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ENANTIOSELECTIVE RECEPTORS *VIA* ONE-BEAD ONE-COMPOUND COMBINATORIAL LIBRARIES

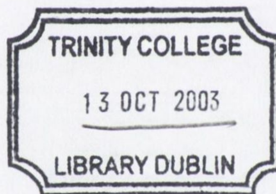
by

Patricia Amy Kelly

A Thesis submitted to the University of Dublin for the Degree of Doctor of Philosophy

Department of Chemistry,
University of Dublin,
Trinity College.

August 2002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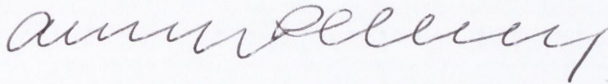

Patricia Amy Kelly

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Summary

Chapter 1 describes the overall aim of this project, which is to extend the range of steroid-based receptors synthesised by the Davis group, using the combinatorial chemistry approach instead of traditional design and synthesis methods. The importance of an efficient method of screening combinatorial libraries for "hits", and the application of such a technique to the discovery of steroid-based enantioselective receptors, are described in **Chapter 2**. The target assay, wherein a solid-phase bound combinatorial library of receptors is exposed to racemic guest, of which each enantiomer is differentially colour-tagged, is also described. **Chapter 3** describes a method whereby a semi-quantitative estimate of the enantioselectivity of individual bead-bound receptors may be obtained, by use of isotopically differentiated enantiomers.

Chapter 4 details the synthesis of dye-tagged amino acids *N*-Boc-L-Tyr (O-Disperse Red 1), *N*-Boc-D-Tyr (O-Disperse Red 1) and *N*-Boc-L-Tyr (O-Disperse Blue 3) in addition to *N*-deutero-protected amino acids *N*-Ac-d_3-L-Phe and *N*-Boc-d_9-L-Phe.

The development of a 2-phase extraction technique between solid-phase bound steroidal receptor and solution phase "pseudoracemic" guest is described in **Chapter 5**. Bound guest, the enantiomers of which are isotopically differentiated, may be identified by electrospray ionisation mass spectrometry, where-from a semi-quantitative estimate of selectivity may be obtained.

Chapter 6 details the screening of a dipeptide combinatorial library with colour-tagged racemic amino acid guest. Active members were picked and treated with isotopically labelled "pseudo-enantiomers", so that bound guest could be quantified by mass spectrometry.

Chapter 7 contains a description of experimental procedures and techniques employed during this project.

Chapter 8 contains a full list of references, by chapter.

Appendix A contains important mass spectra, which are referred to in **Chapters 5** and **6**. HPLC chromatographs of dye-tagged amino acids are contained in **Appendix B.**

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Abbreviations

α	Optical rotation
Ac	Acetyl
amu	atomic mass unit
b	broad
Bn	Benzyl
Boc	*tertiary*-Butoxycarbonyl
$(Boc)_2O$	*tertiary*-Butyldicarbonate
Bu	butyl
Cbz	benzyloxycarbonyl
CDI	1,1-Carbonyldiimidazole
CI	Chemical Ionisation
COSY	Correlatedspectroscopy
cPr	cyclopropanoyl
CSP	Chiral Stationary Phase
d	doublet
DBU	1,8 Diazobicyclo[5.4.0]undec-7-ene
DCC	*N*, *N*-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	Dicyclohexylurea
DEAD	Diethylazodicarbonate
DEPT	Distortionless enhancement by polarization transfer
DIPEA	N, N-Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
e.e.	Enantiomeric excess
EI	Electron Impact
eq.	equivalents
ESIMS	Electrospray Ionisation Mass Spectrometry
FABMS	Fast Atom Bombardment Mass Spectrometry
HMBC	Heteronuclear multiple bond correlation

HMQC	Heteronuclear multiple quantum coherence
HPLC	High Performance Liquid Chromatography
hr	hour
HRMS	High Resolution Mass Spectrometry
IPA	Propan-2-ol
IR	Infra Red
J	coupling constant
K_a	Association constant
Ks	Solvation constant
LC/MS	Liquid Chromatography/Mass Spectrometry
lit	literature
m	multiplet
m.p.	Melting Point
MS	Mass Spectrometry
m/z	mass to charge ratio
NMR	Nuclear Magnetic Resonance Spectroscopy
nOe	nuclear Overhauser effect
Ph_3P	Triphenylphosphine
ppm	parts per million
q	quartet
R_f	Disance travelled relative to solvent front
R_t	Retention time
rt	room temperature
s	singlet
sec	seconds
sx	sextet
t	triplet
TBA	Tetrabutylammonium
TBAF	Tetrabutylammonium fluoride
TEA	Tetraethylammonium
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
tlc	Thin Layer Chromatography
TMA	Tetramethylammonium

TNBS	2, 4, 6-trinitrobenzenesulfonic acid
TOCSY	Total correlation spectroscopy
UV	ultra-violet

CHAPTER 1

1.1 Molecular recognition

Molecular recognition is the study of non-covalent interactions between molecules. It describes the structures and functions of the entities formed by association of two or more chemical species or "supermolecules".[1, 2] The forces involved in neutral molecular recognition and complexation are non-covalent intermolecular interactions including, hydrogen bonding, π-stacking, dipole-dipole interactions, dipole-induced dipole interactions and induced dipole-induced dipole interactions. The two molecules involved in such an interaction are called the "host and guest" or "receptor and substrate". The host and guest come together when they have steric and interactional complementarity. In other words, they must structurally fit together and have complementary binding sites. As non-covalent binding is considerably weaker than covalent binding, multiple interactions are necessary between host and guest. In the case of enantioselectivity, it is generally accepted that a receptor must have at least three points of contact with the guest, with at least one being stereochemically dependent. Thermodynamics also plays a rôle as a host will selectively bind a specific guest only when a larger difference in binding free energy is observed for that system than for the host with any other potential guest. A key factor in molecular recognition is preorganisation, which refers to the conformation of the host before binding. If the host does not undergo a significant conformational change upon binding, it is described as preorganised. A preorganised host will undergo a reduced free energy change prior to complexation compared with one that needs to adopt a binding conformation in order to fit a guest and, therefore, is favoured. These governing factors must coincide for efficient binding to occur.

1.2 Preamble

Receptors may be divided into three groups; cation receptors, anion receptors and receptors for the recognition of neutral molecules. The recognition of cations has

been well documented in the literature since the naissance of molecular recognition and, indeed, it was for their pioneering contribution to this field that Pedersen, Lehn and Cram were awarded the Nobel Prize in Chemistry in 1987. The observation of inclusion complexes between crown ethers and cationic substrates, by Pedersen in 1971, was probably the first instance of a molecular receptor-substrate complex, from which the chemistry of crown ethers developed rapidly and crown ethers became very popular as receptors for cations.[3] As these compounds have the property of forming complexes with positive ions, in particular metallic and ammonium ions, and as their size can be manipulated in order to fit a particular cation, an obvious use of crown ethers is in the separation of mixtures of cations. Crown ethers are also well utilized in organic synthesis, phase transfers and, in the case of chiral crown ethers, the resolution of racemic mixtures.

A series of cation receptors followed the crown ether, including multi-cyclic molecules known as cryptands, which can envelop the enclosed ion in 3 dimensions, thereby binding it even more tightly than the monocyclic crown ether.[1] Cram developed a range of receptors, known as spherands, whose cavities, as the name suggests, can be occupied only by spherical entities.[2, 4] These receptors contain rigid, pre-organised cavities, thereby leading to a strong improvement in binding strength and selectivity. Podands[5] are hosts, which are also justified by their name, in that they have two or more arms emanating from a central structure, by which simple alkali metal cations are bound. The importance of the hosts described is the ability to bind guests specifically and this is achieved *via* ion-dipole attractions between the ionic guest and hetero atoms in the rigid host.

Although, as described above, the recognition of cations has been well documented in the literature,[1, 2, 6] that of anions is an area of ongoing research. Of particular interest to this project is the synthesis of receptors to bind amino acids and their derivatives.

Nature uses amino acids as building blocks for peptides[7] and as molecular messengers[8] within living organisms. Amino acids can assume three forms, depending on pH: anionic, zwitterionic and cationic. As charge distribution is an important factor in molecular recognition, it is important to know in which form the amino acid is to be bound when synthesising a potential receptor so that, as appropriate, an anion, cation or

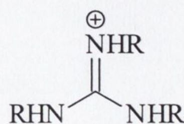
neutral molecule host can be designed. To sidestep this problem initially, much of the work in this area has been performed on derivatised amino acids in which either the carboxylate or ammonium functionality has been protected. *N*-α-protected-α-amino acids are of synthetic and biological importance.[9] They are more lipophilic than free amino acids and, being structurally larger, it is thought that they may offer a greater potential for displaying significant enantioselectivities with particular synthetic hosts. Enantioselective recognition of *N*-protected-α-amino acids in which a carboxylic acid/carboxylate moiety is present is the overall aim of this project.

Some important examples of carboxylate receptors from research literature are described later in this chapter. Particular attention is paid to guanidinium and urea motifs, as carboxylate binding sites, which have been incorporated into rationally designed enantioselective carboxylate binders. Described within the latter end of the chapter are examples of receptors, designed and synthesised in this laboratory, to bind amino acid carboxylates selectively. These receptors are based on the steroidal cholic acid backbone and take account of all of the prerequisites for enantioselective host-guest interactions.

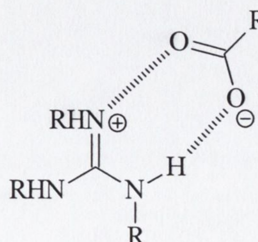
1.3 Positively charged selective hosts

1.3.1 Contribution by de Mendoza *et al.*

The first example of enantioselective recognition of an anionic species by an abiotic receptor was reported in 1988 by Schmidtchen *et al.*[10] They reported that the guanidinium group **1.1**, with a pK_b of 13.5, formed characteristic pairs of well-organised, strong, zwitterionic hydrogen bonds with carboxylates, as shown in **1.2.**

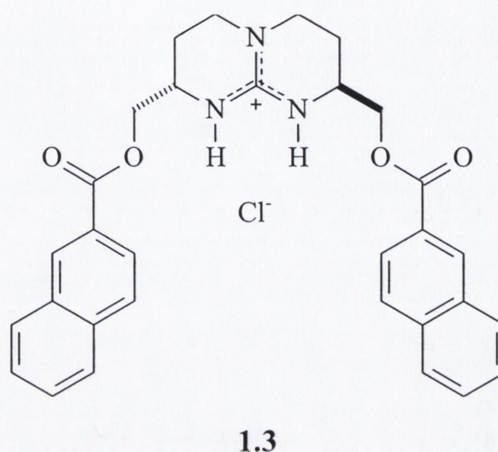


1.1



1.2

1.1 was clearly a good starting point for carboxylate recognition. de Mendoza *et al* incorporated the charged unit into receptors **1.3**-*RR* and **1.3**-*SS,* in a 10 step synthesis from arginine.[11]



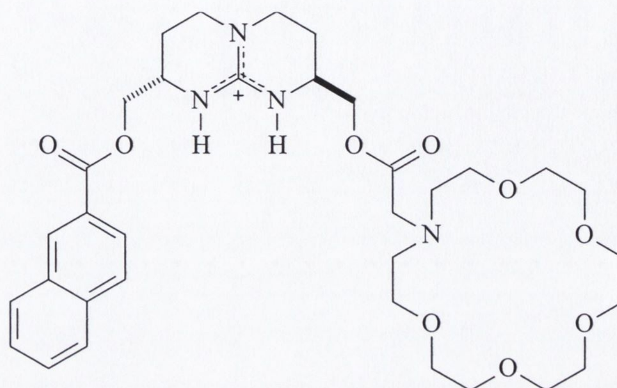
1.3

It was found that **1.3** quantitatively extracted sodium *p*-nitrobenzoate from water, with significant shifts in ^{1}H NMR being seen for the relevant protons of the complex. Extractions from water into $CHCl_3$ of zwitterionic valine, phenylalanine and tryptophan were attempted but these were unsuccessful. The *N*-Ac and *N*-Boc derivatives of sodium tryptophan were extracted, however. **1.3**-*SS* afforded two diastereomeric salts in each case, with diastereomeric excess of ~ 17 % for the L-tryptophan derivative. NMR titration of the TEA salts of *N*-Ac-tryptophan gave stability constants, $K_s = 1051\ M^{-1}$ and $534\ M^{-1}$, for the L and D enantiomers, respectively, which was consistent with the diastereomeric excesses observed.

Zwitterionic amino acids are difficult to extract from water as the electron densities at the carboxylate and ammonium moieties are affected by their mutual vicinity, resulting in self-complexation. In addition to this, the two moieties are highly solvated and, in order for a receptor to appreciably extract a zwitterionic amino acid into the organic phase, it must be able to compensate for the loss of energy due to desolvation of water molecules.

Using this information, de Mendoza *et al.* designed and synthesised an analogue of **1.3**, incorporating a crown ether into one of the "legs" to ensure non-complementary binding sites for the carboxylate (guanidinium) and the ammonium moieties (crown

ether).[12] Two other notable features of the resulting receptor, **1.4,** are an aromatic planar surface (naphthalene ring), designed to be complementary to aromatic amino acids and a chiral structure for enantioselective recognition (*RR* and *SS*).



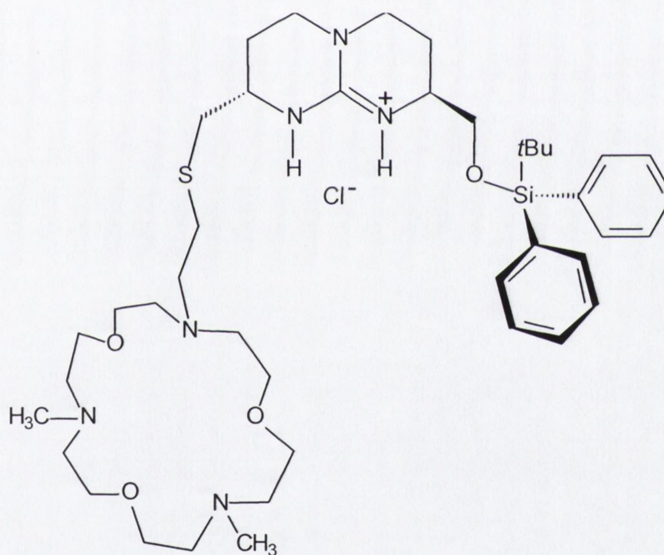
1.4

1.4-*SS* was found to extract L-tryptophan and L-phenylalanine from water into DCM with extraction efficiencies of up to ~ 40 %, by NMR. Chiral recognition was confirmed by observation that the corresponding D-enantiomers were not extracted. Conversely, **1.4**-*RR* extracted D-phenylalanine and D-tryptophan but not the L-enantiomers. Enantioselectivities were determined by HPLC analysis of diastereomeric dipeptides prepared from the extracts of racemic samples of phenylalanine and tryptophan and a suitable optically pure L-leucine derivative. These results gave remarkable e.e.'s of 99 % and 96 % for **1.4**-*SS* with L-tryptophan and L-phenylanine, respectively. However, a later publication corrected these figures noting that some D-enantiomer had, in fact, been extracted. Direct HPLC measurements on the racemic extraction experiments revealed that the L-enantiomer had in fact been extracted in a ratio of ~ 8:1.[13]

Computational molecular modelling on the binding mode of tryptophan suggested that the guanidinium group contributes about one half of the total binding enthalpy. The aza-crown ether provides about one third, while aromatic π-stacking with the indole moiety accounts for about one sixth of the total binding enthalpy.

1.3.2 Contribution by Schmidtchen *et al.*

Following on from de Mendoza's work (**Section 1.3.1**), Schmidtchen *et al.* synthesised the polytopic host **1.5**. This receptor comprises a chiral bicyclic guanidinium attached to both a tri-aza crown ether *via* a hydrophobic thio-ether bridge and to a bulky silyl ether which should convey lipophilicity.[14] The authors noted that **1.5** was designed to function by two-point interaction with a guest and so would not be expected to exhibit enantioselectivity. They found, however, that the inherent chirality of the host resulted in the enantioselective transfer of zwitterionic racemic phenylalanine from aqueous solution into DCM in favour of the L-enantiomer. The observed e.e. was 40 % and was almost independent of pH in the range from 9.1 to 10.5.



1.5

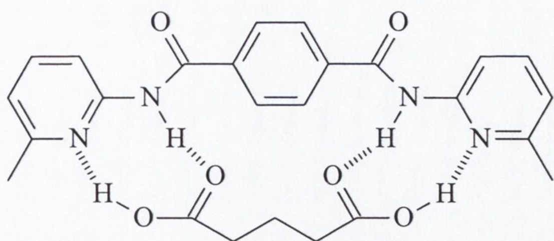
The guanidinium moiety, therefore, has been shown to be an efficient complementary binding site for the carboxylate group within a preorganised receptor molecule. The charged receptor into which it is incorporated can perform the important task of selectively extracting amino acids from the aqueous into the organic phase. Recent examples of the guanidinium moiety being exploited as a binding site for carboxylates include Kilburn's "tweezer" receptors.[15, 16]

1.4 Neutral carboxylate receptors

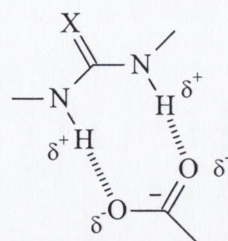
In contrast to receptors **1.4** and **1.5**, the idea of using an electroneutral receptor for carboxylate binding has emerged. Effective neutral receptors should have certain advantages over their charged counterparts in applications such as chiral stationary phases for HPLC and enantioselective phase transfer. The aminopyridine and urea moieties have both been explored as important motifs in electroneutral carboxylate binders.

1.4.1 Contribution by Hamilton *et al.*; comparison of carboxylate recognition by aminopyridine and urea groups.

A neutral receptor formed by spanning two 2-amino-6-methylpyridine groups across a terephthaloyl spacer was synthesised by Hamilton *et al.*[17] It forms complex **1.6,** which has $K_a = 640\ \mathrm{M}^{-1}$, with glutaric acid. However, addition of DMSO to complex **1.6** in 5 % THF/$CDCl_3$ led to strong solvation of the hydrogen bond donor sites, and an almost complete disruption of the binding, as a result of competing with glutaric acid for binding sites. The authors suggested using urea as an alternative to the amino-pyridine motif for carboxylate binding in competitive, polar and/or protic solvents. In the urea-carboxylate complex **1.7**, both hydrogen bond donors are situated on the host creating more favourable interactions than those in **1.6**. Addition of TMA acetate to a (DMSO)-d_6 solution of 1,3-dimethylurea resulted in > 1 ppm downfield shifts of the urea proton resonance, consistent with the formation of bi-dentate hydrogen bonded complex **1.7a**. K_a of 43 M^{-1} was observed for **1.7a**. However, the K_a value obtained for the more acidic thiourea **1.7b** was 10 times greater.

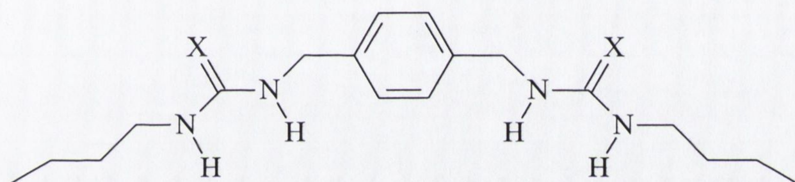


1.6

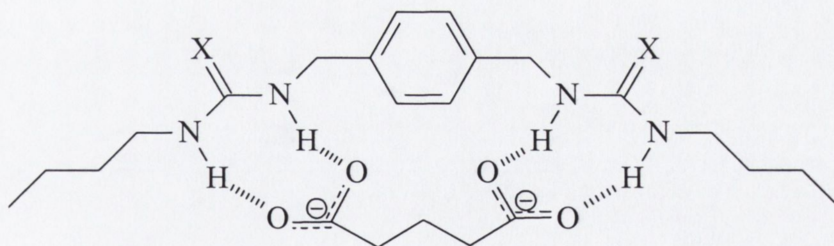


1.7a: X = O
1.7b: X = S

The urea moiety was incorporated into dicarboxylate receptors **1.8a** and **1.8b**, which are analogous to **1.6**. In contrast to **1.6**, **1.8a** was found to bind TBA glutarate in the polar solvent (DMSO)-d_6. ^{1}H NMR shifts and nOe calculations suggest **1.9a** and **1.9b** as the structures of the complexes formed between **1.8a** and **1.8b** and glutarate.



1.8a: X = O
1.8b: X = S

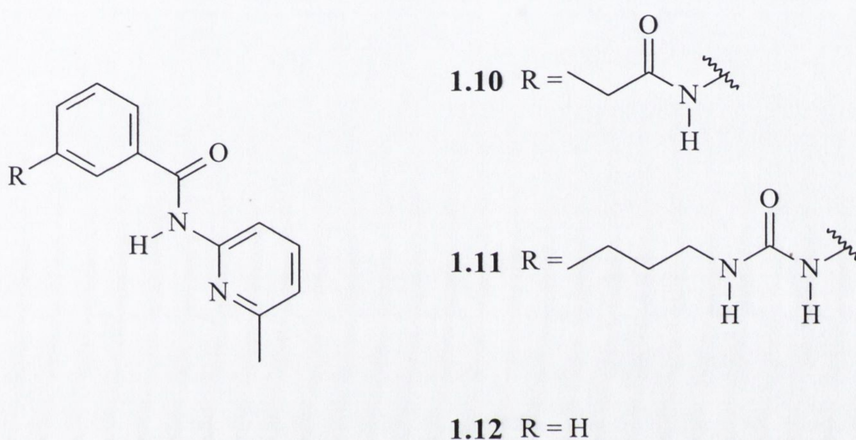


1.9a: X = O
1.9b: X = S

As anticipated, the thiourea complex **1.9b** exhibited an increased stability compared with **1.9a**, with K_a of 1 x 10^4 M^{-1}, which was 15 times greater than that for the urea.

1.4.2 Contribution by Hamilton *et al.*; carboxylate binding by neutral urea-based receptors

In addition to the work described in **Section 1.4.1**, Hamilton *et al.* designed and synthesised receptors **1.10**, **1.11** and **1.12** to bind carboxylates.[18] **1.10** contains an amide, which contributes one binding site complementary to the carboxylate. **1.11** features a urea, which provides two complementary binding sites to the carboxylate, while **1.12** was synthesised to serve as a control in binding experiments.



The abilities of **1.10**, **1.11** and **1.12** to bind *N*-Ac-proline were investigated. The authors reported large downfield shifts of the pyridine amino proton (1.49 ppm) and phenyl amino proton (1.19 ppm), consistent with intermolecular binding. Intermolecular nOes were routinely observed between the methine proton α to the amino acid and both the pyridine methyl and phenyl-2H, thus confirming the proximity of these protons within the complex. The authors also suggested that the results strongly support structures in which one and two hydrogen bonds are involved in the binding of **1.10** and **1.11**, respectively, to the amide carbonyl of the substrate, the latter of which being shown in **Figure 1.1**.

Figure 1.1: Binding of *N*-Ac-L-Pro to **1.11**

NMR titrations of **1.10**, **1.11** and **1.12** with *N*-Ac-L-Pro gave K_a values of 410, 2600 and 50 M^{-1} respectively indicating that K_a increases with number of hydrogen bonds present, as shown in **Table 1.1**.

When *N*-butyryl-D-valine was titrated against **1.10**, **1.11** and **1.12**, it was found, by nOe experiments, that the acyl group was positioned close to the propionyl substituent of **1.10**. From this information, **1.13**, with a stereogenic centre close to the binding region for the acyl group of the substrate, was synthesised in an effort towards chiral recognition. **1.13** was tested against **1.14** and captopril **1.15** and the results of these experiments are shown in **Table 1.1**. Modest chiral discrimination towards the *R* isomer was observed for both **1.14** and **1.15**, demonstrating the ability of the urea group to form complexes with carboxylates.

1.13

1.14

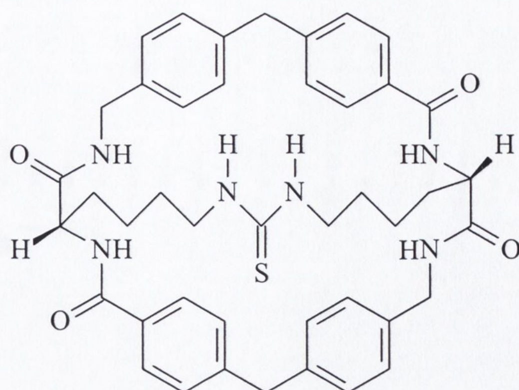
1.15

Table 1.1 K_a values for carboxylate receptors with various guests

Receptor	Substrate	K_a (M^{-1})
1.12	*N*-Ac-L-Pro	50
1.10	*N*-Ac-L-Pro	410
1.11	*N*-Ac-L-Pro	2600
1.10	*N*-butyryl-D-Val	415
1.10	1.15	430
***R*-1.13**	1.15	500
***S*-1.13**	1.15	210
***R*-1.13**	*S*-1.14	680
***S*-1.13**	*S*-1.14	350

1.4.3 Contribution by Kilburn *et al.*

Kilburn *et al*. reported a series of neutral macrocycles designed to bind amino acids and *N*-protected amino acids and peptides.[19, 20, 21, 22] These are, in general, rigid macrocycles with a specific carboxylate binding site (either aminopyridine or thiourea) incorporated at the base of the macrocycle cavity and additional amide functionality around the rim of the structure, such as macrobicycle **1.16**.[19] The carboxylate binding site is provided by a thiourea at the base of the cavity. Chirality and amide functionality are introduced by two lysine derivatives while a biarylmethyl unit rigidifies the rim of the macrobicycle. Binding studies were attempted between **1.16** and various acylated amino acids as their TBA salts but the amino proton signals were shifted down to the aromatic region which already contained aromatic resonances, thus hindering determination of binding constants by NMR titrations. Instead, binding constants were determined by extraction of guest by **1.16** from aqueous to organic phase and the results are shown in **Table 1.2**.



1.16

Table 1.2: Association constants for **1.16** with various TBA carboxylates in $CDCl_3$

Substrate (TBA salt)	**K_a / M^{-1}**
N-Ac-Gly	68,600
N-Ac-L-Ala	16,900
N-Ac-D-Ala	14,600
N-Ac-L-Phe	22,000
N-Ac-D-Phe	13,300
N-Ac-L-Glu	11,100
N-Ac-L-Asp	9,600
N-Ac-L-His	5,800
N-Ac-L-Lys	130,000

It should be noted that *N*-Ac-L-Lysine was particularly strongly bound by **1.16**, suggesting a favourable interaction between the free amino acid residue and the macrobicycle. Glycine was also strongly bound but the remaining guests exhibited reduced binding. The authors suggested that this was due to the amino acid side chain not being tolerated due to steric reasons. The binding results indicated little enantioselectivity for the amino acid derivatives tested. Binding of simple carboxylates such as benzoate and hexanoate was of comparable strength to that of the amino acid

derivatives, suggesting that binding was occurring almost entirely between guest and the thiourea moiety.

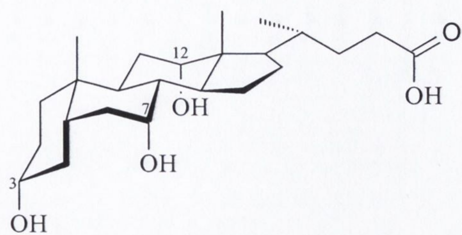
An interesting feature of the ^{1}H NMR spectra of the 1:1 mixtures of **1.16** and the TBA salts of *N*-Ac-alanine was that the D- and L- enantiomers showed markedly different shifts suggesting substantially different binding modes for the two. The D-Ala derivative showed very little change in chemical shift from the unbound substrate to the substrate in the 1:1 complex, while all four alanine resonances were shifted upfield in the 1:1 complex between **1.16** and L-alanine, compared with unbound L-alanine. The authors suggested that the D-enantiomer was bound to the urea on the outside of the macrobicycle while the L-enantiomer was bound within the cavity. This was further substantiated by nOe studies and molecular modelling.[21] While enantioselctivity was not affected by this occurrence, it is an important feature of receptor design and it gives some clues as to how various features may promote or block a particular binding mode.

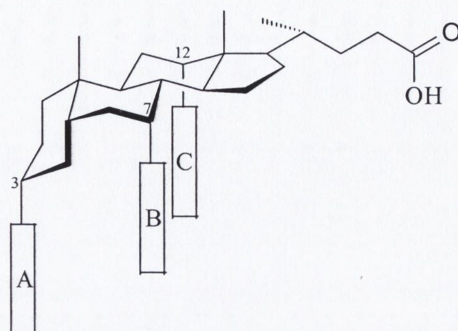
As guanidinium has emerged as an important moiety in charged carboxylate receptors, so the urea moiety has become an important motif in uncharged receptors.

1.5 Work within the Davis group

The workhorse within this group for the design and synthesis of receptors is cholic acid **1.17.**[23] The steroidal structure fulfils many of the criteria, described in **Section 1.1,** required for a good enantioselective receptor. Cholic acid presents itself as a rigid, chiral backbone with three hydroxy groups all of which are arranged on the same side of the steroid. It is cheap, readily available, of biological importance and has well-known chemistry. The hydroxy groups in the 3-α, 7-α, and 12-α positions of the molecule may be differentially transformed to give 3 "legs" A, B and C within which a cavity with which to bind guests may exist, as shown in **1.18**. As described in **Section 2.3**, cholic acid may be attached to solid phase and groups A, B, and C may be introduced in a combinatorial fashion. The technique of combinatorial chemistry and synthesis ofa combinatorial library is discussed in **Section 2.2**. Screening of such a combinatorial library for enantioselective members is the aim of this project. Two screening assays were attempted and these methods are described in **Chapters 2** and **3**. Development

and application of these methods and results and findings are described in **Chapters 5** and **6**.

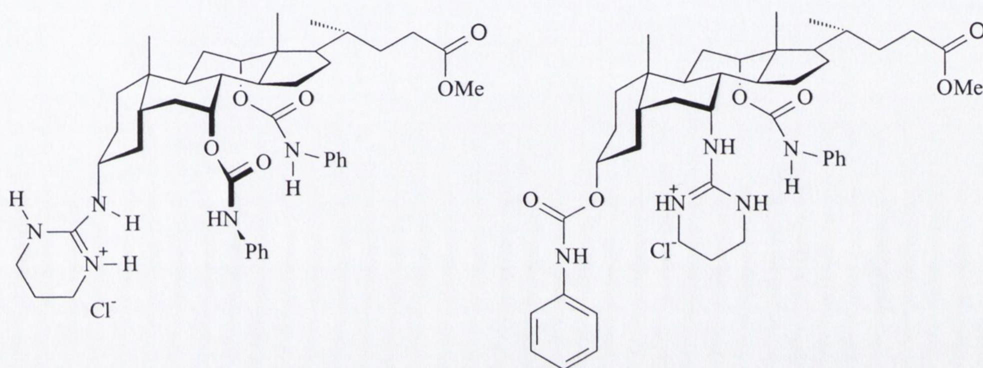




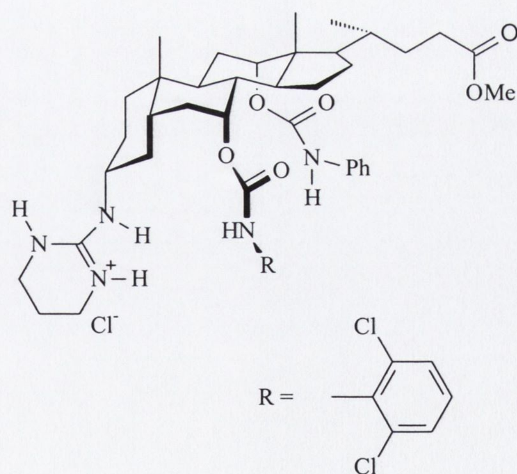
1.17 **1.18**

1.5.1 Contribution by Lawless

Lawless synthesised a series of steroidal receptors with a guanidinium moiety in the 3-α or 7-α position of cholic acid.[24] The charged appendage was designed to form a specific electroneutral complex with the ionic centre of a particular guest. The receptors were synthesised to extract *N*-protected-α-amino acids from the aqueous phase. Some examples of these receptors are **1.19**, **1.20** and **1.21**



1.19 **1.20**



1.21

Solutions of **1.19**, **1.20** and **1.21** were capable of extracting N-protected amino acids from neutral and basic aqueous solutions into $CHCl_3$, presumably through exchange of carboxylate for chloride.[25, 26] Enantioselectivities and extraction efficiencies were determined from ^1H NMR spectra. A typical extraction procedure involved shaking the receptor (6.4 mM) in $CHCl_3$ with the racemic *N*-protected amino acid (7.7 mM) in buffered aqueous phase (pH 7.4; 0.1 M KH_2PO_4/K_2HPO_4). Measurements of L/D ratios were carried out using ^1H NMR with each of the enantiomers giving rise to a discrete resonance due to the formation of diastereomeric complexes with the receptor. By integration, a direct comparison of how much of each enantiomer had been extracted relative to the other, could be obtained. The appropriate resonance could be assigned to each enantiomer by performing the extraction using each of the single enantiomers. Some of the more significant results are shown in **Table 1.3**.

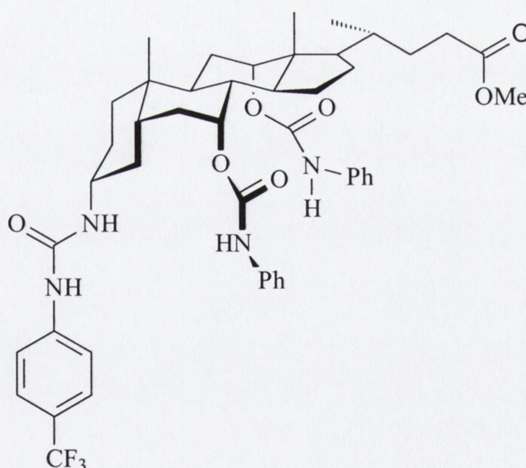
Table 1.3: Extraction efficiencies and e.e.'s for receptors **1.19, 1.20** and **1.21** with various *N*-protected amino acids as their TBA carboxylates.

Receptor	Substrate (as the carboxylate)	Ratio (L/D) extracted	Enantiomeric excess (%)	Substrate extracted
1.19	*N*-Ac-DL-alanine	7:1	75	52 %
1.19	*N*-Ac-DL-phenylalanine	7:1	75	87 %
1.19	*N*-Ac-DL-valine	7:1	75	71 %
1.19	*N*-Ac-DL-tryptophan	7:1	75	83 %
1.20	*N*-Ac-DL-valine	13:2	73	50 %
1.20	*N*-Ac-DL-tryptophan	9:2	63	75 %
1.20	*N*-Boc-DL-histidine	4:1	60	51 %
1.20	*N*-Boc-DL-valine	3:1	50	92 %
1.20	*N*-Cbz-DL-alanine	3:1	50	92 %
1.21	*N*-Ac-DL-phenylalanine	9:1	80	93 %
1.21	*N*-Ac-DL-methionine	9:1	80	93 %
1.21	*N*-Ac-DL-valine	9:1	80	89 %
1.21	*N*-Ac-DL-alanine	6:1	71	76 %
1.21	*N*-Ac-DL-tryptophan	6:1	71	92 %
1.21	*N*-Ac-DL-proline	4:1	60	74 %
1.21	*N*-Boc-DL-histidine	4:1	60	46 %

It should be noted that **1.21** generally showed higher extraction abilities which may be due to the greater acidity of the dichlorophenyl carbamoyl amino proton in the 7-α position. **1.19** exhibited consistency in its ability to differentiate between enantiomers, irrespective of side-chain bulk. Extraction of polar *N*-protected-asparagine was not observed in any case.

1.5.2 Contribution by Dresen

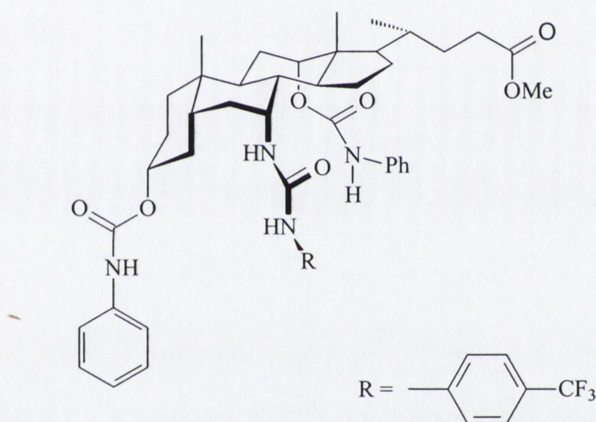
Dresen synthesised receptor **1.22** by incorporating a urea group in the 3-α position and two phenylcarbamates in the 7-α and 12-α positions of cholic acid.[27] It was hoped that this neutral receptor would extract *N*-protected-α-amino acids from the aqueous to the organic phase in the same manner as had Lawless' guanidinium receptors **1.19**, **1.20** and **1.21**. *N*-Ac-DL-valine was used as the guest in these experiments. By modifying the extraction procedure of Lawless, Dresen discovered that a lipophilic cation, in this case, TBA, and a reservoir of *N*-Ac-valine in the aqueous phase were required to effect carboxylate extraction. When the experiment was modified to use 3 equivalents of TBA *N*-Ac-DL-valinate and 10 equivalents of N-Ac-DL-valine in the aqueous phase with 1 equivalent of **1.22** in the organic phase, extraction of N-Ac-valine was observed with an L/D ratio of 4:1 (62 % e.e.). As with Lawless' experiments, the L/D ratio was measured by integration of the α-methine proton of valine in the ^{1}H NMR spectrum, which was split due to formation of diastereomeric complexes.



1.22

1.5.3 Contribution by Hurley

Further to Dresen's work, Hurley incorporated a urea group into the 7-α position of cholic acid and appended phenylcarbamates to the 3-α and 12-α positions **1.23**. Hurley also further developed the existing extraction method of Lawless and Dresen and introduced analysis of these extractions by chiral HPLC.[28] In contrast to the earlier method, 1 equivalent of lipophilic guest, rather than3equivalents, was used relative to receptor. 9 equivalents of sodium *N*-Ac-amino acid carboxylate were also present in the aqueous phase (0.1 M KH_2PO_4/K_2HPO_4; pH 7.4), which was stirred with one mole equivalent of receptor in $CHCl_3$.



1.23

Hurley attempted to compare the binding power of the neutral receptors with that of the guanidinium receptors, in particular **1.20** and **1.22**. To remain consistent with earlier work, NMR binding studies were initially attempted. When **1.20** formed a complex with *N*-Ac-D-Val however, the resonance of the methine proton α to the amino acid became obscured by the broad 3-β proton resonance of the steroid. Variable temperature experiments shifted the peak to an appropriate viewing position but the signal to noise ratio became too high to obtain a useful peak integration. Changing to *N*-Ac-DL-Phe as guest, the complex resonances were found to be poorly separated and hence could not be integrated. As a result, it was decided to try chiral HPLC as an

analytical tool to measure the selectivity of these extractions. As chiral chromatography separated the enantiomers of *N*-Ac-Phe effectively (α = 1.23), an extraction of *N*-Ac-DL-Phe with **1.20** was performed using the method described and the extract analysed by HPLC. The results of some of these extractions are presented in **Table 1.4**. It may be noted that the result for **1.22** with *N*-Ac-DL-Phe compares favourably with Lawless' result for **1.20** with the same guest (**Section 1.5.1**), although the two sets of results were obtained using different extraction conditions.

The urea has potential as a complementary binding site in uncharged receptors of this type, although the enantioselectivities and extraction efficiencies observed for these systems, are, in general, lower than those observed for the charged receptors. Despite the long run times (~2 hrs), chiral HPLC has been developed as another analytical tool available for the analysis of liquid-liquid extractions.

Table 1.4 : Extraction efficiencies and e.e.'s for receptors **1.20** and **1.22** with *N*-Ac-DL-Val and *N*-Ac-DL-Phe

Receptor	Substrate	Analytical Technique	L/D ratio	Enantiomeric excess (%)
1.20	*N*-Ac-DL-Val	^{1}H NMR	3-4:1	50-60
1.20	*N*-Ac-DL-Phe	^{1}H NMR	-	-
1.22	*N*-Ac-DL-Phe	^{1}H NMR	ca 4:1	61
1.22	*N*-Ac-DL-Phe	HPLC	ca 3:1	47
1.22	*N*-Ac-DL-Phe	HPLC	ca 7:1	74
1.20	*N*-Ac-DL-Phe	HPLC	ca 12:1	84

1.6 Conclusion

The best extraction efficiencies and enantioselectivities have been observed for charged receptors. This is unsurprising, as the positively charged moiety was deliberately incorporated into the receptor to form a specific electroneutral complex with an ionic guest. This rational approach of "design and synthesis" has played a key rôle in

molecular recognition, as the chemist has attempted to subtly mould a host to best fit a guest. But the chemist has recently broadened the subject from rational design to include the more serendipitous field of combinatorial chemistry wherein a mixture of compounds is synthesised and tested in the hope of finding a "hit". Can the precise science of molecular recognition successfully partake of the unusual science of combinatorial chemistry? And, if so, can the receptors described above be superseded by a hit discovered within a receptor library?

1.7 Aim of the project

The aim of this project was to develop a method by which enantioselective hits could be identified from combinatorial receptor libraries. The libraries were based on a cholic acid scaffold and featured either a urea or a guanidinium group in the 3-α position of the steroid, with variability introduced in the 7-α and 12-α positions (**Section 2.2**). A reliable screening assay is essential for the successful use of any library. A 2-pronged approach was attempted. Library was first exposed to dye tagged *N*-protected tyrosine such that selective members would appear the same colour as the dye-tagged *N*-protected tyrosine enantiomer that was selected. This would indicate the direction of selectivity. It was attempted to measure the selectivity of individual members by exposing them to a pair of *N*-protected amino acid enantiomers, one of which was *N*-deuterio-protected and therefore differentiable from the other by mass spectrometry. Mass spectrometric analysis of guest decomplexed from a receptor should give a measure of the receptor's selectivity.

CHAPTER 2

2.1 Preamble

Combinatorial chemistry has recently emerged as a powerful alternative to rational or orthodox synthesis.[1, 2, 3.] It is a technique by which large numbers of structurally similar and often related molecules may be synthesised in a time and resource-effective manner. An overview of combinatorial chemistry and, in particular, the one-bead one-compound combinatorial library is described herein. All members of such a library must be screened *en masse* and effective ways of doing so are described in this chapter.

Many examples from the research literature of one-bead one-compound library screening involve testing one visually tagged receptor against a solid-phase library of visually inactive guests, e.g. peptides. Any selective host can be identified by eye and picked manually. The research literature contains some examples of significant sequence selectivity discovered through such a screen. This project, however, aims to screen a library of receptors against visually-tagged guest. As the library is to be screened for enantioselectivity, a racemic guest is required. Each enantiomer may be dyed a different colour so that selectivity may be identified in a visual screen.

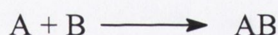
The usefulness of combinatorial chemistry in the discovery and development of new compounds and the application of what may be considered an irregular science to the very structured discipline of molecular recognition are discussed also in this chapter.

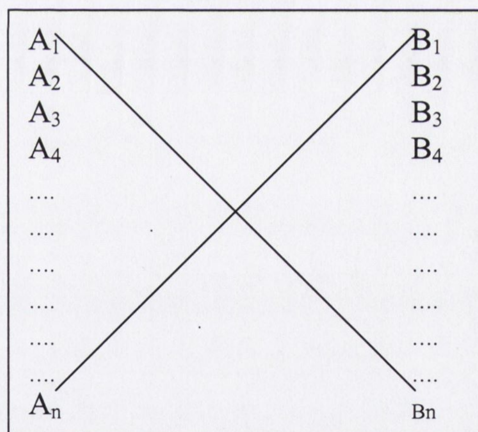
2.2 Combinatorial chemistry

Combinatorial chemistry is a relatively new technique developed by peptide chemists, *ca* 1990 to allow the preparation of a large number of structurally related compounds in the time which is usually required to prepare just a few compounds by orthodox methods. Combinatorial chemistry was considered very attractive to the pharmaceutical industry, because conventional synthetic procedures are very cost and labour intensive. Combinatorial methods allow a large diversity of compounds to be generated in a relatively short time. Simultaneous development of high throughput screening methods

allowed rapid screening of combinatorial libraries for biologically active compounds. Unlike conventional synthetic protocols, which were aimed at the production of a single compound with previously specified structure, the combinatorial approach enables the generation of searchable chemical libraries containing chemically related molecules. Chemical libraries can be divided into two major types: primary libraries used in random screening and focused libraries. The former type is aimed at the discovery of new lead compounds and the members of the library are generated randomly. Focused libraries, on the other hand, are constructed purposely around the structure of a lead compound and are targeted at the optimisation, or tuning of its properties.

Conventional or orthodox chemistry and combinatorial chemistry may be contrasted in the following way: If the coupling of monomer A with monomer B gives the product AB (orthodox synthesis), combinatorial synthesis can take a range of reagents A_1 - A_n and react those with $B_1 - B_n$ to make any combination $A_{1\text{-}n}B_{1\text{-}n}$.





Orthodox Synthesis **Combinatorial Synthesis**

Combinatorial chemistry has its origins in peptide chemistry and was performed originally as peptide synthesis on Merrifield resin.[4, 5, 6.] It may also be performed in solution but there has been a preferential focus on the use of solid-phase chemistry, in part due to its origins and in part due to practical advantages such as the easy washing away of excess reagents and by-products.

There are three main steps involved in combinatorial chemistry: preparation of the library, screening of library components and determination of the chemical structure of active compounds.

Solid-phase libraries are often prepared by "split-and-mix" synthesis. The split-and-mix method was developed by Furka and, as the name suggests, it consists of splitting, coupling and mixing the solid phase resin. Figure **2.1** illustrates split-and-mix by summarising the preparation of a library which contains all the possible trimers that can be constructed from the 3 monomer units A, B and C: 3 solid phase-bound monomers A, B and C are mixed together and split into 3 equal portions, each containing A, B and C. Portion 1 is reacted with monomer A, portion 2 is reacted with monomer B and portion 3 is reacted with monomer C, to yield 9 dipeptides. These dipeptides are again mixed together and split into three equal portions, all containing each of the 9 dipeptides. Coupling is again performed with A, B and C, to yield all 27 possible trimers, as illustrated in **Figure 2.1**.

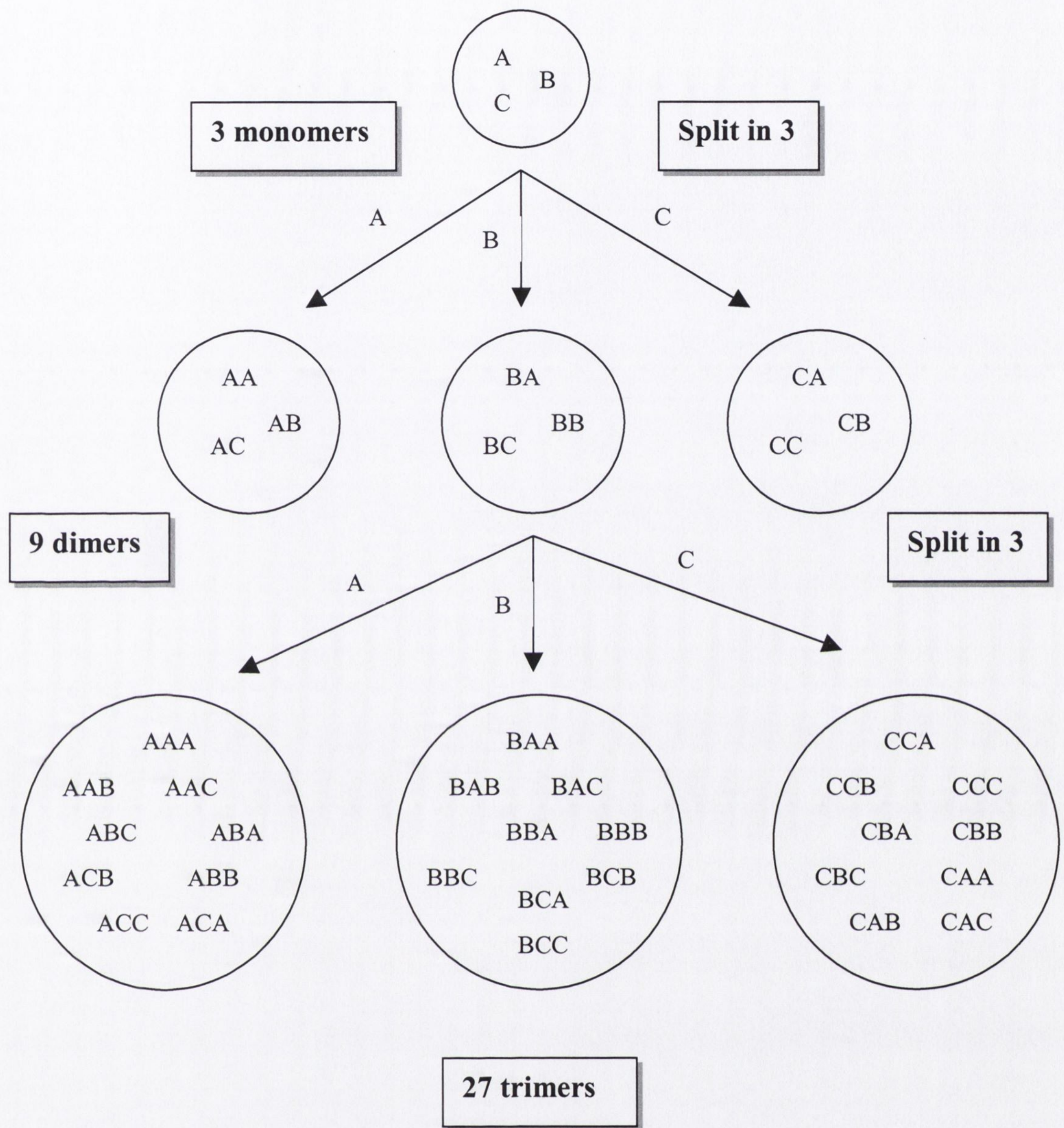


Figure 2.1: Split-and-mix Synthesis.

2.2.1 Contribution by Lam

Lam recognised that, because each bead produced by split-and-mix synthesis encountered only one amino acid at each coupling stage and as the reaction was driven to completion, that only one type of peptide was displayed on and within each bead, and described this as a "one-bead one-compound" library.[8] As each member of the one-bead one-compound library is intimately mixed with all other members, all members must be screened together. Lam also noted that in the case of peptide libraries, with an appropriate detection method such as those used in immunological research, one could detect, and therefore isolate, a peptide bead that interacted specifically with a protein target, for example an antibody.

The authors synthesised a pentapeptide library based on 19 amino acids to give 19^5 individual members, each one on a single solid-phase bead. To screen the library for bead-bound peptides capable of binding particular acceptor molecules, they coupled these acceptor molecules to a visually active target like an enzyme (alkaline phosphatase) or fluorescein. When the visually active target was added in soluble form to the solid-phase peptide library, it was found typically that a few beads were intensely stained and visible to the naked eye. By micromanipulation, beads could be picked and the bead-bound peptides sequenced.

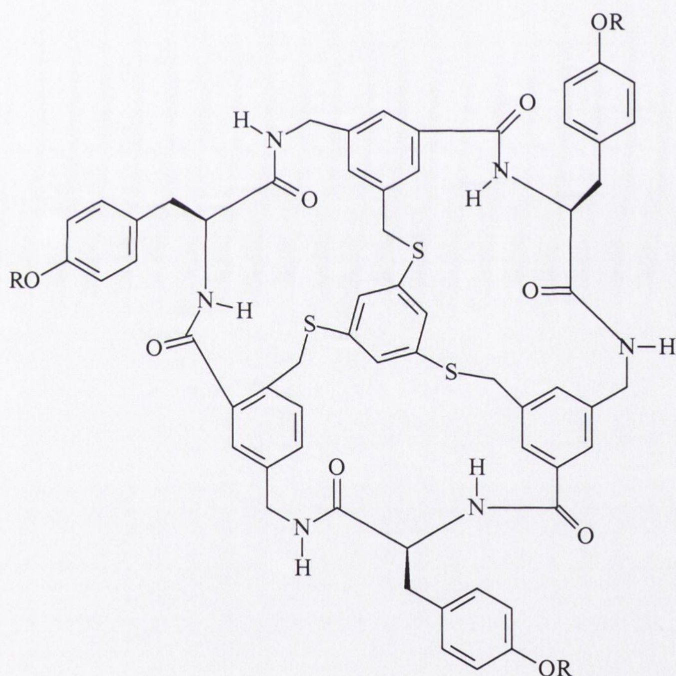
The 19^5-member library was screened against a monoclonal antibody and 6 reactive beads (from ~ 2 million screened) were picked and sequenced. The library was recycled and screened against streptavadin. 28 members were picked and sequenced, 23 of which contained the same triplet sequence. The authors noted that this showed that the concept of synthesising and visually screening a million-member on-bead library was feasible and also that this type of library was recyclable. They concluded by saying that the one-bead-one-peptide concept and its applications not only provide important new tools with which to search for specific ligands of potential therapeutic value but also that such information should enhance fundamental understanding of interactions between ligands and acceptor molecules.

Rational design, it appears, may be incorporated into combinatorial chemistry when knowledge of receptor structure is used to design a library of compounds of

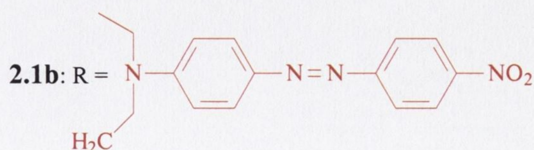
specific structure type. This is important in molecular recognition when combinatorial chemistry can be used for lead optimisation rather than lead discovery.

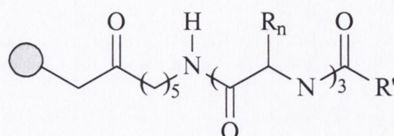
2.2.2 Contribution by Still *et al.*; screening of a one-bead one-compound tripeptide library against a visually-labelled receptor.

As part of ongoing research into selective binding, Still *et al.* replaced the allyl protecting groups of receptor **2.1a**,[9] which had previously been identified as a selective binder of *N*-protected-*N*-methylamides of α-amino acids, with a colour dye tag Disperse Red 1 to give **2.1b**.[10] The red receptor was then used to probe a tripeptide library, **2.2**, which had been synthesised by split-and-mix on individual Merrifield resin beads.



2.1a : R = $CH_2CH{=}CH_2$





Library **2.2**

Library **2.2** (~ 10^6 beads from 15^4 members) was treated with dilute **2.1b** (50 μM) in $CHCl_3$. The authors reported that after more than 24 hours that ~ 10 % of the beads had developed pink-red colourations, while ~ 0.5 % of the beads were stained deep red. 50 deep red beads were picked and decoded (tagging had been used during synthesis to identify peptides). Electron-capture gas chromatography was used to analyse the tag complement of each bead and thus define the structure of each tight-binding substrate. The authors remarked on the surprising sequence selectivity of the picked bead-bound receptors, including exclusive binding of L-amino acids found at the terminal AA_3 site. High selectivity at the AA_2 site for L-proline and L-alanine was also observed.

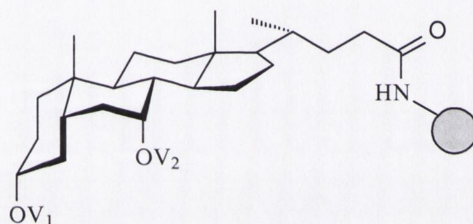
Several decoded library members were resynthesised as their *n*-hexylamides to measure their association with **2.1b** in $CHCl_3$ by NMR titrations. It was reported that the colour assay mimicked solution-phase binding.

Receptor concentration was then lowered to 10 μM so that only the most tightly binding substrates could be found, deeply staining only ~ 0.02 % of the library. The sequence of cyclopropanoyl-L-Ala-L-Pro-L-Ala emerged as common among the picked beads and when this sequence was synthesised as the dodecylamide and titrated against **2.1b** in $CHCl_3$, remarkably high selectivity (ΔG > 5.0 kcal/mol) was observed. Unsurprisingly, the diastereoisomer cyclopropanoyl-D-Ala-L-Pro-L-Ala-$NHC_{12}H_{15}$ showed no evidence of binding under the same conditions.

In a later publication, Still described further examples, and summarised the general approach, of synthesising libraries of peptidic guests to probe specific visually tagged receptor hosts.[11]

2.2.3 Contribution by Still *et al.* screening of a one-bead one-compound peptidosteroidal library against colour-coded peptides

In contrast to screening one receptor with a library of guests (**Section 2.2.2**), Still *et al.* reported a method by which a library of diverse receptors was created and screened with a specific guest.[12] An encoded peptidosteroidal receptor library **2.3** was synthesised by split-and-mix. 10 amino acids were incorporated into 4 different positions on 2 legs of the steroid to give a 10^4 member library. The library was tested against 4 closely related peptides, each of which was attached to the dye Disperse Red 1 *via* a linker: **2.4a**, **2.4b**, **2.4c** and **2.4d**.



2.3

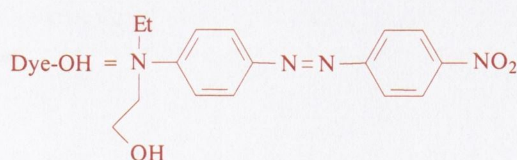
AA_n = Ala, Val, Leu, Phe, Pro, Ser (*t*-Bu), Thr (*t*-Bu), Lys (BOC), Asp (*t*-Bu), Glu (*t*-Bu)

2.4a = dye-CO$(CH_2)_3$CO-Gly-Gly-L-Phe-L-Leu

2.4b = dye-CO$(CH_2)_3$CO-L-Tyr-D-Ala-Gly-L-Phe-L-Leu

2.4c = dye-CO$(CH_2)_3$CO-L-Tyr-Gly-Gly-L-Phe-L-Leu

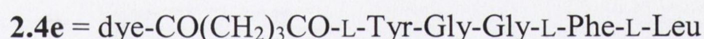
2.4d = dye-CO$(CH_2)_3$CO-L-Tyr-Gly-Gly-L-Phe-L-Met

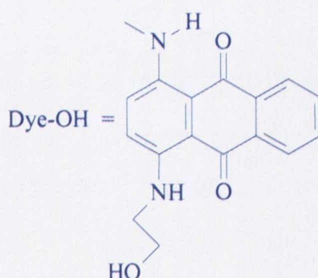


Disperse Red 1

2.3 was screened initially with **2.4c** by agitating the mixture for 48 hours to establish equilibrium. After this time, it was found that ~ 1 % of the beads had turned bright red among many beads with light orange colourations. As before, ~ 50 bright red beads were picked and decoded by gas chromatography to identify $AA_1 - AA_4$ for each of the receptors. Similar screenings were performed for **2.3** with **2.4a**, **2.4b** and **2.4d**. It was found that **2.4c** and **2.4d** were both bound by closely related subsets of the library and that receptors selected by **2.4c** and **2.4d** had a strong preference for Pro at the AA_2 site. This did not hold for **2.4a** and **2.4b**, both of which were found to bind receptors with less selectivity at particular AA_n sites. The receptor populations selected by **2.4a** and **2.4b** and **2.4c**/**2.4d** all differ from one another, establishing that different substrates were bound by different populations of receptor library **2.3**.

This assay uncovered information as to how tightly a given substrate was bound by a receptor. To find out which receptor bound which substrate most selectively however, Still *et al.* developed a two-colour assay. Different coloured dyes were attached to substrates to be distinguished and the library screened for bead-bound receptors which picked up only one colour. In order to perform this test, guest **2.4e**, an analogue of **2.4c**, was synthesised.


2.4e = dye-$CO(CH_2)_3CO$-L-Tyr-Gly-Gly-L-Phe-L-Leu



Disperse Blue 3

When **2.3** was treated with a mixture of **2.4c** and **2.4e,** a variety of purplish to reddish-purple beads were reported to have been observed as well as a few very blue beads. When the blue beads were decoded, the sequences for $AA_1 - AA_4$ were found to be closely related to the sequences found for the single-colour assay performed with **2.4c**. In particular AA_1 - AA_4 = Phe-Pro-Pro-Leu and AA_1 - AA_4 = Asp-Pro-Pro-Val were

observed. Several of the purple beads also gave similar sequences to one-another. "Blue" sequences Phe-Pro-Pro-Leu and Asp-Pro-Pro-Val were resynthesised and their relative binding of **2.4b** and **2.4c** measured. Both receptors were found to bind **2.4c** more powerfully than **2.4b** with selectivities of $\Delta G_{2.4b\text{-}2.4c}$ = -1.0 and -1.6 kcal/mol respectively. When the "purple" sequences Phe-Ala-Pro-Val and Phe-Lys-Phe-Pro were resynthesised, neither was found to have any measurable selectivity with **2.4b** nor with **2.4c**. The library was found not to have any members which could distinguish between **2.4d** and **2.4e** by this assay.

The authors concluded by noting that synthetic receptors had previously been tailored to fit desired substrates using preorganisation as the guiding principle. The ability of diverse generation and screening rather than rational design had been demonstrated in this work. They added that the best approach would appear to result from a combination of good design with efficient methods for diversity generation and screening.

2.3 Aim of this project

As stated in **Section 1.7**, the aim of this project was to develop an efficient screening procedure for the identification of enantioselective members of steroid-based receptor libraries. The libraries in question were based on cholic acid which was attached to the solid phase resin Tentagel® *via* the C-24 side chain. From the knowledge gained in previous work, it was decided to incorporate either a guanidinium or urea group in the 3-α position of the steroid. In addition to this element of rational design, variability was introduced by a *N*-Boc-dipeptide or *N*-Boc-tripeptide in each of the 7-α and 12-α positions, as shown in **Figure 2.2.**[13] The libraries were prepared by a co-worker, Riedner, in sufficient quantity (1 g resin) to allow the work described herein to be undertaken.

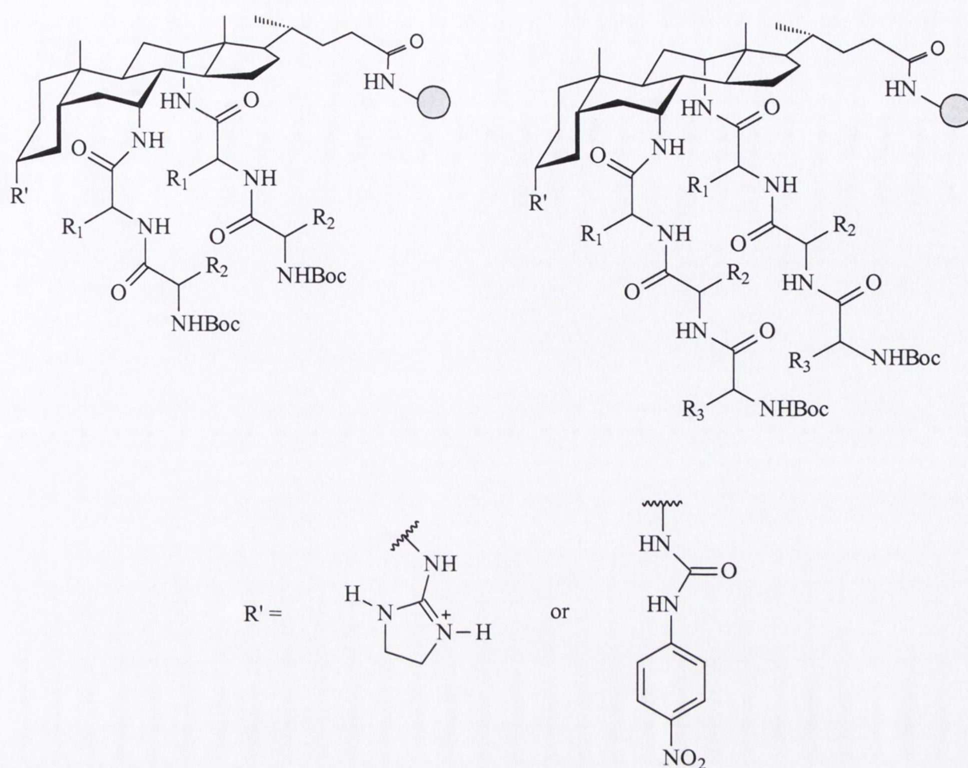
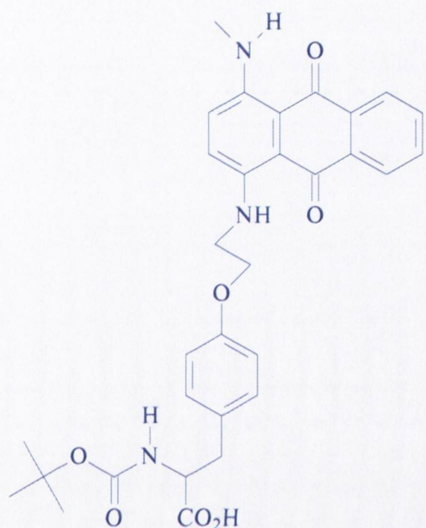


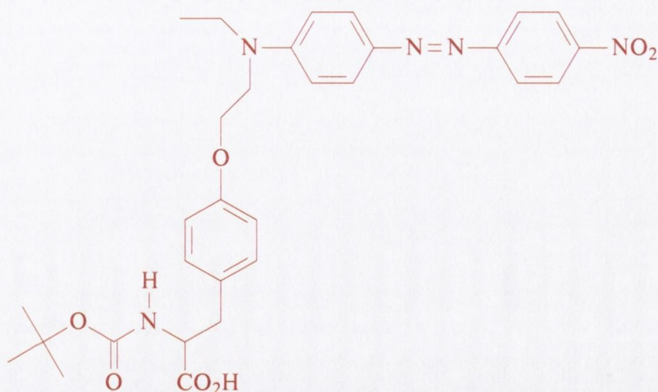
Figure 2.2: Dipeptide and Tripeptide split-and-mix libraries based on cholic acid

To screen effectively for enantioselectivity, the library needed to be treated with equimolar racemic guest so that the 2 enantiomers could compete for thermodynamic equilibrium with the library. To ascertain which of the enantiomers was bound in any case, the enantiomers were required to be distinguishable and this was achieved by dye-tagging. For example, a library that was treated with equimolar D-red and L-blue guest should exhibit any D-selective bead-bound receptors as red beads and any L-selective bead-bound receptors as blue beads. In such an assay, one would expect to observe mostly brown (unselective) beads and a few highly coloured red and blue members. These beads could be picked and subjected to further testing and/or sequenced to identify the selective receptor.

It was decided to tag the enantiomers of an *N*-protected-α-amino acid with different coloured dyes for this purpose. *N*-Boc-tyrosine was chosen because its aromatic R group has a hydroxy functionality through which a dye moiety may be attached. The red and blue aromatic dyes Disperse Red 1 and Disperse Blue 3 were

employed as colour tags for attachment to *N*-Boc-tyrosine to give **2.5** and **2.6**. Both tags are aromatic dyes and there is potential for these moieties to become involved in binding. For this reason, the dye moiety was positioned remote from the chiral centre. Furthermore, coloured beads were picked and subjected to further testing for selectivity to investigate whether the colour change resulted from binding of the chiral centre as desired. This findings are discussed in **Section 6.4**.

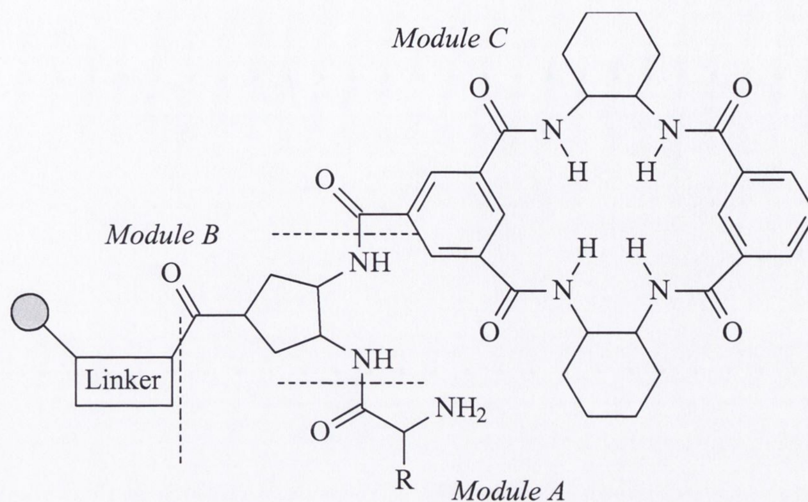




2.5 *N*-Boc-Tyr (O-Disperse Blue 3) **2.6** *N*-Boc-Tyr (O-Disperse Red 1)

2.4. Contribution by Still *et al.*; irreversible binding between a one-bead one-compound library of receptors and dye-labelled enantiomers.

While this work was ongoing, Still reported the enantioselective screening of a receptor library with differentially dyed enantiomers.[14] In a search for an efficient chiral selector, modular library **2.7** was synthesised. Module A (15 different D- and L-amino acids) carries a nucleophilic/basic amine. Module B (*RR* and *SS* enantiomers) is a turn element that directs modules A and C towards one another and module C (*RRRR* and *SSSS*) provides a large functionalised surface. This yields a 60-member library, including both enantiomers of each chemically distinct member. The library was prepared by split-and-mix synthesis on polystyrene beads. The probe molecules L- and D-proline, and their pentafluorophenyl esters, were attached *via* varied linkers (succinyl and isophthaloyl) to the aromatic dyes Disperse Blue 3 and Disperse Red 1, to yield dy-tagged guests **2.8a** and **2.8b**, as shown in **Figure 2.3**.



2.7

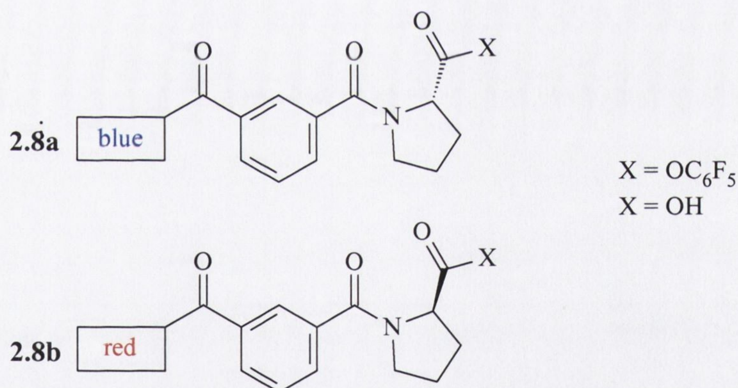


Figure 2.3: Still's modular split-and-mix library, **2.7**, and dye-labelled guest, **2.8**.

Most of the work published involved enantioselective acylation using the pentafluorophenyl derivatised guests. When library **2.7** was allowed to equilibrate with **2.8ab** (X = OC_6F_5; both the isophthaloyl and succinyl linked guests were used, separately) and then examined under a microscope, beads with distinct red and blue colourations were observed among many brown beads. The hues of the beads indicated

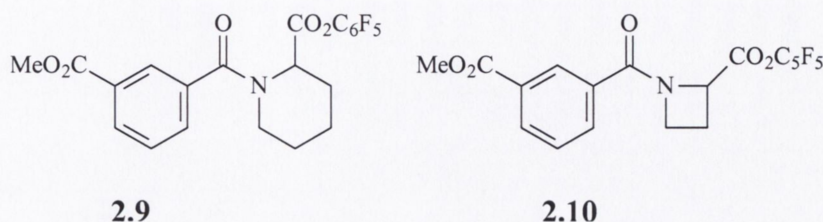
varying strengths of enantioselectivity with the isophthaloyl-linked proline derivatives generally better resolved than their succinyl-linked counterparts. The authors reported that control experiments verified that the observed selectivities did not result from differential binding of the different dye moieties. The most apparently enantioselective beads (i.e. the reddest and bluest ones) were picked and decoded. They were resynthesised on gram scale and treated with excess guest. The bound dye was then released by treatment with NaOMe and quantified by HPLC, thus providing a determination of the % e.e. of proline derivative that had been bound by the beads. The findings are listed in **Table 2.1** and show enantioselectivities between 45 % and 75 % for these acylation experiments.

Table 2.1: % Enantioselection of library members for Pro-based probes Dye-suc-**2.8** and Dye-Iso-**2.8**

Library member	Suc-2.8	Iso-2.8
L-His-(*SS*)B-(*RRRR*)C	49 % ee for D2	4 % ee for D2
D-His-(*RR*)B-(*SSSS*)C	51 % ee for L2	2 % ee for L2
L-Asp-(*SS*)B-(*RRRR*)C	44 % ee for D2	11 % for D2
D-Asp-(*RR*)B-(*SSSS*)C	48 % ee for L2	7 % ee for L2
LAsn-(*SS*)B-(*SSSS*)C	51 % ee for L2	73 % ee for D2
DAsn-(*RR*)B-(*RRRR*)C	39 % ee for D2	81 % ee for L2

It was noted that for every red bead found in the assay, a blue bead carrying the enantiomeric library member was also found, which supports the idea that the dye moieties are not responsible for the observed selectivities. In a further experiment, excess un-dyed MeO-Iso-Pro-OC_6F_5 was stirred with library member D-Asn-(*RR*)B-(*RRRR*)C in $CHCl_3$ while the quantity and % e.e. of the proline derivative remaining in the solution after filtration of the beads was monitored. The authors noted that the 80 % e.e. at 60 % conversion observed in this kinetic experiment corresponded with the 75 % e.e. seen for this receptor and the dye-labelled proline derivative.

This chiral selector resin was also found to resolve other cyclic amino acid derivatives. Isophthaloyl proline homologues **2.9** and **2.10** were resolved by the kinetic resolution and filtration method described above.



Preliminary experiments involving reversible enantioselective binding of **2.8ab** (X = OH) by **2.7** are reported to have given some reddish and blueish beads.

2.5 Conclusion

A one-bead one-compound library, in which all members are intimately mixed, may be tested successfully with a colour screen. The concept may be applied to a library of receptors which may be screened with different guests which are colour coded. Enantioselective acylations have been observed by this method, but the successful dual-colour screening of a library of receptors with dye-labelled enantiomers, for reversible selective binding has not, to the author's knowledge, been reported. The test proposed in **Section 2.3** differs from Still's attempts (**Section 2.4**) in some ways, including library size and position of chiral centre. The dye tag was deliberately positioned remote from the chiral centre of the amino acid derivative in an effort to limit any interference by the aromatic moiety during screening. This differs from library **2.7** in that linkers were used to attach, and thereby partition, the aromatic dye moitie from the chiral centre. The di-peptide and tri-peptide libraries described in **Section 2.3** are 144 and 1440 member libraries respectively, and therefore contain many more members than library **2.7**. Application of this assay to a one-bead-one-compound combinatorial library is discussed in **Chapter 6**.

The proposed screening assay is designed to indicate the direction of selectivity of library members. Although the intensity of an observed colour change may indicate the extent of binding, a measure of enantioselectivity cannot be obtained by this

method. Instead, a second screening assay was developed to measure enantioselectivity of bead-bound receptors by exposing those members picked from a colour screen to a pair of *N*-protected amino acids, one of which is *N*-deuterio protected. Analysis of bound guest by mass spectrometry should give a measure of enantioselectivity. This concept is discussed in **Chapter 3**; development of the assay is discussed in **Chapter 5** and application of the assay to members of a one-bead one-compound combinatorial library is described in **Chapter 6**.

CHAPTER 3

3.1 Preamble

In **Chapter 2,** a dual-colour screen to identify enantioselective hits in a steroidal receptor library was described. This concept was previously reported to have identified enantioselective acylations in a library of bead-bound receptors (**Section 2.4**). There are, however, certain drawbacks to the visual assay, which will be described fully in the following section. One of these drawbacks is resynthesis of hits, in particular when a resynthesised member is found to show low activity. Quantification of enantioselectivity of seemingly active members could be used to avoid unnecessary resynthesis. The colour assay itself is not a good measure of selectivity, as it is subjective. A measurement of selectivity on the single bead scale could circumvent synthesis of all members of a screened library of apparent activity. This is a novel concept and one which requires selectivity measurements on a very small scale.

A highly sensitive analytical technique was required to perform single bead analyses, as a single synthesis bead contains only 100 pmol of active sites. Enantioselectivity of these host-guest systems in the solution phase is normally measured using solution-phase extraction coupled with NMR or chiral HPLC. Neither technique is sensitive enough for a single bead solid-liquid extraction, however. As is described in this chapter, mass spectrometry became an obvious candidate due to its high sensitivity. Lack of mass difference between enantiomers was overcome by deuterio-labelling of the protecting group of one enantiomer to give a pair of "pseudoenantiomers" separable and quantifiable by mass spectrometry.

Recent reports of isotopic differentiation of enantiomers coupled with mass spectrometry to identify chiral host-guest complexes are discussed herein. The concept of mass spectrometric determination of enantioselectivity by single bead extraction of isotopically differentiated enantiomers has not been reported before, to the author's knowledge. Coupled with the dual-colour assay, this method could be used to determine enantioselectivity of bead-bound receptors and identify any very active library members.

3.2 Drawbacks to the dual-colour assay

Although the dual-colour assay described in **Section 2.3** is elegant in its simplicity, it has some drawbacks. First of all, there is some possibility of the aromatic dye moiety becoming involved in binding. Still *et al.* investigated the sequence-selective binding of simple dye molecules in water and reported that Disperse Red 1 was a good label choice in organic solvents like $CHCl_3$ as it showed little tendency to bind simple protected or de-protected peptides.[1] To limit further the possibility of the dye label becoming bound, it was deliberately positioned remote from the chiral centre of the amino acid guest. A second disadvantage is that in a visual screen the extent and direction of binding observed is difficult to ascertain. For example, whether a bead has a light red, orange or light brown colour, and the extent of intensity of hue, is subjective. The analyst needs to perform further testing to determine how selective any member is and this has been done to date by resynthesis and testing, often in the solution-phase. In research literature described in **Sections 2.21**, **2.22** and **2.23**, on average 50 coloured beads were picked from 100,000 beads during each screen, and subsequently decoded and resynthesised in the solution phase, in order to perform NMR binding studies.[2.10] This is time-consuming and laborious especially in the case of resynthesis of any false hits that have been selected.

It would be preferable to resynthesise and test only those members for which there was good evidence of efficient selective binding. This can be realised through a more effective screening assay and this work aims to develop such an assay which would allow only very effective binding candidates through to the final stages of resynthesis and testing.

3.3 Addition to the dual-colour assay

A test was required which would complement the dual-colour assay and which could be performed on single beads and small collections of single beads. This restriction required some consideration as a typical synthesis bead contains ~ 100 pmol of active sites and binds ~ 20 ng guest (for a typical *N*-Ac-α-amino acid). As described in

Section 3.1, traditional methods for determination of enantioselectivity are not adequately sensitive to measure the activity on a single bead. The highly sensitive and accurate technique chosen for the single bead assay is mass spectrometry. Mass spectrometry, of course, does not ordinarily differentiate between enantiomers due to the lack of any mass difference. The research literature, however, contains many recent examples where chiral host-guest recognition has been detected by mass spectrometry. Typically, one enantiomer is isotopically labelled so that a discernable mass difference may be observed between the two enantiomers. Labelling normally involves incorporating a few (1-3) deuterium atoms into one of the two enantiomers to yield a pair of isotopically differentiated "pseudoenantiomers".

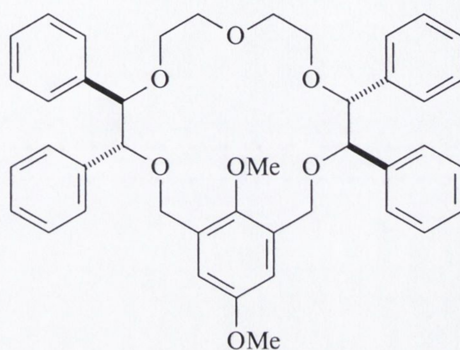
3.4 Chiral molecular recognition in mass spectrometry

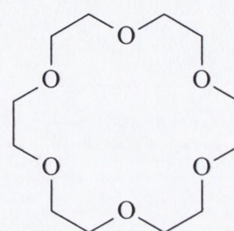
3.4.1 Contribution by Sawada *et al.*; direct measurement of chiral host-guest complexes in FABMS.

Sawada first reported the successful detection by a direct method of chiral recognition in host-guest complexes in 1994.[2] The analytical method used was fast atom bombardment mass spectrometry (FABMS). Complexes were formed between chiral crown ether hosts and amino ester guests, one enantiomer of which was a methyl-d_3 ester. An equimolar solution of labelled and unlabelled guest was complexed with chiral target host. Complex ions produced by a chiral crown ether host (H) and isotopically labelled racemic guest (G) (e.g. a 1:1 mixture of G_r and $[^2H_n]G_s$) have different masses and appear as a pair of complex ion peaks $[(H + G_r)^+$ and $(H + [^2H_n]G_s^+])$. Since the complex ions are identified by the aid of an isotope tag, both the degree and the direction of chiral recognition properties of hosts towards guests can be directly evaluated. The ratio value of the corresponding peak intensities observed in one spectrum is $I[(H + G_r)^+]/ I[(H + [^2H_n]G_s)^+]$ or "IRIS". When the IRIS value is greater than unity the *R* enantiomer is more strongly bound. Conversely, when IRIS < 1.0 the *S* enantiomer is more strongly bound. When IRIS = 1.0 no selectivity is observed, ie the value of IRIS indicates the degree of chiral recognition. This supersedes previous methods, which required several measurements against a standard.[4,]

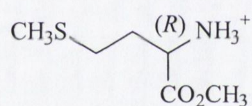
[5] In this experiment one measurement yields information as to the extent of enantioselectivity in a host-guest complex.

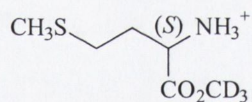
A series of crown ether hosts with isotopically labelled guests was tested.[3] Included were chiral crown ethers **3.1** (*RRRR* and *SSSS*) and the achiral standard **3.2** as hosts and **3.3**, **3.4** and **3.5**, as their hydrochloride salts, as guests.

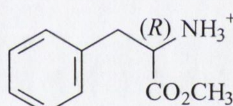


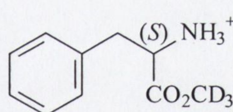


3.1 **3.2**

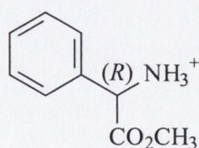


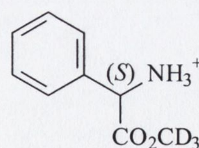






3.3 **3.4**

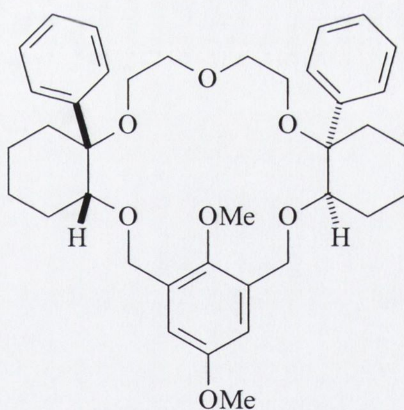




3.5

When mass spectra were obtained of the complexes formed between **3.1**-*RRRR* and guests **3.3**, **3.4** and **3.5,** the lower molecular weight complex was observed as the

more intense peak on each occasion. The measurement of each excess indicated that **3.1**-*RRRR* exhibited a high degree of *R*-enantiomer predominance towards guests **3.3**, **3.4** and **3.5** (IRIS = 1.57, 1.93 and 1.94 respectively). Conversely, host **3.1**-*SSSS* formed complexes preferentially with the heavier guest, i.e. the *S* enantiomer of **3.3**, **3.4** and **3.5** (IRIS = 0.65, 0.52 and 0.48 respectively). In addition, hosts **3.1**-*RRRR* and **3.1**-*SSSS* were found to have a "cross-chiral relationship" as expected for the highly structured complexes formed between enantiomeric hosts, as discussed in **Section 3.3.1**. The degree of *R*-enantiomer preference of **3.1**-*RRRR* was found to be equal to the degree of *S*-enantiomer preference of **3.1**-*SSSS* for guests **3.3**, **3.4** and **3.5**: $\text{IRIS}_{3.1\text{-}(RRRR)} \times \text{IRIS}_{3.1\text{-}(SSSS)} = 1.0$. The control host **3.2** was found to give IRIS = 1.0 for guests **3.3**, **3.4** and **3.5**, as expected. Of the various crown ether and acyclic hosts tested, crown ether **3.6**-*RRRR* was found to exhibit a remarkably high degree of chiral recognition toward various guests. IRIS values of 5.35 and 4.37 were observed for **3.3** and **3.4,** respectively.



3.6

These results indicate that the enantioselectivities of crown ether hosts towards amino ester guests may be measured by FABMS. The authors also reported that the IRIS values measured for these host-guest complexes correlated well with the K_R/K_S values obtained from ^{1}H NMR titrations in $CDCl_3$, e.g. **3.6**-*RRRR* with guest **3.3** gave $K_R/K_S = 4.9$ and IRIS = 5.35. In conclusion, routine FABMS yielded a measure of

enantioselectivity for host-guest complexes between crown-ether hosts and isotopically-labelled amino acid ester-ammonium ion guests.

3.4.2 Contribution by Sawada *et al.*; detection of chiral host-guest complexes using ESIMS

Further to the work described above, Sawada *et al.* reported the observation of chiral host-guest complexes in electrospray ionisation mass spectrometry (ESIMS).[6] The behaviour of non-covalently bound complexes when detected in ESIMS is considered to be similar to their behaviour in the solution phase. The technique has also the ability to detect hydrogen bonded adducts of host-guest complexes in a pure organic or aqueous-organic medium.

Hosts **3.1**, **3.2** and **3.6** were tested with guests **3.3** and **3.4**. As before, **3.2** exhibited an IRIS value of ~ 1.0 with "pseudoracemic" **3.3** and **3.4**. **3.1**-*RRRR* and **3.1**-*SSSS* showed slight *R*- and *S*- preferences, respectively, but the values observed were lower than the values measured in FABMS. The cross-chiral relationship between **3.1**-*RRRR* and **3.1**-*SSSS* was upheld. **3.6**-*RRRR*, which had shown very high *R*-selectivity in FABMS, exhibited markedly reduced values for **3.3** (1.47) and **3.4** (1.36). **3.6**-(*SSSS*) was also tested and was found to exhibit a cross-chiral relationship with **3.6**-(*RRRR*), as expected.

The host-guest complexes observed in ESIMS gave reduced IRIS values compared with the same complexes observed in FABMS. The authors attempted to account for this by suggesting that in the electrospray ionisation processes of solvent evaporation and ion evaporation were responsible. They suggested that the original thermodynamic system was particularly disturbed by intermolecular interactions (amino ester ammonium ion-ammonium ion interaction) under the highly concentrated ionisation conditions used.

3.4.3 Applications of the mass spectrometry enantiomer-labelling concept

Subsequent examples of the use of isotopically labelled enantiomers coupled with mass spectrometry for the purpose of chiral recognition have been reported.[10, 11] Van

Dorrsalaer *et al.* tested a series of spiroacetal polyethers for chiral recognition. Using Sawada's ESIMS method (**Section 3.3.3**) they examined for complexes between a series of spiroacetal polyethers and isotopically labelled phenylglycine methyl ester and phenylglycinol hydrochlorides.[7] Reetz *et al.* used the concept to develop a method of high-throughput screening of enantioselective catalysts.[8] Siuzdak *et al.* measured enantiomeric excesses by kinetic resolution using mass spectrometry and the enantiomeric labelling concept.[9]

3.5 Application of the mass spectrometic enantiomer labelling method to steroidal receptors.

The mass spectrometry enantiomer labelling method described in Section **3.4** is clearly a useful method for the determination of the selectivity of any host which, upon complexing a guest can survive mass spectrometric analysis of that complex. An attempt at using this method to idenfity complexes formed between steroidal receptor and isotopically labelled guest is described in Section **6.5**. If successful, such a method would be a quick and useful indicator of the selectivity of any receptor synthesised in the lab.

3.6 The single bead assay

In contrast to Sawada's work, which involved the observation of chiral host-guest complexes by mass spectrometry, an on-bead assay wherein the host remained bead-bound but de-complexed guest could be quantified by mass spectrometry, was required for this project. A system in which thermodynamic complexation occurred between bead-bound receptor and solution-phase guest, and whereby selectively bound guest could subsequently be cleaved and examined by mass spectrometry, was needed. Such an assay was to be applicable to a single solid-phase bead which had been pre-screened using a colour assay. Chiral recognition on a single bead, evaluated by mass spectrometry has not, to the author's knowledge, been reported previously.

For this assay to be a successful second screen to the colour assay, the two tests need to be closely related. As an aromatic amino acid was dye-tagged, so an aromatic amino acid was chosen to be *N*-(deuterio)-protected. *N*-protected tyrosine was replaced

with *N*-protected phenylalanine, however, as the hydroxy group was no longer required. Solution-phase assays relevant to this work have been performed in this group previously, between steroidal receptors and *N*-protected Phe.[1.23, 1.24, 1.25, 1.26.]

The second screen should help validate the first (i.e. any red bead from a screen using D-red and L-blue guest should show a preference for the lighter enantiomer from an equimolar solution of D-[H_n] and L-[^2H_n]). In addition to this, the relative peak intensities observed in mass spectrometry should point to the degree to which one enantiomer has been bound in preference to the other, i.e. a direct reading of enantioselectivity from the mass spectrum should be observed. The concept was to expose beads to excess "pseudoracemic" guest and, after equilibration, remove excess guest so that the only remaining guest was that which had been selectively bound by bead-bound receptor. Mass spectrometric analysis of this material should indicate the direction and extent of enantioselectivity exhibited by the bead-bound receptor. Any candidate of impressive selectivity could then be sequenced by Edman degradation and resynthesised for further investigation.

3.7 Aim of the project

As described in **Section 2.3**, the overall aim of this work was to develop an effective screening technique to identify enantioselective members of a combinatorial library of steroidal receptors. An initial screen, involving equilibration of the one-bead one-compound library with red and blue dye-tagged *N*-Boc-tyrosine, was previously described. Any highly coloured red and blue members observed in such a screen could be isolated. The direction of selectivity would be indicated by the colour change. The coloured guest could then be cleaved and the beads subjected to a second screen, involving equilibration between bead and equimolar "pseudoracemate". Mass spectrometric analysis of guest that was decomplexed from the bead after equilibration should verify the direction and indicate the extent of enantioselectivity of the bead-bound receptor. As the bead was isolated to perform thesecond assay, the receptor thereon could be sequenced by Edman degradation to identify its structure.

Development of the assay between bead-bound receptor and "pseudoracemic" guest is described in **Chapter 5**. Application of this assay to picked members of a combinatorial library is described in **Chapter 6**.

CHAPTER 4

4.1 Preamble

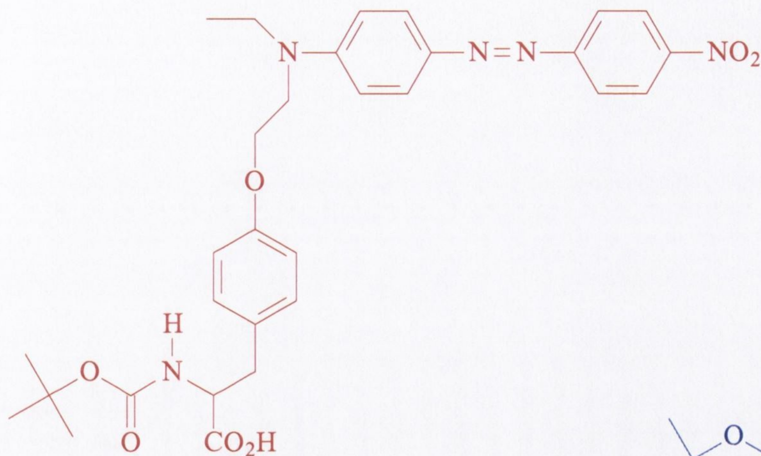
As described in **Section 2.1**, all members of a split-and-mix library are intimately mixed and must be screened together. An efficient way of doing this is to use a visual screen whereby active members may be identified by eye and picked manually. This simple concept may be applied to the enantioselective screening of a library of receptors if each enantiomer is differentially tagged so that not only active members but selective members can be identified. If different colours are used, the direction of selectivity can be ascertained by use of a simple visual screen. It was decided, therefore, to tag opposite enantiomers of an amino acid with a red and a blue dye so that any selective bead-bound receptor could be immediately identified by a red or blue colour change.

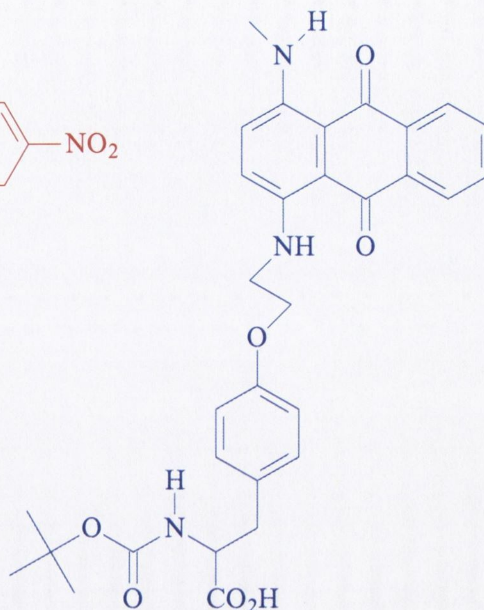
N-protected-tyrosine was chosen as the amino acid to be dye-tagged as it has a hydroxyl group remote from the chiral centre which is suitable for coupling the amino acid to a tag. Furthermore, the aromatic moiety may enhance binding. Most of the related work in this laboratory (**Section 1.5**) describes interactions between steroidal receptors and *N*-protected-α-amino acids. Colour-tagged *N*-Boc-protected-tyrosine emerged as the target guest. The use of an *N*-Boc-protected amino acid is consistent with some of Lawless' work (**Section 1.5.1**) and furthermore, the *N*-Boc group may be removed easily if a dye-tagged zwitterionic amino acid is required.

A second screen, involving the use of isotopically-labelled enantiomers coupled with mass spectrometry, was also developed. As they differ by a certain number of deuterium atoms in the protecting group, the "pseudoenantiomers" may be quantified by mass spectrometry. The purpose of the second screen was to measure the degree of selectivity of receptors selected in the initial colour screen. To this end, N-deuterio-protected amino acids, *N*-Ac-d_3-L-Phe **4.6L** and *N*-Boc-d_9-L-Phe **4.7L** were prepared.

The syntheses of red and blue dye-tagged *N*-Boc-Tyr, **2.6D, 2.6L** and **2.5L** are described in the first half of this chapter. This is followed by a description of the syntheses of *N*-(deuterio-protected) amino acids, in particular *N*-Ac-d_3-L-Phe **4.6L** and *N*-Boc-d_9-L-Phe **4.7L**, in the latter half of this chapter.

4.2 Synthesis of blue and red dye-tagged *N*-Boc-Tyrosine, 2.5L, 2.6L and 2.6D



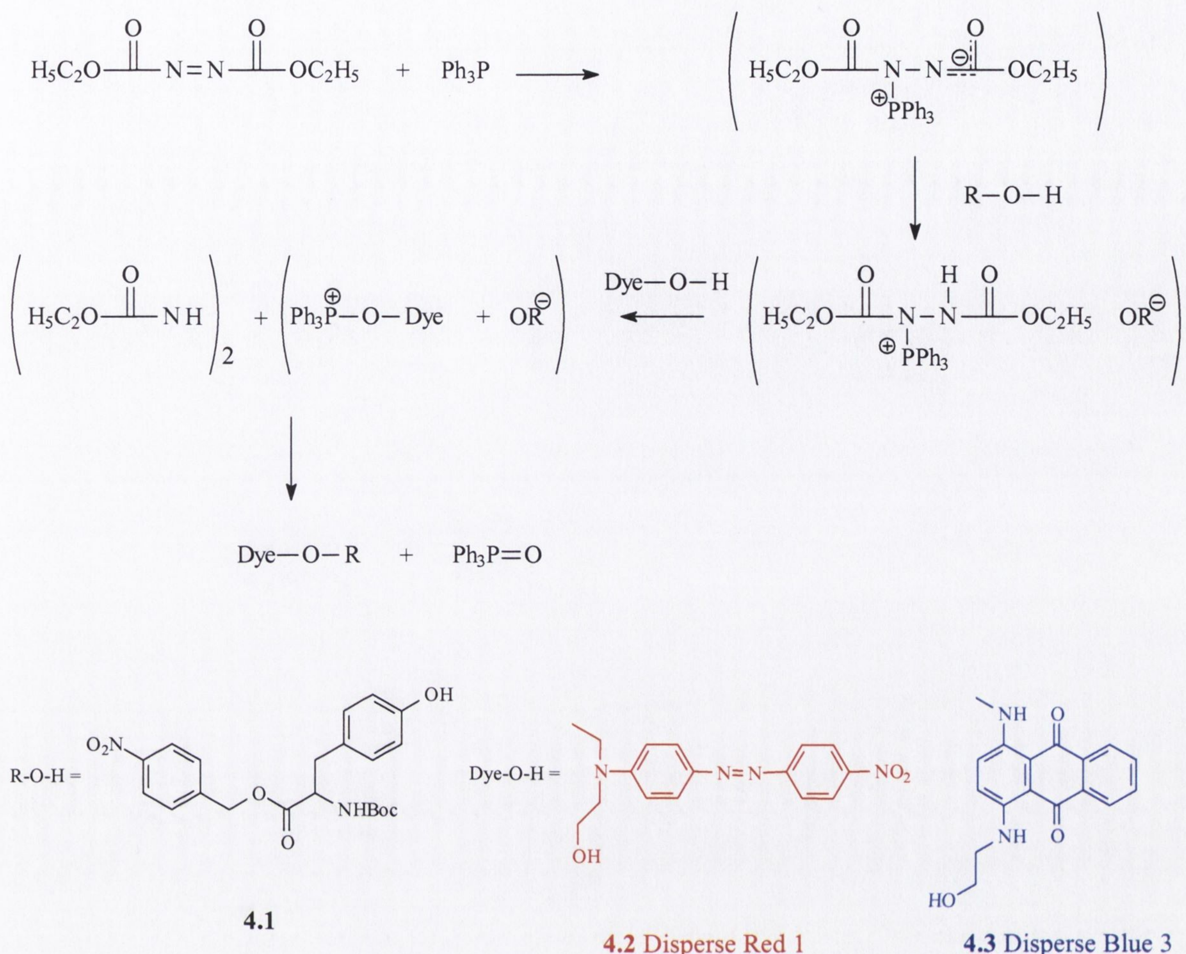


2.6L *N*-Boc-L-Tyr (O-Disperse Red 1)
2.6D *N*-Boc-D -Tyr (O-DisperseRed 1)

2.5 *N*-Boc-L-Tyr (O-Disperse Blue 3)

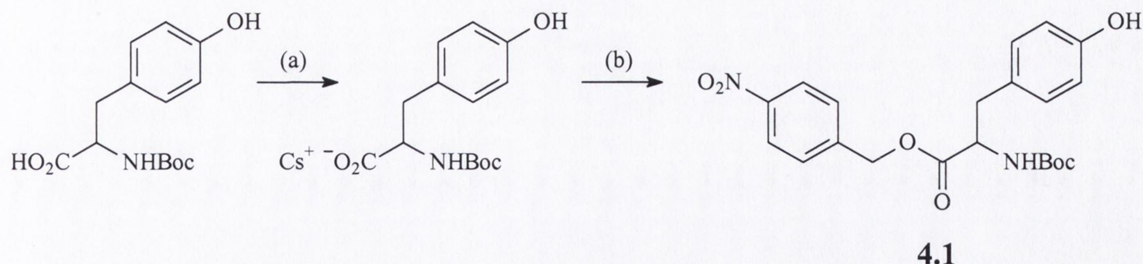
4.2.1 Amino acid carboxylate protection

Dye-tagged amino acid derivatives were obtained by condensing *p*-nitrobenzyl *N*-Boc tyrosine **4.1L** and **4.1D** with Disperse Red 1 **4.2** and by condensing **4.1L** with Disperse Blue 3 **4.3**. The *p*-nitrobenzyl protecting group was subsequently removed to yield the free acids, **2.6L**, **2.6D** and **2.5L**. The condensation was performed using Mitsunobu-type conditions[1], in which the phosphonium ion believed to form between DEAD and Ph_3P is protonated by addition of **4.1,** as shown in **Scheme 4.1**. Addition of the aromatic dye to this system leads to reduced DEAD and formation of an alkoxyphosphonium ion, S_N2 type displacement of which with deprotonated **4.1** leads to formation of the desired product and triphenylphosphine oxide.



Scheme 4.1: Mitsunobu reaction between 4.1 and aromatic dyes 4.2/4.3.

The carboxylic acid of *N*-Boc-Tyr required protection prior to condensation with the organic dye. Benzyl ester protection of the carboxyl group was employed using the method of Wang *et al.*, by which amino acid benzyl esters are prepared easily *via* cesium salts, by reaction with alkyl halides under neutral conditions at room temperature (**Scheme 4.2**).[2] This method was reported to give high yields with no racemisation, which was a crucial factor for this synthesis. The Cs salt was obtained as a white solid by removal of solvent under pressure by rotary evaporation, without raising the temperature of the mixture above 30 °C. When *p*-nitrobenzyl bromide was added to the Cs salt in DMF, a precipitation of CsBr was immediately observed and the ester **4.1** was obtained in > 90 % yield.



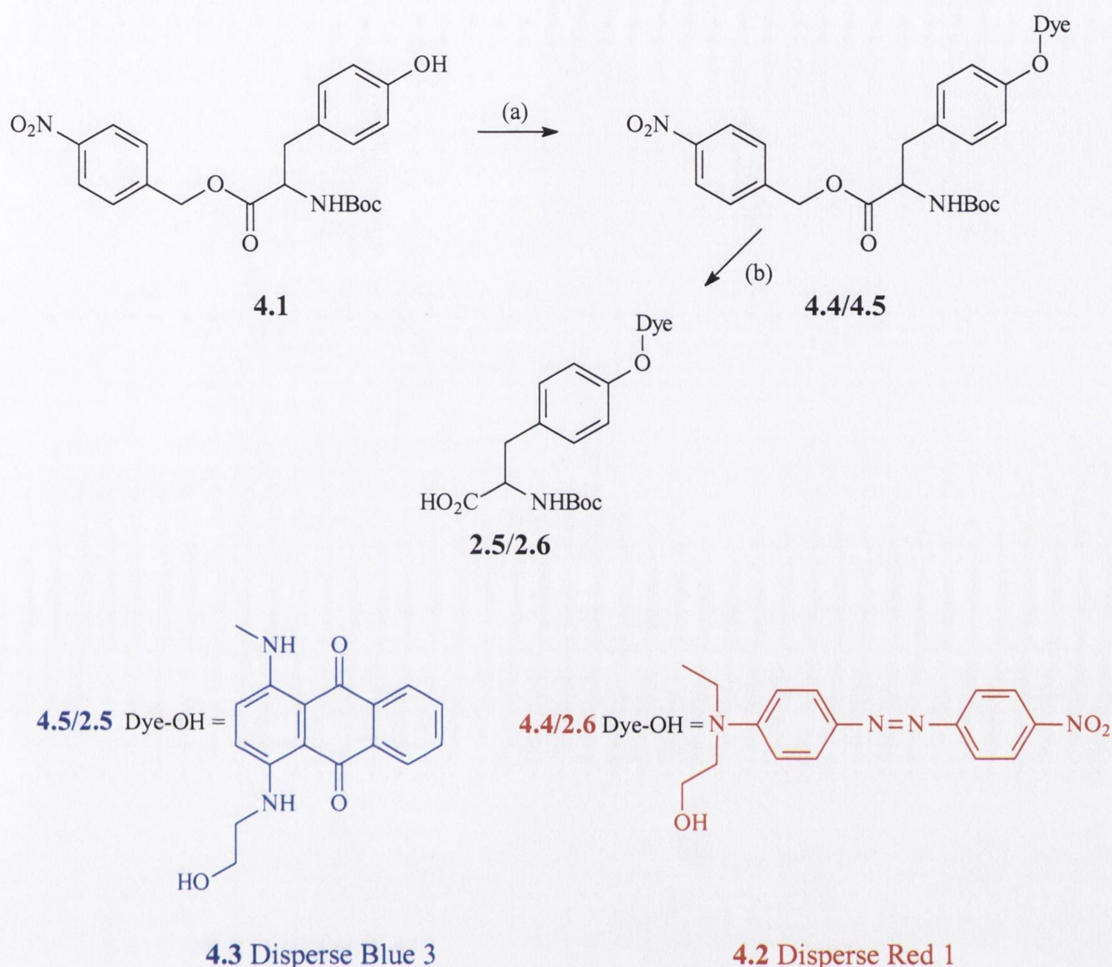
Scheme 4.2 (*Reagents, conditions and yields*) (a) aq. Cs_2CO_3 (20 % w/v), MeOH, H_2O; (b) *p*-nitrobenzyl bromide, DMF; r.t.; 1 hr; 93 %

It was necessary to remove the *p*-nitrobenzyl protecting group after coupling of **4.1** to the dye moiety. The method of Rinehart *et al.*, which uses Bu_4NF in polar solvent to cleave nitrobenzyl esters from amino acids, was employed.[3] Bu_4NF (1.0 M in THF) is commercially available. Furthermore, *N*-Boc groups are preserved under these conditions.

4.1L and **4.1D** were obtained in good yield, by separate preparations from the appropriate enantiomer of *N*-Boc-tyrosine. The ^1H and ^{13}C NMR spectra obtained thereof support the structures of these molecules, as do the high resolution mass spectra and ir spectra. Microanalytical assays, which were obtained of **4.1L** and **4.1D** which had not been recrystallised but which were pure by nmr and tlc, gave adequate results for the carbon and nitrogen contents. The hydrogen contents were 6.33% for the L enantiomer and 6.29 % for the D enantiomer, compared with a calculated content of 5.81 %, however. The analyses were repeated twice to give failing hydrogen contents, and when this was discussed with the Microanalytical Laboratory Technician, it was discovered that, at that time, several samples were failing hydrogen content and that there was an issue with the standard. The optical rotations of both enantiomers were recorded, as enantiomers should have equal but opposite optical rotations. *p*-Nitrobenzyl *N*-Boc-L-tyrosine had an $[\alpha]_D$ of -3.1 but a value of +3.9 was recorded for *p*-nitrobenzyl *N*-Boc-D-tyrosine. The $[\alpha]_D$ values of the commercially available *N*-Boc-tyrosine used in each synthesis was not recorded. The values reported indicate that equal quantities of the material would not yield a racemate, i.e. that at least one of the enantiomers was not enantio-pure. The data suggest that the conditions used to protect enantio-pure *N*-Boc-Tyr impacted the enantiopurity of the amino acid derivative. The

specific rotation data were collected retrospective to forward-processing these products by reaction with Disperse Red 1 and Disperse Blue 3, however, and, as discussed in **Section 4.2.2**, the specific rotation values recorded for the products of those transformations were not found to be equal but opposite in magnitude.

4.2.2 Mitsunobu-type coupling of dye substrate to 4.1



Scheme 4.3 (*Reagents*) (a) Ph_3P, DEAD, Dye-OH, THF; (b) Bu_4NF (1.0 M in THF), 7 eq. THF.

The Mitsunobu-type condensation between dye substrate and **4.1** was attempted initially between **4.2** and *p*-nitrobenzyl *N*-Boc-L-Tyr **4.1L**. A recent publication by Wennemers *et al.* described a similar transformation whereby **4.2** was attached to the phenyl hydroxy group of *N*-Boc-Tyr methyl ester by a Mitsunobu reaction, for the purpose of dye-tagging a library of diketopiperazine receptors.[4] A notable feature of work with these dyes is that the highly coloured material was followed easily by tlc and in flash column chromatography.

It was found that **4.2** tended to form symmetrical ethers with itself in the presence of Ph_3P and DEAD. To prevent this side-product from forming, it was necessary to add **4.2** (1 eq.) in THF to the other reagents: **4.1L** (1 eq.), Ph_3P (1 eq.) and DEAD (1 eq.) in THF. Under these conditions, it is thought that the protected amino acid is deprotonated prior to addition of the dye. By tlc, the yield of desired product was observed to increase when a further addition of Ph_3P and DEAD (0.5 eq. apiece) was made. Although a further such addition led to an increase in yield, this was accompanied by the formation of some undesired red symmetrical ether. Consequently, the reaction was completed and the reaction mixture purified by flash column chromatography. Although the eluant, hexane/ethyl acetate (3:2), removed Ph_3P, oxidised Ph_3P, DEAD, reduced DEAD and the undesired red ether, the desired product co-ran with **4.1L** so that it was necessary to perform flash column chromatography a second time, eluting with 4 % MeOH/DCM. The recovered red material was further purified by recrystallisation from $CHCl_3$/hexane. **4.4L** was recovered in ~ 40 % yield. The yield was impacted negatively by completing the reaction when the symmetrical ether was observed to form. The same procedure was followed in the synthesis of **4.4D** from **4.1D**.

The ^1H and ^{13}C nmr spectra of **4.4L** and **4.4D** were consistent with the structures of the desired products and compared favourably, where expected, with the spectra of **4.1** and Disperse Red 1. Assignment of resonances to the methyl, butyl and aromatic groups, as listed in **Chapter 7**, were routine. Assignment of the 5 methylene groups, the secondary amine and the methine group, in the case of the D enantiomer, used the following rationale: There is a doublet at 3.04 ppm, integrating to 2 H. The H, H-COSY spectrum shows that this resonance has H-H coupling to a multiplet at 4.60 ppm, which integrates to 1 H. The hmqc spectrum shows that the resonances at 3.04 ppm and 4.60 ppm couple to resonances at 54.69 and 37.48 ppm, respectively, in the ^{13}C spectrum. The resonance at 4.60 ppm is also coupled to a doublet at 4.96 ppm in the 1 H spectrum, which does not exhibit coupling with any signal in the ^{13}C spectrum, and is assigned to the amino proton. The doublet at 3.04 ppm and multiplet at 4.60 ppm were assigned to the CH_2CH and CH_2CH protons, respectively. There is a quartet at 3.66 ppm, integrating to 2 H, which has a coupling constant of 7 Hz. The quartet exhibits H-H coupling to the methyl group at 1.3 ppm, which also has a coupling constant of 7 Hz.

The quartet at 3.6 ppm is, therefore, ascribed to the ethyl methylene group. There are 2 groups of triplets, at 3.89 and 4.17 ppm, which share a coupling constant of 5.5 Hz and are observed to couple to each other in the H, H-COSY spectrum. Furthermore, coupling was recorded, in the hmbc spectrum between both the methyl group and the ethyl methylene group and the triplet at 3.89 ppm. As the N*C*H_2CH_2 group is in greater proximity to the ethyl group than is the OC*H_2*CH_2 group, the triplet at 3.89 ppm is ascribed to the N*C*H_2CH_2 group. The triplet at 4.17 ppm, is ascribed to the O*C*H_2CH_2 group. The triplets at 3.89 and 4.17 ppm, respectively, are coupled with resonances at 49.84 and 65.31 ppm in the ^{13}C hmqc spectrum respectively. As in the ^{1}H spectrum of *p*-nitrobenzyl N-Boc-Tyr, there is an AB quartet at 5.22 ppm, which integrates to 2 H and is assigned to the benzyl CH_2. There is coupling between this group and the resonance at 65.31 in the ^{13}C hmqc spectrum. The resonance at 65.31 exhibits coupling with both this group and the O*C*H_2CH_2 group in the ^{13}C hmqc spectrum. The hmqc spectrum indicates that 2 separate carbon atoms both resonate at 65.31 ppm; they are the O*C*H_2CH_2 and benzyl *C*H_2 moieties. The same rationale was used to assign resonances in the spectra of the L enantiomer.

The melting points of **4.4L** and **4.4D** were found to be slightly different, being 126-128 °C and 128-129 °C, respectively. The specific rotation values of **4.3L** and **4.3D** were found to be – 17 and + 22 respectively. Enantiomers should, of course, have equal but opposite specific rotations. These data indicate that one, or both; of the enantiomers is not enantiopure. **Section 4.2.1** bore discussion of the specific rotation values of *p*-nitrobenzyl *N*-Boc-L-tyrosine and *p*-nitrobenzyl *N*-Boc-D-tyrosine, which were -3.1 and +3.9, respectively. In both cases, the specific rotation of the L enantiomer is about 1.3 times greater for the L enantiomer than the D. This suggests that enantio-purity was impacted during benzyl protection of N-Boc-tyrosine, but that the conditions used to attach the dye moiety to the protected amino acid had no further impact on enantio-purity. Microanalytical data were not collected for these molecules, which were further reacted to yield **2.5L** and **2.6D**, which were used in the on-bead assays proposed in **Chapter 2**. Microanalytical data were collected for **2.5L** and **2.6D.**

4.2.3 Removal of the *p*-nitrobenzyl protecting group.

The method of Rinehart, which was described in **Section 4.2.1,** was used to deprotect **4.4L**. The crude product was purified by flash column chromatography, eluting with $CHCl_3$/ammonia-saturated MeOH (4:1). The recovered material was recrystallised from $CHCl_3$/hexane to yield **2.6L** in ~ 35 % yield. The yield of red-tagged N-Boc-L-Tyr was rather low, but adequate as micromolar quantities were required *per* on-bead assay. The opposite enantiomer **2.6D** was obtained by the same preparation.

^1H and ^{13}C NMR were used to identify the desired molecules. The spectra compared and contrasted favourably with those for the esterified analogues, **4.4L** and **4.4D**. As for **4.4L** and **4.4D**, assignment of the resonances for the methoxy, *t*-butyl and aromatic moieties were routine. The methylene, methine and secondary amine groups of **2.6L** were assigned as follows: There are 4 resonances which are inverted in the ^{13}C DEPT spectrum. These correspond to the four CH_2 groups in the molecule. In the H, H-COSY spectrum, a broad multiplet at 3.05 ppm, integrating to 2 H, which exhibits H-H coupling to both the CH and tyrosine NH resonances, was ascribed to the $CHCH_2$ methylene group. It is coupled with a resonance at 36.87 in the hmqc ^{13}C spectrum, which is found to invert in the DEPT 135 spectrum. Two triplets, both with coupling constants of 5.5 Hz, occur at 3.86 and 4.19 ppm in the ^1H spectrum. Both resonances integrate to 2 H and are coupled in the hmqc spectrum to carbon resonances at 49.85 and 65.34 ppm, respectively, both of which resonances may be assigned to methylene protons as *per* inversion in the DEPT 135 spectrum. These resonances are coupled in the H, H-COSY spectrum and, by comparison with the spectra of **4.4D** are believed to correspond to the CH_2CH_2N and CH_2CH_2O methylene group. In the hmbc spectrum, the further up-field group is found to exhibit, in common with the ethyl CH_2, coupling with the quaternary carbon at 151.29 ppm. As this quaternary carbon has a hmqc relation with the dye moiety, the methylene group at 3.86 ppm must be in proximity to both the ethyl group and the dye moiety and is therefore more likely to be the CH_2CH_2N methylene group than the CH_2CH_2O methylene group, which may, in turn, be assigned to the resonance at 4.19 ppm. The corresponding groups occurred at comparable positions in the ^1H spectrum of **4.4D**. A broad peak at 4.53 ppm, integrating to 1 H, exhibits a bond to the methylene peak at 3.05 ppm ($CHCH_2$) in the H, H-COSY

spectrum and to the peak at 54.39 in the ^{13}C spectrum in the hmqc spectrum and is consistent with the methine proton. There is a doublet at 4.95 ppm, integrating to 1 H, which is consistent with the NH proton. The remaining inverted peak, at 46.17 in the DEPT spectrum, may be assigned to the ethyl methylene group. In the hmqc spectrum, it is coupled to the quartet at 3.61 ppm, which integrates to 2 H and has a coupling constant of 7 Hz. This resonance exhibits H-H coupling to the methyl group at 1.30 ppm, which also has a coupling constant of 7 Hz. High resolution mass spectra and microanalytical data, as listed in **Chapter 7**, support the identity of these molecules.

The pair of enantiomers was analysed by chiral HPLC using a Daicel Chiralpak AD column. Analysis was performed in normal phase, eluting with heptane/IPA (4:1) at a flow rate of 0.8 mL/min. Peaks were detected at $\lambda = 254$ nm. A peak at 4 minutes was observed in each spectrum and was ascribed, by an experienced user, to the filters used prior to injection to the HPLC. This peak was also observed in "blank" injections of heptane and IPA. The spectrum of IPA is shown in **Appendix B5**. The peaks therein were considered extrinsic to the samples of interest. **Appendix B1** is the spectrum of **2.6D** and, apart from the aforementioned peak at 4 minutes, a single peak is observed to elute at 4.8 minutes. This peak is deemed to represent **2.6D**. **Appendix B2** is the spectrum for **2.6L**. A peak is observed at 5.3 minutes and is thought to represent **2.6L**. An inversion of the baseline, followed by a peak at about 8 minutes is also observed in this spectrum. Finally, **2.6L** and **2.6D** were combined to give the spectrum in **Appendix B3**. Apart from the peak at 4 minutes, there are 2 main peaks, one at about 4.8 minutes and the second at about 5.3 minutes. As for **Appendix B2**, the second peak is followed by an inversion of the baseline and a small peak at 8 minutes. **Appendices B2** and **B3** indicate that **2.6L** is impure. The purity of the esterified analogues, **4.4L** and **4.4D**, and the starting materials, **4.1L** and **4.1D,** were brought under scrutiny in **Sections 4.2.1** and **4.2.2**, as the respective specific rotations of each set of "enantiomers" were not equal and opposite. The HPLC traces in **Appendix B** indicate that the L enantiomer of red dye-tagged *N*-Boc-Tyr is impure. The specific rotation of **2.6L** and **2.6D** are – 48 and + 40, respectively. Those of the precursors showed a greater value for the D enantiomer than for the L, but this is not maintained for the free acids. If optically active, the impurity associated with the L enantiomer, would impact the specific rotation of the material. This impurity cannot be quantified from the HPLC trace, as the strength of the

chromophore is unknown. There is, however, a 20% difference between the specific rotations of **2.6L** and **2.6D** and, as **2.6D** appears to be pure, it may be inferred, by comparison, that **2.6L** is about 20 % impure.

HPLC traces were not obtained for the precursors, **4.1L, 4.1D, 4.4L and 4.4D**. The specific rotations indicated that at least one enantiomer was impure, with the difference in specific rotation remaining unchanged as **4.1** was converted to **4.4**. The difference in specific rotation did change, however, for **2.6L** and **2.6D**, after the esterified dye-tagged amino acids were treated with strong base. Although both enantiomers were synthesised under the same conditions, the HPLC data indicate that the integrity of the L enantiomer has been compromised.

4.2.4 Purification of Disperse Blue 3 4.3

Although **4.2** is commercially available as the pure dye, **4.3** was purchased as a 20 % component of a mixture of coloured materials. This material was previously used by colleagues in the group of Gennari at Università di Milano, and their purification method thereof was followed.[5] By tlc, the commercially available mixture appeared to contain about 7 blue, pink and purple components. When eluted in DCM/acetone (4:1), the major fractions appeared at R_f = 0.15 (intense blue), 0.25 (purple), 0.35 (intense blue), 0.42 (pink), 0.55 (purple), and 0.65 (blue). The mixture was purified by flash column chromatography, eluting with DCM/acetone (4:1) and the blue spot with R_f = 0.35 isolated. This required further purification by recrystallisation from $CHCl_3$/hexane.

The isolated material was compared directly, by tlc, with that prepared by the Gennari group, and found to have an identical R_f. The nmr spectra described in **Chapter 7** correlated with those recorded by the group of Gennari. In the ^1H NMR spectrum, the methyl and 2 methylene groups are seen as a doublet and 2 quartets, respectively. The 2 aromatic protons of the 1,4-diaminophenyl moiety give doublets at 7.15 and 7.26 ppm, both of which have coupling constants of 9.5 Hz. The remaining aromatic protons occur further downfield and the amino protons occur as a quartet at 10.57 ppm (N*H*Me) and a triplet at 10.83 ppm (N*H*CH_2).

The purification was typically performed with 1 g of impure dye to yield **4.3** as a blue solid (70 – 80 mg). This corresponds to an overall yield of less than 10 % or, based on 20 % purity of starting material, a yield of 40 %. The laborious and poor-yielding preparation of starting material was also the experience of colleagues within the European TMR network.[6] It was decided to couple **4.3** to the L-enantiomer of **4.1** only.

4.2.5 Reaction of 4.3 with 4.1L

4.3 (1 eq.) in THF was added to **4.1L** (1eq.), Ph_3P (1 eq.) and DEAD (1 eq.) in THF and the reaction was followed by tlc. As for the synthesis of **4.4L**, further additions of Ph_3P (0.5 eq.) and of DEAD (0.5 eq.) were made after 90 minutes, which led to an increase in

the yield of desired product. The reaction mixture was purified by flash column chromatography, eluting with hexane/ethyl acetate (1:1). The recovered material was further purified by recrystallisation to give **4.5L** as a blue solid (40 % yield). A specific rotation value of –14 was obtained for this molecule. The opposite enantiomer, against which this value could be compared was not prepared, however.

^{1}H and ^{13}C NMR spectra of **4.5L** were compared and contrasted with those of **4.3** and **4.1L**. A single peak at 1.39 ppm, which integrates to 9 H and has coupling, in the hmqc spectrum, to a peak at 28.29 ppm in the ^{13}C spectrum, was assigned to the *t*-butyl group. The doublet at 3.12 ppm, which integrates to 3 H and is observed in the hmqc spectrum to couple to the peak at 29.71 ppm was assigned to the methyl group. A quartet and a triplet, which are observed to couple with each other in the COSY H-H spectrum, occur at 3.83 and 4.20 respectively. In the hmqc spectrum, the quartet is observed to couple to the peak at 42.20 ppm in the ^{13}C spectrum, which itself inverts in the DEPT 135 spectrum. The triplet is observed to couple to the peak at 66.99 ppm in the ^{13}C spectrum, which is also observed to invert in the DEPT 135 spectrum. Both are methylene protons, the CH_2CH_2N group resonating in the more up-field position as a quartet and the $CH_2\mathit{CH}_2O$ resonating as a triplet at 4.20 ppm. As for **4.1** and **4.4**, an AB quartet was observed at 5.19 ppm and assigned to the benzyl $C\mathit{H}_2$. A doublet, integrating to 2 H is observed to resonate at 3.03 ppm. The hmqc spectrum shows that this peak is coupled to a peak at 37.51 ppm in the ^{13}C spectrum, which is inverted in the DEPT 135 spectrum. The H-H COSY spectrum showed coupling between the resonance at 3.03 ppm and a multiplet at 4.20 ppm, which integrates to 1 H. The resonances at 3.03 ppm and 4.20 ppm were assigned to the $CHC\mathit{H}_2$ and $C\mathit{H}CH_2$ protons, respectively. The methine resonance at 4.20 ppm, was observed, in the hmqc spectrum, to couple to the resonance at 54.72 ppm in the ^{13}C spectrum, and, in the H-H COSY spectrum, to the broad doublet at 4.96 ppm. The latter doublet, which integrates to 1 H and does not couple to any carbon atoms, was assigned to the NH proton. Noteable features of the aromatic protons in the ^{1}H spectrum include 1 proton of the dye moiety occurring with the $CHCl_3$ peak at 7.26ppm. This proton occurs as a doublet and is coupled to the peak at 122.97 ppm in the ^{13}C spectrum. Another single aromatic proton of the dye moiety occurs with the benzyl aromatic protons at 7.39 ppm; this proton also occurs as a doublet and is coupled to the carbon atom at 123.42 ppm. The

corresponding resonances for Disperse Blue 3 occurred as doublets at comparable chemical shifts. The remaining protons of the dye moiety occur at 7.70 ppm and 8.33 ppm. The benzyl aromatic protons are observed at 7.39 ppm (occurring with an aromatic dye proton) and 8.18 ppm. The tyrosine moiety's protons occur as two doublets further up-field. A microanalytical assay was not obtained for this material, which was de-protected to yield **2.5L**, on which the work described in **Chapter 6** was performed. Microanalytical data were collected for **2.5L**.

4.2.6 Removal of the *p*-nitrobenzyl protecting group.

The procedure described in **Section 4.2.3** to deprotect **4.4L** was followed to attempt to deprotect **4.5L**. Bu_4N (7 eq.) was added to **4.5L**. The reaction mixture was monitored by tlc for formation of blue baseline material and, following the standard procedure, an acid work-up was performed. It was found that the subsequent purification by flash column chromatography was not a successful method of recovering the free acid, as the material, once applied to the column, streaked as it eluted. Unlike the esterified analogue, which was observed to elute as a well-defined blue ring, the acid streaked as the eluant progressed down the column such that the entire length of silica gel appeared blue at once. To circumvent this, the reaction mixture was concentrated under reduced pressure and purified by flash column chromatography. Unlike the free acid, which was found not to elute well using normal phase flash column chromatography, the TBA salt was effectively eluted in $CHCl_3$/ammonia-saturated MeOH (4:1). The desired fractions were combined, concentrated under reduced pressure, redissolved in ethyl acetate and washed with $KHSO_4$ (1 M) and brine to yield a blue solid. This was further purified by recrystallisation to deliver **2.5L** as a blue solid (~ 35 % yield).

The ^1H and ^{13}C spectra for this molecule compared and contrasted favourably with the precursor **4.5L**. As for **4.5L**, a singlet integrating to 9 H occurs upfield, which may be assigned to the *t*-butyl group. Two doublets, integrating to 2 H and 3 H occur very close together at 2.95 and 3.05 ppm, respectively. The doublet at 2.95 ppm is coupled to a carbon resonance at 36.38 ppm, which is inverted in the DEPT 135 spectrum, thereby indicating a methylene group. As it occurs as a doublet, and is therefore split by only one proton in close proximity, this resonance was assigned to the

$CHCH_2$ methylene group. The hmqc spectrum shows that the doublet at 3.05 ppm is coupled to a resonance at 29.49 ppm. As the group integrates to 3 H, it may be assigned to the methyl group. As for the ^{1}H spectrum of **4.2L**, there is a quartet at 3.73 ppm and a triplet at 4.20 ppm, which are coupled to each other. Both sets of peaks integrate to 2 H and are coupled to peaks at 42.20 ppm and 66.99 ppm, respectively in the ^{13}C spectrum, both of which peaks are inverted in the DEPT 135 spectrum. As for **4.2L**, the further up-field quartet is assigned to the $NH\mathit{C}H_2CH_2$ methylene group while the $O\mathit{C}H_2CH_2$ methylene group occurs as a triplet further downfield. Finally, there is a broad multiplet at 4.53 ppm, with H-H coupling to both the methylene group at 2.95 ppm and a broad doublet at 5.02 ppm. Integrating to 1 H, it may be assigned to the $C\mathit{H}CH_2$ methine group with the carbon atom being assigned to the peak at 54.43 ppm, with which the proton is coupled in the hmqc spectrum. The doublet at 5.02 ppm to which it is coupled, also integrates to 1 H and may be assigned to the CHN*H* secondary amine proton. The aromatic region of the ^{1}H spectrum occurs as anticipated by comparison with **4.5L**. In particular, one proton of the dye moiety occurs as a doublet at 7.26 ppm and a second such proton occurs at 7.10 ppm on the shoulder of the more downfield of the pair of doublets ascribed to the tyrosine moiety's protons. These protons are coupled to carbon atoms at 122.97 and 123.41 ppm, respectively, in the hmqc spectrum. The remaining secondary amine protons occur downfield as a quartet at 10.62 ppm (N*H*Me) and a triplet at 10.97 ppm (N*H*CH).

As for **2.6L** and **2.6D**, Chiral HPLC (Daicel Chiralpak AD column) was performed in normal phase, eluting with heptane/IPA (4:1) at a flow rate of 0.8 mL/min. The HPLC is exhibited in **Appendix B4**, which, apart from the peaks associated with the blank IPA injection in **Appendix B5**, shows a single peak at 5.4 mins, which supports the purity of **2.5L**. This product was synthesised from **4.1L**, the specific rotation of which, or the specific rotation of **4.1D**, by comparison therewith, was indicated as not being enantio-pure. In **Section 4.2.2**, it was argued that **4.1L** was the impure enantiomer. As **2.5L** was also synthesised from **4.1L**, the enantio-purity thereof must also be questioned. A specific rotation value of – 17 was obtained for this molecule. The D enantiomer, against which this value could be compared, was not synthesised, however, as the starting material, Disperse Blue 3 required a lengthy and low-yielding preparation. A sharp melting point of 128-130 °C was recorded for the

molecule, however, which is indicative of high purity. The microanalytical assay gave acceptable results for C and N content, but an unacceptably high result for hydrogen content. This effect was observed for other samples, as described in Section **4.2.1**. The high resolution mass spectrum provided an accurate mass for this molecule.

2.5L was used, with **2.6D**, to perform the on-bead colour assay described in **Chapter 2**, for which the enantiopurity of these molecules is very important. This work, and an assessment of the impact of using substrate which is not enantio-pure, is discussed in **Chapter 6**.

4.3 Synthesis of *N*-(deuterio-protected) α-amino acids

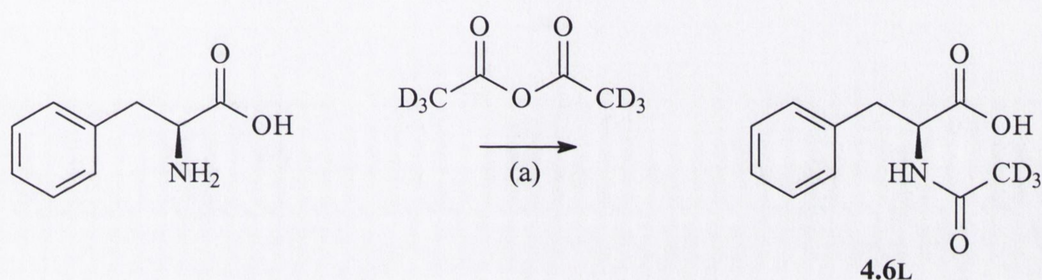
To complement the colour-tagged *N*-Boc-protected amino acids, isotopically differentiated *N*-labelled amino acids were prepared to give "pseudo-enantiomeric" guest, differentiable by mass spectrometry. As described in **Section 3.4,** it was decided to use *N*-(deuterio-labelled) phenylalanine for this work. A lot of related work, including relevant solution phase extractions, was performed previously in this laboratory on *N*-Ac-amino acids. Furthermore, the acetyl group is one of the smallest *N*-protecting groups and has relatively few hydrogen atoms. The fewer the deuterium atoms needed to replace hydrogen atoms, the cheaper the transformation. It was decided, therefore, to develop the isotopically differentiated enantiomer extraction method using isotopically differentiated *N*-Ac-DL-Phe. The L-enantiomer was chosen to be protected with the isotopically labelled protecting group as it was less expensive than the D-enantiomer.

4.3.1 Synthesis of *N*-Ac-d_3-L-Phe 4.6L[a]

N-Ac-d_3-L-Phe was synthesised in a 2-phase reaction between (acetic anhyride)-d_6 and L-Phe. 1.3 equivalents of (acetic anhydride)-d_6 compared with 1 equivalent of L -Phe gave the desired product in 83 % yield (**Scheme 4.4**). Reference to **4.6L** was not found in the literature but, for information purposes, the molecule was compared and contrasted with commercially available *N*-Ac-L-Phe from the Aldrich company.[7] Both molecules were found to have an R_f of 0.64, by tlc, when eluted in a solution of $CHCl_3$/MeOH/AcOH 10:2:1 this being the specification for the commercially available *N*-Ac-L-Phe. Nmr spectra of commercially available *N*-Ac-L-Phe compared favourably with those of **4.6L**, with the exception of a singlet at 1.8 ppm for *N*-Ac-L-Phe in the ^1H spectrum, which was absent for **4.6L**, as expected for a molecule bearing a CD_3 group rather than a CH_3 group. N-Ac-L-Phe has a melting point of 171-173 °C. A melting point of 172-174 °C was recorded for a sample of **4.6L**. A specific rotation value of +

[a] Although not enantiomers, *N*-Ac-d_3-L-Phe and *N*-Ac-D-Phe are referred to as **4.6L** and **4.6D**, respectively, herein. Similarly, "pseudoracemates" *N*-Boc-d_9-L-Phe and *N*-Boc-D-Phe are referred to as **4.7L** and **4.7D** respectively.

25 was recorded for the material but the opposite enantiomer, against which this value could be compared was not prepared and nor was reference thereto found in the literature. The elemental analysis of *N*-Ac-d_3-L-Phe gave acceptable results for both carbon and nitrogen content. Hydrogen content is calculated from the thermal conductivity of the water produced during combustion of the sample. As the thermal conductivity of the D_2O, or HDO produced from the deuterated material is less than that of H_2O, such material would not be expected to provide a satisfactory hydrogen content micro-analysis, and this was found to be the case for *N*-Ac-d_3-L-Phe, which appeared to have a hydrogen content of 5.42 %, compared with a calculated hydrogen content of 4.79 %.



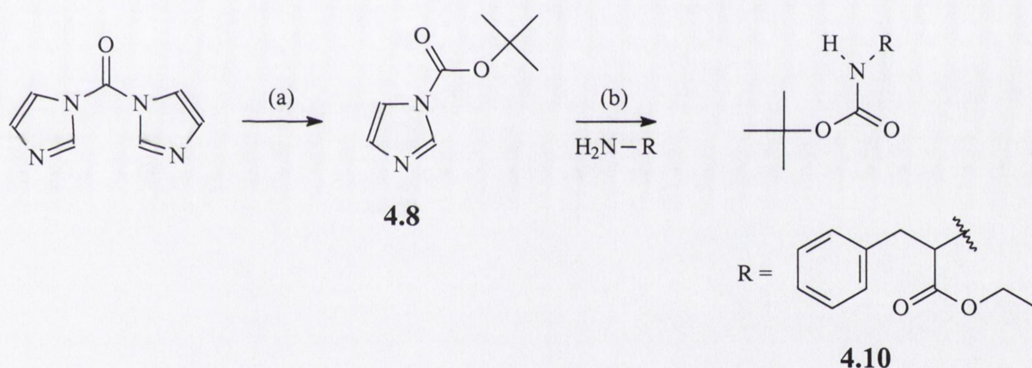
Scheme 4.4 (*Reagents and yield*) (a) aq. $NaHCO_3$ (10 % w/v), THF; 80 %.

4.3.2 Synthesis of *N*-Boc-d_9-L-Phe 4.7L

The solid-liquid extraction method was developed using "pseudoracemic" {*N*-Ac-d_3-L-Phe/*N*-Ac-D-Phe} (**Section 5.2**). The colour assay was designed, however, to use dye-tagged *N*-Boc protected amino acids. To complement the colour assay more fully, isotopically differentiated *N*-Boc-Phe "pseudoenantiomers", in which the L-enantiomer was *N*-(deuterio-Boc)-protected to give *N*-Boc-d_9-L-Phe **4.7L,** were prepared. Although *N*-Boc reagents are readily available, it was necessary to incorporate deuterium into a reagent that could *N*-(deuterio-Boc)-protect an amine. When the *N*-Boc group became a popular protecting group in organic synthesis, a number of reagents that could *N*-Boc-protect an amine were described in the research literature. Commercially available

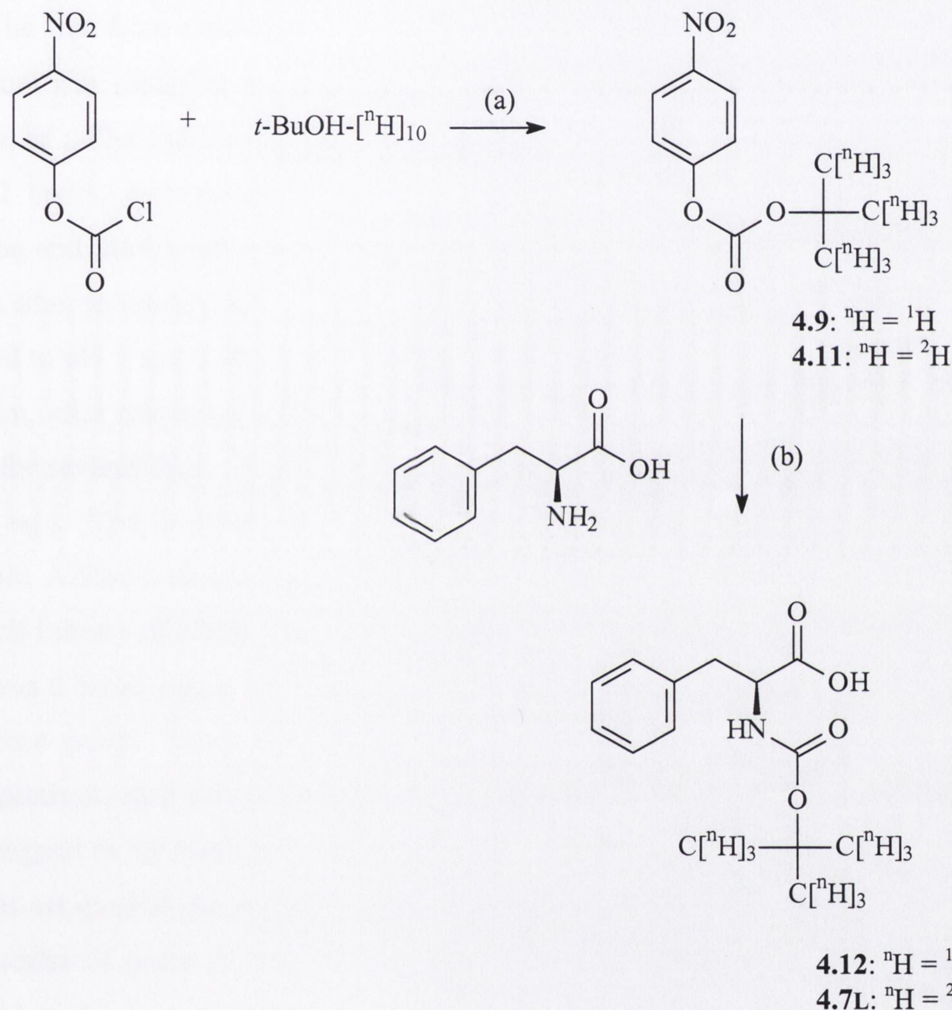
reagents, including Boc_2O, are used routinely for this purpose today. Alternative examples from the literature include tri-phosgene[8], 1-(*t*-butyloxycarbonyl) imidazole **4.8**[9,10] and *t*-butyl *p*-nitrophenyl chloroformate **4.9**.[11]

Reaction of commercially available CDI with *t*-BuOH should give 1-(*t*-butyloxycarbonyl) imidazole **4.8**, as shown in **Scheme 4.5**. This reagent should react with L-Phe to give the *N*-Boc-protected amino acid. Replacement of *t*-BuOH with (*t*-BuOH)-d_{10}, and subsequent reaction with L-Phe, should give the desired product **4.7L**. Indeed, **4.8** was obtained from reaction of CDI with *t*-BuOH in the presence of DBU in DCM at about 0 °C. The product was purified by flash column chromatography to give **4.8** with a yield of 42 %. When L-Phe ethyl ester hydrochloride (1 eq.) was treated with **4.8** (1.1 eq.) and aqueous $NaHCO_3$ (10 % w/v) in THF, *N*-Boc-protected amine, **4.10** was recovered in 23 % yield, thereby demonstrating that **4.8** was an appropriate reagent for the *N*-Boc protection of amino acid derivatives.



Scheme 4.5 (a) *t*-BuOH, DBU, DCM; 0 °C, 42 % (b) aq. $NaHCO_3$ (10 % w/v),THF, reflux 35 °C

t-Butyl *p*-nitrophenyl carbonate **4.9** was described as an efficient *N*-Boc-reagent by Anderson in 1957.[11] The material can be obtained from *p*-nitrophenyl chloroformate which, in turn, can be derived from phosgene or commercially acquired. It was anticipated that, if an efficient reagent for the *N*-Boc protection of amino acids could be obtained from reaction of *p*-nitrophenyl chloroformate with *t*-BuOH, that the deuterated analogue **4.11** should yield the desired product **4.7L** (**Scheme 4.5**).



Scheme 4.5 (Reagents, conditions and yields) (a) pyridine, DCM; 67 % (b) *t*-BuOH, Na_2CO_3, H_2O, reflux, 30 °C; 62 %.

A literature procedure, involving reaction of *p*-nitrophenyl chloroformate (2 eq.) with *t*-BuOH (1 eq.) in the presence of pyridine (2.5 eq.) in DCM, was followed to yield the desired product **4.9** as a white solid.[11] Following purification by flash column

chromatography, a yield of 65 % was recorded for the white solid, which had a melting point of 78 - 80° C, compared with the reported value of 79 - 80 °C.[11] The yield compared well with the literature yield of 72 %.

Following literature procedure, **4.9** (1.5 eq.), L-Phe (1 eq.) and Na_2CO_3 (2.5 eq.) were stirred together in a 2-phase solution of *t*-BuOH and H_2O.[11] The resulting yellow mixture was heated gently to 30 °C until all of the solids dissolved, and maintained at this temperature while the reaction was monitored by tlc. It was anticipated that L-Phe would be free from racemisation at this temperature and under these conditions. The procedure was modified so that **4.9** was dissolved in *t*-BuOH at 30 °C and added to a solution of L-Phe and Na_2CO_3 in H_2O and the yellow, 2-phase solution stirred at 30 °C. After 2 hours, *p*-nitrophenolate dihydrate, which had precipitated, was removed by filtration and the filtrate was adjusted to pH 6 with HCl (0.5 N), before extraction into diethyl ether to remove any remaining **4.9** and *p*-nitrophenol. The aqueous portion was adjusted to pH 1 and **4.12** was extracted into diethyl ether. The product persisted as an oily film, after removing volatiles as azeotropes with toluene and DCM and drying *in vacuo* for several days. A yield of 53 % was obtained using **4.9** (1.5 eq.) relative to L-Phe (1 eq.). The ^{1}H NMR spectrum of **4.12** was compared with that of commercially available *N*-Boc-L-phenylalanine and the spectrum of *N*-Boc-phenylalanine in the "Aldrich Library of NMR Spectra".[12] The spectra compared favourably. In both cases, there was a broad signal at 3.2 ppm, integrating to 2 H, which may be assigned to the methylene group. There were a pair of broad peaks at about 4.6 ppm and 4.9 ppm in each spectrum, each integrating to 1 H. The peak at 4.6 ppm was a broad quartet and was assigned to the methine proton. The further downfield group appeared as a doublet and was assigned to the amino proton. Finally, a singlet at 1.4 ppm, integrating to 9 H, and a series of peaks at about 7.3 ppm, integrating to 5 H, in each spectrum may be assigned to the *t*-butyl and aromatic protons, respectively. An $[\alpha]_D$ value of +25 was recorded for this material, which correlates with the value of +25 quoted for commercially available *N*-Boc-L-Phe.[13]

The procedure was repeated, replacing *t*-BuOH with (*t*-BuOH)-d_{10}, as illustrated in **Scheme 4.5**. The recovered white solid **4.11,** although not recrystallised, was found to have a similar melting point to the non-deuterated analogue **4.9**. The ^{1}H NMR spectrum of **4.11** was compared and contrasted with that of **4.9**. Both spectra gave 2

doublets in the aromatic region, as expected for the *p*-nitrophenyl aromatic protons, with one doublet (8.3 ppm) further downfield than the other (7.4 ppm), due to the influence of the electron-withdrawing nitro group on adjacent protons. No resonances were observed in the up-field region of the spectrum of **4.11**, compared with the spectrum of **4.9** which had a single peak at $\delta = 1.4$ ppm, which integrated to 9 H and corresponded to the $C(CH_3)_3$ resonance. The $C(CD_3)_3$ group of **4.11**, on the other hand, should not have been, and was not, observed in the ^1H NMR spectrum. A yield of 67 % was recorded for **4.11**.

As for **4.9**, **4.11** was reacted with L-Phe in a 2-phase reaction. The product **4.7L** was obtained as an oily film. The recovered product was compared with **4.12**. The ^1H NMR spectra compared well in the aromatic region. In the up-field region, a multiplet, integrating to 2 H was observed at 3.20 ppm and 2 broad signals at 4.62 ppm and 4.97 ppm, both integrating to 1 H and assignable to the methine and amino protons respectively, were observed, which compared favourably with the same region of the spectrum of **4.12**. The spectrum of **4.12** exhibited a single peak at ~ 1.4 ppm which integrated to 9 H and corresponded to the $C(CH_3)_3$ resonance. As *t*-BuOH was used as solvent in this reaction, the corresponding area of the spectrum of **4.7L** was examined carefully. A peak was observed at 1.4 ppm, which had an integration of ~ 4 H. When the product was dried *in vacuo* this peak was no longer apparent, indicating that it had been a solvent peak (*t*-BuOH). The yield (62 %) was improved compared with the analogous reaction, but reduced compared with the literature yield for *N*-Boc-L-Phe (72 %). Because of the cost of incorporating deuterium, and as only small quantities of material were required, the reported yield was thought to be adequate. As the product remained as an oily film after drying *in vacuo* for several days, a microanalytical assay of this material was not obtained. The $[\alpha]_D$ value was +18 (c = 0.1 in ethanol).

4.4 Tetraalkylammonium salts of 2.6L, 2.6D, 2.5L, 4.6L, 4.6D, 4.7L and 4.7D.

The coloured and deuterated substrates were employed in on-bead testing as their tetraalkylammonium salts. Using the method of Dresen, tetrabutylammonium salts were prepared by vigorous stirring of a 2-phase reaction of the derivatised amino acid in the aqueous phase and tetrabutylammonium chloride in chloroform.[1.27]

Tetraethylammonium salts were obtained by the method of Dresen, by titrating the derivatised amino acid (**4.1**) against an aqueous solution of TEAOH (3.5% w/v) until a pH of 7.2 was reached.[1.27] In the case of the more lipophilic N-Boc-protected amino acid, the method of stirring **4.7L** with TEACl in a 2-phase reaction was employed. In general, oils were isolated, except for coloured substrates, the tetralkylammonium salts of which were recovered as solids. NMR data of the salts were obtained.

4.5 Conclusion

The dye-tagged amino acids, *N*-Boc-D-Tyr (O-Disperse Red 1) **2.6D**, and both *N*-Boc-L-Tyr (O-Disperse Red 1) and *N*-Boc-L-Tyr (O-Disperse Blue 3) **2.6L** and **2.5L**, respectively, were synthesised. The chiral HPLC spectrum of **2.6L**, combined with the comparison of its specific rotation with that of **2.6D**, indicates that this material is about 20% impure. **Chapter 2** proposed the use of these materials in on-bead screenings for enantioselectivity of split-and-mix libraries. The purity of these molecules is important in the application of such an assay, and this is discussed in **Chapter 6**.

The isotopically labelled *N*-protected amino acids, *N*-Ac-d_3-L-Phe **4.6L** and *N*-Boc-d_9-L-Phe **4.7L** were also synthesised. These materials may be coupled with *N*-Ac-D-Phe and *N*-Boc-D-Phe, respectively, to give pairs of discretely labelled "pseudoenantiomers", which are differentiable by mass spectrometry and have potential use in measuring the selectivity of on-bead receptors *via* an on-bead screening assay.

CHAPTER 5

5.1 Preamble

Common methods for the determination of enantioselectivity include extraction/NMR, extraction/polarimetry, HPLC and UV experiments. A combinatorial library of bead-bound receptors, if it is to be screened on the solid phase, requires an alternative method to determine selectivity. A single solid phase bead has ~ 100 pmol of active sites. Therefore, determination of the selectivity of a receptor bound to such a solid phase bead requires a very sensitive technique. Mass spectrometry is such a technique but does not differentiate between enantiomers as they have no mass difference. The discrete labelling of enantiomers by incorporation of a few deuterium atoms into the protecting group of one enantiomer gives rise to separate peaks for each enantiomer by mass spectrometry, however. The synthesis of such pairs of "pseudoenantiomers" was described in **Chapter 4**.

Solution phase extractions between receptor and racemic guest are a routine method of enantioselectivity determination. The development of a viable solid-liquid phase extraction method to determine enantioselectivity is described in this chapter. A receptor of known structure was bound to the solid phase. Conditions were developed under which thermodynamic equilibration was observed between the solid-phase bound receptor and coloured guest. Using the same conditions, the solid-phase bound receptor was exposed to "pseudo-racemic" guest. Bound "pseudo-racemic" guest was cleaved and analysed by electrospray mass spectrometry to give a measure of the solid-phase bound receptor's selectivity.

5.2 Extraction Methods

Although enantioselectivity determination *via* solution phase extractions is well developed in this laboratory, the concept of using a solid phase-bound receptor with a solution phase guest needed exploration. In particular, the use of isotopically labelled guest to be determined by mass spectrometry was novel. Receptor **5.1** was synthesised and attached to polystyrene-based Wang® resin solid phase beads by Barry.[1] **5.1**, with a

phenyl urea in the 3-α position and phenyl carbamates in the 7-α and 12-α positions, is an analogue of **1.22**. **1.22** was found to extract TBA *N*-Ac-DL-valinate with an L/D ratio of ~ 4:1 (**Section 1.5**). Barry synthesised receptor **5.2** by coupling **5.1** to methoxy propylamine. **5.2** was found to extract TBA *N*-Ac-DL-valinate with 57 % e.e., which corresponds to a ratio of 4:1.

5.1 **5.2** Ar = —C₆H₄—CF_3

5.1 was coupled to Wang® resin (100 μM), by Barry[1], to give **5.3** (**Scheme 5.1**). The uniform modified beads were used to investigate solid-liquid extractions of isotopically labelled guest, TBA {*N*-Ac d_3-L-Phe/*N*-Ac-D-Phe}.

5.3 Ar = —C₆H₄—CF_3

Scheme 5.1[1]: (*Reagents and conditions*) (a) pyridine, DDC, DCM, DMAP. After shaking for 24 hrs beads were transferred to filter fitted with frit. Washings and filtrations performed with DMF x 2, MeOH, DCM, MeOH/DCM 50:50.

5.2.1 Conditions for the solid-liquid extraction method

To develop conditions in which reversible binding could take place between **5.3** and guest, the properties of the solid support were considered. A typical polystyrene synthesis bead has a loading of 1 mmol/g. 2×10^6 polystyrene beads weigh ~ 1 g. Each bead therefore contains 500 pmol active sites. If all of these sites exhibit receptor, each bead contains 500 pmol of receptor. If an experiment uses 1000 beads, 500 nmol of receptor is present. This is equivalent to 0.5 mL of a 1 mM solution. Allowing for a 10-fold excess of guest, 5 mL of 1 mM guest should be used for an experiment on 1000 beads and 5 μL of 1 mM guest should be allowed per single bead. These calculations were used in the work herein.

A notable feature of polystyrene beads is that they can swell up to 8 times their volume in solvents such as chloroform, in which case the concentration of active sites becomes reduced from 1 M to 0.125 M. This is important when performing on-bead assays as receptor sites become isolated, thereby increasing the ability *per* site to interact with guest. This feature is also attractive to the analyst as the size increase becomes useful during manipulation of the microscopic entities. When swollen to a volume of 8 mg/L, a bead can contain as much as $8/(2 \times 10^{-6})$ mL = 4×10^{-6} mL solvent. A bead which is exposed to substrate at 1 mM concentration, therefore, contains 4 pmol of dissolved species. For a bead-bound receptor, which is binding quantitatively at 1 mM substrate, the amount of free substrate compared with bound substrate will be less than 1 %. A low concentration of background substrate is important when determining the quantity of bead-bound substrate. A proportion of background guest less than 1 % is thought to be adequately low not to impact on evaluation of bound substrate.

Although the ability of **5.3** to bind TBA {*N*-Ac d_3-L-Phe/*N*-Ac-D-Phe (1:1)} (TBA **4.6DL**) selectively was ultimately of interest, TBA **2.6L** was used to find suitable conditions under which **5.3** would bind guest. As TBA **2.6L** is highly coloured, activity with the solid phase, such as complex formation, decomplexation and complexation times, could simply be observed by eye, and noted. In **Chapter 4**, **2.6L** was deemed to be about 20 % impure. The purpose of these experiments is to ascertain under what conditions **5.3** binds chiral guest. The enantiopurity of that guest is not of concern at this stage. Indeed, the experiments described in **Section 5.2.3** involve subjecting **5.3** to

different ratios of "pseudo-enantiomers" rather than enantio-pure material, to investigate the solid phase-bound receptor's ability to select one enantiomer over another.

When **5.3** (100 beads) was treated with TBA **2.6L** (1 mM in $CHCl_3$; 0.5 mL), all beads acquired a uniform bright red colour. The experiment (**Expt 5.1**) was performed in a small petri dish of 2 cm diameter, which was placed within a jar, the bottom of which was covered with $CHCl_3$, and the lid replaced tightly to ensure that the system was in an atmosphere of solvent. After 4 hours, the beads were transferred to a filtration tube fitted with a frit and filtered to remove excess guest. A wash was performed by shaking the beads in $CHCl_3$ (0.5 mL) within the filtration tube, which was sealed at both ends with rubber septa. The liquid was subsequently filtered, to give a slightly red coloured filtrate. Further washing with $CHCl_3$ was found not to cleave any dyed substrate. When left overnight in $CHCl_3$, the beads remained bright red and the solvent colourless, indicating that the guest had been irreversibly bound in this solvent system. It appeared that irreversible binding was achieved in $CHCl_3$. As thermodynamic, rather than kinetic, control is required for this study, it was necessary to change the solvent to a more competitive alternative. When the complexed beads were exposed to 1 % MeOH/$CHCl_3$, coloured guest was decomplexed after about 1 hr. Unlike using 100% $CHCl_3$, the employment of MeOH (1 %) in the solution phase was sufficient to compete with guest for binding sites, thereby promoting reversible binding, as desired. The binding experiment was repeated using 1 % MeOH/$CHCl_3$ as solvent (**Expt 5.2**). When 100 beads of type **5.3** were exposed to TBA **2.6L** (1 mM) in 1 % MeOH/$CHCl_3$, all beads acquired a uniform red colour within 35 minutes, as shown in **Figure 5.1**. When no further change was observed in the appearance of the system after a further 25 minutes, the beads were filtered and exposed to 1 % MeOH/$CHCl_3$ (0.5mL). After about one hour, guest was observed to have decomplexed by exposure to this solvent system. As guest was bound and decomplexed in 1 % MeOH/$CHCl_3$, it appeared that thermodynamic equilibrium was occuring in the presence of 1 % MeOH. Although 1 % MeOH/$CHCl_3$ was found to de-complex guest with time, a decomplexing solution which would cleave complexed guest efficiently but leave receptor bead-bound, was sought. As MeOH had been discovered to compete efficiently for binding sites, the proportion thereof was increased to 50% in $CHCl_3$. The use of $CHCl_3$ was continued, to maintain swelling of the beads and consequent exposure of binding sites to the

competitive MeOH molecules. MeOH/ $CHCl_3$ (1:1; 0.5 μL per bead) was observed to cleave coloured guest immediately upon shaking, such that once the system was filtered, the beads appeared colourless and the supernatant phase red (**Expt 5.3**). When the beads were recycled and re-tested, they were found to exhibit similar behaviour as before, indicating that they were recycleable.

An equilibration time of 35 minutes was observed for **5.3** with TBA **2.6L** (1 mM in 1 % MeOH/$CHCl_3$; 5 μL per bead). No apparent change was observed when beads were inspected after a further 25 minutes. MeOH/$CHCl_3$ (1:1; 5 μL per bead) cleaved bound guest efficiently. DCM was found to exhibit very similar behaviour to $CHCl_3$ in this type of experiment (**Expt 5.4**). The percentage of MeOH in DCM was varied from 1 % to 10 %, but decomplexing times of about 1 hour were recorded in each case (**Expt 5.5**). Due to $CHCl_3$ being a considerably less volatile solvent than DCM, the former was favoured as solvent for these experiments, in particular because use of volatile DCM led to desolvation of beads while the beads were being inspected under the microscope. The concentration of guest was also varied. 0.1 mM guest was applied to the beads and although all beads exhibited colour after equilibration, it was more an orangey colour than the preferable definite red hue exhibited by beads subsequent to exposure to 1 mM guest (**Expt 5.6**).

The conditions of 5 μL of 1 mM guest per bead, which were prompted by calculations, provided conditions of thermodynamic equilibration upon employment of a competitive solvent system of 1 % MeOH in $CHCl_3$. These conditions were employed for experiments between **5.3** and TBA **4.6** and between library **6.1** and guest.

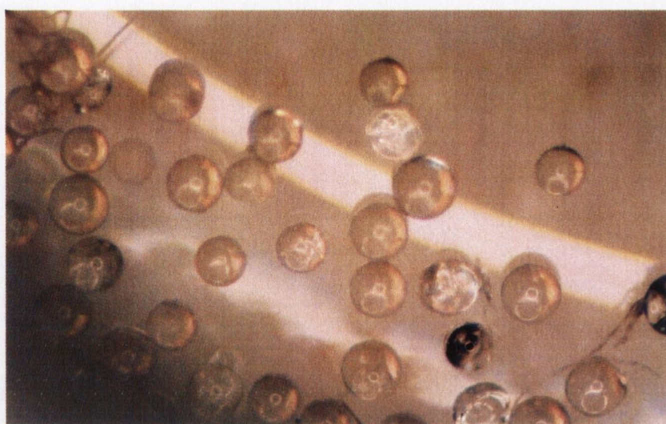


Figure 5.1 a

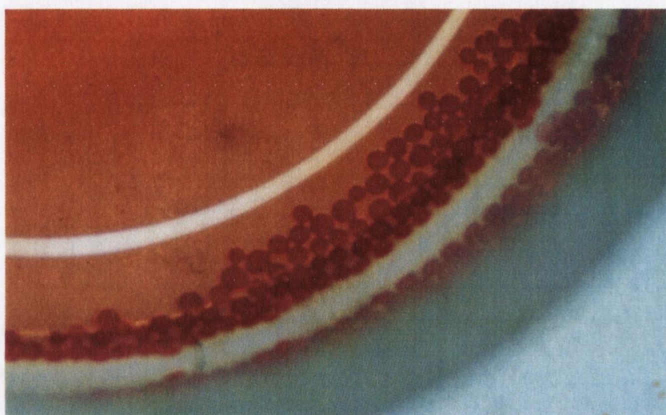


Figure 5.1 b

Figure 5.1 c

Figure 5.1 (**a**) Solid phase-bound receptor **5.3**; (**b**) **5.3** after equilibration with TBA **2.6L** (1 mM; 5 μl per bead); (**c**) **5.3** after equilibration with TBA **2.6L** and filtration of excess guest.

5.2.2 Preparation of the guest

Previously in this laboratory, solution phase extractions between neutral receptors, in particular **1.22** and **5.2**, and guest, used the guest as its lipophilic TBA salt. The TBA salts of **4.6L** and commercially available **4.6D** were synthesised separately by vigorous stirring of each amino acid with TBACl in 2-phase solutions of $CHCl_3/H_2O$ (6:1). H_2O was removed from each salt as an azeotrope with toluene, which was subsequently removed as an azeotrope with DCM. The salts were dried *in vacuo* for several hours but both remained as viscous oils. It was attempted to prepare an equimolar solution of the pair of "pseudoenantiomeric" salts using standard solutions of the individual TBA salts. The resulting solution was analysed for equimolarity by mass spectrometry and LC/MS but the peaks corresponding to the pair of phenylalanate ions were not of equal height. It was thought that the hygroscopic salts had too high a water content, despite drying *in vacuo*, to allow preparation of an equimolar mixture from the two separate standard solutions. It was found, however, that the two standard solutions could be mixed to give a sample with equal peak heights for the "pseudoracemate" by both mass spectrometry and LC/MS. As the actual concentrations of the initial solutions remained undetermined however, an alternative preparation was desired.

Both **4.6L** and commercially available **4.6D** were dried *in vacuo* for several hours. An equimolar quantity of each white powder was mixed together and both a mass spectrum and a ^1H NMR spectrum were obtained of the resulting mixture. A pair of peaks of equal height, with a mass difference of 3 amu, was observed in the mass spectrum (**Expt 5.7**). A ^1H NMR spectrum of the mixture exhibited a methyl peak at δ =1.8 ppm which integrated to 1.5 H compared with an integration of 5 H for the aromatic protons, as expected for **4.6DL**. The TBA salt of this mixture was then prepared by stirring **4.6DL** with TBACl in a two-phase solution of $CHCl_3/H_2O$ (6:1). H_2O was removed as an azeotrope with toluene, which was subsequently removed as an azeotrope with DCM, and the salt dried *in vacuo*. Mass spectrometric analysis of this salt indicated a pair of peaks of equal height corresponding to the pseudoracemic ions, as shown in **Appendix A2**, while ^1H NMR showed the expected integrations of 1.5 H and 5 H for the acetyl methyl and aromatic peaks, respectively.

Direct injection electrospray ionisation mass spectrometry was favoured over LC/MS for analysis of guest in these experiments for a number of reasons. First of all, the guests could be observed in positive or negative mode using direct injection ESIMS and, as carboxylate ions were of interest, it was preferable to use negative mode whereby the "pseudoracemic" carboxylate ions were observed at 206 and 209 m/z. Trifluoroacetic acid was routinely used in the mobile phase of the only available LC/MS spectrometer and this led to reduced signal for the desired peaks in negative mode. The carboxylates could be observed more easily in the positive mode, wherein they were protonated to give a pair of peaks at 208 and 211 m/z. These peaks were usually accompanied by a pair of peaks at 230 and 233 m/z corresponding to the desired guest bound to sodium and a third pair at 249 and 252 m/z corresponding to the desired guest bound to acetonitrile. Usually, the latter pair was the most prominent in a spectrum of the equimolar "pseudoracemate". The peak ratio for this pair, however, was not found to relate directly to the peak ratio for the pair at 208 and 211m/z (**Appendix A1**). Secondly, the lipophilic TBA counterion was retained strongly on the LC column. A water-acetonitrile gradient, in which the TBA cation did not elute well, was used routinely as mobile phase in the LC/MS system. The corresponding peak, at 243 m/z in positive mode, was easily observed but, as it was retained on the column, was observed also in other users' spectra. H_2O/acetonitrile (1:1) was used as mobile phase in direct injection ESIMS but there was no LC column present in the system on which the TBA cation could elute. Furthermore, LC/MS has relatively long run times. A standard run takes 25 minutes compared with less than 5 minutes for a direct injection ESIMS run. Finally, the sensitivity of LC/MS is reduced compared with direct injection ESIMS. Samples are usually prepared as 1 mg/mL for LC/MS to ensure that enough of the sample reaches the mass spectrometer, for analysis, after elution on the HPLC column. A concentration of 1 pg/μL is ample for the ESIMS spectrometer. Sensitivity was important for this work as information was collected from a single bead (~ 20 ng guest per bead). Although a very useful technique for analysis of mixtures, LC/MS is less suitable for down-scaling of known samples.

While developing the method, and before use of the electrospray mass spectrometer became available, the TEA salt of **4.6DL** was prepared. The TEA cation, which is much less lipophilic than the TBA cation, was eluted efficiently in the mobile

phase of the LC/MS, making it the more favourable candidate for this analytical technique. The TEA salt of an equimolar solution of **4.10L** and **4.10D** was also prepared and was found to give a pair of peaks of equal height at 266 and 275 in LC/MS$^+$.

Another area of on-going research in this laboratory is the analysis of solution phase extractions of isotopically labelled guest by MS or LC/MS. As a validation test, Hurley performed a series of experiments wherein the % of **4.6L** in a mixture was varied relative to a standard quantity of **4.6D**.[1.28] It was hoped that the L/D ratio in each case could be determined by mass spectrometry. A mass spectrum was obtained of each mixture and remarkable consistency was observed between the theoretical and observed results over a range of values. This was an important result as it showed that mass spectrometry could be used to measure different ratios of "pseudoenantiomers" and, in turn, selectivity.

5.2.3 The solid-liquid extraction

A solution of TBA **4.6DL** was prepared in 1 % MeOH/$CHCl_3$ such that the solution was 0.5 mM in each guest and 1 mM overall (**Appendix A2**). Solid phase receptor **5.3** was placed in a small pot (roughly 100 beads were counted under the microscope), to which the guest (1 mM; 0.5 mL; 10-fold excess) was added (**Expt 5.8**). The pot was placed within a jar, the bottom of which was filled with 1 % MeOH/$CHCl_3$, and the lid tightly replaced. The system was left for 4 hrs after which time the beads were transferred to a filter fitted with a frit. Excess guest was removed and the beads were washed with $CHCl_3$ (0.5 mL). Washing was performed within the filter, which was sealed at each end with a rubber septum, by shaking for 30 sec. The filtrate was retained for mass spectrometric analysis and was found to contain a pair of intense peaks, corresponding to the "pseudoracemate". A second wash was performed ($CHCl_3$; 0.5 mL) and analysed. The "pseudoracemic" peaks were observed again, but at considerably reduced intensity. Mass spectrometric analysis of a third wash indicated that excess guest had been effectively removed in the first two washes as virtually no racemate was observed in the spectrum. Only baseline peaks were exhibited at the appropriate m/z for the "pseudoracemate".

The beads were then shaken in decomplexing solution MeOH/$CHCl_3$ (1:1; 0.5mL) for 3 minutes. The solution was filtered and diluted for mass spectrometric analysis (**Appendix A3**). A pair of peaks was observed at 206 and 209 m/z, as expected, but a ratio of 1:2 was exhibited for the lighter carboxylate ion of **4.6D** to the heavier carboxylate ion of **4.6L**, (**Figure 5.2**). The selectivity of L over D is consistent in direction with solution phase findings for **1.22** and the other receptors described in **Section 1.5**. The degree of selectivity is reduced however, compared with that of **1.22** and of **5.2**, both of which gave enantioselectivities of 4:1 with TBA *N*-Ac-DL-valinate. There is no solution phase result for an extraction between **5.2** and TBA *N*-Ac-DL-phenylalaninate, although Hurley found that **1.22** extracted *N*-Ac-DL-Phe with an L/D ratio of 3:1 by chiral HPLC and 4:1 by ^{1}H NMR studies.

This experiment was repeated several times to give the same ratio of 2:1 L/D on every occasion.

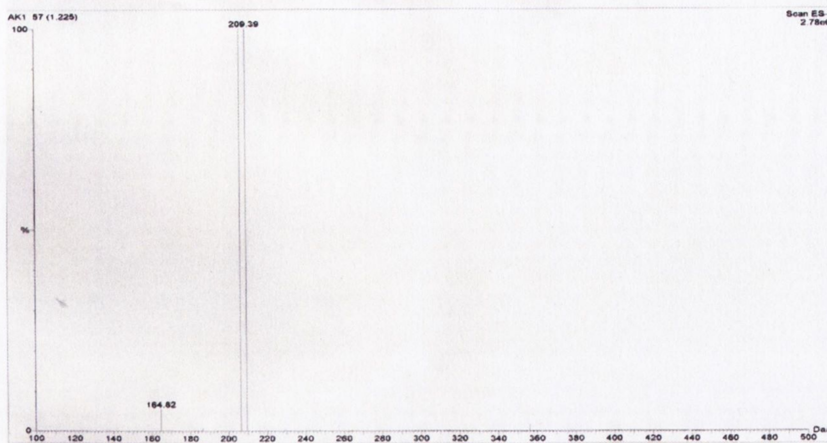


Figure 5.2 a

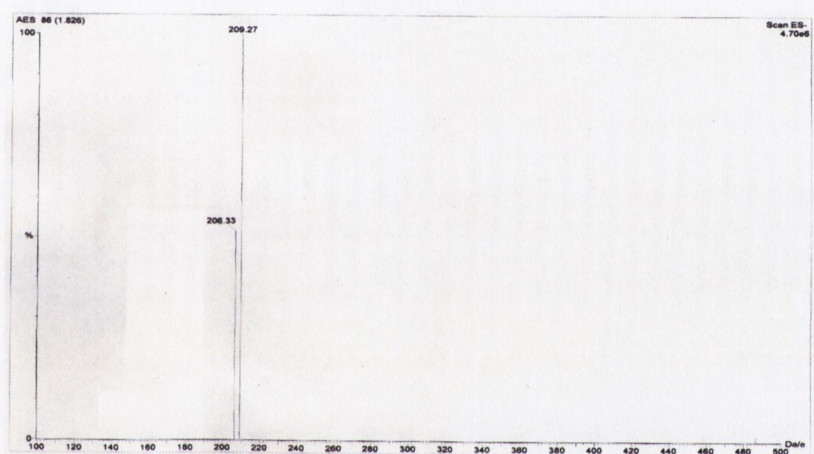


Figure 5.2 b

Figure 5.2: (**a**) TBA **4.6DL**; (**b**) **4.6D/4.6L** decomplexed from solid phase receptor **5.3**.

After decomplexation, the beads were treated with decomplexing solution a second time to investigate whether any guest had remained bound on the first occasion. A mass spectrum of this solution showed only a trace of guest at baseline intensity. The first decomplexation appears to cleave virtually all guest bound by the bead-bound receptor.

Two portions, each of 10 beads, were removed from a 100-bead batch when the experiment was repeated (**Expt 5.9**). The first portion was placed in a filtration tube, washed and decomplexed, as before, but this time the experiment was performed on a scale 1/10 of that of the original experiment which had been performed on 100 beads.

The second batch was placed in a melting point capillary tube which was sealed at one end. Washings and decomplexation were performed by syringe of solvent (5 μL per bead) into the capillary. This was performed under a low-power microscope. The capillary was inverted 20 times to "shake" the beads and the solution was removed by syringe under a low-power microscope to ensure that the clear, colourless beads remained within the capillary. Both portions of 10 beads gave an L/D ratio of 2:1. This was an important result as it indicated that manipulations could be performed successfully within a capillary under the microscope and that the experiment could be scaled down 10-fold without significant loss of sensitivity. Ultimately, this procedure was to be performed on single solid phase beads.

5.2.4 The single bead experiment

The Wang® resin synthesis beads to which **5.1** was bound are 100 μm in diameter and are large enough to be seen by the naked eye. They can be picked up individually on a metal spatula and, with the aid of a low-power microscope, they are particularly easy to manipulate.

Calculations indicate that a typical synthesis bead contains about 100 pmol of active sites. Providing that all binding sites exhibit receptor, a single bead extraction should yield 100 pmol of guest in 5 μL of decomplexing solution. This solution must be made up to 20 μL to constitute a single ESIMS injection. Based on these figures, a 100 pmol solution of TBA **4.6DL** in MeOH/$CHCl_3$ (50/50; 20 μL) was prepared and analysed by $ESIMS^-$. A pair of peaks of equal height was observed at 206 and 209 m/z, corresponding to the "pseudoracemic" anions (**Appendix 20**). This indicated that the instrument was sensitive enough to register guest on the scale of a single polystyrene-based synthesis bead.

In order to perform a single bead experiment, a batch of 100 beads was equilibrated with guest, filtered and washed (**Expt 5.10**). Three individual beads were transferred to individual capillaries and treated with decomplexing solution (5 μL), by syringe, under the microscope. After inverting each capillary 20 times, each solution was removed by syringe and placed in an individual vial. A further addition of MeOH (15 μL) was added to each solution of guest to comprise a single ESIMS injection (20

μL). Mass spectrometric analysis indicated an L/D ratio of 2:1 for each single bead. A variation of this procedure, whereby single beads were placed in individual capillaries from the outset, to undergo the extraction individually, was also performed (**Expt 5.11**). An L/D ratio of 2:1 was observed for each single bead. The solid-liquid single bead extraction appears to be viable, feasible and reliable. Furthermore, using these particular synthesis beads and a low-power microscope, the extraction is surprisingly manageable and user-friendly.

As a batch of TEA **4.6DL** had been prepared, the extraction was performed between **5.3** and it on both a 100 bead and a single bead scale, as per **Expt 5.8**. An L/D ratio of 2:1 was observed again on both scales for this system (**Appendix A4**). The experiment was performed also between **5.3** and TEA **4.10DL**. The peak at 274m/z, corresponding to the heavier enantiomer **4.10L**, was observed in excess compared with **4.10D** at 265 m/z (**Figure 5.3**). The direction of selectivity was as expected but the L/D ratio was reduced to 1.7:1 (**Appendix A5**). Neither **5.1** nor any of the neutral receptors described in **Section 1.5** have been tested with *N*-Boc-protected amino acids. Lawless, however, discovered that the selectivity of certain charged receptors was reduced when *N*-Ac-amino acid guest was replaced with *N*-Boc amino acid guest.[1.20]

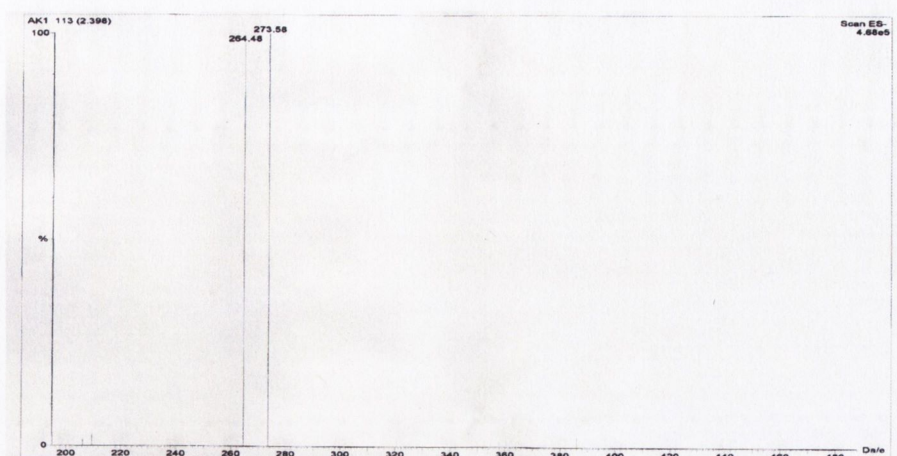


Figure 5.3 a

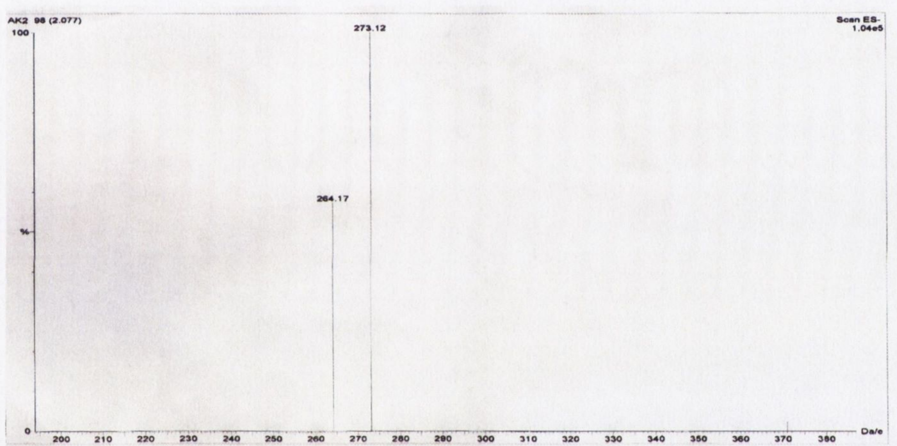


Figure 5.3 b

Figure 5.3 (**a**) Equimolar TEA **4.10DL** (**b**) **4.10D/4.10L** decomplexed from solid phase receptor **5.3**.

5.2.5 Validation of the selectivity of 5.3

5.3 was observed to give a consistent result in solid-liquid extractions with a solution of TBA **4.6DL**. In order to validate this result, a mixture of **5.3** was treated with some biased solutions of TBA **4.6L/4.6D**. As **5.3** is L-selective, solutions that were depleted in **4.6L** were prepared to investigate how well **5.3** could extract the under-represented,

yet apparently favoured, L-enantiomer. A solution of TBA {**4.6L/4.6D** (1:5)} was prepared, as described in **Expt 5.12,** and analysed by mass spectrometry (**Appendix A6**). An extraction was performed between **5.3** (100 beads) and guest TBA {**4.6L/4.6D** (1:5)} (1 mM in 1 % MeOH/$CHCl_3$; 0.5 mL), as per **Expt 5.8**. Decomplexed guest was analysed by $ESIMS^-$ (**Appendix A7**). A ratio of 2:5 L/D was observed for **5.3** with TBA {**4.6L/4.6D** (1:5)} (**Figure 5.4**). This is consistent with a receptor that extracts an equimolar guest with a ratio of 2:1 L/D. The same result was observed when the extraction was performed on a single bead.

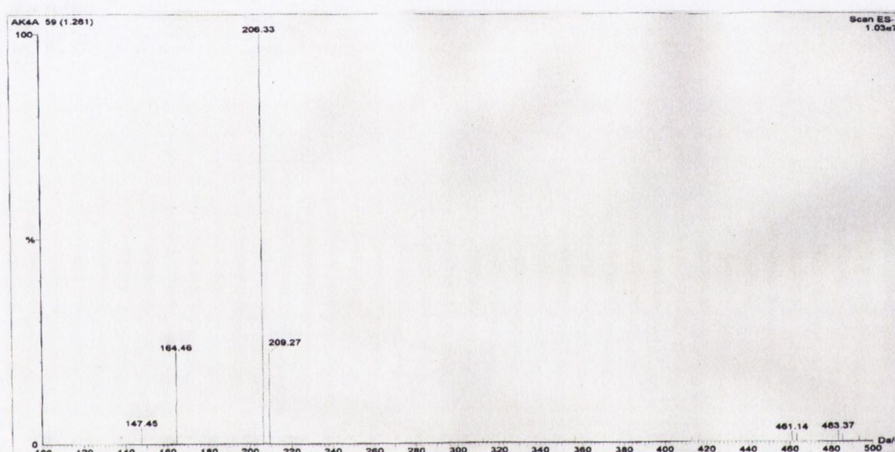


Figure 5.4 a

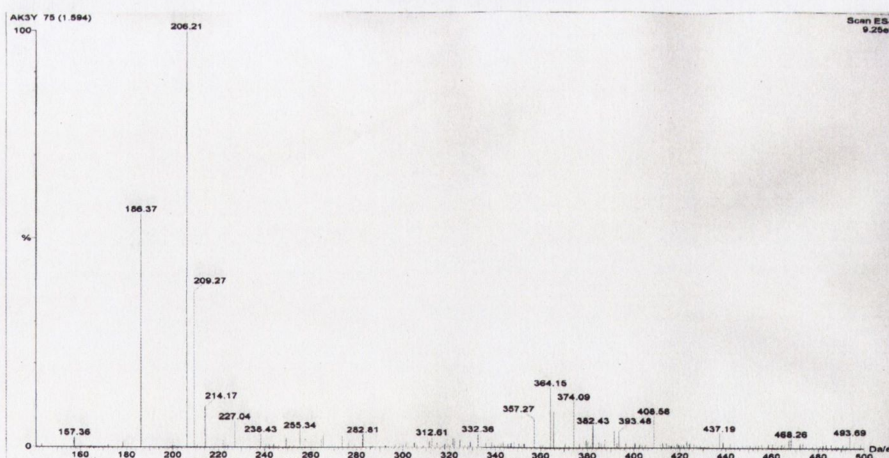


Figure 5.4 b

Figure 5.4 (**a**) TBA {**4.6L/4.6D**(1:5)}; (**b**) {**4.6L/4.6D**} decomplexed from a single bead of type **5.3**.

Finally, a solution of TBA {**4.6L/4.6D** (1:10)} was prepared, as described in **Expt 5.13** (**Appendix A8**). **5.3** was expected to extract the under-represented enantiomer in a ratio of 1:5 L/D. Indeed, this ratio was observed when extractions were performed as per **Expt 5.8** on both the 100-bead and single bead scale (**Figure 5.5**; **Appendix A9**).

Figure 5.5 a

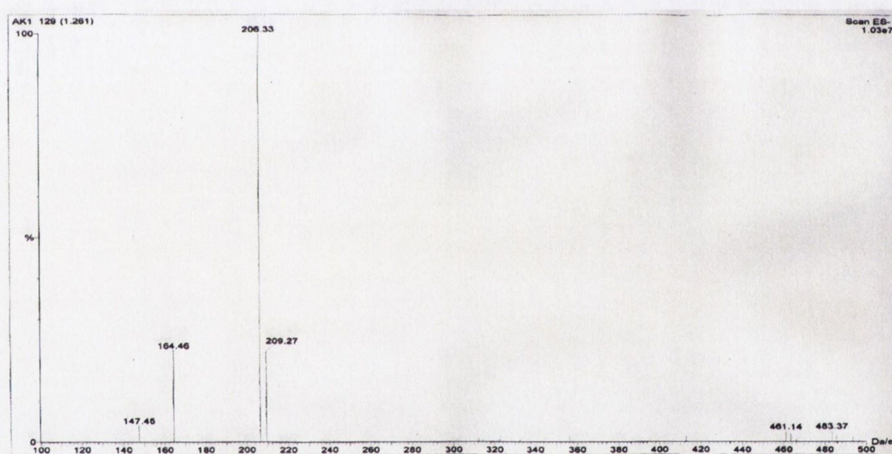


Figure 5.5 b

Figure 5.5 (**a**) TBA {**4.6L/4.6D** (1:10)}; (**b**) Decomplexed {**4.6L/4.6D**} from solid phase receptor **5.3**

A plot of % L/D versus enantioselectivity is shown in **Figure 5.6**. These results validate the observed selectivity of **5.3** by the single bead extraction method.

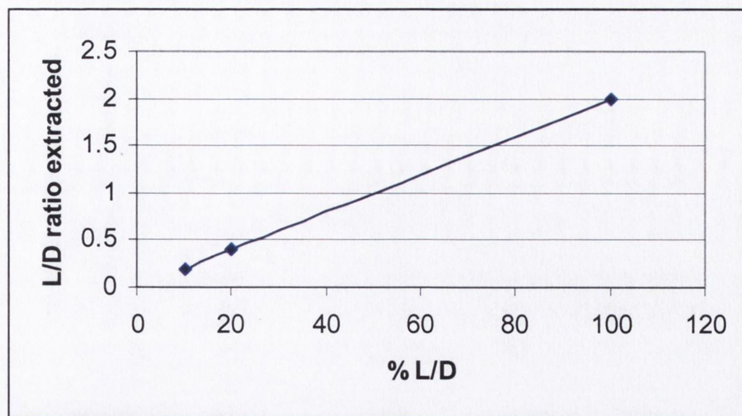


Figure 5.6: L/D ratio extracted by **5.3** versus % of **4.6L/4.6D** guest

5.3 Conclusion

A solid-liquid extraction, to determine the enantioselectivity of bead-bound receptors, was developed. Guest was isotopically labelled, by incorporation of deuterium into the *N*-protecting group of one enantiomer, so that enantioselectivity could be measured by mass spectrometry. Selectivity was verified by varying the % of L-enantiomer relative to D. The method was successfully applied to single beads.

CHAPTER 6

6.1 Preamble

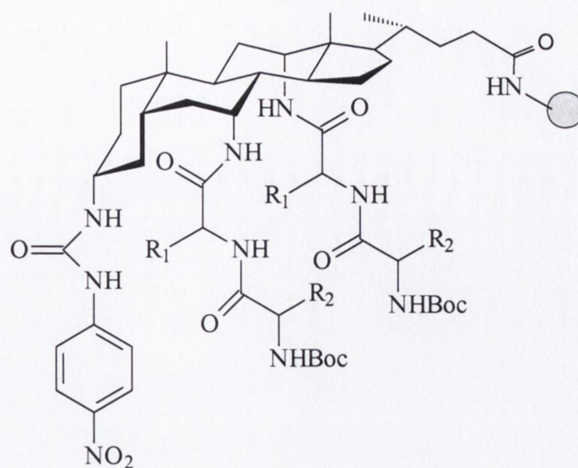
As all members of a one-bead one-compound combinatorial library are mixed intimately they must be tested together. This can be achieved by use of a colour screen wherein each enantiomer of guest is labelled a different colour. To this end, dye-tagged substrates **2.5L**, **2.6D** and **2.6L** were synthesised. A combinatorial library of receptors was screened against an equimolar solution of **2.5L** and **2.6D**. The concentration of guest employed ensured that each bead underwent some colour change, i.e. bound guest. The conditions used and colour changes observed are described in this chapter.

Although the dual-colour assay provides an indication of direction of selectivity, the extent of selectivity remains unknown, although it may be estimated by the observed hue of a bead. Library members with exceptional selectivity are desired. To sequence and re-synthesise all blue and red members of a sizeable library would be time-consuming and laborious. Instead, it was anticipated that the ESIMS assay described in **Chapter 5**, which provided a measure of the selectivity of **5.3** over a range of L/D guest ratios, may be used to obtain a semi-quantitative estimate of the selectivity of picked members. The application of this method to library members is described herein.

6.2 The dual-colour assay

6.2.1 The solid-phase steroidal library

The library used to develop the dual-colour assay was the dipeptide urea library **6.1** described in **Section 2.3**. This steroidal library was synthesised by split-and-mix synthesis on Tentagel® synthesis beads (1 g), by Riedner.[2.13] Each synthesis bead contains only one receptor on and within it. The twelve amino acids coupled to both the 7-α and 12-α positions are listed in **Table 6.1**. As there are 12 amino acids in each of two positions, **6.1** is a 144-member library.

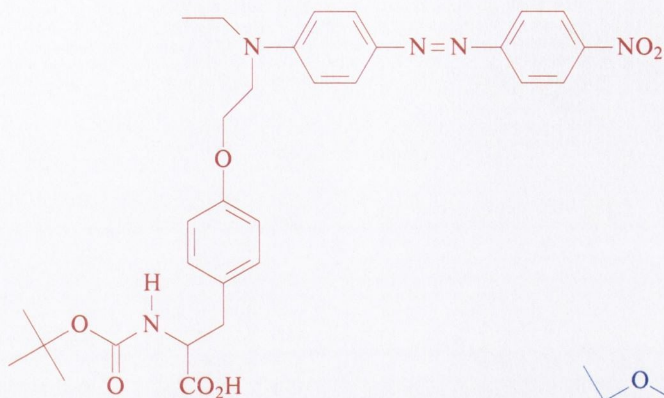


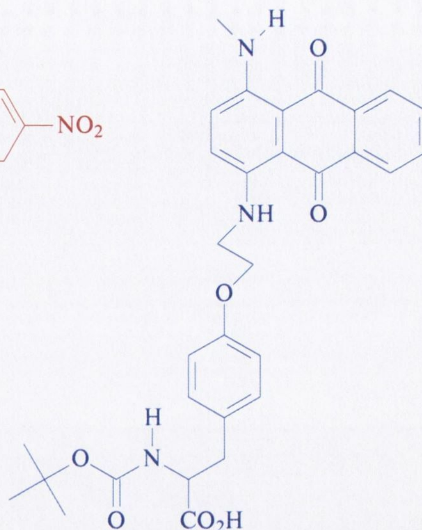
6.1

AMINO ACID 1 (AA_1)	AMINO ACID 2 (AA_2)
Ala-OH	Ala-OH
Glu(Ot-Bu)-OH	Glu(Ot-Bu)-OH
Gln-OH	Gln-OH
Gly-OH	Gly-OH
Leu-OH	Leu-OH
Lys(*N*HBoc)-OH	Lys(*N*HBoc)-OH
Met-OH	Met-OH
Phe-OH	Phe-OH
Pro-OH	Pro-OH
Ser(O*t*-Bu)-OH	Ser(O*t*-Bu)-OH
Trp-OH	Trp-OH
Val-OH	Val-OH

Table 6.1: AA_1 and AA_2 in Dipeptide library **6.1**

6.2.2 Preparation of the guest





2.6 **2.5**

The red salts TBA **2.6L** and TBA **2.6D** and the blue salt TBA **2.5L** were prepared by stirring each salt (1 eq.) with TBACl (1 eq.) and NaOH (1 eq.) in a 2-phase system of $CHCl_3/H_2O$. Each salt was extracted into the organic phase and dried *in vacuo*.

6.2.3 Development of the colour assay

It was anticipated that the conditions developed to perform the ESIMS assay between bead-bound receptor **5.3** and "pseudo-enantiomeric" guest **4.6DL** would also be suitable conditions under which to perform the colour assay between bead-bound library **6.1** and **coloured guest**. As **6.1** is a 144-member library, screening assays were performed on about 1000 beads (degeneracy = 7) to ensure that each member was represented. In order not to waste library on preliminary work, initial single-colour experiments, to establish appropriate conditions, were performed on ~ 100 beads

The red salt TBA **2.6L** (1 mM in 1 % $MeOH/CHCl_3$; 0.5 mL) was added to **6.1** (~ 100 beads) in a small pot. This was placed within a jar, the bottom of which was covered with 1 % $MeOH/CHCl_3$, and the lid replaced tightly to maintain an atmosphere

of solvent. The beads were examined under a microscope at 5-minute intervals. After 35 minutes, three intensely red beads were observed among the remaining beads, the colours of which were rather difficult to ascertain in the red solution. Swirling of the pot to remove the supernatant phase to one side revealed that all the beads had acquired some colour, with hues varying from light orange to intense red. The appearance of the beads did not change noticeably when they were returned to the capped jar and the system left for a longer time, or even overnight. The beads were transferred to a filtration tube fitted with a frit and filtered. To remove any remaining excess guest, washing was performed with $CHCl_3$ (0.5 mL), by shaking the beads within the filtration tube, both ends of which were sealed with rubber septa. The beads were then transferred to a Petri dish using $CHCl_3$ and examined under the low-power microscope. The $CHCl_3$ environment facilitated examination of the beads as they swell considerably in this solvent. When the beads dried out, their colour became distorted so that intensely red beads, for example, became almost black in appearance (**Figure 6.2**). The beads were returned to the filtration tube, filtered, and shaken with decomplexing solution (MeOH/$CHCl_3$ 1:1; 0.5 mL). The beads became colourless almost immediately as the solvent became coloured. The supernatant phase was filtered and the test repeated on the beads. The same colour changes were observed, indicating that the beads were recycleable. This experiment was routinely run as **Expt 6.1**, as detailed in **Chapter 7**.

As discussed in **Chapter 4**, **2.6L** is about 20 % impure. The most intensely red beads are binding not just the dye-labelled protected amino acid but also the impurity associated with **2.6L**. The variety of red hues nevertheless indicates that selective binding is occurring.

Other guest concentrations were tried. For example, TBA **2.6L** (0.1 mM in 1 % MeOH/$CHCl_3$; 0.5 mL) was applied to **6.1** (100 beads), using the method described in **Expt 6.1**. Only a few beads became orange-coloured, presumably the most tightly binding ones. Such a concentration could be used to find the most tightly binding substrate. In a selectivity screen however, the most selective, which is not necessarily the most tightly binding member, is sought. It is important therefore, that all beads bind some guest so that competition can occur. As with the mass spectrometric assay, the

theoretical calculations (1 mM guest; 5 μL per bead) provide optimum conditions under which reversible binding can occur.

Manipulation of these particular synthesis beads (Tentagel®) was trickier than manipulation of **5.3** (Wang® resin beads; 100 μM diameter). Tentagel® is composed of about 80 % polyethylene glycol grafted to cross-linked polystyrene. Tentagel is a popular solid phase for synthesis as the reaction milieu within the resin is generally considered to be more closely related to ether and THF than other resins on offer, thereby having potential for compatibility with a large range of reactions.[2.2] From the perspective of manipulating these beads beneath a microscope, however, the beads are smaller (90 μM diameter) than polystyrene-based Wang® resin beads and can swell to only 4 times their size in water, as opposed to polystyrene beads, which can swell to 8 times their size. Indeed, in $CHCl_3$, when examined under a microscope, the polystyrene-based Wang® resin beads appeared a lot larger than the Tentagel® beads. The concentration of active sites in Tentagel® is about 0.28 M, which is reduced compared with Wang® resin, and is reduced further, to 0.07 M, when swollen. This characteristic, and the environment provided by Tentagel® beads led to their choice as the solid phase for the synthesis of the library. However, the size of the bead, even upon solvation, leads to difficulty upon manipulation thereof. Moreover, the Tentagel® beads tended to stick to each other and to the glass surfaces with which they came into contact. It was easiest to collect them in individual capillaries when they were partially swollen in $CHCl_3$ (**Figure 6.2b**). In this state, the beads did not group together in solvent pools. Nor did they become very sticky, which occurred when all solvent evaporated (**Figure 6.2c**), and which made single beads very difficult to address. Although the interior diameter of the syringe needle was chosen to be smaller than that of a bead, care was required not to remove any beads on the outer surface of the syringe needle. All manipulations were performed under a microscope (**Figure 6.1**).

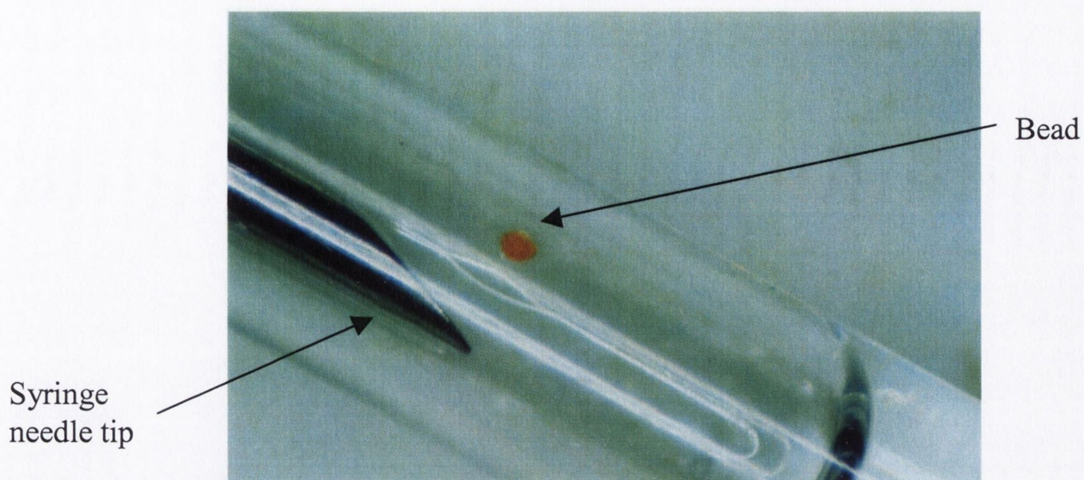


Figure 6.1 A member of split-and-mix library **6.1** within a melting point capillary tube. This bead has an orange colour. The tip of a microsyringe, which was used to transfer solution in and out of the tube, is shown.

The colour experiment, **Expt 6.1**, was repeated using TBA **2.5L** (1 mM in 1 % MeOH/$CHCl_3$; 0.5 mL). A few highly coloured (blue) beads were observed, as expected. When the blue supernatant phase was removed, by filtration, the beads were observed to have varying blue hues, from very pale blue to intensely coloured blue. This was considered to be a parallel result to the single-colour red test.

6.2.4 The dual-colour assay

Standard solutions of TBA **2.5L** and red TBA **2.6D** were prepared and equimolar quantities were mixed together to give a brown solution which was concentrated under pressure to ensure thorough mixing of the guests. The brown substrate was made up to a 1 mM solution (0.5 mM in each guest; 1 mM overall in 1 % MeOH/$CHCl_3$), 5 mL of which was which was applied to dipeptide urea receptor library **6.1** (1000 beads) in a Petri dish, which was placed within a jar, the bottom of which was filled with 1 % MeOH/$CHCl_3$, and the lid tightly replaced (**Expt 6.2**). After 45 minutes, the dish was removed and examined under the microscope. By swirling the Petri dish, a few red and blue beads could be observed in the brown mixture. The dish was replaced and the system left for a further 3 hours. After this time, the beads were transferred to a filtration tube and the brown supernatant phase removed by filtration. To remove unbound guest, the beads were washed with $CHCl_3$ (2.5 mL x 2) by shaking within the filtration tube and filtering. The beads were then placed in a clean Petri dish, transferring with $CHCl_3$, and examined under the microscope. Some red and blue beads, of varying hues, were observed among a majority of brown beads, also of varying hues. All of the blue beads were very distinctive, as were the highly coloured red beads. It was very difficult to distinguish between light red, orange and light brown beads, however (**Figure 6.2**).

The intensely blue, red and some brown beads were picked in individual capillaries and labelled. 1000 beads of **6.1**, which is a 144-member library, were used to perform a screening experiment, such that a degeneracy of about 7 should have been present. 4 intensely red beads and 4 intensely blue beads were picked, however. All beads bearing the same receptor should have behaved in the same manner when exposed to guest, such that for every red or blue bead there should have been 6 other red or blue beads, exhibiting the same receptor. The identification of only 4 intensely red and blue beads from 1000 beads suggests that the assay was not effective in identifying identical members. Quantification of de-complexed substrate after exposure of each picked bead to isotopically labelled guest should indicate whether picked beads exhibit the same selectivites, which would be a necessary characteristic of identical members.

Figure 6.2 a

Figure 6.2 b

Figure 6.2 c

Figure 6.2 A portion of library **6.1** after equilibration with {TBA **2.5L**/TBA **2.6D** (1:1)}. (**a**) The beads are solvated in $CHCl_3$; (**b**) Almost all solvent has evaporated. (**c**) The beads have dried.

6.3 Application of the single bead assay to picked library members

The single bead assay described in **Chapter 5**, which used isotopically differentiated enantiomers coupled with ESIMS⁻ to determine enantioselectivity, was used to determine the enantioselectivity of picked library members. The library had been treated with blue-tagged L-guest and red-tagged D-guest. Therefore, any blue bead should contain an L-selective receptor and, when exposed to equimolar TBA **4.6DL**, should have a preference for **4.6L**. Conversely, any red bead should contain a D-selective receptor and should have a preference for **4.6D**. The degree of enantioselectivity exhibited by any bead-bound receptor should be determined by the intensity ratio of the observed "pseudoracemic" peaks. This could be investigated further by treating a bead with a solution of guest biased towards one enantiomer. For example, **5.3** was found to be L-selective and the degree of selectivity was checked by exposing **5.3** to guest enriched in the D-"pseudoenantiomer". **5.3** extracted the under-represented L-enantiomer with an L/D ratio of 2:1 each time (**Section 5.2.5**). The same methodology should be applicable to members of **6.1**. Any members with pronounced selectivity could be sequenced and re-synthesised for further testing, while time and labour could be saved by avoiding the re-synthesis of members with lower selectivity.

6.3.1 Analysis of single brown beads.

Beads that became brown when subjected to the colour assay were thought to be unselective as they bound similar amounts of each dye-labelled enantiomer. Such beads, when exposed to TBA **4.6DL** were expected to bind equal amounts of each "pseudoenantiomer". A mass spectrum of the guest recovered in such an extraction should give peaks of about equal intensity.

Decomplexing solution ($MeOH/CHCl_3$ (1:1); 5 μL) was added to each brown bead in its capillary, which was inverted a few times until the coloured guest had been cleaved, as described in **Expt 6. 3**. Washing was then performed with $CHCl_3$ (5 μL x 3) to remove the highly competitive decomplexing solvent. Each bead was treated with equimolar TBA **4.6DL** (1 mM in 1 % $MeOH/CHCl_3$; 5 μL) and left to equilibrate within a small, capped sample tube which contained a little solvent, to maintain an atmosphere

of solvent, for 4 hrs. After this time, excess guest was removed by syringe. Each bead was washed with $CHCl_3$ (5 μL x 3) to remove any remaining unbound guest, by inverting the capillary 20 times and removing the solution by syringe. Decomplexing solution was added to each capillary for about 3 minutes during some of which time each bead was "shaken" by inversion of the capillary. As coloured guest had been observed to cleave very easily from each bead using this solution, it was thought that immersion for 3 minutes should cleave the isotopically-labelled guest fully. Each solution was removed and made up to 20 μL with MeOH to comprise a single ESIMS injection. The mass spectra obtained for the three brown beads exhibited peaks of about equal height at 206 and 209 m/z, corresponding to the "pseudoenantiomers". One of these spectra is shown in **Figure 6.3**. (See also **Appendix A10**, **A11**, **A12** for other examples). An interesting point was that the peaks were reduced in intensity compared with the signals obtained for the single beads of **5.3**. This related to the lower loading of Tentagel® beads compared with Wang® resin beads, and is discussed in **Section 6.4**.

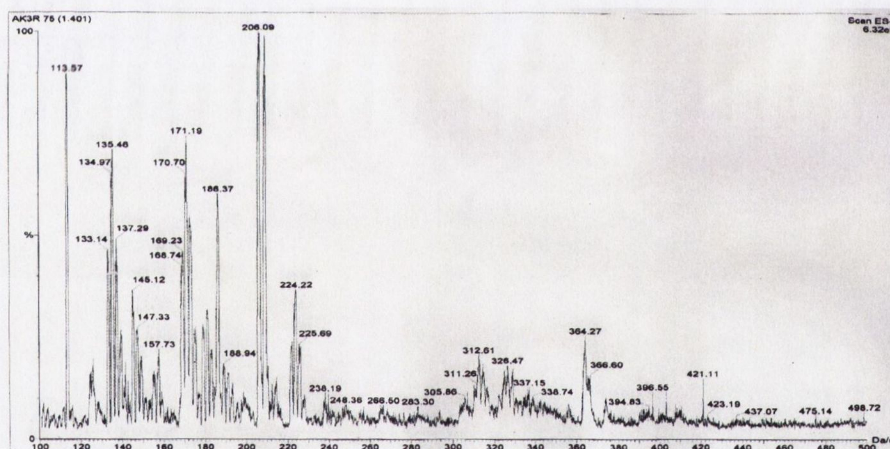


Figure 6.3: 4.6D/4.6L decomplexed from a "brown" member of library **6.1.**

The bead-bound receptors were un-selective, as expected for brown beads. A second decomplexation was performed to investigate whether any guest had remained bead-bound. When this extract was analysed by ESIMS⁻ virtually no signal was observed at the expected m/z values. Only a small amount of baseline material was observed. It appeared that all guest had been cleaved in the first decomplextion step.

The beads were recycled and subjected to the assay again. They were found to give the same result by mass spectrometry, indicating that they were recycleable.

6.3.2 Analysis of single red beads.

As for the brown beads, the red beads (4) were picked in individual capillaries. Decomplexing solution was added to each individual bead to cleave coloured guest. Once this was removed and each bead washed, isotopically labelled guest was added, as before, and each capillary placed within a small, capped sample tube in an atmosphere of solvent. After 4 hours, excess guest was removed from each capillary and each bead was washed with $CHCl_3$. Bound guest was then cleaved and analysed by mass spectrometry.

Red beads selectively bound **2.6D** from an equimolar solution of Blue TBA **2.5L** and Red TBA **2.6D**. Receptors on red beads were D-selective according to the colour assay and, when exposed to equimolar TBA **4.6DL,** were expected to select the lighter enantiomer. Indeed, the mass spectra of each of the 4 beads screened indicated a preference for the lighter enantiomer (**Appendix A13**, **A14**, **A15**). L/D ratios of 1:1.3 (for 2 beads), 1:1.2, and a maximum of 1:1.4 were observed. Although the direction of enantioselectivity, in each case, was consistent with the findings from the colour assay, the findings were modest compared with the impressive red hues of the picked beads. Different L/D ratios suggest that different receptors were on the picked beads, such that beads carrying identical receptors were not identified by the colour assay. That the selectivites recorded by ESIMS varied for each of the intensely coloured red beads, without any very impressive selectivity being recorded, indicates that the the colour changes did not arise from binding of the chiral moiety. It is likely that the aromatic dye moiety was bound in preference to the chiral moiety. This is discussed in **Section 6.4**. The intensity of the signal observed for guest decomplexed from individual members of library **6.1** was reduced compared with the intensity observed for individual beads of type **5.3**. As can be seen in **Figure 6.4**, the peaks in question, at 206 and 209 m/z, have a similar peak height to undesired peaks (arising from impurities) in the spectrum. This is also discussed in **Section 6.4**.

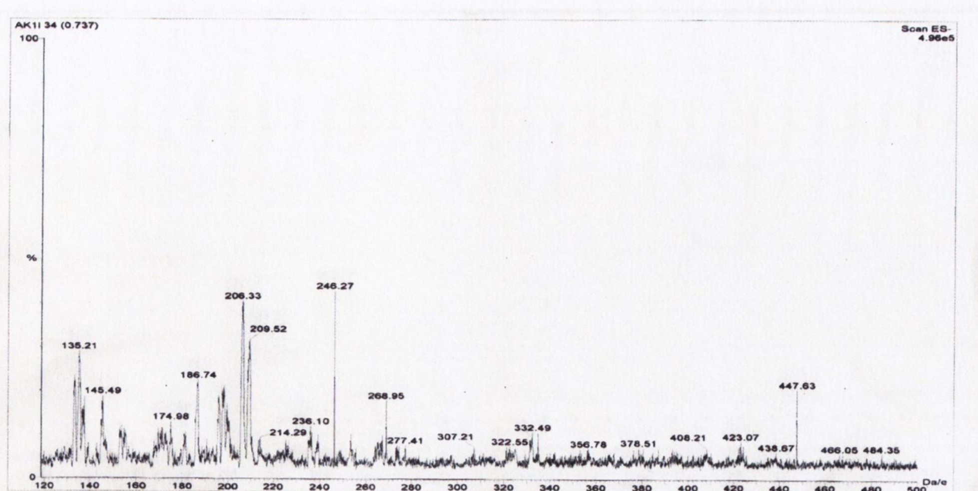


Figure 6.4: {**4.6D/4.6L**} decomplexed from a "red" member of library **6.1.**

6.3.3 Analysis of single blue beads

The single bead assay (**Section 6.3.2**) was applied to the picked blue beads (4). These bead-bound receptors were L-selective according to the colour test and were expected to bind the heavier "pseudo-enantiomer" **4.6L** selectively from an equimolar solution of TBA **4.6DL**. The mass spectrum obtained in each case exhibited such a preference although, as for the red members, the degree of selectivity observed was not very high in any case (**Figure 6.5**). L/D ratios of 1.1:1 (for 2 beads), 1.2:1 and a maximum of 1.4:1 were observed. (**Appendix A15, A16, A17**). As for the red beads, the results from the ESIMS assay are considered not to correlate very well with the intensity observed for the blue beads and this is discussed in **Section 6.4**.

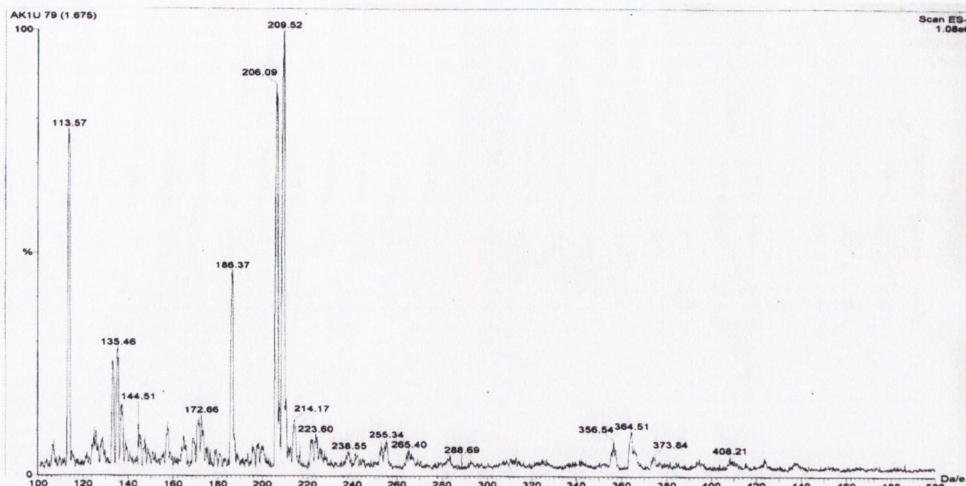


Figure 6.5: 4.6D/4.6L decomplexed from a "blue" member of library **6.1.**

6.3.4 Binding of equimolar {[2.5L + TBACl]/[2.6D + TBACl]} by 6.1

On one occasion when guest was being prepared, TBACl was added to **2.5L** in $CHCl_3$ and H_2O with the omission of base and, separately, TBACl was added to **2.6D** in $CHCl_3$ and H_2O, also with the omission of base **(Expt 6.4)**. An equimolar solution of [**2.5L** + TBACl] and [**2.6D** + TBACl] (1 mM in 1 % MeOH/$CHCl_3$; 5 mL) was added to **6.1** (1000 beads). Some red and blue beads were observed among a majority of brown beads of differing hues and intensities. A portion of this library is shown in **Figure 6.6**.

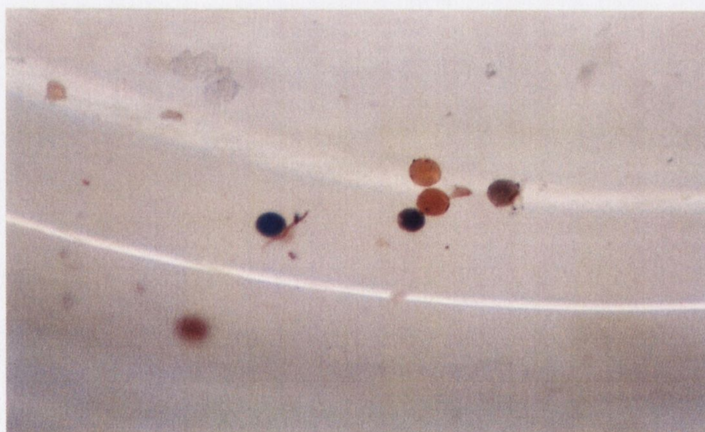


Figure 6.6

6.4 Discussion

The dual-colour assay allowed both red and blue beads to be picked from a background of brown beads. As the conditions used allowed thermodynamic equilibration, reversible binding was observed for steroidal library **6.1**. It was anticipated that the assay would be supported by findings from a second assay between picked beads and "pseudo-enantiomers" **4.6DL**. This second assay provided reliable results for another system (**5.3**) previously, and was validated by a series of experiments upon that system (**Section 5.2.5**). Both the colour and "pseudo-enantiomers" tests provided a measure of the direction of selectivity for single bead-bound steroidal receptors. However, the intensely coloured beads were expected to show considerable enantioselectivity but this was not reflected in the findings of the mass spectrometric assay between **6.1** and isotopically labelled "pseudo-enantiomers".

The enantioselectivities quantified for each bead of each colour were not the same, indicating that the same receptor was not bound to each picked bead, as identical members should bind guest in an identical fashion. There was an assumption that the ESIMS assay developed between polystyrene-bound **5.3** and isotopically labelled guest would also provide a measure of the enantioselectivity of the members of Tentagel®-bound **6.1,** notwithstanding the differences between the solid phases employed.

That all picked red and picked blue beads did not have the same selectivities for **4.6LD** and that the expected number of identical beads were not observed to bind guest, suggests that the binding between the picked members of **6.1** and coloured guest was not the anticipated enantioselective binding of the carboxylate functionalities of **2.5L** and **2.6D**. Reasons for this occurring might include impurity of substrate, and binding by receptor of the dye moiety instead of the carboxylate functionality. In the first instance, the chiral HPLC spectra of **2.6L** and **2.5D** both gave, apart from the peak observed in the blank spectrum **Appendix B5**, a single peak. It is possible that impurities are present beneath the peak in either spectrum, although this is not evident from the spectra. If, for example, the red guest was not enantio-pure, an exclusively D-selective receptor should nevertheless appear red as it would bind **2.6D** only. On the other hand, an L-selective receptor would appear brown as it would bind **2.6L** and **2.5L**. The converse is true for a system in which the blue guest appears as a racemate. In the

case of both guests occurring as racemates, all beads should appear brown. As described above, both guests are thought to be enantiopure, such that, in a library with a degeneracy of 7, for every selective member, 7 beads should appear coloured. This did not occur, which suggests that enantioselective binding did not occur. This observation is supported by the results from the isotopically labelled mass spectrometric assay, which suggests that not only were non-identical beads picked, but that members with un-impressive selectivity were picked. Beads appeared coloured due to selective binding of one coloured guest over another, but the results from the ESIMS assay indicate that the apparent selectivity is not for the chiral centre. Rather, it is for another portion of the guest molecule. As both guests share the tyrosine moiety but bear different dye moieties, the latter must be implicated. Although achiral, both dyes bear aromatic groups that could be bound by **6.1**, and it appears that this could be responsible for the observed activity. The dye moiety was positioned remote from the chiral centre to avoid such binding occurring, and many beads may indeed have bound carboxylate, but it is likely that the picked beads favoured the aromatic dye moiety to the chiral centre.

The observed selectivities of intensely coloured beads were disappointing but the time-consuming sequencing and re-synthesis of apparently efficient receptors was avoided by the secondary testing of these beads against isotopically labelled guest.

An apparent difference between the spectra of guest decomplexed from **5.3** and **6.1** was that the intensity of signal for guest decomplexed from individual members of library **6.1** was reduced compared with the intensity observed for guest decomplexed from individual members of **5.3**. The Tentagel® beads which were used to synthesise library **6.1** have a loading of 0.28 mmol/g, which is reduced compared with the Wang® resin beads used to synthesise **5.3,** which have a loading of ~ 1 mmol/g. **6.1** should therefore be capable of binding only 28 % of the material bound by **5.3**. This was evident by comparison of the signal given by guest de-complexed in each case. The reduced signal from single members of library **6.1** was close to the threshold of the mass spectrometer's sensitivity and the observed peaks had a similar height to undesired peaks (arising from impurities) in the spectrum. Indeed, upon processing a sample from a single member of **6.1**, subtraction of background scans from sample scans was observed to alter the un-processed mass spectrum considerably, because the intensity of

signal from the sample was so close to intensity of the background. As a result, the mass spectra shown for single members of **6.1** are unprocessed, unlike the spectra shown for **5.3**, processing of which did not noticeably alter raw data. A more sensitive machine would give more intense signals for single bead extractions. Alternatively, libraries could be synthesised on solid phase beads with greater loading capacities, for example polystyrene Wang® resin beads. In the context of this project, a further advantage of employing Wang® resin beads is that a reliable ESIMS assay has been developed between Wang® resin-bound receptors and isotopically labelled guest.

The library beads were rather tricky to manipulate, particularly once the colour had been cleaved. This made the ESIMS assay rather time-consuming as it required several steps under the microscope. A more efficient procedure would be to treat all red beads together and all blue beads together, in a filtration tube rather than a capillary, until the final step, which involves decomplexation of isotopically labelled guest and is the only step that requires beads to be separate and labelled. This would be a particular advantage when screening larger libraries, wherein a large number of beads of each colour were selected.

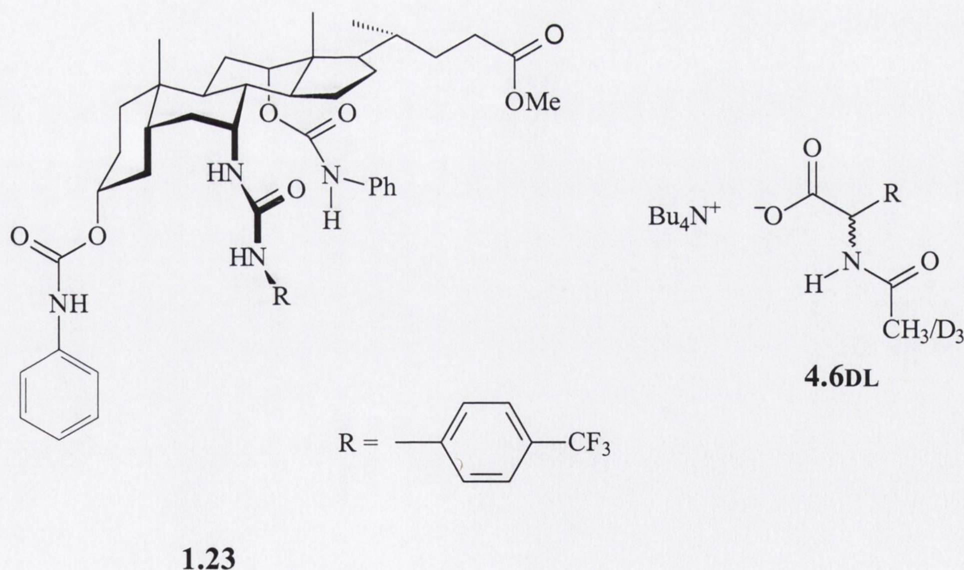
No other libraries have been tested, to date, using this system. Future work includes the testing of a library of tripeptides analogous to **6.1**, but containing 1440 members, and therefore requiring 10 times as much guest as did **6.1**, for a single screen. Now that the method is developed, it is hoped that some highly active members will be found from the urea, or indeed, the guanidinium libraries shown in **Figure 2.2**.

6.5 Chiral Complexes in ESIMS

The work of Sawada and co-workers, in which a series of chiral complexes were observed in both FABMS and ESIMS, was described in **Section 3.3**. Both crown ether and acyclic hosts were found to form complexes with "pseudoracemic" guest, which were robust enough to survive, and be identified by, mass spectrometry. The ability of complexes formed between steroidal receptors and *N*-protected amino acids, to be quantified by mass spectrometry in a manner similar to the method of Sawada, was investigated. If this was successful, "pseudoracemate" could be used to provide a quick measurement of the enantioselectivity of a particular host. The technique involves a

solution phase assay that requires a very small quantity of host (< 1 mg) and provides a relatively quick result.

Receptor **1.23**[1.28] (0.5 mg; 5.9 x 10^{-7} mol), was stirred with a 10-fold excess of TBA **4.6DL** for 15 minutes. The mixture was then prepared for mass spectrometry by dilution to an appropriate concentration. Analysis was performed in $ESIMS^{-}$[a]. The molecular ion [**1.23** – 1] was observed as were [**1.23** + chloride] and [**1.23** + acetate]. Neither of the desired peaks [**1.23** + **4.6D**] at 1054 m/z nor [**1.23**+ **4.6L**] at 1057 m/z was observed, as shown in **Appendix A19**. A relatively intense peak at 1112 m/z was thought to have arisen from complexation by **1.23** of the dodecyl sulfate ion present in detergent. Use of new glassware, which had never been exposed to detergent, led to loss of this peak and to loss of a peak at 265 m/z, corresponding to the dodecyl sulfate anion. Despite exclusion of detergent, other anions including bromide and iodide were observed to have been complexed by **1.23**. Conditions including cone voltages, flow rate and temperature were varied to obtain suitable conditions in which the complex could survive. If a complex was formed between **1.23** and **4.6L** and/or **4.6D**, the anion was replaced by more strongly binding anions present in the mass spectrometer, as neither complex ion was observed.



[a] Unlike the on-bead extraction experiments described in **Chapters 5** and **6**, for which a VG Quattro electropspray mass spectrometer was used to determine guest ratios, at the University of Bristol, the experiments described in **Section 6.5** were performed using a communal Micromass LCT elecrospray spectrometer at Trinity College Dublin.

Sawada and co-workers reported that observed selectivities were reduced in ESIMS compared with FABMS, and accounted for this with the actual process of ionisation. The experiments between **1.23** and TBA **4.6DL** were attempted only in ESIMS, as it was the only available machine. As the machine was communal, the exclusion of various anions was particularly difficult. These experiments may be more successful if performed on a machine that has fewer users, or indeed in FABMS. Were the desired complexes to be observed, the technique could be used to ascertain the enantioselectivity of any receptor. Applying the concept to LC/MS could allow several receptors to be tested at once. Furthermore, with a more sophisticated system such as LC/MS/MS, it may be possible to screen a whole library of receptors, in solution, together.

6.6 Conclusion

Selective binding was observed between a one-bead one-compound combinatorial library of steroidal receptors and dual colour-tagged *N*-protected amino acids. Library members with apparent activity were isolated and subjected to a solid-liquid extraction with isotopically differentiated guest. A measure, by ESIMS, of direction and degree of selectivity on the single bead scale was attempted. When treated with equimolar TBA **4.6DL**, single red beads, which had been found to be selective for TBA **2.6D** in the dual-colour assay, were found to bind **4.6D** with some selectively, while blue beads, which had been found to be selective for TBA **2.5L** in the dual-colour assay, were found to bind **4.6L** with some selectively. The direction of selectivity for each picked bead, according to this assay, was consistent with the findings for the colour assay but the degree of selectivity observed was not very high (1.4:1 and 1:1.4 D/L were the highest ratios observed, by ESIMS). It is thought that the aromatic dye tag became involved in binding. Manipulation of single members of a one-bead one-compound combinatorial library was successful.

Although it is disappointing that no receptor of impressive selectivity was isolated, it is somewhat satisfying that the device by which candidates from **6.1** were eliminated was the analytical method developed and described in **Chapter 5**.

CHAPTER 7

Experimental: General procedures

NMR spectra were recorded using a Bruker DPX 400 spectrometer, Jeol Delta/GSX 270 MHz spectrometer or a Jeol Eclipse 400 MHz spectrometer. Chemical shifts (δ) are reported in parts per million relative to trimethylsilane as standard and coupling constants (*J*) are quoted in Hz. Electrospray mass spectrometry was performed using a Micromass Visons VG Quattro 1 spectrometer (Bristol) and a Micromass LCT spectrometer (Trinity). High resolution mass spectra were collected using a Micromass Autospec spectrometer by the EI technique. LC/MS spectra were performed using a Micromass 600 LC/MS spectrometer.

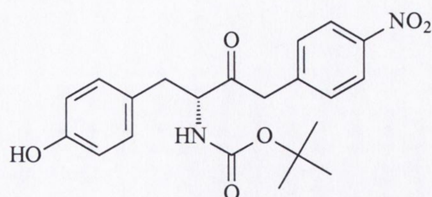
IR spectra were collected using a Perkin Elmer Spectrum One FT-IR spectrometer. Melting points were performed with a Gallenkamp melting point apparatus and are uncorrected. Chiral HPLC was performed using a Daicel Chiralpak AD column, a Gilson model 303 pump and a Dynamax Rainin UV detector at 254 nm. Optical rotations were recorded with a Perkin Elmer 241 MC polarimeter, irradiating with the sodium D line (λ = 589 nm) and $[\alpha]_D$ are given in units of 10^{-1} degcm^{2}g^{-1}.

Solid-phase beads were examined under an Olympus SZ40 microscope using an Olympus Highlight 2100 light source. Photographs were recorded with an Olympus SC35 camera which was attached to the microscope.

Chemicals were purchased from the Aldrich chemical company, Acros Chemicals and the Novabiochem chemical company (amino acids). Tlc was performed using Merck DC Alufolien Kieselgel 60 F_{254} 0.2 mm plates and Merck DC-Fertigplatten SILG-25 UV_{254} 0.25 mm plates. Compounds were visualised by uv light, phosphomolybdic acid (5 % in EtOH), $KMnO_4$ (1 % w/v solution in aqueous base) and by eye, as appropriate. Flash column chromatography was performed with Merck Kieselgel 60 400-230 mesh silica, by the technique of Still.[1]

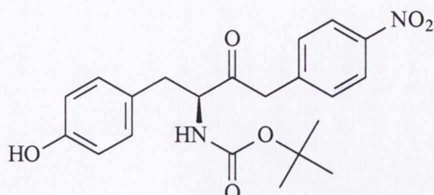
Solvents were distilled prior to use, where appropriate, and dried, if necessary, by standard techniques described by Perrin.[2]

p-Nitrobenzyl N-Boc-L-tyrosine 4.1L



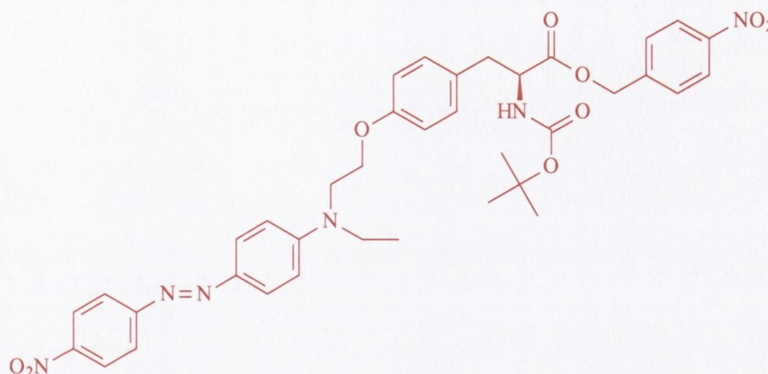
Aqueous Cs_2CO_3 (20% w/v) was added to a solution of N-Boc-L-tyrosine (1 g; 3.55 mmol) in MeOH (2 mL) and H_2O (1.5 mL) until pH 7.5 was reached (pH paper). MeOH was removed under reduced pressure and H_2O removed as an azeotrope with toluene, which was, in turn, removed as an azeotrope with DCM, while maintaining the temperature of the rotary evaporator water bath below 30 C. A white solid ensued, which was dried *in vacuo*. The cesium salt (1.47 g; 3.55 mmol) was then dissolved in DMF (11.8 mL) and p-nitrobenzyl bromide (0.81 g; 3.72 mmol) was added slowly, during which precipitation of CsBr changed the clear yellowish solution to a beige mixture. After 90 mins when, by tlc, all of the starting material appeared to have been consumed, DCM (30 mL) was added to the reaction mixture which was then washed with H_2O (10 mL x 2). The organic phase was dried over anhydrous $MgSO_4$ and evaporated to dryness. The crude product was purified by flash column chromatography, eluting with hexane/ethyl acetate (3:2). **4.1L** was isolated as a white solid (1.3 g; 91 %). R_f = 0.55 in hexane/ethyl acetate (3:2); $[\alpha]_D$ = -3.1 (c = 0.1 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.44 (s, 9 H; C(CH_3)$_3$), 3.02 (d, $J_{H,H}$ = 6.5, 2 H; CH_2CH), 4.62 (m, 1 H; CH), 4.98 (br d, J = 8.5 H; NH), 5.22 (AB quartet, $J_{A,B}$ = 13.5, 2 H; O_2NBn CH_2), 6.72 (d, $J_{H,H}$ = 8.5, 2 H; ArCH), 6.97 (d, $J_{H,H}$ = 8.5, 2 H; ArCH), 7.40 (d, $J_{H,H}$ = 9.0, 2 H; O_2NBn ArCH), 8.21 (d, $J_{H,H}$ = 9.0, 2 H; O_2NBn ArCH); ^{13}C (100 MHz $CDCl_3$) δ = 28.47 (C(CH_3)$_3$), 37.21 (CH_2), 54.47 (CH), 65.33 (O_2NBn CH_2), 80.18 (C(CH_3)$_3$), 114.51 (ArCH), 123.39 (ArCH), 127.55 (ArCH), 129.66 (ArCH), 141.30 (q-C), 147.29(q-C), 154.24 (q-C), 155.40 (q-C) 171.61 (C=O), 177.38 (C=O); IR (Solid state) ν_{max} = 3385 cm^{-1} (NH), 1753 cm^{-1} (C=O), 1682 cm^{-1} (CONH), 1516cm^{-1}, 1346cm^{-1} (N=O); elemental analysis calcd (%) for $C_{21}H_{24}N_2O_7$ (416.43): C 60.57, H 5.81, N 6.73; found C 60.86, H 6.33, N 6.79; HRMS (EI) mass calcd for $C_{21}H_{24}N_2O_7$ (416.158351); mass found (416.157440).

p-Nitrobenzyl N-Boc-D-tyrosine 4.1D



Aqueous Cs_2CO_3 (20% w/v) was added to a solution of N-Boc-D-tyrosine (2 g; 7.1 mmol) in MeOH (4 mL) and H_2O (3 mL) until pH 7.5 was reached (pH paper). MeOH was removed under reduced pressure and H_2O removed as an azeotrope with toluene, which was, in turn, removed as an azeotrope with DCM. A white solid ensued which was dried *in vacuo*. The cesium salt (2.93 g; 7.1 mmol) was then dissolved in DMF (24 mL) and p-nitrobenzyl bromide (1.61 g; 7.5 mmol) added slowly while precipitation of CsBr changed the clear, yellowish solution to a beige mixture. After 90 mins, DCM (60 mL) was added to the reaction mixture which was then washed with H_2O (20 mL x 2). The organic phase was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash column chromatography, eluting with hexane/ethyl acetate (3:2). **4.1D** was isolated as a white solid (2.6 g; 92 %). R_f = 0.55 in hexane/ethyl acetate 3:2; $[\alpha]_D = +3.9$ (c = 0.1 in $CDCl_3$); ^{1}H NMR (400 MHz, $CHCl_3$) δ = 1.45 (s, 9 H; $C(CH_3)_3$), 3.03 (d, $J_{H,H}$ = 6.5, 2 H; CH_2CH), 4.63 (m, 1 H; CH), 4.99 (br d, $J_{H,H}$ = 8.5; 1 H; NH), 5.22 (AB quartet, $J_{A,B}$ = 13.5, 2 H; O_2NBn CH_2), 6.72 (d, $J_{H,H}$ = 8.5, 2 H; ArCH), 6.97 (d, $J_{H,H}$ = 8.5, 2 H; ArCH), 7.40 (d, $J_{H,H}$ = 9.0, 2 H; O_2NBn ArCH), 8.21 (d, $J_{H,H}$ = 9.0, 2 H; O_2NBn ArCH); ^{13}C (100 MHz, $CDCl_3$) δ = 27.79 ($C(CH_3)_3$), 37.39 (CH_2), 54.67 (CH), 64.55 (O_2NBn ArC), 79.90 ($C(CH_3)_3$), 115.01 (ArCH), 123.52 (ArCH), 127.91 (ArCH), 129.83 (ArCH), 141.34(Ar-C), 147.38 (Ar-C), 154.24 (q-C), 155.54 (Ar-C), 171.72 (C=O), 177.50 (Ar-C); IR (Solid state) ν_{max} = 3350 cm^{-1} (NH), 1752 cm^{-1} (C=O), 1680 cm^{-1} (CONH), 1516 cm^{-1}, 1344 cm^{-1} (N=O); elemental analysis calcd (%) for $C_{21}H_{24}N_2O_7$ (416.43): C 60.57, H 5.81, N 6.73; found C 60.89, H 6.29, N 6.84. HRMS (EI) mass calcd for $C_{21}H_{24}N_2O_7$ (416.158351); mass found (416.160019).

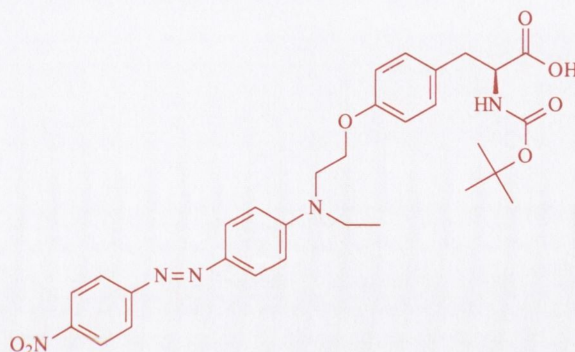
N-Boc-L-tyrosine (O-Disperse Red 1)-O-p-nitrobenzyl ester 4.4L



Disperse Red 1 (79 mg; 0.25 mmol) in dry THF (1.5 mL) was added slowly, under Ar, to a solution of **4.1L** (100 mg; 0.25 mmol), Ph_3P (65 mg; 0.25 mmol) and DEAD (40 µl; 0.25 mmol) in THF (1 mL), and the reaction mixture stirred at room temperature under Ar. After 90 mins, additions of Ph_3P (32 mg; 0 125 mmol) and DEAD (20 µL; 0.125 mmol) were made. Final additions of Ph_3P (32 mg; 0.125 mmol) and DEAD (20 µL; 0.125 mmol) were made after a further 20 mins and the reaction mixture stirred overnight. The reaction mixture was then concentrated under reduced pressure and purified by flash column chromatography, eluting with hexane/ethyl acetate (3:2). A second purification by flash column chromatography, eluting with 4 % MeOH/DCM, was performed, and the crude product recrystallised from $CHCl_3$/hexane to give **4.4L** as a red solid (64 mg; 37% yield). $R_f = 0.6$ (hexane/ethyl acetate 1:1); m.p. 126 – 128 °C; $[\alpha]_D = -17$ (c = 0.1 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ = 1.32 (t, $J_{H,H}$ = 7.0, 3 H; CH_3), 1.43 (s, 9 H; $C(CH_3)_3$), 3.04 (d, $J_{H,H}$ = 6.5, 2 H; CH_2CH), 3.66 (q, $J_{H,H}$ = 7.0, 2 H; CH_2CH_3), 3.89 (t, $J_{H,H}$ = 5.5, 2 H; CH_2CH_2N), 4.17 (t, $J_{H,H}$ = 5.5, 2 H; CH_2CH_2O), 4.60 (m, 1 H; C*H*), 4.96 (br d, $J_{H,H}$ = 8.0, 1 H; N*H*), 5.22 (AB quartet, $J_{A,B}$ = 13.0, 2 H; O_2NBn CH_2), 6.78 (d, $J_{H,H}$ = 8.5, 2 H; OPh ArC*H*), 6.86 (d, $J_{H,H}$ = 9.0, 2 H; ArC*H*), 7.01 (d, $J_{H,H}$ = 8.5, 2 H; OPh ArC*H*), 7.38 (d, $J_{H,H}$ = 9.0, 2 H; O_2NBn ArC*H*), 7.97 (t, $J_{H,H}$ =9.0, 4 H; ArC*H*), 8.19 (d, J_{HH} = 9.0 Hz, 2 H; O_2NBn ArC*H*), 8.35 (d, $J_{H,H}$ = 9.0 Hz, 2 H; ArC*H*); ^{13}C NMR (100 MHz, $CDCl_3$) δ =12.32 (CH_3), 28.29 ($C(CH_3)_3$), 37.48 (CH_2CH), 46.18 (CH_2CH_3), 49.84 (CH_2CH_2N), 54.69 ($CHCH_2$), 65.31 ($OCHCH_2$),

65.31 (O_2NBn Ar*C*), 80.07 (q-C), 111.41 (Ar*C*H), 114.51 (OPh Ar*C*H), 122.62 (ArCH), 123.72 (O_2NBn ArCH), 124.68 (Ar*C*H), 126.20 (Ar*C*H), 128.24 (Ar*C*), 129.96 (Ar-CH), 130.33 (ArCH), 142.39 (O_2NBn Ar*C*), 143.75 (Ar*C*), 147.38 (Ar*C*), 147.71 (O_2NBn Ar*C*), 151.22 (OPh Ar*C*), 154.52 (C=O), 156.77 (Ar*C*), 157.64 (OPh Ar*C*), 171.74 (Ar*C*); IR (Solid state) ν_{max} = 3323 cm^{-1} (NH), 1738 cm^{-1} (C=O) 1600, 1587 cm^{-1} (CONH), 1513, 1338 cm^{-1} (N=O), 1135 cm^{-1} (R-O-R); HRMS (EI) mass calcd for $C_{37}H_{40}N_6O_9$ (712.285677); found (712.286926).

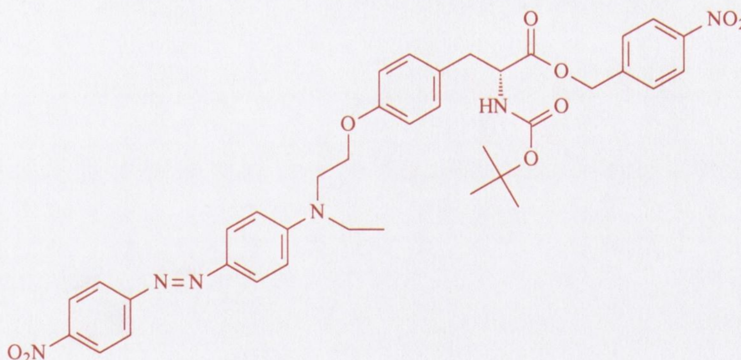
_N_-Boc-L-tyrosine (O-Disperse Red 1) 2.6L



To **4.4L** (64 mg, 0.086 mmol) in THF (0.5 mL) was added Bu_4NF (1.0 M in THF; 270 μL; 0.27 mmol) in THF (0.5 mL). The reaction was monitored by tlc, eluting with hexane/ethyl acetate (3:2), and after 10 mins a high proportion of red baseline material was observed, as expected for **2.6L**. A further addition of TBAF (270 μL; 0.27 mmol) was made and after 20 mins only a small amount of red starting material remained. A final portion of TBAF (90 μL; 0.092 mmol) was then added and, after a further 15 minutes, H_2O (3 mL), followed by ethyl acetate (5 mL), was added to the reaction mixture. The red material was extracted into the organic phase and washed with aqueous $KHSO_4$ (1 M; 1 mL x 3) and brine (1 mL x 3). The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude red product was purified by flash column chromatography, eluting with $CHCl_3$/ammonia-saturated MeOH (4:1). The combined fractions from this step were then recrystallised from $CHCl_3$/hexane to yield **2.6L** as a red solid (29 mg; 33 %). R_f = 0.45 ($CHCl_3$/ammonia-saturated MeOH 4:1); m.p. 111–113 °C; $[\alpha]_D$ = - 48 (c = 0.1 in

$CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ =1.30 (t, $J_{H,H}$ = 7.0, 3 H; CH_3,), 1.42 (s, 9 H; $C(CH_3)_3$), 3.05 (m, 2 H; CH_2CH), 3.61 (q, $J_{H,H}$ = 7.0, 2 H; CH_2CH_3), 3.86 (t, J_{HH} = 5.5, 2 H; NCH_2CH_2), 4.19 (t, $J_{H,H}$ = 5.5, 2 H; OCH_2CH_2), 4.53 (m, 1 H; CH), 4.95 (d, $J_{H,H}$ = 8.5, 1 H; N*H*), 6.83 (d, $J_{H,H}$ = 8.6, 2 H; OPh ArC*H*), 6.83 (d, $J_{H,H}$ = 9.0, 2 H; ArC*H*), 7.12 (d, $J_{H,H}$ = 8.6, 2 H; ArC*H*), 7.93 (t, $J_{H,H}$ = 8.5, 4 H; ArC*H*), 8.34 (d, $J_{H,H}$ = 9.0, 2 H; OPh ArC*H*); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 12.35 (CH_3), 28.28 ($C(CH_3)_3$), 36.87 ($CHCH_2$), 46.17 (CH_2CH_3), 49.89 (NCH_2CH_2), 54.39 ($CHCH_2$), 65.34 (OCH_2CH_2), 80.58 ($C(CH_3)_3$, 111.49 (ArCH), 114.65 (OPh ArCH), 122.62 (ArCH), 124.68 (ArCH), 126.28 (ArCH), 128.40 (q-C), 130.50 (OPh ArCH), 143.82 (q-*C*), 147.49 (q-*C*), 151.29 (q-*C*), 156.80 (q-*C*), 157.69 (q-*C*), 173.89 (C=O), 177.73 (C=O); IR (Solid state) ν_{max} = 3341 cm^{-1} (NH), 2928 cm^{-1} (CO_2H), 1705 cm^{-1} (C=O), 1598 cm^{-1} (CONH), 1510, 1335 cm^{-1} (N=O), 1130 cm^{-1} (R-O-R); R_t = 5.3 mins (Chiralpak AD column; 0.8 mL/min; Heptane/ IPA 4:1 254 nm); elemental analysis calcd (%) for $C_{30}H_{35}N_5O_7$ (577.63) C 62.38, H 6.11, N 12.12 found; C 62.49, H 6.37, N 12.15; HRMS (EI) mass calcd for $C_{30}H_{35}N_5O_7$ (577.253469); mass found (577.253693).

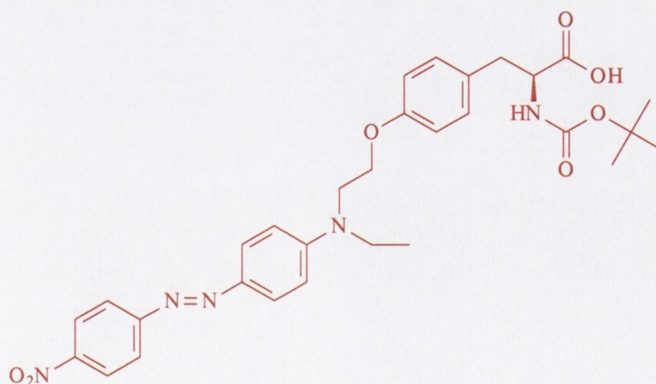
N-Boc-D-tyrosine (O-Disperse Red 1)-O-*p*-nitrobenzyl ester 4.4D



Disperse Red 1 (79 mg; 0.25 mmol) in dry THF (1.5 mL) was added slowly, under Ar, to a solution of **4.1D** (100 mg; 0.25 mmol), Ph_3P (65 mg; 0.25 mmol) and DEAD (40 μL; 0.25 mmol) in THF (2.5 mL) and the reaction mixture stirred at room temperature under Ar. Further additions of Ph_3P (32 mg; 0.125 mmol) and DEAD (20 μL; 0.125 mmol) were made after 30 mins and final additions of Ph_3P (32 mg; 0.125 mmol) and DEAD (20 μL; 0.125 mmol) made after another 20 mins. After a further 30 mins, the

reaction mixture was concentrated under reduced pressure and purified by flash column chromatography, eluting with hexane/ethyl acetate (3:2). A second purification by flash column chromatography, eluting with 4% MeOH/DCM, was performed and the recovered product recrystallised from $CHCl_3$/Hexane to give **4.4D** as a red solid (73 mg; 42 % yield). $R_f = 0.6$ (hexane/ethyl acetate 1:1); m. p. 128-129°C; $[\alpha]_D = +22$ (c = 0.1 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.32 (t, 3 H, $J_{H,H}$ = 7.0; CH_3), 1.44 (s, 9 H; $C(CH_3)_3$), 3.04 (d, $J_{H,H}$ = 6.5, 2 H; CH_2CH), 3.66 (q, $J_{H,H}$ = 7.0, 2 H; CH_2CH_3), 3.89 (t, $J_{H,H}$ = 5.5, 2 H; CH_2CH_2N), 4.17 (t, $J_{H,H}$ = 5.5, 2 H; CH_2CH_2O), 4.60 (m, 1 H; C*H*), 4.96 (d, $J_{H,H}$ = 8.0, 1 H; N*H*), 5.22 (AB quartet, $J_{A,B}$ = 13.6, 2 H; O_2NBnCH_2), 6.78 (d, $J_{H,H}$ = 8.5, 2 H; OPh ArC*H*), 6.86 (d, $J_{H,H}$ = 9.0, 2 H; ArC*H*), 7.01 (d, $J_{H,H}$ = 8.5, 2 H; OPh ArC*H*), 7.38 (d, $J_{H,H}$ = 9.0, 2 H; O_2NBn ArC*H*), 7.97 (t, $J_{H,H}$ = 9.0, 4 H; ArC*H*), 8.19 (d, $J_{H,H}$= 9.0, 2 H; O_2NBn ArC*H*), 8.35 (d, $J_{H,H}$ = 9.0, 2 H; ArC*H*); ^{13}C NMR(100 MHz, $CDCl_3$) δ =12.32 (CH_3), 28.28 ($C(CH_3)_3$), 37.48 (CH_2CH), 46.18 (CH_2CH_3), 49.84 (CH_2CH_2), 54.69 (OCH_2), 65.31 ($NHCH_2$), 65.31 (O_2NArCH), 80.07 ($C(CH_3)_3$), 111.41 (ArCH), 114.51 (ArCH), 122.62 (ArCH), 123.72 (ArCH), 124.68 (ArCH), 126.32, (ArCH), 128.21 (Ar*C*), 129.46 (ArCH), 130.33 (ArCH), 142.39 ($O_2NBnArC$), 143.75 (Ar*C*), 147.38 (Ar*C*), 147.71 (Ar*C*), 151.22 (Ar*C*), 154.82(C=O), 156.77(Ar*C*), 157.61 (q-C), 171.74 (C=O); IR (Solid state) ν_{max} = 3457, 3353 cm^{-1} (NH), ν_{max} = 1738 cm^{-1} (CO_2R), 1678 cm^{-1} (CONH), 1510, 1336 cm^{-1} (N=O), 1130 cm^{-1} (R-O-R).

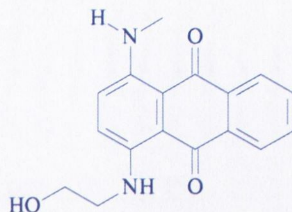
Red *N*-Boc-D-tyrosine (O Disperse Red 1) 2.6D



To **4.4D** (74 mg, 0.1 mmol) in THF (0.5 mL) was added Bu_4NF (1.0 M in THF; 320 μL; 0.32 mmol) in THF (0.5 mL), and the reaction monitored by tlc. After 30 mins, a

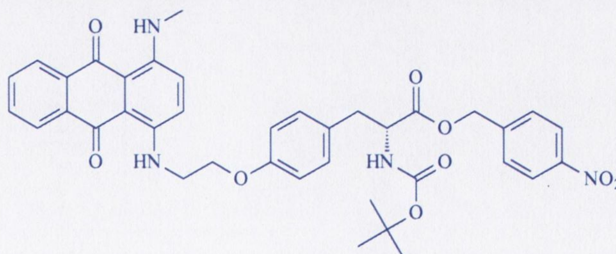
second addition of TBAF (320 μL; 3.2 mmol) was made and after a further 15 mins, H_2O (3 mL) was added to the reaction mixture, followed by ethyl acetate (5 mL). The red product was extracted into the organic phase and washed with aqueous $KHSO_4$ (1 M; 1 mL x 3) and brine (1 mL x 3). The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography, eluting with $CHCl_3$/ammonia-saturated MeOH (4:1). The combined fractions from this step were then recrystallised from $CHCl_3$/hexane to yield **2.6D** as a red solid (20 mg; 33 %). R_f = 0.45 ($CHCl_3$/ammonia-saturated MeOH 4:1); m. p. 112 – 114°C; $[\alpha]_D$ = + 40 (c = 0.1 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ =1.30 (t, $J_{H,H}$ = 7.0, 3 H; CH_3,), 1.41 (s, 9 H; $C(CH_3)_3$), 3.06 (m, 2 H; CH_2CH), 3.63 (q, $J_{H,H}$ = 7.0, 2 H; CH_2CH_3), 3.87 (t, $J_{H,H}$ = 5.5, 2 H; NCH_2CH_2), 4.19 (t, $J_{H,H}$ = 5.5, 2 H; OCH_2CH_2), 4.56 (m, 1 H; *CH*), 4.93 (br d, $J_{H,H}$ = 8.5, 1 H; N*H*), 6.83 (d, $J_{H,H}$ = 8.6, 2 H; OPh ArC*H*), 6.83 (d, $J_{H,H}$ = 9.0, 2 H; ArC*H*), 7.11 (d, $J_{H,H}$ = 8.6, 2 H; ArC*H*), 7.94 (t, $J_{H,H}$ = 8.5, 4 H; ArC*H*), 8.34 (d, $J_{H,H}$ = 9.0, 2 H; OPh ArC*H*); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 12.36 (CH_3), 28.28 ($C(CH_3)_3$), 36.87 ($CHCH_2$), 46.26 (CH_2CH_3), 49.97 (NCH_2CH_2), 54.43 ($CHCH_2$), 65.35 ($OHCH_2CH_2$), 80.63 ($C(CH_3)_3$), 111.71 (Ar*C*H), 114.66 (OPh Ar*C*H), 122.52 (Ar*C*H), 124.71 (Ar*C*H), 126.65 (Ar*C*H), 128.47 (q-C), 130.52 (OPh Ar*C*H), 143.62 (Ar*C*), 147.45 (Ar*C*), 151.86 (Ar*C*), 156.80 (Ar*C*), 157.61 (Ar*C*), 174.03 (q-C); IR (Solid state) ν_{max} = 3341 cm^1 (NH), 2928 cm^{-1}(CO_2H), 1705 cm^{-1} (C=O), 1598 cm^{-1} (CONH), 1510, 1335 cm^{-1} (N=O), 1130 cm^{-1} (R-O-R); elemental analysis calcd (%) for $C_{30}H_{35}N_5O_7$ (577.63) C 62.38, H 6.11, N 12.12 found; C 62.53, H 6.32, N 12.20; HRMS (EI) mass calcd for $C_{30}H_{35}N_5O_7$ (577.253648); mass found (577.253250); R_t = 4.8 min (Chiralpak AD column; 0.8 mL/ min; Heptane/IPA 4:1; 254 nm)

Purification of Disperse Blue 3 4.3[8.3]



Commercially available Disperse Blue 3 (20 % dye content; 1.0 g) was dissolved in acetone (25 mL) and filtered. The filtrate was concentrated under reduced pressure and the residue purified by flash column chromatography, using a column with a diameter of 9 cm, eluting with DCM/acetone (4:1). The blue spot at $R_f = 0.35$ was isolated and concentrated *in vacuo* to give a blue powder which was recrystallised from hexane/$CHCl_3$ to yield **4.3** as a blue solid (68 mg; 6.8 % yield). $R_f = 0.35$ (DCM/acetone 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 3.08$ (d, $J_{H,H} = 5.0$ Hz, 3 H; CH_3), 3.61 (q, $J_{H,H} = 5.5$ Hz, 2 H; CH_2CH_2NH), 3.95 (q, $J_{H,H} = 5.5$ Hz, 2 H; CH_2CH_2OH), 7.15 (d, $J_{H,H} = 9.5$, 1 H; Ar*CH*), 7.26 (t, $J_{H,H} = 9.5$, 1 H; Ar*CH*), 7.68 (apparent septet, 2 H; Ar*CH*), 8.31 (m, 2 H; Ar*CH*), 10.57 (br q, 1 H; N*H*CH_3,), 10.83 (br t, 1 H; N*H*CH_2,); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 28.77$ (CH_3), 44.24 (CH_2CH_2OH), 61.37 (CH_2CH_2NH), 122.29 (Ar*C*H), 124.93 (Ar*C*H), 131.18 (Ar*C*H), 139.08 (Ar*C*H) IR (Solid state) $\nu_{max} = 3412$ cm^{-1} (NH), 1739 cm^{-1} (C=O), 1574, 1524 cm^{-1} (CONH); HRMS (EI): mass calcd for $C_{17}H_{16}N_2O_3$ (296.116092); mass found (296.115372).

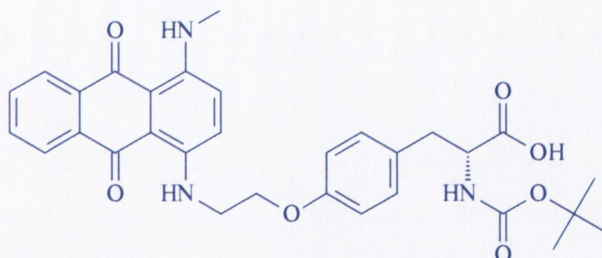
***N*-Boc-L-tyrosine (O-Disperse Blue 3)-O-*p*-nitrobenzyl ester 4.5L**



DEAD (76 μL; 0.48 mmol) was added drop-wise to **4.1L** and Ph_3P (142 mg; 0.48 mmol) in THF (1 mL), under Ar, at 20°C, to give a clear, orange coloured solution, to

which Disperse Blue 3 (127 mg; 0.48 mmol) in THF (5 mL) was added drop-wise, with stirring. The reaction mixture was left to stir under Ar and monitored by tlc. After 90 mins, Ph_3P (71 mg; 0.24 mmol) and DEAD (38 μl; 0.24 mmol) were added to the blue reaction mixture which, after a further 30 mins, was concentrated under reduced pressure and purified by flash column chromatography, eluting with hexane/ethyl acetate (1:1), to yield a blue solid (200 mg), which required further purification by recrystallisation from $CHCl_3$/hexane to yield **4.5L** as a blue solid (116 mg; 36 % yield). R_f = 0.74 ($CHCl_3$/Acetone 4:1); m. p. 189-190 °C; $[\alpha]_D$ = -14 (c = 0.1 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ = 1.39 (s, 9 H; $C(CH_3)_3$), 3.03 (d, $J_{H,H}$ = 5.5, 2 H; $CHCH_2$), 3.12 (d, 3 H, $J_{H,H}$ = 5.0; $NHCH_3$), 3.83 (q, $J_{H,H}$ = 5.5, 2 H; $NHCH_2CH_2$), 4.20 (t, $J_{H,H}$ = 5.5, 2 H; OCH_2CH_2), 4.60 (m, 1 H; C*H*), 4.96 (d; $J_{H,H}$ = 8.5, 1 H; N*H*CH), 5.19 (AB quartet, $J_{H,H}$ = 14.0, 2 H; O_2NBnCH_2), 6.83 (d, $J_{H,H}$ = 8.5, 2 H; O_2NBn ArC*H*), 7.01 (d, $J_{H,H}$ = 8.5, 2 H; O_2NBn ArC*H*), 7.26 (under $CHCl_3$ peak, 1 H; ArC*H*), 7.39 (d, 2 H; ArC*H*), 7.39 (d, 1 H; ArCH), 7.70 (apparent septet, 2 H; ArC*H*), 8.18 (d, $J_{H,H}$ = 9.5, 2 H; O_2NArCH), 8.33 (m, 2 H; ArC*H*); 10.59 (br q, 1 H; N*H*Me),10.60 (br t, 1 H; N*H*); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 28.29 ($C(CH_3)_3$), 29.71 (CH_3), 37.51 ($CHCH_2$), 42.20 (CH_2CH_2N), 54.72 ($CHCH_2$), 65.32 (O_2NArCH), 66.99 (CH_2CH_2O), 77.21 ($C(CH_3)_3$), 110.06 (Ar*C*), 110.49 (Ar*C*), 114.85 (Ar*C*H), 122.97 (O_2NBn Ar*C*H), 123.42 (Ar*C*H), 126.04 (Ar*C*H), 126.11 (Ar*C*H), 128.50 (Ar*C*H), 128.56 (Ar*C*H), 130.30 (O_2NBn Ar*C*H), 132.08 (Ar*C*H), 132.21 (Ar*C*H), 134.38 (Ar*C*), 134.50 (Ar*C*), 142.20 (O_2NBn Ar*C*), 145.70 (Ar*C*), 147.07 (Ar*C*), 147.74 (Ar*C*), 155.10 (Ar*C*), 157.75 (Ar*C*), 160.91 (q-*C*), 171.76 (q-*C*), 182.59 (q-*C*), 182.99 (q-*C*); IR (Solid state) ν_{max} = 3443 cm^{-1} (NH), 1732 cm^{-1} (CO_2R), 1689 cm^{-1} (CONH), 1526, 1346 cm^{-1} (N=O), 1164 cm^{-1} (R-O-R); HRMS (EI) calcd for $C_{38}H_{38}N_4O_9$ (694.23879); mass found (694.264084).

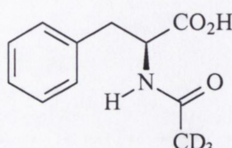
***N*-Boc-L-tyrosine (O-Disperse Blue 3) 2.5L**



TBAF (1 M in THF; 735 μL; 0.74 mmol) in THF (0.5 mL) was added to **4.5L** (100 mg; 0.15 mmol) in THF (1.5 mL) and the reaction mixture monitored by tlc. After 10 mins, a further addition of TBAF (1M in THF; 350 μL; 0.35 mmol) was made. The reaction mixture was concentrated under reduced pressure after a further 20 minutes and the crude residue purified by flash column chromatography, eluting with $CHCl_3$/ammonia-saturated MeOH (4:1). The TBA salt of **2.5L** was isolated, concentrated under reduced pressure, re-dissolved in ethyl acetate (10 mL) and washed with $KHSO_4$ (1 M; 2 mL x 3) and brine (2 mL x 3). The organic phase was dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to give a blue solid which was recrystallised from hexane/$CHCl_3$ to yield **2.5L** as a blue solid (30 mg; 36 %). R_f = 0.81 ($CHCl_3$/ammonia-saturated MeOH 4:1); m. p. 128-130°C; $[\alpha]_D$ = -17 (c = 0.1 in $CHCl_3$) ^1H NMR (400 MHz, $CDCl_3$) δ = 1.42 (s, 9 H; $C(CH_3)_3$), 2.95 (d, 2 H, $CHCH_2$), 3.05 (d, $J_{H,H}$ = 5.5, 3 H; CH_3), 3.73 (q, $J_{H,H}$ = 5.5; 2 H; $NHCH_2CH_2$), 4.20 (t, $J_{H,H}$ = 5.5; 2 H; OCH_2CH_2), 4.53 (m, 1 H, CH), 5.02 (d, $J_{H,H}$ = 8.0, 1 H, NH), 6.86 (d, $J_{H,H}$ = 8.5, 2 H; OPh Ar*CH*), 7.10 (d, $J_{H,H}$ = 8.5, 2 H; OPh Ar*CH*), 7.15 (shoulder of 7.10, d, $J_{H,H}$ = 10.0; 1 H; Ar*CH*), 7.26 (d, $J_{H,H}$ = 10.0; 1 H; Ar*CH*), 7.49 (apparent septet, 2 H; Ar*CH*), 8.24 (m, 2 H; Ar*CH*), 10.62 (q, 1 H; N*H*Me), 10.97 (t, 1 H, J_{HH} = 5.5; NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.32 ($C(CH_3)_3$), 29.49 (CH_3), 36.38 ($CHCH_2$), 42.20 (NCH_2CH_2), 54.43 (CH), 66.99 (CH_2CH_2O), 80.17 ($C(CH_3)_3$), 109.89 (Ar*C*), 110.29 (Ar*C*), 114.78 (ArCH), 126.01 (Ar*C*H), 126.10 (Ar*C*H), 122.97 (Ar*C*H), 123.41 (Ar*C*H), 130.53 (Ar*C*H), 132.02 (Ar*C*H), 132.15 (Ar*C*H), 134.32 (Ar*C*), 134.46 (Ar*C*), 145.79 (Ar*C*), 147.64 (Ar*C*), 155.44 (Ar*C*), 157.59 (Ar*C*), 174.82 (C=O), 175.08 (C=O), 182.34 (C=O), 182.72 (C=O); (Solid state) ν_{max} = 2963 cm^{-1} (CO_2H), 1705 cm^{-1} (C=O), 1573 cm^{-1} (CONH), 1510, 1336 cm^{-1} (N=O), = 1161 cm^{-1} (R-O-R); elemental analysis calcd

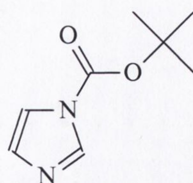
(%) for $C_{31}H_{33}N_3O_7$ (559.61): C 66.53, H 5.94, N 7.51; found C 66.68, H 6.38, N 7.56; HRMS (EI) calcd for $C_{31}H_{33}N_3O_7$ (559.231850); mass found (559.233032); R_t = 5.4 mins (Chiralpak AD column; 1.0 mL/min; Heptane/IPA 4:1; 254 nm);

N-Acetyl-d$_3$-L-phenylalanine 4.6L



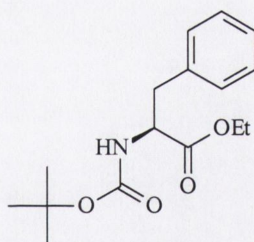
To a mixture of (Acetic Anhydride)-d_6 (200 μL; 2.1 mmol) and L-phenylalanine (269 mg; 1.63 mmol) in THF (2 mL) was added aqueous $NaHCO_3$ (10% w/v; 1.3 mL), to give exothermic dissolution. The resulting 2-phase reaction mixture was stirred vigorously for 90 mins. Organic solvent was then removed under reduced pressure and the aqueous phase adjusted to pH 1. This led to precipitation of **4.6L** as a white solid, which was filtered and dried *in vacuo*. The material was recrystallised from $CHCl_3$/hexane to give a white crystalline solid (283 mg; 83 % yield). R_f = 0.64 ($CHCl_3$/MeOH/AcOH 10:2:1); $[\alpha]_D$ = + 25 (c = 0.1 in $CHCl_3$); ^{1}H NMR (400 MHz, DMSO): δ = 2.85 (dd, $J_{A,B}$ = 13.5, $J_{A,X}$ = 9.6, 1 H; CH_2), 3.05 (dd, $J_{A,B}$ = 13.5, $J_{B,X}$ = 4.6, 1 H; CH_2), 4.33 (m, 1 H; CH), 7.12 (m, 5 H; Ar*CH*), 8.22 (d, 1 H, $J_{H,H}$ = 8.0; NH), 12.5 (s, 1 H; OH); ^{13}C NMR (100 MHz, DMSO) δ = 26.09 (CD_3), 35.74 (CH_2), 51.24 (CH), 124.19 (ArCH), 126.38 (Ar*C*H), 127.76 (ArCH), 135.77 (q-C), 168.17 (C=O), 171.94 (C=O); IR (Solid state) ν_{max} = 3327 cm^{-1}, 1546 cm^{-1} (NH), 2912 cm^{-1} (CO_2H), 1694 cm^{-1} (CONH); elemental analysis calcd (%) for $C_{11}H_{10}D_3NO_3$ (207.22): C 62.84, H 4.79, N 6.66; found C 62.81, H 5.42, N 6.43; HRMS (EI) mass calcd $C_{11}H_{10}D_3NO_3$ (210.108373); found 210.107746.

1-(*t*-Butyloxycarbonyl) imidazole 4.8[4.6]



t-BuOH (200 μL; 2.07 mmol) and DBU (619 μL; 2.07 mmol) were added to CDI (369 mg; 2.07 mmol) in DCM (3 mL), under Ar, in an ice-water bath, to give a clear, slightly yellow coloured solution. The solution was stirred for 1 hr, after which time it was concentrated under reduced pressure and purified by flash column chromatography, eluting with hexane/ethyl acetate (3:1). **4.8** was recovered as a white solid (150 mg; 42 % yield). R_f = 0.54 (hexane/ethyl acetate 3:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.64 (s, 9 H; C(CH_3)$_3$), 7.06 (d, 1 H), 7.39 (d, 1 H), 8.09 (s, 1 H; 2*CH*); ^{13}C NMR (100MHz, $CDCl_3$) δ = 27.4 (C(CH_3)$_3$), 85.12 (*C*(CH_3)$_3$; 116.2 (C*H*), 129.8 (*C*H), 136.2 (*C*H), 146.63 (*C*=O).

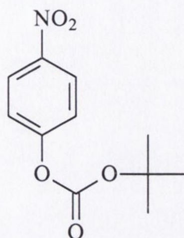
***N*-Boc-L-phenylalanine ethyl ester 4.10**



Aqueous $NaHCO_3$ (10 % w/v; 200 μL) was added to a mixture of L-Phe ethyl ester hydrochloride (50 mg; 0.2 mmol) and 1-(*t*-Butyloxycarbonyl) imidazole (37 mg; 0.22 mmol) in THF (1 mL) and stirred at 35 °C, under reflux conditions, overnight. The solution was then concentrated under reduced pressure and purified by flash column chromatography, eluting with ethyl acetate/hexane (3:1). **4.10** was recovered as a white solid (16 mg; 23 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.25 (t, $J_{H,H}$ = 7.0, 3 H; CH_2CH_3), 1.44 (s, 9 H, C(CH_3)$_3$), 3.11 (d, $J_{H,H}$ = 6.5, 2 H; $CHCH_2$), 4.18 (q, $J_{H,H}$ = 7.0, 2 H; CH_2CH_3), 4.57 (m, 1 H; CH), 4.99 (br d, 1 H; NH), 7.26 (m, 5 H; ArC*H*); ^{13}C NMR (100 MHz; $CDCl_3$) δ = 13.62 (CH_3), 27.83 (C(CH_3)$_3$), 37.99 (CH_2CH_3), 54.02

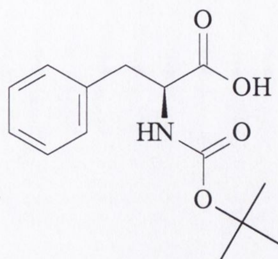
(CH), 60.82 (CH_2CH), 85.08 (q-C), 126.49 (Ar*C*H), 128.00 (Ar*C*H), 128.89 (Ar*C*H), 135.66 (Ar*C*), 166.54 (*C*=O); 171.39 (*C*=O).

t-Butyl-p-nitrophenyl carbonate 4.9[4.11]



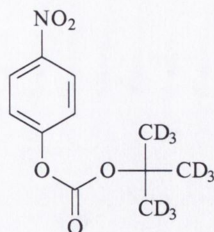
A solution of p-nitrophenylchloroformate (806 mg; 4.0 mmol) in DCM (4 mL) was added gradually, under Ar, to a solution of t-BuOH (200 μL; 2.0 mmol) and pyridine (404 μL; 5.0 mmol) in DCM (3 mL), resulting in an immediate precipitation of pyridinium hydrochloride. After 30 mins, the reaction mixture was filtered to remove the precipitate. The filtrate was washed with HCl (0.5 N; 2 mL x 3), the organic phase extracted, dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The resulting crude product was purified by flash column chromatography, eluting with hexane/ethyl acetate (7:2), to yield **4.9** as a hard white solid (313 mg; 65 % yield). R_f = 0.76 (hexane/ethyl acetate 7:2); m.p. 78 - 80°C (lit. [4.7] 79 - 80°C); ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 1.44$ (s, 9 H, t-Bu), 7.39 (d, $J_{H,H} = 9.0$, 2 H; Ar*CH*), 8.29 (d, $J_{H,H} = 9.0$, 2 H; Ar*CH*); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 27.17$ (C(CH_3)$_3$), 84.35 (*C*(CH_3)$_3$), 121.42 (Ar*C*H), 124.71 (Ar*C*H), 150.06 (q-C), 155.27 (q-C), 167.33 (C=O).

***N*-Boc-L-phenylalanine 4.12**[4.8]



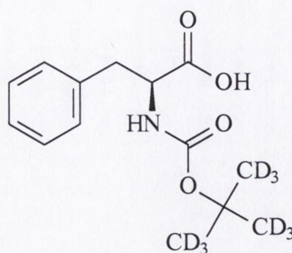
To **4.9** (181 mg; 0.9 mmol) in *t*-BuOH (1 mL), at 30 °C, was added a solution of L-phenylalanine (100 mg; 0.6 mmol) and Na_2CO_3 (159 mg; 1.5 mmol) in H_2O (0.6 mL), to yield a clear, yellow, 2-phase solution, which was left to reflux at 30°C for 2 hrs. After this time, *t*-BuOH was removed under reduced pressure, precipitated *p*-nitrophenolate dihydrate removed by filtration and the filtrate adjusted to pH 6 with HCl (0.5 N) before extraction into diethyl ether (5 mL x 4) to remove any remaining **4.9** and *p*-nitrophenol. The aqueous portion was adjusted to pH 1 and **4.12** was extracted into diethyl ether (5 mL x 4). The combined organic extract was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to give an oily residue. After drying *in vacuo* for 2 days, the product remained as an oily film (84 mg; 53 %). R_f = 0.4 ($CHCl_3$/ammonia-saturated MeOH 4:1); $[\alpha]_D$ = +25 (c = 0.1 in ethanol);[4.12] ^1H NMR (400 MHz, $CDCl_3$) δ = 1.23 (s, 9 H; C(CH_3)$_3$), 3.20 (m, 2 H; CH_2), 4.63 (br q, 1 H, C*H*), 4.97 (br d, 1 H; N*H*), 7.30 (m, 5 H; ArC*H*); ^{13}C NMR (100 MHz, $CDCl_3$) 27.81 (C(CH_3)$_3$), 37.34 (CH_2), 53.83 (CH), 88.65 (*C*(CH_3)$_3$), 125.84 (ArCH), 126.62 (Ar*C*H), 128.53 (Ar*C*H), 150.10 (q-C), 175.71 (C=O), 209.35 (C=O); IR: ν_{max} = 3307cm^{-1} (NH), 2978 cm^{-1} 2978 (CO_2H), 1645 cm^{-1} (CONH)

t-Butyl-d$_9$ p-nitrophenyl carbonate 4.11



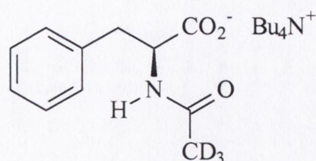
A solution of p-nitrophenylchloroformate (536 mg; 2.66 mmol) in DCM (3 mL) was added gradually, under Ar, to a solution of t-BuOH-d$_{10}$ (100 μL; 1.33 mmol) and pyridine (270 μL; 3.33 mmol) in DCM (3 mL), resulting in an immediate precipitation of pyridinium hydrochloride. After 30 mins, the reaction mixture was filtered to remove the precipitate. The filtrate was washed with HCl (0.5 N; 1 mL x 3, the organic phase was extracted, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The resulting crude product was purified by flash column chromatography, eluting with hexane/ethyl acetate (7:2), to give **4.11** as a hard white solid (222 mg; 67 % yield). R_f = 0.76 (hexane/ethyl acetate 7:2); m.p. 78 - 80°C; ^{1}H NMR (400 MHz, CDCl$_3$) δ = 7.38 (d, $J_{H,H}$ = 9.0, 2 H; ArC*H*), 8.28 (d, $J_{H,H}$ = 9.0, 2 H; ArC*H*); ^{13}C NMR (100 MHz, CDCl$_3$) δ = 30.46 (C(*C*D$_3$)$_3$, 84.35 (*C*(CD$_3$)$_3$), 121.22 (Ar*C*H), 124.58 (Ar*C*H),150.36 (q-C), 155.63 (q-C), 168.02 (q-C); IR (Solid state) ν_{max} = 1752 cm^{-1} (C=O), 1521 cm^{-1}, 1347 cm^{-1} (N=O); elemental analysis calcd (%) for $C_{11}H_4D_9NO_5$ (248.28): C 53.21, H 1.62, N 5.85; found C 53.46, H 4.92, N 5.67: HRMS (EI) mass calcd (248.135863); found 248.135376.

***N*-Boc-d$_9$ L-phenylalanine 4.7L**



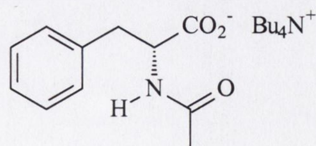
To **4.11** (90 mg; 3.6 mmol) in *t*-BuOH (2 mL), at 30 °C, was added a solution of L-phenylalanine (40 mg; 2.4 mmol) and Na_2CO_3 (64 mg; 6 mmol) in H_2O (1 mL), to yield a clear, yellow, 2-phase solution, which was refluxed at 30°C for 2 hrs. After this time, *t*-BuOH was removed under reduced pressure, precipitated *p*-nitrophenolate dihydrate was removed by filtration and the filtrate adjusted to pH 6 with HCl (0.5 N) before extraction into diethyl ether (5 mL x 4) to remove any remaining **4.11** and *p*-nitrophenol. The aqueous portion was adjusted to pH 1 and **4.7L** was extracted into diethyl ether (5 mL x 4). The combined organic extract was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to give an oily residue. After drying *in vacuo* for 2 days, **4.7L** remained as an oily film (41 mg; 62 %). R_f = 0.4 ($CHCl_3$/ammonia-saturated MeOH 4:1); $[\alpha]_D$ = +17 (c = 0.1 in ethanol); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.20 (m, 2 H; CH_2), 4.62 (br q, 1 H; C*H*), 4.97 (br d, 1 H; N*H*), 7.30 (m, 5 H; ArC*H*); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 30.26 (CD_3), 36.35 (CH_2), 54.12 (CH), 69.40 (*C*(CD_3)$_3$), 125.82 (ArCH), 127.01 (Ar*C*H), 128.53 (ArCH), 155.11 (q-C), 175.71 (C=O), 209.35 (C=O); IR (Solid state) ν_{max} = 3307 cm^{-1} (NH), 2978 cm^{-1} (CO_2H), 1645 cm^{-1} (CONH).

Tetrabutylammonium *N*-Ac-d$_3$-L-phenylalaninate (TBA 4.6L)



A solution of TBACl.H_2O (63 mg; 0.21 mmol) in $CHCl_3$ (6.5 mL) was added to a solution of **4.6L** (50 mg; 0.24 mmol) and NaOH (9.6 mg; 0.24 mmol) in H_2O (1 mL). The 2-phase mixture was stirred vigorously for 4 hrs after which time the phases were allowed to separate and the organic phase isolated. Further extractions were performed between the aqueous phase and $CHCl_3$ (6.5 mL x 3), the combined organic phase dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. H_2O was removed as azeotropes with toluene (6 mL x 3) and then DCM (6 mL x 3) before drying *in vacuo* for 10 hrs and storing under Ar. TBA **4.6L** was recovered as a slightly yellow coloured viscous oil (94 mg; 87 %). ^1H NMR (400 MHz, $CDCl_3$) δ = 1.01 (t, $J_{H,H}$ = 7.5, 12 H; CH_3), 1.45 (sx, $J_{H,H}$ = 7.5, 8 H; CH_2CH_3), 1.63 (m, 8 H; $CH_2CH_2CH_3$), 3.26 (dd, $J_{A,B}$ = 13.0, $J_{A,X}$ = 5.0, 1 H; CH_2), 3.32 (shoulder of 3.36, 1 H; CH_2); 3.36 (m, 8 H; $CH_2NH_3^+$), 4.40 (m, 1 H; CH), 6.69 (d, $J_{H,H}$ = 8.0, 1 H; N*H*), 7.12 (m, 5 H; ArC*H*), ^{13}C NMR (100 MHz, $CDCl_3$) δ = 12.70 (CH_3), 18.65 (CH_2CH_3), 21.98 (CD_3), 22.68 ($CH_2CH_2CH_3$), 37.92 (CH_2CH), 55.45 (CH), 57.83($CH_2NH_3^+$), 124.79 (Ar*C*H), 126.69 (Ar*C*H), 129.54 (Ar*C*H), 138.90 (Ar*C*), 169.93 (C=O), 173.8 (C=O).

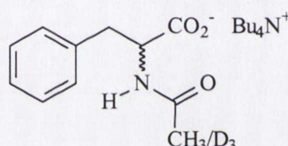
Tetrabutylammonium *N*-Ac D-phenylalaninate (TBA 4.6 D)



A solution of TBACl.H_2O (64 mg; 0.22 mmol) in $CHCl_3$ (6.5 mL) was added to a solution of **4.6D** (50 mg; 0.24 mmol) and NaOH (9.6 mg; 0.24 mmol) in H_2O (1 mL). The 2-phase mixture was stirred vigorously for 4 hrs after which time the product was extracted into the organic phase. After further $CHCl_3$ extractions (6.5 mL x 3), the

combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. H_2O was removed as azeotropes with toluene (6 mL x 3) and then DCM (6 mL x 3) before drying *in vacuo* for 10 hrs and storing under Ar. TBA **4.6D** was obtained as a slightly yellow coloured viscous oil (83 mg; 74 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.01 (t, $J_{H,H}$ = 7.5, 12 H; CH_3), 1.44 (sx, $J_{H,H}$ = 7.5, 8 H; CH_2CH_3), 1.66 (m, 8 H; $CH_2CH_2CH_3$), 1.88 (s, 3 H; CH_3), 3.25 (dd, $J_{A,B}$ = 13.0, $J_{A,X}$ = 5.2, 1 H; CH_2), 3.30 (shoulder of 3.32, 1 H; CH_2); 3.32 (m, 8 H; $CH_2NH_3^+$), 4.43 (m, 1 H; CH), 6.71 (d, $J_{H,H}$ = 8.0, 1 H; N*H*), 7.24 (m, 5 H; ArC*H*); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 13.05 (CH_3), 18.82 (CH_2CH_3), 22.79(CH_3), 23.52 ($CH_2CH_2CH_3$), 37.23 (CH_2CH), 55.64 (CH), 58.52 ($CH_2NH_3^+$), 124.94 (Ar*C*H), 127.10 (Ar*C*H), 128.91 (Ar*C*H), 139.14 (Ar*C*), 170.93 (C=O), 175.24 (C=O).

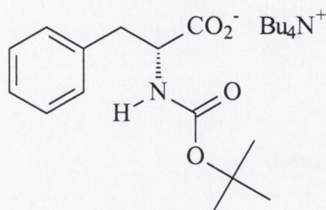
Tetrabutylammonium {*N*-Ac-D-Phe/*N*-Ac-d$_3$-L-Phe} (TBA 4.6DL)



A solution of TBACl.H_2O (51 mg; 0.17 mmol) in $CHCl_3$ (6 mL) was added to a solution of **4.6L** (20 mg; 0.095 mmol), **4.6D** (19.7 mg; 0.095 mmol) and NaOH (7.6 mg; 0.17 mmol) in H_2O (1 mL). The 2-phase mixture was stirred vigorously for 4 hrs after which time the phases were allowed to separate and the organic phase isolated. Further extractions were performed between the aqueous phase and $CHCl_3$ (6 mL x 3), the combined organic phase dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. H_2O was removed as azeotropes with toluene (6 mL x 3) and then DCM (6 mL x 3) before drying *in vacuo* for 10 hrs and storing under Ar. TBA **4.6DL** was recovered as a slightly yellow coloured viscous oil (64 mg; 85 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.01 (t, $J_{H,H}$ = 7.5, 12 H; CH_3), δ = 1.45 (sx, $J_{H,H}$ = 7.5, 8 H; CH_2CH_3), 1.63 (m, 8 H; $CH_2CH_2CH_3$), 1.77 (s, 1.5 H; CH_3), 3.28 (dd, $J_{A,B}$ = 13.0, $J_{A,X}$ = 5.2, 1 H; CH_2), 3.33 (shoulder of 3.36, 1 H; CH_2), 3.36 (m, 8 H; $CH_2NH_3^+$), 4.40 (m, 1 H; CH), 6.69 (d, $J_{H,H}$ = 8.0, 1 H; N*H*), 7.12 (m, 5 H; ArC*H*); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 13.01 (CH_3), 19.38 (CH_2CH_3), 22.68 (CH_3), 23.63 ($CH_2CH_2CH_3$), 37.51

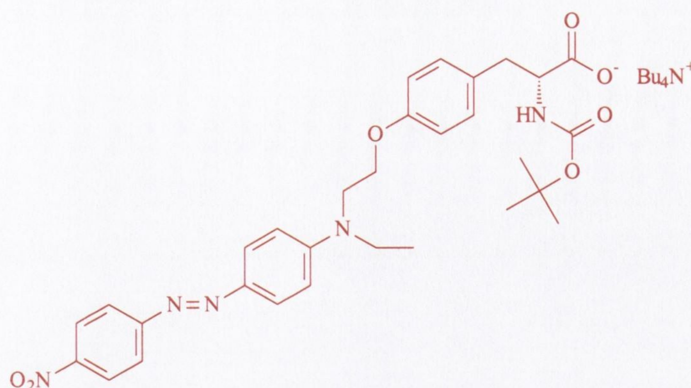
(CH_2CH), 55.52 (CH), 58.45 ($CH_2NH_3^+$), 125.14 (Ar*C*H), 127.18 (Ar*C*H), 129.53 (ArCH), 138.90 (Ar*C*), 169.93 (C=O), 173.8 (C=O).

Tetrabutylammonium *N*-Boc-D-phenylalaninate (TBA 4.7D)



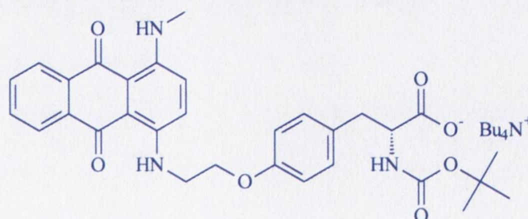
A solution of TBACl.H_2O (50.6 mg; 0.17 mmol) in $CHCl_3$ (8 mL) was added to **4.7D** (50 mg; 0.19 mmol) and NaOH (7.5 mg; 0.19 mmol) in H_2O (1.5 mL). The 2-phase mixture was stirred vigorously for 4 hrs after which time the phases were allowed to separate and the organic phase isolated. Further extractions were performed between the aqueous phase and $CHCl_3$ (8 mL x 3), the combined organic phase dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. H_2O was removed as azeotropes with toluene (8 mL x 3) and then DCM (8 mL x 3) before drying *in vacuo* for 10 hrs and storing under Ar. TBA **4.7D** was recovered as an oily film (72 mg; 75 %). ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 0.98$ (t, $J_{H,H} = 7.5$, 12 H; CH_2CH_3), 1.35 (s, 9 H; $C(CH_3)_3$), 1.41 (sx, $J_{H,H} = 7.5$, 8 H; CH_2CH_3), 1.61 (br m, 8 H; $CH_2CH_2CH_3$), 3.11 (m, 2 H; $CHCH_2$), 3.26 (m, 8 H; $CH_2NH_3^+$), 4.19 (br q, 1 H; C*H*), 5.60 (br d, 1 H; N*H*), 7.25 (m, 5 H; ArC*H*); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 13.15$ (CH_3), 19.41 (CH_2CH_3), 23.35 ($CH_2CH_2CH_3$), 27.76 ($C(CH_3)_3$), 37.12 ($CHCH_2$), 57.40 (CH), 58.34 ($CH_2NH_3^+$), 124.61 (Ar*C*H), 127.16 (Ar*C*H), 129.48 (Ar*C*H), 137.85 (q-C), 138.75 (q-C), 155.21 (C=O), 177.71 (C=O).

Tetrabutylammonium Red *N*-Boc L-Tyr (TBA 2.6L)



A solution of NaOH (0.7 mg; 0.017 mmol) in H_2O (200 μL) was added to a solution of **2.6L** (10 mg; 0.017 mmol) and TBACl (5.1 mg; 0.017 mmol) in $CHCl_3$ (1 mL) and stirred for 45 mins. The red product was then extracted into the organic phase, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness, before drying *in vacuo* for 5 hrs to give TBA **2.6L** as a red solid (13 mg; 91 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.99 (t, $J_{H,H}$ = 7.5, 12 H; CH_3), 1.26 (t, $J_{H,H}$ = 6.9, 3 H; CH_2CH_3), 1.42 (s, 9 H, $C(CH_3)_3$), 1.44 (sx, $J_{H,H}$ = 7.5, 8 H; $CH_2CH_2CH_3$), 1.89 (m, 8 H; $CH_2CH_2CH_3$), 3.08 (m, 2 H; CH_2CH), 3.28 (m, 8 H; $CH_2NH_3^+$), 3.56 (q, $J_{H,H}$ = 7.0, 2 H; CH_2CH_3), 3.80 (t, $J_{H,H}$ = 5.5, 2 H; CH_2CH_2N), 4.15 (t, $J_{H,H}$ = 5.6, 2 H; CH_2CH_2O), 4.59 (m, 1 H; C*H*), 4.99 (d, 1 H; NH), 6.73 (d, $J_{H,H}$ = 9.2, 2 H; OPh ArC*H*), 6.80 (d, $J_{H,H}$ = 8.9, 2 H; ArC*H*), 7.19 (d, $J_{H,H}$ = 8.6, 2 H; OPh ArC*H*), 7.91 (t, $J_{H,H}$ = 8.9, 4 H; ArC*H*), 8.32 (d, $J_{H,H}$ = 8.9, 2 H; ArC*H*).

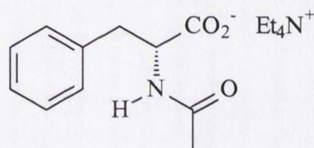
Tetrabutylammonium Blue *N*-Boc-L-Tyr (TBA 2.5L)



A solution of NaOH (1.1 mg; 0.028 mmol) in H_2O (350 μL) was added to a solution of **2.5L** (16 mg; 0.028 mmol) and TBACl (8.4 mg; 0.028 mmol) in $CHCl_3$ (1.5 mL) and stirred for 45 mins. The blue product was then extracted into the organic phase, dried

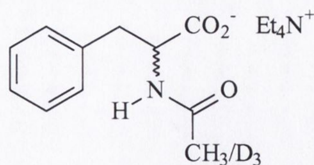
over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure before drying *in vacuo* for 5 hrs to give TBA **2.5L** as a blue solid (22 mg; 96 %). ^{1}H NMR (400 MHz; $CDCl_3$) δ = 0.99 (t, $J_{H,H}$ = 7.5, 12 H; $CH_2CH_2CH_3$), 1.37 (s, 9 H; $C(CH_3)_3$), 1.41 (sx, $J_{H,H}$ = 7.5, 8 H; $CH_2CH_2CH_3$), 1.64 (m, 8 H; $CH_2CH_2CH_3$), 3.12 (m, 5 H; $NHCH_3$, $CHCH_2$), 3.32 (m, 8 H; $CH_2NH_3^+$), 3.81 (q, $J_{H,H}$ = 5.5, 2 H; OCH_2CH_2), 4.20 (t, $J_{H,H}$ = 5.5, 2 H; NCH_2CH_2), 4.62 (br, 1 H; $CHCH_2$), 4.98 (d, $J_{H,H}$ = 8.5; 1 H $NHCH_3$), 6.75 (d, 2 H; ArCH), 7.17 (d, 2 H; ArCH), 7.20 (shoulder of 7.17, 1 H; ArCH) 7.26 (under $CHCl_3$ peak; 1 H, ArCH); 7.70 (m, 2H; ArCH), 8.32 (m, 2 H; ArCH), 10.66 (q, 1 H; NHMe), 10.99 (t, 1 H; $NHCH_2$).

Tetraethylammonium N-Ac-D-phenylalaninate (TEA 4.6D)



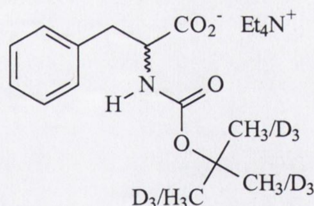
A solution of **4.6D** (50 mg; 0.24 mmol) in H_2O/MeOH (4:1) (1 mL) was titrated against an aqueous solution of TEAOH (3.5 % w/v) until pH 7.2 was reached (pH meter). The solution was concentrated under reduced pressure and H_2O was removed as azeotropes with toluene and then DCM. The resulting residue was dried *in vacuo* for several hrs to yield TEA **4.6D** as an oil (80 mg; 99.3 %). ^{1}H NMR (400 MHz, DMSO) δ = 1.28 (t, $J_{H,H}$ = 7.3, 12 H; CH_2CH_3), 1.88 (s, 3 H; CH_3), 3.16 (dd, $J_{A,B}$ = 13.0, $J_{A,X}$ = 5.4, 1 H; CH_2), 3.26 (dd, $J_{A,B}$ = 13.0, $J_{B,X}$ = 7.8, 1 H; CH_2), 3.35 (q, $J_{H,H}$ = 7.3, 8 H; CH_2CH_3), 4.39 (m, 1 H; CH_2CH), 7.10 (m, 5 H; ArCH), 8.33 (d, $J_{H,H}$ = 8.0, 1 H; NH).

TEA {N-Ac-D-Phe/N-Ac-d$_3$-L-Phe} (TEA 4.6DL)



A solution of **4.6D** (20 mg; 0.1 mmol) and **4.6L** (19.7 mg; 0.1 mmol) in H_2O/MeOH (4:1) (1 mL) was titrated against an aqueous solution of TEAOH (3.5% w/v) until pH 7.2 was reached (pH meter). The solution was concentrated under reduced pressure and H_2O was removed as azeotropes with toluene and then DCM. The resulting residue was dried *in vacuo* for several hrs to yield TEA **4.6DL** as an oil (67 mg; 99.2 %). ^1H NMR (400 MHz, DMSO) δ = 1.35 (t, $J_{H,H}$ = 7.3, 12 H; CH_2CH_3), 1.91 (s, 1.5 H; CH_3), 3.21 (dd, $J_{A,B}$ = 12.9, $J_{A,X}$ = 5.3, 1 H; CH_2), 3.31 (dd, $J_{A,B}$ = 12.9, $J_{A,X}$ = 8.3, 1 H; CH_2), 3.40 (q, $J_{H,H}$ =7.3, 8 H; CH_2CH_3), 4.43 (m, 1 H; CH_2CH), 7.13 (m, 5 H; Ar*CH*), 8.33 (d, $J_{H,H}$ = 8.0, 1 H; NH).

TEA {*N*-Boc-D-Phe/*N*-Boc-d$_9$-L-Phe} (TEA 4.7DL)



4.7D (9.6 mg; 0.036 mmol), **4.7L** (10 mg; 0.036 mmol), NaOH (0.28 mg; 0.072 mmol) and TEACl (12 mg; 0.072 mmol) in $CHCl_3$ (1mL) and H_2O (0.1 mL) were mixed together to give a 2-phase solution. The solution was stirred vigorously for 1 hr after which time the phases were allowed to separate and the organic phase isolated. After further $CHCl_3$ extractions (1 mL x 3), the combined organic extract was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The resulting oil was dried *in vacuo* for several hrs to yield TEA **4.7DL** as a viscous, slightly yellow coloured oil (24 mg; 84 %). ^1H NMR (400 MHz, DMSO) δ = 1.33 (t, $J_{H,H}$ =10.7, 12 H;

CH_2CH_3), 1.43 (s, 4.5 H; $C(CH_3)_3$), 3.22 (m, 2 H; CH_2), 3.39 (q, $J_{H,H}$ = 10.7, 8 H; CH_2CH_3), 4.30 (br q, 1 H; CH), δ = 5.46 (br d, 1 H; NH), 7.23 (m, 5 H; ArCH).

Expt 5.1: Exposure of 5.3 to TBA 2.6L (1 mM in $CHCl_3$)

TBA **2.6L** (1 mM in $CHCl_3$; 0.5 mL) was applied to **5.3** (100 beads) in a Petri dish of diameter 2 cm. The system was placed in a jam-jar, the bottom of which was covered with $CHCl_3$ and the lid tightly replaced. The system was left for 4 hrs, after which time it was removed and inspected. The beads appeared red and the solution appeared a light red colour. The beads were transferred to a filtration tube fitted with a frit and filtered to remove excess guest. The beads were washed with $CHCl_3$ (0.5 mL) by shaking the filtration tube, which was sealed at both ends with rubber septa. The filtrate was observed to have a red hue. Two further washes were performed with $CHCl_3$ (5 mL x 3), but the recovered $CHCl_3$ was colourless in each case, while the beads remained bright red. A solution of 1 % MeOH in $CHCl_3$ (0.5 mL) was applied to the beads within the capillary tube and the guest was observed to have decomplexed from the solid phase after 1 hr such that, after filtration, the beads appeared colourless and the supernatant phase red.

Expt 5.2: Exposure of 5.3 to TBA 2.6L (1 mM in 1 % MeOH/$CHCl_3$).

TBA **2.6L** (1 mM in 1 % MeOH/$CHCl_3$; 0.5 mL) was applied to **5.3** (100 beads) in a Petri dish of diameter 2 cm. The system was placed in a jam-jar, the bottom of which was covered with 1 % MeOH/$CHCl_3$ and the lid tightly replaced. The system was inspected at 5 minute intervals and, after 35 minutes, all beads were observed to have acquired a uniform red colour. The beads were replaced in the jar and examined after a further 25 minutes but no change in appearance was noted. The beads were transferred to a filtration tube fitted with a frit and filtered to remove excess guest. 1 % MeOH/$CHCl_3$ (0.5 mL) was added to the beads in the filtration tube, which was sealed at both ends with rubber septa. After 1 hr the supernatant phase was observed to have become red and the beads colourless.

Expt 5.3: Exposure of 5.3 to TBA 2.6L (1 mM in 1 % MeOH/$CHCl_3$); cleavage with MeOH/$CHCl_3$ (1:1)

TBA **2.6L** (1 mM in 1 % MeOH/$CHCl_3$; 0.5 mL) was applied to **5.3** (100 beads) in a Petri dish of diameter 2 cm. The system was placed in a jam-jar, the bottom of which was covered with 1 % MeOH/$CHCl_3$ and the lid tightly replaced. After 1 hr, when all beads were observed to have a uniform red colour, the beads were transferred to a filtration tube fitted with a frit and filtered to remove excess guest. The beads were washed with $CHCl_3$ (0.5 mL) to remove un-bound guest. MeOH/$CHCl_3$ (1:1; 0.5 mL) was added to the beads in the filtration tube, which was sealed at both ends with rubber septa. Upon shaking the filtration tube, the coloured guest was immediately observed to enter the solution phase, rendering the solid phase colourless. The coloured solution was filtered and the beads washed twice more with MeOH/$CHCl_3$ (1:1; 0.5 mL). The beads were isolated and observed to be colourless.

Expt 5.4: Exposure of 5.3 to TBA 2.6L (1 mM in DCM)

TBA **2.6L** (1 mM in DCM; 0.5 mL) was applied to **5.3** (100 beads) in a Petri dish of diameter 2 cm. The system was placed in a jam-jar, the bottom of which was covered with DCM and the lid tightly replaced. The system was left for 1 hr, after which time it was removed and inspected. The beads appeared red and the solution appeared a light red colour. The beads were transferred to a filtration tube fitted with a frit and filtered to remove excess guest. The beads were washed with DCM (0.5 mL) by shaking the filtration tube, which was sealed at both ends with rubber septa. The filtrate was observed to have a red hue. Two further washes were performed with DCM (5 mL x 3), but the recovered DCM was colourless in each case, while the beads remained intensely red.

Expt 5.5: Exposure of 5.3 to TBA 2.6L in varying percentages of MeOH in DCM; de-complexation with varying percentages of MeOH in DCM.

TBA **2.6L** (1 mM in 1 % MeOH/DCM; 0.5 mL) was applied to 5.3 (100 beads) in a Petri dish of diameter 2 cm. The system was placed in a jam-jar, the bottom of which was covered with 1 % MeOH/DCM, and the lid tightly replaced. The system was left for 1 hr, after which time it was removed and inspected. The beads appeared red and the supernatant phase a light red colour. The beads were transferred to a filtration tube fitted with a frit and filtered to remove excess guest. 1 % MeOH/DCM (0.5mL) was added to the beads in the filtration tube, which was sealed at both ends with rubber septa. After 1 hr, the solution was observed to have become red and the beads colourless.

The experiment was repeated using 2 %, 5 % and 10 % MOH/DCM in all of which cases, guest was observed to complex as for 1 % MeOH/DCM and, during decomplexation, to be drawn into the solution phase over a period of about 1 hr.

Expt 5.6: Exposure of 5.3 to TBA 2.6L (0.1 mM in 1 % MeOH/$CHCl_3$).

TBA **2.6L** (0.1 mM in 1 % MeOH/$CHCl_3$; 0.5 mL) was applied to **5.3** (100 beads) in a Petri dish of diameter 2 cm. The system was placed in a jam-jar, the bottom of which was covered with 1 % MeOH/$CHCl_3$ and the lid tightly replaced. After 1 hr, all beads were observed to have acquired a uniform light red to orange colour. The beads were transferred to a filtration tube fitted with a frit and filtered to remove excess guest. MeOH/$CHCl_3$ (1:1; 0.5 mL) was added to the beads in the filtration tube, which was sealed at both ends with rubber septa. The beads became colourless and the solution light red immediately.

Expt 5.7: Preparation of tetrabutylammonium salt of equimolar 4.6DL

To a mixture of *N*-Ac-d_3-L-Phe (40 mg; 0.19 mmol) and *N*-Ac-D-Phe (39.4 mg; 0.19 mmol), both of which had been separately dried *in vacuo* for several hours, was added MeOH (10mL), to yield a solution which was stirred at room temperature for 5 minutes before concentrating under pressure to yield a white solid, **4.6DL**, which was dried *in vacuo* for 4 hours (79.4 mg). R_f =0.64 ($CHCl_3$/MeOH/AcOH 10:2:1); ^{1}H NMR (DMSO): δ = 1.77 (s, 1.5 H; CH_3), 2.86 (dd; $J_{A,B}$ = 13.5, $J_{A,X}$ = 9.6; CH_2), 3.06 (dd; $J_{A,B}$ = 13.5, $J_{A,X}$ = 4.6; CH_2), 4.37 (q, J = 4.9, 1 H; CH); 7.26 (m, 5 H; ArCH), 8.22 (d, 1 H, J = 8.2; NH); ESMS$^-$: peaks of equal intensity at 206 and 209 amu. **4.6DL** (39.7 mg) was dissolved, with stirring, in a solution of NaOH (7.6 mg; 0.17 mmol) and H_2O (1 mL). A solution of TBACl.H_2O (51 mg; 0.17 mmol) in $CHCl_3$ (6mL) was added to the aqueous solution and the 2-phase mixture stirred vigorously for 4 hr after which time the phases were allowed to separate and the organic phase isolated. Further extractions were performed between the aqueous phase and $CHCl_3$ (6 mL x 3), the combined organic phase dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. H_2O was removed as azeotropes with toluene (6 mL x 3), which was subsequently removed as an azeotrope with DCM (6 mL x 3) before drying *in vacuo* for 10 hrs and storing under Ar. TBA **4.6DL** was recovered as a slightly yellow coloured viscous oil (64 mg; 85 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.01 (t, $J_{H,H}$ = 10.7, 12 H; CH_3), δ = 1.45 (sx, 8 H; CH_2CH_3), 1.63 (m, 8 H; $CH_2CH_2CH_3$), 1.77 (s, 1.5 H; CH_3), 3.28 (dd, $J_{A,B}$ = 13.0, $J_{A,X}$ = 5.2, 1 H; CH_2), 3.33 (shoulder of 3.36, 1 H; CH_2), 3.36 (m, 8 H; $CH_2NH_3^+$), 4.40 (m, 1 H; CH), 6.69 (d, $J_{H,H}$ = 8.0, 1 H; NH), 7.12 (m, 5 H; Ar*CH*); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 13.01 (CH_3), 19.38 (CH_2CH_3), 22.68 (CH_3), 23.63 ($CH_2CH_2CH_3$), 37.51 (CH_2CH), 55.52 (CH), 58.45 ($CH_2NH_3^+$), 125.14 (Ar*C*H), 127.18 (Ar*C*H), 129.53 (ArCH), 138.90 (Ar*C*), 169.93 (C=O), 173.8 (C=O); ESMS$^-$: equal intensity peaks at 206 and 209 amu.

Expt 5.8: Exposure to, and decomplexation of, TBA 4.6DL from 5.3 (100 beads).

TBA **4.6DL** (1 mM in 1 % MeOH/$CHCl_3$; 5 μL per bead; 0.5 mL) was applied to ~ 100 beads **5.3** in a Petri dish of diameter 2 cm. The beads had been placed in the dish under a microscope and roughly counted by eye. The system was placed in a jam-jar, the bottom of which was covered with 1 % MeOH/$CHCl_3$, and the lid tightly replaced. The system was left for 4 hrs after which time the beads and supernatant phase were transferred to a plastic filtration tube fitted with a frit, and filtered to remove excess guest. $CHCl_3$ (5 μL per bead; 0.5 mL) was then added to the beads in the filtration tube, which was subsequently sealed at both ends with rubber septa, and shaken for 30 sec to remove any remaining unbound guest. The washing was repeated twice and bound guest was then decomplexed by shaking the beads in MeOH/$CHCl_3$ (1:1; 5 μL per bead; 0.5 mL), within the sealed filtration tube, for 3 mins, and filtering. The filtrate was analysed by electrospray mass spectrometry. H_2O/AcCN (1:1), which had been degassed, was used as mobile phase, at a flow rate of 0.04 mL/min, which gave a time delay of 30 sec between time of injection and observation of sample peaks in the real-time mass spectrum. Source temperature was 80 °C and cone voltage was 30 V. A scan time of 1.0 sec in the range of 100 - 500 m/z was used to identify **4.6DL**. Two peaks, with a ratio of 1:2 were observed at 206 and 209 amu.

Expt 5.9: Exposure to, and decomplexation of, TBA 4.6DL from 5.3 (10 beads).

5.3 (100 beads) were exposed to TBA **4.6DL** (1 mM in 1 % MeOH/$CHCl_3$; 5 μL per bead; 0.5 mL) and filtered as per **Expt 5.8**. 10 beads were then removed and placed in a filtration tube, washed and decomplexed as before, with the quantities of wash and decomplexing solutions scaled down 10-fold compared with **Expt 5.8**. Another 10 beads were placed in a melting point capillary which had been sealed at one end. These beads were washed with $CHCl_3$ (5 μL per bead x 3) by adding the solution to the capillary via syringe and inverting the syringe to shake the beads. Adding the solution in the same fashion, inverting 20 times and removing the solution by syringe performed decomplexation. The decomplexing solution was analysed by mass spectrometry. Both solutions gave peaks in a ratio of 1:2 at 206 and 209 amu.

Expt 5.10: Mass spectrometry assay 3; Decomplexation of TBA 4.6DL from 5.3 (single beads)

As per **Expt 5.9**, 3 single beads were isolated in single melting point capillaries after equilibration, filtering and washing of 100 beads which had been exposed to TBA **4.6DL** (1 mM in 1 % MeOH/$CHCl_3$; 5 μL per bead; 0.5 mL). To each capillary was added MeOH/$CHCl_3$ (1:1; 5 μL), prior to inversion 20 times and removal of the solution from each capillary. Each volume was made up to 15 μL with MeOH to comprise a single ESMS injection, which was performed as per **Expt 5.9**, to give a pair of peaks in a ratio of 1:2, at 206 and 209 amu, on each occasion.

Expt 5.11: The single bead assay.

To **5.3** (1 bead) in a melting point capillary sealed at one end was added TBA **4.6DL** (1 μM; 5mL). The capillary was placed within a capped sample tube, the bottom of which was covered with 1 % MeOH/$CHCl_3$, for 4 hr, after which time the solution was removed from the capillary by syringe and the bead washed 3 times with $CHCl_3$ (5 μL), each time by inversion of the capillary 20 times. Decomplexing solution (MeOH/$CHCl_3$ 1:1; 5 μL) was subsequently added to the bead in the capillary, which was inverted 20 times, prior to removal of the solution, which was made up to 20 μL with MeOH (15 μL) prior to ESMS injection. Peaks at 206 and 209 amu, in a ratio of 1:2 were observed.

Expt 5.12: Preparation of [4.6L/4.6D (1:5)]

To a mixture of *N*-Ac-d_3-L-Phe (8.1 mg; 0.04 mmol) and *N*-Ac-D-Phe (40 mg; 0.19 mmol), both of which had been separately dried *in vacuo* for several hours, was added MeOH (10 mL), to yield a solution which was stirred at room temperature for 5 minutes before concentrating under pressure to yield a white solid, which was dried *in vacuo* for

4 hours (48 mg). By ESMS$^-$ analysis, this mixture gave peaks at 206 and 209 amu in a ratio of 5:1. A solution of TBACl.H_2O (61 mg; 0.21 mmol) in $CHCl_3$ (6 mL) was added to the white solid which had been dissolved in an aqueous solution of NaOH (8 mg; 0.2 mmol) in H_2O (1 mL) and the 2-phase mixture stirred vigorously for 4 hr after which time the phases were allowed separate and the organic phase isolated. Further extractions were performed between the aqueous phase and $CHCl_3$ (6 mL x 3), the combined organic phase dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. H_2O was removed as azeotropes with toluene (6 mL x 3), which was subsequently removed as an azeotrope with DCM (6 mL x 3) before drying *in vacuo* for 10 hrs and storing under Ar. TBA [**4.6L/4.6D** (1:5)] was recovered as a slightly yellow coloured viscous oil (64 mg; 85 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.01 (t, $J_{H,H}$ = 10.7, 12 H; CH_3), δ = 1.45 (sx, 8 H; CH_2CH_3), 1.63 (m, 8 H; $CH_2CH_2CH_3$), 1.77 (s, 2.5 H; CH_3), 3.28 (dd, $J_{A,B}$ = 13.0, $J_{A,X}$ = 5.2, 1 H; CH_2), 3.33 (shoulder of 3.36, 1 H; CH_2), 3.36 (m, 8 H; $CH_2NH_3^+$), 4.40 (m, 1 H; CH), 6.69 (d, $J_{H,H}$ = 7.3, 1 H; NH), 7.12 (m, 5 H; Ar*CH*); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 13.01 (CH_3), 19.37 (CH_2CH_3), 22.66 (CH_3), 23.60 ($CH_2CH_2CH_3$), 37.53 (CH_2CH), 55.52 (CH), 58.46 ($CH_2NH_3^+$), 125.10 (Ar*CH*), 127.19 (Ar*CH*), 129.53 (ArCH), 138.92 (Ar*C*), 169.94 (C=O), 173.75 (C=O); ESMS$^-$: peaks at 206 and 209 amu, in a ratio of 1:2.

Expt 5.13: Preparation of [4.6L/4.6D (1:10)]

To a mixture of *N*-Ac-d$_3$-L-Phe (8.1 mg; 0.04 mmol) and *N*-Ac-D-Phe (78 mg; 0.38 mmol), both of which had been separately dried *in vacuo* for several hours, was added MeOH (10 mL), to yield a solution which was stirred at room temperature for 5 minutes before concentrating under pressure to yield a white solid, which was dried *in vacuo* for 4 hours (86 mg). By ESMS$^-$ analysis, this mixture gave peaks at 206 and 209 amu in a ratio of 10:1. A solution of TBACl.H_2O (61 mg; 0.21 mmol) in $CHCl_3$ (6 mL) was added to the white solid which had been dissolved in an aqueous solution of NaOH (8 mg; 0.2 mmol) in H_2O (1 mL) and the 2-phase mixture stirred vigorously for 4 hr after which time the phases were allowed separate and the organic phase isolated. Further extractions were performed between the aqueous phase and $CHCl_3$ (6 mL x 3), the

combined organic phase dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. H_2O was removed as azeotropes with toluene (6 mL x 3), which was subsequently removed as an azeotrope with DCM (6 mL x 3) before drying *in vacuo* for 10 hrs and storing under Ar. TBA [**4.6L/4.6D** (1:10)] was recovered as a slightly yellow coloured viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.03 (t, $J_{H,H}$ = 10.7, 12 H; CH_3), δ = 1.45 (sx, 8 H; CH_2CH_3), 1.63 (m, 8 H; $CH_2CH_2CH_3$), 1.77 (s, 1.8 H; CH_3), 3.28 (dd, $J_{A,B}$ = 13.0, $J_{A,X}$ = 5.2, 1 H; CH_2), 3.33 (shoulder of 3.36, 1 H; CH_2), 3.36 (m, 8 H; $CH_2NH_3^+$), 4.41 (m, 1 H; CH), 6.68 (d, $J_{H,H}$ = 7.3, 1 H; NH), 7.12 (m, 5 H; Ar*CH*); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 13.03 (CH_3), 19.37 (CH_2CH_3), 22.69 (CH_3), 23.60 ($CH_2CH_2CH_3$), 37.55 (CH_2CH), 55.53 (CH), 58.47 ($CH_2NH_3^+$), 125.10 (Ar*CH*), 127.21 (Ar*CH*), 129.53 (ArCH), 138.92 (Ar*C*), 169.95 (C=O), 173.75 (C=O); ESMS$^-$: peaks at 206 and 209 amu, in a ratio of 5:1.

Expt 6.1: Exposure of 6.1 (100 beads) to 2.6L (1 mM in 1 % MeOH/CHCl$_3$; 0.5 mL)

2.6L (1 mM in 1 % MeOH/$CHCl_3$; 0.5 mL) was applied to **6.1** (~ 100 beads) in a Petri dish of diameter 2 cm. The beads had been placed in the dish under a microscope and roughly counted by eye. The system was placed in a jam-jar, the bottom of which was covered with 1 % MeOH/$CHCl_3$, and the lid tightly replaced. The system was left for 4 hr after which time the dish contents were transferred to a filtration tube, and filtered. $CHCl_3$ (0.5mL) was added to the beads in the filtration tube, which was sealed at both ends with rubber septa. The beads were shaken and the solvent filtered, to remove any unbound guest. The beads were then transferred to a Petri dish, using $CHCl_3$ to effect the transfer and maintain solvation of the beads, which were examined under a low power microscope. All beads exhibited a red hue, which varied from an orange stain to an intense red, which was observed for 3 beads. The beads were returned to the filtration tube, filtered and shaken with decomplexing solution (MeOH/$CHCl_3$ 1:1; 0.5mL). The beads were observed to become colourless, immediately, as the supernatant phase was observed to colour. The supernatant phase was filtered.

Expt 6.2: Equilibration of 6.1 with TBA 2.5L/TBA 2.6D (1:1)

An equimolar solution of TBA **2.5L** and TBA **2.6D** (1 mM in 1 % MeOH/$CHCl_3$; 5 μL per bead; 5 mL) in 1 % MeOH/$CHCl_3$ was applied to dipeptide library **6.1** (1 mg; ~ 1000 beads) in a Petri dish of diameter 5 cm. The Petri dish was placed within a jam-jar, the bottom of which was covered with 1 % MeOH/$CHCl_3$, the lid tightly replaced and the system left for 4 hrs. After this time, the beads and supernatant solution were transferred to a filtration tube fitted with a frit and excess guest removed by filtration. To remove any remaining unbound guest, the beads were washed by shaking in $CHCl_3$ (2.5 mL x 2) for 30 sec, within the filtration tube, which had been sealed at both ends with rubber septa. The beads were subsequently washed back into the Petri dish with $CHCl_3$, and examined under a microscope. Some blue and red beads were observed among a majority of brown coloured beads. Individual melting point capillary tubes were used to pick the red and blue beads. Some brown beads were also picked.

Expt 6.3. Extractions between members of 6.1 and TBA 4.6DL

Procedures were performed on single beads within individual capillaries, by micro-syringe, and under a microscope.

Coloured guest was decomplexed from the bead by addition of $CHCl_3$/MeOH (1:1: 5 μL), by syringe, to the bead in its capillary and inversion of the capillary 20 times before removal of solution, by syringe. The bead was washed with $CHCl_3$ (5 μL x 3) by syringe of solvent into the capillary tube and inversion of the tube 20 times, before removal of solution by syringe. TBA **4.6DL** (0.1 mM in 1 % MeOH/$CHCl_3$; 5 μL) was then added to the capillary which was left within a capped sample tube, the bottom of which was covered with 1 % MeOH/$CHCl_3$, for 4 hrs, after which time excess solution was removed by syringe. Bound guest was decomplexed by shaking the bead in MeOH/$CHCl_3$ (5 μL) for 3 mins and recovering the solution by syringe. The recovered solution was made up to 20 μL with MeOH and analysed by mass spectrometry, using the same conditions as described in **Expt 5.11**.

Expt 6.4

TBACl. H_2O (7.1 mg; 0.024 mmol) was added to **2.5L** (15 mg; 0.026 mmol) in $CHCl_3$ (500 μL), and H_2O (200 μL) and stirred for 45 mins. The blue product was extracted into the organic phase, dried over anhydrous $MgSO_4$, concentrated under pressure and dried *in vacuo* for 5 hours. TBACl. H_2O (7.1 mg; 0.024 mmol) was added to **2.6D** (14 mg; 0.024 mmol), in $CHCl_3$ (600 μL) and H_2O (200 μL) and stirred for 45 mins. The red product was extracted into the organic phase, dried over anhydrous $MgSO_4$, concentrated under pressure and dried *in vacuo* for 5 hours.

CHAPTER 8 - References

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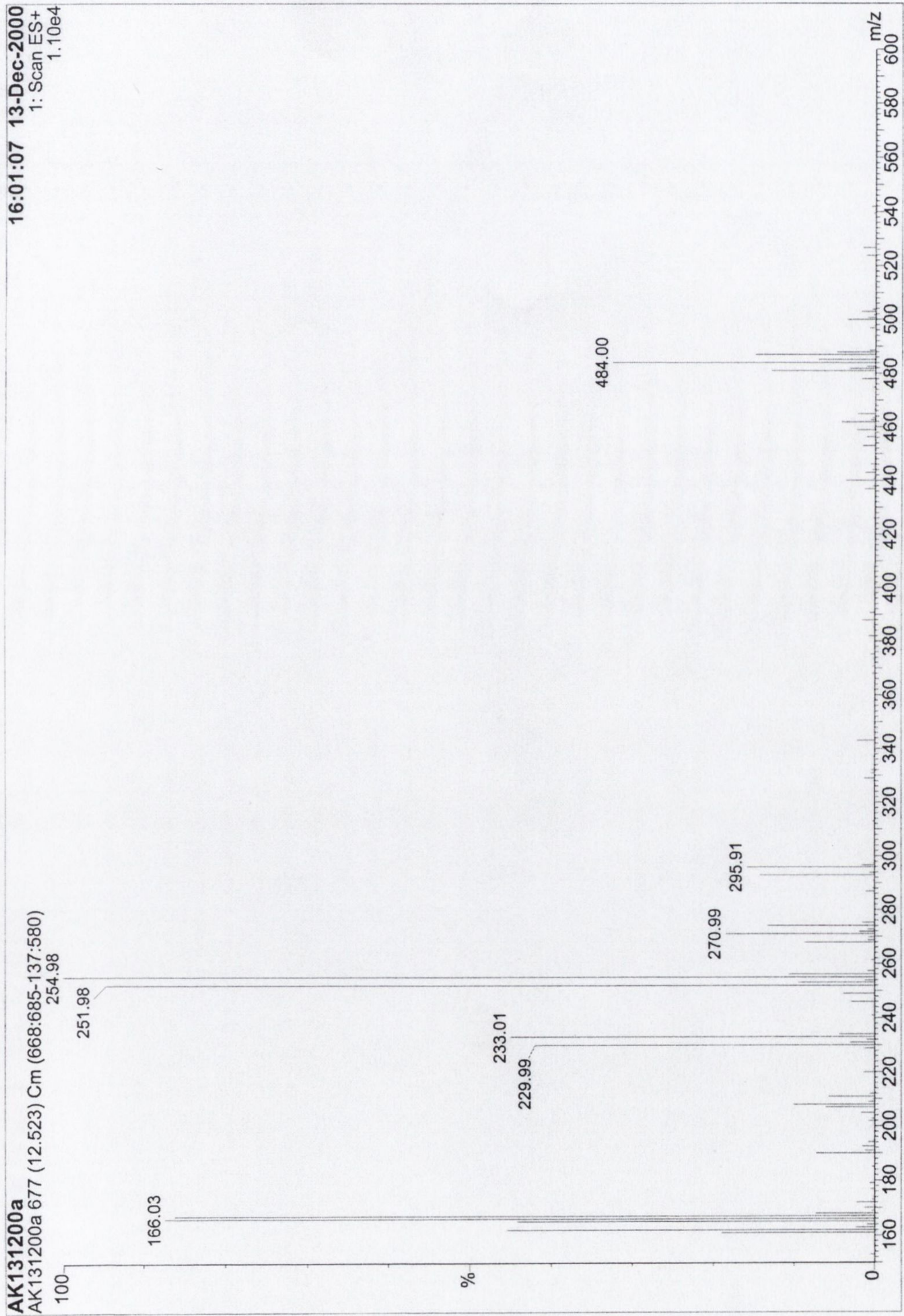
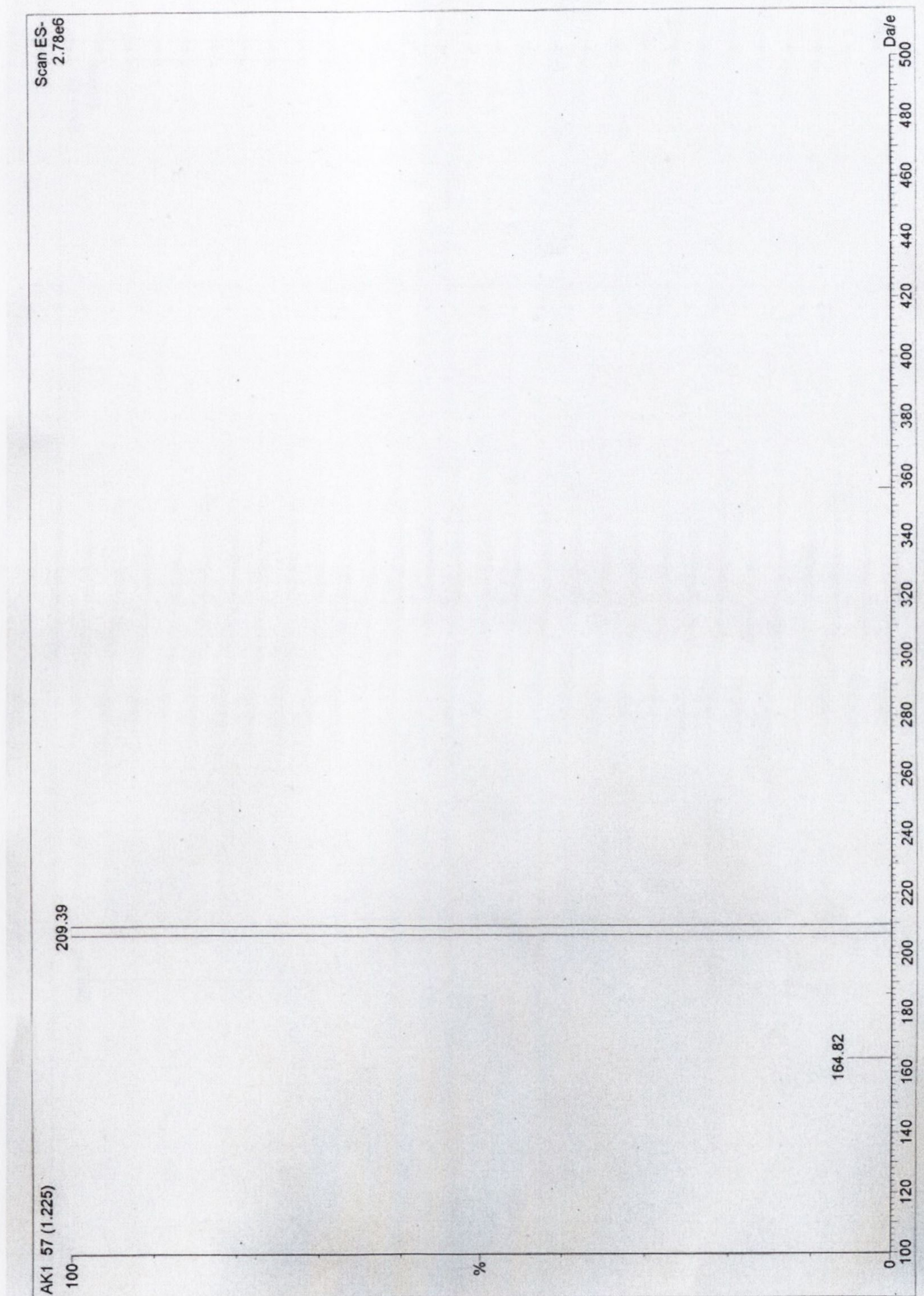
APPENDIX A

Appendix A1: TBA **4.6DL** analysed by LC/MS$^+$

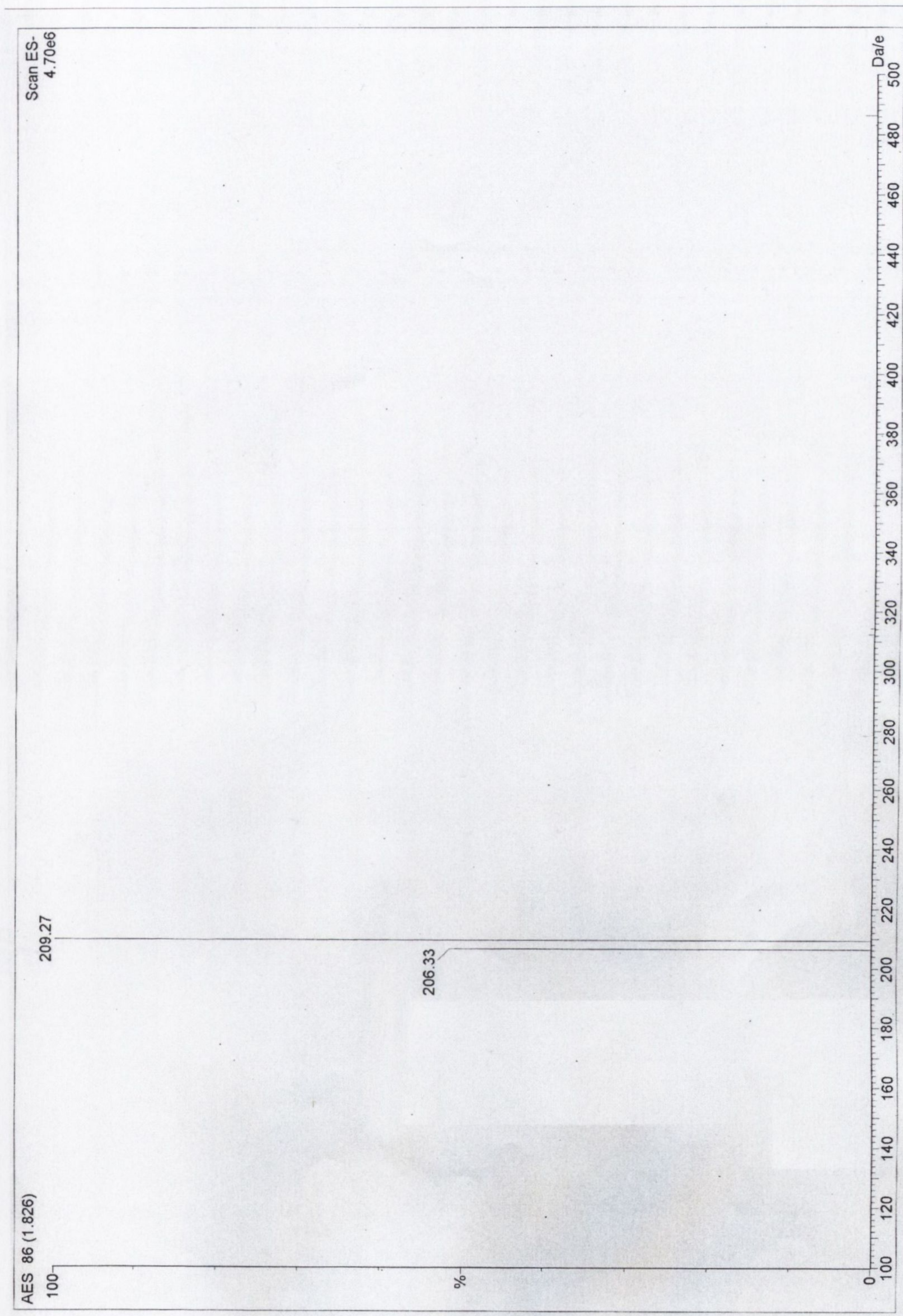


Appendix A2: TBA **4.6DL** analysed by ESIMS[-a]

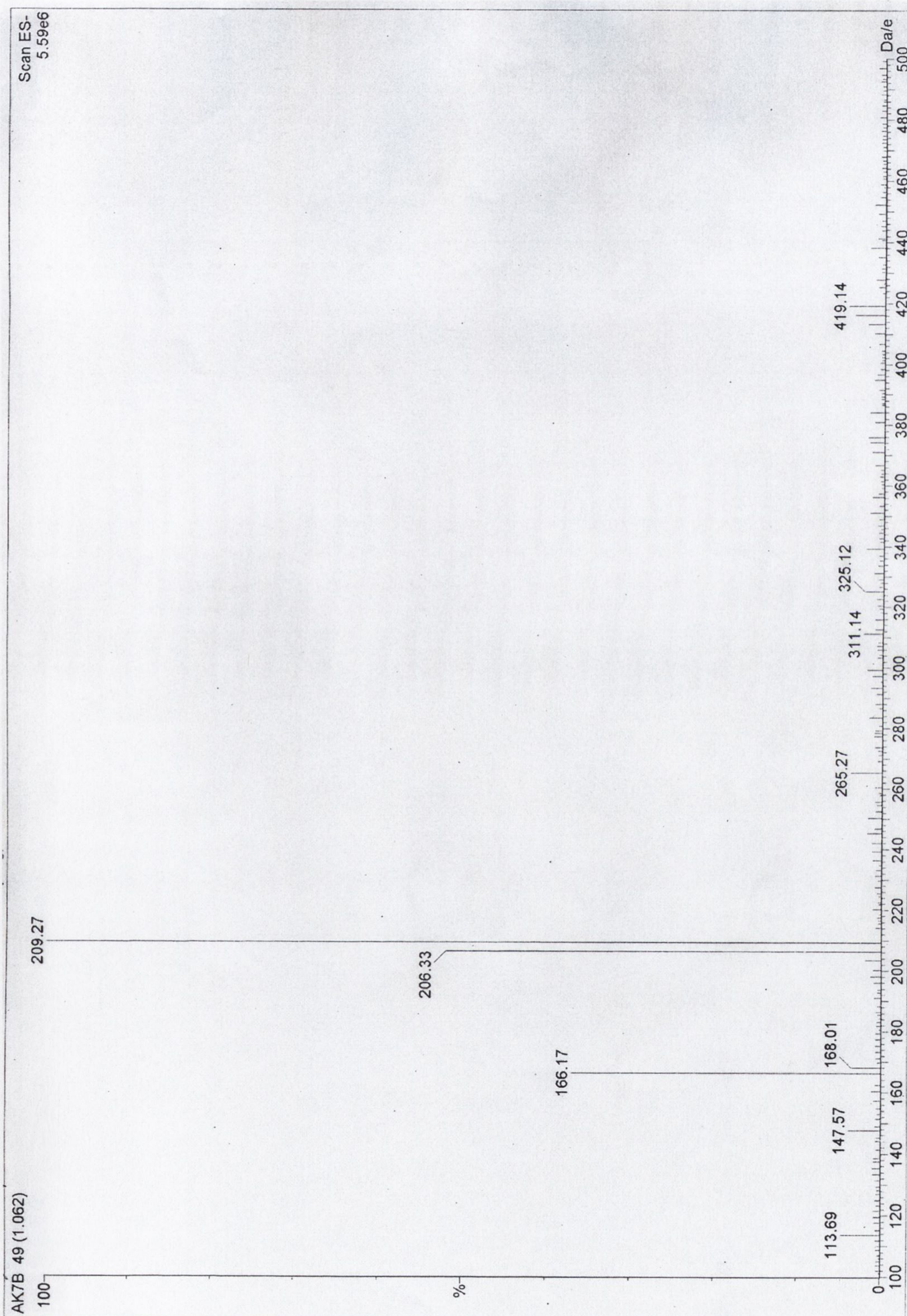


[a] ESIMS refers to direct injection electrospray mass spectrometry using the VG Quattro instrument throughout **Appendix A**, with the exception of appendices **A1** and **A19**.

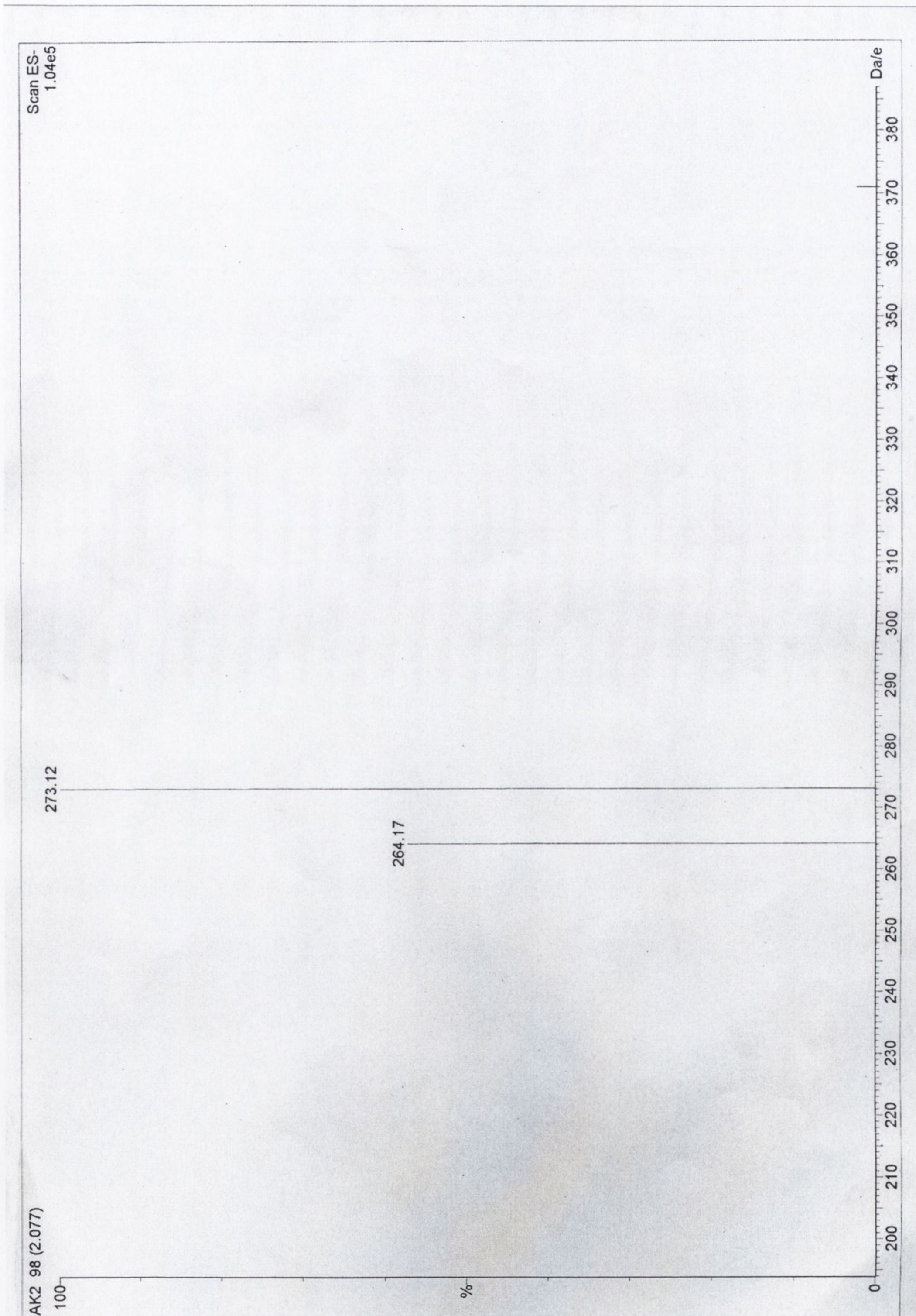
Appendix A3: ESIMS$^-$ spectrum of **4.6D/4.6L** decomplexed from **5.3** (100 beads) which had been exposed to TBA **4.6DL**



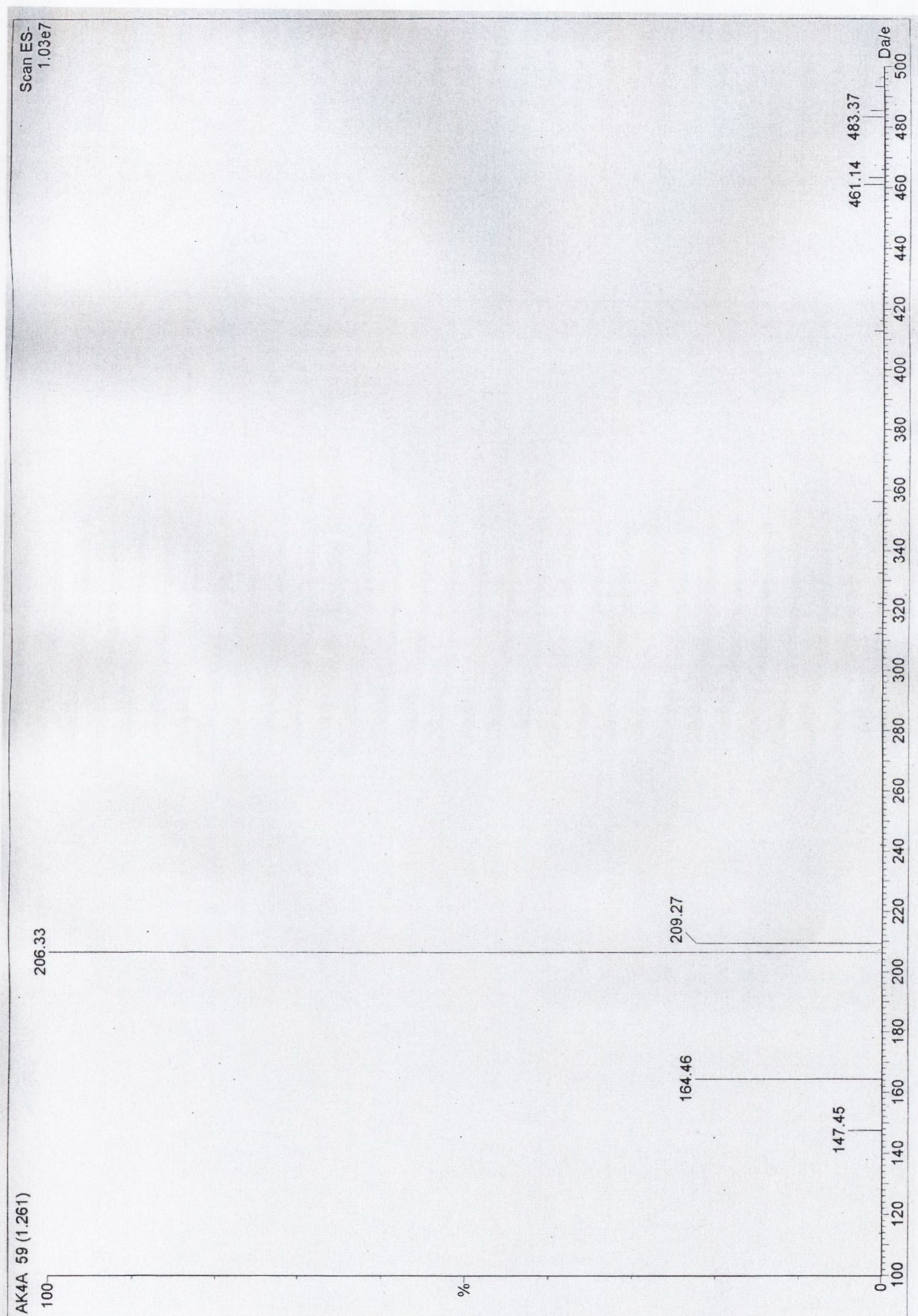
Appendix A4: ESIMS$^-$ spectrum of **4.6D/4.6L** decomplexed from **5.3** (100 beads) which had been exposed to TEA **4.6DL**



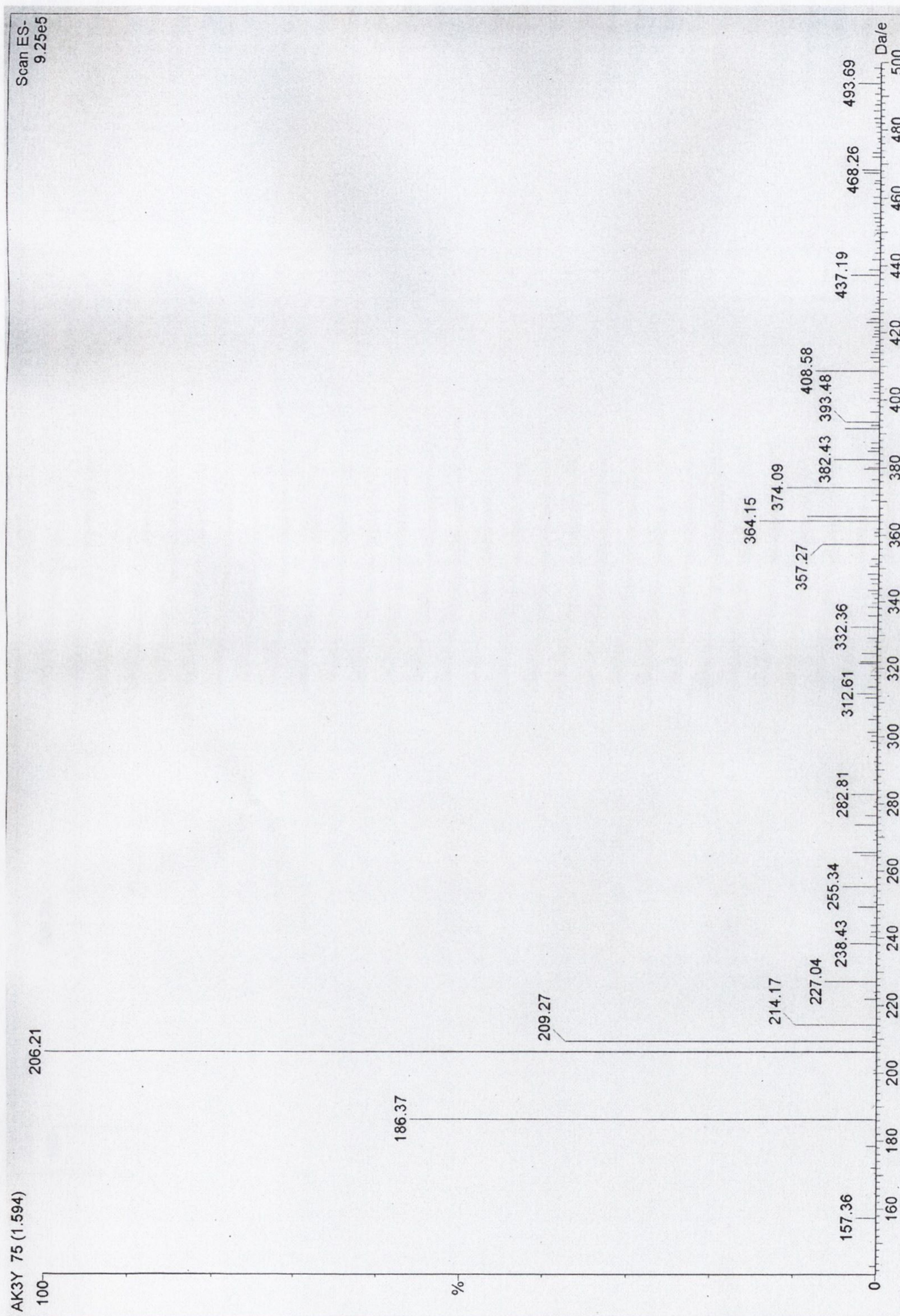
Appendix A5: ESIMS$^-$ spectrum of **4.7D/4.7L** decomplexed from **5.3** (100 beads) which had been exposed to TBA **4.7DL**



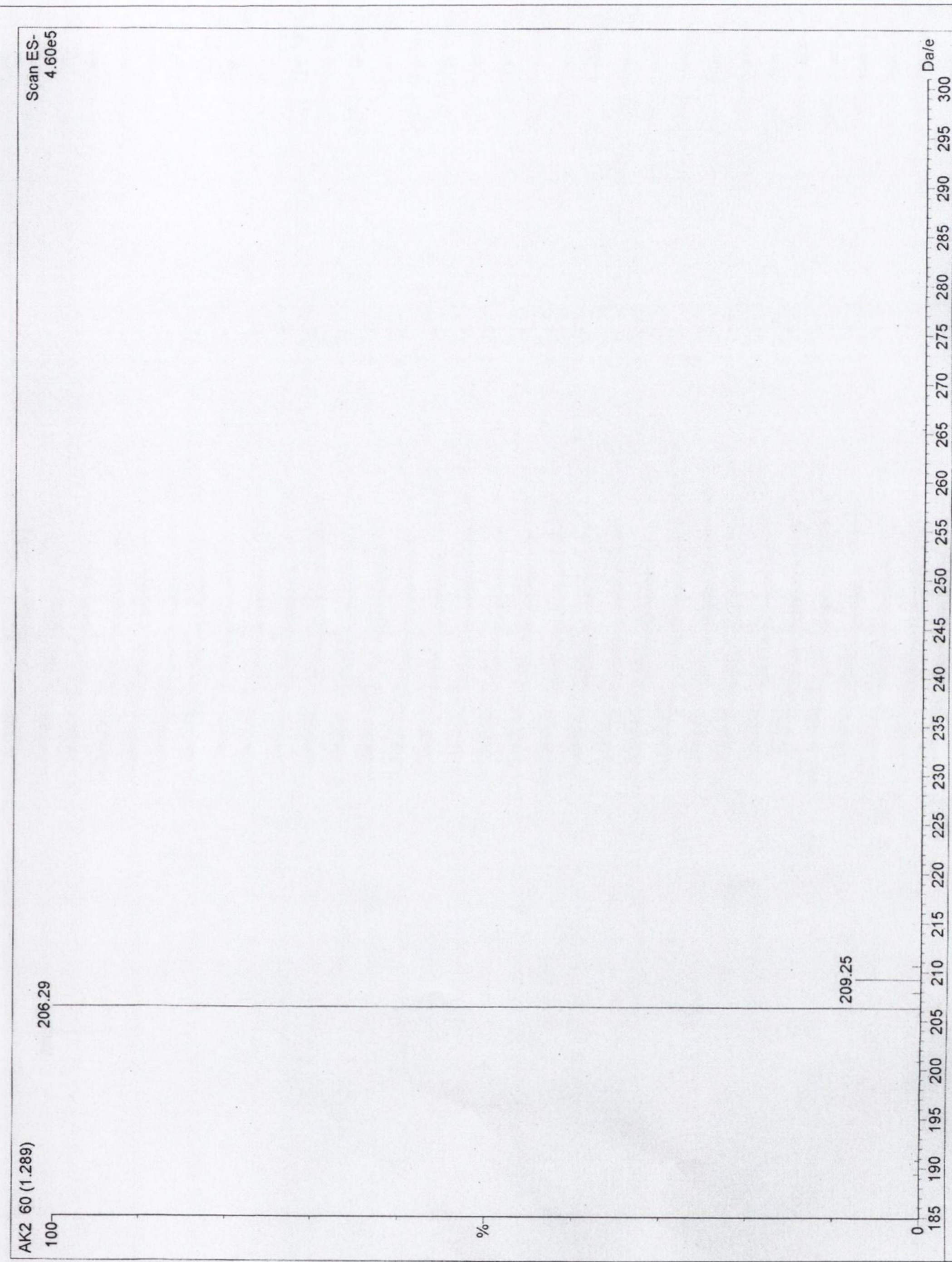
Appendix A6: ESIMS$^-$ spectrum of TBA **4.6L/4.6D** (1:5)}



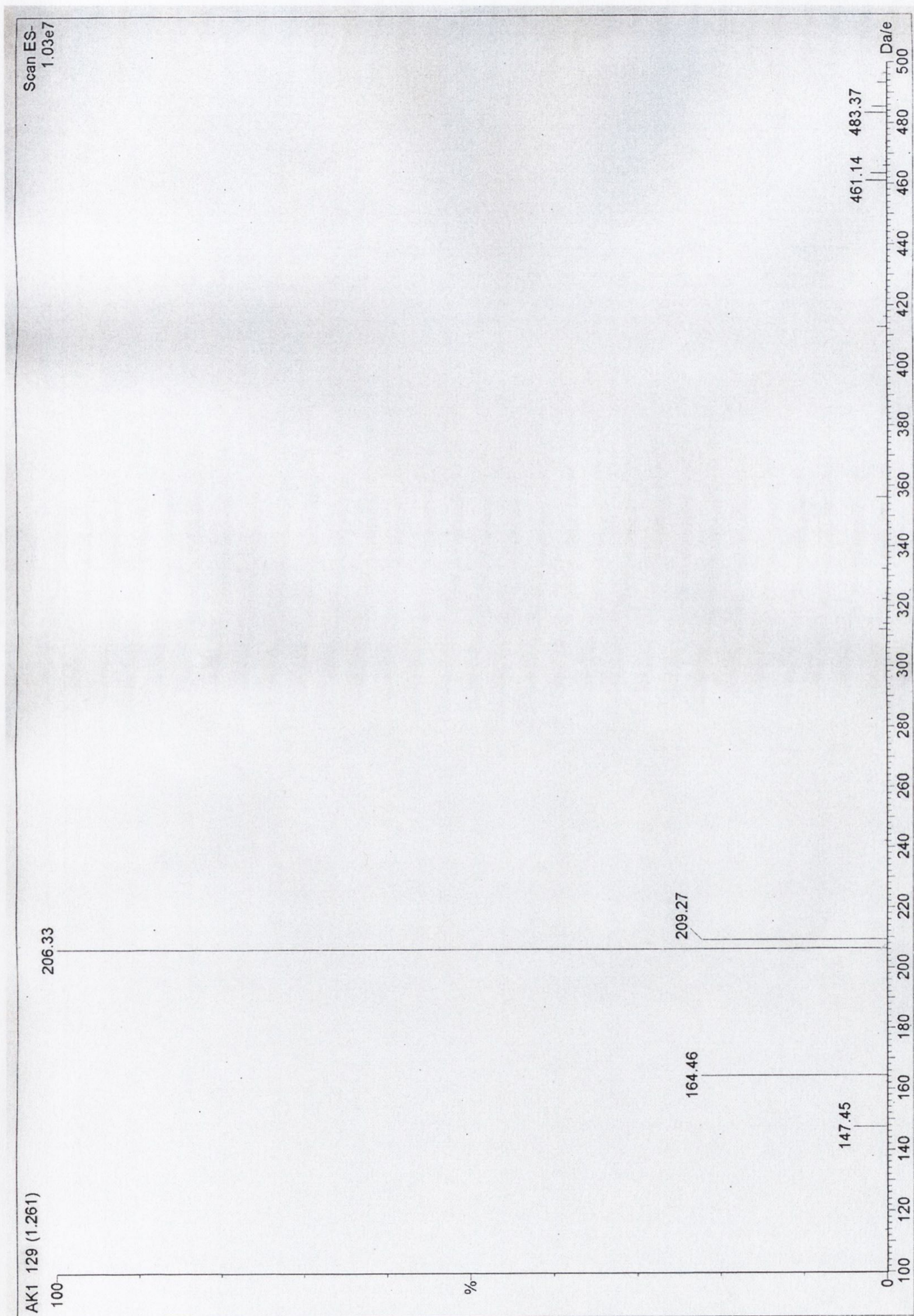
Appendix A7: ESIMS$^-$ spectrum of **4.6D/4.6L** decomplexed from **5.3** which had been exposed to **4.6L/4.6D** (1:5)



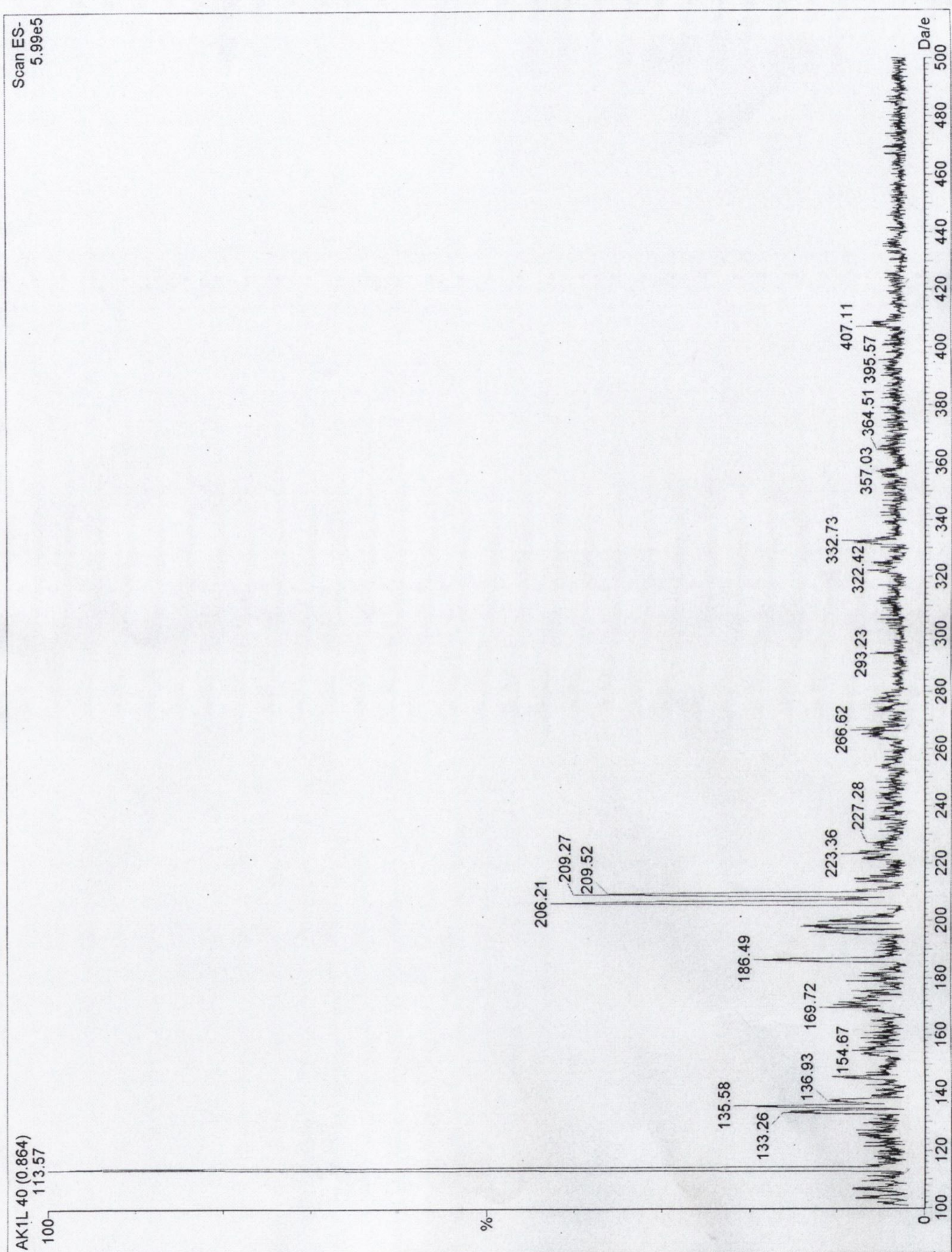
Appendix A8: ESIMS$^-$ spectrum of TBA **4.6L/4.6D** (1:10)



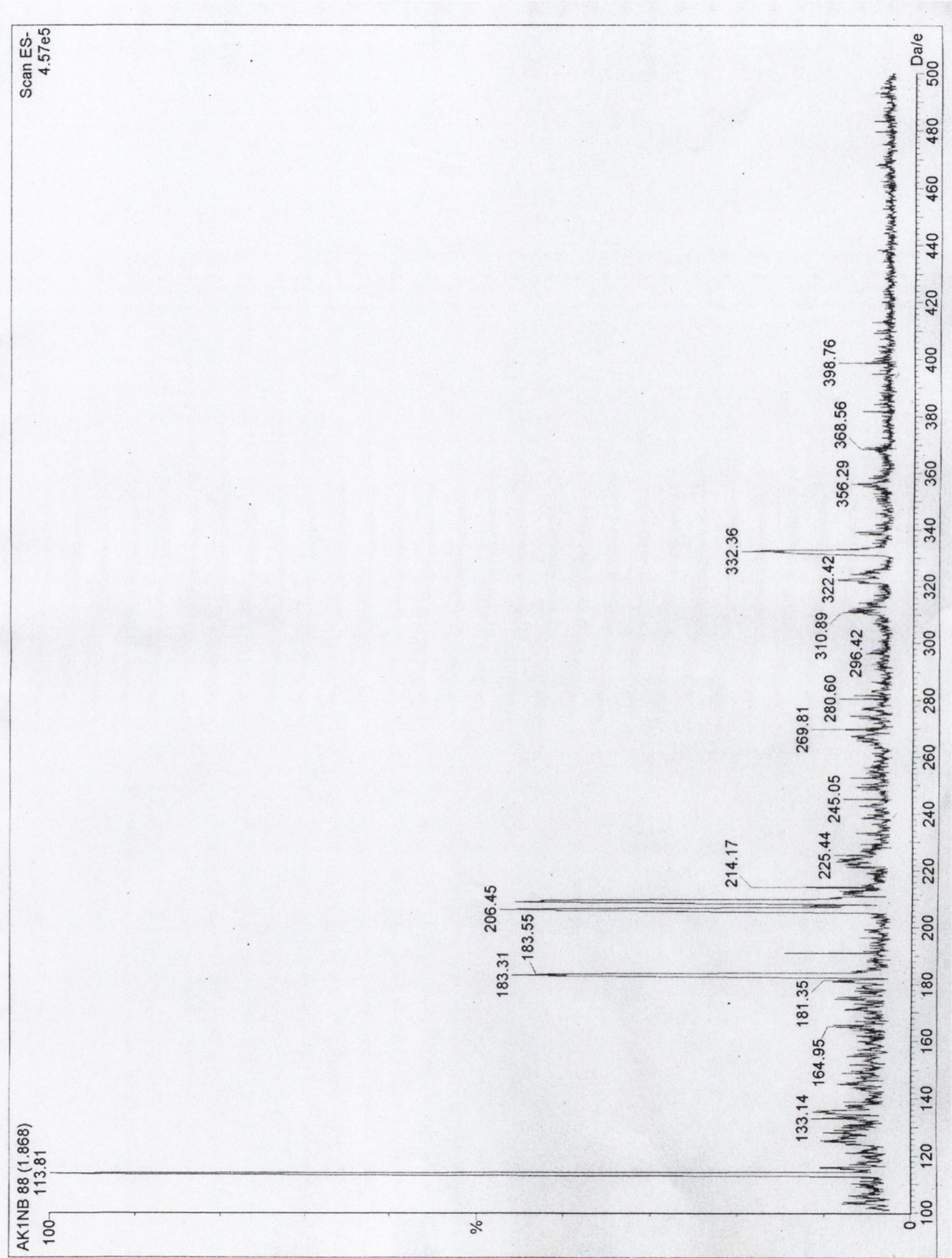
Appendix A9: ESIMS$^-$ spectrum of TBA **4.6L/4.6D** decomplexed from **5.3** which had been exposed to **4.6L/4.6D** (1:10)



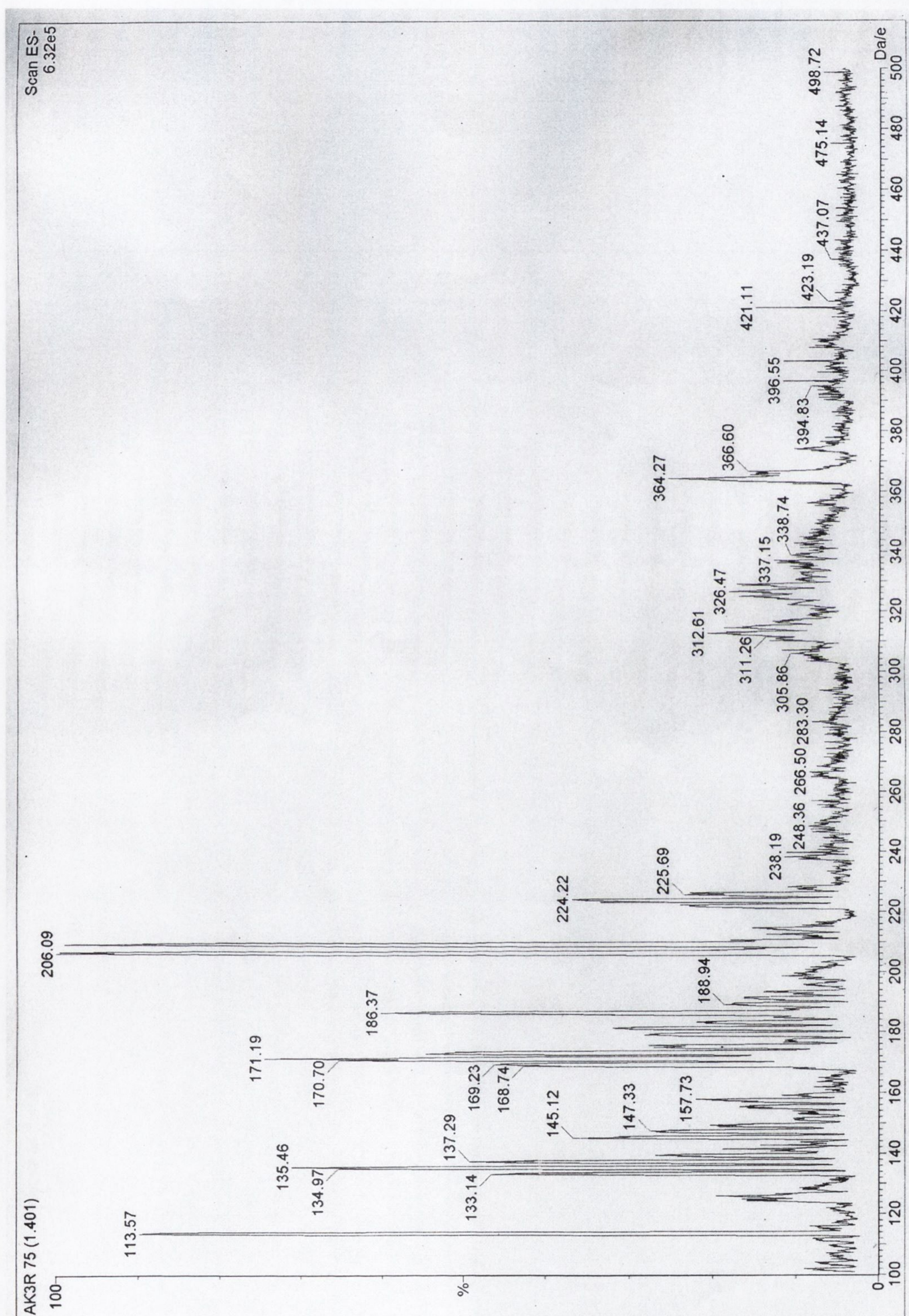
Appendix A10 **4.6L/4.6D** decomplexed from a single "brown" member of library **6.1** which had been exposed to TBA **4.6DL**



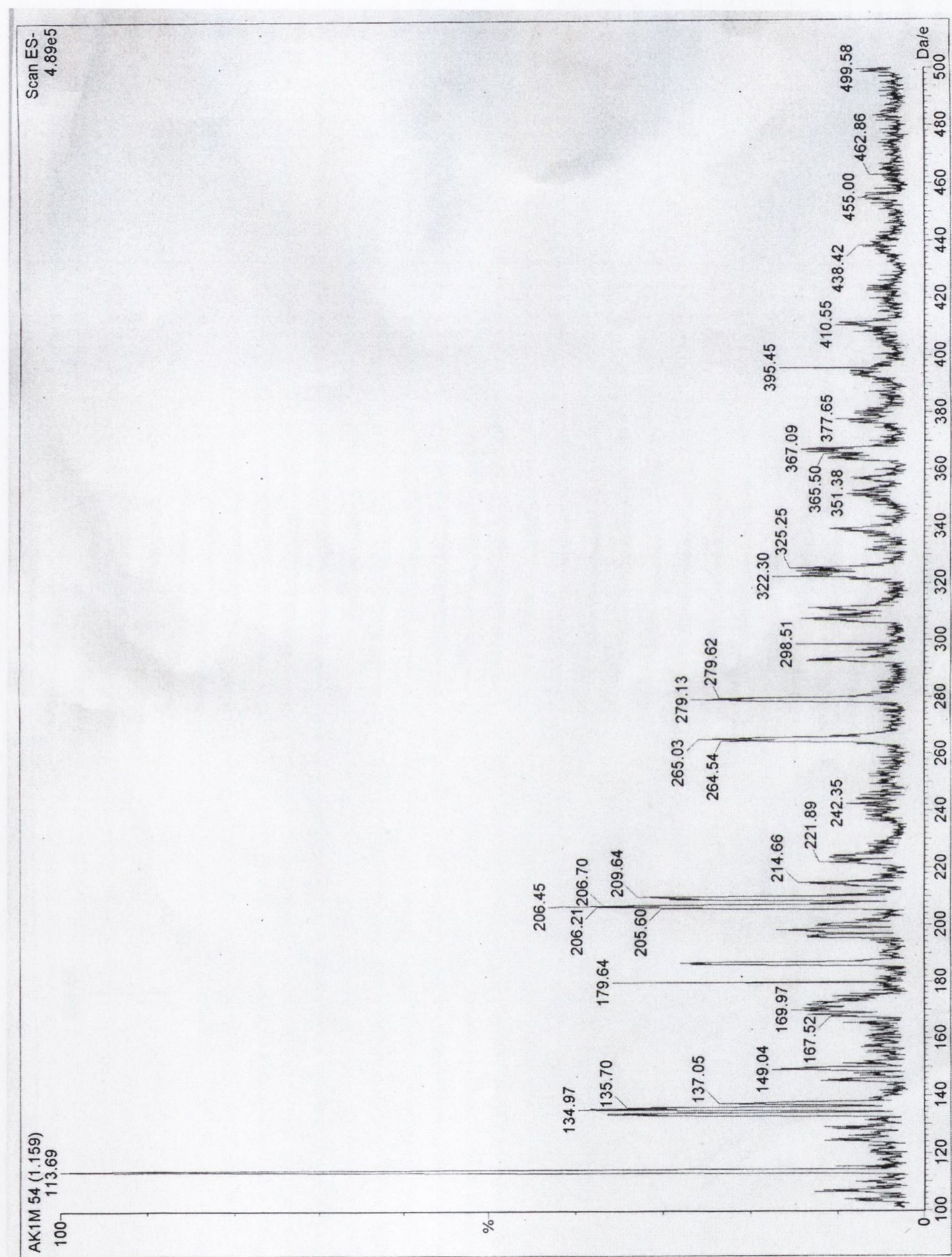
Appendix A11: **4.6L/4.6D** decomplexed from a single "brown" member of library **6.1** which had been exposed to TBA **4.6DL**



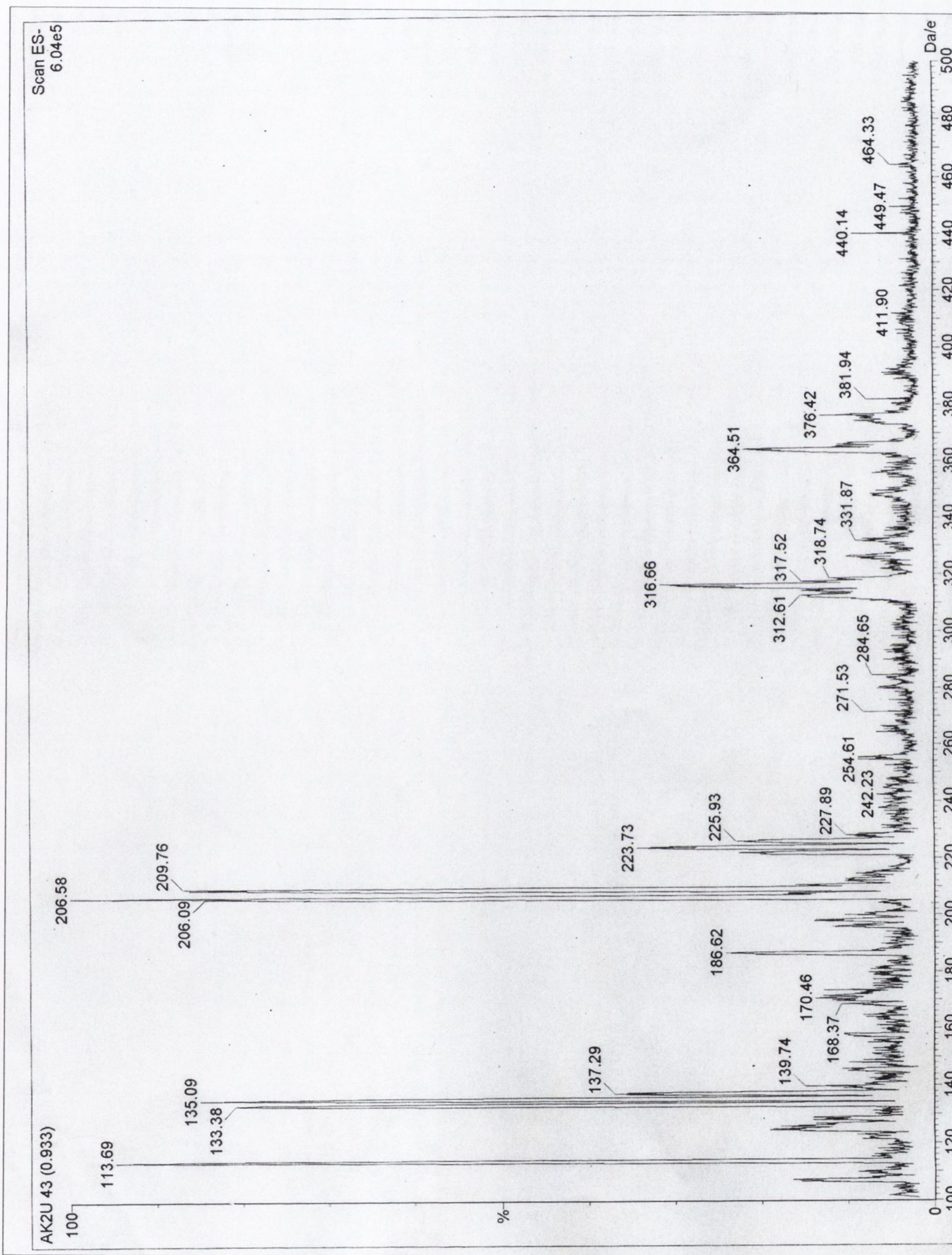
Appendix A12: **4.6L/4.6D** decomplexed from a single "brown" member of library **6.1** which had been exposed to TBA **4.6DL**



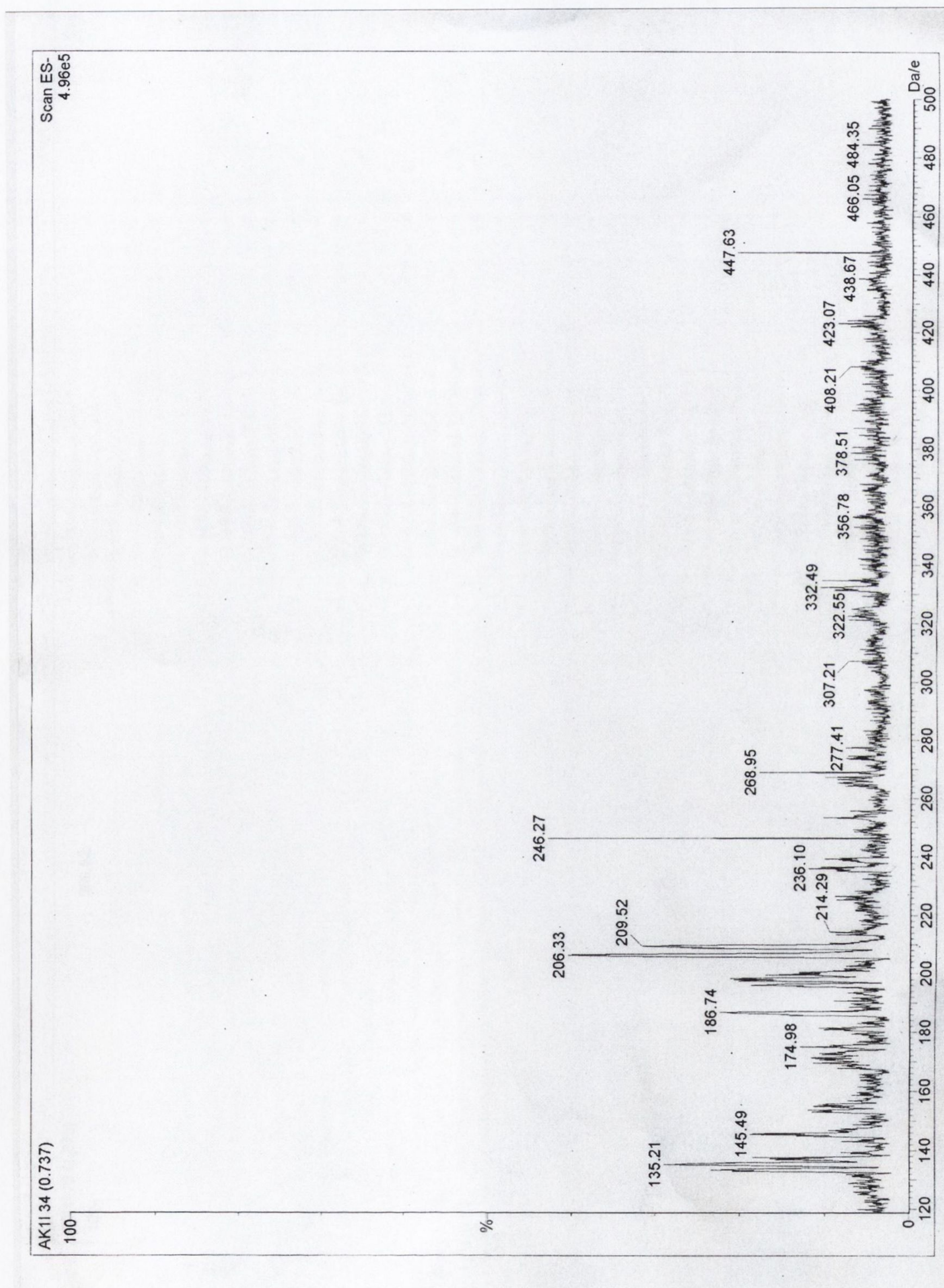
Appendix A13: **4.6L/4.6D** decomplexed from a single "red" member of library **6.1** which had been exposed to TBA **4.6DL**



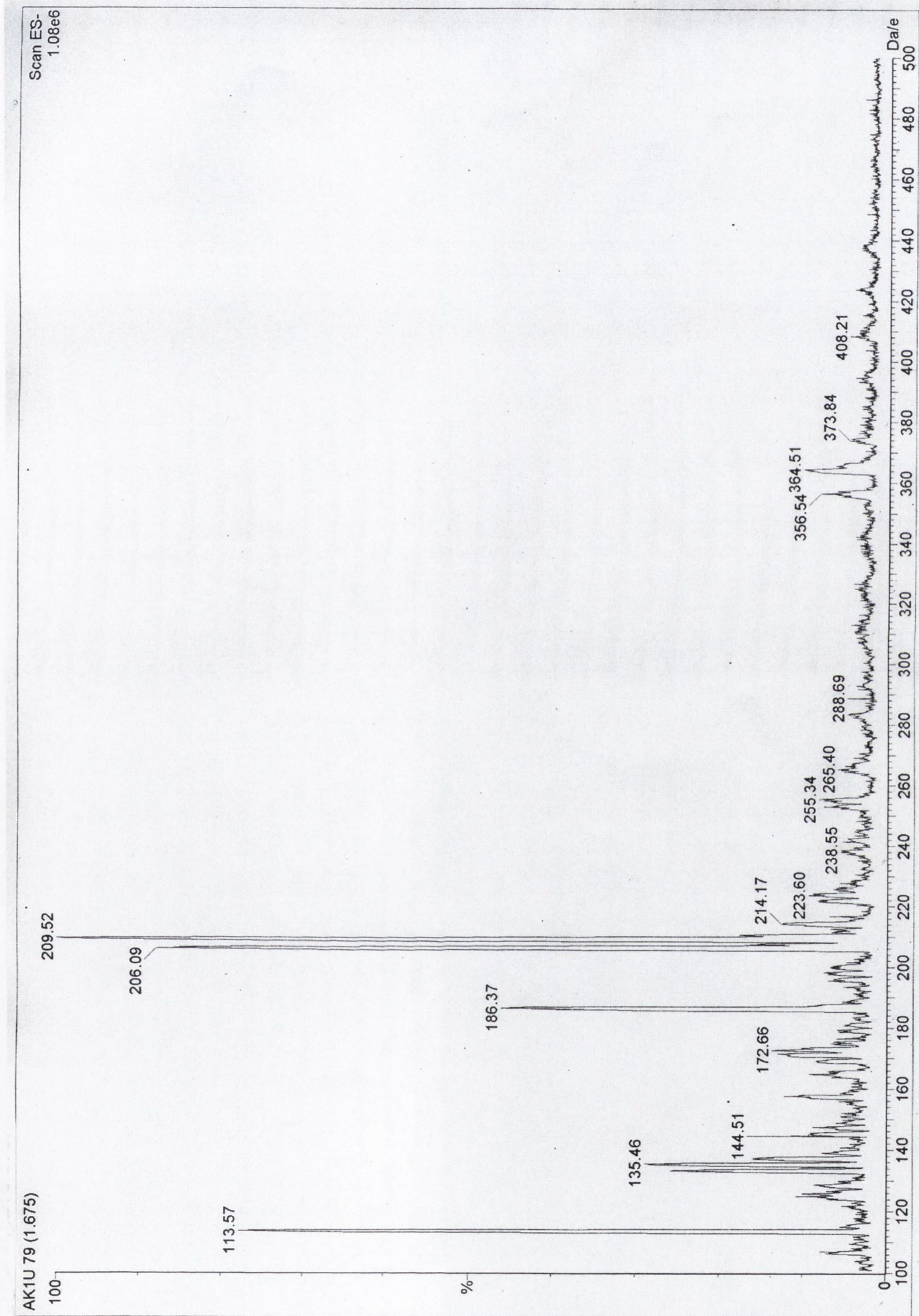
Appendix A14: **4.6L/4.6D** decomplexed from a single "red" member of library **6.1** which had been exposed to TBA **4.6DL**



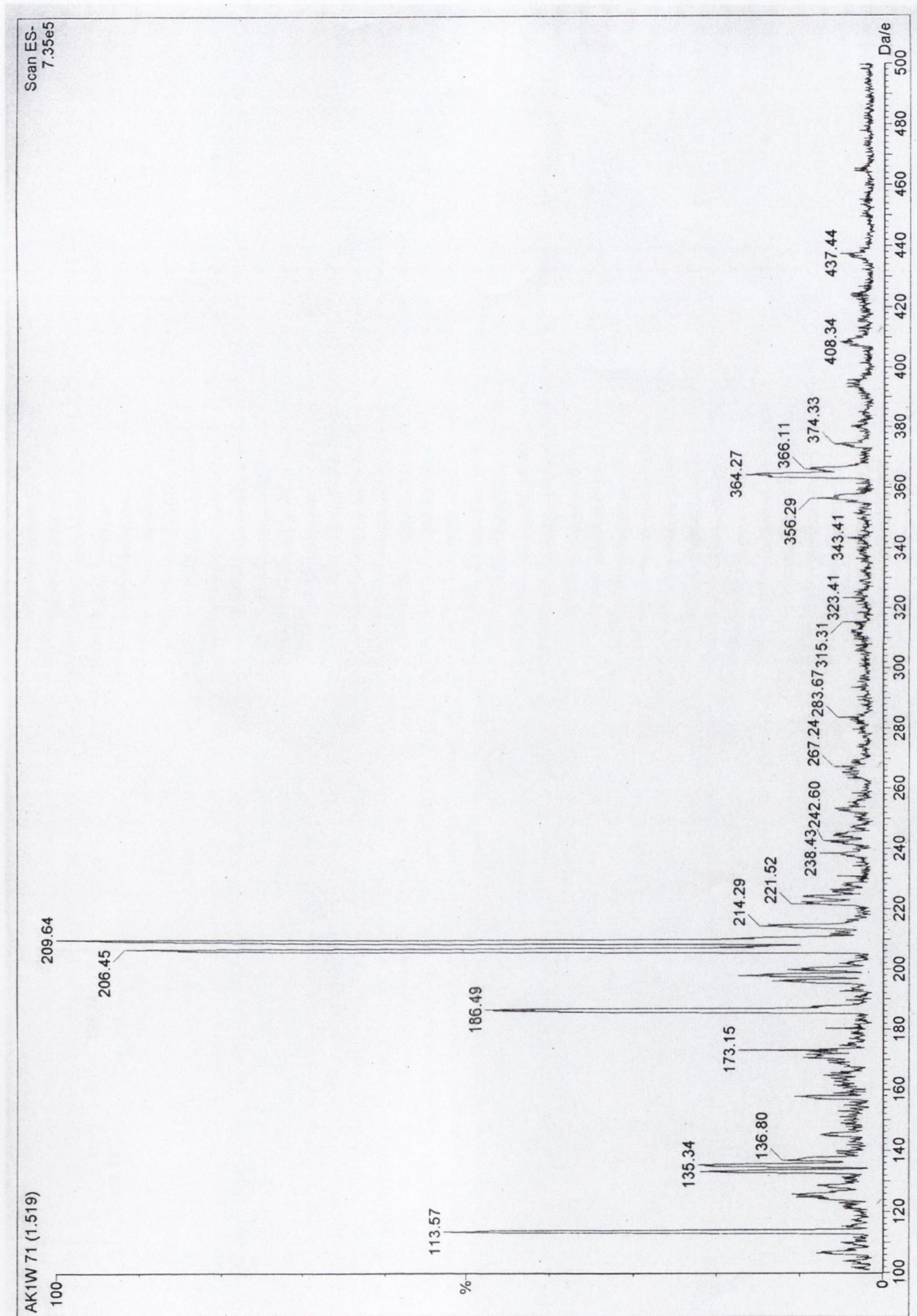
Appendix A15: **4.6L/4.6D** decomplexed from a single “red” member of library **6.1** which had been exposed to TBA **4.6DL**



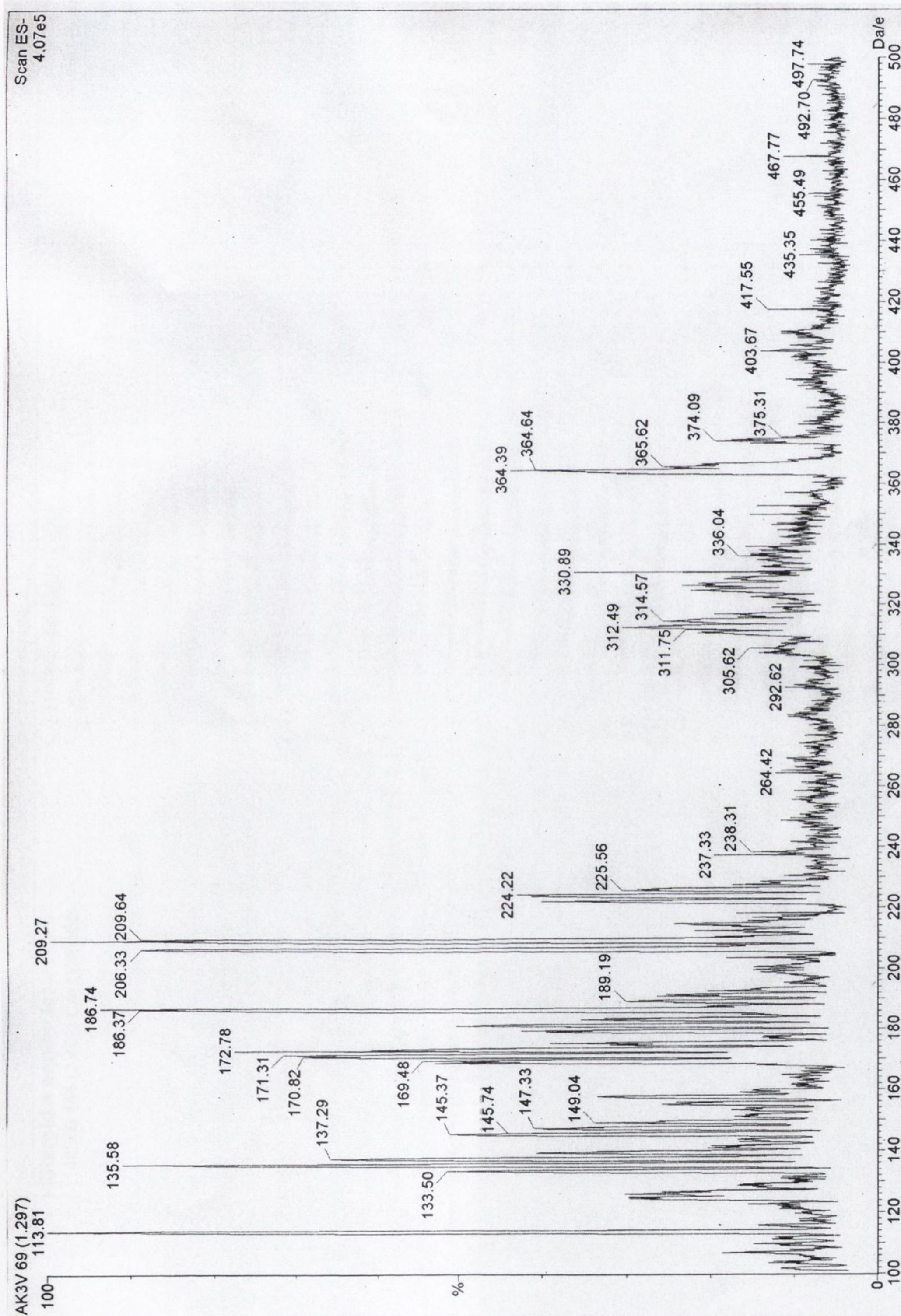
Appendix A16: **4.6L/4.6D** decomplexed from a single "blue" member of library **6.1** which had been exposed to TBA **4.6DL**



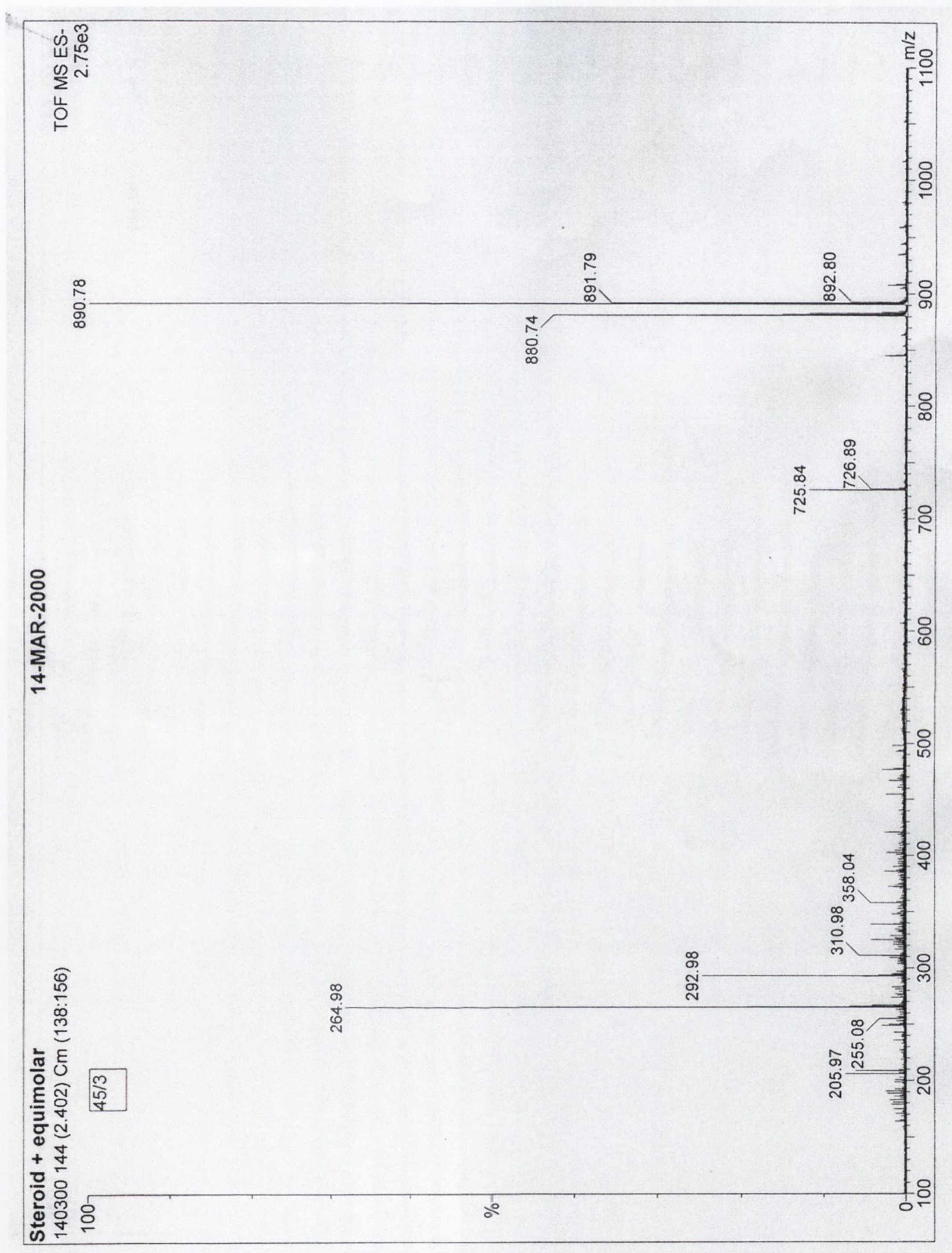
Appendix A17: **4.6L**/**4.6D** decomplexed from a single "blue" member of library **6.1** which had been exposed to TBA **4.6DL**



Appendix A18: **4.6L/4.6D** decomplexed from a single "blue" member of library **6.1** which had been exposed to TBA **4.6DL**

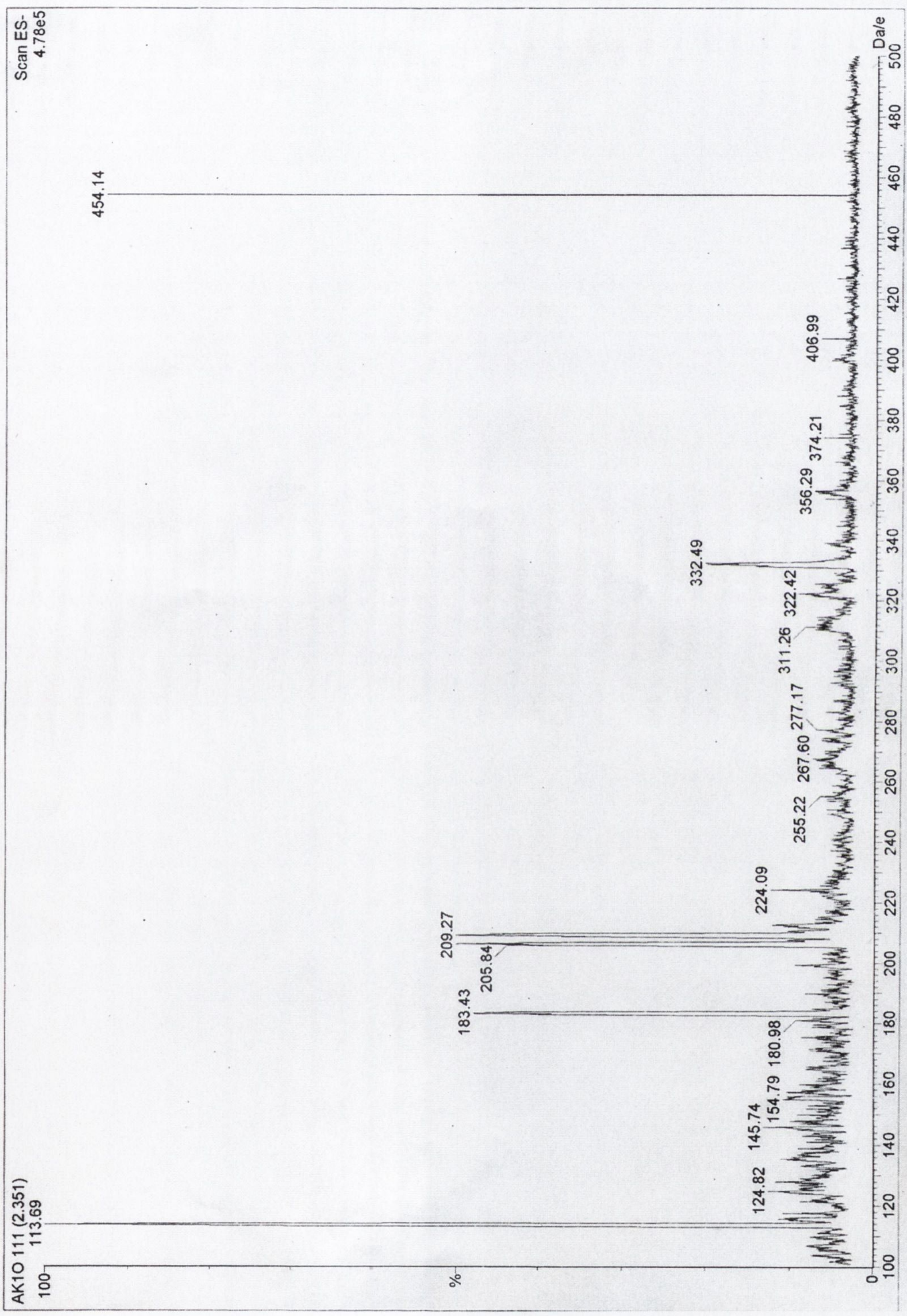


Appendix A19: ESIMS[+b] spectrum of a mixture of receptor **6.2** and excess TBA **4.6DL**



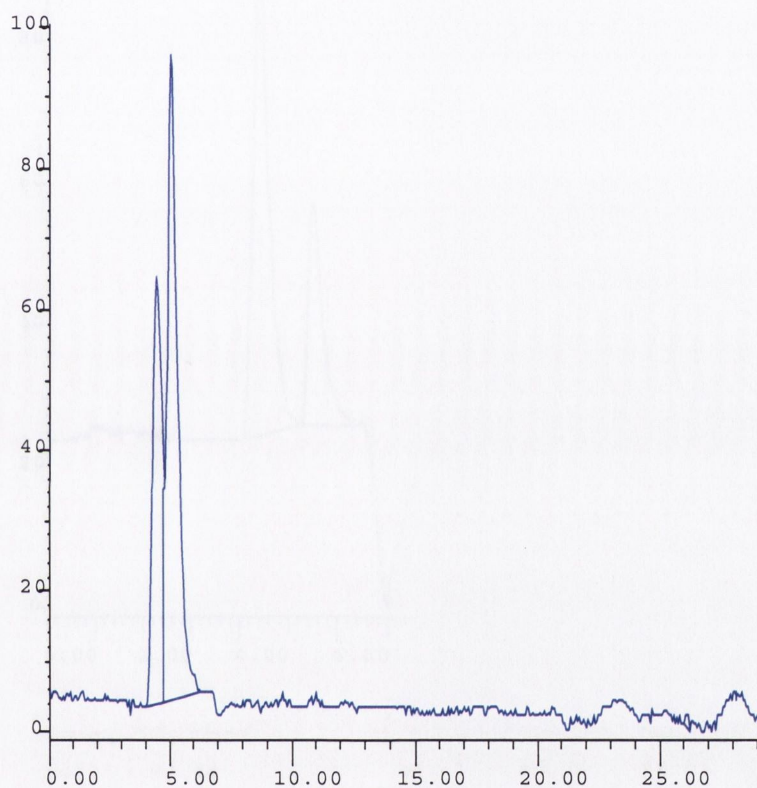
b In **Appendix A19**, ESIMS refers to direct injection electrospray spectroscopy using the Micromass LCT instrument.

Appendix 20: TBA **4.6DL** (5 μM)

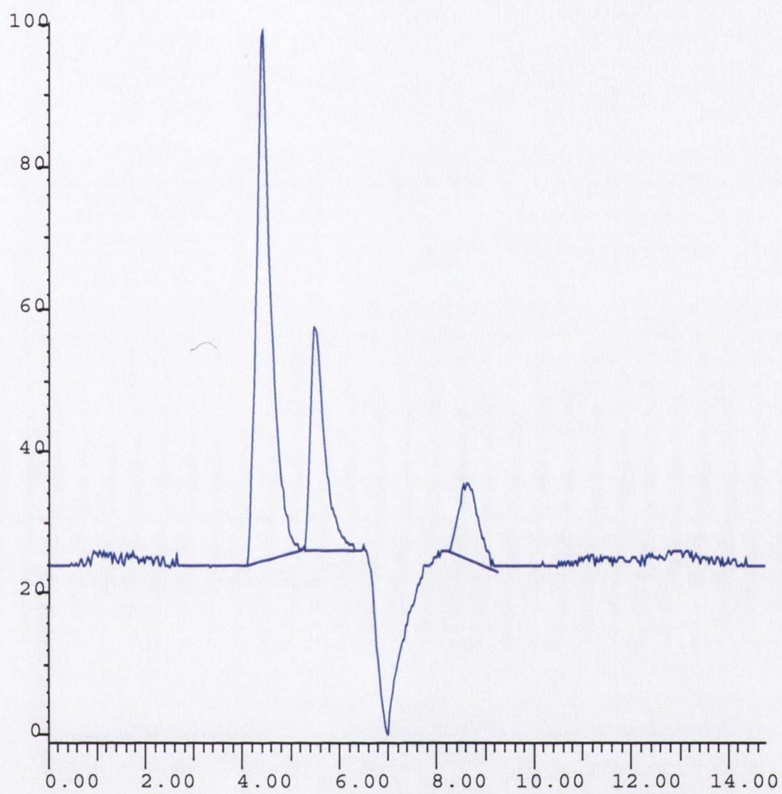


APPENDIX B

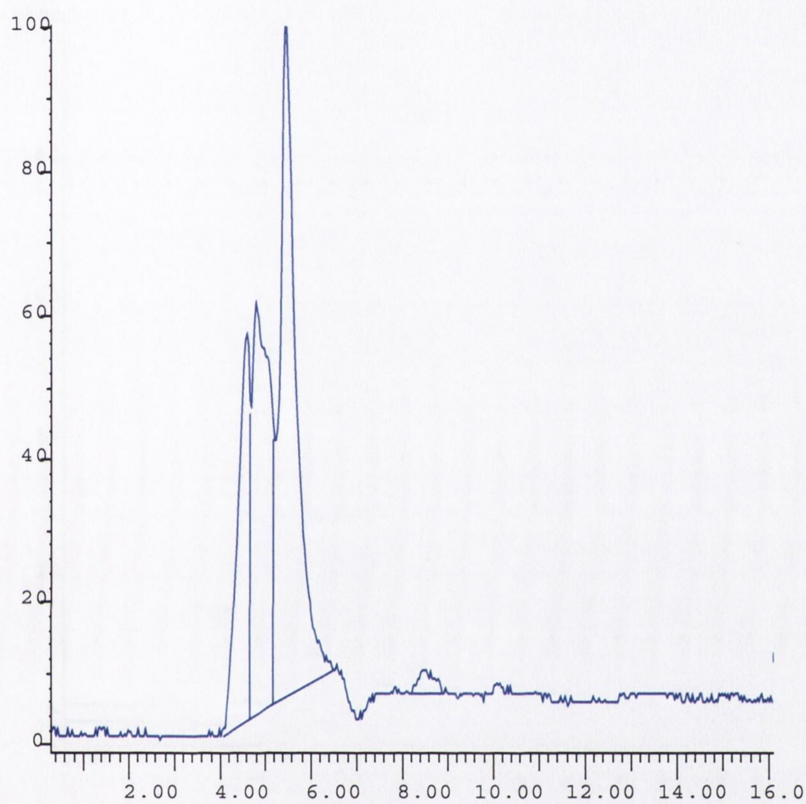
Appendix B1: Chiral HPLC spectrum of **2.6D**. (Daicel Chiralpak AD column; 2 mg/mL; 0.8 mL/min; Heptane/IPA (80/20); 254 nm).



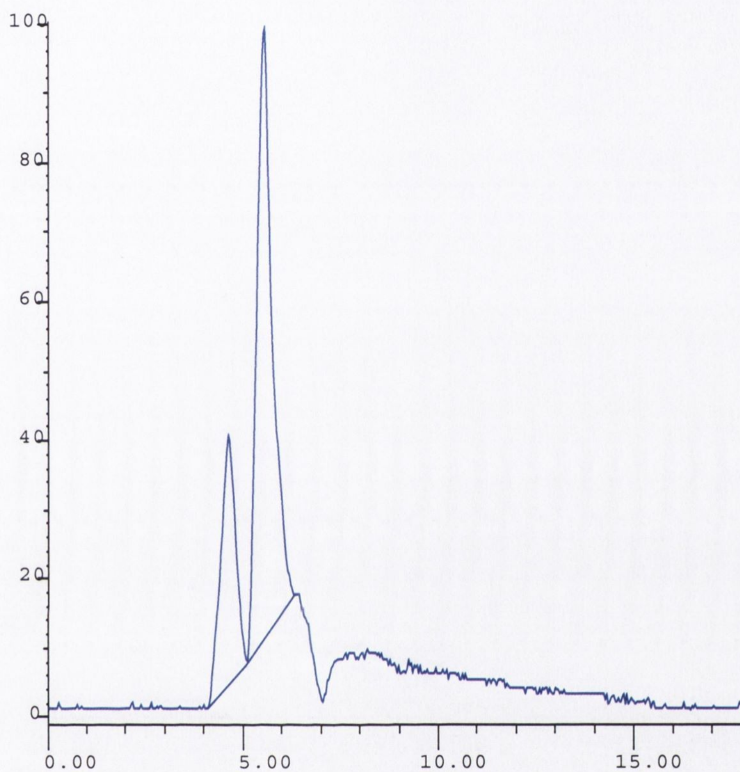
Appendix B2: Chiral HPLC spectrum of **2.6L**. (Daicel Chiralpak AD column; 1 mg/mL; 0.8 mL/min; Heptane/IPA (80/20); 254 nm).



Appendix B3: Chiral HPLC spectrum of **2.6DL**. (Daicel Chiralpak AD column; 0.8 mL/min; Heptane/IPA (80/20); 254 nm)



Appendix B4: Chiral HPLC spectrum of **2.5L,** (Daicel Chiralpak AD column; 1 mg/mL; 0.8 mL/min; Heptane/IPA (80/20); 254 nm).



Appendix B5: Chiral HPLC spectrum of IPA (Daicel Chiralpak AD column: Heptane/IPA (80/20)).