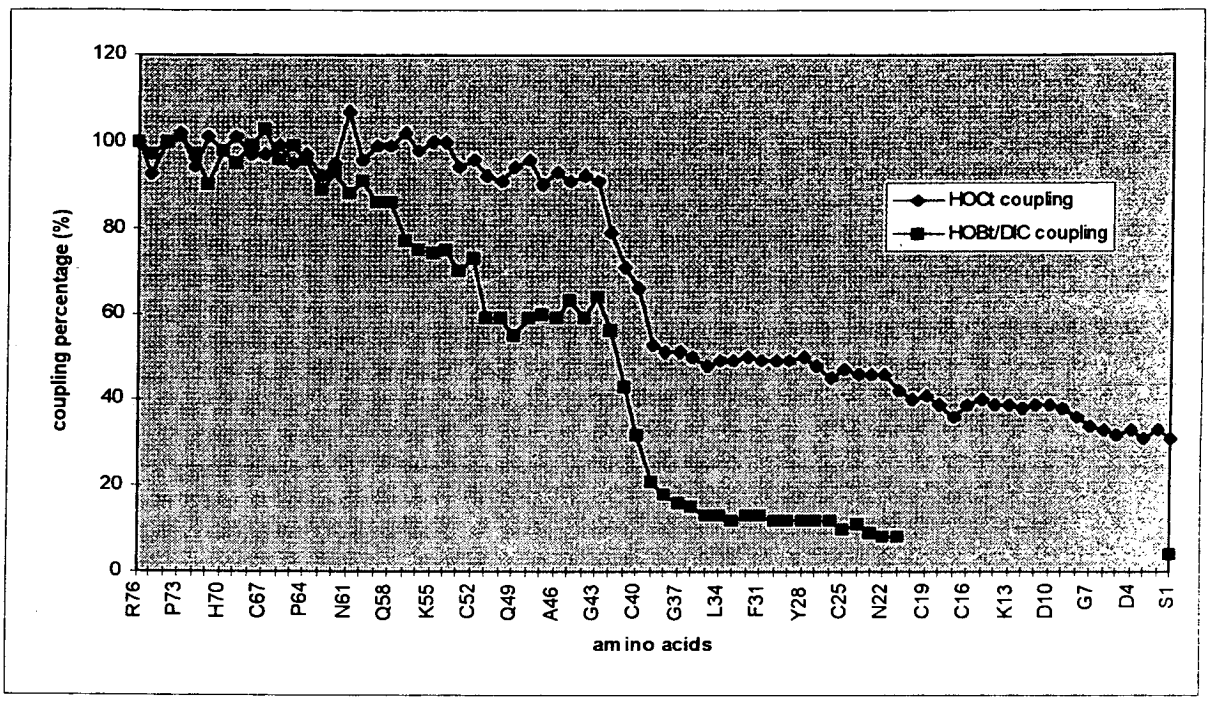


Figure 4.2.5 The coupling percentage using HOCt;
A comparison to the HOBt/DIC coupling

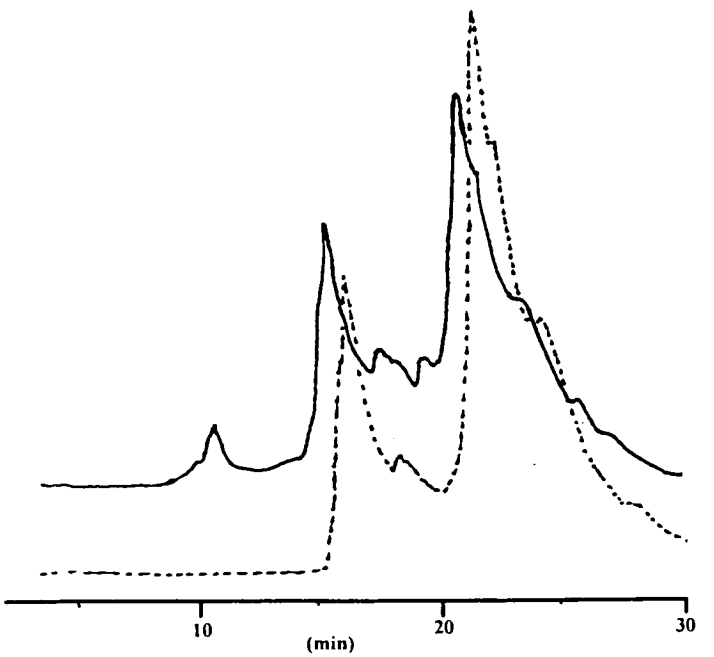


Figure 4.2.6 The crude Tbfmoc peptide of the 2nd synthesis
RP C8 (size A), (— 214nm, ····· 364nm), 1ml/min.

The coupling yield dropped again at the same residues which indicated that these are the difficult sequences. However, an overall yield of 30% was afforded which is 25% better than the first synthesis where the amino acid was coupled by HOBt active ester and DIC symmetrical anhydride methodology. The peptide was reacted with Tbfmoc and the Tbfmoc-peptide cleaved as before. Comparison of the HPLC traces of the crude peptides from HOCt and HOBt synthesis showed that HOCt synthesis was much better (Figures 4.2.1 and 4.2.6).

The crude peptide was fully denatured in 6M guanidine.HCl containing 0.2M DTT. The mixture was incubated at 37°C for 1h and purified by G50 Sephadex gel filtration. The corresponding portions were collected and lyophilised. The Tbfmoc group was removed by incubation of the peptide in 6M guanidine.HCl (0.1M Tris pH 8.5, 1mM EDTA, 0.2M DTT) and the reaction was monitored by dual wavelength HPLC (Figure 4.2.7).

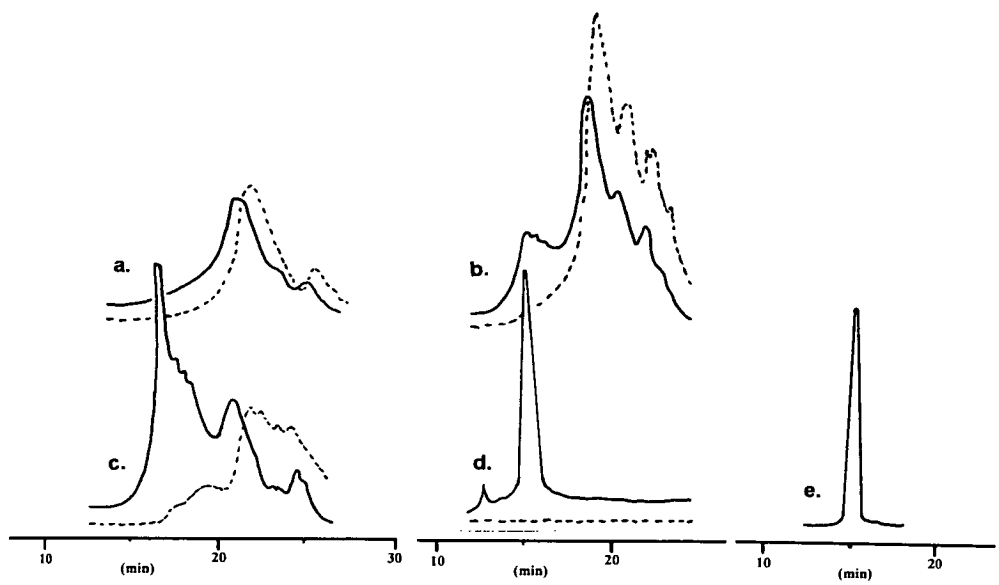


Figure 4.2.7 HPLC monitoring of the deprotection of Tbfmoc, 2nd synthesis
RP C8 (size A), (— 214nm, ····· 364nm), 1ml/min.
a. 10min; b. 60min; c.120min; d.180min; e. after gel filtration

The mixture was eluted from a G50 Sephadex column and the corresponding portions were collected and folded in 0.1M Tris buffer in the presence of GSSG and GSH. The folding process was monitored by HPLC (Figure 4.2.8) which indicated that

the reaction was complete in 3 days. The mixture was purified by HPLC and the peptide was lyophilised to give 15mg product.

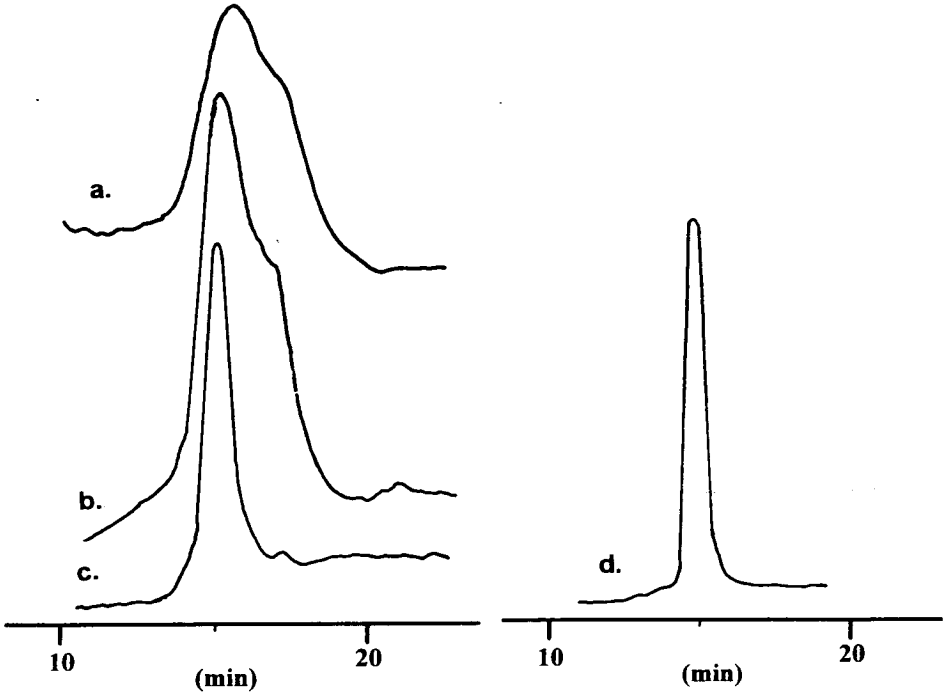


Figure 4.2.8 HPLC monitoring of the oxidation and folding process
RP C8 (size A), 214nm, 1ml/min.
a. 30min; b. 2 days; c. 3 days. d. after purification

4.3 Studies of the disulphide patterns

4.3.1 Introduction

The unambiguous assignment of the ω–agatoxin disulphide bonds is critical for understanding the molecular basis of the biological activity. Unfortunately, no general method exists for elucidating disulphide connections. Sequential enzymatic and chemical cleavage with subsequent analysis of the disulphide bond containing fragments have been employed most often for localisation of disulphide connections in peptides. Success in utilising this methodology alone is problematic, particularly with highly folded cysteine rich peptides such as the ω–agatoxins. This has been shown clearly in the assignment of the four disulphide bonds in the ω–agatoxin IVB and IVC.[16] Since twelve cysteines are present in the ω–agatoxin IIIA, the assignment of the six disulphide bonds would be even

more difficult. However, with the disulphide pattern of IV type ω–agatoxins already known, efforts have been made to predict the disulphide pattern in the ω-agatoxins.[18] As shown in Table 4.1.1, among the twelve cysteines in ω-agatoxin IIIA, eight are present in a very similar pattern as shown in ω-agatoxin IVA ($Cys^{2,9,18,19,25,27,38,40}$), despite the dissimilarity in the sequences. Supposing the same disulphide pattern in the Type IV ω-agatoxins also applied to the ω-agatoxin IIIA, this still leaves 4 cysteines with three possible disulphide patterns, which are:

(a) 2-19, 9-25, 18-40, 27-38, 16-67 and 52-59.

(b) 2-19, 9-25, 18-40, 27-38, 16-52 and 59-67;

(c) 2-19, 9-25, 18-40, 27-38, 16-59 and 52-67;

According to a calculation based on the known data, (a) is regarded as the most likely pattern and (b) is the second, while (c) is the least possible.[18]

4.3.2 Trypsin digestion of the synthetic ω-agatoxin IIIA

An attempt was made to study the disulphide patterns in the synthetic peptide. Trypsin was chosen for the digestion of ω-agatoxin IIIA as it cleaves basic amino acids (Arg, Lys). The possible fragments with the predicted disulphide patterns are shown in Figure 4.3.1.

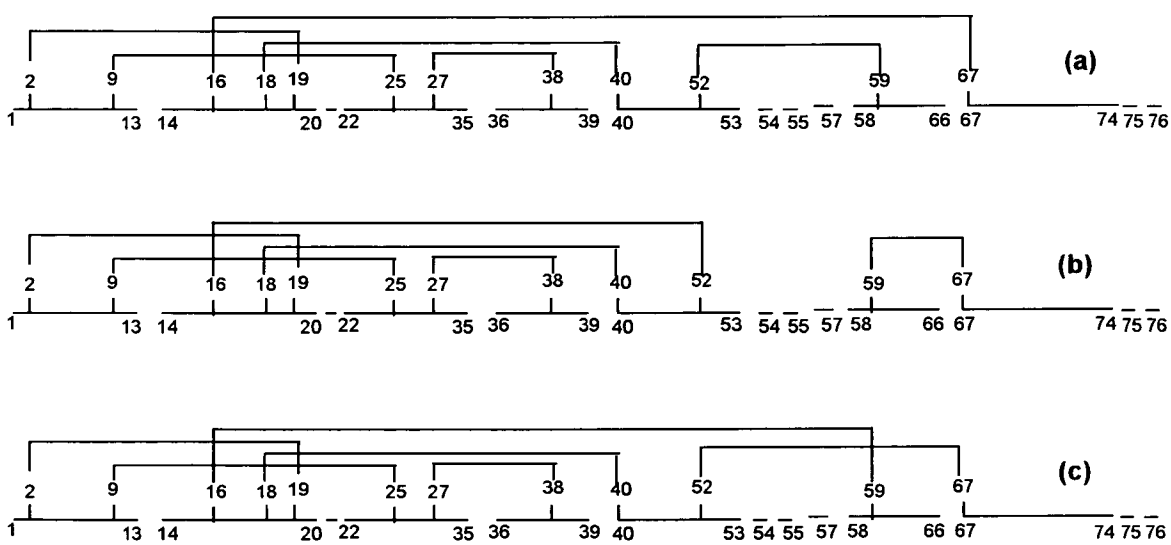


Figure 4.3.1 Possible fragments by trypsin digestion with the predicted disulphide patterns

As seen in Figure 4.3.1, all fragments are connected with each other due to the presence of multiple disulphide connections except one in pattern (b). This means that two fragments can be isolated if pattern (b) is the correct pattern; and only one fragment can be isolated if (a) or (c) is the correct pattern. The HPLC of the enzyme digestion showed three major peaks and the MS of the mixture is shown in Table 4.3.1. The fragments were separated on HPLC, but the fractions collected failed to show any useful information on the MS.

Since no MS signal corresponding to the most simple fragment connected by Cys^{59} and Cys^{67}, pattern (b) is unlikely to be present in the synthetic ω-Aga IIIA. However, the most sensitive signals obtained on MS were at 1472 and 1492, which indicated the presence of a fragment and its sodium salt. According to the MS of the tryptic fragments, this signal is likely to be fragment [40-53], thus a connection between Cys^{40} and Cys^{52} is likely to be present, which does not corresponding to any of the predicted pattern. Obviously, an extensive investigation is required in order to further define the disulphide pattern in ω–AgaIIIA.

Table 4.3.1 The MS of enzyme digestion fragments and the possible sequences

M+1	Possible sequences and the corresponding MS
1472.8/1492.4	40-53 (1469.7, Na^+ 1490.7); 36-39 + 58-66 (1463.6)
2141.8	1-20 (2134.8)
2168,2189	
2299.2	1-13 + 14-21 (2309.9) or 1-21(2290.9)
2578.7	22-35 + 67-74 (2560.2)
3179.5/3189.0	36-53 + 56-66 (3141.4)
3214/3220/3226	
4882.7	1-13 + 14-21 + 22-35 + 67-74 (4870.0)

However, it was found later that the synthetic peptide was a mixture of numerous conformations (Figure 4.3.3), therefore further investigations into folding and oxidation conditions should be carried out before a proper assignment can be made.

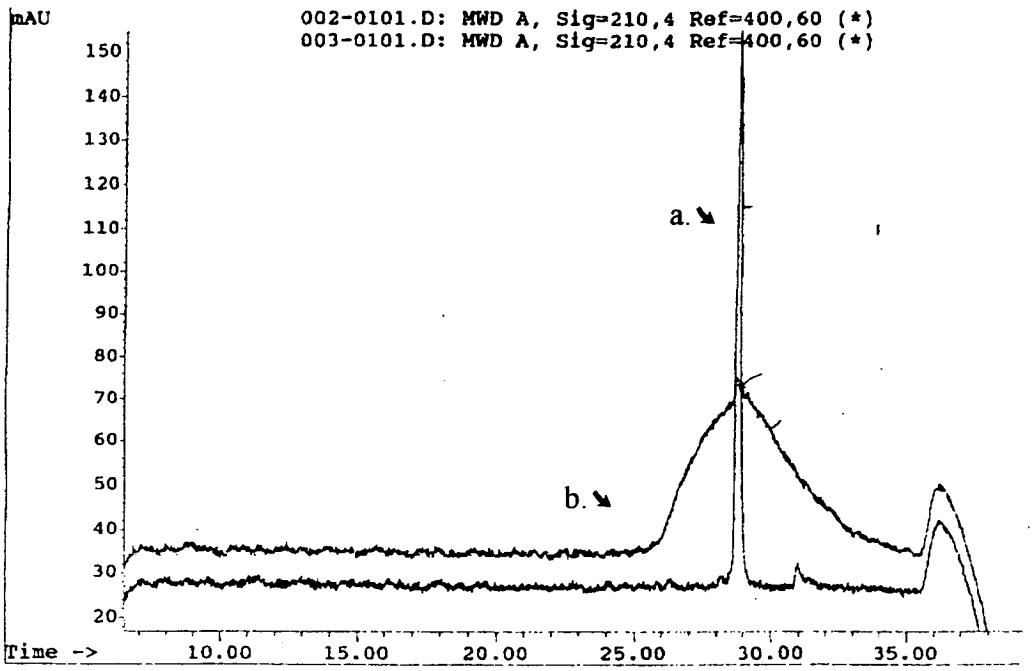


Figure 4.3.3 Comparison of the synthetic and native ω-agatoxin IIIA on HPLC[18]

a. native ω-AgaIIIA; b. synthetic ω-AgaIIIA
A: 0.1%TFA/5%CH_3CN in H_2O, B: 0.1% TFA/5% H_2O/CH_3CN,
Gradient (t,B%): (0,5), (30,35), (35,80).

4.4 Biological activity of the synthetic ω-agatoxin IIIA

The synthetic ω-agatoxin IIIA was tested in cell lines expressing recombinant R-type Ca^{2+} channels using whole-cell voltage-clamp techniques (Figure 4.4.1). The IC_{50} of the synthetic peptide was ~1μM, while that of the native ω–agatoxin IIIA was 20nM. The binding and functional activities of the synthetic ω-AgaIIIA for the N-type of Ca^{2+} channels are 8.9nM and 170nM respectively, 47 and 27 fold less potent than that of the native ω-AgaIIIA (Table 4.4.1). These results were very interesting, considering that the synthetic peptide was a mixture. Although the activity of the synthetic peptide was 50

fold less potent than the native ω–agatoxin IIIA, it was still potent enough, and potentially very useful in the biological studies. However, when this work was under the way, a synthetic peptide, SNX-111, corresponding to the structure of the ω-conopeptide MVIIA from *Conus magus*, was found to be highly effective neuroprotective agent in animal models of transient global ischemia.[19] Therefore, this work was not carried any further.

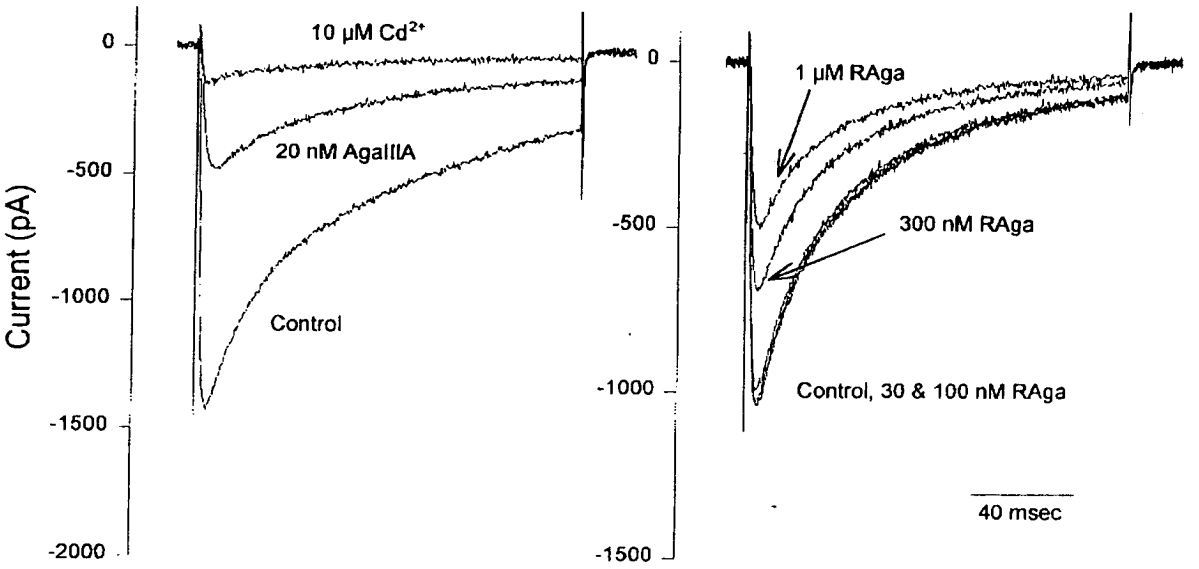


Figure 4.4.1 Comparison of native ω-AgaIIIA and synthetic ω-AgaIIIA(RAga) on R-type Ca^{2+} channel currents

Table 4.4.1 The binding and functional activity of synthetic and native ω-AgaIIIA

peptide	Binding IC_{50}(nM)	Functionality IC_{50}(nM)
Native ω-AgaIIIA	0.19	6.2
Synthetic ω-AgaIIIA	8.9	170

4.5 Experimental

4.5.1 General

All general experiments were the same as described in Section 3.4.1 unless otherwise stated.

4.5.2 The first synthesis of ω-agatoxin IIIA

4.5.2.1 Synthesis of ω-agatoxin IIIA utilising HOBt/DIC coupling method

The loading of the commercial dibenzosuberenyl resin (0.65mmol/g) is too high for the synthesis of large peptides such as ω-agatoxin IIIA. The first amino acid was therefore coupled manually to reduce the initial loading. Arg(Pmc)OH (1.0mmol) was dissolved in DMF and DIC (0.50mmol) was added. The mixture was sonicated at RT for 10 min. then added to the Fmoc deprotected dibenzosuberenyl resin (1.0g). The resulting mixture was sonicated for 1h at RT. The loading of FmocArg(Pmc) was tested as described in Section 3.4.1 and it was found to be 0.223mmol/g.

The synthesis was carried out at 0.11mmol scale and the subsequent amino acids were coupled once as the DIC symmetrical anhydride and then as the HOBt active ester. The coupling efficiency was monitored by the absorption of the Fmoc-piperidine adduct (302nm). The Fmoc of the last amino acid was left intact in order to check the loading manually.

The completed peptide resin (52.1mg) was deprotected with 20% piperidine/DMF (10ml) and the UV absorption at 302nm was observed (A_{302}= 0.11). The loading of the last amino acid was 2.4 $x10^{-3}$mmol/g according to the formula given in Section 3.4.1.

Loading =10xA_{302}/9Wt=10x0.11/9x52.1=2.4x10^{-3} mmol/g

The theoretical loading of the peptide is 0.053mmol/g. The overall yield is therefore calculated as follows:

2.4x10^{-3} / 0.053 = 0.042 = 4.5%

4.5.2.2 Purification and folding of ω-agatoxin IIIA

TbfmocCl (45.8mg, 0.1mmol) and DIEA (15ml, 0.09mmol) were dissolved in dry DCM (~50ml) and the peptide on resin (1.5g) was added. The mixture was sonicated at RT for 2h in the absence of light. The mixture was then filtered and washed with DCM, dioxane, and DCM again. The resin was dried and the Tbfmoc loading was tested by sonicating 13.8mg of the Tbfmoc resin in 20% piperidine/dioxane (10ml) for 15min. The absorbance at 364nm was 0.100 and the loading of Tbfmoc was calculated as follows:

$$\textbf{Loading(mmol/g)} = 0.613 \times A_{364} / 13.8 = 0.613 \times 0.100 / 13.8 = 4.4 \times 10^{-3} \textbf{ mmol/g}$$

The resin bonded peptide was then cleaved with TFA: EDT: thioanisole: H_2O (10:1:0.5:0.5) and 0.75g of phenol at RT for 5h. The resin was filtered off and the filtrate was reduced *in vacuo* to remove TFA. The residue was precipitated with ether, filtered and washed with ether. The crude peptide was purified by RP HPLC using dual wave length, 214nm/364nm, (RP C8, size D; Gradient (t, CH_3CN%): (2,10), (10,30), (35,50)). The portions which absorbed at both wave lengths (214nm/364nm) were collected and lyophilised. The peptide obtained (~10mg) was incubated at 37 °C in 6M guanidine.HCl, containing 0.1M Tris buffer (pH 8.5, 37°C), 1mM EDTA and DTT(50mg) to remove the Tbfmoc group. The reaction was monitored by dual wave length HPLC (RP C8, size B) and it was complete in about 3h. The mixture was chromatographed on G50 Sephadex column, eluting with 30% acetic acid. The peptide portions were lyophilised to give the fully reduced peptide (c.a. 5mg). The reduced peptide was dissolved in 6M guanidine.HCl (0.1M Tris pH8.0, 1mM EDTA, 0.5ml) and diluted rapidly into 50ml 1M guanidine.HCl (0.1M Tris pH8.0, 1mM EDTA, 0.5mM GSSG / 5mM GSH). This solution was stirred gently at RT for 3 days until a sharp peak appeared on HPLC. The mixture was adjusted to pH 4.0 with acetic acid and loaded onto a RP C8 HPLC column through the A pump and washed with 2% CH_3CN until all the salt had eluted. A shallow gradient (2%-60% CH_3CN over 60 min) was then applied. The peptide portion was collected and lyophilised to give the final peptide (3mg). Ellmans' test was negative.

4.5.3. The second synthesis of ω-agatoxin IIIA

4.5.3.1 Synthesis of ω-agatoxin IIIA using HOCt coupling method

A second synthesis was carried out using HOCt double coupling methodology. The same FmocArg(Pmc) resin (0.5g, loading 0.223mmol/g) was used and an overall coupling yield of ~30% was afforded according to the Fmoc deprotection file. The coupling of the last amino acid was tested by sonicating the peptide resin (34.6mg) in 20% piperidine/DMF (10ml) for 10 min. The UV absorption at 302nm was 0.516 and the loading was calculated in the same way as described in section 4.5.2 to give a final loading of 0.018mmol/g and an overall yield of 34%.

4.5.3.2 Purification and folding of ω-agatoxin IIIA

TbfmocCl (68.7mg, 0.15mmol) and DIEA (20μl, 0.11mmol) were dissolved in dry DCM (~50ml) and the peptide resin (1.6g) was added. The mixture was sonicated at RT for 2h in the absence of light. The mixture was then filtered and washed with DCM, dioxane, and DCM again. The resin was dried and the Tbfmoc loading was checked by sonicating 10.5mg of the resin in 20% piperidine/dioxane (10ml) for 10min. The absorbance at 364nm was 0.274. The Tbfmoc loading was calculated the same way as described in Section 4.5.2 to give a loading of 0.019 mmol/g.

The resin bound peptide was then cleaved with TFA: EDT: thioanisole: H_2O (10:1:0.5:0.5) and 0.75g of phenol at RT for 5h. The resin was filtered off and TFA was removed *in vacuo*. The residue was precipitated with ether, filtered and washed with ether. The crude peptide was dissolved in 20ml 6M guanidine.HCl plus DTT (200mg) and incubated at 37°C for 1h and the mixture was chromatographed on G50 Sephadex column, which was eluted with 30% acetic acid. The portions which absorbed at wavelengths 226nm and 364nm were collected and lyophilised to give the Tbfmoc-peptide (90mg).

The Tbfmoc-peptide (90mg) was dissolved in 6M guanidine.HCl (0.1M Tris pH 8.5, 1mM EDTA, 500mg DTT, 50ml) and incubated at 37°C to remove the Tbfmoc group. The reaction was monitored by dual wave length HPLC (RP C8, size B). The absorbance at 364nm disappeared and the peak sharpened gradually. The reaction was

complete in 3h and the mixture was chromatographed on a G50 Sephadex column eluting with 30% acetic acid. The peptide portions were collected and lyophilised to give 50 mg crude peptide.

The crude peptide was dissolved in 6M guanidine.HCl (0.1M Tris pH8.0, 1mM EDTA, 200ml) and the milky solution was diluted rapidly into a mixture of 1M guanidine.HCl (0.1M Tris pH8.0, 1mM EDTA, 0.5mM GSSG / 5mM GSH, 500ml) with 100ml pure water to give a final concentration of 1M guanidine.HCl. The mixture was stirred gently at RT and monitored by HPLC. Aggregation was observed after 20h, and further 400ml of the same 1M guanidine.HCl solution was added. The mixture was stirred for another 2 days until a sharper peak was observed on HPLC. The mixture was purified by HPLC under the same conditions as described in Section 4.5.2 to give 15mg (11%) final product.

MS m/s: found 8516.9, requires $C_{347}H_{113}O_{114}S_{12}$: 8517.7.

Amino acid analysis (6M HCl, 110°C, 48h): Asp/Asn 10(9.12), Thr 1(0.89), Ser 7(4.89), Glu/Gln 6(7.11), Pro 2(-), Gly 8(8.00), Ala 2(2.18), Cys 12(9.07), Val 2(2.13), Ile 3(2.63), Leu 2(2.41), Tyr 4(2.39), Phe 2(2.27), His 1(1.01), Lys 7(6.66), Arg 7(6.25).

HPLC Rt 15min (RP C8, size A,(t,B%): (2,10), (32,90);

HPLC Rt 15min (Vydac C8 size B,(t,B%):(2,10), (32,90)).

4.5.4 Enzyme digestion

The peptide (0.5mg) was dissolved in 0.5 ml 0.1M NH_4OAC (pH7.0) which containing 0.1mg trypsin. The mixture was incubated at 37 °C and monitored by HPLC. A sample of the solution (200ml) was taken after 2h and the reaction was terminated by addition of 10μl 6M HCl. The remaining digest was terminated after 7h. Both samples were identical on HPLC and were analysed by MS. The mixture was then separated on analytical HPLC (Hichrom C18, size B, 10-70% over 60 min). The fractions were lyophilised but they failed to give any signal on MS.

4.5.5 Biological assay[17]

The biological assay was carried out at Parke Davis Pharmaceutical Reasearch at Michigan.

4.6 References

[1] Olivera, B.M. (1994), *Ann. Rev. Biochem.* **63,** 823-867.

[2] Bindockas, V.P., Venema, V.J., & Adams, M.E. (1991), *J. Neurophysiology,* **66,** 590-601.

[3] Sher, E., Clementi, F. (1991), *Neuroscience,* **42,** 301-307.

[4] Miller, R.J. (1987), *Science*, **235**, 46-52.

[5] Ertel, E.A., Warren, V.A., Adams, M.E., Griffin, P.R., Cohen, C.J. & Smith, M.M. (1994), *Biochemistry,* **33,** 5098-5108.

[6] Olivera, B.M., Imperial, J.S., Cruz, L.J., Bindokas, V.P., Venema, V.J. & Adams, M.E. (1991) *Ann. New York Acad. Sci.,* **635,** 114-122.

[7] Venema, V.J., Swiderek, K.M., Lee, T.D., Hathaway, G.M. & Adams, M.E. (1992), *J. Biol. Chem.* **267,** 2610-2615.

[8] Shon, K.-J., Hasson, A., Spira, M.E., Cruz, L.J., Gray, W.R., & Olivera, B.M. (1994), *Biochemistry,* **33,** 11420-11425.

[9] Mintz, I.S., Venema, V.J., Swiderek, K.M., Lee, T.D., Bean, B.P. & Adams, M.E. (1992), *Nature (London),* **355**, 827-829.

[10] Mintz, I.S., Venema, V.J., Adams, M.E. & Bean, B.P. (1991), *Proc. Natl. Acad. Sci. USA,* **88,** 6628-6631.

[11] Cohen, C.J., Ertel, E.A., Smith, M.M., Venema, V.J., Adams, M.E. & Leibowitz, M.D. (1992), *Mol. Pharmacol.* **42,** 947-961.

[12] Yu, H., Rosen, M.K., Saccomano, N.A., Phillips, D., Volkmann, R.A. & Schreiber, S.L. (1993), *Biochemistry,* **32,** 13123-13129.

[13] Adams, M.E., Mintz, I.M, Reily, M.D. Thanabal, V & Bean, B.P (1993), *Mol. Biol.* **44,** 681-688.

[14] Hong, S.J. & Chang, C.C. (1995), *J. Physiol. (London),* **482,** 283-290.

[15] Kim, J., Konishi, S., Iwai, H., Kohno, T., Gouda, H., Shimada, I., Sato, K. & Arata, Y. (1995), *J. Mol. Biol.* **250,** 659-671.

[16] Nishio, H., Kumagaya, K.Y., Kubo, S., Chen, Y.N., Momiyama, A., Takahashi, T., Kimura, T. & Sakakibara, S. (1993), *Biochem. Biophys. Res. Commun.* **196,** 1447-1453.

[17] Heck, S.D., Kelbaugh, P.R., Kelly, M.E., Thadeio, P.F., Saccomano, N.A., Stroh, J.G. & Volkman, R. A. (1994), *J. Am. Chem. Soc.* **116,** 10426-10436.

[18] Parke Davis Pharmceutical Research Ltd. *Personal communication.*

[19] Valentino, K., Newcomb, R., Gadbois, T., Singh, T., Bowersox, S., Bitner, S. (1993), *Proc. Natl. Acad. Sci. USA,* **90,** 7894-7897.

Appendix I Sequences of ET-1, ET-3, and full length ET-1 analogues

Pept.	Structure
	1 5 10 15 21
ET-3	CysThrCysPheThrTyrLysAspLysGluCysValTyrTyrCysHisLeuAspIleIleTrp
ET-1 (60)	CysSerCysSerSerLeuMetAspLysGluCysValTyrPheCysHisLeuAspIleIleTrp
1C	**Aib**Ser**Aib**SerSerLeu**Nle**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
1	**X** Ser**Aib**SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
1B	**X** Ser**Aib**SerSerLeu**Nle**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
2	**X** Ser**Ala**SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
2B	**X** Ser**Ala**SerSerLeu**Leu**AspLysGlu**Ala**ValTyrPhe **X** HisLeuAspIleIleTrp
26	**X** Ser**Ala**SerSerLeu**Leu***asp*LysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
21	**X** Ser**Ala**SerSerLeu**Leu**AspLysGlu**Aib**ValTyr*phe* **X** HisLeuAspIleIleTrp
24	**X** Ser**Ala**SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** *phe*LeuAspIleIleTrp
22	**X** Ser**Ala**SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeu*asp*IleIleTrp
23	**X** Ser**Ala**SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** *phe*Leu*asp*IleIleTrp
25	**X** Ser**Ala**SerSerLeu**Leu***asp*LysGlu**Aib**ValTyrPhe **X** *phe*LeuAspIleIleTrp
56	**Aib**Ser**Aib**SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeu**ThrMle**IleTrp

Notes:

Aib: α-aminoisobutyric acid;
X : Cys(Acm);
Nle: norleucine;
Mle: γ-methylleucine;
Amino acids in lower case ***italic*** are the D-isomers, and those in bold are replacement positions.

Appendix II Sequence of ET-1 and its truncated analogues

Pept.	Structure
	1 5 10 15 21
ET-1	CysSerCysSerSerLeuMetAspLysGluCysValTyrPheCysHisLeuAspIleIleTrp
15A	Ser **X** SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
15-1	Ser**Ala**SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
14	**Ala**SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
13	SerSerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
12	SerLeu**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
11	Leu**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
40	Leu**Leu**AspLysGlu**Ala**ValTyrPhe **X** HisLeuAspIleIleTrp
54	Leu**Leu**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
47	Leu**Leu**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeu**Thr**IleIleTrp
51	Leu**Leu**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeu**Leu**IleIleTrp
38	Leu**Leu**Asp**Glu**Glu**Aib**ValTyrPhe**Aib**HisLeu**ThrMle**IleTrp
33	**Leu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
33A	**AcLeu**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
72	**LeuAla**LysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
53	**Leu**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
62	**Mle**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
64	**Mle***asp*LysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
67	**Nle**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
61	**Val**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
61A	**AcVal**AspLysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
63	**Val***asp*LysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
63A	**AcVal***asp*LysGlu**Aib**ValTyrPhe**Aib**HisLeuAspIleIleTrp
32	**Nle**AspLysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
43	**Leu**AspLysGlu**Aib**ValTyr**Tyr** **X** HisLeuAspIleIleTrp
34	**Leu***asp*LysGlu**Aib**ValTyrPhe **X** HisLeuAspIleIleTrp
65	**Leu**AspLysGlu**Ala**ValTyrphe **X** HisLeuAspIleIleTrp

(to be continued)

Appendix II Sequence of ET-1 and its truncated analogues (continued)

Pept.	Structure
	1 5 10 15 21
52	Asp Lys Glu **Aib** Val Tyr Phe **Aib** His Leu Asp Ile Ile Trp
31	Asp Lys Glu **Aib** Val Tyr Phe **X** His Leu Asp Ile Ile Trp
41	Asp Lys Glu **Ala** Val Tyr Phe **X** His Leu Asp Ile Ile Trp
41a	**Ac** Asp Lys Glu **Ala** Val Tyr Phe **X** His Leu Asp Ile Ile Trp
39	Asp Lys Glu *ala* Val Tyr Phe **X** His Leu Asp Ile Ile Trp
55	Asp **Glu** Glu **Aib** Val Tyr Phe **Aib** His Leu Asp Ile Ile Trp
46	Asp Lys Glu **Aib** Val Tyr Phe **Aib** His Leu **Thr** Ile Ile Trp
50	Asp Lys Glu **Aib** Val Tyr Phe **Aib** His Leu **Leu** Ile Ile Trp
66	Asp Lys Glu **Aib** Val Tyr Phe **Aib** His Leu **Mle** Ile Ile Trp
37	Asp Lys Glu **Aib** Val Tyr Phe **Aib** His Leu **Thr Mle** Ile Trp
37A	**Ac** Asp Lys Glu **Aib** Val Tyr Phe **Aib** His Leu **Thr Mle** Ile Trp
36	Asp **Glu** Glu **Aib** Val Tyr Phe **Aib** His Leu **Thr Mle** Ile Trp
36A	**Ac** Asp **Glu** Glu **Aib** Val Tyr Phe **Aib** His Leu **Thr Mle** Ile Trp
68	*asp* Lys Glu **Aib** Val Tyr Phe **Aib** His Leu Asp Ile Ile Trp
69	Asp *lys* Glu **Aib** Val Tyr Phe **Aib** His Leu Asp Ile Ile Trp
59	Asp Lys *glu* **Aib** Val Tyr Phe **Aib** His Leu Asp Ile Ile Trp
58	Asp Lys Glu **Aib** *val* Tyr Phe **Aib** His Leu Asp Ile Ile Trp
57	Asp Lys Glu **Aib** Val *tyr* Phe **Aib** His Leu Asp Ile Ile Trp
70	Asp Lys Glu **Aib** Val Typ *phe* **Aib** His Leu Asp Ile Ile Trp
71	Asp Lys Glu **Aib** Val Tyr Phe **Aib** *his* Leu Asp Ile Ile Trp
30	Lys Glu **Aib** Val Tyr Phe **X** His Leu Asp Ile Ile Trp
29	Glu **Aib** Val Tyr Phe **X** His Leu Asp Ile Ile Trp
28	**Aib** Val Tyr Phe **X** His Leu Asp Ile Ile Trp
35A	**Ac** Val Tyr Phe **X** His Leu Asp Ile Ile Trp

Notes:

Aib: α-aminoisobutyric acid;
X : Cys(Acm);
Nle: norleucine;
Mle: γ-methylleucine;
Amino acid in lower case ***italic*** are the D-isomers, and those in bold are replacement positions.

Appendix III Sequences of BigET analogues

peptide	structure
	1 5 10 15 20 25 30 35 38 39
BigET(45)	CysSerCysSerSerLeuMetAspLysGluCysValTyrPheCysHisLeuAspIleIleTrpValAsnThrProGluHisValValProTyrGlyLeuGlySerProArgSer
5	**X** Ser **X** SerSerLeuMetAspLysGlu **X** ValTyrPhe **X** HisLeuAspIleIleTrpValAsnThrProGluHisValValProTyrGlyLeu**LeuGlySerProArgSer**
18	**X** Ser **X** SerSerLeuMetAspLysGlu **X** ValTyrPhe **X** HisLeuAspIleIle **W--V** AsnThrProGluHisValValProTyrGlyLeuGlySerProArgSer
S18	CysSerCysSerSerLeuMetAspLysGluCysValTyrPheCysHisLeuAspIleIle **W--V** AsnThrProGluHisValValProTyrGlyLeuGlySerProArgSer
7	ValTyrPhe **X** HisLeuAspIleIle **W--V** AsnThrProGluHisValValProTyrGlyLeuGlySerProArgSer
6	IleIle **W--V** AsnThrProGluHisValValProTyrGlyLeuGlySerProArgSer
16	IleIleTrpValAsnThrProGluHisValValProTyrGlyLeuGlySerProArgSer
17	IleIleTrp **X'**AsnThrProGluHisValValProTyrGlyLeuGlySerProArgSer
S17	IleIleTrp**Pen**AsnThrProGluHisValValProTyrGlyLeuGlySerProArgSer
3	ValAsnThrProGluHisValValProTyrGlyLeu**LeuGlySerProArgSer**

(to be continued)

Appendix III Sequences of BigET analogues

(continued)

peptide	structure
	1 5 10 15 20 25 30 35 38 39
83	HisLeuAspIleIleTrpValAsnThrProGluHisValValProTyrGlyLeuGlySerProArgSer
84	HisLeuAspIleIleTrpValAsnThrProGluHisValValProTyrGlyLeuGly
79	HisLeuAspIleIleTrp **X** AsnThrProGluHisValValProTyrGlyLeuGly
80	HisLeuAspIleIleTrp**Cys**AsnThrProGluHisValValProTyrGlyLeuGly
81	HisLeuAspIleIleTrp **X'**AsnThrProGluHisValValProTyrGlyLeuGly
82	HisLeuAspIleIleTrp**Pen**AsnThrProGluHisValValProTyrGlyLeuGly
27	IleIleTrpValAsnThrProGluHisValValProTyrGlyLeuGly
76	IleIleTrp **X** AsnThrProGluHisValValProTyrGlyLeuGly
75	IleIleTrp**Cys**AsnThrProGluHisValValProTyrGlyLeuGly
77	IleIleTrp **X'**AsnThrProGluHisValValProTyrGlyLeuGly
78	IleIleTrp**Pen**AsnThrProGluHisValValProTyrGlyLeuGly
9	IleIleTrpValAsnThrProGluHisValValProTyrGlyLeu**Leu**
10	IleIleTrpValAsnThrProGluHisValValProTyr**Leu**Gly**Leu**
8	Glu HisValValProTyrLeuGly**Leu**

Notes: X: Cys(Acm); X': Pen(Acm), W-V: Trp(CH_2NH)Val; Amino acids in **bold** are replacement positons.

Appendix IV
HPLC conditions and amino acid analysis data for the ET-1 analogues

Pept. No.	HPLC Analytical[a]	HPLC Preparative[b]	Amino acid analysis 6M HCl, 110°C
60 (ET-1)	RP C18(A) 18min VydacC18(B) 21min Hichrom C18 (B) 22min	RPC18(C) (60,2), (120,50). Repurified: RP C18 (2,10), (6,25), (30, 40).	Asp 2(2.05), Ser 3(2.81), Glu 1(1.16), Cys 4(3.22), Val 1(1.03), Met 1(0.92), Ile 2(1.67), Leu 2(1.98), Tyr 1(0.87), Phe 1(0.98), His 1(1.00), Lys 1(0.99). 24hrs.
1	RP C18(A) 18min VydacC18(B) 21min	RPC18(C) (2,10), (10,35), (25,50).	Asp 2(2.45), Ser 3(2.21), Glu 1(1.16), Cys 2(1.60), Val 1(1.05), Ile 2(1.65), Leu 3(2.98), Tyr 1(0.30), Phe 1(0.93), His 1(0.97), Lys 1(0.98). 40hrs.
1B	RP C8(A) 22min	RPC18(C) (2,10), (10,30), (25.42).	Asp 2(2.01), Ser 3(2.38), Glu 1(1.17), Cys 2(1.33), Val 1(0.99), Ile 2(1.62), Leu 2(1.94), Nle 1(1.02), Tyr 1(0.49), Phe 1(0.93), His 1(0.98), Lys 1(0.96). 36hrs.
1C	Vydac C18(B) 23min	RPC18(C) (2,10), (10,30), (26,45).	Asp 2(1.82), Ser 3(2.70), Glu 1(1.25), Val 1(1.27), Ile 2(1.45), Leu 2(1.84), Tyr 1(1.00), Phe 1(0.99), His 1(1.00), Lys 1(1.04), Nle 1(0.98). 36hrs
2	Vydac C18(B) 21min	RPC18(C) (2,10), (10,35), (22, 45).	Asp 2(2.07), Ser 3(2.10), Glu 1(1.04), Ala 1(0.91), Cys 2(1.19), Val 1(0.96), Ile 2(1.65), Leu 3(2.84), Tyr 1(0.53), Phe 1(0.90), His 1(0.98), Lys 1(0.84). 40hrs.
2B	RP C18(A) 22min	RPC18(C) (2,10), (10,30), (25,40).	Asp 2(2.06), Ser 3(2.15), Glu 1(1.13), Ala 2(1.88), Cys 2(1.41), Val 1(1.18), Ile 2(1.83), Leu 3(3.00), Tyr 1(0.28), Phe 1(1.00), His 1(1.04), Lys 1(1.04). 40hrs.
21	RP C8(A) 18min	RPC18(C) (2,10), (10,30), (24,38).	Asp 2(2.01), Ser 3(2.59), Glu 1(1.13), Ala 1(0.99), Cys 2(1.51), Val 1(1.01), Ile 2(1.19), Leu 3(2.97), Tyr 1(0.61), Phe 1(0.97), His 1(1.01), Lys 1(1.01). 30hrs.
22	Vydac C18(B) 18min	RPC18(C) (2,10), (10,30), (24,38).	Asp 2(2.03), Ser 3(2.40), Glu 1(1.16), Ala 1(0.99), Cys 2(1.14), Val 1(0.94), Ile 2(1.36), Leu 3(2.76), Tyr 1(0.65), Phe 1(0.98), His 1(0.91), Lys 1(0.92). 30hrs.
23	RP C18(A) 20min	RPC18(C) (2,10), (10,30), (26,45).	Asp 2(2.10), Ser 3(2.44), Glu 1(1.19), Ala 1(0.98), Cys 2(1.53), Val 1(1.07), Ile 2(1.20), Leu 3(2.94), Tyr 1(0.61), Phe 2(2.08), Lys 1(1.04). 30hrs.
24	RP C18(A) 20min	Vydac C18(D) (2,10), (12,40), (22,58).	Asp 2(2.03), Ser 3(2.60), Glu 1(1.02), Ala 1(1.11), Cys 2(1.34), Val 1(1.07), Ile 2(1.59), Leu 3(2.92), Tyr 1(0.41), Phe 2(1.89), Lys 1(1.01). 30hrs.
25	RP C18(A) 18min	Vydac C18(D) (2,10), (10,50), (18,57).	Asp 2(2.02), Ser 3(2.24), Glu 1(1.12), Ala 1(1.05), Cys 2(1.24), Val 1(1.04), Ile 2(1.47), Leu 3(2.88), Tyr 1(0.53), Phe 2(1.95), Lys 1(1.02). 30hrs.
26	RP C18(A) 18min	RPC18(C) (2,10), (10,35), (25,45).	Asp 2(2.09), Ser 3(2.32), Glu 1(1.15), Ala 1(1.02), Cys 2(1.30), Val 1(0.99), Ile 2(1.54), Leu 3(2.83), Tyr 1(0.57), Phe 1(0.92), His 1(1.01), Lys 1(1.01). 30hrs.

56	Hichrom C18 (B) 20min	RPC18(C) (2,10), (10,30), (32,50)	Asp 1(0.91), Ser 3(2.70), Glu 1(1.25), Val 1(1.27), Ile 1(0.97), Leu/Mle 4(3.64), Tyr 1(1.00), Phe 1(0.99), His 1(1.00), Lys 1(1.04), Nle 1(0.98). 36hrs
15A	RP C18(A) 18min	RPC18 (2,10), (10,30), (26,42)	Asp 2(2.05), Ser 3(2.40), Glu 1(1.16), Cys 2(1.18), Val 1(0.98), Ile 2(1.42), Leu 3(2.86), Tyr 1(0.65), Phe 1(1.01), His 1(0.99), Lys 1(0.98). 48hrs.
15-1	RP C18(A) 18min	RPC18(C) (2,10), (10,30), (8), (27,43).	Asp 2(2.01), Ser 3(2.47), Glu 1(0.85), Ala 1(0.93), Cys 1(0.48), Val 1(1.33), Ile 2(1.60), Leu 3(3.00), Tyr 1(0.87), Phe 1(1.01), His 1(1.04), Lys 1(0.98). 48hrs.
14	RP C18(A) 18min	RPC18(C) (2,10), (10,30), (24,50).	Asp 2(2.03), Ser 2(1.61), Glu 1(0.94), Ala 1(1.08), Cys 1(0.82), Val 1(1.23), Ile 2(1.82), Leu 3(3.09), Tyr 1(0.83), Phe 1(0.97), His 1(1.03), Lys 1(0.96). 24hrs.
13	RP C18(A) 18min	RPC18(C) (2,10), (10,30), (24,50).	Asp 2(2.00), Ser 2(1.52), Glu 1(0.86), Cys 1(0.57), Val 1(1.27), Ile 2(1.65), Leu 3(2.99), Tyr 1(0.82), Phe 1(0.94), His 1(1.03), Lys 1(0.96). 24hrs.
12	RP C18(A) 18min	RPC18(C) (2,10), (10,30), (24,50).	Asp 2(2.07), Ser 1(0.78), Glu 1(1.02), Cys 1(0.27), Val 1(1.17), Ile 2(1.75), Leu 3(2.86), Tyr 1(0.88), Phe 1(1.03), His 1(1.04), Lys 1(0.99). 24hrs.
11	RP C18(A) 18min	RPC18(C) (2,10), (10,30), (24,50).	Asp 2(2.08), Glu 1(0.83), Cys 1(0.74), Val 1(1.29), Ile 2(1.61), Leu 3(3.01), Tyr 1(0.72), Phe 1(1.04), His 1(1.07), Lys 1(1.08). 24hrs.
40	RP C18(A) 16min	RPC18(C) (2,10), (10,28), (26,43).	Asp 2(1.99), Glu 1(1.36), Ala 1(1.00), Cys 1(0.77), Val 1(1.01), Ile 2(1.56), Leu 3(2.88), Tyr 1(0.63), Phe 1(1.03), His 1(0.89), Lys 1(1.10). 26hrs.
54	RP C18(A) 19min	RPC18(C) (2,10), (10,30), (28,38).	Asp 2(1.98), Glu 1(1.19), Val 1(0.99), Ile 2(1.16), Leu 3(3.02), Tyr 1(0.99), Phe 1(0.99), His 1(1.05), Lys 1(1.01). 24hrs.
47	RP C18(A) 20min	RPC18(C) (2,10), (8,26), (30,40).	Asp 1(0.98), Thr 1(1.03), Glu 1(1.21), Val 1(1.01), Ile 2(1.24), Leu 3(3.04), Tyr 1(0.99), Phe 1(1.00), His 1(1.05), Lys 1(1.01). 24hrs.
51	RP C18(A) 19min	RPC18(C) (2,10), (10,32), (26,43).	Asp 1(0.96), Glu 1(1.16), Val 1(0.99), Ile 2(1.32), Leu 4(3.92), Tyr 1(0.89), Phe 1(0.99), His 1(1.03), Lys 1(1.00). 24hrs.
38	RP C18(A) 19min	RPC18(C) (2,10), (10,32), (28,44).	Asp 1(1.04), Thr 1(1.02), Glu 2(2.31), Val 1(0.98), Ile 1(0.91), Leu/Mle 4(3.50), Tyr 1(1.00), Phe 1(1.01), His 1(1.00). 20hrs.
33	RP C18(A) 16min	RPC18(C) (2,10), (10,25), (22,35).	Asp 2(2.21), Glu 1(1.23), Cys 1(0.60), Val 1(1.09), Ile 2(1.47), Leu 2(1.94), Tyr 1(0.94), Phe 1(1.04), His 1(1.01), Lys 1(1.01). 20hrs.
33A	RP C18(A) 20min	RPC18(C) (2,10), (10,28), (24,38).	Asp 2(1.96), Glu 1(1.20), Cys 1(0.83), Val 1(1.03), Ile 2(1.34), Leu 2(1.94), Tyr 1(0.84), Phe 1(1.03), His 1(1.00), Lys 1(1.01). 20hrs.
72	RP C18(A) 24min	RPC18(C) (2,10), (10,28), (24,38).	Asp 1(1.08), Glu 1(1.21), Ala 1(1.03), Val 1(1.02), Ile 2(1.38), Leu 2(1.97), Tyr 1(0.91), Phe 1(1.01), His 1(0.99), Lys 1(1.03). 20hrs.
53	Vydac C18(B) 20min	RPC18 (2,10), (10,28), (30,40).	Asp 2(2.18), Glu 1(1.21), Val 1(1.04), Ile 2(1.49), Leu 2(1.96), Tyr 1(0.93), Phe 1(1.03), His 1(0.99), Lys 1(1.02). 20hrs.

62	RP C18(A) 16min Hichrom C18 (B) 20min	RPC18(C) (2,10), (10,30), (26,40).	Asp 2(2.13), Glu 1(1.17), Val 1(0.99), Ile 2(1.52), Leu/Mle 2(1.92), Tyr 1(0.91), Phe 1(1.00), His 1(1.00), Lys 1(0.99). 20hrs.
64	RP C18(A) 17min Hichrom C18 20min	RPC18(C) (2,10), (10,30), (26,40).	Asp 2(2.09), Glu 1(1.21), Val 1(0.99), Ile 2(1.43), Leu/Mle 2(1.89), Tyr 1(0.88), Phe 1(1.00), His 1(1.02), Lys 1(1.01). 20hrs.
67	Hichrom C18 (B) 20min	RPC18(C) (2,10), (10,28), (26,40).	Asp 2(2.21), Glu 1(1.16), Val 1(1.00), Ile 2(1.44), Leu 1(0.98), Nle 1(0.96), Tyr 1(0.89), Phe 1(1.01), His 1(0.99), Lys 1(0.98). 20hrs.
61	RP C18(A) 18min	RPC18(C) (2,10), (10,28), (28,40).	Asp 2(2.20), Glu 1(1.21), Val 2(2.03), Ile 2(1.42), Leu 1(0.98), Tyr 1(0.90), Phe 1(0.98), His 1(1.01), Lys 1(0.98). 24hrs.
61A	RP C18(A) 20min	RPC18(C) (2,10), (10,30), (28,42).	Asp 2(2.18), Glu 1(1.12), Val 2(2.04), Ile 2(1.39), Leu 1(0.99), Tyr 1(0.88), Phe 1(1.01), His 1(1.02), Lys 1(0.99). 24hrs.
63	Hichrom C18 (B) 17min	RPC18(C) (2,10), (10,32), (28,43).	Asp 2(2.13), Glu 1(1.18), Val 2(1.98), Ile 2(1.41), Leu 1(0.99), Tyr 1(0.92), Phe 1(0.98), His 1(1.00), Lys 1(0.99). 24hrs.
32	RP C18(A) 17min	RPC18(C) (2,10), (8,25), (26,38).	Asp 2(1.97), Glu 1(1.13), Cys 1(0.81), Val 1(0.99), Ile 2(1.37), Leu 1(0.93), Nle 1(0.97), Tyr 1(0.86), Phe 1(0.96), His 1(0.96), Lys 1(0.99). 20hrs.
43	Vydac C18(B) 16min	RPC18 (2,10), (10,28), (26,35).	Asp 2(2.03), Glu 1(1.23), Cys 1(0.84), Val 1(0.99), Ile 2(1.42), Leu 2(1.98), Tyr 2(1.68), His 1(0.97), Lys 1(1.00). 20hrs.
34	Vydac C18(B) 17min	RPC18(C) (2,10), (10,28), (26,36).	Asp 2(2.05), Glu 1(1.20), Cys 1(0.82), Val 1(1.03), Ile 2(1.37), Leu 2(1.99), Tyr 1(0.82), Phe 1(1.03), His 1(1.01), Lys 1(1.02). 20hrs.
65	Hichrom C18 (B) 20min	RPC18(C) (2,10), (10,30), (27,48).	Asp 2(1.96), Glu 1(1.16), Cys 1(0.76), Val 1(1.02), Ile 2(1.46), Leu 2(1.98), Tyr 1(0.79), Phe 1(0.99), His 1(1.01), Lys 1(1.02). 20hrs.
52	RP C18(A) 16min	RPC18 (2,10), (10,28), (24,38).	Asp 2(2.18), Glu 1(1.21), Val 1(1.04), Ile 2(1.49), Leu 1(0.99), Tyr 1(0.93), Phe 1(1.03), His 1(0.99), Lys 1(1.02). 20hrs.
39	Vydac C18(B) 19min	RPC18(C) (2,10), (10,28), (30,40).	Asp 2(1.22), Glu 1(1.14), Ala 1(1.13), Cys 1(0.81), Val 1(1.08), Ile 2(1.48), Leu 1(1.10), Tyr 1(0.67), Phe 1(1.04), His 1(1.02), Lys 1(1.01). 30hrs.
31	RP C18(A) 18min	RPC18(C) (2,10), (8,25), (22,33)	Asp 2(1.91), Glu 1(1.15), Cys 1(0.95), Val 1(1.01), Ile 2(1.29), Leu 1(0.99), Tyr 1(0.84), Phe 1(0.97), His 1(0.97), Lys 1(0.99). 20hrs.
41	RP C18(A) 18min Hichrom C18 (B) 19min	RPC18(C) (2,10), (10,30), (25,42)	Asp 2(2.01), Glu 1(0.80), Ala 1(1.20), Cys 1(0.72), Val 1(1.00), Ile 2(1.43), Leu 1(1.00), Tyr 1(0.60), Phe 1(0.93), His 1(0.91), Lys 1(0.90). 36hrs.
41A	RP C18(A) 20min	RPC18(C) (2,10), (10,30), (28,45)	Asp 2(1.94), Glu 1(1.13), Ala 1(0.94), Cys 1(0.88), Val 1(1.01), Ile 2(1.24), Leu 1(1.02), Tyr 1(0.76), Phe 1(0.98), His 1(1.02), Lys 1(1.00). 30hrs.
50	RP C18(A) 18min	RPC18(C) (2,10), (10,32), (24,43)	Asp 1(1.06), Glu 1(1.16), Val 1(0.94), Ile 2(1.22), Leu 2(1.90), Tyr 1(0.95), Phe 1(0.98), His 1(1.02), Lys 1(0.97). 20hrs.
46	RP C18(A) 19min	RPC18 (2,10), (8,26), (30,40)	Asp 1(1.08), Thr 1(1.05), Glu 1(1.18), Val 1(1.00), Ile 2(1.42), Leu 1(0.98), Tyr 1(0.93), Phe 1(1.01), His 1(0.99), Lys 1(1.02). 20hrs.

66	RP C18(A) 20min Hichrom C18 (B) 18min	RPC18(C) (2,10), (10,34), (30,45)	Asp 1(1.03), Glu 1(1.20), Val 1(1.00), Ile 2(1.54), Leu/Mle 2(1.89), Tyr 1(0.91), Phe 1(1.00), His 1(0.99), Lys 1(1.04). 20hrs.
55	Vydac C18(B) 17min	RPC18(C) (2,10), (10,28), (25,38)	Asp 2(1.90), Glu 2(2.29), Val 1(0.99), Ile 2(1.19), Leu 1(1.03), Tyr 1(0.99), Phe 1(0.97), His 1(1.04). 20hrs.
37	Vydac C18(B) 17min	RPC18(C) (2,10), (10,30), (25,40)	Asp 1(1.00), Thr 1(1.07), Glu 1(1.20), Val 1(1.00), Ile 1(0.94), Leu/Mle 2(1.89), Tyr 1(1.00), Phe 1(1.00), His 1(0.99), Lys 1(1.05). 20hrs.
37A	Vydac C18(B) 21min	RPC18(C) (2,10), (10,32), (26,42)	Asp 1(1.16), Thr 1(1.18), Glu 1(1.22), Val 1(1.00), Ile 1(0.98), Leu/Mle 2(1.92), Tyr 1(1.01), Phe 1(0.99), His 1(0.97), Lys 1(1.00). 20hrs.
36	Vydac C18(B) 18min	RPC18(C) (2,10), (10,32), (22,42)	Asp 1(0.98), Thr 1(1.02), Glu 2(2.34), Val 1(1.01), Ile 1(0.95), Leu/Mle 2(1.89), Tyr 1(0.93), Phe 1(0.99), His 1(1.00). 20hrs.
36A	Vydac C18(B) 20min	RPC18(C) (2,10), (10,32), (26,46)	Asp 1(0.96), Thr 1(1.04), Glu 2(2.30), Val 1(0.93), Ile 1(0.98), Leu/Mle 2(1.92), Tyr 1(0.91), Phe 1(0.98), His 1(1.00). 20hrs.
68	RP C18(A) 23min	RPC18 (2,10), (10,30), (25,40)	Asp 2(2.08), Glu 1(1.21), Val 1(1.04), Ile 2(1.45), Leu 1(0.97), Tyr 1(0.87), Phe 1(1.02), His 1(0.99), Lys 1(1.01). 20hrs.
69	RP C18(A) 19min	RPC18 (2,10), (10,30), (25,40)	Asp 2(2.02), Glu 1(1.18), Val 1(1.02), Ile 2(1.48), Leu 1(0.98), Tyr 1(0.85), Phe 1(1.01), His 1(1.00), Lys 1(1.01). 20hrs.
59	Hichrom C18 (B) 17min	RPC18 (2,10), (10,32), (27,43)	Asp 2(1.98), Glu 1(1.22), Val 1(1.05), Ile 2(1.42), Leu 1(0.99), Tyr 1(0.81), Phe 1(0.99), His 1(1.00), Lys 1(1.01). 20hrs.
58	Hichrom C18 (B) 18min	RPC18 (2,10), (10,32), (26,42)	Asp 2(2.07), Glu 1(1.19), Val 1(1.02), Ile 2(1.38), Leu 1(1.00), Tyr 1(0.81), Phe 1(1.00), His 1(0.99), Lys 1(1.01). 20hrs.
57	Hichrom C18 (B) 18min	RPC18 (2,10), (10,28), (26,38)	Asp 2(2.13), Glu 1(1.20), Val 1(1.04), Ile 2(1.40), Leu 1(0.99), Tyr 1(0.87), Phe 1(1.01), His 1(0.99), Lys 1(1.00). 20hrs.
70	Hichrom C18 (B) 22min	VydacC18(D) (2,10), (10,32), (28,45)	Asp 2(2.04), Glu 1(1.24), Val 1(1.02), Ile 2(1.35), Leu 1(0.98), Tyr 1(0.93), Phe 1(1.02), His 1(1.00), Lys 1(1.01). 20hrs.
71	Hichrom C18 (B) 24min	RPC18(C) (2,10), (10,28), (26,40)	Asp 2(2.08), Glu 1(1.22), Val 1(1.03), Ile 2(1.46), Leu 1(1.00), Tyr 1(0.84), Phe 1(1.02), His 1(0.99), Lys 1(1.01). 20hrs.
30	RP C18(A) 17min	RPC18(C) (2,10), (10,25), (24,36)	Asp 1(0.99), Glu 1(1.11), Cys 1(0.83), Val 1(1.01), Ile 2(1.32), Leu 1(0.96), Tyr 1(0.82), Phe 1(0.98), His 1(0.99), Lys 1(0.99). 20hrs.
29	RP C18(A) 17min	RPC18(C) (2,10), (10,25), (25,38)	Asp 1(1.04), Glu 1(1.11), Cys 1(0.78), Val 1(0.96), Ile 2(1.35), Leu 1(0.97), Tyr 1(0.83), Phe 1(0.95), His 1(0.95). 20hrs.
28	RP C18(A) 17min	RPC18(C) (2,10), (10,28), (26,40).	Asp 1(0.98), Cys 1(0.93), Val 1(0.97), Ile 2(1.39), Leu 1(1.01), Tyr 1(0.83), Phe 1(1.00), His 1(1.00). 20hrs.
35A	Vydac C18(B) 22min	RPC18(C) (2,10), (10,28), (30,40).	Asp 1(0.98), Cys 1(0.76), Val 1(1.04), Ile 2(1.45), Leu 1(0.99), Tyr 1(0.88), Phe 1(1.01), His 1(0.99). 20hrs.

Notes: a. Analytical column and retention time;
b. Gradient in (t,B%)

Appendix V
HPLC conditions and amino acid analysis data for the BigET-1 analogues

Pept. No.	HPLC Analytical[a]	HPLC Preparative[b]	Amino Acid Analysis 6M HCl, 110°C
45	RP C8 16min Vydac C18 17min	RP C18 (2,10), (10,30), (28, 40).	Asp 3(2.73), Thr 1(0.98), Ser 5(3.83), Glu 2(2.18), Pro 3(3.25), Gly 2(2.14), Cys 4(2.59), Val 4(4.33), Met 1(0.64), Ile 2(1.53), Leu 3(2.96), Tyr 2(0.92), Phe 1(1.03), His 2(1.98), Lys 1(1.00), Arg 1(0.99). 48hrs.
O45 (BET)	RP C8 16min Vydac C18 17min	RP C8 (60,2), (120, 60) Repurified: RP C8 (2,10), (6,25), (20, 38).	Asp 3(3.23), Thr 1(1.02), Ser 5(4.13), Glu 2(2.15), Pro 3(3.20), Gly 2(2.10), Cys 4(2.45), Val 4(4.24), Met 1(0.71), Ile 2(1.49), Leu 3(2.98), Tyr 2(1.01), Phe 1(1.06), His 2(2.01), Lys 1(1.01), Arg 1(1.00). 48hrs.
5	RP C8(A) 18min	RPC18(C) (2,10), (10,33), (22,43).	Asp 3(2.79), Thr 1(0.97), Ser 5(3.57), Glu 2(2.03), Pro 3(3.32), Gly 2(2.16), Cys 4(2.29), Val 4(4.41), Met 1(0.69), Ile 2(1.63), Leu 4(3.86), Tyr 2(0.87), Phe 1(1.12), His 2(1.99), Lys 1(0.99), Arg 1(0.99). 48hrs.
18	RP C18(A) 18min RP C8(A) 16min	RPC18(C) (2,10), (38,50).	Asp 3(2.98), Thr 1(0.93), Ser 5(3.62), Glu 2(2.35), Pro 3(3.36), Gly 2(1.99), Cys 4(1.98), Val 3(2.97), Met 1(0.78), Ile 2(1.39), Leu 3(2.94), Tyr 2(0.77), Phe 1(1.09), His 2(1.98), Lys 1(1.01), Arg 1(1.06). 48hrs.
O18	RP C18 17min RP C8 16min	RP C18 (60, 0), (110, 60). Repurified: RP C18 (2,10), (40,55).	Asp 3(2.92), Thr 1(0.95), Ser 5(3.78), Glu 2(2.33), Pro 3(3.21), Gly 2(1.99), Cys 4(1.90), Val 3(3.02), Met 1(0.86), Ile 2(1.19), Leu 3(2.89), Tyr 2(0.47), Phe 1(1.21), His 2(1.91), Lys 1(1.01), Arg 1(1.08). 48hrs.
7	RP C18(A) 17min	RPC18(C) (2,10), (10,30), (26,42).	Asp 2(1.89), Thr 1(0.94), Ser 2(1.73), Glu 1(1.12), Pro 3(3.34), Gly 2(2.00), Cys 1(0.31), Val 3(2.70), Ile 2 (1.26), Leu 2(2.00), Tyr 2(1.64), Phe 1(1.01), His 2(1.99), Arg 1(1.09). 20hrs.
6	RP C18(A) 16min	RPC18(C) (2,10), (10,25), (25,40).	Asp 1(0.94), Thr 1(0.98), Ser 2(1.79), Glu 1(1.17), Pro 3(3.84), Gly 2(2.10), Val 2(1.76), Ile 2(0.96), Leu 1(1.07), Tyr 1(1.05), His 1(1.03), Arg 1(0.97). 20hrs.
16	RP C18(A) 14min	RPC18(C) (2,10), (26,35).	Asp 1(0.99), Thr 1(0.92), Ser 2(1.63), Glu 1(1.06), Pro 3(2.71), Gly 2(2.04), Val 3(2.73), Ile 2(1.31), Leu 1(1.02), Tyr 1(0.98), His 1(1.01), Arg 1(0.97). 30hrs.
17	RP C18(A) 14min	RPC18(C) (2,10), (26,35).	Asp 1(1.09), Thr 1(0.94), Ser 2(1.78), Glu 1(1.00), Pro 3(2.98), Gly 2(2.21), Val 2(1.99), Ile 2(1.04), Leu 1(1.02), Tyr 1(0.90), His 1(1.05), Arg 1(0.96). 30hrs.
S17	RP C18(A) 14min	RPC18(C) (2,10), (30,40).	as pept. 17, not tested
3	RP C18(A) 14min	RPC18(C) (2,10), (28,42).	Asp 1(0.99), Thr 1(0.83), Ser 2(1.28), Glu 1(1.19), Pro 3(3.57), Gly 2(1.92), Val 3(2.81), Leu 2(1.96), Tyr 1(0.89), His 1(0.99), Arg 1(0.99). 20hrs.

Pept. No.	HPLC Analytical[a]	HPLC Preparative[b]	Amino Acid Analysis 6M HCl, 110°C
83	Hichrom C18 (B) 20min	RPC18(C) (2,10), (10,28), (25,40).	Asp 2(1.89), Thr 1(0.97), Ser 2(1.87), Glu 1(1.13), Pro 3(3.32), Gly 2(2.06), Val 3(3.21), Ile 2(1.63), Leu 2(1.89), Tyr 1(0.47), His 2(1.99), Arg 1(0.99). 48hrs.
84	Hichrom C18 (B) 16min	Vydac C18(D) (2,10), (10,30), (20,38).	Asp 2(1.91), Thr 1(0.98), Glu 1(1.12), Pro 2(2.12), Gly 2(2.07), Val 3(3.12), Ile 2(1.63), Leu 2(1.91), Tyr 1(0.54), His 2(1.99). 40hrs.
79	Hichrom C18 (B) 18min	RPC18(C) (2,10), (10,25), (21,33).	Asp 2(1.98), Thr 1(0.97), Glu 1(1.15), Pro 2(2.05), Gly 2(2.04), Cys 1(0.76), Val 2(1.97), Ile 2(1.59), Leu 2(1.97), Tyr 1(0.89), His 2(1.98). 24hrs.
80	Hichrom C18 16min	RPC18(C) (2,10), (10,25), (22,31).	Asp 2(2.02), Thr 1(0.98), Glu 1(1.21), Pro 2(2.10), Gly 2(2.08), Cys 1(0.65), Val 2(1.98), Ile 2(1.56), Leu 2(1.98), Tyr 1(0.87), His 2(1.98). 24hrs.
81	Hichrom C18 (B) 19min	RPC18(C) (2,10), (10,25), (20,33).	Asp 2(2.04), Thr 1(0.97), Glu 1(1.23), Pro 2(2.11), Gly 2(2.06), Val 2(2.02), Ile 2(1.48), Leu 2(1.98), Tyr 1(0.83), His 2(1.99). 24hrs.
82	Hichrom C18 (B) 20min	RP C18(C) (60,2), (72,30), (82,38).	as pept. 81, not tested
27	RP C18(A) (B) 18min	RPC18(C) (2,10), (10,28), (16,33).	Asp 1(0.99), Thr 1(0.97), Glu 1(1.10), Pro 2(1.98), Gly 2(2.07), Val 3(2.89), Ile 2(1.74), Leu 1(1.06), Tyr 1(1.04), His 1(0.97). 30hrs.
76	Hichrom C18 (B) 18min	RPC18(C) (2,10), (8,22), (24,31).	Asp 1(1.02), Thr 1(0.98), Glu 1(1.18), Pro 2(2.01), Gly 2(2.06), Cys 1(0.68), Val 2(1.98), Ile 2(1.54), Leu 1(1.02), Tyr 1(0.96), His 1(0.99). 24hrs.
75	Hichrom C18 (B) 18min	RPC18(C) (2,10), (8,22), (24,31).	Asp 1(0.98), Thr 1(0.97), Glu 1(1.13), Pro 2(2.01), Gly 2(2.05), Cys 1(0.76), Val 2(1.97), Ile 2(1.64), Leu 1(1.02), Tyr 1(0.97), His 1(0.98). 24hrs.
77	Hichrom C18 (B) 18min	RPC18(C) (2,10), (8,22), (28,33).	Asp 1(1.06), Thr 1(0.92), Glu 1(1.19), Pro 2(2.13), Gly 2(2.05), Val 2(1.98), Ile 2(1.47), Leu 1(1.02), Tyr 1(0.91), His 1(1.00). 24hrs.
78	Hichrom C18 (B) 16min	RPC18(C) (60,2), (68,28), (83,42).	as pept 77, not tested
9	RP C18 18min	RPC18(C) (2,10), (28,50).	Asp 1(1.01), Thr 1(1.02), Glu 1(1.00), Pro 2(1.67), Gly 1(1.12), Val 3(2.89), Ile 2(1.54), Leu 2(2.06), Tyr 1(0.97), His 1(1.09). 20hrs.
10	RP C18(A) 18min	RPC18 (2,10), (28,50).	Asp 1(1.03), Thr 1(0.95), Glu 1(0.74), Pro 2(1.68), Val 3(3.14), Ile 2(1.70), Leu 3(3.14), Tyr 1(0.99), His 1(1.01). 20hrs.
8	RP C18(A) 15min	RPC18(C) (2,10), (28,35).	Glu 1(1.09), Pro 1(0.96), Gly 1(1.07), Val 2(1.96), Leu 2(2.06), Tyr 1(0.86), His 1(1.06). 20hrs.

Notes: a. Analytical column and retention time;
b. Gradient in (t,B%)