UNIVERSITY OF EDINBURGH

College of Science and Engineering
School of Chemistry

Approaches to High Throughput Physical Organic Chemistry

By

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Doctor of Philosophy

December 2007

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ABSTRACT

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Over the past ten years, the development of High Throughput (HT) synthetic chemistry techniques has allowed the rapid preparation of libraries of hundreds to thousands of compounds. These tools are now extensively used for drug and material discovery programmes. The subsequent development of analytical capabilities to carry out qualitative and quantitative assessment of the compounds generated by HT synthesis as well as their HT screening has led to a dramatic broadening of the scope of HT techniques, ranging from image based analysis techniques to mass spectrometry (MS).

Based on the latter, a range of solid phase and solution phase analytical constructs was developed to enable the qualitative and quantitative assessment of mixtures of small compounds, using positive electrospray MS as the sole analytical tool. A version of the construct allowed HT reactivity profiling to be carried out on a range of ten carboxylic acids, ten aldehydes and ten isonitriles in the Ugi 4-component condensation reaction. The effect of various parameters such as the concentration of the monomers on the reactivity was investigated. The elaboration of a HT Hammett parameter assessment method was made possible by the development of an electrophilic version of the construct. The value of the Hammett ρ value was afforded by means of combinatorial Hammett plots and σ values were successfully evaluated in a HT mode for around thirty anilines with substituents in the *meta* and *para* position of the aromatic ring. Finally, analytical constructs were used in an attempt to evaluate enzyme reaction kinetics via the labelling of peptides and small drug fragment with coded constructs, to afford affinity determinations between the enzyme (protease) and peptidic or fragment based substrates.

DECLARATION

I, Christophe Portal, declare that the thesis entitled Approaches to High Throughput Physical Organic Chemistry and the work presented in it are my own.

I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

Date:_____

Signed:_____

PREFACE

The research work described in this thesis was carried out under the supervision of Prof. Mark Bradley at the University of Southampton (Jan. 2004 – Feb. 2005) and the University of Edinburgh (March 2005 – Dec. 2006).

Part of the work presented herein has been published as:

- "High Throughput Physical Organic Chemistry: Analytical constructs for monomer reactivity profiling" Portal, C.; Launay, D.; Merritt, A.; Bradley, M. *J. Comb. Chem.* **2005,** *7*, 554-560.

- "High Throughput Physical Organic Chemistry: Hammett parameters evaluation" Portal, C.; Bradley, M. *Anal. Chem.* **2006,** *78*, 4931-4937.

- "Approaches to High Throughput Physical Organic Chemistry" Portal, C.; Bradley, M. *Org. Biomol. Chem. (Emerging Areas)* **2007,** *5*, 587-592.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor, Professor Mark Bradley, for giving me this great opportunity of carrying out a PhD in his research group and believing in me throughout these three years. His concern, enthusiasm and encouragement were indispensable for the successful completion of this work. I also would like to thank my advisors Bruno Linclau and Mike Greaney for their help throughout this thesis.

I would like to thank all members of the Bradley group (both past and present) who made this time so special and enjoyable. Many thanks to Edgar the "German posh-doc" for all the laughter during Friday nights' pool games, and for accepting to proof read this thesis. Thanks to all the members of the CCE, Alessandra, Gianluca, Eric, Luciano and Delphine for their daily good humour and their dedication in the lab organisation. Special thanks to the members of the "cheminar" group for all these unforgettable organic chemistry evenings (and the group therapy as well).

Antonio and Luciano, it has been a great pleasure to share flat with you guys. I really appreciated the contribution of you two to make daily life so special (squash, good food, poker and limoncello, etc.). I would like to wish Delphine the "blondinette" all the best for the end of her PhD; thanks for your friendship and your help.

Finally, I would like to thank my parents and my sisters for their never-ending love and support throughout my study. Thanks for the incredible help you gave me, especially during hard times. I could not have written this thesis without you.

TABLE OF CONTENTS

ABBREVIATIONS

Amino acids

Arg:	arginine
Cys:	cysteine
Ile:	isoleucine
Leu:	leucine
Lys:	lysine
Pro:	proline
Ser:	serine
Thr:	threonine
Val:	valine

General

aro:	aromatic
ACC:	7-amino-4-caboxymethylcoumarin
AMC:	7-amino-4-methylcoumarin
APCI:	atmospheric pressure chemical ionisation
ATR:	attenuated total reflexion
b.p.:	boiling point
Boc:	*tert*-butyloxycarbonyl
Boc:	*tert*butoxycarbonyl
br:	broad
calcd	calculated
caspase	cysteine aspartic acid protease
cHx	cyclohexyl
CLND:	chemioluminescent Nitrogen Detector
COSY:	correlation spectroscopy
d:	doublet (NMR)
Da:	Dalton
DBU:	diaza(1,3)bicyclo[5.4.0]undecane
DCU:	dicyclohexylurea
Dde:	*N*-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl]
DEPT:	distortionless enhanced polarization transfer
DIC:	diisopropylcarbodiimide
DIPEA	*N*, *N*-diisopropylethylamine

DMA:	dimethylacrylamide
DMAP	4-dimethylaminopyridine
DMF:	*N*,*N*-dimethylformamide
DMSO:	dimethylsulfoxide
DNA:	deoxyribonucleic acid
DTT:	1,4-dithiothreitol
EDTA:	ethylenediaminetetraacetatic acid
ELSD:	evaporative light scattering detector
eq.:	equation
equiv.:	equivalent(s)
ESI:	electrospray ionisation
FAB:	fast atom bombardment
FAM:	6-carboxyfluorescein
Fmoc:	9-fluorenylmethoxycarbonyl
FRET:	fluorescence resonance energy transfer
FTIR:	Fourier transform infrared
HATU:	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HIV:	human immunodeficiency virus
HLPS:	high loading polystyrene
HOBt:	hydroxybenzotriazole
HPLC:	high performance liquid chromatography
HR:	high resolution
HT:	high throughput
HTS:	high throughput screening
Hz:	Hertz
IR:	infrared
***J*:**	scalar coupling constant (NMR)
LLPS:	low loading polystyrene
m:	multiplet (NMR) or medium (IR)
MAS	magic angle spinning
MCR:	multi component reaction
MeOH:	methanol
Mp:	melting point
NMR:	nuclear magnetic resonance
PEG:	poly (ethylene glycol)
Pfp:	pentafluorophenyl
PNA:	peptide nucleic acid
ppm:	part per million
PS:	polystyrene
PTFE:	polytetrafluoroethylene
q:	quartet
QMS:	quadrupole mass spectrometer
QSAR:	quantitative structure activity relationship
quint:	quintuplet (NMR)
s:	singlet (NMR) or strong (IR)
SAR:	structure activity relationship
SDS-PAGE:	sodium dodecylsulfate polyacrylamide gel electrophoresis

SPE:	solid phase extractor
SPPS:	solid phase peptide synthesis
t	triplet (NMR)
TAMRA:	tetramethyl-6-Carboxyrhodamine
TEA	triethylamine
TFA:	trifluoroacetic acid
TIC:	total ion count
TIS:	triisopropylsilane
TLC:	thin layer chromatography
TMS:	trimetylsilyl
t_R:	retention time
Trityl	triphenylmethyl
tris	trishydroxymethylaminomethane
Ugi-4CC:	Ugi 4-component condensation
UV:	ultra violet
UV-Vis:	ultra violet and visible
w:	weak
δ:	chemical shift (NMR)
ν:	IR frequency (cm^{-1})

Chapter One

Introduction

1.1. HT Chemistry and Physical Organic Chemistry

Since the early days of organic chemistry, scientists have carried out chemical transformations, carefully studying their outcome as a function of many experimental parameters so as to better understand what might enhance a reaction, both in terms of the quality of the product obtained and the time required to get that given product. Firstly, observations would focus on both the characteristics of the reagents involved in the transformation (functionalities, substitution, solubility, etc.) as well as the experimental settings (time, temperature, pressure, etc.). This preliminary approach would allow qualitative guidelines for chemical reactions to be determined, but they became rapidly insufficient since they lacked precision. The need thus arose for these qualitative observations and empirical rules to be applicable on a more rigourous footing, allowing physical and mathematical tools to be employed, to build up relationships between experimental conditions and the result of an experiment. Physical organic chemistry is the discipline that is concerned with the study of various rules that are in use in organic chemistry, and that allow prediction and/or explanation of the outcome of a reaction (rate, preferential formation of a product, etc.) using parameters related to the reagents (substituents, aromaticity, etc.) or the reaction conditions employed (pressure, temperature, etc.).

Since the first detailed studies carried out on simple substitution chemistries in the mid 1930s,[1] to the establishment of many well acknowledged free energy relationships that are now commonly used in organic synthesis,[2-5] physical organic chemistry has come a long way. It has provided a platform from which fundamental rules of chemistry have developed, supplying explanations concerning molecular reactivity and allowed detailed mechanistic understanding to be gained. Many examples of free energy relationships can be quoted that illustrate this, among which the famous Hammett equation and its extended forms (the Taft equation for example) stand out.[2-4] These relationships allow the composition of an equilibrium or the rate

of a chemical transformation to be predicted on the basis of parameters depending on the structure of the reagents such as the electronic contributions of substituents. The Bronsted equation, giving a correlation between acid strength (K_a) and catalytic activity is also noteworthy here.[5] The analysis and understanding of isotope effects, which has established the variation in the reaction rate of a chemical transformation when an atom in one of the reactants is replaced by one of its isotopes, also provides a range of subtle tools to probe reaction mechanisms.[6] It has been shown that the magnitude of the isotope effect is much greater in the case where the isotope is involved in a bond that is formed or broken in the reaction (primary isotope effect). The development of all these relationships has resulted in physical organic chemistry having a significant impact on the way in which synthetic and mechanistic organic chemistry has evolved.

However, physical organic chemistry has often been tarnished (unfairly) with a "reputation" of tedium and repetition, with the vision of days if not weeks spent hunched in front of a high performance liquid chromatography (HPLC) system, analysing single reactions. As they essentially arise from experimental data, the generation of physical organic chemistry relationships indeed requires extensive amounts of data to be analysed, which necessitates not only performing the reactions of interest but also carefully analysing the evolution of the composition of the reaction mixtures, to identify possible intermediates, by-products and products and determine their rates of formation and disappearance, in order to work out sensible correlations. For instance the elaboration of the Hammett's free energy relationship, based on experimental results, necessitated a tremendous amount of data based on the composition of the ionisation equilibrium of benzoic acids, which had been compiled by Dippy over several years.[7] This can be contrasted with high throughput (HT) organic chemistry which has developed at a remarkable rate over the past decade, allowing chemists to rapidly and efficiently generate libraries of hundreds to thousands of compounds.[8] Combinatorial techniques such as split and mix strategies or parallel synthesis along with automation have indeed greatly helped the efficient preparation of compounds with enhanced diversity and in reduced time frames. The subtle boon provided by microwave heating also deserves to be noted here, since it allows not only unprecedented and highly accurate controlled heating of reactions,

but also (and importantly from a physical organic chemistry view-point) reproducible and known reaction times, temperatures and pressures in a manner normally impossible with traditional heating methods in organic synthesis.[9] This tool will be a great asset in the perspective of increasing the speed of physical organic chemistry studies. In addition to its contribution to the fast preparation of new chemical entities, combinatorial chemistry had triggered the development of analytical tools in order to meet the challenge of analysing as rapidly as possible large numbers of compounds, the increase in throughput of analytical capability being required not only to afford characterisation and assess purity of the final compounds but also to monitor the progress of large numbers of reactions. These improvements (see section 1.3.) could serve physical organic chemistry by allowing a dramatic speed up of the assessment of the effect of structural changes on a given compound on the kinetics or on the equilibrium of a reaction. The following section presents the progress in some analytical techniques associated with the assessment of reactions carried out in a HT manner, firstly by the adaptation of existing techniques to HT, such as HPLC, mass spectrometry (MS) and infrared (IR) based techniques and secondly by the development of new specific HT tools such as MS labels and analytical constructs.

1.2. Introduction to high throughput synthesis

1.2.1. Solid phase chemistry and library preparation

Since the genesis of modern organic chemistry, classical organic reactions have been carried out in solution phase, implying the separation of the desired product from reagents and by-products after the reaction. This purification step can however turn out to be incredibly time consuming, and this is why the concept of solid phase chemistry was introduced by Merrifield.[10] By the attachment of one of the reagents to a solid support, the purification step becomes limited to a filtration and washing of the support. This methodology, which has changed radically the synthesis of peptides,[11] however remained predominantly limited to this field until the introduction of combinatorial techniques (so called as the process was initially based on the combination of pools of reagents). Two ways of combining these pools of

reagents can be used to achieve chemical diversity. "Split-and-mix" synthesis (**figure 1.1 (a)**), first developed by Furka, Lam and Houghten has now been used extensively in the pharmaceutical industry for the generation of thousands of compounds.[12-14] At each step, all beads are split equally into the number of reactions carried out (3 in the example). After reaction and washing, the beads are mixed together and then separated again into the next number of vessels required (3 again). The process continues until the last step with each bead containing a different compound (one bead / one compound). The advantage of this technique is the ability to generate exponentially growing numbers of compounds (3^n after n steps) while keeping the number of reactions quite low ($3 \times n$ in the case of n steps). With split and mix, however, all compounds end up in the same mixture.

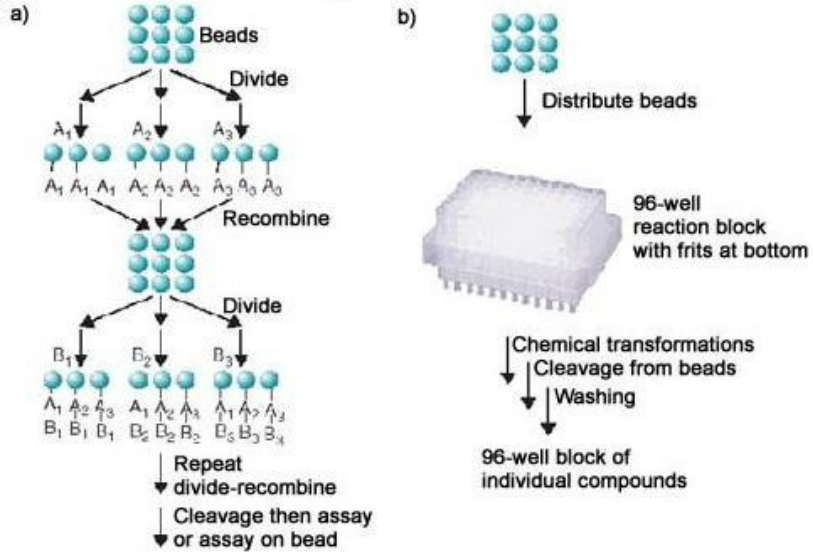


Figure 1.1: Differences between (a) split and mix and (b) parallel synthesis (reproduced with permission)[15]

The second approach is "parallel synthesis", where discrete compounds end up distributed in 96 or 384-well plates (**figure 1.1 (b)**). The technique is based on the distribution of diverse reagents having the same reactive functionality (*i.e.* carboxylic acids, amines, etc.) at each step of the synthesis. At the end of the sequence, each well will have followed a specific order, different from all other wells. Final compounds can therefore be identified thanks to the known sequence they followed.

1.2.2. Analysis of products arising from parallel synthesis

With the development of parallel synthetic capability, the need for the development of HT analytical tools has become more and more pressing. Two issues indeed need to be tackled by analytical chemists: (i) the ability to characterise the huge number of final compounds generated by library synthesis and (ii) the possibility to monitor reactions *in situ*. Quality control of final libraries is indeed required to ensure that the synthetic steps were successful and that the compounds targeted are sufficiently pure to be taken to HT screening (HTS). As all analyses are carried out after the final cleavage from the solid support, the use of classical analytical tools, such as HPLC, nuclear magnetic resonance (NMR), etc. is possible. Several important advances have thus been realised to increase the throughput of existing analytical techniques and analyse libraries of compounds while reducing the time frame as much as possible. These advances have concerned all areas of analytical chemistry and almost every spectroscopic method has undergone modifications to try to meet the requirements imposed by HT synthesis in terms of throughput. For example, most HPLC and NMR systems are now fitted with autosamplers, allowing the routine analysis of compounds with increased speed. Ultraviolet-visible (UV-Vis) spectroscopic analyses can now be carried out directly on 96-well plates thanks to the use of microplate readers. However, the throughput of these techniques is still limited and therefore they are typically used for the analysis of final compounds. Further improvement of the analytical capability is thus necessary to achieve HT reaction assessment.

1.3. High throughput reaction assessment

Systematic analysis of reaction mixtures to check the advancement of reactions and identify the intermediates would certainly be of great help to supply feedback regarding the quality of the intermediates and ascertain that the reaction conditions are well suited for all substrates undergoing the transformation. In addition, these reaction assessment data would not only be useful for synthetic chemists for the qualitative and quantitative assessment of the composition of their reaction mixtures, but also for physical organic chemists since this analysis would supply data regarding reaction kinetics, the existence of intermediates and their lifetimes, and the structural properties of the final compounds, for instance the presence of several isomers, unexpected by-products, etc. Furthermore, the existence of a HT reaction assessment technique could allow HT enzyme kinetics to be carried out and open a raft of application regarding substrate specificity profiling of inhibition assays.
The development of analytical tools to assess the composition of reaction mixtures in a HT manner in a qualitative as well as a quantitative manner was first realised using existing techniques.[16] In this section, the most efficient methods that enable the rapid evaluation of the composition of reaction mixtures are summarised.

1.3.1. Adaptation of GC and HPLC to HT

Because of their proven accuracy and reliability, gas chromatography (GC) and HPLC were amongst the first tools to be adapted to HT. Apparatus became more and more accessible, with the integration of automation to allow samples to be injected automatically so that the instruments could be used 24 h a day. User friendly software contributed to turn GC and HPLC into efficient routine reaction mixture analysis tools.

1.3.1.1. GC as a HT reaction assessment tool

Parallel automated GC has been used in many examples for the analysis and the screening of libraries, such as for the development of new efficient chiral ligands for copper-catalyzed enantioselective Michael additions.[17] However, this screening

method was not truly HT, as each sample required at least 15 minutes to analyse. A significant improvement made to GC methods in terms of rapidity was introduced by Reetz.[18] Two GC instruments, equipped with chiral columns, were connected to a sample manager and a computer. Using this unit, the yield and enantiomeric excess of a mixture could be determined in about 2 minutes provided that the separation parameters (solvent, temperature, etc) were optimized for each new chiral compound.

1.3.1.2. HPLC as a HT reaction assessment tool

Similarly but with more success because of their applicability to a broader range of compounds, HPLC based techniques were developed to serve as reaction assessment means. Chiral HPLC analysis has been used to determine enantiomeric excesses (*ee*'s) in racemic mixtures in order to allow the screening of libraries of catalysts. However, HPLC methods have failed to be applicable as a general HTS tool since they are too time consuming and lack the possibility to be generalised as gradients have to be adapted to each kind of substrates to obtain suitable separation of enantiomers.

1.3.2. Spectroscopic methods for HT reaction assessment

1.3.2.1. UV-Vis spectroscopy

The concept of reaction assessment based on the variation of the UV-Vis absorption of the reaction mixture is probably one of the oldest qualitative reaction assessment techniques in synthetic chemistry. Since the early days of organic chemistry, chemists have indeed been able to qualitatively evaluate reaction conversions thanks to colour changes that often occur with the formation of the product.[19] Thin layer chromatography (TLC) has enabled qualitative reaction assessments to be rapidly carried out on reaction mixtures, based on the UV absorption properties of the products (or the coloured spots generated by the use of one of the multiple available TLC stains).[20] Regarding solid phase chemistry, colorimetric tests such as the ninhydrin Kaiser test are routinely used to monitor the conversion of primary amines on solid supports.[21] Cho *et al.* developed a self indicating resin that enabled the monitoring of reactions at the level of a single bead.[22] Substitution of around 5 % of

the reactive sites of an amino functionalised resin with a bromophenol blue derivative allowed the rapid determination of the presence of free amines on the resin, the beads turning from dark blue to yellow as the reaction progresses as a result of intrabead interactions.

With the development of UV-Vis spectrophotometers, organic chemists are now able to monitor reactions thanks to absorption at a given wavelength. UV-Vis reaction assessment is mostly based on the product of the reaction itself displaying spectral properties that the reagents did not have (*i.e.* absorption at a specific wavelength). Multiple enzymatic assays are based on this concept: an extensive number of so-called "chromogenic substrates" have indeed been developed for the fast and efficient assessment of enzymatic reactions. A representative example is the use of *para*-nitrophenol esters to monitor the activity of esterases.[23] The free phenolate displays a strong absorbance at 410 nm, allowing enzyme activity to be obtained directly from the study of the absorbance of the reaction mixture at this specified wavelength. The fact that UV-Vis methods are non destructive and instantaneous, makes them particularly suitable for HT applications: evaluation of reaction conversions can be carried out in real time by analysing of the reaction mixture. The increasing accuracy of detectors and the reduced amounts of material required to carry out UV-Vis spectroscopy have allowed analyses to be run directly on the reactions vessels used for HT synthesis. The now widespread use of 96-well plates and the development of microplate readers have enabled the fast assessment of the UV-Vis spectroscopic properties of multiple reaction mixtures for direct HT reaction assessment. The use of coloured or UV active species in a way that the spectroscopic properties of reactants do not interfere with those of the product has seen widespread application. Among the most recent examples is the HTS of a rare earth catalyst library for the selective catalytic reduction of NO_x.[24] This process allowed selective NO_x reduction using hydrocarbons in an oxidizing atmosphere, as it is the case in exhaust gas environment. 80 different combinations of the three elements Co, Ce and In in different mass ratios from 0.5 to 5 % could be assessed using a 24-channel microreactor coupled to a scanning UV absorption array system. According to the variation in UV light intensity, from the known UV absorption of NO, it was possible to determine the relative quantities of the reactants or the products before,

during, and after the catalytic reaction, and therefore evaluate the efficiency of the screened catalysts.[24]

In a slightly different approach, the formation of the product could be monitored by indirect means, using for example an auxiliary whose colour change indicated progression of a reaction. Based on this idea, Morken built a HTS method to assess the efficiency of a set of catalysts in allylic alkylation reactions.[25] 1-Naphtol, one of the products of the reaction, underwent rapid electrophilic aromatic substitution with Fast Red diazonium salt. The evaluation of the efficiency of catalysts was based on several metal-ligand combinations, made possible by the detection of the red colour in the reaction mixture. While simple visual analysis of the 96-well plate was sufficient to differentiate efficient from inactive catalysts, parallel UV analysis was used to differentiate catalysts of similar activity and provided a means to subtract background absorption due to ligand or metal salts. This operation enabled an accurate evaluation of the amount of product generated by the reaction and thus the efficiency of the catalyst, allowing the discovery of the first non-phosphane iridium catalysts for allylic alkylation.

Recently, the concept of quantitative TLC has been introduced as a HT reaction assessment technique, to evaluate the potency of numerous catalysts in the Sonogashira reaction.[26] The method was based on the appearance of a yellow coloured compound, whose presence can easily be detected on a TLC plate (**figure 1.2**). A preliminary study allowed the elaboration of a calibration curve that correlated the intensity of the coloured spots obtained after elution on the plate of the expected product at different concentrations. The use of image analysis gave a correlation between the spot intensities and the amount of product on the plate. Assays were carried out in a 96-well plate format, each well containing a different catalyst candidate. Each resulting mixture was spotted in equal amounts on a TLC plate and eluted. Correlation between the volume of the spots obtained by image analysis of the region of interest on the TLC plate and the yield of the reaction gave an assessment of the catalytic power of the catalyst under investigation.

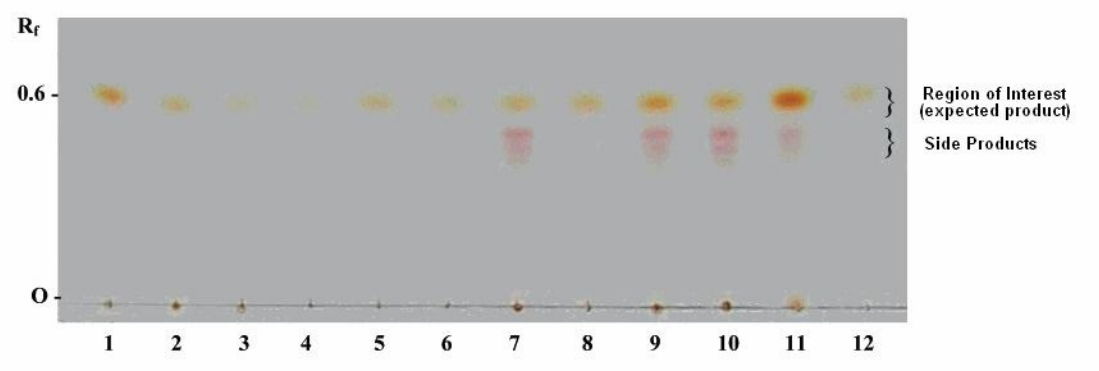


Figure 1.2: Image analysis based quantitative TLC analysis (reproduced with permission)[26]

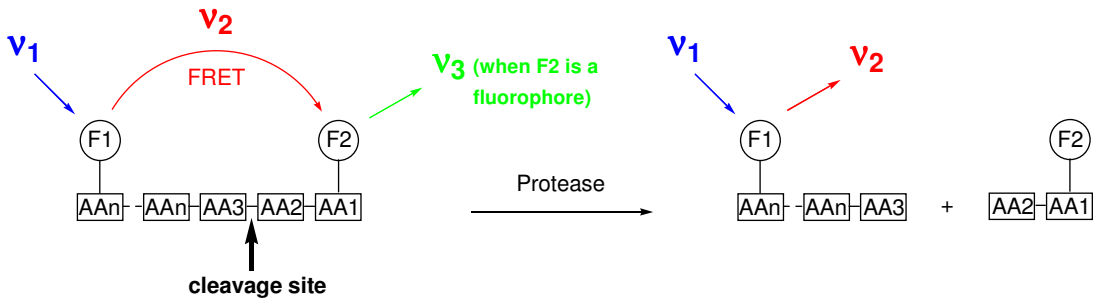
This technique not only allowed several catalytic activities to be evaluated rapidly in a very reliable manner, but also the rapid detection of any unexpected by-product, therefore supplying precious information regarding the mechanism of the reaction, the stability of the intermediates, etc. Quantitative TLC is a very efficient means of qualitatively and quantitatively analysing reaction mixtures in a HT manner. The approach permitted an investigation of all combinations between 11 metal salts and 7 different ligands, and revealed the efficiency of a new Ruthenium based catalyst in the Sonogashira reaction: $RuCl_2$(1-Me,4-*i*PrC_6H_4)(PPh_2). The technique was versatile since it was applicable to any reaction generating a coloured compound. Furthermore, quantitative TLCs succeeded in the HT reaction assessment where the use of a microplate reader failed, because of the presence of several by-products with a strong red colour from the other constituents of the mixture.

1.3.2.2. Fluorescence spectroscopy

A different way of looking at the spectroscopic properties of reaction mixtures to carry out HT reaction assessment is fluorescence spectroscopy. Some structures have the ability when excited at a given wavelength to emit light at a wavelength remote to the excitation one.[27] Instead of looking at the amount of light absorbed by the compound, attention can be focused on the amount of light re-emitted, *i.e.* its fluorescence. With the emergence of spectrofluorometry has appeared the concept of fluorogenic substrates. Similarly to chromogenic substrates, these compounds exhibit fluorescence only after a given transformation has occurred, allowing quantitative monitoring of the transformation. As for absorbance measurements, this

spectroscopic technique has been adapted to microplate formats and it has been used as a HT reaction assessment technique. A huge number of fluorogenic substrates have been reported to date, and amongst the best known are amino-coumarin derivatives. For example, 7-aminocoumarins are fluorescent species whose emission is quenched when the amino functionality is substituted, as is the case when involved in an amide bond for example. The hydrolysis of the amide bond is therefore accompanied by an important increase of fluorescence, allowing the hydrolysis kinetics to be monitored. Based on this observation, 7-aminocoumarin derivatives have been extensively used as means of protease kinetics assessment by linking the amine to the C terminus of a peptide (see section 4.1.2.4).[28,29] For example, the use of positional scanning libraries based on the absolute substrate specificity of caspases for the aspartic acid residue allowed the complete substrate specificity (for one side of the cleavage site) of all 15 known caspases to be rapidly determined using peptide coumarin fluorogenic substrates.[30]

One alternative to overcome the limitations imposed by the use of fluorogenic aminocoumarins is to tag the peptidic sequence of interest or any species whose cleavage is being looked into with two fluorophores that possess the property to form a fluorescence resonance energy transfer (FRET) system (or a quenched system). FRET relies on the combination of two fluorophores such that the emission wavelength of the first one (fluorescence donor F1) overlaps the excitation wavelength of the second one (fluorescence acceptor, F2) provoking an emission of light at the wavelength of the acceptor (**scheme 1.1**) and a dramatic decrease of the detectable fluorescence of the donor (F2 can also be a quencher, *i.e.* a species absorbing light at the emission wavelength of the fluorophore donor F1 but without re-emission).



Scheme 1.1: Principle of FRET based protease assays

Any cleavage along the peptidic sequence induces the separation of the donor and the acceptor, resulting in the generation of the fluorescence of the donor as well as the disappearance of the emission of the acceptor. FRET peptides have been used a lot for the specificity profiling of proteases using a microplate approach. Similarly, FRET peptides have been used along with split and mix strategies in numerous HT proteases assays, as described in section 4.2.2.3.

The use of fluorescence as a HT reaction assessment technique is not limited to enzymatic kinetics. A HTS assay for atom transfer catalysis was developed by Sames using a species that generated a highly fluorescent product upon oxygen atom transfer.[31] The emission wavelength indeed shifted significantly (up to 90 nm) upon epoxidation, allowing detection of product at approximately 3 % conversion. Similarly, a carbon atom transfer species was elaborated, which allowed detection at less than 1 % conversion. Such sensitivity permitted the examination of single-bead reactions in a HT array format (1536 wells per plate), and provided a broad detection window ranging from single to high turnover numbers. Thousands of metal complexes were evaluated in a single screening experiment.

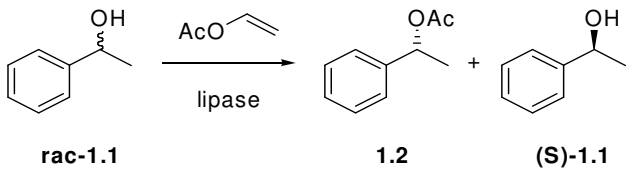
As for UV-Vis techniques, the fluorometric based detection could be carried out indirectly, using fluorescent probes. In the late 1990s, Miller described a fluorometric method for screening many catalysts.[32] The principle of the method was based on the use of a molecular sensor which fluoresced upon detection of the desired reaction product. The big asset of the approach was that it could be applied both in a spatially addressed solution-phase assay as well as in a single-bead-single-catalyst assay.[32]

In spite of the numerous applications of UV-Vis and fluorescence based methods for HT reaction assessment, some limitations reduce the scope of these techniques. To be able to record measurements of the UV-Vis absorption or emission of a reaction mixture in an efficient manner, interference with the signal must be kept as low as possible. The reliability of these techniques therefore relies on the use of specific materials that do not absorb or emit at any of the wavelengths being used for the monitoring. Similarly, constituents of the reaction mixtures other than those directly

concerned by the method must be inert regarding the irradiating and/or the emitted signals. Additionally, many chromophores and fluorophores suffer from being quite intolerant to certain reaction conditions, preventing them from being used (for example the intolerance of some cyanines for low pH values). Photobleaching can also be an issue since it limits long term analysis. These drawbacks have driven some people into developing other techniques that could be used with a broader range of substrates and conditions.

1.3.2.3. IR thermography and arrays of thermistors

One method of monitoring reactions that has received attention is the use of thermal measurements. As the change in temperature of a transformation with time reflects both its thermodynamics and kinetics, the HT measurement of the temperature of a reaction mixture is a valuable source of information regarding a reaction's profile. Temperature can be monitored in a number of ways, most of which are not compatible with HT techniques, such as the use of calorimeters. However, some methods have been successfully developed and/or adapted to achieve HT monitoring of reaction mixtures. The most common way for HT measurement of temperature is IR thermography which consists of an IR sensitive camera capable of assessing the temperature of a reaction mixture at a given time allowing very precise evaluation of a reaction mixture's temperature profile (the sensitivity of these cameras can reach a precision of 0.02 °C). A representative example of the use of IR thermography is the method developed by Reetz for looking at enantioselective reactions involving biocatalysts or chiral transition metal catalysts.[33] The work was based on the use of a modified microtitre plate where reactions were carried out and temperature changes in the reaction wells measured periodically. The acetylation of 1-phenylethanol **1.1** (**Figure 1.3**) was one of the transformations studied with this technique with the catalytic efficiency of several lipases investigated in a HT manner.



rac-1.1 **1.2** **(S)-1.1**

Scheme 1.2: Enzymatic resolution of rac-1.1

For each enzyme, enantioselectivity was screened by performing the reaction in three separate wells, respectively containing **rac-1.1**, **(S)-1.1** and **(R)-1.1** (**scheme 1.2**). The comparison of the temperature changes in the three wells, allowed conclusions to be drawn regarding the enantioselectivity of the enzyme.

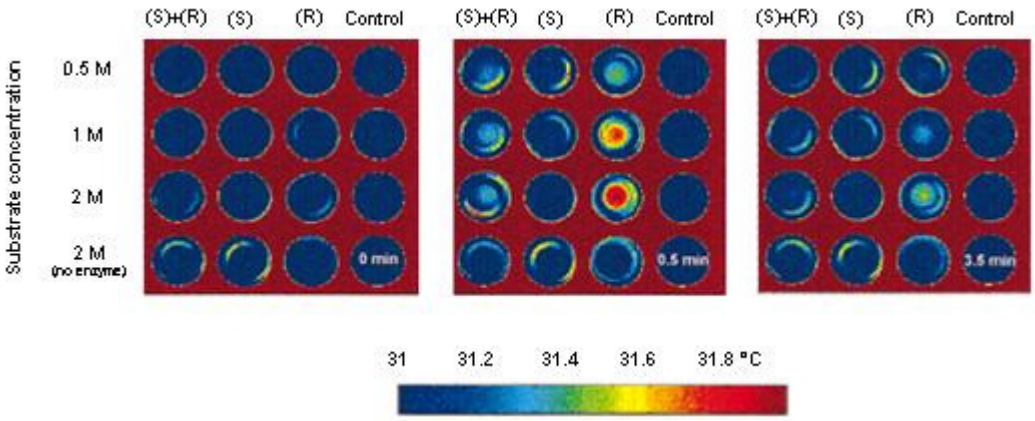


Figure 1.3: IR thermography for the HT assessment of the reaction on scheme 1.2 (reproduced with permission)[33]

IR thermography allowed qualitative stereoselectivity data to be obtained simultaneously for a large number of different lipases. The IR thermography based methodology was subsequently applied to enantioselective transition metal catalysis: the potency of cobalt and chromium based species was assayed in the ring opening hydrolysis of epoxides, demonstrating the versatility of the approach.[33] The fact that the technique is applicable to a wide range of substrate represents one of its strongest assets. Fluorescence based techniques indeed require the incorporation of one or several fluorophores or pro-fluorophores to the substrate which not only require preparation but also can influence the behaviour of the investigated material. On the contrary, IR thermography affords reaction assessment on the actual substrate of the reaction. Based on an analogous technique, on bead IR thermographic imaging was used in the late 1990s by Morken.[34] This work targeted the discovery of new polymer bound catalysts. The principle of the approach resided in the increase of temperature provoked at the surface of a resin bead holding an active catalyst. An acyl transfer reaction from acetic anhydride to ethanol was investigated: after mixing the reagents

and the solid supported catalysts together, the floating hot beads were picked and decoded (**figure 1.4**).

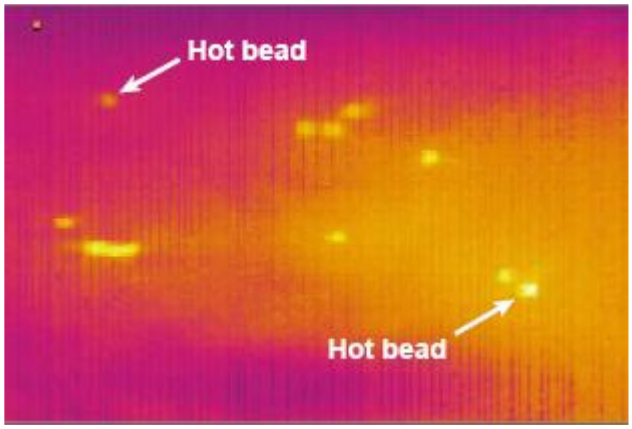


Figure 1.4: IR thermographic image of 14 visible hot beads in the presence of several thousand noncatalyst beads (reproduced with permission)[34]

Among 3150 screened solid supported catalysts, the 23 most active beads were selected and the activity of the resin bound catalysts was investigated individually. The results obtained showed that, to a rough approximation, the HT assay was representative of the catalytic efficiency, making the technique promising in terms of new catalyst discovery. It is indeed quite easy to think about generalisation of such a method to any reaction requiring the use of catalysts since most of these are exothermic. Further development of the technique might allow catalytic reaction kinetics to be followed directly on bead.

However, some important issues such as rates of energy transfer and weak signal to noise have been pointed out recently with IR thermography that seriously limit the method. Furthermore, reaction mixtures need to be IR transparent for imaging to be made. For instance, the aforementioned on bead IR thermographic screening had to be carried out in chloroform for the resin beads to float at the surface of the solvent to make it possible the obtaining of good quality IR images. To overcome these limitations, Sutherland developed the concept of an array of thermistors as an alternative to IR thermography for catalytic activity screening.[35] The method relied on the use of thermistors to allow the monitoring of temperature changes of chemical and biochemical reactions. The apparatus was based on the use of a 96-well plate, with an array of thermistors immersed into the centre of all wells to measure

temperature variations in each well. Thermistor arrays offer an attractive alternative to IR thermography since they are more sensitive to temperature changes and they allow measurements to be carried out in non IR transparent materials. Furthermore, as the apparatus conceived by Sutherland *et al.* is fully automated, the replacement of the thermistors by similar arrays of probes could allow reaction assessment through the measurement of pressure, pH, etc.

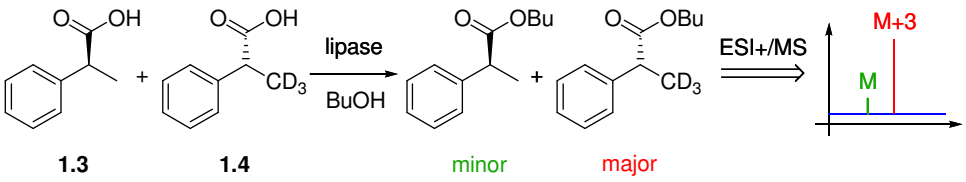
1.3.3. MS for HT reaction assessment

MS seems to be have major limitations as an analytical technique for HT reaction assessment since it does not *a priori* give quantitative data nor does it seem to be applicable to the determination of *ee'*s (since it cannot provide any chiral information). However, MS has proven to be very successful thanks to the application of a variety of different techniques (see below). MS indeed offers unrivalled speed of analysis and great sensitivity and has seen many applications in areas ranging from the analysis of peptide and proteins (for example proteomics and serum profiling) to libraries of small organic compounds.[36] Qualitative reaction assessment is indeed straightforward since the disappearance or the appearance of compounds can be monitored to check the conversion of a chemical transformation provided the compounds have good MS ionisation properties. However, the technique has obvious limitations for the analysis of compounds that have the same molecular weight, such as enantiomers or diastereomers, compounds that have poor ionisation properties, or mixtures of compounds where one of the constituents strongly dominates the ionisation of all the others. Different attempts to overcome these limitations have been reported by the introduction of concepts such as pseudo-enantiomers (or pseudo-diastereomers) or analytical constructs.

1.3.3.1. Pseudo-enantiomers and pseudo-diastereomers

The impossibility to directly determine *ee'*s by MS analysis of a mixture has led to the introduction of the concept of pseudo-enantiomers which solves this dilemma and allows MS methods to be used for HT determination of *ee'*s. The approach involves "isotopic tagging" whereby one of the enantiomers of a compound is synthesised or

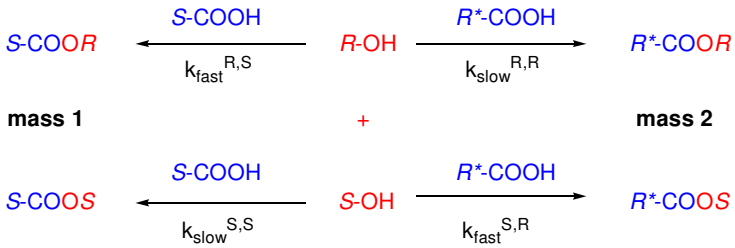
chemically modified to allow the incorporation of an isotopic label (for example a CD_3 replacing a CH_3 group) such that the mass of the molecule is related to the absolute configuration of the stereocentre, allowing mass differentiation in the mass spectrum. Modification is logically carried out as far as possible from the region of the molecule where the transformation of interest is supposed to take place, and ideally should not induce any change in the physicochemical properties of the compound, such as its solubility or hydrophobicity, etc. This "tagged" enantionomer is mixed in a 1:1 manner with the unlabelled enantiomer. The two compounds are said to be "pseudo-enantiomers", since one of the two isomers is "heavier" than the other thanks to the presence of the CD_3 group. The stereoselectivity of any chemical transformation can thus be assessed by MS, provided once again that it is not affected by the modified group. Pseudo-diastereomers can be considered in the same way.[37] Using this approach, Reetz developed a HT screening method to examine the enantioselectivity of a number of catalysts.[38] Thanks to the replacement of the terminal methyl group on the R enantiomer of 2-phenylpropionic acid **1.3** by a deuterated equivalent, the pseudo enantiomer **1.4** was generated, allowing an investigation of a lipase catalysed stereoselective esterification, as shown in **scheme 1.3**.



Scheme 1.3: Use of a pseudo-racemate for HT stereoselectivity evaluation

As a proof of concept, Reetz demonstrated that the results obtained *via* the use of pseudo-enantiomers compared very well to those obtained with classical *ee* determination techniques, proving that the labelling of one of the enantiomers did not induce changes in reaction selectivity. The pseudo enantiomer MS method allowed the evaluation of 1000 *ee*'s a day, generating valuable data regarding the activity and the selectivity of the lipases.

Guo developed a related approach to allow the HT *ee* determination of alcohols (also applicable to amines).[39] The alcohols under investigation, *R*-OH and *S*-OH were coupled to a pseudo-racemate of a carboxylic acid. This pseudo-racemate was a mixture of two N-acyl prolines, with the S enantiomer acylated by a benzoyl group (*S*-COOH) and the R enatiomer with a *para*-methyl benzoyl group (*R**-COOH). Among the four different products that could arise from the different combinations of carboxylic acids and alcohols, two were preferentially formed since each one of the pseudo-enantiomers reacted preferentially with a given enantiomer of the alcohol (**scheme 1.4**).



Scheme 1.4: Use of a pseudo-racemate for HT *ee*'s evaluation

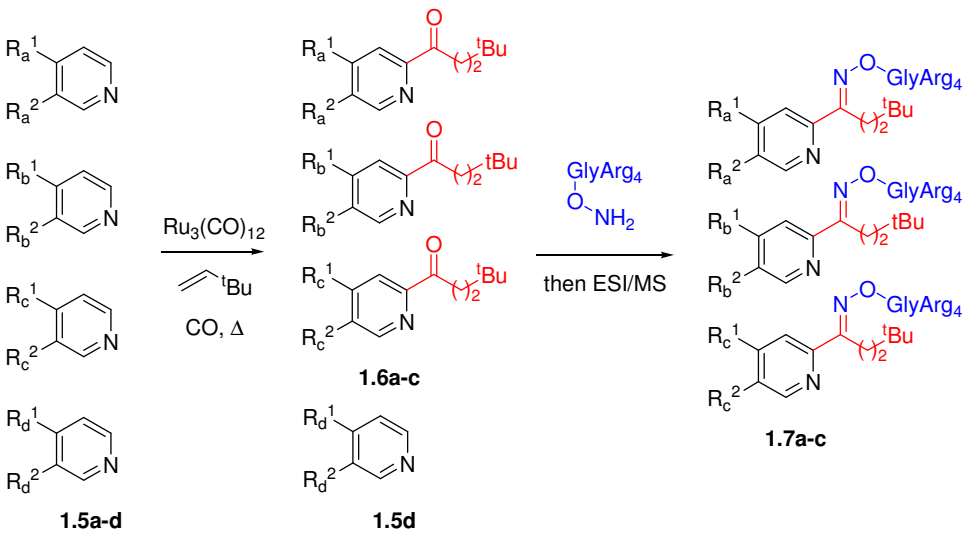
Electrospray MS (ESI/MS) analysis of the final mixture therefore directly gave the ratio between the two products, data that could be used to determine the *ee* of the starting substrate.[37]

With this approach the issue of analysing enantiomers via MS was solved. Compounds analysed by this technique however still needed to be easily detectable by MS *i.e.* have good ionisation properties. This issue represents another important limitation to the development of HT MS based reaction assessment techniques. Several MS tagging strategies have therefore been developed to ensure ionisation of the compounds injected into the MS apparatus.

1.3.3.2.MS "Tagging"

To allow the most to be made of MS techniques, and apply them to as broad a range of chemistries and compounds as possible, especially those with poor ionisation abilities, a variety of MS tagging strategies have been successfully applied. One representative example of this is the work reported by Szewczyk and the development of a solution phase MS labelling method for HT reaction evaluation and

optimisation.[40] The method (**scheme 1.5**) consists of a one pot acylation of a library of pyridine based substrates **1.5a-d** with 3,3-dimethylbut-1-ene and carbon monoxide, in the presence of $[Ru_3(CO)_{12}]$. The "tag", composed of four arginine residues and an *N*-terminal alkoxyamine (H_2N-O-$GlyArg_4$) was used to selectively label the products of the reaction mixture (**1.6a-c**) by oxime formation with any ketone functionality generated in the reaction. The "tag" not only guaranteed the ionisation of the products **1.7a-c** for MS detection, but also dominated it, allowing quantitative conclusions to be drawn from the integration of the MS peak areas, by comparison to an internal reference (2-pyridinecarboxaldehyde labelled with the (H_2N-O-$GlyArg_4$) tag). Furthermore, thanks to the tag, only peaks corresponding to the products of the reaction would be identified whereas unreacted reagents etc, would not be detected.



Scheme 1.5: MS tagging approach for HT reaction evaluation and optimisation

This approach allowed rapid evaluation of around 30 substrates to define structure-reactivity relationships and reaction compatibility of functional groups. One big asset of this method is that it is virtually applicable to any reaction that generated a carbonyl group to allow attachment of the tag.

1.3.4. The concept of analytical constructs

In the area of solid phase synthesis, the concept of analytical constructs has been added to the list of tools for HT reaction assessment. Analytical constructs indeed consist of applying the concept of MS tagging to solid supported compounds: these solid phase dual linkers necessarily incorporate a strongly ionisable moiety that enhances the ionisation properties of compounds so that they can be easily detected by MS. They can also contain features whose aim is to improve the efficiency of the analytic step, such as mass splitters to allow easy detection of the compounds from the background noise, UV chromophores to allow quantitative conclusions to be drawn, etc. Analytical constructs have been used mainly to identify products and monitor solid phase reactions,[41] but they have also proven useful in other applications, such as functional group compatibility studies,[42] and linker development.[43-45]

1.3.4.1. Solid phase analytical constructs: genesis of the concept

Merrifield was the first to introduce the concept of dual solid phase linkers for solid phase chemistry in the late 1970s.[46] He prepared a multi-detachable resin support, thanks to the presence of a solid phase linker with two differentiable cleavage points. The first cleavage point triggered the release of the desired peptides, either fully protected or unprotected (depending on the use of acidic or nucleophilic species). The second cleavage point permitted *via* a photocleavage the liberation of the peptide still attached to the linker, allowing its reattachment to another solid support for further elongation. A few years after, Geysen introduced the concept of "analytical constructs" by making use of a double linker in the development of several MS based encoding strategies for the rapid identification of products arising from a three step solid phase synthesis process (**figure 1.5**).[47] The initial aim of the code, based on the use of different ratios of isotopically differentiated amino acids was the determination of the first monomer (monomer A). The third monomer was determined by subtracting the sum of the masses of the known first monomer and that of the third monomer (defined by the final pool from which the particular bead was taken) from the total mass. The idea behind this coding strategy was that unlike

peptide or oligonucleotide based coding strategies used until then,[48, 49] the MS label method did not require elements to be added to the code at each step of the synthesis: it could be read instantly and did not require any sequencing, saving up time in the decoding process.

In spite of the arguable efficiency of this strategy, the key feature of this approach was that MS analyses were greatly enhanced by the presence of the MS label. The code, which was only supposed to give information about the nature of monomer A, happened to act as an analytical enhancer for all MS studies, carried out on the species liberated by cleavage of linker 1. From then on, cleavage of linker 2 was exclusive to the release of the product after the analytical step.

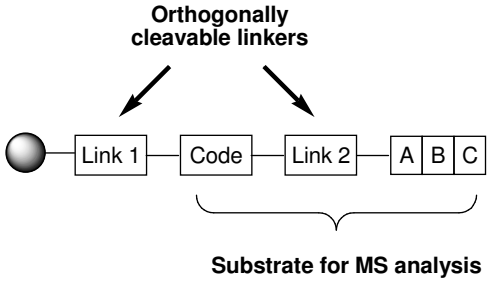


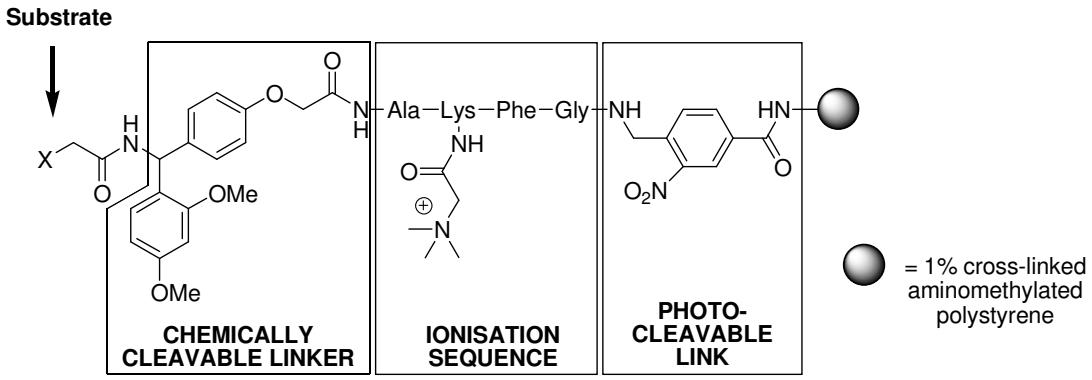
Figure 1.5: Principle of the analytical constructs developed by Geysen

The concept of "peak splitter" was also introduced by Geysen at this occasion. It consists of an approximately 1:1 ratio of two otherwise identical entities that therefore differ in mass, giving a characteristic shape to the peaks of interest and thus allowing extraction of the peaks of interest from the background noise of the mass spectrum.

1.3.4.2. Further improvements

Based on Geysen's exploratory work, several MS based solid phase dual linkers were developed almost all gathered under the generic name of "analytical constructs". Carrasco developed a solid phase analytical construct for the monitoring of nucleophilic substitutions, palladium-based coupling reactions, and solid phase peptide synthesis (SPPS) using both Boc- and Fmoc- based chemistries.[50] Deploring the inefficient ionisation of numerous analytes arising from solid phase chemistry reactions in an attempts to monitor conversions, Carrasco elaborated the synthetic

construct **1.8** that allowed for the direct analysis of the attached species as well as their chemical cleavage as desired. The compound, described in **figure 1.6** and based on some early work that showed that it was possible to directly carry out matrix assisted laser desorption ionisation (MALDI) analysis of peptides bound to a solid support *via* a photolabile α-methylphenacyl-ester linker,[51] also featured a chemically cleavable linker, allowing the release of the substrate of interest. The core of the construct was prepared with a modified peptide sequence that "guaranteed" the ionisation of the moiety released by the photocleavage through the presence of a quaternary ammonium species attached to the side chain of a Lysine residue.



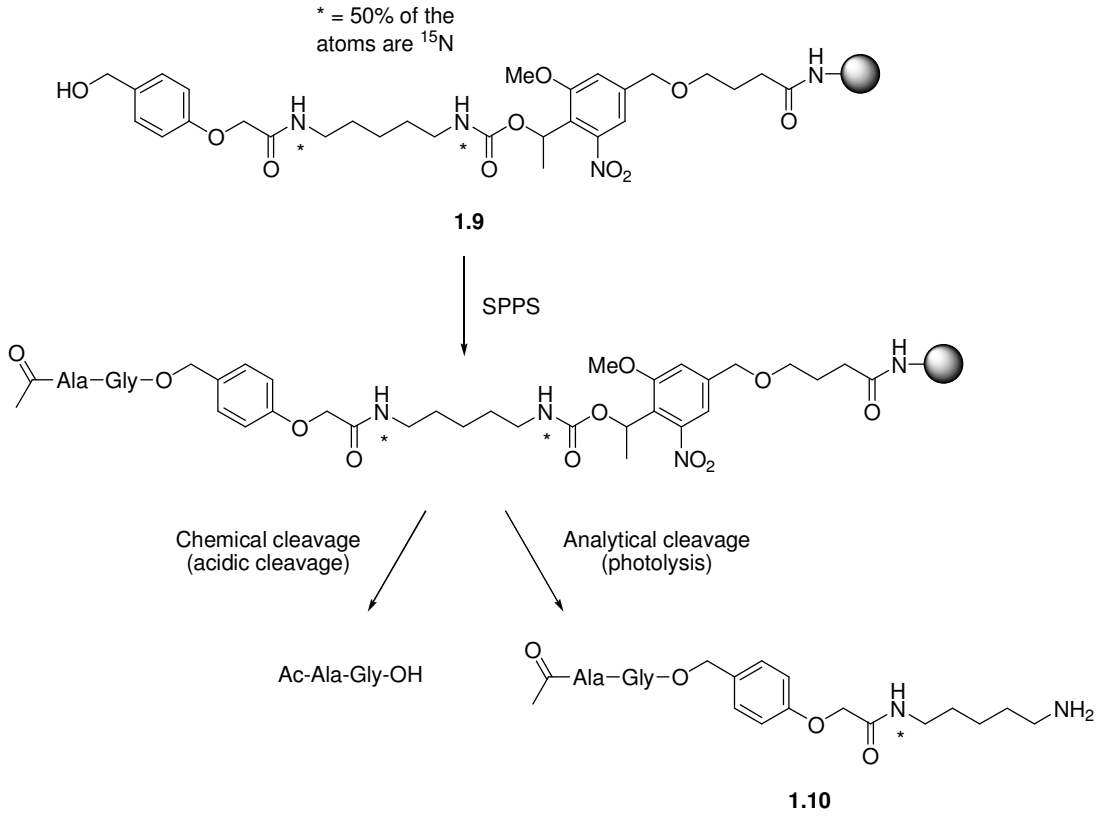
1.8

Figure 1.6: Analytical construct developed by Carrasco

Analytical construct **1.8** was used to follow the nucleophilic displacement of a bromoacetamide moiety (X = Br) by potassium cyanide (10 equiv.). Direct soft MALDI analysis of a few beads indeed enabled the rapid qualitative analysis of the solid supported species, giving the evolution of the conversion of the reaction in a HT manner (in comparison, the monitoring of the reaction without the use of the analytical construct would take much more time since it would require cleavage from the solid support prior to analysis. Moreover, it would not be guaranteed that the afforded cleaved species would be detected by MS). Ultimately, the action of concentrated trifluoroacetic acid (TFA) on the solid supported compounds triggered the cleavage of the product of the reaction.

Another example of analytical constructs was given by McKeown through the development of a photolabile carbamate based dual linker **1.9** (**scheme 1.6**).[52] The

construct, used for the monitoring of SPPS, allowed the liberation *via* photolysis of compound **1.10** constituted of the prepared peptide (Ac-Ala-Gly-OH in the example) attached to a moiety that incorporated an ionisable diamine based construct where 50 % of the nitrogen atoms were ^{15}N. Therefore, ESI/MS analysis of the dipeptide linked to the construct was much clearer than the one of the dipeptide alone: the ionisable sequence guaranteed good ionisation and the presence of the peak splitter allowed for easy identification.



Scheme 1.6: Analytical construct developed by McKeown

The rapidity of the analysis and the increased readability of the MS spectrum afforded by the analytical construct approach permitted rapid monitoring of SPPS.

Most of the further developments in the field of analytical constructs have concerned the implementation of the moiety used as an MS sensitiser as well as the introduction of a chromophore to the analytical construct, allowing UV-based quantification to be carried out after HPLC separation of the different constituents of the mixture on construct bound species. One extensively used example of these improvements is the analytical construct first introduced by Congreve.[53-55] Construct **1.11** consists of an

analytical enhancer, including a secondary amine based MS sensitiser, an isotopic mixture based mass splitter, and an anthracene moiety for quantitative purposes as shown in **figure 1.7**. This analytical enhancer was linked to the reactive moiety (an amine functionality) *via* a modified Rink linker on one side and to the solid support on the other side *via* a sulfonamide linkage.

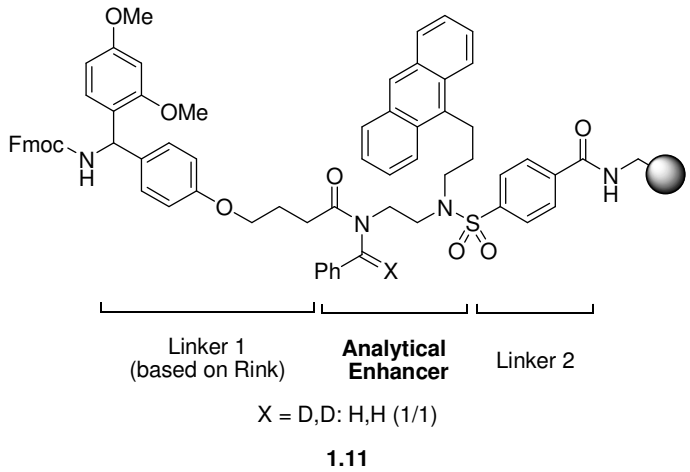


Figure 1.7: Analytical construct developed by Congreve

Cleavage of linker 2, afforded by 2-mercaptoethanol and diaza(1,3)bicyclo[5.4.0]undecane (DBU) allowed the release into the solution of the unreacted starting material and the product (or by-product). Their rapid qualitative analysis was carried out by MS thanks to the strongly ionisable secondary amine and the easy-to-spot relevant peaks thanks to the peak splitter. Quantitative conclusions regarding the composition of the cleavage mixture were obtained after HPLC analysis, at a wavelength where the absorption of the anthracene was close to its maximum (386 nm). Hence, the integration of the peak areas accounted for the quantity of each constituents of the mixture, identified thanks to their masses. Finally, traditional acid mediated Rink linker cleavage could release the products of the reaction from the solid support. The use of these analytical constructs proved to be very efficient not only for the conversion assessment of solid supported reaction,[53] thus allowing reaction conditions optimisation,[55] but also the development of new solid phase linkers. Zaramella also reported a construct where dabsyl and dansyl moieties were used as the UV chromophores for quantification purposes;[56] the

construct proved to be very useful in identifying and quantifying compounds arising from solid phase chemistries.

Hence, analytical constructs that incorporate chromogenic moieties turned analytical constructs from purely qualitative to quantitative tools for solid supported reactions. However, this supposes the systematic use of HPLC to separate the peaks of interest in order to integrate them, their identification being possible thanks to the MS data.

This not only supposes long analysis times but also the need to adjust HPLC gradients to each mixture of compounds analysed in order to ensure good separation of all compounds, which dramatically reduces the throughput of the analysis. Moreover, it imposes a limitation on the nature of the substrate and the product since their UV absorptions (at 386 nm) should not interfere with the anthracene moiety for the peak areas.

1.4. ESI+/MS Quantitative analytical constructs for HT Physical Organic Chemistry

Analytical constructs have thus demonstrated their power in the HT determination of the composition of mixtures of compounds from a qualitative point of view. Regarding quantitative aspects, there is still room for progress since the few methods for the HT determination of the composition of mixtures that have been reported until now are far from being universal and that some important limitations must be addressed regarding these techniques. The elaboration of a new family of analytical constructs that would enable HT identification of mixtures of products attached to it as well as their quantification would be of tremendous interest. Such a tool would allow HT reaction assessment to be carried out and therefore open the door to a raft of applications in the field of physical organic chemistry. As a matter of fact, being able to measure the qualitative and quantitative composition of a reacting mixture at any moment while carrying out a transformation gives access to a substantial amount of useful information regarding the kinetics of a reaction and thus its mechanism.

1.4.1. HT determination of the composition of a mixture of compounds by ESI+/MS analytical constructs

1.4.1.1. Principle of the method

HT ESI+/MS quantitative analytical constructs **1.12** (**figure 1.8**) were concieved on the basis of what was has been done in the past.[40, 41, 50] Linkage to the solid support was achieved by the use of the acid labile Rink linker. The use of a lysine amino acid provided one arm with an amine functionality (side chain) for further modification to get the desired reactive site and another one (α-amino functionality) for the attachment of the analytical enhancer. The latter was composed of a tetralkyl ammonium species, not only acting as a MS sensitiser but also as an ionisation leveller. The ion thus guaranteed the detectability of the construct by ESI+/MS and gave it quantitative properties. In order for the MS peaks corresponding to the species attached to the analytical construct to have a characteristic aspect, a *para*-bromophenyl moiety was also incorporated in the structure, resulting in the characteristic bromine pattern.

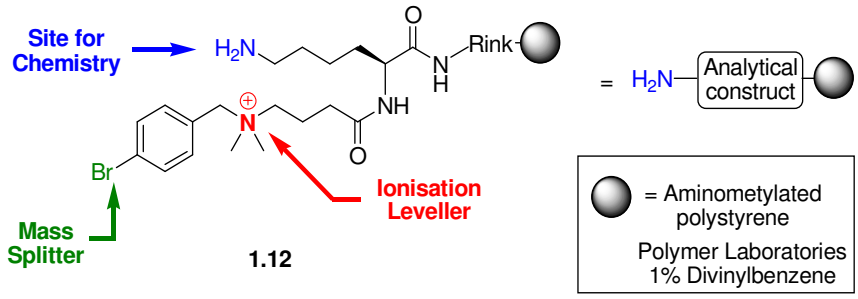


Figure 1.8: Amino quantitative analytical constructs

By strongly dominating the ionisation of the whole compound after cleavage from the solid support, the positively charged moiety indeed tolerates a wide range of species attached at the chemistry site without having an effect on the ionisation ability of the construct (see section 1.4.3.2 for limitations). Therefore, as the MS response is exclusively correlated to the tetralkylammonium ion, the intensity of the afforded peaks is independent of what is attached to the construct and is only proportional to the quantity of product in the cleavage mixture. (**figure 1.9**)

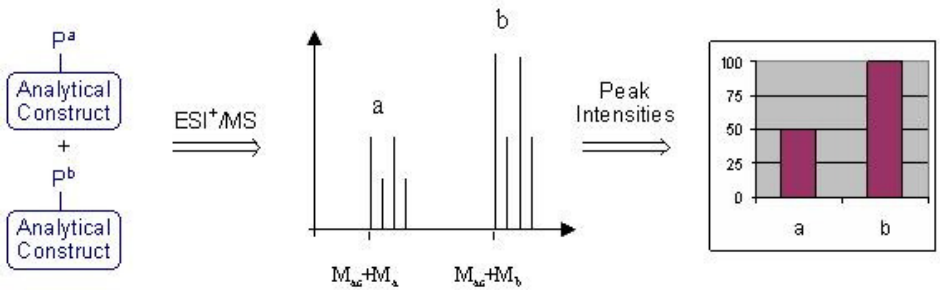


Figure 1.9: Principle of the ESI+/MS quantitative analytical construct

1.4.1.2. Extraction of ESI+/MS data

In order to ensure the highest accuracy in the quantitative assessment of the composition of mixtures of analytical construct bound compounds, a protocol was elaborated to afford the extraction of the relative ion count for each compound of interest, as shown in **figure 1.10**.

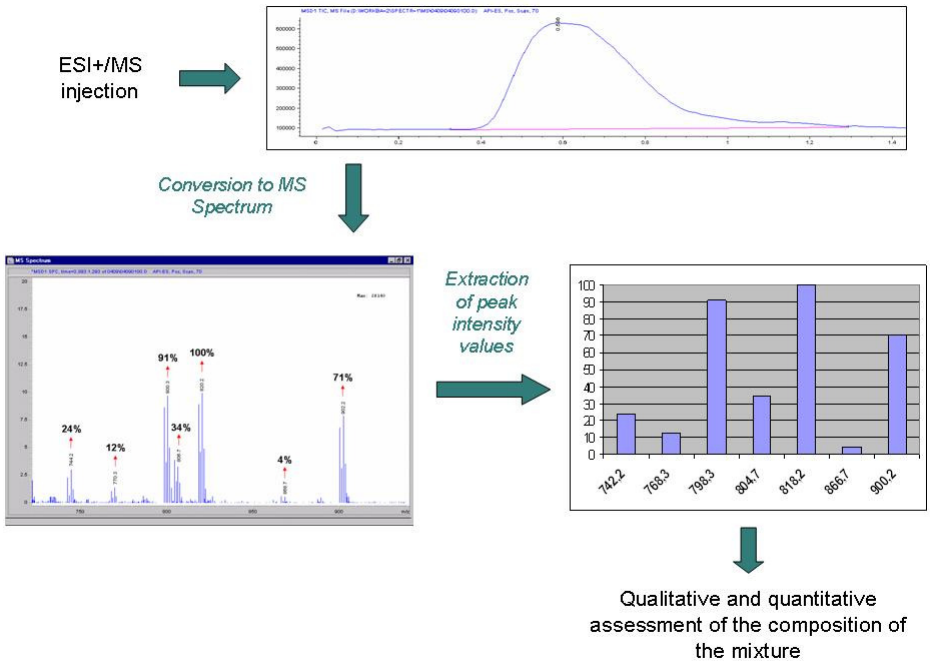


Figure 1.10: Treatment of ESI+/MS data to afford the composition of the mixture

After performing the ESI+/MS analysis of the mixture of cleaved compounds, the total ion current (TIC) trace was converted to a relative ion abundance graph *via* the Chemstation software. The software allowed the extraction of the peak intensities to a comma separated value file, allowing each peak to be compared to the others.

These values permitted each compound in the mixture to be quantified and compared to the major one, used as a reference. Alternatively, an internal reference could be used (see section 4.3.2).

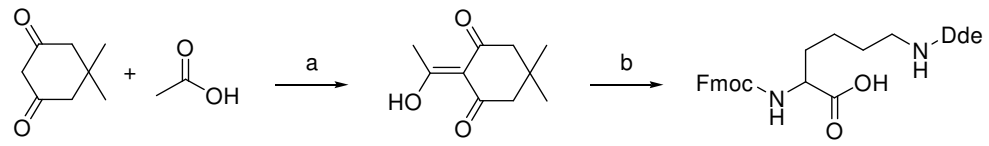
Quantitative analytical constructs therefore allowed the straightforward detection of the peaks of interest from the background noise thanks to the aryl bromide moiety. These peaks could subsequently be easily identified thanks to the knowledge of their molecular weights. The soft ionisation technique (ESI+/MS) avoided fragmentation and the presence of the ionisation dominating quaternary ammonium ion avoided the formation of multiply charged ions.

1.4.2. Preparation of quantitative analytical constructs

1.4.2.1. Fmoc-Lys(Dde)-OH **1.13**

The choice of Fmoc-Lys(Dde)-OH **1.13** as a base upon which to build the analytical construct was motivated by the presence on this amino acid of three linkage points.[57] The carboxylic acid functionality was used to afford attachment to the solid support whereas the two orthogonally protected amine functionalities could be substituted differentially, the α–amino group serving as a linkage point for the analytical enhancer and the ε-amino group as a site for chemistry. The protecting groups used were fully orthogonal so they could be removed selectively at will by a nucleophile for the Dde group and a base for the Fmoc group.[58]

Fmoc-Lys(Dde)-OH **1.13** was synthesised by reacting Dde-OH **1.14** with Fmoc-Lys, as summarised in **scheme 1.7**.[57] The protocol described by Chhabra for the preparation of Dde-OH **1.14** from dimedone **1.15** and acetic acid **1.16,** making use of dicyclohexylcarbodiimide (DCC) as the coupling reagent and 4-dimethylaminopyridine (DMAP),[59] was successfully applied to afford the cyclic enol in good yield and purity. Dde-OH **1.14** was subsequently condensed to the side chain amine residue of Fmoc-Lys-OH to give the protected amino acid **1.13** in good yield and purity.

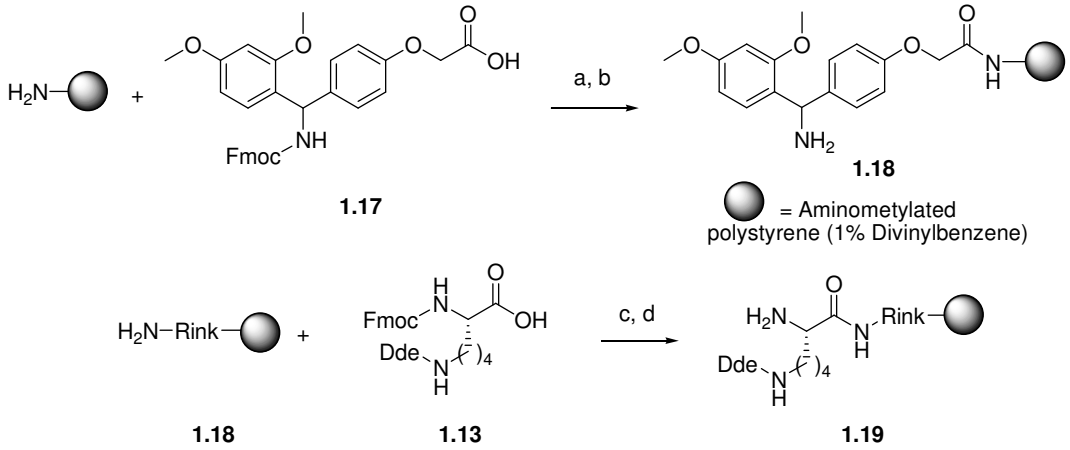


1.15 **1.16** **1.14** **1.13**

Scheme 1.7: Preparation of Fmoc-Lys(Dde)-OH. Reagents and conditions: (a) DCC (1 equiv.), DMAP (0.1 equiv), DMF, 36 h, 93 %, (b) Fmoc-Lys-OH (0.5 equiv.), TFA (0.1 equiv.), EtOH, reflux, 60 h, 75 %

1.4.2.2. Lys-(Dde) Rink amine resin **1.19**

The coupling of the Fmoc-Rink linker **1.17** to aminomethylated polystyrene resin afforded Rink amine resin **1.18**. Amino acid **1.13** was then coupled to afford, after Fmoc deprotection, resin **1.19** (**scheme 1.8**): these two coupling steps were carried out using diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) and were carefully monitored to completion, in order to ensure the highest quality of the resin at the end of the synthetic process.

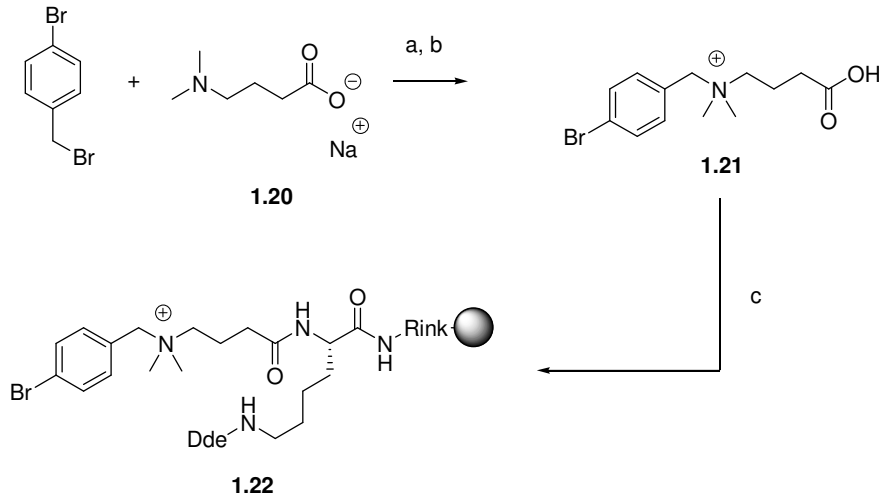


1.18 **1.17** **1.18** **1.18** **1.13** **1.19**

Scheme 1.8: Preparation of Lys-(Dde) Rink resin 1.19 from aminomethylated polystyrene resin. Reagents and conditions: (a) DIC (1.5 equiv.), HOBt (1.5 equiv), CH_2Cl_2/DMF (7:3, v/v), 15 h, (b) Piperidine in DMF (1:4 v/v), 2 × 5 min (c) DIC (1.5 equiv.), HOBt (1.5 equiv), CH_2Cl_2/DMF (7:3, v/v), 15 h, (d) Piperidine in DMF (1:4 v/v), 2 × 5 min.

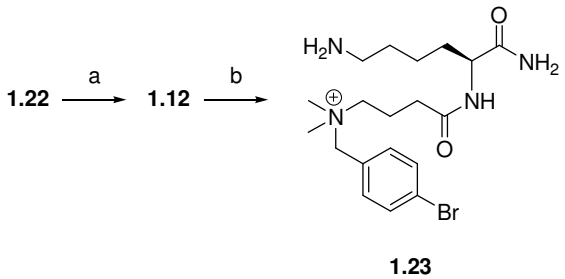
1.4.2.3. Analytical enhancer and final assembly of the construct

Sodium *N,N*-dimethyl-4-aminobutyrate **1.20** was generated from its hydrochloric salt to enable it to undergo the nucleophilic substitution described in **scheme 1.9**. The use of an ion exchange resin was used to afford the intermediate **1.21** which was coupled to resin **1.19**, as shown in **Scheme 1.8**, using DIC and HOBt to yield **1.22**.



Scheme 1.9: Final assembling of the construct. Reagents and conditions: (a) CH_2Cl_2, 15 h (b) Amberlite 200, MeOH, 30 min, 98 % for two steps (c) Resin **1.19** (0.5 equiv.), DIC (0.5 equiv.), HOBt (0.5 equiv), CH_2Cl_2/DMF (7:3, v/v), 15 h.

The Dde group on resin **1.22** was subsequently removed using hydroxylamine and imidazole to yield the analytical construct resin **1.12** (**scheme 1.10**).[60] Complete validation of this solid phase route was achieved by full characterisation of the cleaved compound **1.23**.

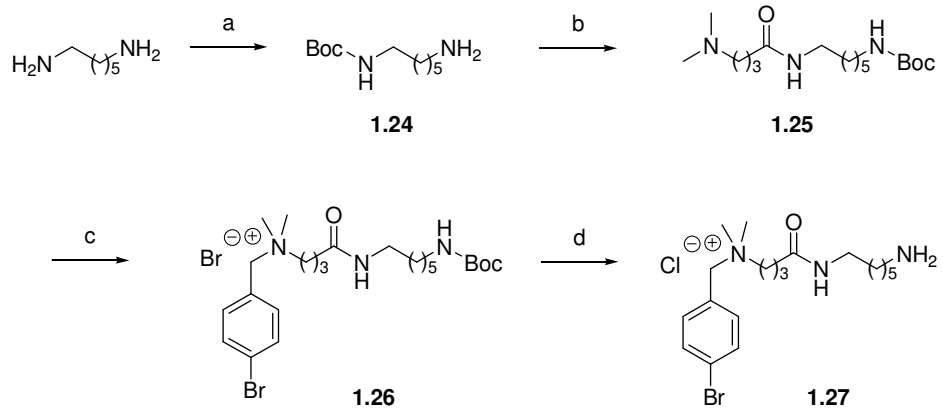


Scheme 1.10: Final Dde deprotection and cleavage for characterisation purposes. Reagents and conditions: (a) NH_2OH (1 equiv.), Imidazole (0.75 equiv.), CH_2Cl_2/NMP (1:5, v/v), 15 h (b) TFA in CH_2Cl_2 (1:4, v/v), 15 min.

The amino functionalised construct **1.12** obtained could be used without any further modification for Ugi monomer reactivity profiling purposes. Thanks to the set of transformations available on amines, it also provided a very versatile starting point to generate other functionalities that were subsequently needed. It also allowed the introduction of linkers such as a polyethyleneglycol (PEG) based linker, or 6-aminohexanoic linker to adapt the physicochemical properties of the constructs.

1.4.2.4. Solution phase variations

A solution phase version of the analytical construct was prepared using a similar strategy (see **scheme 1.11**). However, given the difficulty of handling the quaternary ammonium salt **1.21**, the synthesis had to be adapted in order to carry out the nucleophilic displacement at a later stage, as described in **scheme 1.11**. 1,6-Diaminohexane was submitted to a selective protection of one of the amine functionalities to generate compound **1.24**.[61] Coupling to the hydrochloride salt of *N,N*-dimethylaminobutyric acid yielded compound **1.25** which could undergo nucleophilic displacement to generate salt **1.26**. Boc group deprotection and deprotonation of the resulting amine yielded solution phase analytical construct **1.27**.



Scheme 1.11: Synthesis of the solution phase amino analytical construct. Reagents and conditions: (a) Boc_2O (0.125 equiv.), Dioxane, 22 h, 85 % (b) **1.21** (1.1 equiv.), DCC (1.1 equiv.), CH_2Cl_2/DMF (7:3, v/v), 12 h, then aq. $NaHCO_3$, 89 % (two steps), (c) 4-Bromobenzyl bromide (1.1 equiv.), CH_2Cl_2, 3 h, (d) 2M HCl in Et_2O, 5 min, then Amberlyst A27, MeOH, 89 %.

1.4.3. Proof of concept and limitations of the method

The quantitative properties of the ESI+/MS analytical constructs had been demonstrated in the past by the preparation of a series of compounds **1.28** to **1.31** shown in **figure 1.11**.[62] The compounds were synthesised in parallel by coupling using **1.12** as the resin bound amine entry followed by cleavage of the compounds from the solid support.

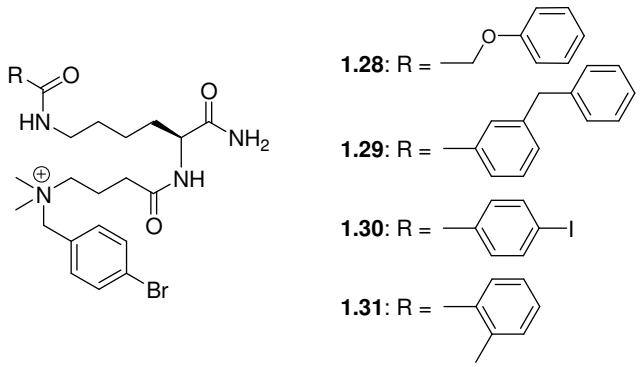


Figure 1.11. Compounds used for the preliminary validation the method

All products were purified and analysed one by one by HPLC. Quantification was then performed using the peak areas found on a precalibrated evaporative light scattering detector (ELSD),[63] while MS quantification was achieved by calculation of the abundance of the compound using the MS trace and the intensity of the peak, as explained in section 1.4.1. The intensities of the ESI+/MS peaks obtained for the analyses of different concentrations of solutions of **1.28**, **1.29**, **1.30** and **1.31** were plotted vs. their respective ELSD areas. The linearity between the two various methods of analysis was found to be excellent, which gave the first proof of the efficiency of the ESI+/MS based quantification. However, this study suffered from the concern that the use of single compounds did not show the ability of ESI+/MS based analytical constructs to accurately assess the quantitative composition of mixtures of compounds. Furthermore, the similarity between the compounds used in this preliminary work and the fact that no highly ionisable group other than the quaternary ammonium were included to the structures still left a doubt regarding the limitations of the MS quantification.

1.4.3.1. ESI+/MS based quantification of mixtures of compounds

A mixture of compounds **1.32** and **1.33** (**figure 1.12**, mixture 1) was prepared by a microwave assisted Ugi 4-component condensation (4-CC) using resin **1.12** as the amine entry, cyclohexyl isonitrile, hydrocinnamaldehyde and two different carboxylic acids, according to a literature protocol.[64] The mixture was subsequently analysed by ESI+/MS as well as HPLC/ELSD in order to afford determination of the composition of the mixture by two methods. Similarly, a second mixture of compounds **1.34** and **1.35** (**figure 1.12**, mixture 2) was prepared using benzyl isonitrile and analysed.

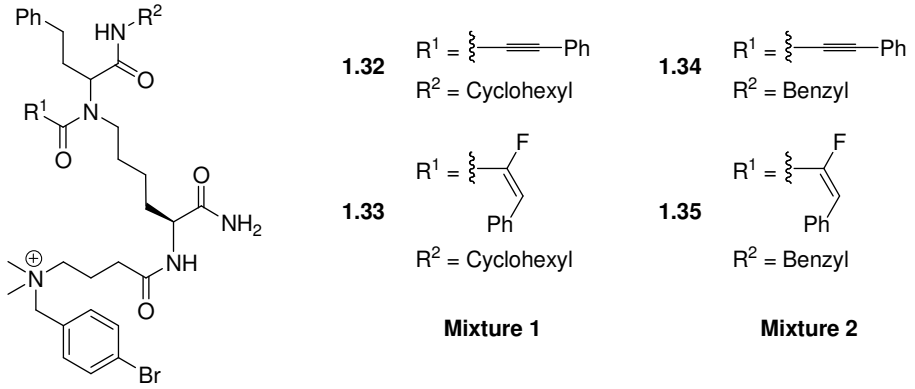


Figure 1.12. Compounds used to validate the quantitative properties

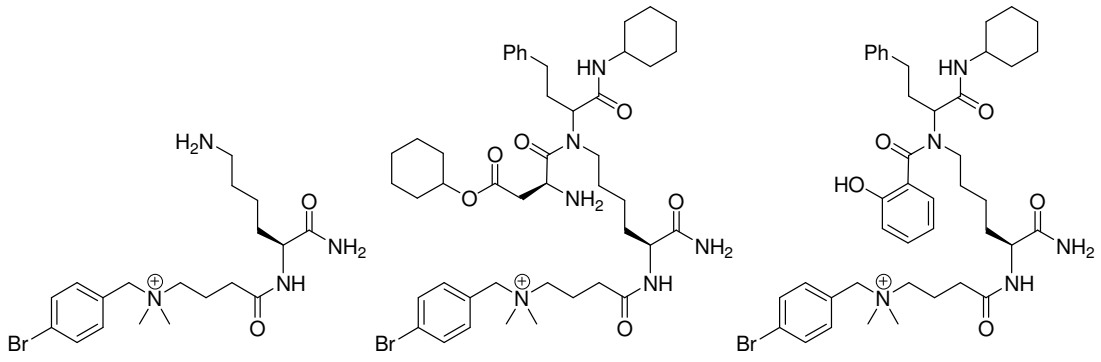
The choice of the ELSD detector was motivated by the results reported in a study carried out by Fang who demonstrated that ELSD can be used as a "universal" detector for rapid quantification in combinatorial chemistry.[63] As presented in **table 1.1**, the composition of the two mixtures as determined by the two methods were within the limits of experimental error described in Fang's studies.

Table 1.1: Determination of the composition of the two mixtures of the two analytical construct bound α-(acylamino) amides by ESI+/MS and ELSD

Quantification of the mixture	Mixture 1		Mixture 2	
	1.32	**1.33**	**1.34**	**1.35**
HPLC/ELSD	71 % ± 6 %	29 % ± 3 %	73 % ± 6 %	27 % ± 3 %
ESI+/MS	73 % ± 7 %	27 % ± 3 %	80 % ± 8 %	20 % ± 2 %

1.4.3.2. Quantification of mixtures of strongly ionisable compounds

In order to evaluate the ionisation properties of the analytical enhancer (quaternary ammonium), a mixture of two compounds with increased diversity was prepared *via* a Ugi-4CC. Resin **1.12** was used as the amine entry of the multicomponent reaction, cyclohexyl isonitrile and hydrocinnamaldehyde respectively being used as the isonitrile and the aldehyde species. The first carboxylic acid Boc-Glu(cHx)OH was chosen to have an ionisable functionality on it, such as an amine (protected during the reaction) and an ester that could disturb the domination of the ionisation by the quaternary ammonium. The second carboxylic acid was 2-hydroxybenzoic acid. The total quantity of carboxylic acid (0.5 equiv.) was deliberately lower than the amount that would be required to achieve complete conversion of the starting amine **1.12**, in order to yield a final mixture of three compounds, corresponding to the cleaved starting amine **1.23**, and the two Ugi-4CC products **1.36** and **1.37** (**figure 1.13**).



1.23 **1.36** **1.37**

Figure 1.13. Second series of compounds used to validate the quantitative properties

As before, the mixture of compounds was analysed by ESI+MS as well as HPLC/ELSD. The composition of the mixture obtained by the two methods are presented in **table 1.2**.

Table 1.2: determination of the composition of a mixture of compounds 1.23, 1.36 and 1.37 by ESI+/MS and ELSD

Quantification of the mixture	**1.23**	**1.36**	**1.37**
HPLC/ELSD	45 % ± 4 %	10 % ± 1 %	45 % ± 4 %
ESI+/MS	54 % ± 5 %	6 % ± 1 %	40 % ± 4 %

One can notice that despite the increased diversity introduced among the three compounds **1.23**, **1.36** and **1.37**, ESI+/MS quantification of the mixture still correlated quite well with HPLC/ELSD. ESI+/MS analytical constructs are therefore suitable for qualitative and quantitative analysis of mixtures of compounds bound to them, even in cases when the compounds differ dramatically by the presence of ionisable groups, such as esters, free alcohols and amines.

It must be pointed out that the two studies presented in sections 1.4.3.1 and 1.4.3.2 were carried out on mixtures of only two or three compounds owing to the difficulty in achieving good HPLC separation of multiple compounds linked to the analytical construct. The quaternary ammonium indeed seemed to govern the overall retention of the molecules and prevented more compounds from being correctly separated even by the use of very slow gradients and small particle size HPLC columns.

1.4.3.3. Limitations to ESI+/MS based quantification

The first limitation on the use of quantitative analytical constructs for ESI+/MS based quantification of mixtures of compounds was quite obviously the fact that the compounds to be analysed had to differ in their molecular weights by at least 4 units for the bromine patterned peaks not to interfere with each other. As quantification relied on the shape of the MS peaks the starting materials had to be free of any atom that would induce a drastic change in peak shape. Therefore, certain compounds could not be used with the method, such as compounds incorporating halogens (especially bromine or chlorine), etc.

Finally, despite the large variety of mixtures of molecules that can be analysed (section 1.4.3.2.), ESI+/MS quantitative properties fail to achieve sufficient

reliability in the case to many ionisable groups are attached to the construct, such as in the case of peptides or charged species (see section 4.4.1.2)

1.5. Conclusions

Combinatorial Chemistry and HT synthesis has enabled huge numbers of chemical transformations to be carried out in a reduced time frame. The rapid preparation of libraries of compounds is now routinely carried out and analytical chemistry has had to meet the challenge of increasing its throughput to enable the rapid analysis of the reaction mixtures generated by HT synthetic techniques, not only to evaluate the purity of the compounds prepared, but also to understand how these transformations proceed with time, allowing conclusions to be drawn about their kinetics, the presence of key intermediates, etc. The real time analysis of a reaction mixture indeed supplies priceless information, which can be used to explain the mechanism of a reaction and determine its rate limiting step, to explain the presence of unexpected by-products, dead end intermediates, etc. Several analytical techniques have thus emerged to allow the HT analysis of mixtures of compounds and although some of them have demonstrated great efficiency in achieving qualitative conclusions, quantitative conclusions still remain challenging. To try and address this limitation, solid phase and solution phase ESI/MS based quantitative analytical constructs were prepared and have shown to display good efficiency in achieving the HT qualitative and quantitative assessment of mixtures of highly diverse compounds. In the following chapters, different applications of these constructs will be presented, to show how they contribute to the development of HT physical organic chemistry.

Chapter Two

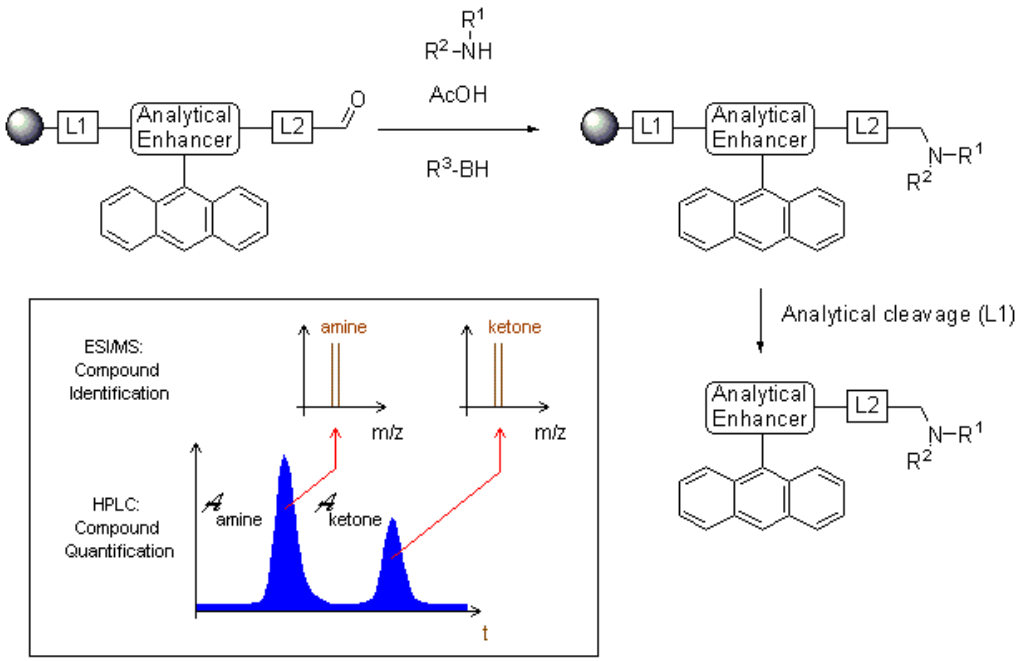
High Throughput Ugi 4-Component Condensation Monomer Reactivity Profiling Using Quantitative Analytical Constructs

2.1. Introduction

Combinatorial chemistry and high throughput (HT) techniques are now widely acknowledged as key technologies for speeding up the discovery of new therapeutic agents. Over the past few years, these techniques have undergone major developments that have enabled the efficient preparation of tens of thousands of compounds, allowing increasingly huge libraries of new chemical entities to be produced in a reduced time frame.[65, 66] However, shifting from processes that generate a limited number of compounds to library production, where great numbers of molecules are prepared under identical conditions, has brought unexpected drawbacks. Whereas it was firstly assumed that the purity of all compounds would be satisfactory and that they would be present in comparable concentrations across the library, reality turned out to be somewhat different, with the desired compounds often existing as minor products or not existing at all.[67] From these observations, two methods of dealing with the problem of impure libraries have come to the fore. The first consists of the systematic purification of the whole library prior to screening, ensuring sufficient purity of the compounds and allowing screening steps to be carried out with uniform purity and identical concentration. Members that show activity can then be compared and subjected to structure activity relationship (SAR) determination in order to determine the key structural features of the final compound. The disadvantage of this process quite obviously comes from the dramatic reduction of throughput since purification is performed on all reaction mixtures, including those that do not require it. Furthermore in many cases, the purification step does not lead to satisfactory results. The second option is the direct utilisation of the crude mixtures for HT screening (HTS). However in case of hits, the active compound has to be identified from amongst the components of the mixture and re-synthesized.

Moreover, as the concentration of compounds is variable, screening results cannot be compared directly across the library.

Quality control of libraries has thus turned out to be a key component for HT synthesis and many solutions have been proposed by means of analytical methods based on high performance liquid chromatography (HPLC) systems coupled to multiple detectors such as evaporative light scattering detector (ELSD), chemoluminescent nitrogen detector (CLND), etc, to evaluate as rapidly as possible the quality of the libraries.[68, 69] Compounds without satisfactory purity are simply discarded, saving the trouble and the money of taking them to HTS. Although efficient in guaranteeing the quality of the molecules that are screened against the target, this method results in a significant part of the work ending up in the chemical waste container, and prevents many compounds from being screened despite the fact that they could have generated useful information. At this point the question of anticipating synthetic failure to allow crude compounds to be generated in desired purity has to be raised. What if reagents could be tested prior to synthesis, instead of embarking through a process where a high percentage of combinations will fail because of insufficient reactivity of the monomers under the investigated conditions? The main reason for the poor quality of libraries is indeed attributable to the fact that reaction conditions are the same for the preparation of the whole library whereas the reactivity of the monomers used may vary dramatically from one to another: some of them not reactive enough to achieve satisfactory conversion whereas others generate numerous by-products. Ideally, reaction conditions should be adapted to the reactivity of each building block, provided that it is known prior to library preparation. This would allow, after discarding the unreactive species, monomers to be gathered in groups of similar reactivity, guaranteeing optimal reaction condition for a given set of components. However, the question is how to accomplish such a task, given that a complete combinatorial rehearsal would be both impractical and laborious, and that the synthesis of smaller arrays of compounds is often not representative of the diversity of the whole set of monomers. In 2004, Parr *et al.* described the use of a quantitative solid-phase analytical construct to evaluate the reactivity of amines toward reductive amination (**scheme 2.1**).[55]



Scheme 2.1: Parr's LC/MS analytical construct

An analytical construct (see section 1.3.4) incorporating an aldehyde functionality was reacted in a reductive amination reaction with a set of amines on a one by one basis in the presence of borane as a reducing agent. Thanks to an anthracene moiety on the analytical construct, the quantitative composition of the reaction mixture (product(s) and starting material) was easily evaluated by HPLC after "analytical" cleavage (linker L1). As the presence of the analytical enhancer guaranteed good ionisation of all species, correlation of each peak with the corresponding species (product or reagent) was performed by mass spectrometry (MS) analysis. The afforded qualitative and quantitative data thus permitted yields to be calculated, therefore leading to the assessment of the reactivity of 84 computationally selected amines towards the reaction. The information collected were useful not only for monomer selection, but also provided a balanced data set for reactivity prediction.[55] However, two factors seriously limited the interest of the method. Firstly, the reactions were carried out separately for each monomer tested to obtain discreet compounds, requiring the workup of every reaction mixture. Moreover, the analytical step involved an HPLC analysis that was 30 min long, dramatically limiting the speed of reactivity profiling.

To address the need to evaluate the reactivity of large sets of monomers in a HT manner under identical reaction conditions, a positive electrospray (ESI+) MS

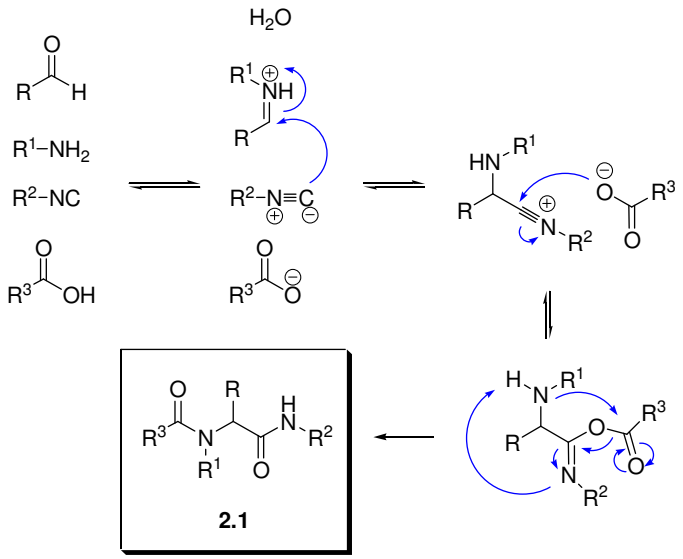
analytical construct based method was developed. In this chapter a HT reactivity assessment method for the Ugi 4 component condensation (4CC), based on ESI+/MS as the sole analytical tool and allowing the simultaneous evaluation of mixtures of carboxylic acids, aldehydes, and isonitriles will be described.

2.2. Principle of the method

A general HT method was developed to assess the reactivity of building blocks in the Ugi-4CC, utilising a quantitative analytical construct. Ten members of each family of monomers (*i.e.* carboxylic acids, aldehydes and isonitriles) were selected for their different features (bulkiness of the reagent, variation around the reactive functionality) so as to have different reactivity in the Ugi-4CC.

2.2.1. The Ugi-4CC

The Ugi-4CC belongs to the category of multicomponent reactions (MCRs). An MCR is a transformation which delivers a product by the reaction of at least three components, with the product showing essential structural elements of all the starting materials. MCRs, especially those using isonitriles, have a tremendous synthetic potential, since elaborated structures can be built up from simple reagents, in a single pot reaction.[70] MCRs are of specific interest in HT synthesis since they can easily be automated, enabling the fast generation of libraries of compounds with increased complexity form simple building blocks.[71] Moreover, MCRs are more convergent than standard synthetic pathways using sequences of uni- and bimolecular reactions: they guarantee a limited number of steps and generally proceed in high yields. One of the most widely used MCRs along with the Passerini reaction,[72] is the Ugi-4CC.[73] This condensation generates α-(acylamino) amides **2.1** from an aldehyde, an amine, a carboxylic acid and an isonitrile (**scheme 2.2**).



Scheme 2.2: Mechanism of the Ugi-4CC

The Ugi-4CC has been extensively used during the past few years: parallel solution phase versions of the reaction have been described,[74] as well as solid phase examples.[64] In the latter case, it is typically the amine functionality that is displayed on solid phase, leading to compounds being attached to the solid support *via* the nitrogen atom of the tertiary amide.

2.2.2. Description of the method

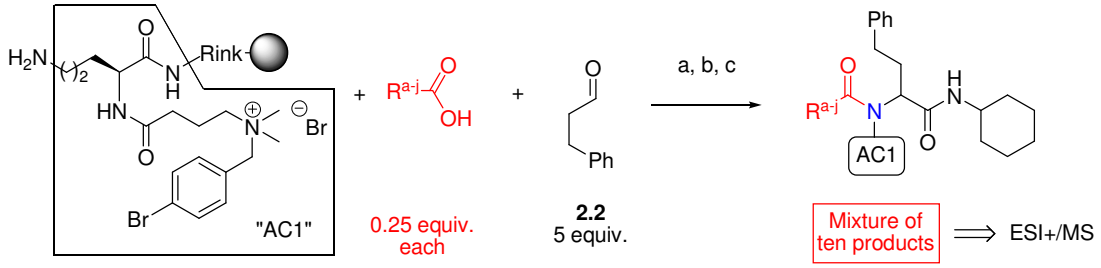
Two different kinds of assays were carried out. The first one, a solid phase assay, used construct **1.12** as the amine entry of the 4CC and the second one used the solution phase analytical construct **1.27** as the amine entry. Monomer reactivity profiling was performed under comparable conditions for both solid and solution phase methods, as described below.

The description concerns the HT assessment of carboxylic acid reactivity but the method was similarly adapted to aldehydes and isonitriles. Hydrocinnamaldehyde **2.2** and cyclohexyl isonitrile **2.3** were used as the two defined components for the investigation of carboxylic acid reactivity, because of their known good reactivity in the reaction (preliminary study). In order for them not to be limiting factors, they were used in excess compared to the starting amine (5 equiv. each). Regarding the investigated monomers, several restrictions had to be observed since the method was

based on ESI+/MS as the analytical tool: the monomers that were chosen for the study had to be free of any substituent that would make the final compounds fall into one of the limitations described in section 1.4.3.3 (no atom that would change the bromine pattern, at least 4 Da separating the molecular weight of each monomer, etc.). Four different concentrations of monomers were investigated, corresponding to 0.10 equiv, 0.25 equiv., 0.50 equiv., and 0.75 equiv. of each carboxylic acid used, relative to the starting amine **1.12**.

2.2.2.1. The solid phase method

The resin was first swollen in MeOH/CH_2Cl_2 and mixed with the aldehyde and the ten carboxylic acids, and shaken for 10 min. The isonitrile was then added. Microwave irradiation was performed for 30 min at 120 °C as described by Tempest *et al.* for solid phase Ugi-4CCs.[64] After filtration, the resin bound α-(acylamino) amides were washed and tifluoroacetic acid (TFA) cleavage followed by ESI+/MS injection gave the qualitative and quantitative composition of the mixture that could then be correlated to monomer reactivities (**scheme 2.3**).



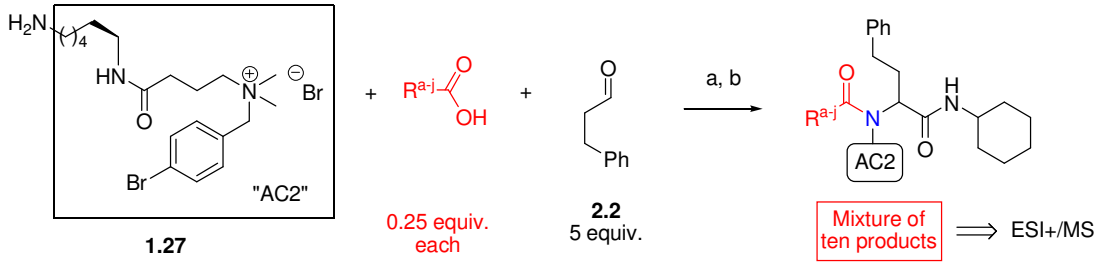
Scheme 2.3: Procedure for the reactivity assessment of 10 carboxylic acids in the Ugi-4CC using a solid phase analytical construct (1.12). . Reagents and conditions: (a), CH_2Cl_2/MeOH (1:1 v/v), 10 min, (b) cycloheyxyl isonitrile **2.3** (5 equiv.), CH_2Cl_2/MeOH (1:1 v/v) , 120°C, 30 min, (c) TFA in CH_2Cl_2 (1:4 v/v), 15 min

The assessment of the composition of the final reaction mixture from the ESI+/MS data was performed according to the procedure described in section 1.4.1. Quantitative data thus obtained allowed:

- The calculation of the conversion of the reaction (compared to the starting analytical construct bound amine)
- The assessment of the relative reactivities of the carboxylic acids in the assay

2.2.2.2. The solution phase method

For the solution phase investigation, the aldehyde, the carboxylic acids and the starting amine were pre-mixed together with MeOH/CH_2Cl_2, followed by the addition of the isonitrile. Microwave irradiation was performed for 15 min at 120 °C since the solution phase reaction turned out to proceed at a much higher rate than the solid phase one, allowing reduction of the reaction time. Direct ESI+/MS injection of the crude reaction mixtures allowed qualitative and quantitative determination of the composition that could be correlated to monomer reactivities. The procedure is summarised in **scheme 2.4**.



Scheme 2.4: Procedure for the reactivity assessment of 10 carboxylic acids in the Ugi-4CC using a solution phase analytical construct (1.27). Reagents and conditions: (a), CH_2Cl_2/MeOH (1:1 v/v), 10 min, (b) cycloheyxyl isonitrile **2.3** (5 equiv.), CH_2Cl_2/MeOH (1:1 v/v) , 120°C, 15 min

Assessment of the composition of the mixture was carried out as for the case of solid phase analytical constructs (section 2.2.2.1), to afford conversions as well as the relative reactivities of the monomers used in the study.

2.3. Solid phase Ugi 4-CC monomers reactivity profiling

2.3.1. Carboxylic Acids

The first study was carried out using the solid phase method. A set of ten building blocks with carboxylic acid functionalities was chosen among the ones available in the laboratory (**figure 2.1**). The choice was not only guided by the requirements presented in section 2.2.2 but also by a rapid preliminary reactivity screening in order

for the selected ten monomers to have different levels of reactivity. 0.10, 0.25, 0.50 and 0.75 equivalent of each acid were reacted together in a Ugi-4CC and analysed by ESI+/MS. The procedure was repeated three times to assess the reproducibility of the method.

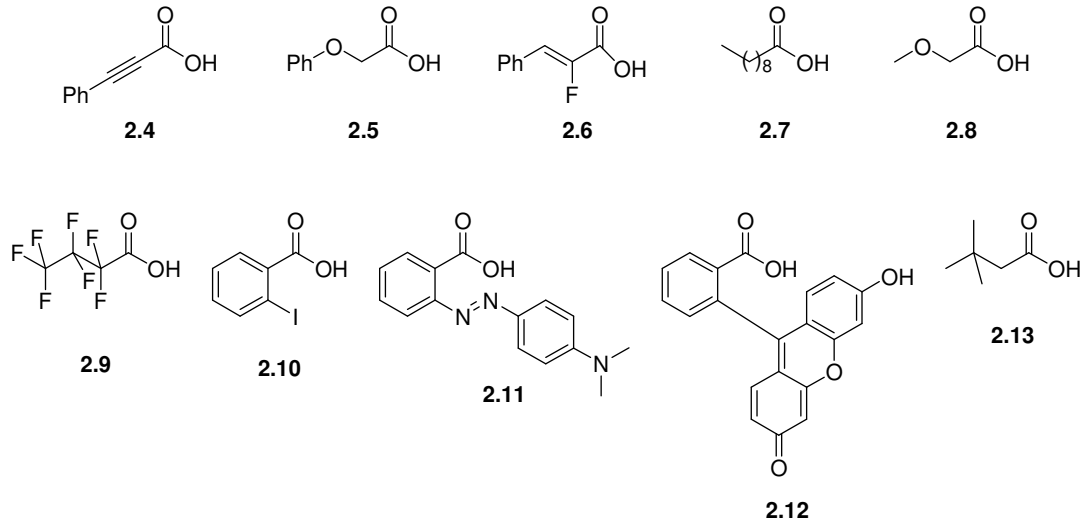
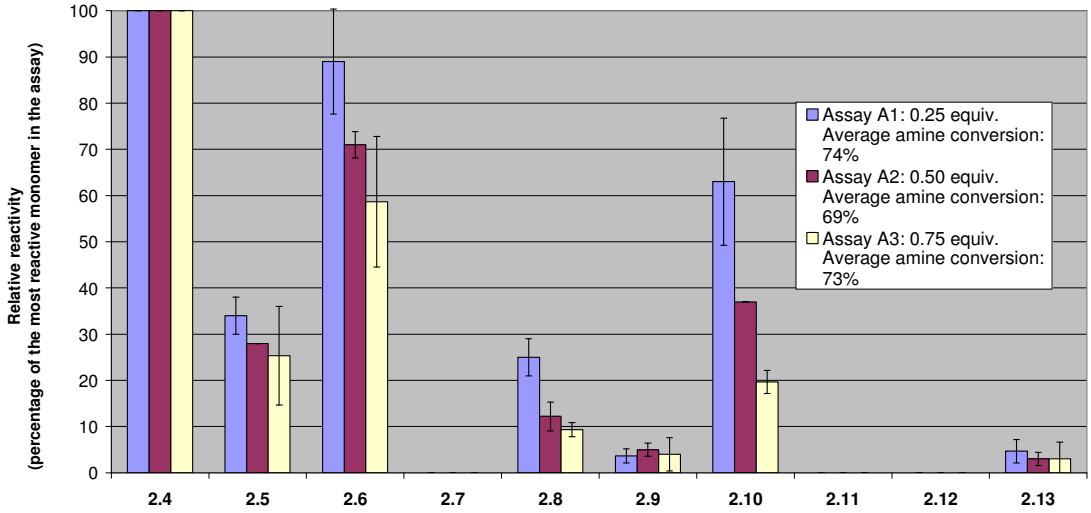


Figure 2.1: Carboxylic acids chosen for the Ugi-4CC monomer reactivity profiling

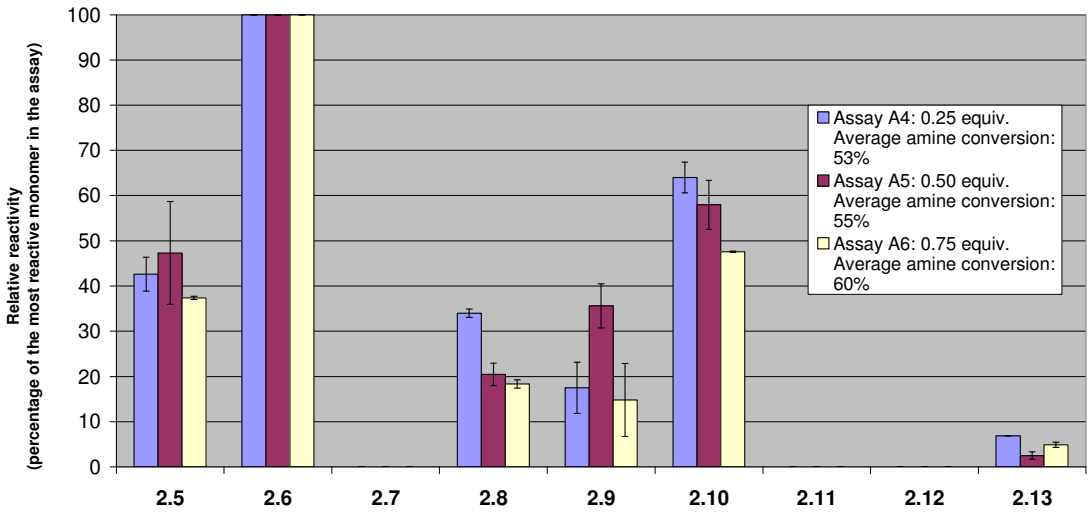
The results obtained are shown in **graph 2.1** (along with the standard deviation). In spite of the great care that had been taken to perform the monomer reactivity profiling with 0.10 equiv. of each carboxylic acid, the results obtained turned out to be unreliable: because of the very low conversion of the resin bound amine, it was difficult, even by extraction of the ESI+/MS peaks, to obtain meaningful data. Therefore, they will not be presented here.



Graph 2.1: Reactivity profiling for ten carboxylic acids

The relative quantity of monomers did not influence the conversion of the starting amine, which ranged from 69 to 74 %. Phenylpropiolic acid **2.4** was found to have the best reactivity in all the experiments carried out. The study also clearly showed that phenylpropiolic acid **2.4** dominated the reactivity of all monomers. Increasing the concentration of carboxylic acids from 0.25 to 0.75 equivalents indeed induced a significant decline in the observed reactivity of the other species, illustrating the fact that phenylpropiolic acid **2.4** was much more potent than other species. The most bulky compounds, methyl red **2.11** and fluorescein **2.12** did not form any product. Quite surprisingly, decanoic acid **2.7** did not form any product either, although there was no reason to think that this species would not have displayed a good reactivity in the reaction. (A study presented in section 2.5 was carried out with different linear fatty acids to examine this matter).

In order to investigate the domination of the reactivity by the most "potent" monomer previously mentioned, the reactivity profiling method was carried out using the same conditions, but removing the most reactive carboxylic acid, *i.e.* phenylpropiolic acid **2.4**. Results (**graph 2.2**) showed conservation of the reactivity ranking, with α-fluorocinnamic acid **2.6** now being the most reactive carboxylic acid, followed by 2-iodobenzoic acid **2.10**, phenoxyacetic acid **2.5** and methoxyacetic acid **2.8**. Methyl red **2.11**, fluorescein **2.12** and decanoic acid **2.7** still did not form any product. The conversion of the starting amine dropped by around 20 %, which was expected since the best building block had been withdrawn from the experiment.



Graph 2.2: Reactivity profiling for nine carboxylic acids (most reactive acid removed)

2.3.2. Aldehydes

Similarly, a set of ten aldehydes (**figure 2.2**) was chosen for reactivity profiling in the Ugi-4CC.

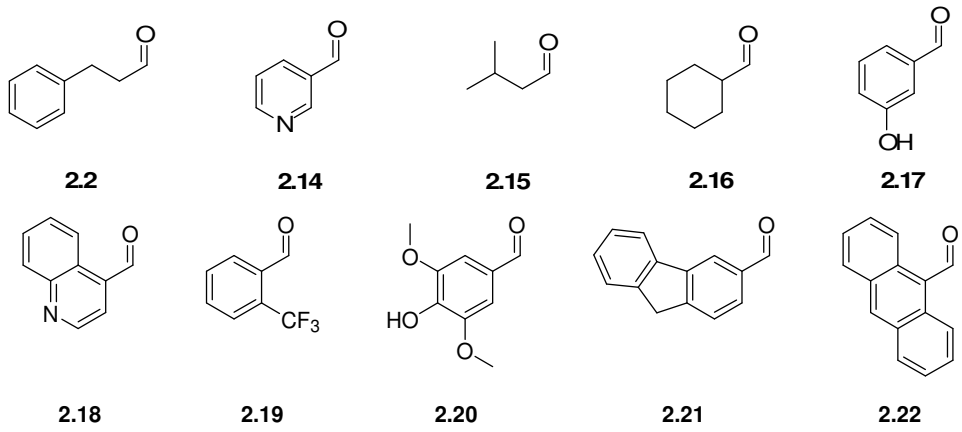
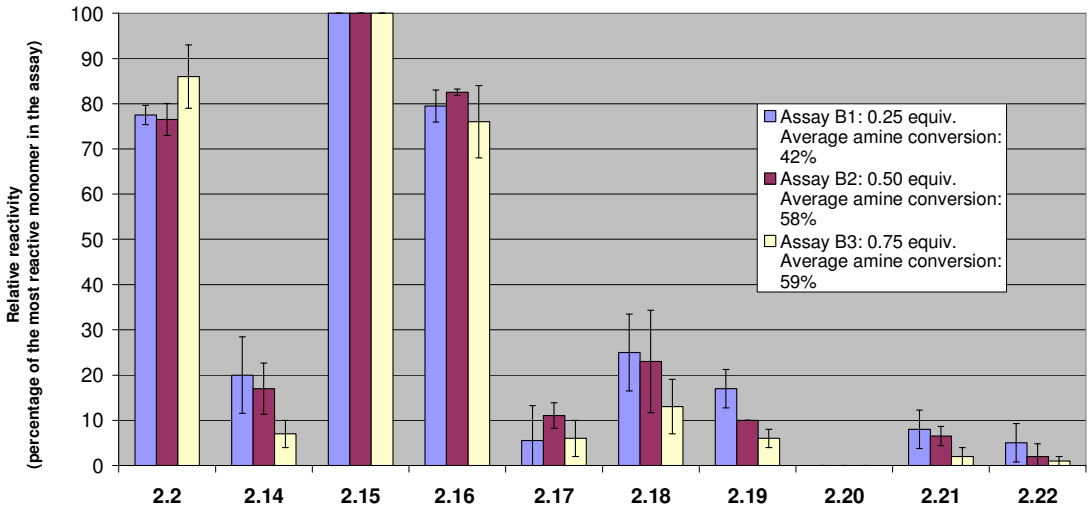


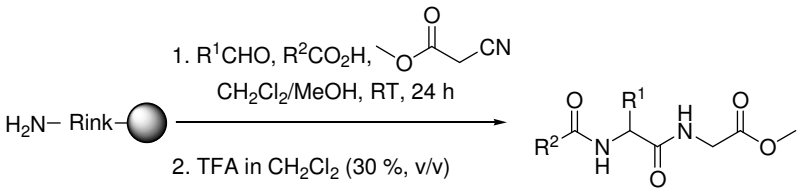
Figure 2.2: Aldehydes chosen for Ugi-4CC monomer reactivity profiling

The procedure utilised cyclohexyl isonitrile **2.3** and phenylpropiolic acid **2.4** (5 equiv. each) and the analytical construct **1.12**. The reactivity results obtained are presented in **graph 2.3**.



Graph 2.3: Reactivity profiling for ten aldehydes

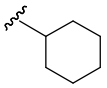
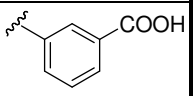
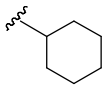
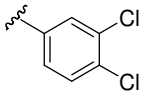
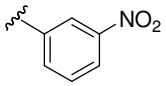
The conversions (based on starting amine) typically ranging from 40 % to 60 % were found to be a little lower than the ones obtained for the carboxylic acids. The relative reactivities measured by the HT analytical construct method did not appear to be dependant on the concentration of starting monomers, as it was the case with the acids. The best aldehyde in the assay did appear to dominate the reactivity of the other members at higher concentrations. What clearly came out of this study was the superiority of aliphatic aldehydes in terms of reactivity compared to aldehydes where the carbonyl moiety was conjugated to an aromatic ring. Hydrocinnamaldehyde **2.2** and cyclohexane carboxaldehyde **2.16** displayed a reactivity of around 80 % compared to the best aliphatic monomer 3-methylbutyraldehyde **2.15**. These results correlated perfectly to the literature, and especially the work performed by Tempest *et al.* regarding a series of solid phase Ugi-4CCs (**scheme 2.5**).[64] The author reported the yields corresponding to the preparation of a library 96 α-(acylamino) amides arising from the use of 8 different aldehydes and 12 carboxylic acids (**table 2.1**).



Scheme 2.5: Solid phase Ugi-4CCs performed by Tempest *et al.* [64]

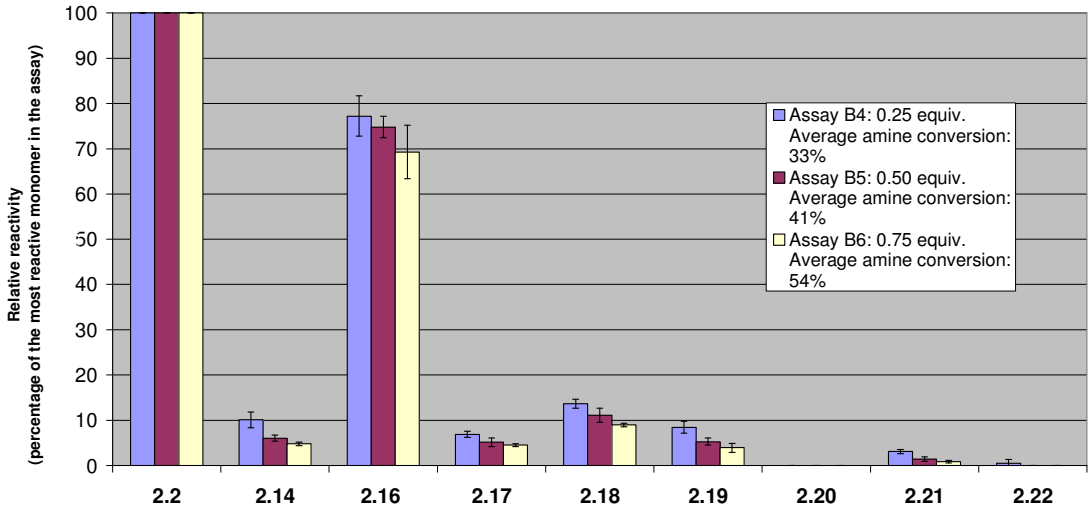
The best isolated yields afforded by Tempest from a series of Ugi reactions performed with several aldehydes were achieved for the aliphatic species whereas aromatic compounds were found to give lower amounts of product (see **table 1.1**)

Table 2.1 : Isolated yields obtained by Tempest with the synthesis of a library of Ugi products

Isolated Yields (%)		Acid - R^2 =			
		H			
Aldehyde R^1 =		59	95	79	95
		67	70	59	71
		46	14	11	31
		5	0	24	30

The ESI+/MS analytical construct approach allowed verification of the lower reactivity of aromatic aldehydes in a HT manner, without having to perform the preparation of discrete compounds, which allowed saving a substantial amount of time.

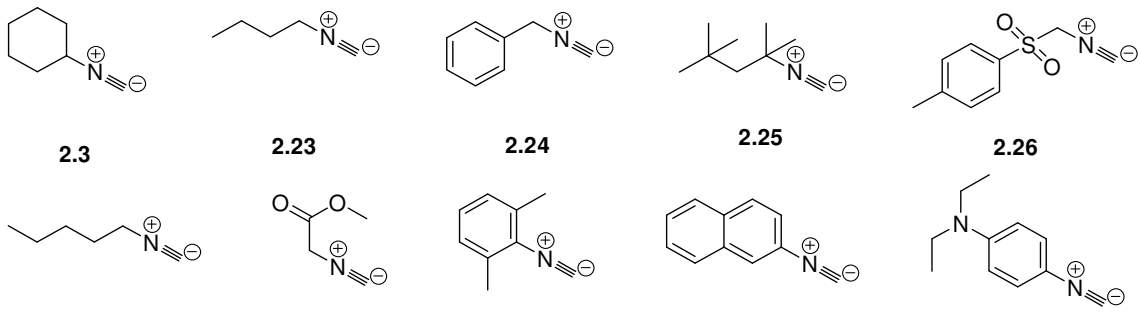
The removal of 3-methylbutyraldehyde **2.15** from the set of monomers and the reaction of the nine remaining ones under the same conditions also showed conservation of the reactivity ranking, with the remaining aliphatic aldehydes hydrocinnamaldehyde **2.2** and cyclohexane carboxaldehyde **2.16** showing much better reactivity than the others. Again, the overall conversion of the reaction dropped slightly, especially at lower concentrations of aldehydes.



Graph 2.4: Reactivity profiling for nine aldehydes (most reactive species removed)

2.3.3. Isonitriles

With the same objective of profiling their reactivity in the Ugi-4CC, a range of ten isonitriles were selected from commercially available materials (**figure 2.3**).



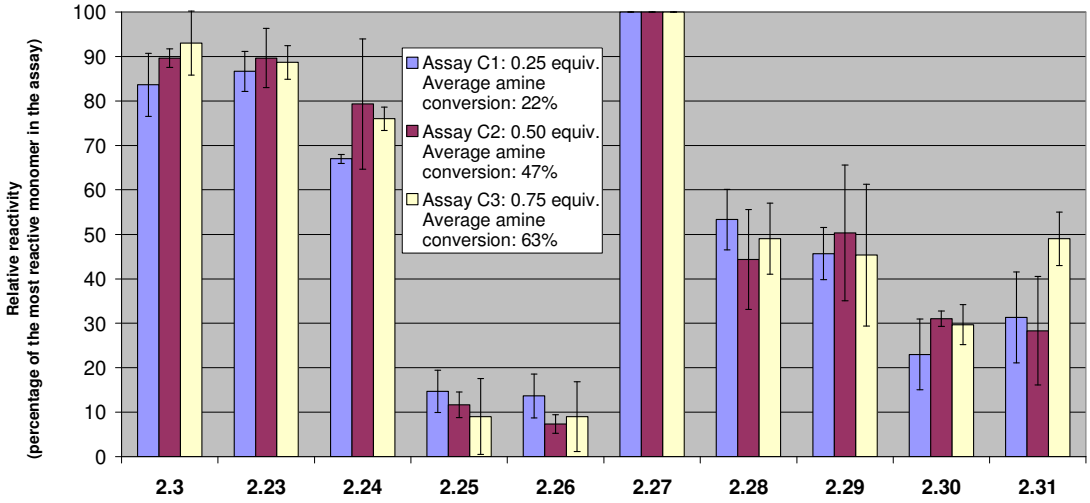
2.3 **2.23** **2.24** **2.25** **2.26**

2.27 **2.28** **2.29** **2.30** **2.31**

Figure 2.3: Isonitriles chosen for the Ugi-4CC monomer reactivity profiling

Using the same methodology with hydrocinnamaldehyde **2.2**, phenylpropiolic acid **2.4**, and the analytical construct **1.12**, the reactivity of a group of ten isonitriles (**figure 2.3**) was assessed. The reactivity results obtained are shown in **graph 2.5**. One very important observation that came out of the study was that the quantity of monomers, which previously did not appear to influence much the conversion of the starting amine here turned out to be have a greater effect. In the case where 0.25 equiv. of each monomer was used, the conversion of the reaction was about 20 %.

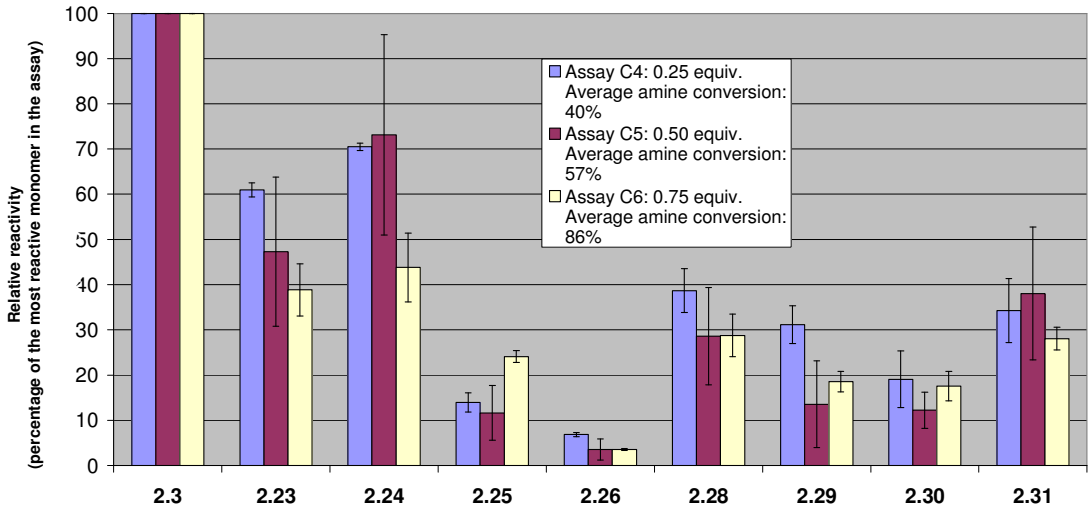
This value was doubled in the case of 0.50 equiv., and increased again for 0.75 equiv., to reach around 60 %. However, the relative reactivities remained totally unchanged. These results indicated that the limiting step of the mechanism of the Ugi-4CC is the nucleophilic attack of the isonitrile that occurs on the iminium ion generated from the solid phase analytical construct **1.12** and hydrocinnamaldehyde **2.2**.



Graph 2.5: Reactivity profiling for ten isonitriles

The study also showed that overall there were small differences in the reactivity of monomers; four of the building blocks chosen (**2.3**, **2.23**, **2.24** and **2.27**) had excellent reactivities in the Ugi-4CC. Four others showed good reactivity (**2.28**-**31**), and the two most bulky components turned out to be quite unreactive. In general, the reactivity of isonitriles in the reaction seems to be governed only by their bulkiness.

In the same way as for carboxylic acids and aldehydes, the best monomer of the previous assay was removed and a Ugi-4CC carried out under the same conditions. The reactivity ranking (**graph 2.6**) once again turned out to be conserved, and cyclohexyl isonitrile **2.3** was the most reactive species.



Graph 2.6: Reactivity profiling for nine isonitriles (most reactive species removed)

Here again, the conversion of the starting amine appeared to be correlated to the concentration of the starting building blocks under investigation. However, the average conversions that were measured with nine isonitriles instead of ten were significantly higher than expected, around 20 % above the ones obtained in the case of ten monomers. Such a result is quite surprising since they were, if anything, expected to drop but not to increase. The study was repeated for both cases (ten monomers and nine monomers) and the same trend was observed. No clear explanation could be given regarding this experimental fact.

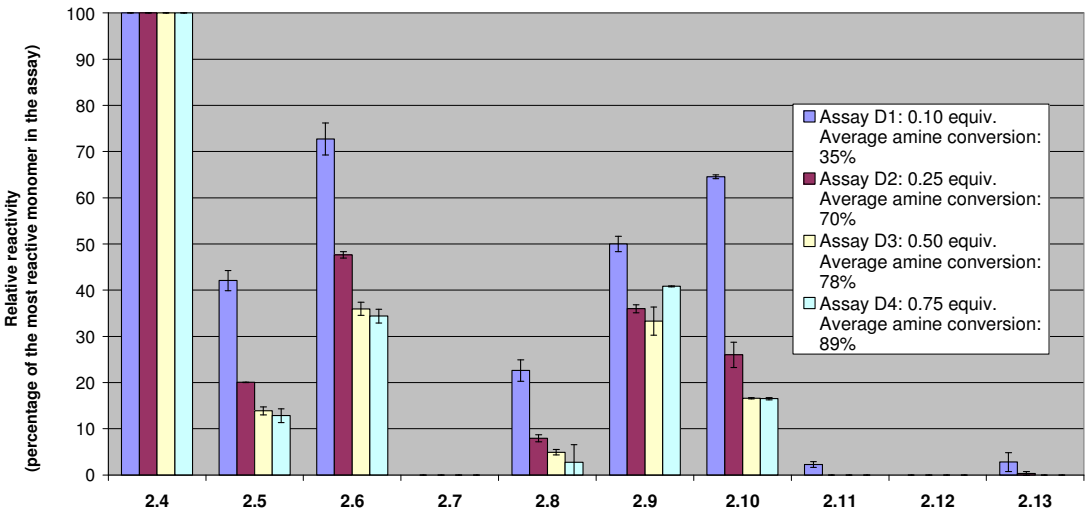
2.4. Solution phase Ugi 4-CC monomers reactivity profiling

After having assessed the reactivity of three sets of building blocks as monomers in the microwave assisted Ugi-4CC where the amine functionality is linked to a solid support, a solution phase equivalent of the method was developed, as explained in **scheme 2.4**. The solid phase study raised several issues that were worth of investigation. Firstly, the elaboration of a solution phase reactivity test that would be equivalent to the one developed on solid support could allow conclusions to be drawn regarding the effect of the heterogeneity induced by the solid support on the reactivity of building blocks. One could indeed imagine that the steric hindrance induced by the polymer would decrease the reaction rate for bulky species. Secondly,

the use of solution phase quantitative analytical constructs allowed increased throughput of the method since neither washing of the resin nor cleavage are necessary.

2.4.1. Carboxylic Acids

The same ten carboxylic acids as the ones used for the solid phase monomer reactivity profiling method were investigated in solution phase (**figure 2.2**). The monomers were reacted with hydrocinnamaldehyde **2.2** and cyclohexyl isonitrile **2.3** (5 equiv. each) using various quantities of the investigated building blocks (0.10, 0.25, 0.50 and 0.75 equiv., three experiments being carried out for each case). The reactivity results obtained are displayed in **graph 2.7**.



Graph 2.7: Solution phase reactivity profiling for ten carboxylic acids

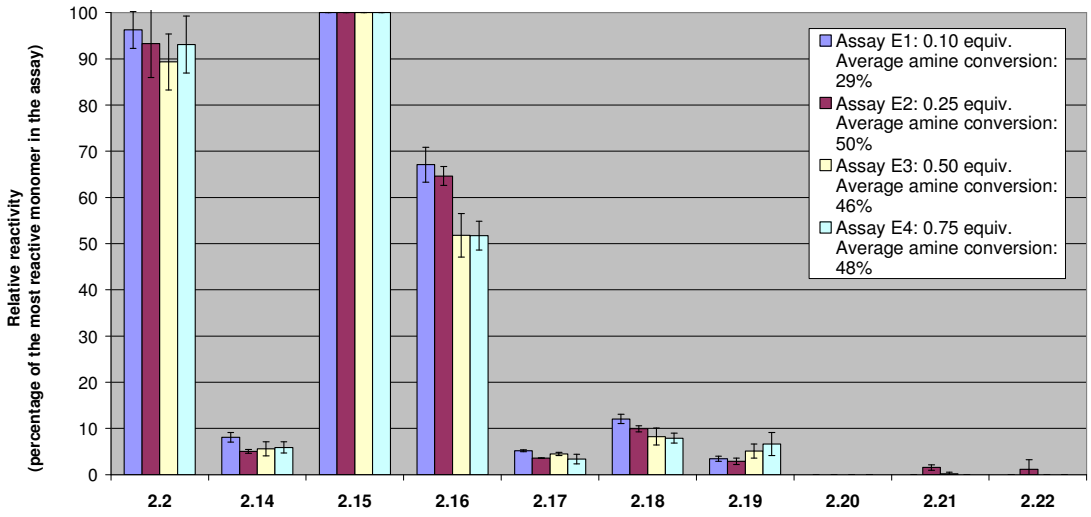
As for the case of the solid phase assays, the conversion of the starting amine did not appear to be affected by the quantity of carboxylic acids, apart from the lowest amount (0.10 equiv.) The conversions obtained turned out to be comparable to what was afforded in the case of the solid phase study, and the reactivity ranking was pretty similar to the previous method. Phenylpropiolic acid **2.4** and α-fluorocinnamic acid **2.6** turned out to be the two most "potent" monomers, and 2-iodobenzoic acid **2.10** and phenoxyacetic acid **2.5** again showed moderate reactivity. However, it has to be said that heptafluorobutyric acid **2.9** also showed moderate reactivity in the

Ugi-4CC, and thus appeared to be more reactive than in the case of the solid supported reaction. This may be explained by the greater influence of steric effects in the case of the use of polymer supported reagents, affecting the reactivity of heptafluorobutyric acid **2.9** or the inability of the fluorous "tail" of compound **2.9** to enter the PS. Methyl red **2.10** and fluorescein **2.11** and decanoic acid **2.6** did not show significant activity throughout the study, as it was already the case for the solid phase study.

The use of the solution phase analytical construct **1.27** allowed the investigation of the case where the quantity of each monomer was 0.10 equiv., making the total quantity of monomers used be equal to the starting amine. The results obtained in this specific case therefore deserve specific attention, and will be discussed in section 2.4.4.

2.4.2. Aldehydes

The series of ten aldehydes previously described (**figure 2.2**) underwent a solution phase Ugi-4CC with the analytical construct **1.27,** cyclohexyl isonitrile **2.3** and phenylpropiolic acid **2.4**. The conclusions regarding the four monomer quantities investigated (performed in triplicate), are summarised in **graph 2.8**.

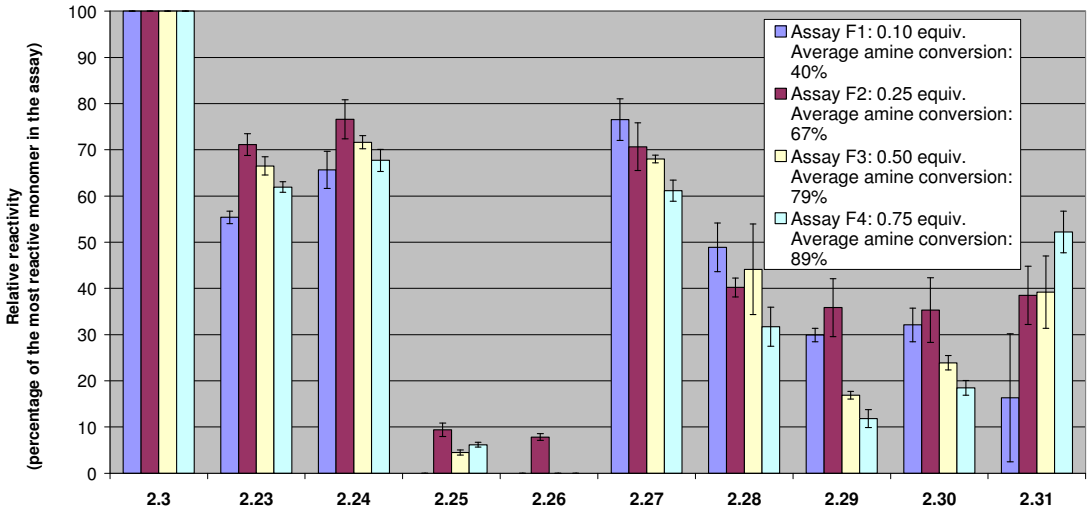


Graph 2.8: Solution phase reactivity profiling for ten aldehydes

As it was the case previously, 3-methylbutyraldehyde **2.15** appeared as the most reactive building block of the series. Hydrocinnamaldehyde **2.2** showed very good reactivity, followed by cyclohexane carboxaldehyde **2.16**. Again, the three aliphatic aldehydes turned out to be much more reactive than others. The case of 0.1 equiv. showed no change on the reactivity results obtained, corroborating what was observed previously regarding the non domination of the reactivity by the most powerful monomers of the set.

2.4.3. Isonitriles

To finish, the solution phase assays were carried out with the set of ten isonitriles (**figure 2.3**). Once again, the conversion of the starting amine appeared to be directly linked to the amount of isonitrile used in the assay. The reactivity ranking that was observed (**graph 2.9**) turned out to be the same as obtained with previous studies, bulky species being essentially unreactive.



Graph 2.9: Solution phase reactivity profiling for ten isonitriles

As observed before, no member of the set of isonitriles dominated the reactivity and no link seemed to exist between building block reactivity and the quantity of the monomers used.

2.4.4. The case of 0.1 equiv. of each monomer

The reactivity results obtained in the specific case where 0.1 equiv. of each monomer was used (graphs 2.7, 2.8 and 2.9) brought some interesting results that need to be discussed. Since the total quantity of monomers used in this case was equal to 1 equiv. of the starting amine, each monomer was given the "opportunity" to react and generate the corresponding α-(acylamino) amide: the effect of the domination of the reactivity by the most powerful monomer was thus suppressed, allowing reactivity assessment to be made independently of the other monomers in the assay. In other terms, in the specific case of 0.1 equiv. of all monomers compared to the starting amine, it is no longer the relative reaction rates that were looked at but the conversion of all monomers. The experiment was therefore equivalent to ten parallel Ugi-4CCs, with final assessment of the yield for each reaction. The data afforded by the case 0.1 equiv. on graphs **2.7**, **2.8** and **2.9** indeed account for the efficiency of the investigated monomer to generate the expected product, which actually is the information that is needed to keep or discard a given compound from a set of monomers foreseen to be used to build a library of discrete compounds. As each monomer could theoretically react to lead to the final product, the fact that the conversion of a given compound was different from zero but lower that 100 % means that the compound was reactive enough to lead to the product, but that a certain percentage of the reagent is lost, either because it underwent degradation prior to reaction, because the product itself got degraded subsequently to the reaction, or it had insufficient time to react.

In the case of aldehydes for instance, hydrocinnamaldehyde **2.2** and 3-methylbutyraldehyde **2.15** can be considered as efficient building blocks since they both generated the expected product in comparable quantities (excellent yields have been reported using 3-methylbutyraldehyde **2.15** in microwave assisted Ugi-4CC.)[75] All other monomers turned out to be unreactive, since no or few products were detected. In the case of cyclohexane carboxaldehyde **2.16**, only half of the starting material is converted to the corresponding α-(acylamino) amide: degradation is likely to have happened at some level of the mechanism (building block and/or product) since not all the monomer led to the product.

A similar explanation can be given for carboxylic acids, where five of the monomers (compounds **2.5**, **2.6**, **2.8**, **2.9** and **2.10**) seem to have undergone degradation at some stage of the reaction. Bulky isonitriles fail to generate the corresponding Ugi product, and all other monomers did not reach the same conversion as cyclohexyl isonitrile, meaning that part of the product or the monomer itself is lost at some point of the mechanism.

This property of ESI+/MS based analytical constructs to allow assessment of the ability of a given monomer to generate the expected product in the Ugi-4CC represents a reliable technique that can be used to improve the quality of the final library by rapidly evaluating the ability of a set of several building blocks to generate the α-acylamino amide in acceptable yield.

2.5. Fatty acid investigation

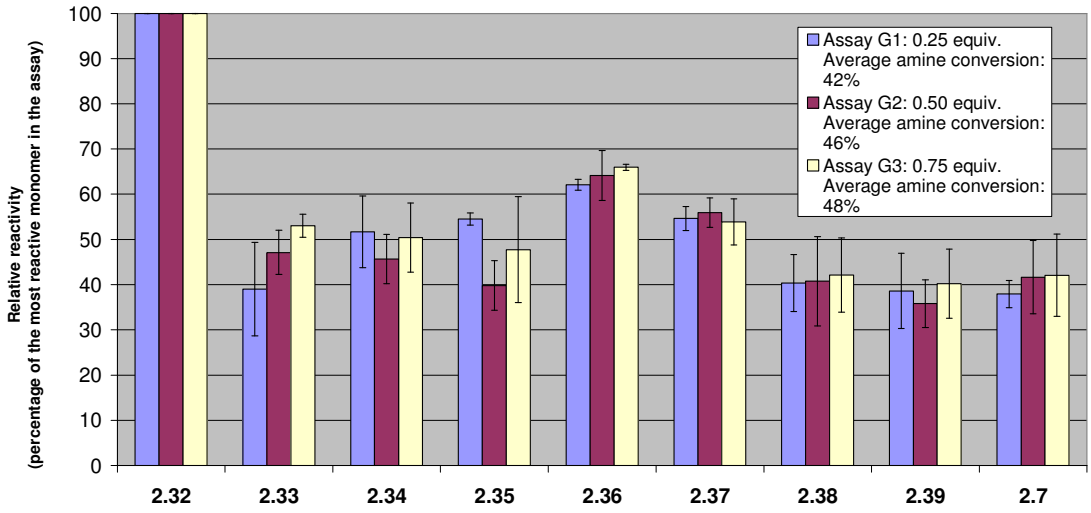
As noticed in the reactivity profiling of decanoic acid, no product was generated by this monomer in the Ugi-4CC. Therefore, a complementary study was carried out looking at the reactivity of nine fatty acids **2.7** and **2.32-39** (**Figure 2.4**).

2.32

2.33 (n = 1) **2.37** (n = 5)
2.34 (n = 2) **2.38** (n = 6)
2.35 (n = 3) **2.39** (n = 7)
2.36 (n = 4) **2.7** (n = 8)

Figure 2.4: Set of nine linear fatty acids investigated for their reactivity in the Ugi-4CC

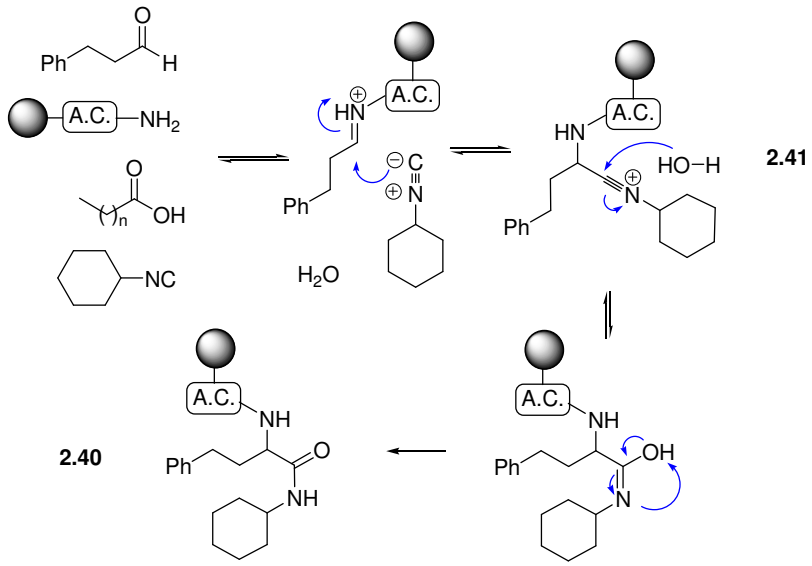
It was interesting to investigate if the chain length had any effect on the reactivity of the corresponding carboxylic acid, and the availability of a HT technique to carry out these assays enabled this to be done very rapidly. The study was carried out with the solid phase analytical construct **1.12**, the other components being hydrocinnamaldehyde **2.2** and cyclohexyl isonitrile **2.3**, under the same conditions as described in section 2.2.2. Three different quantities were evaluated (in triplicate) and the results obtained are presented in **graph 2.10**.



Graph 2.10: Solid phase reactivity profiling for nine fatty acids

The reactivity of fatty acids appeared to be globally poor, since the conversion of the starting amine did not exceed 50 %. All acids appeared to have similar reactivity, apart from acetic acid, which appeared to be twice as reactive as the other compounds.

It has to be said that the data presented in **graph 2.10** were afforded using anhydrous solvents (MeOH and CH_2Cl_2) for the microwave assisted reaction. Initially, using normal grade solvents, a similar reactivity chart was obtained, but no starting amine was detectable at the end of the reaction. It was first thought that the conversion of the resin bound substrate was complete until an unexpected brominated species (m/z = 670.5) was discovered by ESI+/MS analysis. This mass corresponded to product **2.40**, (which had never been observed in previous assays) and corresponds to the attack of water instead of a carboxylate anion on the intermediate **2.41** (**scheme 2.6**).



Scheme 2.6: Postulated side reaction to explain the formation of by-product 2.40

2.6. Conclusions

The analytical construct reactivity assessment method has shown to be very powerful and efficient in assessing the relative reactivity of building blocks for three families of components for the Ugi-4CC. The assays allowed the reactivities to be measured with high precision, (low values of standard deviations obtained), at reduced cost (limited amounts of solvents and chemical), in a very limited time frame (typically less than one hour from the stock solutions to the reactivity results for a set of monomers).

The study allowed reactivity ranking to be established among three sets of carboxylic acids, aldehydes and isonitriles in the Ugi-4CC, with the starting amine either attached to a solid support or in solution. The investigation of different concentrations permitted a study of competition effects between the monomers of the same category. This allowed very important conclusions to be drawn, such as:

- The general unreactivity of all bulky species for all families of monomers that were totally unreactive or generated very low amounts of the corresponding product. Harsher reaction conditions may be required to ensure correct

reactivity of those monomers: these conditions may be established in a HT manner using the ESI+/MS analytical construct based approach.

- The superiority of aliphatic aldehydes as entries of the Ugi-4CC, as previously reported in the literature.[64]
- The domination of the reactivity by the most "potent" carboxylic acid monomers at high concentrations.
- The link between the amount of isonitrile and the conversion of the reaction.

The increased accuracy brought by the use of solution phase analytical constructs made possible an investigation of cases where the total amount of monomer used was identical to the amount of starting material, suppressing competition effects. This allowed an investigation of the ability of a given monomer to generate the expected α-acylamino amide under specific reaction conditions.

It has to be mentioned that the study presented above was limited to ten monomers to examine the applicability of the method. However, the ESI+/MS window could allow many more species to be tested at the same time, provided the sensitivity of the detector was high enough to identify all products. Realistically, the method would be easily amenable to up to 40 compounds per assay using the same spectrometer and this number could even be increased using a more sensitive MS system. The results obtained also clearly illustrate what has been said regarding the non homogeneous composition of final mixtures in split and mix synthesis despite the equimolar composition of the starting solution of monomers. It has clearly been shown that the final composition of the mixture is far from being the equimolar one expected. The ESI+/MS analytical construct method could be used to optimise reaction conditions after gathering the monomers in groups of similar reactivity. It could also be used with any reaction meeting the requirements described above for monomer reactivity profiling, reaction rehearsal and condition optimisation.

Chapter Three

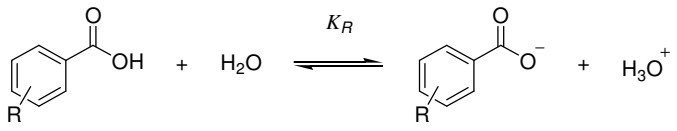
High Throughput Hammett Parameter Assessment Using Quantitative Analytical Constructs

3.1. The Hammett equation

The "Hammett equation" and the associated σ and ρ parameters were developed around 70 years ago by Louis Hammett.[2] At first, the equation allowed simple correlations to be made between the rate constants (or equilibrium constants) of two similar reactions happening on a functional group attached to a benzene ring (e.g. the carboxylic acid functionality of benzoic acids), in terms of the electronic contributions caused by substituents in the *meta* or *para* position of the ring. Thereafter, it has been adapted to more general cases so as to be applicable to *ortho* substituents for instance, as well as substrates which did not necessarily contain benzene rings, giving rise to many other useful free energy relationships (Taft, Yukawa-Tsuno, etc.).[3, 4] Thus, Hammett's parameters have turned out to be really powerful in a broad range of applications such as reaction prediction, QSAR studies, etc.[76, 77] The Hammett equation and the associated "σ" and "ρ" parameters can thus be seen as a keystone of physical organic chemistry, and the elaboration of a HT method which would allow rapid Hammett parameter evaluation, would be worthy of interest.

3.1.1. The Hammett relation and other related free energy relationships

In 1937, Hammett introduced a set of equations, based upon experimental results, that allowed the quantitative assessment of the effect of a substituent X in the *meta* or the *para* position of a benzene ring upon the rate or the equilibrium of a reaction, where the reacting group was located in a side chain attached to the ring. The classical example of this was the equilibrium of ionisation of benzoic acids as described in **scheme 3.1**.[2]



R=X or H
R *meta* or *para*

Scheme 3.1: Equilibrium of ionization of benzoic acids

The relation, as first proposed (**eq 3.1**) involved K_X the equilibrium constant (or rate constant) for a substituted reactant, K_H the corresponding quantity for the unsubstituted reactant, d the distance between the substituent and the reacting group, and ε the dielectric constant of the medium in which the reaction occurred. The quantities A, B_1 and B_2 were constants independent of temperature and solvent.

$$-RT\ln\left(\frac{K_X}{K_H}\right)=\frac{A}{d^2}\left(\frac{B_1}{\varepsilon}+B_2\right)=\Delta G \qquad \textbf{eq. 3.1}$$

In the case of equilibrium constants, **eq 3.1** allowed the evaluation of the free energy change of the reaction, and in the case of rate constants, it afforded the change in free energy of activation. The Hammett equation therefore belongs to the category of free energy relationships. Subsequently to this, Hammett simplified the relationship: as the only term in **eq 3.1** to depend on the substituent itself is A, and as the other constituents of the formula were either constants or related to the reaction, the relation could be rearranged to give the Hammett equation as it is best known, separating the substituent component from those of the reaction (**eq. 3.2**).

$$\log\left(\frac{K_X}{K_H}\right)=\rho\sigma_X \qquad \textbf{eq 3.2}$$

$$\text{where } \rho=\frac{1}{d^2T}\left(\frac{B_1}{\varepsilon}+B_2\right) \text{ and } \sigma_X=-\frac{A_X}{2.303R}$$

Under this new from, σ_x represents the intrinsic electronic effects of the substituent X in comparison to hydrogen. The second parameter, ρ, accounts for the sensitivity of the reaction to the substituent contribution σ. As Hammett's approach was totally experimental and the only accessible data was the $\rho\sigma_X$ product, an arbitrary value of $\rho = 1$ was taken for the ionisation of benzoic acids in water at 25ºC. From the extensive records kept by Dippy regarding the composition of this specific reaction

for various relevant benzoic acids,[7, 78-80] numerous σ_X were then calculated.[2] Subsequently, the methodology was extended to other reactions, provided they still respected the restrictions imparted by Hammett. **Eq 3.1** and therefore **eq 3.2** are indeed limited in Hammett's analysis to polar effects (resonance and inductive effects) of the substituents: substitution at the *ortho* position of aromatic rings for instance could not be envisaged in the study. Within those requirements, other ρ values for various transformations were calculated.

Hence, thanks to the elaboration of databases of ρ and σ values, **eq. 3.2** turned out to be successful in predicting compositions of equilibria (or reaction rates); once the composition of the equilibrium for an unsubstituted compound K_H (or its kinetic equivalent k_H) is known, **eq 3.2** enabled K_X (or k_X) to be deduced for an analogue substituted in the *meta* or *para* position whose σ_X was known. However, Hammett's initial limitations in terms of the diversity of substrates for which the equation applied turned out to be very restrictive. Since the formula was established for *meta* and *para* substituted benzenes, the experimental values of σ_X only accounted for polar electronic effects of the substituents, *i.e.* field/inductive and resonance effects. Phenomena such as steric effects were not taken into account in the calculation of σ's and the Hammett equation failed to be applicable to more general cases, and therefore needed to be extended.

3.1.2. Generalisation of Hammett linear free energy relationship

In order to enlarge the categories of substrates it covered, modifications to the Hammett equation were made to include other properties induced by the substituent X that it would be useful to consider, such as steric interactions or enhanced resonance effects. The name "extended Hammett equation" was therefore given to any multiparametric extension of **eq 3.2** that could be written as in **eq 3.3** where P is a given property such as an equilibrium or a rate constant, h the value of the property P when X is replaced by hydrogen, σ_i the property to consider, α_i representing the "weight" of parameter *i* in the final result.

$$P = \sum_{i=1}^{n} \alpha_i \sigma_i + h \qquad \textbf{eq 3.3}$$

Several examples of extended Hammett equations are available in the literature. One of the most famous was the one developed in the late 1950s by Taft, who studied the effect of the R group on the hydrolysis (or esterification) rates for aliphatic esters (RCOOR'), or benzoic esters, where R is an *ortho* substituent.[3, 4] The results obtained following this work allowed the elaboration of a free energy relationship (**eq. 3.4**) that took into account both polar and steric effect of substituents, respectively named σ* and E_s (ρ* and δ analogous to ρ in the Hammett equation).

$$\log\left(\frac{k_X}{k_H}\right) = \rho^*\sigma^* + \delta E_s \qquad \textbf{eq 3.4}$$

σ* was found to be identical to σ_p's and σ_m's defined and calculated by Hammett respectively in the case of *para* and *meta* substituents. The Taft equation therefore extended Hammett's analysis, including the steric considerations that had been left aside by only considering *meta* and *para* substituents of the benzene rings.

3.1.3. Properties and use of Hammett parameters

3.1.3.1. Hammett's σ value

Hammett's σ value allows a quantification of the electronic effects provoked by a substituent on a benzene ring (*meta* or *para* position) on a reaction centre attached to the ring. Given how the Hammett relation was originally defined, positive values of σ are characteristic of electron-withdrawing substituents, whereas negative values are for electron-donating groups. The measurement of σ_m's and σ_p's, has been afforded in almost all cases by experimental data (kinetic studies or equilibrium composition assessment) on a reaction where the ρ value was known and comparison with unsubstituted substrates, according to **eq. 3.5**. This has allowed the construction of huge databases of σ values.[81]

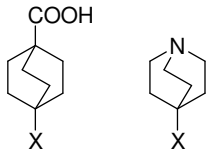
$$\sigma_X = \frac{1}{\rho}\log\left(\frac{K_X}{K_H}\right) \qquad \textbf{eq. 3.5}$$

Following the evaluation of σ's, much effort has been put into factoring the value into its component parts. Wide agreement has been achieved by splitting it into

field/inductive effect σ_I (alternatively named σ_F, σ_L, or F) and resonance contributions σ_R (also known as R).[82] Regarding σ_p, **eq. 3.6** was elaborated.

$$\sigma_P = \alpha \times \sigma_I + \sigma_R \qquad \textbf{eq. 3.6}$$

Several assessments of the value of α were carried out,[83] and in most correlations, α did not differ much from 1: σ_P can therefore be considered as the exact sum of the inductive and the resonance effect. The inductive component σ_I can be defined either by evaluation of the ionisation constant of bicyclooctane carboxylic acids **3.1**,[84] or quinuclidines **3.2**,[85] since there was little possibility for resonance or polarization interactions in such systems (**figure 3.1**).



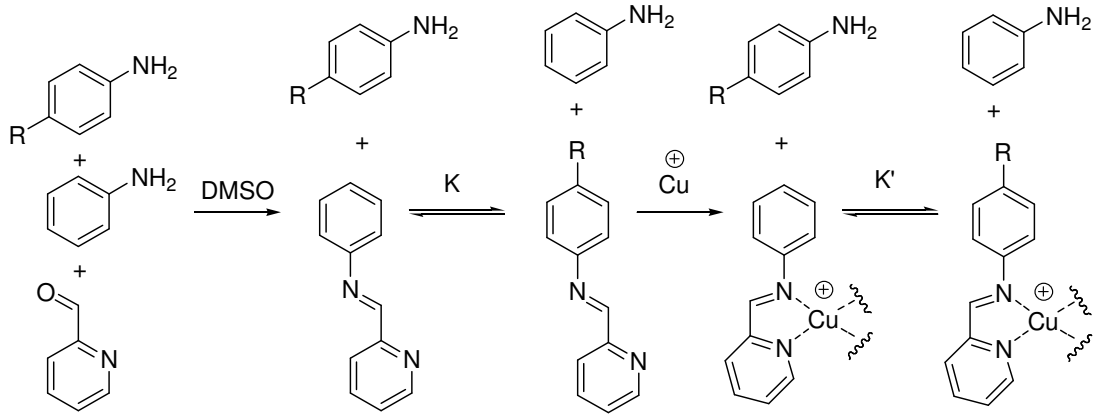
3.1 **3.2**

Figure 3.1: Bicyclooctane based compounds used to evaluate σ_I's

Fluorine NMR turned out to be quite efficient in assessing the value of the resonance electronic effect σ_R.[86] This was done by comparison of the chemical shifts of two fluorobenzenes, both substituted with the group of interest, one in the *meta* and the other one in the *para* position.

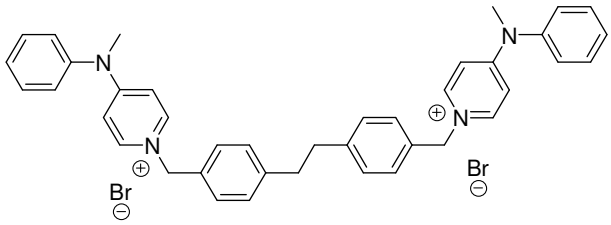
Given its ability to quantify electronic effects for a great number of substituents, and despite the limitations previously evoked, Hammett σ values have seen much application over the past decades. It has been used, as mentioned earlier, as a tool for predicting compositions of equilibria (or reaction rates). Brown showed for example amazingly good correlations between the reaction rate of aromatic electrophilic substitutions as well as electrophilic side chains reactions and the σ value for different *meta* substituted benzene derivatives, allowing predictions of the outcome of the reaction to be made on the basis of substituents constants.[87] More recently, *para* substituent electronic effects values have been used along with the Hammett equation to afford the desired degree of selectivity as well as high yields in an imine exchange reaction.[76] The composition of the equilibrium *K* between two substituted and unsubstituted 2-pyridylimines obtained by mixing together a *para* substituted

aniline, aniline and pyridine carboxaldehyde, could be successfully predicted from the σ value of the substituent (**figure 3.2**). By playing with the change of the equilibrium constant *K'* for the different successive reversible reaction induced by the introduction or the removal of Cu^{I} to the reaction mixture, controlled construction or destruction of cyclic structures was possible. The driving force provided by substituent effects could thus be used to control and shape the constitution of a dynamic combinatorial library, allowing the generation and destruction of molecular diversity within mixtures.



Scheme 3.2: Equilibria between different substituted and unsubstituted 2-pyridilimines with ans without the presence of Cu^{I}

σ values have turned out to be useful in many other applications: Campos *et al.* used σ values (from literature as well as some values afforded by a ^{13}C NMR based method developed in house) as electronic molecular descriptors in a QSAR study of bispyridinium bromides based anticancer compounds, leading to lead compound **3.3**.[77]

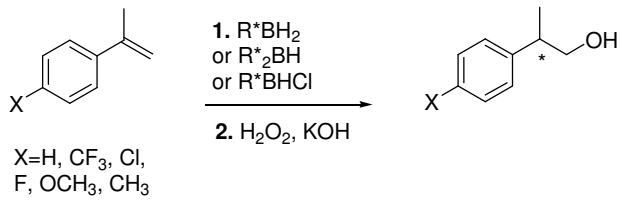


3.3

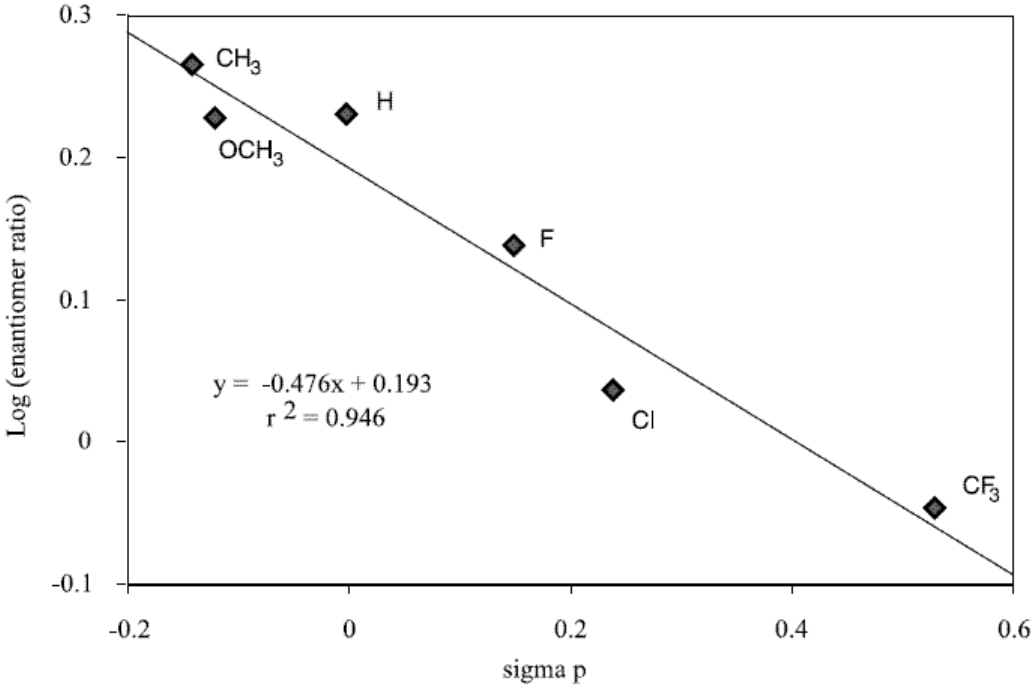
Figure 3.2: Antitumour *bis*-quaternary ammonium compound discovered with the help of σ_p's as QSAR molecular descriptors

3.1.3.2. Hammett's ρ value

As mentioned earlier, the ρ value was the starting point of Hammett's analysis with the choice of a unity value defined for the reaction of ionisation of *meta* or *para* benzoic acids in water. A positive value for ρ thus indicates that the reaction is favoured by electron-withdrawing substituents (or the equilibrium shown in **scheme 3.1** lies to the right hand side). Additionally, the sign of the ρ value supplies information regarding the reaction center at the transition state: a negative ρ value indeed means that the reaction center is electron deficient during transition state. In such a case, the presence of electrodonating substituent thus lowers the energy of the transition state, and consequently decreases the energy of activation of the reaction and increases its rate. The absolute value of ρ gives a statement of how much the reaction under investigation is likely to be affected by variations of the electronic effects of a group in the *meta* or the *para* position. ρ is entirely dependent on the type of reaction studied, as well as the conditions under which it is carried out. It is strongly influenced by the temperature, solvent, etc. The measurement of the sensitivity of a given reaction to electronic effects is afforded by the construction of so called "Hammett plots": for a series of substrates of the reaction differing only by a substituent X on the benzene ring, kinetic studies are carried out. With the values of the rates constants *k* for all substrates, a graph can be constructed by plotting $\log\left(\frac{k_X}{k_H}\right)$ versus σ_X. In the case where the reaction under investigation follows the Hammett equation *i.e.* the reaction is governed by electronic effects, the slope of the line obtained gives a quantitative assessment of the influence of X on the rate of the reaction (similar investigations can be carried out on the values of equilibrium constants in case the reaction is an equilibrium). Thanks to Hammett plots (**graph 3.1**) and the determination of ρ values, it has been demonstrated that para electronic effects, for example, have a significant influence on the asymmetric hydroboration of 2-phenyl-2-methylprop-2-enes (**scheme 3.3**).[88]



Scheme 3.3: Asymmetric hydroboration reactions for Hammett plot construction



Graph 3.1: Hammett plots obtained for a series of 2-phenyl-2-methylprop-2-enes with different *para* substituents as described in scheme 3.1 (reproduced with permission).[88]

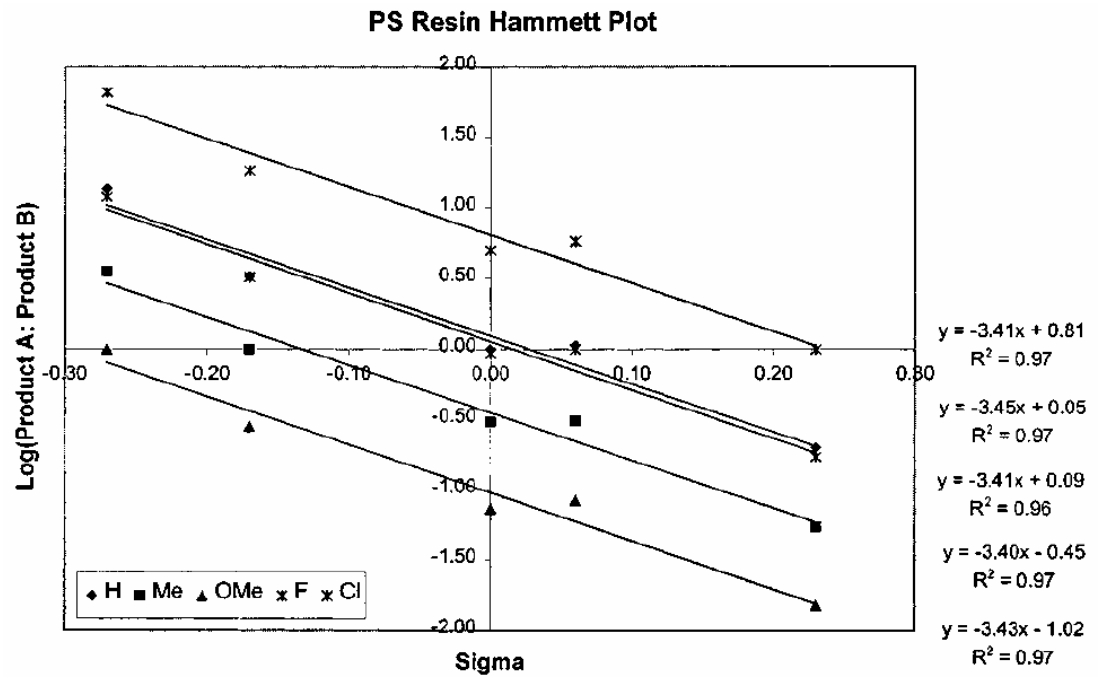
In this case, the ρ value obtained was -0.476, meaning that the formation of one enantiomer was favoured by electron-donating substituents in the *para* position of the aromatic ring. Since it is possible to quantify how much the electronic contributions could affect the kinetics of a reaction, Hammett plots can be used to optimise reaction conditions. Once determined the conditions that minimise the value of ρ, they can be used to carry out the reaction since they guarantee the best outcome. As an example of this, Gerritz developed a method based on the construction of Hammett plots to evaluate the efficiency of a range of solid supports in an acylation reaction.[89] Since the ρ value is influenced by the solvent and solid supports act like solvents,[90] it is possible to evaluate them by assessing the ρ parameter. The reaction used to do this was amide bond formation arising, as

described in **scheme 3.4**, from the competitive displacement of a solid supported Pfp ester by two different anilines, substituted in the *para* position by a group whose σ_p was known (*p*-H, *p*-OCH_3, *p*-CH_3, *p*-F, *p*-Cl). After the cleavage of the product from the solid support, the assessment of the relative quantity of products was performed by NMR spectroscopy. A "traditional" Hammett experiment would compare every member of a series of substituted benzene derivatives, (*i.e.* the *para* substituted anilines) to the standard benzene derivative (aniline), one by one. In contrast, the "combinatorial" Hammett experiment reported in the study comprises 10 competition experiments (carried out in duplicate) in which all possible binary combinations of five anilines are compared. The graph is then obtained by plotting $\log\left(\frac{k_X}{k_Y}\right)$ afforded for a fixed Y against σ_X: the slope of the line obtained is ρ, as explained in **eq 3.7**.

$$\log\left(\frac{k_X}{k_Y}\right) = \rho\sigma_X - \rho\sigma_Y = Ax + B \qquad \textbf{eq 3.7}$$

where $A = \rho$, $x = \sigma_X$, $B = \rho\sigma_Y = \text{constant}$

A value of ρ of -3.45 was determined using the data obtained *via* **Graph 3.2** as well as complementary experiments for the acylation reaction using PS Resin as the solid support. This value confirms the fact that the reaction is favoured by the presence of electron-donating groups at the *para* position of the aniline. The use of a combinatorial approach to afford quantitation of ρ permitted a dramatic increase in the speed of the method since the five lines required only ten experiments.



Graph 3.2: Combinatorial Hammett plot for one set of competition experiment conducted with PS resin (reproduced with permission)[89]

The use of "combinatorial Hammett plots" allowed the testing of seven different supports, and comparison to solution phase experiment results, whose ρ value (ρ = -3.09) was assessed by a similar method. The use of several different solid supports in the same reaction vessel with variations in the polymer used, the grafting, the presence of an hydrophilic linker, etc. (LLPS crowns, MA/DMA crowns, PS resin, PTFE tubes, PS lanterns, HLPS crowns, PS-PEG resin) allowed an evaluation of their efficiency in the same conditions. The solid support that turned out to be the most efficient towards the reaction considered was PS-PEG resin. The value of ρ of -3.09 calculated for this specific case was the same as the one assessed in the case of solution phase experiment. LLPS crowns showed increased sensitivity to substituent effects: the calculated value of ρ of -3.52 means that anilines, especially the ones with electron withdrawing groups will be harder to react using this support.

Thus, the Hammett equation, which was originally only concerned with specific chemical transformation involving a narrow range of aromatic structures, can give rise to many relations applicable to a much broader range of substrate and reactions, allowing Hammett σ and ρ values to be widely used in various areas of chemistry. However, despite the introduction of the concept of combinatorial Hammett plots, there was a need for the development of a method to evaluate Hammett parameters in

a HT manner. Until now the bottleneck has been the analytical step, and obtaining kinetic data or equilibrium compositions is impeded by the fact that very few techniques can rapidly identify compounds in mixtures. The study presented herein demonstrates the speed, efficacy and accuracy of a HT ESI+/MS based approach, using the quantitative analytical construct **3.5** derived from **1.12**, for the rapid determination of Hammett parameters (**figure 3.3**).

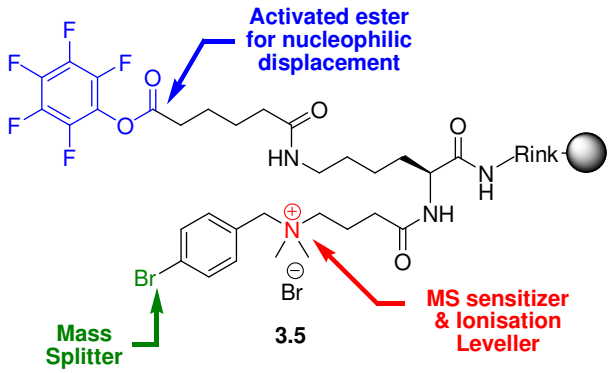


Figure 3.3: Analytical construct used in the HT determination of Hammett parameters

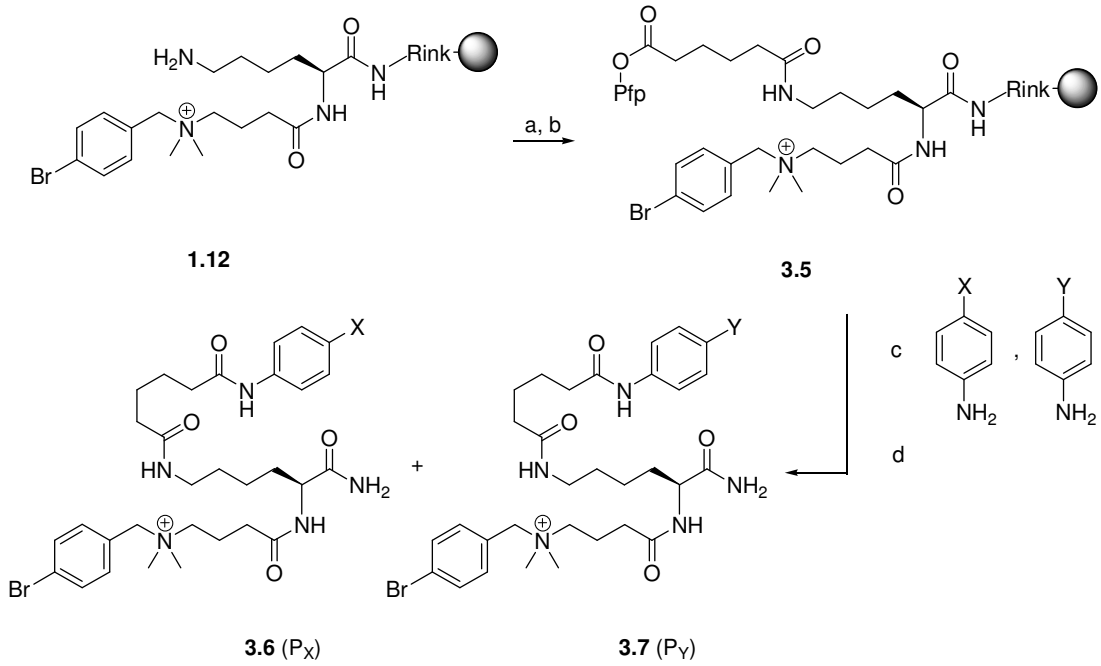
3.2. HT evaluation of Hammett ρ values

3.2.1. Description of the approach

The concept that was used to achieve the HT evaluation of Hammett parameters was adapted from what has been carried out so far in the field, with the novelty brought in by the use of analytical constructs allowing the rapid and straightforward ability to draw analytical conclusions with very high accuracy. A competitive reaction was needed, which would bring into play two species that differ only in the *meta* or *para* substituent on a benzene ring where a reactive site would be attached. The two products generated by this reaction would need to remain attached to the analytical construct. Anilines were first studied, as Gerritz had used those, for the competitive displacement of a Pfp ester moiety that could be placed on the construct. On the basis of analytical construct **1.12**, resin **3.5** was prepared according to **scheme 3.4**.

To afford the carboxylic ester functionality that was required, an amide bond formation was carried out between adipic acid (1,6-hexanedioic acid) and the free

amine of construct **1.12**. A trans-esterification reaction was then carried out using pentafluorophenyl trifluoroacetate to afford the desired active ester **3.5**. Resin **3.5** was then used as an acylating agent to form, after cleavage of the linker, two amides **3.6** and **3.7**, thanks to the attack of an equimolar mixture of anilines on the Pfp moiety, as described in **scheme 3.4**.



Scheme 3.4: Preparation of the active ester and utilisation for the acylation of anilines
Reagents and conditions: (a) Adipic acid (8 equiv.), DIC (8 equiv.), HOBt (8 equiv), CH_2Cl_2/DMF (7:3, v/v), 2 x 30 min; (b) Pentafluorophenyl trifluoroacetate, pyridine, DMF, 6 h, (c) Equimolar mixture of two anilines, DMF/Pyridine (1:1, v/v), 24 h; (d) TFA/DCM, (1:5 v/v), 15 min.

It should be noted that the two anilines were introduced in large excess compared to the analytical construct to ensure that the reaction was not controlled by anything other than the reactivity of the species involved (no diffusion limitation, shortage of one of the reagents, etc). ESI+/MS analysis of the mixture of compounds **3.6** and **3.7** and subsequent treatment of the data obtained, as mentioned in section 1.4.1., allowed the ratio between the two compounds to be calculated. As the reactions were considered irreversible and the anilines were introduced in equimolar quantities, this ratio directly correlates with the rate ratio of the competitive reactions, as it appears in **eq. 3.7**. Thus, carrying out this procedure for all possible combinations of a set of five anilines allows the construction of a "combinatorial Hammett plot" for the

assessment of the value of ρ for the reaction described in **scheme 3.4**, using the solid supported reagent **3.5**.

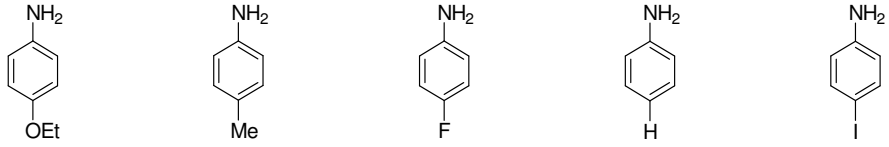
3.2.2. Construction of Combinatorial Hammett plots

The intention in these initial studies was to assess the robustness, reliability and accuracy of the approach and obtain the ρ parameter for the specific transformation (**scheme 3.3**). As the ESI+/MS analytical step will be carried out on the mixture of amides arising from the acylation of the two anilines, the choice of the set of compounds that were used to build the combinatorial Hammett plot had to meet the following requirements:

- The anilines chosen to be part of the study should not interfere with the properties of the construct, as explained in section 1.4.3.3. For instance, no Cl or Br atom could be tolerated on any of the anilines. Similarly, the anilines were chosen so that the difference in molecular weight would be at least 4 Da.
- The range of values of the σ_X's of the *para* anilines should be as broad as possible.
- It had to be kept in mind that a difference of ±1 in the absolute value of the σ_X's, for a reaction where the value of ρ would be -3.5, (as expected given the values obtained by Gerritz) would mean an expected ratio between the amides of approximately 3/1000, which would be at the limit of detection of the mass spectrometer.

Given these requirements, a set of five anilines was chosen, as described in **figure 3.3**, where the values of their σ_p's is also indicated. It should be noted here that instead of the classical σ_p's, the analogs σ_p^-'s had to be used: $\sigma_p^{\pm}$ values indeed account for the enhanced conjugation that can occur between a *para* substituent and the reaction centre, as can happen in the case of anilines and phenols.[81] This trend had been observed by Hammett himself and reported at an early stage of his research. The constants σ_p^- are defined for substituents which delocalize a negative charge, and σ_p^+ for substituents that would delocalize positive charges; they are overall

similar to σ_p's but for a few groups such as NMe_2 (σ_p = -0.83, σ_p^- = -0.12), they can vary dramatically. In the case of anilines, as enhanced delocalisation can happen between some of the *para* substituents and the lone pair on the nitrogen, the use of σ_p^- was preferred to σ_p.

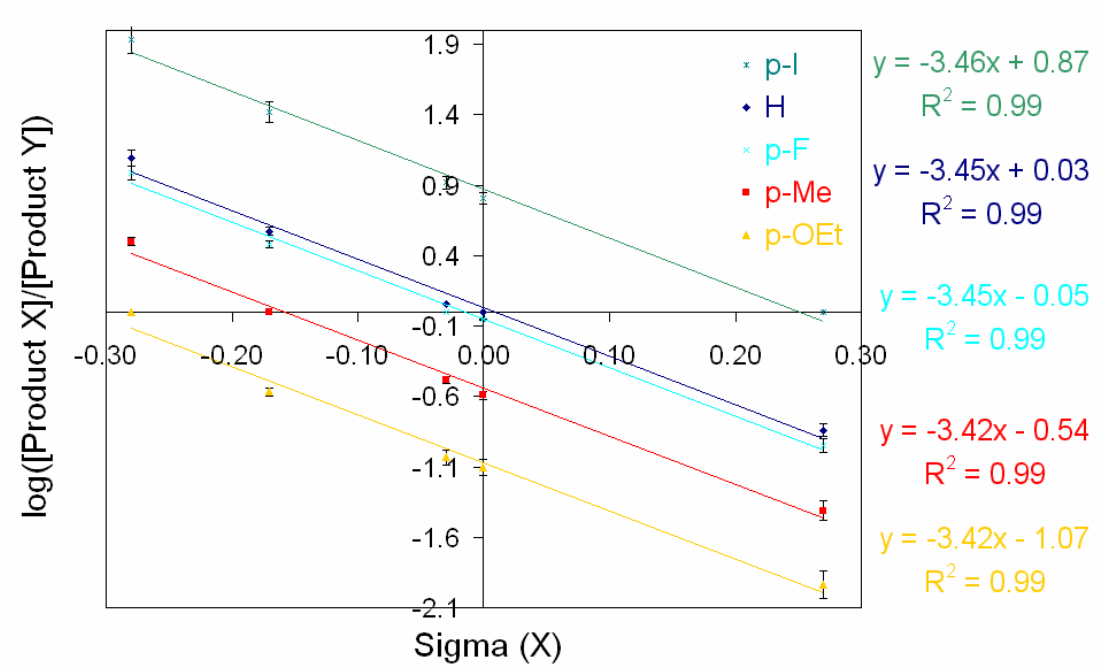


3.8	**3.9**	**3.10**	**3.11**	**3.12**
p-OEt aniline	*p*-Me aniline	*p*-F aniline	Aniline	*p*-I aniline
σ_p^- = -0.28	σ_p^- = -0.17	σ_p^- = -0.03	σ_p^- = 0	σ_p^- = 0.27
σ_p = -0.24	σ_p = -0.17	σ_p = 0.06	σ_p = 0	σ_p = -0.18

Figure 3.4: *Para* substituted anilines used for the construction of Hammett plot

The method thus consisted of competitive amide bond formation reactions being carried out for all possible combinations of *para*-ethoxyaniline **3.8**, *para*-methylaniline **3.9**, *para*-fluoroaniline **3.10**, the unsubstituted aniline **3.11** and *para*-iodoaniline **3.12**. The ten reactions were performed in a HT manner, enabling all combinations to be carried out at the same time, and in quadruplicate in order to determine reproducibility and ensure the accuracy of the methodology. This aspect was fundamental, not only because the throughput of the method must be as high as possible, but more importantly to minimize experimental error. Because of the dependence of the ρ value on the temperature, all reactions had to be carried out under the same conditions, which was guaranteed by the fact that all 40 reactions were conducted simultaneously. After the reaction sequences, quantitative composition of the mixtures by ESI+/MS was performed as explained in section 1.4.3.1. The Hammett plots were constructed as follows: for each aniline Y, all possible values of $\log\left(\frac{k_X}{k_Y}\right)$, as well as the "zero" value (for X = Y) were plotted against the $\sigma_{p\text{-}X}^-$'s and the slope of the line calculated by linear regression. It must be mentioned that very small standard deviations were observed and that the experimental values obtained for the combinatorial Hammett plot (**graph 3.3**) can

therefore be considered as highly reliable. Very good regression coefficients were obtained (R = 0.99 for all assays), giving confirmation that electronic effects had a direct influence on the reactivity of the starting aniline and therefore on the reaction rates. The value of the ρ parameter (ρ = -3.44) was calculated by taking an average of the five slopes obtained. The negative sign for ρ meant that, for the reaction considered, the reaction center is electron deficient.



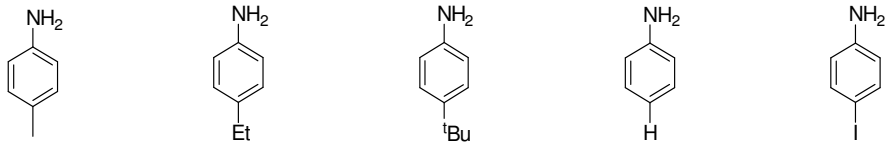
Graph 3.3: Combinatorial Hammett plot for the assessment of the ρ value

This value was very satisfactory since it was in perfect agreement to what had been obtained by Gerritz in the case of a study of solid supports:[89] ρ = -3.45 was obtained for PS resin, which is the resin that was used for the experiments reported here.

Despite good results, with 40 reactions to do only to assess the value of the reaction parameter, the speed of the method seemed a bit limited. One pot combinatorial Hammett plots were thus developed to be able to assess the value of ρ by means of a single reaction.

3.2.3One pot combinatorial Hammett plot

In order to significantly increase the speed of the construction of combinatorial Hammett plots, a single pot assay was carried out in quadruplicate, with five different anilines in large excess but in equimolar quantities, as shown in **figure 3.5**. It must be said that this assay was conducted after the assessment of the value of σ_p's using quantitative analytical constructs presented in section 3.3.2.had been performed. Therefore values used here are those obtained experimentally, since they turned out to give slighlty better correlations coefficients.



3.13	**3.9**	**3.14**	**3.11**	**3.12**
p-Me aniline	*p*-Et aniline	*p*-^{t}Bu aniline	Aniline	*p*-I aniline
σ_p^- = -0.17	σ_p = -0.15	σ_p^- = -0.13	σ_p^- = 0	σ_p^- = 0.27

Figure 3.5: *para* substituted anilines used for the construction of the single pot Hammett plots

Cleavage was performed to yield a mixture of five amides with different *para* substituents whose ESI+/MS analysis afforded the spectrum represented in **figure 3.6**.

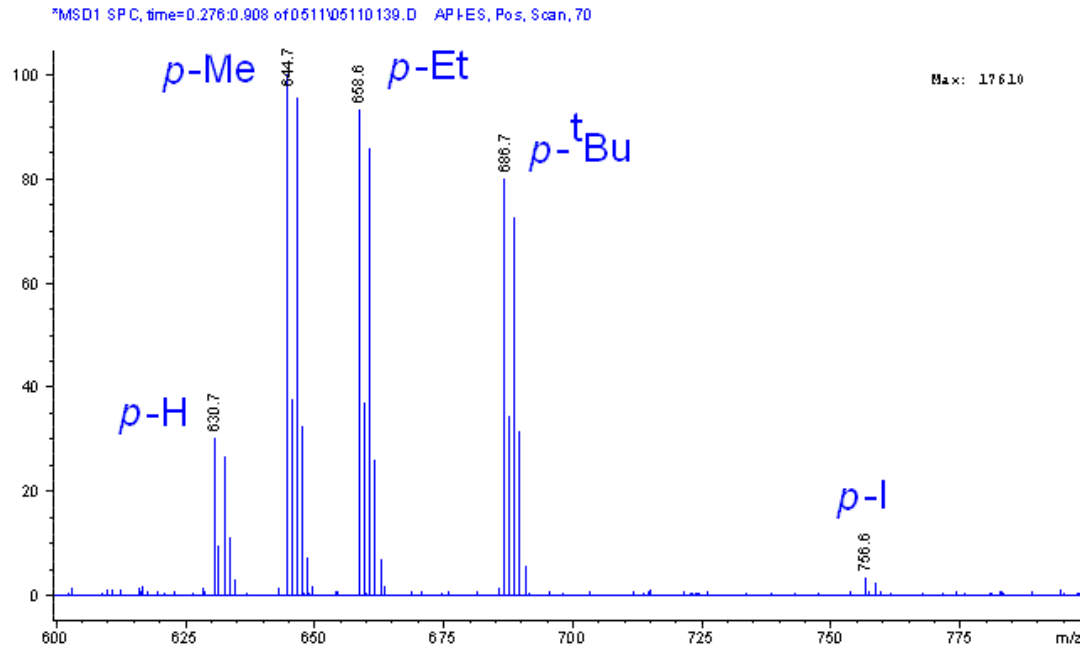
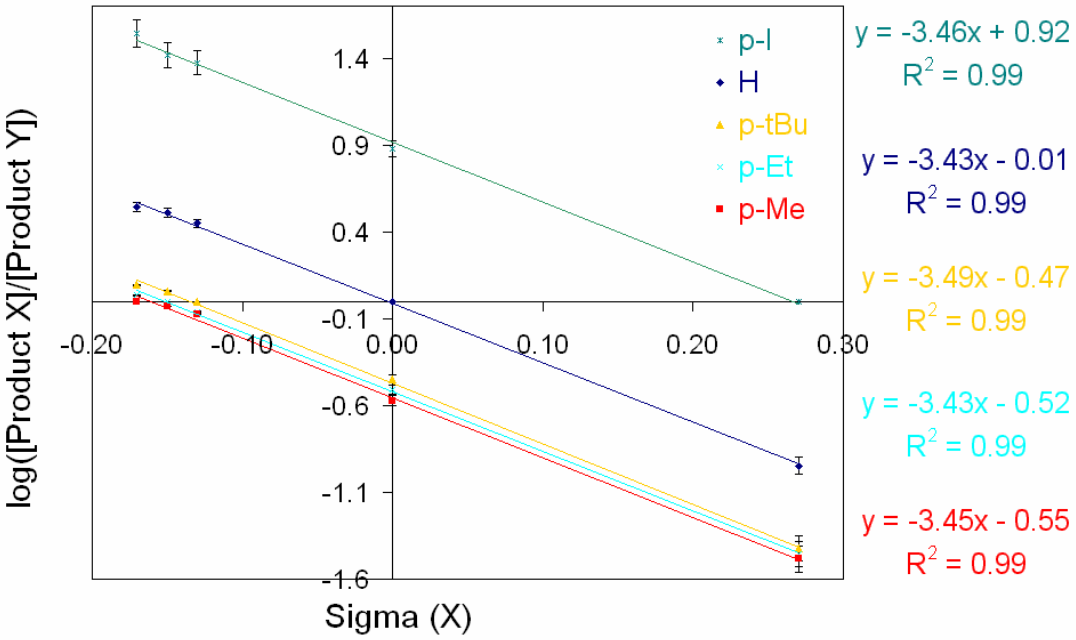


Figure 3.6: ESI+/MS spectrum obtained for the analysis of the mixture of amides obtained for the single pot assay using five anilines

All five products were present in different quantities, due to the different effects of the *para* group on the reactivity of the starting aniline. The extraction of the peak intensities and the correlation to the relative quantities of the corresponding products permitted a combinatorial Hammett plot to be constructed, as previously described (**graph 3.4**).



Graph 3.4: One pot combinatorial Hammett plot for the assessment of the ρ value

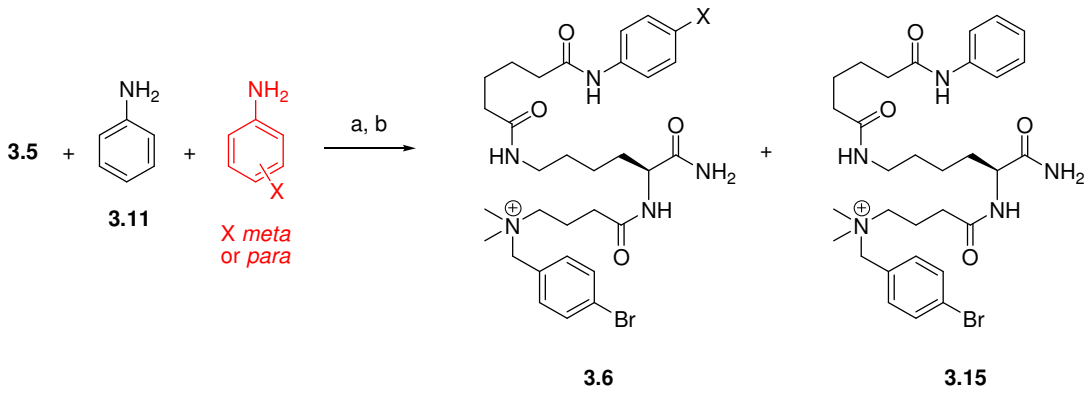
All slopes were obtained with very satisfactory correlation coefficients (R = 0.99 for all assays), demonstrating the high reliability of the MS tool to assess competitive reaction rates with essentially identical ρ values obtained for the reaction. The average value of ρ = -3.45 was essentially identical to what was obtained in the case of the combinatorial Hammett plot described in section 3.2.2. However here, instead of carrying out forty tedious reactions, workups and ESI+/MS analyses, only four were performed. One pot combinatorial Hammett plots allowed the reliable evaluation of Hammett ρ value in a HT manner since they afforded the same amount of data as the classical approach and yet reduced the number of steps required to afford this data by ten.

With this value of ρ = -3.45 now known, it was then possible to take the method to the next step, consisting of the HT assessment of the Hammett σ parameter.

3.3. HT evaluation of Hammett σ^- values

3.3.1. Description of the approach

With the value of the reaction parameter ρ known for the conditions described in **scheme 3.4**, HT σ value evaluation could be carried out by competition experiments between the aniline of interest and the unsubstituted analogue **3.11**, as described in **scheme 3.5**. Equimolar quantities of the two species were added in large excess compared to the analytical construct resin **3.5**. After reaction, the resin was washed and the mixture of amides **3.6** and **3.15** cleaved from the solid support.



Scheme 3.5: Amide bond formation using 3.5 as acylating reagent for the assessment of Hammett parameters. Reagents and conditions: (a) DMF/Pyridine (1:1, v/v), 24 h; (b) TFA/DCM, (1:5 v/v), 15 min

ESI+/MS analysis was then carried out and treatment of the MS intensities as described in section 1.4.3.1 gave rise to the the ratio between the two products, which equals $\frac{k_X}{k_H}$. The assessment of the σ_X^- value was then made possible using the Hammett equation, transformed as it appears in **eq. 3.8**.

$$\sigma_X^- = \frac{1}{\rho}\log\left(\frac{k_X}{k_H}\right) \qquad \textbf{eq. 3.8}$$

For each aniline under investigation, four similar assays were carried out to allow the assessment of standard deviations and to determine the repeatability of the method.

In the case of the evaluation of the σ parameters for *p*-Me aniline, the ESI+/MS spectrum obtained is presented in **figure 3.7**.

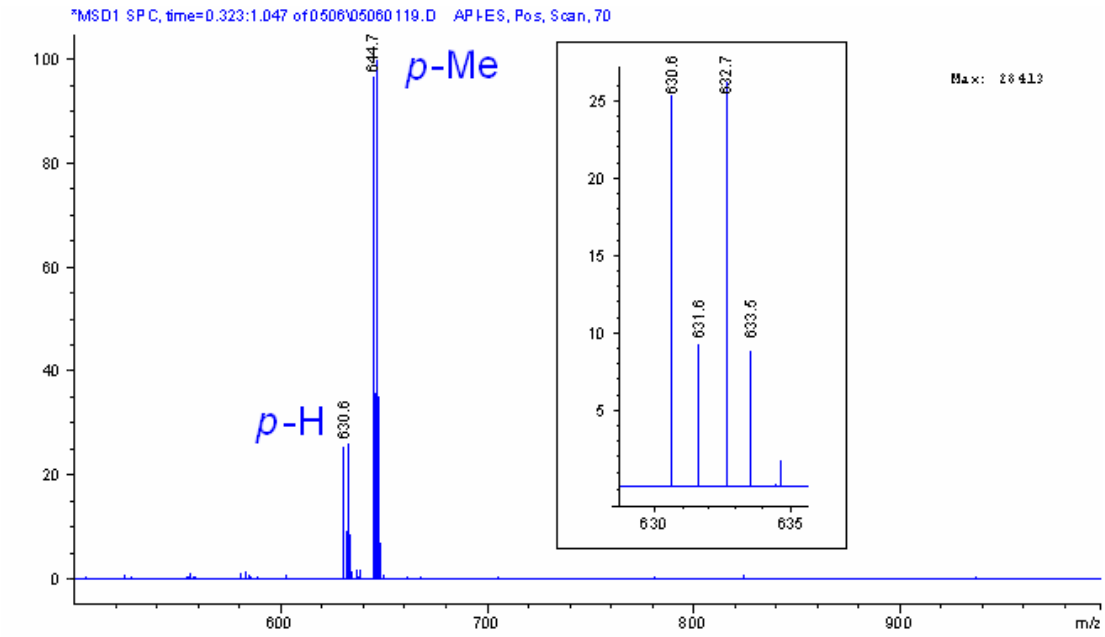


Figure 3.7: Example of ESI+/MS spectrum obtained in the case of a competition experiment between *p*-Me aniline (X = Me) and aniline in the conditions described in scheme 3.5.

From the values of the peak intensities of the spectrum in **figure 3.7**, as well as the data of the other three assays, the assessment of the value of $\sigma_{p\text{-}Me}^{-}$ was -0.17±0.01. This value was in perfect agreement with literature values of $\sigma_{p\text{-}Me}^{-}$ of -0.17.[81] HT assessment of σ_X^{-} was thus carried out for various anilines, with groups at the *para* as well as the *meta* position. The Hammett equation indeed also applies in the latter case, allowing the use of **eq. 3.8** for the evaluation of the electronic parameter for *meta* substituent.

Several different anilines holding substituents in the *para* or the *meta* position, with as much diversity as possible were therefore assayed: this allowed the evaluation of the robustness and reliability of the ESI+/MS Hammett substituent parameter assessment method, and the determination of the limitations to the method.

3.3.2. HT evaluation of σ_p^-'s

The first series of anilines to be tested were *para*-alkyl and *para*-aryl anilines (*p*-Me as presented earlier, *p*-Et, *p*-^{t}Bu, *p*-iPr, *p*-Trityl) as well as four halogenated substrates (*p*-F, *p*-Cl, *p*-I, *p*-CF_3), following a protocol described in **scheme 3.5**. Among these nine groups, four were electron donating and were therefore expected to enhance the nucleophilicity of the amine functionality of the aniline. The results that were obtained after ESI+/MS and analysis of the peak intensity values turned out to be excellent in comparison with literature,[81] as presented in **table 3.1**.

Table 3.1: Assessment of σ_p^-'s using the HT analytical construct method for the first series of compounds

Substituent	σ_p lit.[81]	σ_p^- lit.[81]	σ_p^- exp.
Me	-0.17	-0.17	-0.17±0.01
Et	-0.15	-0.19	-0.15±0.01
^{t}Bu	-0.20	-0.13	-0.13±0.02
iPr	-0.16	-0.16	-0.16±0.01
Trityl	0.02	N/A	-0.05±0.01
F	0.06	-0.03	-0.02±0.01
Cl	0.23	0.19	0.19±0.01
I	0.27	0.27	0.27±0.01
CF_3	0.54	0.65	0.64±0.01

As **Table 3.1** shows, the standard deviations obtained were low enough to permit reliable evaluation of the electronic contribution of the investigated groups on the aniline rings, by means of Hammett's σ_p^- calculation. The values obtained experimentally were in very good agreement with literature values.[81] All values indeed did not differ from the expected σ_p^-'s by more than 0.01, apart from the case of *p*-Et substituent that surprisingly fitted better with the value of σ_p. Attention has to be drawn on the last entry of the table: the extremely deactivating character of the *p*-CF_3 group indeed resulted in a ESI+/MS spectrum as appears in **figure 3.8**. The ratio between the two solid phase amides obtained and cleaved from the resin was less than 1/150, and yet the MS method allows this to be determined directly. It is

important to note here the key advantage of the sensitivity of the ESI+/MS method with a level of detection hardly achievable by any other analytical tool.

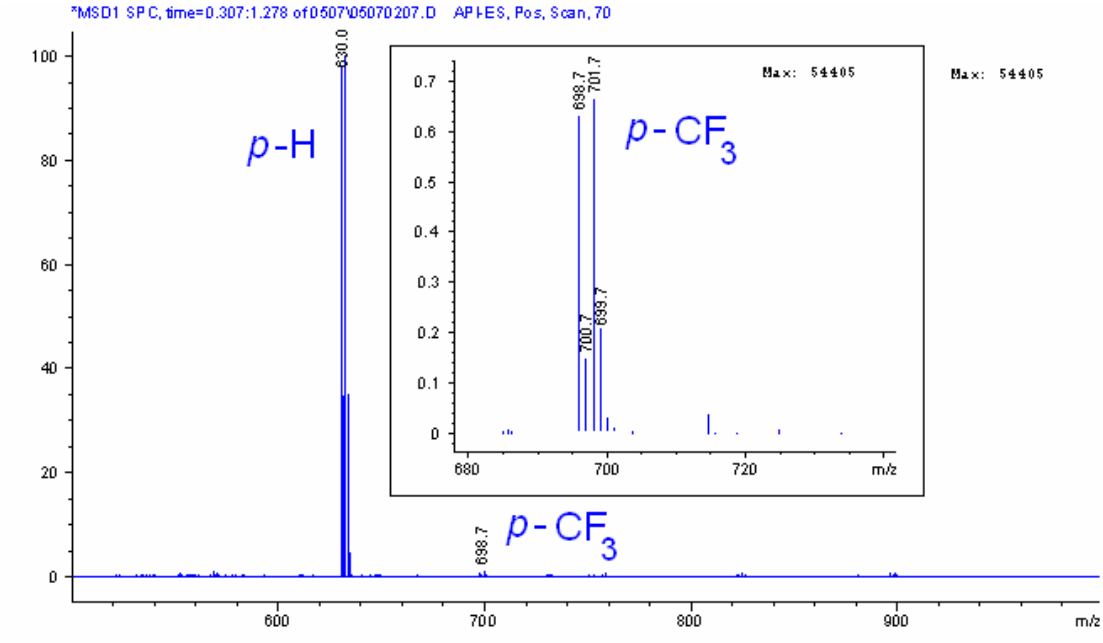


Figure 3.8: Example of ESI+/MS spectrum obtained in the case of a competition experiment between *p*-CF_3 aniline (X = CF_3) and aniline in the conditions described in scheme 3.5.

Moreover, it is important to note the inclusion in the study of *p*-Cl aniline, a species that would change the shape of the ESI+/MS peak of the final amide. It seemed interesting to check if the quantitative properties of the analytical constructs were kept in the case of a change in peak aspect. The results that were obtained were in good agreement with expectations, which seemed to indicate that the possible addition to the study of species that would induce a change on the shape of the peak of the final amide, provided it was known beforehand. It was indeed fundamental to take into account all relevant ESI+/MS peaks induced by the product for efficient quantitation, *i.e.* in case of *p*-Cl substituent one peak more than other substituents.

Given the excellent results obtained with the first series of anilines, a second series of substrates was put under investigation, involving more complex substituents, again at the *para* position of the aromatic ring of the aniline. A range of electron donating (*p*-OH, *p*-OMe, *p*-OEt, *p*-O*n*Pr) and electron withdrawing (*p*-OCF_3, *p*-$OCHF_2$) oxygenated substrates were therefore evaluated in the same manner. Two other

species, *p*-SMe and *p*-NMe_2 were also included to the study to confirm the applicability of the HT method to a broad range of substrates. The results obtained are shown in **table 3.2**.

Table 3.2: Assessment of σ_p^-'s using the HT analytical construct method for a series of *para*-alkoxy anilines, *p*-SMe and *p*-NMe_2 anilines

Substituent	σ_p lit.[81]	σ_p^- lit.[81]	σ_p^- exp.
OH	-0.33	-0.37	-0.37±0.01
OMe	-0.27	-0.26	-0.27±0.01
OEt	-0.24	-0.28	-0.31±0.01
O*n*Pr	-0.32	N/A	-0.31±0.01
OCF_3	0.35	0.27	0.27±0.01
$OCHF_2$	0.18	0.11	0.09±0.01
SMe	0.00	0.06	0.05±0.01
NMe_2	-0.83	-0.12	-0.13±0.01

Similarly to the alkyl and halide derivatives, the results obtained were found to be in good agreement to the values reported in the literature for these substituents.[81] Once again, the standard deviations obtained for these assays were very small since they did not exceed 0.01. The divergence from literature values did not exceed 0.02, apart from the case of *p*-OEt were it reached 0.03.

It has to be noted here that the evaluation of the electronic effects afforded by the HT method unquestionably accounted for the extra conjugation that some *para* substituents can have with the nitrogen of the aniline: in the case of the *p*-NMe_2 substituent for example, this value of σ_p is shifted from -0.83, which is characteristic of a very strong activating group to -0.12 which makes the NMe_2 group a moderate activator of the nucleophilicity of the amine when in the *para* position.

Using the same methodology, the assessment of the σ value was undertaken for substituents in the *meta* position of the aromatic ring of anilines.

3.3.3. HT evaluation of σ_m's

Various *meta* substituted anilines were reacted one by one with the analytical construct **3.5** in the presence of an equimolar quantity of aniline, as it appears in **scheme 3.5**. The workup and analysis of the data generated by the HT protocol (all reactions were run in parallel) was carried out as for *para* substituents.

Investigations were carried out on substituents similar to the ones described in the case of *para* substituents. Alkylated substrates *m*-Me, *m*-Et and *m*-^{t}Bu as well as halides *m*-F, *m*-Cl (since the study of compounds modifying ESI+/MS peak shape was allowed) and m-I and the alkyl halide *m*-CF_3 were thus included in the study. Additionally, *m*-OH as well as some alkoxy substituents, *m*-OMe and *m*-OEt, *m*-OCF_3 and *m*-$OCHF_2$ were assayed. Finally, *m*-SMe and *m*-NMe_2 were also included in the series of *meta* substituted anilines. The results obtained by the ESI+/MS method are gathered in **table 3.3**.

Table 3.3: Assessment of σ_m's using the HT analytical construct method

Substituent	σ_m lit.[81]	σ_m exp.
Me	-0.07	-0.04±0.01
Et	-0.07	-0.05±0.01
^{t}Bu	-0.10	-0.12±0.02
F	0.34	0.31±0.01
Cl	0.37	0.36±0.01
I	0.35	0.31±0.01
CF_3	0.43	0.40±0.01
OH	0.12	0.05±0.01
OMe	0.12	0.07±0.02
OEt	0.10	0.05±0.01
OCF_3	0.31	0.36±0.01
$OCHF_2$	0.31	0.29±0.01
SMe	0.15	0.12±0.01
NMe_2	-0.16	-0.13±0.01

The agreement between the assessment of electronic effects for *meta* substitution of anilines and the literature turned out to be a little less good than it was in the case of *para* substituents. The ESI+/MS based method was still highly reproducible since the standard deviations that were calculated for all assays (**table 3.2**) did not exceed 0.01. However, the quality of data obtained in terms of agreement with literature was less good than in the case of *para* substituents. Most results indeed turned out to be different from expected values by typically 0.03, sometimes overestimated, sometimes underestimated. Generally speaking, the evaluation of the Hammett substituent parameter for alkoxy substituents at the *meta* position did not bring results that compared perfectly with what has been reported until now.[81] However, emphasisis has to be put on the fact that the literature values that were used as comparison points are the results of experimental determination as well: there might be some imprecision on these values since the case of *meta* substituents has been covered by fewer experiments than *para* substituents. Secondly, the biggest gap between the litterature value of σ_m and experimental one afforded by the ESI+/MS technique was equal to 0.07 (for the *m*-OH substituent). This means an experimental difference, in terms of the amount of amide detected and the theoretical amount of product, of only 5 %.

3.3.4. Scope and limitations of the method

Limitations arise for two main reasons. First, the method of assessment is based on an acylation reaction using a quantitative analytical construct, with ESI+/MS as a detection method, imposes some restrictions on the nature of the candidates for the Hammett parameters assessment. Thus, the investigated aniline must comply with the requirements imposed by the reaction conditions, since the value of the reaction parameter ρ = -3.45 was evaluated for those conditions and only makes sense under the conditions specified in **scheme 3.5**. This prevented for example, *p*-COO^- aniline from being tested since this compound happens to be insoluble in the reaction solvent. Furthermore, because of the use of an ESI+/MS based method, charged substituents were excluded from the study, because of the possibility of multiple charged compounds in the final MS spectrum. Substituents prone to partial ionisation

such as thiol based ones had to be removed from the study due to the fact that they could get partially ionised under the reaction conditions, indeed creating a species (*p*-S^- aniline in the case of *p*-SH aniline for example) which behaves completely differently in the acylation. For instance, the evaluation of the Hammett parameter for the *p*-SH group gave a value of σ_p^- of -0.18 whereas a value of 0.15 was expected. Even if only a small percentage of the thiol group happens to be ionised in the reaction conditions, this leads to a completely inconsistent experimental value of σ_p^-. Compounds that were readily deprotonated, such as substituents incorporating carboxylate functionalities, like *p*-CH_2COO^-, *p*-$(CH_2)_2COO^-$ and *m*-CH_2COO^-, could not be evaluated either despite some hope that it might be feasible (these substituents would remain deprotonated during the acylation reaction and the final acidic cleavage would generate the final amide with the carboxylic acid protonated, avoiding the formation of a double charged species). Eventually, the expected product was detected only under its single charged form following ESI+/MS, but the subsequent analysis of the data generated gave inconsistent values of σ_p^- (0.13 instead of -0.16 for *p*-CH_2COO^-, 0.02 instead of -0.07 regarding *p*-$(CH_2)_2COO^-$ and 0.17 instead of 0.07 in the case of *m*-CH_2COO^-). It should be mentioned here that this had been already observed by Hoefnagel who reported that the simple Hammett equation poorly applies in the case of charged substituents.[91] Since the ESI+/MS analytical construct method has been built on the simple Hammett equation, as mentioned in section 3.2.1, such a deviation from literature values is thus not surprising.

The second limitation is due to the logarithmic character of the Hammett equation itself. It was indeed impossible to assess relative quantities of compounds whose ratios were less than 1/150 with enough reliability. This prevented direct assessment of Hammett substituent parameters for any compound whose σ^- values were over 0.65. At this level of detection, ESI+/MS already demonstrates quite eloquently its superiority over other analytical techniques. However, going beyond this limit induces a vanishing of the peak of interest into the background noise and prevents any reliable intensity measurement. This drawback however could be solved by the replacement of aniline as the reference by a substituted aniline species that has a

reactivity closer to that of the investigated compound (provided it is known in advance).

3.4. Conclusions

The use of an acylating agent linked to quantitative analytical constructs allowed the evaluation of Hammett parameters in a HT manner. Firstly, the introduction of the concept of one pot combinatorial Hammett plots afforded fast and reliable evaluation of Hammett's reaction constants using five anilines in one pot. The values obtained were consistent with previous assessments carried out in a more classical manner, under comparable reaction conditions. One pot combinatorial Hammett plots allowed data to be generated in a single assay with the same amount of data as 10 reactions being generated. Once this value was assessed, HT Hammett σ parameter determination was undertaken for a panel of thirty anilines with substituents in the *para* and the *meta* positions of the aniline. The method could be used on substituents with a broad diversity since alkyl, halides, alkoxy and nucleophilic species were all successfully assayed. For all substituents, the reproducibility of the technique turned out to be excellent. The quantitative analytical constructs method allows the fast and reliable assessment of electronic effects, and therefore permits the rapid evaluation of Hammett parameters for new "customised" groups, where literature values are not available. Furthermore, the result of the superposition of several different substituents in the *meta* ant the *para* positions of the aromatic ring can be assessed by means of quantitative analytical constructs allowing the the linearity of the Hammett relationship to be investigated.

Chapter Four

Approaches to High Throughput Protease Kinetics Assessments Using Quantitative Analytical Constructs

4.1. Introduction

Large scale genomic and proteomic analysis has recently revealed the huge number of proteins existing in nature. On top of those directly encoded by the human genome, whose initial sequencing suggested the presence of 30000-40000 genes,[92] have to be added those resulting from post-translational modifications. The establishment of the function of these proteins is and will be one of the biggest challenges of research in years to come. It is indeed fundamental to understand the mechanism and the target of a given protein to be able then to interact with it in order to influence a biological response, such as a disease or a malfunctioning. In order to start undertaking this huge task, scientists have begun to gather proteins into groups of similar function, determined by sequence similarity.[93] Doing so, general methods can be applied to investigate as efficiently as possible these proteins, allowing the elaboration of specific High Throughput (HT) tools to be used on a given family of proteins in order to increase the efficiency and the speed of their analysis and the understanding of their mode of action. Among all these families of proteins, enzymes are among the most interesting to investigate because of their function in many biological processes. In the huge family of enzymes, proteases occupy a major place.

4.1.1. Proteases: an important family of enzymes

Proteases are a class of enzymes that occur naturally in all organisms and constitute 1-5% of the gene content. Proteases catalyse the hydrolysis of amide bonds between two amino acids, with high specificity regarding the sequence of these amino acids. There are currently six known classes of proteases: serine proteases, threonine proteases, cysteine proteases, aspartic acid proteases, metalloproteases and glutamic acid proteases. The mechanism to cleave a peptide bond involves the attack at the

carbonyl group by a nucleophile, which can be either a water molecule (in the case of aspartic acid, metallo- and glutamic acid peptidases) or an amino acid side chain (in the case of serine, cysteine and threonine peptidases). In the latter case, the cleavage involves a catalytic triad, where a histidine residue is used to activate the serine, cysteine or threonine residue. Proteases have numerous functions in a living organism. Thanks to proteases and selective peptide cleavage, biological cascades can be triggered or stopped, ensuring the regulation of biological processes, such as homeostasis.[94] Protease activated receptors for instance play an important role in the control blood coagulation, whose malfunctioning can have dramatic consequences. Proteases also regulate the amount of active hormone being released from the available "stock" of prohormones since their action generates active peptide hormones regulating many physiological processes.[95] In the case of apoptosis, certain caspases deactivate proteins responsible for cell maintenance and DNA repair, therefore leading to cell death.[96] Many other examples could be given as proteases are essential to many key processes of living organisms and are necessary to cellular life and activity.[95]

From a therapeutic point of view, proteases account for 5-10 % of all drug targets, with applications in hypertension and cancer,[97] HIV and other viral processes,[98] neurodegenerative diseases,[99] etc. They therefore represent an increasingly interesting target,[100] and much effort has concentrated in investigating protease substrate specificity. This knowledge indeed helps a better understanding of the mode of action of proteases.

4.1.2. Protease substrate specificity

A characteristic function of proteases is their ability to discriminate among many potential substrates, termed the substrate specificity of a protease. Substrate specificity is a critical factor that maintains the fidelity of the biological processes in which a protease acts. For researchers, substrate specificity can serve as a handle by which a protease can be discriminated from others in its class, even in cases in which a large degree of structural homology exists. Moreover, the knowledge of the substrate specificity of a protease can greatly enhance the understanding of its

function. The protease's natural substrate can therefore be identified and the action of the enzyme can be more easily understood in a cascade of reactions. From another point of view, the discovery of new selective substrates of the protease can also be very interesting since it allows the elaboration of protease inhibitors (by the use of peptidomimetic strategies for instance to build up the candidate).

A specific nomenclature is given to a peptide that incorporates a scissile amide bond: each amino acid of the sequence is denominated by the letter P, with a number being attributed to it, according to its proximity to the cleavage centre. Depending on whether it is located at the N part or the C part of the cleavable bond that is designated, a "prime" is added to the denomination (N part). A similar principle is used with the letter S for the enzyme subpockets accommodating the amino acids of the proteolytic substrate previously mentioned (**figure 4.1**).

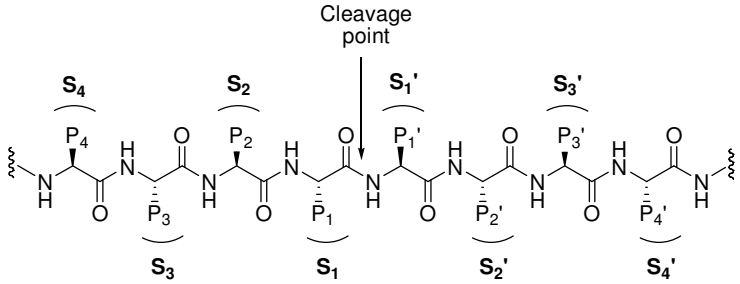


Figure 4.1: Standard nomenclature for protease substrate cleavage

This specificity of a protease not only concerns the two amino acids involved directly in the cleavage (P_1, P_1') but also the positions next to them (P_2, P_2', P_3, P_3', etc.) which can make a protease highly selective for a specific determined sequence. The investigation of the substrate specificity of a protease consists of the assessment of its preference regarding the P and P' amino acid positions of a peptide chain. This allows establishing either a unique substrate for very specific proteases, or trends regarding the affinity of amino acids for the S and S' subpockets (hydrophilicity, size allowance, etc.) for more general proteases. Several techniques have been used to analyse protease substrate specificity: the development of tools allowing the production of huge numbers of different peptide based species has indeed made possible the HT screening of parallel or split and mix libraries against a given protease. The most common of those techniques are explained below.

4.1.2.1. Direct use of peptide pools

The development of tools permitting the preparation of pools of synthetic peptides such as combinatorial methods,[101] has made possible the direct screening of mixtures of peptides.[102] After reaction, the substrates that present an affinity for the enzyme are left with a free amino group and therefore submitted to Edman degradation,[103] a technique that allows sequence determination for any peptide with an unprotected α-amino group. This method provides information about the so called "prime side" specificity of the enzyme (**figure 4.1**), the first round of sequencing giving information on the P_1' position, the second on the P_2', etc.

4.1.2.2. Use of irreversible inhibitors

The use of an irreversible inhibition strategy and positional scanning permitted Nazif to determine the substrate specificity of serine proteases.[104] Assays were carried out on individual or competing substrates by replacing the "prime part" of the peptide by a vinyl sulfone moiety, using an invariant asparagine at the P_1 position to direct the attack of the protease. The peptides sequence whose amino acids in the P_2, P_3, etc. positions had an affinity for the protease ended up covalently attached to the active site. The effectiveness of the peptide vinyl sulfone as an inhibitor and therefore as a substrate mimic was visualized by the addition of a general radiolabelled inhibitor subsequently to the assay, which covalently modified any remaining active site nucleophiles of the protease. The degree of affinity was quantified by separation of the protease *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and determination of the decrease in radioactivity compared to the standard, which corresponded to 100 % radiolabelled inhibitor.

4.1.2.3. FRET based peptides substrates

The use of fluorescence resonance energy transfer (FRET) for internal fluorescence quenching is very efficient in determining protease substrate specificity. The basis of the approach is the preparation of a given peptide labelled on one end with a fluorophore donor and on the other end with an acceptor quenching the fluorescence of the donor. Upon cleavage of the peptide, a significant increase of fluorescence is

observed at the wavelength of the donor in the case where the sequence corresponds to the enzyme specificity and subsequent sequencing of the selected peptide by Edman degradation affords the substrate specificity of the enzyme target.[105] This concept has been applied by numerous research groups, making use of polymers allowing enzymatic assays to be performed directly on the solid support, affording for instance the substrate specificities of the serine protease subtilisin Carlsberg,[106] *Escherichia coli* leader peptidase and napsin A.[107] The combination of the FRET detection method with peptide nucleic acid (PNA) tagging of the different peptidic sequences on a microarray slide as carried out by Diaz-Mochon, has significantly increased its power.[108] The encoding of each amino acid by a PNA triplet indeed allowed 10,000 peptides arising from split and mix solid phase synthesis to be screened with chymopapain and subtilisin, using the carboxyfluorescein (FAM) and Tetramethyl-6-carboxyrhodamine (TAMRA) FRET system (**figure 4.2**). The use of PNA codes allowed the enzymatic assays to be carried out on the cleaved labelled peptides in solution, and followed by hybridisation to a deoxyribonucleic acid (DNA) microchip that permitted the straightforward identification of each successful sequence. Moreover, the use of the microarray format allowed minimal consumption of enzyme (60 pmole) and substrate (3.5 nmole).

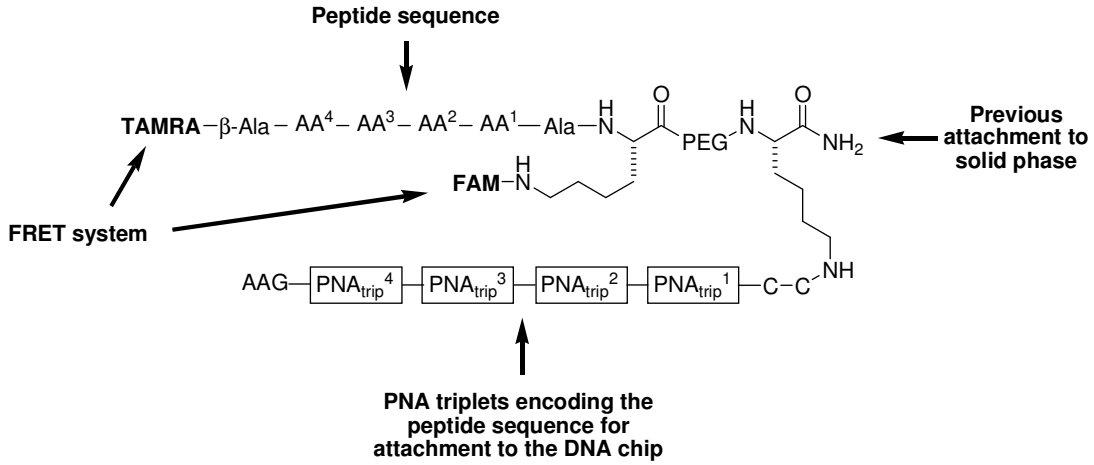
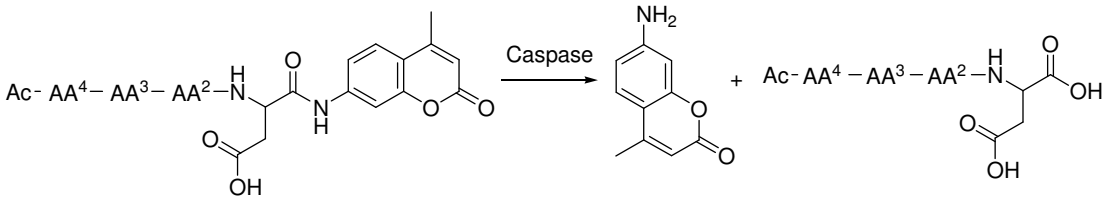


Figure 4.2: general structure of the PNA encoded FRET based peptide library

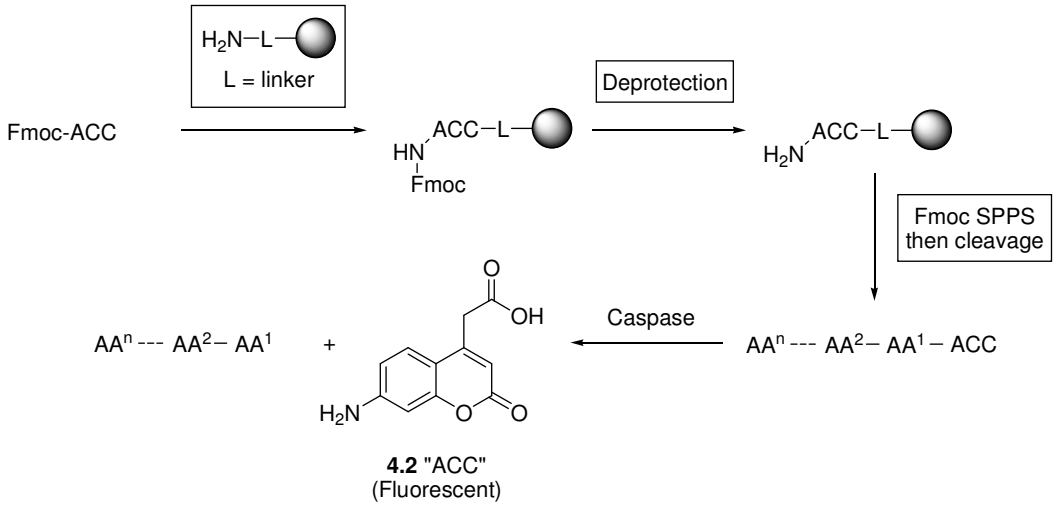
4.1.2.4. Solution phase fluorogenic substrates

Profiling of the substrate specificity of proteases regarding the "nonprime" side of the peptide was also carried out by the use of fluorogenic substrates. Similarly to the method using irreversible inhibitors, a specific amino acid whose affinity with the enzyme is known has to be used for the P_1 position, to direct cleavage. Instead of other amino acids on the "prime" side of the peptide, a fluorophore whose spectroscopic properties are partly or totally quenched by the attachment to the peptide is used. Typically a coumarin derivative such as 7-amino-4-methyl coumarin (AMC) **4.1** is used to cap the C terminus of the peptide. Amide bond cleavage releases the free fluorophore, allowing simple and continuous determination of cleavage rates for single substrates. Numerous examples of the use of AMC peptides have been reported: this so called "positional scanning" method carried out in microwells afforded, for instance, substrates specificities for positions P_2, P_3 and P_4 (AA^2, AA^3 and AA^4) for nine human caspases (**scheme 4.1**) using aspartic acid at position P_1 (AA_1).[30]



Scheme 4.1: Fluorogenic AMC peptide cleavage

The elaboration by Ellman of the bifunctional fluorophore 7-amino-3-carboxymethylcoumarin (ACC) **4.2** permitted the direct attachment of the fluorophore to a solid support, and full solid phase peptide synthesis (SPPS) of the substrate by Fmoc strategy. The method thus allowed variations to be made on the P_1 position (AA^1) as shown in **scheme 4.2**.[29] Furthermore, the use of ACC fluorophore **4.2** showed kinetic profiles comparable to AMC with up to three fold fluorescence increase.



Scheme 4.2: Principle of the preparation of fluorogenic ACC peptides

Despite harsh conditions being required to achieve completion of the first coupling to the aniline because of its poor nucleophilicity,[109] applications of these fluorogenic substrates have been successfully made to profile substrate specificity of the "nonprime" side for multiple proteases, such as serine proteases.[110] A library of 160,000 tetrapeptides substrate sequences completely randomising each of the P_1, P_2, P_3 and P_4 positions was used by Choe *et al.* to carry out substrate profiling on multiple cysteine proteases on a microtitre plate format.[111]

Nowadays, the full investigation of a given protease ("prime" and "nonprime" side) is most commonly afforded by completing the information afforded by the use of coumarin based peptide substrates by the determination of the specificity of the "prime" side, using other techniques, such as FRET based positional scanning on pentapeptides, where the three first positions are fixed as determined by the first part of the study.[112] In the same idea, the P_1, P_2 and P_3 positions of tripeptidyl-peptidase were investigated, thus allowing the subsequent evaluation of the P_1' and P_2' positions to be carried out by the biased preparation of pentapeptides and the subsequent analysis of the mixtures by LC/MS/MS.[113]

In order to increase the throughput of fluorogenic based substrate specificity profiling, Salisbury reported the use of ACC peptides with proteolytic assays directly carried out on a surface. The bifunctional character of the ACC fluorophore **4.2** indeed permitted its attachment to the slide by means of a specific linker attached to the fluorophore *via* an amide bond and to the glass slide *via* an oxime formation with aldehydes functionalities of the glass slide.[114] Substrate specificity was obtained and

compared well to solution phase assays for a variety of proteases. Similarly, nanodroplet microarrays for direct enzyme assays have also been reported, where the cleavage experiments were also based on the release of the ACC fluorophore **4.2**, but were carried out directly on the array, by delivering the enzyme directly to the fluorogenic peptides.[115]

4.1.3. Development of protease inhibitors

Besides affording a better understanding of the enzyme itself, the knowledge of the substrate specificity of a protease allows the elaboration of new inhibitors of this enzyme. A species (either peptidic or non peptidic) that shows affinity for the active site of a protease can indeed be turned into an inhibitor by the use of known pharmacophores.[116] As most affinity studies are carried out with peptides, inhibitors have been mainly developed on the basis of preferred peptides for the enzyme, subsequently using a peptidomimetic approach to ensure sufficient drug like properties.

4.1.3.1. Peptididomimetic inhibitors

To be effective drugs, protease inhibitors need to have minimal peptide character, high stability to nonselective proteolytic degradation, good membrane permeability, long bloodstream and cell lifetime to ensure homogeneous tissue distribution, low susceptibility to elimination, high selectivity for the targetted protease, and good bioavailability (preferably by oral delivery). These properties usually require the compounds to have a low molecular weight (*i.e.* $\leq$ 1000 Da). Protease inhibitors have been traditionally developed by screening for lead compounds with subsequent optimization or by empirical substrate-based methods, involving truncating polypeptide substrates to short peptides (< 10 amino acids), replacing the cleavable amide bond by a noncleavable isostere, and optimizing inhibitor potency through trial and error structural modifications that progressively reduce the peptide nature of the molecule.[117] Regarding proteases, inhibitors have been elaborated based on P and P' characteristics elucidated thanks to techniques previously described.[118] This approach allows local and global conformational parameters to be rapidly defined,

which offers a basis for modifications to be carried out to increase their potency, such as cyclisation, turn mimetics, isostere replacement, etc. Among the drugs that have been discovered using this methodology is Ritonavir, an HIV-1 protease inhibitor.

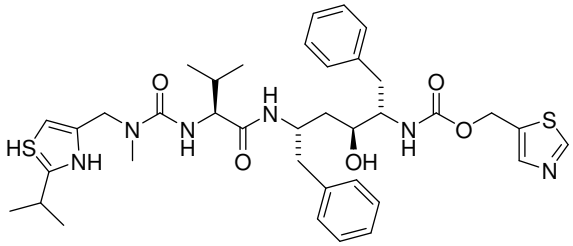
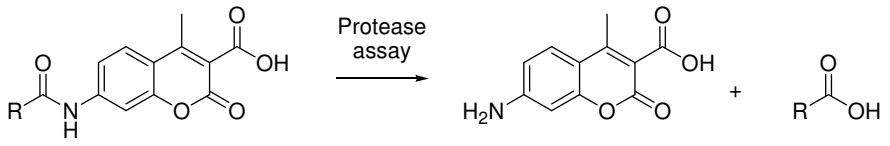


Figure 4.3: example of peptidomimetic HIV-1 protease inhibitor (rotinavir)

4.1.3.2. Fragment based inhibitors

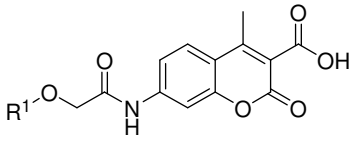
Fragment based approaches have also shown a certain efficiency in the discovery of new lead compounds in drug discovery.[119] With proteases, a novel fragment based inhibitor development approach has been elaborated. The method consists of the use of non peptidic low molecular weight fragments and the measurement of their affinity for the target protease. The big advantage of this process is that the broad range of tools previously described for substrate specificity profiling can be used likewise for any potential drug fragment incorporating an amide bond. Subsequent introduction of a mechanism based pharmacophore at the place of the cleavable amide bond virtually allows conversion of the substrate into an inhibitor. The work performed by Wood demonstrates well the power of the fragment based discovery of non-peptidic protease inhibitors.[116] From the initial screening of a library of 105 *N*-acylaminocoumarins carried out to determine their affinity for cathepsin S using an AMCA fluorogenic substrate (**scheme 4.3**), phenoxyacetyl derivatives as well as 1,4-disubstituted-1,2,3-triazole derivatives were found to exhibit encouraging inhibition of the protease.

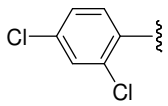
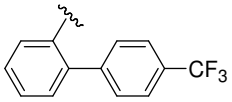
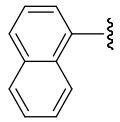
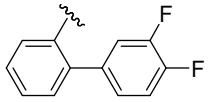
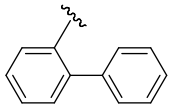
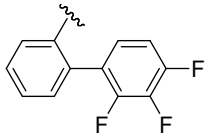
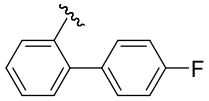


Scheme 4.3 : Principle of the fluorogenic assays for fragment affinity assessment

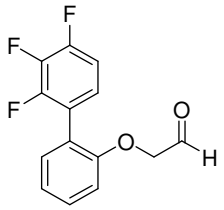
So called substrate activity screening was then carried out to improve substrate affinities. In the case of phenoxyacetyl derivatives, the preparation of analogues of the initial AMCA fluorogenic substrate hit **4.4** was carried out and these were subsequently screened against the target (**table 4.1**). Key features to achieve improvement of the affinity were determined thanks to experimental data, such as the diphenyl motif (**4.6**), the necessity of fluorine substituents (first a trifluoromethyl group (**4.7**) then a direct substitution of the second aromatic ring, (**4.8** and **4.9**)). The final compound **4.10** afforded a substantial (10,000 fold) improvement of the affinity compared to the initial hit **4.4**.

Table 4.1 : principle of the fluorometric assay for fragment affinity assessment



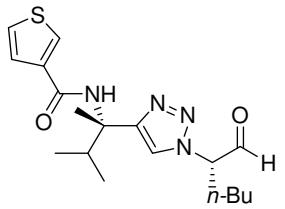
Entry	R^1	Rel k_{cat}/K_m	Entry	R^1	Rel k_{cat}/K_m
4.4		1	**4.8**		1077
4.5		7	**4.9**		1308
4.6		65	**4.10**		10385
4.7		866			

An analoguous approach was carried out on the 1,4-disubstituted-1,2,3-triazole fragments. The final use of the aldehyde pharmacophore to create an inhibitor by replacement of the AMCA fluorophore **4.3** led to the discovery of non-peptidic nanomolar aldehyde cathepsin S inhibitors, such as **4.11** and **4.12** (**figure 4.4**).[116]



4.11

Ki = 0.49 ± 0.05 μM



4.12

Ki = 9 ± 3 nM

Figure 4.4: Inhibitors elaborated by Wood's structure activity screening

Patterson recently replaced the aldehyde on compound **4.11** by a nitrile functionality: this resulted in selective inhibition of cathepsin S, with no significant inhibition of cathepsins B and L.[120] The substrate activity screening method also proved to be successful in identifying a low molecular weight non-peptidic inhibitor of chymotrypsin, with selectivity over a panel of other serine proteases including the closely related cathepsin G.[121]

4.2. Positive electrospray mass spectrometry (ESI+/MS) analytical construct based fragment activity screening method

Despite a proven efficiency, fragment based affinity studies using profluorescent substates present some disadvantages, the main one being the need to use microtitre plates to monitor the increase of fluorescence in a HT manner. The use of this format and the fact that candidates have to be screened as individual compounds raises the problem of reproducibility, since such a great number of experiments are hard to carry out under the exact same conditions and experimental error can lead to dramatic variations in experimental data. Ideally, compounds would have to be checked for affinity towards the protease target in a format that would allow all candidates to be assayed in the same pot; which would confer high reproducibility to the assay as well as increased throughput. As explained below, the use of encoded analytical constructs and ESI+/MS might allow this to be done.

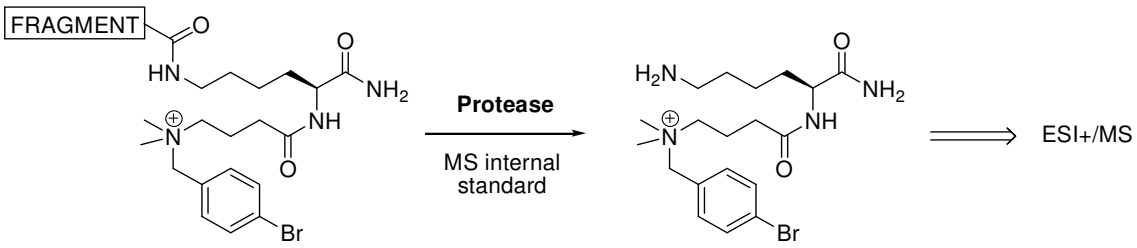
4.2.1. Principle of the method

4.2.1.1. Choice of the cathepsin S protease

The enzyme that was chosen to build up the ESI+/MS quantitative analytical construct based protease affinity study was cathepsin S. Cathepsin S is a cysteine protease present in the human spleen; it is implicated in processes regulating antigen presentation and the subsequent immune response. It is involved in multiple autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis and happens to be overexpressed in human malignant tumor tissues and cells, which makes it a choice target for substrate specificity profiling and new inhibitors development.[122] Cathepsin S acts catalytically through the nucleophilic addition of the thiolate of Cysteine-25 (formed as an ion-pair with Histidine-164) to the carbonyl of the peptide bond followed by the hydrolysis of the intermediate to yield the degraded peptide products. Most inhibitors of cathepsin S reported in the literature depend on the chemical interaction of an electrophilic "warhead" with the cysteine thiolate of the active site and in principle the enzyme can be inactivated either irreversibly or reversibly by such inhibitors. In the perspective of learning more about the specificity of this enzyme that could be used to develop new inhibitors, the establishment of a new HT technique could be of great interest.

4.2.1.2. Description of the ESI+/MS method

The idea that lies behind the ESI+/MS based HT enzymatic affinity assessment was adapted form the work carried out by Wood, Patterson and Salisbury,[116, 120, 121] relying on affinity measurement by increase of fluorescence triggered by the release of a coumarin fluorophore bound to a given drug fragment. It was envisaged that such kinetic measurements could be carried out in a comparable manner by ESI+/MS, with the release of a specific quantitative analytical construct instead of the fluorescent species accounting for the rate of cleavage of the amide bond between the construct (developed on the basis of **1.12**) and the given fragment by the protease (**scheme 4.4**.)

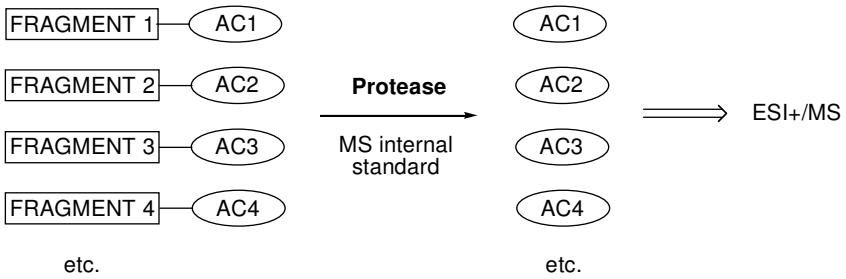


Scheme 4.4: Principle of fragment/protease affinity measurement for using ESI+/MS analytical constructs

ESI+/MS monitoring of the quantity of cleaved construct as a function of the time, by means of regular injections into the spectrometer and comparison of the peak intensity to an internal reference would thus afford kinetic data and indicate the level of affinity of the fragment for the protease.

4.2.2. HT single pot affinity assessment

The advantage of the ESI+/MS based method over the fluorophore based one is the possibility to use several different species in the same pot, by means of different analytical constructs labelling of the fragments for future deconvolution (**scheme 4.5**). The use of different quantitative analytical constructs would allow each compound to be followed by release of the specific tag.



Scheme 4.5: One-pot affinity measurement for different fragments

Regular ESI+/MS injections and comparison of the peak intensities to an internal standard would then give the cleavage rate for all the different fragments that have affinity for the target in the same pot, allowing rapid selection of the substrates that are worth keeping in the study to achieve better affinity.

The different constructs used to encode all fragments would be built on the basis of compound **1.12**: the addition of different spacers to the amine functionality of the

lysine side chain of **1.12**, provided their mass differ by 4 Da at least (see section 1.4.3.3) could allow such encoding. The use of linear carbon chains for instance would be one option to consider, with encoding being achieved by the number of carbon atoms in the chain (**figure 4.5**).

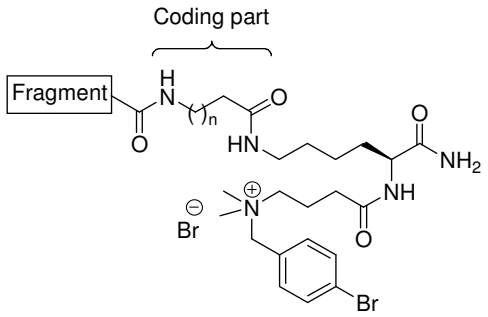


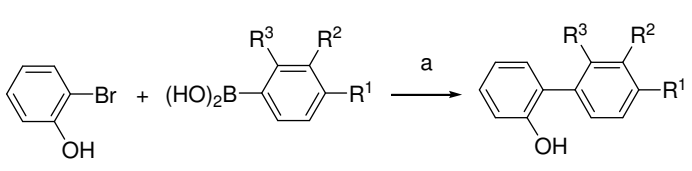
Figure 4.5: analytical constructs coding strategy for differentiation of the fragments

4.3. Phenoxyacetyl fragments: preparation and affinity tests

A set of four phenoxyacetyl derivatives shown by Wood to have moderate to good affinity for cathepsin S,[116] as well as a similar trifluoromethoxy derivative, were prepared to assess the reliability of the analytical construct based enzymatic kinetics assessment.

4.3.1. Preparation of the labelled fragments

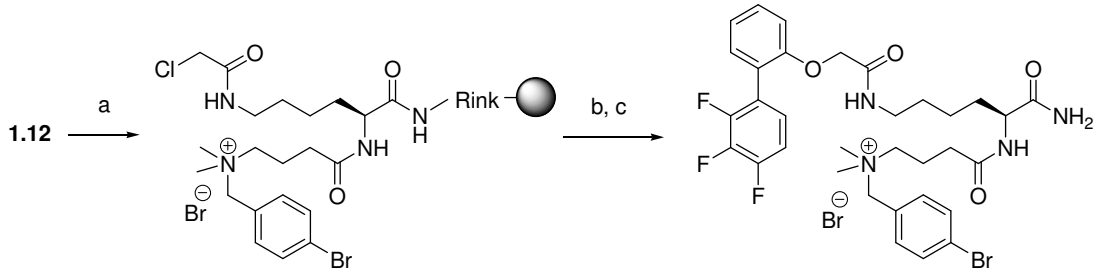
In order to have a few phenoxyacetyl fragments, 5 Suzuki coupling reactions were performed to afford the biphenyl-2-ol derivatives **4.13**, **4.14**, **4.15**, **4.16** and **4.17** in moderate yield (23 % to 66 %).



4.13: $R^1=R^2=R^3=F$
4.14: $R^1=R^2=R^3=H$
4.15: $R^1=CF_3$, $R^2=R^3=H$
4.16: $R^1=R^2=F$, $R^3=H$
4.17: $R^1=OCF_3$, $R^2=R^3=H$

Scheme 4.6: Preparation of the phenoxyacetyl fragments. Reagents and conditions: (a) $Pd(PPh_3)_4$ (0.05 equiv), Na_2CO_3 (4 equiv.), Benzene/MeOH/H_2O, (20:4:1 v/v/v), 15 h, 23 % to 66 %.

For preliminary testing purposes, the solid phase preparation of the 2,3,4-trifluorophenyl fragment bound to the analytical construct was undertaken (**scheme 4.6**). The key amide bond was generated by peptide coupling between chloroacetic acid and the amine functionality of the lysine side chain of analytical construct **1.12**. At this point, the loading of resin **4.18** was evaluated by microanalysis (Chlorine, 0.44 mmol/g). Subsequent nucleophilic displacement of the chlorine atom by the bicyclic phenol **4.13** and subsequent trifluoroacetic acid (TFA) cleavage yielded compound **4.19** ready for enzymatic assays. It is important to note here that as the preliminary tests only consider a single species, no coding spacer was used for the preparation of the construct.



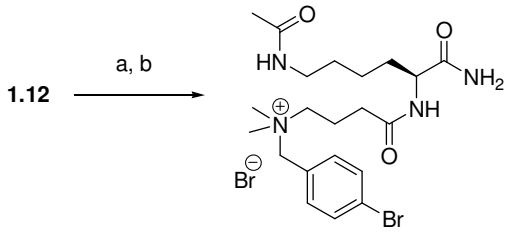
4.18 **4.19**

Scheme 4.6: Preparation of the construct bound 2',3',4'-trifluorodiphenol derivative 4.19 Reagents and conditions: (a) chloroacetic acid (3 equiv.), DIC (3 equiv.), HOBt (3 equiv.), CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (b) $CsCO_3$ (3 equiv.), KI, (2 equiv.) 70°C, 15 h, (c) TFA in DCM, (1:4, v/v), 15 min, 93%.

4.3.2. Enzymatic screening of 2,3,4-trifluorobiphenoxyacetyl fragment

The 2,3,4-trifluorobiphenoxyacetyl fragment prepared should have a fairly good affinity for cathepsin S since the the corresponding aldehyde inhibitor **4.11** was reported by Wood to be quite good (K_i = 0.49 µM).[116] Compound **4.19** was assayed against the protease under conditions similar to the ones reported by Wood: the enzyme concentration was 0.6 nM, and kinetics were carried out for six concentrations of substrate **4.19** (2 µM, 5 µM, 10 µM and 15 µM, 50µM, 100 µM). This set of conditions also took into account that given the activity of cathepsin S (1.96 µmol/nmol/min for its original Leu-Arg dipeptide substrate), the range of substrate concentrations guaranteed a kinetics window that would be large enough to allow the sampling and quenching (MeOH) of 20 µL of the reaction mixture (total

volume: 3 mL) every 30 s for ESI+/MS analysis as total consumption of the substrate should not take less than 10 min. For each substrate concentration, an equimolar amount of compound **4.20** was introduced in the reaction mixture. This compound, prepared by capping the amine functionality on analytical construct **1.12** (**scheme 4.7**) was used as an internal standard for MS peak intensity measurements.



4.20

Scheme 4.7: Preparation of the ESI+/MS internal standard. Reagents and conditions: (a) acetic anhydride (20 equiv.), DIPEA (10 equiv.), DMF, 30 min, (b) TFA in DCM, (1:4, v/v), 15 min.

The assay buffer consisted of a 100 mM solution of pH 6.1 sodium phosphate buffer with 100 mM of sodium chloride, 1 mM of dithiothreitol (DTT), 1 mM of potassium ethylenediamine tetraacetate (EDTA), and 0.001% of Tween-20.

The method of injection as well as the MS analysis parameters had to be slightly modified on this occasion to match the requirements brought by the use of ESI+/MS analytical construct. First, the injection of crude reaction mixtures to the mass spectrometer was made with an isovolumic mixture of methanol and water, guaranteeing complete solubility of all species in solution. Secondly, as the crude mixture contains the enzyme as well as many highly ionisable species (reaction buffer), only a MS window of a few hundreds Da (400 – 700 Da) was considered for quantitative analysis, in order to include the substrate of the reaction **4.19** (691.2 Da) the reference compound **4.20** (469.2 Da) as well as the quantitative analytical construct **1.23** (427.1 Da) resulting from the enzymatic cleavage.

Unfortunately, the results of the ESI+/MS data for all the concentrations assayed did not show any cleaved product (expected mass 427.1 Da). Only the MS reference **4.20** and the substrate **4.19** were detected. The ESI+/MS spectrum is given in **figure 4.6** as a representative example.

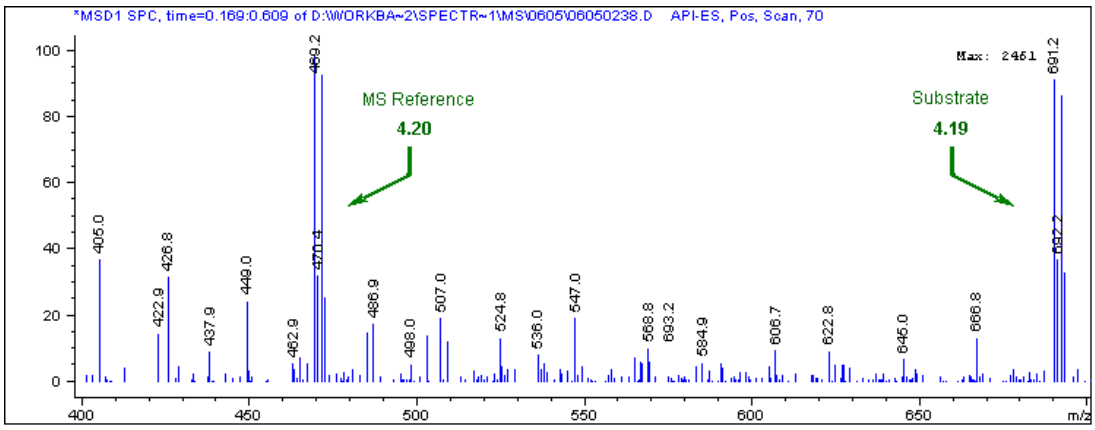


Figure 4.6: Typical ESI+/MS spectrum for the kinetics of cleavage of 4.19 in the presence of reference 4.20 (example obtained after 15 h)

As this first experiment involved a fragment reported to have only a low affinity for the target, this absence of cleavage could mean several things. The fact that no enzymatic activity could be measured could indeed be attributed to the proximity of the analytical construct with the fragment that would prevent favourable interactions with the enzyme to occur or could be due to another problem with the method. It was therefore decided to verify the reliability of the approach by performing enzyme kinetics using a well known substrate of cathepsin S instead of a low affinity fragment.

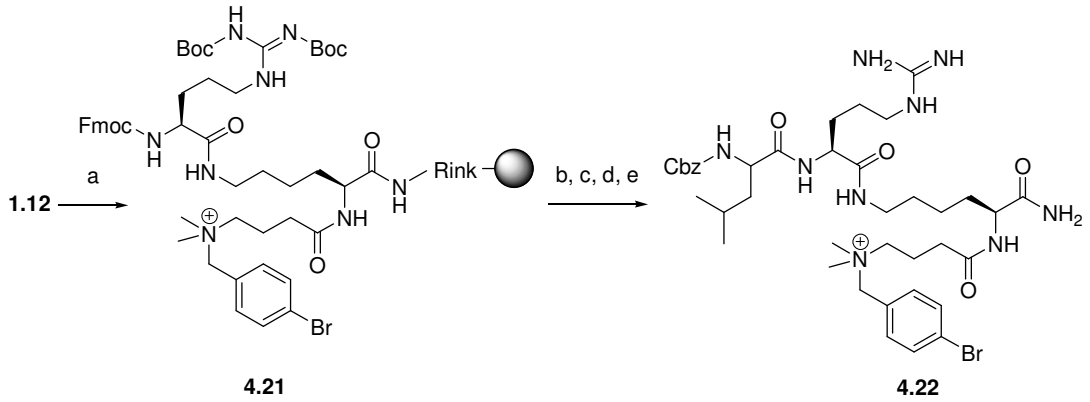
4.4. Preparation and affinity tests of dipeptidic substrates on cathepsin S using the ESI+/MS method

For cathepsin S, typical activity screening methods are carried out using a Leu-Arg dipeptide; cathepsin S is indeed specific for arginine (Arg) residues in the P_1 position and leucine (Leu) in the P_2 position.[123] Typically, a coumarin type fluorophore is attached to the C terminal end of arginine, proteolytic activity releasing the fluorescent aminocoumarin. Leucine is commonly protected on the N terminus, and therefore the typical substrate used for spectrofluorometric assays is Cbz-Leu-Arg-AMC. Using the same approach as described previously, determination of the activity of cathepsin S was undertaken using ESI+/MS, by means of Leu-Arg dipeptide, with a quantitative analytical construct replacing the fluorogenic coumarin, thus enabling ESI+/MS kinetics.

4.4.1. Analytical construct bound Cbz-Leu-Arg

4.4.1.1. Preparation of the substrate

The dipeptidic substrate **4.22** was prepared from the solid phase analytical construct **1.12** by Fmoc SPPS, as described in **scheme 4.8**.



Scheme 4.8: Preparation of the dipeptide analytical construct. Reagents and conditions: (a) Fmoc-Arg(Boc)$_2$, coupling reagent (see **table 4.2**), (b) Acetic Acid (10 equiv.), DIC (10 equiv.), HOBt (10 equiv.), CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (c) Piperidine in DMF (1:4 v/v), 2 × 5 min, (d) Cbz-Leu (3 equiv.), DIC (3 equiv.), HOBt (3 equiv.), CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (e) TFA/TIS/CH_2Cl_2, (90:5:5, v/v/v), 15 min.

However, the first coupling step turned out to be difficult to carry out: the reaction indeed proceeded very slowly and gave rise to many unidentified by-products severely compromising the purity of the product peptide and therefore hampering the following of the synthesis. Several experimental conditions were used to carry out the coupling between arginine and analytical construct **1.12** as summarised in **table 4.2**.

Table 4.2 : coupling condition for loading arginine onto the analytical construct 1.12

Entry	Amino acid	Coupling Reagents	Conditions	Notes
1	Fmoc-Arg(Boc)$_2$ (3 equiv.)	DIC/HOBt	2 × 30 min, DMF/CH_2Cl_2 (7:3 v/v)	- no starting material left - many unidentified by-products
2	Fmoc-Arg(Boc)$_2$ (3 equiv.)	DIC/HOBt	15 h DMF/CH_2Cl_2 (7:3 v/v)	- incomplete conversion - many unidentified by-products
3	Fmoc-Arg(Boc)$_2$ (5 equiv.)	DIC/HOBt	2 × 30 min DMF/CH_2Cl_2 (7:3 v/v)	- no starting material left - product + minor by-products
4	Fmoc-Arg(Boc)$_2$ (5 equiv.)	HATU/2,4,6-Collidine	2 × 15 h DMF	- incomplete conversion - many unidentified by-products
5	Fmoc-Arg(Boc)$_2$ (5 equiv.)	DIC/HOBt	20 min	- incomplete conversion - many unidentified by-products

Entry 3 gave the best results since the purity of the cleaved product, after capping of the unreacted amine functionalities reached 72 % (254 nm) as shown in **figure 4.7**. Thus, after capping the unreacted amines, the deprotection of the Fmoc protecting group was realised and the next coupling carried out.

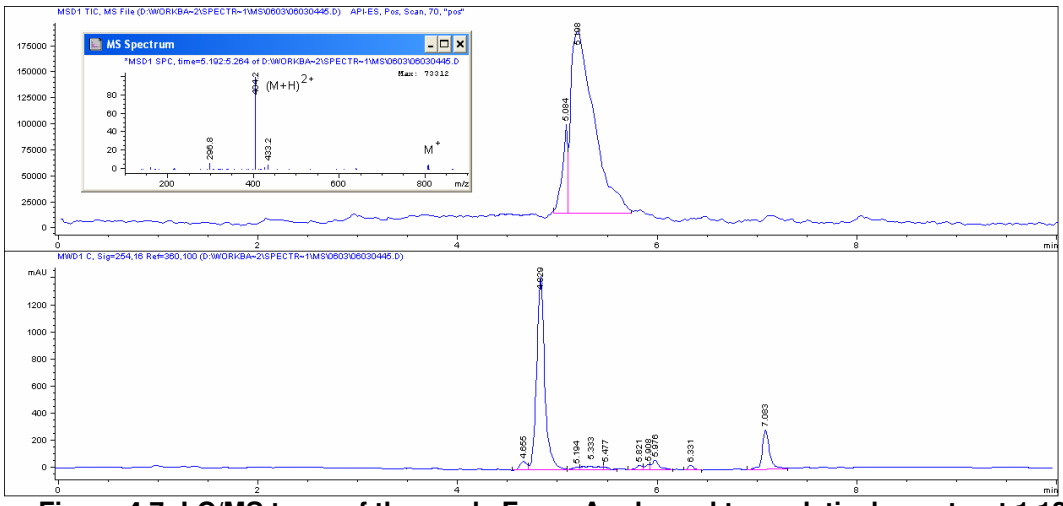


Figure 4.7: LC/MS trace of the crude Fmoc-Arg bound to analytical construct 1.12

The loading of Cbz-Leu proceeded to completion using the standard conditions decribed in **scheme 4.7**. Dipeptide **4.22** was cleaved from the resin and purified by semi-preparative HPLC.

4.4.1.2. Substrate activity measurements

Dipeptide **4.22** was assayed with cathepsin S under conditions similar to the ones used for compound **4.20**. Kinetics were carried out at several concentrations of substrate (5 µM, 10 µM, 30 µM, 50 µM, 80 µM and 100 µM) since the value of K_m determined by Ellman for Cbz-Leu-Arg-AMC was 23 µM. The enzyme concentration in these assays was 0.1 nM.

For each substrate concentration, an equimolar amount of compound **4.20** was introduced into the reaction mixture to be used as an internal standard for MS peak intensity measurements. The assay buffer and the sampling of the reaction mixture were similar to what was described in section 4.3.2. Unfortunately and despite several repetitions of the protocol, no enzymatic activity was detected by ESI+/MS. It should be noted here that the starting material **4.22** was detected only under its $(M+H)^{2+}$ form in the MS analysis, meaning that one of the limitations of quantitative analytical construct regarding the quanification of highly ionisable compounds described in section 1.4.3.3 has been reached with this compound. This would not have had any impact in the study since the principle of the quantification is based on the formation of the cleaved compound **1.12** but it gave a good example of a case where the quaternary ammonium failed to dominate the ionisation.

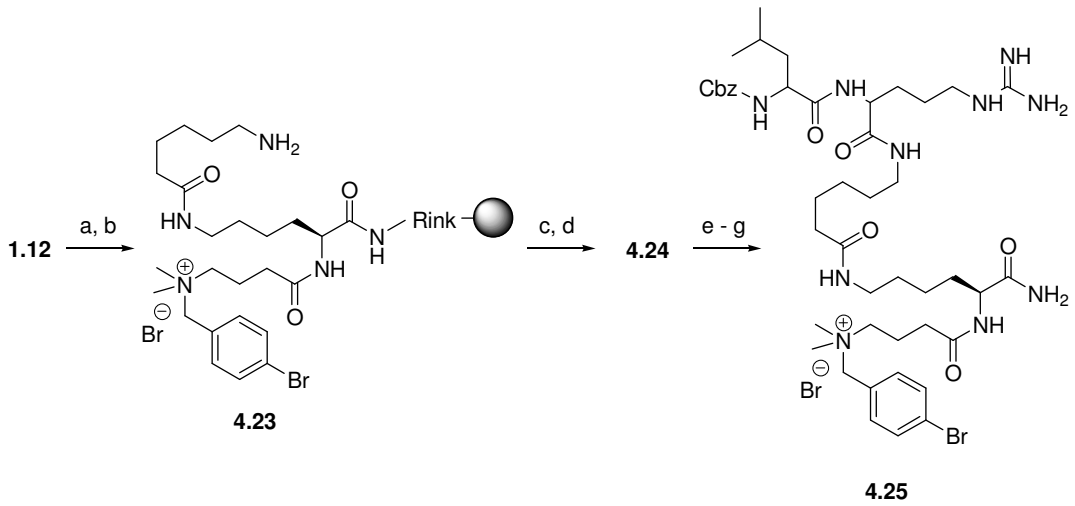
4.4.2. Use of spacers

As the proximity of the cleavable amide bond to the quaternary ammonium functionality of the analytical construct might be the reason why the protease was inactive on dipeptide **4.22**, it was decided to add a spacer between the analytical construct and the Cbz-Leu-Arg dipeptide. Several possibilities were tested.

4.4.2.1. Use of 6-aminohexanoic acid as a spacer

Analytical construct **4.25** was synthesised on solid phase by a method similar to what was described in section 4.4.1.1. Condensation of Fmoc-6-aminohexanoic acid to analytical construct **1.12** prior to peptide synthesis, as mentioned in **scheme 4.8** permitted the introduction of a hexanoic acid type spacer. Here again, the coupling with arginine turned out to be very slow and did not to go to completion. Capping of

the unreacted amine functionalities was therefore carried out. The protecting group for the side chain of arginine was changed to Pbf, since this option was less costly and turned out to give results similar to Boc, despite longer deprotection times (3-4 h instead of 30 min). The final product was purified by semi-preparative HPLC.

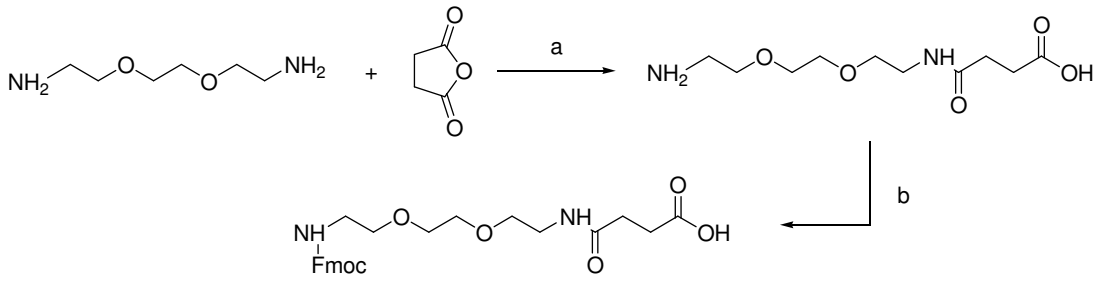


Scheme 4.8: Incorporation of a spacer in the preparation of the dipeptide analytical construct 4.25. Reagents and conditions: (a) Fmoc-6-aminohexanoic acid (3 equiv.), DIC (3 equiv.), HOBt (3 equiv.), CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (b) Piperidine in DMF (1:4 v/v), 2 × 5 min, (c) Fmoc-Arg(Pbf) (5 equiv.), DIC (5 equiv.), HOBt (5 equiv.), CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (d) Ac2O (20 equiv.), DIPEA (10 equiv.), HOBt (1 equiv.), DMF, (e) Piperidine in DMF (1:4 v/v), 2 × 5 min, (f) Cbz-Leu (3 equiv.), DIC (3 equiv.), HOBt (3 equiv.), CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (g) TFA/TIS/DCM, (90:5:5, v/v/v), 15 min.

Dipeptide **4.25** was assayed under the same conditions as described in section 4.4.1.2, apart from the fact that only one concentration of 10 µM of the substrate was investigated, to check if any cleavage took place. Cleavage indeed happened, but the rate of the reaction was dramatically lower than expected. In the conditions studied, it should have taken no more than 9 min for the consumption of the substrate to be complete, whereas the actual total conversion took around 8 h. Given the progress achieved by the introduction of a spacer, some more work was undertaken on this matter. Given that the flexibility of the linear carbon chain was questionable and that its hydrophobic properties could account for the slowing down of the enzymatic reaction, the use of a hydrophilic spacer, such as a polyethylene glycol (PEG) derivative was chosen.

4.4.2.2. Use of a PEG spacer

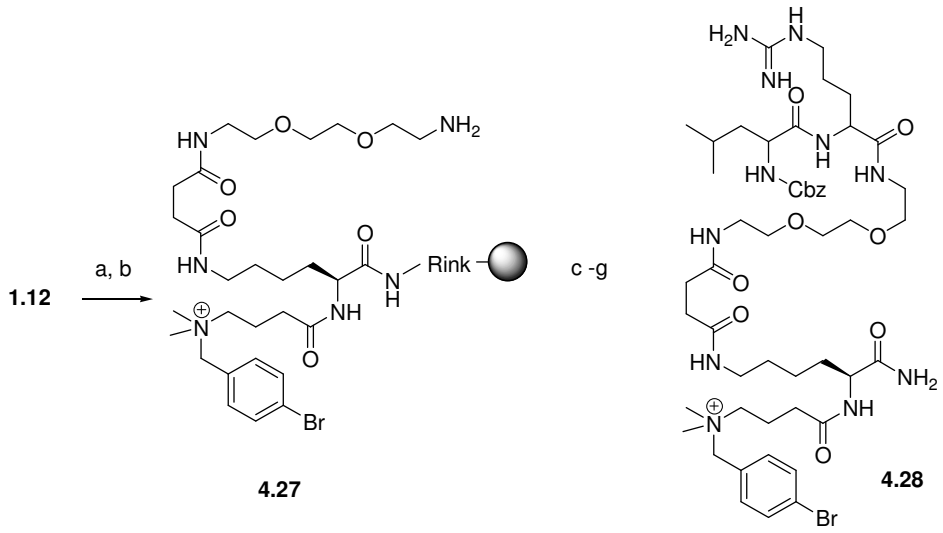
Similarly to what was described in section 4.4.2.1, the preparation of a Cbz-Leu-Arg dipeptide bound to an analytical construct incorporating a PEG derivative **4.26** was undertaken. The preparation of **4.26**, previously described by Song,[124] was achieved by nucleophilic attack of 2,2'-(Ethylenedioxy)bis(ethylamine) on succinic anhydride and subsequent Fmoc protection of the free amine for use in Fmoc based peptide couplings (**scheme 4.9**).



4.26

Scheme 4.9: Preparation of the hydrophilic spacer 4.26. Reagents and conditions: (a) MeCN, 3 h, (b) Fmoc-OSu (1.3 equiv.), DIPEA (1.3 equiv.), 15 h, 87 % (two steps)

Spacer **4.26** was condensed to analytical construct **1.12**, and after Fmoc deprotection arginine was coupled with the same difficulties as previously mentioned. Again, the synthesis was taken to the next step (Leu coupling) after unreacted amines were capped. Acid mediated cleavage of the crude dipeptide followed by semi-preparative HPLC afforded the desired product **4.28** with a purity of 89 % (**scheme 4.10**).



Scheme 4.10: Incorporation of a spacer in the preparation of the dipeptide analytical construct. Reagents and conditions: (a) compound **4.26** (3 equiv.), DIC (3 equiv.), HOBt (3 equiv.) CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (b) Piperidine in DMF (1:4 v/v), 2 × 5 min, (c) Fmoc-Arg(Pbf) (5 equiv.), DIC (5 equiv.), HOBt (5 equiv.), CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (d) Ac_2O (20 equiv.), DIPEA (10 equiv.), HOBt (1 equiv.) DMF (e) Piperidine in DMF (1:4 v/v), 2 × 5 min, (f) Cbz-Leu (3 equiv.), DIC (3 equiv.), HOBt (3 equiv.), CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (g) TFA/TIS/DCM, (90:5:5, v/v/v), 15 min.

Kinetics were carried out with cathepsin S as described in section 4.4.2.1. Cleavage occurred, but once again turned out to be much slower than expected, although faster than the previous assay: the complete consumption of a 10 µM quantity of substrate took around 4 h. As the suspicion of an effect of the presence of the MS reference compound **4.20** on the cleavage rates was raised, an assay was carried out with cathepsin S at a concentration of 0.1 nM, with a quantity of substrate **4.28** of 10 µM but without compound **4.20**. The same issue was observed since it took again several hours for the consumption of **4.28** to be complete, as checked by ESI+/MS analysis.

At this point, two key features of the method had to be verified in order to have a better idea of what was wrong with the use of ESI+/MS analytical construct as tools for enzymatic kinetics measurements. The first one was the quality of the cathepsin S: in order to assess that the protease substrate had the same proteolytic activity as mentioned by the supplier. The second key point was to use the same substrates as used previously with another protease that would also accommodate the substrate, such as trypsin to check the hydrolysis kinetics and verify that the obtained cleavage rates are as low as in the case of cathepsin S.

4.5. Preparation and affinity tests of dipeptidic substrates on cathepsin S using the ACC fluorophore method

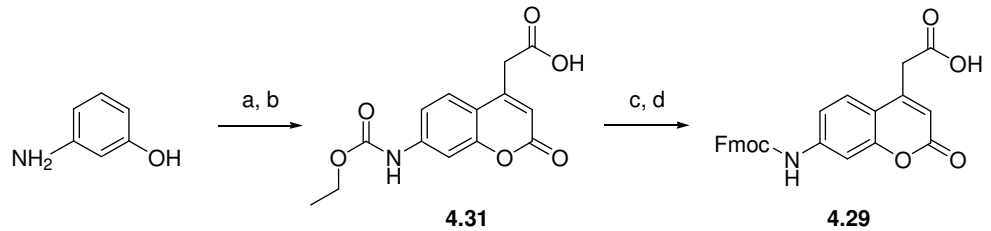
In order to assess the proteolytic activity of the sample of cathepsin S that was used for the assays described in section 4.4, the utilisation of a coumarin type fluorophore (such as AMC, ACC, AMCA, see section 4.1.2.4.) bound to a Cbz-Leu-Arg dipeptide to carry out fluorescence based kinetics was used. However, given the high price of the commercial Cbz-Leu-Arg-AMC and the synthetic interest in the preparation of the fluorophore and its attachment of the peptide, Cbz-Leu-Arg-ACC was synthesised.

4.5.1. Choice of the fluorophore

The choice of the ACC fluorophore was motivated by its resemblance with AMC in terms of enzymatic tests: as mentioned in section 4.1.2.4, ACC fluorogenic peptides exhibit kinetic behaviour comparable to AMC based ones.[29] As the only available data regarding the K_m for the Cbz-Leu-Arg dipeptide with cathepsin S (K_m = 23 µM) was carried out with an AMC fluorophore, the use of ACC could then allow data comparison. Besides, the use of ACC allows the peptide to be prepared on solid phase, by standard solid phase peptide synthesis.[116]

4.5.2. Preparation of the fluorophore and loading onto the solid support

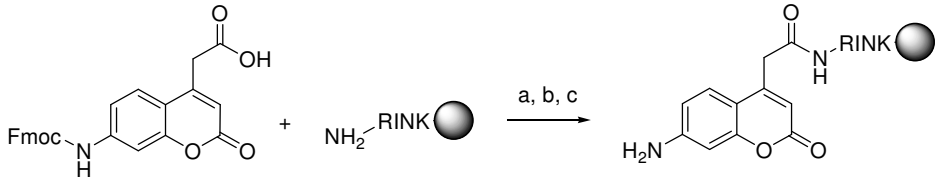
The Fmoc-ACC fluorophore **4.29** was prepared from 3-aminophenol, after amine protection with ethyl chloroformate and a Pechman type condensation of 1,3-acetonedicarboxylic acid to achieve construction of the coumarin **4.31** in moderate yield (47 %). Deprotection of the amino functionality and re-protection with the Fmoc group as shown in **scheme 4.11** afforded Fmoc-ACC **4.29** in excellent purity (99 %).



4.31 **4.29**

Scheme 4.11: Preparation of the Fmoc-ACC flurorophore 4.29. Reagents and conditions: (a) Ethyl chloroformate (0.5 equiv.), EtOAc, 99 % (b). 1,3 acetonedicarboxylic acid (1.1 equiv.), H_2SO_4/H_2O (7:3 v/v), 30 min, 47% (c) Aqueous NaOH, reflux, 16h, 99%, (d) *i*-Pr_2EtN (2.2 equiv.), TMS-Cl (2.2 equiv), then Fmoc-Cl (1.1 equiv.), CH_2Cl_2, reflux, 3h, 91%

As observed by Ellman, the loading of the fluorophore onto polystyrene Rink resin did not reach total conversion after two rounds of coupling: the conditions needed to be repeated a third time to achieve 89 % substitution, as determined by Fmoc test (loading s = 0.49 mmol/g).[125] The unreacted amine functionalities were then capped using acetic anhydride under standard conditions (**scheme 4.12**).



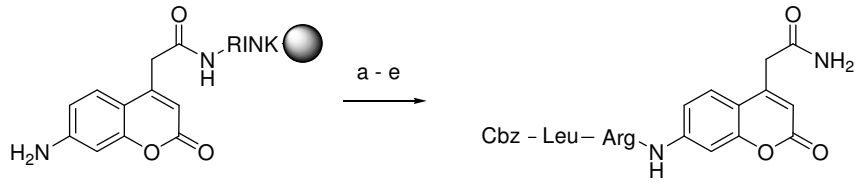
4.29 **4.33**

Scheme 4.12: Loading of the Fmoc-ACC flurorophore 4.29 on Rink amine resin. Reagents and conditions: (a) Rink amine resin (0.5 equiv.), DIC/HOBt, CH_2Cl_2/DMF, 7;3 v/v, 3 × 30 min, (b) Acetic Anhydride (20 equiv), Pyridine (10 equiv.), 30 min, (c) Piperidine in DMF (1:4 v/v), 2 × 5 min, 30 min

4.5.3. Preparation of the dipeptide

As already reported in the literature,[29] the attachment of the first amino acid to the solid phase amine functionality of the ACC fluorophore was tedious to carry out. The work carried out by Maly showed that specific coupling conditions ((O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 2,4,6-collidine) were required, and even if moderate to good yields were reported for almost all amino acids, a few species were described for which even after two rounds of acylation the loading of the amino acid onto the solid phase was only 50 % (such as Arg, Pro, Thr, Ile and Val).[29] Capping of the remaining unreacted amine functionalities was thus required to avoid multiplication of by-

products. The specific coupling conditions described above (HATU and 2,4,6-collidine) were used for the loading of arginine with the aim of preparing Cbz-Leu-Arg-ACC **4.34**, followed by the capping of unreacted amine groups. Condensation of Cbz-Leu then proceeded smoothly after deprotection of the Fmoc group using standard DIC/HOBt activation conditions.



4.32 **4.34**

Scheme 4.13: Solid phase preparation of dipeptide 4.34. Reagents and conditions: (a) Fmoc-Arg(Pbf) (5 equiv.), HATU (5 equiv.), 2,4,6-collidine (5 equiv.), DMF, 2 × 24 h, (b) Acetic Anhydride (20 equiv), Pyridine (10 equiv.), 30 min, (c) Piperidine in DMF (1:4 v/v), 2 × 5 min (d) Cbz-Leu (3 equiv.), DIC (3 equiv.), HOBt (3 equiv.), CH_2Cl_2/DMF (7:3 v/v), 2 × 30 min, (e) TFA/TIS/DCM, (90:5:5, v/v/v), 15 min (12 % overall).

Cleavage from the resin afforded the crude labelled dipeptide **4.34**. Semi preparative HPLC gave the desired compound Cbz-Leu-Arg-ACC **4.34** (**figure 4.8**).

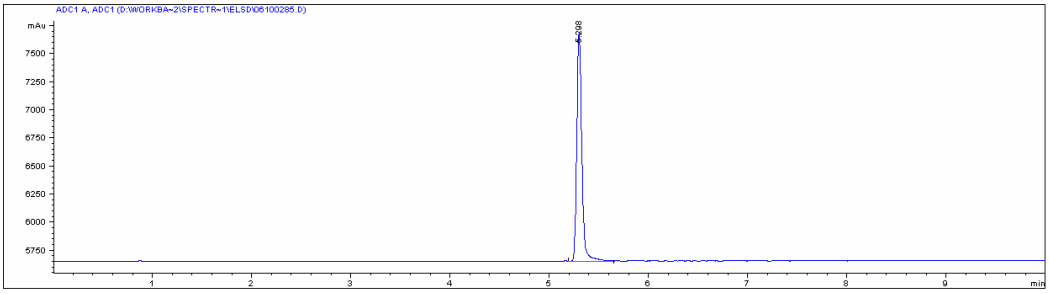


Figure 4.8: HPLC/ELSD trace of the final Cbz-Leu-Arg-ACC dipeptide 4.34

It has to be noted that subsequently to this work, the problem of the preparation of ACC labelled peptides with arginine as the first amino acid was tackled by Beythien *et al.*[109] Based on the observation that low yields were generally afforded in the solid phase synthesis of fluorogenic peptide substrates with arginine as the fisrt amino acid, a method was developed to improve the efficiency of the loading of the amino acid onto the solid support. This work particularly stresses the difficulty to introduce arginine on solid supported weak nucleophiles, such as the phenol functionality on the solid supported coumarins such as **4.32**.

4.5.4. Spectrofluorometric assays using Cbz-Leu-Arg-ACC 4.34

Substrate **4.34** was assayed with cathepsin S (0.6 nM) at a concentration of 10 µM, and the cleavage of the ACC fluorophore **4.2** was monitored by spectrofluorometry, at an excitation wavelength of 355 nm and an emission wavelength of 450 nm. Despite several attempts, no exploitable fluorescence curve could be produced: kinetics only resulted in a three fold increase in the fluorescence, whereas much more was expected. However, after all kinetics should be normally finished, *i.e.* ten minutes, ESI+/MS analysis were carried out and no Cbz-Leu-Arg-ACC **4.34** could be detected whereas this compound has got strong ionisation properties: the amide bond cleavage of **4.34** was therefore complete at the end of the expected time, giving elements according to which the enzyme sample was as active as it should be and that a problem in the assay conditions (enzyme buffer, etc.) was unlikely to be the cause of the slow kinetics observed with the ESI+/MS analytical construct method. However, this needed to be confirmed by the utilisation of the analytical construct bound Cbz-Leu-Arg dipeptide with another protease also specific to arginine on the P_1 position, such as trypsin.

4.6. Preparation and affinity tests of dipeptidic substrates on trypsin using ESI+/MS method

4.6.1. Principle of the method

4.6.1.1. Choice of trypsin

Trypsin is a serine protease, found in the digestive system. Trypsin catalyses the hydrolysis of peptides with a quite broad specificity regarding the P_1 position of its substrates. The aspartate residue (Asp 189) located in the catalytic pocket S_1 is responsible for attracting and stabilizing positively-charged lysine and/or arginine, and is thus responsible for this specificity. Trypsin is therefore an excellent test enzyme to assess the efficacy of the ESI+/MS based technique, as dipeptides **4.22**, **4.25** and **4.28** prepared previously contains an arginine amino acid in the P_1 position.

4.6.1.2. Description of the assays

Compounds **4.22**, **4.25** and **4.28** were assayed with trypsin in an attempt to evaluate the K_m value for these substrates. Kinetics were carried out in trishydroxymethylaminomethane (tris) buffer (0.1 M, pH = 8.2), at an enzyme concentration of 0.8 µM, using six different concentrations of substrate: 0.1 mM, 0.5 mM, 0.75 mM, 1 mM, 1.25 mM and 1.5 mM. The assays were run in the presence of the amine capped analytical construct **4.20** used as a reference at the same concentration of 0.75 mM in each kinetic experiment. The results obtained confirmed that the analytical constructs induced dramatic slowing down of the enzymatic activity: all kinetics indeed took more than five hours for the complete hydrolysis of compound **4.28** to take place. Furthermore, the afforded kinetic data did not allow calculation of the value of K_m for compound **4.28**: kinetic profiles, shown in **figure 4.9** obtained therefore turned out to be unexploitable.

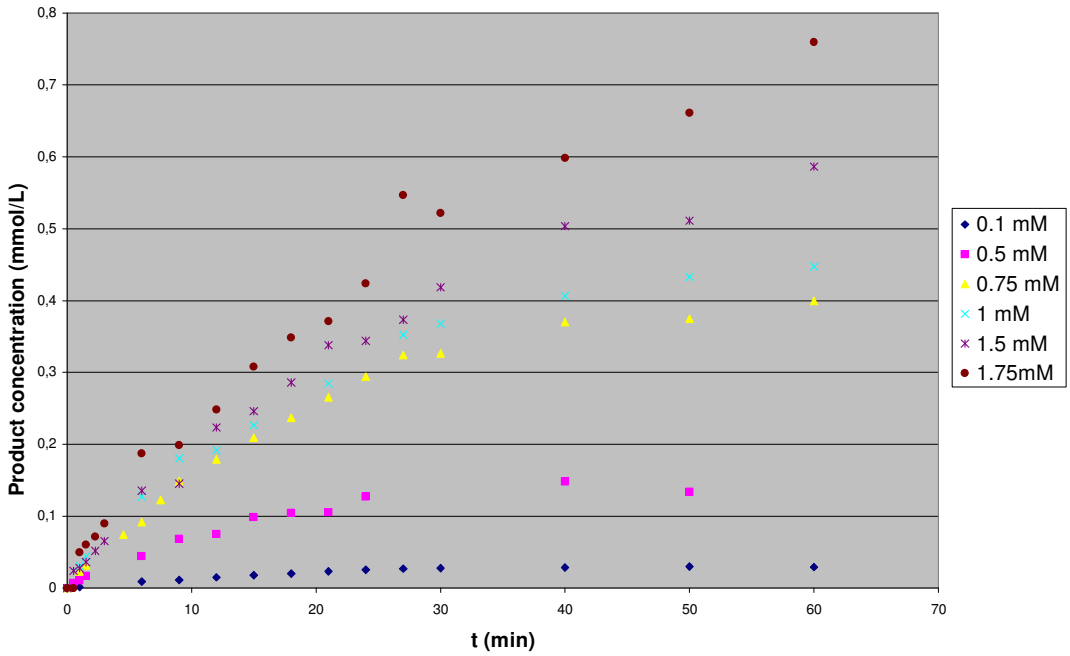


Figure 4.9: Trypsin kinetics using compound 4.28 as a substrate at the indicated concentrations

4.7. Conclusions

A new method of carrying out protease kinetics was conceived, on the basis of the liberation of ESI+/MS quantitative analytical constructs. This method was supposed to allow the HT affinity measurement of non peptidic fragments for a given protease, which could have led to non peptidic inhibitors by the use of a known pharmacophore. Unfortunately, neither the affinity of non peptidic fragment nor that of a known Leu-Arg dipeptide substrate for the protease could be efficiently measured using the ESI+/MS method. It indeed appeared that the presence of the construct attached to the substrates dramatically slowed down all kinetics and no meaningful data could be afforded. One reason to explain this effect could be the presence in the analytical enhancing unit of a quaternary ammonium which could interact with the protein target and especially some negatively charged amino acids slowing down the approach of the substrate to the active pocket. Although it did not give the expected increase in fluorescence, the use of a Leu-Arg dipeptide substrate linked to a fluorogenic moiety confirmed the good activity of the chosen protease on this specific substrate. Furthermore, even the use of a more tolerant peptidase such as trypsin on the dipeptide construct did not allow meaningful data to be collected because of slow cleavage times. Therefore, it appears that the use of quaternary ammonium based quantitative analytical constructs for carrying out enzyme kinetics might not be possible. However, the use of another MS sensitiser and quantitation moiety could help solving this problem.

Chapter Five

Experimental Section

5.1. General Section

5.1.1. General Information

All solvents and reagents were obtained from commercial suppliers and used without purification, unless otherwise stated.

All solution-phase reactions were stirred magnetically, unless otherwise stated, and followed by high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) where appropriate, using aluminium-coated Silica Gel 60 (Macheray Nagel: 0.20 mm layer). TLC visualisation was performed using short wavelength ultra violet (UV) light (254 nm) and/or $KMnO_4$ oxidation.[126]

Solid phase reactions were carried out in polypropylene syringes equipped with polyethylene frits and Teflon stopcocks.

Microwave assisted synthesis was performed using a Smith synthesiser from Personal Chemistry (Biotage).

Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker ARX-250 or Bruker ARX-360 spectrometers in the solvents indicated at 298 K. Chemical shifts are reported on the δ scale in ppm and were referenced to residual non deuterated solvent resonances for ^{1}H and the deuterated solvent for ^{13}C.

Infra red (IR) spectra were obtained with neat compounds on a Fourier transform IR Bruker Tensor Spectrometer, with 16 scans, resolution $\pm$ 4 cm^{-1} fitted with a Specac single reflection diamond attenuated total reflexion (ATR) Golden Gate accessory.

Analytical HPLC spectra were obtained using an Agilent 1100 series system. Four different HPLC grade eluents were used, at a flow rate of 1 mL/min:

- Eluent A: water + 0.1 % formic acid
- Eluent B: methanol + 0.1 % formic acid
- Eluent C: water + 0.01 % trifluoroacetic acid (TFA)
- Eluent D: acetonitrile + 0.04 % TFA.

The columns used were a Gemini C18 110A (column 1) from Phenomenex (100×4.60 mm, 5 µm) or a Luna C18 (column 2) from Phenomenex (150×4.60 mm, 5 µm) or a Luna C18 (column 3) from Phenomenex (150×4.60 mm, 3 µm). Typical sample concentration was around 1 mg/mL. The following methods were used:

- Method 1: (column 1, 10 min, eluents A and B): 95 % to 5 % A over 6 min, then 5 % A for 1 min, then 5 % to 95 % A over 3 min

- Method 2: (column 2, 15 min, eluents A and B): 95 % A for 1 min, then 95 % to 5 % A over 7 min, then 5 % A for 1 min, then 5 % to 95 % A over 6 min.

- Method 3: (column 1, 10 min, eluents C and D): 95 % to 5 % C over 6 min, then 5 % C for 1 min, then 5 % to 95 % C over 6 min.

- Method 4: (column 2, 15 min. eluents C and D): 95 % C for 1 min, then 95 % to 5 % C over 7 min, then 5 % C for 1 min, then 5 % to 95 % A over 6 min.

- Method 5: (column 3, 60 min. eluents C and D): 100 % C for 5 min, then 100 % to 70 % C over 40 min, then 70 % C for 5 min, then 70 % to 95 % C over 10 min.

Analytical HPLC/evaporative light scattering detector (ELSD) spectra were obtained with the same system (using the same eluents, methods and flow rates) coupled to a Polymer Lab 100 ES ELSD.

Analytical HPLC / mass spectrometry (MS) spectra were obtained using an Agilent 1100 system (same eluents, methods and flow rate) coupled to an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS) using an electrospray (ESI) ion source or an atmospheric pressure chemical ionisation (APCI) ion source. The same typical concentration and methods as for HPLC/ELSD were used

Direct MS injections (ESI/MS or APCI/MS) were performed by diluting the sample in a mixture of A and B (95:5 v/v, 1.5 min) with a typical sample concentration of about 1 µg/mL on the LC/MS system previously described, by-passing the HPLC column, unless otherwise stated.

Semi-preparative HPLC purifications were performed on an Agilent Technologies 1100 modular HPLC equipped with an automated fraction collection triggered by

absorbance at 220 nm. The column used was a Waters X-Terra Prep RP18 (150 × 19.0 mm, 5 µm), with eluents A and B at 5 mL/min, with a gradient of 95 % A to 95 % B over 20 min, then 95 % B for 5 min.
High Resolution (HR) MS analyses were carried out by the MS Department of the University of Edinburgh, using fast atom bombardment (FAB).
Melting points (Pyrex capillaries) were determined using a Gallenkamp melting point apparatus and are uncorrected.
Elemental analyses were carried out by Medac Ltd, U.K.

5.1.2. General Experimental Methods

Kaiser Ninhydrin Test[21]

The following qualitative test was used to determine the presence of free primary amine functionalities on resin beads. Reagent A and B were prepared as follows:

Reagent A: Potassium cyanide (65 mg, 1 mmol) was dissolved in water (100 mL). A sample of this solution (2 mL) was diluted with freshly distilled pyridine (98 mL). Phenol (40 g, 420 mmol) was dissolved in absolute ethanol (10 mL). The two solutions were mixed together to give reagent A.
Reagent B: Ninhydrin (2.5 g, 14 mmol) was dissolved in absolute ethanol (50 mL).

Procedure: Reagent A (3 drops) and reagent B (1 drop) were added to a small sample of resin (< 0.5 mg) in a small test tube. The mixture was heated at 100 °C for 5 min. The presence of resin bound free amine was indicated by a blue colour.

Fmoc test resin loading determination

To a known mass of resin (30 mg) was added a solution of piperidine in DMF (1:4 v/v, 10 mL). The resin was allowed to stand for 15 min and the solution was filtered and a fraction of the filtrate (1 mL) was diluted with piperidine in DMF (1:4 v/v,

9 mL). The absorbance at 302 nm was recorded and the loading was calculated from **eq. 5.1**:[125]

$$\text{Loading (mol/g)} = \frac{A_{302} \times V}{\varepsilon_{302} \times m} \qquad \textbf{eq. 5.1}$$

Where: A_{302} = absorbance at 302 nm

ε_{302} = molar extinction coefficient (7800 $M^{-1}cm^{-1}$)

V = diluted volume (10 mL)

m = mass of resin (mg)

Solid phase Fmoc group deprotection

The deprotection of the Fmoc group was performed by swelling the resin (*e.g.* 500 mg) in a solution of piperidine in DMF (1:4 v/v, 15 mL) for 5 min. The resin was washed with DMF (3 × 15 mL) and the deprotection procedure was repeated. The resin was washed with DMF (3 × 20 mL), CH_2Cl_2 (3 × 20 mL), methanol (3 × 20 mL), and diethyl ether (3 × 20 mL).

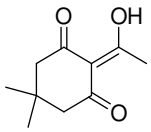
Activation of acidic ion exchange resin (Amberlite 200)

After washing Amberlite 200 (10 g) with ethanol/methanol (1:1 v/v, 3 × 100 mL), the resin was rinsed with water (4 × 100 mL), washed with 1 N aqueous hydrochloric acid (1 × 100 mL), and rinsed again with water (2 × 100 mL). It was then washed with 1 N aqueous NaOH (1 × 100 mL), rinsed with water (2 × 100 mL), and the acid form was generated by swelling the resin in concentrated aqueous hydrochloric acid in water (37 % v/v, 2 × 100 mL). The beads were then washed with methanol (5 × 100 mL) to remove water.

5.2. Experimental to chapter one

5.2.1. Preparation of the solid phase analytical construct

Dde-OH (1.14)[59]



1.14

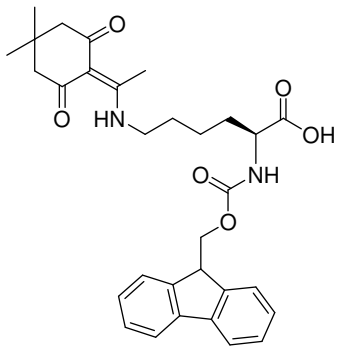
The protocol followed was reported by Chhabra *et al.*[59]

Dimedone **1.15** (2.0 g, 14.3 mmol) was dissolved in *N*,*N*-dimethylformamide (DMF, 35 mL) with acetic acid **1.16** (0.82 mL, 14.3 mmol, 1 equiv.), dicyclohexylcarbodiimide (DCC) (2.95 g, 14.3 mmol, 1 equiv.) and 4-dimethylaminopyridine (DMAP, 174 mg, 1.43 mmol, 0.1 equiv.). The reaction was followed by analytical TLC (CH_2Cl_2/MeOH, 95:5 v/v) and revealed to be complete after 36 h (R_f = 0.97). Precipitating dicyclohexylurea (DCU) was filtered. DMF was removed *in vacuo* and the residue was dissolved in ethyl acetate (35 mL). Remaining DCU was removed on a celite plug and the organic layer was washed with 1 M aqueous $KHSO_4$ (3 × 30 mL) and then dried over $MgSO_4$. The solvent was removed *in vacuo* to afford Dde-OH as an orange oil (2.41 g, 93 %).

HPLC (method 2): t_R = 9.9 min
Purity: 96 % (UV 254 nm)
ESI+/MS: m/z = 183.2 $(M+H)^+$
IR ν (cm^{-1}): 3307 (vw, br, ν_{O-H}), 1659 (vs, $\nu_{C=O}$), 1543 (s, $\nu_{C=O}$)
^{1}H NMR (250 MHz, $CDCl_3$): 2.59 (3H, s, CH_3), 2.52 (2H, s, $CH_2C=O$), 2.34 (2H, s, $CH_2C=O$), 1.06 (6H, s, $(CH_3)_2$)
^{13}C NMR + DEPT 135 + DEPT 90 (62.5 MHz, $CDCl_3$): 202.4 (C(OH)), 197.9 (C=O), 195.2 (C=O), 112.4 (C(O)C=C(OH), 52.4(CH_2), 47.0 (CH_2), 30.6 (CH_3C(OH)), 28.5 (CH_3), 28.2 (CH_3)

All analyses agreed with the literature.[59]

Fmoc-Lys(Dde)-OH (1.13)[57]



1.13

The protocol followed was reported by Chhabra *et al*.[57]

To a stirred suspension of Fmoc-Lys-OH (2.02 g, 5.4 mmol, 1 equiv.) in ethanol (45 mL) was added Dde-OH **1.14** (2 g, 10.8 mmol, 2 equiv.) and TFA (42 µL, 0.54 mmol, 0.1 equiv.) at room temperature. The mixture was refluxed for 60 h and the reaction monitored by TLC (ethyl acetate/hexane 95:5 v/v, R_f = 0.71). The solvent was removed *in vacuo* and the orange residue dissolved in ethyl acetate (75 mL). The organic solution was washed with 1 M aqueous $KHSO_4$ (2 × 75 mL). After drying and concentrating *in vacuo*, the yellow oil was triturated with hexane to remove unreacted Dde-OH. Product **1.13** was precipitated with CH_2Cl_2-hexane and the title material was afforded as an off white powder (2.4 g, 84 %).

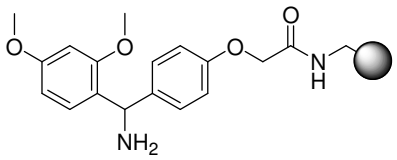
HPLC (method 2): t_R = 11.2 min
Purity: 98 % (UV 254 nm)
ESI+/MS : m/z = 533.3 $(M+H)^+$
IR ν (cm^{-1}): 3304 (w, br, ν_{N-H}), 1715 (vs, $\nu_{C=O}$), 1630 (m, $\nu_{C=O}$), 1534 (vs, br, $\nu_{C=O}$)

^{1}H NMR (300 MHz, $CDCl_3$): 13.43 (1H, s, COOH), 9.13 (1H, bs, C(O)NH), 7.73 (2H, d, J^3 = 7.1 Hz, H_{ar}), 7.56 (2H, d, J^3 = 6.6 Hz, H_{ar}), 7.39 (2H, t, J^3 = 6.7 Hz, H_{ar}), 7.28 (2H, d, J^3 = 7.4 Hz, H_{ar}), 5.85 (1H, bs, J^3 = 8.0 Hz, CH_2NH), 4.43 (1H, m, CH), 4.35 (2H, d, J^3 = 6.8 Hz, CH_2 Fmoc), 4.18 (1H, t, J^3 = 6.7 Hz, CH Fmoc), 3.37 (2H, m, CH_2NH), 2.52 (3H, s, CH_3 Dde), 2.35 (4H, s, 2 × CH_2 Dde), 1.96 (2H, m, CH_2 lys) 1.72 (2H, m, CH_2 lys), 1.51 (2H, m, CH_2 lys), 1.00 (6H, s, $(CH_3)_2$ Dde)

^{13}C NMR + DEPT 135 + DEPT 90 (75 MHz, $CDCl_3$): 198.7 (C(O) Dde), 174.8 (C(O)OH), 174.5 ((C=CNH)), 156.6 (C(O)NH), 144.2 (C_{ar} Fmoc), 141.6 (C_{ar} Fmoc), 128.1 ((CH_{ar} Fmoc)), 127.4 (CH_{ar} Fmoc), 125.5 (CH_{ar} Fmoc), 120.35 (CH_{ar} Fmoc), 108.2 (C=CNH), 67.4 (CH_2 Fmoc), 53.8 (HOOC-CH), 52.7 (2 × CH_2 Dde), 47.5 (CH Fmoc), 43.7 (CH_2NH), 32.4 (CH_2lys), 31.9 (CH_2lys), 30.5 ($C(CH_3)_2$), 28.8 (($C(CH_3)_2$)), 23.0 (CH_2lys), 18.6 (CH_3 Dde)

All analyses agreed with the literature.[57]

Rink amine resin (1.18)



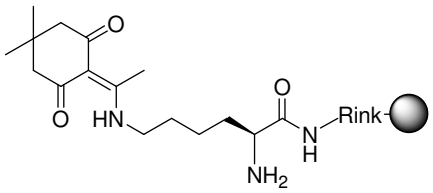
1.18

The coupling method was reported by Konig *et al.*[127]

The Fmoc-Rink Linker carboxylic acid **1.17** (860 mg, 1.6 mmol, 1.5 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 5 mL). Hydroxybenzotriazole (HOBt, 216 mg, 1.6 mmol, 1.5 equiv.) was added and after complete dissolution, diisopropylcarbodiimide (DIC, 248 µL, 1.6 mmol, 1.5 equiv.) was introduced to the mixture. After 20 min of stirring, the solution was added to aminomethylated polystyrene resin (1 % crosslinked, 1.08 g, s = 1.01 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 5 mL). The reaction was stirred for 1 h, until a qualitative ninhydrin test (see section 5.1.2) was negative. The resin was washed with DMF (3×20 mL), CH_2Cl_2 (3×20 mL), methanol (3×20 mL), and diethyl ether (3×20 mL). The deprotection of the Fmoc group was performed as decribed in section 5.2.1. and overnight drying *in vacuo* afforded the title resin **1.18** as a white coloured material (1.4 g, 99 %).

IR ν (cm^{-1}): 3427 (w, br, ν_{N-H}), 1682 (m, $\nu_{C=O}$), 1209 (s, ν_{C-O})

Lys(Dde) Rink resin (1.19)

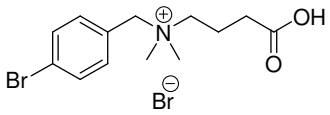


1.19

Fmoc-Lys(Dde)-OH **1.13** (2 g, 3.7 mmol, 1.5 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 20 mL). HOBt was added (499 mg, 3.7 mmol, 1.5 equiv.). After complete dissolution, DIC (576 µL, 3.7 mmol, 1.5 equiv.) was added. After 20 min of stirring, the solution was added to Rink amine resin **1.18** (3.3 g, s = 0.77 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 15 mL). The reaction was stirred for 1 h, until a qualitative ninhydrin test (see section 5.1.2) was negative. The resin was washed with DMF (3×40 mL), CH_2Cl_2 (3×40 mL), methanol (3×40 mL), and diethyl ether (3×40 mL). The resin was dried *in vacuo* overnight to afford a bright yellow resin. The deprotection of the Fmoc group was performed as described in section 5.1.2. before drying *in vacuo* overnight to afford **1.19** as a bright yellow resin (4.1 g, 96 %).

IR ν (cm^{-1}): 3327 (w, br, ν_{N-H}), 1676 (s, $\nu_{C=O}$), 1644 (m, $\nu_{C=O}$), 1603 (m, $\nu_{C=O}$), 1575 (m, $\nu_{C=O}$)

_N_-(4-Bromobenzyl)-_N_-(3-carboxypropyl)-_N,N_-dimethylammonium bromide (1.21)

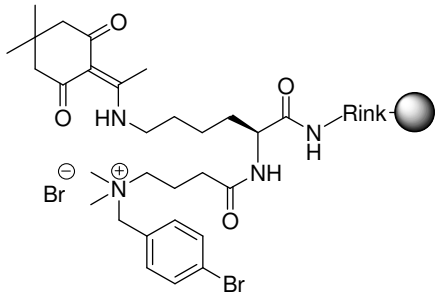


1.21

Sodium *N,N*-dimethyl-4-aminobutyrate **1.20** was generated from its hydrochloric salt (1.5 g, 9 mmol) by dissolving it in water (10 mL) and by addition of 1 N aqueous NaOH (18 mL). After removal of the water by freeze-drying, the white salt was suspended in CH_2Cl_2 (30 mL). 4-bromobenzyl bromide (3.38 g, 13.5 mmol, 1.5 equiv.) in CH_2Cl_2 (20 mL) was added dropwise over 30 minutes. The mixture was stirred for 1 h at room temperature. After removing the solvent *in vacuo*, water was added (10 mL) and precipitating 4-bromobenzyl bromide was filtered off. Freeze drying of the filtrate afforded a white powder which was stirred in MeOH (50 mL) in the presence of activated Amberlite 200 (see section 5.1.2) for 30 min. Removal of the solvent *in vacuo* afforded the acid form of **1.21** as a white amorphous powder (3.5 g, 98 %).

HPLC (method 1): t_R = 3.8 min
Purity: 97 % (ELSD)
Mp: 148-150°C
ESI+/MS: m/z = 300.0 (M^+)
ESI+/HRMS: m/z = 300.05957 (M^+) (calcd 300.05992)
IR ν (cm^{-1}): 1593 (vs, $\nu_{C=O}$)
^{1}H NMR + COSY (250 MHz, D_2O): 7.71 (2H, d, J^3 = 8.5 Hz, CH_{ar}), 7.44 (2H, d, J^3 = 8.5 Hz, CH_{ar}), 4.46 (2H, s, CH_2), 3.28 (2H, m, N^+CH2), 3.01 (6H, s, N^+(CH_3)$_2$), 2.27 (2H, t, J^3 = 6.8 Hz, CH_2COOH), 2.10 (2H, m, CH_2)
^{13}C NMR + DEPT 135 + DEPT 90 (62.5 MHz, D_2O): 181.1 (COOH), 134.9 (CH_{ar}), 132.7 (CH_{ar}), 126.5 (C$_{ar}$), 125.2 (C$_{ar}$), 67.4 (N^+CH_2), 64.2 (N^+CH_2), 50.0 ((CH_3)$_2$), 34.0 (CH_2COOH), 19.6 (CH_2)

Dde-amine analytical construct resin (1.22)

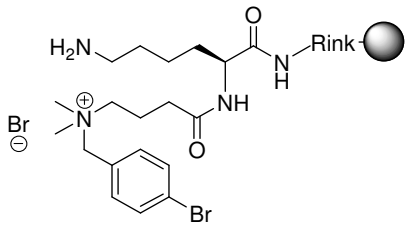


1.22

Quaternary ammonium salt **1.21** (1 g, 2.8 mmol, 2 equiv.) was suspended in CH_2Cl_2/DMF (7:3 v/v, 10 mL). HOBt was added (378 mg,, 2.8 mmol, 2 equiv.). After complete dissolution, DIC (441 µL, 2.8 mmol , 1 equiv.) was introduced. After 20 min of stirring, the solution was added to Lys(Dde) Rink resin **1.19** (2 g, s = 0.66 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 10 mL). The reaction was stirred over 1 h. The resin was washed with DMF (3 × 30mL), CH_2Cl_2 (3 × 30 mL), methanol (3 × 30 mL), and diethylether (3 × 30 mL). The coupling procedure was repeated. After 1 h, a negative qualitative ninhydrin test (see section 5.1.2) was carried out. The washing procedure was repeated and the resin dried *in vacuo* overnight to afford **1.22** as a buff coloured resin (2.55 g, 98 %).

IR ν (cm^{-1}): 3400 (w, br, ν_{N-H}), 1663 (s, br, $\nu_{C=O}$), 1601 (m, $\nu_{C=O}$), 1572 (m, br, $\nu_{C=O}$)

Amine analytical construct resin (1.12)



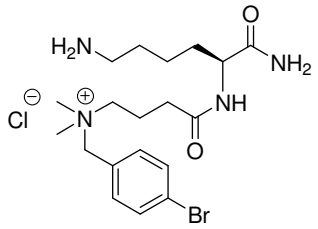
1.12

Dde deprotection was carried out according to the method described by Diaz-Mochon *et al.*[60]

Hydroxylamine hydrochloride (2.5 g, 3.60 mmol, 1 equiv.) and imidazole (1.83 g, 0.75 equiv.) were suspended in *N*-Methyl-2-Pyrrolidone (NMP, 10 mL) and the mixture was sonicated until complete dissolution. The solution was diluted with CH_2Cl_2 (2 mL) and the Dde-amine analytical construct resin **1.22** (1.2 g, s = 0.51 mmol/g) was swollen in it. After 3 h, the deprotecting solution was removed by filtration and the resin was washed with DMF (3 × 20 mL), CH_2Cl_2 (3 × 20 mL), methanol (3 × 20 mL), and diethylether (3 × 20 mL), and dried *in vacuo* overnight to afford the title compound **1.12** as a buff coloured resin (1 g, 99 %).

IR ν (cm^{-1}): 3400 (w, br, ν_{N-H}), 1660 (s, br, $\nu_{C=O}$), 1603 (m, $\nu_{C=O}$)

[3-(5-Amino-1-carbamoyl-pent-1-ylcarbamoyl)-propyl]-(4-bromobenzyl)-dimethylammonium chloride (1.23)



1.23

To afford complete characterisation of the analytical construct **1.23**, cleavage from the resin was performed by swelling resin **1.12** (900 mg, 0.61 mmol/g, 1 equiv.) in a solution of TFA in CH_2Cl_2 (1:4 v/v, 12 mL) for 15 min. After removal of the solvents *in vacuo*, **1.23** was obtained as an oily solid (150 mg, 90 %).

HPLC (method 1): t_R = 1.5 min

Purity: 100 % (ELSD)

ESI+/MS: m/z = 427.1 (M^+)

FAB+/HRMS: m/z = 427.17063 (M^+) (calcd 427.17086)

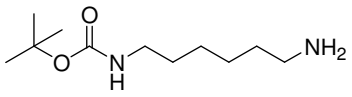
IR ν (cm^{-1}): 3420 (w, br, ν_{N-H}), 1678 (s, br, $\nu_{C=O}$)

^{1}H NMR (250 MHz, D_2O): 7.61 (2H, d, J^3 = 8.4 Hz, CH_{ar}), 7.34 (2H, d, J^3 = 8.4 Hz, CH_{ar}), 4.38 (2H, s, $N^+CH_2C_{ar}$), 4.15 (1H, t, J^3 = 5.9 Hz, CHC(O)NH_2), 3.20 (2H, m, N^+CH_2), 2.95 (6H, s, $(CH_3)_2$), 2.91 (2H, t, J^3 = 8.4 Hz, CH_2NH_2), 2.35 (2H, t, J^3 = 7.1 Hz, CH_2C(O)NH), 2.07 (2H, m, $CH_2CH_2N^+$), 1.60 (4H, m, 2 × CH_2lys), 1.34 (2H, m, CH_2lys)

^{13}C NMR + DEPT 135 (62.5 MHz, D_2O): 177.0 (C(O)NH_2), 174.6 (C(O)NH), 134.8 (CH_{ar}), 132.6 (CH_{ar}), 126.3 (C_{ar}), 125.2 (C_{ar}), 67.4 ($N^+CH_2C_{ar}$), 63.1 ($N^+CH_2CH_2$), 53.9 (CHC(O)NH_2), 49.9 ((CH_3)$_2$), 39.4 (CH_2NH_2), 31.4 (CH_2C(O)NH), 30.6 (CH_2lys), 26.5 (CH_2CH), 22.3 (CH_2), 18.4 (CH_2lys)

5.2.2. Preparation of the solution phase amino analytical construct

(6-Aminohexyl)-carbamic acid *tert*-butyl ester (1.24)



1.24

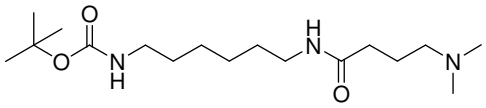
The procedure followed was the one described by Krapcho.[61]

A solution of di-*tert*-butyl-dicarbonate (15 g, 69 mmol, 1 equiv.) in dioxane (225 mL) was added over a period of 2.5 h to a solution of 1,6-diaminohexane (63.8 g, 552 mmol, 8 equiv.) in dioxane (150 mL). The mixture was allowed to stir for 22 h and the solvent was removed *in vacuo*. Water (300 mL) was added and the insoluble bis-substituted product was collected by filtration. The filtrate was extracted with CH_2Cl_2 (3 × 200 mL), and the organic layer was backwashed with water. The solvent was removed *in vacuo* to yield **1.24** as an oil (12.7 g, 85 %).

HPLC (method 2): t_R = 4.2 min
Purity: 96 % (ELSD)
ESI+/MS: m/z = 217.2 $(M+H)^+$
^{1}H NMR (250 MHz, $CDCl_3$): 4.68 (1H, s, br, NH), 3.07 (2H, dt, br, J^3 = 6.4 Hz, CH_2NH), 2.66 (2H, dt, J^3 = 6.9 Hz, CH_2NH_2), 1.79 (2H, s, br, NH_2), 1.41 (13H, s, br, $(CH_3)_3$ + $CH_2CH_2NH_2$ + $CH_2CH_2NHC(O)$), 1.30 (4H, m, 2 × CH_2)
^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$): 156.4 ($C(O)O^tBu$), 79.4 ($C(CH_3)_2$), 42.3 (CH_2NH_2), 40.9 ($CH_2NHC(O)$), 33.8 (CH2), 30.0 (CH2), 28.4 ($C(CH_3)_3$), 26.6 (2 × CH_2)

All analyses agreed with the literature.[61]

_N_-[6-(4-Dimethylaminobutaroylamino)-hexyl] carbamic acid *tert*-butyl ester (1.25)

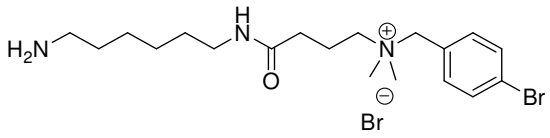


1.25

4-dimethylaminobutyric acid hydrochloride salt (4.26 g, 25.4 mmol, 1 equiv) and DCC (5.24 g, 1 equiv.) were dissolved in CH_2Cl_2/DMF (7:3 v/v, 75 mL). After 20 min of stirring, (6-Amino-hexyl)-carbamic acid *tert*-butyl ester **1.24** (4.71 g, 22.9 mmol, 0.9 equiv.) was added and the mixture was allowed to stir for 12h. The reaction mixture was then cooled to -18°C and filtered twice through a celite plug to remove DCU. After evaporation of the solvent, the crude oil was dissolved in ethyl acetate and washed with 1 N aqueous $NaHCO_3$ to remove any unreacted acid. Product **1.25** was afforded as a yellow oil (7.46 g, 89 %).

HPLC (method 1): t_R = 4.7 min
Purity: 97 % (ELSD)
ESI+/MS: m/z = 330.0 $(M+H)^+$
ESI+/HRMS: m/z = 330.26744 $(M+H)^+$ (calcd 330.26784)
IR ν (cm^{-1}): 3250 (w, ν_{N-H}), 1681 (m, $\nu_{C=O}$), 1208 (m, ν_{C-O})
^{1}H NMR (250 MHz, $CDCl_3$): 7.03 (1H, s, br, $CH_2C(O)N\underline{H}$), 4.60 (1H, s, br, $OC(O)N\underline{H}$), 3.20 (2H, dt, br, J^3 = 6.8 Hz, $C\underline{H}_2NHC(O)CH_2$), 3.07 (2H, dt, br, J^3 = 6.4 Hz, $C\underline{H}_2NHC(O)O$), 2.86 (2H, t, J^3 = 6.9 Hz, $C\underline{H}_2N(CH_3)_2$), 2.63 (6H, s, $CH_2N(C\underline{H}_3)_2$), 2.43 (2H, t, J^3 = 7.1 Hz, $C\underline{H}_2C(O)NH$), 2.02 (2H, quint, J^3 = 7.0 Hz, $C\underline{H}_2CH_2C(O)NH$), 1.46 (2H, m, $C\underline{H}_2$), 1.37 (9H, s, $C(C\underline{H}_3)_3$), 1.31 (4H, m, 2 × $C\underline{H}_2$)
^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$): 171.6 ($CH_2\underline{C}(O)NH$), 155.9 ($O\underline{C}(O)NH$), 57.6 ($\underline{C}H_2N(CH_3)_2$), 43.6 (($\underline{C}H_3)_2$), 39.3 ($CH_2$), 32.9 ($\underline{C}H_2NHC(O)$), 29.9 ($\underline{C}H_2$), 29.3 ($\underline{C}H_2$), 28.4 ($\underline{C}H_3)_2$), 26.4 ($\underline{C}H_2$), 26.2 ($\underline{C}H_2$), 21.3 ($CH_2$)

_N_-(4-Bromobenzyl)-_N_-[3-(6-aminohexylcarbamoyl)-propyl]-dimethylammonium bromide (1.27)



1.27

[6-(4-Dimethylamino-butyrylamino)-hexyl]-carbamic acid *tert*-butyl ester **1.25** (2 g, 8.7 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (20 mL) and 4-bromoethylbenzyl bromide was added (2.4 g, 9.6 mmol, 1.1 equiv.) The mixture was stirred for 3 h and the solvent removed *in vacuo*. The crude oil was triturated in toluene to remove unreacted bromide. The oil was dried *in vacuo* to afford product **1.26** which was directly reacted in the next step.

Deprotection of the tert-butyloxycarbonyl (Boc) group was performed by suspending the oil in a solution of 2 M HCl in diethyl ether (10 mL) for 5 min to afford a white solid. The title compound was obtained as an oil after suspending the salt in MeOH (10 mL) in the presence of Amberlyst A-27 (3 g , 89 %)

HPLC (method 1): t_R = 3.1 min

Purity: 100 % (ELSD)

ESI+/MS: m/z = 398.5 M^+

ESI+/HRMS: 398.17915 (calcd 398.17970)

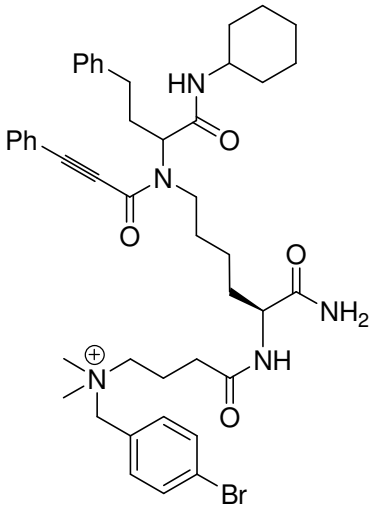
^{1}H NMR (250 MHz, d^6-DMSO): 8.12 (1H, t, J^3 = 5.4 Hz, C(O)N<u>H</u>), 7.71 (2H, d, J^3 = 8.3 Hz, C<u>H</u>$_{ar}$), 7.55 (2H, d, J^3 = 8.4 Hz, C<u>H</u>$_{ar}$), 4.62 (2H, s, C<u>H</u>$_2$C$_{ar}$), 3.04 (2H, m, $N^+C\underline{H}_2CH_2$), 2.99 (6H, s, (C<u>H</u>$_3$)$_2$), 2.72 (2H, m, C<u>H</u>$_2$NH$_2$), 2.56 (4H, m, CH$_2$N<u>H</u>$_2$ + C<u>H</u>$_2$C(O)NH), 2.15 (2H, m, C<u>H</u>$_2$NHC(O)), 1.99 (2H, m, C<u>H</u>$_2$CH$_2$NHC(O)), 1.55 (2H, m, C<u>H</u>$_2$CH$_2N^+$), 1.35 (2H, m, C<u>H</u>$_2$CH$_2$NH$_2$), 1.27 (4H, m, 2 × C<u>H</u>$_2$).

^{13}C NMR + DEPT 135 (75 MHz, d^6-DMSO): 170.7 (<u>C</u>(O)NH), 137.5 (CH$_{ar}$), 132.2 (<u>C</u>H$_{ar}$), 127.9 (<u>C</u>$_{ar}$), 124.4 (<u>C</u>$_{ar}$), 65.3 (<u>C</u>H$_2$C$_{ar}$), 63.2 (<u>C</u>H$_2$C$_{ar}$), 49.4 (C(<u>C</u>H$_3$)$_2$), 38.7 (<u>C</u>H$_2$NH$_2$), 31.9 (<u>C</u>H$_2$), 29.2 (<u>C</u>H$_2$), 27.2 (<u>C</u>H$_2$), 26.2 (<u>C</u>H$_2$), 25.9 (<u>C</u>H$_2$), 20.4 (<u>C</u>H$_2$), 18.7 (<u>C</u>H$_2$).

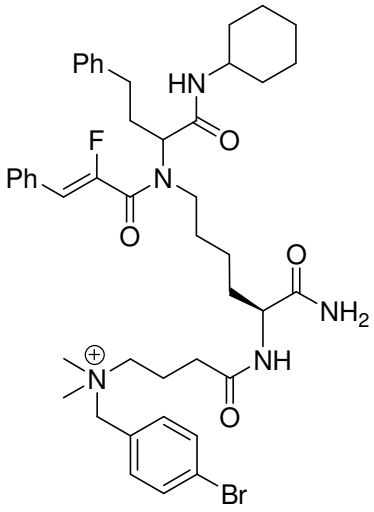
5.2.3. Experimental procedure for the validation of the quantitative properties of ESI+/MS analytical constructs

5.2.3.1. Preparation and analsis of mixtures of compounds

Preparation and analysis of the mixture of compounds 1.32 and 1.33



1.32



1.33

Stock solutions (152 mM) of the following species were prepared:

- Hydrocinnamaldehyde: (200 μL, 1.52 mmol) was dissolved in MeOH/CH_2Cl_2 (1:1 v/v, 9.8 mL).
- *Cyclo*hexyl isonitrile: (189 μL, 1.52 mmol) was mixed with MeOH/CH_2Cl_2 (1:1 v/v, 9.811 mL).

Stock solutions (76.2 mM) of the two carboxylic acids were prepared, by dissolving each acid (0.762 mmol) in MeOH/CH_2Cl_2 (1:1 v/v, 10 mL), which corresponded to the following:

- Phenylpropiolic acid: 111 mg
- α-fluorocinnamic acid: 127 mg

Analytical construct resin **1.12** (25 mg, s = 0.61 mmol/g, 1 equiv.) was swollen in CH_2Cl_2/MeOH (1:1 v/v, 1 mL) in a 5 mL microwave vial. To the resin were added phenylpropiolic acid and α-fluorocinnamic acid (0.5 mL of each stock solution corresponding to 2.5 equiv. of each acid), and hydrocinnamaldehyde (0.5 mL of stock solution, 5 equiv.). The volume of the reaction mixture was adjusted to 4.5 mL by the addition of CH_2Cl_2/MeOH (1:1 v/v). The vial was sealed and placed on a linear shaker for 10 min before cyclohexyl isonitrile was added (0.5 mL of the stock solution, 5 equiv.). The mixture was heated under microwave irradiation at 120°C for 30 min. The resin was transferred to a solid phase extraction tube and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), methanol (5 × 3 mL), and diethylether (5 × 3 mL).

Cleavage of products from the resin was carried out with a solution of TFA in CH_2Cl_2 (1:4 v/v, 12 mL) for 15 min. After removal of the solvents *in vacuo*, the crude mixture of compounds was dissolved in HPLC grade MeOH (10 mL). A sample of the mixture (1 mL) was transferred to an HPLC vial for ESI+/MS analysis (injection volume: 1 µL) and HPLC/ELSD analysis (method 5, injection volume 100 µL).

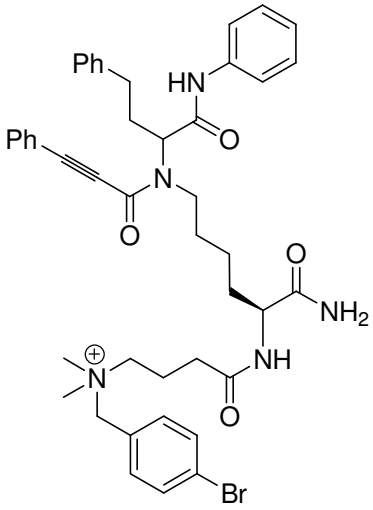
The ion chromatogram was extracted from the MS trace obtained (total ion current, TIC) using the Chemstation software (Rev. A.08.03 [847], Agilent Technologies). Peaks which did not display the characteristic bromine pattern were discarded and each of the remaining peaks was attributed to the corresponding Ugi 4-CC product. The relative intensities of the MS peaks were exported to a comma separated values file, for treatment with Microsoft Excel (Office Excel 2003 SP2) to allow determination of the composition of the mixture.

ELSD quantification was carried out by calcutating the area of each peak, identified thanks to HPLC/MS analysis of the mixture using the same method.

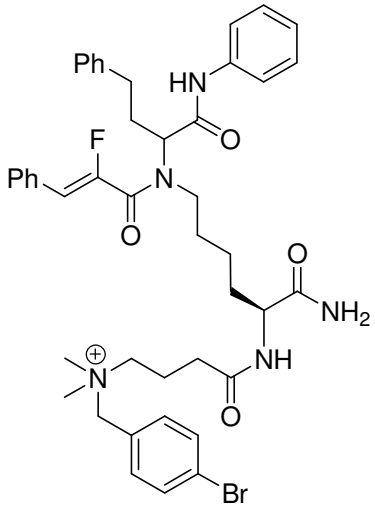
HPLC (method 5): t_R = 16.1 min (compound **1.32**, 71 %), t_R = 16.9 min (compound **1.33**, 29 %)

ESI+/MS: m/z = 798.3 (**1.32**, M^+, 73 %), m/z = 818.3 (**1.33**, M^+, 27 %)

Preparation and analysis of the mixture of compounds 1.34 and 1.35



1.34



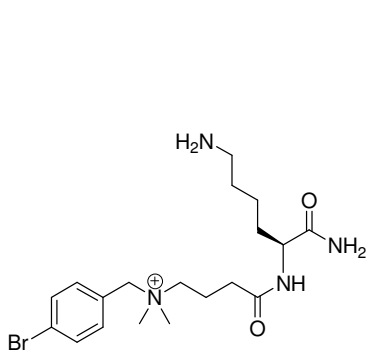
1.35

The procedure was repeated replacing cyclohexyl isonitrile by benzyl isonitrile to prepare and analyse a mixture of compounds **1.34** and **1.35**.

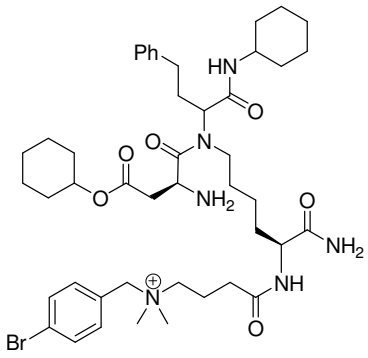
HPLC (method 5): t_R = 16.3 min (compound **1.34**, 73 %), t_R = 17.2 min (compound **1.35**, 27 %)

ESI+/MS: m/z = 793.3 (**1.34**, M^+, 80 %), m/z = 812.3 (**1.35**, M^+, 20 %)

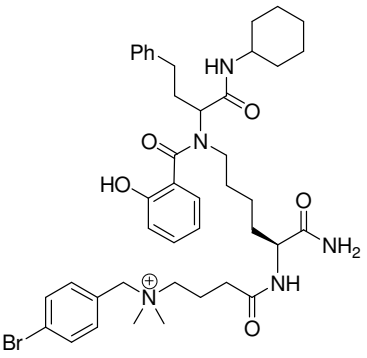
5.2.3.2. Preparation and analsis of mixtures of ionisable compounds



1.23



1.36



1.37

Stock solutions (76.2 mM) of the two carboxylic acids were prepared, by dissolving each acid (0.762 mmol) in MeOH/CH_2Cl_2 (1:1 v/v, 10 mL), which corresponded to the following:

- Boc-L-Glu(CHx)OH: 174 mg
- 2-hydroxybenzoic acid: 105 mg

The procedure decribed in section 5.2.3.1 was carried out using 50 µL of each stock solution of carboxylic acid (corresponding to 0.25 equiv. of each carboxylic acid) to prepare and analyse a mixture of compounds **1.36** and **1.37**.

HPLC (method 5): t_R = 4.8 min (compound **1.23**, 45 %), t_R = 9.3 min (compound **1.36**, 10 %), t_R = 10.4 min (compound **1.37**, 45 %)
ESI+/MS: m/z = 427.1 (**1.23**, M^+, 54 %), m/z = 867.4 (**1.36**, M^+, 6 %), m/z = 790.3 (**1.37**, M^+, 40 %)

5.3. Experimental to chapter two

5.3.1. General procedure for solid phase microwave assisted Ugi 4-CCs

The following procedure was used for the monomer reactivity profiling of carboxylic acids (0.25 equiv.) and then adapted in the case of 0.50 and 0.75 equiv. of each carboxylic acid. Similar protocols were then used for aldehydes and isonitriles. Experimental conditions are inspired from a described procedure for microwave assisted Ugi 4CC.[128]

Stock solutions (152 mM) of the following species were prepared:

- Hydrocinnamaldehyde **2.2**: (200 μL, 1.52 mmol) was dissolved in MeOH/CH_2Cl_2 (1:1 v/v, 9.8 mL).
- Cyclohexyl isonitrile **2.3**: (189 μL, 1.52 mmol) was mixed with MeOH/CH_2Cl_2 (1:1 v/v, 9.811 mL).

Stock solutions (76.2 mM) of carboxylic acids were prepared, by dissolving each acid (0.762 mmol) in MeOH/CH_2Cl_2 (1:1 v/v, 10 mL), which corresponded to the following:

- Phenylpropiolic acid **2.4**: 111 mg
- Phenoxyacetic acid **2.5**: 116 mg
- α-fluorocinnamic acid **2.6**: 127 mg
- Decanoic acid **2.7**: 131 mg
- Methoxyacetic acid **2.8**: 69 mg
- Heptafluorobutyric acid **2.9**: 163 mg
- 2-Iodobenzoic acid **2.10**: 189 mg
- Methyl red **2.11**: 205 mg
- Fluorescein **2.12**: 253 mg
- 3,3-dimethylbutyric acid **2.13**: 88 mg

Analytical construct resin **1.12** (25 mg, s = 0.61 mmol/g, 1 equiv.) was swollen in CH_2Cl_2/MeOH (1:1 v/v, 1 mL) in a 5 mL microwave vial. To the resin was added the

mixture of 10 carboxylic acids (50 μL of each stock solution corresponding to 0.25 equiv. of each acid), and hydrocinnamaldehyde **2.2** (0.5 mL of stock solution, 5 equiv.). The volume of the reaction mixture was adjusted to 4.5 mL by the addition of CH_2Cl_2/MeOH (1:1 v/v). The vial was sealed and placed on a linear shaker for 10 min before cyclohexyl isonitrile **2.3** was added (0.5 mL of the stock solution, 5 equiv.). The mixture was heated under microwave irradiation at 120°C for 30 min. The resin was transferred to a solid phase extraction tube and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), methanol (5 × 3 mL), and diethylether (5 × 3 mL).

Cleavage of products from the resin was carried out with a solution of TFA in CH_2Cl_2 (1:4 v/v, 12 mL) for 15 min. After removal of the solvents *in vacuo*, the crude mixture of compounds was dissolved in HPLC grade MeOH (10 mL). A sample of the mixture (1 mL) was transferred to an HPLC vial for ESI+/MS analyses (injection volume: 1 μL)

The ion chromatogram was extracted from the MS trace obtained (total ion current, TIC) using the Chemstation software (Rev. A.08.03 [847], Agilent Technologies). Peaks which did not display the characteristic bromine pattern were discarded and each of the remaining peaks was identified either as the cleaved starting material **1.23**, a product of the reaction, or as a by-product. The relative intensities of all MS peaks were exported to a comma separated values file, for treatment with Microsoft Excel (Office Excel 2003 SP2). 100 % reactivity was attributed to the most intense peak among the products. The peak intensities of other products were calculated as a function of the latter, affording percentages of reactivity. The conversion of the reaction was obtained by calculating the ratio between the peak intensity of the remaining starting material and the sum of the peak intensities of products and by-products.

5.3.2. General procedure for solution phase microwave assisted Ugi 4-CCs

The following procedure was used for the solution phase monomer reactivity profiling of carboxylic acids (0.25 equiv.) and adapted to the case of 0.10, 0.50 and 0.75 equiv. of each carboxylic acid. Similar protocols were the used for the aldehyde and isonitrile studies.

Stock solutions with the following concentrations were prepared:

- Solution phase analytical construct **1.27**: 13.75 mM
  110 mg (1.52 mmol) in MeOH/CH_2Cl_2 (1:4 v/v, 20 mL)
- Hydrocinnamaldehyde **2.2**: 152 mM
  200 µL (1.52 mmol) in MeOH/CH_2Cl_2 (1:4 v/v, 9.8 mL)
- Cyclohexyl isonitrile **2.3**: 152 mM
  189 µL (1.52 mmol) in MeOH/CH_2Cl_2 (1:4 v/v, 9.811 mL)

Stock solutions of each carboxylic acid were prepared as decribed in section 5.3.1.

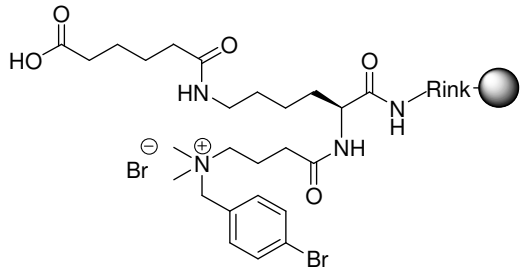
Solution phase analytical construct **1.27** (1 mL of stock solution, 13.75 µmol, 1 equiv.) was introduced into a 5 mL microwave vial. The mixture of 10 carboxylic acids (50 µL of each stock solution corresponding to 0.25 equiv. of each acid) was added, and hydrocinnamaldehyde **2.2** (0.5 mL of stock solution, 5 equiv.). The volume of the reaction mixture was adjusted to 4.5 mL by the addition of CH_2Cl_2/MeOH (1:1 v/v). The vial was sealed and placed on a linear shaker for 10 min before cyclohexyl isonitrile **2.3** was added (0.5 mL of the stock solution, 5 equiv.). The mixture was heated under microwave irradiation at 120°C for 15 min.
The crude mixture of compounds was dissolved in HPLC grade MeOH (10 mL). 1 mL of mixture was transferred to an HPLC vial for ESI+/MS analysis (injection volume: 1 µL).

A method similar to the one decribed in section 5.3.1. was used to afford the conversion of the reaction as well as relative reactivity values.

5.4. Experimental to chapter three

5.4.1. Preparation and characterisation of the analytical construct

Adipic acid analytical construct resin (3.4)



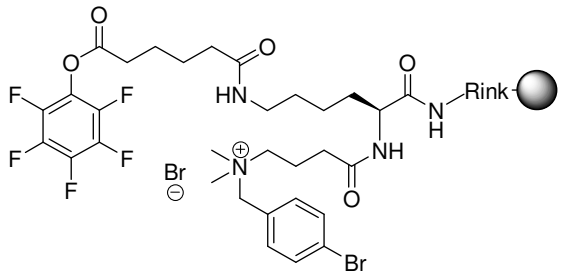
3.4

The protocol followed was reported by Gerritz *et al*.[89]

Adipic Acid (709 mg, 4.9 mmol, 8 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 10 mL). HOBt was added (659 mg, 4.9 mmol, 8 equiv.) and after complete dissolution, DIC (765 µL, 4.9 mmol, 8 equiv.) was introduced. After 10 minutes, to the solution was added amine analytical construct resin **1.12** (1 g, s = 0.61 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 5 mL). The reaction was stirred over 30 minutes. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL). The coupling step was repeated. After 30 minutes, a negative qualitative ninhydrin test (see section 5.1.2) was carried out and the washing step was repeated.
The resin was dried *in vacuo* overnight to afford **3.4** as a buff coloured resin (1.2 g, 95 %).

IR ν (cm^{-1}): 3407 (w, br, ν_{N-H}), 1667 (s, br, $\nu_{C=O}$), 1598 (m, $\nu_{C=O}$)

Pentafluorophenyl ester analytical construct resin (3.5)



3.5

The protocol followed was reported by Gerritz *et al*.[89]

The adipic acid analytical construct resin **3.4** (2 g, s = 0.49 mmol/g) was swollen in a 0.5 M solution of Pfp trifluoroacetate (860 µL, 10 mmol, 10 equiv.) and 0.5 M pyridine (400 µL, 10 mmol, 10 equiv.) in DMF (18.74 mL). The mixture was stirred over 6 h and the resin was washed with DMF (3 × 30 mL), CH_2Cl_2 (3 × 30 mL) and diethylether (3 × 30 mL). The resin was dried *in vacuo* overnight to afford **3.5** as a buff coloured resin (2.3 g, 98 %).

IR ν (cm^{-1}): 3415 (w, br, ν_{N-H}), 1725 (m, $\nu_{C=O}$), 1659 (s, br, $\nu_{C=O}$)

5.4.2. Experimental procedures for Hammett parameters assessment

General procedure for competitive amide bond formation

The following general procedure was used for the competitive amide bond formation, to assess reaction rates regarding the displacement of Pfp ester by a mixture of substituted anilines. The following describes the method for competition experiment between aniline **3.11** and *para*-toluidine **3.9** (*para*-methyl aniline).

Stock solutions (5 M) of the following species were prepared in DMF/pyridine (1;1 v/v, 5 mL)

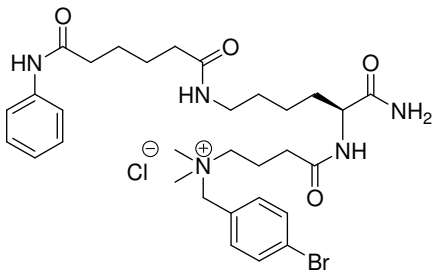
- aniline **3.11** (232 mg, 2.49 mmol)
- *para*-toluidine **3.9** (268 mg, 1 equiv.)

Pfp ester analytical construct resin **3.5** (10 mg, s = 0.42 mmol/g) was swollen in a mixture of the stock solutions (5 mL of each aniline solution). The mixture was stirred for 24 h. The resin was washed with DMF (3 × 2 mL), CH_2Cl_2 (3 × 2 mL), methanol (3 × 2 mL), and diethylether (3 × 2 mL).

Cleavage of the products from the resin was carried out with a solution of TFA in CH_2Cl_2 (1:4 v/v, 12 mL) for 15 min. After removal of the solvents *in vacuo*, the crude mixture of compounds was dissolved in HPLC grade MeOH (10 mL). A sample of the solution (1 mL) was transferred to an HPLC vial for ESI+/MS to be carried out (injection volume: 1 μL).

Quantitative assessment of the composition of the mixture was carried out by extracting MS data as described in section 5.2.3.1.

_N_-(4-Bromobenzyl)-_N_-{3-[1-carbamoyl-5-(5-pentafluorophenyloxycarbonyl-pentanoylamino)-pentylcarbamoyl]-propyl}-dimethylammonium chloride (3.15)



3.15

Pfp ester analytical construct resin **3.5** (1 g, s = 0.42 mmol/g) was swollen in a 0.5 M solution of aniline **3.11** (466 mg, 3.58 mmol, 8.5 equiv.) in DMF/pyridine (1:1 v/v, 10 mL) and shaken overnight. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL). The product was cleaved form the resin with TFA in CH_2Cl_2 (1:4 v/v, 15 min). The solvents were the removed *in vacuo* to afford compound **3.15** as an oily solid (247 mg, 83 %)

HPLC (method 1): t_R = 6.1 min

Purity: 98 % (ELSD)

IR ν (cm^{-1}): 3405 (w, br, ν_{N-H}), 1654 (s, br, $\nu_{C=O}$), 1546 (w, $\nu_{C=O}$)

ESI+/MS: m/z = 630.2 (M^+)

FAB+/HRMS: m/z = 630.26602 (M^+) (calcd 630.26549)

^{1}H NMR + COSY + NOESY (250 MHz, d^6-DMSO): 9.89 (1H, s, br C(O)N<u>H</u>CHC(O)NH_2), 7.72 (2H, d, J^3 = 8.3 Hz, C<u>H</u>$_{ar}$), 7.58 (2H, d, J^3 = 8.3 Hz, C<u>H</u>$_{ar}$), 7.50 (2H, d, J^3 = 7.9 Hz, C<u>H</u>$_{ar}$aniline), 7.39 (2H, s, br, C(O)N<u>H</u>$_2$), 7.27 (2H, t, J^3 = 7.9 Hz, C<u>H</u>$_{ar}$aniline), 7.00 (1H, t, J^3 = 7.9 Hz, C<u>H</u>$_{ar}$aniline), 4.50 (2H, s, $N^+C\underline{H}_2C_{ar}$), 4.16 (1H, m, C<u>H</u>C(O)NH_2), 3.24 (1H, m, C(O)N<u>H</u>CH_2), 3.02 (2H, m, C<u>H</u>$_2$), 2.94 (6H, s, (C<u>H</u>$_3$)$_2$), 2.26 (4H, m, 2 × C<u>H</u>$_2$), 2.04 (4H, m, 2 × C<u>H</u>$_2$), 1.51 (6H, m, 3 × C<u>H</u>$_2$), 1.36 (2H, m, C<u>H</u>$_2$), 1.22 (2H, m, C<u>H</u>$_2$)

^{13}C NMR + DEPT 135 + DEPT 90 (90 MHz, d^6-DMSO): 175.3 (<u>C</u>(O)NH_2), 173.3 (<u>C</u>(O)NHPh), 172.6 (<u>C</u>(O)NHCH), 172.1 (<u>C</u>(O)N<u>H</u>CH_2), 140.8 (<u>C</u>H$_{ar}$), 136.6 (<u>C</u>H$_{ar}$), 133.4 (<u>C</u>H$_{ar}$), 130.1 (<u>C</u>H$_{ar}$), 128.9 (<u>C</u>H$_{ar}$), 125.6 (<u>C</u>H$_{ar}$), 124.4 (<u>C</u>H$_{ar}$), 120.5 (<u>C</u>H$_{ar}$), 66.8 ($N^+\underline{C}H_2C_{ar}$), 64.6 ($N^+\underline{C}H_2CH_2$), 53.8 (<u>C</u>HC(O)$NH_2$), 50.6 ((<u>C</u>H$_3$)$_2$), 39.8

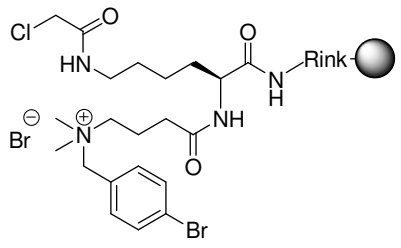
($\underline{C}H_2NHPh$), 37.7 ($\underline{C}H_2$), 36.8 ($\underline{C}H_2$), 33.2 ($\underline{C}H_2$), 32.8 ($\underline{C}H_2$), 30.4 ($\underline{C}H_2$), 26.5 ($\underline{C}H_2$), 26.4 ($\underline{C}H_2$), 24.4 ($\underline{C}H_2$), 19.7 ($\underline{C}H_2$)

5.5. Experimental to chapter four

5.5.1. Preparation of phenoxyacetyl fragments

5.5.1.1. Preparation of chloroacetamide analytical construct resin

Chloroacetamide analytical construct resin (4.18)



4.18

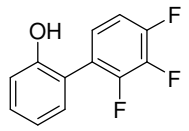
Chloroacetic acid (70 mg, 0.75 mmol, 3 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 3 mL). HOBt was added (100 mg, 0.75 mmol, 3 equiv.) and after complete dissolution, DIC (118 μL, 0.75 mmol, 3 equiv.) was introduced. After 20 min of stirring, the solution was added to the analytical construct resin **1.12** (0.5 g, s = 0.5 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 2 mL). The reaction was stirred over 30 min. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL). The coupling procedure was repeated. After 30 min, a negative qualitative ninhydrin test (see section 5.1.2) was carried out. The washing procedure was repeated and the material dried *in vacuo* overnight to afford the title compound **4.18** as a buff coloured resin (0.53 g, 98 %).

Loading (chlorine elemental analysis): 0.44 mmol/g (theoretical 0.46 mmol/g)
IR ν (cm^{-1}): 3405 (w, br, ν_{N-H}), 1712 (m, $\nu_{C=O}$), 1681 (s, br, $\nu_{C=O}$)

5.5.1.2. Preparation of the biphenol derivatives

The following biphenol compounds were prepared:

2-(2'-3'-4'-trifluorophenyl)phenol (4.13)[116]



4.13

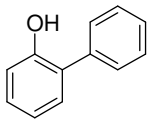
The synthesis of diphenol compounds was adapted from the synthesis described by Wood.[116] The preparation of 2'-3'-4'-trifluoro-biphenyl-2-ol **4.13** is given as a representative example.

To a solution of 2-bromophenol (692 mg, 4 mmol, 1 equiv.) in benzene, MeOH and water (20:4:1 v/v/v, 12.5 mL) was added Na_2CO_3 (1.69 g, 16 mmol, 4 equiv.), 2,3,4-trifluorophenylboronic acid (704 mg, 4 mmol, 1 equiv.) and $Pd(PPh_3)_4$ (231 mg, 0.2 mmol, 0.05 equiv.) under a nitrogen atmosphere. The reaction mixture was heated at reflux for 15 h and then allowed to cool to room temperature. After evaporation of solvents *in vacuo* and dissolution in EtOAc (20 mL), a filtration was performed to remove solids and the organic layer was washed with water (20 mL) and subsequently dried with Na_2SO_4. The residue obtained after filtration and evaporation of the solvent *in vacuo* was purified by silica gel chromatography with EtOAc in hexane (15:85 v/v) to give the desired bisphenyl compound **4.13** as a pale yellow powder (340 mg, 38 %).

HPLC (method 1): t_R = 7.5 min
Purity: 98 % (254 nm)
APCI-/MS: 213.0 $(M-H)^-$
Mp: 49 °C

^{1}H NMR + COSY (250 MHz, $CDCl_3$): 7.48 (1H, td, J^3 = 7.7 Hz, J^4 = 1.8 Hz, CH_{ar}), 7.38 (1H, d, J^3 = 7.7 Hz, CH_{ar}), 7.32-7.15 (3H, m, CH_{ar}), 7.13-6.09 (1H, m, CH_{ar}), 5.02 (1H, s, OH)
^{19}F NMR (235 MHz, $CDCl_3$): -135 (F_{para}), -140 (F_{ortho}), -161 (F_{meta}).

2-Phenylphenol (4.14)[116]

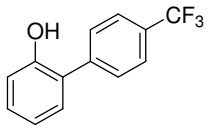


4.14

A protocol similar to the one previously described yielded **4.14** as a pale yellow powder (160 mg, 23 %).

HPLC (method 1): t_R = 8.6 min
Purity: 99 % (254 nm)
Mp: 58 °C
^{1}H NMR (250 MHz, $CDCl_3$): 7.70-7.68 (2H, m, CH_{ar}), 7.54-7.35 (5H, m, CH_{ar}), 7.08 (1H, dd, J^3 = 7.9 Hz, CH_{ar}), 6.88 (1H, d, J^3 = 7.7 Hz, CH_{ar}), 4.98 (1H, s, OH)

2-(4'-trifluoromethylphenyl)phenol (4.15)[116]



4.15

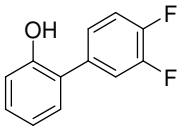
A protocol similar to the one previously described yielded compound **4.15** as a dark orange powder (438 mg, 46 %).

HPLC (method 1): t_R = 7.9 min
Purity: 100 % (254 nm)
APCI-/MS: 237.0 $(M-H)^-$

Mp: 61 °C

^{1}H NMR (250 MHz, $CDCl_3$): 7.67 (2H, d, J^3 = 8.1 Hz, CH_{ar}), 7.58 (2H, d, J^3 = 8.1 Hz, CH_{ar}), 7.24-7.20 (2H, m, CH_{ar}), 7.00-6.90 (2H, m, CH_{ar}), 5.09 (1H, s, br, OH)

2-(3'-4'-difluorophenyl)phenol (4.16)[116]



4.16

A protocol similar to the one previously described yielded **4.16** as a dark pink powder (428 mg, 52 %)

HPLC (method 1): t_R = 7.6 min

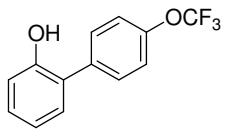
Purity: 99 % (254 nm)

APCI-/MS: 205.0 $(M-H)^-$

Mp: 52 °C

^{1}H NMR (250 MHz, $CDCl_3$): 7.39-7.27 (2H, m, CH_{ar}), 7.26-7.22 (4H, m, CH_{ar}), 7.04-6.93 (2H, m, CH_{ar}), 5.04 (1H, s, br, OH)

2-(4'-trifluoromethoxyphenyl)phenol (4.17)[116]



4.17

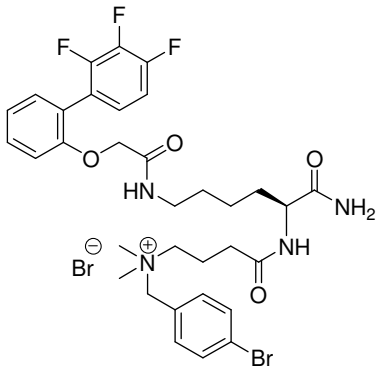
A protocol similar to the one previously described yielded compound **4.17** as grey powder (617 mg, 66 %).

HPLC (method 1): t_R = 7.9 min
Purity: 98 % (254 nm)
APCI-/MS: 253.0 $(M-H)^-$
Mp: 40 °C
^{1}H NMR (250 MHz, $CDCl_3$): 7.52 (1H, d, J^3 = 8.9 Hz, CH_{ar}), 7.32 (1H, d, J^3 = 8.9 Hz, CH_{ar}), 7.22-7.27 (2H, m, CH_{ar}), 6.93-7.03 (2H, m, CH_{ar}), 5.02 (1H, s, OH)

All analyses agreed with the literature. [116]

5.5.1.3. Solid phase immobilisation of the biphenol derivatives

(4-Bromobenzyl)-(3-{(S)-1-carbamoyl-5-[2-(2',3',4'-trifluoro-biphenyl-2-yloxy)-acetylamino]-pentylcarbamoyl}-propyl)-dimethylammonium bromide (4.19)



4.19

Chloroacetamide analytical construct resin **4.18** (0.6 g, s = 0.48 mmol/g, 1 equiv.) was swollen in DMF (5 mL) and cesium carbonate (334 mg, 1.03 mmol, 3 equiv.) was added with potassium iodide (113 mg, 687 mmol, 2 equiv.). 2'-3'-4'-trifluoro-biphenyl-2-ol **4.13** (385 mg, 1.72 mmol, 5 equiv.) was added and the suspension was gently stirred at 70˚C for 2 h. The resin was washed with DMF (3 × 5 mL), CH_2Cl_2 (3 × 5 mL), methanol (3 × 5 mL), and diethylether (3 × 5 mL). The compound was cleaved from the resin (0.7 g, 0.48 mmol/g) with TFA in CH_2Cl_2 (1:4 v/v, 10 mL) for 15 min to afford **4.19** as an oily product (244 mg, 93 %).

HPLC (method 1): t_R = 7.6 min

Purity: 99 % (ELSD)

ESI+/MS: m/z = 691.2 (M^+)

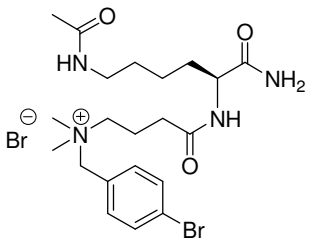
FAB+/HRMS: m/z = 691.21083 (M^+) (calcd 691.21068)

^{1}H NMR (250 MHz, D_2O): 7.63-7.52 (3H, m, CH_{ar}), 7.36-7.31 (3H, m, CH_{ar}), 7.28-7.12 (3H, m, CH_{ar}), 7.09 (1H, m, CH_{ar}), 4.40 (2H, s, $N^+CH_2C_{ar}$), 4.24 (2H, s, C(O)CH_2O), 4.16 (1H, m, CHC(O)NH_2), 3.18 (2H, m, $N^+CH_2CH_2$), 2.97 (6H, s, $N^+(CH_3)_2$), 2.90 (2H, t, J^3 = 8.0 Hz, CH_2CHC(O)NH_2), 2.33 (2H, t, J^3 = 6.9 Hz, CH_2C(O)NH), 2.04 (2H, m, CH_2), 1.40 (4H, m, 2 × CH_2lys), 1.24 (2H, m, CH_2lys)

^{13}C NMR + DEPT 135 (62.5 MHz, D_2O): 178.2 (C(O)NH_2), 173.2 (C(O)NHCH), 170.1 (C(O)$NHCH_2$), 154.7 (OCH_{ar}), 150.1 (C(F)H_{ar}), 145.7 (C(F)H_{ar}), 141.3 (C(F)H_{ar}), 131.2 (CH_{ar}), 128.8 (CH_{ar}), 127.2 (CH_{ar}), 126.3 (CH_{ar}), 125.8 (CH_{ar}), 123.1 (CH_{ar}), 117.1 (CH_{ar}), 106.9 (CH_{ar}), 67.5 (OCH_2C(O)NH), 66.7 ($N^+CH_2C_{ar}$), 63.5 ($N^+CH_2CH_2$), 52.8 (CHC(O)NH_2), 50.7 ($(CH_3)_2$), 42.9 (CH_2NH_2), 33.1 (CH_2C(O)NH), 30.3 (CH_2lys), 23.3 (CH_2CH), 21.7 (CH_2), 19.8 (CH_2lys)

5.5.2. Preparation of MS reference compound

N-capped analytical construct (4.20)



4.20

Acetic acid (94 µL, 1.65 mmol, 3 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 6 mL). HOBt was added (222 mg, 1.65 mmol, 3 equiv.). After complete dissolution, DIC (260 µL, 1.65 mmol, 3 equiv.) was introduced. After 20 min of stirring, the solution was added to the analytical construct resin **1.12** (0.9 g, s = 0.61 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 2 mL). The reaction was stirred over 30 min. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL). The coupling procedure was repeated. After 30 min, a negative qualitative ninhydrin test (see section 5.1.2) was carried out. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL) and dried *in vacuo* overnight to afford a buff coloured resin. Cleavage from the solid support was carried out using TFA in CH_2Cl_2 (1:4 v/v, 10 mL) for 15 min. The crude product was precipitated in cold Et_2O and dissolved in water to afford, after freeze-drying compound **4.20** as a crystalline white solid (477 mg, 87%)

HPLC (method 1): t_R = 3.6 min

Purity: 98 % (220 nm)

ESI+/MS: m/z = 469.2 (M^+)

FAB+/HRMS: m/z = 469.18131 (M^+) (calcd 469.18088)

^{1}H NMR (250 MHz, D_2O): 7.49 (2H, d, J^3 = 8.5 Hz, CH_{ar}), 7.26 (2H, d, J^3 = 8.5 Hz, CH_{ar}), 4.48 (2H, s, $N^+CH_2C_{ar}$), 4.18 (1H, t, J^3 = 6.1 Hz, CHC(O)NH_2), 3.44 (2H, m,

$N^+C\underline{H}_2$), 3.08 (6H, s, $(C\underline{H}_3)_2$), 2.95 (2H, t, J^3 = 8.4 Hz, $C\underline{H}_2NHC(O)CH_3$), 2.35 (2H, t, J^3 = 6.4 Hz, $C\underline{H}_2C(O)NH$), 2.15 (2H, m, $C\underline{H}_2$), 1.78 (3H, s, CH_3), 1.49 (4H, m, 2 × $C\underline{H}_2$lys), 1.36 (2H, m, $C\underline{H}_2$lys)

$^{13}\underline{C}$ NMR + DEPT 135 (62.5 MHz, D_2O): 178.3 ($\underline{C}(O)NH_2$), 173.5 ($\underline{C}(O)NH$), 168.7 ($CH_3\underline{C}(O)NH$), 131.5 ($\underline{C}H_{ar}$), 130.3 ($\underline{C}H_{ar}$), 126.1 ($\underline{C}_{ar}$), 125.4 ($\underline{C}_{ar}$), 66.2 ($N^+\underline{C}H_2C_{ar}$), 63.8 ($N^+\underline{C}H_2CH_2$), 51.7 ($\underline{C}HC(O)NH_2$), 50.8 (($\underline{C}H_3)_2$), 39.4 ($\underline{C}H_2NHC(O)CH_3$), 32.6 ($\underline{C}H_2C(O)NH$), 29.5 ($\underline{C}H_2$lys), 25.4 ($\underline{C}H_2CH$), 23.2 ($\underline{C}H_2$), 22.8 ($\underline{C}H_3$), 20.3 ($\underline{C}H_2$lys)

5.5.3. Conditions for cathepsin S assays

Cathepsin S buffer (pH = 6.1):[116] Na_2HPO_4 (1.029 g, 6.64 mmol), NaH_2PO_4 (5.13 g, 32.9 mmol), NaCl (2.92 g, 48.7 mmol), EDTA dipotassium salt (184 mg , 0.5 mmol), DTT (77 mg, 0.5 mmol) and tween-20 (5 µL) were dissolved in dionised water (0.5 L)

Cathepsin S: the original enzyme sample (cathepsin S, Human, Recombinant, *E. coli*, Calbiochem, 25 µg in 100 µL pH 6.5 buffer (35 mM potassium phosphate, 35 mM Sodium acetate, 2 mM DTT, 2 mM EDTA, 50 % ethylene glycol), specific activity 80,000 mU/mg protein) was separated into 10 Eppendorf tubes (10 µL each). The afforded solution was diluted with cathepsin S buffer (1.49 mL) to give a 68 nM solution.

General procedure for enzyme kinetic analysis

The following procedure was used for carrying out the kinetic analysis at a concentration of substrate **4.19** of 2 µM and reference **4.19** of 2 µM.

Substrate **4.19** (24 µL of a 250 mM solution) and MS Reference **4.20** (24 µL of a 250 µM solution) were introduced into an Eppendorf tube. The volume of the reaction mixture was made to 3 mL with cathepsin S buffer and was placed in a water bath at 37 °C. After 10 min, cathepsin S was added (24 µL of a 68 nM

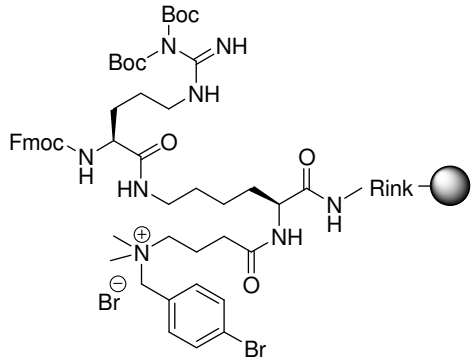
solution). The tube was vortexed periodically and every 30 s during the next 30 min, a sample of the reaction mixture (20 µL) was taken and immediately diluted with MeOH (40 µL) to quench the enzymatic activity and placed into an LC/MS vial. The crude reaction mixture (30 µL) was analysed by ESI+/MS (injection flow 2 mL/min; solvents A and B (1:1 v/v; mass window: 400-700 Da; time of acquisition 45 s).
The peak of interest (427.1 Da) could not be observed even after a few days.

The procedure was adapted to give the following substrate and reference concentrations: 5 µM, 10 µM and 15 µM, 50µM, 100 µM. All experiments were run in duplicate.
The peak of interest was again not observed.

5.5.4. Preparation of dipeptide analytical constructs

5.5.4.1. Preparation of analytical construct bound Cbz-Leu-Arg

Fmoc-Arg(Boc$_2$)-analytical construct resin (4.21)



4.21

Fmoc-Arg(Boc$_2$)-OH (298 mg, 0.5 mmol, 5 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 3 mL). HOBt was added (68 mg, 0.5 mmol, 5 equiv.) and after complete dissolution, DIC (72 µL, 0.5 mmol, 5 equiv.) was introduced. After 20 min of stirring, the solution was added to the analytical construct resin **1.12** (200 mg, s = 0.5 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 2 mL). The reaction was stirred over 30 min. The resin was washed with DMF (3 × 5 mL), CH_2Cl_2 (3 × 5 mL),

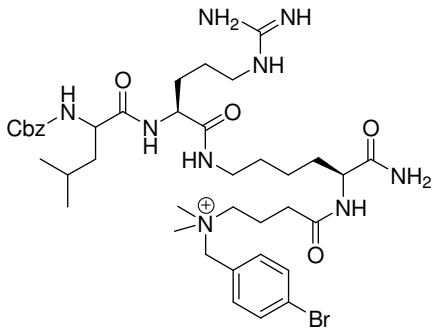
methanol (3 × 5 mL), and diethylether (3 × 5 mL). The coupling procedure was repeated. After 30 min, a negative qualitative ninhydrin test (see section 5.1.2) was carried out. The resin was washed with DMF (3 × 5 mL), CH_2Cl_2 (3 × 5 mL), methanol (3 × 5 mL), and diethylether (3 × 5 mL) and was dried *in vacuo* overnight to afford **4.21** as a buff coloured resin (255 mg, 97 %).

Capping of the unreacted amine functionalities was realised as follows. The resin was swollen in a mixture of acetic anhydride (188 µL, 2 mmol, 20 equiv.) and diisopropylethylamine (DIPEA, 165 µL, 1 mmol, 10 equiv.) in DMF (3 mL) for 30 min.The resin was washed with DMF (3 × 5 mL), CH_2Cl_2 (3 × 5 mL), methanol (3 × 5 mL), and diethylether (3 × 5 mL) and was dried *in vacuo*.

To check the efficiency of the coupling, a cleavage step was performed on a resin sample (20 mg) by swelling it in a mixture of triisopropylsilane (TIS) and CH_2Cl_2 in TFA (5:5:90 v/v/v, 500 µL) for 15 min. After evaporation of the solvent *in vacuo,* the white solid was triturated in cold Et_2O to afford the desired compound.

HPLC (method 4): t_R = 4.6 min
Purity: 72 % (254 nm)

Cbz-Leu-Arg Analytical construct (4.22)



4.22

The deprotection of the Fmoc group on resin **4.21** (255 mg, s = 0.43 mmol/g, 1 equiv.) was perfrmed as described in section 5.1.2 and the resin was dried *in vacuo* overnight.

Cbz-Leu-OH (265 mg, 1 mmol, 10 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 3 mL). HOBt was added (153 mg, 1 mmol, 10 equiv.) and after complete dissolution, DIC (144 µL, 1 mmol, 10 equiv.) was introduced. After 20 min of stirring, the solution was added to the deprotected resin in CH_2Cl_2/DMF (7:3 v/v, 2 mL). The reaction was stirred for 30 min. The resin was washed with DMF (3 × 5 mL), CH_2Cl_2 (3 × 5 mL), methanol (3 × 5 mL), and diethylether (3 × 5 mL). After 30 min, a negative qualitative ninhydrin test (see section 5.1.2) was carried out. The resin was washed with DMF (3 × 5 mL), CH_2Cl_2 (3 × 5 mL), methanol (3 × 5 mL), and diethylether (3 × 5 mL) and was dried *in vacuo* overnight.

The cleavage step was performed by swelling the resin in a mixture of TIS and CH_2Cl_2 in TFA (5:5:90 v/v/v, 5 mL) for 15 min. After evaporation of the solvent *in vacuo,* the white solid was precipitated in cold Et_2O to afford the crude compound **4.22**. Semi preparative HPLC gave **4.22** in good purity (30 mg, 30 %).

HPLC (method 4): t_R = 5.4 min
Purity: 95 % (254 nm)
ESI+/MS: m/z = 416.0 $(M+2H)^{2+}$)
FAB+/HRMS: m/z = 830.39238 (M^+) (calcd 830.39282)

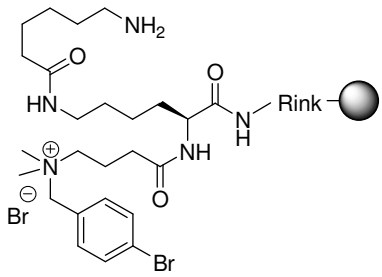
5.5.4.2. Enzymatic assays with compound **4.22**

Enzymatic assays at an enzyme concentration of 0.1 nM and the following substrate and MS references concentrations: 5 µM, 10 µM, 30 µM, 50 µM, 80 µM and 100 µM were carried out as described in section 5.5.3.

The expected MS peak (427.1) could not be detected even after several hours.

5.5.4.3. Use of aminohexanoic acid spacer

Aminohexanoic acid analytical construct resin (4.23)

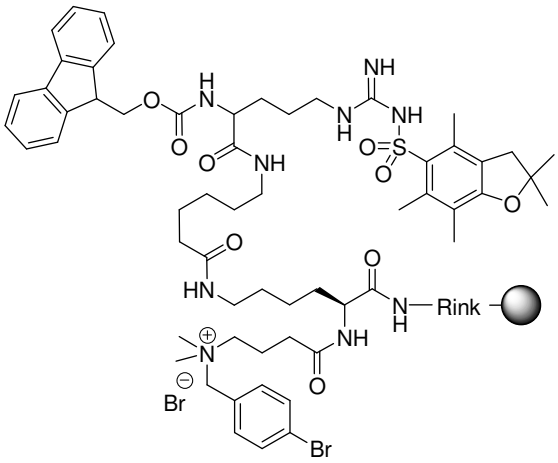


4.23

Fmoc-Aminohexanoic acid (883 mg, 2.5 mmol, 10 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 3 mL). HOBt was added (338 mg, 2.5 mmol, 10 equiv.) and after complete dissolution, DIC (358 µL, 2.5 mmol, 10 equiv.) was introduced. After 20 min of stirring, the solution was added to the analytical construct resin **1.12** (0.5 g, s = 0.5 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 2 mL). The reaction was stirred over 30 min. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL). The coupling procedure was repeated. After 30 min, a negative qualitative ninhydrin test (see section 5.1.2) was carried out. The washing procedure was repeated.

The deprotection of the Fmoc group was performed as described in section 5.1.2. and the resin was dried *in vacuo* to afford compound **4.23** as a buff coloured resin (0.52 g, 99 %).

Fmoc-Arg(Pbf)-aminohexanoic-analytical construct resin (4.24)

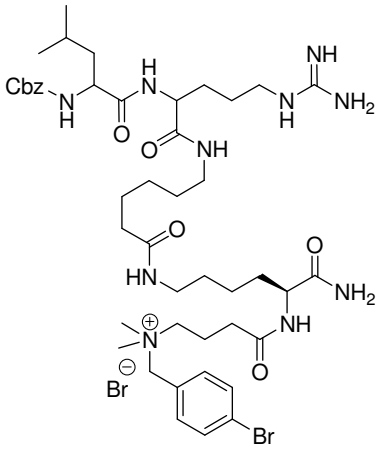


4.24

Fmoc-Arg(Pbf)-OH (810 mg, 1.25 mmol, 5 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 3 mL). HOBt was added (169 mg, 1.25 mmol, 5 equiv.) and after complete dissolution, DIC (179 µL, 1.25 mmol, 5 equiv.) was introduced. After 20 min of stirring, the solution was added to the aminohexanoic analytical construct resin **4.23** (520 mg, s = 0.47 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 5 mL). The reaction was stirred over 30 min. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL). The coupling and washing procedures were repeated and the resin was dried *in vacuo*.

Capping of the unreacted amine functionalities was realised as follows. The resin was swollen in a mixture of acetic anhydride (467 µL, 5 mmol, 20 equiv.) DIPEA (412 µL, 2.5 mmol, 10 equiv.) and HOBt (38 mg, 0.25 mmol, 1 equiv.) in DMF (6 mL). After 30 min, a negative qualitative ninhydrin test (see section 5.1.2) was carried out. The usual washing procedure was carried out and the resin was dried *in vacuo* overnight to afford the title compound as a buff coloured material (650 mg, 97 %).

Cbz-Leu-Arg(Pbf)-aminohexanoic-analytical construct (4.25)



4.25

The deprotection of the Fmoc group was performed as described in section 5.1.2. Cbz-Leu-OH (672 mg, 2.5 mmol, 10 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 3 mL). HOBt was added (338 mg, 2.5 mmol, 10 equiv.) and after complete dissolution, DIC (358 µL, 2.5 mmol, 10 equiv.) was introduced. After 20 min of stirring, the solution was added to the deprotected resin in CH_2Cl_2/DMF (7:3 v/v, 2 mL). The reaction was stirred over 30 min. A negative qualitative ninhydrin test (see section 5.1.2) was carried out and the resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL) and was dried *in vacuo* overnight to afford the title compound as a buff coloured resin (780 mg, 96 %).

The cleavage step was performed by swelling the resin in a mixture of TIS and CH_2Cl_2 in TFA (5:5:90 v/v/v, 500 µL) for 15 min. After evaporation of the solvent *in vacuo,* the white solid was triturated in cold Et_2O to afford the crude compound **4.25**. Semi preparative HPLC afforded **4.25** in good purity (50 mg, 22 %).

HPLC (method 4): t_R = 5.7 min
Purity: 99 % (ELSD)
ESI+/MS: m/z = 471.4 $((M+H)^{2+})$
ESI+/HRMS: m/z = 943.47614 $(M+H)^+$ (calcd 943.47688)

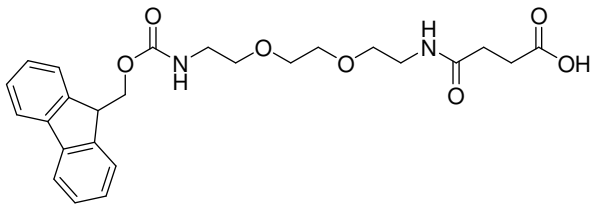
5.5.4.4. Enzymatic assays with compound **4.25**

Enzymatic assays at an enzyme concentration of 0.1 nM and substrate and MS references concentrations of 5 µM, 10 µM, 30 µM, 50 µM, 80 µM and 100 µM were carried out as descrined in section 5.5.3.

The expected MS peak (427.1) started to be detected after two hours of kinetics. The total consumption of **4.25** took around 8 hours to take place.

5.5.4.5. Introduction of the "PEG" spacer

4-(2-(2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)ethoxy)ethoxy)ethylamino)-4-oxobutanoic acid (4.26)[124]



4.26

2,2'-(Ethylenedioxy)bis(ethylamine) (2.96 mL, 20 mmol, 1 equiv.) was dissolved in CH_3CN (50 mL) and succinic anhydride (2.02 g, 20 mmol, 1 equiv.) was added dropwise over an hour. The reaction was allowed to proceed for an additional 3 h at room temperature. After the waxy product settled, the solvent was discarded and the product was rediluted in a mixture of H_2O and acetonitrile (1:1 v/v, 100 mL) and placed in an ice bath for 30 min. Fmoc-OSu (8.77 g, 26 mmol, 1.3 equiv.) in CH_3CN (100 mL) was added and enough DIPEA was added to maintain the pH around 8-9 throughout the reaction. After stirring for 10 h at room temperature, the solvent were removed *in vacuo*. The resulting product was dissolved in 5% aqueous $NaHCO_3$ (100 mL) and washed with EtOAc (100 mL). The aqueous layer was acidified to pH 2 with 1 N aq. HCl and extracted three times with EtOAc (50 mL). The organic layer obtained was washed with distilled water (100 mL) and dried over Na_2SO_4. After

evaporation of the solvent *in vacuo*, compound **4.26** was obtained as an oily solid (8.2 g, 87 %).

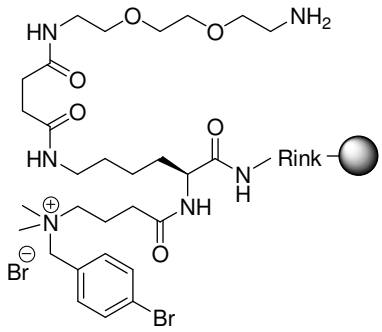
HPLC (method 2): t_R = 2.4 min

Purity: 96 % (220 nm)

ESI-/MS: 939.0 $(M-H)^-$, 469.3 $(2M-H)^-$

^{1}H NMR (250 MHz, d_6-DMSO): 12.06 (1H, s, COOH), 7.88 (2H, d, J^3 = 7.2 Hz, H_{ar}), 7.68 (2H, d, J^3 = 6.6 Hz, H_{ar}), 7.40 (2H, t, J^3 = 6.9 Hz, H_{ar}), 7.32 (2H, d, J^3 = 6.6 Hz, H_{ar}), 4.28 (2H, d, J^3 = 6.4 Hz, CH_2 Fmoc), 4.21 (1H, t, J^3 = 6.0 Hz, CH Fmoc), 3.49 (2H, bs, CH_2NH), 3.38 (4H, m, 2 × CH_2), 3.15 (4H, m, 2 × CH_2), 2.4 (2H, t, J^3 = 6.6 Hz, $CH_2CONHCH_2$), 2.3 (2H, t, J^3 = 6.2 Hz, CH_2COOH).

Amino PEG analytical construct resin (4.27)

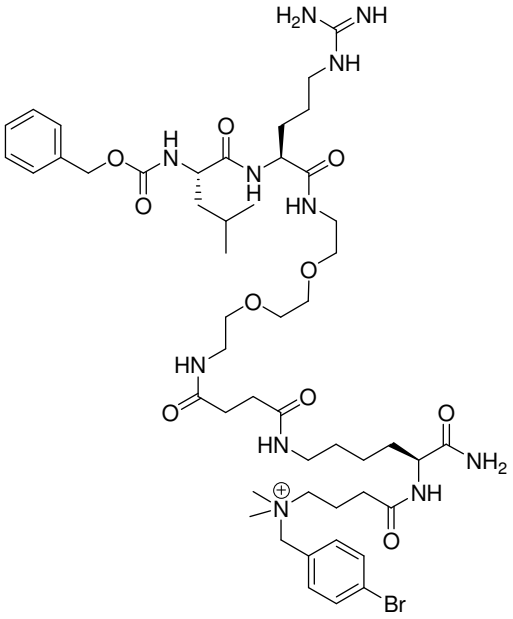


4.27

Carboxylic acid **4.26** (1.17 mg, 2.5 mmol, 10 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 3 mL). HOBt was added (338 mg, 2.5 mmol, 10 equiv.) and after complete dissolution, DIC (358 µL, 2.5 mmol, 10 equiv.) was introduced. After 20 min of stirring, the solution was added to the analytical construct resin **1.12** (0.5 g, s = 0.5 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 2 mL). The reaction was stirred over 30 min. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL). The coupling procedure was repeated. After 30 min, a negative qualitative ninhydrin test (see section 5.1.2) was carried out. The washing procedure was repeated. The deprotection of the Fmoc

group was carried out as decribed in section 5.1.2. and the afforded resin was dried *in vacuo* to afford the title material as a buff coloured resin (0.54 g, 97 %).

Cbz-Leu-Arg PEG analytical construct (4.28)



4.28

The same experimental coupling and cleavage conditions as for those used in the case of resin **4.23** were used to prepare the Cbz-Leu-Arg dipeptide in the case of the PEG based spacer (see section 5.5.4.2).

Crude **4.25** was subsequently purified by semi preparative and obtained in good purity (50 mg, overall yield: 18 %).

HPLC (method 3): t_R = 4.7 min
Purity: 96 % (220 nm)
ESI+/MS: m/z = 530.9 ($(M+H)^{2+}$)
FAB+/HRMS: m/z = 1060.51913 (M^+) (calcd 1060.51948)

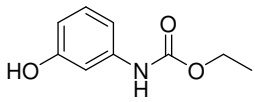
5.5.4.6. Enzymatic assays with compound **4.28**

Enzymatic assays at an enzyme concentration of 0.1 nM and a substrate and MS references concentrations of 10 µM were carried out as decribed in section 5.5.3. Total consumption of **4.28** took around 4 hours to take place (as assessed by ESI+/MS).

5.5.5. Preparation of Cbz-Leu-Arg-ACC

5.5.5.1. Preparation of Fmoc-ACC and loading onto the resin

3-*N*-(Carbethoxy)aminophenol (4.30)[29]

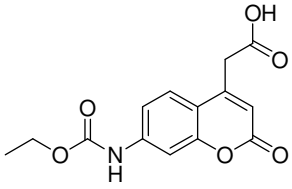


4.30

3-aminophenol (20 g, 183 mmol, 2 equiv.) was stirred in ethyl acetate (70 mL) and heated to reflux for 30 min. Ethyl chloroformate (9.94 g, 91.6 mmol, 1 equiv.) was then added dropwise over a period of 1 h. The mixture was cooled to room temperature and the white precipitate that formed was filtered and washed with ethyl acetate (3 × 40 mL) and petroleum ether (3 × 40 mL). The filtrates were combined and the solvents were removed *in vacuo*. Product **4.30** was afforded as a white powder (16.5 g, 99 %).

HPLC (method 3): t_R = 4.8 min
Purity: 100 % (254 nm)
ESI+/MS: 182.1 $(M+H)^+$
^{1}H NMR (250 MHz, CD_3OD): 9.03 (1H, s, br, OH), 7.05 (1H, t, J^3 = 8.0 Hz, CH_{ar}), 7.02 (1H, s, br, CH_{ar}), 6.81 (1H, ddd, J^3 = 8.0 Hz, J^4 = 2.0 Hz, J^4 = 0.8 Hz, CH_{ar}), 6.45 (1H, ddd, J^3 = 8.0 Hz, J^4 = 2.0 Hz, J^4 = 0.9 Hz, CH_{ar}), 4.16 (2H, q, J^3 = 7.1 Hz, CH_2CH_3), 1.29 (3H, t, J^3 = 7.1 Hz CH_2CH_3)

7-*N*-(Carbethoxy)aminocoumarin-4-acetic acid (4.31)[29]

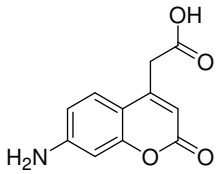


4.31

3-*N*-(Carbethoxy)aminophenol **4.30** (15 g, 82.8 mmol, 1 equiv.) was dissolved in H_2SO_4/H_2O (7:3 v/v, 500 mL) and cooled in an ice bath. The mixture was then rapidly stirred and 1,3-acetonedicarboxylic acid (13.3 g, 91.2 mmol, 1.1 equiv.) was added in portions. The reaction mixture was allowed to warm up to room temperature and was stirred for 8 h. It was then poured onto ice (600 g) and stirred for 30 min. The white precipitate formed was washed with diethyl ether (3×400 mL) and recrystallised from CH_3CN. **4.31** was dried and collected as a white solid (11.4 g, 47 %).

HPLC (method 1): t_R = 6.8 min
Purity: 99 % (254 nm)
ESI+/MS: 292.2 $(M+H)^+$, 314.2 $(M+Na)^+$
^{1}H NMR (250 MHz, CD_3OD): 10.17 (1H, s, COOH), 7.62 (1H, d, J^3 = 8.0 Hz, CH_{ar}), 7.57 (1H, d, J^3 = 2.0 Hz, CH_{ar}), 7.38 (1H, dd, J^3 = 8.0 Hz, J^4 = 2.0 Hz, CH_{ar}), 6.33 (1H, s, $CH_{\alpha}C$=O), 4.17 (2H, q, J^3 = 7.1 Hz, CH_2CH_3), 3.86 (2H, s, CH_2COOH),1.26 (3H, t, J^3 = 7.1 Hz CH_2CH_3)

7-aminocoumarin-4-acetic acid "ACC" (4.2)[29]



4.2

7-*N*-(Carbethoxy)aminocoumarin-4-acetic acid **4.31** (10 g, 34.3 mmol, 1 equiv) was mixed with NaOH (13.7 g, 343 mmol, 10 equiv.) in water (90 mL) and stirred at reflux for 16 h. After cooling to room temperature, the pH was brought to 2 by adding H_2SO_4. The yellow precipitate was filtrered off to afford the title compound **4.2** as a yellow powder (7.14 g, 95 %)

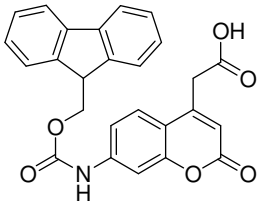
HPLC (method 1): t_R = 3.6 min

Purity: 99 % (254 nm)

APCI/MS: 220.2 $(M+H)^+$, 242.2 $(M+Na)^+$

^{1}H NMR (250 MHz, d_6-DMSO): 7.33 (1H, d, J^3 = 8.7 Hz, CH_{ar}), 6.55 (1H, dd, J^3 = 8.7 Hz, J^3 = 2.2 Hz, CH_{ar}),), 6.42 (1H, d, J^3 = 2.1 Hz, CH_{ar}), 6.16 (2H, s, NH_2), 5.98 (1H, s, CH_αC=O), 3.73 (1H, s, CH_2COOH)

7-*N*-(Fluorenylmethoxycarbonyl)aminocoumarin-4-acetic acid (4.29)[29]

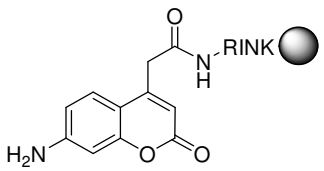


4.29

7-aminocoumarin-4-acetic acid **4.2** (6 g, 27.4 mmol, 1 equiv.), DIPEA (10.5 mL, 60.2 mmol, 2.2 equiv.), and freshly distilled trimethylsilane chloride (TMS-Cl, 7.65 mL, 60.2 mmol, 2.2 equiv, b.p. = 87 °C) were dissolved in CH_2Cl_2 (50 mL) and taken to reflux for 3 h. The reaction mixture was cooled in an ice bath, and Fmoc-OSu (7.79 g, 30.1 mmol, 1.1 equiv.) was added portionwise, while maintaining the pH around 8.5 by addition of DIPEA. After overnight stirring, methanol was added to the reaction mixture (150 mL) under rapid stirring. The precipitate that formed was washed with methanol (2 × 75 mL) and dried *in vacuo* to afford the title compound as an off white powder (7.38 g, 61 %).

HPLC (method 4): t_R = 8.2 min
Purity: 99 % (254 nm)
APCI/MS: 442.3 $(M+H)^+$
^{1}H NMR (250 MHz, d_6-DMSO): 12.83 (1H, s, NH), 10.20 (1H, s, CH_2COOH), 7.91 (2H, d, J^3 = 7.2 Hz, CH_{ar} (Fmoc)), 7.76 (2H, d, J^3 = 7.2 Hz, CH_{ar} (Fmoc)), 7.62 (1H, d, J^3 = 8.7 Hz, CH_{ar} (coumarin)), 7.55 (1H, s, CH_{ar} (coumarin)), 7.46-7.32 (5H, m, CH_{ar}), 6.34 (1H, s, $CH_{\alpha}C=O$), 4.56 (2H, d, J^3 = 6.2 Hz, CH_2CH (Fmoc)), 4.34 (1H, t, J^3 = 6.2 Hz, CH_2CH (Fmoc)), 3.86 (1H, s, CH_2COOH).
Analytical data were conformed to what has been reported in the literature.[29]

ACC Rink amide resin (4.32)



4.32

7-*N*-(Fluorenylmethoxycarbonyl)aminocoumarin-4-acetic acid **4.29** (278 mg, 0.63 mmol, 3 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 3 mL). HOBt was added (85 mg, 0.61 mmol, 3 equiv.) and after complete dissolution, DIC (90 µL, 0.61 mmol, 3 equiv.) was introduced. After 20 min of stirring, the solution was added to Rink amine resin **1.18** (250 mg, s = 0.85 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 3 mL). The reaction was stirred over 30 min. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL). The coupling and washing procedures were repeated twice and the resin was dried *in vacuo* overnight.

Capping of the unreacted amine functionalities was realised as follows. The resin was swollen in a mixture of acetic anhydride (397 µL, 4.2 mmol, 20 equiv.) and pyridine (168 µL, 2.1 mmol, 10 equiv.) in DMF (3 mL). The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL) and was dried *in vacuo*.

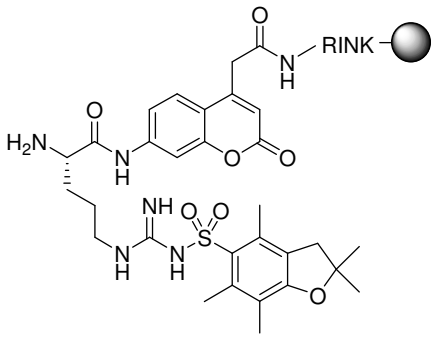
The deprotection of the Fmoc group was done as described in section 5.1.2. and the material dried *in vacuo* to afford resin **4.32** (0.26 g, 89 %).

Loading (determined by Fmoc test): 0.49 mmol/g (theoretical 0.55 mmol/g)

IR ν (cm^{-1}): 3412 (w, br, ν_{N-H}), 1720 (m, $\nu_{C=O}$), 1690 (s, br, $\nu_{C=O}$)

5.5.5.2. Preparation of Cbz-Leu-Arg-ACC

Arg-ACC Rink amide resin (4.33)



4.33

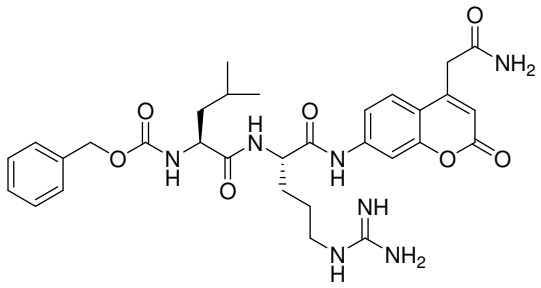
Fmoc-Arg(Pbf)-OH (681 mg, 1.05 mmol, 5 equiv.) was dissolved in DMF (3 mL). O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 400 mg, 1.05 mmol, 5 equiv.) and 2,4,6-collidine (139 µL, 1.05 mmol, 5 equiv.) were added. The solution was added to resin **4.32** (230 mg, s = 0.73 mmol/g, 1 equiv.) in DMF (3 mL). The reaction was stirred overnight. The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL). The coupling and washing procedures were repeated and the resin was dried *in vacuo* overnight.

Capping of the unreacted amine functionalities was realised as follows. The resin was swollen in a mixture of acetic anhydride (397 µL, 4.2 mmol, 20 equiv.) and pyridine (168 µL, 2.1 mmol, 10 equiv.) in DMF (3 mL). The resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL) and was dried *in vacuo*.

The deprotection of the Fmoc group was done according to the procedure described in section 5.2.1 and the material dried *in vacuo* to afford resin **4.33** (0.21 g, 70 %).

IR ν (cm^{-1}): 3407 (w, br, ν_{N-H}), 1720 (m, $\nu_{C=O}$), 1690 (s, br, $\nu_{C=O}$), 1646 (s, $\nu_{C=N}$)

Cbz-Leu-Arg-ACC (4.34)[121]



4.34

Cbz-Leu-OH (279 mg, 1.05 mmol, 5 equiv.) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 3 mL). HOBt was added (142 mg, 1.05 mmol, 5 equiv.) and after complete dissolution, DIC (132 µL, 1.05 mmol, 5 equiv.) was introduced. After 20 min of stirring, the solution was added to resin **4.33** (210 mg, s = 0.56 mmol/g, 1 equiv.) in CH_2Cl_2/DMF (7:3 v/v, 2 mL). The reaction was stirred over 30 min. A negative qualitative ninhydrin test (see section 5.1.2) was carried out and the resin was washed with DMF (3 × 10 mL), CH_2Cl_2 (3 × 10 mL), methanol (3 × 10 mL), and diethylether (3 × 10 mL) and was dried *in vacuo* overnight.

The cleavage step was performed by swelling the resin in a mixture of TIS and CH_2Cl_2 in TFA (5:5:90 v/v/v, 500 µL) for 15 min. After evaporation of the solvent *in vacuo,* the white solid was triturated in cold Et_2O to afford the crude compound **4.34**. Semi preparative HPLC afforded **4.34** in excellent purity (19 mg, 12 %).

HPLC (method 1): t_R = 5.3 min
Purity: 100 % (ELSD)
ESI+/MS: m/z = 622.5 ($(M+H)^+$)

All analyses were conform to literature.[121]

5.5.5.3. Cathepsin S assays with fluorogenic substrate **4.34**

Fluorometric assays were performed in a 3 mL reaction volume at a concentration of fluorogenic substrate **4.34** of 10 µM in cathepsin S buffer (prepared as described in section 5.4.3.) The reactions was started by addition of cathepsin S (for a final

enzyme concentration of 0.6 nM) followed by short vigorous stirring and the fluorescence of the solution was measured by spectrofluorometry using an excitation wavelength of 355 nm and an emission wavelength of 450 nm. The change of fluorescence intensity was followed over 60 minutes. All experiments were run in duplicate. The expected fluorescence increase was not observed and no exploitable fluorescence curve could be obtained.

5.5.5.4. Trypsin assays with substrates **4.22**, **4.25** and **4.28**

Trishydroxymethylaminomethane (tris) buffer (0.1 M, pH = 8.2):[116] Tris base (6.05 g, 0.05 mol) was dissolved in deoionised water (500 mL). The pH was adjusted to 8.2 by adding 1 M HCl in water.

Enzymatic assays at an enzyme concentration of 0.8 µM and a substrate **4.22** and MS reference **4.20** concentration of 0.1 mM were carried out as decribed in section 5.5.3 in tris buffer using trypsin as the enzyme.
The protocol was adapted to achieve substrate and MS reference concentration of 0.5 mM, 0.75 mM, 1 mM, 1.25 mM and 1.5 mM.

The same protocol was used with compounds **4.25** and **4.28** using the same MS reference **4.20**. All experiments were run in duplicate.

The kinetic profiles obtained were much slower than expected and did not allow calculation of K_m for any of the three substrates.

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APPENDIX: Publications

High Throughput Physical Organic Chemistry: Analytical Constructs for Monomer Reactivity Profiling

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Received January 27, 2005

A polymer-supported analytical construct was used to quantify the reactivity of a range of monomers in the Ugi four-component condensation using positive electrospray ionization mass spectrometry (MS) as a quantitative analytical tool. The construct incorporated a bromo group to act as a peak splitter and a quaternary ammonium to act as a MS sensitizer and ionization leveler, thereby allowing direct quantitation of the cleaved adducts by MS. The relative reactivities of 10 carboxylic acids were quantified by the relative levels of product generated as determined by MS and 10 isonitriles, and 10 aldehydes were investigated in the same way. The effect of concentration variations on monomers reactivity and product profiles were rapidly determined using this approach, and the method opens up the way for studying, in a single pot, multiple reactions with a broad range of monomers under identical and self-consistent reaction conditions.

Introduction

Over the past few years, combinatorial synthesis has become a very fast and efficient technique for preparing a range of pharmacologically active compounds.[1,2] However, despite the ability to quickly provide a huge number of potential drug candidates, both split and mix or discrete compounds library syntheses are often not as efficient as desired to enable the production of a highly diverse set of pure compounds. Indeed, many combinations of reactants do not lead to the desired compound as the main product, which represents a major waste of time and resources. One of the main reasons for this reaction failure is that the vast variety of monomers that need to be chosen in order to ensure maximum diversity of the library also increases the differences in reactivity between the different building blocks, and even though optimization of reaction conditions are generally undertaken before full-scale library synthesis, this cannot cover all combinations of monomers implicated. Ideally, each monomer should be tested to ensure it is reactive enough to produce the desired product in good yield and purity to have it immediately ready for screening.

A method to evaluate the reactivity of monomers was recently reported by Parr et al. who described the use of a quantitative solid-phase analytical construct to evaluate the reactivity of amine monomers toward reductive amination.[3] The concept of analytical constructs, first introduced by Geysen,[4] was modified to include a ultraviolet (UV) chromophore used to allow quantitative deductions; however, this method requires both mass spectrometry (MS) and liquid chromatography/UV (LC/UV) analysis after cleavage from the resin to identify and quantify the products and link their concentration to monomer reactivities.

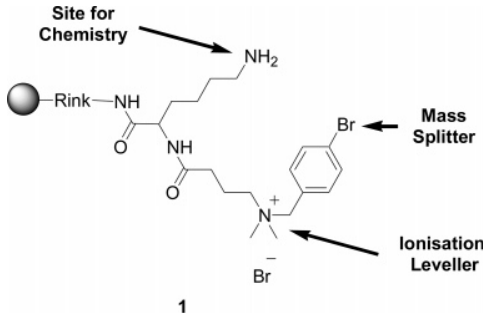

Figure 1. Resin-bound analytical construct **1**.

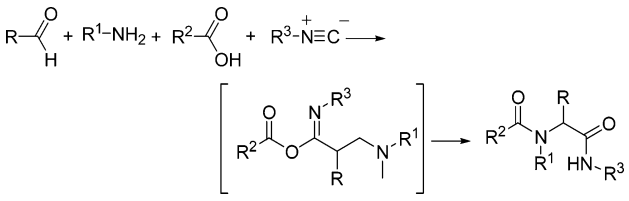

Figure 2. The Ugi 4CC.

The methodology described herein allows the rapid profiling of the reactivity of a set of monomers by means of a single positive electrospray ionization MS (ESI+/MS) analysis. To do this, an analytical construct elaborated on the basis of the work of Carrasco was used.[5] A reactive functionality present on the construct was allowed to react with an array of monomers, and cleavage yielded a mixture of products, each attached to the common construct, on which ESI+/MS analysis could be performed. This analysis was made quantitative thanks to the presence on the construct of a quaternary ammonium species. As reported by Szewczyk,[6] such a group dominates ionization of the global structure

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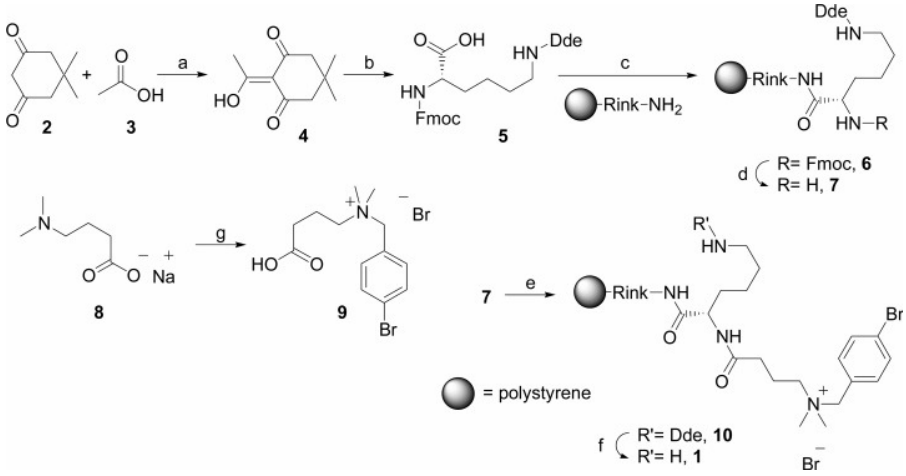
10.1021/cc050013e CCC: $30.25
Published on Web 06/25/2005

Scheme 1. Preparation of the Analytical Construct[a]



[a] Reagents and conditions: (a) DCC, DMAP, CH_2Cl_2, 36 h, 75%; (b) TFA (0.1 equiv), EtOH, reflux, 60 h, 54%; (c) Rink amine resin (s = 0.85 mmol/g, prepared from aminomethylated polystyrene (Polymer Laboratories, 1.11 mmol/g, 75−150 μm, 1−2% divinylbenzene), DIC, HOBT, CH_2Cl_2/DMF (7:3, v/v), 12 h; (d) 20% piperidine, DMF, 30 min; (e) **9**, DIC, HOBT, CH_2Cl_2/DMF (7:3, v/v), 12 h; (f) 80% NH_2OH·HCl/imidazole, in *N*-methyl pyrrolidone/CH_2Cl_2 (1:1 v/v), 3 h; (g) (1-bromo-4-bromomethyl) benzene, CH_2Cl_2, 1 h, then Amberlite 200, CH_2Cl_2, 30 min, 98%.

and therefore links the intensity of the peak to the quantity of the corresponding compound in the mixture. The presence of an aryl bromide provided a known isotope pattern for the molecular ions allowing rapid identification of the products (see Figure 1).[7]

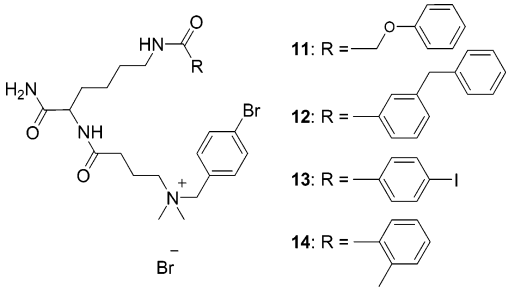
Since the intensities of the ESI+/MS peaks correlate to relative amounts of the various products, each value obtained can therefore be attributed to the reactivity of monomers. Thus, one ESI+/MS analysis gives a quantification of the reactivity of each monomer. The analytical construct used in this study had an amine functionality that was used as the amine entry for an Ugi four-component condensation (4CC). This multiple component reaction has the advantage of building quite complex α-acylamino amides from simple building blocks (Figure 2);[8] the technique has first been used to validate the methodology and then to evaluate the reactivity of 10 common aldehydes, 10 carboxylic acids, and 10 isonitriles in the Ugi 4CC.

Results and Discussion

Preparation of the Construct. The 4,4-dimethyl-2,6-dioxocyclohex-1-ylidene (Dde) protecting group was prepared from dimedone **2** and acetic acid **3**[9] and condensed with Fmoc-Lys-OH to give Fmoc-Lys-(Dde)-OH **5**.[10] Coupling to polystyrene Rink amine resin and subsequent deprotection of Fmoc group afforded resin **7**. The analytical enhancer was prepared from sodium 4-dimethylamino-butyrate **8** which was alkylated with (1-bromo-4-bromomethyl) benzene to give ammonium salt **9**. Coupling to resin **7** gave resin **1**, after Dde deprotection (Scheme 1).[11]

Proof of the Method. To validate the methodology, it had to be proved that the MS method was quantitative and that ionization of the analytical construct, once cleaved, was dominated by the quaternary ammonium ion (i.e., independent of the product bound to it). To achieve this proof compounds **11**, **12**, **13**, and **14** were synthesized in parallel via an Ugi 4CC, using **1** as the resin bound amine entry (Chart 1).

Chart 1. Compounds Used to Validate the Method



Following cleavage of the compounds from the solid support, each product was purified by semipreparative high-performance (HP)LC and analyzed through an online detection system comprising (i), a chemiluminescent nitrogen detector (CLND) (ii), an evaporative light scattering detector (ELSD) (iii), a diode array detector (DAD) (iv), and an electrospray ionization mass spectrometer (ESI/MS). Quantitation was then determined according to the peak areas for CLND[12] and precalibrated ELSD,[13] while MS quantification was achieved by calculation of the abundance of the compound by using the MS trace and the intensity of the concerned peak.

The spectrum obtained by ESI+/MS analysis demonstrated all the properties expected; thus each compound was well ionized and detectable, and in each case, only the molecular peak was present and gave the expected $^{79}Br/^{81}Br$ patterns, which allowed them to be rapidly differentiated from any background noise (Figure 3).

The intensities of the ESI+/MS peaks obtained for the analyses of different concentrations of solutions of **11**, **12**, **13**, and **14** were plotted vs their respective ELSD areas and CLND areas (Chart 2). The linearity between the various methods analyses was very good (regression coefficients were 0.997 for ELSD vs MS and 0.994 for MS vs concentrations as determined by CLND).

Additionally, the method was validated by checking the effect of other potentially ionisable groups bound to the construct. Two products were prepared in a single-pot Ugi

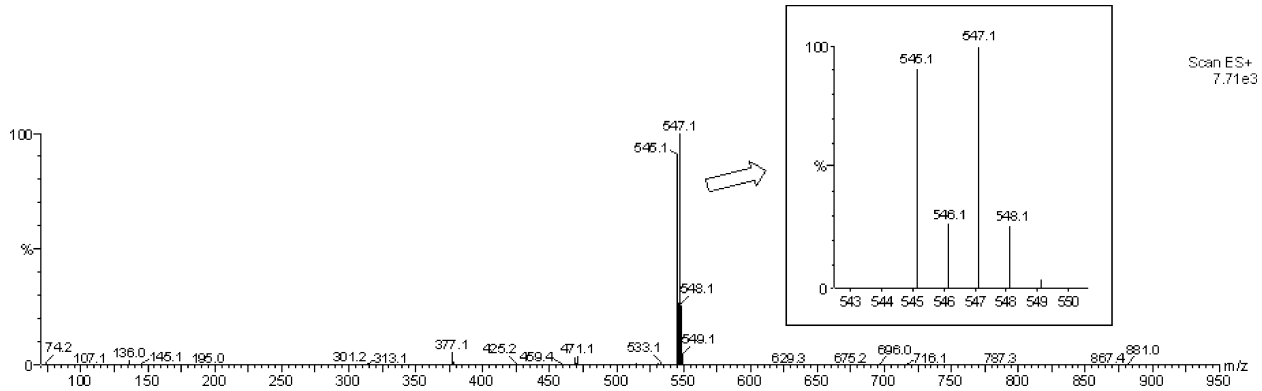


Figure 3. MS spectrum obtained for compound **14** and an expansion showing the bromine isotope pattern.

Chart 2. Proof of the Linearity between ESI+/MS and ELSD and CLND Analyses

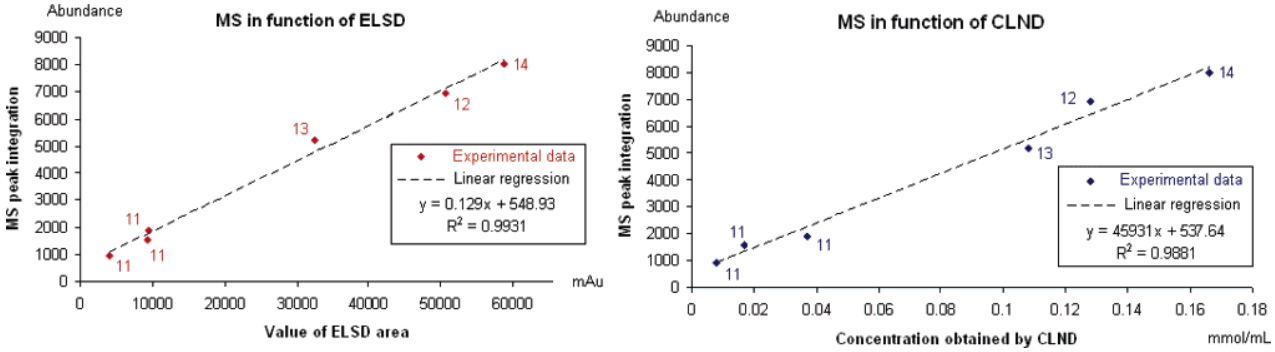
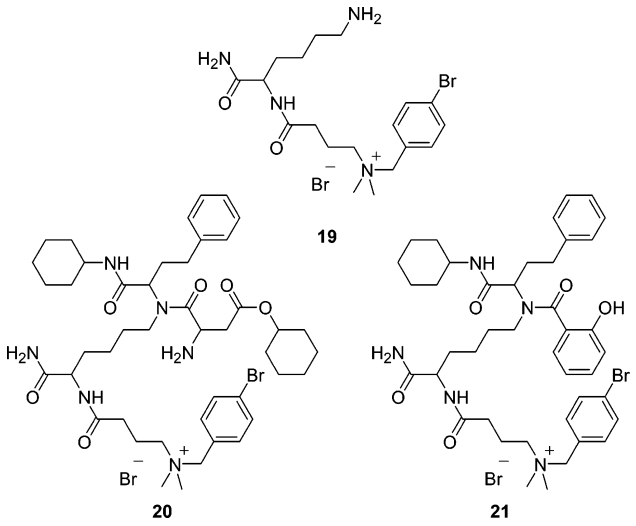


Chart 3. Mixture of Products Analyzed by ESI+/MS and ELSD



4CC, using **1** as the resin bound amine entry, hydrocinnamaldehyde **15**, cyclohexyl isonitrile **16**, Boc-Asp(OChex)-OH **17**, and salicylic acid **18**, to give a mixture of unreacted amine **19** and products **20** and **21** (Chart 3).

The composition of the mixture was analyzed by ELSD and ESI+/MS. As shown in Table 1, the results obtained demonstrate the ability of the method to quantify products in a mixture, independently to what is attached to the construct.

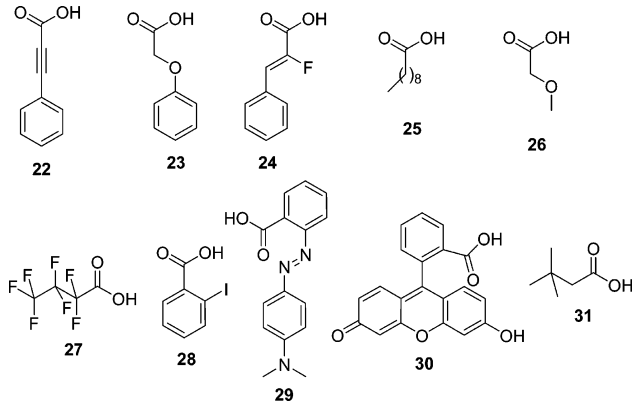
Since both studies confirmed that the method was quantitative, further investigations could be undertaken.

Carboxylic Acid Reactivity Profiling. Because the method was quantitative, it was possible to use it to determine

Table 1. Composition of the mixture of **19**, **20** and **21** determined by ESI+/MS and ELSD

	composition of the mixture	
compound	ESI+/MS	ELSD
19	45%	54%
20	10%	6%
21	45%	40%

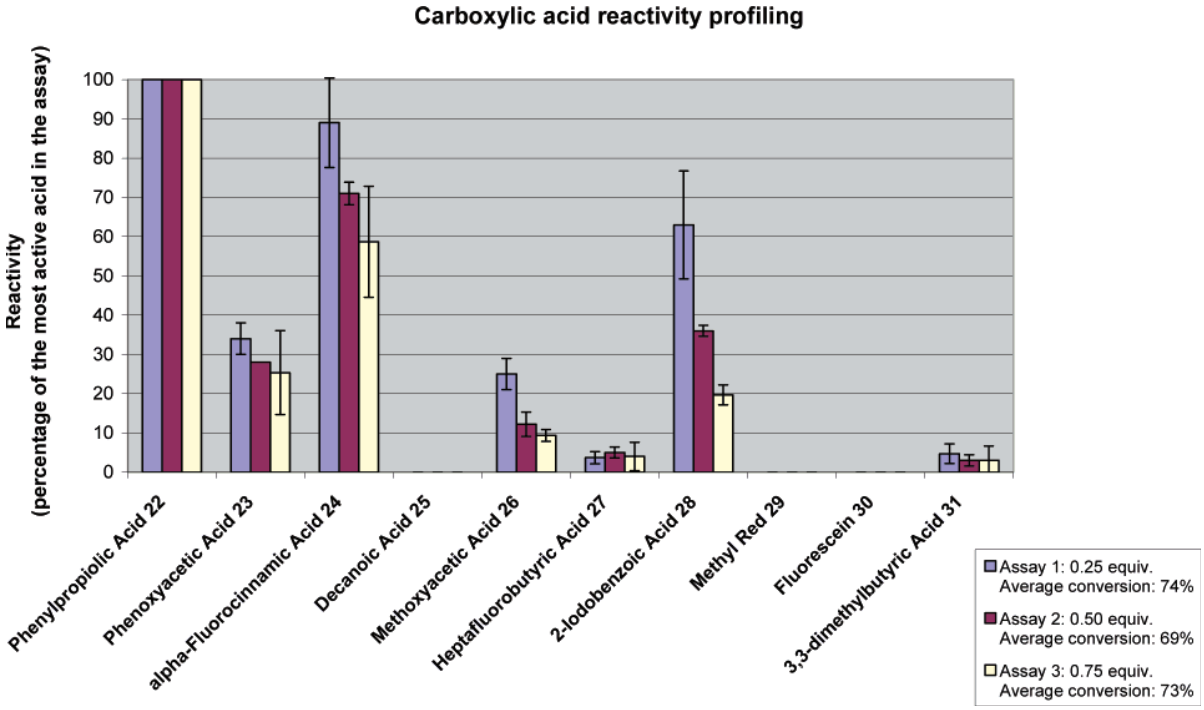
Chart 4. The Ten Carboxylic Acids Used in Ugi 4CC



the composition of a mixture of different compounds bound to the analytical construct. Thus by reacting the solid supported amine analytical construct with an isonitrile, an aldehyde, and 10 different carboxylic acids (as shown in Chart 4), 10 Ugi 4CCs would occur and a mixture of products result.

The reactivity of each acid in the assay could then be measured after cleavage by means of a single ESI+/MS analysis (the other species chosen for the reaction were

Chart 5. Values and Standard Deviations Obtained for the Carboxylic Acid Reactivity Profiling

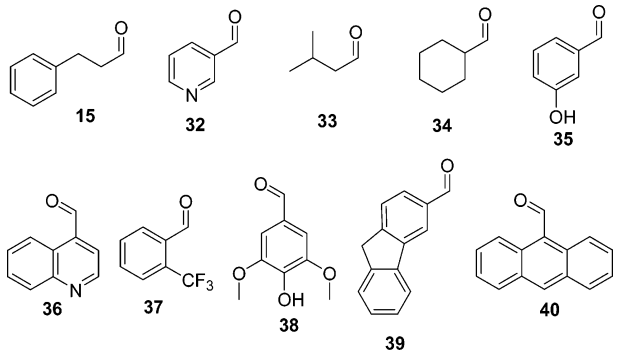


hydrocinnamaldehyde **15** and cyclohexyl isonitrile **16**, both components having good reactivity in the Ugi 4CC. Five equivalents (61 mM) were used relative to the amine entry in order to guarantee it would not be the limiting factor in the reaction (pseudo first order). The reactions were performed in methanol/CH_2Cl_2 (1:1, v/v) under microwave irradiation for 30 min at 120 °C.[14] Several concentrations of acid were used in order to establish if the reactivity of one of the acids would dominate the others', thus 0.25 equiv (3.0 mM), 0.5 equiv (6.1 mM), and 0.75 equiv (9.1 mM) of each acid were used respectively for assays 1, 2, and 3. After each reaction, the products were cleaved from the Rink linker using a solution of trifluoroacetic acid (TFA) in CH_2Cl_2 (20% v/v).

The ESI+/MS data obtained for each assay was treated with the Masslynx software, which allowed the peaks that presented a bromine pattern to be extracted from the background noise. After measurement of the intensity of each peak, a value was attributed to each acid relating to its reactivity (three assays were performed for each value of the monomer concentration). Thus, the reactivity of each monomer as a percentage of the most active in the assay is reported in Chart 5 as well as the standard deviation. By use of the intensity of the MS peak relative to the starting amine, the conversion was also calculated for each assay.

Clearly, phenylpropiolic acid **22** had the highest reactivity, followed by α-fluorocinnamic acid **24** and 2-iodobenzoic acid **28**. Bulky structures such as methyl red **29** and fluorescein **30** were revealed to be essentially unreactive, as well as decanoic acid **25**, which was quite surprising. At high concentrations the reactivity of phenylpropiolic acid **22** in some cases dominated the chemistry even though the relative order of reactivity was unchanged, with, for example, the values measured for α-fluorocinnamic acid **24** and 2-iodobenzoic acid **28** being lowered in case of 0.75 equivalent of each acid. To be able to give a representative value of the reactivity of each acid, it was therefore necessary to maintain the amount of each acid low enough to avoid saturating the experiment and losing the competitive effect.

Chart 6. The Ten Aldehydes Used in Ugi 4CCs



It also came out of this study that each acid had a completely different behavior with regards to the reaction. A library synthesis that would involve the 10 acids picked for this study would be inefficient as more than 50% of the reaction mixtures would result in no product or dramatically poor yields; thus with this technique, it becomes possible to exclude unreactive monomers from library synthesis.

Aldehyde Reactivity Profiling. The same study was carried out on 10 aldehydes (Chart 6). Five equivalents of phenylpropiolic acid **22** and five equivalents of cyclohexyl isonitrile **16** were used for each assay (61 mM), and again, the amount of aldehyde was varied. Thus 0.25 equiv (3.0 mM), 0.5 equiv (6.1 mM), and 0.75 equiv (9.1 mM) of each aldehyde were used for assays 4, 5, and 6, respectively.

The average conversions for assays 4, 5, and 6 were lower than the ones observed in case of the acids. A similar process as described above allowed the reactivity of each aldehyde

Chart 7. Values and Standard Deviations Obtained for the Aldehyde Reactivity Profiling

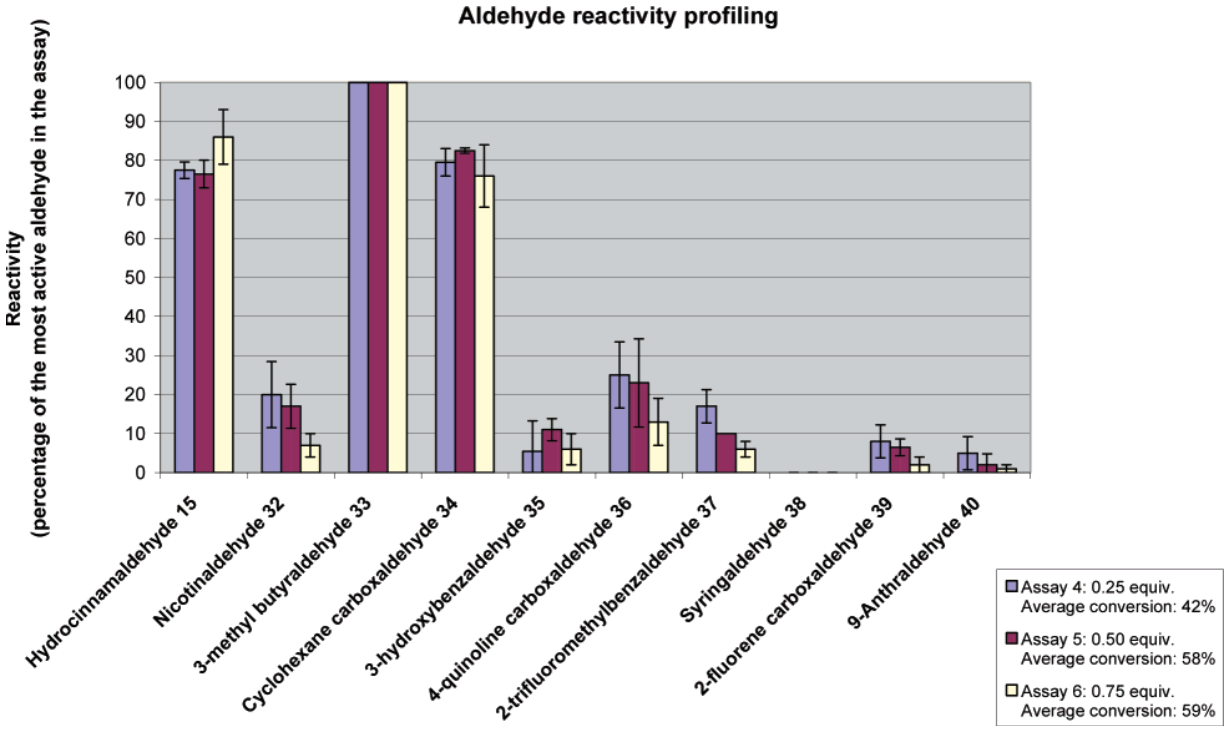
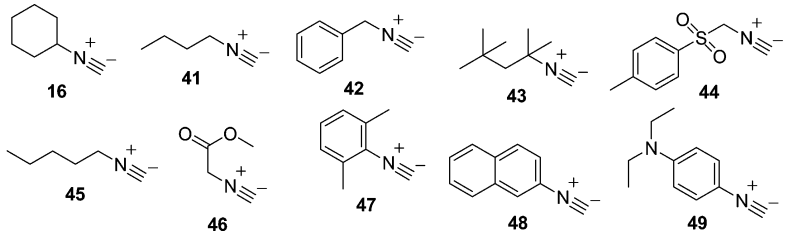


Chart 8. The Ten Isonitriles Used in Ugi 4CCs



to be determined from the ESI+/MS spectra. The same conclusions as those observed by Tempest[15] and Kim[16] in more traditional experiments were drawn: aliphatic aldehydes as 3-methyl butyraldehyde **33** and cyclohexane carboxaldehyde **34** had very good reactivities (Chart 7). Hydrocinnamaldehyde **15** also showed good activity. Nicotinaldehyde **32**, 4-quinoline carboxaldehyde **36**, and 2-trifluoromethylbenzaldehyde **37** had only a reactivity of around 10 percent of the best monomer, while syringaldehyde **38** did not react in any of the three assays. None of the aldehydes dominated the reactivity as determined by the flat effect of increasing the quantity of monomer in the reaction.

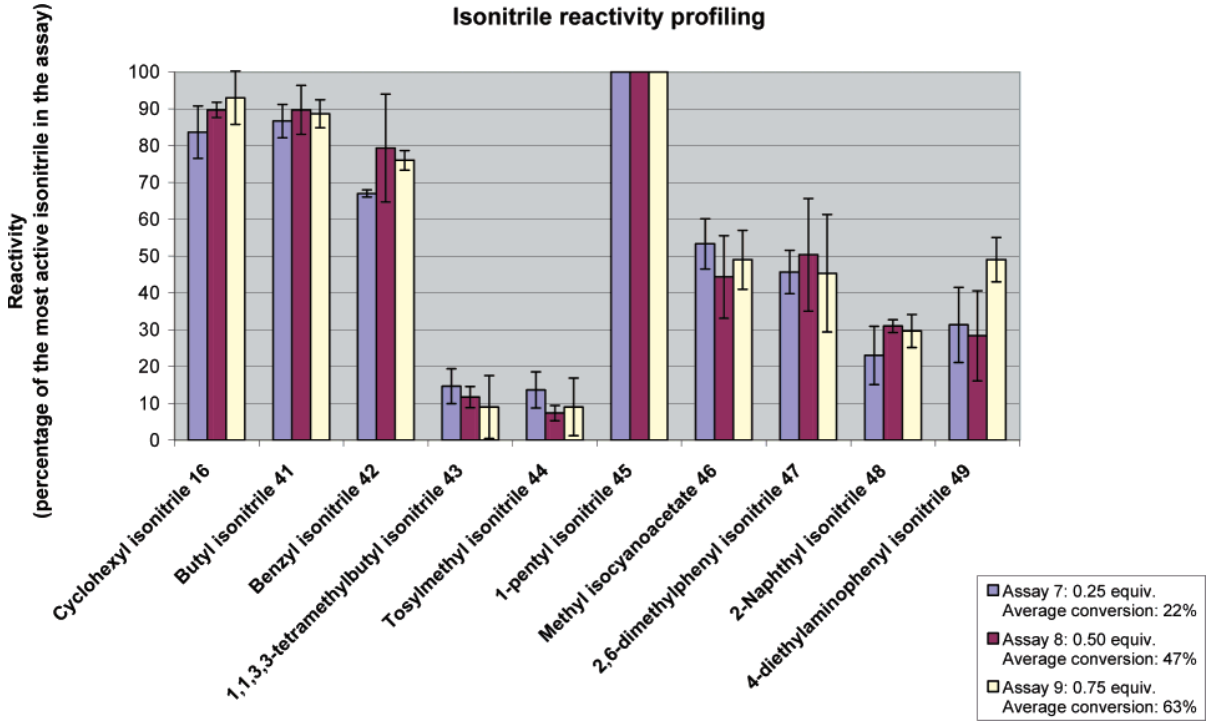
Isonitrile Reactivity Profiling. The same procedure was applied for the reaction of the 10 isonitrile monomers, listed in Chart 8.

For these assays, phenylpropiolic acid **22** and hydrocinnamaldehyde **15** were used, respectively, as the acid and the aldehyde entries of the Ugi 4CC (5 equiv of each, 61 mM). Butyl isonitrile **41**, cyclohexyl isonitrile **16**, and benzyl isonitrile **42** had good reactivity compared to the reference, which was 1-pentyl isonitrile **45** (Chart 9). The amount of isonitrile used for assays 7, 8, and 9, which was 0.25 equiv (3.0 mM), 0.5 equiv (6.1 mM), and 0.75 equiv (9.1 mM) did not affect the reactivity. However it came that the conversion of the starting amine was very dependent on the quantity of isonitrile involved in the reaction as showed on Chart 9. 1,1,3,3-Tetramethylbutyl isonitrile **43** and tosylmethyl isonitrile **44** showed poor reactivity toward the Ugi 4CC and are not advisable for combinatorial synthesis under the described conditions.

Conclusion

In conclusion, a high throughput tool to quantify the reactivity of combinatorial chemistry monomers has been developed. An analytical construct was built up and has proven to be very reliable to quantify products in a mixture in a HT manner. This property allowed the rapid quantification of the reactivity of carboxylic acids, aldehydes, and isonitriles monomers during a series of Ugi 4CCs; the quantitative study of the monomers reported here only took a few hours per family (three concentrations tested), allowing fast discrimination of unreactive compounds, so that the synthetic process can be undertaken with a panel of building blocks having the same level of reactivity. The values of the reactivities obtained were comparable to those already reported in the literature,[15,16] and the use of this construct can extended to rapidly evaluate the reactivity of monomers in a broad spread of reactions making it useful in undertaking general monomer reactivity profiling as well as high throughput physical organic chemistry.

Chart 9. Values and Standard Deviations Obtained for the Isonitrile Reactivity Profiling



Experimental

Instrumentation. NMR spectra were recorded on a Bruker AC-300 spectrometer in the solvents indicated at 298 K. Chemical shifts are reported on the δ scale in ppm and were referenced to residual solvents resonances. IR spectra were obtained with neat compounds on a Fourier transform infrared (FTIR) Perkin-Elmer 2000 Spectrometer (Beaconsfield, Bucks, England) coupled with an AutoIMAGE FTIR microspectrometer (Beaconsfield, Bucks, England), 32 scans, resolution $\pm$ 8 cm^{-1}. HPLC/ELSD analyses were obtained using an Agilent 1100 series system (eluent A, water + 0.1% formic acid; eluent B, methanol + 0.1% formic acid; gradient, 95−5% A over 10 min then 5−95% A over 3 min) coupled to a Polymer Lab 100 ES ELS Detector. Eluents used were analytical grade. ESI+/MS analyses were carried out on an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS) using electrospray positive ionization. Reactions under microwave irradiation were performed in a SmithSynthesizer from Biotage. The version of the Masslynx software that was used is version 2.2 build 9.

Preparation of Dde−OH (4).[9] Dimedone (**2**) (11.5 g, 82 mmol) was dissolved in DMF (175 mL) with acetic acid (**3**) (4.95 g, 1 equiv), DCC (17 g, 1 equiv), and (dimethylamino)-pyridine (DMAP) (10 g, 1 equiv). The reaction was finished over 36 h. Precipitating dicyclohexylurea (DCU) was removed by filtration, and the solvent was evaporated in vacuo. After dissolution in ethyl acetate, the organic phase was dried with $MgSO_4$ and the solvent evaporated to afford Dde−OH as an orange oil (11.9 g, 75%). IR ν (cm^{-1}): 3270, 2927, 1719, 1679, 1504−1450, 1208, 785−699. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 13.98 (1H, s), 2.59 (3H, s), 2.52 (2H, s), 2.34 (2H, s), 1.06 (6H, s). ^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$, δ, ppm): 202.4, 197.9, 195.2, 112.3, 52.5, 46.9, 30.8, 28.5, 28.2.

Preparation of N_α-Fmoc-N_ϵ-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl]-lysine (Fmoc-Lys(Dde)-OH (5).[10] Trifluoroacetic acid (84 μL, 1.1 mmol) was added to a stirred suspension of Fmoc-Lys-OH (4.04 g, 10.7 mmol) and Dde−OH (4) (4 g, 2 equiv) in ethanol (90 mL) at room temperature. The mixture was then refluxed for 60 h (reaction monitored with analytical thin-layer chromatography (ethyl acetate/hexane 95:5 v/v, R_f = 0.21). The solvent was evaporated and the orange residue dissolved in ethyl acetate (150 mL). The organic solution was washed with 1 M aqueous $KHSO_4$ (2 × 175 mL). After drying and concentrating in vacuo, the yellow oil was triturated three times with hexane to remove unreacted Dde−OH to give **5** as a white crystalline solid (3.1 g, 54%). ESI+/MS: m/z = 533.3 $(M + H)^+$. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 13.32 (1H, s), 7.74 (2H, d, J^3 = 7.3 Hz), 7.58 (2H, d, J^3 = 6.8 Hz), 7.37 (2H, t, J^3 = 7.1 Hz), 7.27 (2H, t, J^3 = 7.3 Hz), 5.79 (1H, d, J^3 = 7.9 Hz), 4.37 (1H, m), 4.19 (2H, m), 3.38 (1H, m), 2.55 (2H, s), 2.36 (3H, s), 1.97 (2H, m), 1.96−1.53 (6H, m), 1.00 (6H, s). ^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$, δ, ppm): 198.2, 174.5, 174.0, 156.2, 143.9, 143.8, 141.3, 127.7, 127.0, 125.1, 120.0, 107.9, 67.1, 53.4, 52.4, 43.3, 32.0, 31.6, 30.1, 28.4, 28.2, 22.6, 22.4, 18.1, 14.1.

Preparation of Rink Amine Polystyrene Resin. The Fmoc-Rink Linker (970 mg, 1.8 mmol) was dissolved in CH_2Cl_2 (7 mL) and DMF (3 mL). Hydroxybenzotriazole was added (243 mg, 1.5 equiv), followed after 10 min of stirring by DIC (279 μL, 1.5 equiv). After 20 min of stirring, aminomethylated polystyrene resin (1.08 g, s = 1.11 mmol/g) in CH_2Cl_2/DMF (15 mL, 7:3 v/v) was added. The reaction was stirred over 15 h until a ninhydrin test was negative. The resin was washed with DMF (15 mL, 3 times), CH_2Cl_2,

(15 mL, 3 times), methanol (15 mL, 3 times), and diethyl ether (15 mL, 3 times). Fmoc group deprotection was performed by treating the resin with Piperidine/DMF solution (20% v/v) for 30 min. The resin was washed with DMF (15 mL, 3 times), CH_2Cl_2, (15 mL, 3 times), methanol (15 mL, 3 times), and diethyl ether (15 mL, 3 times). The resin was dried in vacuo overnight to afford white colored Rink amine resin (1.5 g). IR ν (cm^{-1}): 3250, 2922, 1681, 1503−1452, 1208, 785−699.

Preparation of Resin 7. Fmoc−Lys(Dde)−OH **5** (2 g, 3.7 mmol) was dissolved in CH_2Cl_2/DMF (40 mL, 7:3 v/v). Hydroxybenzotriazole was added (499 mg, 2 equiv), followed after 10 min of stirring by DIC (576 μL, 2 equiv). After 20 min of stirring was added Rink amine resin (3.3 g, s = 0.85 mmol/g) in CH_2Cl_2/DMF (7:3 v/v, 50 mL). The reaction was stirred over 15 h until a ninhydrin test was negative. The resin was washed with DMF (40 mL, 3 times), CH_2Cl_2, (40 mL, 3 times), methanol (40 mL, 3 times), and diethyl ether (40 mL, 3 times). Resin **6** was dried in vacuo overnight to afford a buff-colored resin (4.2 g). Fmoc group deprotection was performed as previously described; the resin was then dried in vacuo overnight to afford the title resin **7**.

Preparation of (4-Bromo-benzyl)-(4-carboxy-butyl)-dimethylammonium Bromide (9). NaOH (4 g, 100 mmol) in ethanol (15 mL) was added to a stirred solution of *N*,*N*-dimethylaminobutyric acid (8.35 g, 50 mmol) in CH_2Cl_2 (100 mL). Solvent was evaporated, and the acid was dissolved in CH_2Cl_2. 4-Bromoethylbenzyl bromide (13.75 g, 1.1 equiv) was added dropwise during 30 min. The mixture was stirred over 30 min at room temperature. The salt was filtered and washed twice with CH_2Cl_2 (20 mL) and then stirred in CH_2Cl_2 on a linear shaker in the presence of the activated Amberlite 200 to give a white salt. (98%). ESI+/MS: m/z = 300.0 (M^+). ESI+/HRMS: $C_{13}H_{19}NBr_2O_2$ calculated m/z = 300.0594 (M^+) measured m/z = 300.0593. IR ν (cm^{-1}): 3024, 2967, 1725, 1573, 501. ^{1}H NMR (300 MHz, d^6-DMSO, δ, ppm): 7.76 (2H, d, J^3 = 8.4 Hz), 7.66 (2H, d, J^3 = 8.4 Hz), 4.69 (2H, s), 3.35 (2H, m), 3.03 (6H, s), 2.06 (2H, m), 1.98 (2H, m). ^{13}C NMR + DEPT 135 (75 MHz, d^6-DMSO, δ, ppm): 175.9, 136.1, 132.7, 128.6, 124.9, 65.6, 65.5, 50.0, 34.3, 20.0.

Preparation of Resin 1. Acid **9** (1 g, 2.8 mmol) was dissolved in CH_2Cl_2 (14 mL) and DMF (6 mL). HOBt was added (383 mg, 2 equiv) and after 10 min of stirring, DIC (441 μL, 2 equiv) was added. After 20 min of stirring, resin **7** (2 g, s = 0.66 mmol/g) in CH_2Cl_2/DMF (7:3 v/v, 30 mL) was added. The reaction was stirred over 15 h until a ninhydrin test was negative. The resin was washed with DMF (30 mL, 3 times), CH_2Cl_2, (30 mL, 3 times), methanol (30 mL, 3 times), and diethyl ether (30 mL, 3 times). The resin was dried in vacuo overnight to afford resin **10** as a buff colored resin (3 g). Polystyrene bound analytical construct **1** was afforded through deprotection of resin **10** by swelling it in 80% NH_2OH·HCl/imidazole in *N*-methyl pyrrolidone/CH_2Cl_2 (1:1 v/v) for 3 h.[11]

Microwave-Assisted Solid-Supported Ugi 4CCs: Example of Carboxylic Acid Reactivity Study. Resin (75 mg, **1**, s = 0.61 mmol/g) was swollen with CH_2Cl_2/MeOH (50% v/v, 1 mL) in a 5-mL microwave vial. To the resin was added the mixture of 10 carboxylic acids in CH_2Cl_2/MeOH (50% v/v, 0.5 mL) and then hydrocinnamaldehyde (**15)** (41 mg, 5 equiv) in solution in CH_2Cl_2/MeOH (50% v/v, 0.5 mL). The vial was sealed and placed on a linear shaker for 30 min before cyclohexyl isonitrile **(16)** was added (47 μL, 5 equiv). The mixture was then microwave irradiated for 30 min at 120 °C. The resin was washed with DMF (1 mL, 5 times), CH_2Cl_2, (1 mL, 5 times), methanol (1 mL, 5 times), and diethyl ether (1 mL, 5 times). The products were then cleaved form the resin with 1 mL of trifluoroacetic acid/CH_2Cl_2 (20% v/v, 1 mL, 15 min). Toluene was added to the mixture before solvents were removed in vacuo to prevent products from being in the presence of concentrated trifluoroacetic acid.

Acknowledgment. We thank the Combinatorial Centre of Excellence partners: GSK, AstraZeneca, Pfizer, Eli-Lilly and Evotech OAI, and the EPSRC.

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CC050013E

Anal. Chem. **2006,** *78,* 4931−4937

High-Throughput Physical Organic Chemistry — Hammett Parameter Evaluation

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High-throughput analysis techniques were developed to allow the rapid assessment of a range of Hammett parameters utilizing positive electrospray mass spectrometry (ESI$^+$-MS) as the sole quantitative tool, with the core of the approach being a so-called "analytical construct". Hammett substituent parameters were determined for a range of meta- and para-substituted anilines by high-throughput (HT) assessment of relative reaction rates for competitive amide bond formation reaction with up to five parameters determined in a single pot reaction. Sensitivity of the reaction to substituents' effects (materialized by Hammett's ρ parameter) was determined in the first instance, with HT Hammett's σ substituent parameter assessment then carried out successfully for over 30 anilines, with excellent correlation observed between the HT ESI$^+$-MS method of determination and literature values.

High-throughput (HT) tools and technologies have had a tremendous impact on a variety of chemistry-related areas, ranging from the discovery of new materials and catalysts to the enhancement of synthetic and medicinal chemistry capability.[1,2] In these latter areas, a variety of solid-phase synthesis tools have emerged, along with a number of parallel approaches, as a key means of enhancing compound generation and purity. Spurred on by these developments in supported organic synthesis, resins have been exploited in an increasingly wide variety of areas, such as the heterogenization of homogeneous catalysts,[3] the immobilization of pH and oligonucleotide sensors,[4,5] fluorescence resonance energy transfer (FRET)-based sensors for monitoring carbohydrate and glycoprotein binding to lectins,[6] self-indicating catalysts with beads carrying both a catalyst and a pH indicator,[7] and so-called dyad beads carrying both a catalyst and the acceptor half of a Diels−Alder reaction.[8] With so many chemistries now taking place on solid supports, a variety of increasingly sophisticated techniques have been put into place to allow reactions to be analyzed and followed directly on the solid support, including, for example, attenuated total reflectance (ATR) Fourier transform infrared (FT-IR) and magic angle spinning nuclear magnetic resonance (MAS NMR) spectroscopies,[9,10] as well as a host of mass spectrometry (MS)-based approaches.[11−13] In this last area, a great enhancement has been brought about by a series of tools termed "analytical constructs" which have seen widespread application as a means of bead decoding,[14] reaction monitoring,[15,16] reaction evaluation and optimization,[17,18] the development of new solid-phase linkers,[19,20] and studies of their chemical compatibility.[21,22] The "analytical construct" works by releasing from the solid support a molecule that is attached to a MS sensitizing moiety, which ensures uniformity of ionization and, ideally, an isotopic label (inducing peak splitting) to enable the rapid extraction of the desired product information from the background of the mass spectrum.[23] Occasionally, an ultra violet (UV) chromophore has also been included.[17] Typically, these analytical constructs have consisted of a dual solid-phase linker system providing two orthogonal cleavage points between the reactive

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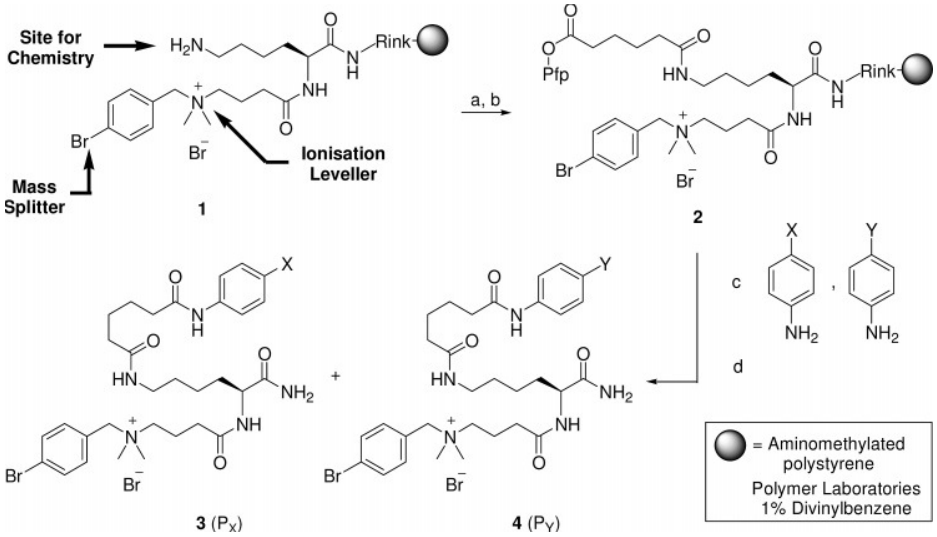
10.1021/ac060596u CCC: $33.50
Published on Web 05/28/2006

Scheme 1. Preparation of the Construct and Competition Experiments for the Assessment of ρ[a]



[a] Reagents and conditions: (a) adipic acid, DIC, HOBT, CH_2Cl_2/DMF (7:3, v/v), 2 × 30 min; (b) pentafluorophenyl trifluoroacetate, pyridine, DMF, 6 h; (c) equimolar mixture of two anilines, DMF/pyridine (1:1, v/v), 24 h; (d) TFA in DCM (20% v/v), 15 min.

site and the solid support,[23] sometimes being achieved by use of photocleavable linkers.[21] Despite recent developments, however, the technique of analytical constructs has remained mainly qualitative, with most attempts at drawing quantitative conclusions involving high performance liquid chromatography (HPLC) separation and UV-based quantitation.[24,25]

We recently reported the preparation and the use of a novel type of analytical construct **1** (see Scheme 1), which allowed the rapid identification and quantification of any compound attached to it by means of positive electrospray MS analysis (ESI^+-MS). A quaternary ammonium species incorporated into the analytical construct served as the MS sensitizer and was proven to have ionization leveling properties such that the global ionization of the construct was dominated by the quaternary ammonium ion and did not depend on what was attached to it, thus allowing quantitative conclusions to be drawn regarding the composition of mixtures of products linked to it merely by measuring the ESI^+-MS intensities.[26]

The proven quantitative properties of the analytical construct **1** allowed the direct assessment of the reactivity of a range of building blocks in the Ugi reaction, by direct ESI^+-MS quantification of each of the products generated when a mixture of starting materials were reacted in the famous four-component condensation.[26] In view of the ability of this analytical construct to allow rapid quantitative assessment for a particular reaction, its use was investigated as a means of determining relative reaction rates and the development of a method for HT Hammett parameter assessment. In 1937, Hammett introduced a set of equations that allowed the quantitative assessment of the effect that various meta or para substituents have on the position of the equilibrium of ionization of benzoic acids in solution. This treatment was extended to assess the effect of substitution on the benzene ring upon the rate (or upon the equilibrium) of a range of reactions and gave rise to two parameters: σ_x being the intrinsic electronic effect of the substituent X in comparison to hydrogen and ρ, the sensitivity of the reaction to the substituent contribution, with positive or negative ρ values accounting for the reaction being favored by electron-donating or -withdrawing effects (eq 1). The value of $\rho = 1$ is defined for the benzoic acid example first studied, in water at 25 °C.[27,28]

$$\log\left(\frac{K_X}{K_H}\right) = \rho\sigma_X \qquad (1)$$

These parameters have since been widely studied, with much work having been focused on the parameter for para substituents σ_p, to split it into two distinct components: the inductive (or field) and resonance contributions, respectively, σ_I (alternatively noted σ_F, L or F) and σ_R (noted σ_R, R), with $\sigma_p = \sigma_I + \sigma_R$.[29] To quantify the inductive contribution, σ_I, various methods have been explored, from the composition of the ionization equilibrium of compounds, in which the substituent is placed on a nonaromatic ring (bicyclooctane carboxylic acids or quinuclidines),[30,31] to the study of fluorine NMR chemical shifts.[32] The resonance parameter is most often afforded by deduction. It should also be noted that in the case of para substituents conjugated with the reaction center, as for para-substituted phenols and anilines, a new constant $\sigma_p^{\pm}$ has been introduced (σ_p^- constants defined for substituents that delocalize a negative charge, and σ_p^+ for substituents that would delocalize positive charges).[33] For instance, in the case of

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anilines, for substituents in which the lone pair of the nitrogen can be delocalized, σ_p^- should be used instead of σ_p to account for electronic effects. Until now, a number of very different methods have afforded an extensive amount of data quantifying more or less precisely the electronic effect for a given substituent, and despite minor differences, general agreement on these values has resulted, such that they are used efficiently in numerous applications to understand reactivity and regioselectivity of given reactions[34] and to predict reaction selectivity.[35] Although extended forms of the Hammett equation incorporating hydrophobic,[36] steric, and hydrogen bonding effect have been developed,[37,38] σ parameters as defined by Hammett are still used as molecular descriptors in quantitative structure activity relationships (QSAR).[39,40]

The study presented herein demonstrates the speed, efficacy, and accuracy of a HT ESI$^+$-MS-based approach using the quantitative analytical construct derived from **1** for the rapid determination of Hammett parameters. Initial studies were aimed at the determination of a reaction ρ value, from which evaluation of Hammett's σ parameters for several activating and deactivating aromatic substituents, in the meta and para positions could be undertaken. Our work followed an earlier study by Gerritz, who carried out Hammett ρ parameter determination while looking at the acylation of various anilines with resin-bound pentafluorophenol (Pfp) esters, using NMR spectroscopy, quantitatively assessing the effect of the choice of solid support on the ρ parameter.[41] The approach started with the analytical construct **2**, to which a Pfp ester moiety was introduced, as shown in Scheme 1, and the relative rates of competitive displacement of this active ester by two competing reagents (mixtures of meta- or para-substituted anilines and aniline) determined by ESI$^+$-MS. The quantitative properties of the analytical constructs allowed assessment of relative reaction rates in these competitive reactions by determination of the MS ratios of the two resulting products, thus enabling direct evaluation of the Hammett parameters by ESI$^+$-MS.

EXPERIMENTAL SECTION

Instrumentation. All solvents and reagents were obtained from commercial suppliers and used without any purification. Solid-phase reactions were carried out in polypropylene syringes equipped with polyethylene frits and Teflon stopcocks. NMR spectra were recorded on a Bruker ARX-250 or a Bruker ARX-360 spectrometer in the solvents indicated at 298 K. Chemical shifts are reported on the δ scale in parts per million and were referenced to residual solvent resonances. IR spectra were obtained with neat compounds on a FTIR Bruker tensor spectrometer, 16 scans, resolution ± 4 cm^{-1}, fitted with a Specac single-reflection diamond ATR Golden Gate. HPLC/evaporative light scattering detector (ELSD) analyses were obtained using an Agilent 1100 series system (eluent A, water + 0.1% formic acid; eluent B, methanol + 0.1% formic acid. Gradient, 95−5% A over 10 min; flow rate, 1 mL/min) coupled to a Polymer Lab 100 ES evaporative light scattering detector. The column used was a Gemini C18 110A from Phenomenex (100 × 4.60 mm, 5-μm). ESI$^+$-MS analyses were carried out on an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS) using electrospray positive ionization (ESI$^+$). MS injections were performed by elution with A/B (95:5, v/v, 1.5 min). High-resolution (HR) MS analyses were carried out by the MS Department of the University of Edinburgh. Eluents used were HPLC grade. The amine analytical construct resin **1** was prepared according to a procedure already reported.[26]

Preparation of Pentafluorophenyl Ester Analytical Construct Resin, 2.[41] Adipic acid (640 mg, 4.4 mmol) was dissolved in CH_2Cl_2/DMF (7:3 v/v, 5 mL). HOBt was added (594 mg, 4.4 mmol). After 1 min of stirring, DIC (690 μL, 4.4 mmol) was added. After complete dissolution, was added the amine analytical construct resin (1 g, $s = 0.55$ mmol·g^{-1}) in CH_2Cl_2/DMF (7:3 v/v, 5 mL). The reaction was stirred for 30 min. The resin was washed with DMF (10 mL, 3×), CH_2Cl_2 (10 mL, 3×), methanol (10 mL, 3×), and diethyl ether (10 mL, 3×). The coupling step was then repeated, and the resin was washed with DMF (10 mL, 3×), CH_2Cl_2 (10 mL, 3×), methanol (10 mL, 3×, and diethyl ether (10 mL, 3×). The afforded resin was then swollen in a solution of Pfp trifluoroacetate (860 μL, 10 mmol) and pyridine (400 μL, 10 mmol) in DMF (20 mL). The mixture was stirred for 6 h, and the resin was then washed with DMF (20 mL, 3×), CH_2Cl_2 (20 mL, 3×), and diethyl ether (20 mL, 3×), and dried in vacuo overnight to afford the title compound **2** as a buff-colored resin (1.1 g). IR ν (cm^{-1}): 3420 (w, br, ν_{N-H}), 1659 (s, br, $\nu_{C=O}$).

Preparation of *N*-(4-Bromobenzyl)-*N*-{3-[(*S*)-1-carboxy-5-(5-phenylcarbamoylpentanoylamino)-pentylcarbamoyl]-propyl}-*N*′′,*N*′′-dimethylammonium Bromide (3, X = H). To assess the purity of the compound attached to the resin, the displacement of the Pfp functionality of the resin **2** previously prepared was performed by aniline as follows: Pfp ester analytical construct resin **2** (1 g, $s = 0.42$ mmol/g) was shaken in a solution of aniline (466 mg, 60 mmol) in DMF/pyridine (1:1, v/v, 10 mL) overnight. The resin was washed with DMF (10 mL, 3×), CH_2Cl_2 (10 mL, 3×), methanol (10 mL, 3×), and diethyl ether (10 mL, 3×). The product was then cleaved from the resin with trifluoroacetic acid/CH_2Cl_2 (20% v/v, 15 min, 10 mL). The solvent was then removed in vacuo to afford the title compound **3** (X = H) (250 mg, 65%). Purity: 100% (ELSD), ESI$^+$-MS: $m/z = 630.2$ (M$^+$), FAB$^+$/HRMS: $m/z = 630.2660$ (M$^+$ $C_{31}H_{45}BrN_5O_4^+$ requires 630.26). ^{1}H NMR + COSY + NOESY (250 MHz, DMSO-d_6, δ, ppm): 9.89 (1H, s), 8.01 (1H, d, $J^3 = 9.4$ Hz), 7.79 (1H, bt), 7.72 (2H, d, $J^3 = 8.2$ Hz), 7.58 (2H, d, $J^3 = 8.0$ Hz), 7.50 (2H, d, $J^3 = 7.9$ Hz), 7.39 (2H, bs), 7.27 (2H, t, $J^3 = 7.7$ Hz), 7.00 (1H, bt), 4.50 (2H, s), 4.16 (1H, m), 3.24 (1H, m), 3.02 (2H, m), 2.94 (6H, s), 2.26 (4H, m), 2.04 (4H, m), 1.51 (6H, m), 1.36 (2H, m), 1.22 (2H, m). ^{13}C NMR + DEPT 135 + DEPT 90 (90 MHz, DMSO-d_6, δ, ppm): 175.3, 173.3, 172.6, 172.1, 140.8, 136.6, 133.4, 130.1, 128.9, 125.6, 124.4, 120.5, 66.8, 64.6, 53.8, 50.6, 39.8, 37.7, 36.8, 33.2, 32.8, 30.4, 26.5, 26.4, 24.4, 19.7.

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General Procedure for Competitive Amide Bond Formation. The following general procedure was used to access the relative reaction rates of the displacement of Pfp esters by a mixture of substituted anilines. The specific example below used aniline and *para*-toluidine (*para*-methyl aniline).

The analytical construct resin containing the Pfp ester **2** (s = 0.42 mmol/g, 10 mg) was swollen in a solution of aniline (372 μg, 4 mmol) and *para*-toluidine (429 μg, 4 mmol) in DMF/pyridine (1;1, v/v, 0.8 mL). The mixture was stirred for 24 h in a 1-mL polypropylene syringe before the resin was washed with DMF (1 mL, 3×), CH_2Cl_2 (1 mL, 3×), methanol (1 mL, 3×), and diethyl ether (1 mL, 3×). The products were then cleaved from the resin with trifluoroacetic acid/CH_2Cl_2 (20% v/v, 1 mL, 15 min), and the solvents were removed in vacuo, affording a mixture of two products. The crude was dissolved in HPLC water (1 mL) prior to ESI^+-MS analysis.

Procedure for Hammett ρ Parameters Assessment ("Combinatorial" Hammett Plots). Each combination of two anilines (X and Y, from *para*-fluoroaniline, *para*-iodoaniline, *para*-ethoxyaniline, *para*-methylaniline, and aniline) was reacted according to the general procedure described above. For each reaction, an ESI^+-MS analysis of the crude mixture was performed (injection volume, 1 μL). The ion chromatogram was then extracted from the MS trace obtained (total ion current, TIC) using the Chemstation software (Rev. A.08.03 [847], Agilent Technologies), and the relative intensities of all MS peaks were exported to a comma-separated values file. The values I_X and I_Y of the intensities of the peaks corresponding, respectively, to P_X and P_Y were determined and then processed as follows: for a specific aniline (Y), $\log(I_X/I_Y)$ was plotted against the respective values of σ_{pX}^- taken from the literature.[33] The slope of the line was obtained by linear regression, affording ρ. The procedure was repeated for each aniline, giving five values of ρ. All experiments were carried out in quadruplicate.

Procedure for Hammett ρ Parameters Assessment ("One-Pot Combinatorial" Hammett Plot). A solution of five anilines (*para*-fluoroaniline (444 μg, 4 mmol), *para*-iodoaniline (876 μg, 4 mmol), *para*-ethoxyaniline (549 μg, 4 mmol), *para*-methylaniline (429 μg, 4 mmol), and aniline (372 μg, 4 mmol)) in DMF/pyridine (1:1, v/v, 0.8 mL) was added to the Pfp ester analytical construct resin **2** (s = 0.42 mmol/g, 10 mg). The mixture was stirred for 24 h in a 1-mL polypropylene syringe before the resin was washed with DMF (1 mL, 3×), CH_2Cl_2 (1 mL, 3×), methanol (1 mL, 3×), and diethyl ether (1 mL, 3×). The products were cleaved from the resin with trifluoroacetic acid/CH_2Cl_2 (20% v/v, 1 mL, 15 min), the solvent was removed in vacuo, the crude residue was redissovled in HPLC water (1 mL), and the mixture of five products was analyzed by ESI^+-MS analysis (injection volume, 1 μL). The data obtained were processed as described previously to afford the "combinatorial" Hammett plot. All experiments were carried out in quadruplicate.

Procedure for Hammett σ Parameters Assessment. The following procedure is given for the assessment of σ_{pMe}, but all other assessments were carried out in an analogous manner. *para*-Methylaniline was used with aniline according to the general procedure for competitive amide bond formation described above. ESI^+-MS analysis of the crude mixture as described above allowed the intensities of all MS peaks to be exported from the raw data to a comma-separated values file. The values I_{pMe} and I_H of the intensities of the peaks corresponding to P_{pMe} and P_H were processed as follows to afford a value of $\sigma_{pMe}/\sigma_{pMe} = (1/\rho)\log(I_{pMe}/I_H)$. All experiments were carried out in quadruplicate.

RESULTS AND DISCUSSION

Preparation of the Construct. The amino analytical construct resin **1** was prepared as reported previously.[26] Amide coupling to adipic acid followed by trans-esterification using Pfp-trifluoroacetate with pyridine afforded resin **2** (Scheme 1).[41]

Assessment of the Value of ρ Parameter. The first part of the study consisted of determination of the Hammet ρ parameter, providing a measure of the sensitivity of a given reaction to substituent effects and giving information on the nature of the reaction mechanism, with the higher the absolute value of ρ, the greater the influence of different σ's in terms of reaction rates or composition of the final equilibrium. It is strongly influenced by parameters such as temperature, solvent used, etc. In addition, since polymeric supports act as solvents and influence the environment in which the reaction actually takes place,[42] they would, as was observed by Gerritz, be expected to have an influence on the ρ parameter.[41] The intention in these initial studies was to assess the robustness, reliability, and accuracy of the approach and to obtain the ρ parameter for this specific transformation. The method employed consisted of building a "combinatorial" Hammett plot, using literature values of σ_p^- for four different para-substituted anilines, *para*-fluoroaniline, *para*-iodoaniline, *para*-ethoxyaniline, *para*-methylaniline, and aniline itself.[33] Ten competition experiments between any two anilines X and Y afforded 10 mixtures of two amides resulting from the displacement of the Pfp functionality. Each assay was carried out in quadruplicate to determine repeatability and ensure the accuracy of the methodology (Scheme 1).

The mixture of products **3** "P_X" and **4** "P_Y" obtained after cleavage of the Rink linker was analyzed by ESI^+-MS. The intensity of each peak was extracted to an Excel file using Chemstation software (Rev. A.08.03 [847], Agilent Technologies), allowing rapid calculation of the ratio between the two MS peaks for each of the 10 combinations, directly accounting for the relative reaction rates. A typical ESI^+-MS trace (obtained for a mixture of P_H and P_{Me}), with an expansion of the bromine pattern corresponding to one of the products, is shown in Figure 1.

For each aniline Y, "Hammett" lines could be drawn by plotting the four values of $\log([P_X]/[P_Y])$, as well as the "zero" value for X = Y, against the respective values of σ_{pX}^- taken in the literature.[33] Because the starting anilines were used in equimolar ratios in the reaction mixture, the Hammett equation applies (eq 2), and the value of ρ is, thus, obtained by the slope of the lines.

$$\log\left(\frac{[P_X]}{[P_Y]}\right) = \log\left(\frac{k_X}{k_Y}\right) = \rho\sigma_{pX} - \rho\sigma_{pY} \qquad (2)$$

All data obtained gave very satisfactory correlation coefficients, demonstrating the high reliability of the MS tool to assess competitive reaction rates with essentially identical ρ values obtained for the reaction (Figure 1). It should be highlighted that

(42) Czarnik, A. W. *Biotechnol. Bioeng. (Comb. Chem.)* **1998**, *61*, 77−79.

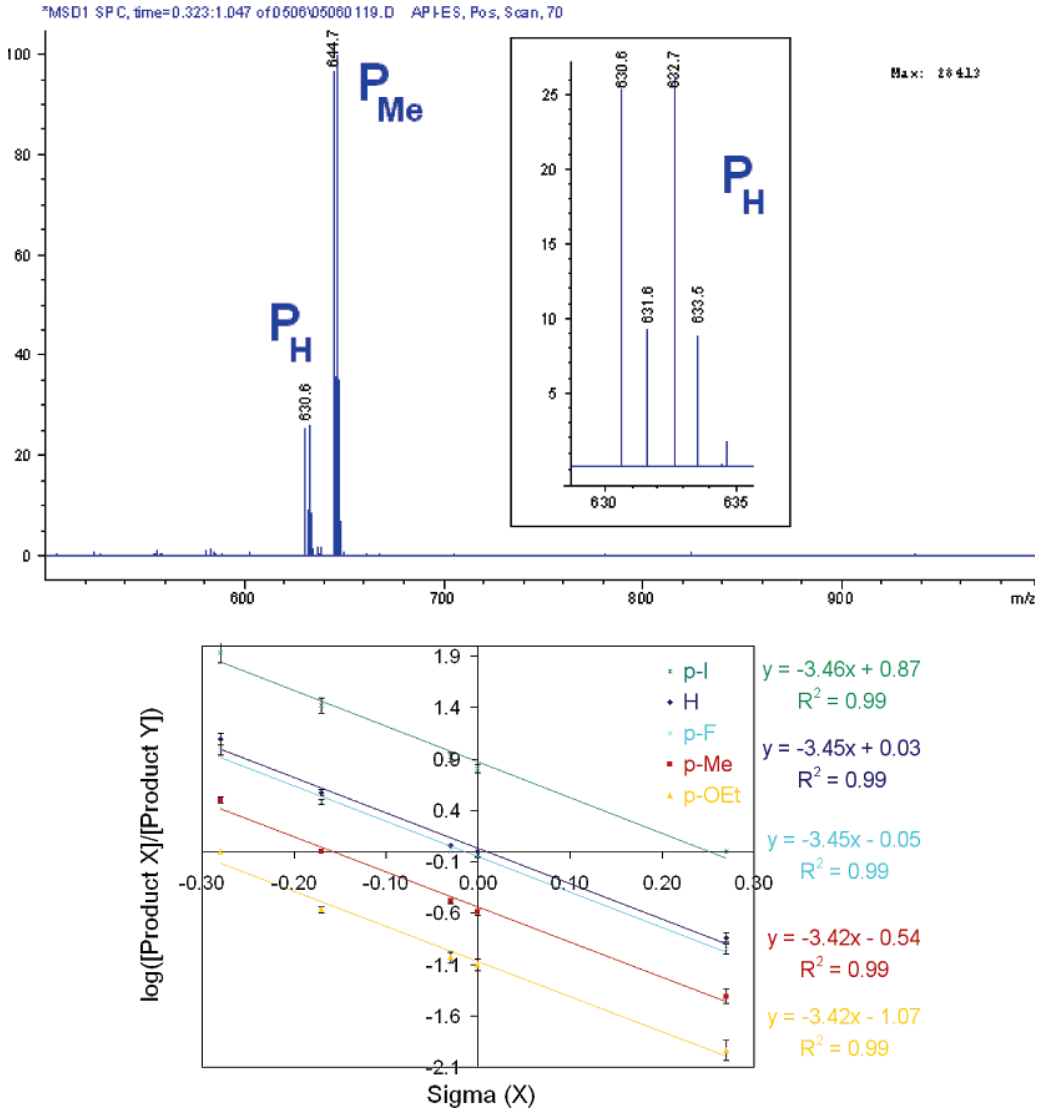

Figure 1. Example of ESI$^+$-MS spectrum of a mixture of **3** and **4** (with X = H, Y = Me) used to build the combinatorial Hammett plot shown below.

these data were the result of 40 assays, with direct analysis of the ESI$^+$-MS information, with each sample run in quadruplicate (standard deviations are indicated on the graph).

The value of ρ obtained was -3.44, agreeing with the results of Gerritz ($\rho = -3.45$), indicating that the reaction is favored by electron-donating groups. Competition experiments were then carried out with five anilines in equimolar quantities, in the same mixture (*para*-methylaniline, *para*-ethylaniline, *para-tert*-butylaniline, *para*-iodoaniline, and aniline) and reacted with the analytical construct **2**, affording in a single ESI$^+$-MS analysis the data required to draw the entire combinatorial Hammett plot (Figure 2). Again, the experiment was carried out in quadruplicate.

The value of ρ obtained (-3.45) was consistent with the previous results, thus allowing assessment of the sensitivity of the support to substituent effects in a single pot assay and from a single MS analysis.

HT σ_p and σ_m Parameter Assessment. Having assessed the value of ρ, HT measurement of the σ parameters could be undertaken. This was carried out by direct competition experiments between the substituted aniline and aniline itself as a reference from which the relative reaction rates could be assessed and σ_X deduced, according to eq 3, by measurement of the ratio between the two products as determined by a single ESI$^+$-MS injection. The experimental procedure was built in such a way that the molar ratios between each aniline of the reaction mixture and the Pfp-ester analytical construct was 100:1, so as not to be limited by any diffusion of the reagents and to keep the concentration of anilines constant, despite consumption by the reaction (again, each assay was carried out in quadruplicate).

$$\sigma_X = \frac{1}{\rho}\log\left(\frac{k_X}{k_H}\right) \qquad (3)$$

The first series of anilines to be tested were *para*-alkyl and *para*-aryl anilines (*p*-Me, *p*-Et, *p*-^{t}Bu, *p*-^{i}Pr, *p*-trityl) and halogenated substrates (*p*-F, *p*-Cl, *p*-I, *p*-CF_3). Deviation was showed to be very weak, and correlation between experimental and literature data (σ_p^-) was very good (Table 1).[33] It is important to note here the sensitivity and level of detection of the method: to assess the

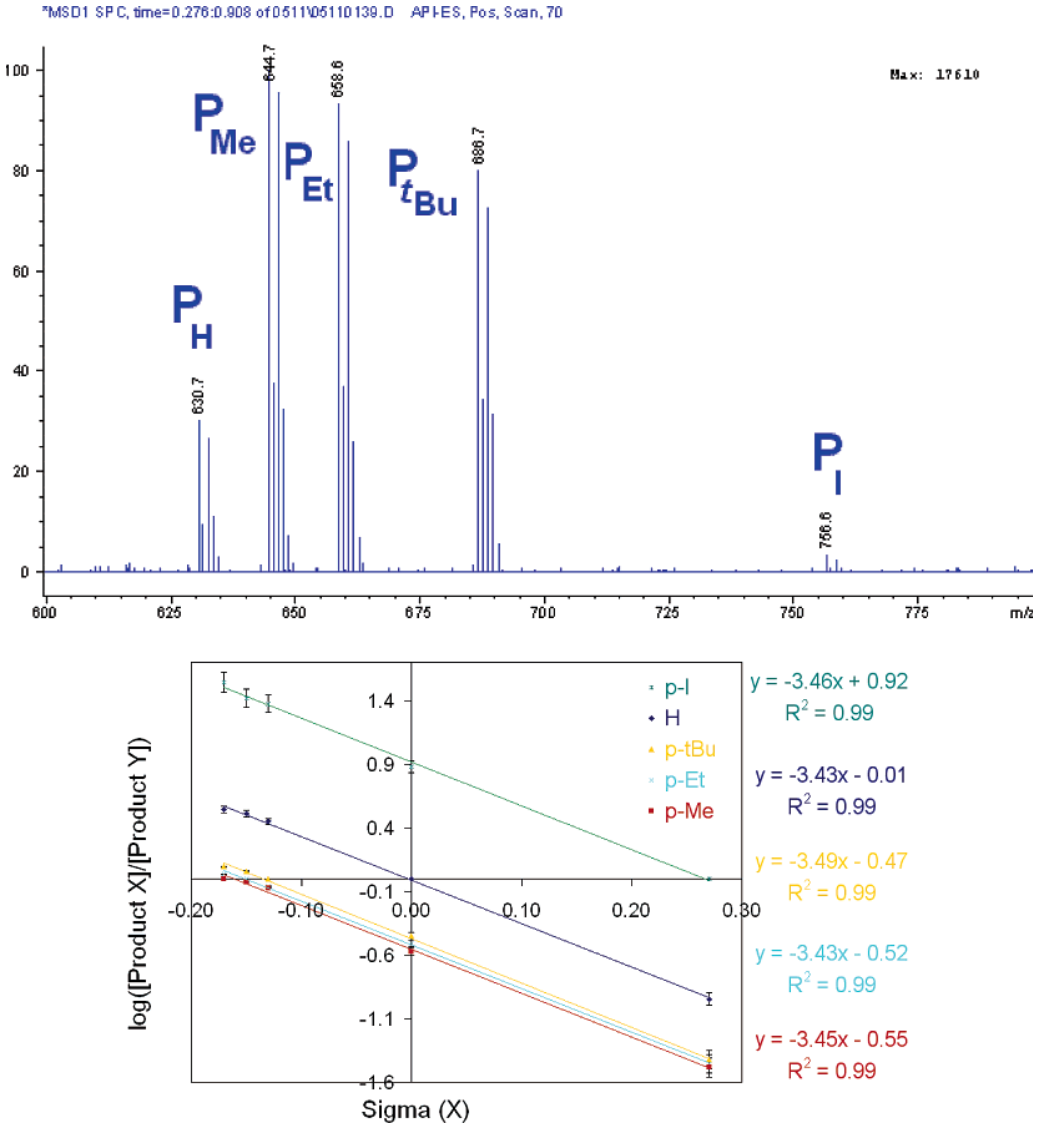

Figure 2. Typical ESI$^+$-MS spectrum (five-aniline assay) and one-pot combinatorial Hammett plot.

Table 1. σ_p Assessment for a Series of Para-Substituted Anilines

substituent	σ_p lit.[33]	σ_p^- lit.[33]	σ_p^- exptl
Me	−0.17	−0.17	−0.17 ± 0.01
Et	−0.15	−0.19	−0.15 ± 0.01
^{t}Bu	−0.20	−0.13	−0.13 ± 0.02
^{i}Pr	−0.16	−0.16	−0.16 ± 0.01
trityl	0.02	N/A	−0.05 ± 0.01
F	0.06	−0.03	−0.02 ± 0.01
Cl	0.23	0.19	0.19 ± 0.01
I	0.27	0.27	0.27 ± 0.01
CF_3	0.54	0.65	0.64 ± 0.01
SMe	0.00	0.06	0.05 ± 0.01
NMe_2	−0.83	−0.12	−0.13 ± 0.01

Hammett parameter for the *p*-CF_3 group, the ratio between the two amide products was less than 1:150, as shown on Figure 3, yet the MS method allows this to be determined directly.

The case of the *p*-CF_3 group demonstrates quite eloquently the power of the MS quantitation tool: such a level of detection using analytical methods such as NMR or HPLC/UV would not be practical. A range of electron-donating (*p*-OH, *p*-OMe, *p*-OEt, *p*-OnPr) and electron-withdrawing (*p*-OCF_3, *p*-$OCHF_2$) oxygenated substrates were evaluated in the same manner and again afforded good results in comparison to literature values (Table 2).

The method was applied to the same alkyl and halo groups in the meta position of the aromatic ring of the anilines. Correlations again proved to be excellent and highly reproducible, as shown by the data in Table 3. The final series looked at deactivating oxygenated substituents as well as *m*-SMe and *m*-NMe_2 groups in the meta position. For this series, correlation between experimental data and literature values was not as good as for *m*-OH, *m*-OMe, *m*-OEt substituents, all showing less deactivation than literature values, but all other groups tested gave excellent results and repeatability (Table 3).

Limitations to the Method. The main limitation to the method of assessment of Hammett parameters by ESI$^+$-MS using quantitative analytical constructs arises from the detection method itself. As shown for *p*-CF_3 aniline, an absolute difference of 0.65 in the value of σ was observed, but this is probably the upper

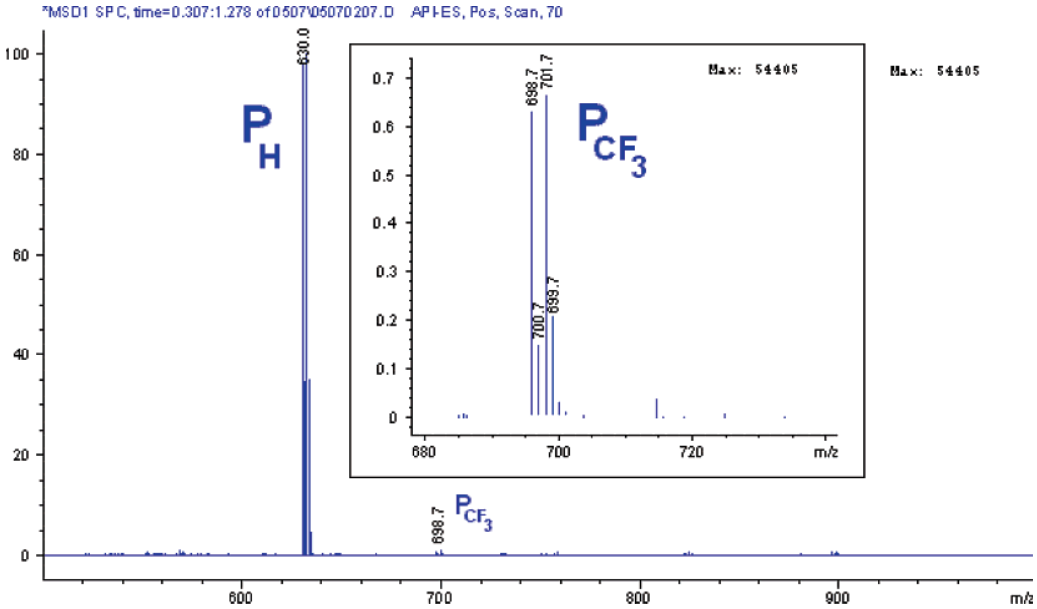

Figure 3. ESI^+-MS spectrum from reactions with aniline and *p*-CF_3 aniline.

Table 2. σ_p^- Assessment for a Series of *para*-Alkoxy Anilines

substituent	σ_p lit.[33]	σ_p^- lit.[33]	σ_p^- exptl
OH	−0.33	−0.37	−0.37 ± 0.01
OMe	−0.27	−0.26	−0.27 ± 0.01
OEt	−0.24	−0.28	−0.31 ± 0.01
OnPr	−0.32	−0.32	−0.31 ± 0.01
OCF_3	0.35	0.27	0.27 ± 0.01
$OCHF_2$	0.18	0.11	0.09 ± 0.01

Table 3. σ_m Assessment for a Series of Meta-Substituted Anilines

substituent	σ_m lit.[33]	σ_m exptl
Me	−0.07	−0.04 ± 0.01
Et	−0.07	−0.05 ± 0.01
^{t}Bu	−0.10	−0.12 ± 0.02
Cl	0.37	0.36 ± 0.01
I	0.35	0.31 ± 0.01
F	0.34	0.31 ± 0.01
CF_3	0.43	0.40 ± 0.01
OH	0.12	0.05 ± 0.01
OMe	0.12	0.07 ± 0.02
OEt	0.10	0.05 ± 0.01
OCF_3	0.31	0.36 ± 0.01
$OCHF_2$	0.31	0.29 ± 0.01
SMe	0.15	0.12 ± 0.01
NMe_2	−0.16	−0.13 ± 0.01

limit that can be reached by the method and direct comparison with unsubstituted anilines. This arises because the relative intensity of peaks is used to draw quantitative conclusions, and the full set of four peaks corresponding to a compound must be clearly be extractible from the background noise of the mass spectrum, which is impossible to achieve with σ values above 0.7. Thus, when assessing σ_p^- values for very deactivating groups, such as NO_2, CN, and COOEt, whose literature values are 1.00, 1.00, and 0.75, respectively, only the unsubstituted product coming from aniline could be detected. These issues are quite common, however, and are usually solved using substituents that, in effect, change the relative starting point (i.e., compare with an electron-withdrawing group rather than H and then reassemble on the same scale). It was also noticed that σ_p values could not be successfully measured for charged substituents, such as COO^-, $(CH_2)COO^-$, and $(CH_2)_2COO^-$, because of a problem of solubility of the corresponding anilines in the solvent used for the assays.

CONCLUSION

The use of a quantitative analytical construct as a HT tool for assessment of relative reaction rates by ESI^+-MS afforded results with good sensitivity and reliability. The value of ρ determined by the construction of a combinatorial Hammett plot required just 10 ESI^+-MS injections (carried out in quadruplicate) and showed the repeatability and the reliability of the experimental procedures. These data could be generated either in a competitive sense, using two anilines at a time, or using five anilines in a single pot assay. This preliminary step allowed successful HT determination of Hammett substituent electronic effects (σ values, again with direct ESI^+-MS analysis of cleaved crude mixtures). The MS approach to Hammett analysis is, thus, rapid, quantitative, and highly reliable and, if the analytical construct can be attached to the molecules of interest, offers a new approach to physical organic chemistry investigations.

ACKNOWLEDGMENT

We thank the Combinatorial Centre of Excellence partners: GSK, AstraZeneca, Pfizer, Eli-Lilly, and Evotech OAI.

Received for review March 31, 2006. Accepted April 19, 2006.

AC060596U

Approaches to high throughput physical organic chemistry

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Received 16th October 2006, Accepted 5th December 2006
First published as an Advance Article on the web 9th January 2007
DOI: 10.1039/b614923e

High throughput (HT) techniques are now extensively used for the synthesis of libraries of several thousands of compounds. More recently, HT methods began to be applied to other areas, such as physical organic chemistry. This has allowed for instance the development of tools for HT reaction assessment, HT kinetic and thermodynamic measurements, and physicochemical property profiling, using a broad set of analytical tools, ranging from mass spectrometry to image analysis based techniques. This article provides an overview of recent HT physical organic chemistry techniques. Special attention is given to the application of quantitative analytical constructs for HT monomer reactivity profiling and HT evaluation of Hammett parameters.

Introduction

Physical organic chemistry has had a fundamental impact on the way in which synthetic and mechanistic organic chemistry has developed. Take for example the detailed studies carried out on simple substitution chemistries in the mid 1930s, and it becomes clear that physical organic chemistry has allowed direct correlations to be established between the structure of organic molecules and their reactivity.[1] It has provided a platform from which fundamental rules of chemistry have developed, supplying explanations concerning molecular reactivity, and allowed detailed mechanistic understanding to be gained. The establishment of linear free energy relationships, based on the work of Hammett for instance, has provided an unequalled understanding of the reactivity of organic compounds with the development of parameters such as σ and ρ, while analysis and understanding of isotope effects has provided a range of subtle tools to probe reaction mechanisms. However, physical organic chemistry has often been tarnished (unfairly) with a "reputation" of tedium and repetition, with the vision of days if not weeks spent hunched in front of a high performance liquid chromatography (HPLC) system, analysing single reactions. This can be contrasted with high-throughput (HT) organic chemistry which has developed at a tremendous rate over the past decade, allowing chemists to rapidly and efficiently generate libraries of hundreds to thousands of compounds. As a direct consequence of these advances in HT synthetic chemistry, analytical chemistry tools have been developed to meet the challenges of being able to monitor the progress of large numbers of reactions *in situ*, to determine the purity of these library members and to enable automated purification and mass spectrometry (MS)

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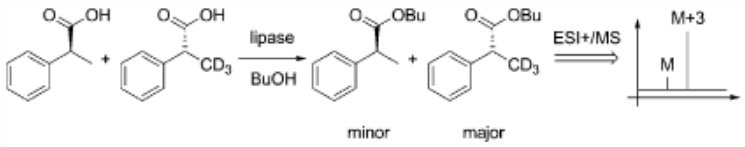


Scheme 1 Use of a pseudo-racemate for HT stereoselectivity evaluation.

based characterisation. As a result of these factors, unparalleled amounts of data are routinely being generated and larger monomer sets than ever before are being used in an increasingly large repertoire of chemistries. Clearly, these approaches open up a raft of opportunities to the physical organic chemist, with the accessibility of huge data collections, the ability to run much larger reaction sets and the ready availability of automation. It is also worth noting here the subtle boon provided by microwave heating, which allows not only unprecedented and highly accurate controlled heating of reactions, but, and importantly from a physical organic chemistry view-point, reproducible and known reaction times, temperatures and pressures in a manner normally impossible with traditional heating methods in organic synthesis.[2] This review will provide an overview of a number of approaches that have been used to enable increased throughput in physical organic chemistry, predominantly by the application of a number of HT tools that allow rapid reaction assessment and analysis, invigorating this whole process.

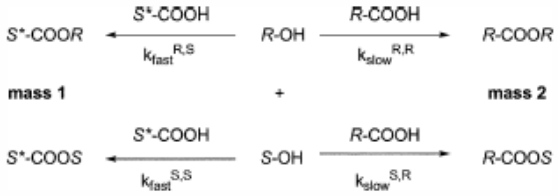
1 MS-based methods for HT reaction assessment

Since MS offers unrivalled speed of analysis and great sensitivity, it is often the tool of choice for the HT analysis of complex mixtures, and has seen application in areas ranging from the analysis of peptides and proteins (for example proteomics and serum profiling) to libraries of small organic compounds. In this review we shall concentrate on methods that focus predominantly on small molecules and that utilise, on the whole, soft ionisation techniques, although other MS based methods are also applicable.

(a) Pseudo-enantiomers and pseudo-diastereomers

For compounds that show good MS ionisation, HT qualitative reaction assessments can be made by the direct injection of reaction mixtures. However, the technique has obvious limitations for the analysis of compounds that have the same molecular weight, such as enantiomers or diastereomers. This has led to the introduction of the concept of pseudo-enantiomers which solves this dilemma and allows MS methods to be used for HT determination of enantiomeric excesses. The approach involves "isotopic tagging" whereby one of the enantiomers of a compound is synthesised or chemically modified to allow the incorporation of an isotopic label (for example a CD_3 replacing a CH_3 group). This "tagged" enantionomer is then mixed in a 1 : 1 manner with the unlabelled enantiomer. The two compounds are said to be "pseudo-enantiomers", since one of the two isomers is "heavier" than the other thanks to the presence of the CD_3 group: they form a racemic mixture whose analysis by electrospray ionisation MS (ESI/MS) is now possible. The stereoselectivity of a chemical transformation can thus be assessed by MS, provided this transformation is not affected by the modified group. Pseudo-diastereomers can be considered in the same way.[3] Using this approach, Reetz *et al.* developed a HT screening method to examine the enantioselectivity of a number of catalysts.[4] This allowed, for example, the evaluation of 1000 enantiomeric excesses (*ee*'s) a day for the lipase catalysed stereoselective esterification of 2-phenylpropionic acid, as shown in Scheme 1. This work also demonstrated that the results obtained *via* the use of pseudo-enantiomers compared very well to those obtained with other techniques, proving that the labelling of one of the enantiomers did not induce changes in reaction selectivity.

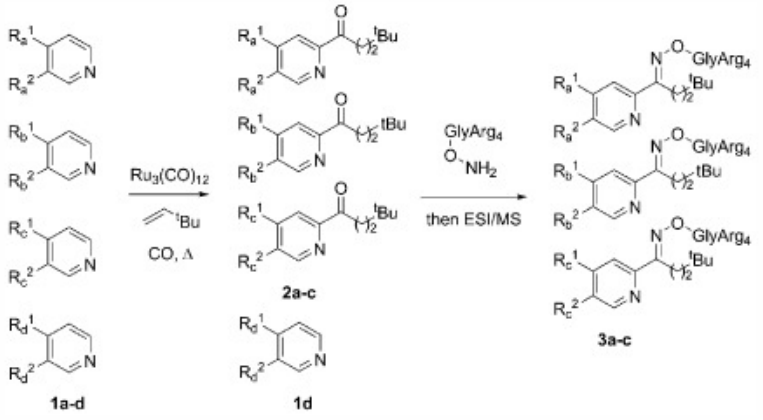
Guo *et al.* developed a related approach to allow the HT *ee* determination of alcohols and amines.[5] Thus, the alcohols (or amines) under investigation, *R*-OH and *S*-OH were coupled to a pseudo-racemate of a carboxylic acid (*R*–COOH and *S**-COOH). The esterification of the various pairs of chiral reagents proceeded with different speeds (each one of the pseudo-enantiomers reacted preferentially with a given enantiomer of the alcohol, as shown in Scheme 2), with ESI/MS analysis of the final mixture making it possible to evaluate the *ee* of the starting mixture of alcohols following Horeau and Nouaille's work.[3]



Scheme 2 Use of a pseudo-racemate for HT *ee* evaluation.

(b) MS "Tagging"

To allow the most to be made of MS techniques, and apply them to as broad a range of chemistries and compounds as possible, especially those with poor ionisation abilities, a variety of MS tagging strategies have been successfully applied. One representative example of this is the work reported by Szewczyk *et al.* and the development of a solution phase MS labelling method for HT reaction evaluation and optimisation.[6] The method, as described in Scheme 3, consists of a one pot acylation of a library of pyridine based substrates **1a–d** with 3,3-dimethylbut-1-ene and carbon monoxide, in the presence of $[Ru_3(CO)_{12}]$. The "tag", composed of four arginine residues and an *N*-terminal alkoxyamine (H_2N–O–$GlyArg_4$) was used to selectively label the products of the reaction mixture (**2a–c**) by oxime formation with any ketone functionality generated in the reaction. The "tag" not only guaranteed the ionisation of the products **3a–c** for MS detection, but also dominated it, allowing quantitative conclusions to be drawn from the integration of the MS peak areas, by comparison to an internal reference (2-pyridinecarboxaldehyde labelled with the (H_2N–O–$GlyArg_4$) tag). Furthermore, thanks to



Scheme 3 MS tagging approach for HT reaction evaluation and optimisation (products **2a–c** are prepared with varying levels of success and their relative levels can be determined *via* ESI/MS following derivatisation).

the tag, only peaks corresponding to the products of the reaction will be identified whereas unreacted reagents *etc.*, are not ionised or detected.

This approach allowed the rapid evaluation of large numbers of substrates to define structure–reactivity relationships and reaction compatibility of functional groups. One big asset of this method is that it is applicable to virtually any reaction that generates a carbonyl group, thus allowing tag attachment.

(c) Analytical constructs

In the area of solid phase synthesis, a number of tools have been developed that have enhanced reaction analysis and those termed "analytical constructs" are perhaps the best known (Fig. 1). These "analytical constructs" incorporate features that allow the rapid and reliable qualitative analysis of reactions by the incorporation of a "MS sensitizer" tag, which guarantees uniform MS ionisation and sometimes also a "mass splitter" for the rapid identification of relevant peaks from the mass spectrum. These analytical constructs have been used mainly to identify products and monitor solid phase reactions,[7] but they have also proven useful in other applications, such as functional group compatibility studies,[8] and linker development.[9–11] Quantitative conclusions have been achieved, either by the incorporation of an ultraviolet (UV) chromophore into the construct, or using MS based quantitation, which clearly enhances the power of the concept. Such quantitative analytical constructs were developed on the basis of a quaternary ammonium species as an MS sensitizer and ionisation leveller and an aryl bromide as a peak splitter (see Fig. 1). Cleavage of mixtures of compounds linked to the construct and their direct positive ESI/MS (ESI+/MS) analysis afforded a set of peaks whose intensities were proportional to the amount of product in the mixture (Fig. 2).[12]

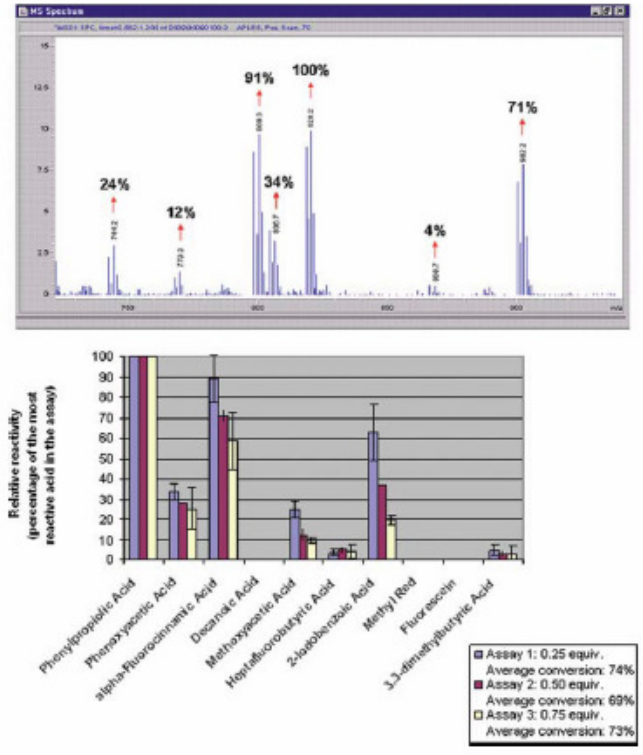


Fig. 2 Top: MS trace (of a crude reaction mixture, single injection) and bottom: monomer reactivity data obtained for the reaction described in Scheme 4.

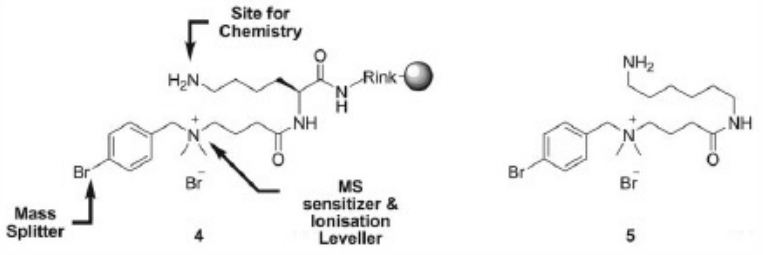


Fig. 1 Solid phase MS analytical construct (**4**) and its solution phase variant (**5**).

(i) HT monomer reactivity profiling. Currently, one of the bottlenecks of HT synthesis is the time and money wasted in the elaboration of libraries where combinations of reactants do not give the desired product in satisfactory yield and/or purity. One way to prevent this would be to "scan" rapidly all monomer combinations to determine if the desired compounds will be generated efficiently before embarking on the production of the complete library. However, it would be incredibly time consuming to test the monomers one by one, and therefore HT methods have been elaborated to achieve this. Quantitative analytical constructs turned out to be an extremely efficient means of evaluating the relative reactivity of a range of ten carboxylic acids in the Ugi-4 component condensation (4CC), with ESI+/MS as the sole analytical tool. To do so, the carboxylic acids **6a–j** were mixed and then reacted with the analytical construct **4**, hydrocinnamaldehyde **7** and cyclohexyl isonitrile **8**, to afford a mixture of α-acylamino amides **9a–j** as shown in Scheme 4.[12]

Identification and quantification of the cleaved products **9a–j** was rapidly achieved thanks to the properties of the analytical construct: quantification was made possible by the ionisation levelling property of the construct, while the relevant peaks were located in a "clean" region of the spectrum thanks to the added mass of the construct and were easily identified due to the bromine isotope pattern. Subsequent correlation of this data to the corresponding monomers **6a–j** allowed an assessment of their relative reactivities (Fig. 2) with the most reactive carboxylic acid being defined as 100% with the reactivity of the other building blocks expressed in relation to this.

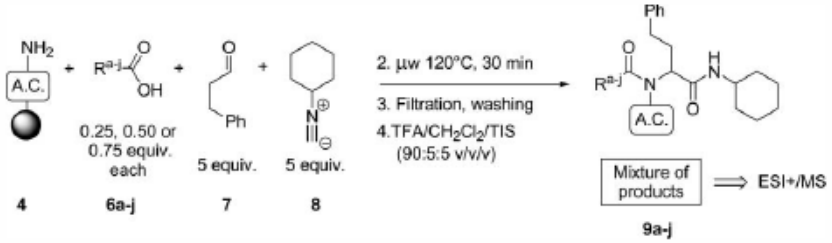
The mixtures of monomers **6a–j** could be studied at various concentrations to study the effect of relative building block concentration on reactivity (Fig. 2). The approach was also extremely efficient in terms of material, since the amount of each monomer used was typically around 100 μg (a few mg for the other components), while less than 2 mL of solvent were used per experiment. Several building blocks turned out to be unreactive in the Ugi-4CC, and would sensibly have to be taken out of the pool of starting materials if the Ugi-4CC were to be used to generate a library. Similar studies were undertaken with mixtures of ten isonitriles and ten aldehydes, where variations in concentration showed no remarkable change in reactivity profiles. The method was also carried out using the solution phase analytical construct **5**, allowing monomer reactivity profiling with just 0.1 eq. of each carboxylic acid.

The relative reactivity profiling of building blocks for a given reaction allows them to be gathered into groups of similar reactivity to ensure good yields and purities when it comes to prepare the final library of α-acylamino amides. Moreover, the fact that only the reactivity of the carboxylic acids happened to be concentration dependent has mechanistic implications. Analysis of these results from a broader perspective, suggests that the versatility of the ESI+/MS analytical construct, makes them applicable to a broad range of reactions, monomer rehearsal and reaction optimisation (Fig. 1).

(ii) HT Hammett parameter assessment. As shown previously, ESI+/MS quantitative analytical constructs allow the rapid investigation of relative reaction rates. In the case of families of substrates ranking enables an evaluation of the effect of substituents across the family. This principle was applied to the competitive displacement of a pentafluorophenyl ester functionality placed on the reactive site of the construct **4** (Fig. 1), by reaction of an equimolar mixture of aniline and various substituted anilines (*meta* or *para*). In such a case, the Hammett equation applies: $\log\left(\frac{k_X}{k_H}\right) = \rho\sigma_x$ and the relative reaction rates, translated by the ratio of the amides generated, depend on the intrinsic electronic effect of the substituent (σ parameter) and how these electronic effects are transmitted to the reaction centre (ρ parameter). One "pot" combinatorial Hammett plots were generated to allow assessment of the value of ρ for the reaction.[13] Having determined this value a HT method was designed to successfully measure σ_X's on more than 30 *para* and *meta* anilines (see Table 1), allowing rapid yet accurate assessment of σ's for any substituent. This is particularly useful for values not reported in the literature (custom-made groups and complicated substituents incompatible with previous methods of evaluation, *etc.*).

Table 1 Values of Hammett σ parameters determined for *meta* and *para* substituents on anilines using the HT MS approach

Substituent	σ_p^- lit.[14]	σ_p^- exp.[15]	σ_m lit.[14]	σ_m exp.[15]
Me	−0.17	−0.17 ±0.01	−0.07	−0.04 ± 0.01
^{t}Bu	−0.13	−0.13 ±0.02	−0.10	−0.12 ± 0.02
Cl	0.19	0.19 ±0.01	0.37	0.36 ± 0.01
I	0.27	0.27 ±0.01	0.35	0.31 ± 0.01
CF_3	0.65	0.64 ±0.01	0.43	0.40 ± 0.01
OH	−0.37	−0.37 ±0.01	0.12	0.05 ± 0.01
OMe	−0.26	−0.27 ±0.01	0.12	0.07 ± 0.02
SMe	0.15	0.12 ±0.01	0.15	0.12 ± 0.01
NMe_2	−0.16	−0.13 ±0.01	−0.16	−0.13 ± 0.01



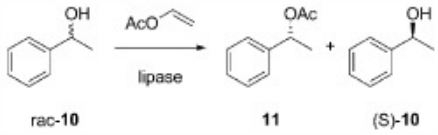
Scheme 4 Solid phase Ugi-4CC using the "analytical construct" resin **4** as the amine component.

2 Image based methods for HT reaction kinetics and thermodynamic assessment

(a) Thermographic imaging

Davies *et al.* reported a general method to allow HT kinetic measurements of chemical reactions to be carried out by monitoring the change in temperature of the reaction mixture by thermographic imaging of microtitre plates.[16] Monitoring the temperature changes in the reaction mixtures permitted thermodynamic data to be obtained, in an effort to evaluate reaction enthalpies. The success of the study was relative since the development of the method required tedious modelling steps and the enthalpies obtained were much less than values obtained *via* reaction calorimetry. In the case of a calorimeter, heat losses to the environment are minimised whereas performing the experiment in an open microtitre plate leads to large and uncontrolled heat losses; however, this work made it possible to afford a correct ranking of the enthalpies in a HT manner and represents an important preliminary piece of work towards HT reaction enthalpy determination.

In the same way infrared (IR) thermography was successfully used as a HT screening method for looking at enantioselective reactions involving biocatalysts or chiral transition metal catalysts.[17] The work was based on the use of a modified microtitre plate where reactions, such as the lipase catalysed enantioselective acylation of 1-phenylethanol (Scheme 5) were carried out and temperature changes in the reaction wells monitored periodically thanks to the presence of an IR camera. Enantioselectivity was screened by performing the reaction in three separate wells respectively containing *rac*-**10**, (*S*)-**10** and (*R*)-**10** and comparing the temperature changes in the three wells, affording qualitative selectivity data. Again, this work is a very important step towards HT assessment of *ee*'s using IR thermography.



Scheme 5 Enzymatic resolution of *rac*-**10**.

On bead IR thermographic imaging was used by Taylor and Morken for the discovery of new polymer bound multifunctional catalysts for acyl transfer reactions from acetic anhydride to ethanol.[18] After mixing the reagents and the catalysts, the hot beads were picked and decoded (Fig. 3).

Among the 3150 catalysts that were screened, 23 beads were selected and the activity of the catalysts was investigated. The results obtained showed that, to a rough approximation, the HT assay was representative of the catalytic efficiency, and that further development of the technique might allow catalytic reaction kinetics to be followed on bead. However there are important issues with these experiments such as rates of energy transfer and the need to have beads that float or reagents that are IR transparent. As an alternative to IR thermography for catalytic activity screening, Connolly and Sutherland used an array of thermistors that allowed the monitoring of temperature changes of chemical and biochemical reactions by direct immersion in the reaction mixture.[19] Thanks to the 96-well plate format, the technique is readily adaptable to problems requiring HT thermal analysis, allowing increased accuracy, as well as measurements to be carried out through non IR transparent materials. The method could also be further developed to monitor other physical parameters by replacing the array of thermistors by an array of miniaturized probes, such as for pH, *etc.*

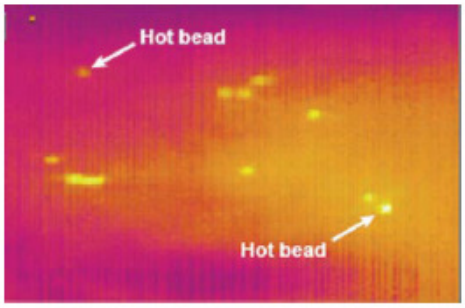


Fig. 3 IR thermographic image of 14 visible hot beads in the presence of several thousand noncatalyst beads (reproduced with permission).

(b) Thin layer chromatography (TLC) based analysis

In the key area of the discovery of new catalysts where the development of HT techniques is essential, colorimetric assays occupy a significant position. Quantitative TLC based assays have for example been developed by Garbacia *et al.* to perform HT screening of the composition of reaction mixtures.[20] By image analysis (with calibration), they were able to deduce the degree of reaction conversion from the intensity of the product spot, under 12 different reaction conditions (11 different metals or no metal). The technique not only enabled monitoring of conversion but also the qualitative assessment of the reaction mixture, making it possible to identify the existence of unexpected products, or reaction intermediates (see Fig. 4). The discovery of a new ruthenium-based catalyst for the Sonogashira coupling was made possible *via* this technique.

Conclusion

HT techniques have already demonstrated their power in enhancing synthetic processes, specifically in the development of new chemical entities. This naturally has led to the development of a wide portfolio of HT analytical tools and techniques many of which can be applied to studies in physical organic chemistry. HT reaction assessment has begun to be possible with the development of quantitative analytical constructs, which have been shown to be particularly powerful in allowing reaction conversion monitoring and these have now been applied in various applications, such as HT determination of enantiomeric excesses, HT monomer reactivity profiling and HT assessment of Hammett parameters. Those tools have shown much promise in terms of ease of use, versatility and throughput, and other applications of these constructs are expected in the near future. Other techniques also offer paths into the area, ranging from sophisticated thermal imaging to the use of simple, yet powerful quantitative parallel TLC analysis. However, whatever tools are applied the conclusion is clear, HT methods will fundamentally change the nature of physical organic chemistry and will offer routes to data generation,

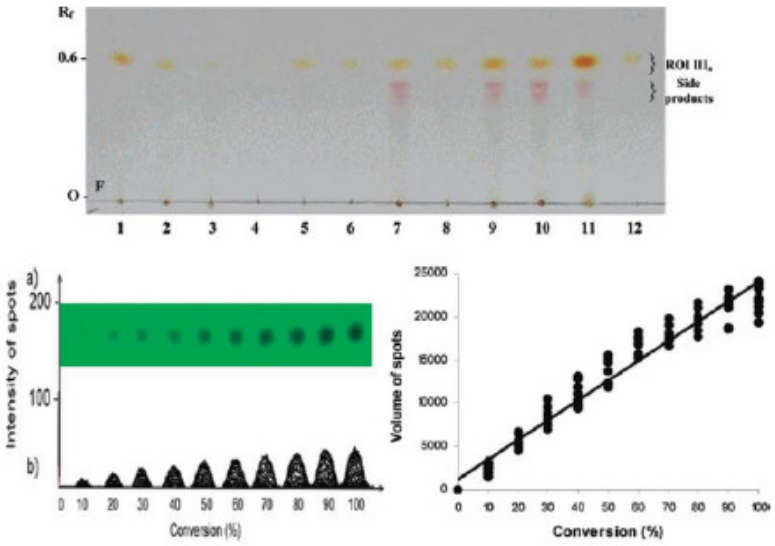


Fig. 4 Image of a TLC plate showing the composition of reaction mixtures (desired product is III_a) generated using different catalysts. Quantification was obtained by image analysis of the region of interest (ROI) and calibration (reproduced with permission).

and tools to understand reactions and mechanisms in a manner that will unshackle the field of physical organic chemistry.

Acknowledgements

We thank the Combinatorial Centre of Excellence partners.

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