Dynamic Peptide Libraries

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This thesis is dedicated to my wonderful parents, my lovely fiancé and my beautiful sister.

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Finally, I would like to thank Stephen Moggach, who is not only a loving and supporting boyfriend but also my best friend. Thank you for never letting me give up in my periods of despair, you have been so patient.

Declaration

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

Abstract

The protease thermolysin has been used directly to synthesise dipeptides from Fmoc-protected amino acids on a $PEGA_{1900}$ solid support. The thermolysin-catalysed solid phase synthesis of longer peptides is reported. Fmoc-protected peptides as long as hexamers (poly-L-leucine) and tetramers (poly-L-phenylalanine and poly-L-tyrosine) were enzymatically synthesised. In each case enzymatic synthesis of peptides resulted in the formation of a library of peptides varying in length, the formation of which was caused by "scrambling" by the enzyme. Due to the reversible nature of the enzyme catalysed peptide bonds, it was believed that the aforementioned solid phase peptide libraries may be exploited to generate dynamic peptide libraries.

From the Juliá-Colonna asymmetric epoxidation it is know that chalcone undergoes non-covalent interactions with the amino terminus of poly-L-leucine. Chalcone was therefore employed as a suitable template for the $PEGA_{1900}$ immobilised poly-L-leucine libraries. However, it was found that the Fmoc-protecting group used in these libraries inhibited binding of chalcone to poly-L-leucine. As an alternative, unprotected poly-L-leucine libraries were generated in solution phase from dileucine using thermolysin. It was found that when the unprotected poly-L-leucine libraries were generated in the presence of chalcone using 90 % acetonitrile in pH 8 buffer as a solvent, that the distribution of the library shifted to favour formation of the hexamer mostly at the expense of the tetramer. Similar results were also observed for libraries generated from diphenylalanine and phenylleucine in the presence of chalcone.

The catalytic activity of poly-L-leucine library members and poly-L-phenylalanine library members in the Juliá-Colonna epoxidation was investigated. These investigations demonstrated that the peptide amplified by chalcone in the dynamic combinatorial libraries displayed an improved catalytic activity in comparison to other library members. This indicates that dynamic peptide libraries may be exploited as a tool for identifying potential catalysts for the Juliá-Colonna asymmetric epoxidation.

Abbreviations

Ac	acetyl
ACN	acetonitrile
Ala	alanine
BPy	4,4'-bipyridine
bs	broad singlet
CA	carbonic anhydrase
CC	combinatorial chemistry
d	doublet
DABCO	diazabicyclo[2.2.2]octane
Dns	diaminonaphthalenesulphonyl
DBU	1,8-diazobicyclo[5.4.0]undec-7-ene
DCC	dynamic combinatorial chemistry
DCL	dynamic combinatorial library
DCM	dichloromethane
DIC	diisopropylcarbodiimide
DIPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DME	1,2-dimethoxyethane
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulphoxide
EDCI	1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
ES	electrospray
ESI	electrospray ionisation
FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
FmocAA	Fmoc protected amino acid
FTICR	fourier transform ion cyclotron resonance

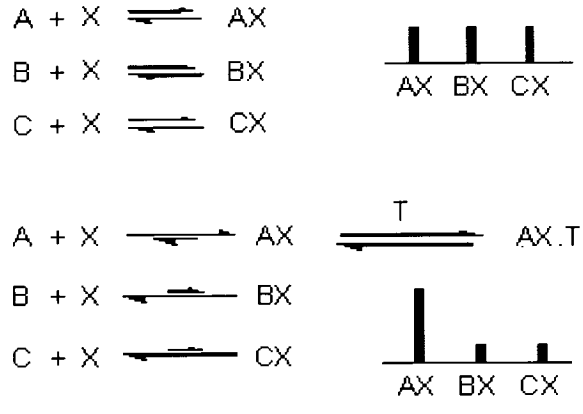
Gly	glycine
HATU	*N,N,N',N'*-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate
HMPA	4-hydroxymethylphenoxyacetic acid
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
IR	infra-red
LC-MS	liquid chromatography – mass spectrometry
Leu	leucine
Lys	lysine
m	multiplet
mp	melting point
MS	mass spectrometry
MTT	methyltrityl
NA	neuraminidase
NANA	*N*-acetylneuramic acid aldolase
NMR	nuclear magnetic resonance
PABA	*para* amino benzoic acid
PEGA	polyethylene glycol functionalised polyacrylamide
PS	poly styrene
q	quartet
quint	quintet
Rt	retention time
RT	room temperature
RP	reversed phase
RPPC	relative percentage product composition
s	singlet
t	triplet
TBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium

	tetrafluoroborate
TFA	trifluoroacetic acid
TFE	trifluoroethanol
THF	tetrahydrofuran
TIC	total ion count
TIS	triisopropylsilyl
TLC	thin layer chromatography
TMS	trimethylsilyl
TSA	transition state analogue
Tyr	tyrosine
UV	ultra violet
WGA	wheat germ aldolase

Contents

1. Introduction

The concept of combinatorial chemistry (CC) was established in the early 1980s [1]. CC involves the generation of compounds from structurally similar building blocks followed by screening for activity against target molecules. To date combinatorial chemistry has grown from conception to a major tool in molecular recognition, finding application from drug to catalyst discovery [2-4]. Traditionally synthesis and screening of compounds has been carried out in separate steps. However, during the mid 1990s the concept of Dynamic Combinatorial Chemistry (DCC) was proposed independently by the groups of Sanders [5]and Huc and Lehn [6]. In contrast to combinatorial chemistry, the bonds linking the building blocks in DCC are reversibly formed. In theory, this results in products that are formed from all possible combinations of building blocks giving rise to a library of interconverting members termed a Dynamic Combinatorial Library (DCL). DCLs are under thermodynamic control, therefore stable library members display larger concentrations in comparison to members of low stability. Introduction of a template into such a library can result in a molecular recognition event with a library member. This can lead to the stabilisation of that member which subsequently induces a shift in equilibrium to favour the "active member" at the expense of the "inactive members" (Scheme 1).



Scheme 1 The DCC concept: reversible reactions performed with a limiting amount of **X** generate a mixture of **AX**, **BX** and **CX**. The binding of **AX** to template **T** causes perturbation of the equilibrium involving **A** and **X** to give the overall amplification of **AX** at the expense of the other library members [7].

Templating of DCLs therefore allows the synthesis and screening of a library of compounds in a "one pot" reaction where the active library member is identified by amplification.

The field of DCC has been extensively [8-15] reviewed during which time Huc and Lehn have divided DCLs and templating into a number of subclasses.

1.1 Templating

Huc and Lehn [6] proposed that templating fell into two categories: casting and moulding (Figure 1). Casting comprises of a host, such as an enzyme, protein or macromolecule, which acts as a template. The host induces the self-assembly of a guest substrate from a library of interconverting fragments. Moulding comprises of a guest substrate such as a cation or small molecule as a template. In this case, the guest induces the self assembly of the host from a library of interconverting fragments. Indeed, these two approaches to templating represent the main difference in the pioneering work carried out by Sanders and Huc and Lehn.

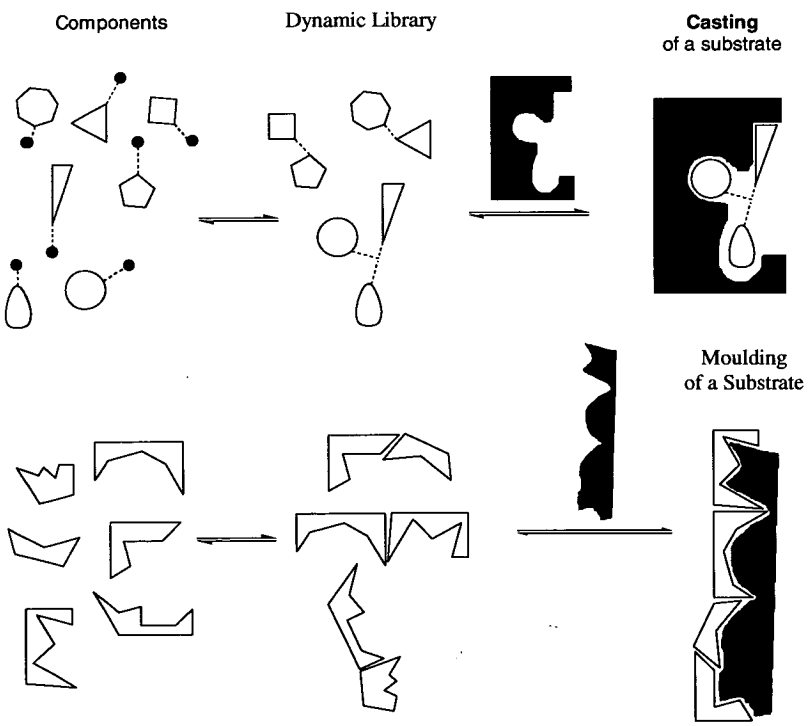
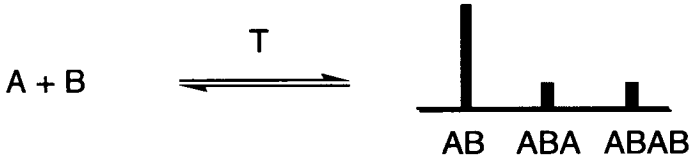


Figure 1 Diagrammatic representation of casting and moulding

1.2 Classes of Dynamic Combinatorial Libraries

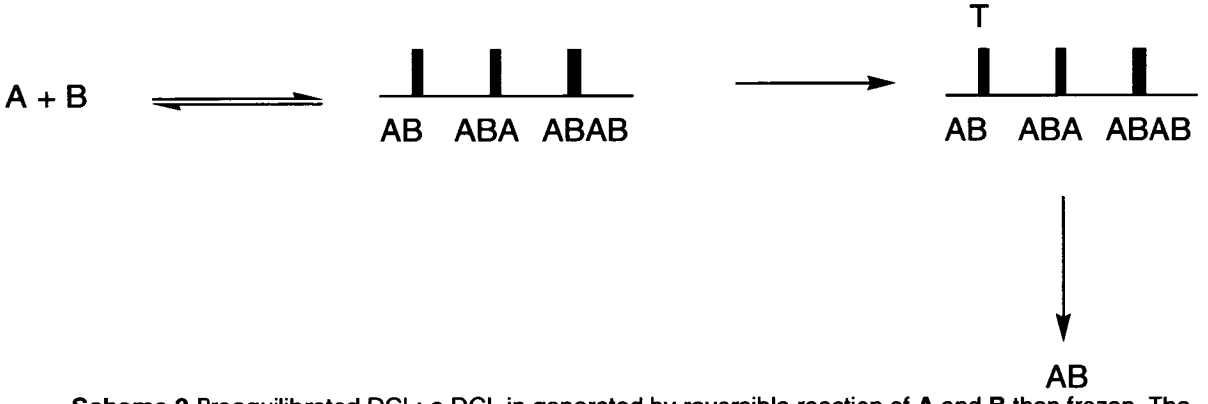
In a later review, Ramström and Lehn reported three different approaches to the generation and screening of DCLs [9]. Each approach shares a reversible linking reaction for generation but differ in screening method.

The first and most common of these three approaches was termed "Adaptive DCLs". Adaptive DCLs represent DCC in its purist form in which the libraries are generated in the presence of template. As described earlier, the best binders are identified by amplification (Scheme 2).



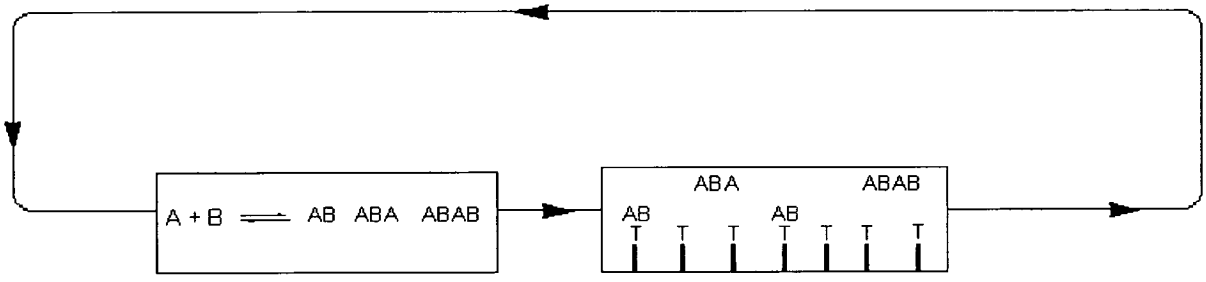
Scheme 2 Adaptive DCL: a DCL generated by reversibly reacting **A** and **B** in the presence of template **T** to give a library where the best binder **AB** is amplified at the expense of other members.

The second approach is a mixture of DCC and CC methods termed "Preequilibrated DCLs". This involves the generation of a library of compounds through reversible bonds (Scheme 3). The library is then "frozen" and screening is performed under a different set of static conditions. In this case no amplification is observed and therefore screening and deconvolution is carried out as for traditional CC.



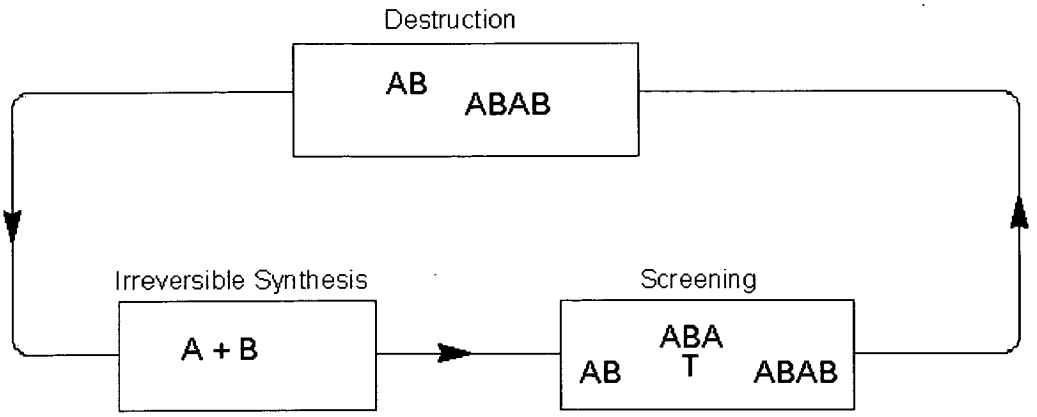
Scheme 3 Preequilibrated DCL: a DCL in generated by reversible reaction of **A** and **B** then frozen. The frozen library is then screened for activity against target **T**.

The third approach was termed "Iterative DCLs" [16]. This is where a DCL is generated in one compartment then allowed to interact with target separately or in the same reaction chamber (Scheme 4). Target species are immobilised so that the best binders are retained as complexes and separated from the poor binders. The inactive members are then transferred back to the reaction chamber where they undergo rereaction. Several cycles of reaction and screening result in the accumulation of the best binders.



Scheme 4 Iterative DCL: a DCL in generated by reversible reaction of **A** and **B** then allowed to interact with immobilised target **T**. The poorest binders may undergo rereaction.

A further fourth approach has been since developed by Corbett *et al* [17, 18] termed Pseudodynamic Combinatorial Chemistry. This approach differs from those previously described as it comprises of the irreversible synthesis and destruction of library members in the presence of a target. Compounds are irreversibly synthesised then allowed to interact with the target (Scheme 5). The best binders form a complex with the target. A destruction reaction rapidly cleaves the unbound compounds and slowly cleaves weakly bound compounds. Compounds that are strongly bound to the target are cleaved very slowly. Some of the building blocks released in the destruction undergo further cycles of synthesis, screening and destruction. Several cycles results in the accumulation of the best binders at the expense of the worst binders.



Scheme 5 Pseudodynamic Combinatorial Library: a static library is generated through reversible reaction of **A** and **B**. The library is then screened for activity against target **T**. The poorest binders are hydrolysed to original building blocks and may undergo further synthesis, screening and destruction cycles.

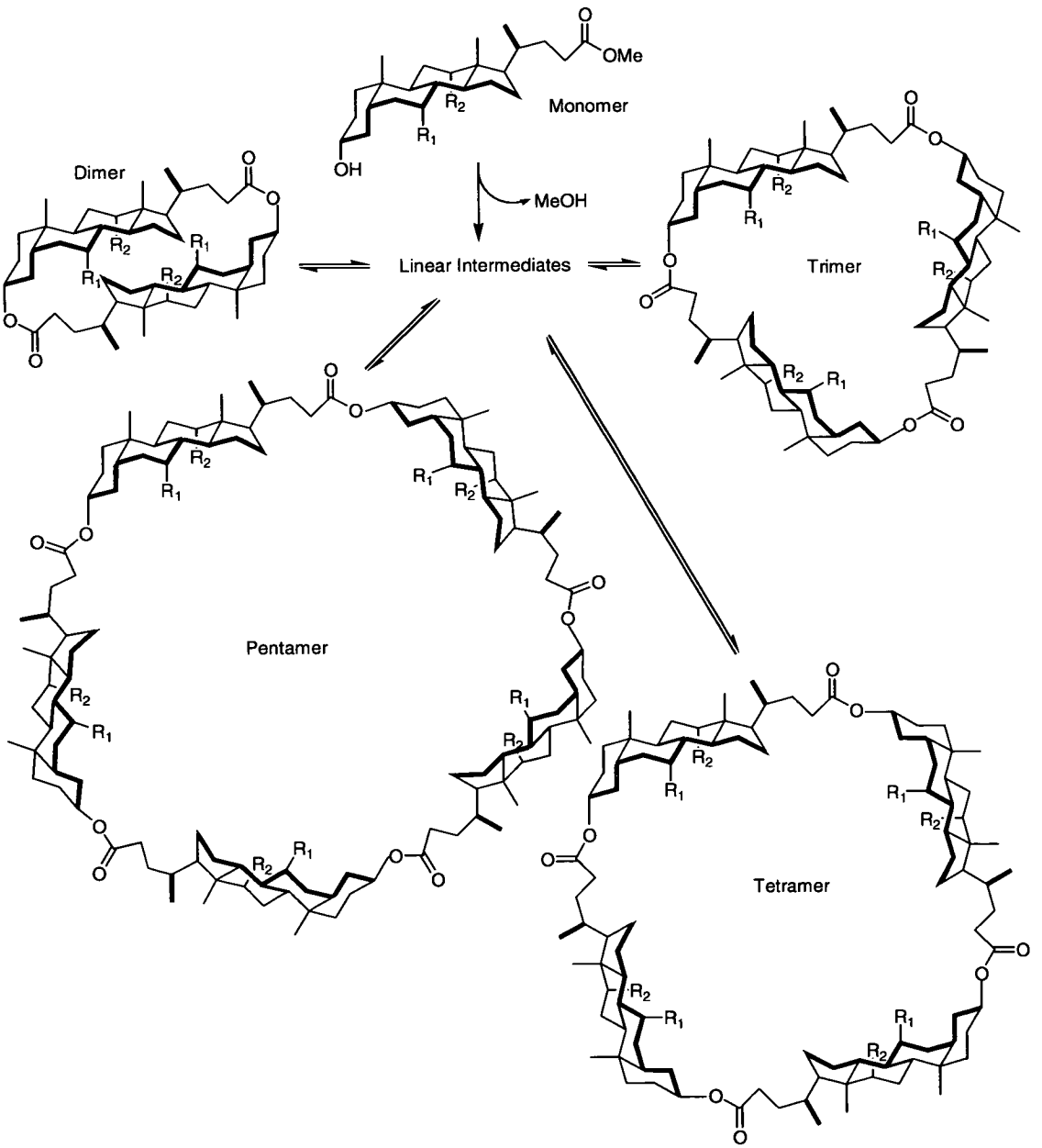
1.3 Reversible Reactions

To date there have been many literature reports of the generation of DCLs. Initially, research was focused on expanding the range of reactions, however, recent studies have shown a greater focus on influencing the distribution of libraries with a template. As templating of DCLs has become more commonplace a number of key requirements have been identified for the reversible reaction [13]. Firstly, the rate of exchange must be rapid enough to ensure the proof-reading of all possible structures on a suitable time scale. Secondly the conditions required for exchange must be tolerant to a wide range of functional groups and be compatible with those required for molecular recognition events. Once the library has been generated in the presence of a template it must be possible to "switch off" or "freeze" interconversion. If this is not done the removal of the template will result in the redistribution of the DCL to its original values. Freezing may be facilitated by adjustment of the pH, removal of reagents or by covalent modification of library members. Covalent trapping of library members may, however, result in a reduced binding activity. To date the diversity of reversible reactions employed for the construction of DCLs is narrow. The majority of exchange processes employed so far have been reversible covalent reactions. Such linking reactions include hydrazone exchange, imine exchange, reversible biocatalysis and disulfide exchange. Olefin metathesis has also shown potential for the generation of DCLs but reports are

limited [19, 20]. More recent reports include reversible Diels-Alder reactions [21], the reversible Michael addition of thiols [22] and thioester exchange [23, 24].

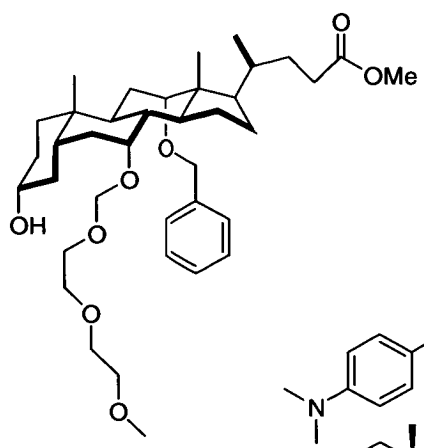
1.3.1 Ester Exchange

The pioneering work carried out by Sanders and co-workers was based on trans-esterfication as a reversible reaction [5]. Sanders and co-workers demonstrated that a library of cyclic oligocholates could be generated by the "living" macrolactonisation of a building block derived from cholic acid (Scheme 6).



Scheme 6 An equilibrating mixture of cyclic steroid oligomers exchanging through esterification

Building blocks were functionalised with an alcohol and methyl ester. Exchange was affected under harsh conditions by catalysis with potassium methoxide in the presence of dicyclohexyl-18-crown-6 in toluene under reflux. A library of cyclic oligocholates varying from trimers to pentamers was generated. Control experiments confirmed the chemistry to be reversible and under thermodynamic control [25]. The authors were also able to demonstrate that the chemistry was general and a wide range of dynamic molecular scaffolds could be synthesised. Such scaffolds include those based on xanthen [26], cinchona [27] and ephedra [28]. However the harsh conditions required for transesterification precluded the use of a wide range of functional groups and therefore limited molecular recognition. Due to this, very few examples of templated macrolactone based libraries have been reported. However, Brady and Sanders [29] observed that generation of DCLs from building block **1** in the presence of alkali sodium ions resulted in an increase in larger macrocyles. Lukeman and Sanders [30] made a later attempt at templating of a macrolactone library incorporating pyridine and dimethylalanine monomers **2** and **3**. Pyridyl units were incorporated to exploit the use of coordination chemistry as a tool for templating. Despite the successful formation of the desired library, templating was ineffective. Exposure of the library to Pt(II) and Zn(II) porphyrins resulted in an inhibition of library formation. As a consequence no macrolactones were formed and only reduction by-products of the template were observed. As a result of these studies milder conditions for library generation were sought.



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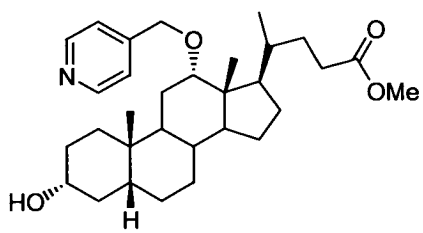

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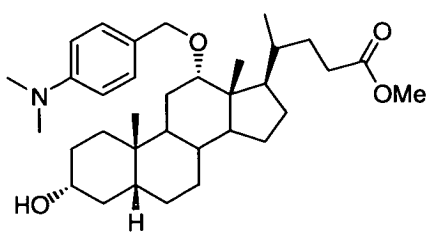

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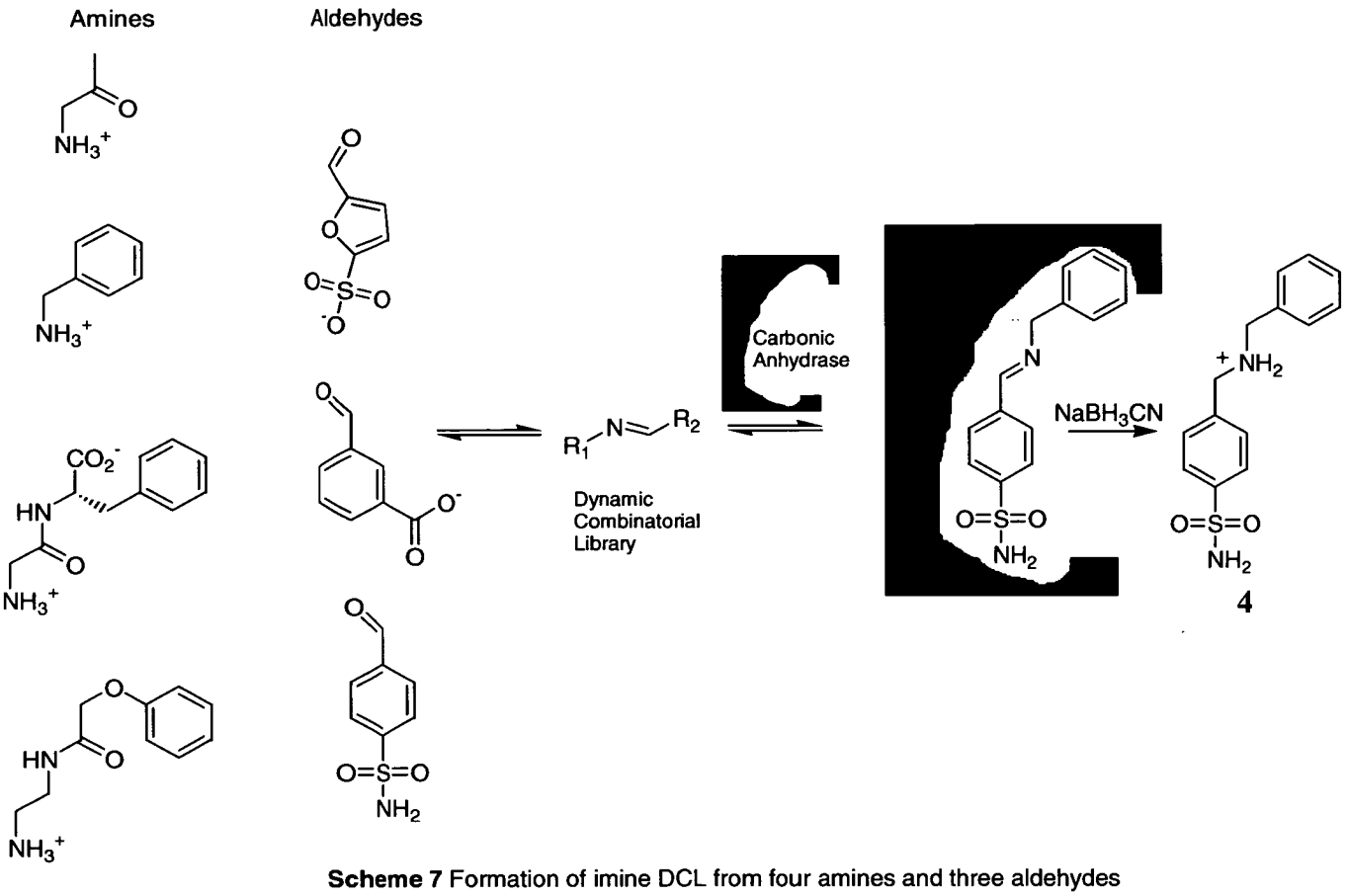
Kaiser and Sanders [31] developed milder conditions for the generation of ester-based libraries by employing palladium-catalysed allyl transesterification. The authors were able to apply the Pd(0)-catalysed exchange to the synthesis of a cyclic zinc porphyrin dimer in the presence of a bidentate pyridyl ligand template. This offered a milder alternative to base catalysis.

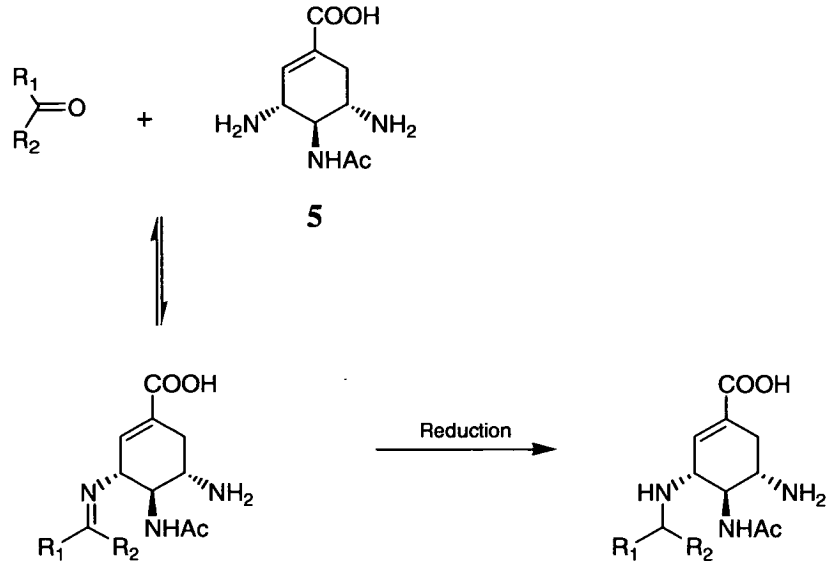
1.3.2 Exchange Over the C=N Double Bond

Exchange over the labile C=N double bond has been the subject of work by several groups. Imine exchange was the first reversible reaction to be explored by Huc and Lehn [6]. This pioneering paper described a "casting process" based on the recognition assembly of inhibitors of carbonic anhydrase (CA). A library of 12 imines was prepared from a library of 7 parent amines and aldehydes in the presence of CA (Scheme 7). As imines are sensitive to hydrolysis and exchange freely at room temperature, often without the need for catalysis, the reaction required freezing by covalent modification. Such freezing also facilitated the isolation and analysis of the final equilibrated mixture. The library was frozen by reduction of the imines to amines with $NaBH_3CN$. A two fold increase in **4** was observed in the presence of CA in comparison to the control experiment. A second experiment in which the library was generated in the presence of CA and inhibitor 4-sulfamoylbenzoate, showed a drastically diminished effect on the library distribution. This suggests that the binding site was responsible for ligand amplification.



Scheme 7 Formation of imine DCL from four amines and three aldehydes

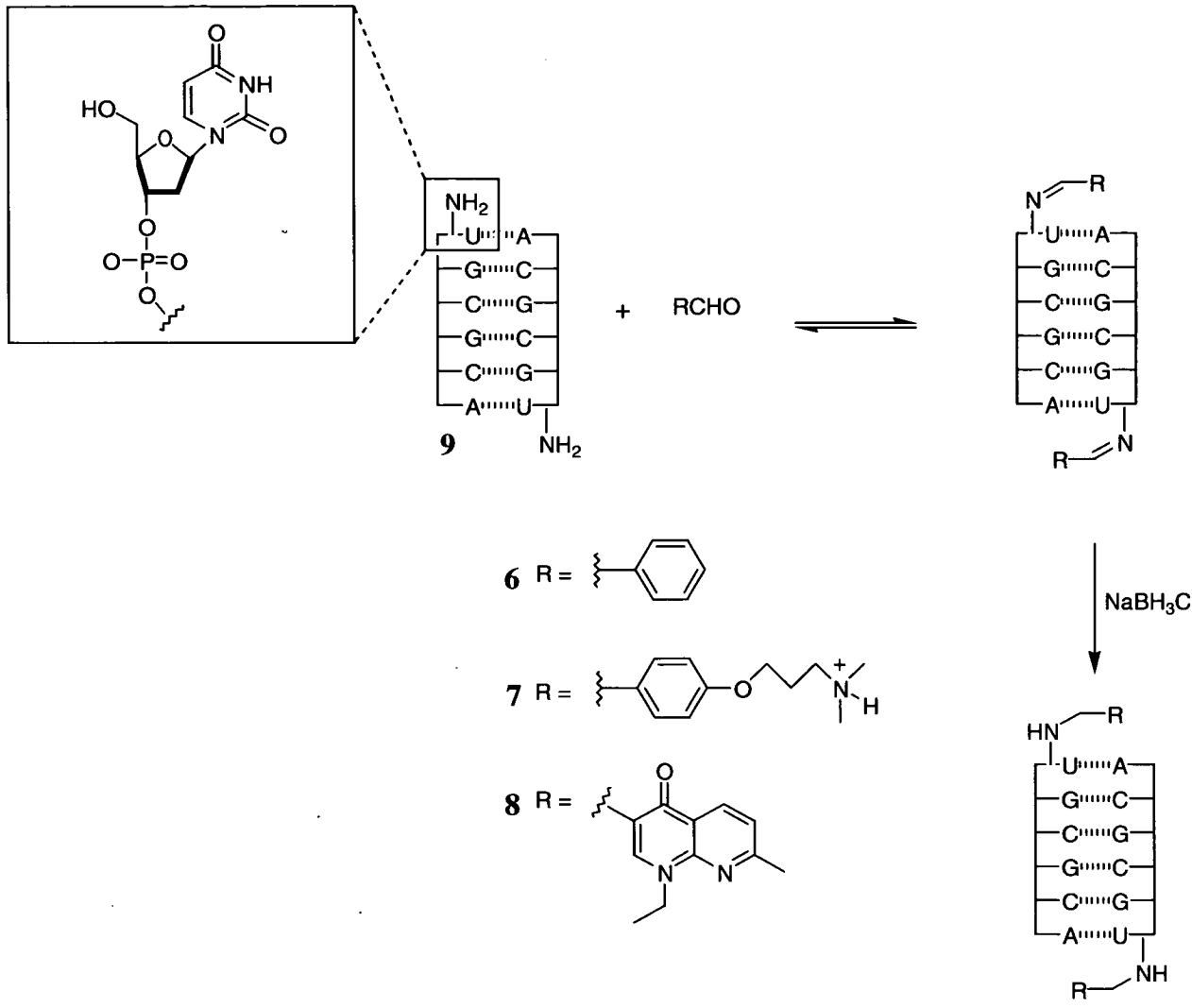
A more recent example of a casting process used to identify potent enzyme inhibitors has been described by Eliseev and co-workers [32, 33]. The authors employed neuraminidase (NA), a key enzyme in the influenza virus, as a molecular trap for a library of imines. Imine libraries were generated from a molecular scaffold, diamine **5**, and a mixture of ketones then subsequently reduced to amines (Scheme 8).



Scheme 8 Formation of imine DCL from diamine and ketones

A strategy was followed where little or no components of the reduced library could be detected in the absence of target. Library analysis was subsequently simplified as target induced hits were clearly observed. Despite the success in identifying ligands using this process, it was found that the inhibitory properties of the stabilised library did not reflect those of the transient components.

Bugaut *et al* described an alternative application of imine based DCLs. The authors demonstrated the simultaneous identification of the type and location of an appended residue capable of stabilising oligonucleotide complexes [34, 35]. A library of 15 mono- and bi-conjugated oligonucleotides was generated from a pool of 3 residues in the form of aldehydes **6** – **8** and an oligonucleotide bearing two reactive amino groups **9** (Scheme 9). Addition of a complementary oligonucleotide allowed the amplification and identification of the most stable appended residue.

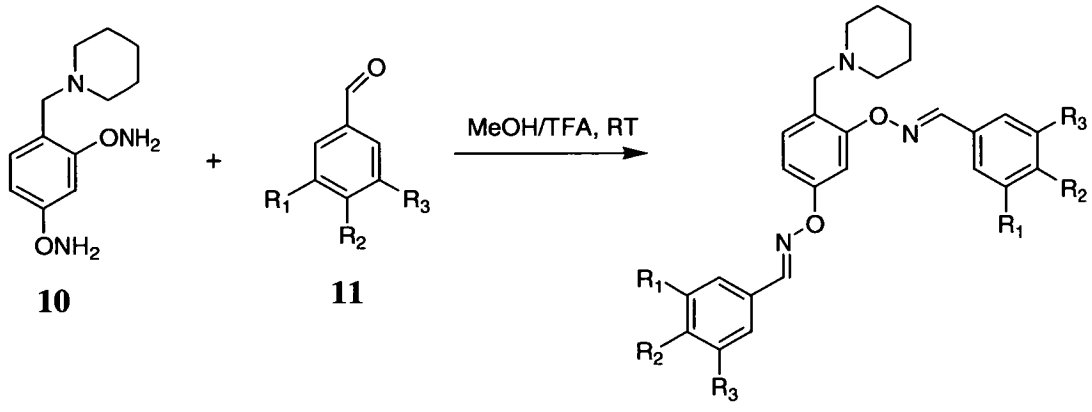


Scheme 9 Formation of an imine DCL that simultaneously identifies the type and location of a stabilising residue

Overall, imine exchange has been well investigated and applied to a reasonably wide range of systems. However, reduction of imines to kinetically stable amines changes both geometry and electronics which has been found affect the binding properties of the target compound in some systems [32, 33]. As a consequence stabilised imines in the form of oximes and hydrazones have gained an increased interest.

Oximes and hydrazones have the advantage of being hydrolytically stable thus eliminating the need for a reduction step. Eliseev and co-workers have investigated *O*-aryl and *O*-alkyl oximes for the generation of a DCL [36]. Initial studies by the author involved the generation of a library of oxime ethers from aromatic scaffold **10**. Libraries

were formed by reaction of the two aromatic aminooxy groups of **10** with eleven aromatic aldehydes of general structure **11** (Scheme 10).

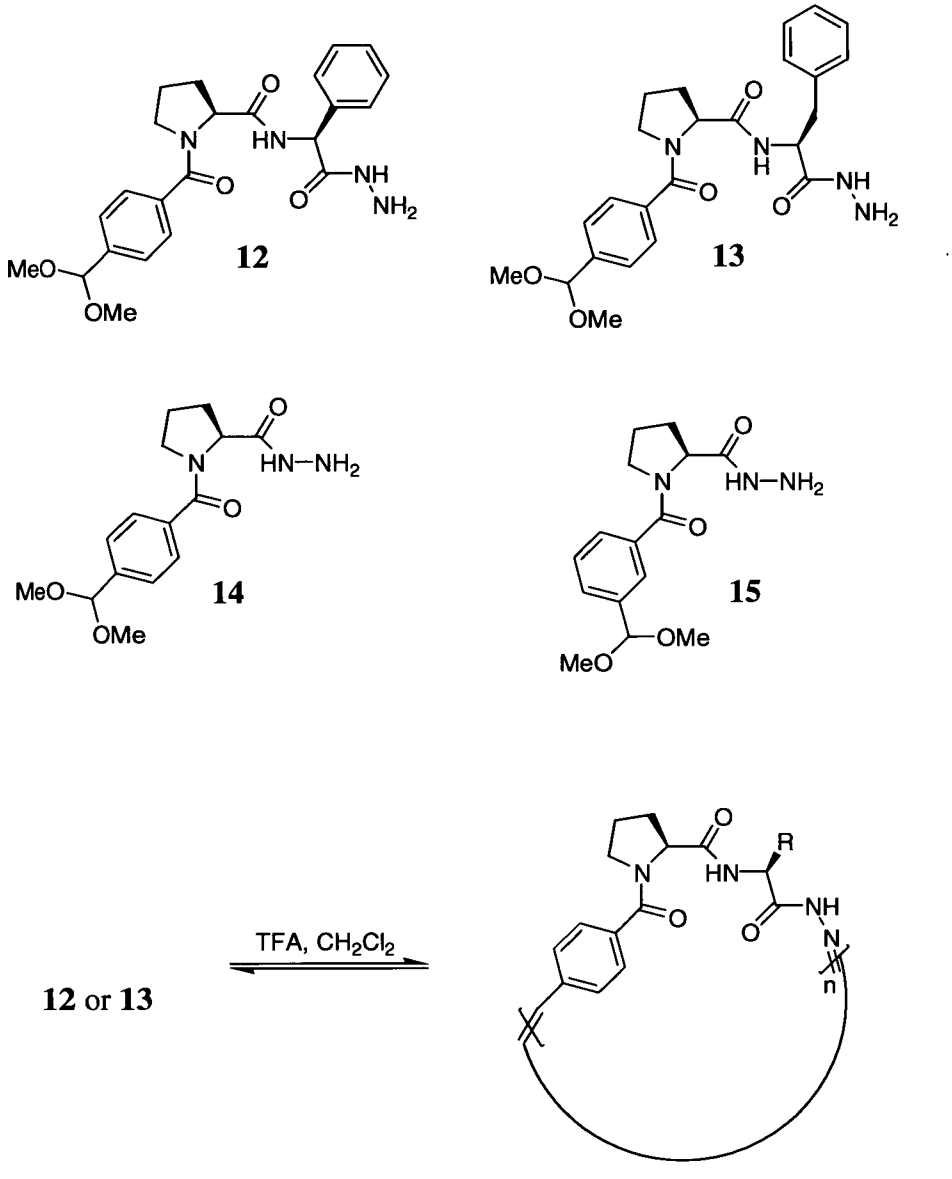


Scheme 10 Formation of an oxime-based DCL

Reaction of all 55 pair wise combinations of aromatic aldehydes in separate wells demonstrated the diversity of the reaction as all the expected products were formed. A library using all the building blocks was not attempted as similarities in mass did not allow for unambiguous characterisation of regioisomers. This problem was later addressed by the authors who employed a combined synthetic/analytical approach involving the synthesis of isotopically labelled aminooxy scaffolds [37]. Further to this compounds were also analysed by tandem mass spectrometry, in some cases, omitting the need for tagging [38]. An extensive kinetic study carried out by the authors demonstrated that exchange may be carried out in water at elevated temperature of 60 °C [39]. It was also shown that exchange was slow or negligible under physiological conditions, thus such conditions could be used for freezing the reaction. However, to date, no examples of templated oxime libraries have been published.

In contrast hydrazone exchange has grown in popularity to become one of the most applied linking reactions in DCC. The majority of these applications have been to the generation of pseudo-peptide macrocycles by Sanders and co-workers. Hydrazone exchange has been shown to proceed under mildly acidic conditions where exchange is frozen by removal of the acid [40]. Libraries of a wide diversity have been generated from amino acid derived building blocks **12** – **15**. Each building block comprised of an

amino acid core incorporating a protected aldehyde moiety and a hydrazide functionality. Treatment of **12 – 15** in DCM with TFA resulted in both the deprotection of the dimethoxy acetal and the subsequent hydrazone exchange.

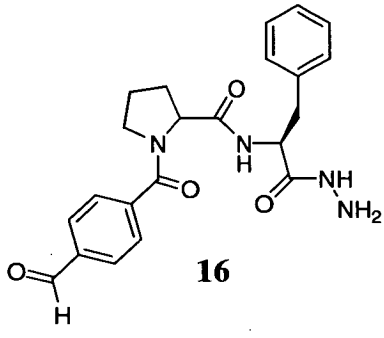


Scheme 11 Formation of a hydrazone based DCL

Treatment of individual monomers **12** and **13** with TFA yielded a library containing a wide range of cyclic species varying from a cyclic monomer to a cyclic 13-mer (Scheme 11). Even wider diversity was achieved from mixtures of monomers. A combination of just two amino acid building blocks resulted in the formation of over 130 cyclic species detectible by mass spectrometry. The reversibility of the reaction has been demonstrated

by mixing libraries generated from individual monomers to give a DCL of mixed cyclic species.

Templating of hydrazone DCLs has proven to be very successful. Indeed, some shifts in product distribution have been the largest reported. The first example was reported by Furlan *et al* [41]. A library of 10 macrocycles comprising of cyclic dimers to cyclic undecamers was generated from building block **13**. The distribution of the library was influenced by [18]crown-6. Generation of the library in the presence of [18]crown-6 resulted in a decrease in the concentration of larger macrocyles. HPLC analysis revealed that **16** had been amplified. **16**, which was previously undetectable in the library, was found to constitute 67 % of the final mixture. However isolation of **16** was not possible as it reacted with itself to form hydrazones.



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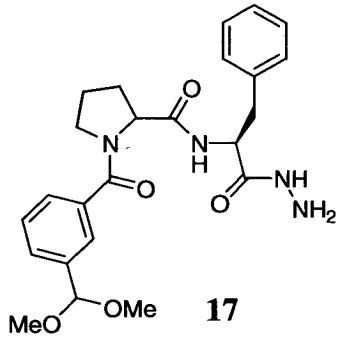

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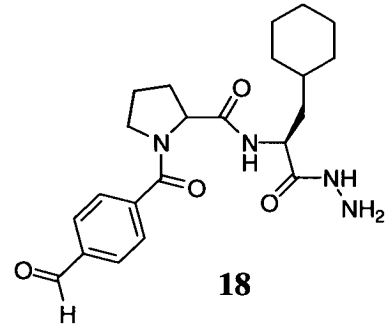

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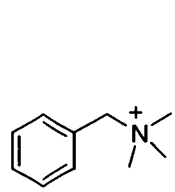
The distribution of the same library was also influenced with NaI and KI which amplified of the cyclic trimer [42]. More recent work has also demonstrated the metal induced amplification of macrocyclic libraries generated from **17** and **18** [43]. Amplified receptors constituted 95 % and above for the final mixtures. Receptors could be subsequently isolated by semi-preparative HPLC. Further examples include templating of libraries generated from building block **15** with trimethylammonium salts **19 – 22** [44, 45].



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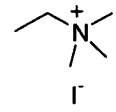

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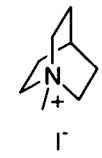

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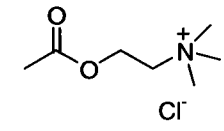

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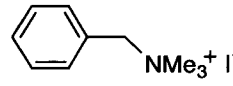
Shifts towards the cyclic trimer were observed at the expense of cyclic dimer. The final concentration of the cyclic trimer in the mixture varied from 10 – 90 % depending on the template used. Such large shifts allowed ready isolation of the pure trimer by silica chromatography. The affect that **19** – **22** had on the library was found to correlate well with relative affinities to the isolated receptor. In a similar experiment a catenane was amplified and isolated in a 67 % yield by templating of a library generated from **13** with **22** [46]. Impressively the catanane was not found to be detectable in libraries generated in the absence of template.

Simultaneous selection, amplification and isolation of pseudo-peptide receptors has been achieved by employing immobilised trimethylammonium salts as templates [47]. Roberts *et al* reasoned that commercially available Amberlist-27 resin (A27) **23** could be used as a solid phase analogue of the trimethylammonium salt, benzyltrimethyl ammonium iodide **24**.



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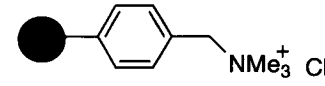

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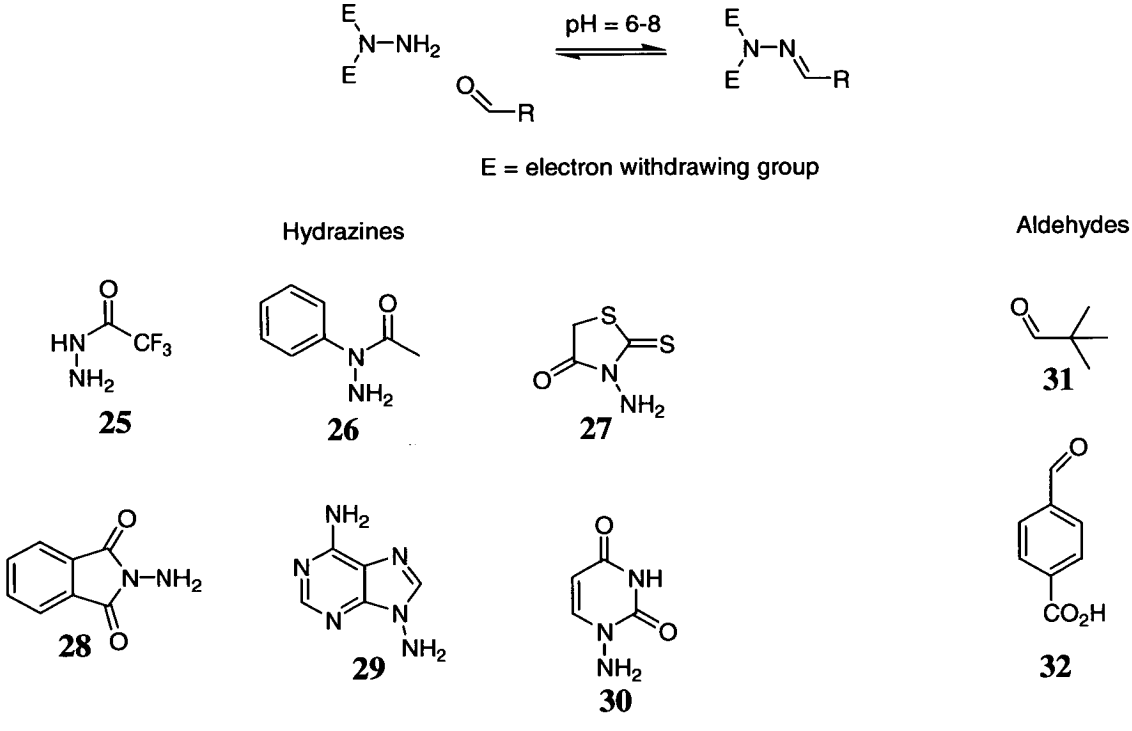
15 was cyclised in the presence of **23**. HPLC analysis showed the cyclic trimer had been selected from the library at the expense of the other members. The pure receptor was isolated in a 40 % yield by washing **23** with $CHCl_3$ to remove impurities. The trimer was subsequently eluted from **23** by washing with MeOH, a solvent known to disrupt hydrogen bonding between the template and receptor.

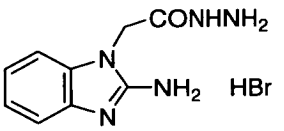
Sanders and co-workers have expanded the classes of compounds that may be generated using hydrazone exchange by generating macrocyclic libraries from steroid based hydrazone scaffolds [48]. However, no examples of templating such libraries have been reported to date. Huc and co-workers have also carried out work directed towards expanding classes of compounds generated through hydrazone exchange [49]. The authors demonstrated that hydrazones could be formed in water at neutral pH from hydrazines bearing electron withdrawing groups **25** – **30** and aromatic or aliphatic aldehydes **31** and **32** (Scheme 12). Generation of these libraries under such conditions allows the potential to template hydrazone libraries with biologically important hosts. As yet, no such templating has been described by the authors.



Scheme 12 Hydrazone DCL generated from hydrazines bearing electron withdrawing groups

Lehn and co-workers have however, described templating of acyl hydrazone DCLs with enzymes using a preequilibrated approach. Work carried out by the authors has shown that libraries of compounds may be rapidly generated in aqueous media [50, 51]. The dynamic properties are controlled under similar conditions to those reported by Sanders and co-workers. Hydrazone formation and exchange take place at low pH (3-7) and is stopped at a high pH (>7). DCLs containing 440 different constituents were generated

from 16 hydrazides and **33 – 48** 5 mono or dialdehyde building blocks **49 – 53**. In a separate step the library was screened toward inhibition of the bifunctional enzyme HPr kinase/phosphate. Testing of the complete library confirmed that it contained active species that were inhibitors of the enzyme. In order to identify the inhibitors from the pool of library members a dynamic deconvolution protocol relying on the dynamic features of the library was utilised. This method was based on the removal of single building blocks from a complete library. This results in the redistribution of the remaining components and suppression of all constituents containing the removed component from the equilibrating pool to give sub libraries. Thus, on testing of the sub libraries a decrease in the inhibitory effect reveals the importance of the removed component. Subsequently compound **32-55-32** was found to be and inhibitor of HPr kinase/phosphate.



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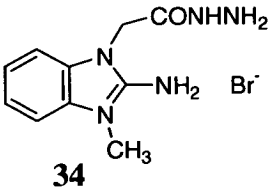

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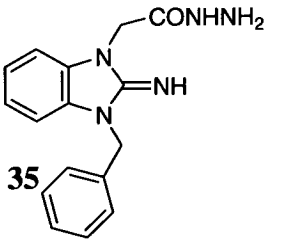

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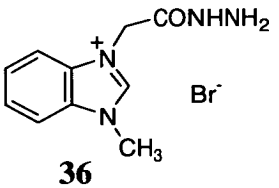

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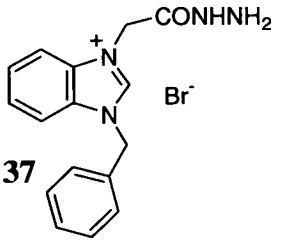

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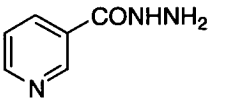

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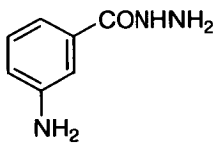

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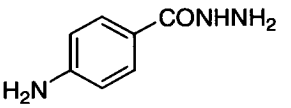

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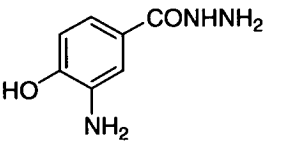

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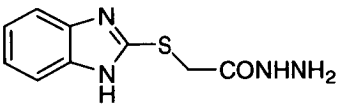

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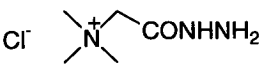

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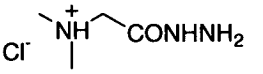

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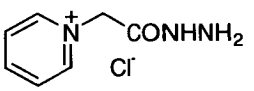

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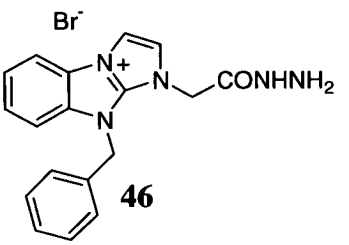

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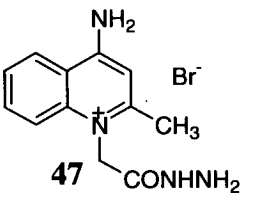

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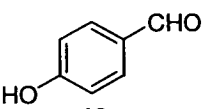

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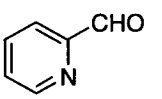

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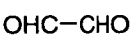

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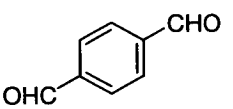

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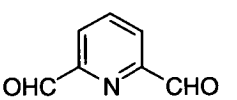

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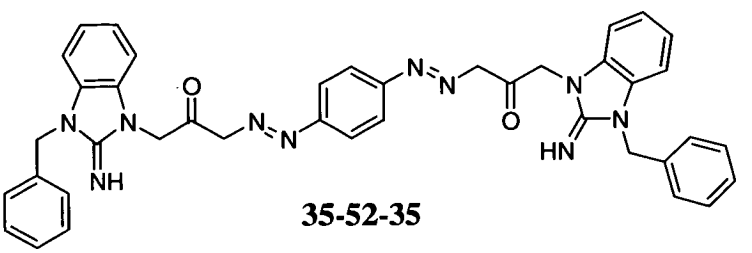
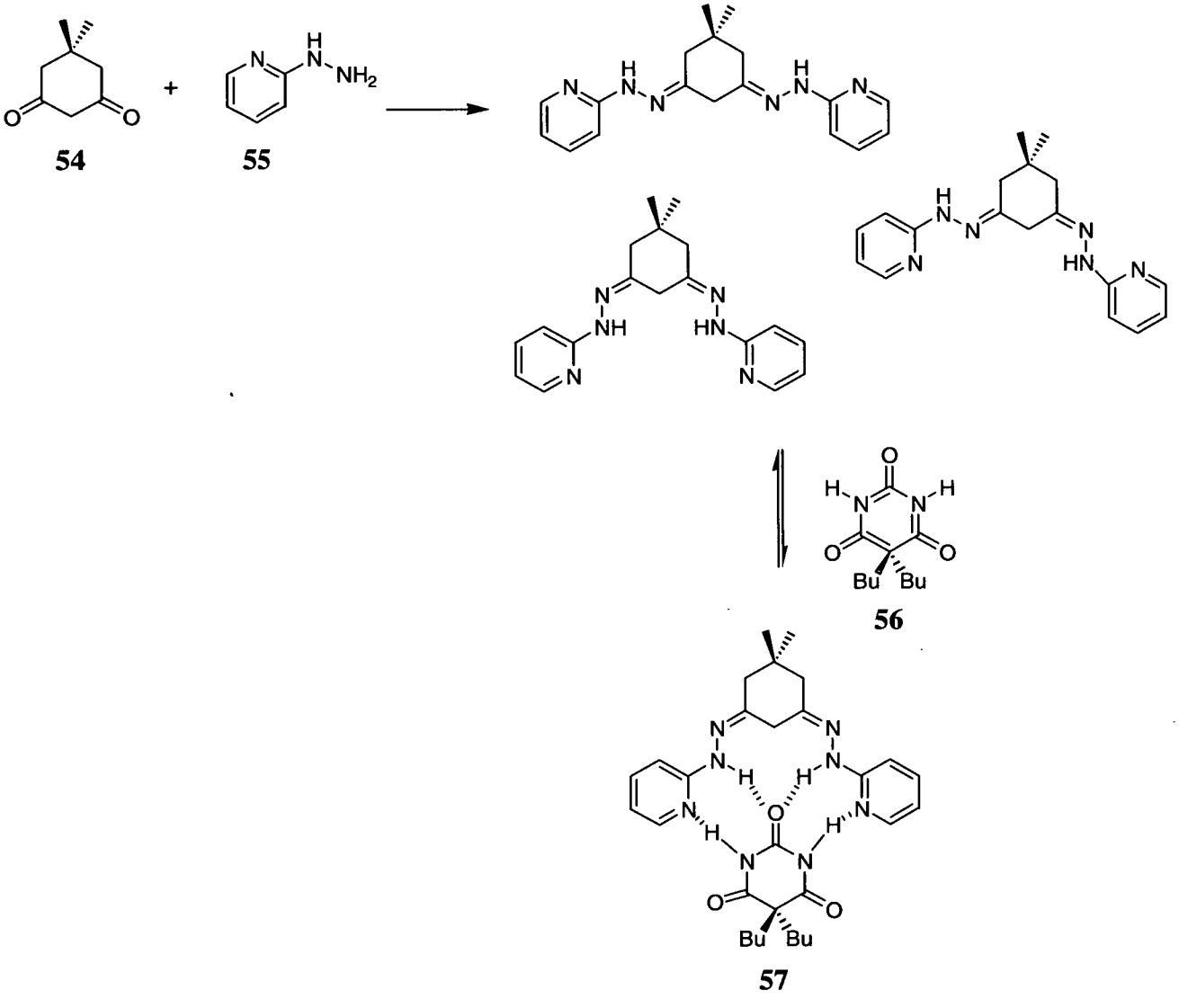


35-52-35

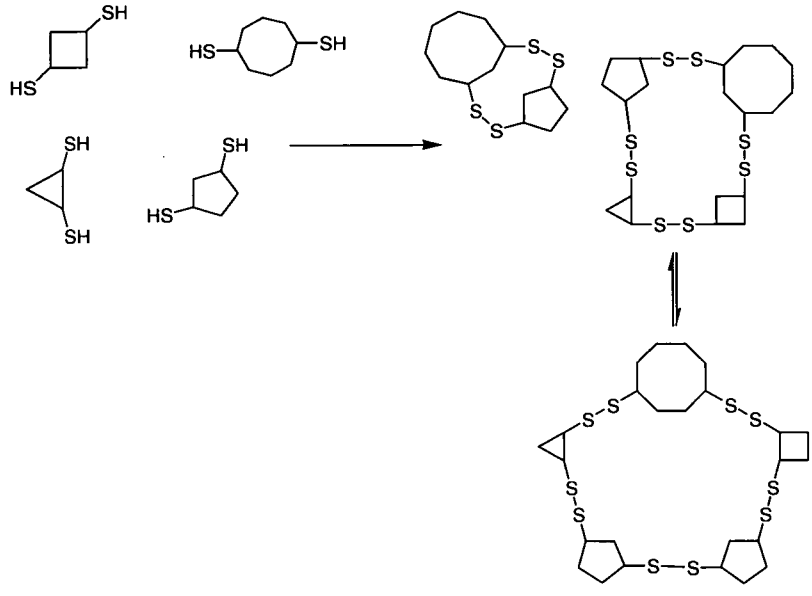
Hydrazone DCLs may also be envisaged as dynamic conformational or confirgurational libraries. Such libraries have been the subject of work by Berl *et el* [52]. The authors formed a library of dihydrazone isomers by condensation of **54** and **55** (Scheme 13). Progressive addition of dibutylbarbiturate **56** to the equilibration mixture of isomers lead to the amplification of the (Z/Z) isomer **57**.



Scheme 13 Induced fit selection from a constitutional/configurational library of hydrazones

1.3.3 Disulfide Exchange

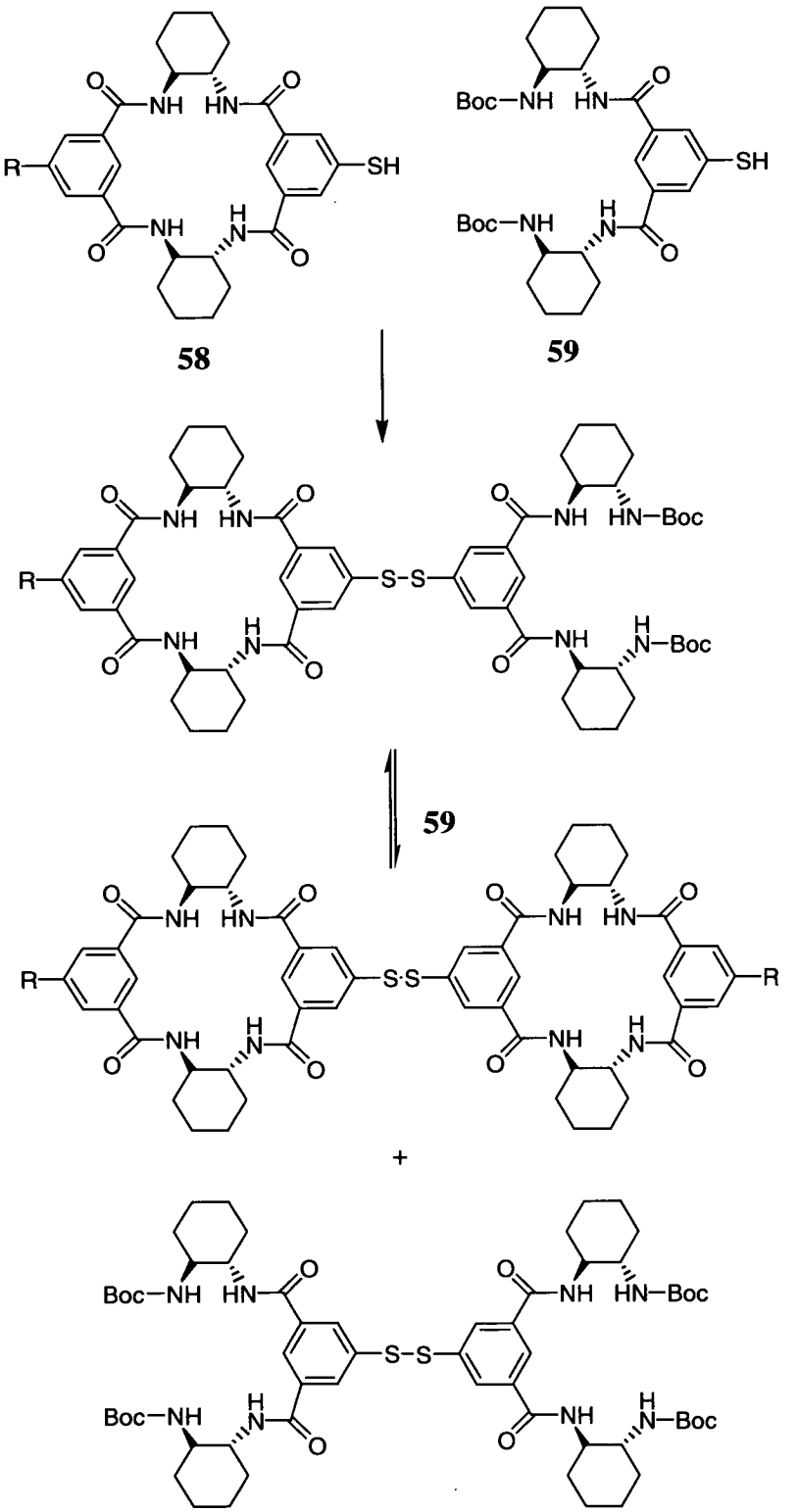
To date disulfide exchange has proven to be one of the most investigated and applied reversible reactions to the generation of DCLs. Disulfides form readily by oxidation of thiols, mediated by exposure to air or oxidising reagents. Exchange then takes place under neutral of mildly basic conditions (pH 7- 9) in the presence of catalytic thiolate (Scheme 14). Exchange is switched off by either removal of the thiolate of adjustment of the pH to acidic conditions. The resulting stable disulfides may be directly analysed by HPLC.



Scheme 14 Formation of disulfide based DLC

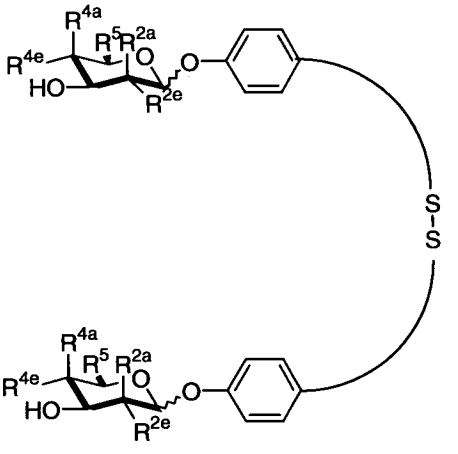
Hioki and Still initially investigated the application of disulfide exchange for the generation and subsequent templating of DCLs [53]. A method was investigated related to a moulding process based on previous work by the authors in which linked oligomers of isophthalic acid and trans-1,2-diamines were identified as highly selective receptors for the tripeptide (D)Pro(L)Val(D)Val. Libraries were generated from thiol building blocks **58** and **59** based on these receptor cores. The mixed disulfide was formed by I_2 mediated oxidation of **58** and **59** (Scheme 15). Disulfide **58**-SS-**59** was then equilibrated in the presence of **59**-SH/Et_3N. This resulted in homodisulfide products **58**-SS-**58** and **59**-SS-**59** where the equilibrium constant of the system was found to be 1.8. When the library was generated in the presence of three equivalents of polymer bead supported

(D)Pro(L)Val(D)Val, the equilibrium was shifted in favour of the receptor **58**-SS-**58** (Keq = 32). Employment of an immobilised template allowed isolation of the receptor in a 97.5 % purity by extraction from the polymer support.



Scheme 15 Two thiols **58**-SH and **59**-SH and their subsequent DCL formation

Ramström and Lehn [54] and Otto *et al* [55] simultaneously demonstrated that disulfide DCLs may also be generated in water. This allowed the introduction of biomolecules as templates. Ramström and Lehn introduced the carbohydrate binding lectin concanavolin A immobilised on sepharose beads to a library of interconverting disulfide linked sugars (Scheme 16). Both adaptive and preequilibrated methods were employed for screening of libraries. Introduction of an immobilised template into a preequilibrated library allowed binding species to be "fished out" of the final mixture. Active members could thus be identified by quantitative analysis of the solution. Library members that were found to have a decreased concentration were identified as the active member. HPLC analysis of solution phase species and species eluted from beads revealed an amplification of the manose-manose dimer and dimers containing one manose moiety. A more pronounced effect was observed for the preequilibrated library.

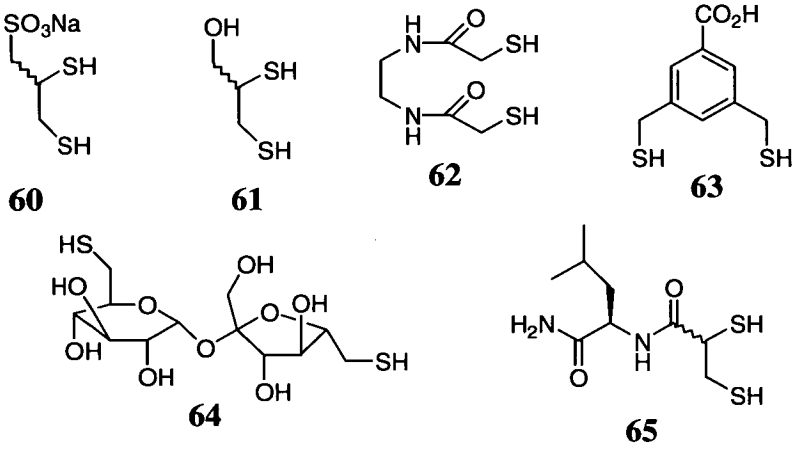


Compound	R^{2a}	R^{2e}	R^{4a}	R^{4e}	R^{5}
Man/Man	OH	H	H	OH	CH_2OH
GalC2/GalC2	H	OH	OH	H	CH_2OH
GalC3/GalC3	H	OH	OH	H	CH_2OH
Glc/Glc	H	OH	H	OH	CH_2OH
Ara/Ara	H	OH	OH	H	H
Xyl/Xyl	H	OH	H	OH	H

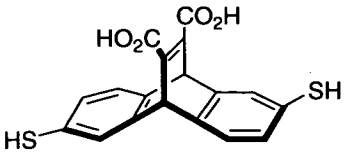
Scheme 16 Structures of some disulfide linked sugar dimers formed in the disulfide library by Lehn and Ramström

Otto *et al* [55] established that macrocylic libraries of large diversity may be generated from neutral or charged dithiol building blocks including carbohydrates and α-amino

acids. In this example libraries were generated from building block **60** –**65** and subsequently analysed by (FTICR)-ESI-MS. Mass/charge ratios for 119 library members were observed.



Otto *et al* later established this method as not only suitable for the identification of receptors but also as a means of synthesising those receptors [56]. Equilibration of building block **63**, **66** and **67** resulted in 45 different macrocylic receptors identified by ESI-MS. The true number may be higher due to the occurrence of stereoisomers and sequence isomers. Analysis of the library by HPLC gave quantitative information on the distribution. Libraries were then generated in the presence of guest molecules **68** and **69**. For libraries influenced with **68** an increase in a heterotrimer containing two units of **66** and one unit of **67**. **69** gave an increase of a homotrimer comprising of **66**. As **66** was introduced as a racemic mixture, receptors were also obtained as a mixture of stereoisomers. HPLC analysis showed amplification was not stereoselective. Receptors were synthesised by the generation of a biased library that contained only building blocks present in the receptor. Synthesis of the heterotrimer was achieved in a 65 % yield by mixing building blocks **66** and **67** in the presence of **68**. Similarly homotrimer host was produced in a 95 % yield by oxidising **66** in the presence of **69**.



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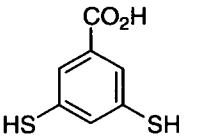

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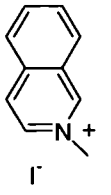

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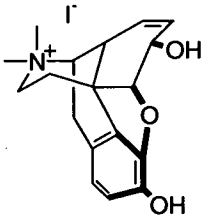

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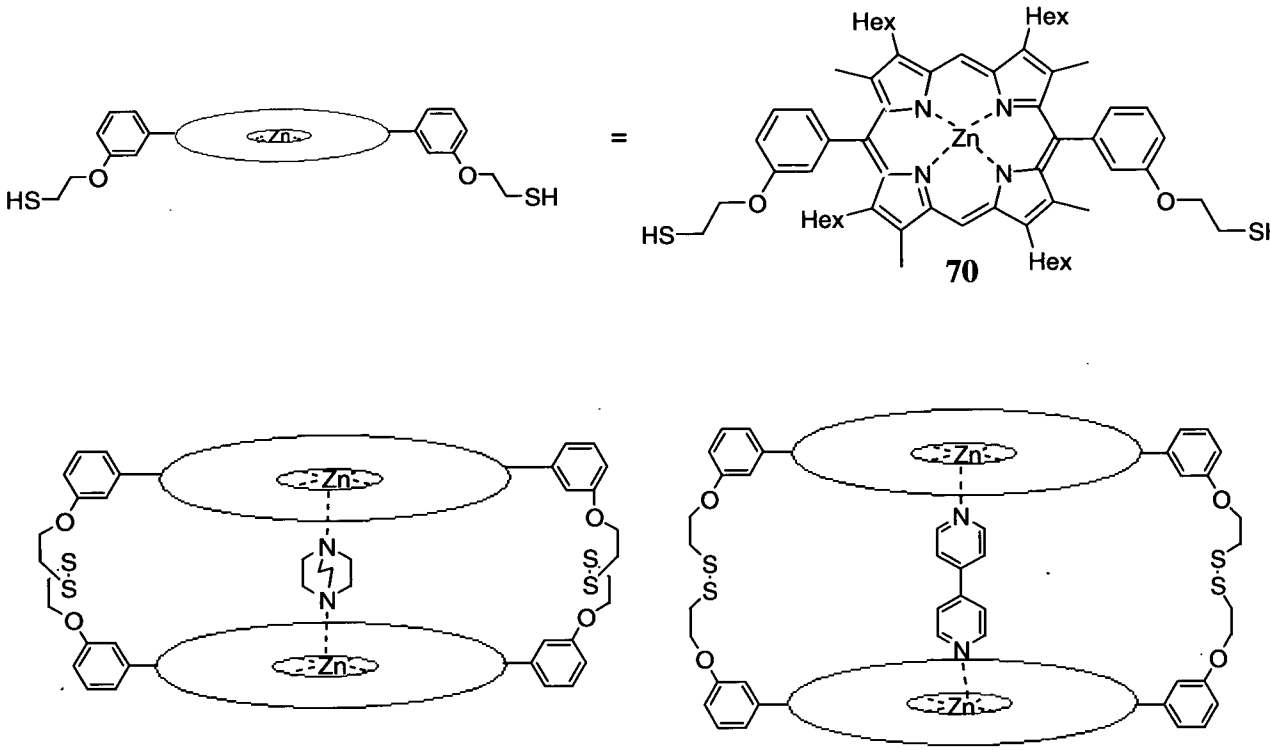
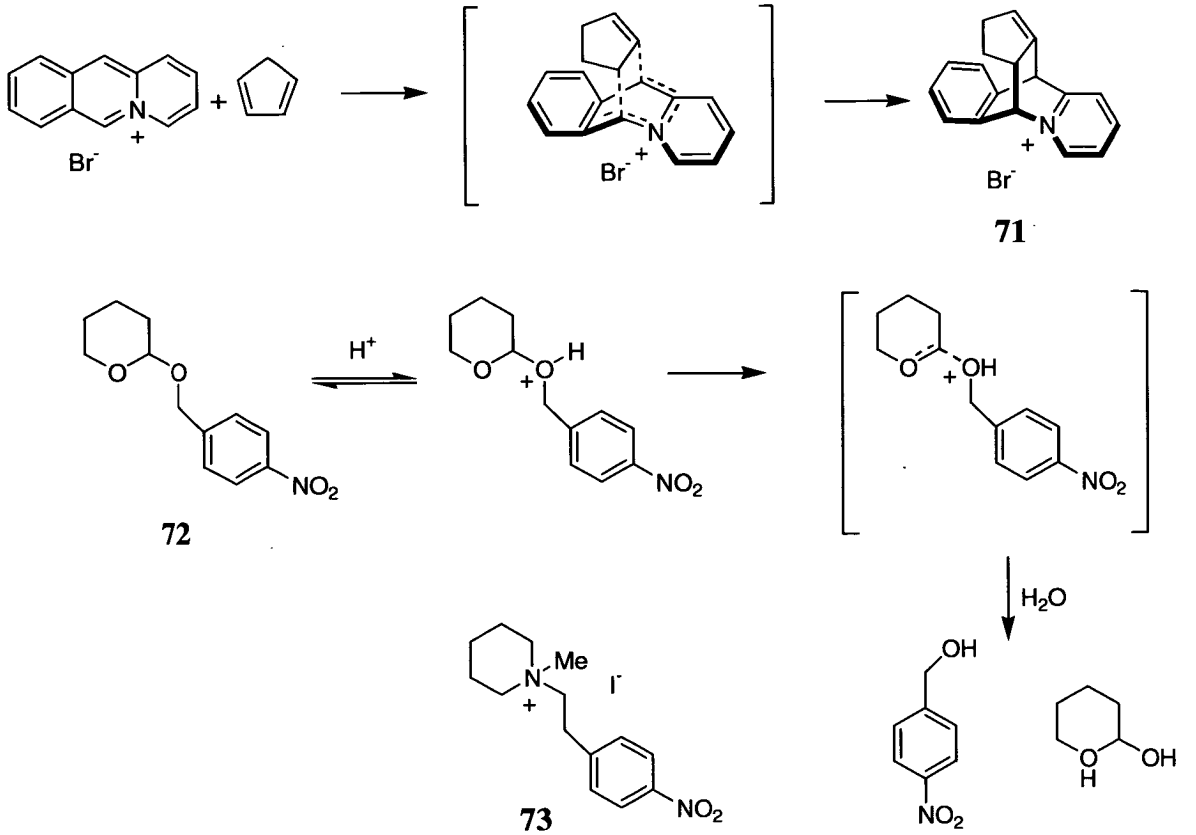
The groups of Otto and Sanders have further applied the simultaneous identification and synthesis of receptors to several systems. These include the anion induced amplification of dimers of neutral cyclic peptides bridged by disulfide linkers [57]. In this example two new receptors were obtained with unprecedented binding efficiencies. Diastereoselective amplification and synthesis of a cyclic tetramer generated from a racemic mixture of building block **66** has also been achieved by templating with NMe_4I [58].

In extension to this, the class of macrocyclic structures available via disulfide exchange has been widened to encompass porphyrins. Examples include macrocycles constructed of solely metalloporphyrins [59] and macrocycles incorporating π electron-rich aromatic and metalloporpyrin or porphyrin building blocks [60]. One particularly interesting case displayed the amplification of a flexible cyclic metalloporphyrin dimer with templates of varying size [61]. Flexible macrocycles were prepared from building block **70**. 1,4-Diazabicyclo(2.2.2)octane (DABCO) or the larger 4,4'-bipyridine (BPy) were introduced as templates. This resulted in the encapsulation of both templates by the dimer, thus demonstrating the accordion-like features of the receptor (Scheme 17).



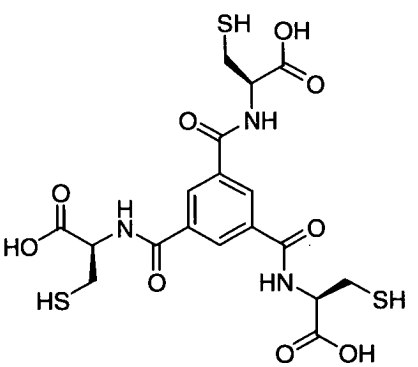
Scheme 17 Encapsulation of DABCO and BPy in cyclic porphyrin dimer

DCLs generated through disulfide exchange have also found application in catalyst and, potentially, drug targeting. For catalyst discovery templates that mimic transition states of chemical or biotransformations are introduced to a DCL. Receptors that bind that stable transition-state analogue (TSA) may potentially catalyse the corresponding reaction. Otto, Sanders and co-workers have successfully amplified macrocycles from libraries using a TSA [62]. This has led to the discovery of macrocyclic catalysts for the Diels-Alder reaction between acridizinium bromide and cyclopentadiene (Scheme 18). The authors reasoned that the product **71** would be a good TSA. Introduction of **71** into a library generated from building blocks **63**, **66** and **67** resulted in the amplification of a macrocyclic trimer of **66**. In a similar experiment a macroyclic catalyst that mimics glycosidase function by hydrolysing acetal **72** was identified [63] (Scheme 18). Introduction of ammonium salt **73** as a TSA to a disulfide library also resulted in the amplification of the macrocyclic trimer of **66.** The trimeric host was found to catalyse both reactions, however, a modest efficiency was displayed in both cases.



Scheme 18 Similarity between transition state analogue and transition state

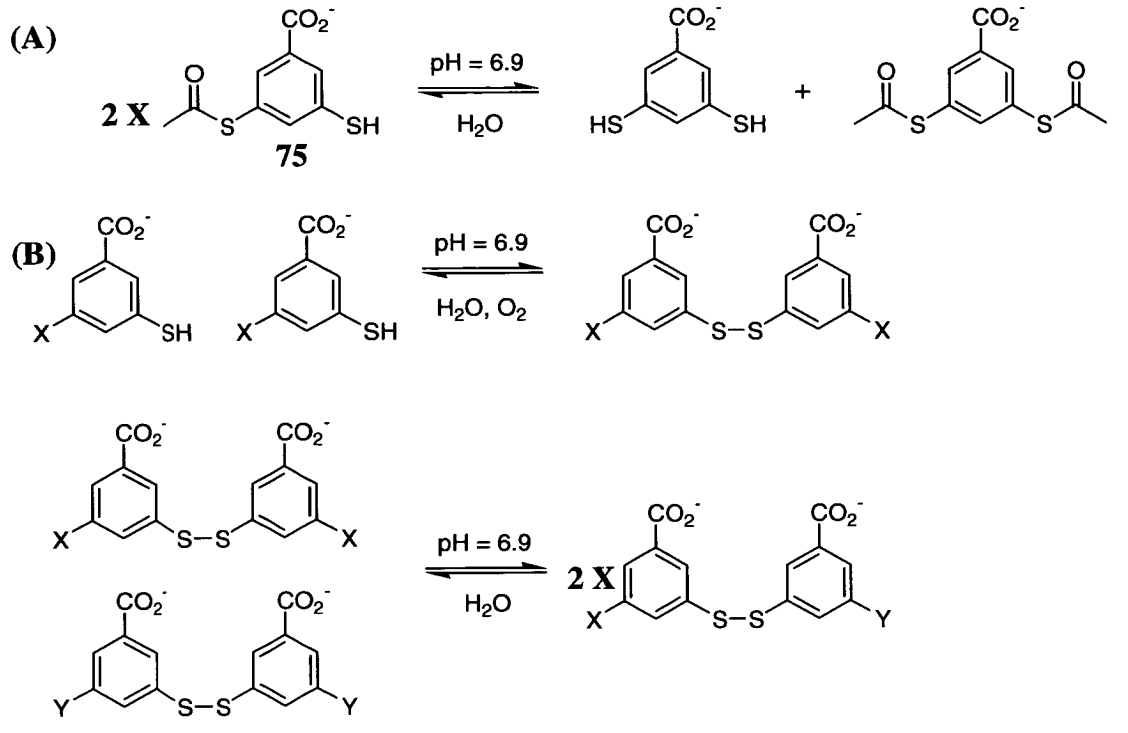
Molecular encapsulation in disulfide cages has been the subject of work by Otto and co-workers [64]. Libraries of disulfide cages were generated from dithiols **67** and **63** and trithiol **74**. Although, so far, no templating of cages has been attempted, it is envisaged by the authors that such systems may be employed in drug targeting where the disulfide cages could potentially find application in redox-controlled guest release.



74

1.3.4 Orthogonal Libraries

Additional degrees of library diversity have been achieved in DCLs by using two simultaneous exchange processes to connect building blocks. Lehn and co-workers have reported the use of double level "orthogonal" dynamic combinatorial libraries [65, 66]. These libraries employed imine formation in parallel with reversible metal coordination where the two different exchange processes could be activated and deactivated independently [66]. More recently a double-level "communicating" library has been reported by Sanders and co-workers [67]. This library features two simultaneous covalent exchange reactions in aqueous solution at neutral pH. This library was generated from building block **75** by the one pot exchange of disulfide and thioester linkages. **75** contained two exchangeable groups: a monotopic thioester group which acted as a labile capping group through thioester exchange and a ditopic dithiol group which could be potentially involved in chain elongation through disulfide exchange or termination through thioester exchange. Thioester exchange (Scheme 19A) and disulfide exchange (Scheme 19B) were addressed sequentially. Thioester exchange was initially addressed by dissolving **75** in buffer in aqueous solution in the absence of oxygen. Thiol condensation and simultaneous disulfide exchange were then activated by exposing the mixture to atmospheric oxygen. A library of 11 members was generated where 8 oligomers combining disulfide and/or thioester functionalities were unambiguously identified by LC-MS. Indeed the resulting library was found to contain both cyclic and linear species. This is believed to be the first example of interconversion between open and closed species.



Scheme 19 Building blocks involved in thioester exchange **(A)** and further disulfide oligomerisation **(B)**

1.3.5 Enzyme Catalysis in the Generation of DCLs

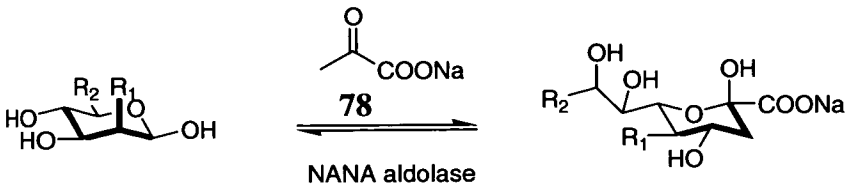
Enzyme catalysed reactions have had a limited application in DCC and very few enzymatic approaches have been published to date. Enzyme catalysed reactions offer a number of advantages related to the generation of DCLs:

- Enzyme catalysed reactions are characteristically reversible under aqueous conditions.
- Reactions may be frozen by simply inactivating or removing the enzyme.
- Products of enzyme catalysed reactions are usually stable. Distribution of library members may therefore be analysed directly without the need for a derivatisation step.
- Diverse libraries may be produced by broad specificity enzymes, many of which are commercially available.

The application of enzyme catalysed reactions to DCL generation was first investigated by Swann *et al* [68]. The authors demonstrated pools of diverse peptides could be

generated through peptide bond exchange mediated by low specificity proteases. Incubation of peptides Tyr-Gly-Gly and Phe-Leu with thermolysin under conditions that catalysed by both synthesis and hydrolysis generated a mixture of 15 peptides. The library was templated with a monoclonal antibody capable of binding Tyr-Gly-Gly-Phe-Leu. Unfortunately the antibody was saturated by the amount of Tyr-Gly-Gly-Phe-Leu formed in the reaction. Antibody saturation coupled with analytical problems meant that amplification of Tyr-Gly-Gly-Phe-Leu could not be directly proven. However, the library was found to modestly inhibit the binding of a radiolabelled β-endorphin to the antibody, thus the authors concluded that Tyr-Gly-Gly-Phe-Leu was weakly amplified.

Lins *et al* later described the generation of DCL through enzyme-catalysed carbon-carbon bonds [7, 69]. *N*-Acetylneuramic acid aldolase (NANA aldolase) was chosen as the enzyme for library formation. NANA aldolase catalysed the cleavage of sialic acid **77a** to ManAc **76a** and sodium pyruvate **78**. In the presence of excess **78** the equilibrium may be driven towards synthesis of the aldol product. Sialic acid aldolase was therefore used to generate a small library of sialic acid analogues **77a-c** from substrates **76a-c** in the presence of excess sodium pyruvate **78** (Scheme 20). Libraries were generated by simply incubating equimolar amounts of **76a-c** with two equivalents of **78** in the presence of NANA aldolase in pH 7.5 at 37 °C. After 16 hours the reaction was stopped by thermal inactivation of the enzyme. This method was, however, not successful and the enzyme was still found to be active. More stringent thermal denaturation (5 min at 95 °C) was found to decompose aldol products so libraries were therefore "frozen" by diluting aliquots of the reaction mixture and analysing them directly by HPLC.



76a R_1 = NHAc, R_2 = CH_2OH

76b R_1 = OH, R_2 = CH_2OH

76c R_1 = OH, R_2 = H

77a R_1 = NHAc, R_2 = CH_2OH

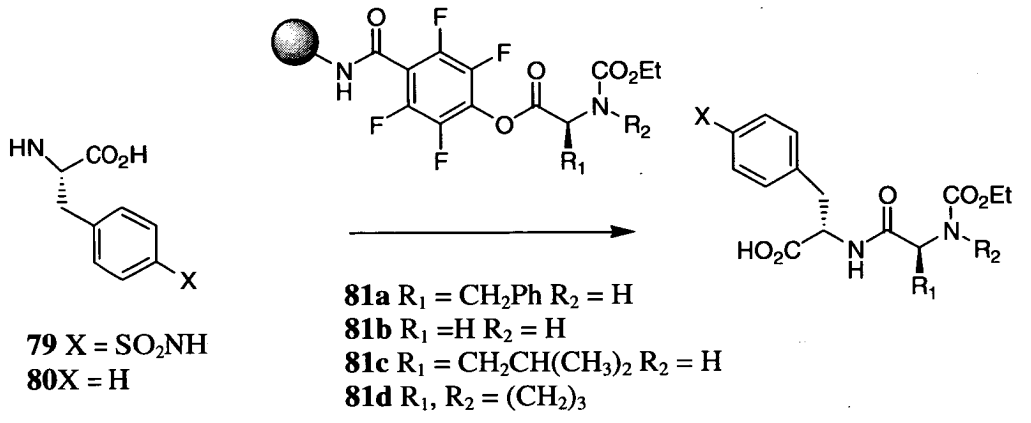
77b R_1 = OH, R_2 = CH_2OH

77c R_1 = OH, R_2 = H

Scheme 20 NANA aldolase catalyses the cleavage of sialic acid **77a** to ManAc **76a** and sodium pyruvate **78**; in the presence of **78**, aldol products **77a-c** may be generated from **76a-c**

Distribution of the aldol products **77a-c** was affected by the introduction of plant lectin Wheat Germ Agglutinin (WGA) which is known to specifically bind **77a** with a modest affinity. A relative amplification of 80 % was observed for **77a** together with a relative suppression of 80 % for **77b**. It was noted that **77c** was not suppressed during the course of the reaction indicating that WGA may have a weak affinity for **77c**. To test this hypothesis the authors generated a two member library of **77b** and **77c** in the presence of WGA. A modest amplification of **77c** over **77b** was observed. The authors concluded that the amplification of **77c** may be due to experimental error.

One further example of enzymatic reactions in DCC encompasses research by Corbett and co-workers [17, 18, 70]. This work does not demonstrate the enzyme as a tool for the generation of DCLs but as a tool for the destruction of poorly binding substrates in pseudo-DCLs (Section 1.2). The authors constructed a pseudo-dynamic library of eight dipeptides, the enzyme carbonic anhydrase (CA) as a molecular trap and the non-specific protease Pronase to destroy the poor binders by irreversible hydrolysis. Peptides were irreversibly synthesised in water under alkaline conditions (pH 8-10) by reaction of nucleophiles **79** and **80** with four immobilised active ester resins **81a-d** (Scheme 21).



Scheme 21 Irreversible synthesis of dipeptide library

Synthesis, screening and hydrolysis were divided into separate chambers to prevent modification of the CA. A three chambered vessel was prepared by suspending two dialysis bags in a surrounding solution (Figure 2). Chamber one (the synthesis chamber) contained the tentagel immobilised active esters; chamber two (the screening chamber) contained the carbonic anhydrase trap and chamber three (the hydrolysis chamber) contained pronase. Addition of nucleophiles **79** and **80** generated the dipeptide library. Dipeptides then diffused into the screening chamber where the best binders were trapped by CA. The poor binders then diffused into the hydrolysis chamber where nuleophiles **79** and **80** were regenerated by cleavage of the dipeptides with pronase. **79** and **80** were then free to diffuse back into the synthesis chamber to repeat the cycle. After four sixteen hour cycles a selectivity of 100:1 in favour of the strongest binder was observed.

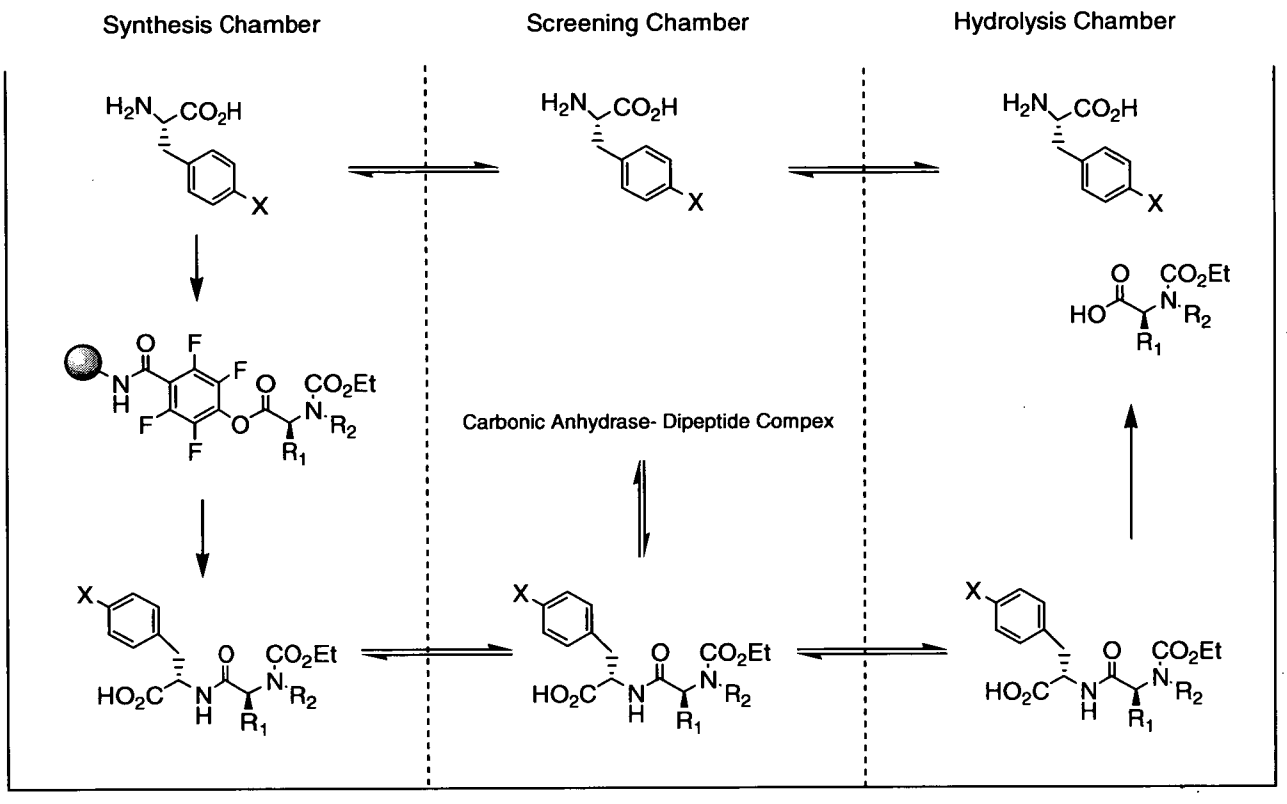


Figure 2 Diagrammatic representation of pseudodynamic combinatorial library experiment

1.4 Project Aim

Currently, the range of reversible covalent reactions employed for the generation of DCLs is narrow. Due to the increasing application of DCLs to screening processes such as receptor, drug and catalyst discovery, there is an increasing demand to widen the range of reversible covalent reactions. As already discussed enzymatic transformations offer a number of advantages for the generation of DCLs including the fact that they are characteristically reversible under aqueous conditions. Despite this, the use of enzyme catalysis for library generation has been largely overlooked and only work by ourselves [7, 69] and Swann [68] has been reported. Although the work carried out by Swann demonstrated that a library of large diversity could be generated by incubating small peptides with thermolysin, amplification of library members with an antibody could not be conclusively proven. A significant problem in work by Swann *et al* has been the low abundance of peptides in comparison to free amino acid building blocks. Generally, one would expect the equilibrium of the reaction to be biased towards hydrolysis rather than synthesis. However, some work within our research group had demonstrated that

peptide libraries could be generated in good amounts even in aqueous media when the substrates were immobilised on a solid support. The aim of the present project was to exploit these findings and enzymatically synthesise interconverting peptide libraries on a solid support. In the context of applications to dynamic combinatorial chemistry the influence of potential templates on the library distribution would be investigated.

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2. Protease-Catalysed Synthesis of Peptide Libraries on a Solid Support

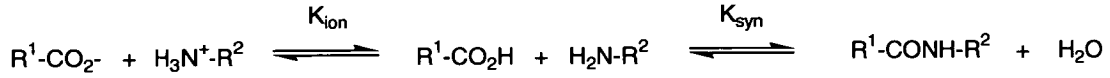
2.1 Introduction

Direct protease-catalysed synthesis of peptides from amino acid building blocks is widely used as an alternative to chemical synthesis. This is largely due to a number of advantages connected to the use of proteases. Advantages include the fact that proteases are highly stereo- and regiospecific catalysts which are active under mild conditions (pH 6-8), are easy to handle, are widely available and mostly inexpensive. Further to this, proteases are among the best characterised enzymes and much current knowledge of protein structure and function originates from these investigations [1]. However, in aqueous media the position of equilibrium of the protease synthesis/hydrolysis reaction is far over in the direction of hydrolysis (Scheme 22).

Scheme 22 The synthesis/hydrolysis equilibrium of a protease

Altered reaction conditions are therefore required to synthesise peptides in good yields. As protease-catalysed synthesis of peptides from free amines and carboxylic acids is thermodynamically unfavourable in dilute aqueous media, various "low water" reactions have been developed to shift the equilibrium towards synthesis. An approach which has received the most study is the manipulation of the ionisation equilibrium by replacement of water with organic solvents [2-6]. In some cases water is completely replaced by organic solvents and in others quantities of water are retained for enzyme activity. Addition of organic solvents results in a decrease in the dielectric constant of the media. As the thermodynamic barrier to synthesis is predominantly determined by the energy required for the transfer of a proton from the nucleophile to the negatively charged carboxylate of the acyl donor, decreasing the dielectric constant of the media results in a reduced acidity of the carboxylate acyl donor (Scheme 23). This, in turn,

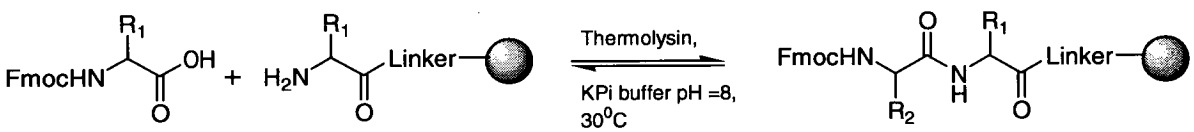
increases the equilibrium constant for proton transfer (K_{ion}) and therefore promotes synthesis.



Scheme 23 Protease-catalysed peptide synthesis by control of ionisation equilibrium

Employment of organic solvents has proven to be popular and many applications have been reported in the literature. A drawback of this approach is that enzyme activity is often significantly lowered and substrate solubility can be limiting. "Low water" reactions have also been reported in aqueous media where products are precipitated from aqueous substrate suspensions [7, 8]. However, product precipitation is not always favoured for these "solid-to-solid" reactions.

Previous work within our group had demonstrated that the synthesis/hydrolysis equilibrium of the protease thermolysin could be shifted towards synthesis in aqueous media by immobilising the substrate on a $PEGA_{1900}$ solid support [9] (Scheme 24)



Scheme 24 Thermolysin-catalysed peptide synthesis on a solid support

There are thought to be several reasons for the success of this shift:

1. A large excess of acyl donor to supported substrate can be used to push the equilibrium towards synthesis.
2. Ionization of the supported amine is suppressed by the overall positive charge of the resin.
3. Improved solvation of the protected amino acid in the PEGA micro environment is thought to result in a higher local concentration at the site of catalysis.

Further study has shown the latter of these reasons to be the largest contributing factor [10].

At the start of the present project only dipeptides had been synthesised using such methodology. Our aim was to investigate the application of solid phase enzymatic synthesis to longer peptides. It was expected that as the peptide chain was elongated and extended away from the PEGA microenvironment into solution, that a tendency towards hydrolysis would be observed. It was thought that the enzyme would hydrolyse the peptide chain at various points resulting in a library of peptides varying in length. It was also expected that due to hydrolysis the peptide chains would reach a maximum length. We were interested in the length of chain at which hydrolysis would occur and also what the maximum achievable length of peptides would be. Three peptide chains were enzymatically synthesised in order to observe if effects were general or restricted to certain peptides. Fmoc protected substrates were chosen according to the substrate specificity of thermolysin which favours bulky hydrophobic side chains [11]. Substrates screened as FmocAA were:

1. Fmoc-L-Leu **82**
2. Fmoc-L-Phe **83**
3. Fmoc-L-Tyr **84**

2.2 Attachment of Substrate to Solid Support

PEGA resin (Figure 3), developed by Meldal and co-workers [12], is one of the most commonly used supports for enzymatic transformations. PEGA resin is a copolymer of polyethylene glycol and polyacrylamide where the resin may be prepared using a variety of PEG lengths. The highly polar PEG chains allow the resin to swell in water as well as a wide range of organic solvents. Further to this the flexibility of the PEG chains allow the diffusion of large macromolecules into the bead. Studies have shown that for enzyme hydrolysis the reactive sites of $PEGA_{1900}$ are accessible to enzymes up to 35 kDa [13]. For synthesis it appears that the resin is accessible to enzymes with a molecular

weight of up to 50 kDa [14]. Recently, we have used two photon microscopy to demonstrate that thermolysin (35 kDa) is able to diffuse to the core of the $PEGA_{1900}$ bead in a period of 45 minutes [15]. Given the previous successful combination of $PEGA_{1900}$ and thermolysin in the enzymatic synthesis of dipeptides, the same combination was used for the synthesis of longer peptides.

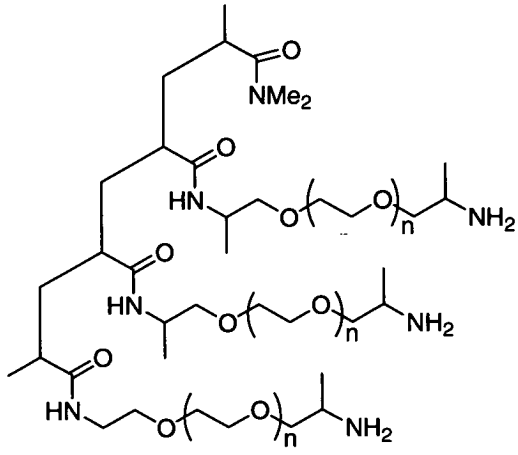
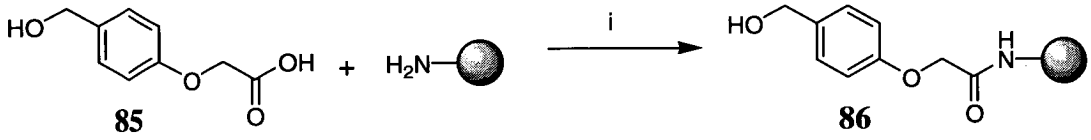


Figure 3 General structure of PEGA resin

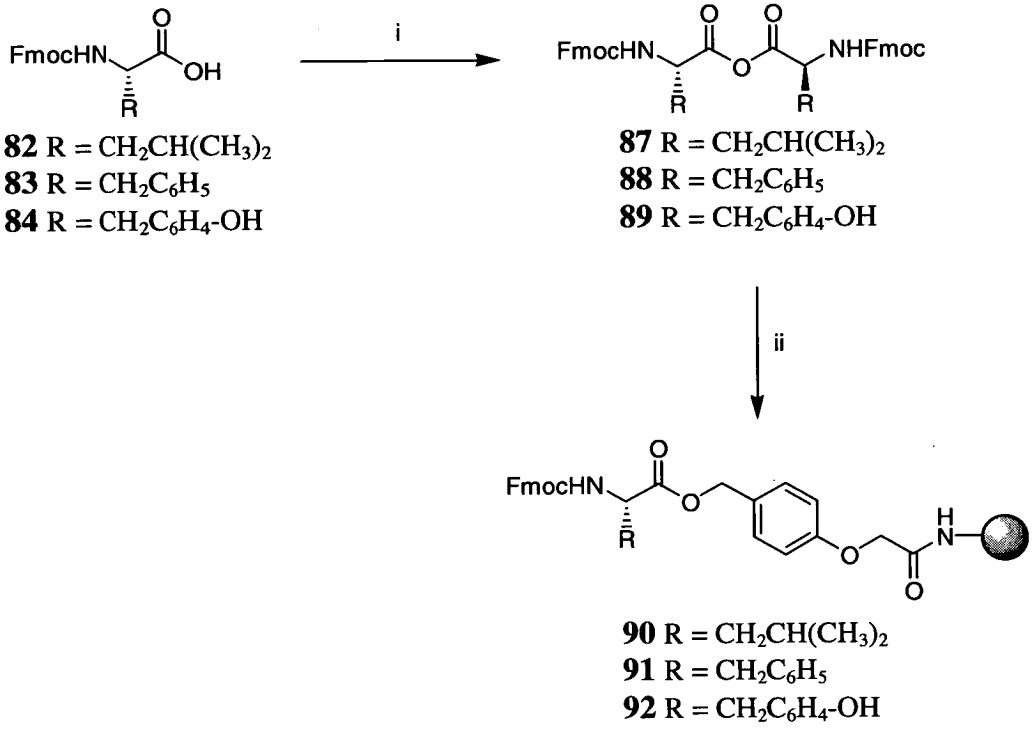
Wang linker **85** was coupled to the amino functionalised resin using standard amide coupling reagents DIC and HOBt to give **86** (Scheme 25). The reaction was deemed complete when no colour change was observed by the ninhydrin test which monitors the presence of primary amine groups on the resin.



Scheme 25 *Reagents and conditions:* DIC, HOBt, DMF, 18 hrs, RT

Previously within our group, the first amino acid was coupled to the Wang linker *via* an ester bond formed using DIC and HOBt [15]. The DIC, HOBt method of coupling was found to be unreliable and gave varied loadings. A more reliable protocol proved to be *via* the symmetrical anhydride. Symmetrical anhydrides **87-89** were preformed by treatment of Fmoc amino acids **82-84** with DIC. **87-89** were then added to **86,** which

had been preswollen in DMF with a catalytic amount of DMAP, to give the desired ester bonds **90-92** (Scheme 26).



82 R = $CH_2CH(CH_3)_2$
83 R = $CH_2C_6H_5$
84 R = $CH_2C_6H_4$-OH

87 R = $CH_2CH(CH_3)_2$
88 R = $CH_2C_6H_5$
89 R = $CH_2C_6H_4$-OH

90 R = $CH_2CH(CH_3)_2$
91 R = $CH_2C_6H_5$
92 R = $CH_2C_6H_4$-OH

Scheme 26 *Reagents and conditions:* (i) DIC, DMF, 1 hr, RT (ii) **86**, DMAP, 18 hr, RT

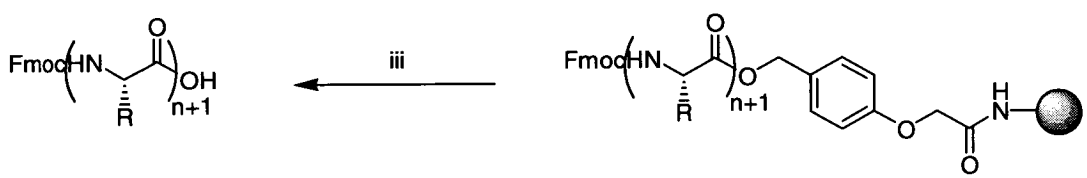
The loading of the **90-92** was calculated by acidic cleavage of the ester followed by quantitative reversed-phase HPLC analysis. Typically a loading of 60 μmols g^{-1} was achieved.

2.3 Enzymatic Synthesis of Longer Peptides

Enzymatic couplings were carried out in a stepwise synthesis by deprotection of the Fmoc with 20% piperidine in DMF then incubation of the Fmoc protected acyl donor and the resin bound substrate in the presence of thermolysin, overnight at 30°C (Scheme 27). Reactions were stopped by filtering and washing the resin. Deprotection and activation steps were repeated until a maximum chain length was reached. After each coupling step product was cleaved from a small amount of resin and analysed by RP-LC-MS.

90 R = $CH_2CH(CH_3)_2$ n = 1
91 R = $CH_2C_6H_5$ n = 1
92 R = $CH_2C_6H_4$-OH n = 1
93 R = $CH_2CH(CH_3)_2$, n = 2
94 R = $CH_2CH(CH_3)_2$, n = 3
95 R = $CH_2CH(CH_3)_2$, n = 4
96a-c R = $CH_2CH(CH_3)_2$, n = 3-5
97a-d R = $CH_2CH(CH_3)_2$, n = 3-6
99 R = $CH_2C_6H_5$ n = 2
100a-b R = $CH_2C_6H_5$ n = 2-3
101a-c R = $CH_2C_6H_5$ n = 2-4
103 R = $CH_2C_6H_4$-OH n = 2
104a-b R = $CH_2C_6H_4$-OH n = 2-3
105a-b R = $CH_2C_6H_4$-OH n = 3-4

ii



93 R = $CH_2CH(CH_3)_2$, n = 1
94 R = $CH_2CH(CH_3)_2$, n = 2
95 R = $CH_2CH(CH_3)_2$, n = 3
96a-c R = $CH_2CH(CH_3)_2$, n = 2-4
97a-d R = $CH_2CH(CH_3)_2$, n = 2-5
98a-d R = $CH_2CH(CH_3)_2$, n = 2-5
99 R = $CH_2C_6H_5$ n = 1
100a-b R = $CH_2C_6H_5$ n = 1-2
101a-c R = $CH_2C_6H_5$ n = 1-3
102a-c R = $CH_2C_6H_5$ n = 1-3
103 R = $CH_2C_6H_4$-OH n = 1
104a-b R = $CH_2C_6H_4$-OH n = 1-2
105a-b R = $CH_2C_6H_4$-OH n = 2-3

Scheme 27 *Reagents and conditions:* (i) 20% piperidine in DMF (ii) **82**, **83** or **84**, thermolysin, KPi buffer pH = 8, 30°C (iii) 95% TFA in H_2O

90 was deprotected by treatment of the resin with 20 % piperidine in DMF then incubated with **82** in the presence of thermolysin (Scheme 27). Product was cleaved from the resin with 95 % TFA in H_2O then analysed by LC-MS (Figure 4). In the reaction mixture containing **90** as a starting material Fmoc(Leu)$_2$ **93** was observed in a 99 % conversion yield. In order to synthesise Fmoc(Leu)$_3$ **94**, **93** was deprotected and then incubated with **82** in the presence of thermolysin followed by acidic cleaveage of the product from the support. LC-MS analysis showed that **94** had been formed in a 99% conversion yield. Similarly, Fmoc(Leu)$_4$ **95** was generated in a 99 % conversion yield by deprotection and incubation of **94** with **82** in the presence of thermolysin.

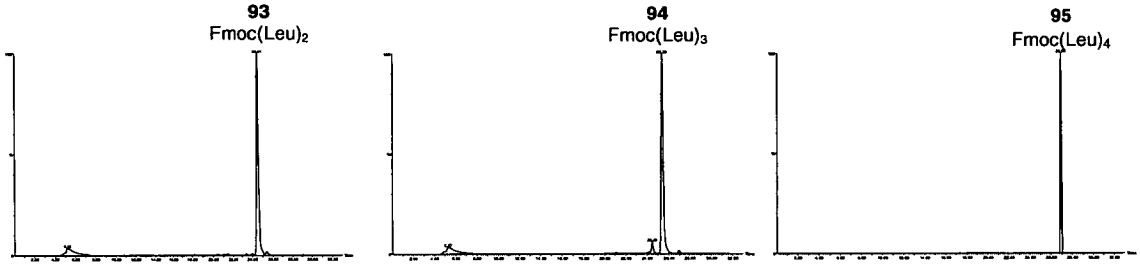


Figure 4 Chromatograms of Fmoc(Leu)$_2$ **93**, Fmoc(Leu)$_3$ **94** and Fmoc(Leu)$_4$ **95**

However, on incubation of deprotected **95** with **82** and thermolysin a library of Fmoc protected poly-L-leucine peptides **96a-c** varying in length were formed (Figure 5). LC-MS analysis of the products cleaved from the resin showed Fmoc(Leu)$_3$ **96a**, Fmoc(Leu)$_4$ **96b** and the desired Fmoc(Leu)$_5$ **96c** had been formed. The peptide chains were further extended by incubation of deprotected **96a-c** with **82** in the presence of thermolysin. As expected, several products **97a-d** were observed by LC-MS in the cleavage mixture. As well as Fmoc(Leu)$_6$ **97d**, Fmoc(Leu)$_3$ **97a**, Fmoc(Leu)$_4$ **97b** and Fmoc(Leu)$_5$ **97c** were formed.

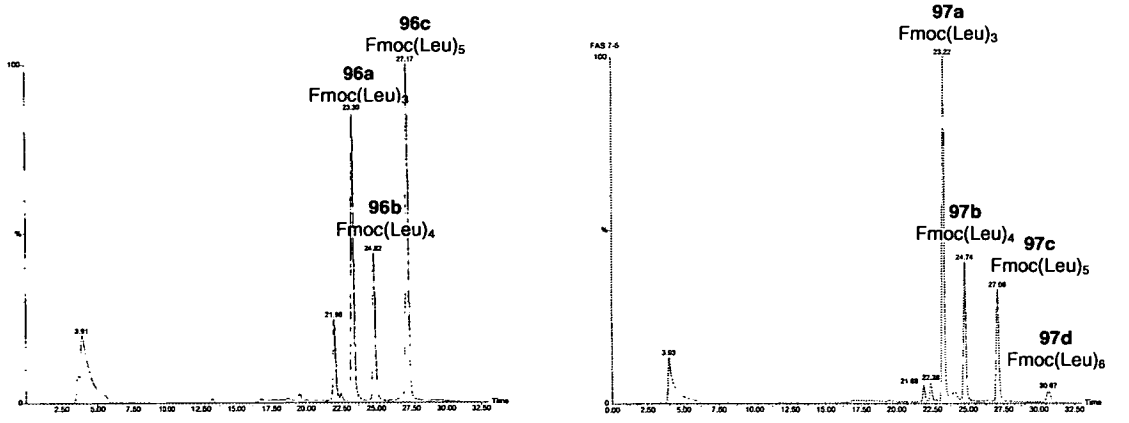


Figure 5 Chromatograms of Libraries **96** and **97**

In each case, the library members of **96** and **97** were not evenly distributed and some of the members were more predominant than others. The distributions of these have been quoted as a percentage of the total peak integration of all the library members. This is referred to as the Relative Percentage Product Composition (RPPC). RPPC values have been summarised on the tables below (Tables 1 and 2).

Table 1 Table of RPPC values for Library **96**

Library Member	RPPC
96a Fmoc(Leu)$_3$	35.9 %
96b Fmoc(Leu)$_4$	19.1 %
96c Fmoc(Leu)$_5$	45.0 %

Table 2 Table of RPPC values for Library **97**

Library Member	RPPC
97a Fmoc(Leu)$_3$	54.8 %
97b Fmoc(Leu)$_4$	22.0 %
97c Fmoc(Leu)$_5$	19.9 %
97d Fmoc(Leu)$_6$	3.4 %

Further extension of the peptide chains was attempted to produce a library that included Fmoc(Leu)$_7$ as a member. The deprotected library **97** was incubated with **82** in the presence of thermolysin. Analysis of products cleaved from the resin showed that Fmoc(Leu)$_7$ had not been formed and the hexamer Fmoc(Leu)$_6$ was the maximum achievable length for poly-L-leucine peptide chains. However, an Fmoc protected library **98a-d** containing the same library members as **97a-d** had been generated. The distribution of **98** differed from that of **97** (Table 3).

Table 3 Table of RPPC values for attempted Library **98**

Library Member	RPPC
98a Fmoc(Leu)$_3$	34.6 %
98b Fmoc(Leu)$_4$	39.8 %
98c Fmoc(Leu)$_5$	19.2 %
98d Fmoc(Leu)$_6$	6.5 %

For libraries **97** and **98**, Fmoc(Leu)$_3$ and Fmoc(Leu)$_4$ were found to be the most dominant products followed by Fmoc(Leu)$_5$ and Fmoc(Leu)$_6$. Fmoc(Leu)$_4$ and Fmoc(Leu)$_5$ were found to be present in higher amounts in **98** than in **97**.

2.3.2 Fmoc Protected Poly-L-Phenylalanine

91 was deprotected with 20 % piperidine in DMF and incubated with **83** in the presence of thermolysin (Scheme 27). The product was cleaved from the resin by treatment with 95 % TFA in H_2O then analysed by LC-MS. LC-MS analysis showed that Fmoc(Phe)$_2$ **99** had been formed in a 99 % conversion yield. Immobilised **99** was then deprotected and incubated with **83** presence of thermolysin. Acidic cleavage of the products from the support and analysis by LC-MS showed a mixture of peptides Fmoc(Phe)$_2$ **100a** and Fmoc(Phe)$_3$ **100b** had been generated in a 43.9 % and 56.1 % conversion yield respectively. As expected, deprotection of **100** followed by thermolysin mediated coupling of **83** also resulted in the generation of a mixture of compounds **101a-c**. LC-MS analysis of the products cleaved from the resin showed that a three member library had been formed. Library members comprised of Fmoc(Phe)$_2$ **101a**, Fmoc(Phe)$_3$ **101b** and Fmoc(Phe)$_4$ **101c**. RPPC values were calculated from the peak areas of the compounds and summarised in the table below (Table 4).

Table 4 Table of RPPC values for Library **101**

Library Member	RPPC
101a $Fmoc(Phe)_2$	19.0 %
101b $Fmoc(Phe)_3$	30.1 %
101c $Fmoc(Phe)_4$	50.9 %

In an attempt to generate a library that also contained $Fmoc(Phe)_5$ as a library member, **101** was deprotected then incubated with **83** in the presence of thermolysin. Acidic cleavage of products from resin followed by LC-MS analysis of the cleavage mixture showed that $Fmoc(Phe)_5$ had not been formed and that the tetramer $Fmoc(Phe)_4$ **101c** was the maximum achievable length for poly-L-phenylalanine peptides. Instead, an Fmoc protected library **102a-c** was observed that contained the same library members as **101a-c**. Calculation of the RPPC values of **101a-c** showed that, as for **97** and **98**, that libraries **101a-c** and **102a-c** differed in terms of product distribution (Table 5).

Table 5 Table of RPPC values for attempted Library **102**

Library Member	RPPC
102a $Fmoc(Phe)_2$	25.7 %
102b $Fmoc(Phe)_3$	55.2 %
102c $Fmoc(Phe)_4$	19.1 %

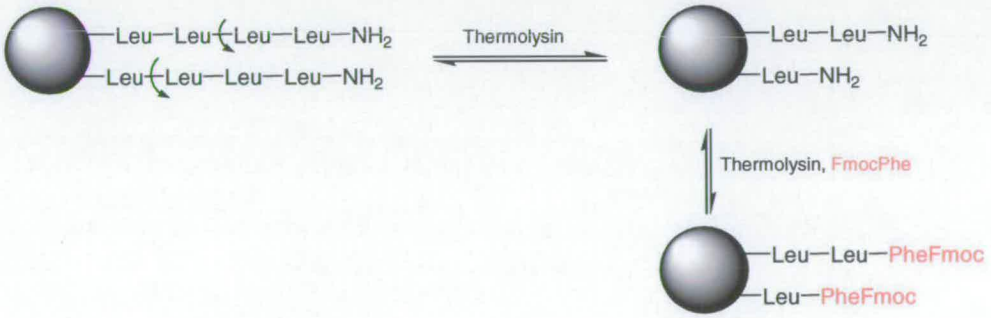
2.3.3 Fmoc Protected Poly-L-Tyrosine

92 was deprotected by treatment of the resin with 20 % piperidine in DMF then incubated with **84** in the presence of thermolysin (Scheme 27). Cleavage of the products with 95 % TFA in H_2O followed by LC-MS analysis showed that $Fmoc(Tyr)_2$ **103** was formed in a 99 % conversion yield. Incubation of deprotected **103** with **84** in the presence of thermolysin generated a mixture of peptides **104a-b**. LC-MS analysis of the products cleaved from the resin showed that $Fmoc(Tyr)_2$ **104a** and $Fmoc(Tyr)_3$ **104b** had been formed in a 47.5 % and 52.5 % conversion yield respectively. **104** was then deprotected and incubated with **84** in the presence of thermolysin. LC-MS analysis of the products cleaved from the resin revealed, as expected, that a mixture of products **105a-b** had been formed. A two member library of $Fmoc(Tyr)_3$ **105a** and $Fmoc(Tyr)_4$

105b was observed by LC-MS in a 47.5 % and 52.5 % conversion yield respectively. The deprotected mixture **105** was incubated with **84** in the presence of thermolysin in an attempt extend the peptide chains further. Products were cleaved from the resin and analysed by LC-MS. However, it was found that Fmoc(Tyr)$_5$ had not been generated and the maximum achievable length of poly-L-tyrosine was the tetramer Fmoc(Tyr)$_4$.

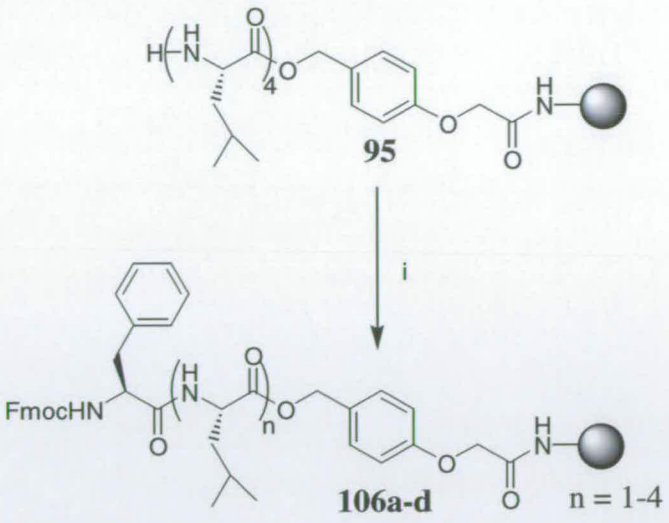
2.4 Investigation of Enzymatic Scrambling

As longer peptides are assembled on solid support by the above enzyme catalysis, they become potential substrates for thermolysin. Based on the known specificity of thermolysin, the smallest would be predicted to be an unprotected tripeptide or an Fmoc protected dipeptide. Larger oligomers would present several potential cleavage sites. If both synthesis and cleavage happen in the presence of excess FmocAA, the result might be a so-called "scrambled" library of different lengths and compositions (if different building blocks are used) (Scheme 28)



Scheme 28 Enzymatic scrambling of peptide chain

Scrambling was investigated further by incubating deprotected resin bound **95** in the presence of FmocPhe and thermolysin (Scheme 29).



Scheme 29 *Reagents and conditions:* FmocPhe **83**, thermolysin, KPi buffer pH = 8, 30°C

The peptides FmocPheLeu **106a**, FmocPhe(Leu)$_2$ **106b**, FmocPhe(Leu)$_3$ **106c** and FmocPhe(Leu)$_4$ **106d** were observed by LC-MS after cleavage from the support (Figure 6). **106a – 106c** may only be formed if the peptide chain is first cleaved and FmocPhe is subsequently coupled by the enzyme.

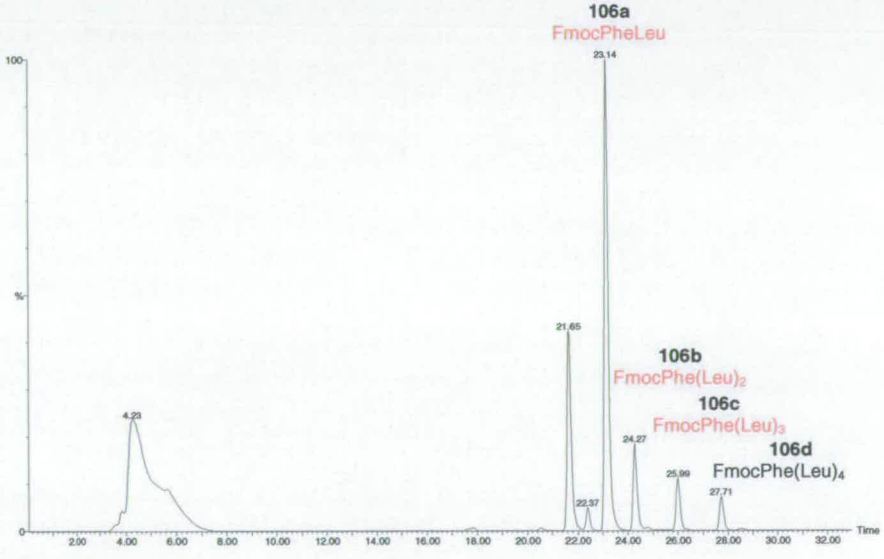
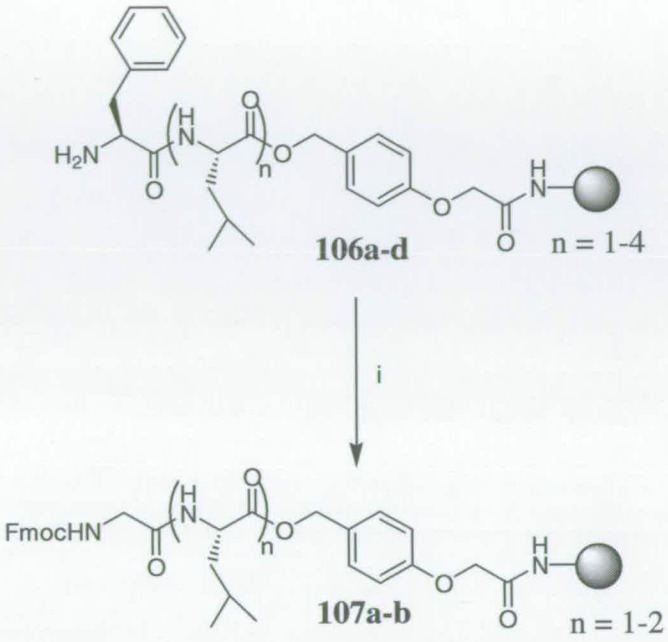


Figure 6 Chromatogram of Library **106a-d**

To scramble the library further a third substrate was introduced into the system. FmocGly was incubated with the deprotected library **106** (Scheme 30). LC-MS analysis of products cleaved from the resin showed results to be disappointing. Instead of

forming several peptides containing the residues Leu, Phe and Gly, a library consisting of only two members was observed. These were identified as FmocGlyLeu **107a** and FmocGly(Leu)$_2$ **107b**. This is further evidence that peptide bonds are reversibly formed in the reaction mixture.



Scheme 30 *Reagents and conditions:* FmocGly, thermolysin, KPi buffer pH = 8, 30°C

2.5 Summary and Conclusions

Overall, it has been conclusively demonstrated that peptide chains longer than dipeptides may be synthesised by thermolysin on PEGA$_{1900}$ with FmocAA building blocks. For all of the peptide chains synthesised in this manner a library of peptides varying in length were observed as well as a maximum achievable length. For poly-L-leucine peptides **93** – **98**, only the desired peptides were formed for the synthesis of Fmoc(Leu)$_2$ **93**, Fmoc(Leu)$_3$ **94** and Fmoc(Leu)$_4$ **95**. On synthesis of Fmoc(Leu)$_5$, hydrolysis of the peptide chain at various points occurred and peptide library **96a-c** was observed. It was found on generation of library **98** that the hexamer Fmoc(Leu)$_6$ was the maximum achievable length for poly-L-leucine peptides using this approach. For poly-

L-phenylalanine peptides **99 – 102** hydrolysis was found to occur on synthesis of the $Fmoc(Phe)_3$ resulting in the two member library **100a-b**. Generation of library **102a-c** demonstrated that the tetramer $Fmoc(Phe)_4$ was the maximum achievable length for poly-L-phenylalanine. Synthesis of poly-L-tyrosine peptides **103 – 105a-b** also demonstrated that hydrolysis occurred on synthesis of the trimer $Fmoc(Tyr)_3$ to give the two member library **104a-b**. Further to this the tetramer $Fmoc(Tyr)_4$ **104b** was the found to be the maximum achievable length for poly-L-tyrosine peptides. Generation of libraries **106** and **107** from mixtures of building blocks confirmed the reversible nature of the peptide bonds and that libraries were indeed formed as a direct result of hydrolysis. The observed hydrolysis effects were general in the sense that all generated peptides were found to eventually form libraries. However, effects differed in terms of the length of peptide at which hydrolysis occurred and also for the maximum achievable length of peptides. This library formation provides an excellent basis for application in DCC.

2.6 References

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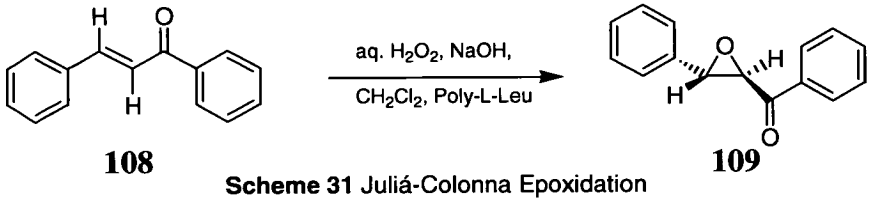
3. Templating of Immobilised Dynamic Peptide Libraries

3.1 Introduction

To successfully influence the distribution of the immobilised dynamic peptide libraries developed in Chapter 2, a template was sought. Chalcone **108** was chosen as a suitable template for the poly-L-leucine libraries as poly-L-leucine is known to catalyse the asymmetric epoxidation of **108** in the Juliá-Colonna epoxidation [1, 2] (Scheme 31).



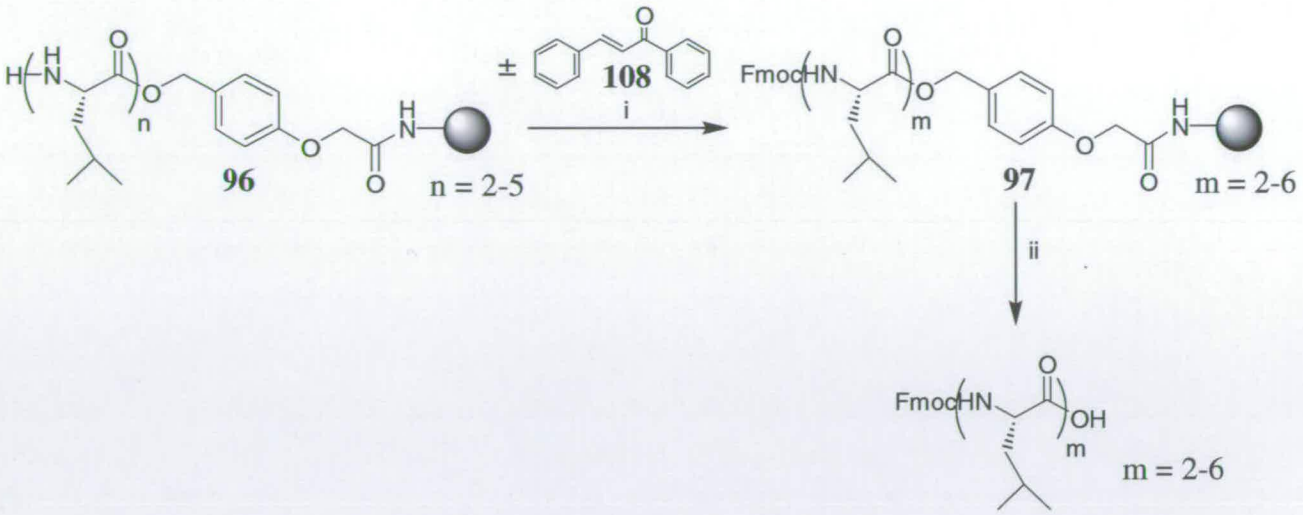
108 **109**

Scheme 31 Juliá-Colonna Epoxidation

Considerable work has been conducted to investigate the mode of binding of **108** to poly-L-leucine. Recent studies had shown that peptides with a length forming one helical turn are sufficient for catalysis, and peptides as small as pentamers gave optimum enantiomeric excesses [3]. This work suggested that **108** would have some affinity for small poly-L-leucine compounds (but perhaps not mono- or dileucine) and hence the poly-L-leucine **108** system was considered to be excellent for proof-of-principle studies.

3.2 Attempted Templating of Fmoc(Leu)$_6$ Library 97

The Fmoc(Leu)$_6$ library **97** was generated from library **96** in the presence and absence of **108** (Scheme 32). Products were cleaved from the support with 95 % TFA in H_2O then analysed by LC-MS. Comparison of the LC-MS chromatograms of both libraries showed there to be no difference in distribution. Two main reasons were identified for the lack of shift. Firstly, **108** had a limited solubility in aqueous media and might not be soluble in sufficient amounts required for binding. Secondly, the amino terminus in **97** is protected and literature suggests that unprotected *N*-termini provided a better binding pocket (Figure 7) [4, 5].



Scheme 32 *Reagents and conditions*: (i) FmocLeu, thermolysin, KPi Buffer pH = 8, 18 hr, 30°C (ii) 95 % TFA in H_2O

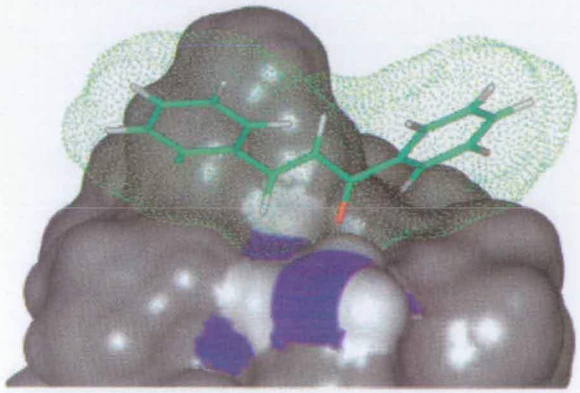


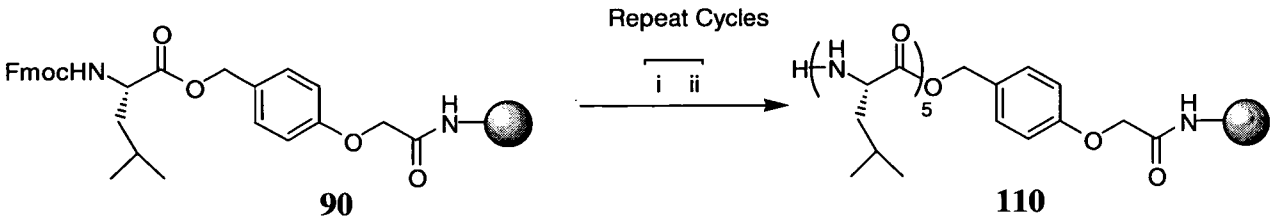
Figure 7 Chalcone **108** bound to the N terminus of the α-helix, NH of the termini and of the two proceeding amino acids are shown in blue/white [3]

3.3 Employment of Organic Co-Solvents to Aid Template Solubility

Thermolysin is tolerant to a wide range of organic solvents, so it was envisaged that a co-solvent could be employed to aid solubilisation of **108**. Enzymatic coupling of FmocLeu to chemically synthesised PEGA bound $(Leu)_5$ **110** was used as a test reaction to ascertain if enzymatic synthesis on solid phase in the presence of organic solvents

would be possible and also to determine in the enzyme would scramble **110** and give the same distribution as for the Fmoc(Leu)$_6$ library **97**.

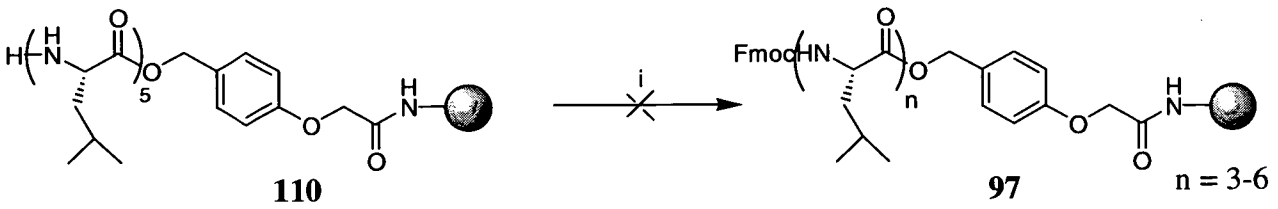
110 was synthesised from **90** by deprotection of the Fmoc with 20% piperidine in DMF followed by coupling of FmocLeu with standard peptide coupling reagents TBTU, HOBt and DIPEA (Scheme 33). Coupling and deprotection cycles were repeated until **110** was obtained.



90 **110**

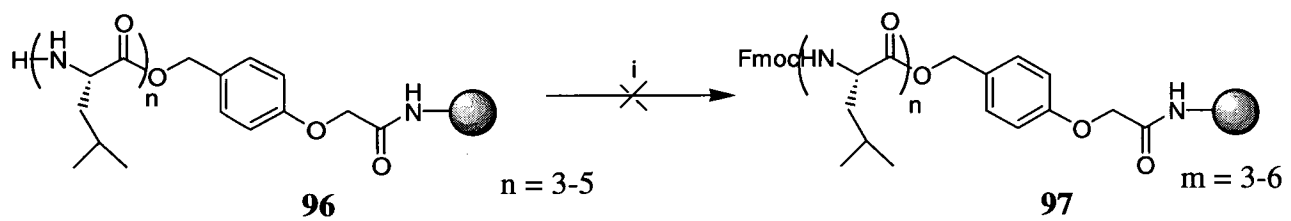
Scheme 33 *Reagents and conditions:* (i) 20 % Piperidine in DMF, 1 hr, RT (ii) FmocLeu, TBTU, HOBt, DIPEA, DMF, 2 hr, RT

Enzymatic coupling of the FmocLeu was then carried out by incubating FmocLeu with **110** in the presence of thermolysin in 90% ACN in pH8 KPi buffer and 5% DMSO in pH8 KPi buffer (Scheme 34). Products were cleaved from the support with 95 % TFA in H_2O then analysed by LC-MS. Analysis by LC-MS showed only starting material **110** to be present. As thermolysin is known to be active in 90 % ACN [6, 7], the failure of the reaction was attributed to the use of **110** as a substrate. The problem was investigated further by enzymatically coupling FmocLeu to **110** in buffer only. Again, only starting material was observed by LC-MS. A possible explanation of these observations may be that pentaleucine may form a thermodynamically stable helix and thereby resist scrambling.



Scheme 34 *Reagents and Conditions:* (i) FmocLeu, thermolysin, 90 % ACN in KPi Buffer pH = 8, 18 hrs 30°C, or FmocLeu, thermolysin, 5 % DMSO in KPi Buffer pH = 8, 18 hrs, 30°C, or FmocLeu, thermolysin, KPi Buffer pH = 8, 18 hrs, 30°C

Given that **110** was found to be a poor substrate, co-solvent reactions were repeated using the enzymatic coupling of FmocLeu to library **96** as a test reaction (Scheme 35). LC-MS analysis of the cleaved products still revealed only starting material to be present. As the enzymatic coupling of FmocLeu to **96** is known to give **97** when the reaction is carried out in buffer, the failure of the reaction was attributed to the presence of the organic co-solvents. The organic co-solvent is believed to have an unfavourable effect on the system by either decreasing substrate concentration at the site of catalysis by improving solvation of the acyl donor in solution, or by hampered enzyme diffusion caused by decreased swelling of the $PEGA_{1900}$.



Scheme 35 *Reagents and conditions:* (i) FmocLeu, thermolysin, 90% ACN KPi Buffer pH = 8, 18 hr, 30°C or FmocLeu, thermolysin, 10% DMSO KPi Buffer pH = 8, 18 hr, 30°C

3.4 Formation of Adducts between Chalcone and $(Leu)_5$

As organic co-solvents were found to inhibit library formation, introduction of **108** into the system should therefore have no effect on library distribution. However, when **108** was incubated with **110** in 90 % ACN in pH 8 buffer two additional peaks were observed by LC-MS in the cleavage mixture (Figure 8). ES-MS data for both peaks showed a combined mass for $(Leu)_5$ and **108** (Figure 9). This indicates that covalently

bound adducts of $(Leu)_5$ and **108** were formed. Formation of such an adduct in the absence of oxidants had never been reported in the Juliá-Colonna epoxidation and may provide insight into the mechanism of the reaction.

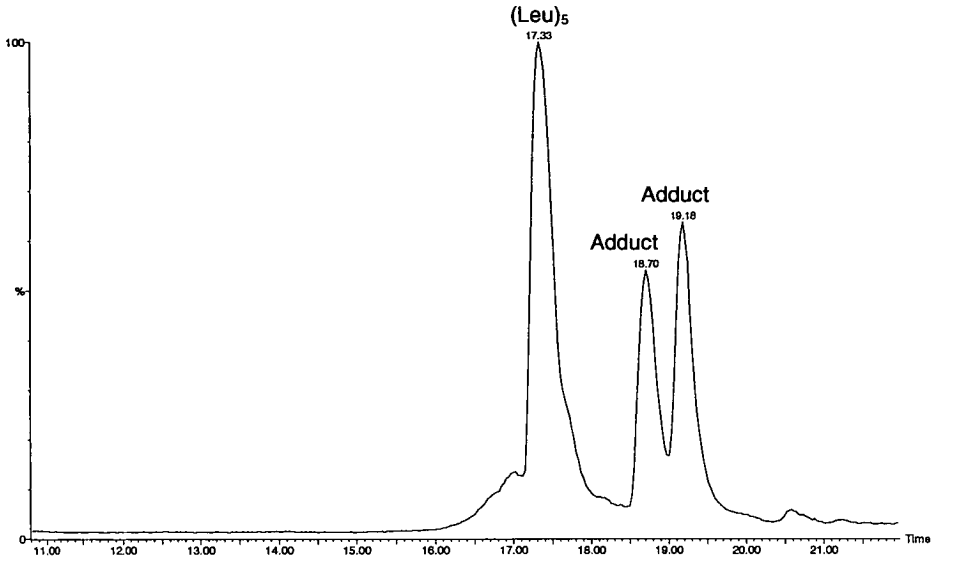


Figure 8 HPLC trace of $(Leu)_5$.**108** adducts

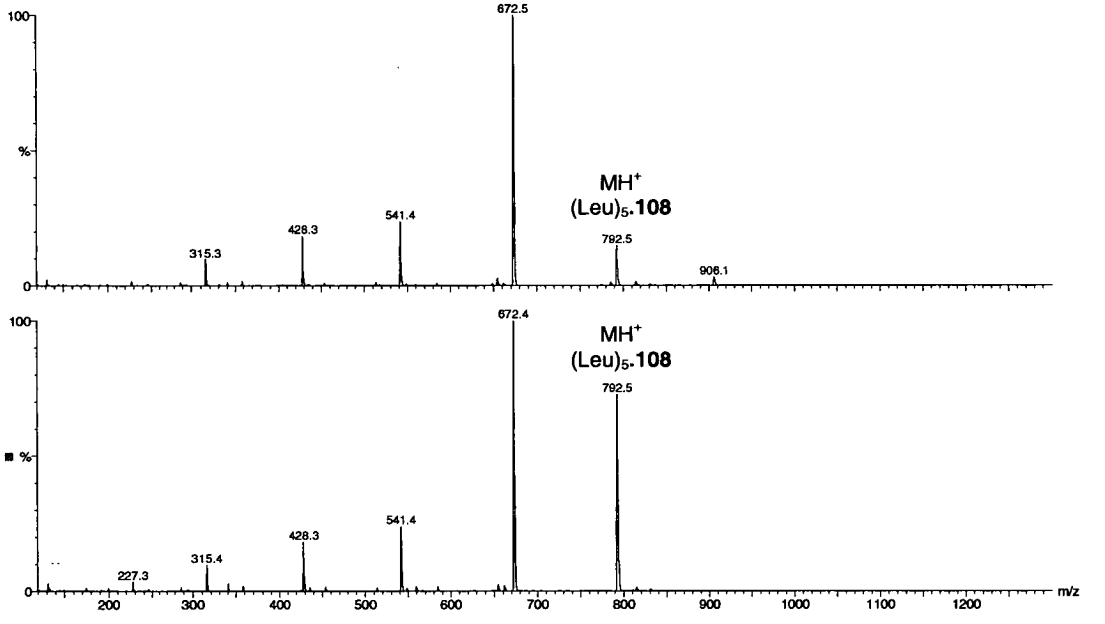
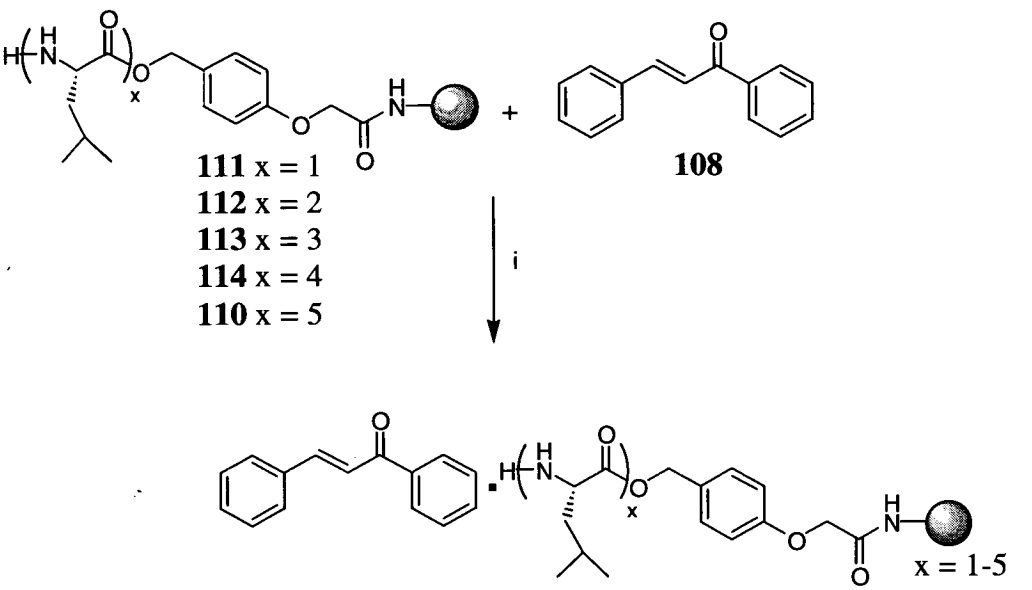


Figure 9 Mass spectrum of $(Leu)_5$.**108** adducts

This observation of adducts was further investigated by incubating **108** with PEGA immobilised peptides $(Leu)_1$ **111**, $(Leu)_2$ **112**, $(Leu)_3$ **113**, $(Leu)_4$ **114** and $(Leu)_5$ **110** in order to ascertain if formation of the poly-L-leucine.**108** adduct was specific for certain

peptide lengths. Immobilised $(Leu)_1$ – $(Leu)_5$ were synthesised from **90** using standard peptide coupling procedures according to Scheme 33. **108** was then incubated with **111**, **112**, **113**, **114** and **110** individually in 90% ACN in pH8 buffer (Scheme 36). Products were cleaved from resin with 95 % TFA in H_2O then analysed by LC-MS. Poly-L-leucine.**108** adduct was only observed for pentaleucine (Table 6). This suggests that one helical turn of the peptide chain is necessary for formation of the poly-L-leucine.**108** adduct. One might also speculate that the same factors that influence catalytic properties are relevant in adduct formation, although this needs further investigation.

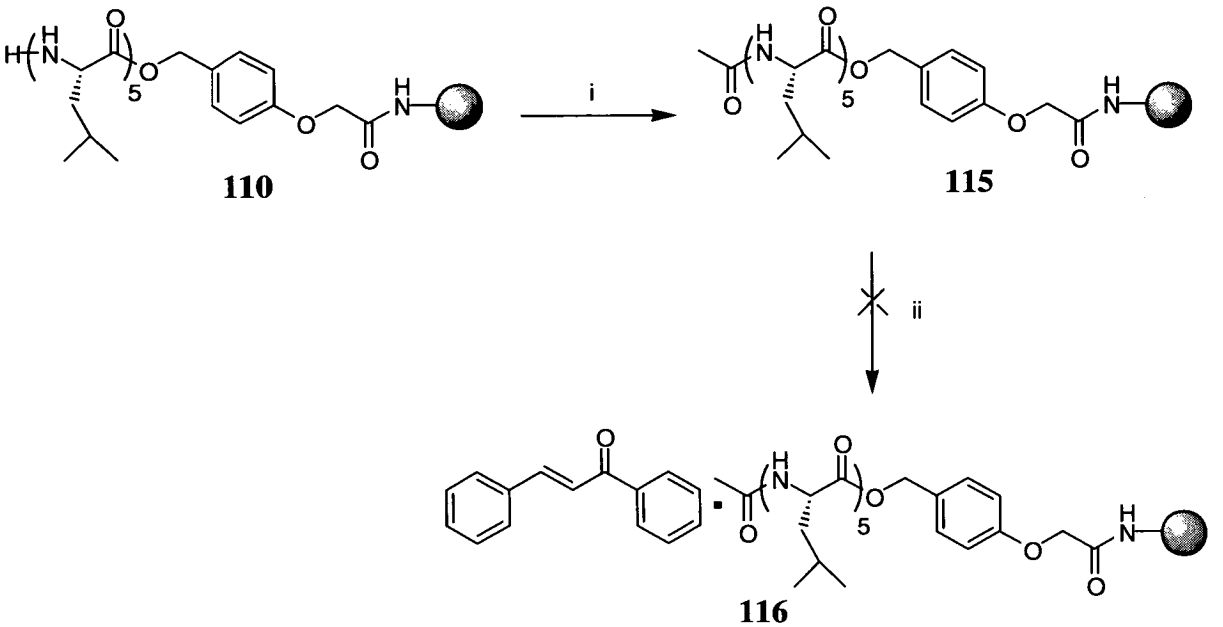


Scheme 36 *Reagents and Conditions:* (i) 90% ACN in KPi Buffer pH = 8, 18 hrs, 30°C.

Table 6 Table detailing formation of $(Leu)_x$.**108** adduct

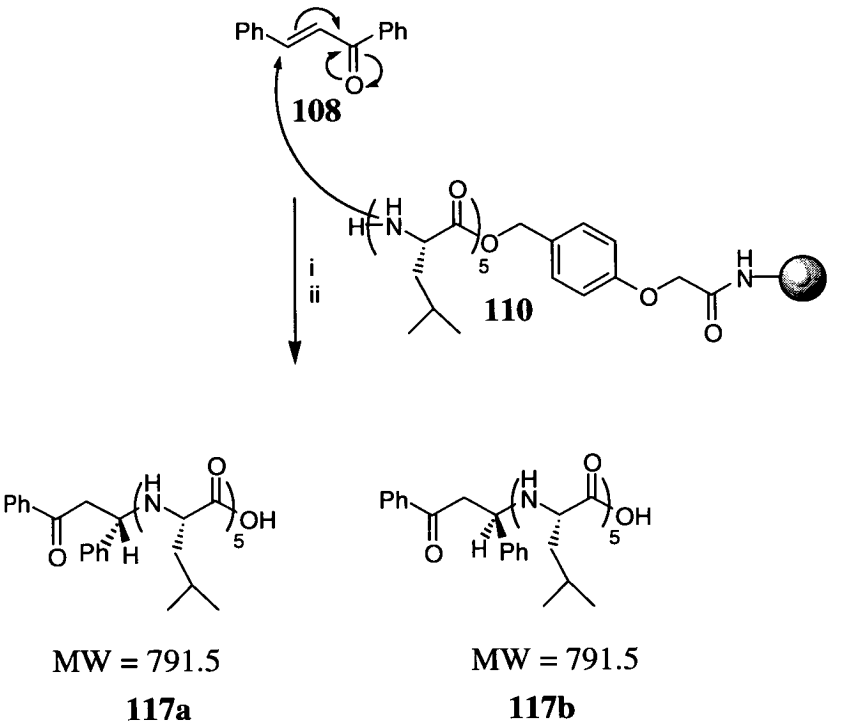
Poly-L-Leucine Oligomer	Formation of $(Leu)_x$.**108** Adduct
111 $(Leu)_1$	No
112 $(Leu)_2$	No
113 $(Leu)_3$	No
114 $(Leu)_4$	No
110 $(Leu)_5$	Yes

To gain further insight into the nature of the adduct formed, peptide **110** was treated with acetic anhydride to give acetylated $(Leu)_5$ **115** (Scheme 37). **108** was then incubated with the acetylated product **115** in 90 % ACN in buffer. Analysis of the cleavage cocktail by LC-MS showed only the presence of **115** confirming that adduct **116** had not been formed. This indicates that the free amine is essential for the adduct formation.



Scheme 37 *Reagents and conditions:* (i) Acetic anhydride, DIPEA, DMF, 1 hr, RT (ii) 90% ACN in KPi buffer pH = 8, 18 hr, 30°C.

Isolation of the adduct was attempted by mass directed purification. Unfortunately insufficient product was isolated for NMR studies. Based on data so far, we propose that a covalent bond is formed by nucleophilic attack of the amino terminus on the β carbon of the α, β-unsaturated ketone in a Michael addition to give the ketone **117** (Scheme 38). The presence of two peaks with identical mass could be explained by the formation of two diastereoisomers **117a** and **117b**.



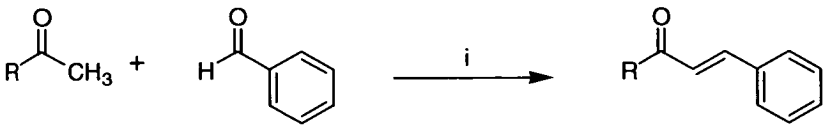
Scheme 38 Proposed structure of $(Leu)_5$.chalcone adduct. *Reagents and conditions:* (i) 90% ACN in KPi Buffer pH = 8, 18 hrs, 30°C (ii) 95 % TFA in H_2O

3.5 Replacement of Chalcone with Hydrophilic Analogues

Given that organic co-solvents inhibit library formation a template was sought that was compatible with conditions under which our libraries were formed. Several hydrophilic analogues of chalcone were synthesised as potential templates.

3.5.1 Synthesis of Chalcone Analogues

An aminophenyl derivative **118** and a furanyl derivative **119** were synthesised according to literature procedures [8, 9] from commercially available acetophenones **120** and **121** and benzaldehyde **122** *via* the Murphy and Wattanasin method of aldol condensation [10] (Scheme 39).

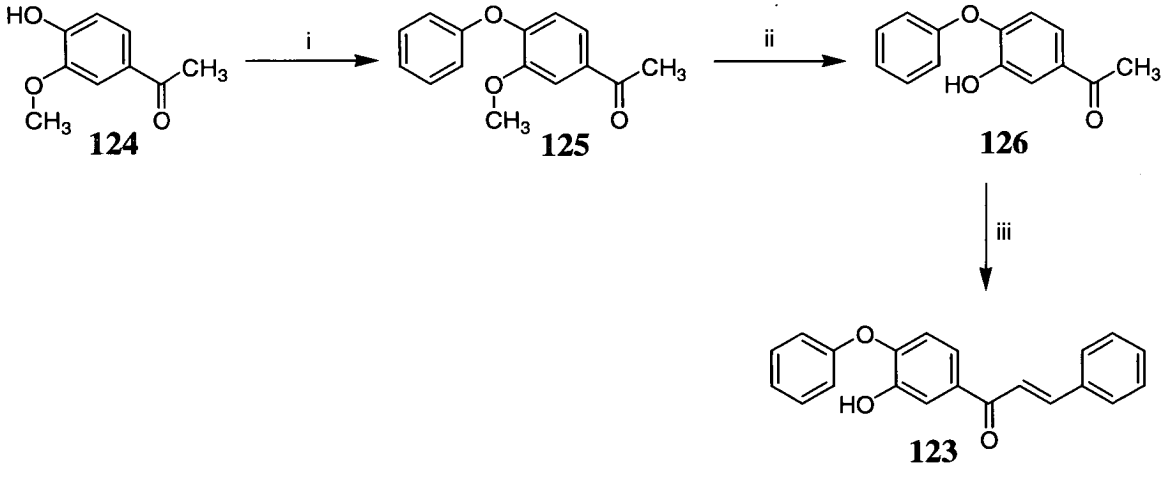


120, 121 **122** **118, 119**

118, 120 R = C_6H_5-NH_2
119, 121 R = C_4H_4O

Scheme 39 *Reagents and conditions:* (i) NaOH, EtOH, 18 hr, 0°C

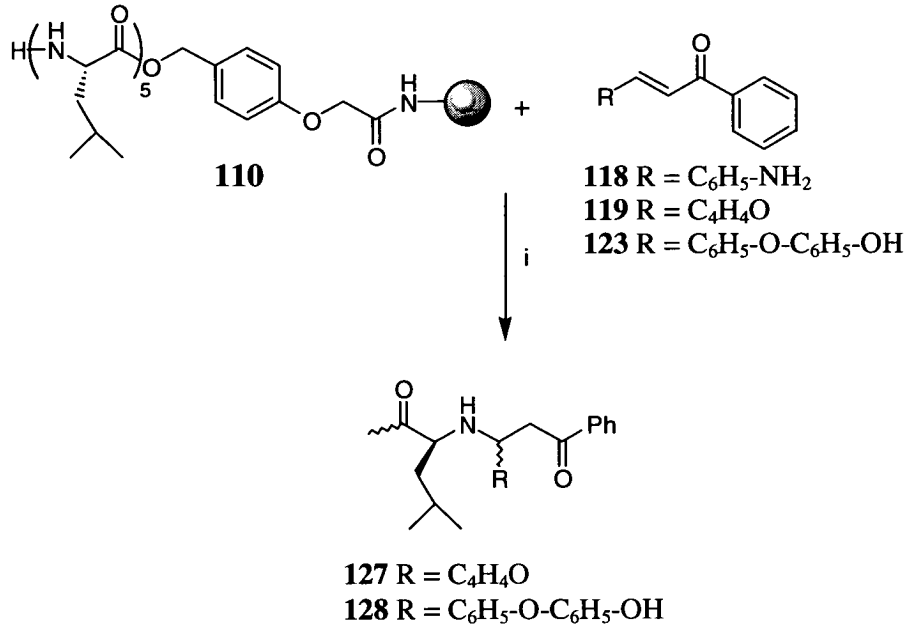
The phenoxyphenyl derivative **123** was synthesised in 3 steps from commercially available 3-methoxy-4-hydroxyacetophenone according to Gilmore *et al* [11] (Scheme 40). Phenylboronic acid was coupled to the hydroxy moiety of **124** using copper II acetate and triethylamine in DCM saturated with air. The methoxy group of **125** was demethylated with boron tribromide to give the phenol **126**. The acetophenone **126** was then converted to the desired α,β-unsaturated enone **123** via a Murphy and Wattanasin condensation with **122**.



Scheme 40 *Reagents and conditions:* (i) $Cu(OAc)_2$, $PhB(OH)_2$, Et_3N, CH_2Cl_2, air, 48 hr, rt (ii) BBr_3, CH_2Cl_2., 3 hr, -78 to 0°C (iii) **122** NaOH, EtOH, 18 hr, 0°C.

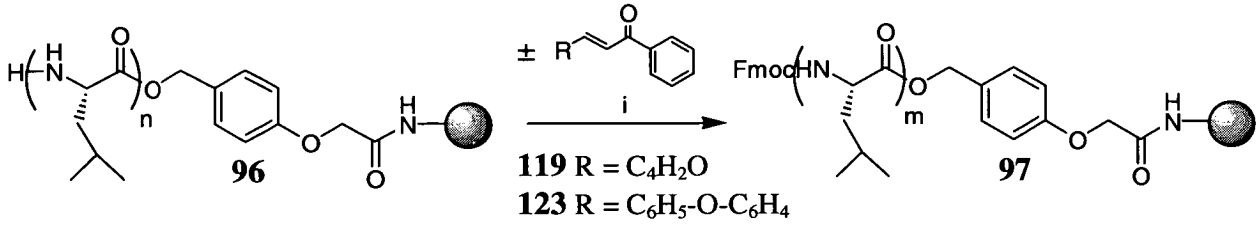
Compatibility of templates **118, 119** and **123** with library conditions was tested by reacting templates with **110** (Scheme 41). It was reasoned that if templates were indeed compatible with the conditions of library formation then analogues of adduct **117** would be formed. Templates **118, 119** and **123** were individually incubated with **110** under the aqueous conditions required for successful library formation. **119** and **123** gave the

desired adduct with proposed structures **127** and **128.** However, LC-MS analysis showed that **118** did not form the expected adduct with **110**.



Scheme 41 *Reagents and conditions:* (i) KPi buffer pH = 8, 18 hr, 30°C

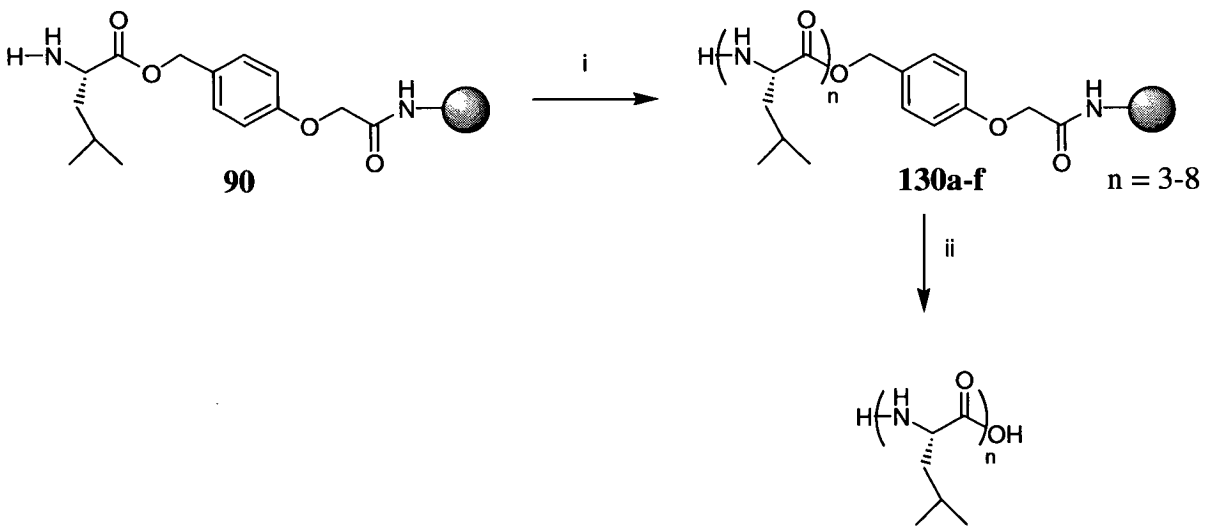
Given the fact that **119** and **123** successfully formed adducts with **110** under conditions required for the formation of libraries, **119** and **123** were introduced to library **97** as templates. Library **97** was generated from library **96** in the presence and absence of **119** and **123** (Scheme 42). Again, comparison of the LC-MS chromatograms of the cleaved libraries generated in the presence and absence of **119** and **123** showed there to be no difference in product distribution. As **119** and **123** have been shown to be compatible with library formation, the lack of shift was attributed to the inhibited binding of **119** and **123** by the Fmoc protecting group at the amino terminus of the peptide chain.



Scheme 42 *Reagents and conditions:* (i) FmocLeu, KPi buffer pH = 8, 18 hr, 30°C

3.6 Generation of Libraries from $(Leu)_2$

Since the lack of shift in distribution of Fmoc-protected libraries such as **97** was thought to be due to Fmoc protection, the generation of an unprotected library was required. Thermolysin is known to be an endopeptidase [12] and therefore requires protected substrates for synthesis such as the Fmoc protected substrates employed for synthesis so far. Alternatively, in order to generate an unprotected library short oligomers such as $(Leu)_2$ **129** may be employed as substrates in place of FmocLeu. $(Leu)_2$ **129** was therefore incubated with PEGA bound leucine **90** in the presence of thermolysin (Scheme 43). After 18 hours the resin was thoroughly washed followed by acidic cleavage of the products from the resin. Results were promising and an unprotected library of poly-L-leucine **130a-f** varying from trimers to octamers was observed by LC-MS (Figure 10).



Scheme 43 *Reagents and conditions*: (i) $(Leu)_2$ **129**, thermolysin, KPi buffer pH = 8, 18 hr, 30°C (ii) 95 % TFA in H_2O

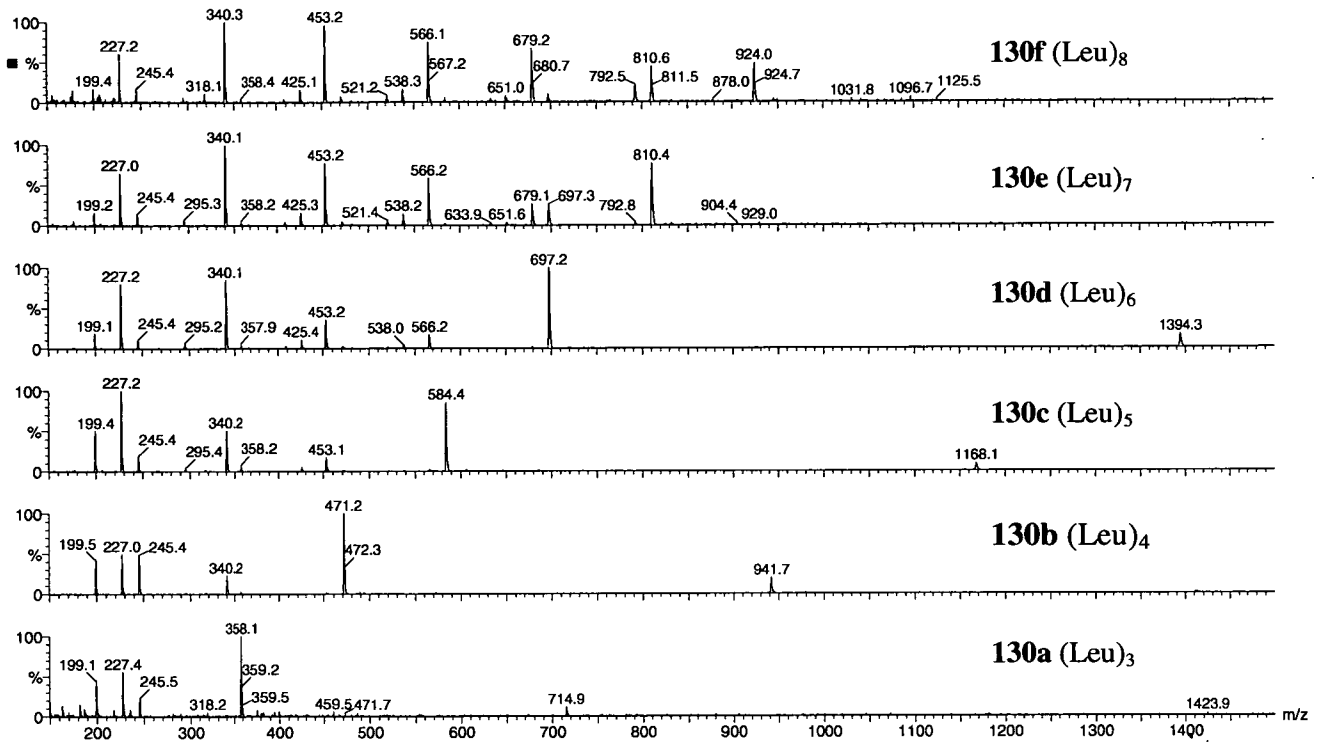


Figure 10 Mass spectra of unprotected poly-L-leucine library members **130a-f**

The RPPC of the library was calculated from TIC data as for Chapter 2, Section 2.3.1, and summarised in the table below (Table 7).

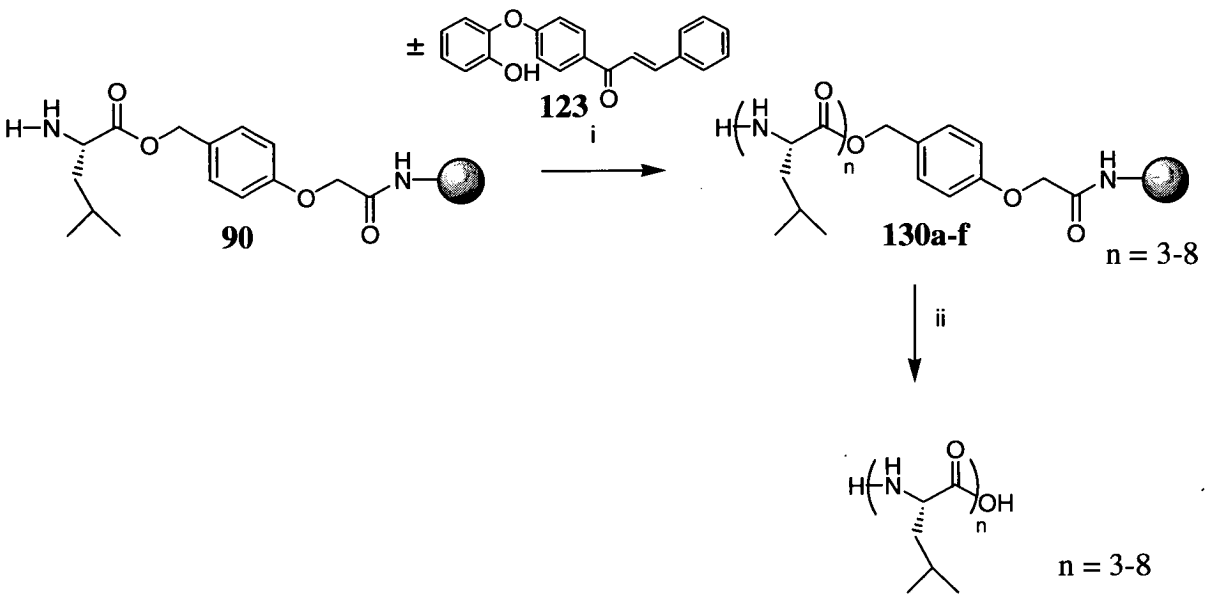
Table 7 Table of RPPC values for poly-L-leucine library members **130a-f**

Library Member	RPPC
130a $(Leu)_3$	1.0 %
130b $(Leu)_4$	26.6 %
130c $(Leu)_5$	7.1 %
130d $(Leu)_6$	55.1 %
130e $(Leu)_7$	8.5 %
130f $(Leu)_8$	1.8 %

$(Leu)_6$ **130d** was found to be the most dominant product in the final mixture followed by $(Leu)_4$ **130b**. $(Leu)_5$ **130c** and $(Leu)_7$ **130e** are the next most prevalent products and

were present with similar RPPCs. $(Leu)_3$ **130a** and $(Leu)_8$ **130f** were the least dominant products and also had similar compositions.

Library **130** was generated in the presence of target **123**. This was done in duplicate and also in parallel with library free of **123** (Scheme 44).



Scheme 44 *Reagents and conditions*: (i) $(Leu)_2$, thermolysin, KPi buffer pH = 8, 18 hr, 30°C (ii) 95 % TFA in H_2O

Quantitative LC-MS analysis of the products cleaved from the resin showed the results for these experiments to be promising (Figure 11). The templated library showed a 7 % increase in the composition of **130c** $(Leu)_5$ and a 13.5 % shift towards **130d** $(Leu)_6$ mostly at the expense of **130b** $(Leu)_4$ which decreased by 28% (Table 8 and 9). The RPPC of library free of template showed good agreement with previous data. Interestingly, no adducts between library members and **123** were observed.

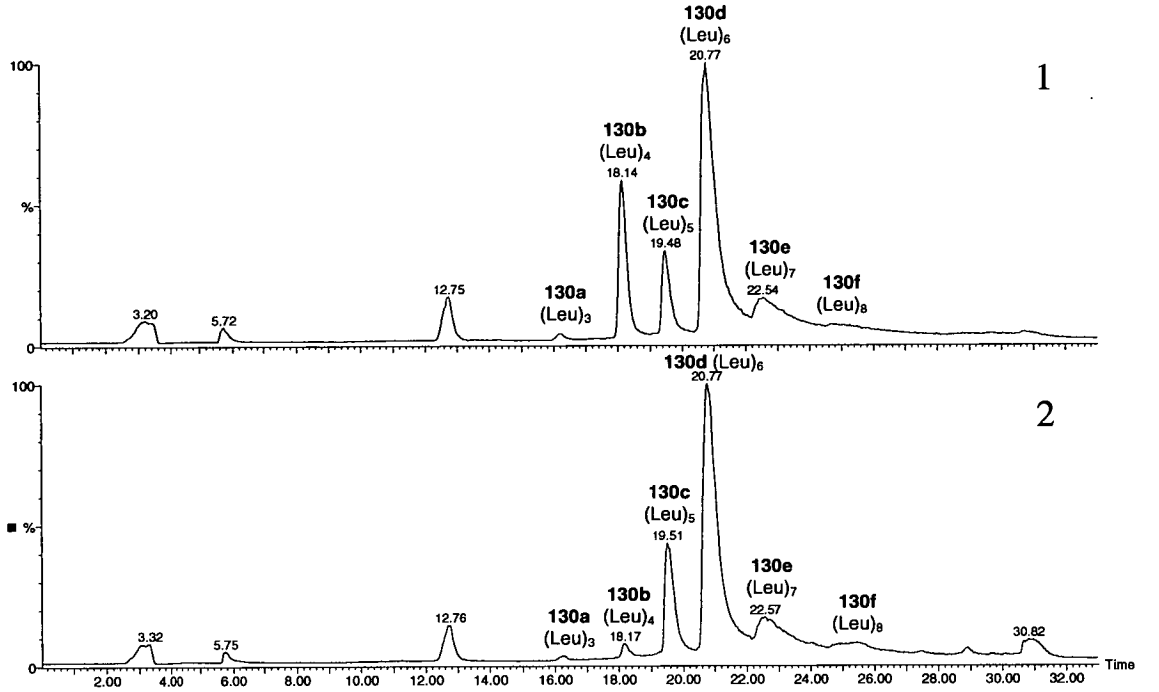


Figure 11 (1) Poly-L-leucine library **130** generated in the absence of **123** (2) Poly-L-leucine library **130** generated in the presence of **123**

Table 8 Average RPPC of library **130** generated in the presence and absence of **123**

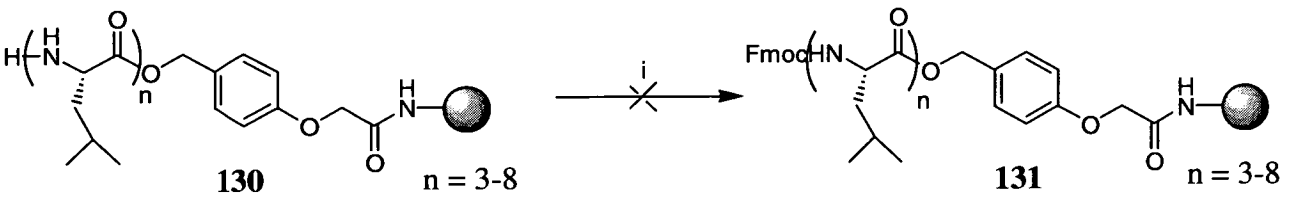
Library Member	RPPC of library **130** generated in the absence of **123**	RPPC of library **130** generated in the presence of **123**
130a $(Leu)_3$	2.2 %	1.0 %
130b $(Leu)_4$	29.9 %	1.9 %
130c $(Leu)_5$	9.1 %	16.1 %
130d $(Leu)_6$	48.5 %	68.3 %
130e $(Leu)_7$	8.0 %	10.0 %
130f $(Leu)_8$	2.2 %	2.7 %

3.7 UV Tagging of Unprotected Libraries

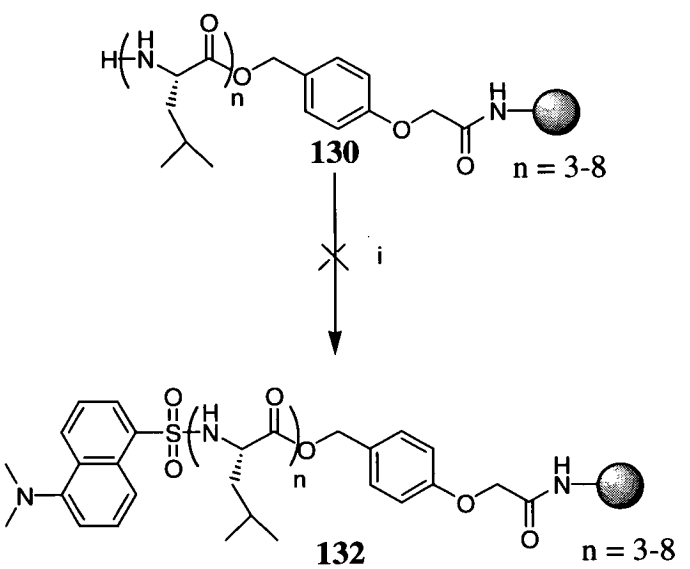
The signal-to-noise ratios in LC-MS chromatograms of the poly-L-leucine libraries at 214 nm was poor due to a background TFA signal. In order to improve sensitivity, incorporation of a UV-active tag that absorbs in alternative UV region was investigated.

3.7.1 Tagging at the Amino Terminus of Peptide Libraries

Initially attachment of UV active tags at the amino terminus was attempted by treatment of **130** with Fmoc chloroformate (Scheme 45) and dansyl chloride (Scheme 46). However, neither of these methods yielded the desired tagged libraries.

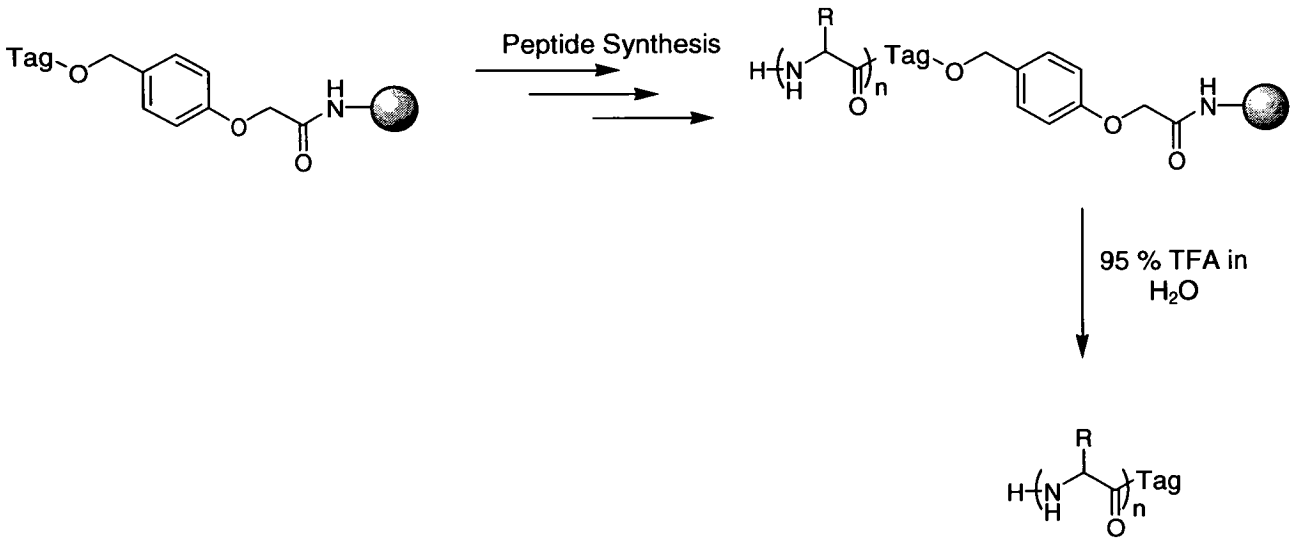


Scheme 45 *Reagents and Conditions* : (i) Fmoc-Cl, $NaHCO_3$, H_2O, Dioxane, 18 hr, rt



Scheme 46 *Reagents and Conditions*: (i) Dns-Cl, DIPEA, DMF, 12 hr, rt

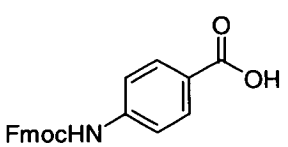
An alternative strategy of incorporating tags at the carboxy terminus after the Wang linker was investigated (Scheme 47). All subsequent peptides would therefore be synthesised onto the tag ensuring all products cleaved from the resin contained a chromophore.



Scheme 47 Alternative strategy incorporating UV active tag after Wang linker

3.7.2 Tagging at the Carboxy Terminus of Peptide Libraries

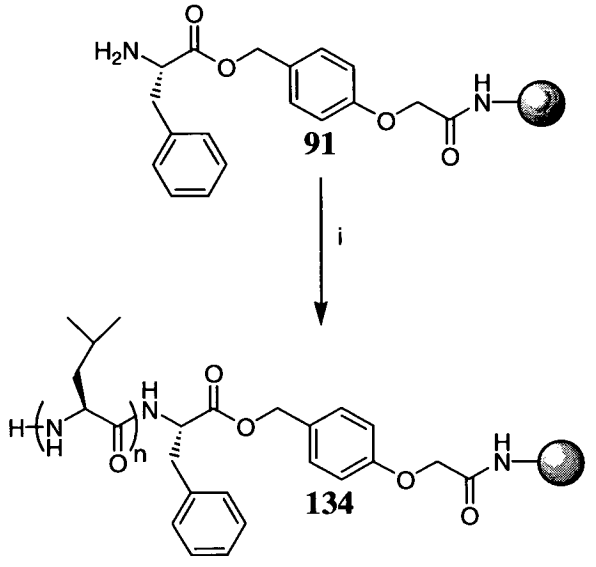
Fmoc *para* amino benzoic acid **133** was identified as a suitable tag at the carboxy terminus. **133** was attached to the Wang linker of **86** *via* the symmetrical anhydride method described in Chapter 2, Scheme 25. The Fmoc group was removed from the resin bound **133** by treatment of the resin with 20 % piperidine in DMF. FmocLeu was then coupled to the deprotected **133** using standard peptide coupling reagents TBTU, HOBt and DIPEA in DMF. The resulting product was cleaved from the resin by treatment with 95% TFA in H_2O followed by analysis by LC-MS. Although the product gave a good UV response, the MS data was poor. Further investigation found that PABA gave an improved response under negative ionisation. This renders PABA an unsuitable tag as it is difficult to completely remove all the TFA from the cleavage mixture thus limiting mass spectrometry to positive ionisation. **133** was therefore replaced by phenylalanine which is known to respond under positive ionisation.



133

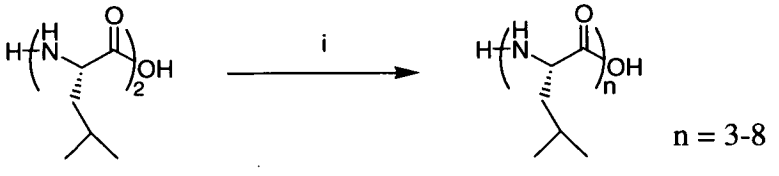
$(Leu)_2$ **129** was incubated with PEGA bound phenylalanine **91** in the presence of thermolysin (Scheme 48). LC-MS analysis of products cleaved from the resin showed

that a poly-L-leucine library had been generated. However, MS data revealed that the library was untagged and **134** had not been formed. Analysis of these unexpected results suggested that a poly-L-leucine library had formed simultaneously in solution.



Scheme 48 *Reagents and conditions:* $(Leu)_2$, Thermolysin, KPi buffer pH = 8, 18 hr, 30°C

To confirm this $(Leu)_2$ **129** was incubated with thermolysin in the absence of **91** (Scheme 49) using the same conditions described in Scheme 24. As expected a library of poly-L-leucine oligomers **135** was observed by LC-MS.



129 **135**

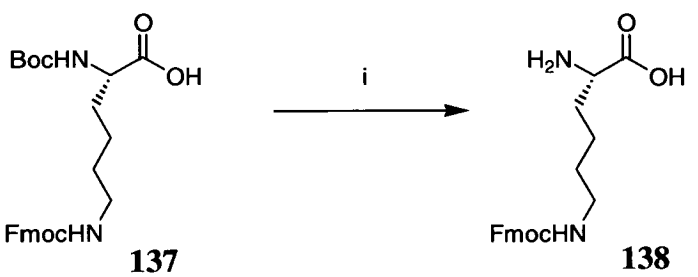
Scheme 49 *Reagents and conditions*: (i) Thermolysin, KPi buffer pH = 8, 18 hr, 30°C

It was thought that the signals for **134** were masked as **135** may have been produced simultaneously in a higher concentration and co-eluted with the tagged **134**. These results were unexpected since the resin had been washed very thoroughly after the solid phase synthesis and yet **135** formed in the solution phase seemed to stick to the resin and proved difficult to wash away. More rigorous washing procedures were therefore employed. **134** was first washed with 5 % TFA in 1:1 ACN/H_2O and then 6M

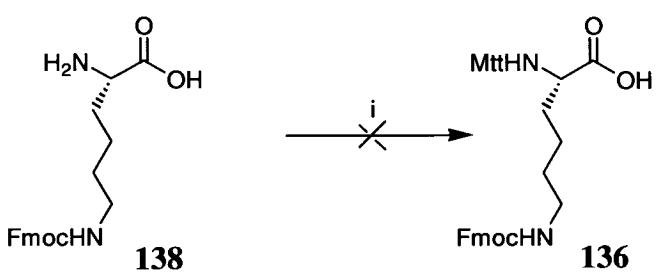
guanidine.HCl. Analysis of the cleavage mixture still showed residual untagged **135** and no **134** was observed.

To prevent co-elution of **134** with **135** the design of the tag was revised to contain a bulky hydrophobic side chain. Phenylalanine was therefore replaced with N^{α}-Mtt-N^{ε}-Fmoc-lysine **136**. It was envisaged that the Mtt protecting group may be selectively removed with 2% TFA in DCM without compromising the Wang linker. The Fmoc side chain protecting group may then remain acting both as a UV chromophore and increasing the hydrophobicity of the resin bound poly-L-Leucine.

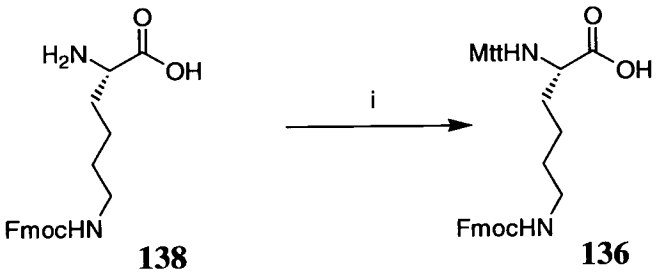
136 was prepared in a two step synthesis. N^{ε}-Fmoc-Lys **138** was obtained from **137** by removal of the Boc group with 50:50 TFA in DCM (Scheme 50). Reaction of the resulting amine **138** with Mtt chloride was attempted using conditions described in Novabiochem [13] (Scheme 51). However, coupling was unsuccessful and for this reason conditions previously applied by Barlos *et al* [14] were employed (Scheme 52). In a "one pot" synthesis the acid moiety was temporarily protected with trimethylsilane. Mtt chloride was then coupled to amine followed by mild hydrolysis of the ester with methanol to give **136**.



Scheme 50 *Reagents and conditions* (i) 50:50 TFA:DCM, 1 hr, rt

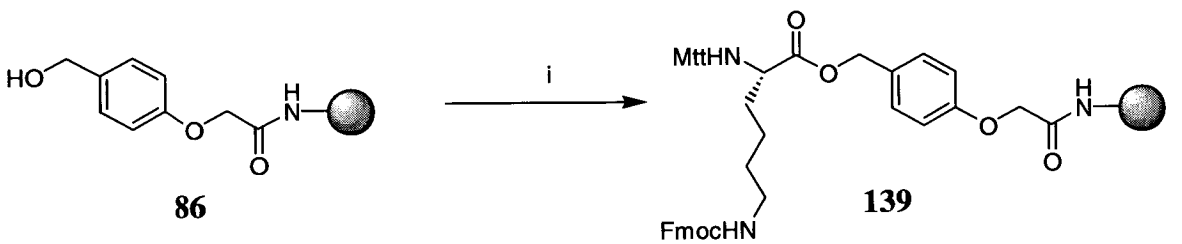


Scheme 51 *Reagents and conditions*: (i) Mtt-Cl, Et_3N, DCM, 24 hr, rt



Scheme 52 *Reagents and conditions*: (i) 1. Me_3SiCl, Et_3N, DCM 2. Mtt-Cl, Et_3N 3. MeOH

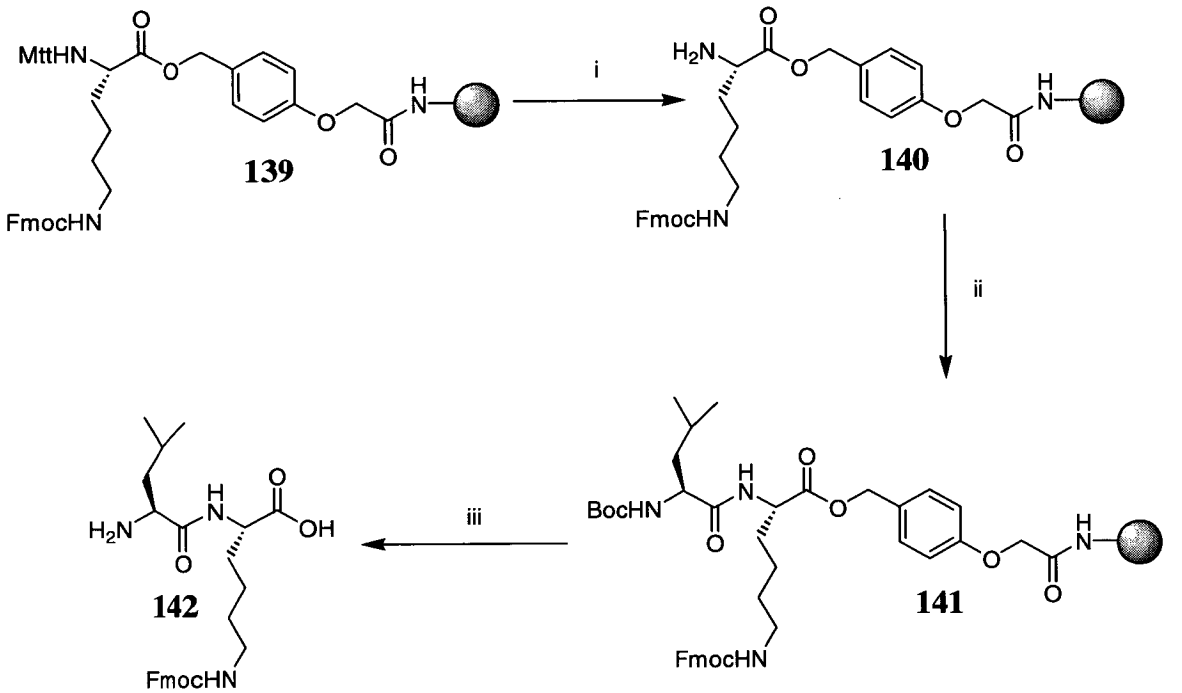
As trityl protected amino acids are known to encounter difficulties during anhydride formation [15], **136** was coupled to resin **86** using *in situ* coupling reagents DIC, HOBt and DMAP (Scheme 53). The methyl red-diphenyldichlorosilane test confirmed all Wang hydroxyl groups had reacted.



Scheme 53 *Reagents and conditions*: (i) DIC, HOBt, DMAP, DMF, 18 hr, rt

There had been few literature reports of trityl protected amino acids being used in conjunction with PEGA resin, therefore compatibility of trityl chemistry on PEGA was tested by synthesis of dipeptide **142**. The Mtt protecting group of **139** was removed with 2% TFA in DCM to give **140** (Scheme 54). BocLeu was subsequently coupled to **140** using standard peptide coupling reagents TBTU, HOBt amd DIPEA in DMF. **141** was treated with 95% TFA in H_2O in order to simultaneously deprotect and cleave products from the resin to give **142**. LC-MS analysis of the cleavage mixture showed the synthesis to be unsuccessful. Given the apparent success of attachment of **136** to the solid support synthesis failure was attributed to insufficient deprotection of Mtt from **139**. Consequently, to improve the efficiency, deprotection was repeated to include a TIS scavenger. **139** was therefore treated with 2:5:93 TFA:TIS:DCM. The deprotection step was then carried out a second time to ensure complete removal of the Mtt moiety to give **140**. BocLeu was then coupled to **140** using TBTU, HOBt and DIPEA to give **141**. **141** was again treated with 95 % TFA in H_2O to give **142**. LC-MS analysis of the

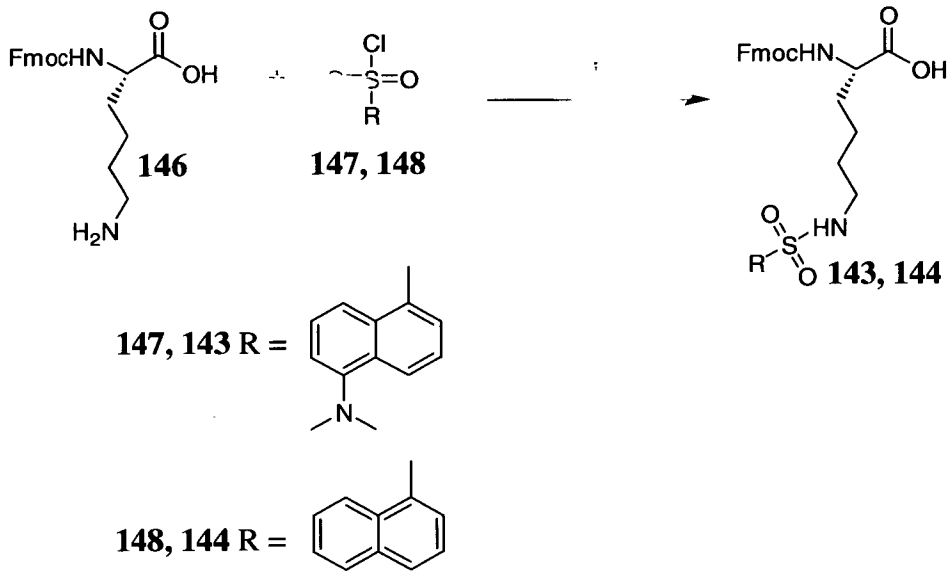
cleavage mixture showed that **142** had still not been formed. As a result both loading of **136** onto resin and deprotection of **139** were readdressed. HATU was used in place of DIC as an alternative for coupling hindered **136** to **86**. Deprotection of **139** using 2:5:93 TFA:TIS:DCM was also increased from two cycles to five. Again, BocLeu was coupled to the resulting **140** using TBTU, HOBt and DIPEA to give **141**. Treatment of **141** with 95 % TFA in H_2O and subsequent LC-MS analysis showed that **142** had not been formed. Work carried out by Matysiak *et al* has shown some trityl based protecting groups to be stable on solid supports containing PEG chains[16]. It was therefore concluded that the Mtt group could not be removed due to its increased stability facilitated by the PEG chains of the PEGA resin.



Scheme 54 *Reagents and conditions*: (i) 2% TFA in DCM, 2 min, rt (ii) BocLeu, TBTU, HOBt, DIPEA, DMF, 2 hr, rt (iii) 95% TFA in H_2O

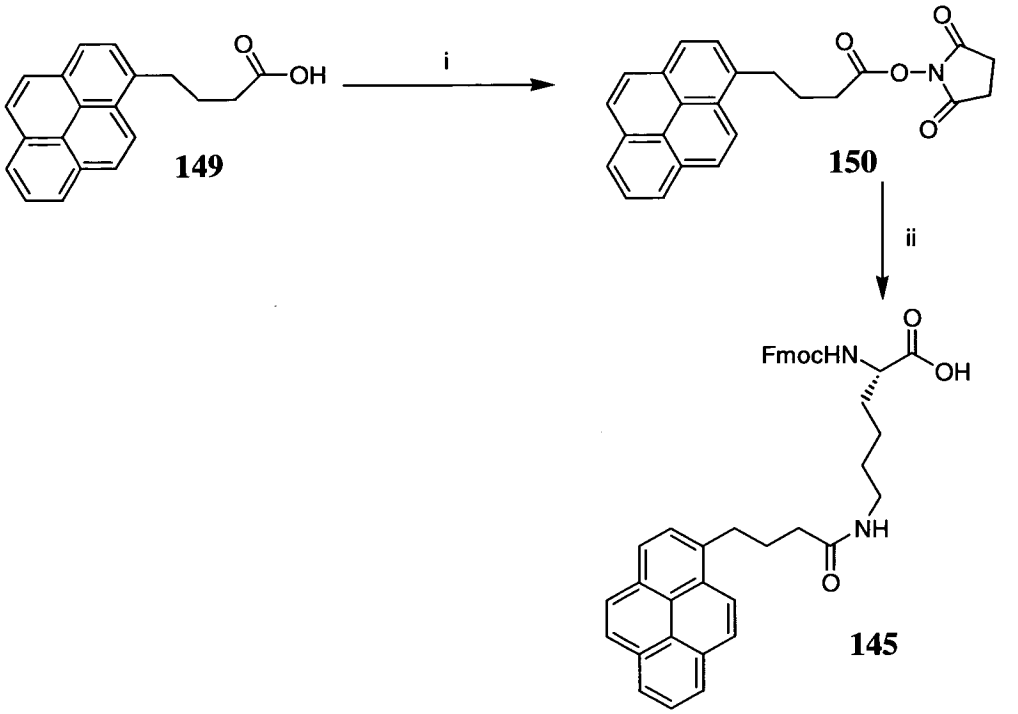
As the Mtt protecting group had been shown to be incompatible with PEGA resin, tags were redesigned to employ N^{α}-Fmoc protection of Lys together with a "permanent" covalent bond at the side chain. Side chains were chosen that contained UV active, bulky, hydrophobic groups. Three potential N^{α}-Fmoc protected lysine tags were synthesised: FmocLys(Dns) **143**, FmocLys(Npsyl) **144** and FmocLys(Pyrene) **145**.

Tags **143** and **144** were prepared by reaction of N^{α}-FmocLys **146** with commercially available sulphonyl chlorides **147** and **148** to give the desired sulphonamide bonds (Scheme 55).



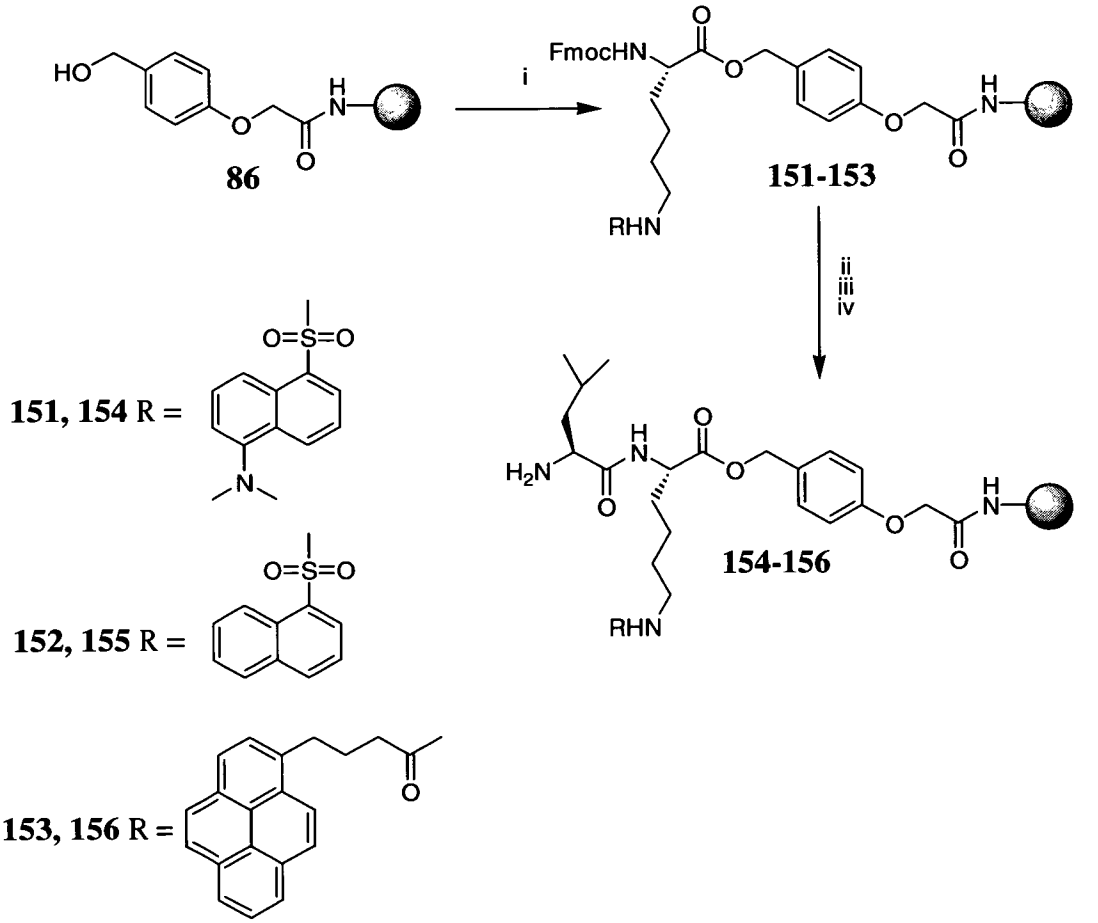
Scheme 55 *Reagents and conditions*: (i) Na_2CO_3, 70:30 THF:H_2O, 1 hr rt

Tag **145** was synthesised using a modified two step literature procedure [17] (Scheme 56). Active ester **150** was prepared by EDCI mediated condensation of 4-pyrenebutanoic acid **149** with *N*-hydroxysuccinimide . **150** was then reacted with N^{α}-FmocLys **146** to give the desired amide bond **145**.



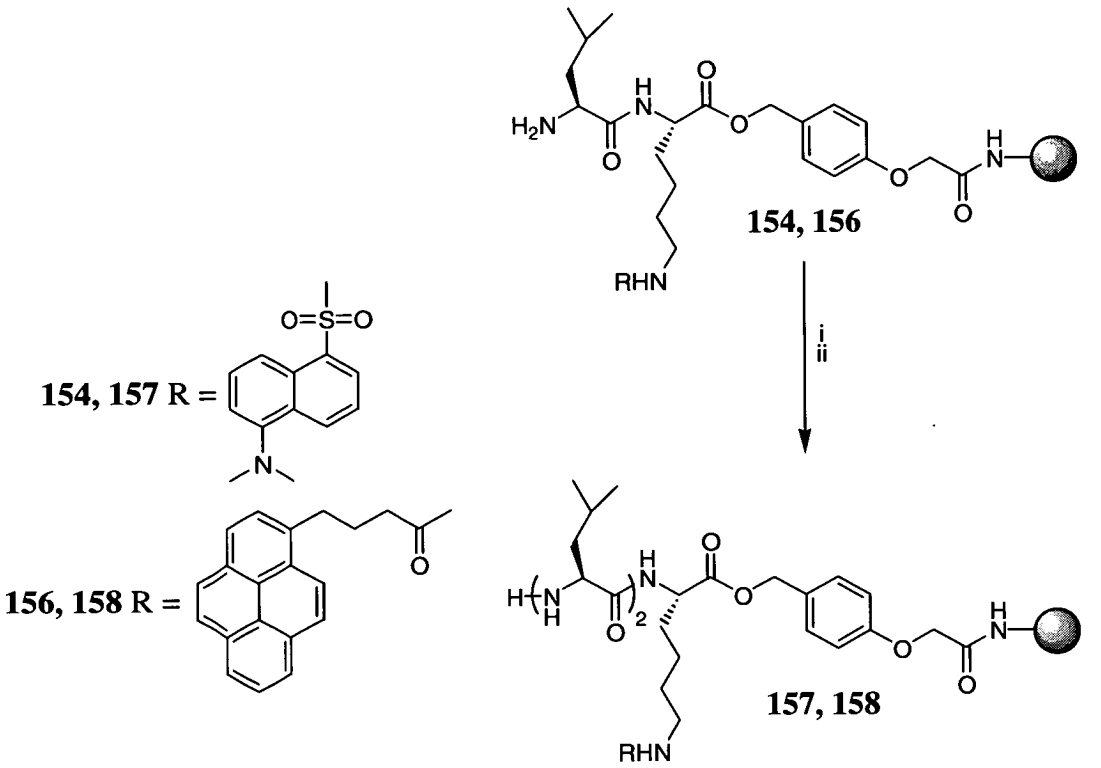
Scheme 56 *Reagents and Conditions*: (i) *N*-Hyroxysuccinimide, EDCI, DMF, 16hr, rt (ii) FmocLys, Na_2CO_3, 70:30 THF:H_2O, 1 hr, rt

143-145 were coupled to resin **86** *via* the preformed symmetrical anhydride (Scheme 57). **143-145** were treated with DIC then added to **86** which had been swollen in DMF in the presence of DMAP to give **151-153**. **151-153** were then deprotected with 20 % piperidine in DMF and FmocLeu was subsequently coupled using TBTU, HOBt and DIPEA. The Fmoc of the Leu residue was then removed with 20 % piperidine in DMF to give tagged dipeptides **154-156**. A portion of resin **154-156** was treated with 95% TFA in H_2O to release product from the support. LC-MS analysis of the cleavage cocktail confirmed the presence of **154** and **156**.



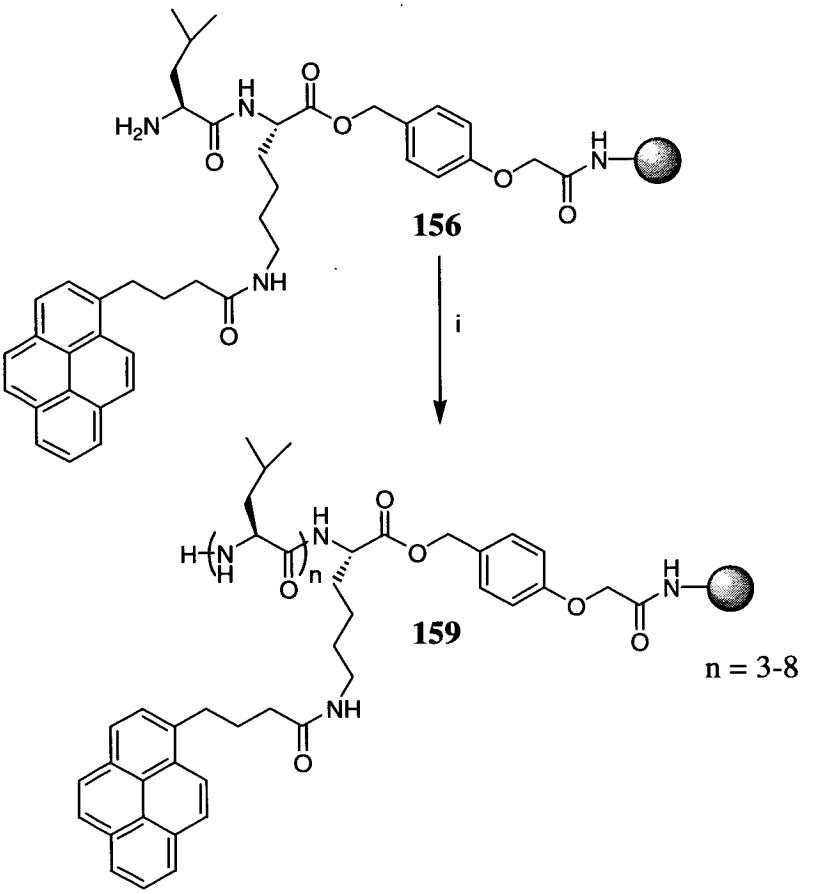
Scheme 57 *Reagents and conditions*: (i) **143**, **144** or **145**, DIC, DMAP, DMF (ii) 20 % piperidine in DMF (iii) FmocLeu, TBTU, HOBt, DIPEA, DMF (iii) 20% piperidine in DMF

To test if enzymatic synthesis on **154** and **156** was possible, FmocLeu was incubated with **154** and **156** in the presence of thermolysin (Scheme 58). The resulting products were deprotected with 20 % piperidine in DMF to give tagged dileucine **157** and **158**. Acidic cleavage and subsequent LC-MS analysis showed both tripeptides **157** and **158** had been formed.



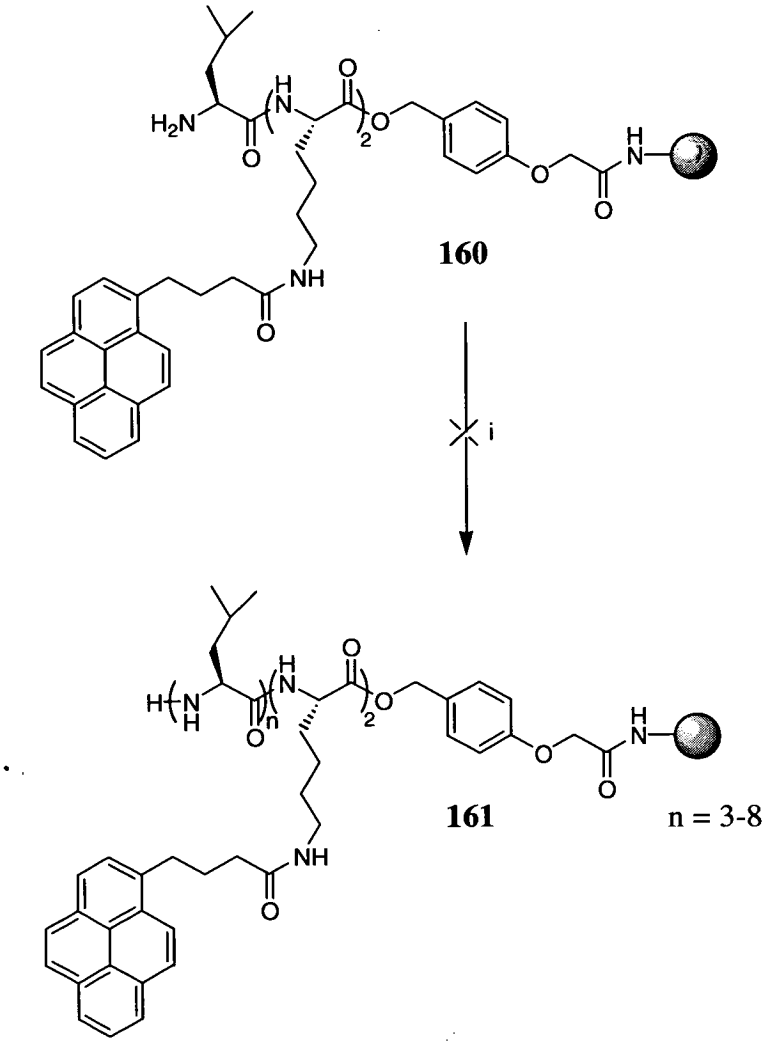
Scheme 58 *Reagents and conditions*: (i) FmocLeu, thermolysin, KPi buffer pH = 8, 18 hr, 30°C (ii) 20 % piperidine in DMF

Given that enzymatic synthesis on **154** and **156** was successful, generation of tagged unprotected poly-L-Leu library **159** was attempted and $(Leu)_2$ **129** was incubated with **156** in the presence of thermolysin (Scheme 59). LC-MS analysis of cleaved products showed a mixture of **156** and the untagged poly-L-leucine library **135** that had been simultaneously formed in solution to be present in the final reaction mixture. **159** was found not to be present. Co-elution of **156** and poly-L-leucine library **135** solution phase peptides was, however, still apparent and it was thought that the signals for **159** may be masked by those of **135**.



Scheme 59 *Reagents and conditions*: (i) $(Leu)_2$, thermolysin, KPi buffer pH = 8, 18 hr, 30°C

In order to further increase the hydrophobicity of the tag, two **145** residues were coupled to **86** after the Wang linker followed by a leucine residue to give **160**. $(Leu)_2$ **129** was then incubated with **159** in the presence of thermolysin in an attempt to generate library **161** (Scheme 60). LC-MS analysis showed the tag **160** and free poly-L-leucine library **135** were now separated by HPLC (Figure 12). However, **161** was not observed indicating that only the untagged solution phase poly-L-leucine library **135** could be generated from $(Leu)_2$.



Scheme 60 *Reagents and conditions*: (i) $(Leu)_2$, thermolysin, KPi buffer pH = 8, 18 hr, 30°C

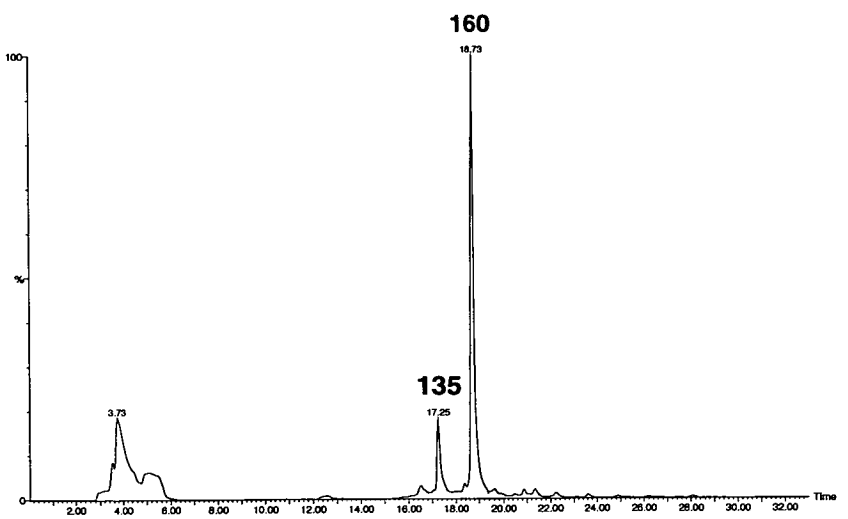


Figure 12 HPLC trace of separated tag **160** and solution phase poly-L-Leu library **135**

Highly concentrated suspensions of amino acids are known to undergo solid to solid transfer reactions [18, 19]. In such reactions the synthesis/hydrolysis equilibrium of a protease is shifted towards synthesis by precipitation of products from the solution. It seemed reasonable that such a reaction may compete with the formation of immobilised **161** resulting in the formation of only **135** in the reaction mixture. This was investigated further by decreasing the number of $(Leu)_2$ equivalents in the reaction described in Scheme 60 from ten to five and two. In both cases, **161** was not observed thus confirming that an unprotected poly-L-leucine library could not be formed on solid phase using the present conditions.

3.8 Summary and Conclusions

N^{α}-Fmoc protected poly-L-leucine library **97** could be generated in the presence and absence of **108**. Comparison of these libraries generated in the presence and absence of **108** showed that **108** did not influence the distribution of the library. It was thought that **108** was not sufficiently soluble in aqueous buffer to facilitate binding of **108** to poly-L-leucine and that Fmoc-protection may inhibit the binding of **108**. Organic solvents were employed to aid the solubilisation of **108** but were found to inhibit the formation of library **97**. However, it was found that an apparently covalent bond between PEGA bound $(Leu)_5$ **110** and **108** was formed to give an adduct of proposed structure **117**. On further investigation it was found that the poly-L-leucine.**108** adduct was not formed with PEGA bound poly-L-leucine oligomers smaller than $(Leu)_5$ or acetylated PEGA bound $(Leu)_5$ **115**. It was speculated that the same factors that influence the catalytic properties of poly-L-leucine may also be relevant in adduct formation. Given that PEGA bound libraries were limited to generation in aqueous buffer, template **108** was replaced with hydrophilic analogues **118**, **119** and **123**. Adduct formation was further employed as a probe to test if hydrophilic analogues **118**, **119** and **123** were sufficiently soluble in aqueous buffer to be used as templates. **119** and **123** were found to form adducts of proposed structure **127** and **128** and were therefore employed as templates.

To overcome binding problems associated with Fmoc protection the synthesis of unprotected poly-L-leucine libraries was the attempted using $(Leu)_2$ as substrate in place

of FmocLeu. However, poly-L-leucine libraries **159** and **161** could not be detected on solid phase. It is interesting to note that poly-L-leucine libraries were only formed in solution. The solution phase formation of these libraries was further investigated.

3.9 References

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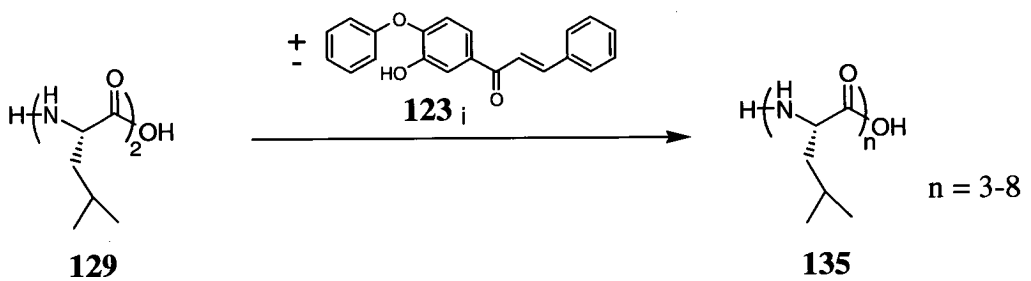
4. Solution Phase Dynamic Peptide Libraries

4.1 Introduction

Chapter 3 demonstrated that an unprotected poly-L-leucine library **135** could be formed in solution. Section 3.6 indicated that the equilibrium of this library may have been successfully shifted by generation of the poly-L-leucine library in the presence of **123**. These results have prompted our investigation into the generation of dynamic peptide libraries in solution phase and identification of best peptide binders by templating.

4.2 Templated Solution Phase Peptide Library in Buffer

Poly-L-leucine library **135** was generated from $(Leu)_2$ **129** with thermolysin in the presence and absence of **123** (Scheme 61).



129 **135**

Scheme 61 *Reagents and conditions:* Thermolysin, KPi buffer pH = 8, 18 hr, 30°C

Reactions were stopped after 18 hours by inactivation of the enzyme by acidification of the reaction mixture and analysed by LC-MS (Figure 13). The relative percentage product composition of the products was calculated from TIC data as for Chapter 2, Section 2.3.1 and summarised in the tables below (Table 9 and 10).

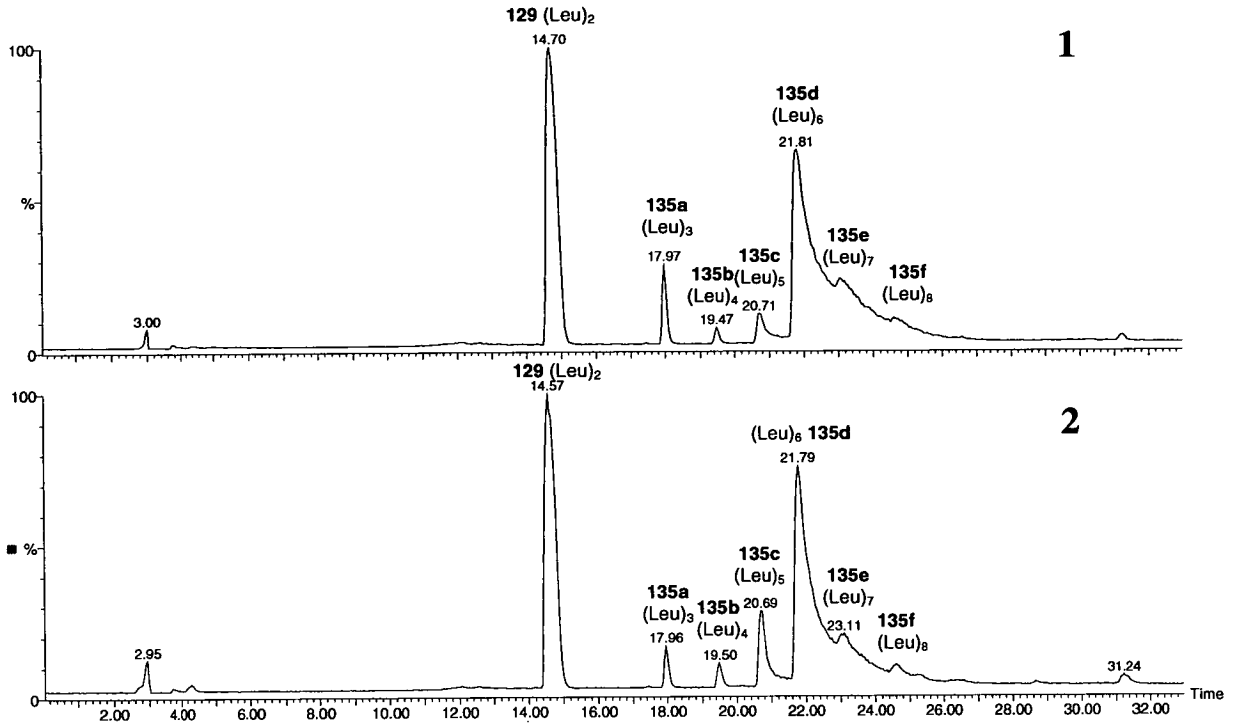


Figure 13: (1) HPLC trace of poly-L-leucine library **135** generated in the absence of **123** (2) HPLC trace of poly-L-leucine library **135** generated in the presence of **123**

Table 9 RPPC of library **135** generated in the absence **123**

Library Member	RPPC	RPPC of Repeated Library
135a $(Leu)_3$	38.5 %	43.4 %
135b $(Leu)_4$	3.3 %	3.8 %
135c $(Leu)_5$	3.6 %	3.7 %
135d $(Leu)_6$	43.6 %	44.4 %
135e $(Leu)_7$	6.2 %	3.9 %
135f $(Leu)_8$	4.8 %	0.8 %

Table 10 RPPC of library **135** generated in the presence of **123**

Library Member	RPPC
135a $(Leu)_3$	20.3 %
135b $(Leu)_4$	6.9 %
135c $(Leu)_5$	16.6 %
135d $(Leu)_6$	50.0 %
135e $(Leu)_7$	2.4 %
130f $(Leu)_8$	3.9 %

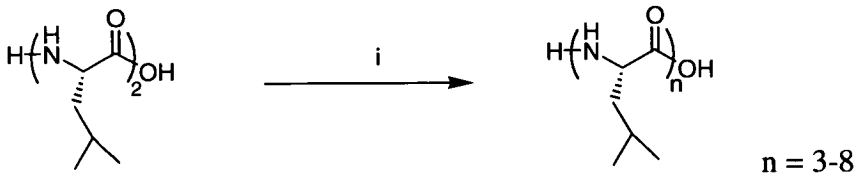
The results differed slightly from those observed in Section 3.6 but were still promising. **135** generated in the absence of **123** showed $(Leu)_3$ **135a** and $(Leu)_6$ **135d** to be the most dominant products. $(Leu)_4$ **135b**, $(Leu)_5$ **135c**, $(Leu)_7$ **135e** and $(Leu)_8$ **135f** were present in small but similar amounts. **135** generated in the presence of **123** showed a 6.4 % increase in the composition of $(Leu)_6$ **135d** and a 13.0 % increase in $(Leu)_5$ **135c** mostly at the expense of $(Leu)_3$ **135a** which decreased by 18.2 %.The compositions of $(Leu)_4$ **135b**, $(Leu)_7$ **135e** and $(Leu)_8$ **135f** remained low and varied slightly.

Generation of **135** in the absence of **123** was repeated in order to establish reproducibility of RPPC values. The reaction was analysed by LC-MS and the RPPC was calculated from TIC data (Table 9). Repeated data was found to be in good agreement with previous data.

4.3 Employment of Organic Co-Solvents to Aid Template Solubilisation

Previously within this project dynamic peptide libraries had been limited to templating with hydrophilic targets due to the adverse effects of organic solvents in enzymatic solid phase synthesis (Section 3.3). As the limiting factor of the resin was no longer present there was an opportunity to reemploy organic co-solvents for target solubilisation.

Ulijn *et al* [1, 2] have reported that plotting the concentration of organic co-solvents in enzymatic synthesis against activity generates a " U-shaped" curve where either low or high concentrations of co-solvents were most favourable. Generation of poly-L-leucine libraries was attempted in three different solvent systems. $(Leu)_2$ **129** was incubated in the presence of thermolysin at 30 °C in 90 % ACN, THF and methanol in pH8 KPi buffer (Scheme 62). Reactions were stopped after 18 hours by acidification of the reaction mixture and analysed by LC-MS.



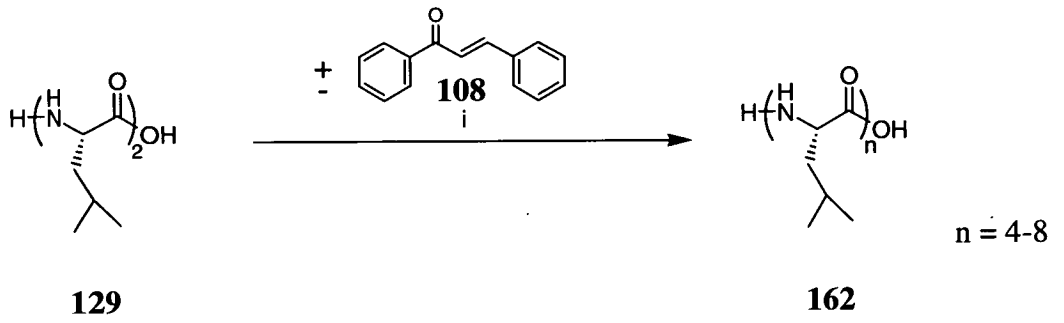
129 **162**

Scheme 62 *Reagents and conditions:* (i) [1] Thermolysin, 90 % ACN in pH 8 KPi buffer, 18 hr, 30 °C [2] Thermolysin 90% THF in pH 8 KPi buffer, 18 hr, 30 °C or [3]Thermolysin, 90 % methanol in pH 8 KPi buffer, 18 hr, 30 °C

A poly-L-leucine library **162** comprising of six leucine peptides varying from trimers to octamers were observed for reactions carried out in ACN. A similar library was observed for reactions carried out in THF, however, chromatograms were also found to contain a number of unidentifiable peaks. Reactions carried out in methanol were not successful and only starting material was observed by LC-MS. Methanol is thought to inhibit the library formation by disruption of hydrogen bonding in the products [3]. Based on these results a solvent system of 90 % ACN in buffer was employed for all subsequent reactions.

4.4 Templated Solution Phase Dynamic Peptide Libraries in 90 % ACN

Given that poly-L-leucine library **162** could be successfully generated in 90 % ACN, screening of templates was no longer limited to hydrophilic analogues of chalcone. This was further investigation by generating **162** from $(Leu)_2$ **129** with thermolysin in the presence and absence of **108** using 90 % ACN in buffer as a solvent for the reaction (Scheme 63).



129 **162**

Scheme 63 *Reagents and conditions:* Thermolysin, 90 % ACN in KPi buffer pH = 8, 18 hours, 30 °C

Reactions were stopped after 18 hours by acidification of the reaction mixture and analysed by LC-MS. Poly-L-leucine libraries were formed for both reactions where libraries comprised of five peptides varying from tetramers to octamers (Figure 14).

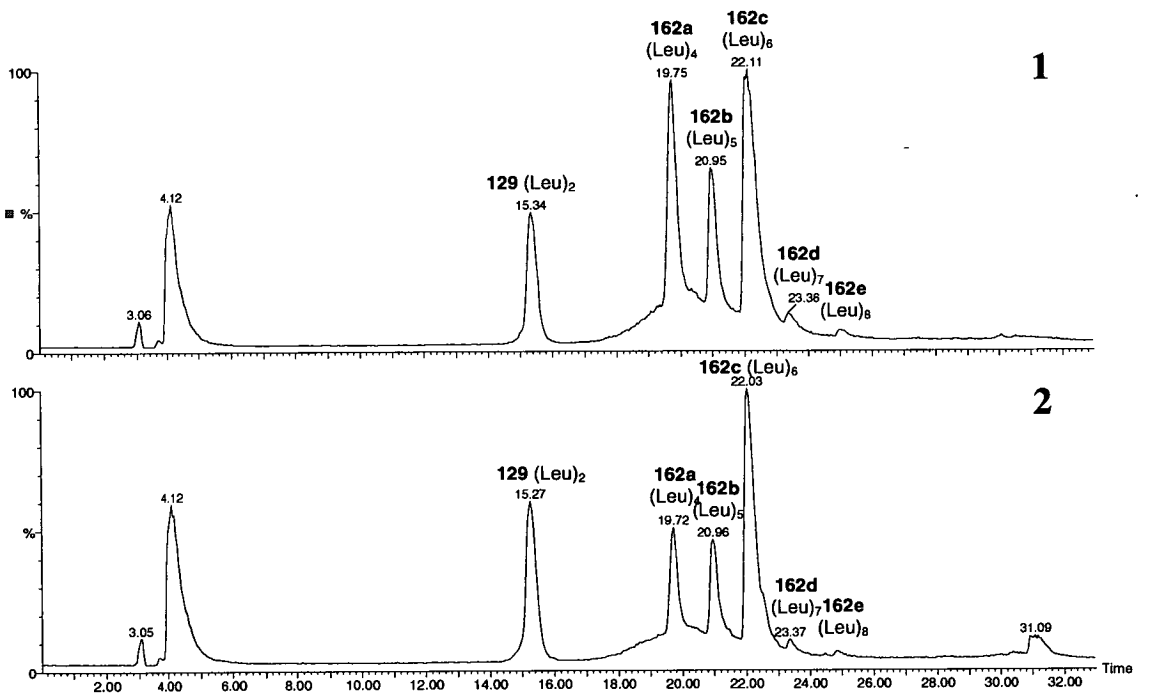


Figure 14 (1) HPLC trace of poly-L-leucine library **162** generated in the absence of **108** (2) HPLC trace of poly-L-leucine library **162** generated in the presence of **108**

The relative percentage product composition of each library was calculated from TIC data and summarised in the tables below (Table 11 and 12)

Table 11 Table of RPPC of library **162** generated in the absence of **108**

Library Member	RPPC
162a $(Leu)_4$	31.7 %
162b $(Leu)_5$	12.3 %
162c $(Leu)_6$	52.8 %
162d $(Leu)_7$	2.3 %
162e $(Leu)_8$	0.9 %

Table 12 Table of RPPC of library **162** generated in the presence of **108**

Library Member	RPPC
162a $(Leu)_4$	16.6 %
162b $(Leu)_5$	11.5 %
162c $(Leu)_6$	68.3 %
162d $(Leu)_7$	2.7 %
162e $(Leu)_8$	1.0 %

Library **162** generated in the absence of **108** showed $(Leu)_6$ **162c** to be the most dominant product followed by $(Leu)_4$ **162a** then $(Leu)_5$ **162b**. $(Leu)_7$ **162d** and $(Leu)_8$ **162e** were present in trace amounts. Library **162** generated in the presence on **108** showed a 15.5 % increase in $(Leu)_6$ **162c** mostly at the expense of $(Leu)_4$ **162a** which decreased by 15.1 %.

To determine if the data was reproducible, both reactions were repeated as described in Scheme 63. The relative percentage product composition was summarised in the tables below (Table 13 and 14).

Table 13 Table of RPPC of repeated library **162** generated in the absence of **108**

Library Member	RPPC
162a $(Leu)_4$	34.1 %
162b $(Leu)_5$	14.8 %
162c $(Leu)_6$	49.5 %
162d $(Leu)_7$	1.3 %
162e $(Leu)_8$	0.4 %

Table 14 Table of RPPC of repeated library **162** generated in the presence of **108**

Library Member	RPPC
162a $(Leu)_4$	20.9 %
162b $(Leu)_5$	15.7 %
162c $(Leu)_6$	61.2 %
162d $(Leu)_7$	1.8 %
162e $(Leu)_8$	0.4 %

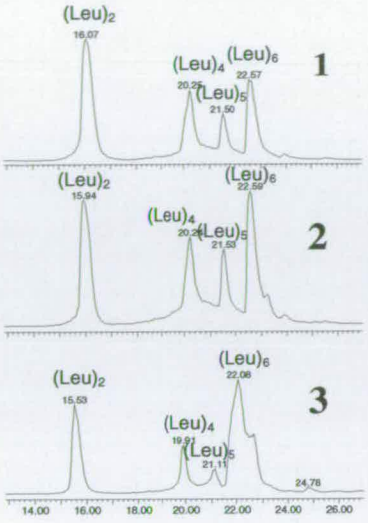
Repeated data gave good agreement with previous data and showed a shift towards $(Leu)_6$ **162c** at the expense of $(Leu)_4$ **162a**. However, the shift was slightly less pronounced with a 11.7 % increase in $(Leu)_6$ **162c** and a 13.2 % decrease in $(Leu)_4$ **162a**. Interestingly, despite the good agreement in composition of the products, quantities of unreacted $(Leu)_2$ **129** were found to vary when quantitatively analysed by LC-MS.

To determine is shifts were statistically significant, library **162** was generated in the absence of **108** once more and the standard deviation of $(Leu)_6$ **162c** was calculated. The mean RPPC value for **162c** was found to be 49.7 % and the standard deviation of **162c** was found to be 49.7 % ± 2.1 %. As the average shift was 15.1 %, which is more than three times greater than the error, the shift was therefore out with statistical control and confirmed to be significant. However, this significance is based on the assumption that the data for **162c** follows a Gaussian distribution.

4.5 Effect of Chalcone Concentration on Shift of Equilibrium

The effect of template concentrations on library distribution has been well investigated [4-6]. Studies by Severin *et al* have shown that at high concentrations of template library members other than the best binder may be amplified [7]. These considerations and the need to optimise the observed shift prompted us to study the effect of increasing the concentration of **108** on the degree of shift observed in the libraries.

Previously, **108** and analogues of **108** had been introduced to **162** at concentrations equivalent to the concentration of $(Leu)_2$ **129** (0.02 M). Concentrations of **108** were increased to values of 0.1 M, 0.5 M and 1 M. Reactions were carried out in duplicate using conditions described for Section 4.4, Scheme 35 and subsequently analysed by LC-MS (Figure 15). The average relative percentage product composition of each reaction was calculated from TIC data and plotted on the bar chart shown below (Figure 15).



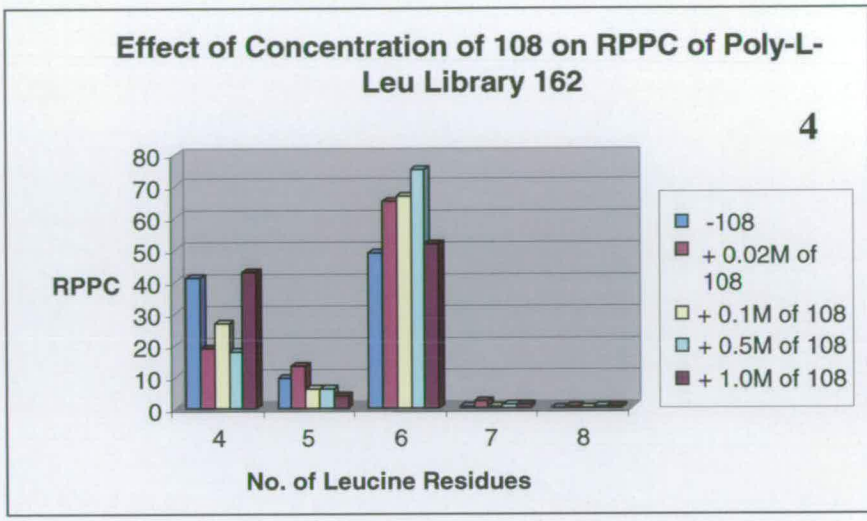
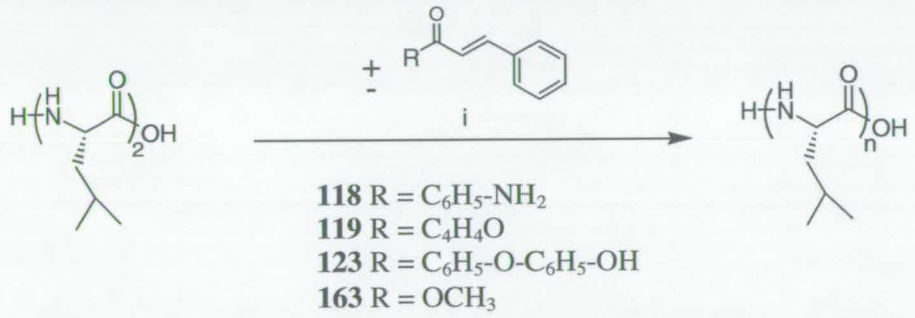


Figure 15: (1) HPLC trace of poly-L-leucine library **162** generated in the absence of **108** (2) HPLC trace of poly-L-leucine library **162** generated in the presence of 0.1M of **108** (3) HPLC trace of poly-L-leucine library **162** generated in the presence of 0.5M **108** (4) Bar chart representation of average distribution of poly-L-leucine library **162** at varied concentrations of **108**.

At concentrations of at 0.1 M **108** a slightly increased shift of 17.9 % towards $(Leu)_6$ **162c** was observed. Concentrations of 0.5 M also showed a biased towards $(Leu)_6$ **162c** but with a much larger shift of 26.1 %. At 1 M **108**, the composition of library was largely unchanged and the amount of $(Leu)_6$ **162c** was within the experimental error of **162c** generated in the absence of **108**. It was also noted that no other library members were amplified. A possible explanation for these findings is that the concentration of **108** is so high that binding is no longer selective for $(Leu)_6$ **162c** and instead non-selective binding to all library members occurs resulting in no effect on the library distribution. In conclusion, concentrations of **108** up to 0.5 M gave shifts towards $(Leu)_6$ **162c** where 0.5 M gave the largest shift.

4.6 Screen of Chalcone Analogues

A template screen of chalcone analogues was carried out to determine if shifts towards $(Leu)_6$ **162c** were compound dependant or if other library members would be amplified. **162** was generated from $(Leu)_2$ **129** with thermolysin in the presence of chalcone analogues **118**, **119** and **123** and also commercially available methyl cinnamic ester **163** (Scheme 64). Each reaction was carried out as for Section 4.4 where **118** was introduced at a concentration of 0.02 M and **119**, **123** and **163** were introduced at 0.5 M . Each library was carried out in duplicate and analysed by LC-MS.



Scheme 64 *Reagents and conditions:* (i) Thermolysin, pH 8 KPi buffer, 18 hr, 30 °C.

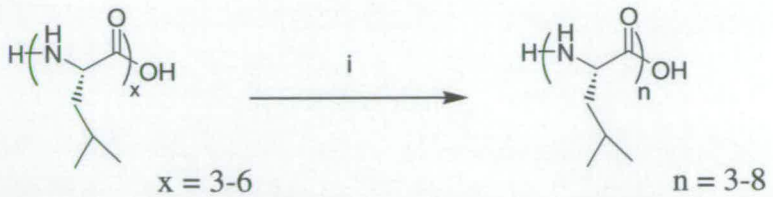
Libraries generated in the presence of **119** gave a significant average shift of 17.3 % towards $(Leu)_6$ **162c**. Libraries generated in the presence of **123** also demonstrated a significant shift towards $(Leu)_6$ **162c** which gave an average of 19.5 %. It is interesting to note that when **162** was generated in the presence of **123** in buffer only (Section 4.2), the major shift was towards $(Leu)_5$ and only a comparatively small shift towards $(Leu)_6$ was observed. However, when the same reaction was carried out in 90 % ACN in pH8 KPi buffer no such simultaneous shift was observed in **162.** This indicates that affinities for library members is solvent dependant.

162 generated in the presence of **118** and **163** gave shifts of 1 % and 5.3 % respectively towards $(Leu)_6$ **162c**. Both of these shifts were found to be less than three times the calculated error of 2.1 % and therefore were not significant. It was predicted that **163** would have no effect on the composition as it is known not to be epoxidised in the Juliá-

Colonna epoxidation [8]. However, **118** is known to be epoxidised under such conditions and was therefore expected to exert a change in library distribution. The lack of shift may be related to the failure of **118** to form an adduct with $(Leu)_5$ **110** in Section 3.5. In conclusion not all analogues of chalcone have an effect on the composition of library **162**. However, analogues that did induce a shift showed a preference for $(Leu)_6$ **162c**.

4.7 Investigation into the State of Equilibration of Poly-L-Leucine Libraries

It was noted that leucine oligomers comprising of an even number of monomers are present in larger amounts in library **162** compared to oligomers containing an odd number of residues. Although tetramers and hexamers may be thermodynamic products due to the formation of stable α-helices it is also highly likely that they may be kinetic products as they are more easily formed. This uneven distribution in products suggests that library **162** is not under the control of a true thermodynamic equilibrium. To determine if the distribution of the library was a true thermodynamic equilibrium, alternate poly-L-leucine oligomers $(Leu)_3$ **184**, $(Leu)_4$ **174**, $(Leu)_5$ **175** and $(Leu)_6$ **176** (these compounds were chemically synthesised in Chapter 5, Scheme 72) were employed as starting material and individually incubated with thermolysin (Scheme 65). All oligomers were incubated at the same monomer concentration of 0.04 M and the distributions of the resulting libraries monitored by LC-MS over a period of two weeks. After this time autolysis of the enzyme occurred and unambiguous characterisation of leucine oligomers was no longer possible. Fresh enzyme was added to the reaction mixture every two days to ensure constant interconversion of the library members. It was expected that if **162** was indeed a true equilibrium then a consistent library distribution would be observed independent of the length of oligomer used a starting material. RPPC values were calculated from TIC data and summarised in the bar charts below (Figure 16).



184 x = 3 **174** x = 4
175 x = 5 **176** x = 6

162

Scheme 65 *Reagents and conditions:* (i) Thermolysin, 90 % ACN in pH 8 KPi buffer, 18 hr, 30 °C

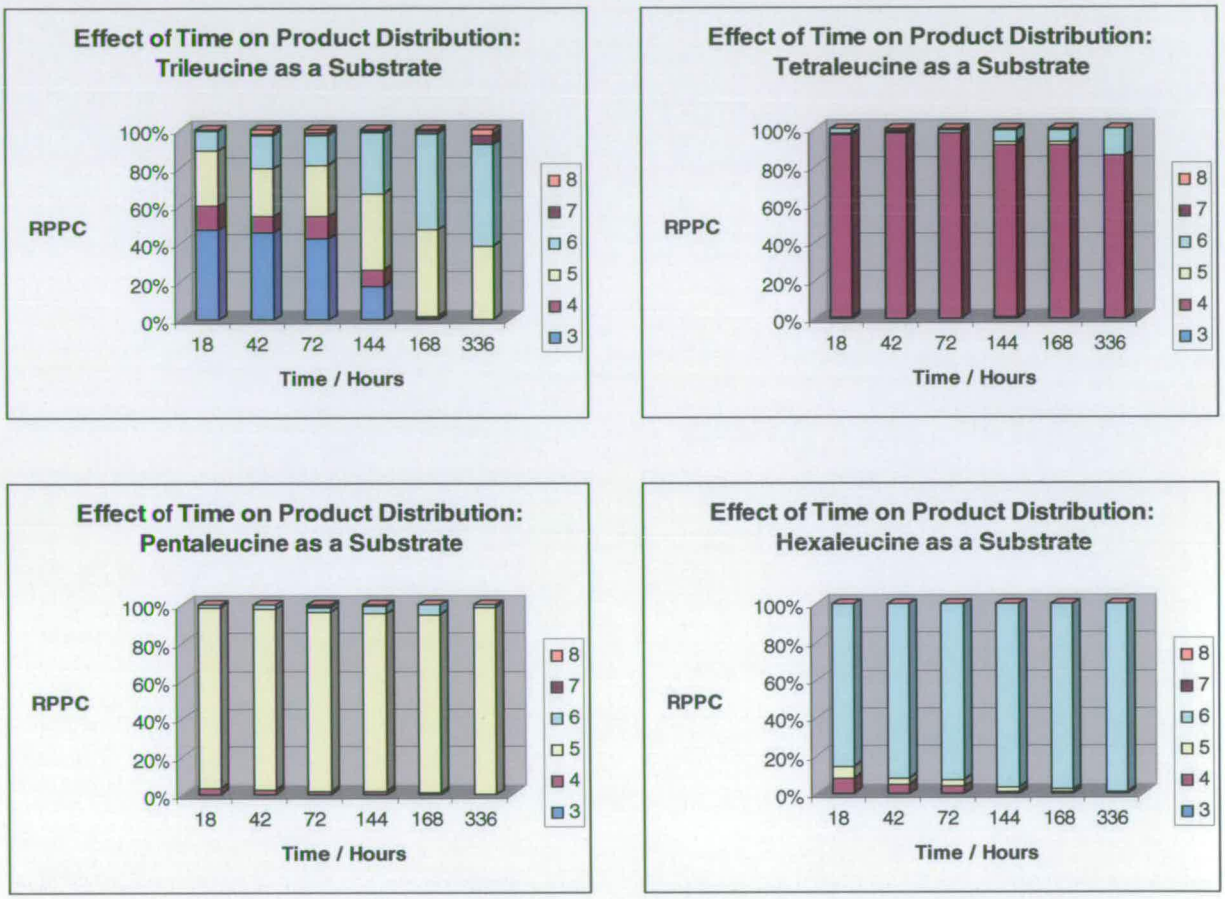
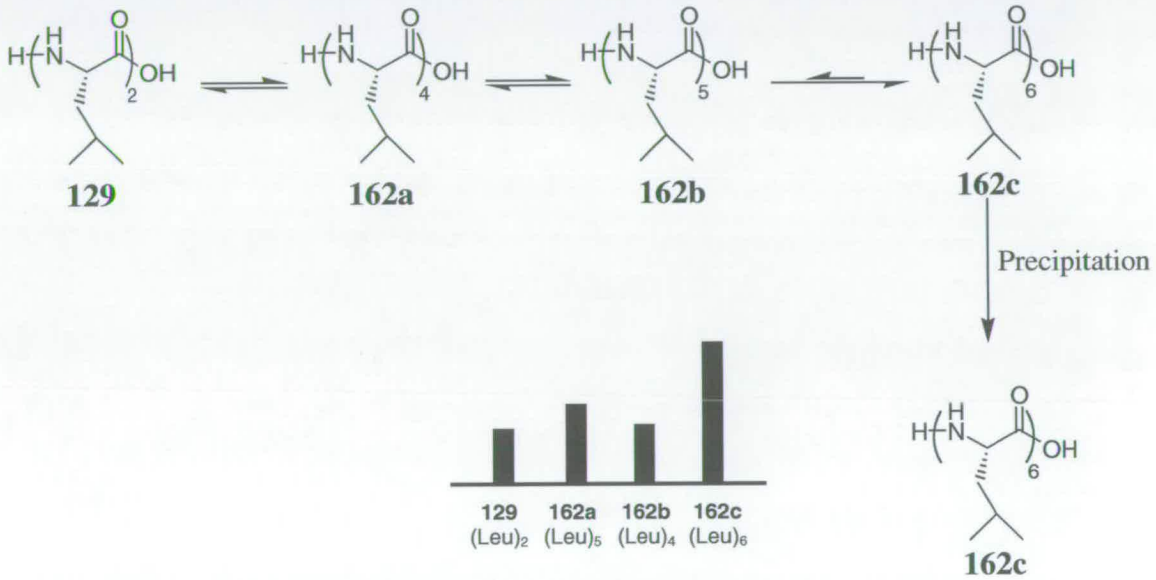


Figure 16: Bar charts showing product distributions of libraries generated from various substrates over a period of two weeks.

Interestingly, library distributions varied greatly depending on the length of leucine oligomers used as starting material, thus confirming that the library distribution was not under thermodynamic control. Further to this, libraries generated from larger oligomers

showed very little evidence of scrambling by the enzyme. This suggests poor solubility of the larger library members. However, a general trend was observed in which the RPPC of $(Leu)_6$ **162c** increased throughout the course of each reaction. The increasing dominance of $(Leu)_6$ **162c** was thought to be caused by two factors. Firstly, $(Leu)_6$ **162c** may precipitate at a greater rate due to an increased hydrophobicity compared to the other library members (Scheme 66). This results in the accumulation and removal of $(Leu)_6$ **162c** from the reaction mixture thus causing the equilibrium to shift in favour of the synthesis of $(Leu)_6$ **162c**. Secondly, $(Leu)_6$ **162c** could also be the most thermodynamically stable member as it may adopt a stable α-helical structure to a greater extent than other library members.



Scheme 66 Library members **162a**, **162b** and **162c** in equilibrium with each other and starting material **129**. **162c** precipitates from solution causing an accumulation of **162c** in the library.

The extent of the precipitation of $(Leu)_6$ **162c** was investigated by LC-MS analysis of the supernatant of library **162** generated from $(Leu)_2$ **129** in the absence of **108**. The bar chart displayed in Figure 17 shows the distribution of the entire library compared to that of the supernatant. On comparison it was observed that $(Leu)_2$ **129** and $(Leu)_4$ **162a** were more prevalent in the supernatant than in the entire library. $(Leu)_5$ **162b** was found to be only slightly decreased in the supernatant and a dramatic reduction in $(Leu)_6$ **162c** was observed. This confirms that precipitation synthesis of $(Leu)_6$ **162c** does occur. It should, however, be noted that the library is biased towards $(Leu)_6$ **162c** in the presence of **108**. As the literature indicates that **108** has a preferred affinity for stable helices [9-11],

the accumulation of $(Leu)_6$ **162c** is attributed to both thermodynamic stability and precipitation driven synthesis. In conclusion the library is thought to be under some kinetic control and not equilibrated. Some more systematic studies into the compositions of the supernatant with time are currently being investigated by another group member Valeria Barratini.

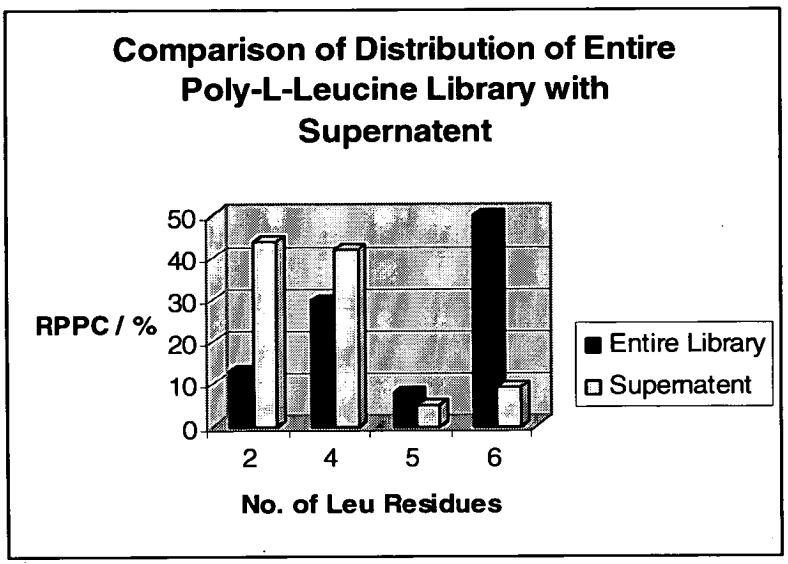


Figure 17: Bar chart comparing the distribution of entire poly-L-leucine library **162** with supernatant

4.8 Investigation of Inhibition of Thermolysin by Chalcone

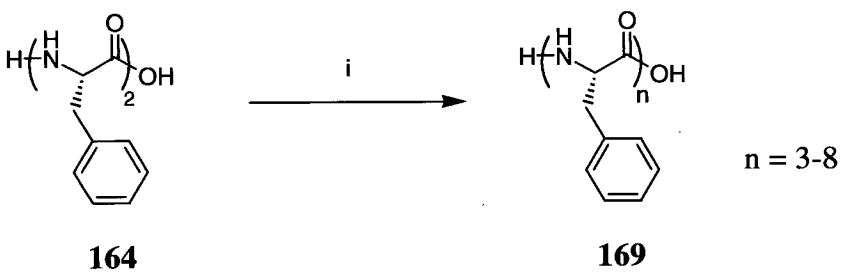
It seemed reasonable that **108** may have an effect on the activity of the enzyme and therefore be responsible for the shift in distribution. This was further investigated by preincubating thermolysin in 90 % ACN in buffer the presence and absence of **108** overnight. Thermolysin was filtered and washed then used to generate **162** from $(Leu)_2$ **129** as described in Section 4.4, Scheme 63. Analysis by LC-MS showed the same distribution for both libraries. The possibility that the binding of **108** to thermolysin was responsible for the shifts was therefore discounted.

4.9 Peptide Library Formation From Dipeptides Other Than Dileucine

The asymmetric epoxidation of chalcone is not only catalysed by poly-L-leucine but also by the peptides poly-L-alanine and poly-L-valine [12]. For this reason and also to investigate the diversity of the libraries formed, we attempted to generate libraries from the dipeptides PhePhe **164**, PheLeu **165**, LeuPhe **166**, GlyGly **167** and AlaAla **168**.

*4.9.1 Libraries Generated from PhePhe **164***

Commercially available PhePhe **164** was incubated in 90 % ACN in pH 8 KPi buffer in the presence of thermolysin at 30 °C (Scheme 67). The reaction was carried out in duplicate and stopped after 18 hours by acidification of the reaction mixture.



164 **169**

Scheme 67 *Reagents and conditions:* (i) Thermolysin, 90 % ACN in KPi buffer pH = 8, 18 hr, 30 °C

Analysis of the acidified reaction mixture by LC-MS revealed a six membered poly-L-phenylalanine library **169** had been formed comprising of peptides varying from trimers to octamers where the relative percentage composition of the products was calculated from TIC data and summarised in the tables below (Table 15).

Table 15 Table of RPPC of library **169** generated in the absence of **108**

Library Member	RPPC	RPPC of Duplicate Library
169a $(Phe)_3$	11.1 %	11.4 %
169b $(Phe)_4$	56.3 %	53.3 %
169c $(Phe)_5$	8.8 %	7.0 %
169d $(Phe)_6$	22.0 %	25.0 %
169e $(Phe)_7$	1.4 %	2.6 %
169f $(Phe)_8$	0.1 %	0.7 %

Table 16 Table of RPPC of library **169** generated in the presence of **108**

Library Member	RPPC
169a $(Phe)_3$	8.6 %
169b $(Phe)_4$	38.0 %
169c $(Phe)_5$	6.8 %
169d $(Phe)_6$	42.1 %
169e $(Phe)_7$	4.2 %
169f $(Phe)_8$	0.3 %

$(Phe)_4$ **169b** was found to be the most dominant product followed by $(Phe)_6$ **169d**, $(Phe)_3$ **169a** and $(Phe)_5$ **169c**. $(Phe)_7$ **169e** and $(Phe)_8$ **169f** were found to be present in trace amounts. The RPPC of duplicate reactions were found to be in good agreement.

Library **169** was then generated in the presence and absence of 0.5 M **108**, where each reaction was carried out in duplicate. Comparison of the LC-MS (Figure 19) chromatograms and RPPC values (Table 16) of the libraries showed an 18.6 % increase in $(Phe)_6$ **169d** mostly at the expense of $(Phe)_4$ **169b** which decreased by 16.8 % when **169** was generated in the presence of **108**. The standard deviation of $(Phe)_6$ **169d** in **169** generated in the absence of **108** was found to be 21.1 ± 3.2 %. Assuming a Gaussian distribution, as the increase of $(Phe)_6$ **169d** was three times greater than the error, the shift was found to be significant.

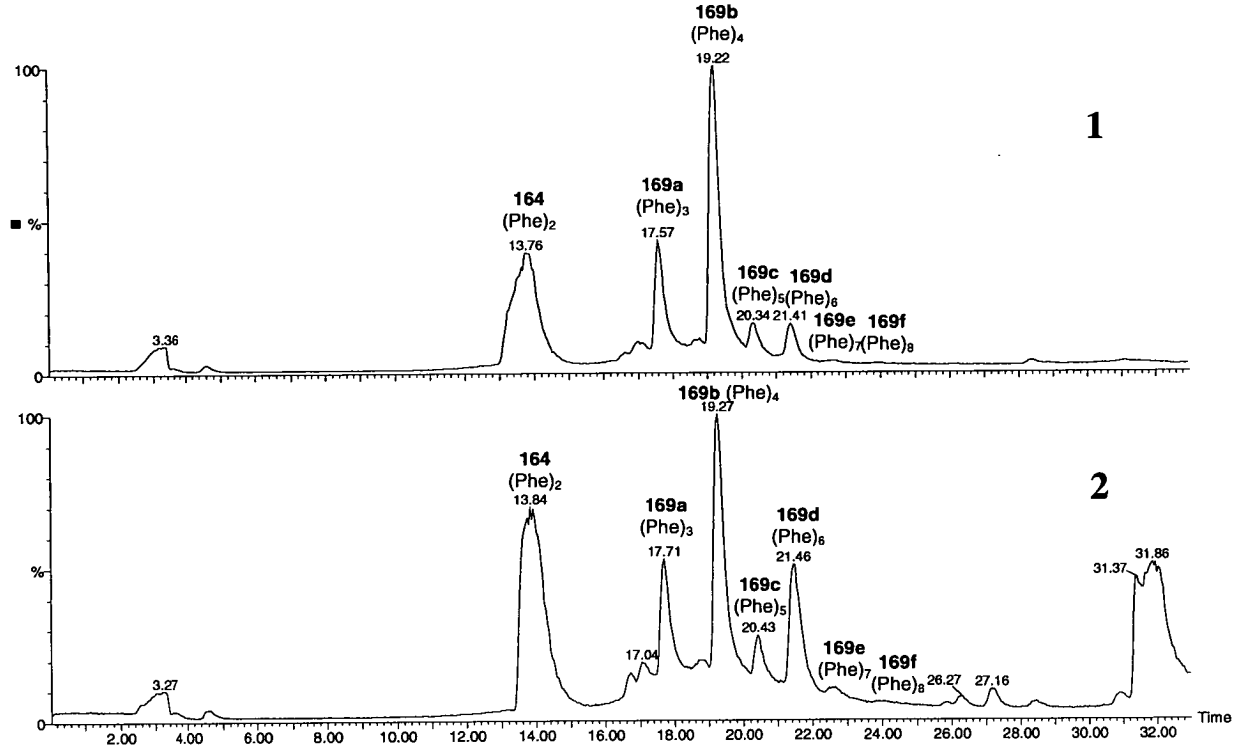
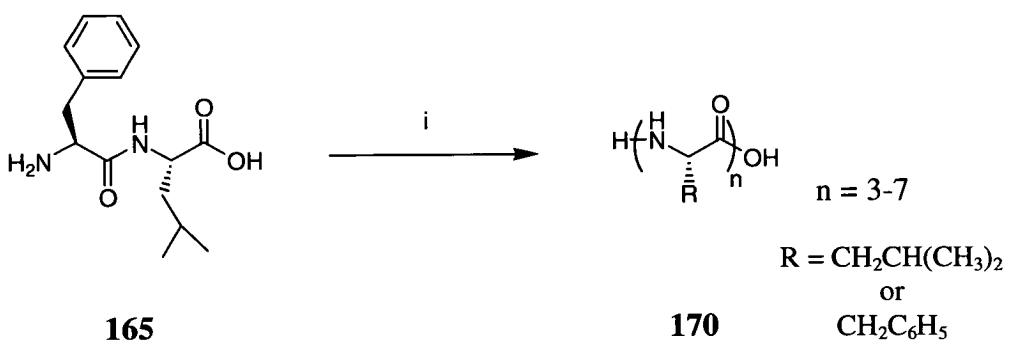


Figure 18: (1) HPLC trace of poly-L-phenylalanine library **169** generated in the absence of **108** (2) HPLC trace of poly-L-phenylalanine library **169** generated in the presence of **108**

*4.9.2 Libraries Generated from PheLeu **165***

Commercially available PheLeu **165** was incubated in 90 % ACN in pH8 KPi buffer in the presence of thermolysin at 30 °C (Scheme 68). The reaction was stopped after 18 hours by acidification of the reaction mixture then analysed by LC-MS.



165 **170**

Scheme 68 *Reagents and conditions:* (i) Thermolysin, 90 % ACN in KPi buffer pH = 8, 18 hr, 30 °C

It was found that a five membered library **170** of peptides was formed comprising of peptides varying from trimers to heptamers. All peptides consisted of several phenylalanine residues and one leucine residue. This was an unexpected result as it was predicted that the enzyme would simply couple the dipeptides thus resulting in predominantly tetramers and hexamers consisting of equal numbers of phenylalanine and leucine residues. This result suggests that thermolysin transfers single phenylalanine residues which is unusual behaviour for an endopeptidase.

170 was then generated in the presence and absence of **108** using conditions as described in Scheme 68. Each reaction was carried out in duplicate then quantitatively analysed by LC-MS in order to establish reproducibility (Figure 19). The RPPC of each library was calculated from TIC data. The average RPPC of duplicates was calculated and summarised in the tables below (Table 17 and 18).

Table 17 Table of RPPC of library **170** generated in the absence of **108**

Library Member	RPPC
170a Trimer	22.1 %
170b Tetramer	30.9 %
170c Pentamer	36.1 %
170d Hexamer	10.2 %
170e Heptamer	0.8 %

Table 18 Table of RPPC of library **170** generated in the presence of **108**

Library Member	RPPC
170a Trimer	12.0 %
170b Tetramer	15.9 %
170c Pentamer	30.7 %
170d Hexamer	31.5 %
170e Heptamer	10.0 %

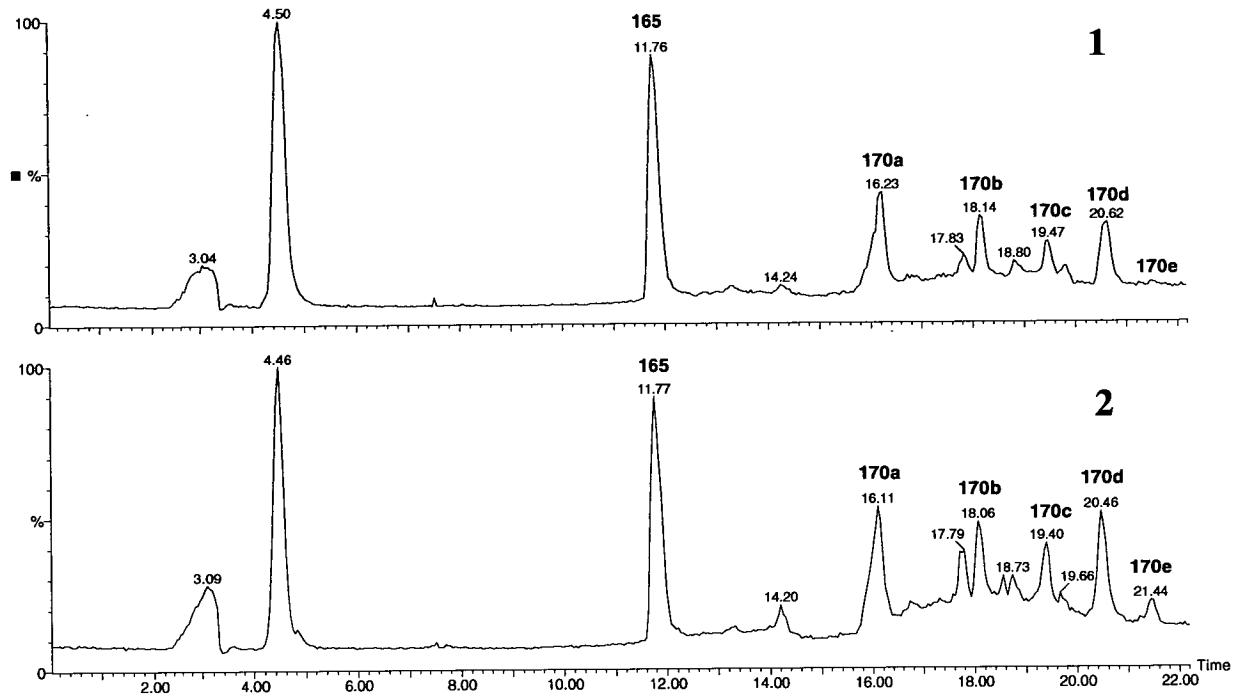
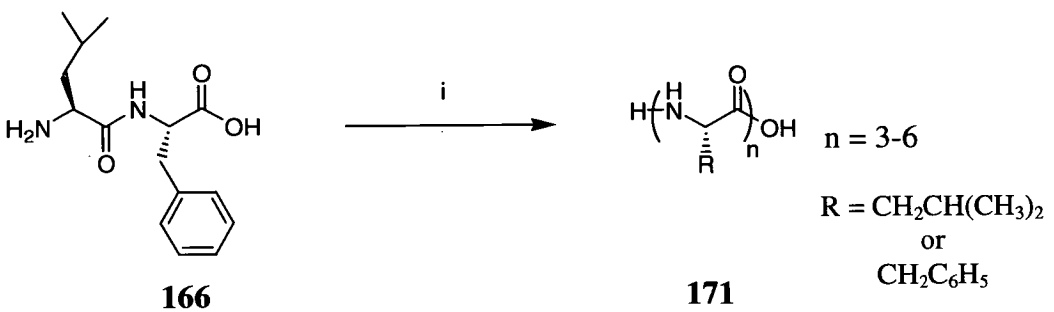


Figure 19: (1) HPLC trace of peptide library **170** generated from **165** in the absence of **108** (2) HPLC trace of peptide library **170** generated from **165** in the presence of **108**

Library generated in the absence of **108** showed the pentamer **170c** to be the most dominant product followed by the tetramer **170b**, trimer **170a** and hexamer **170d** in ascending order. Only a trace of heptamer **170c** was present. Library generated in the presence of **108** showed a shift towards the higher oligomers. A 21.3 % increase in the hexamer **170d** and a 9.2 % increase in the heptamer **170e** were both observed. The standard deviations of the RPPCs of the hexamer **170d** and heptamers **170e** in **170** generated in the absence of **108** were calculated and found to be 10.2 ± 4.3 % and 0.75 ± 0.75 % respectively. In both cases the increase was greater than three times the error and, assuming a Gaussian distribution, the shifts were therefore found to be significant.

LeuPhe **166** was synthesised on commercially available polystyrene resin preloaded with FmocPhe *via* a Wang linker. The Fmoc was removed with 20 % piperidine in DMF. FmocLeu was coupled to the resin using standard peptide coupling reagents TBTU, HOBt and DIPEA in DMF as described in Scheme 33, Chapter 3. The Fmoc was removed after coupling and the resin was washed with DMF, methanol and DCM. The resin was shrunk by washing with diethyl ether then dried over night in a vacuum oven. **166** was cleaved from the resin by treatment with 95 % TFA in H_2O. **166** was then precipitated from the cleavage mixture with cold diethyl ether, redissolved in H_2O:ACN (1:1) then lypholised to give the product as a white fluffy powder.

166 was subsequently incubated in 90 % ACN in pH8 KPi buffer in the presence of thermolysin at 30 °C (Scheme 69). The reaction was stopped after 18 hours by acidification of the reaction mixture then analysed by LC-MS.



166 **171**

Scheme 69 *Reagents and conditions:* (i) Thermolysin, 90 % ACN in KPi buffer pH = 8, 18 hr, 30 °C

A four membered library of peptides was formed where peptides varied from trimers to hexamers. One trimer containing two phenylalanine residues was observed by LC-MS. Two pentamers were observed: one consisting of three phenylalanine and two leucine residues and one consisting of three leucine and two phenylalanine residues. Peptides containing an even number of monomers, i.e. tetramers and hexamers, contained equal numbers of leucine and phenylalanine residues as expected.

171 was then generated in the presence and absence of **108** using conditions as described for Scheme 69. All reactions were carried out in duplicate, analysed by LC-MS and the RPPC of each library was calculated from TIC data. The results for this were disappointing. RPPC values for duplicate reactions of **171** generated in the presence of **108** were found to be in good agreement with each other (Table 19).

Table 19 Table of RPPC of library **171** generated in the absence of **108**

Library Member	RPPC	RPPC of Duplicate Library
171a PLP	6.0 %	4.0 %
171b LPLP	9.4 %	7.2 %
171c LPLPL	3.5 %	4.5 %
171d PLPLP	3.5 %	5.0 %
171e LPLPLP	78.4 %	79.4 %

Duplicate reactions of library generated in the absence of **108** were found to be in very poor agreement. These reactions were therefore repeated a further four times. The RPPC values between each reaction was still found to vary greatly. The average RPPC of **171** generated in the presence and absence of **108** was calculated and summarised in the tables below (Table 20 and 21).

Table 20 Table of RPPC of library **171** generated in the absence of **108**

Library Member	RPPC
171a PLP	7.8 %
171b LPLP	30.2 %
171c LPLPL	4.1 %
171d PLPLP	3.6 %
171e LPLPLP	46.7 %

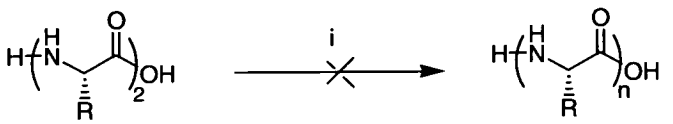
Table 21 Table of RPPC of library **171** generated in the presence of **108**

Library Member	RPPC
171a PLP	5.0 %
171b LPLP	8.3 %
171c LPLPL	4.0 %
171d PLPLP	4.3 %
171e LPLPLP	79.0 %

On first glance it appears that there has been a 32.3 % increase in the hexamer **171e**. However, the standard deviation of the RPPC of hexamer **171e** in **171** generated in the absence of **108** was found to be 46.7 ± 11.9 %. As the increase in hexamer **171e** in less than three times the standard deviation the shift is within statistical control and therefore found not to be significant. It was noted that during the reaction there was a great deal of aggregation between the peptide and enzyme. This practical aspect may be responsible for the variation in library distribution.

4.9.4 Libraries Generated From GlyGly 172 and AlaAla 173

Commercially available GlyGly **167** and AlaAla **168** were incubated in 90% ACN in pH 8 KPi buffer in the presence of thermolysin at 30 °C (Scheme 70). Reactions were stopped after 18 hours by acidification of the reaction mixture.



167 R = H
168 R = CH_3

172 R = H
173 R = CH_3

Scheme 70 *Reagents and conditions:* (i) Thermolysin, 90 % ACN in KPi buffer pH = 8, 18 hr, 30 °C

Analysis by LC-MS revealed that libraries had not been formed and only starting material was observed in the HPLC trace. It was noted that the side chains of glycine and alanine are less hydrophobic than phenylalanine and leucine. For this reason it was thought that precipitation of products and therefore synthesis of products was not favoured.

Generation of libraries **172** and **173** was reattempted by repeating reactions described in Scheme 70 in the presence of **108** as it seemed reasonable that **108** would bind to peptides that may be formed in solution and encourage library formation. Again, analysis by LC-MS showed only starting material in the reaction mixture. Higher substrate concentrations of both dipeptides may be required for library formation but due to time constraints this was not attempted.

4.10 Summary and Conclusions

A dynamic poly-L-leucine library **135** was generated from $(Leu)_2$ **129** in the presence of thermolysin in aqueous buffer. Generation of the library in the presence of **123** resulted in an increase in the composition of $(Leu)_5$ **135c** and $(Leu)_6$ **135d** at the expense of $(Leu)_3$. Poly-L-leucine libraries were also successfully generated in organic solvents. A solvent system of 90 % ACN in buffer was chosen as the standard conditions for the generation of poly-L-leucine library **162**. Employment of organic solvents allowed all hydrophobic as well as hydrophilic analogues of chalcone to be used as templates. Generation of poly-L-leucine libraries in the presence of **108** in 90 % ACN in buffer resulted in the amplification of $(Leu)_6$ **162c** at the expense of $(Leu)_4$ **162a**. A 0.5 M concentration of **108** was found to give the most pronounced shift of $(Leu)_6$ **162c**. Introduction of **119** and **123** as templates also resulted in amplification of $(Leu)_6$ **162c** at the expense of $(Leu)_4$ **162c**. Studies, however, found the library not to be a true thermodynamic equilibrium. Dynamic peptide libraries were also successfully generated from the dipeptides PhePhe **164**, PheLeu **165** and LeuPhe **166**. Generation of libraries from **164** and **165** in the presence of **108** gave statistically significant shifts towards higher oligomers at the expense of lower oligomers.

4.11 References

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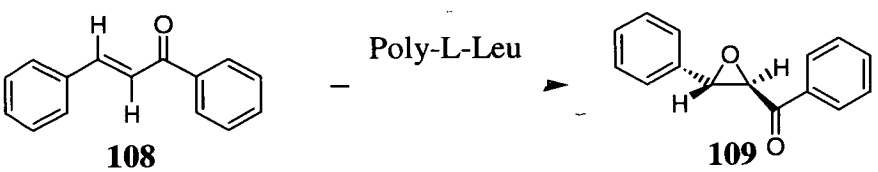
5. Correlation of Shifts to Catalytic Activity of Peptides

5.1 Introduction

Chapter 4 demonstrated that when several dynamic peptide libraries were generated in the presence of **108** and some analogues of **108**, an increase in composition of higher oligomers was observed. This shift suggested that higher oligomers would bind more tightly to **108** than lower oligomers. Given that the poly-L-leucine oligomers are also used as catalysts in the Juliá-Colonna epoxidation (Scheme 71) we were interested to see if the shift would correlate to an increased catalytic activity of the polypeptides. If there was a correlation, DCLs could be used as a possible tool for identifying new catalytically active peptides for the Juliá-Colonna epoxidation. With this in mind the catalytic activity of a series of library members of libraries **162** and **169** were compared in the Juliá-Colonna epoxidation to ascertain if library members amplified by **108** in the DCL experiments gave the best catalytic activity.



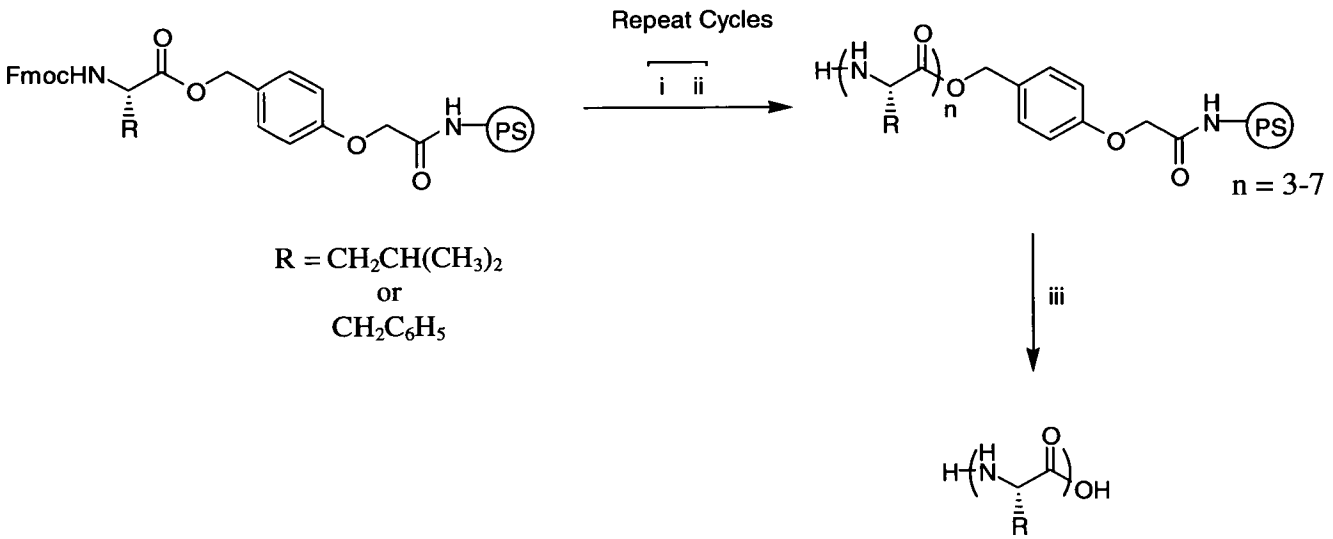
108 **109**

Scheme 71 Juliá-Colonna epoxidation

5.2 Synthesis and Purification of Peptides

Libraries **162** and **169** formed insufficient quantities of polypeptides for isolation and use as catalysts in the Juliá-Colonna epoxidation. Members of libraries **162** and **169** that were not commercially available were therefore chemically synthesised. Peptides $(Leu)_4$ **174** -$(Leu)_6$ **176**, $(Phe)_3$ **177** -$(Phe)_7$ **181** and the single peptide LFLFLF **182** were manually synthesised on commercially available polystyrene resin preloaded with Fmoc protected amino acid *via* a Wang linker. The Fmoc was deprotected with 20 % piperidine in DMF (Scheme 72). Fmoc protected amino acids were subsequently coupled using TBTU, HOBt and DIPEA. Deprotection and coupling steps were repeated until the desired length of peptide was reached. The Fmoc was removed after

the final coupling step and the resin was washed with DMF, methanol and DCM. The resin was shrunk by washing with diethyl ether then dried in a vacuum oven. **174-182** were then cleaved from the support by treatment of the resin with 95 % TFA in H_2O. **174-182** were then precipitated from the cleavage mixture with cold diethyl ether.



174 R = $CH_2CH(CH_3)_2$ n = 4 **178** R = $CH_2C_6H_5$ n = 4
175 R = $CH_2CH(CH_3)_2$ n = 5 **179** R = $CH_2C_6H_5$ n = 5
176 R = $CH_2CH(CH_3)_2$ n = 6 **180** R = $CH_2C_6H_5$ n = 6
177 R = $CH_2C_6H_5$ n = 3 **181** R = $CH_2C_6H_5$ n = 7
182 R = $CH_2CH(CH_3)_2$ and $CH_2C_6H_5$ n = 6

Scheme 72 *Reagents and conditions:* (i) 20% Piperidine in DMF, 1 hr, RT (ii) FmocAA, TBTU, HOBt, DIPEA, DMF, 2 hr, RT (iii) 95% TFA in H_2O, 4 hr, rt

Analysis of peptides by LC-MS showed the desired product as a single peak for the poly-L-phenylalanine series **177-181**, LFLFLF **182** and $(Leu)_4$ **174**. No further purification of these peptides was therefore required. LC-MS analysis of $(Leu)_5$ **175** and $(Leu)_6$ **176** revealed a mixture of the desired product and shorter deleted peptides. Purification of **175** and **176** by preparative HPLC proved problematic as peptides were very hydrophobic and therefore only dissolved in acidified organic solvents. Addition of

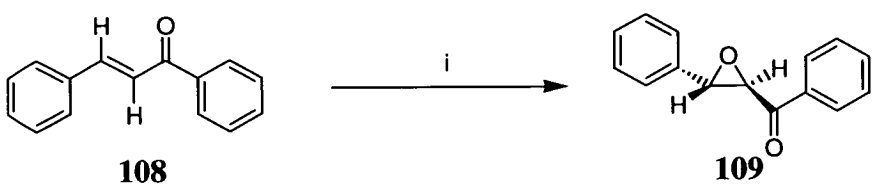
even drop amounts of H_2O or aqueous buffer resulted in either precipitation of products or formation of a gel.

As mixtures of organic and aqueous solvents were unsuitable for peptide solubilisation an alternative solvent was sought. Kissin *et al* [1] and Tiburu *et al* [2] had reported the dissolution and subsequent purification of hydrophobic peptides in neat fluorinated alcohols. **175** and **176** were consequently dissolved in trifluroethanol and TFA then purified by preparative reversed-phase chromatography. A gradient comprising of ACN and H_2O was employed for elution of **175** and **176**. Peptides were purified with a 60 % recovery.

5.3 Investigation of Protocols Suitable for Probing Catalytic Activity

There were several different methods of carrying out the Juliá-Colonna epoxidation by which catalytic activity could be probed. Methods fell into two main catagories: triphasic and biphasic. Triphasic conditions employed an aqueous nucleophillic base and oxidant, water immiscible organic solvent and an insoluble poly-L-leucine catalyst [3]. Biphasic conditions employed a non-nucleophilic base, oxidant, organic solvent and insoluble poly-L-leucine catalyst [4, 5].

Initially we were interested in employing conditions similar to those required for formation of libraries **162** and **169**. A biphasic protocol employing a urea-hydrogen peroxide complex, DBU and ACN as a solvent was therefore investigated (Scheme 73).

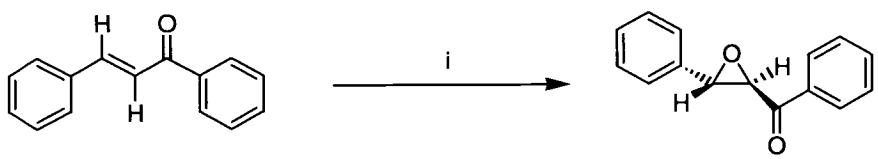


108 **109**

Scheme 73 *Reagents and conditions:* (i) Urea-hydrogen peroxide, DBU, ACN, ±poly-L-leucine-1,3-diaminopropane, 23 hr, rt

The reaction was carried out using commercially available poly-L-leucine-1,3-diaminopropane as a catalyst. In order to monitor background formation of **109** a reaction was carried out in parallel where the catalyst was omitted. TLC analysis showed the catalysed reaction to be incomplete after 22 hours. A second aliquot of oxidant was therefore added to both reactions. The catalysed reaction was found to go to completion after a further hour. However, the uncatalysed reaction was also found to go to completion after this period of time. This protocol was a therefore not suitable for our comparative studies and alternative conditions were sought.

Literature reports had detailed that reaction times may be reduced to as little as twenty minutes when the reaction was carried out in THF [4]. It seemed reasonable that, given this reduced reaction time, that a reduction in the background formation of **109** would also be observed. Consequently, catalysed and uncatalysed reactions were repeated using literature conditions that employed a urea-hydrogen peroxide complex and DBU in THF (Scheme 74). Again, commercially available poly-L-leucine-1,3-diaminopropane was employed as a catalyst.



108 **109**

Scheme 74 *Reagents and conditions:* (i) Urea-hydrogen peroxide, DBU, THF, ±poly-L-leucine-1,3-diaminopropane, 2 hr, rt

TLC analysis showed the catalysed reaction to be complete after 2 hours. The reaction carried out in the absence of catalyst was still found to form **109** but at a reduced amount. Analysis of the uncatalysed reaction by chiral HPLC revealed **109** had been formed as a racemate in a 50 % conversion yield. Given the reduced background formation of **109**, Scheme 74 was tested to probe the catalytic activity of the poly-L-leucine series. Commercially available $(Leu)_1$ **183**, $(Leu)_2$ **129** and $(Leu)_3$ **184** and previously synthesised $(Leu)_4$**174** - $(Leu)_6$ **176** were used in place of poly-L-leucine-1,3-diaminopropane to catalyse the reaction. Conversion yields were determined by NMR

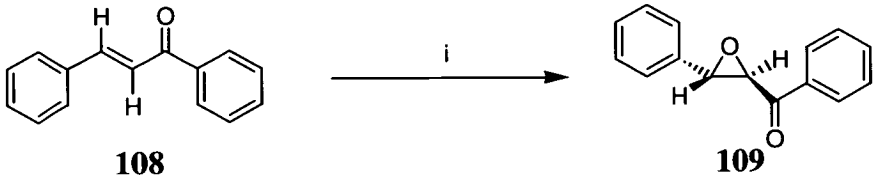
and enantiomeric excesses were determined by chiral HPLC and tabulated below (Table 22).

Table 22 Table of conversion yields and enantiomeric excesses for Juliá-Colonna reactions (Scheme 74) catalysed with $(Leu)_1$-$(Leu)_6$ oligomers

No. of Leucine Residues	Conversion Yield	e.e.
0	26 %	Racemic
1	21 %	Racemic
2	28 %	Racemic
3	47 %	Racemic
4	30 %	Racemic
5	32 %	Racemic
6	30 %	Racemic

Similar conversion yields for the Juliá-Colonna reaction were observed for reactions catalysed with $(Leu)_2$ **129**, $(Leu)_4$ **174**, $(Leu)_5$ **175** and the uncatalysed reaction. A slightly lower yield was observed for reactions catalysed with $(Leu)_1$ **183** and an unexpectedly higher yield was observed with $(Leu)_3$ **184**. As **109** had been formed in such similar amounts in both the uncatalysed and catalysed reactions, it was decided that this method was also unsuitable for the comparison of catalytic activity.

Alternative biphasic conditions developed by Allen *et al* [5] had been reported to be suitable as a probe for catalytic activity due to an initial lag in the reaction rate of **109** formed in the absence of catalyst. The protocol reported by Allen *et al* employed sodium percarbonate as a base and oxidant as a cheaper alternative to urea-hydrogen peroxide complex and DBU. A mixture of DME and H_2O was employed as a solvent to facilitate the dissolution of the sodium percarbonate and release of the hydrogen peroxide (Scheme 75).

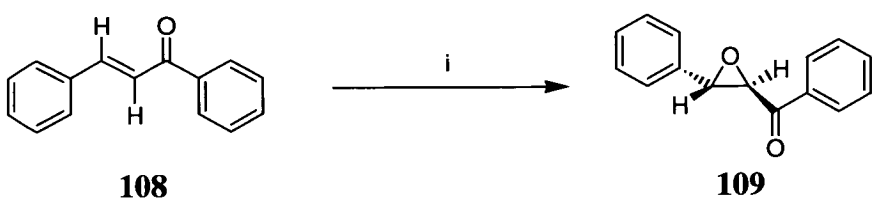


108 **109**

Scheme 75 *Reagents and conditions:* (i) $Na_2CO_3.1.5H_2O_2$, DME, H_2O, ± poly-L-leucine-1,3-diaminopropane, 2hr, rt

This method was investigated as a probe for catalytic activity by carrying out reactions in the presence and absence of commercially available poly-L-leucine-1,3-diaminopropane catalyst. TLC analysis of reactions showed a strong signal for **109** in the uncatalysed reaction in the time taken for the catalysed reaction to reach completion. The protocol described in Scheme 75 was therefore not suitable for the present investigations.

As the background formation of **109** under biphasic conditions seemed to be a general problem, triphasic conditions were examined for probing catalytic activity. Reactions carried out comprised of aqueous sodium hydroxide base and hydrogen peroxide oxidant with immiscible DCM for solubilisation of substrate [6]. Reactions were stirred at room temperature for 24 hours in the presence and absence of poly-L-leucine-1,3-diaminopropane catalyst (Scheme 76). Analysis of the products of the catalysed reaction by chiral HPLC showed **109** had been formed in a 98 % conversion yield and 95 % e.e.. HPLC analysis of the products for the uncatalysed reaction revealed only **108** to be present, thus confirming that no background formation of **109** had taken place.



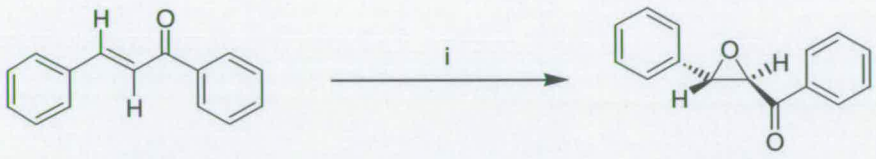
108 **109**

Scheme 76 *Reagents and conditions:* (i) aq. H_2O_2, aq. NaOH, DCM, ±poly-L-leucine-1,3-diaminopropane.

As no there was no background formation of **109**, these triphasic conditions were used to probe the catalytic activity of the poly-L-leu and poly-L-phe series.

5.4 Comparison of Catalytic Activity of Poly-L-Leucine Series

Peptides $(Leu)_1$ **183**, $(Leu)_2$ **129,** $(Leu)_3$ **184** and $(Leu)_4$ **174** -$(Leu)_6$ **176** were used to catalyse the epoxidation under triphasic conditions (Scheme 77). DCM followed by aqueous sodium hydroxide and hydrogen peroxide were added to a series of round bottomed flasks each charged with a poly-L-leucine oligomer. Reactions were started by addition of **108** to each flask then stirred at room temperature for 24 hours. Reactions were then stopped by removal of the poly-L-leucine peptides by filtration. Epoxidations were repeated three times for each catalyst. Conversion yields and enantiomeric excesses were determined by chiral HPLC. Averages were calculated and summarised in the bar chart below (Figure 20).



108 **109**

Scheme 77 *Reagents and conditions:* (i) aq. H_2O_2, aq. NaOH, DCM, $(Leu)_n$ (n = 1-6), 24 hours, rt

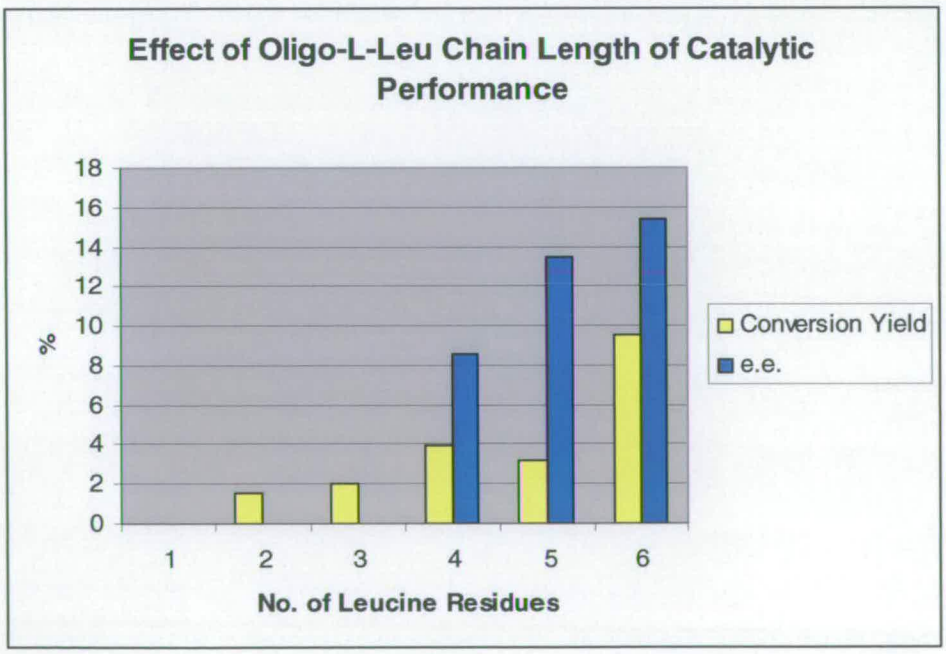


Figure 20: Bar chart showing effect of oligo-L-leu chain length on catalytic performance

109 was not observed for the reaction catalysed by Leu **183**. However, **109** was present for reactions catalysed with $(Leu)_2$ **129**, $(Leu)_3$ **184** and $(Leu)_4$ **174** - $(Leu)_6$ **176**. Although conversion yields and enantiomeric excesses were low, $(Leu)_6$ **176** was found to give an improved catalytic activity compared to other library members. This was a promising result as $(Leu)_6$ **176** had also been identified as the member in library **162** that was amplified by **108** in the DCL experiment (Scheme 63).

5.5 Comparison of Catalytic Activity of Poly-L-Phenylalanine Series

$(Phe)_3$ **177** - $(Phe)_7$ **181** were used to catalyse the Juliá-Colonna epoxidation under triphasic conditions as described in Scheme 77. Conversion yields and e.e.s were determined by chiral HPLC and summarised in the bar chart below (Figure 21).

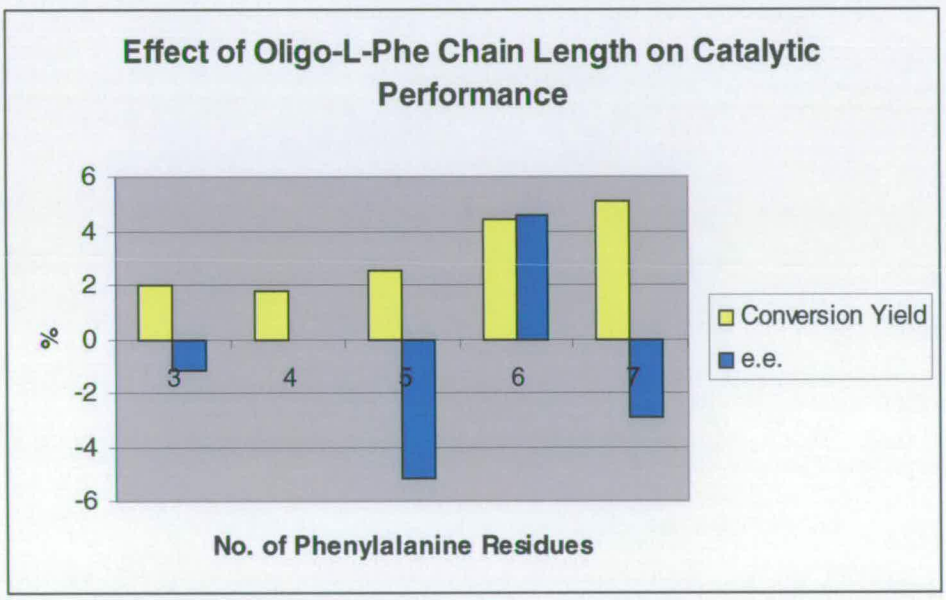


Figure 21: Bar chart showing effect of oligo-L-phe chain length on catalytic performance

Generally, conversion yields and e.e.s were found to be lower than for leucine peptides. However, results were still found to be promising as $(Phe)_6$ **180**, which had been amplified by **108** in library **169**, showed an improved conversion yield and e.e. compared to smaller library members. $(Phe)_7$ **181**, which was also found to be slightly

amplified by **108** in library **169**, showed an improved yield but poor e.e. The reduced catalytic activity of the poly-L-phe series was consistent with the smaller shift toward the active member observed for library **169**.

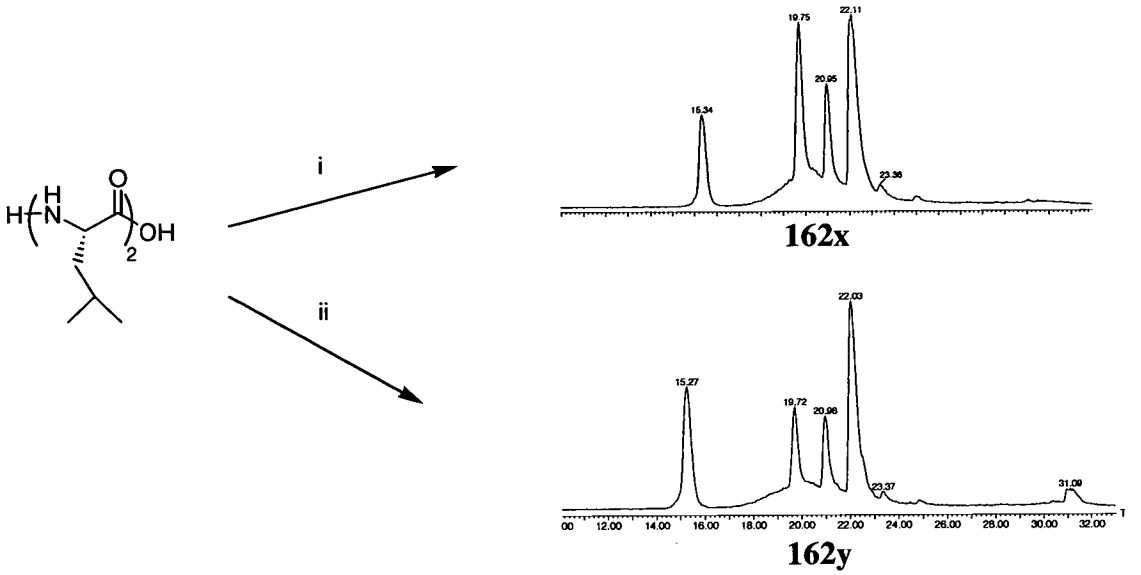
5.6 Investigation of Catalytic Activity of the Active Member

LFLFLF **183** was the only member of library **171** tested for catalytic activity. This was done to demonstrate that the active member identified by a templated DCL would show catalytic activity and that synthesis and testing of all library members was not necessary. **183** was used to catalyse the epoxidation under the triphasic conditions as described in Scheme 77. Analysis by chiral HPLC showed that **183** catalysed the reaction but gave a poor conversion yield of 2.3 % and an e.e. of 6.3 %. This confirmed that there appears to be some correlation between catalytic activity of peptides and amplification of library members in the presence of **108**.

5.7 Comparison of Catalytic Activity of Templated Poly-L-Leucine Library to Library Free of Template

It has been reported in the literature that DCC may also be used as a tool for quantitatively producing compounds [7]. This application was investigated by employing the perturbed and unperturbed poly-L-leucine libraries **162** generated in Chapter 4 as catalysts in the Juliá-Colonna epoxidation. It was expected that perturbed **162** would show an improved catalytic effect compared to unperturbed **162** due to the higher proportion of $(Leu)_6$ present.

162 was generated from $(Leu)_2$ **129** with thermolysin in the presence and absence of **108** to give the unperturbed library **162x** and perturbed library **162y** (Scheme 78).



Scheme 78 *Reagents and conditions:* (i) Thermolysin, 90 % ACN in KPi buffer pH = 8, 18 hours, 30 °C (ii) **108**, thermolysin, 90 % ACN in KPi buffer pH = 8, 18 hours, 30 °C

Excess thermolysin was removed from **162x** and **162y** by filtration. **108** was then extracted from **162y** with diethyl ether. **162x** and **162y** were then lypholised and employed as catalysts under triphasic conditions as described in Scheme 77. Analysis by chiral HPLC showed a 6.6 % conversion yield of the racemate for the reaction catalysed with **162x**. **162y** gave a slightly increased conversion yield of 7.6 % and an e.e. of 5.4 %. Not surprisingly, the catalytic activity is smaller than that observed when manually synthesised and purified $(Leu)_6$ **176** was used as a catalyst.

As it could not be certain that the enzyme was completely removed from **162x** and **162y** the catalytic activity of thermolysin was also investigated using the triphasic conditions described in Scheme 77. Aqueous sodium hydroxide and hydrogen peroxide and DCM were therefore added to thermolysin. The reaction was then started by adding **108** and the mixture and stirred for 24 hours. Surprisingly a 5.6 % conversion yield and 17.9 % e.e was observed on analysis by chiral HPLC. As slightly higher conversion yields and much lower e.e.s were observed for **162x** and **162y** we were satisfied that most of the enzyme had been removed and therefore had not contributed to the catalytic activity.

The catalytic activity of enzymes other than thermolysin and inhibited thermolysin were also invesitgated. This was done to determine, firstly, if catalysis was exclusive to the enzyme thermolysin and, secondly, if the active site of thermolysin was involved in the mechanism of catalysis. Bovine serum albumin, chymotrypsin and thermolysin inhibited with EDTA were used to catalyse the reaction under triphasic conditions. Conversion yields and e.e.s were determined by chiral HPLC and tabulated below (Table 23).

Table 23 Table of conversion yields and enantiomeric excesses of reactions catalysed by enzymes

Catalyst	Conversion Yield	e.e.
BSA	6.8 %	13.7 %
Chymotrypsin	4.2 %	8.4 %
Inhibited Thermolysin	3.8 %	1.9 %

Chymotrypsin and BSA were indeed found to catalyse the formation of **109**. The catalytic effect of thermolysin was thus confirmed to be a general protein effect and not exclusive to thermolysin. Inhibited thermolysin was also found to catalyse the reaction but gave a reduced e.e. and conversion yield compared to thermolysin free of inhibitor. This suggests that the active site may play a role in the catalysis mechanism but is not solely responsible for activity.

In conclusion, **162x** and **162y** were found to catalyse the Juliá-Colonna epoxidation. Although **162y** did show an improvement in catalytic activity compared to **162x,** better results were observed when manually synthesised $(Leu)_6$ **176** was employed as a catalyst.

5.8 Summary and Conclusions

Triphasic conditions were found to be the most suitable procedure for the Juliá-Colonna epoxidation for probing catalytic activity. All active members investigated were found to be catalytically active under triphasic conditions. Overall, active members showed an improved catalytic activity when compared to other members of the same library. This

suggests that the dynamic peptide libraries developed in this project may be exploited as a tool for identifying possible catalysts for the Juliá-Colonna epoxidation. Libraries **162x** and **162y** were also found to catalyse the epoxidation where **162x** showed a slightly improved activity over **162y**. This suggests that dynamic peptide libraries may be further exploited as a means of synthesising catalyst.

5.9 References

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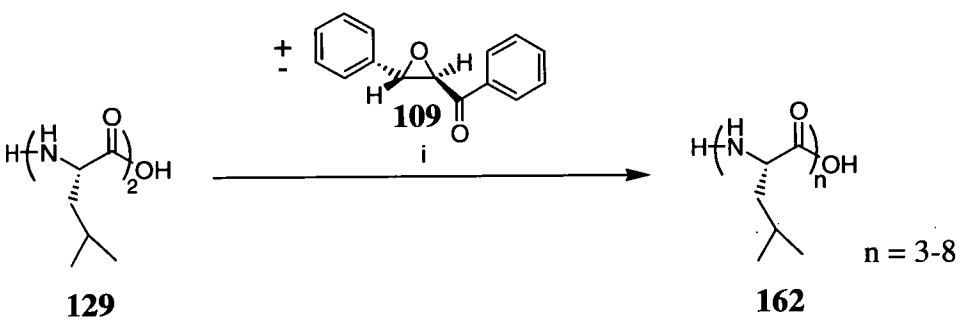
6. Suggestions for Future Work

The enzymatic generation of peptide libraries such as poly-L-leucine library **162** and poly-L-phenylalanine library **169** in solution phase and influencing the distribution of those libraries using **108** as a template has been very successful. It also appears from our work that the active members identified in these libraries display the best catalytic activity in the Juliá-Colonna epoxidation when compared to other members. However, selection for peptides that bind starting materials/substrates is not likely to lead ultimately to efficient catalysts. A way forward may be to use transition state analogues [1, 2] although these might be difficult to design for the Juliá-Colonna epoxidation. Perhaps a next step might be to investigate libraries that originate from templating with product **109** as an alternative (Scheme 79).

The state of equilibrium needs to be investigated more thoroughly. Equilibrium studies carried out in Chapter 4 have shown that precipitation of library members occurred which may act as kinetic trap for hydrophobic members. Despite this complication it might be possible that those library members in solution may reach a thermodynamic equilibrium. It may therefore be more valuable to examine the extent of amplification in the supernatant over the entire final mixture.



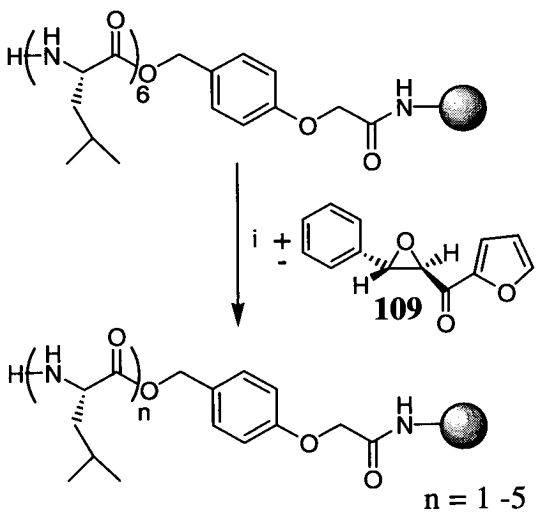
129 **162**

Scheme 79 *Reagents and conditions*: (i) Thermolysin, 90 % ACN in KPi buffer pH = 8, 18 hr, 30 °C

Additional layers of diversity may be added to dynamic peptide libraries by employing more that one protease of varying specificity for library generation. Additional complexity may be achieved by adding enzymes of varying function, for example, a

protease and an enzyme capable of glycosylating the side chains of the resulting peptides.

The generation of peptide libraries on a solid support was also found to be successful. However, the distribution of these libraries was not successfully influenced in the presence of analogues of **108** due to poor molecular recognition hampered by Fmoc protection. As hydrolysis of resin bound peptides has been shown by our group to be favoured in high dilution [3], an alternative templating strategy may be to hydrolyse peptide chains in the presence of a template (Scheme 80). Examination of the PEGA bound hydrolysis products may reveal amplification of the best binders. Care should be taken on quantification that solution phase hydrolysates do not stick to the resin and give false values. To overcome this the tagging strategy described in Chapter 3 should be employed. Further to this a preequilibrated approach could be applied to solid phase libraries. Libraries could be generated on a solid support, deprotected, then, in a separate step, screened for activity. For example, active members may be identified using formation of adducts similar to **117** that were observed in Chapter 3. Using such a method of screening would also involve investigation of the catalytic activity of PEGA bound poly-L-leucine.



Scheme 80 ***Reagents and conditions***: (i) Thermolysin, KPi buffer pH = 8, 30 °C

6.1 References

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7. Experimental

7.1 General Experimental

7.1.1 Instrumentation

^{1}H and ^{13}C NMR were recorded on Brüker AC250 or Brüker DPX-360 instruments.

Electrospray mass spectrometry (ES-MS) was performed using a Micromass platform II instrument. Fast atom bombardment (FAB) mass spectrometry was performed using a Kratos MS50TC instrument.

Infrared spectroscopy (IR) was carried out using a Perkin Elmer Paragon 1000 FT-IR spectrometer with the frequencies (ν) measured in wavenumbers (cm^{-1}). Solid samples were measured as KBr discs.

Optical rotations were performed on an Optical Activity AA1000 polarimeter from Optical Activity Ltd (sodium 589 nm detection). Sample concentration was measured in g/100 ml and $[\alpha]^{20}_{D}$ values were quoted in 10^{-1} deg cm^{-1}.

Melting points were recorded on a Gallenkamp melting point apparatus and were uncorrected.

A 360° Stuart Scientific SB1 blood rotator was used for sample agitation. Enzymatic reactions that required heating were carried out in a Stuart Scientific S160 incubator.

7.1.2 Solvents and Reagents

All reagents were standard laboratory grade and used as supplied unless otherwise stated. Benzaldehyde was purchased form Sigma Aldrich and distilled before use. Where solvent has been described as anhydrous it was purchased as anhydrous grade. For HPLC methods: UV grade acetonitrile and water; HPLC grade ethanol, hexane and

trifluroacetic acid were used. Peptide synthesis grade DMF was obtained from Rathburn. Thermolysin was purchased from Sigma as a lyophilised powder containing approximately 20% buffer salts as calcium and sodium acetate (91 units/mg protein). $(Leu)_2$ and $(Leu)_3$ were purchased from Bachem. All other dipeptides were purchased from Aldrich unless otherwise stated.

7.1.3 Chromatography

Analytical thin layer chromatography (TLC) was performed using Merck aluminium backed plates coated with silica gel $60F_{254}$. Identification was carried out using UV fluorescence (254 nm) and potassium permanganate dip. Flash column chromatography was carried out with a variety of glass columns using silica gel 60H (Merck 9385, 0.04-0.063 mm, 230-400 mesh).

7.1.4 High Performance Liquid Chromatography (HPLC)

7.1.4.1 Liquid Chromatography – Mass Spectrometry (LC-MS)

Reversed phase LC-MS was carried out on a Waters™ 2790 Separation Module, a Waters™ 486 Tuneable Absorbance Detector measuring at 254 nm or 214 nm coupled to a Micromass Platform II spectrometer with MassLinx Version 3.5 software. A Phenomenenex Luna 5μ C18(2) 250 x 2.00 mm column was used as the stationary phase eluting with H_2O (0.1% TFA)/ ACN (0.1% TFA) at a flow rate of 0.2 ml min^{-1}. Samples were injected at a volume of 100μl via a 100μl loop. Retention times (Rt) were quoted in mins. Two gradients were employed (Tables 24 and 25). Fmoc protected peptides were analysed using gradient 1 and unprotected peptides were analysed using gradient 2.

Table 24 Gradient 1

Time (mins)	H_2O/0.1% TFA (%)	ACN/0.1% TFA (%)
0	90	10
6	80	20
8	80	20
13	20	80
20	15	85
22	15	85
25	15	85
27	50	50
30	90	10
33	90	10

Table 25 Gradient 2

Time (mins)	H_2O/0.1% TFA (%)	ACN/0.1% TFA (%)
0	90	10
6	80	20
8	80	20
23	30	70
25	15	85
27	50	50
30	90	10
33	90	10

7.1.4.2 Chiral HPLC

Normal phase HPLC was performed on a Waters™ 600 controller/pump utilising a 486 tuneable detector and equipped with a Chiracel-ODH column with dimensions 250 x 4.6 mm. Samples were injected via a 20 µl loop and a flow rate of 1 ml/min. An isocratic method of 95 % hexane and 5% ethanol was employed for elution. A wavelength of 254 nm was used for sample detection and Millenium[32] software was used for processing data.

7.1.4.3 Preparative HPLC

Reversed phase preparative HPLC was performed on a Waters™ 600 controller/pump utilising a Waters™ 996 photodiode array. A Phenomenenex Luna 5μ C18(2) 250 x 10 mm column was employed as the stationary phase eluting H_2O (0.1% TFA)/ACN (0.1% TFA) at a flow rate of 4.0 ml min^{-1}. Samples were injected manually via a 2000 μl loop. A wavelength of 254 nm was used for sample detection and Millenium[32] software was used for processing data. The following gradient was employed for sample elution (Table 26):

Table 26 Gradient 3

Time (min)	H_2O/0.1% TFA (%)	ACN/0.1% TFA
0	90	10
6	80	20
27	10	90
30	10	90
33	90	10

7.2 General Solid Phase

7.2.1 Resins

All enzyme catalysed reactions were performed on amino functionalised polyethylene glycol dimethyl acrylamide$_{1900}$ (PEGA$_{1900}$). PEGA$_{1900}$ was supplied by Polymer Laboratories with a loading of 0.2 $mmolg^{-1}$ as a 10 % suspension in methanol. Large scale chemical synthesis of peptides was carried out on preloaded polystyrene resin purchased from Merck Biosciences.

7.2.2 Solid Phase Reactions

Solid phase reactions were carried out in 2, 10 or 25 ml plastic isolute SPE filtration columns with leur top caps and 20 μm porosity frits purchased from Cranford Scientific. Reactions were spun on a blood rotator for the amount of time and temperature stated to effect thorough mixing of resin and reagents.

7.2.3 General Washing Protocols

A typical wash cycle for 1 g of resin consists of DMF (3 ml x 2), DMF/MeOH (1:1) (3 ml x 2), THF (3ml x 2), DCM (3ml x 2), DMF (3ml x 2). The resin was then washed with the solvent of the next intended reaction. All washings were carried out under vacuum using a VacMaster™ 12 wash station.

7.2.4 Qualitative Determination of Resin Bound Free Amines: Ninhydrin Test

A small sample of resin (~10 mg) was treated with two drops of reagents A, B and C. The resin was then heated for 2-5 min. The appearance of a dark blue colour indicated the presence of free amines and therefore incomplete coupling. No colour change verified complete coupling.

Reagents: (A) ninhydrin (500 mgs) in EtOH (10 mls); (B) liquefied phenol (80 g) in EtOH (20 ml); (C) 0.001M potassium cyanide (2 ml) diluted to 100 ml with pyridine.

7.2.5 Qualitative Determination of Resin Bound Free Hydroxyls: Methyl Red – Diphenyldichlorosilane Test

A small sample of resin (~ 10 mg) was placed in a 2 ml isolute tube together with 10% TEA in DCM (200 μl). Diphenyldichlorosilane was added (100 μl) and the mixture was left to stand for 10 min. The resin was filtered and washed twice with 10% TEA in DCM. The resin was then suspended in 0.75% w/v methyl red (300 μl) and left to stand for 10 min. The beads were washed with DMF/DCM (1:1) until the resulting solution

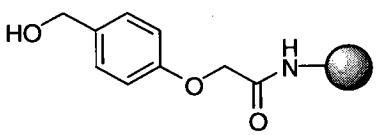
was colourless. Colourless beads indicated no free hydroxyls and orange beads indicated the presence of free hydroxyls.

7.2.6 General Procedure for Acidic Cleavage

Resin was incubated with 95% TFA in H_2O and spun on a blood rotator for 4 hrs. Resin was then filtered under vacuum and washed with 95% TFA in H_2O. Washings were either diluted with ACN/H_2O (1:1) and analysed directly by LC-MS gradient 1 or the peptide was precipitated with cold diethyl ether.

7.3 Thermolysin-Catalysed Fmoc Solid Phase Peptide Synthesis

7.3.1 Preparation of 4-Hydroxymethyl-phenoxyacetamide polyethylene glycol dimethyl acrylamide$_{1900}$ ***86***



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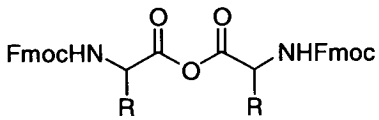
$PEGA_{1900}$ resin (5.0g, 0.1 mmol) was washed using standard protocol 7.2.3. 4-hyroxymethyl-phenoxyacetic acid (54.7 mg, 0.3 mmol) and HOBt (81.1 mg and 0.6 mmol) were added to the wet PEGA resin together with enough dry DMF (4 ml) to form a moveable gel. DIC (93.9 µl, 0.6 mmol) was added to the resulting gel and vortexed to effect thorough mixture then spun at room temperature for 18 hrs. The resin was filtered and washed according to standard protocol 7.2.3. Subsequent analysis by the ninhydrin test (Section 7.2.4) showed there to be no free amines so coupling was deemed complete.

7.3.2 General Procedure for Preparation of Symmetrical Anhydride



DIC (5 equivalents to resin) was added to a solution of Fmoc protected amino acid (10 equivalents to resin) in dry DMF. The solution was stirred under an inert atmosphere at room temperature for 1 hour. The symmetrical anhydride was used in the subsequent coupling reaction without any further purification.

7.3.3 General Procedure for Attachment of Substrates to Resin

DMAP (1.2 mg, 0.01 mmol) was added to a suspension of **86** (5.0 g, 0.1 mmol) in dry DMF (4 ml). The mixture was spun at room temperature for 1 hour. A solution of crude symmetrical anhydride (general procedure 7.3.2) in dry DMF was added to the resin. The mixture was then vortexed and spun on a blood rotator overnight at room temperature. Resin was then filtered and washed according to standard protocol 7.2.3.

7.3.4 Determination of Loading of 4-Hydroxymethyl-phenoxyacetamide polyethylene glycol dimethyl acrylamide$_{1900}$

Loadings were calculated from calibration curves determined by RP-HPLC. Standards of known concentration were eluted using gradient 1 and calibration curves were drawn by plotting concentration against peak area to give a straight line. Substrate was then cleaved from a known mass of resin using method 7.2.6 and washings were diluted to a known volume with ACN/H_2O (1:1). The number of moles in the resulting solution was then calculated using the straight line equation of the calibration curve. The loading of the substrate was then calculated using equation 1:

$$\text{Loading} = \frac{\text{Number of Moles}}{\text{Mass of Resin}}$$

Equation 1

7.3.5 General Procedure for Deprotection of Fmoc

Resin was treated with enough 20% piperidine in DMF to form a moveable gel and spun for 1 hour at room temperature. The resin was filtered and washed according to protocol 7.2.3.

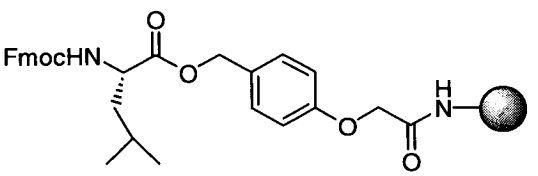
7.3.6 Preparation of pH8 Potassium Phosphate Buffer

K_2HPO_4 (17.42 g, 0.1 mol) was dissolved in H_2O (100 ml) to give a 1M solution. KH_2PO_4 (13.60 mol, 0.1 mol) was dissolved in H_2O (100 ml) to give a 1M solution. The pH was adjusted to 8 by mixing 1 M K_2HPO_4 (94 ml) with 1 M KH_2PO_4 (6 ml). The resulting solution was then diluted to 1000 ml to give 0.1M pH 8 potassium phosphate buffer.

7.3.7 General Procedure for Thermolysin-Catalysed Solid Phase Peptide Synthesis

Fmoc protected amino acid (10 equivalents to resin) and thermolysin (10 mgs) was added to the deprotected resin bound substrate (200 mg – 1500 mg) together with pH 8 potassium phosphate buffer (1 ml per 200 mg wet resin). The mixture was vortexed to effect thorough mixing then spun on a blood rotator at 30°C. Resin was then filtered and washed according to standard protocol 7.2.3.

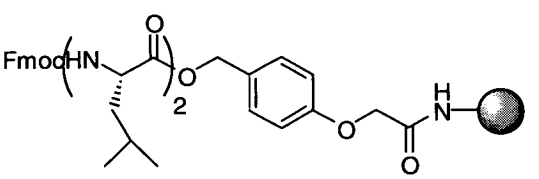
7.3.8 Attachment of Fmoc-L-Leu to 4-Hydroxymethyl-phenoxyacetamide polyethylene glycol dimethyl acrylamide$_{1900}$ ***90***



90

Fmoc-L-Leucine was attached to **86** via the preformed symmetrical anhydride using methods described in Sections 7.3.2 and 7.3.3. Product was cleaved from a small portion of beads as described in Section 7.2.6. Loading was determined as for Section 7.3.4. Loading 39 $\mu molg^{-1}$.

7.3.9 Enzymatic Synthesis of PEGA$_{1900}$ Immobilised Fmoc(Leu)$_2$ ***93***

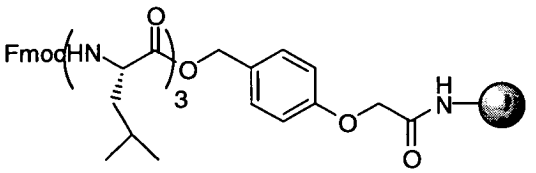


93

90 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocLeu was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then

analysed directly by LC-MS using gradient 1. Fmoc(Leu)$_2$ Rt = 24.5 min; MS(ES) m/z = 467.3 (MH^+ requires 467.3), 489.3 (MNa^+).

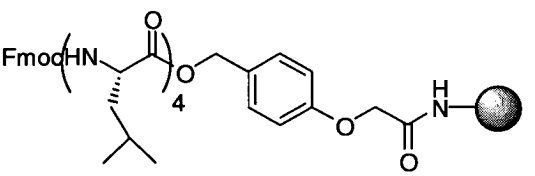
7.3.10 Enzymatic Synthesis of PEGA$_{1900}$ Immobilised Fmoc(Leu)$_3$ **94**



94

93 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocLeu was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. Fmoc(Leu)$_3$ Rt = 25.4 min; MS(ES) m/z = 580.3 (MH^+ requires 580.3), 602.2 (MNa^+).

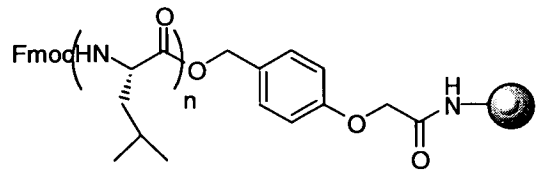
7.3.11 Enzymatic Synthesis of PEGA$_{1900}$ Immobilised Fmoc(Leu)$_4$ **95**



95

94 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocLeu was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. Fmoc(Leu)$_4$ Rt = 26.7 min; MS(ES) m/z = 693.4 (MH^+ requires 693.4).

7.3.12 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised Fmoc(Leu)$_5$ Library **96**

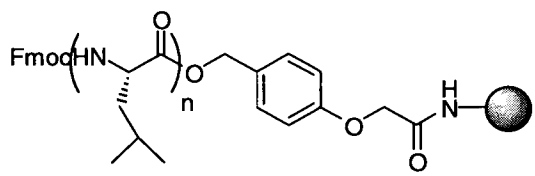


n = 3-5

96a-c

95 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocLeu was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. **96c** Fmoc(Leu)$_5$ Rt = 27.2 min; MS(ES) m/z = 806.8 (MH^+ requires 806.5). **96b** Fmoc(Leu)$_4$ Rt = 24.8 min; MS(ES) m/z = 693.5 (MH^+). **96a** Fmoc(Leu)$_3$ Rt = 23.3 min; ES(MS) = 580.3 (MH^+).

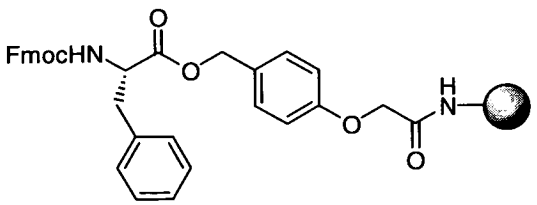
7.3.13 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised Fmoc(Leu)$_6$ Library **97**



97a-d n = 3-6

96 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocLeu was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. **97d** Fmoc(Leu)$_6$ Rt = 30.7 min; MS(ES) m/z = 919.7 (MH^+ requires 919.6). **97c** Fmoc(Leu)$_5$ Rt = 27.1 min; MS(ES) m/z = 805.6 (MH^+). **97b** Fmoc(Leu)$_4$ Rt = 25.2 min; MS(ES) m/z = 693.4 (MH^+). **97a** Fmoc(Leu)$_3$ Rt = 23.2 min; MS(ES) m/z = 580.3 (MH^+).

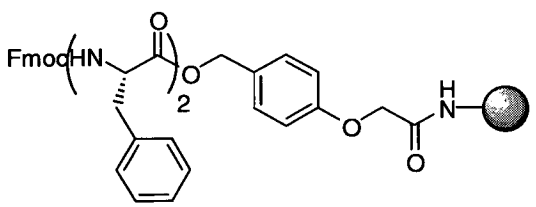
7.3.14 Attachment of Fmoc-L-Phe to 4-Hydroxymethyl-phenoxyacetamide polyethylene glycol dimethyl acrylamide$_{1900}$ ***91***



91

Fmoc-L-Phenylalanine was attached to **86** via the preformed symmetrical anhydride using methods described in Sections 7.3.2 and 7.3.3. Product was cleaved from a small portion of beads as described in Section 7.2.6. Loading was determined as for 7.3.4. Loading 65 $\mu molg^{-1}$.

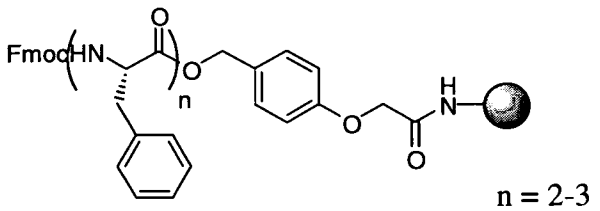
7.3.15 Enzymatic Synthesis of PEGA$_{1900}$ Immobilised Fmoc(Phe)$_2$ ***99***



99

91 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocPhe was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. Fmoc(Phe)$_2$ Rt = 22.6 min; MS(ES) m/z = 535.2 (MH^+ requires 535.2).

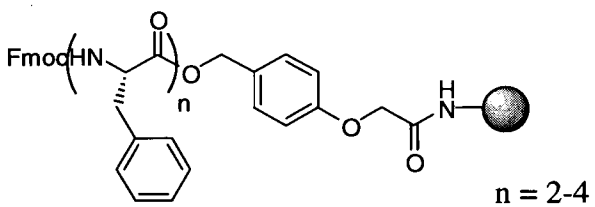
7.3.16 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised Fmoc(Phe)$_3$ Library ***100***



100a-b

99 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocPhe was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. **100b** Fmoc(Phe)$_3$ Rt = 23.7 min; MS(ES) m/z = 682.1 (MH^+ requires 682.3). **100a** Fmoc(Phe)$_2$ Rt = 22.7 min; MS(ES) m/z = 535.1 (MH^+).

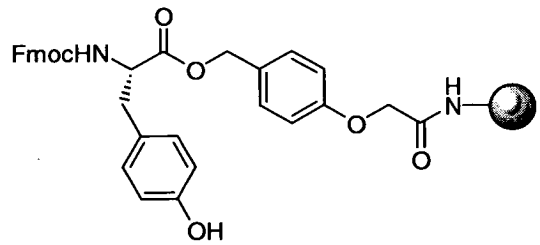
7.3.17 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised Fmoc(Phe)$_4$ Library ***101***



101a-c

100 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocPhe was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. **101c** Fmoc(Phe)$_4$ Rt = 24.6 min; MS(ES) m/z = 829.6 (MH^+ requires 829.4). **101b** Fmoc(Phe)$_3$ Rt = 23.5 min; MS(ES) m/z = 682.1 (MH^+). **101a** Fmoc(Phe)$_2$ Rt = 22.5 min; MS(ES) m/z = 535.1 (MH^+).

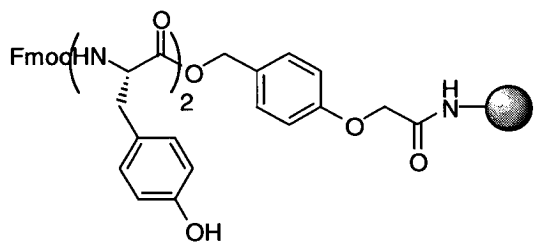
7.3.18 Attachment of Fmoc-L-Tyr to 4-Hydroxymethyl-phenoxyacetamide polyethylene glycol dimethyl acrylamide$_{1900}$ ***92***



92

Fmoc-L-Tyrosine was attached to **86** via the preformed symmetrical anhydride using methods described in Sections 7.3.2 and 7.3.3. Product was cleaved from a small portion of beads as described in Section 7.2.6. Loading was determined as for 7.3.4. Loading 23 $\mu molg^{-1}$.

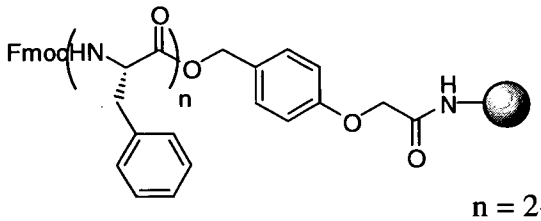
7.3.19 Enzymatic Synthesis of PEGA$_{1900}$ Immobilised Fmoc(Tyr)$_2$ ***103***



103

92 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocTyr was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. Fmoc(Tyr)$_2$ Rt = 20.8 min; MS(ES) m/z = 567.1 (MH^+ requires 567.2).

*7.3.20 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised $Fmoc(Tyr)_3$ Library **104***

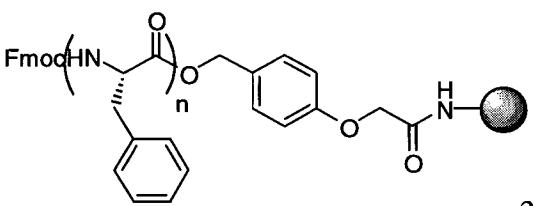


n = 2-3

104a-b

103 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocTyr was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. **104b** $Fmoc(Tyr)_3$ Rt = 20.4 min; MS(ES) m/z = 730.6 (MH^+ requires 730.3). **104a** $Fmoc(Tyr)_2$ Rt = 20.3 min; MS(ES) m/z = 567.2 (MH^+).

*7.3.21 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised $Fmoc(Tyr)_4$ Library **105***

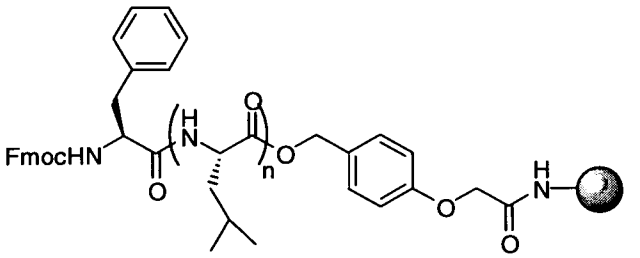


n = 3-4

105a-b

90 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocTyr was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. **105b** $Fmoc(Tyr)_4$ Rt = 20.4 min; MS(ES) m/z = 893.7 (MH^+ requires 893.3). **105a** $Fmoc(Tyr)_3$ Rt = 22.3 min; MS(ES) m/z = 730.3 (MH^+).

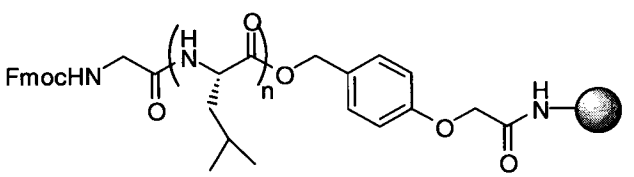
*7.3.22 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised FmocPhe(Leu)$_n$ Library **106***



106a-d n = 1-4

95 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocPhe was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. **106d** FmocPhe(Leu)$_4$ Rt = 26.0 min; MS(ES) m/z = 840.5 (MH^+ requires 840.5). **106c** FmocPhe(Leu)$_3$ Rt = 24.3 min; MS(ES) m/z = 727.5 (MH^+ requires 727.4). **106b** FmocPhe(Leu)$_2$ Rt = 23.1 min; MS(ES) m/z = 614.4 (MH^+ requires 614.3). **106a** FmocPheLeu Rt = 22.6 min; MS(ES) m/z = 501.1 (MH^+ requires 501.2).

*7.3.23 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised FmocGly(Leu)$_n$ Library **107***



107a-b n = 1-2

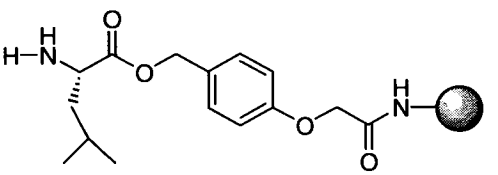
106 was deprotected as described in Section 7.3.5. Enzymatic coupling of FmocGly was carried out as described in Section 7.3.7. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN/H_2O (1:1) then analysed directly by LC-MS using gradient 1. **107b** FmocGly(Leu)$_2$ Rt = 20.7 min; MS(ES) m/z = 524.4 (MH^+ requires 524.4). **107a** FmocGlyLeu Rt = 20.0 min; MS(ES) m/z = 411.5 (MH^+ requires 411.2).

7.4 Chemical Solid Phase Peptide Synthesis

7.4.1 General procedure for removal of Fmoc and Amide Bond Formation

Resin (corresponding to 200 mg – 5 g) was deprotected according to section 7.3.5. A solution of Fmoc-amino acid (10 equiv), TBTU (10 equiv), HOBt (10 equiv) and DIPEA (10 equiv) in DMF (1-5 ml) was added to the resin. The reaction mixture was spun on a blood rotator for 2 hr at room temperature. The resin was filtered then washed according to standard protocol 7.2.3.

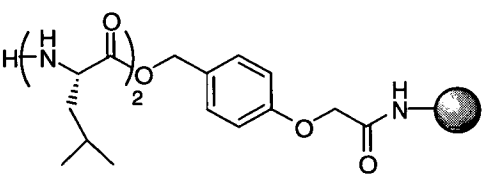
*7.4.2 Synthesis of $PEGA_{1900}$ Immobilised Leu **111***



111

90 was deprotected according to Section 7.3.4. The resin was filtered and washed according to standard protocol 7.2.3.

*7.4.3 Synthesis $PEGA_{1900}$ immobilised $(Leu)_2$ **112***

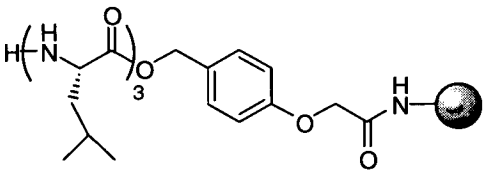


112

90 was deprotected then FmocLeu was coupled according to Section 7.4.1. The Fmoc was removed after coupling. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) then analysed

by LC-MS using gradient 1. $(Leu)_2$ Rt = 15.0 min; MS(ES) m/z = 245.5 (MH^+ requires 245.2).

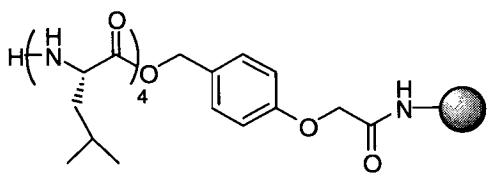
7.4.4 Synthesis of $PEGA_{1900}$ immobilised $(Leu)_3$ ***113***



113

90 was treated with two deprotection and coupling cylces of FmocLeu according to Section 7.4.1. The Fmoc was removed after the final coupling. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) then analysed by LC-MS using gradient 1. $(Leu)_3$ Rt = 16.3 min; MS(ES) m/z = 358.6 (MH^+ requires 358.3).

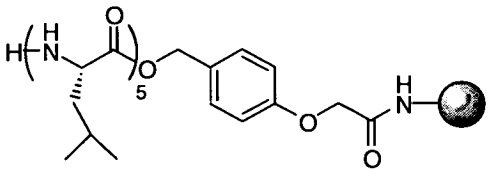
7.4.5 Synthesis of $PEGA_{1900}$ immobilised $(Leu)_4$ ***114***



114

90 was treated with three deprotection and coupling cylces of FmocLeu according to Section 7.4.1. The Fmoc was removed after the final coupling. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) then analysed by LC-MS using gradient 1. $(Leu)_4$ Rt = 17.0 min; MS(ES) m/z = 471.4 (MH^+ requires 471.4)

*7.4.6 Synthesis of $PEGA_{1900}$ immobilised $(Leu)_5$ **110***

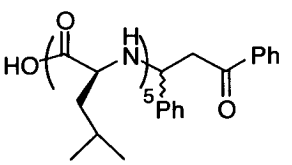


110

90 was treated with four deprotection and coupling cylces of FmocLeu according to Section 7.4.1. The Fmoc was removed after the final coupling. Product was cleaved from a small portion of beads as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) then analysed by LC-MS using gradient 1. $(Leu)_5$ Rt = 17.3 min; MS(ES) = 584.6 (MH^+ requires 584.4).

7.5 Adduct formation

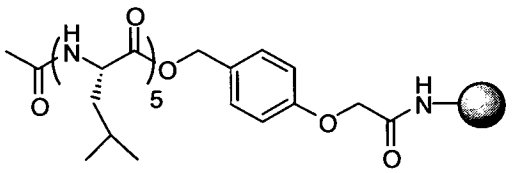
*7.5.1 Preparation of (2E)-1-Phenyl-3-phenyl-prop-2-ene-1-one.$(Leu)_5$ Adduct **117***



117

A solution of chalcone (7.2 mg, 3.5 x 10^{-5} mol) in 90% ACN in pH 8 KPi buffer (1 ml) was added to **110** (200 mg, 2 x 10^{-6} mol). The reaction mixture was vortexed then spun on a blood rotator overnight (18 hr) at 30°C. The resin was filtered and washed according to standard protocol 7.2.3. Products were then cleaved from the resin according to standard protocol 7.2.6. Washings were diluted with ACN:H_2O (1:1) and analysed directly by LC-MS using gradient 1. (2*E*)-1-Phenyl-3-phenyl-prop-2-ene-1-one.$(Leu)_5$ Rt = 18.3 min; MS(ES) m/z = 792.5 (MH^+ requires 792.5). (2*E*)-1-Phenyl-3-phenyl-prop-2-ene-1-one.$(Leu)_5$ Rt = 19.2 min; MS(ES) m/z = 792.7 (MH^+).

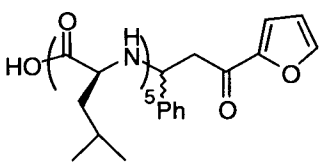
*7.5.2 Synthesis of Ac(Leu)$_5$ **115***



115

A solution of acetic anhydride (27.6 µl, 2.5 x 10^{-5} mol) and DIPEA (24 µl, 2.5 x 10^{-4} mol) in DMF (2 ml) was added to **110**. The reaction mixture was vortexed then spun on a blood rotator for 1 hour at room temperature. The resin was filtered and washed according to standard protocol 7.2.3. Product was cleaved form a small portion of resin as described in Section 7.2.6. Washings were diluted with ACN:H_2O and analysed directly by LC-MS using gradient 1. Ac(Leu)$_5$ Rt = 20.4 min; MS(ES) m/z = 626.4 (MH^+ requires 626.4).

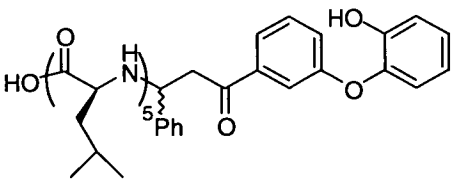
*7.5.3 Preparation of (2E)-1-(furan-2-yl)-3-phenyl-prop-2-ene-1-one.(Leu)$_5$ Adduct **127***



127

A suspension of **119** (8.5 mg, 3.5 x 10^{-5} mol) in pH8 KPi buffer (1 ml) was added to **110** (200 mg, 2 x 10^{-6} mol). The mixture was vortexed then incubated in a blood rotator overnight (18 hr) at 30°C. The resin was filtered and washed according to standard protocol 7.2.3. Product was then cleaved from the resin as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) then analysed directly by LC-MS. (2*E*)-1-(furan-2-yl)-3-phenyl-prop-2-ene-1-one.(Leu)$_5$ Rt = 18.4 min; MS(ES) m/z = 782.9 (MH^+ requires 782.5). (2*E*)-1-(furan-2-yl)-3-phenyl-prop-2-ene-1-one.(Leu)$_5$ Rt = 18.6 min; MS(ES) m/z = 782.7 (MH^+)..

*7.5.4 Preparation of (2E)-1-(3-hydroxy-4-phenoxyphenyl)-3-phenyl-prop-2-en-1-one .$(Leu)_5$ Adduct **128***

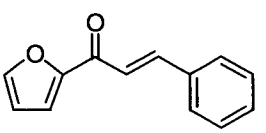


128

A suspension of **123** (8.5 mg, 3.5 x 10^{-5} mol) in pH 8 KPi buffer (1 ml) was added to **110** (200 mg, 2 x 10^{-6} mol). The mixture was vortexed then spun on a blood rotator overnight (18 hr) at 30°C. The resin was filtered and washed according to standard protocol 7.2.3. Product was then cleaved from resin as described in Section 7.2.6. Washings were diluted with ACH:H_2O (1:1) then analysed directly by LC-MS. (2*E*)-1-(3-hydroxy-4-phenoxyphenyl)-3-phenyl-prop-2-en-1-one.$(Leu)_5$ Rt = 18.9 min; MS(ES) m/z = 900.8 (MH^+ requires 900.5) (2*E*)-1-(3-hydroxy-4-phenoxyphenyl)-3-phenyl-prop-2-en-1-one.$(Leu)_5$ Rt = 19.3 min; MS(ES) m/z = 900.7 (MH^+).

7.6 Synthesis of Hydrophilic Analogues of Chalcone

*7.6.1 Synthesis of (2E)-1-(furan-2-yl)-3-phenyl-prop-2-ene-1-one **119** [1]*

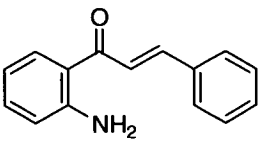


119

Benzaldehyde (5.6 ml, 56.6 mmol) was added to a solution of 2-acetylfuran (6.23 g, 56.6 mmol) in absolute ethanol (50 ml). The reaction mixture was cooled to 0°C. Six NaOH pellets were added and the reaction mixture was stirred overnight (18 hr) at 0 °C. The cream precipitate was filtered, washed with water and recrystallised from ethanol to give the *title compound* **119** as cream crystals (3.75 g, 33 % yield). ν_{max}

(KBr)/cm^{-1} 3148-3040 (Ar H), 1656 (CO); δ_H ($CDCl_3$; 250 MHz) 7.89 (1H, d, *J* 15.9, C*H*β), 7.64-7.68 (3H, m, *CH*ar), 7.46 (1H, d, *J* 15.9, C*H*α), 7.41-7.43 (3H, m, C*H*ar), 7.34 (1H, d, *J* 3.6, C*H*ar), 6.60 (1H, dd, *J* 3.6, 1.7, C*H*ar); δ_C ($CDCl_3$; 63 MHz) 177.9(*C*O), 153.6 (*C*ar), 146.4 (*C*Har) 143.9 (*C*Hβ), 135.6 (*C*ar)130.5 (*C*Har), 128.8 (2 x *C*Har), 128.4 (2 x *C*Har), 121.0 (*C*Har), 117.4 (*C*Hα), 112.4 (*C*Har); MS(ES) m/z = 198.5 (M requires 198.1), 220.5 (MNa^+). ^{1}H NMR and ^{13}C NMR consistent with literature data [1].

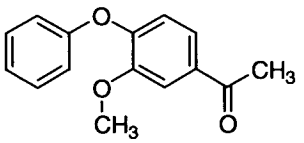
7.6.2 Synthesis of (2E)-1-(2-aminophenyl)-3-phenyl-prop-2-ene-1-one ***118*** [2]



118

Benzaldehyde (2.8 ml, 25.3 mmol) was added to a solution of 2-aminoacetophenone (2.5 g, 18.5 mmol) in absolute methanol (30 ml). The reaction mixture was cooled to 0 °C. Six NaOH pellets were added and the reaction mixture was stirred overnight (18 hr) at 0°C. The bright yellow precipitate was filtered and recrystallised from ethanol to give the *title compound* **118** as yellow crystals (3.27 g, 85 % yield). ν_{max} (KBr)/cm^{-1} 3288-3441 (NH_2), 3025-3077 (ArH), 1645 (CO); δ_H($CDCl_3$; 250 MHz) 7.87 (1H, dd, *J* 8.4, 1.6, C*H*ar), 7.75 (1H, d, *J* 15.6, C*H*β), 7.59-7.67 (3H, m, C*H*ar, C*H*α), 7.39-7.44 (3H, m, C*H*ar), 7.27-7.33 (1H, m, C*H*ar), 6.67-6.73 (2H, m, C*H*ar), 6.33 (2H, bs, N*H*$_2$); δ_C ($CDCl_3$; 63 MHz) 192.1 (*C*O), 151.4 (*C*ar), 143.3 (*C*Hβ), 135.0 (*C*ar), 134.7 (*C*Har), 131.4 (*C*Har), 130.4 (*C*Har), 129.3 (2 x *C*Har) 128.6 (2 x *C*Har), 123.5 (*C*Har), 119.5 (*C*ar), 117.7 (*C*Hα), 116.3 (*C*Har); MS(ES) m/z = 223.8 (M requires 223.1). ^{1}H NMR consistent with literature data [2].

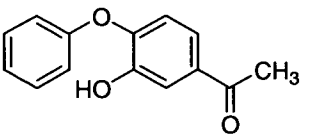
7.6.3 Synthesis of 3-Methoxy-4-phenoxyacetophenone **125** [3]



125

Activated molecular sieves (3 g, 4A°), phenylboronic acid (20 g, 164 mmol), copper (II) acetate (7.98 g, 40 mmol) and anhydrous pyridine (13.1 ml, 160 mmol) were added successively to a stirred solution of 4-hydroxy-3-methoxyacetophenone (6.70 g, 40 mmol) in anhydrous DCM (400 ml) under N_2. Dried air was bubbled through the solution and the resulting black mixture was stirred at room temperature for 48 hr. The reaction mixture was filtered through celite. The filtrate was washed with Na_2-EDTA solution (2M, 500 ml), dilute aqueous HCl acid (2M, 500ml) and extracted with DCM (2 x 300 ml). The combined organic extracts were washed with brine (2 x 250 ml) and dried over $MgSO_4$. Solvent was removed under reduced pressure to afford the crude product as brown solid. The crude material was purified by column chromatography (Si; diethyl ether: petroleum ether, 1:1) to give the *title compound* **125** as a pale cream solid (3.39 g, 34 % yield). Rf = 0.6 (diethyl ether: petroleum ether, 1:1); ν_{max} (KBr)/ cm^{-1} 2840-3077 (Ar H), 1674 (CO); δ_H ($CDCl_3$; 250 MHz) 7.64 (1H, d, *J* 2.0, C*H*ar), 7.50 (1H, dd, *J* 8.3, 2.0, C*H*ar), 7.36-7.39 (2H, m, C*H*ar), 7.15 (1H, tt, *J* 7.5, 1.1, C*H*ar), 7.01-7.05 (2H, m, C*H*ar), 6.88 (1H, d, J 8.3, C*H*ar), 3.94 (3H, s, OCH_3), 2.56 (3H, s, $COCH_3$); δ_C ($CDCl_3$; 63 MHz) 196.7 ($COCH_3$), 156.1 (*C*ar), 150.3 (2 x *C*ar), 132.9 (*C*ar), 129.7 (2 x *C*Har), 123.8 (*C*Har), 122.5 (*C*Har), 118.8 (2 x *C*Har), 117.8 (*C*Har), 111.4 (*C*Har), 56.0 (OCH_3), 26.3 ($COCH_3$); MS(ES) m/z = 242.6 (M requires 242.1). ^1H NMR and ^{13}C NMR consistent with literature data [3].

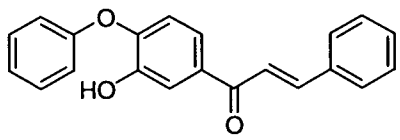
7.6.4 Synthesis of 3-Hydroxy-4-phenoxyacetophenone **126** [3]



126

A solution of **125** (2.44 g, 10.08 mmol) in anhydrous DCM (7.5 ml) was added dropwise to a solution of boron tribromide (1M, 2.83 ml, 30.25 mmol) in DCM (30.25 ml) under N_2. The resulting green solution was stirred at -78°C for 1 hr. The temperature was then adjusted to 0°C and stirred for 2 hr. The reaction mixture was transferred slowly via canula to a solution of vigorously stirring saturated sodium hydrogen carbonate (280 ml) at 0°C (on neutralisation HBr gas is evolved). The resulting mixture was stirred at 0°C for 1 hr until no more gas was evolved. The mixture was extracted with DCM (3 x 250 ml). The combined extracts were washed with brine (2 x 250 ml) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure to afford a red/orange oil which crystallised on standing. The crude product was purified by column chromatography (Si; petroleum ether: ethyl acetate, 80:20) to give the *title compound* **126** as a pale cream solid (1.6 g, 70 % yield). Rf = 0.2 (petroleum ether:ethyl acetate, 80:20); ν_{max} (KBr)/cm^{-1} 3224 (bd, OH), 1660 (CO); δ_H ($CDCl_3$; 250 MHz) 7.65 (1H, d, *J* 2.1, C*H*ar), 7.42 (1H, dd, *J* 8.5, 2.1, C*H*ar), 7.40-7.41 (2H, m, C*H*ar), 7.24 (1H, tt, *J* 7.5, 1.2, C*H*ar), 7.07-7.10 (2*H*, m, C*H*ar), 6.82 (1H, d, *J* 8.5, C*H*ar), 5.91 (1H, bs, OH), 2.57 (3H, s. $COCH_3$); δ_C ($CDCl_3$; 63 MHz) 196.8 ($COCH_3$) 155.1 (*C*ar), 148.4 (*C*ar), 146.6 (*C*ar), 133.1 (*C*ar), 130.3 (2 x *C*Har), 124.7 (*C*Har), 121.3 (*C*Har), 119.3 (2 x *C*Har), 116.4 (*C*Har), 116.0 (*C*Har), 26.4 ($COCH_3$); MS(ES) m/z = 228.5 (M requires 228.1). ^1H NMR and ^{13}C NMR consistent with literature data [3].

7.6.5 Synthesis of (2E)-1-(3-hydroxy-4-phenoxyphenyl)-3-phenyl-prop-2-en-1-one ***123***[3]

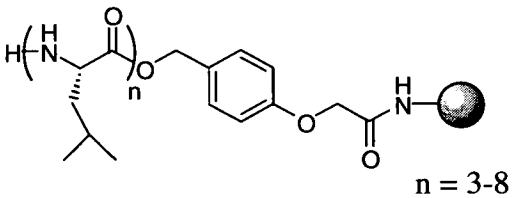


123

Benzaldehyde (0.85 ml, 8.4 mmol) was added to a solution of **126** (1.28 g, 5.6 mmol) in absolute ethanol (10 ml). The reaction mixture was cooled to 0°C. 6 NaOH pellets were added and the reaction mixture was stirred at 0°C overnight (18 hr). The resulting bright orange precipitate was filtered and purified by column chromatography (Si; ethyl acetate: n-hexane, 1:3) to give the *title compound* **123** as a pale yellow crystals (1 g, 37 % yield). Rf = 0.5 (ethyl acetate:n-hexane, 1:3); ν_{max} (KBr)/cm^{-1} 3341 (bd, OH), 1657 (CO); δ_H ($CDCl_3$; 250 MHz) 7.82 (1H, d, *J* 15.7, C*H*β), 7.75 (1H, d, *J* 2.1, C*H*ar), 7.61-7.63 (2H, m, C*H*ar), 7.54 (1H, dd, *J* 8.5, 2.1, C*H*ar), 7.50 (1H, d, *J* 15.7, C*H*α), 7.38-7.43 (4H, m, C*H*ar), 7.19-7.22 (2H, m, C*H*ar), 7.09-7.13 (2H, m, C*H*ar), 6.88 (1H, d, *J* 8.5, C*H*ar), 5.88 (1H, bs, O*H*); δ_C ($CDCl_3$; 63 MHz) 188.8 (*C*O), 155.2 (*C*ar), 148.3 (*C*ar), 146.7 (*C*ar), 144.5 (*C*Hβ) 134.2 (*C*ar), 134.1 (*C*ar), 130.4 (C*H*ar), 130.1 (2 x C*H*ar), 128.9 (2 x C*H*ar), 128.4 (2 x C*H*ar), 124.7 (C*H*ar), 121.6 (C*H*ar), 121.5 (C*H*ar), 119.3 (2 x C*H*ar), 116.6 (C*H*α), 116.1 (*C*Har); MS(ES) m/z = 316.5 (M requires 316.1). ^1H NMR and ^{13}C NMR consistent with literature data [3].

7.7 Unprotected Poly-L-Leu Libraries

7.7.1 Attempted Synthesis of Immobilised Poly-L-Leu Library ***130***



n = 3-8

130a-f

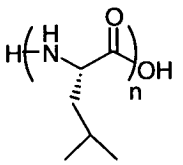
90 (200 mg, 2 x 10^{-6} mol) was deprotected as described in Section 7.3.5. Enzymatic coupling of $(Leu)_2$ to resin was carried out as described in Section 7.3.7. Product was cleaved from a small portion of resin as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) then analysed directly by LC-MS using gradient 2. **130a** $(Leu)_3$ Rt = 16.5 min; MS(ES) m/z = 358.1 (MH^+ requires 358.3). **130b** $(Leu)_4$ Rt = 18.3 min; MS(ES) m/z = 471.2 (MH^+ requires 471.4). **130c** $(Leu)_5$ Rt = 19.6 min; MS(ES) m/z = 254.4 (MH^+ requires 584.4). **130d** $(Leu)_6$ Rt = 20.1 min; MS(ES) m/z = 697.2 (MH^+ requires 697.5). **130e** $(Leu)_7$ Rt = 22.6 min; MS(ES) m/z = 810.6 (MH^+ requires 810.6). **130f** $(Leu)_8$ Rt = 25.0 min; MS(ES) m/z = 923.5 (M requires 923.7).

7.7.2 Attempted Templating of Immobilised Poly-L-Leu Library ***130a-f***

90 (200 mg, 2 x 10^{-6} mol) was deprotected as described in standard protocol 7.3.5. A saturated solution of **123** (11 mg, 3.5 x 10^{-5} mol) in pH8 KPi buffer (1 ml) was added to deprotected **90** together with $(Leu)_2$ (5 mg, 2 x 10^{-5} mol) and thermolysin (10 mg). The reaction mixture was vortexed and incubated on a blood rotator overnight (18 hr) at 30°C. Product was cleaved from the resin as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) then analysed directly by LC-MS using gradient 2. **130a** $(Leu)_3$ Rt = 16.3 min; MS(ES) m/z = 358.1 (MH^+). **130b** $(Leu)_4$ Rt = 18.2 min; MS(ES) m/z = 471.1 (MH^+). **130c** $(Leu)_5$ Rt = 19.6 min; MS(ES) m/z = 584.4 (MH^+). **130d** $(Leu)_6$ Rt = 20.8 min; MS(ES) m/z = 697.3 (MH^+). **130e** $(Leu)_7$ Rt = 22.2 min. MS(ES) m/z = 810.6 (MH^+). **130f** $(Leu)_8$ Rt = 24.5 min; MS(ES) m/z = 923.8 (M).

7.8 Tagging of Unprotected Libraries

7.8.1 Generation of Solution Phase Poly-L-Leucine Library 135

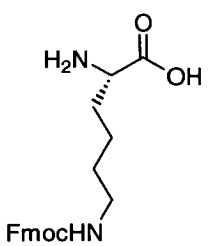


n = 3-8

135a-f

Thermolysin (10 mg) was added to a suspension of $(Leu)_2$ (10 mg, 4 x 10^{-5} mol) in pH8 KPi buffer (2 ml). The mixture was vortexed to effect thorough mixing then spun on a blood rotator overnight (18 hr) at 30°C. 1M HCl was added to the reaction mixture dropwise until the pH tested acidic. A 100 µl aliquot of the reaction mixture was diluted to 2 ml with ACN:H_2O (1:1) then analysed directly by LC-MS using gradient 2. **135a** $(Leu)_3$ Rt = 18.3 min; MS(ES) m/z = 358.4 (MH^+). **135b** $(Leu)_4$ Rt = 19.8 min. MS(ES) m/z = 471.3 (MH^+). **135c** $(Leu)_5$ Rt = 21.0 min; MS(ES) m/z = 584.4 (MH^+). **135d** $(Leu)_6$ Rt = 21.1 min; MS(ES) m/z = 697.4 (MH^+). **135e** $(Leu)_7$ Rt = 23.6 min. MS(ES) m/z = 810.4 (MH^+). **135f** $(Leu)_8$ Rt = 25.2 min; MS(ES) m/z = 924.0 (MH^+).

7.8.2 Synthesis of N^{ε}-Fluorenylmethyloxycarbonyl-L-lysine 138

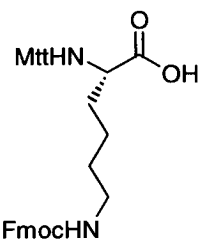


138

N^{α}-Boc-N^{ε}-Fmoc-L-Lysine (3 g, 6.4 mmol) was dissolved in a 1:1 mixture of TFA:DCM (20 ml) and stirred for 1 hour at room temperature. Solvent was removed under reduced pressure to give a clear oil which was triturated into to cold ether. The resulting white solid was recrystalised from methanol to give the *title compound* **138** as

a fluffy white solid (2.36 g, 99 % yield). mp 182-184 °C; ν_{max} (KBr)/cm^{-1} 3314 (NH_2) , 3065-2936 (Ar H), 1692 (OC*O*NH); δ_H (DMSO; 360 MHz) 7.88 (2H, d, *J* 7.6, C*H*ar) 7.68 (2H, d, *J* 7.6, C*H*ar) 7.40 (2H, t, *J* 7.6, C*H*ar) 7.32 (2H, t, *J* 7.6, C*H*ar) 4.28 (2H, d, *J* 6.5, CHCH_2O) 4.20 (1H, t, *J* 6.5, C*H*CH_2O) 3.48 (1H, t, *J* 6.2, C*H*α) 2.96 (2H, m, C*H*ε) 1.60-1.80 (2H, m, C*H*β) 1.20-1.45 (4H, m, C*H*γ, C*H*δ); δ_C (DMSO; 90 MHz) 172.4 (Lys *C*O) 157.6 (Fmoc *C*O) 145.4 (2 x *C*ar) 142.2 (2 x *C*ar) 129.08 (2 x *C*Har)128.5 (2 x *C*Har) 126.6 (2 x *C*Har) 121.6 (2 x *C*Har) 66.7 (CH*C*H_2O) 54.6 (*C*Hα) 48.2 (*C*HCH$_2$O) 41.2 (*C*H$_2$ε) 31.7 (*C*H$_2$β) 30.6 (*C*H$_2$δ) 23.5 (*C*H$_2$γ); $[\alpha]_D^{22}$ + 15° (c = 1, MeOH 0.6% TFA); MS(FAB) Found: MH^+ 369.18082, $C_{21}N_2O_4H_{24}$ requires MH^+ 369.18143.

7.8.3 Synthesis of N^{α}-Methyltrityl-N^{ε}-fluorenylmethyloxycarbonyl-L-lysine ***136***

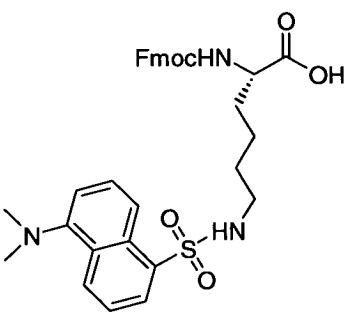


136

To a stirred suspension of **138** (3.81 g, 10.3 mmol) in $CHCl_3$ (25 ml), Me_3SiCl (1.31 ml, 10.3 mmol) was added. The reaction was heated under reflux for 2 hours then allowed to cool to room temperature. Et_3N (2.89 ml, 20.6 mmol) was added to the mixture at a rate sufficient to maintain a gentle reflux. A solution of Mtt-Cl (3.02 g, 10.3 mmol) in $CHCl_3$ (10 ml) was added dropwise and the resulting solution was stirred overnight (16 hr) at room temperature. Methanol (50 mmol) was added and the reaction mixture was allowed to stir for a further 30 min. Solvent was removed under reduced pressure to give a residue which was partitioned between diethyl ether (70 ml) and a solution of precooled citric acid (70 ml). Unreacted **138** crystalised and was removed by filtration. The organic layer was washed with brine (2 x 30 ml), saturated $NaHCO_3$ (2 x 30 ml) and water (2 x 30 ml). The organic layer was dried ($MgSO_4$) and the solvent removed under reduced pressure. The crude product was purified by column chromatography (Si; $CHCl_3$:MeOH: Et_3N, 92.5:5:2.5) to give the *title compound* **136** as a white foam (1.75 g,

27 % yield). mp 59-61 °C; ν_{max} (KBr)/cm^{-1} 3415-3323 (NH) 3057-2863 (ArH) 1712 (OC*O*NH); δ_H ($CDCl_3$; 360 MHz) 7.67 (2H,d, *J* 7.3, C*H*ar) 7.51 (2H, d, *J* 7.3, C*H*ar) 7.43 (4H, d, *J* 8.0, C*H*ar) 7.28-7.32 (4H, m, C*H*ar) 7.18-7.23 (2H, m, C*H*ar) 7.09-7.14 (4H, m, C*H*ar) 7.03-7.05 (2H, m, C*H*ar) 6.90 (2H, t, *J* 8.0, C*H*ar) 4.27 (2H, m, CHCH_2O) 4.11 (1H, t, *J* 6.9, C*H*CH_2O) 3.14 (1H, m, C*H*α) 3.02 (2H, m, C*H*ε) 2.18 (3H, s, CH_3) 1.38-1.40 (2H, m, C*H*β) 1.26-1.30 (4H, m, C*H*γ, C*H*δ); δ_C ($CDCl_3$; 90 MHz) 185.7 (Lys *C*O) 162.2 (Fmoc*C*O) 153.0 (2 x *C*ar) 149.9 (*C*ar)149.8 (2 x *C*ar) 147.0 (2 x *C*ar) 141.0 (*C*ar) 134.9 (4 x *C*Har) 134.7 (2 x *C*Har) 134.0 (2 x *C*Har) 133.4 (2 x *C*Har) 133.2 (4 x *C*Har) 132.8 (2 x *C*Har) 131.7 (2 x *C*Har) 130.9 (2 x *C*Har) 125.7 (2 x *C*Har) 76.9 (CH*C*H_2O) 72.2 (trityl *C*) 63.0 (*C*Hα) 53.0 (*C*HCH$_2$O) 46.5 (*C*H$_2$ε) 40.6 (*C*H$_2$β) 35.5 (*C*H$_2$δ) 28.0 (C*H*$_2$γ) 26.7 (*C*H$_3$); $[\alpha]_D^{22}$ +16° (c = 2, $CHCl_3$); MS(FAB) Found: MH^+ 625.30581 $C_{41}N_2O_4H_{40}$ requires MH^+ 625.30663.

7.8.4 Synthesis of N^{α}-Fluorenylmethyloxycarbonyl-N^{ε}-diaminonaphthalenesulphonamide-L-lysine **143**

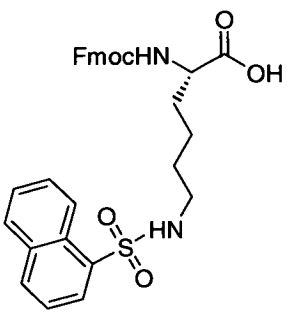


143

A solution of Dns-Cl (2 g, 5.4 mmol) in THF (10 ml) was added to a stirred solution of N^{α}-FmocLys (2 g, 5.4 mmol) and Na_2CO_3 (2.30 g, 21.7 mmol) in THF (28 ml) and H_2O (12 ml). The reaction mixture was stirred vigorously at room temperature for 1 hour. The product was extracted with ethyl acetate (2 x 50 ml). The combined organic layers were washed with brine (2 x 50 ml) and H_2O (2 x 50 ml) then dried over $MgSO_4$. Solvent was removed under reduced pressure to give a yellow oil which was purified by column chromatography (Si; $CHCl_3$:MeOH:AcOH, 92.5:5:2.5). Fractions containing the *title compound* were combined and washed with 0.1 M HCl (5 x 20 ml) then dried over

$MgSO_4$. Solvent was removed under reduced pressure to give the *title compound* **143** as a yellow foam (2.68 g, 82 % yield). Rf = 0.2 ($CHCl_3$:MeOH:AcOH, 92.5:5:2.5) mp = 60-62 °C; ν_{max} (KBr)/cm^{-1} 3294 (NH), 3064-2789 (Ar H), 1711 (OC*O*NH), 1142 (SO_2); δ_H (DMSO 360 MHz) 8.45 (1H, d, *J* 8.3, C*H*ar) 8.31 (1H, d, *J* 8.3, C*H*ar) 8.10 (1H, dd, *J* 7.2, 1.1, C*H*ar) 7.52-7.93 (7H, m, C*H*ar) 7.24-7.39 (4H, C*H*ar) 4.25-4.30 (2H, m, CH_2CHO) 4.2 (1H, t, *J* 7.2, CH_2CHO) 3.79-3.81 (1H, m, C*H*α) 2.81 (6H, s, 2 x CH_3N) 2.73-2.78 (2H, m, C*H*ε) 1.22-1.32 (6H, m, C*H*β, C*H*γ, C*H*δ) ; δ_C (DMSO; 90 MHz) 173.3 (Lys *C*O) 155.9 (Fmoc *C*O) 151.4 (*C*ar) 143.9 (2 x *C*ar) 140.9 (2 x *C*ar) 136.2 (*C*ar) 129.6 (*C*Har)129.2 (*C*ar) 128.8 (*C*ar) 128.4 (*C*Har) 127.9 (*C*Har) 127.7 (2 x *C*Har) 127.2 (2 x *C*Har) 125.4 (2 x *C*Har) 123.8 (*C*Har) 120.2 (2 x *C*Har) 119.3 (*C*Har) 115.3 (*C*Har) 65.3 ($CHCH_2O$) 53.4 (*C*Hα) 46.5 ($CHCH_2O$) 44.9 (2 x CH_3) 42.0 (CH_2ε) 30.0 (CH_2β) 28.6 (CH_2δ) 22.5(CH_2γ); $[\alpha]_D^{22}$ +2.5° (c = 2, $CHCl_3$); MS(FAB) Found MH^+ 602.23386 $C_{33}H_{35}O_6N_3$ requires MH^+ 602.23248.

7.8.5 Synthesis of N^{α}-Fluorenylmethyloxycarbonyl-N^{ε}-naphthalenesulphonamide-L-lysine ***144***

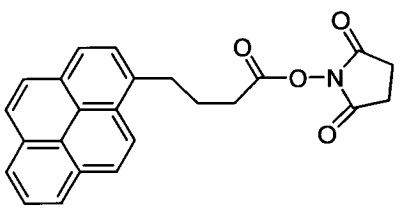


144

A solution of naphthalenesulphonyl chloride (1.76 g, 5.4 mmol) in THF (10 ml) was added to a stirred solution of N^{α}-FmocLys (2 g, 5.4 mmol) and $NaCO_3$ (2.30 g, 21.7 mmol) in THF (28 ml) and H_2O (12 ml). The reaction mixture was stirred vigorously at room temperature for one hour. The product was extracted with ethyl acetate (2x 50 ml). The combined organic layers were washed with brine (2 x 50 ml) and H_2O (2 x 50 ml) then dried over $MgSO_4$. Solvent was removed under reduced pressure to give a clear oil which was purified by column chromatography (Si; $CHCl_3$:MeOH:AcOH, 92.5:5:2.5).

Fractions containing the *title compound* **144** were combined and washed with 0.1 M HCl (5 x 20 ml) then dried over $MgSO_4$. Solvent was removed under reduced pressure to give the *title compound* as a white foam (1.88 g, 62 g). Rf = 0.4 ($CHCl_3$:MeOH:AcOH, 92.5:5:2.5); mp = 58-60 °C; ν_{max} (KBr)/cm^{-1} 3277 (NH), 3061-2857 (Ar H), 1709 (OC*O*NH), 1155 (SO_2); δ_H (DMSO; 360 MHz) 8.44 (1H, s, N*H*) 8.11-8.17 (2H, m, C*H*ar) 8.03 (1H, d, J 7.6, C*H*ar) 7.81-7.87 (3H, m, C*H*ar) 7.63-7.71 (4H, m, C*H*ar) 7.58 (1H, d, J 8.3, C*H*ar) 7.39 (2H, t, J 7.6, C*H*ar) 7.30 (2H, t, J 7.6, C*H*ar) 4.27-4.29 (2H, m, CHCH_2O) 4.21 (1H, t, J 7.2, C*H*CH_2O), 3.85-3.87 (1H, m, C*H*α) 2.72-2.77 (2H, m, C*H*ε) 1.28-1.39 (6H, m, C*H*β, C*H*γ, C*H*δ); δ_C (DMSO; 90 MHz) 174.1 (Lys *C*O) 156.3 (Fmoc *C*O) 144.0 (*C*ar) 143.9 (*C*ar) 140.9 (2 x *C*ar) 137.6 (*C*ar) 134.3 (*C*ar) 131.9 (*C*ar) 130.8 (*C*Har) 130.6 (*C*Har) 130.1 (*C*Har) 129.3 (*C*Har) 129.1 (2 x *C*Har) 129.0 (*C*Har) 128.8 (*C*Har) 128.5 (2 x *C*Har) 126.7 (2 x *C*Har) 123.8 (*C*Har) 121.6 (2 x *C*Har) 65.7 (CH*C*H_2O) 53.8 (*C*Hα) 46.8 (*C*HCH_2O) 42.6 (*C*H_2ε) 30.4 (*C*H_2β) 29.0 (*C*H_2δ) 23.7 (*C*H_2γ); $[\alpha]_D^{22}$ +6° (C = 2, $CHCl_3$); MS(FAB) Found 559.19094 $C_{31}H_{30}O_6H_2S$ requires MH^+ 559.19028.

7.8.6 Synthesis of 4-Pyrenebutyric acid N-hydroxysuccinimide ester ***150*** [4]

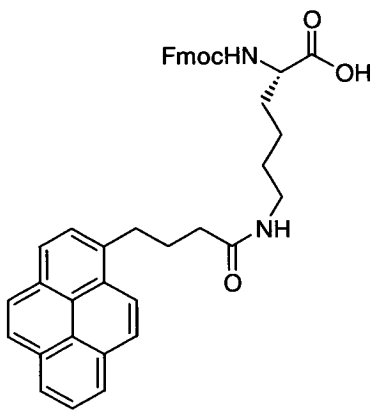


150

A solution of *N*-hydroxysuccinimide (2.39 g, 20.8 mmol) and EDCI (2.99 g, 15.6 mmol) in DMF (10 ml) was added to a solution of 4-pyrenylbutanoic acid (3 g, 10.4 mmol) in DMF (30 ml). The reaction mixture was stirred vigorously at room temperature for 16 hours. Ethyl acetate was added and the organic layer was washed with brine (2 x 50 ml), saturated sodium bicarbonate solution (2 x 50 ml) and H_2O (2 x 50 ml) then dried over $MgSO_4$. Solvent was removed under reduced pressure to give a brown powder which was purified by column chromatography (Si; ethyl

acetate:toluene, 25:75) to give the *title compound* **150** as a light brown powder (3.23 g, 82 % yield). Rf = 0.53 (ethyl acetate:toluene, 25:72); ν_{max} (KBr)/cm^{-1} 3037-2873 (Ar H), 1817 (C*O*NCO), 1785 (C*O*NCO), 1728 (OC*O*); δ_H (DMSO; 250 MHz) 7.60-8.21 (9*H*, m, C*H*ar) 2.83 (2H, t, *J* 7.5, C*H*4) 2.73 (4H, s, CH_2, NHS) 2.61 (2*H*, t, *J* 7.5, C*H*2) 2.38 (2H, quint, *J 7.5* C*H*3); δ_C (DMSO; 63 MHz) 169.0 (2 x NHS *C*O) 168.4 (pyrene *C*O) 134.7 (*C*ar) 131.3 (*C*ar) 130.7 (*C*ar) 130.0 (*C*ar) 128.6 (*C*ar) 127.5 (*C*Har) 127.3 (2 x *C*Har) 126.7 (*C*Har) 125.7 (*C*Har) 124.9 (2 x *C*Har) 124.8 (*C*Har) 124.7 (2 x *C*ar) 123.0 (*C*Har) 32.1 (C$H_2$2) 30.4 (*C*$H_2$4) 26.3 (*C*$H_2$3) 25.5 (2 x NHS *C*H_2); MS(FAB) Found: 386.13562 $C_{24}H_{19}O_4O$ requires MH^+ 386.13923. ^{13}C NMR consistent with literature data [4].

7.8.7 Synthesis of N^{α}-Fluorenylmethyloxycarbonyl-N^{ε}-pyrenebutanoyl-L-lysine ***145*** [4]

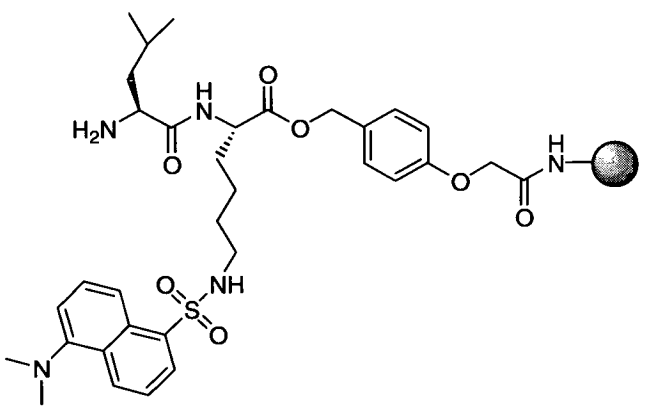


145

A solution of **150** (0.79 g, 2.21 mmol) in THF (10 ml) was added to a stirring solution of N^{α}-FmocLys (0.83 g, 2.25 mmol) and Na_2CO_3 (0.47 g, 4.42 mmol) in THF (21 ml) and water (9 ml). The reaction mixture was stirred at room temperature for 1 hour. The product was extracted with ethyl acetate (2 x 30 ml). The organic layers were combined and washed with brine (2 x 50 ml) and H_2O (2 x 50 ml) then dried over $MgSO_4$. Solvent was removed under reduced pressure to give a brown syrup which was purified by column chromatography (Si; MeOH:DCM, 10:90) to give the *title compound* **145** as a creamy white powder (1.30 g, 92 % yield). Rf = 0.17 (MeOH:DCM, 10:90); ν_{max} (KBr)/cm^{-1} 3312.1 (NH), 3039-2860 (Ar H) 1742 (OC*O*NH), 1684 (C*O*NH); δ_H

(DMSO, 360 MHz) 8.36 (1H, d, *J* 9.4, C*H*ar) 8.25 (2H, d, *J* 7.9, C*H*ar) 8.17-8.26 (2H, m, C*H*ar) 7.91-8.02 (5H, m, C*H*ar) 7.65-7.71 (3H, m, C*H*ar) 7.39 (2H, td, *J* 7.6, 2.9, C*H*ar) 7.30 (2H, tt, *J* 7.6, 1.4, C*H*ar) 4.25 (2H, d, *J* 6.8, CHCH_2O) 4.21 (1H, t, *J* 6.8, C*H*CH_2O) 3.91-3.97 (1H, m, C*H*α) 3.29 (2H, t, *J* 7.2, C*H*ε) 3.08-3.10 (2H, m, C*H*4) 2.23 (2H, t, *J* 7.6 C*H*2) 1.99-2.03 (2H, t, *J* 7.6, C*H*3) 1.35-1.73 (6H, m, C*H*δ, C*H*β, C*H*γ); δ_C (DMSO, 90 MHz) 174.2 (Lys *C*O) 171.9 (Pyr *C*O) 156.3 (Fmoc *C*O) 144.0 (*C*ar) 143.9 (*C*ar) 140.8 (2 x *C*ar) 136.7 (*C*ar) 131.0 (*C*ar) 130.6 (*C*ar) 129.4 (*C*ar) 128.3 (*C*ar) 127.8 (2 x *C*Har) 127.7 (*C*Har) 127.6 (*C*Har) 127.3 (*C*Har) 127.2 (2 x *C*Har)126.6 (*C*Har) 126.3 (*C*Har) 125.4 (2 x *C*Har) 125.1 (2 x *C*Har) 124.9 (*C*Har) 124.4 (*C*ar) 124.3 (*C*ar) 123.6 (*C*Har) 120.2 (2 x *C*Har) 65.7 (CH*C*H_2O) 53.9 (*C*Hα) 46.8 (*C*HCH_2O) 38.4 (*C*H_2ε) 35.2 (*C*$H_2$4) 32.4 (*C*$H_2$2) 30.6 (*C*H_2β) 29.0 (*C*H_2δ) 27.7 (*C*$H_2$3) 23.3 (*C*H_2γ); $[\alpha]_D^{22}$ + 2.0° (c = 1, THF); MS(FAB) Found: 639.28477 $C_{41}H_{38}O_5N_2$ requires MH^+ 639.28590. ^{13}C NMR consistent with literature data [4].

7.8.8 Chemical Synthesis of PEGA$_{1900}$ Immobilised H-LeuLys(Dns) ***154***

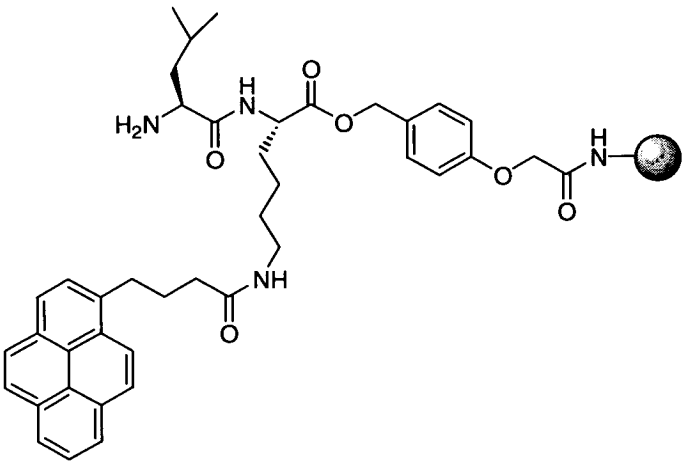


154

143 was attached to **86** *via* the preformed symmetrical anhydride method as described in sections 7.3.2 and 7.3.3. The Fmoc was removed and FmocLeu was subsequently coupled as described in standard protocol 7.4.1. The resin was deprotected after coupling as described in section 7.3.5. Product was cleaved from a small portion of resin as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) and

analysed directly by LC-MS using gradient 1. H-LeuLys(Dns)-OH Rt = 15.9 min; MS(ES) m/z = 493.2 (MH^+ requires 493.2).

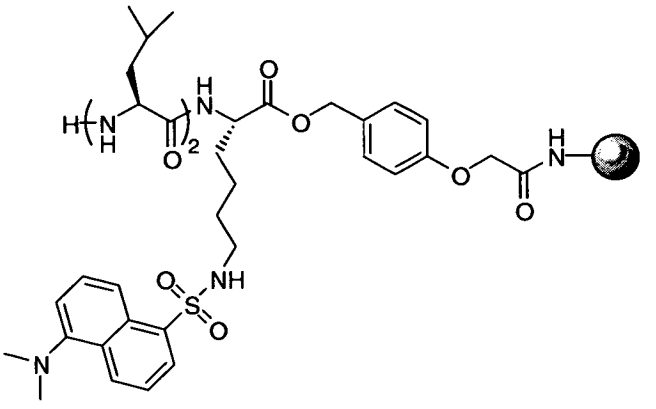
7.8.9 Chemical Synthesis of $PEGA_{1900}$ Immobilised H-LeuLys(Pyrene) **156**



156

145 was attached to **86** *via* the preformed symmetrical anhydride method as described in Sections 7.3.2 and 7.3.3. The Fmoc was removed and FmocLeu was subsequently coupled as described in standard protocol 7.4.1. The resin was deprotected after coupling as described in section 7.3.5. Product was cleaved from a small portion of resin as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) and analysed directly by LC-MS using gradient 1. H-LeuLys(Pyrene) Rt = 17.5 min; MS(ES) m/z = 530.3 (MH^+ requires 530.3)

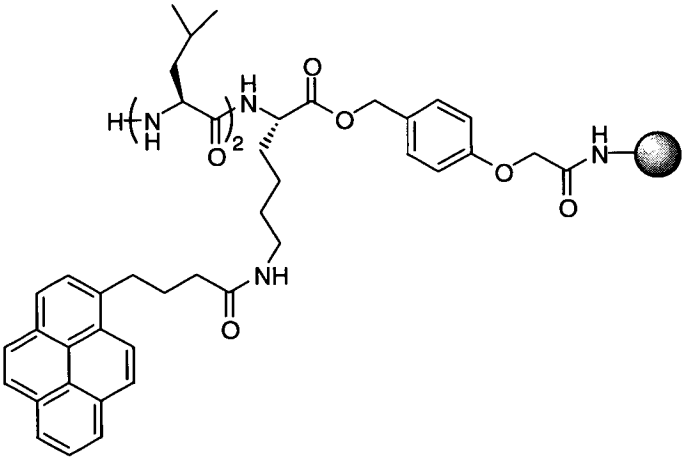
*7.8.10 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised H-$(Leu)_2$Lys(Dns) **157***



157

FmocLeu was enzymatically coupled to **154** as described in Section 7.3.6. The resin was deprotected after coupling as described in section 7.3.5. Product was cleaved from a small amount of resin as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) and analysed directly using gradient 1. H-$(Leu)_2$Lys(Dns) Rt = 16.2 min; MS(ES) m/z = 606.2 (M requires 606.3)

*7.8.11 Enzymatic Synthesis of $PEGA_{1900}$ Immobilised H-$(Leu)_2$Lys(Pyrene) **158***

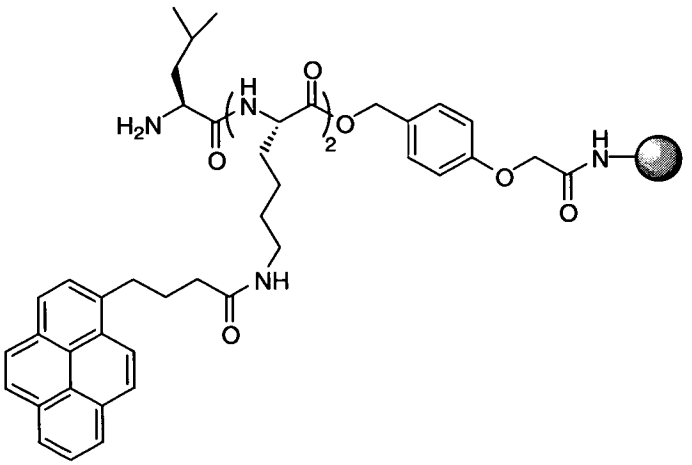


158

FmocLeu was enzymatically coupled to **156** was described in Section 7.3.6. The resin was deprotected after coupling as described in section 7.3.5. Product was cleaved from a

small amount of resin as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) and analysed directly by LC-MS using gradient 1. H-(Leu)$_2$Lys(Pyrene) Rt = 16.6 min; MS(ES) m/z = 643.3 (M requires 643.3)

7.8.12 Chemical Synthesis of PEGA$_{1900}$ Immobilised H-Leu[Lys(Pyrene)]$_2$ **160**



160

145 was attached to **86** via the preformed symmetrical anhydride method as described in Section 7.3.2 and 7.3.3. The resin was deprotected and a further residue **145** was coupled using standard protocol 7.4.1. The deprotection/coupling method was repeated with FmocLeu. Resin was deprotected after the final coupling as described in Section 7.3.5. Product was cleaved from a small portion of resin as described in Section 7.2.6. Washings were diluted with ACN:H_2O (1:1) and analysed directly using gradient 1. H-Leu[Lys(Pyrene)]$_2$. Rt = 18.7 min; MS(ES) m/z = 928.9 (MH^+ requires 928.5).

7.9 Solution Phase Dynamic Peptide Libraries

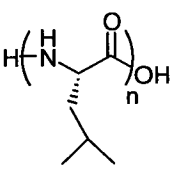
7.9.1 General Procedure for Enzymatic Generation of Peptide Libraries in 90 % ACN

Dipeptide (4 x 10^{-5} mol) was added to ACN (1.8 ml) then sonicated for 1 minute to give a suspension. pH 8 KPi buffer (200 µl) was added to the suspension and sonicated for a further minute. Thermolysin was added and the mixture was vortexed and incubated on a blood rotator overnight (18 hr) at 30 °C. 1 M HCl was added to the reaction mixture dropwise until the pH tested acidic. A 100 µl aliquot of the reaction mixture was diluted to 2 ml with ACN:H_2O (1 ml) then analysed directly by LC-MS using gradient 2.

7.9.2 General Procedure for Templating of Peptide Libraries in 90 % ACN

Dipeptide (4 x 10^{-5} mol) was added to ACN (1.8 ml) then sonicated for 1 minute to give a suspension. pH 8 KPi buffer (200 µl) was added to the suspension and sonicated for a further minute. Thermolysin was added together with template (4 x 10^{-5} – 2 x 10^{-3} mol) and the mixture was vortexed and incubated on a blood rotator overnight (18 hr) at 30 °C. 1 M HCl was added to the reaction mixture dropwise until the pH tested acidic. A 100 µl aliquot of the reaction mixture was diluted to 2 ml with ACN:H_2O (1:1) (1 ml) then analysed directly by LC-MS using gradient 2.

*7.9.3 Enzymatic Generation of Poly-L-Leucine Library **162***



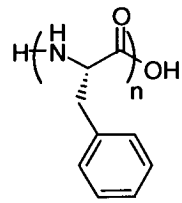
n = 4-8

162a-e

162 was generated in from $(Leu)_2$ (10 mg, 4 x 10^{-5} mol) as described in Section 7.9.1 and templated with chalcone (208 mg, 1.0 mmol), **118** (8.92 mg, 4.0 x 10^{-5} mol), **119** (198 mg, 1.0 mmol) and **123** (316 mg, 1.0 mmol) according to Section 7.9.2. **162a** $(Leu)_4$ Rt = 20.0 min; MS(ES) m/z = 471.4 (MH^+). **162b** $(Leu)_5$ Rt = 21.3 min; MS(ES)

m/z = 584.3 (MH^+). **162c** $(Leu)_6$ Rt = 22.3 min; MS(ES) m/z = 697.6 (MH^+). **162d** $(Leu)_7$ Rt = 23.7 min; MS(ES) m/z = 810.6 (MH^+). **162e** $(Leu)_8$ Rt = 25.3 min; MS(ES) m/z = 924.0 (MH^+).

*7.9.4 Enzymatic Generation of Poly-L-Phenylalanine Library **169***

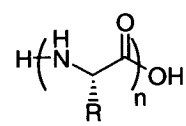


n = 3-8

169a-f

169 was generated from $(Phe)_2$ (12.4 mg, 4 x 10^{-5} moles) as described in Section 7.9.1 and templated with chalcone (208 mg, 1 mmol) as described in Section 7.9.2. **169a** $(Phe)_3$ Rt = 17.7 min; MS(ES) m/z = 460.5 (MH^+ requires 460.2). **169b** $(Phe)_4$ Rt = 19.2 min; MS(ES) m/z = 607.4 (MH^+ requires 607.3). **169c** $(Phe)_5$ Rt = 20.4 min; MS(ES) m/z = 754.6 (MH^+ requires 754.4). **169d** $(Phe)_6$ Rt = 21.4 min; MS(ES) m/z = 901.9 (MH^+ requires 901.4). **169e** $(Phe)_7$ Rt = 22.7 min; MS(ES) m/z = 1049.0 (MH^+ requires 1048.5). **169f** $(Phe)_8$ Rt = 23.9 min; MS(ES) 1196.1 (MH^+ requires 1195.6)

*7.9.5 Enzymatic Generation of Peptide Library From PheLeu **170***



n = 3-7

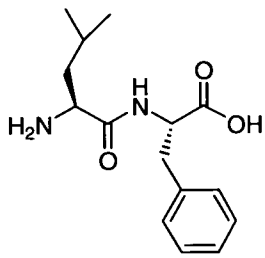
R = $CH_2CH(CH_3)_2$ or $CH_2C_6H_5$

170a-e

170 was generated from PheLeu.HBr (14.4 mg, 4.0 x 10^{-5} mol) as described in Section 7.9.1 and templated with chalcone (208 mg, 1.0 mmol) as described in Section 7.9.2. **170a** $(Phe)_2Leu$ Rt = 16.2 min; MS(ES) m/z = 425.4 (MH^+ requires 425.2). **170b** $(Phe)_3Leu$ Rt = 18.2 min; MS(ES) m/z = 572.5 (MH^+ requires 572.3). **170c** $(Phe)_4Leu$

Rt = 20.5 min; MS(ES) m/z = 719.3 (MH^+ requires 719.4). **170d** $(Phe)_5Leu$ Rt = 21.5 min; MS(ES) m/z = 867.0 (MH^+ requires 867.4). **170e** $(Phe)_6Leu$ Rt = 21.4 min; MS(ES) m/z 1013.8 (MH^+ requires 1013.5).

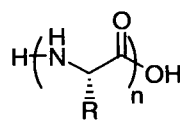
*7.9.6 Synthesis of LeuPhe **166***



166

FmocPhe-Wang-PS resin was deprotected and FmocLeu was coupled according to Section 7.4.1. The Fmoc was removed after the coupling step. **166** was cleaved from the resin as described in Section 7.2.6. TFA was removed under reduced pressure to give a clear oil. The oil was triturated into cold ether to give the *title compound* as white crystals (54 mg, 66 % yield). **166** was analysed using gradient 2. LeuPhe Rt = 13.4 min; MS(FAB) Found: 279.17031, $C_{15}H_{23}N_2O_3$ requires MH^+ 279.17087.

*7.9.7 Enzymatic Generation of Peptide Library From LeuPhe **171***



n = 3-6

R = $CH_2CH(CH_3)_2$ or $CH_2C_6H_5$

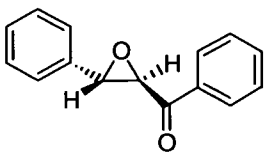
171a-e

171 was generated from **166** (11 mg, 4.0 x 10^{-5} moles) as described in Section 7.9.1 and templated with chalcone (208 mg, 1.0 mmol) as described in Section 7.9.2. **171a** PheLeuPhe Rt = 16.1 min; MS(ES) m/z = 426.0 (MH^+ requires 426.2). **171b** PheLeuPheLeu Rt = 16.2 min; MS(ES) m/z = 539.7 (MH^+ requires 539.3), **171c**

LeuPheLeuPheLeu Rt = 17.4 min; MS(ES) m/z = 652.6 (MH^+ requires 652.4). **171d** PheLeuPheLeuPhe Rt = 17.8 min; MS(ES) m/z = 686.5 (MH^+ requires 686.4). **171e** PheLeuPheLeuPheLeu Rt = 18.91 min; MS(ES) m/z = 799.7 (MH^+ requires 799.5).

7.10 Juliá-Colonna Epoxidations

7.10.1 Synthesis of (-)-2(R),3(S)-Epoxy-1,3-diphenylpropan-1-one ***109***



109

7.10.1.1 Biphasic Conditions: Condition 1

Poly-L-leucine-1,3-diaminopropane (200 mg) was added to a stirred mixture of chalcone (100 mg, 0.48 mmol), Urea.H_2O_2 (54.2 mg, 0.58 mmol) and DBU (87 µl, 0.58 mmol) in anhydrous ACN. The resulting paste was stirred at room temperature. After 22 hours a second aliquot of urea.H_2O_2 (54.2 mg, 0.58 mmol) was added and the mixture was stirred for a further hour. The reaction mixture was filtered and the residue was washed with EtOAc (3 x 5 ml). The organic layer was then washed with water (3 x 5 ml) and dried over $MgSO_4$. Solvent was removed under reduced pressure to give the *title compound* **109** as a white solid.

7.10.1.2 Biphasic Conditions: Condition 2 [5]

Poly-L-leucine-1,3-diaminopropane (200 mg) was added to a stirred mixture of chalcone (100 mg, 0.48 mmol), Urea.H_2O_2 (54.2 mg, 0.58 mmol) and DBU (87 µl, 0.58 mmol) in anhydrous THF. The resulting paste was stirred at room temperature for 2 hours. The reaction mixture was filtered and the residue was washed with EtOAc (3 x 5 ml). The organic layer was then washed with water (3 x 5 ml) and dried over $MgSO_4$.

Solvent was removed under reduced pressure to give the *title compound* **109** as a white solid.

7.10.1.3 Biphasic Conditions: Condition 3 [6]

Sodium percarbonate (56.5 mg, 0.36 mmol) was added to a stirred mixture of chalcone (50 mg, 0.24 mmol) and poly-L-leucine-1,3-diaminopropane (100 mg) in DME (0.5 ml) and water (0.5 ml). The reaction was stirred at room temperature for 2 hours then filtered. The residue was washed with EtOAc (3 x 5 ml). The organic layer was then washed with water (3 x 5 ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure to give the *title compound* **109** as a white solid

7.10.1.4 Triphasic Conditions [7]

DCM (1.6 ml) followed by 30 % aqueous H_2O_2 (1.2 ml) and 24 % aqueous NaOH (0.8 ml) were added to a round bottomed flask charged with poly-L-leucine-1,3-diaminopropane (50 mg). The mixture was left to stir at room temperature for 10 minutes then chalcone (40 mg, 0.19 mmol) was added. The mixture was shielded from light and stirred at room temperature for a further 24 hours. The reaction mixture was filtered and the residue was washed with DCM (3 x 5 ml). The organic layer was washed with water (3 x 5 ml) then dried over $MgSO_4$. Solvent was removed under reduced pressure to give the *title compound* **109** as a white solid

Rf = 0.43 (Si; EtOAc:Pet Ether, 2:8); δ_H ($CDCl_3$; 250 MHz) 7.94 (2H, d, *J* 7.1, C*H*ar) 7.27-7.92 (7H, m, C*H*ar) 4.24 (1H, d, *J* 1.9, C*H*O) 4.01 (1H, d, *J* 1.8, C*H*O); MS(ES) m/z = 225.0 (MH^+ requires 225.1). ^1H NMR consistent with literature data [7].

Note: Samples were analysed by chiral HPLC according to Section 7.1.4.2.

Table 27 Table of retention times of diastereomers

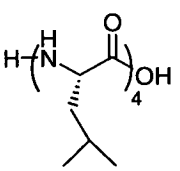
Diastereomer	Rt (Mins)
2(R), 3(S) **2**	11.3
2(S), 3(R) **2**	9.9

7.10.2 General Procedure for Probing Catalytic Activities of Peptides

DCM (1.6 ml) followed by 30 % aqueous H_2O_2 (1.2 ml) and 24 % aqueous NaOH (0.8 ml) were added to a round bottomed flask charged with the peptide oligomer (0.1 eq, 0.048 mmol) then stirred at room temperature for 10 minutes. The reaction was started by adding chalcone (40 mg, 0.19 mmol), shielded from light and left to stir at room temperature for 24 hours. The reaction mixture was filtered and the residue was washed with DCM (3 x 5 ml). The organic layer was washed with water (3 x 5 ml) then dried over $MgSO_4$. Solvent was removed under reduced pressure to give the *title compound* as a yellow oil. Reactions were analysed by chiral HPLC as described in Section 7.1.4.2.

7.11 Solid Phase Peptide Synthesis of Library Members

7.11.1 Synthesis of (Leu)$_4$ ***174***

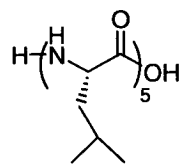


174

FmocLeu-Wang-PS resin was deprotected and FmocLeu was subsequently coupled according to Section 7.4.1. The deprotection/coupling step was repeated twice with

FmocLeu. The Fmoc was removed after the final coupling step. **174** was cleaved from the resin as described in Section 7.2.6. The peptide was precipitated with cold ether to give a suspension. The suspension was centrifuged and the supernatant decanted. The peptide was resuspended in cold ether then centrifuged and the supernatant decanted. This was repeated a further 2 times. The solid obtained was dissolved in ACN:H_2O (6:4) then lyophilised overnight to give the *title compound* **174** as a white solid (106 mg, 89 % yield). **174** was used without any further purification. **174** was analysed by HPLC using gradient 2. $(Leu)_4$ Rt = 20.4 min. MS(FAB) Found: 471.35448, $C_{24}N_4O_5H_{46}$ requires MH^+ 471.35437.

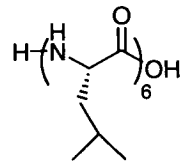
7.11.2 Synthesis of $(Leu)_5$ 175



175

FmocLeu-Wang-PS resin was deprotected and FmocLeu was subsequently coupled according to Section 7.4.1. The deprotection/coupling step was repeated three more times with FmocLeu. The Fmoc was removed after the final coupling step. **175** was cleaved from the resin as described in Section 7.2.6. The peptide was precipitated with cold ether to give a suspension. The suspension was centrifuged and the supernatant decanted. The peptide was resuspended in cold ether then centrifuged and the supernatant decanted. This was repeated a further 2 times. The solid obtained was dissolved in ACN then lyophilised overnight to give the *title compound* **175** as a white solid (121 mg, 82 % yield). 15 mg portions were dissolved in TFE (1.8 ml) and TFA (200 µl) then purified by preparative HPLC (Section 7.1.4.3, gradient 3). Purified peptide was then analysed by LC-MS using gradient 2 and was shown to be predominantly one peak. $(Leu)_5$ Rt = 19.7 min. MS(FAB) Found: 584.43846, $C_{30}N_5O_6H_{57}$ requires MH^+ 584.43837.

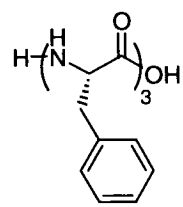
7.11.3 Synthesis of $(Leu)_6$ ***176***



176

FmocLeu-Wang-PS resin was deprotected and FmocLeu was subsequently coupled according to Section 7.4.1. The deprotection/coupling step was repeated four more times with FmocLeu. The Fmoc was removed after the final coupling step. **176** was cleaved from the resin as described in Section 7.2.6. The peptide was precipitated with cold ether to give a suspension. The suspension was centrifuged and the supernatant decanted. The peptide was resuspended in cold ether then centrifuged and the supernatant decanted. This was repeated a further 2 times. The solid obtained was dissolved in ACN then lyophilised overnight to give the *title compound* **176** as a white solid (177 mg, 100 % yield). 10 mg portions were dissolved in TFE (1.6 ml) and TFA (400 µl) then purified by preparative HPLC (Section 7.1.4.3, gradient 3). Purified peptide was then analysed by LC-MS using gradient 2 and was shown to be predominantly one peak. $(Leu)_6$ Rt = 20.9 min. MS(FAB) Found: 697.52261, $C_{36}N_6O_7H_{68}$ requires MH^+ 697.52237.

7.11.4 Synthesis of $(Phe)_3$ ***177***

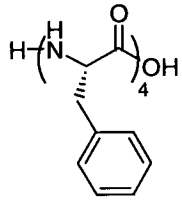


177

FmocPhe-Wang-PS resin was deprotected and FmocPhe was subsequently coupled according to Section 7.4.1. The deprotection/coupling step was repeated once more with FmocPhe. The Fmoc was removed after the final coupling step. **177** was cleaved from

the resin as described in Section 7.2.6. TFA was removed under reduced pressure to give the a clear oil. The oil was partitioned between water and diethyl ether. The aqueous layer was washed with diethyl ether (2 x 10 ml) then lyophilised to *title compound* **177** as a white solid (77 mg. 67 % yield). **177** was used without any further purification. **177** was analysed by HPLC using gradient 2. $(Phe)_3$ Rt = 19.6 min. MS(FAB) Found: 460.22352, $C_{27}N_4O_3H_{30}$ requires MH^+ 460.22363.

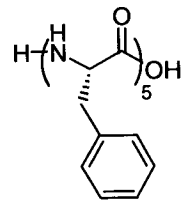
7.11.5 Synthesis of $(Phe)_4$ ***178***



178

FmocPhe-Wang-PS resin was deprotected and FmocPhe was subsequently coupled according to Section 7.4.1. The deprotection/coupling step was repeated twice more with FmocPhe. The Fmoc was removed after the final coupling step. **178** was cleaved from the resin as described in Section 7.2.6. The peptide was precipitated with cold ether to give a suspension. The suspension was centrifuged and the supernatant decanted. The peptide was resuspended in cold ether then centrifuged and the supernatant decanted. This was repeated a further 2 times. The solid obtained was dissolved in ACN:H_2O (1:1) then lyophilised overnight to give the *title compound* **178** as a white solid (86 mg, 88 % yield). **178** was used without any further purification. **178** was analysed by HPLC using gradient 2. $(Phe)_4$ Rt = 20.2 min. MS(FAB) Found: 607.29205, $C_{36}N_4O_5H_{39}$ requires MH^+ 607.29205.

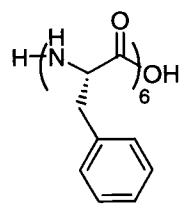
7.11.6 Synthesis of (Phe)$_5$ ***179***



179

FmocPhe-Wang-PS resin was deprotected and FmocPhe was subsequently coupled according to Section 7.4.1. The deprotection/coupling step was repeated three times more with FmocPhe. The Fmoc was removed after the final coupling step. **179** was cleaved from the resin as described in Section 7.2.6. The peptide was precipitated with cold ether to give a suspension. The suspension was centrifuged and the supernatant decanted. The peptide was resuspended in cold ether then centrifuged and the supernatant decanted. This was repeated a further 2 times. The solid obtained was dissolved in ACN:H_2O (1:1) then lyophilised overnight to give the *title compound* **179** as a white solid (114 mg, 90 % yield). **179** was used without any further purification. **179** was analysed by HPLC using gradient 2. (Phe)$_5$ Rt = 19.9 min. MS(FAB) Found: 754.36032, $C_{45}N_5O_6H_{48}$ requires MH^+ 754.36046.

7.11.7 Synthesis of (Phe)$_6$ ***180***

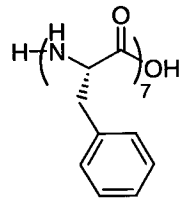


180

FmocPhe-Wang-PS resin was deprotected and FmocPhe was subsequently coupled according to Section 7.4.1. The deprotection/coupling step was repeated four times more with FmocPhe. The Fmoc was removed after the final coupling step. **180** was cleaved from the resin as described in Section 7.2.6. The peptide was precipitated with

cold ether to give a suspension. The suspension was centrifuged and the supernatant decanted. The peptide was resuspended in cold ether then centrifuged and the supernatant decanted. This was repeated a further 2 times. The solid obtained was dissolved in ACN:H_2O (1:1) then lyophilised overnight to give the *title compound* **180** as a white solid (115 mg, 76 % yield). **180** was used without any further purification. **180** was analysed by HPLC using gradient 2. $(Phe)_6$ Rt = 21.8 min. MS(FAB) Found: 901.42852, $C_{54}N_6O_7H_{57}$ requires MH^+ 901.42887.

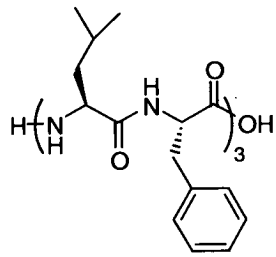
7.11.8 Synthesis of $(Phe)_7$ ***181***



181

FmocPhe-Wang-PS resin was deprotected and FmocPhe was subsequently coupled according to Section 7.4.1. The deprotection/coupling step was repeated five times more with FmocPhe. The Fmoc was removed after the final coupling step. **181** was cleaved from the resin as described in Section 7.2.6. The peptide was precipitated with cold ether to give a suspension. The suspension was centrifuged and the supernatant decanted. The peptide was resuspended in cold ether then centrifuged and the supernatant decanted. This was repeated a further 2 times. The solid obtained was dissolved in ACN:H_2O (1:1) then lyophilised overnight to give the *title compound* **181** as a white solid (128 mg, 73 % yield). **181** was used without any further purification. **181** was analysed by HPLC using gradient 2. $(Phe)_7$ Rt = 23.20 min. MS(FAB) Found: 1048.49554, $C_{63}N_7O_8H_{66}$ requires MH^+ 1048.49729.

7.11.9 Synthesis of LFLFLF 182



182

FmocPhe-Wang-PS resin was deprotected and FmocLeu was subsequently coupled according to Section 7.4.1. The deprotection/coupling step was repeated with FmocPhe followed by FmocLeu twice more. The Fmoc was removed after the final coupling step. **182** was cleaved from the resin as described in Section 7.2.6. The peptide was precipitated with cold ether to give a suspension. The suspension was centrifuged and the supernatant decanted. The peptide was resuspended in cold ether then centrifuged and the supernatant decanted. This was repeated a further 2 times. The solid obtained was dissolved in ACN:H_2O (1:1) then lyophilised overnight to give the *title compound* **182** as a white solid (181 mg, 90 % yield). **182** was used without any further purification. **182** was analysed by HPLC using gradient 2. LFLFLF Rt = 21.44 min. MS(FAB) Found: 799.47517, $C_{45}N_6O_7H_{63}$ requires MH^+ 799.47582.

7.12 References

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