UNIVERSITY OF EDINBURGH

College of Science and Engineering
School of Chemistry

High-throughput synthesis and screening of chiral stationary phase media

By

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Doctor of Philosophy

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ABSTRACT

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Libraries of peptide based chiral stationary phases (CSPs), on silica-supports, were synthesised, packed into columns and tested for their ability to resolve racemates using a range of test analytes. Research focused on varying the CSP end group and results showed that the most important capping group was 3,5-dinitrobenzoyl. On the basis of initial results, CSPs were developed that used 3,5-dinitrophenylalanine; an amino acid variant of the 3,5-dinitrobenzoyl group, in the peptide core. However, these CSP were found to be unsuccessful in separating test analytes.

Possible protein chiral stationary phase ligands were screened using a surface plasmon resonance biosensor (Biacore S51®), using pharmaceutical enantiomers as analytes, to analyse possible binding characteristics, giving results that correlated well with the analogous screenings carried out by HPLC.

A reciprocally designed CSP based on a single enantiomer of anthryl ethanol was synthesised, the key synthetic step involving the use of Sharpless asymmetric dihydroxylation which incorporated the key chiral centre into the molecule. This enantiomer was attached onto a silica support and used as a CSP to separate peptide racemates.

Declaration

The research described in this thesis was carried out by the author under the supervision of Prof. Mark Bradley at the University of Southampton between October 2003 and February 2005 and at the University of Edinburgh between February 2005 and March 2007. No part of this thesis has been previously submitted at this or any other university for any other degree or professional qualification.

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And finally thanks to my family, most importantly Mum, Dad and Tom, who I know will do anything to help me.

"I eat my peas with honey,
I've done it all my life,
It makes the peas taste funny,
But it keeps them on the knife"

- Anon., collected by Edward Lear

Abbreviations

Å	Angstrom
Ac	acetyl
Abu	4-amino butyric acid
AGP	human α-acid glycoprotein
Ahx	6-amino hexanoic acid
Ala	alanine
α	selectivity factor
APS	3-aminopropyl silica
aq	aqueous
Bn	benzyl
bp	boiling point
BSA	bovine serum albumin
br	broad
Bu	butyl
^{t}Bu	tert-butyl
Bz	benzoyl
°C	degrees Celsius
calcd	calculated
CE	capillary electrophoresis
CEC	capillary electrochromatography
CSP	chiral stationary phase
δ	chemical shift in ppm downfield from tetramethylsilane
d	doublet
Dap	diaminopropionic acid
DCM	dichloromethane
DIPEA	N,N-diisopropyl-N-ethylamine
DIC	diisopropylcarbodiimide
DMAP	4-(dimethylamino)-pyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNB	dinitrobenzoyl
eq	equivalent
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ES	electrospray mass spectrometry
Elem	elemental analysis
EM	electromagnetic
Et	ethyl
Fmoc	9-fluorenylmethoxycarbonyl
g	grams
Gly	glycine

h	hours
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
HSA	human serum albumin
Hz	Hertz
^{i}Pr	isopropyl
IPA	2-propanol
IR	infrared
J	coupling constant
k	retention factor
L	litres
Lys	lysine
λ	wavelength
m	multiplet, metres
M	moles *per* litre
Me	methyl
Mem	methoxyethoxyl methyl ether
Mom	methoxy methyl ether
min	minutes
MIP	molecular imprinted polymer
mol	moles
mp	melting point
MS	mass spectrometry
mw	molecular weight
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
Ph	phenyl
Phe	phenylalanine
ppm	parts *per* million
Pr	propyl
1-PrOH	propan-1-ol
2-PrOH	propan-2-ol
PS	polystyrene
Py	pyrenyl
q	quartet
R_f	retention factor (movement of compound/solvent front)
RP-HPLC	reverse-phase high performance liquid chromatography
R_s	resolution factor
rt	room temperature
s	singlet, seconds
Ser	serine
SFC	supercritical fluid chromatography
SM	starting material

SPR	surface plasmon resonance
t	triplet
TLC	thin layer chromatography
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
tlc	thin layer chromatography
t_R	elution time of compound on HPLC
Tyr	tyrosine
UV	ultraviolet (EM spectrum)
Vis	visable (EM spectrum)

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1 Introduction

1.1 Chiral separations

1.1.1 Introduction

In 1815 the French physicist Jean-Baptiste Biot reported that he found α-quartz rotated plane polarized light [1]. This was the first report of a molecule displaying what is now known as chirality. However, the initial ideas of stereochemistry didn't really begin until 1848, when Louis Pasteur reported hemihedral facets on the crystals of racemic sodium ammonium tartrate. He was able to separate the mirror image crystals of the isomers by the use of a magnifying glass and tweezers[2, 3], the first reported chiral separation!

Pasteur found that the physical properties of individual enantiomers differed from one another only in the rotation of a plane polarized light. This led to the postulation that the enantiomers had different three-dimensional arrangements of atoms/groups which at a molecular levels are mirror-images of each other[4]. Following this, the Dutch chemist Jacobus Hendricus van't Hoff[5] and the French chemist Achille Le Bel[6], both independently hypothesised in 1874 that the molecular basis of chirality that Pasteur observed was due to an asymmetric carbon. The asymmetric carbon proposed by Van't Hoff had the correct tetrahedral shape, whereas Le Bel, proposed a square pyramid[3].

The chirality[7] of a molecule can in fact be attributed to any of the following: 1) by virtue of a chiral center, e.g., an sp^3-C covalently bonded to four different groups of atoms; 2) restricted rotation giving rise to perpendicular disymmetric planes, such biaryl antroisomers and unsymmetrically substituted allenes; 3) due to a helical shape, such as helicenes and *trans*-cyclooctene; 4) restricted rotation cause by covalent bonding conformation, such as substituted paracyclophanes; 5) other special cases, such as adamantanes with differing substitutes at the bridgehead positions. A chiral molecule has a non-super-imposable mirror image, called an

enantiomer. Enantiomers have the same internal energies and their physical and chemical properties are tantamount in an achiral environment.

The importance of chirality in the pharmaceutical industry has been recognised for many years, because the pharmacological and pharmakinetic activities may vary between different enantiomers of the same drug[8]. Before 1992 only 10% of all chiral synthetic drugs on the market were sold as single enantiomers. At that time, the Food and Drugs Administration regulations (USA) were changed and stated that for any new stereoisomeric drug to be approved, both enantiomers had to be treated as separate substances in pharmacokinetic and toxicological profiling[9]. In response to this in 1998, 80% of all drugs under development were single enantiomers[8]. Furthermore, by 2000 the worldwide annual sales of single enantiomer products final formulation was $131 billion and by 2005 this had grown to $225, representing 37% of the total final formulation pharmaceutical market of $602 billion [10, 11].

Some pre-existing drugs have been remarketed as single enantiomers, thus (S)-(+)-Ibuprofen reaches therapeutic concentrations in blood in 12 minutes versus 30 minutes for racemic mixture and has now been marketed separately from the racemic drug as a fast acting alternative[12, 13]. (R)-Fluoxetine for the treatment of depression is a pure isomer of Prozac with improved efficacy and also minimizes adverse side effects (anxiety and sexual dysfunction).

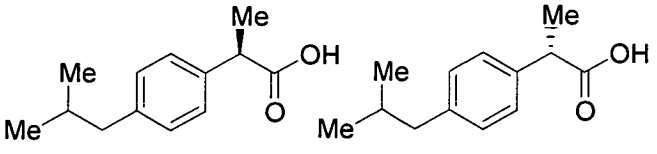


Figure 1 S (left) and R (right) Ibuprofen

There have also been horrendous examples of enantiomer specific toxicological effects. The most well known is that of thalidomide, where R-thalidomide is a sedative and S-thalidomide is teratogenic.[3]

However, although enantiomers have different pharmacological profiles, they have identical physical and chemical properties rendering their separation difficult as conventional techniques of purification cannot be used. As a consequence, pharmaceutical companies have had to find new and improved techniques for both analytical and semi-preparative chiral separations.

Chiral compounds are also important in the agrochemical industries[7, 14], where the usage of non-active isomers (contained in agrochemical racemates), contribute towards environmental pollution and poisoning and are discouraged.[15]. They also have various roles of use within synthesis, specifically asymmetric synthesis[1], such as for the preparation of pure optically active compounds. They are also used in studies for determining reaction mechanisms, as well as reaction pathways. Furthermore enantiomer separations are also important in environmental[16, 17], forensic[18, 19] and nutritional analysis[20].

1.1.2 Chiral separation methods

The market for optically pure products has experienced a huge increase [21], which has stimulated the development of asymmetric synthesis and chiral separation. The application of chiral technologies for pharmaceutical and agrochemical intermediates has been estimated to have generated annual revenues of $8 billion in 2003[22].

Again it was Pasteur that made the first advancements in the field by studying the influence of one chiral compound upon another and introduced the technique of resolution via diastereomer formation[4]. This separation of enantiomers by diastereomer formation is the basis of many modern chromatographic separation techniques.

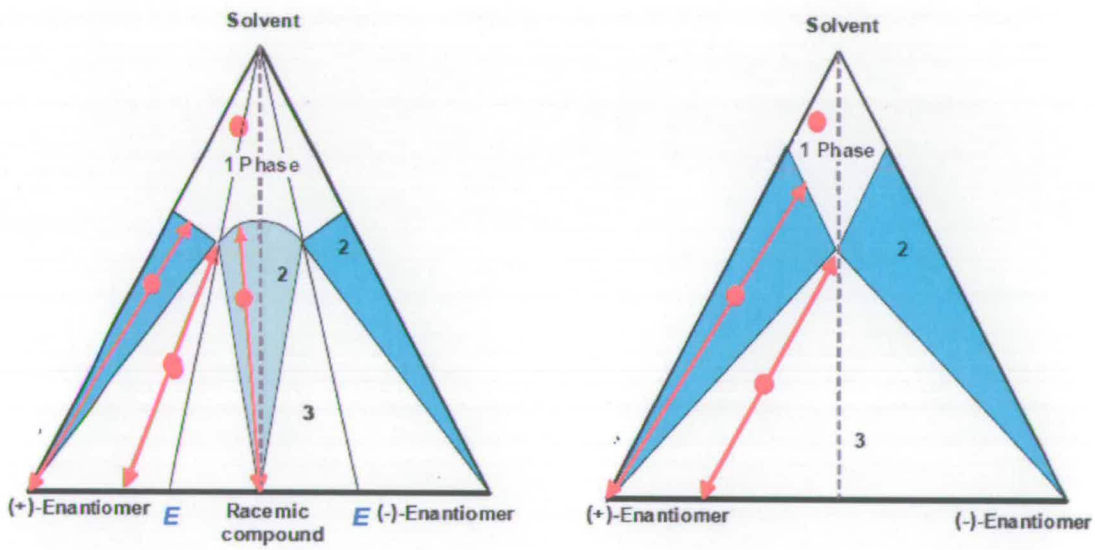
1.1.2.1 *Chiral Derivatisation*

Early methods of separating enantiomers were based on chiral derivatisation[23, 24]. This method involved the reaction of a racemate with an enantiomerically pure chiral derivatising agent (usually a cheap natural product) to form two diastereomers. The diastereomers having different physical and chemical properties could be separated by conventional methods such as chromatography, HPLC and crystallisation. This method is a so called an indirect method. However it is often less favoured, due to the number of extra steps involved, corresponding to extra time, effort and poorer recovery[25]. It is successfully employed by Eli Lilly in the resolution-racemisation-recycle synthesis of the serotonin-norepinephrine reuptake inhibitor, Duloxetine[26] where (S)-Mandelic acid is added to a racemic amine intermediate. The (S,S)-diastereomeric salt formed is insoluble, whereas the (S,R)-diastereomeric salt stays in solution. The (S,S) diastereomer is collected and base hydrolysed to remove the Mandelic acid to the give enantiomerically pure (S) amine.

1.1.2.2 *Chiral Crystallisation*

Enantiomeric enrichment of racemates from solution or melts through chiral crystallisation is possible; however the feasibility of selective crystallization depends strongly on the whims of nature, specifically on the solid-liquid equilibria[27]. Wallach's rule states that racemic crystals are denser than that of the pure enantiomer crystal [28]. It follows that increased density of a crystal normally corresponds to higher crystalline stability and reduced aqueous solubility. Therefore, crystallisation of racemic solutions or melts of these materials form racemic crystals which are homogenous co-crystallisations of both enantiomers, this accounts for 90-95% of molecules[29]. However, there are a small proportion of exceptions to this, where the enantiomer forms more stable crystals than the racemate. In this case the crystallisation process forms crystals that contain just one enantiomer, but the crystals produced are a mechanical mixture of enantiomers giving a so-called racemic conglomerate.

The application of crystallization for the separation or purification of enantiomers requires detailed knowledge of the corresponding binary (melting point) and ternary (solubility) phase diagrams[29]. Figure 2 shows typical ternary diagrams of both racemic compounds and conglomerates. Important features of the diagram indicate the ternary composition required to give pure enantiomers, which are the shaded turquoise areas. A racemic mixture of a conglomerate type material can be directly crystallised into enantiomers at the appropriate solvent concentration and temperature. In the other case (non-conglomerate type), the racemate can only be separated when there is sufficient enantiomeric enrichment in the feed solution. The ternary diagram also shows the conditions at which racemic crystals are formed (light-blue shading). The one-phase areas are near the solvent corner where the solution is too under saturated for crystallisation. The three-phase areas are where a mixture of pure enantiomer and racemic compound crystallise.



Racemic compound Racemic conglomerate

Figure 2 Typical ternary phase diagram of enantiomeric systems under isothermal conditions.

1.1.2.3 *Chiral Capillary Electrophoresis*

Capillary Electrophoresis is a versatile technique of high speed, high sensitivity and low limits of detection. A source vial, destination vial and capillary (20-200 mm i.d.) are filled with an electrolyte such as an aqueous buffer solution. The

sample is then introduced to the capillary inlet via capillary action, pressure, or siphoning and the migration of the analytes is initiated by an electric field which is applied between the source and destination. All ions, both positive and negative, are pulled in the same direction through the capillary by electroosmotic and electrophoretic flow. The analytes separate as they migrate due to their varying electrophoretic mobility. A detector (normally UV/Vis.) placed near the end of the capillary determines the electropherogram.

Chiral recognition mechanisms in capillary electrophoresis are similar to those found in chiral HPLC when using chiral mobile phase additives. Resolution takes place due to the formation of diastereomeric complexes between the chiral selector and the racemate enantiomers. The nature of the interactions between chiral selector and enantiomer depends on the type of chiral selector. Chiral separations are achieved in one of two ways: 1) indirect resolution is performed by diastereomeric complex formation followed by standard resolution by capillary electrophoresis; 2) direct resolution involves the use of chiral selectors in the background electrolyte. There are various types of background electrolyte chiral selectors that have been used, most of which are also used in analogous systems of chiral HPLC as chiral stationary phases. The most common are; macrocyclic antibiotics, proteins and polysaccharides as well as Pirkle type, alkaloids, crown ethers and cyclodextrins. Furthermore, micellular electrokinetic chromatography can be used as an adaptation of capillary electrophoresis, with the addition of sufficient surfactant, to aid the separation of neutral analytes. The disadvantages of this technique are in the scaling up the processes to preparative level and in the trouble of selection of appropriate chiral selectors.

1.1.2.4 *Super-critical Fluid Chromatography*

Supercritical fluid chromatography utilises mobile phases that are used above both the critical temperature (T_c) and the critical pressure (P_c) of the eluent[30]. The most commonly used fluid for SFC is carbon dioxide, as it is relatively inexpensive, non-toxic, non-flammable and readily available. The overall setup of a SFC system is much the same as used for HPLC. Generally SFC is carried out using packed columns, which contain the stationary phase[30, 31]. All columns types used for chiral

separation on HPLC can also be used in SFC, with the exception of protein based CSPs.

SFC does not give an enhancement in enantioselectivity over chiral HPLC as they are analogous techniques in which chiral HPLC separations are achieved on SFC, with few exceptions[32, 33]. However, the reduced viscosities and increased diffusivities of supercritical fluids compared to liquids, mean that the pressure drops across the column and increases the efficiency of the column at higher flow rates[34, 35]. As higher flow rates can be used, separation can be carried out in reduced time. Although, screening on SFC can be carried out more rapidly, it does not remove the trial and error approach associated with method development of chiral separation carried out on HPLC. SFC is complicated by the possible chemical interactions of the carbon dioxide mobile phase with some analytes. In particular amines can react with the carbon dioxide *in situ*, therefore altering the results observed[36, 37].

Enantioseparation can also be carried out with pressures above the critical pressure (P_c), but keeping the temperature below critical (T_c). Although the mobile phase is now a liquid, it still retains some of the preferential properties of a supercritical fluid, this technique is known as subcritical fluid chromatography.

1.1.2.5 *Simulated Moving-bed Chromatography*

Continuous - counter-current chromatography or simulated moving-bed chromatography is an adaptation of traditional HPLC. It was first used in petrochemical and sugar industries for separating very large quantities of materials. The process involves the continuous separation of materials rather than the discontinuous separation of materials in batches, as is the case with HPLC.

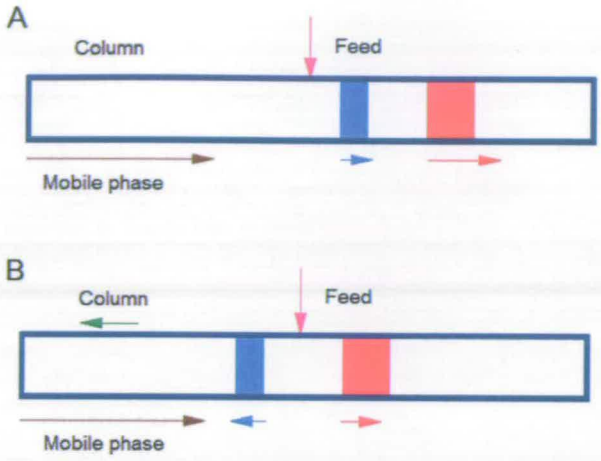


Figure 3 Principle of simulated moving bed chromatography; (A) Analyte mixture feed into middle of column and compounds move at different speeds along the column with mobile phase, (B) Column is moved in the opposite direction to mobile phase with a magnitude between of speeds of the compounds.

A simulated moving-bed system consists of multiple columns connected in series with valves between each column which can be individually opened and closed. The system has multiple pumps controlling the addition of fresh solvent, injecting feed, remove the raffinate and extract used solvent. The valve system between the columns allows the operator to open or close the inlet and outlet of each column simultaneously. The operation of simulated moving-bed chromatography involves many parameters and therefore the design and process optimization are very complicated, therefore these systems use sophisticated computer simulations.

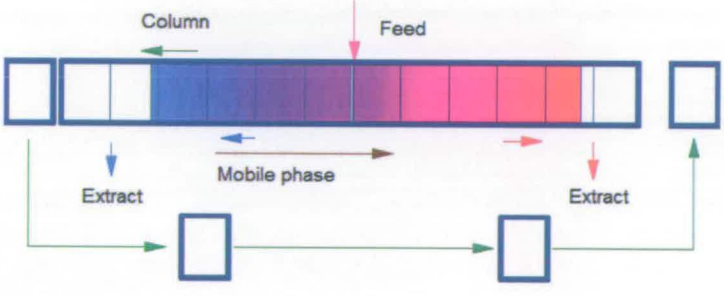


Figure 4 Simulated moving bed chromatography; numerous columns (segments) connected in series with continuous feed of mixture. The mobile phase inlet, feed inlet and compound extract points move each time a column moves from one end to the other.

As well as the advantage that these techniques have for the separation of large quantities of materials; the continuous process also uses less solvent and requires less stationary phase. Furthermore, some racemates have small affinity differences between enatiomers and CSP, where it is not possible to improve resolution by

changing stationary phase or further optimisation of chromatographic conditions. In these cases, simulated moving-bed can be separated by allowing their small retention time differences to accumulate by utilising multiple column passes. However, investment and maintenance cost compared to single column operations are much higher; making simulated moving-bed methods generally unnecessary for non-large scale separations. Also the higher complexity means that each separation has to be extensively simulated.

1.1.2.6 *Molecular Imprinted polymers*

Molecular imprinted polymers can be used as a unique type of chiral selector in HPLC[38-40], TLC, SFC and CEC[41, 42]. Molecular imprinting is method of polymerization to prepare synthetic materials that are able to mimic the molecular recognition found in biological systems[43-45].

The technique involves adding a target molecule with a solution of functional monomers. The two compounds associate to form a 'complex', which is then incorporated by polymerization into a highly cross-linked macroporous polymer matrix. Following this the target is removed to leave sites in the polymer with specific shape and functional group complementarity to the original print molecule. The polymeric material formed can then be used in various chromatographic systems as a chiral selector with its special recognition capacity for target enantiomers[46].

As with the conventional techniques of HPLC and CE, initially screenings are required for the determination of appropriate CSP or electrolyte additive respectively. MIP have the advantage of the made-to-measure approach, where the order of elution is always predictable with the imprinted target enantiomer being more strongly bound. However, MIP's are not suitable for use with low solubility target molecules. There are many other disadvantages to this technique, such the requirement of significant quantities of enantiomeric target material[47], low sample capacity and poor peak shape[48, 49], and non-suitability with reverse phase chromatography[50].

1.1.2.7 *Gas Chromatography*

As with HPLC, Gas Chromatography chiral separations are mainly performed on chiral stationary phases, although indirect methods are also used; where chiral analytes are pre-derivatised with chiral auxiliaries and separated on achiral stationary phases.

The stationary phase of a chiral GC can be formed by a variety of chiral selectors most of which are in common with those used in HPLC. The most common are: amino acid or, metal coordination based and cyclodextrin inclusion type[51] which are normally covalently bound to polysiloxanes[52]. As in HPLC, the analyte enantiomers form transient diastereomeric intermediates with the stationary phase chiral selector, leading to one enantiomer being more strongly bound to the stationary phase and therefore being retained longer on the column/capillary.

The main application of chiral Gas Chromatography is in the precise determination of enantiomeric compositions[53], due to the technique being quick and highly efficient. Also it has the advantage that volatile enantiomers can be analysed directly from the vapour phase of the sample matrix, whereas LC and CE require optimization of numerous chromatographic/electrophoretic conditions, such as solvents, buffer pH, modifiers and gradients. However, due to the conditions in which GC is run, there are many restrictions to the analysis carried out. Most importantly the analyte has to have sufficient volatility, but also have sufficient thermal stability and stereochemical integrity at elevated temperatures[53].

1.1.2.8 *Other methods of chiral separation*

Chromatography direct methods of chiral separation are based on the formation of transient diastereomeric complexes, rather that derivatisation. One approach called, Chiral Mobile Phase Additives consists of adding an enantiomerically pure compound to the HPLC mobile phase to generate transient diastereomeric complexes between the analyte and the chiral mobile phase additive[54]. However

this method is not widely used, because of the constant supply of the often expensive chiral mobile phase additive required.

There are many other methods which are currently not routine for chiral separations; however they may have specific application in which they are appropriate. Potentiometric, amperometric and piezoelectrical enantioselective biosensors have been used for enantioselective analysis of chiral drugs on a miniaturised scale[55-57], although, their sensitivity and biosensor lifetime are low[57]. Another method focuses on the use of chiral selective membranes in electrodialysis, where the chiral selector is a membrane which separates selections of the electrodialysis chamber. This is similar to capillary electrophoresis, although it also has the potential to be used on larger scale separations, however currently the optical enrichment provided is poor[58].

1.2 Chiral chromatography and HPLC

1.2.1 Chiral Stationary Phases

Since 1980 chiral separations have been mainly carried out using a direct method, chiral chromatography. Chiral HPLC columns are made by immobilising single enantiomers onto a stationary phase. Resolution relies on the formation of transient diastereomers on the surface of the column packing. The compound which forms the most stable diastereomer will be most retained, whereas the opposite enantiomer will form a less stable diastereomer and will elute first. The ability of a given CSP to interact differently with enantiomers is called chiral recognition and the principles of chiral recognition have been extensively studied. To achieve chiral recognition and discrimination between enantiomers there needs to be a minimum of three points of interaction, with at least one of these interactions being stereochemically dependent[59], the three point rule[60].

The forces that lead to this interaction are very weak and require careful optimisation by adjustment of the mobile phase and temperature to maximise selectivity. Chromatography is a multi-step method where the separation is a result of the sum of a large number of interactions. Typically a free energy of interaction difference of only 0.03 $kJmol^{-1}$ between the enantiomers and the stationary phase can lead to resolution.

The intermolecular forces involved with chiral recognition are polar/ionic interactions, pi-pi interactions, hydrophobic effects, hydrogen bonding dipole-dipole (Keesom), induced dipole-dipole (Debye) and London dispersion forces[1]. These can be augmented by the formation of inclusion complexes and binding to specific sites such as on peptides or receptor sites in complex phases.

1.2.2 Chiral Stationary Phase Types

Chiral Stationary Phases (CSPs) may be classified according to their interaction mechanism with the racemate. A scheme for classification was first proposed by Wainer[61]:

1.2.2.1 *Type I CSPs (Pirkle or Brush type)*

These differentiate enantiomers by the formation of complexes based on attractive interactions. These may be hydrogen bonds, pi-pi interactions, dipole stacking. The columns within this group are mainly the result of the work of Pirkle[62, 63] and co-workers. Pirkle applied work on chiral NMR to HPLC stationary phases in the 1960's, by a process of reciprocal design, that is immobilising analytes, onto solid-phases, that had given large 'resolutions' on earlier chiral stationary phases in solution.

Pirkle type CSP's fall into three classes: π-electron acceptors/π-electron donors (e.g. Whelk-0, ULMO), π-electron acceptors (e.g. Phenylglycine, β-Gem 1, α-Burke 2, Pirkle 1-J) and π-electron donors (e.g. Naphthylleucine). They are generally made up of small molecules covalently bound to silica, meaning the columns exhibits high durability without the leaching of chiral selector into the mobile phase. Chromatography with Pirkle columns is generally run in normal phase mode, but is also extensively used in reverse phase mode. Further details of synthesis and usage of Type I CSP are in section 1.3.

1.2.2.2 *Type II CSPs*

1.2.2.2.1 Polysaccharide

Most type II phases are based on polysaccharide derivatives. Polysaccharides can be immobilised onto the silica support, via absorption onto the surface of the silica[64, 65]. The chemically modified polysaccharide can be immobilized onto a vinylised silica gel via a radical co-polymerization reaction[66], polycondensation of triethoxysilyl groups[67, 68] or by covalent bonding using an inert linker to the silica[69]. The chiral selector is normally cellulose or amylase based with a large number of

commercial variations based on different derivatisations of the saccharide hydroxyl groups.

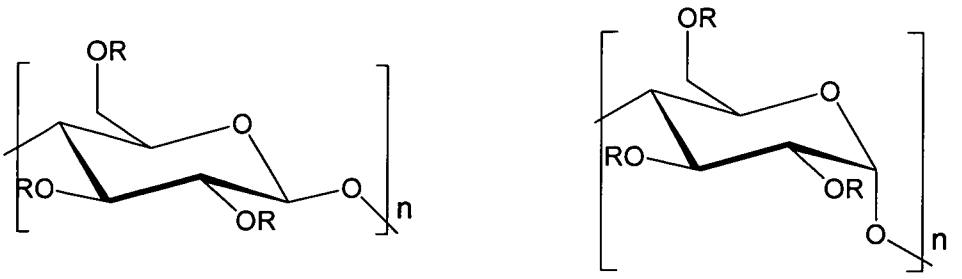


Figure 5 Left: a modified cellulose chiral selector found in type II CSPs. Right: a modified amylase chiral selector found in type II CSPs; the R groups represent the point of variation for many commercialised columns.

There are many different derivatised polysaccharide based CSP available, each with their own specialised uses. These include: 3,5-dimethylphenylcarbamate for both cellulose[70, 71] (e.g. Chiralcel-OD®) and amylose[71-73] (e.g. Chiralpak-AD®), these columns have good generality use and can separate a large range of racemates and are particularly effective for aromatic, amide, carbamate and ester groups as well as alkyl amines, and compounds with multiple stereogenic sites; 1-phenylethylcarbamate for amylase[72] (e.g. Chiralpak-AS®) and 4-methylbenzoate for cellulose[74] are particularly effective for ß lactams, epoxides, acids, natural products and heterocyclic compounds. (Chiralpak-OJ®); acetate for cellulose (Chiralcel-OA®), this is a special column suited for low molecular weight racemates; 4-chlorophenylcarbamate[73] (e.g. Chiralcel-OF®), which is a very retentive column and especially suited for prostaglandins. All of the above column phases are produced by coating the polysaccharide onto silica, which renders some higher polarity solvents incompatible[75], due to problems of leaching. Recently, Chiralpak IA amylose 3,5-dimethylphenylcarbamate and Chiralpak IB cellulose 3,5-dimethylphenylcarbamate columns, with polysaccharides immobilized on silica gel, were launched onto the market; they can be used with a wide range of solvents[76]. They also show some amount of complimentarity in comparison with similar coated columns[77, 78].

There are a number of sites in each derivative which may interact with functional groups on chiral analytes. For example there are points for π-π stacking

interactions, dipole–dipole stacking and hydrogen bonding. These interactions are relatively weak and are considered to be more effective under normal-phase conditions[79]. Although, normal-phase conditions are most popular with polysaccharide based CSP[80, 81], both reverse phase[79, 81] and polar-organic[81] eluents have been successfully employed.

1.2.2.2.2 Synthetic helical polymers

Helical polymers are another type of CSP that can interact with analytes by forming inclusion complexes. However there is limited research into their application. Unlike biological macromolecules of proteins and nucleic acid where the helical nature is determined by the homochirality of their components, the absence of chirality from the building blocks of synthetic helical polymers leads to equal preference of right and left-handed helices[82]. A predominance of one helix can be attained by the used of chiral monomers or via the use of asymmetric polymerisation[82, 83]. Although certain helical polymers can resolve a range of racemates, details of the molecular recognition mechanism are not widely known and advantages of these over polysaccharide based CSP seem limited[84].

1.2.2.3 *Type III CSPs*

This category is for macrocyclic based CSP, with chiral selectors based on cyclodextrins, glycopeptides and crown ethers. They rely on the solute entering into chiral cavities to form inclusion complexes.

1.2.2.3.1 Cyclodextrin CSPs

Cyclodextrins are cyclic oligosaccharides containing six or more D-(+)-glucopyranose units, which are bonded through α-(1,4) linkages. They are the most widely used type of chiral selector in chiral GC; they are also very commonly used in CE. The classic inclusion complex column is the cyclodextrin type of column developed by Armstrong[62]. Although generally they are not first choice in HPLC and SFC, currently there are many commercialised cyclodextrin CSP columns which are successful for enantioseparation using all mobile phases modes. Hydroxypropyl-β-cyclodextrin[85] (e.g. Cyclobond I RSP) and aromatic

derivatised cyclodextrin[86] (e.g. Cyclobond I DMP) are very widely used and effective for many enantioseparations.

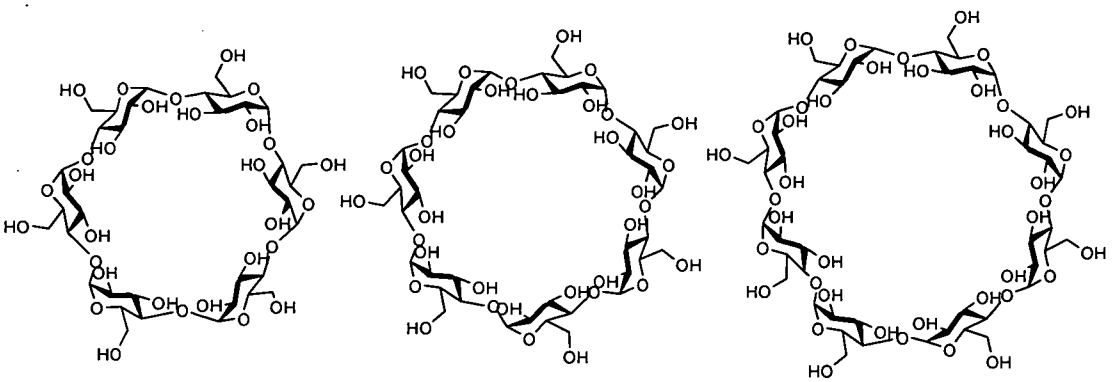


Figure 6 from left to right: α, β and γ-cyclodextrin.

When in reverse phase mode, the interaction mechanism is dominated by formation of hydrophobic inclusion complexes (Figure 7) and hydrogen bonding interaction at the mouth of the cyclodextrin cavity. In normal and polar organic phase modes dipole-dipole forces play a role in the interaction between analyte and the immobilised cyclodextrin.

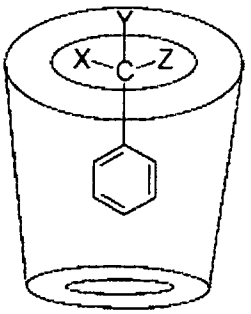


Figure 7 Formation of inclusion complex between analyte enantiomer and cyclodextrin

Cyclodextrin based CSPs can be synthesized by immobilization of monoazido functionalised Cyclodextrins onto the surface of amino functionalised silica gel via stable urea linkages using a Staudinger reaction[87, 88], or by forming a reactive siloxane intermediate using hydrosilylation with triethoxylsilane followed by its coupling with silica gel[89]. The main advantages of cyclodextrin CSP are their ease of preparation, broad applicability and cost. However, the kinetics of host-guest complexation are rather slow and therefore can result in poor chromatographic peak shapes.

1.2.2.3.2 Crown ether CSPs

Crown ethers are macrocyclic polyethers which form stable inclusion complexes with alkali, alkaline-earth and primary ammonium cations. They were first synthesised by Pedersen[90] and then adapted into chiral crown ethers by Lehn[91] and Cram[92, 93]. It was Cram who used the chiral crown ethers for enantioseparation, first using them as a chiral mobile phase additive[94] then later as a CSP[95].

Chiral crown ether CSPs are powerful tools for the separation of chiral molecules containing primary amino groups in reverse phase mode with acidic additives. Chiral recognition is based on the formation of inclusion complexes between the primary ammonium ion and the crown. Crown ethers are covalently bound to silica in CSP synthesis such as in the commercialised column Sumichiral OA-8000 (Figure 8)[96-98]. However, the use of acid in the mobile phase means that the durability of a crown ether column is a concern. This problem has been recently overcome by the use of phenolic substituents in the CSP (Figure 8)[99, 100]. Phenol in the mobile phase is sufficient to form ammonium ions from amine analytes; so it can be used in normal phase mode[101]. The major disadvantage of crown ethers as chiral selectors is their limited range of racemates which can be effectively separated. Currently a large array of primary amines and secondary amines, including β-blockers[102] have been successfully resolved.

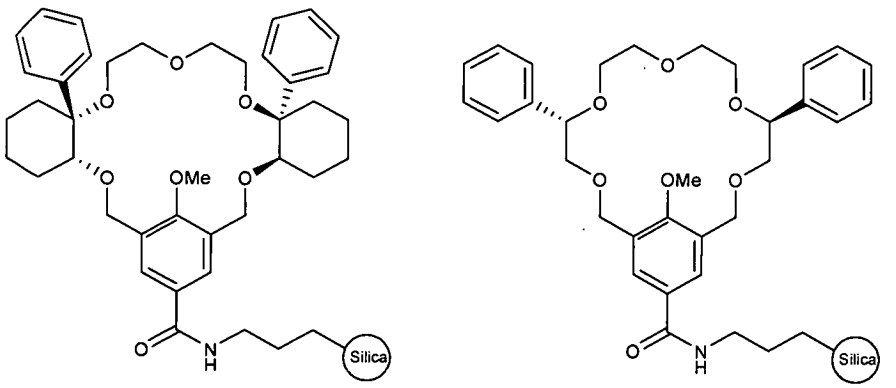


Figure 8 left: Crown ether CSP by Hirose; right: Sumichiral OA-8000 [101].

1.2.2.3.3 Macrocyclic antibiotics

In the past decade, macrocyclic antibiotics have proved to be an exceptionally useful class of chiral selectors for the separation of enantiomers of biological and pharmacological importance by means of HPLC and electrophoresis. In particular

the use of glycopeptides; avoparcin, teicoplanin, ristocetin A and vancomycin (Figure 9) have been used to resolve more racemates than with all the other macrocyclic antibiotics combined[103].

Vancomycin-related antibiotics bind to the bacterial cell wall peptide D-Ala-D-Ala-OH, thus blocking the process of wall-building of Gram-positive bacteria under aerobic and anaerobic conditions[104, 105]. Since the target of these antibiotics is the D-Ala-D-Ala group, in 1994 Armstrong used them in the separation of amino acid enantiomers[106]. Macrocyclic antibiotics possess several characteristics which allow them to serve as chiral selectors. They posses a number of stereogenic centres and functional groups, this allows them to have multiple interactions with chiral analytes. Different antibiotics may be acidic, basic, or neutral, and can have little or no UV–Vis absorbance. The macrocyclic antibiotics can interact by hydrophobic, dipole-dipole, π-π interactions, hydrogen bonding, as well as steric repulsions[107] and ionic interactions[108, 109].

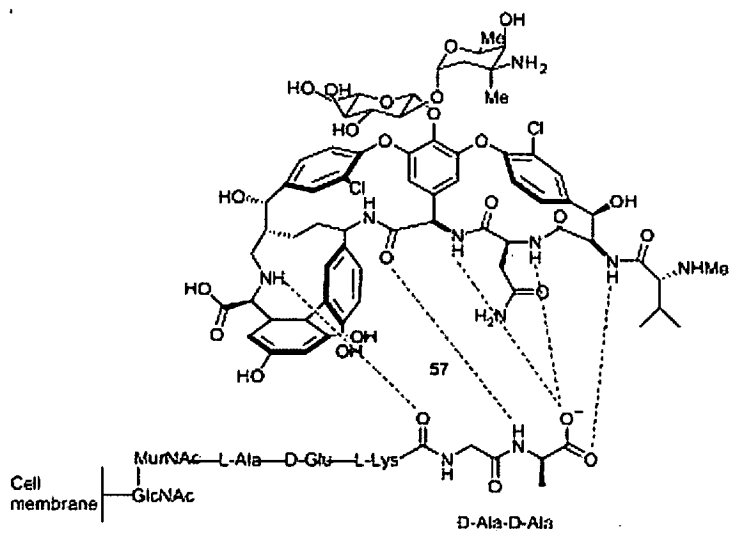


Figure 9 Vancomycin binding to D-Ala-D-Ala.

Macrocyclic antibiotics CSPs are synthesized by the covalent linkage of antibiotics to silica gel using a variety of chemistries. This ensures their stability while retaining their chiral recognition properties[110-112]. As the glycopeptides have hydrophobic, hydrophilic and ionisable moieties, this gives them good solubility in aqueous solution. Furthermore, unlike protein based CSP, glycopeptide based CSP can be used in normal and polar organic mode without irreversible change or

denaturation. They can also be derivatised to alter their enantioselectivity[110, 112]. Also, glycopeptide CSPs in general have good complimentarity to each other; partial separation of an analyte on one can often be completely resolved on another.

1.2.2.4 *Type IV CSPs*

Type IV CSPs separate by means of diastereomeric metal complexes. This technique is also known as Chiral Ligand Exchange Chromatography (CLEC) and was developed by Davankov[113, 114]. It was the first practical approach for the resolution of amino acids on HPLC and is now an especially effective method for separation of amino acids, amino acid derivatives and hydroxy acids.

In ligand exchange chromatography the retention of analytes occurs by the reversible formation of metal complexes by the coordination of the analyte molecule, which acts as a ligand to the metal ion. In traditional CSP based separation, a chiral amino acid-copper complex is bound to silica or a polymeric stationary phase. Racemic amino acids may then be separated by the formation of diastereomeric copper complexes. Copper ions are also included in the mobile phase to ensure there is no loss of copper from the complex. The Cu^{2+} or other metal ions form diastereomeric complexes with the chiral analytes, where the complexation is kinetically labile and separation occurs due to differences in the thermodynamic stabilities between the enantiomers and the divalent cation[115].

As well as CSP based ligand exchange chromatography, amino acids have also been successfully separated using ligand exchange molecules as chiral mobile phase additives and as chiral coated phases, where reverse phase silica is coated with a hydrophobic derivative of the complexing chiral selector[116]. However, ligand exchange chromatography is not a widely used technique. Due to the constraints in the formation of diastereomeric complexes at the metal binding sites, there are strict limitations to which chiral molecules can be successfully separated. Only ionisable analytes with hydroxyl or amino substituents α to a carboxylic acid are appropriate for the formation of the diastereomeric complexes necessary for

resolution[115]. Furthermore, ligand exchange chromatography is further complicated by the necessity of UV absorbing copper salts in the mobile phase[115].

1.2.2.5 *Type V CSPs*

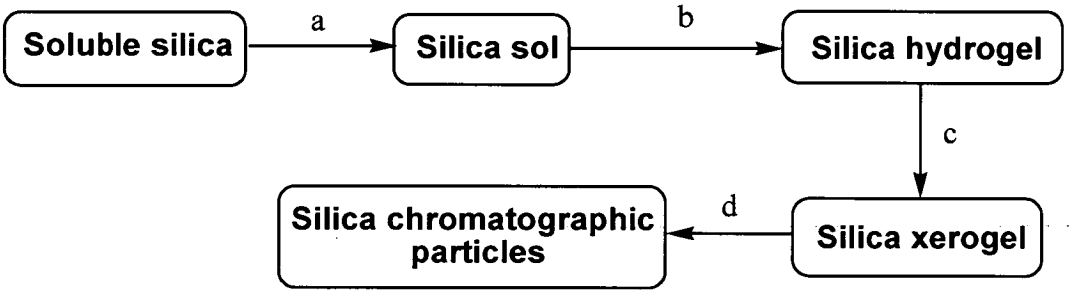
Proteins CSPs are defined as Type V CSPs where separations rely on a combination of hydrophobic and polar interactions. In 1973, it was first demonstrated that protein could be used for chiral resolution, when Stewart[117] used bovine serum albumin on agarose to separate D,L-Tryptophan. Since then, various proteins were being used as chiral selectors in HPLC; glycoproteins such as α_1-acid glycoprotein[118], ovomucoid[119], ovoglycoprotein[120], avadin[121] and riboflavin binding protein[122]; albumins such as bovine serum albumin[123] and human serum albumin[124]; enzymes such as trypsin[125], cellobiohyrolase I[126], lysozyme[127], pepsin[128], α-chymotrypsin[129] and amyloglucosidase[130]; and other proteins such as β-lactoglobulin[131] and ovotransferrin[132] have all been used.

Enantioseparation of analyte racemates using protein CSPs occurs due to multiple possible intermolecular interactions between enantiomer and protein surfaces. Some proteins are specifically good for resolution of acid, basic or neutral racemates, while others are particularly effective for certain types of analytes[133]. To use proteins as chiral selectors, the protein has to be incorporated into the stationary phase whilst also maintaining the protein's natural conformation which is essentially for analyte resolution. This has been achieved in a number of different ways, the most common of which is immobilisation via covalent attachment using silica, agarose or other polymer as the base material. Further details of synthesis and usage of Type I CSP are given in section 1.4.

1.2.3 Silica

Although porous polymer and other materials are used as solid-phase supports in HPLC stationary phase packings, silica is by far the most used material, due to its high mechanical robustness allowing high operating pressures for long periods while displaying low backpressures as well as high column efficiency[134]. Perfusion particles are commonly used for the separation of macromolecules, they contain

very large pores (4000 to 8000 Å) throughout the support and also include a network of smaller interconnecting pores (300 to 1000 Å) between these large through-pores[25]. However, the most appropriate particles are totally porous microspheres which are available in various different diameters, pores sizes and surface areas. They provide high efficiency, sample loading and durability. They can be used for separating small molecules, when particles are 3-15 μm, pore sizes are 70-120 Å and surface area 150-400 m^2g^{-1}, but separation of macromolecules requires larger pores (> 150 Å).



(a) Hydrolysis and polycondensation, (b) Condensation and gelling, (c) Condensation, ageing and dehydration, (d) Hydrothermal treatment and sieving.

Figure 10 Formation of silica particles

Manufacturing of porous silica is most frequently achieved via the sol-gel[135] process starting from sodium silicate solutions, silicon tetrachloride or tetraalkoxysilanes, where the conditions employed can be used to control the particle shape, particle diameter, pore size and surface area generated (Figure 10).

For optimal use in HPLC the silica particles should contain a fully hydroxylated layer of silanol groups (SiOH), with a concentration of about 8 $\mu molm^{-2}$ [136]. These silanol groups can be chemically modified with a wide variety of different functionalities[137-140]; which can be used to alter the polarity of the stationary phase or to tether other molecules to the silica particle. Chemical modifiers include alkyl, aryl, halogen, hydroxyl, amine, carbonyl and carboxylic acid groups, which can be achieved using a number of different methods with the most applicable being through the formation of a siloxane group (Scheme 1)[141-144].

Scheme 1 Functionalising silica surface via siloxane formation.

1.2.4 Liquid Chromatography Variables

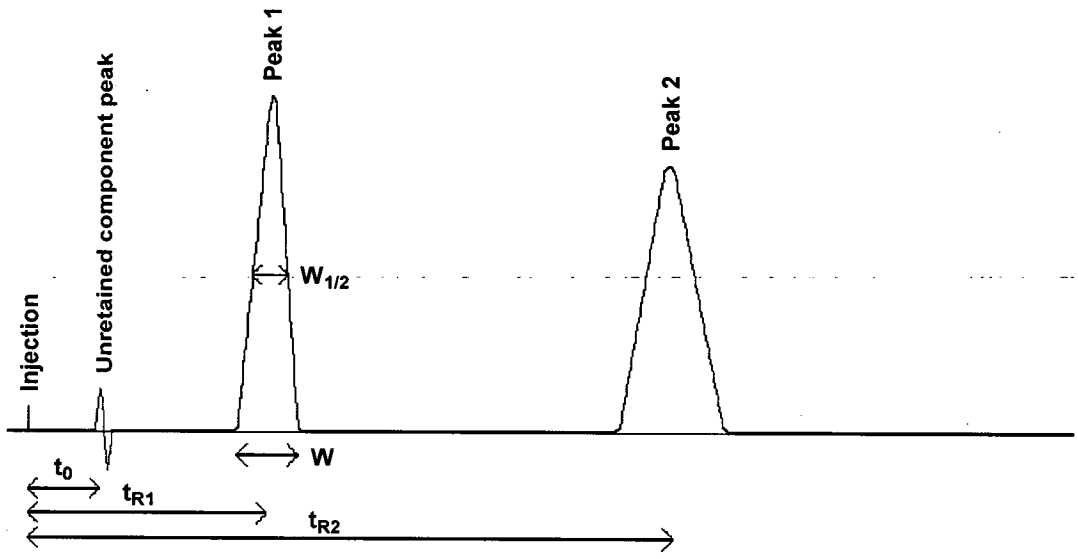


Figure 11 Standard chromatographic conditions.

Retention factor $k = (t_R - t_0) / t_0$

The capacity factor, k, of a sample component is a measurement of the degree to which that component is retained by the column relative to an unretained component, and t_0 is the elution time of the unretained component representing the column void volume.

Selectivity factor $\alpha = k_2 / k_1$

The selectivity parameter α, is a measure of the ability of the column to separate peaks 1 and 2 due to different affinity and therefore retention.

Resolution factor $R_s = 2 (t_{R2} - t_{R1}) / (W_2 + W_1)$

W is the peak width of the component, R_s measures the quality of separation of two adjacent peaks, where W_1 and W_2 are the peak base widths of peak 1 and peak 2 respectively.

Theoretical plates $N = 16 (t_R / W)^2 = 5.54 (t_R / W_{1/2})^2$

$W_{1/2}$ is the width at half the peak height, 16 is a factor carried over from distillation theory. N is the number of theoretical plates, which represents the efficiency of a column. N is the measure of the band spread of a peak throughout the chromatographic system. The smaller the band spread, the larger the N and the more efficient the column is.

1.3 Type I CSPs (Pirkle or Brush)

Low molecular mass selectors linked to a solid support form the type I class of stationary phase (also called brush, Pirkle or donor-acceptor type phases), in which the individual chiral molecules are evenly distributed onto the surface of the silica.

Z^1 = carbonyl hydrogen bond receptor, Z^2 = hydrogen bond receptor, PB = π-base, PA = π-acid

Figure 12 Three-point interaction of Pirkle type CSP with enantiomers

Work by Pirkle in 1979 using rationally designed chiral columns based on the three-point interaction rule was the basis for type I CSPs[145]. Whilst working on NMR shift reagents, he observed that chiral fluoroalcohols such as 2,2,2-trifluoro-9-anthrylethanol interacted with a wide number of solutes to form two diastereomeric two-point chelate-like solvates[146, 147]. There were three points for interaction with the third needed to obtain a stereochemically dependent interaction; these were hydrogen bonding, carbonyl hydrogen bonding and π-π donor-acceptor (Figure 12)[148, 149]. Using this approach Pirkle was able to separate a number of 3,5-dinitrobenzoyl derivatised amines, alcohols and mercaptans. From this work Pirkle used the principle of reciprocity to design 'second-generation CSPs', such as the well known phases of 3,5-dinitrobenzoyl phenylglycine and 3,5-dinitrobenzoyl leucine, which were capable of separating a wide range of analytes containing π-donor groups.

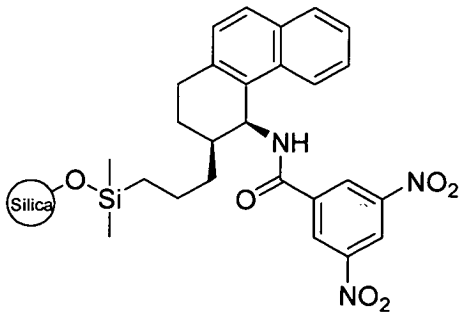


Figure 13 WHELK-O 1 chiral stationary phase

The reciprocity concept was also used in development of other commercially available CSP, such as WHELK-O 1 (Figure 13) which was especially designed for the separation of the non-steroidal anti-inflammatory drug naproxen.

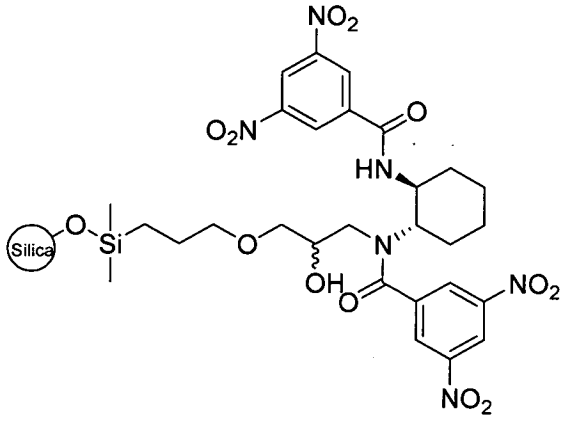


Figure 14 DACH-DNB chiral stationary phase

Another series of CSP have used the trans-1,2-diaminocyclohexane (DACH) molecule as a potential new chiral selector[150, 151], because of beneficial results obtained on using some DACH derivatives as chiral auxiliaries in the field of asymmetric induction. This research has lead to the commercially available DACH-DNB columns which have been used to separate a large range of racemates, further more the DACH was used for a series of hybrid polymeric chiral materials[152].

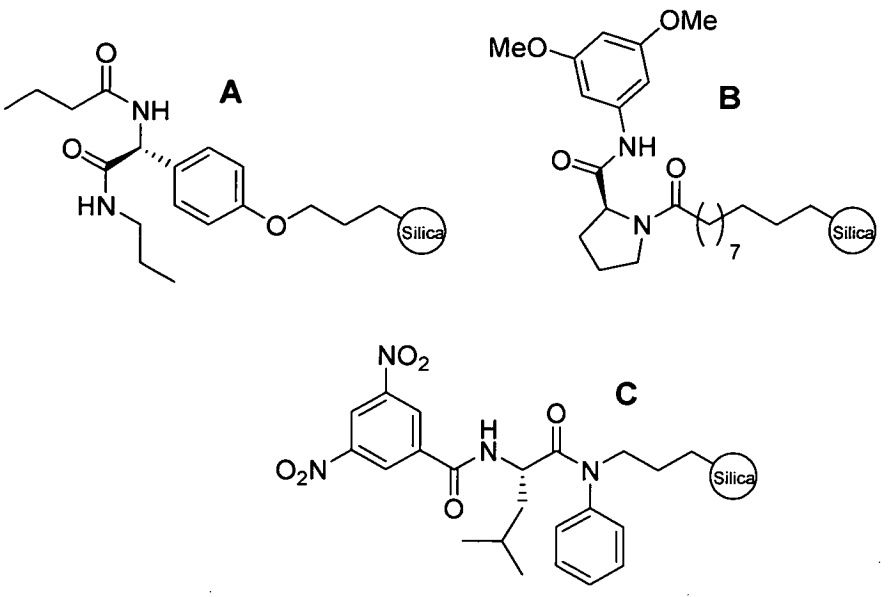


Figure 15 Amino acid based CSPs[153-155].

A large number of CSP have been based on the use of amino acids as they possesses many qualities which make good chiral selectors[153-155]. They inherently have chirality with each enantiomer being readily available, and are appropriate for the solid-phase synthesis which may be appropriate in synthesizing CSP. In recent years there have been extensive examples of totally synthetic chiral selectors being used in chiral stationary phases with mixed results[156-159].

Type 1 CSPs can separate a wide spectrum of racemic compounds, such as alkyl and aryl carbinols, aryl substituted hydantoins, lactams, succinamides, phthalides, sulfoxides, sulfides, amides and imides[14]. For racemic analytes having amino, carboxyl and hydroxyl polar functional groups, they have to be made less polar by derivatisation as they interact too strongly with the CSP when underivatised.

1.4 Protein type V chiral stationary phases

1.4.1 Introduction

Protein-based CSPs are of special interest because of their unique enantioselective properties and because they are capable of separating a wide range of different racemate types[7, 160]. The CSP can be made up of native protein, protein fragments or modified protein and rely on many different types of interaction with the analyte to achieve resolution.

1.4.2 Types of protein CSP

1.4.2.1 *Glycoproteins*

Glycoproteins are proteins that contain oligosaccharide chains (glycans). α_1-acid glycoprotein[118], ovomucoid[119], ovoglycoprotein[120], avadin[121] and riboflavin binding protein[122] are glycoproteins that been used in CSP columns. Human α_1-Acid glycoprotein contains one peptide chain of 181 amino acids and has five polysaccharide moieties, whilst it also contains 14 sialic acid residues (a monosaccharide containing a nine carbon backbone and acid functionality) which gives the protein an acidic character and a isoelectric point of 2.7[161] and was first used as a CSP by Hermansson[118]. AGP based CSP columns have been showed to separate a large range of different compounds[160, 162-164] although the retention and enantioselectivity depend greatly on the chromatographic conditions used, especially the use of modifiers which can affect the hydrogen bonding properties[165] as well as pH and temperature which cause a conformational change in AGP structure[166, 167].

Ovomucoid, ovoglycoprotein and avidin from chicken egg white were first used as chiral selector in CSPs by Miwa[119, 121]. Ovomucoid CSP columns have been utilised in separating drug enantiomers and in assaying of enantiomers in biological fluids[168, 169]. Avidin which has an isoelectric point of 10 has been applied mainly to the separation of acidic and neutral compounds[121, 170, 171]. Riboflavin samples from chicken egg white[122], chicken egg yolk[172] and quail egg white[173] have been

reported as separating a small number of racemates in chromatography although these are more commonly used for capillary electrophoresis.

1.4.2.2 *Albumins*

Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA) have both been used as chiral stationary phases to successfully separate a variety of enantiomeric forms. BSA and HSA are closely related and therefore share many binding characteristics; both have an isoelectric point of 4.7[174]. They have been immobilised onto agarose, silica[175-177], hydroxyethylmethacrylate[178], polystyrene divinylbenzene perfusion beads[179] and polyethylene hollow fibre membranes[180].

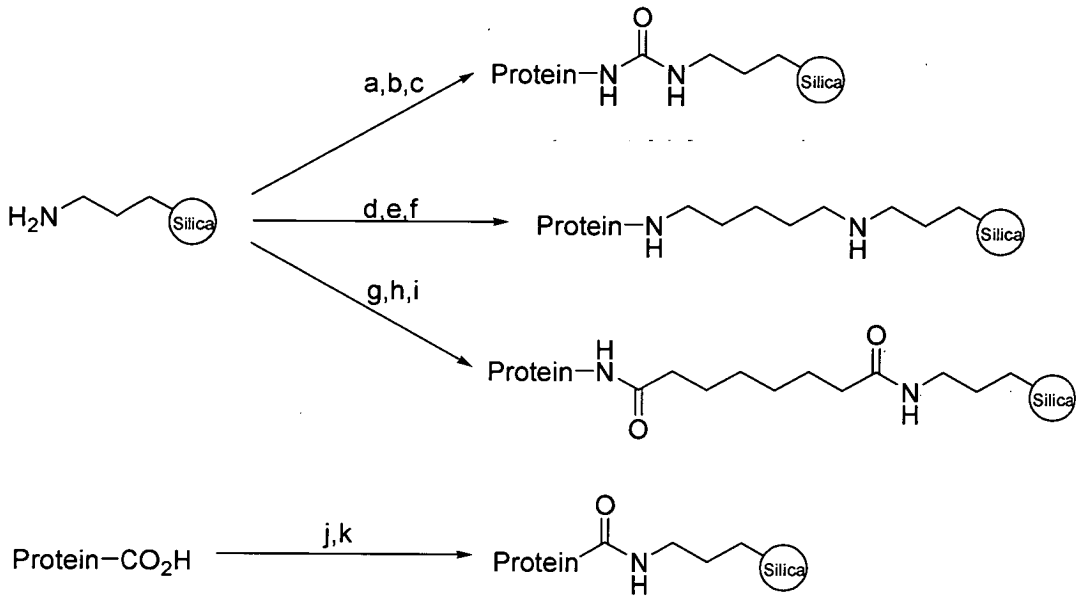
Some CSPs have been based on the use of BSA fragments; Anderson used a 38000 Da fragment that was obtained by enzymatic cleavage which was adsorbed onto silica, but results showed this was poorer than BSA in resolving racemates[181]. Kaginaka used an isolated fragment of mass 35236 which gave mixed results compared to whole BSA[182, 183]. Although BSA and HSA have similar binding characters, there are some differences such as with Warfarin, where (S)-Warfarin elutes before (R)-Warfarin on a column using an HSA based CSP, but the elution order reverses when using a BSA based CSP column[124].

1.4.2.3 *Enzymes and other proteins*

Trypsin and α-chymotrypsin are serine proteases and were first used as CSP by Theolohan[125] and Wainer[129], they have been used to separate amino acids, amino acid derivatives and a range of other compounds[125, 184]. Cellobiohydrolase I is one of four major cellulases produced by the fungus *Trichoderma reesei*[185] and has been used to resolve the enantiomers of a range of compounds especially β-blockers, whereas other cellulases were not good[186, 187]. Marle used fragments of cellobiohydrolase I to determine that the majority of the enantiomeric resolution was attributed to the core structure rather than the flexible region and binding domain[188]. Lysozyme and pepsin have both been used for the separation of basic and uncharged enantiomers[127, 128], although immobilised pepsin has been shown to irreversibly denature above pH 7[128]. Amyloglucosidase, ovotransferrin and β-lactoglobulin have also been used as CSP, but their applicability is limited[130-132].

1.4.3 Synthesis of protein chiral stationary phases

There are two methods for generating CSP by the immobilisation of proteins; either by physical adsorption onto the solid phase or by covalent binding. Although chiral stationary phases have generally been silica-based, many other materials have been used for coating of proteins such as zirconia, anion exchange silica, hollow fibres and poly(vinylimidazole) coated silica. Immobilisation by adsorption is a quick approach to generating a protein CSP and can be done *in situ* by passing protein solution through a packed column, however as protein molecules are linked to the solid-phase by only weak interactions there are significant problems with column stability and long term use.

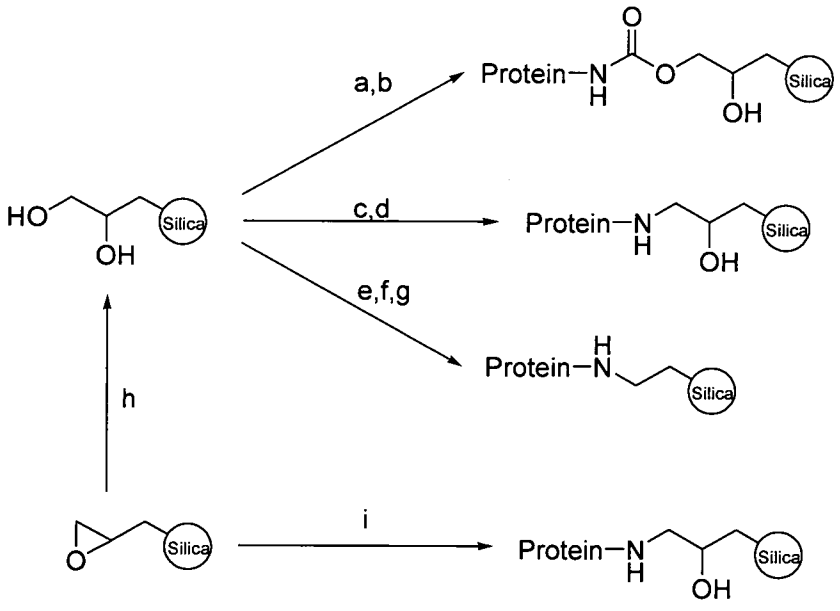


(a) *N,N'*-disuccinimidyl carbonate, (b) protein, (c) ethanolamine/water, (d) gluteraldehyde, (e) protein, (f) $NaBH_3CN$, (g) N,N'-disuccinimidylsuberate, (h) protein, (i) ethanolamine/water, (j) EDC, HSSI (*N*-hydroxysulfosuccinimide), (k) protein.

Scheme 2 Synthesis of protein CSP using aminopropyl silica

The preferred method for immobilisation of proteins is via covalent attachment, which uses derivatised silica material for the binding of proteins. The three most common types of silica for protein immobilisation are amino-silica (normally as aminopropyl silica); diol-silica and epoxy-silica. Each have various means for the attachment of the protein which is normally achieved through either terminal or amino acid side-chain amine or carbonyl groups.

For amino-silica (Scheme 2) the most commonly used method involves the activation of aminopropyl silica with *N,N'*-disuccinimidyl carbonate (DSC), followed by binding of the protein, through side-chains of Lys, Arg and N-terminal amine groups. Another procedure similar to this uses *N,N'*-disuccinimidylsuberate (DSC) and leads to an immobilised protein with a six carbon spacer between the two amide bonds, which leads to increased flexibility and availability of the immobilised protein, but due to the nature of the spacer it may hydrophobically interact with the analyte thus reducing the CSPs resolution. Proteins can also be bound to aminopropyl silica with the use of glutaraldehyde, by the formation of Schiff-bases firstly with aminopropyl silica followed by protein amine groups, the imine groups are then reduced using sodium cyanoborohydride. Another method of immobilisation is via peptide coupling reaction of protein to aminopropyl silica through the proteins carboxyl groups using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (HSSI).



(a) CDI, (b) protein, (c) tresyl chloride, (d) protein, (e) H_5IO_6, (f) protein, (g) $NaBH_4$, (h) HCl (0.1 M), (i) protein.

Scheme 3 Synthesis of protein CSP using diol and epoxy silica

Diol-silica (Scheme 3) also requires activation prior to protein immobilisation; this is most commonly achieved in one of three ways. Diol-silica can be activated with 1,1'-carbonyldiimidazole (CDI), followed by binding of the protein, through side-chain Lys, Arg and N-terminal amine groups. The diol-group can also be activated using tresyl chloride or by oxidation to an aldehyde using periodic acid, followed

by reductive amination using sodium borohydride. Protein immobilisation on epoxy-silica (Scheme 3) can be achieved either via ring opening with HCl (0.1 M) to give diol functionality or by direct binding without the need for activation.

1.5 Surface plasmon resonance

1.5.1 Introduction

Surface plasmon resonance is a phenomenon first discovered by Turbadar[189], when polarized light, under conditions of total internal reflection, strikes an electrically conducting layer at the interface between media of different refractive index.

At an interface between two transparent media of different refractive index (e.g. glass and water), light coming from the side of higher refractive index is partly reflected and partly refracted. Above a certain critical angle of incidence, no light is refracted across the interface, and total internal reflection is observed. While incident light is totally reflected the electromagnetic field component penetrates a short (tens of nanometers) distance into the medium of a lower refractive index creating an exponentially detenuating evanescent wave[190].

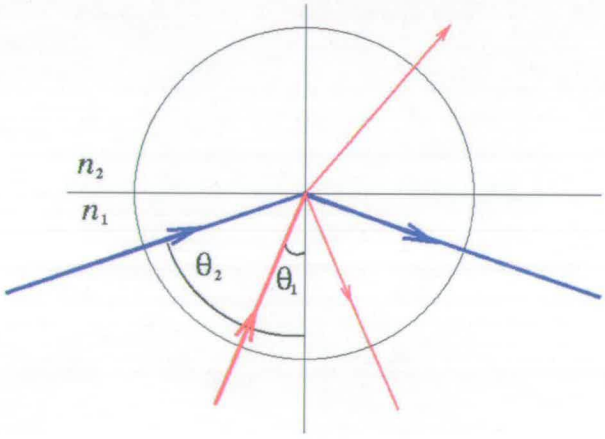
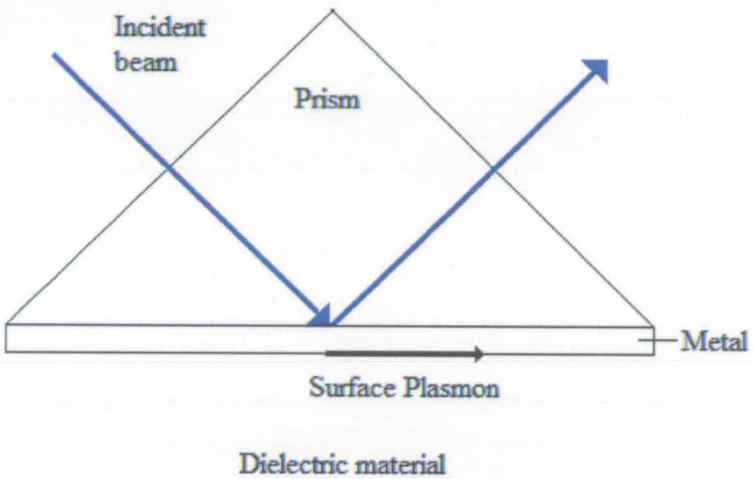


Figure 16 Total internal reflection: n_1 and n_2 are differing refractive index media ($n_1 > n_2$). The incident ray a θ_1 (red) is below the critical angle and is refracted and reflected, whereas ray at θ_2 (blue) is above the critical angle and undergoes total internal reflection.

On a metal surface the collective vibrations of an electron gas/electron density surrounding the atomic lattice sites of the metal are called surface plasmons or surface plasma polaritons. Surface plasmon resonance can occur when a thin layer of the metal (normally gold) is placed at the interface of the two differing refractive index media. Using monochromatic and plane polarised light a resonance energy transfer occurs between evanescent wave and surface plasmons when the photon

and surface plasmon wave vectors match. This produces a reduction in intensity of reflected light at a certain angle. The wave vectors of the metal surface plasmon which determine the angle at which the reflected light intensity minimum occurs, depends on the nature of the metal surface and the medium in close proximity to it. Therefore the resonance conditions are influenced by the refractive index of the bulk media above the metal (gold)[190, 191].



1.5.2 Surface Plasmon resonance biosensors

Surface plasmon resonance biosensors use monochromatic light focused as a wedge-shaped beam on the total internal reflection interface and the angle of minimum reflectance intensity is determined using a detector. The sensor chips use a gold layer as the metal and the surface coating of the sensor chip and the surrounding sample solution is the low refractive index medium. Biomolecular interactions occurring at the sensor surface change the solute concentration and thus the refractive index changes near to the sensor chip and within the evanescent wave penetration range. The angle of incidence required to create the SPR phenomenon (the SPR angle) is therefore altered and it is this change which is measured as a response signal[191, 192].

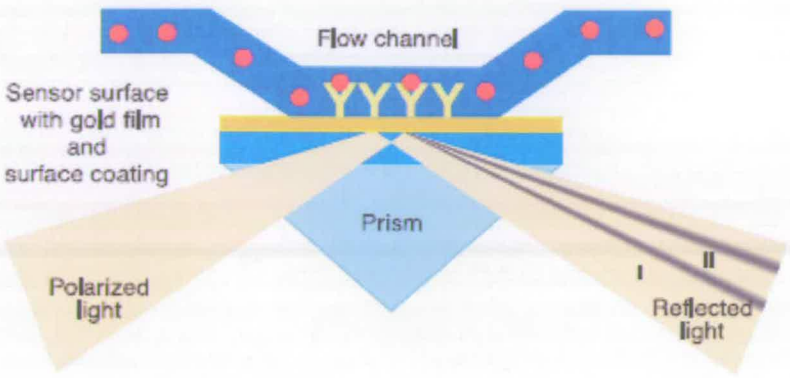


Figure 17 SPR biosensor system measuring interaction.

Immobilised biomolecules on the sensor chip surface interact with the materials flowing over the sensor through a flow channel by binding or dissociation. The interaction causes a change in angle, which is proportional to the mass of bound material and is recorded in a sensorgram. When sample is passed over the sensor surface, the sensorgram shows an increasing response as molecules interact and the response remains constant if the interaction reaches equilibrium. When sample is replaced by buffer, the response decreases as the interaction partners dissociate.

There are numerous manufacturers of SPR biosensors; Biacore International AB (Uppsala, Sweden) is one of the leading companies in SPR technology and have marketed many systems which cover a range of applications{Biacore, Accessed on 10/12/07 #374}. Of the current systems Biacore A100 and Biacore Flexchip focus on automated array SPR measurements, whereas Biacore X100 and Biacore T100 are automated non-array type. Other varieties; Biacore 3000 has an SPR-MALDI interface; Biacore C specialises in determining the active concentrations of protein therapeutics and vaccines; and Biacore X for manual single measurement usage{Biacore, Accessed on 10/12/07 #374}. Other companies such as Sensata Technologies (Attleboro, MA, USA) manufacture the Spreeta model, which can be considered a low cost SPR system and Lumera Corp. (Bothell, WA, USA) which uses SPR to analyse high-density microarrays in a flow cell in its Proteomic Processor model[194].

1.5.3 Uses of SPR biosensors

SPR is a surface sensitive technique which is ideal for studying interactions between immobilised biomolecules and a solution phase analyte. A molecular interaction can be monitored as long as one of the interaction partners can be immobilised onto the sensor chip. It has many advantages over conventional techniques such as fluorescence or ELISA, most significantly because the technique uses measurement based on refractive index changes, meaning that the analyte does not require labelling such as radioactivity or fluorescence, or special characteristics such as scattering bands[195]. It can also be detected directly rather than via multistep detection protocols used in sandwich assay[195, 196]. SPR also is capable of detecting analytes over a wide range of molecular weights and binding affinities.

Data from an SPR biosensor can be used for the determination of many factors: Specificity, whether the binding partners have a binding affinity or not; Kinetics, rates for complex formation and dissociation; Affinity, the strength of binding interaction is determined at equilibrium as a function of sample concentration and concentration, this can be determined using a binding calibration curve. SPR biosensors can provide data for numerous applications, for example: Drug screening, where interactions between small molecules and immobilised proteins give details on potential drug candidates binding characteristics[197-199]; Kinase analysis, where small kinase systems can have affinities, kinetics and thermodynamics fully characterised[200, 201]; Immunoassay sensing, which can provide high sensitivity, selectivity, speed and reliability in carrying out direct[202], sandwich[203], displacement[204] and indirect[205] competitive inhibition assays; Cell signalling pathways, where kinetic and thermodynamic data can help determine inherent pathways[206, 207]; Antibody development, in which potentially therapeutic antibody can be analysed for their immonogenecity with specific expressed proteins[208].

1.6 Reciprocal based chiral stationary phases

In most cases, the processes of selecting successfully effective chiral selectors have been done on the basis of the trial-and-error[209]. However, Pirkle and others have successfully employed the concept of reciprocity of chiral recognition in designing chiral selectors rationally[209-215]. Based on the idea that if an immobilised enantiomer (+)-A as a CSP could separate a given racemate (+)-B and (-)-B, the counter argument should hold that an immobilised enantiomer of (+)-B as a CSP will be able to separate racemate (+)-A and (-)-A.

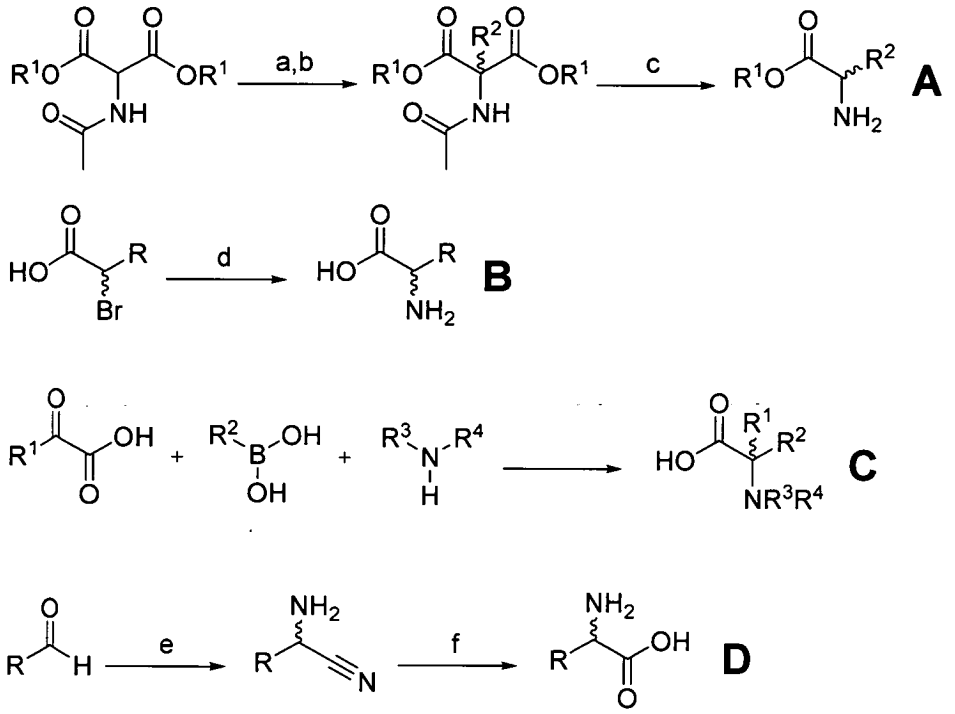
1.6.1 Uses of Reciprocal designed CSPs

Reciprocally designed CSPs have been used for the determination of appropriate chiral selectors for a given target molecule. Pirkle used this method to develop a CSP for separating the anti-inflammatory drug naproxen[153, 216, 217], by immobilising (S)-naproxen and using it to screen potential naproxen chiral selectors. The developed CSP was then also successfully employed in separating other non-steroidal anti-inflammatory drugs[153]. Frechet used a combinatorial approach to discover new, potentially useful chiral stationary phases[210, 218-222] such as using the Ugi multicomponent condensation reaction to create large numbers of analytes and screening these as leads for CSPs[220].

However, the use of reciprocity in chiral resolution should not be relied on to give effective results. In work by Hyan the use of reciprocally designed CSPs gave opposing results to what was expected, firstly with CSPs based 3,5-dinitrobenzoyl-α-amino acid amides and esters[209] and also with proline-3,5-dimethylanilide based CSP[213]. Reciprocal systems of chiral recognition are not intrinsically mirror images of one another, where success of one resolution would not exclude failure of the reciprocal arrangement[223, 224]. As well as this there are numerous other factors which can contribute towards non-reciprocal behaviour such as the structural changes necessary for immobilisation of the reciprocal CSP. The nature of the immobilisation can affect the energies involved in the resolution[153] and differences in CSP loading level.

1.7 Synthesis of non-proteinogenic α-amino acids

As α-amino acids are the fundamental building blocks of proteins as well as peptides there has been enormous amount of research focused on their synthesis. In order to modify the physical and biological properties there have been many reports on the use of non-proteinogenic α-amino acids[225-229].

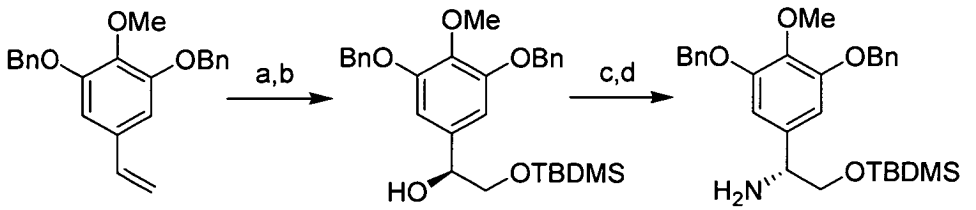


(a) NaOEt, (b) R^2-X, (c) H^+, (d) NH_3, (e) KCN, NH_4Cl, (f) H^+

*Scheme 4 Synthesis of α-amino acids: **A** = alkylation of an acetamidomalonate, **B** = nucleophilic substitution of α-halocarboxylic acid, **C** = Petasis reaction, **D** = Strecker amino acid synthesis.*

There are five methods for obtaining optically active amino acids, these are: Isolation from a natural source; chemical or enzymatic resolution of racemic amino acid derivative; asymmetric synthesis; diastereoselective synthesis; and catalytic enantioselective synthesis[230]. Chemical and enzymatic resolution procedures generally require the synthesis of amino acid functionality first, which can be achieved in a number of different ways, the most common of which are alkylation of an acetamidomalonate[231], nucleophilic substitution of α-halocarboxylic acid, Petasis reaction[232, 233] and Strecker amino acid synthesis{Strecker, 1850 #313;Strecker, 1854 #314}. These methods are summarized in Scheme 4. Depending on the method used to synthesise the amino acid and the substituents present will determine the appropriate method of resolution to give a single optical

isomer. When using the alkylation of acetamidomalonate, this can be achieved by selective deacetylation of the L-enantiomer using enzymes such an acylase[236] or lipase[237].

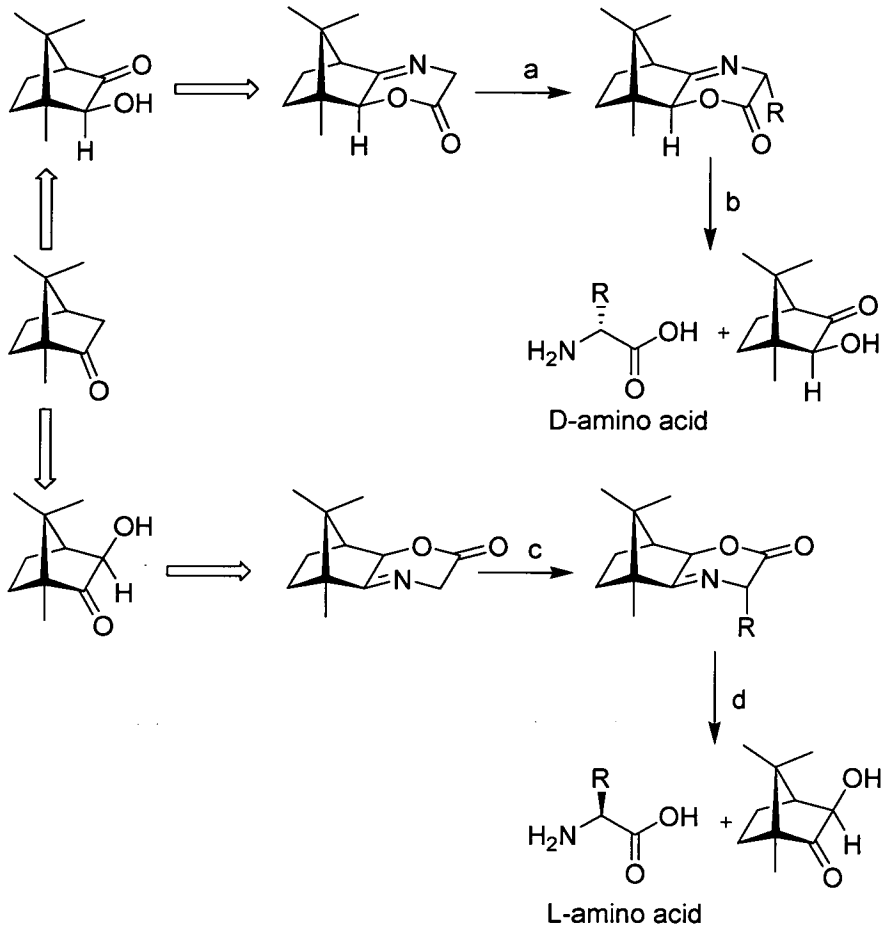


(a) AD-mix-α, (b) TBDMS-Cl, (c) Diphenylphosphoryl azide (DPPA), DEAD-Ph_3P, (d) Ph_3P.

Scheme 5 Section of the synthesis of (R)-(4-methoxy-3,5-dihydroxyphenyl) [238].

Asymmetric synthesis of optically pure non-proteinogenic α-amino acids can be achieved in a number of ways. For example Boger used Sharpless asymmetric dihydroxylation, followed by displacement of the secondary alcohol upon Mitsunobu activation and subsequent reduction to an amine functionality, in the synthesis of the amino acid (R)-(4-methoxy-3,5-dihydroxyphenyl) glycine from Vancomycin[238] (Scheme 5).

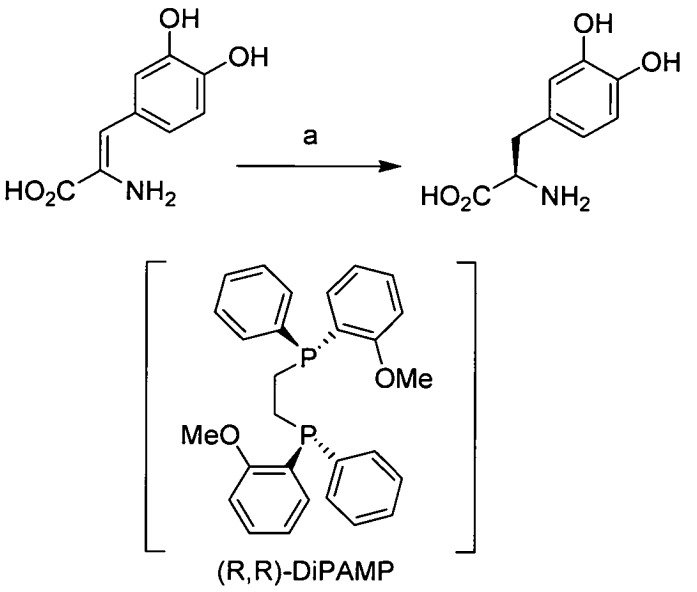
Diastereoselective synthesis uses chiral auxiliaries for inducing optical character into the amino acid. Important reactions in this class include the use of Evans and McDonald auxiliaries[239] and work by Lu, which used a glycine enolate of a (R)-camphor derivative to give either desired stereoisomer, due to a strong preference to form the *endo* product[240, 241] (Scheme 6)



(a)(i) LDA, HMPA/THF, (ii) R-Br, -78 °C, (b)(i) HCl (8.0 M), 87 °C, (ii) EtOH, propylene oxide, (c)(i) LDA, THF, (ii) HMPA, (iii) R-Br, -78 °C, (d) (i) HCl (8.0 M), 87 °C, (ii) EtOH, propylene oxide.

Scheme 6 Chiral Tricyclic Iminolactone Derived from (R)-Camphor

Enantioselective catalysis and the synthesis of chiral α-amino acids has been the subject of extensive research in recent years. There are numerous methods in this vast field which have been successfully employed and each of these can be categorised by the method of introducing chirality: by α-hydrogen, α-amine group, α-side-chain and carbonyl group. The most common is α-hydrogen introduction which involves either: the enantioselective hydrogenation of carbon-carbon double bonds; hydrogenation or hydride addition onto carbon-nitrogen double bonds; protonation of chiral enolates; or by a combined carbonyl hydrogenation-dynamic kinetic resolution of racemic (R)-amino-1,3-dicarbonyl compounds. One of the most important ideas in this area was by Knowles who developed the DiPAMP ligand for use in asymmetric hydrogenation using a rhodium catalyst[242]. This catalyst was used in Monsanto's synthesis of L-DOPA a non-proteinogenic α-amino acid that is used in the treatment of Parkinson's disease[243] (Scheme 7).



(a) H_2, Rh((R,R)-DiPAMP)(COD)

Scheme 7 Knowles-Monsanto synthesis.

1.8 Aims of the project

The development of high-throughput methods to dramatically increase the efficiency of column selection for a broad range of chiral separations could be enhance our knowledge of interactions occurring in the chiral separation process, relating structural changes to the chiral separation observed and by the separation of a range of racemates with small structural changes on a large range of different chiral stationary phase columns.

To achieved this, this PhD describes the preparation of silica bead based CSP libraries; which including common Pirkle type materials including variation of the capping group and peptide composition as well as protein-type CSPs. The generation of an array of inexpensive and general chiral stationary phases could lead to a candidate CSP for separating a high proportion of current pharmaceutical racemates.

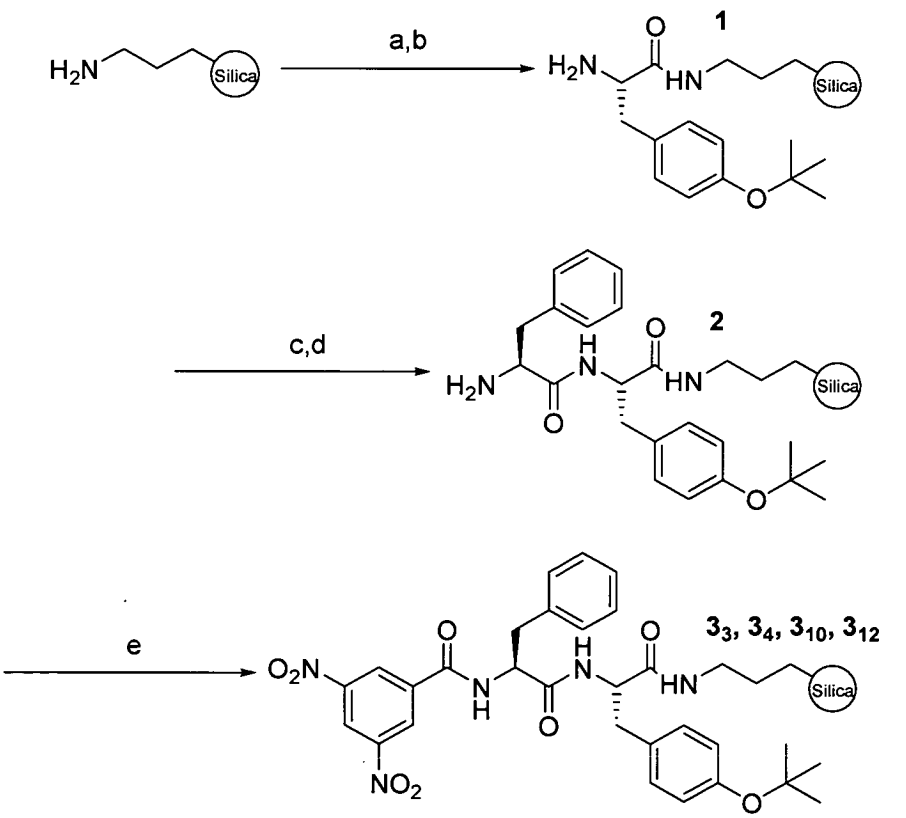
The exploration of other potential high throughput methodologies, such as the Biacore biosensors as a quick screening method for the successful selection of chiral stationary phase was also investigated. In this process comparative screenings of analytes on specific chiral columns and the same chiral selectors immobilised on Biacore surface plasmon resonance chips were carried out.

2 Dipeptide based type I CSPs

2.1 Preliminary Chiral Stationary Phases

2.1.1 Introduction

The dipeptidic amino acid sequence used was developed from the screening of a library of potential dipeptidic CSPs previously described[244]. Synthesis of CSP **(3)** was achieved using various aminopropyl silicas (spherically beaded particles of 3,4,10 and 12 μm), coupled to Fmoc protected amino acids, using diisopropylcarbodiimide (DIC) and N-hydroxybenzotriazole (HOBt). Using a solvent mixture of DCM/DMF (4:1), the peptide coupling could be forced by refluxing for three hours.



(a) Fmoc-Tyr(^{t}Bu)-OH, DIC, HOBt, DCM/DMF (4:1), (b) Piperidine/DMF (5:95), (c) Fmoc-Phe-OH, DIC, HOBt, DCM/DMF (4:1), (d) Piperidine/DMF (5:95), (e) 3,5-dinitrobenzoyl chloride, NEt_3, DCM.

Scheme 8 Synthesis of initial CSP with differing size silica beads.

This was followed by deprotection of the Fmoc group with 5% piperidine in DMF to give **(1)**. The procedure was modified from the standard Fmoc deprotection procedure, which uses 20% piperidine in DMF. Larger concentrations of piperidine causes a breakdown of the silica bead core structure [244]. The modified procedure requires shaking of the beads in 5% piperidine in DMF solution (20 mLg^{-1}) for 30 min. Deprotection monitored by UV spectroscopy detecting the fulvene-piperidine complex at 302 nm.

The second amino acid group, Fmoc-Tyr(^{t}Bu)-OH, was coupled using the same procedure, followed by another Fmoc deprotection to give **(2)**. The primary amine group was then capped with 3,5-dinitrobenzoyl chloride to give the desired CSPs **(3_3)**, **(3_4)**, **(3_{10})** and **(3_{12})**, with bead sizes of 3,4,10 and 12 μm respectively. After each step in the synthesis, the reaction was checked by a TNBS test, which indicates the presence of free primary amine groups and was used for both the coupling and deprotection steps. After each step was complete the reaction mixture was filtered and washed. For silica particles of 10 μm and 12 μm a microfiltration device was used with a 5 μm pore size. For 3 μm and 4 μm particle sized silica a centrifuge procedure was used.

2.1.2 Screening of the Preliminary CSPs

CSP materials were packed into columns of 150 mm length by 2.1 mm i.d. using an Alltech Model 1666 slurry packer. The columns were attached to an HPLC for screening. All CSP columns were screened using a test racemate; 1-(anthracen-9-yl)-2,2,2-trifluoroethanol as it has been shown to separate relatively easily on Type I CSPs and therefore would likely provide positive results to compare[244, 245]. Initial tests showed that all columns gave a separation of the test racemate into its enantiomers, except 3,5-DNB-Phe-Tyr(^{t}Bu)-APS using 4 μm silica **(3_4)**. The 4 μm aminopropyl silica used was supplied by Capital HPLC, a different manufacturer to that of the 3, 10 and 12 μm aminopropyl silicas (Thermohypersil), which may have caused this anomaly. The three CSP columns **(3_3)**, **(3_4)** and **(3_{12})** were each screened under normal phase conditions, using various chromatographic conditions. Each of the three columns were used to separate racemic 1-(anthracen-9-yl)-2,2,2-trifluoroethanol using flow rates of 0.2, 0.3 and 0.4 $mLmin^{-1}$ and heptane/^{i}ProH eluent compositions of 92:8, 95:5 and 98:2.

Results obtained from these initial screenings indicated that selectivity factors were not greatly affected by flow rate or eluent composition for this CSP column type. However, the resolution factor was influenced by both flow rate and eluent composition. In general, slower flow rates gave better resolution, as did less polar eluents, indicating that selectivity factors are a better measure of CSPs inherent enantioseparational powers.

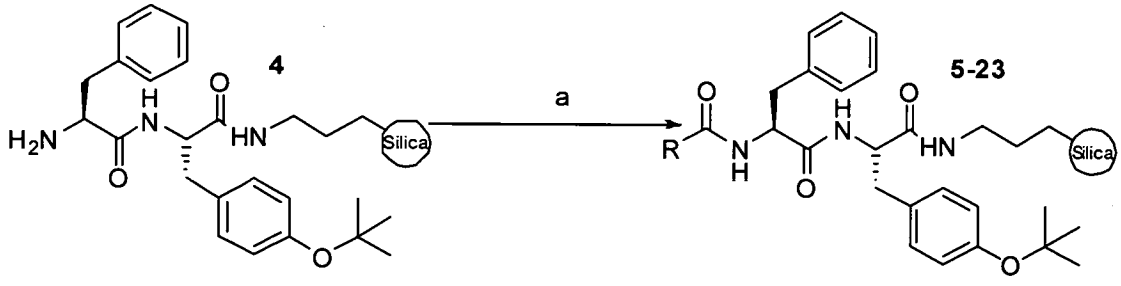
Silica bead size was found to cause negligible differences in the selectivity and resolution factor results. For subsequent experiments it was decided that 10 or 12 μm beaded aminopropyl silica should be used. However, this was a synthetic preference rather than for chromatographic reasons, due to the impracticalities involved in the washing steps, when using the smallest (<5 μm) silica beads.

2.2 Variable head group CSPs

2.2.1 Synthesis of CSPs with varied head groups

In order to evaluate the role of the CSP head (or end) group in chiral separations a library of CSPs was generated. These used the same common core as before (Phe-Tyr(^{t}Bu)-APS), attached to an aminopropyl silica support (spherical beaded, 10 μm).

Synthesis of dipeptide **(4)** attached to a silica support was achieved as previously described for **(2)**, except with the sole use of 10μm silica. Free-amino dipeptide **(4)** was then derivatised with a range of different aryl carboxylic acids (Scheme 9 and Table 1) to give a library of CSPs **(5-23)**.



(a) DIC, HOBt, DCM/DMF (4:1), RO_2H*

* R groups used in synthesis of CSPs **(5-23)** shown in Table 1.

Scheme 9 Synthesis of CSPs with varied head groups.

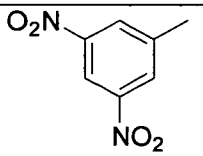
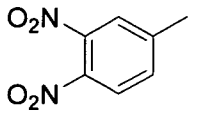
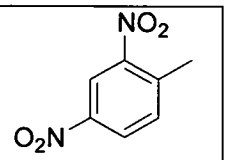
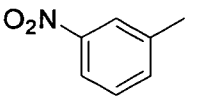
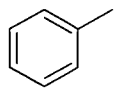
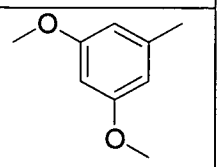
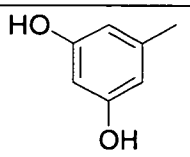
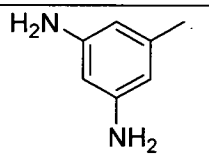
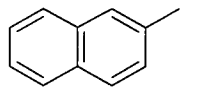
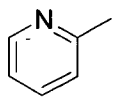
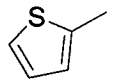
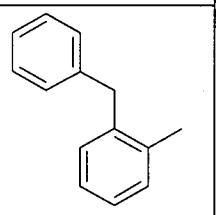
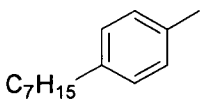
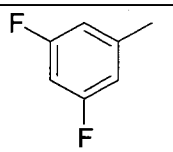
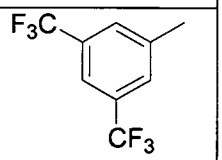
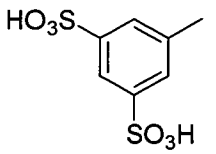
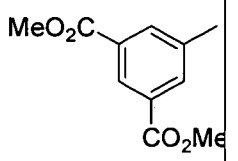
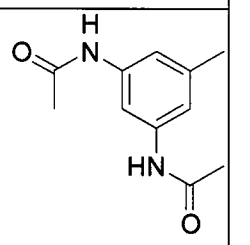
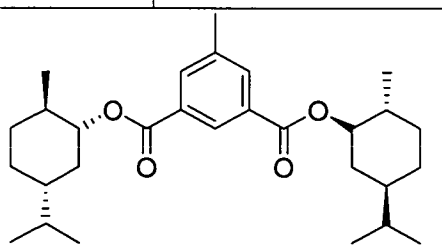
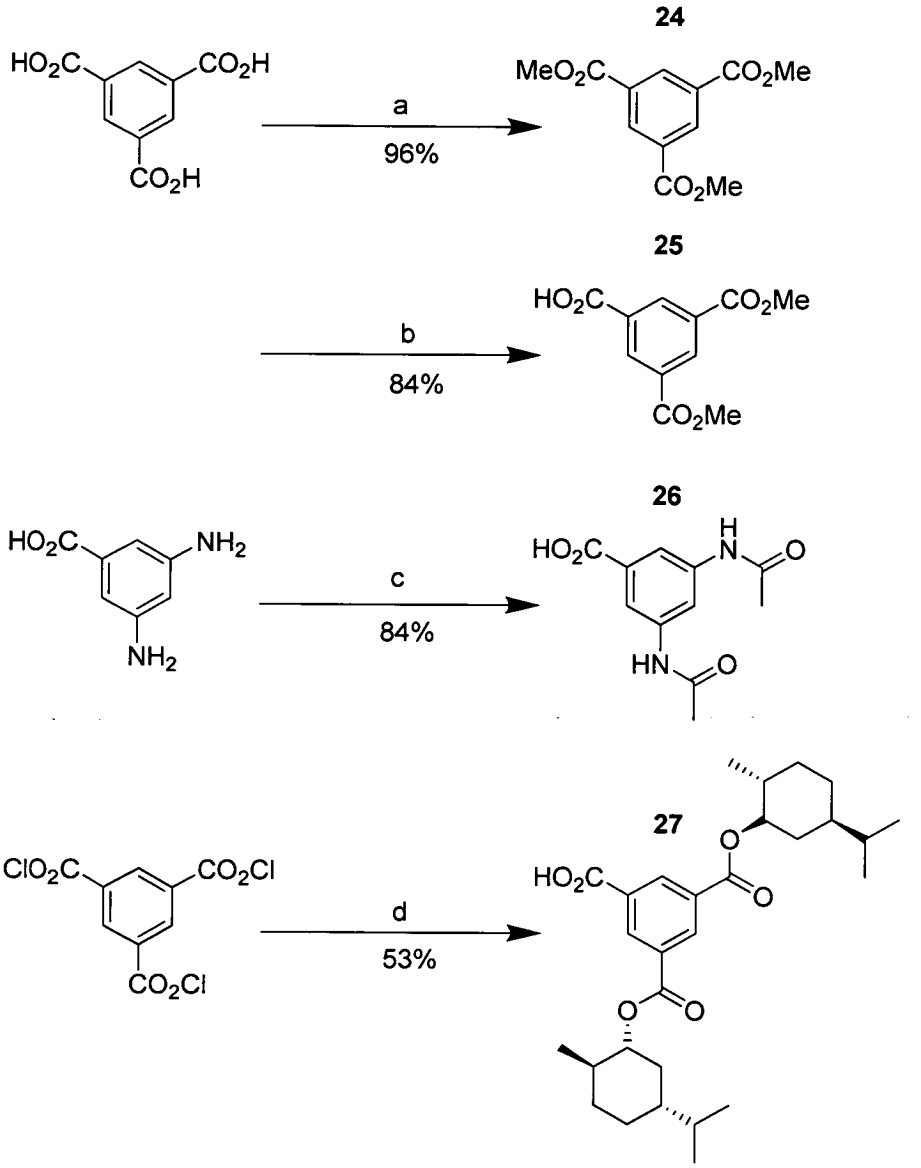
5, R = 	**6, R =** 	**7, R =** 
8, R = 	**9, R =** 	**10, R =** 
11, R = 	**12, R =** 	**13, R =** 
14, R = 	**15, R =** 	**16, R =** 
17, R = 	**18, R =** 	**19, R =** 
20, R = 	**21, R =** 	**22, R =** 
23, R = 		

Table 1 The head groups used for CSPs ***(5-23)***

Three of the carboxylic acids used were not commercially available and were synthesized (Scheme 10). Thus benzene-1,3,5-tricarboxylic acid dimethyl ester **(25)** was synthesized starting from benzene-1,3,5-tricarboxylic acid, which was converted into the trimethyl ester **(24)** using methanol and a catalytic quantity of acid (conc. H_2SO_4), followed by selective saponification (1 eq. NaOH) to give the desired mono-carboxylic acid **(25)**.

3,5-Bis(acetylamido)benzoic acid **(26)** was synthesized directly from 3,5-diaminobenzoic acid using acetyl chloride and pyridine to acylate the aniline groups. Benzene-1,3,5-tricarboxylic acid-bis((1R,2S,5S)-menthol) ester **(27)** was synthesized from benzene-1,3,5-tricarbonyl chloride by the addition of two equivalents of (1R,2S,5S)-(-)-menthol in THF. This was then followed by the quenching of the remaining acid chloride with water, to give the required acid **(27)**.

Each CSP was synthesized on a 1.0 g scale and the materials were packed separately into empty HPLC columns (150 mm length by 2.1 mm i.d.) using an Alltech 1666 slurry packing device. Each CSP in the library was screened with a range of racemates based on the racemate 1-(anthracen-9-yl)-2,2,2-trifluoroethanol.



(a) conc. H_2SO_4(cat.), MeOH, DCM, Reflux (b) NaOH (1eq.), MeOH, (c) AcCl, pyridine, Et_2O, (d)(i) (1R, 2S, 5S)-(-)-Menthol (2eq.), Et_3N, THF, (ii) Water.

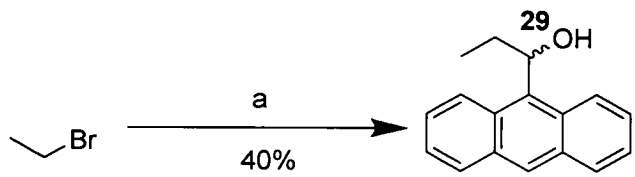
Scheme 10 Synthesis of carboxylic acids end groups (25-27) for CSPs (21-23) respectively.

2.2.2 Racemic analytes

To test the separation ability of the new library of CSPs a range of racemates were required for screening purposes. A small library of test racemates were synthesised based on the structure of 1-(anthracen-9-yl)ethanol (otherwise known as anthrylethanol). A library of racemic analogues based on anthrylethanol was chosen as they were likely to give enough positive separation and will allow a direct comparison for new CSP columns as well as analysing important structural factors of the analyte that contribute towards the ease of its own enantioseparation.

Synthesis was carried out to give a collection of racemic compounds **(29-31)**, **(33)** and **(35-37)** such that each of the three groups attached to the chiral centre of anthrylethanol were altered. The synthesis of three of these was achieved by nucleophilic addition to 9-anthraldehyde **(28)** to give the propyl **(29)**, pentyl **(30)** and phenyl **(31)** anthryl carbinols.

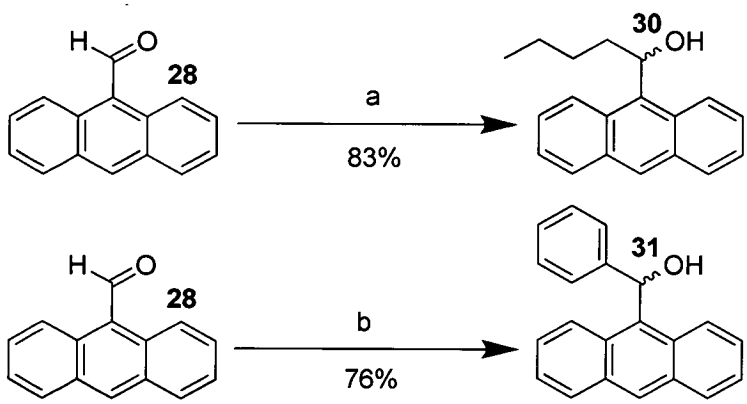
A Grignard reagent was reacted with 9-anthraldehyde to give compound **(29)** was successfully achieved in 40% yield (Scheme 11).



(a)(i) Magnesium turnings, THF, I_2 (cat.), (ii) 9-Anthrylaldehyde, THF, (iii) 10% NH_4Cl.

Scheme 11 Grignard synthesis of (29).

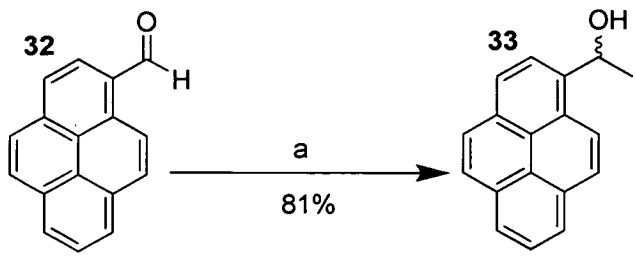
Compounds **(30)** and **(31)** were successfully synthesised via addition of ^{n}BuLi and PhMgBr to 9-Anthraldehyde respectively (Scheme 12).



(a)(i) ^{n}BuLi (2.5M), THF, 0°C, (ii) Water, (b)(i) PhMgBr (1.0M), THF, 0°C, (ii) Water.

Scheme 12 Synthesis of racemates (30) and (31).

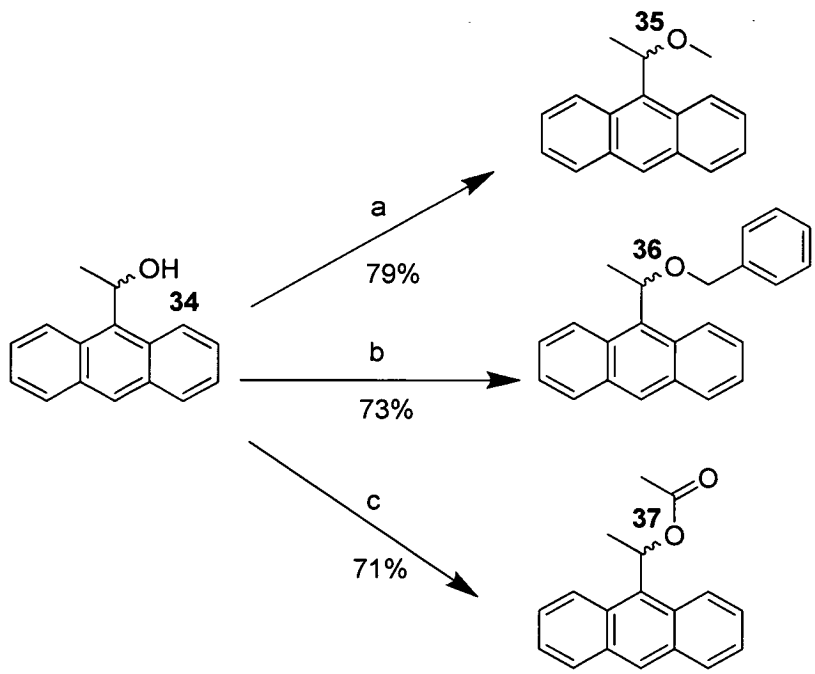
The pyrene **(33)** analogue of anthrylethanol **(34)** was synthesized by the addition of MeLi to the 1-pyrenecarboxaldehyde (Scheme 13).



(a) MeLi (1.4 M), THF, 0 °C

Scheme 13 Synthesis of Pyrene analogue to anthryl ethanol (33).

Three further racemates were prepared by protecting the hydroxyl group of anthrylethanol **(34)** with methyl **(35)**, benzyl **(36)** and acetyl **(37)** groups. This was achieved by deprotonating anthrylethanol with sodium hydride followed by reaction of the hydroxyl group using MeI, BnBr or Ac_2O respectively (Scheme 14).



(a)(i) NaH, THF, 0 °C, (ii) methyl iodide (1.4 M), THF, 0 °C→ rt, (b)(i) NaH, THF, (ii) benzyl bromide, THF, 0 °C→ rt°C , (c) Ac_2O, NEt_3, DMAP.

Scheme 14 Synthesis of anthryl ethanol analogues (35-37).

The final two test racemate analogues were the commercially available 1-(1-Naphthyl) ethanol **(38)** and 1-(9-anthracenyl)-2,2,2-trifluoroethanol **(39)** (Figure 18).

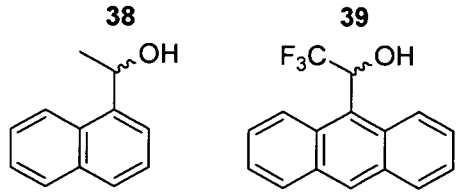


*Figure 18 Naphthyl equivalent racemate **(38)** and 1-(anthracen-9-yl)-2,2,2-trifluoroethanol **(39)**.*

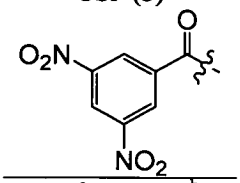
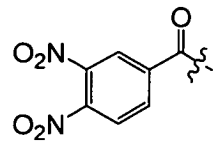
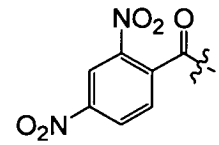
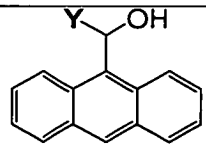
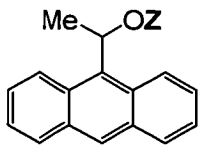
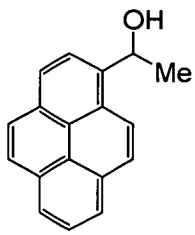
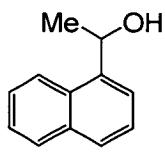
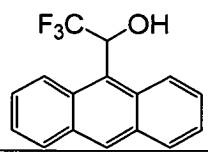
2.2.3 Screening for the varied head group CSPs

2.2.3.1 *Introduction*

Screening of these materials was carried out on an Agilent 1100 HPLC, eluting isocratically with 3% 2-propanol in heptane, with a flow rate of 0.3 $mLmin^{-1}$. Conditions were not optimized for individual racemates or CSPs, as the total number of combinations was already 171 and it was desirable to ensure that all the racemates and CSP screening results were directly comparable.

2.2.3.2 *Screening results for varied head group CSPs*

The most important screening results are shown in Table 2, Table 3 and Table 4 and in Graph 1. These results show that only six of the ten racemates showed separation on any of the columns. The racemic series with capped hydroxyl groups showed no enantioseparation on any member of the CSP library. Racemates with capped hydroxyl groups clearly interact too weakly with the stationary phase and are eluted too quickly for sufficient interactions with the immobilized enantiomer and therefore no observable enantioseparation occurs. This emphasizes the importance of the hydroxyl group in the hydrogen bonding interaction with the chiral stationary phase. Also the anthryl group of the racemate seems very important to the separation. Naphthyl carbinol **(38)**, showed separation on only two of the CSP library, Whereas pyrenyl carbinol **(33)** was not separated on any of the CSP library. Racemates **(29)-(32)** and **(34)** which vary at the alkyl position, showed very similar separation to one another.

No.	Racemate structure	CSP (5) 		CSP (6) 		CSP (7) 	
		k'_1 [c]	α [b]	k'_1 [c]	α [b]	k'_1 [c]	α [b]
							
(34)	Y= Me	4.64	1.55	3.05	1.19	3.73	1.20
(29)	Y = Et	2.81	1.57	3.65	1.08	2.08	1.15
(30)	Y = Bu	1.79	1.45	1.30	1.19	1.70	1.19
(31)	Y = Ph	3.36	1.56	2.86	1.21	3.09	1.21
							
(35)	Z = Me	0.20	1.00	0.10	1.00	0.18	1.00
(36)	Z = Bn	0.85	1.00	0.75	1.00	0.79	1.00
(37)	Z = Ac	1.48	1.00	0.72	1.00	0.58	1.00
(33)		1.25	1.00	3.65	1.00	4.68	1.00
(38)		3.61	1.13	3.85	1.11	2.64	1.00
(39)		3.22	1.54	2.76	1.18	3.73	1.23

[a] *Mobile phase is heptane/2-propanol (97:3), flow rate of 0.3mL/min.*

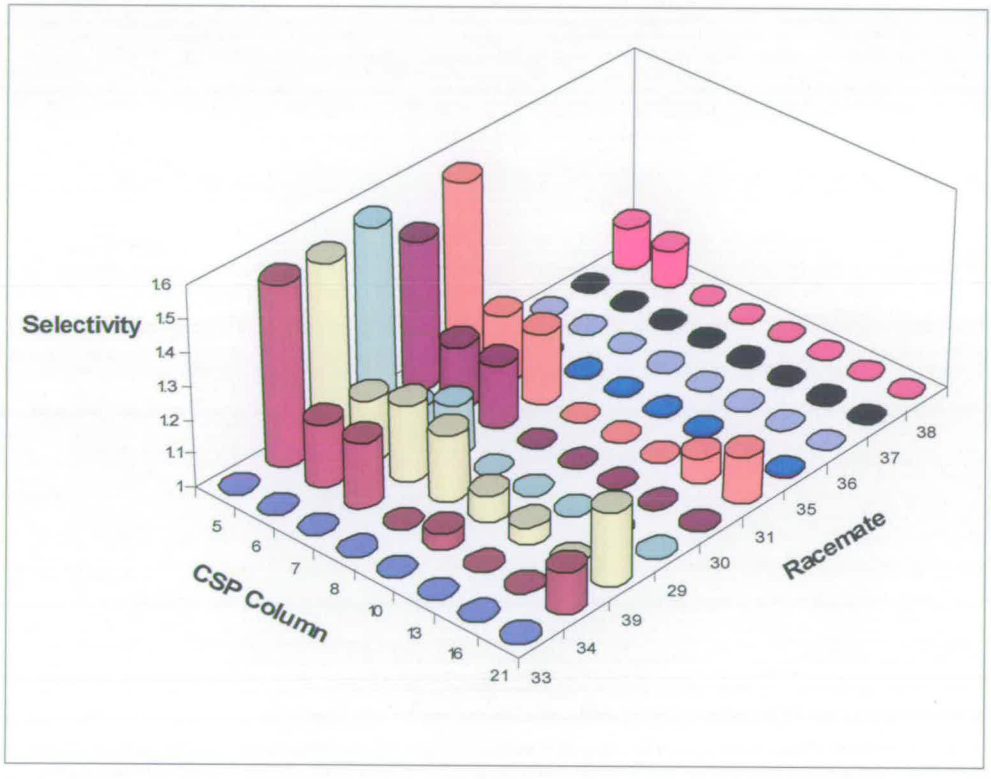
[b] $\alpha = k'_2/k'_1$ = *enantioseparation factor.*

[c] k'_2 *and* k'_1 *are the capacity factors for the second of first eluted enantiomer respectively.*

Table 2 Enantioseparation of racemates ***(29)-(31), (33)-(39)*** *on CSP* ***(5)-(7).***

Of the nineteen CSP columns screened, eight CSPs showed separation of a least one racemate. The 3,5-dinitrobenzoyl **(5)** end group was generally the best at separating the racemate library. When the nitro moietes on the end group were altered, either by position; 2,4-dinitrobenzoyl **(6)** and 3,4-dinitrobenzoyl **(7)**, or by

substitution with a hydrogen atom; 3-nitrobenzoyl **(8)**, the enantioseparation (α value) of the racemates decreased, in some cases to 1.0 (i.e. no observable separation). Other 3,5-disubstitued electron withdrawing end groups, such as 3,5-difluorobenzoyl **(18)**, 3,5-bis(trifluoromethyl)benzoyl **(19)** and 3,5-disulfobenzoyl **(20)** did not have any of the separational properties of the parent 3,5-dinitrobenzoyl end group **(5)**. In fact none of these CSPs showed any separation of any member of the racemate library.



Graph 1 Screening results of columns that show separation of at least one racemate.

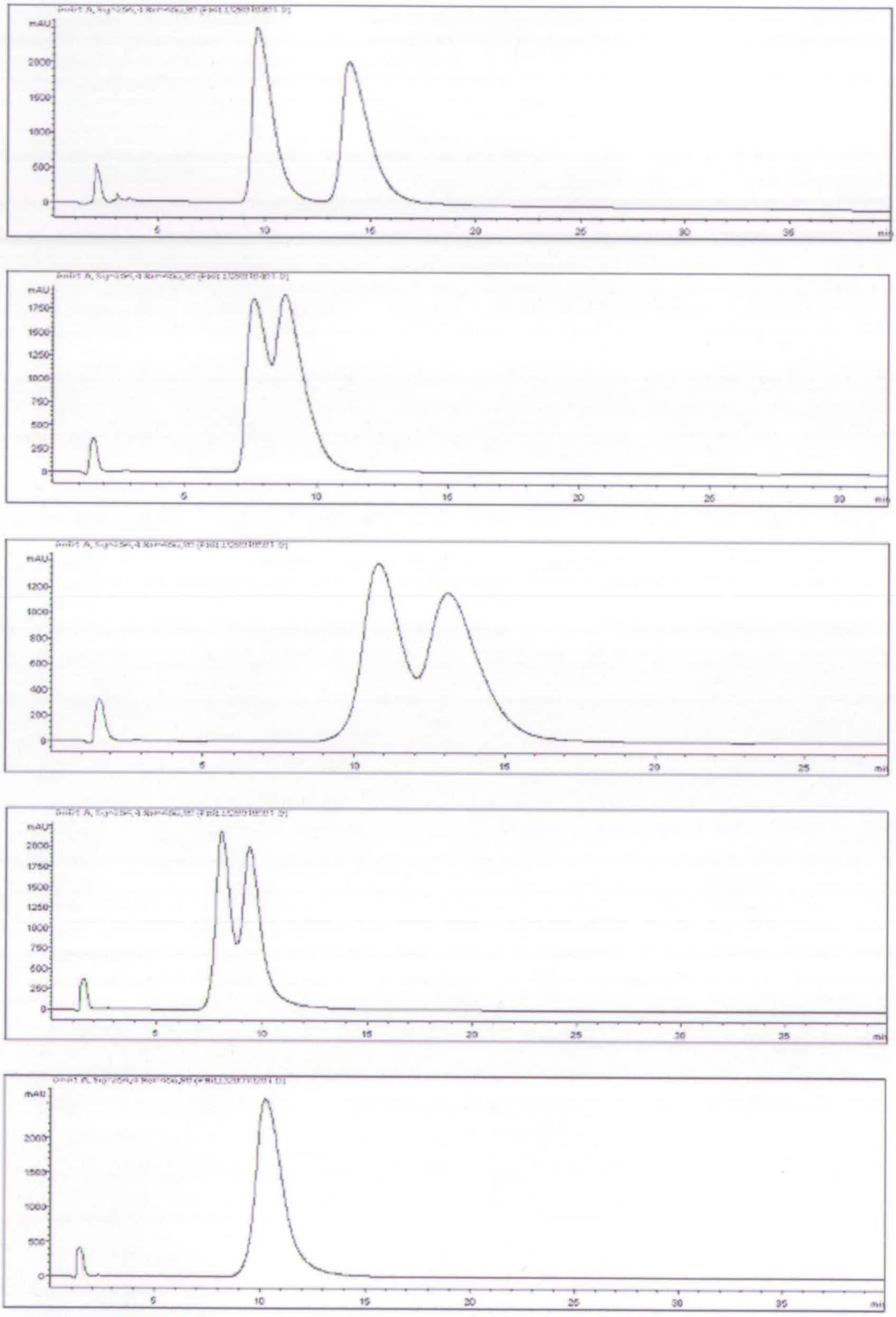
The other end groups showed limited success in enantioseparating the racemates. CSPs with end groups of: 3,5-dimethoxybenzoyl **(10)**, 3,5-bis(methoxycarbonyl)benzoyl **(21)**, 2-naphthoyl **(13)** and 2-Benzylbenzoyl **(16)** each gave separation of at least one racemate in the library. Of the CSPs that showed no enantioseparation ability, some were due to low sample retention by the column, highlighted by low capacity (k') values, notably these were 2-thiophenoyl **(15)** and 3,5-difluorobenzoyl **(18)**. In these cases the low sample retention could have been improved by optimization of mobile phase. However some columns

gave good retention of sample but still without enantioseparation such as 2-pyridoyl **(14)** and 3,5-bis(acetylamido)benzoyl **(22)**.

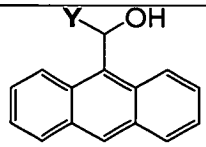
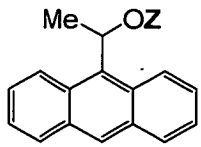
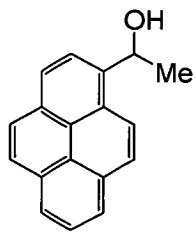
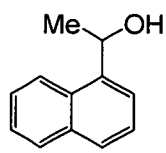
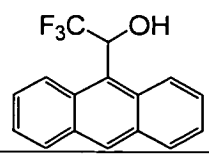
The 3,5-bis(methoxycarbonyl)benzoyl **(21)** capping group gave enantioseparation of three members of the racemate library. This led us to investigate the possibility of using further 3,5-disubstituted ester end groups. 3,5-bis(mentholoxycarbonyl)benzoyl **(23)** was derived from this, with the addition of further chiral information in the menthol ester side groups instead of the methyl ester side groups. However, unlike 3,5-bis(methoxycarbonyl)benzoyl **(21)**, 3,5 bis(mentholoxycarbonyl)benzoyl **(23)** CSP didn't show any enantioseparation of the racemate library. This could be because the large menthol end group substituents may compete for complexation, hindering the racemic analyte's normal mode of interaction with the CSP.

Although the 3,5-dinitrobenzoyl **(5)** end group, which is used in many commercialized CSPs, could not be bettered for enantioselectivity, there were a number of interesting results. Notably the positioning of the nitro groups around the benzoyl end group greatly affected the enantioseparation observed. In 3,5-dinitrobenzoyl end group **(5)**, the positioning of nitro groups gives the most evenly distributed π-electron acceptor. However the 2,4-dinitrobenzoyl **(6)** end group also gave some enantioseparation, whereas there was no separation with the benzoyl **(9)** end group. This indicated that the nitro groups in the 2, 4 and 6 positions of the end group benzoyl ring can also be involved in generating a successful CSP end group. Future work in adding substituents into the 2,4 and 6 positions of the 3,5-dinitrobezoyl end group could augment the enantioseparation of the CSP. Using Alkoxy and alkylester moieties in CSP end groups has been shown to give separation. These as well as further nitro groups, could all be substituted into the 2 and/or 4 and/or 6 positions of the 3,5-dinitrobenzoyl end group, to potentially give CSPs with better enantioseparation. However, the stability of all of these types of end groups is not well known.

All attempts at co-crystallisation of the CSP peptide structure and racemic 1-(anthracen-9-yl)-2,2,2-trifluoroethanol **(39)**, (R)-1-(anthracen-9-yl)-2,2,2-trifluoroethanol and (S)- 1-(anthracen-9-yl)-2,2,2-trifluoroethanol proved fruitless.



Graph 2 Separation of 1-(anthracen-9-yl)-2,2,2-trifluoroethanol ***(39)*** *on CSPs: (from top to bottom)* ***(5)-(9)****.*

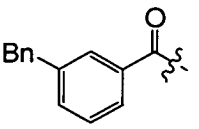
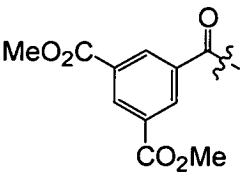
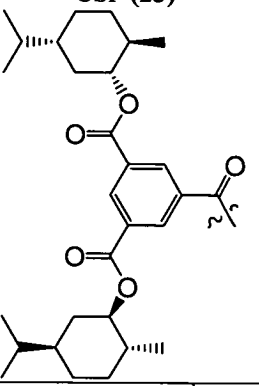
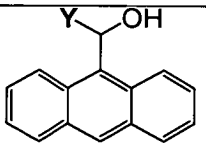
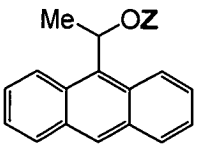
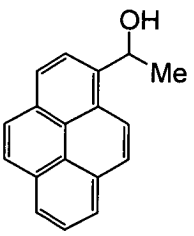
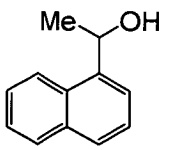
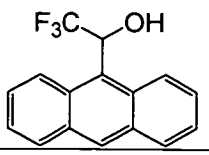
No.	Racemate structure	CSP (8)		CSP (13)		CSP (10)	
		k'_1 [c]	α [b]	k'_1 [c]	α [b]	k'_1 [c]	α [b]
							
(34)	Y= Me	2.85	1.00	3.28	1.05	3.79	1.00
(29)	Y = Et	1.70	1.00	3.80	1.00	4.05	1.00
(30)	Y = Bu	1.26	1.00	3.12	1.00	3.42	1.00
(31)	Y = Ph	2.99	1.00	3.09	1.00	3.36	1.05
							
(35)	Z = Me	0.01	1.00	0.10	1.00	0.04	1.00
(36)	Z = Bn	0.80	1.00	0.72	1.00	0.54	1.00
(37)	Z = Ac	0.50	1.00	0.34	1.00	0.21	1.00
(33)		4.44	1.00	3.02	1.00	3.21	1.00
(38)		1.75	1.00	1.80	1.00	1.93	1.00
(39)		3.08	1.20	3.99	1.08	5.61	1.05

[a] *Mobile phase is heptane/2-propanol (97:3), flow rate of 0.3mL/min.*

[b] $\alpha = k'_2/k'_1$ = *enantioseparation factor.*

[c] k'_2 *and* k'_1 *are the capacity factors for the second or first eluted enantiomer respectively.*

Table 3 Enantioseparation of racemates ***(29)-(31), (33)-(39)*** *on CSPs* ***(8), (10)*** *and* ***(13).***

No.	Racemate structure	CSP (16)		CSP (21)		CSP (23)	
							
		k'_1 [c]	α [b]	k'_1 [c]	α [b]	k'_1 [c]	α [b]
							
(34)	Y= Me	2.19	1.00	3.11	1.13	3.11	1.00
(29)	Y = Et	1.13	1.00	2.06	1.00	2.01	1.00
(30)	Y = Bu	0.84	1.00	1.93	1.00	1.70	1.00
(31)	Y = Ph	2.47	1.08	3.54	1.14	3.09	1.00
							
(35)	Z = Me	0.01	1.00	0.02	1.00	0.10	1.00
(36)	Z = Bn	0.60	1.00	0.84	1.00	0.50	1.00
(37)	Z = Ac	0.14	1.00	0.44	1.00	0.41	1.00
(33)		2.46	1.00	4.88	1.00	4.21	1.00
(38)		1.95	1.00	2.24	1.00	2.32	1.00
(39)		3.75	1.00	5.69	1.22	3.66	1.00

[a] *Mobile phase is heptane/2-propanol (97:3), flow rate of 0.3mL/min.*

[b] $\alpha = k'_2/k'_1$ = *enantioseparation factor.*

[c] k'_2 *and* k'_1 *are the capacity factors for the second or first eluted enantiomer respectively.*

Table 4 Enantioseparation of racemates ***(29)-(31), (33)-(39)*** *on CSPs* ***(16),(21)*** *and* ***(23).***

2.3 CSPs containing two 3,5-dinitrobenzoyl head groups

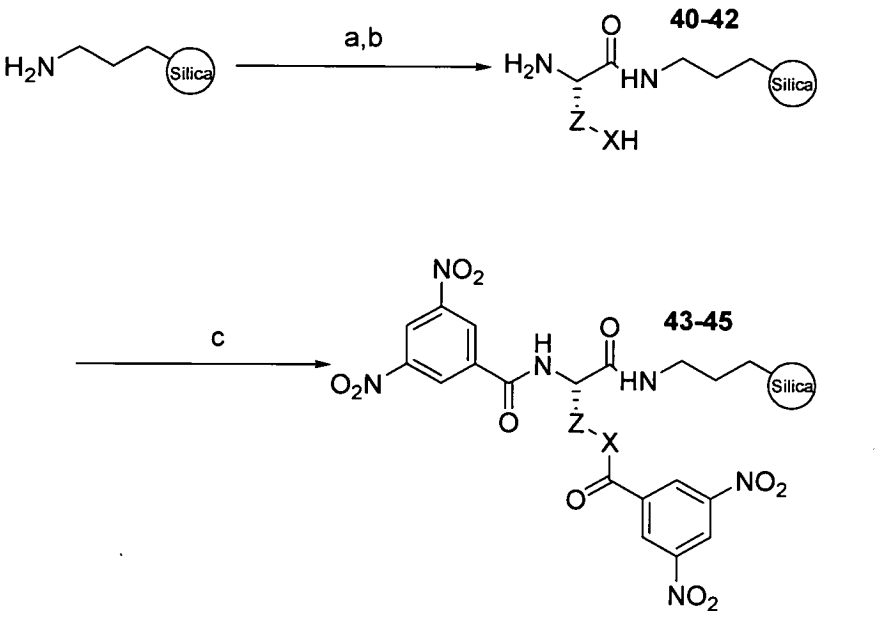
2.3.1 Introduction

Following the screening of a wide range of head groups, the 3,5-dinitrobenzoyl group was found to be by far the most successful. Chiral stationary phases containing two 3,5-dinitrobenzoyl head groups, could lead to beneficial enhancement of enantioseparation or separation of a broader range of racemates, in comparison to that of CSPs with only one 3,5-dinitrobenzoyl head group.

2.3.2 Synthesis of CSPs containing two 3,5-dinitrobenzoyl head groups

Three initial CSPs containing two 3,5-dinitrobenzoyl head groups were synthesised **(43-45)**. Each of these head groups used an amino acid backbone attached to the aminopropyl derived silica beads. However, in order for the CSP to contain the two required 3,5-dinitrobenzoyl groups, different amino acids had to be used, compared to previous procedures. The backbone was simplified to just one amino acid which would then be derivatised with two equivalents of the required 3,5-dinitrobenzoyl group, instead of the original dipeptidic sequence of Tyr(^{t}Bu) and Phe as neither Phe nor Tyr(^{t}Bu) were suitable for this.

CSP **(43-45)** used amino acids Dap (Diaminopropionic acid), Lys and Ser respectively. Synthesis was achieved starting with Aminopropyl silica (10 μm) as shown in Scheme 15. Following a peptide coupling procedure similar to that used before, the appropriately protected amino acid (Ser, Dap or Lys) was coupled to aminopropyl silica. Boc-Dap(Boc)-OH, Fmoc-Lys(Fmoc)-OH and Fmoc-Ser(^{t}Bu)-OH, were used to form compounds **(40-42)** respectively. After successful coupling of the amino acid to the solid support, the protecting groups were removed (both amine and sidechain). Once all the protecting groups had been removed the amine functional group(s) (and hydroxyl for Ser based compound **(42)** were available for coupling. 3,5-Dinitrobenzoic acid was coupled using a similar method. In the case of the Ser based compound **(42)**, the formation of the required ester bond, was achieved by the addition of catalytic amounts of DMAP to the standard coupling reagents. Successful coupling of two 3,5-dinitrobenzoyl groups gave CSPs **(43)**, **(44)** and **(45)**, containing Dap, Lys and Ser respectively.



(40) and **(43)** Z = CH_2, X = NH, (a) Boc-Dap(Boc)-OH, DIC, HOBt, DCM/DMF (4:1), (b) DCM/TFA/Water (50:50:1), (c) 3,5-dinitrobenzoic acid, DIC, HOBt, DCM/DMF (4:1)

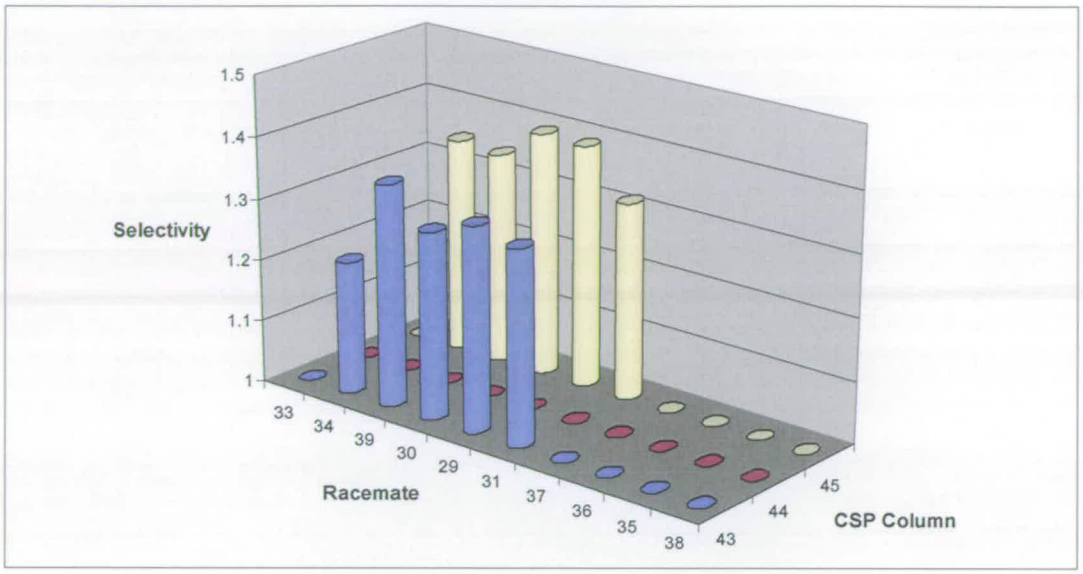
(41) and **(44)** Z = $CH_2CH_2CH_2CH_2$, X = NH, (a) Fmoc-Lys(Fmoc)-OH, DIC, HOBt, DCM/DMF (4:1), (b) Piperidine/DMF (5:95), (c) 3,5-dinitrobenzoic acid, DIC, HOBt, DCM/DMF (4:1)

(42) and **(45)** Z = CH_2, X = O, (a) Fmoc-Ser(^{t}Bu)-OH, DIC, HOBt, DCM/DMF (4:1), (b)(i) Piperidine/DMF (5:95), (ii) DCM/TFA/Water (50:50:1), (c) 3,5-dinitrobenzoic acid, DIC, HOBt, DMAP (cat.), DCM/DMF (4:1)

*Scheme 15 Synthesis of CSPs **(43-45)**.*

2.3.3 Screening of CSPs containing two 3,5-dinitrobenzoyl head groups

Three new CSPs were packed into columns (150 mm length by 2.1 mm i.d.) and screened using the same racemate library as previously described in section 2.2. The screening results obtained (Graph 3) show that CSP columns **(43)** and **(45)** both separated the same five racemates out of the ten member racemate library. These results were similar to the best CSP **(5)** results from the previous CSP library, with the exception that CSP **(5)** also showed small separation of the naphthyl based racemate **(38)**. These results show that within this small library of racemates, the new CSPs containing two 3,5-dinitrobenzoyl groups did not increase the range of racemates that can be separated, nor improve the α-values.



*Graph 3 Screening results of CSP columns **(43-45)** with ten member racemate library.*

Separation of the library by CSPs **(43)** and **(45)** were very similar to CSP **(5)** with the exception of racemate **(38)**. The hydroxyl capped racemates **(35-37)**, still showed no separation, which was not surprising following similarly negative results in previous screenings.

However, the overall chiral selectivity of CSPs **(43)** and **(45)** are lower, in all cases, compared to CSP **(5)**. CSPs **(43)** and **(45)**, have selectivity factors between 1.3-1.4, whereas CSP **(5)**, showed selectivity factors between 1.5-1.6 (for separated racemates). Therefore it was concluded that CSPs **(43)** and **(45)**, containing two 3,5-dinitrobenzoyl groups, showed no chiral stationary phase improvement compared to CSP **(5)** which contained only one 3,5-dinitrobenzoyl group.

CSP **(44)** showed no separation of any of the racemate library. This was quite an intriguing result compared to CSP **(45)**, which showed separation of five of the ten member racemate library. CSP **(44)** and **(45)** are structurally very similar, with CSP **(44)** (containing Lys) having a four carbon side chain, to which one of the 3,5-dinitrobenzoyl group was attached, compared to CSP **(45)** (containing Dap) having a one carbon side chain. These comparative results indicated that the extra length in the carbon side chain linkage in CSP **(44)** is responsible for the loss of chiral separation ability.

2.4 CSPs with achiral spacers

2.4.1 Introduction

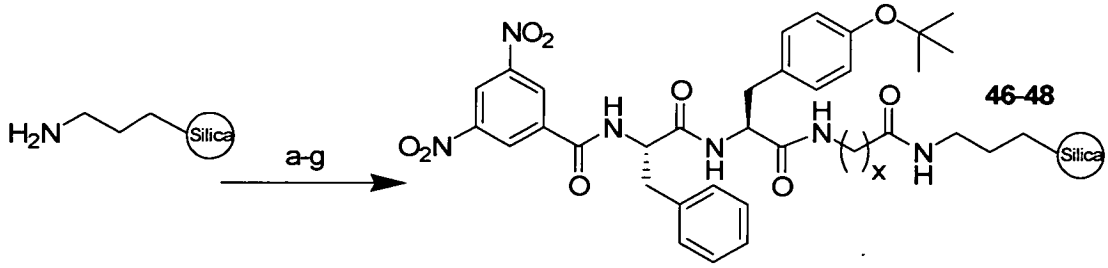
Placing achiral spacers into the original structural framework of CSP **(5)**, would help to confirm the most important structural features of the CSP towards the enantioseparation of racemates.

Synthesis of 'spacered' CSP based upon the structure of CSP **(5)**, was carried out using very similar procedures involved in the synthesis of previous CSPs. These spacers were placed into two positions of the CSP. Group A; spacers between the aminopropyl silica bead and the main peptide chain. Group B; spacers between the end group and the peptide chain attached to the aminopropyl silica bead.

To fit well with the peptide chemistry used in the synthesis of the CSPs, the spacers were also required to be amino acids. Glycine, the non-side-chain derivatised proteogenic amino acid, was used as the 2 carbon spacer. Non-proteogenic amino acids aminobutyric acid and aminohexanoic acid were used for 4 and 6 carbon spacers.

2.4.2 Synthesis of spacered CSPs-group A

Synthesis was achieved starting with aminopropyl silica. To this was coupled an Fmoc protected achiral spacer (Fmoc-Gly-OH, Fmoc-Abu-OH, Fmoc-Ahx-OH). Following the coupling and Fmoc deprotection, was the synthesis of the rest of the spacered CSPs. This involved coupling the amino acid sequence (Tyr(^{t}Bu), Phe) using Fmoc protected amino acid with Fmoc deprotection steps following successful peptide couplings. Following this, end group coupling of the 3,5-dinitrobenzoyl group occurred, which successfully gave three new CSPs **(46)**,**(47)** and **(48)** with glycine, aminobutyric acid and aminohexanoic acid spacers respectively (Scheme 16).

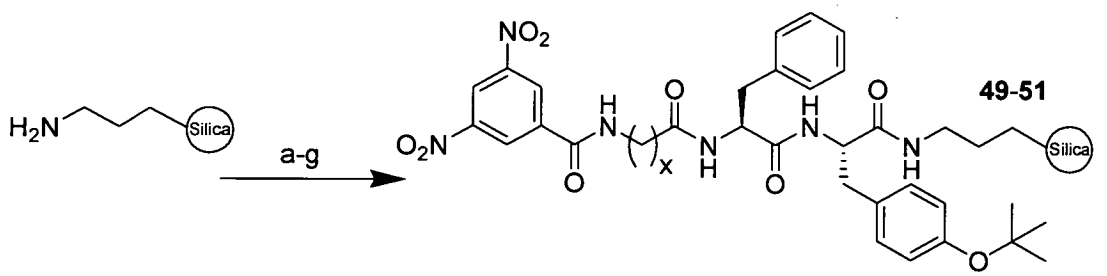


(a) (**46**) X = CH_2, Fmoc-Gly-OH, DIC, HOBt, DCM/DMF (4:1),
(**47**) X = $CH_2CH_2CH_2$, Fmoc-Abu-OH, DIC, HOBt, DCM/DMF (4:1),
(**48**) X = $CH_2CH_2CH_2CH_2CH_2$, Fmoc-Ahx-OH, DIC, HOBt, DCM/DMF (4:1),
(b) Piperidine/DMF (5:95), (c) Fmoc-Tyr(^{t}Bu)-OH, DIC, HOBt, DCM/DMF (4:1),
(d) Piperidine/DMF (5:95), (e) Fmoc-Phe-OH, DIC, HOBt, DCM/DMF (4:1),
(f) Piperidine/DMF (5:95), (g) 3,5-Dinitrobenzoic acid, DIC, HOBt, DCM/DMF (4:1)

Scheme 16 Synthesis of spacered CSP-group A

2.4.3 Synthesis of spacered CSPs-group B

Synthesis of spacered CSP-group B was very similar to that used for spacered CSP-group A. This was achieved by following the identical synthetic sequence for that of compound **(4)**. The free amino-group of was then derivatised with the selection of achiral spacers (Gly, Abu, Ahx) using standard coupling conditions. These three peptide compounds were then coupled with the 3,5-dinitrobenzoyl group, to give the required three CSPs **(49)**, **(50)** and **(51)**, which have glycine, aminobutyric acid and aminohexanoic acid spacers respectively (Scheme 17).



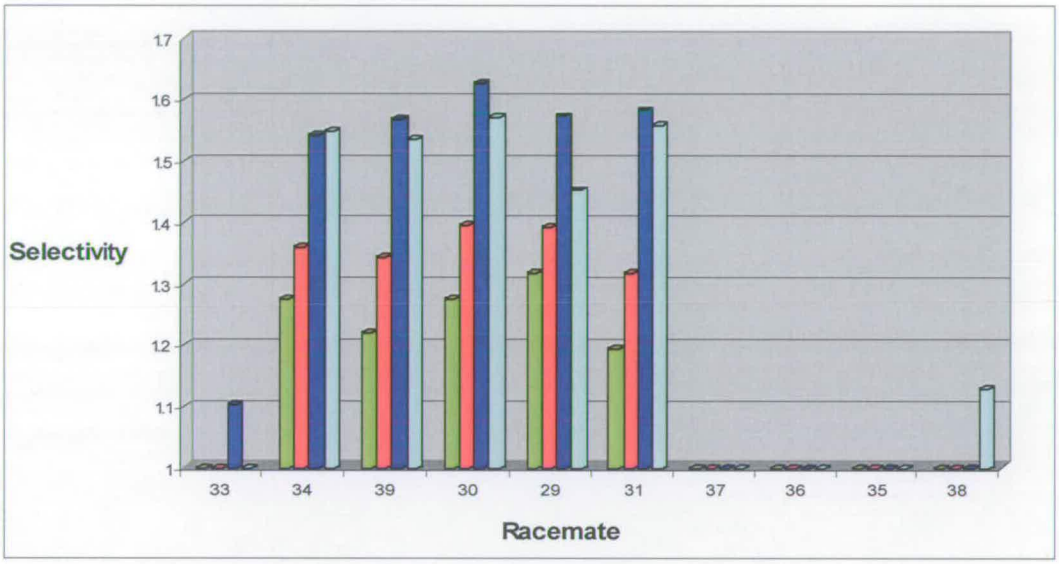
(a) Fmoc-Tyr(^{t}Bu)-OH, DIC, HOBt, DCM/DMF (4:1), (b) Piperidine/DMF (5:95),
(c) Fmoc-Phe-OH, DIC, HOBt, DCM/DMF (4:1), (d) Piperidine/DMF (5:95),
(e) (**49**) X = CH_2, Fmoc-Gly-OH, DIC, HOBt, DCM/DMF (4:1),
(**50**) X = $CH_2CH_2CH_2$, Fmoc-Abu-OH, DIC, HOBt, DCM/DMF (4:1),
(**51**) X = $CH_2CH_2CH_2CH_2CH_2$, Fmoc-Ahx-OH, DIC, HOBt, DCM/DMF (4:1),
(f) Piperidine/DMF (5:95), (g) 3,5-Dinitrobenzoic acid, DIC, HOBt, DCM/DMF (4:1)

Scheme 17 Synthesis of spacered CSP-group B

2.4.4 Screening of spacered CSPs

Screening was carried out on column packed CSPs using the same ten member racemate library as previously synthesized and used for screening.

The CSP-spacered libraries were screened under identical conditions as used previously. Chiral selectivity results are shown for the spacered CSP-group A in Graph 4 and spacered CSP-group B in Graph 5.



Lime = CSP **(46)**, Red = CSP **(47)**, Blue = CSP **(48)**, Light turquoise = CSP **(5)**

Graph 4 Screening results of spacered CSP-group A.

The screening results from spacered CSP-group A **(46-48)**, showed that the separation of the silica from the peptide chain, or effectively lengthening the CSP, had a significant effect on the chiral selectivity of any racemate. Although CSPs **(46-48)** are identical to CSP **(5)** at the peptide end, they differ markedly with respect to silica attachment. With the CSP sequence **(46)**, **(47)**, and **(48)** comes an increasing tether length between the silica bead and the optically active peptide end over the original CSP **(5)**. However, such a significant change in the corresponding chiral selectivity obtained from screening the CSPs was not expected, as previous experiments indicated that the nature of the head group was a key structure feature of CSP **(5)**.

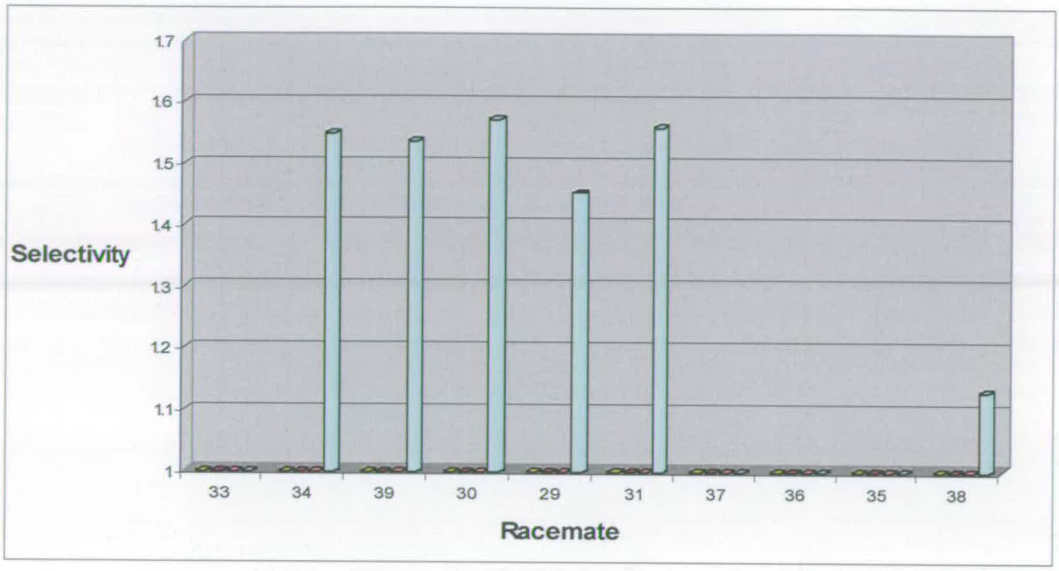
Increasing the distance of the optically active peptide portion of the CSP away from the silica bead, had a detrimental effect on the chiral selectivity, such as CSPs

(47) and **(48)** with the longer achiral spacers (Abu and Ahx). CSP **(47)**, with the intermediate length achiral spacer (Abu, 4 carbon), separated five of the ten racemates successfully. However, all of the chiral selectivity values were slightly lower than that for CSP **(5)**. CSP **(48)** had the longest achiral spacer (Ahx, 6 carbon). Although CSP **(48)** did still successfully separate five of the ten member of the racemates library, the results show that the further increase in achiral spacer length causes a further decrease in chiral selectivity aptitude.

CSP **(47)** has the shortest achiral spacer (Gly, 2 carbon). This CSP generally showed a slightly greater aptitude of chiral selectivity compared to CSP **(5)** and also separated six of the ten member racemate library. However, the racemates separated were not all the same. Racemate **(33)** had a small separation on CSP **(47)** but there was no separation of racemate **(38)**. The results of these two racemates on CSP **(47)** are opposite to those of CSP **(5)**.

The results of CSP **(47)** indicate that a small increase in the overall chain length of CSP, in comparison to CSP **(5)**, has a small positive effect of the chiral selectivity in most cases. This is certainly true for the largest pyrene racemate analogue **(33)**, which had a small separation on CSP **(47)** it compared to zero separation on CSP **(5)**. However, CSP **(5)** gave a small chiral separation of smallest naphthyl racemate analogue **(38)** compared to zero separation of this racemate for CSP **(47)**.

Overall the CSP **(47)** with a Glycine spacer shows slightly better chiral separation aptitude than the original CSP **(5)** with no spacer. However, there was a small increase in the selectivity of some of the racemates separation, but a complete loss of separation for one member of the racemate library.



Yellow = CSP **(49)**, Violet = CSP **(50)**, Rose = CSP **(51)**, Light turquoise = CSP **(5)**

Graph 5 Screening results of spacered CSP-group B.

The screening results from spacered CSP-group B are much clearer to interpret. Graph 5 shows the chiral selectivity of CSPs **(49)-(51)**, and unspacered CSP **(5)** for the same ten member racemate library.

CSPs **(49)-(51)** all showed no separation of any of the member racemate library. This clearly indicates that the insertion of the achiral spacer in between the optical active peptide part of the CSP and the 3,5-dinitrobenzoyl end-group has a very significant effect on the chiral separation ability of the CSP. Even with the smallest spacer (Gly, 2 carbon) used in CSP **(49)**, there was complete loss of chiral separation compared to CSP **(5)**.

To conclude, the results show the importance of the CSP end-group's direct structural attachment to the optically active peptide part of the CSP. The separation of the end group away from the optical active portion removed all detectable chiral separation ability.

2.5 CSPs with switched D/L character of the amino acid

2.5.1 Introduction

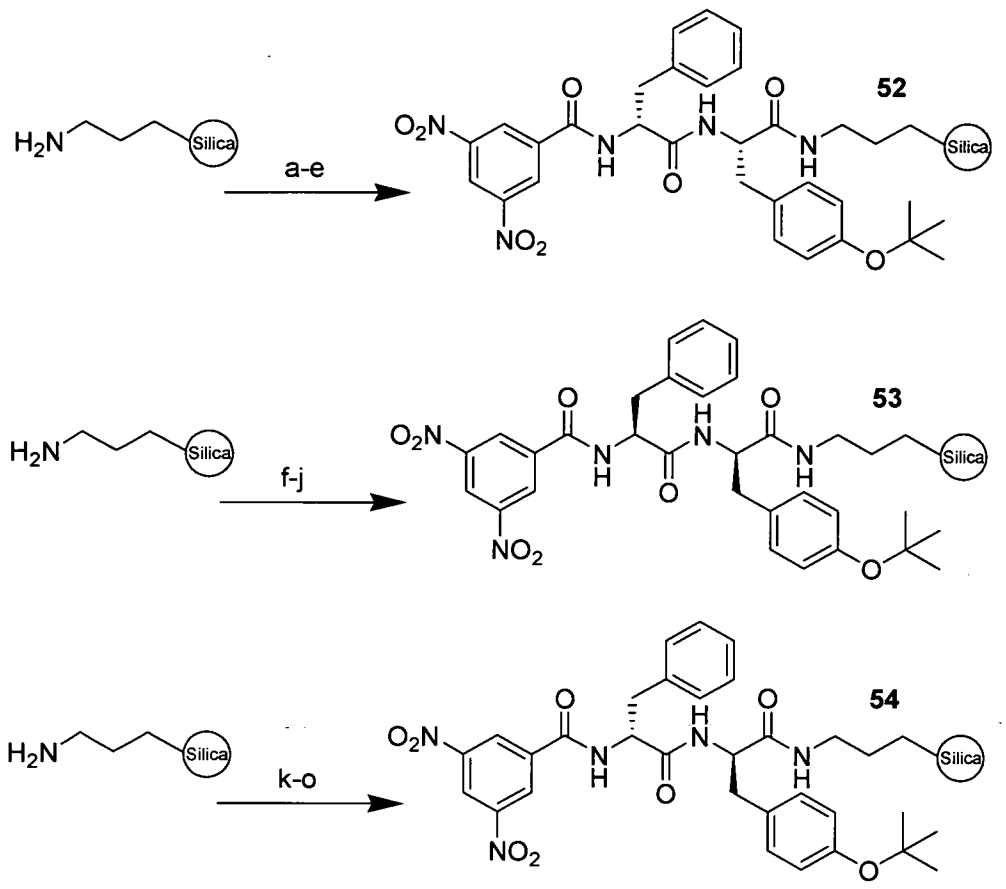
CSPs synthesized thus far have exclusively used the natural L-configuration amino acids. CSP **(5)**, which is a dipeptide, contains two L-configuration amino acids. The opposite enantiomer (with both amino acids with the D-configuration) shows the same magnitude of chiral selectivity, however, the order of elution of the two enantiomers in the racemates will be opposite.

This phenomenon comes from the inherent mechanism which operates in chiral separation. If the CSP-enantiomer, with L-amino acids, forms a stronger transient diastereomeric complex with one of the analyte-enantiomers over the opposite analyte enantiomer, the opposite enantiomer-CSP, will form a similarly stronger transient diastereomeric complex with the opposite analyte-enantiomer, explaining the reversal in elution order observed.

CSP **(5)** contains two amino acids, which give the possibility for two further optical isomers, diastereomeric in relation to the original CSP **(5)**, with each diastereomer containing one D and one L-configured amino acid. Screening of these two new CSPs will give information towards the comparable importance of each amino acid in the CSP. It is also possible that one diastereomer will give an augmented chiral separation ability due to different possible three dimensional orientations of possible transient diastereomeric complexes formed. This situation arises due to the possible non-identical physical nature of diastereomers.

2.5.2 Synthesis of differing amino acid D/L character CSPs

Synthesis was achieved using procedures similar to that for CSP **(5)**, except that this time the Fmoc-D-Tyr(^{t}Bu)-OH and Fmoc-D-Phe-OH were substituted for the L-configured equivalent amino acids where required. The opposite enantiomer to CSP **(5)** was also successfully synthesised.

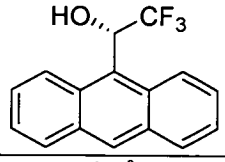
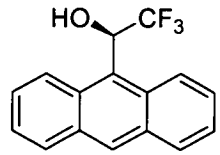


(a) Fmoc-Tyr(^{t}Bu)-OH, DIC, HOBt, DCM/DMF (4:1), (b) Piperidine/DMF (5:95), (c) Fmoc-D-Phe-OH, DIC, HOBt, DCM/DMF (4:1), (d) Piperidine/DMF (5:95), (e) 3,5-dinitrobenzoic acid, DIC, HOBt, DCM/DMF (4:1), (f) Fmoc-D-Tyr(^{t}Bu)-OH, DIC, HOBt, DCM/DMF (4:1), (g) Piperidine/DMF (5:95), (h) Fmoc-Phe-OH, DIC, HOBt, DCM/DMF (4:1), (i) Piperidine/DMF (5:95), (j) 3,5-dinitrobenzoic acid, DIC, HOBt, DCM/DMF (4:1), (k) Fmoc-D-Tyr(^{t}Bu)-OH, DIC, HOBt, DCM/DMF (4:1), (l) Piperidine/DMF (5:95), (m) Fmoc-D-Phe-OH, DIC, HOBt, DCM/DMF (4:1), (n) Piperidine/DMF (5:95), (o) 3,5-dinitrobenzoic acid, DIC, HOBt, DCM/DMF (4:1)

Scheme 18 Synthesis of CSP with differing D/L character.

2.5.3 Screening of CSP with differing D/L character to determine difference in racemic analytes R/S elution order

The first screening for CSPs **(52)-(54)** was to determine how the stereochemistry of the CSP affected the corresponding order of elution of the enantiomers. For screening, the single enantiomers of 1-(anthracen-9-yl)-2,2,2-trifluoroethanol, **(39)** were used as the analyte, to give retention factor values, so that the chiral selectivity factor and elution order could be subsequently be determined. The results are shown in Table 5.

CSP	Short CSP structure	(S)-1-(anthracen-9-yl)-2,2,2-trifluoroethanol  k'_S[a]	(R)-1-(anthracen-9-yl)-2,2,2-trifluoroethanol  k'_R[a]	α[b]
(5)	3,5DNB-L-L-SiO_2	**3.22**[c]	4.95	1.53[d]
(52)	3,5DNB-D-L-SiO_2	7.13	**5.69**[c]	*1.25*[e]
(53)	3,5DNB-L-D-SiO_2	**4.70**[c]	5.77	1.23[d]
(54)	3,5DNB-D-D-SiO_2	5.67	**4.05**[c]	1.40[e]

[a] *Mobile phase is heptane/2-propanol (97:3), flow rate of 0.3 mL/min.*

[b] *α = k'(largest)/k'(smallest) = chiral selectivity factor*

[c] *Highlighted is first eluted enantiomer for corresponding CSP*

[d] *chiral selectivity where S enantiomer is eluted before R enantiomer*

[e] *chiral selectivity where R enantiomer is eluted before S enantiomer*

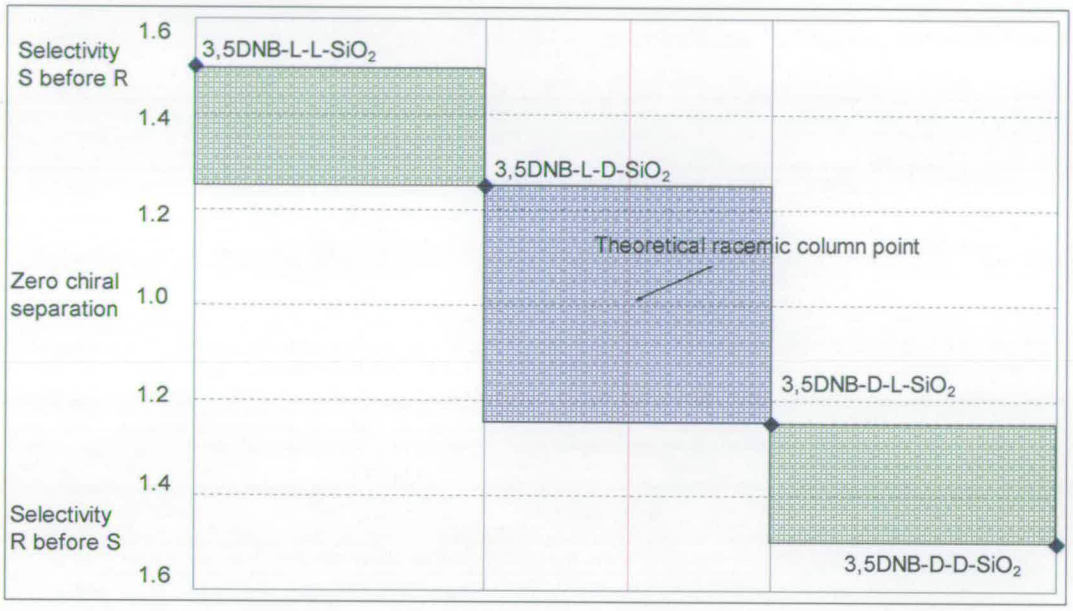
*Table 5 Screening CSPs **(52)-(54)** and **(5)** with single enantiomers of 1-(anthracen-9-yl)-2,2,2-trifluoroethanol to determine elution order.*

Results obtained indicate how each of the amino acid influences the chiral separation of the overall CSP. Two of the CSPs screened showed chiral separation with S being eluted before R. These are the original CSP **(5)** and CSP **(53)**, which contains D-Phe. The other two CSPs, **(52)** and **(54)**, had an opposite elution order (the elution order using CSP **(5)** and CSP **(54)** was also previously examined[244]). These results show that the Phenylalanine in these CSPs has a greater influence over the chiral separation effect, than the Tyrosine residue.

Comparing CSP **(5)** with CSPs **(52)** and **(53)**; where CSP **(53)** has D-Tyr(^{t}Bu) replacing L-Tyr(^{t}Bu) as the first amino acid, gives a drop in the magnitude of the chiral selectivity, but with the elution order staying the same. However, CSP **(52)** (D-Phe replacing L-Phe as the second amino acid) resulted in a very similar drop in the magnitude of chiral selectivity, but with a reversal of elution order. This shows for this particular type of dipeptidic CSP, the second amino acid, Phenylalanine has a stronger influence over the overall chiral separation compared to the second

amino acid Tyr(^{t}Bu). Changing the stereochemistry of Phenylalanine produces the overall changing of the elution order, rather than the changing of Tyr(^{t}Bu), which gives only a reduction of the overall CSP chiral separation ability.

Approximate chiral selectivity values are used in Figure 19 to illustrate how the changing of amino acid two, Phenylalanine, caused a more dramatic effect on the selectivity, than Tyrosine (~twice as much).



[a] *Diagram shows order of elution above and below zero chiral separation (1.0)*

[b] *An elution order of S before R is shown above zero chiral separation arbitrarily.*

[c] *Light green shading shows change of first amino acid, Tyr(^{t}Bu).*

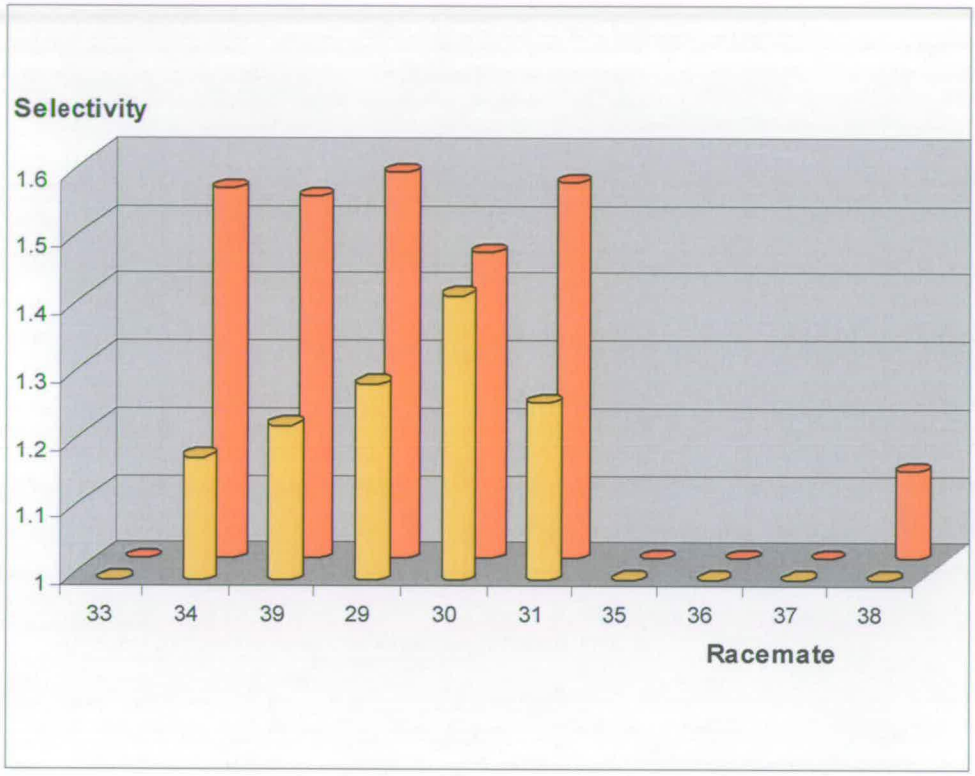
[c] *Light blue shading shows change of first amino acid, Phe.*

Figure 19 Diagram to show the effect the second amino acid, Phenylalanine, has on chiral selectivity.

2.5.4 Screening of CSP with differing D/L character to determine chiral selectivity

Following the screening with 1-(anthracen-9-yl)-2,2,2-trifluoroethanol, all of the CSP were screened using the standard ten member racemate library. It was assumed that CSP **(5)** and CSP **(53)** would separate all of the racemates with the same elution order. The results are plotted in Graph 6, arbitrarily as positive chiral selectivity. Similarly CSPs **(52)** and **(54)**, were assumed to give identical elutions, but this time results were plotted as negative chiral selectivities (Graph 7). This assumption does not indicate the elution order for each individual racemate analyte.

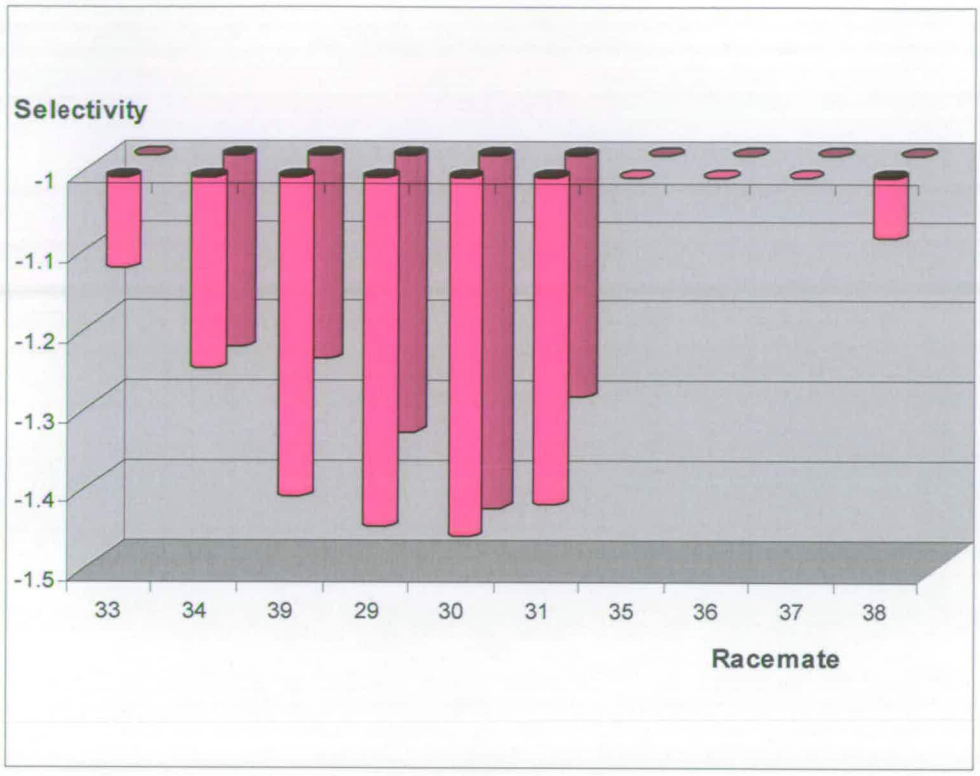
The selectivity given as a ratio without consideration of the elution order and furthermore, it is known that for CSP **(5)**, the R/S elution orders of racemates 1-(anthracen-9-yl)-2,2,2-trifluoroethanol **(39)** and 1-(anthracen-9-yl)ethanol **(34)** are opposite[244].



Light Orange = CSP **(5)**, Gold = CSP **(54)**

*Graph 6 Chiral Selectivity for CSPs **(5)** and **(53)** with standard racemate library*

CSP **(53)** successfully separated five of the ten member of the racemate library. All of the separated racemates gave lower chiral selectivity values than CSP **(5)**. This trend was anticipated and results from the switching of the configuration of amino acid one, from L-Tyr(^{t}Bu) to D-Tyr(^{t}Bu). An almost identical trend was observed with CSP **(5)** compared to CSP **(53)**, similarly caused by the change in amino acid, this time from D-Tyr(^{t}Bu) to L-Tyr(^{t}Bu).



Pink = CSP **(54)**, Plum = CSP **(52)**

*Graph 7 Chiral selectivity for CSPs **(52)** and **(54)** with standard racemate library*

2.6 Utilising experimental design to optimize chiral separation chromatographic conditions

2.6.1 Introduction

In testing numerous CSPs it is vital that identical chromatographic conditions are used for each screen, so that results are directly comparable with each other. Although chiral selectivity factors are strongly influenced by the CSP material, many other chromatographic conditions can alter these values. The most common variables in HPLC were varied using a design of experiments (DOE) approach to confirm which chromatographic conditions were most important when considering optimization of a chiral separation.

2.6.2 The experimental design

The design of experiment was trialled on CSP **(5)** with 1-(9-anthracenyl)-2,2,2-trifluoroethanol **(39)** as the test racemate. DOE was carried out with four numeric

factors; temperature (5°C and 35°C), flow rate (0.1 $mLmin^{-1}$ and 0.7 $mLmin^{-1}$), injection volume (1 μL and 11 μL) and eluent 2-propanol (1% and 5%). These factorials were used in a design resolution of IV (level at which the design is confounded). This required eight runs along with 3 optional centre point runs. The runs are shown in order in Table 6 and Table 7, along with seven responses (t_0/min, t_1/min, t_2/min, α, R_s, Solvent used/mL and total time/min). This experiment gave reliable information on first level effects of the numeric factor on the responses, most importantly, the separation factor (α). Additional information on any strong second level effects was also generated, although these were not conclusive as the combined second level factorials aliases with other equivalent terms, thus making it harder to distinguish second level factors responsible for the given effect. Nevertheless, the second level factors may be determined by discounting the aliased second level factors based on scientific logic[246].

Run number	Temp. /°C	F.R. /$mLmin^{-1}$	Injection vol./μL	Eluent 2-PrOH %	t_0/min.
1	35	0.1	11	1	6.21
2	5	0.7	1	5	0.97
3	5	0.1	1	1	7.31
4	5	0.1	11	5	7.48
5	20	0.4	6	3	1.6
6	35	0.7	11	5	0.88
7	20	0.4	6	3	1.52
8	35	0.7	1	1	0.91
9	5	0.7	11	1	1.09
10	20	0.4	6	3	1.6
11	35	0.1	1	5	6.07

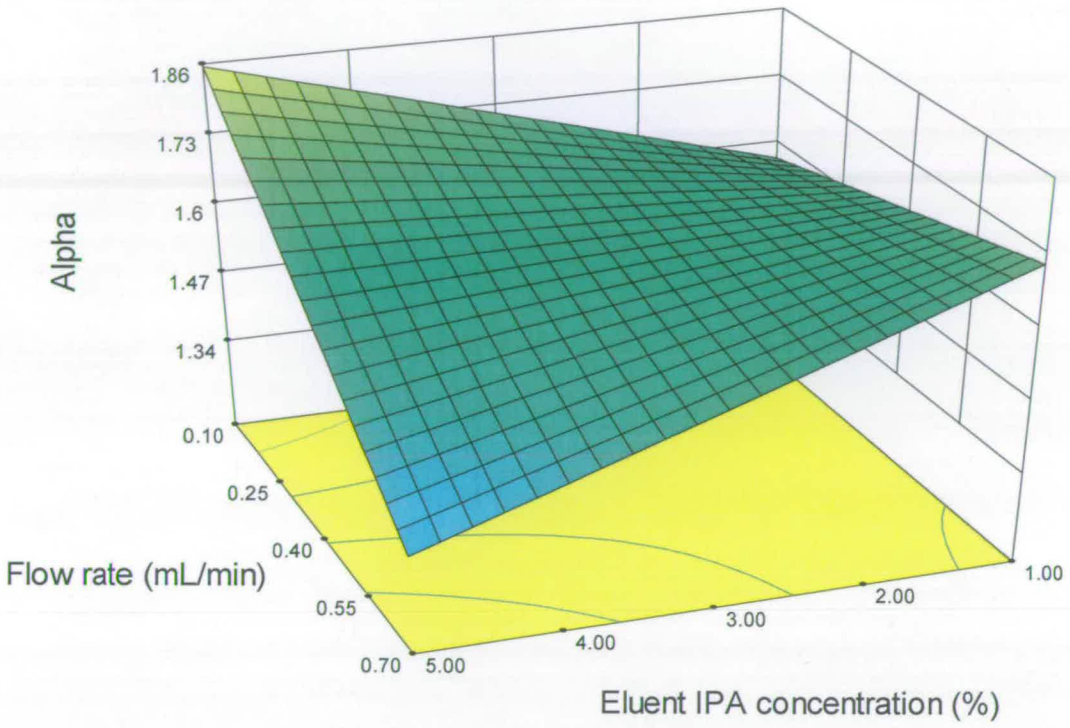
Table 6 1-(9-anthracenyl)-2,2,2-trifluoroethanol ***(39)*** *separation on CSP* ***(5)*** *using experimental design.*

Run number	t_1/min.	t_2/min.	α (sep. factor)	R_s (res. factor)	Solvent used/mL	Total time/min.
1	32.94	39.85	1.26	0.298	6.06	60.62
2	1.86	2.59	1.52	1.707	2.58	3.69
3	38.08	65.51	1.89	3.827	7.63	76.27
4	13.33	19.77	2.1	1.031	3.22	32.22
5	3.61	5.04	1.71	1.121	3.29	8.23
6	1.33	1.41	1.18	0.165	1.95	2.79
7	3.3	4.56	1.71	1.066	2.57	6.42
8	4.35	6.23	1.55	2.014	5.92	8.45
9	5.74	9.85	1.88	1.197	10.02	14.32
10	3.6	5.05	1.73	1.145	7.64	3.06
11	10.43	13.08	1.61	1.374	1.72	17.19

*Table 7 1-(9-anthracenyl)-2,2,2-trifluoroethanol **(39)** separation on CSP **(5)** using experimental design.*

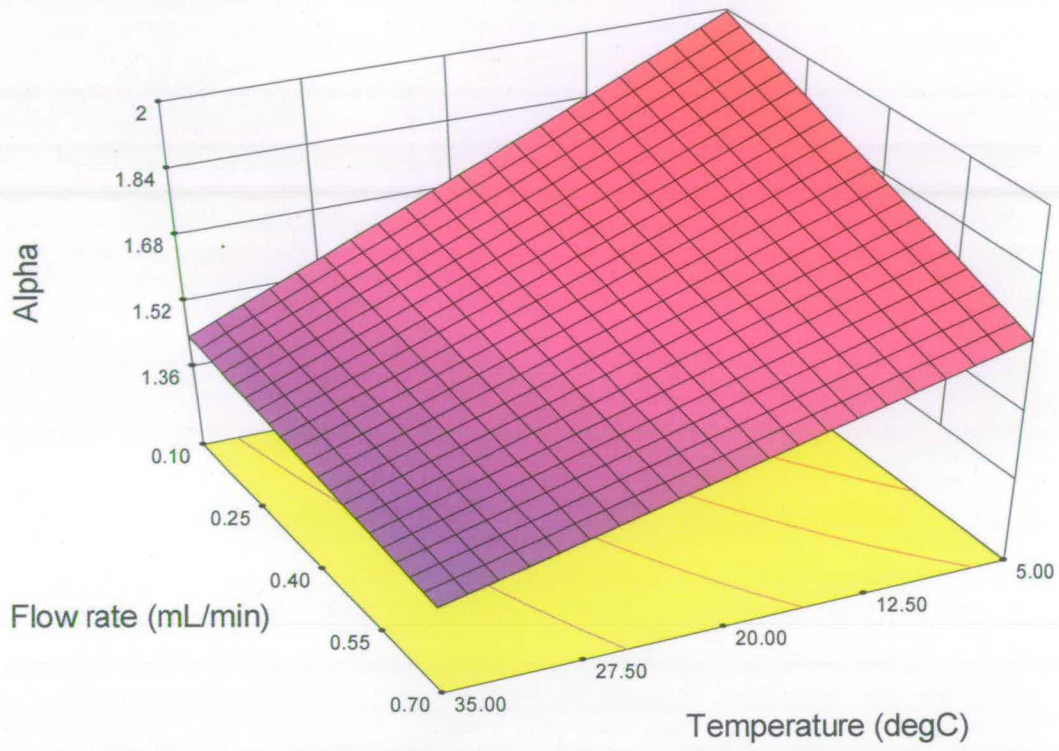
2.6.3 Interpretation of experimental design results

Results obtained from the DOE showed that many factors affect measured responses. The most significant observations come from the separation factor (α). Temperature and Flow rate had strong negative effects, meaning as temperature or flow rate increases (without changing other factors and irrespective of the other factors values) the separation factor will decrease. 2-Propanol concentration only had a very weakly negative effect. This result was surprising, but can be explained by the relatively small range that the eluent 2-propanol concentration was tested over (only between 1-5%). Two important second level effects were also deduced. Flow rate/eluent 2-propanol concentration second level term showed a strong negative effect on the separation factor (α), as shown in Graph 8. This means that higher α values occured when using either high flow rate and low eluent 2-propanol concentration or *vice-versa*. However lower α values occurred when either both factor are high or low. Temperature/flow rate second level term also had a positive effect on the separation factor (α), as shown in Graph 9. Thus when both temperature and flow rate were low, (with other factors constant) the highest α values were observed.



[a] *α values determined with constant Temperature of 20°C and an injection volume of 6 µL.*

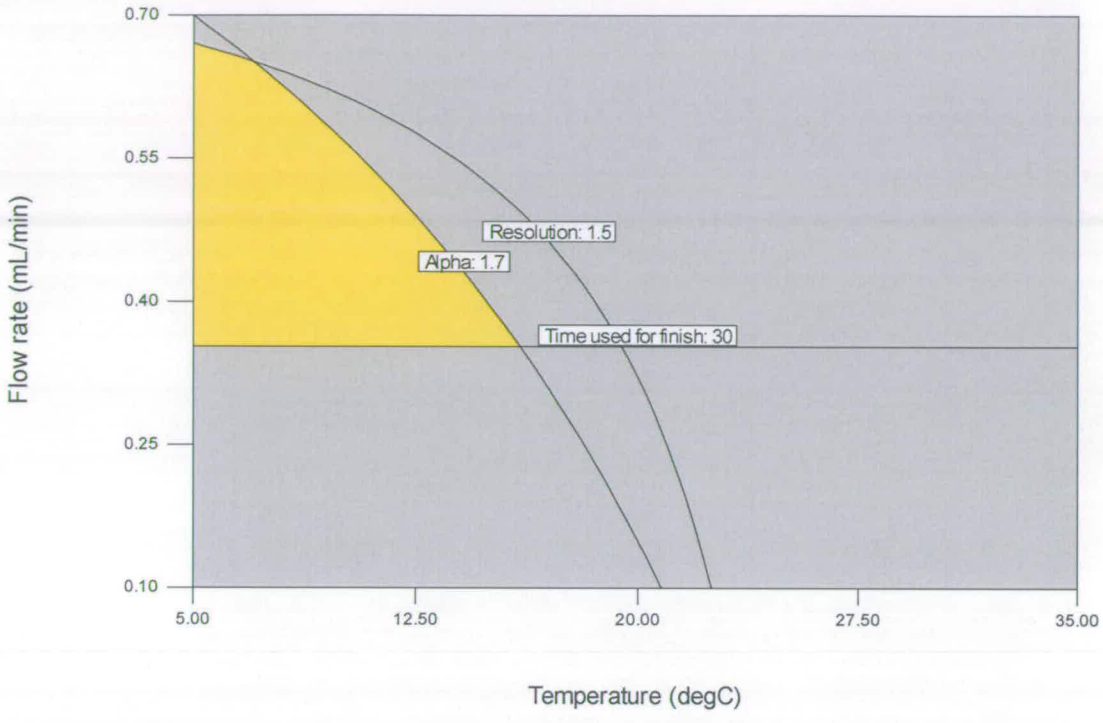
Graph 8 An experimental design graph showing predicted separation factors depending on variable flow rate/mLmin^{-1} and eluent 2-propanol (IPA)% [a]. Plotted using Design-Expert®.



[a] *α values determined with constant injection volume of 6 µL and eluent 2-propanol (IPA) of 3%.*

Graph 9 An experimental design graph showing predicted separation factors depending on variable flow rate/mLmin^{-1} and temperature/°C [a]. Plotted using Design-Expert®.

DOE was also used to determine an optimal condition range for the separation of (R/S)-1-(9-anthracenyl)-2,2,2-trifluoroethanol on CSP **(5)**. Optimatisation ('excellent separation'), restricted responses to favourable values: separation factor (α) $\geq$ 1.7, resolution factor (R_s) $\geq$ 1.5 and total time $\leq$ 30 minutes. Constant actual factors: injection volume (6 µL) and eluent 2-propanol (IPA) concentration (3%) were used, which are the midpoint values. The results are shown in Graph 10.



[a] *'Excellent' separations was restricted to separations factor (α) ≥ 1.7, resolution factor (R_s) ≥ 1.5 and total time ≤ 30 minutes.*

[b] *Graph determined with constant injection volume of 6μL and eluent 2-propanol (IPA) of 3%.*

[c] *'Excellent' separation area shaded in gold.*

Graph 10 An experimental design overlaid optimization plot, showing the predicted 'excellent' separation area using a variable flow rate/mLmin^{-1} and temperature/°C [a,b,c]. Plotted using Design-Expert®.

2.7 Synthesis of new CSP with two 3,5-dinitrobenzoyl end-groups

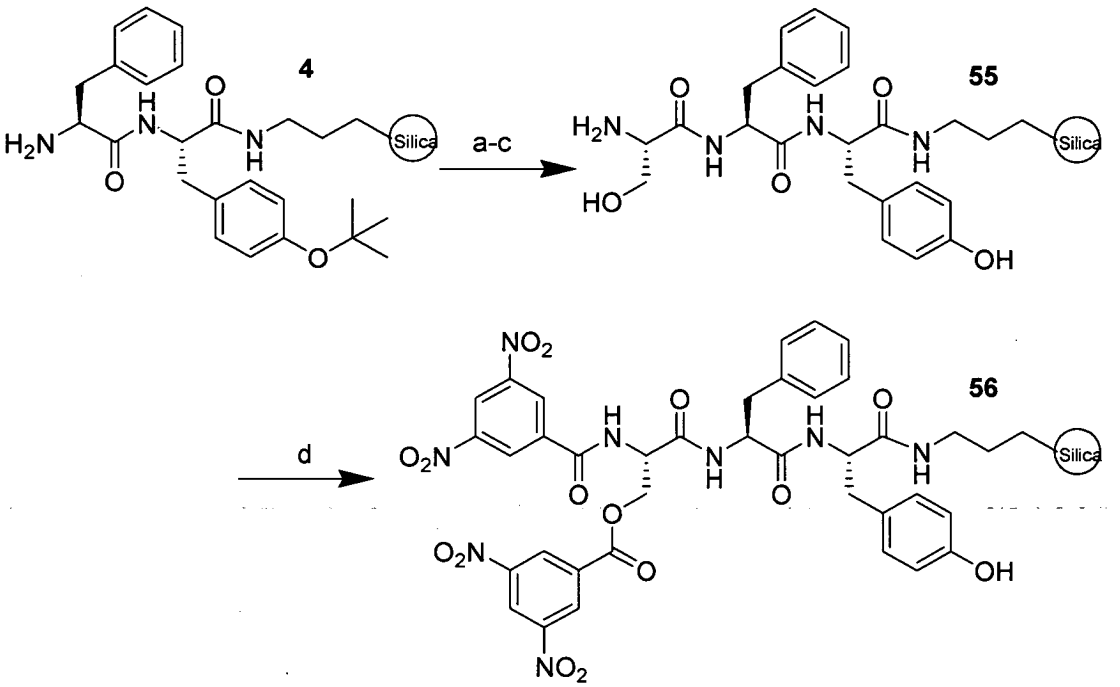
2.7.1 Introduction

One further CSP was synthesised following on from work in section 2.3. This CSP incorporated the two 3,5-dinitrobenzoyl groups in the structure used in CSP **(5)**.

2.7.2 Synthesis of new CSP with two 3,5-dinitrobenzoyl end groups

This was achieved starting with compound **(4)**, which was derivatised into a tripeptidic structure **(55)**, with the coupling of Fmoc-Ser(^{t}Bu)-OH, using standard coupling conditions, followed by Fmoc and ^{t}Bu deprotections. Compound **(55)** was

then derivatised with two 3,5-dinitrobenzoyl groups using a slight modification of standard coupling condition with the addition of a catalytic quantity of DMAP to the reagent mixture.

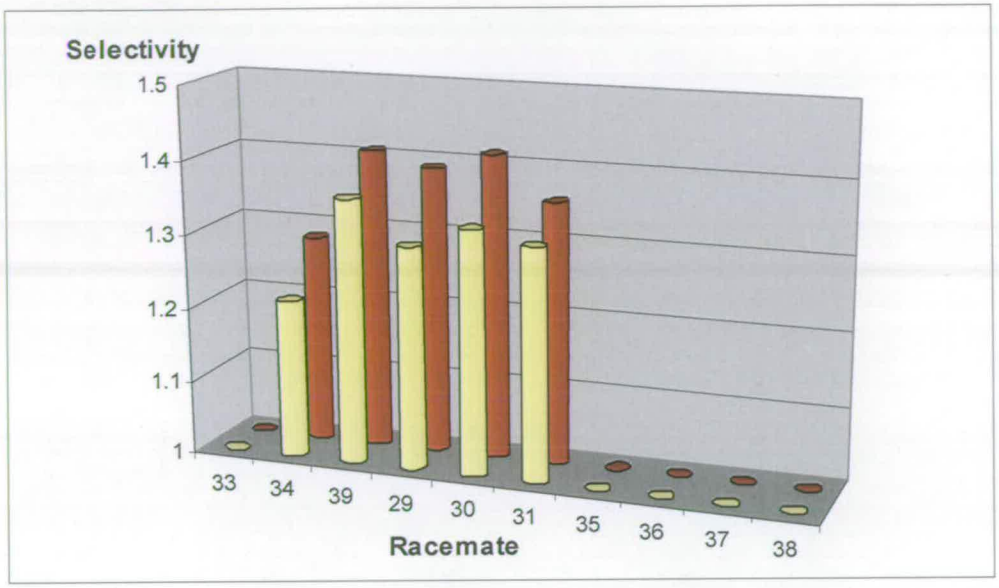


(a) Fmoc-Ser(^{t}Bu)-OH, DIC, HOBt, DCM/DMF (4:1), (b)Piperidine/DMF (5:95), (c) DCM/TFA/Water (50:50:1), (d) 3,5-dinitrobenzoic acid, DIC, HOBt, DMAP (cat.), DCM/DMF (4:1)

Scheme 19 Synthesis of CSP ***(56)***

2.7.3 Screening of new CSP containing two 3,5-dinitrobenzoyl groups

The standard racemic library was used for screening CSP **(56)**. Chiral selectivity values are plotted along with values for CSP **(43)** for comparison in Graph 11.



Brown = CSP **(56)**, Light Yellow = CSP **(43)**

*Graph 11 Chiral selectivity of CSP **(56)** compared to CSP **(43)** on standard racemate library*

Results for CSP **(56)** were similar to that of CSP **(43)**. Five of the ten member racemate library were successfully separated, identical to CSP **(43)**. However, the chiral selectivity for all five was slightly higher for the new CSP **(56)**. The new CSP, which incorporates both the structural features of CSP **(5)** (Phe, Tyr peptide chain) as well as utilising two 3,5-dinitrobenzoyl end groups, did not enhance the enantioselectivity of CSP **(5)** for the library of racemates tested.

2.8 Summary

A 3,5-dinitrobenzoyl end group is utilised in many commercially available CSPs. The importance comes from its ability to interact strongly, mainly through π-π forces, with analyte enantiomers. This strong interaction enhances the scale of transient diastereomeric energies, thus improving the chances for chiral separation.

A library of CSPs with alternative end groups was synthesised and screened against a small library of racemate analytes. The results were compared to identical screenings using a CSP with the 3,5-dinitrobenzoyl end group **(5)**. Although the library of CSPs could not improve upon the separation ability of CSP **(5)**, it did give an important insight to show how important all the features of the 3,5-dinitrobenzoyl group were. Removal or movement around the ring of nitro moieties caused large detrimental effects on the CSP.

For a dipeptidic type CSP, the second amino acid from the silica bead was found to have more influence on the overall chiral separation ability of the CSP. This was found by switching the optical character of the dipeptidic CSP structure.

Incorporating two 3,5-dinitrobenzoyl groups into the CSP structure could also not improve the CSP's ability over that of CSP **(5)** containing one 3,5-dinitrobenzoyl group. This is possibly because the second 3,5-dinitrobenzoyl a group is competing for interaction with the analyte in the same manner as the first 3,5-dinitrobenzoyl groups does.

3 Surface plasmon resonance biosensor screening of chiral stationary phase ligands.

3.1 Protein based CSPs

3.1.1 Introduction

The determination of appropriate chiral stationary phase media, for the separation of a given optically active racemate, is generally a difficult procedure. Historical precedent for separation of structurally similar racemates gives ideas to possible useful CSPs, however, even the smallest structural differences between analytes can show a dramatically different behaviour on the same column.

Separation of any given novel racemic compound thus firstly requires selection of a CSP from hundreds of commercially available CSP columns, sometimes without indication which columns may potentially allow successful separation of the analyte[247].

The determination of an appropriate CSP medium requires a faster approach to the screening of the racemate analyte on chiral stationary phase materials. Standard HPLC setup is limited to the screening of only one CSP at a time therefore, this can be very time consuming process. Of all the commercially available CSPs, there are approximately twenty to thirty that are regarded as those with the highest possible chance of success, with a very high chance that at least one member of these elite CSP columns will successfully separate any give racemate[247].

Screening of a given racemate on all twenty to thirty CSPs is however itself a very time consuming process, without taking into account the infinite number of chromatographic changes that are possible. The parallel screening of all CSPs simultaneously is possible, but is beyond the fiscal means of all but the largest of research budgets and does not overcome the further problems in chromatographic optimisation.

Surface plasmon resonance (SPR) biosensors are primarily used to monitor the interaction between an immobilised biomolecule and a flowing solution of another biomolecule or small molecule. The interaction between the immobilised and free

material is monitored in real time by the biosensor system utilising the surface plasmon resonance phenomena.

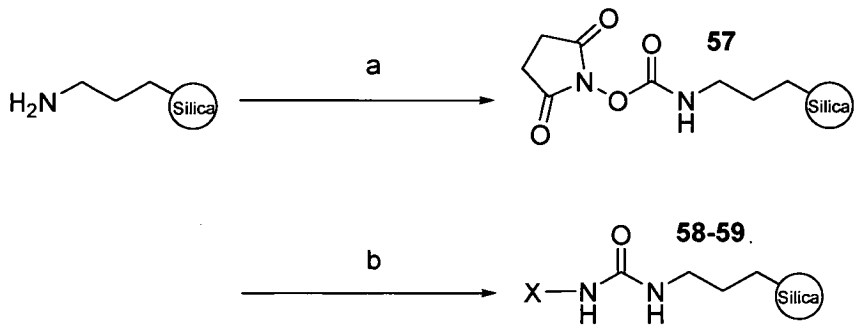
The SPR biosensor has many similarities to that of HPLC. An HPLC trace is the resultant of the sum of many thousands of interactions between immobilised-molecule and analyte-molecule over the whole column. SPR biosensors measure instantaneous real-time interactions between a material immobilised onto a sensor in the device and a solution of analyte flowing over the sensor's surface.

3.1.2 Synthesis of protein CSPs

As SPR biosensors are used to monitor biomolecule-based interactions. It was decided that biomolecule-based CSP materials could be used for initial screenings on HPLC.

There are a small number of biomolecules that have previously been used for CSP columns[174]. Some commercially available CSP columns of this type use cheap readily available proteins such as Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA), and these proteins can be covalently attached to silica to give the required CSPs.

Synthesis was achieved following a literature procedure for synthesising a protein CSP[128]. Aminopropyl silica needed to be chemically activated before the protein could be immobilised to it. This was achieved using a large excess of N,N'-disuccinimidyl carbonate (10 eq.). The silica was then filtered, washed and dried to give the aminopropyl silica with a carbo-succinimidyl activating group **(57)**. To a solution of activated silica **(57)** in phosphate buffer (pH 6.8) was slowly added the protein, BSA or HSA whilst carefully maintaining the pH. The amine groups present on the lysine residues of the protein displace the succinimide group on the silica to form a strong urea bond, thus immobilising the protein to the silica to give the desired CSPs **(58)** and **(59)** having immobilised BSA and HSA respectively which were packed into HPLC columns (150 mm length by 2.3 mm i.d.).



(a) *N,N'*-discuccinimidyl carbonate, MeOH, (b) **(58)** X = BSA, Bovine Serum Albumin, phosphate buffer pH 6.8, **(59)** X = HSA, Human Serum Albumin, phosphate buffer pH 6.8

Scheme 20 Synthesis of protein based CSPs (58) and (59)

3.1.3 Screening of protein CSPs

3.1.3.1 *Initial screening of protein CSPs*

These protein CSP columns were then screened using commercially available small molecule racemic analytes; benzoin, warfarin, ibuprofen, chlorpheniramine, bendroflumethiazide. With the nature of the CSPs involved in this screen, the HPLC was always used in reverse phase mode and initial screenings used phosphate buffer based mobile phases with small quantities of organic modifiers.

The five analytes were run on both CSP columns **(58)** and **(59)**, using six different chromatographic conditions; two phosphate buffer concentrations (10 mM and 50 mM) and three buffer pHs (6.5, 7.0 and 7.5) using a ramped gradient of 1-PrOH as an organic modifier (2% isocratic for t = 0-10 min, 2-10% gradient over t = 10-30 min, 10% isocratic for t = 30-40 min, 10-2% gradient over t = 40-50 min, 2% for t = 50-60 min) at a flow rate of 0.5 $mLmin^{-1}$ flow rate. However, the screening of the CSPs all showed either no separation or ambiguous results. Both the CSPs were resynthesised and screened again using some of the same chromatographic conditions. A number of other combinations of gradients, organic modifiers (2-PrOH, EtOH) and flow rates (0.2-1.0 $mLmin^{-1}$) were also investigated, but none of these combinations gave positive chiral separations.

3.1.4 Screening of commercial protein CSPs

3.1.4.1 *Introduction*

Commercially available protein CSP columns were used at GSK, Stevenage. These columns could be used for the chiral screening of some small molecule enantiomers whilst also gaining valuable knowledge aimed at improving chromatographic conditions.

An immobilised bovine serum albumin column was purchased, from Nagel, Resolvosil BSA-7 (100 mm length x 4.0 mm i.d.) was used, along with a human serum albumin column and a α_1-acid glycoprotein (AGP) column, manufactured by ChromTech AB (Chiral-HSA and Chiral-AGP respectively; both 50 mm length x 4.6 mm i.d.). These columns will be known as BSA-CSP, HSA-CSP and AGP-CSP.

Due to this single enantiomers of the analytes will now be used, as this improves the clarity of the HPLC spectra results. These single enantiomers will also be imperative for the future SPR biosensor screenings. Both Chlorpheniramine and Bendroflumethiazide are both deemed unsuitable for the future screenings, as they are both commercially unavailable for purchase as discrete single enantiomers and suitable semi-preparative chiral separation methods could not be determined. Analytes were initially screened using one of two generic gradient methods. Both methods used a mobile phase combination of ammonium acetate buffer (pH 7.4) (A) and 2-PrOH (B).

Protein gradient method 1

Time/min	Eluent A/%	Eluent B/%
0	100	0
15	75	25
25	75	25
25.1	100	0
30	100	0

Protein gradient method 2

Time/min	Eluent A/%	Eluent B/%
0	100	0
15	50	50
25	50	50
25.1	100	0
30	100	0

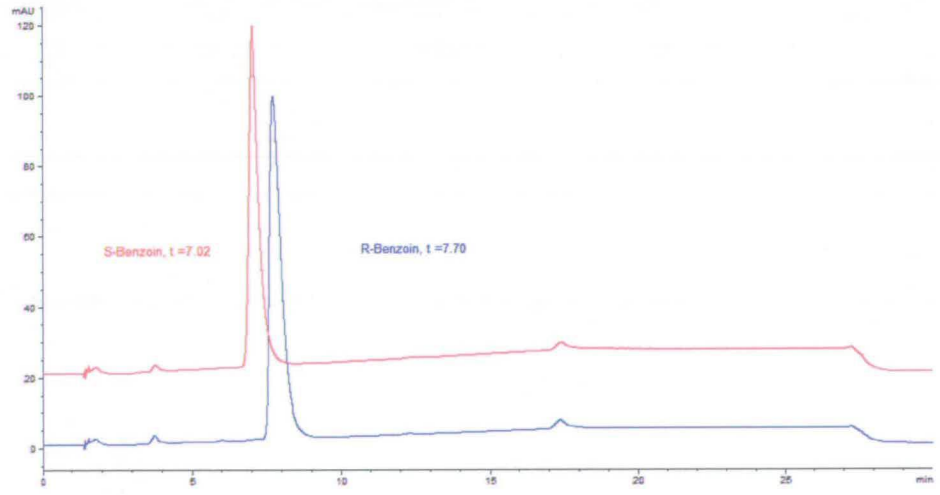
*Table 8 Protein gradient methods 1 and 2. *Eluent A= Ammonium acetate buffer (pH 7.4); Eluent B=2-PrOH.*

The time of sample elution in the generic screening was used as a guide for the alcohol modifier concentration for an individually tailored isocratic mobile phase composition run.

3.1.4.2 *Benzoin*

Figure 20 R- and S-Benzoin

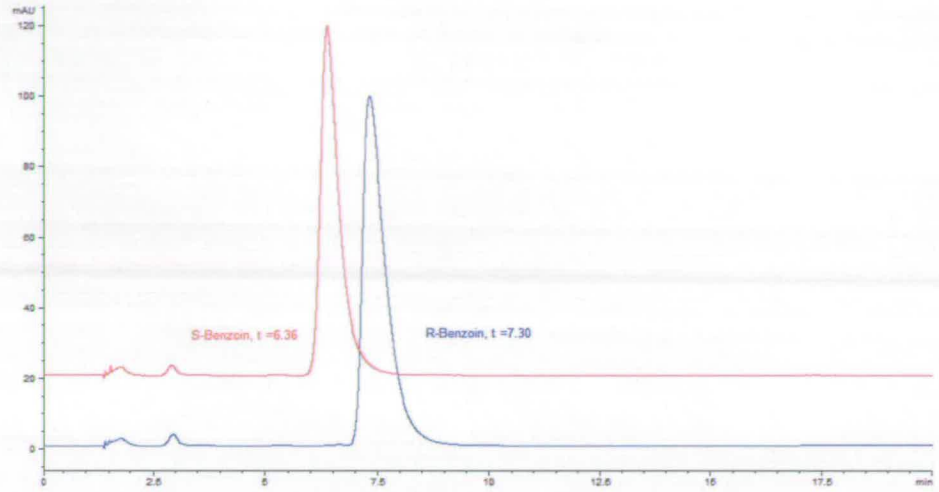
Initial screens of R and S-Benzoin enantiomers on a BSA-CSP column, used protein gradient method 1 (section 3.1.4.1). Results of this screen (Figure 21) indicate that Benzoin bound very weakly, therefore subsequent isocratic screening used a small concentration of 2-PrOH (3%).



*Mobile phase = protein gradient method 1, flow rate = 1.0 $mLmin^{-1}$, viewed at 254 nm

Figure 21 Screening of R and S-Benzoin on BSA-CSP

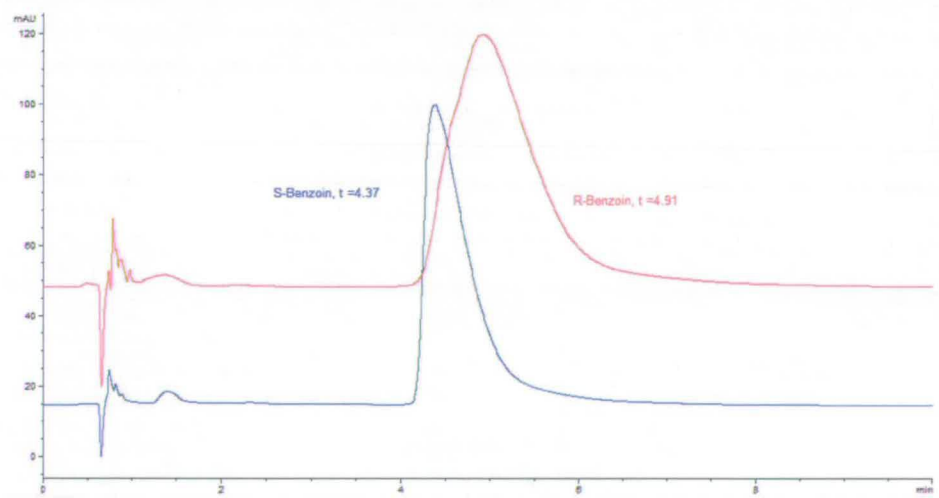
Isocratic screening results (Figure 22) confirmed that the elution order was S-Benzoin eluted before the more strongly retained R-enantiomer, which had already been indicated from the initial gradient screening.



*Mobile phase = Ammonium acetate buffer (pH 7.4)/2-PrOH (97:3), flow rate = 1.0 $mLmin^{-1}$, viewed at 254 nm

Figure 22 Screening of R and S-Benzoin on BSA-CSP (rescreen with isocratic elution)

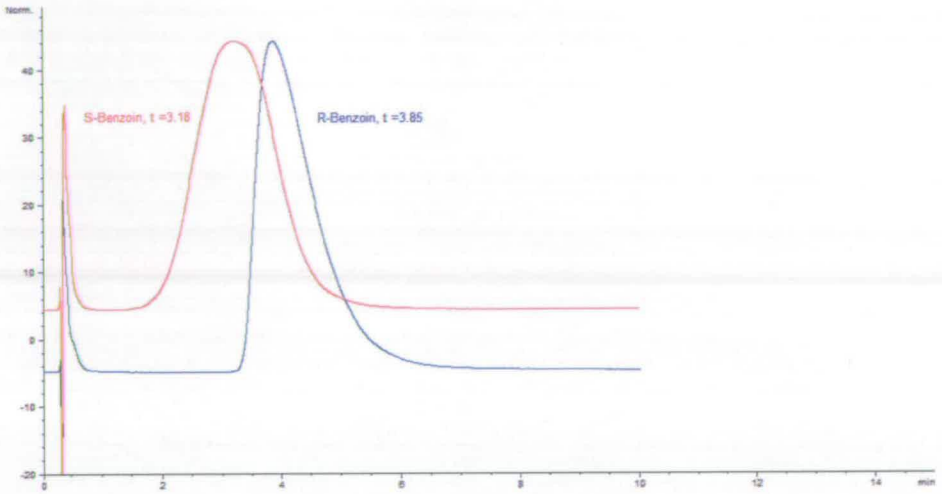
Similar to the gradient screening on BSA-CSP, both HSA-CSP and AGP-CSP indicated a weak interaction between both proteins and the analyte enantiomers, and therefore both were analysed with isocratic runs with ammonium acetate buffer (pH 7.4) as the sole content of the mobile phase.



*Mobile phase = Ammonium acetate buffer (pH 7.4), flow rate = 0.5 $mLmin^{-1}$, viewed at 254 nm

Figure 23 Screening of R and S-Benzoin on HSA-CSP

HSA-CSP screening results (Figure 23) showed that S-Benzoin eluted before R-Benzoin, whereas in the AGP-CSP screen (Figure 24) this order was reversed; with R-Benzoin eluting before S-Benzoin.



*Mobile phase = Ammonium acetate buffer (pH 7.4), flow rate = 1.0 $mLmin^{-1}$, viewed at 254 nm

Figure 24 Screening of R and S-Benzoin on AGP-CSP

3.1.4.3 *Warfarin*

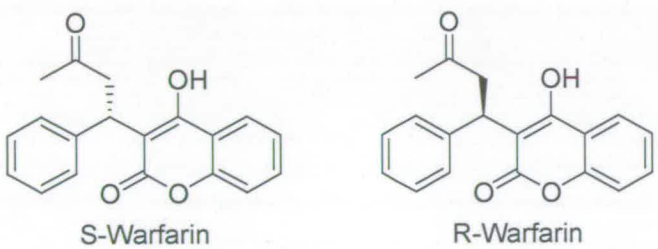
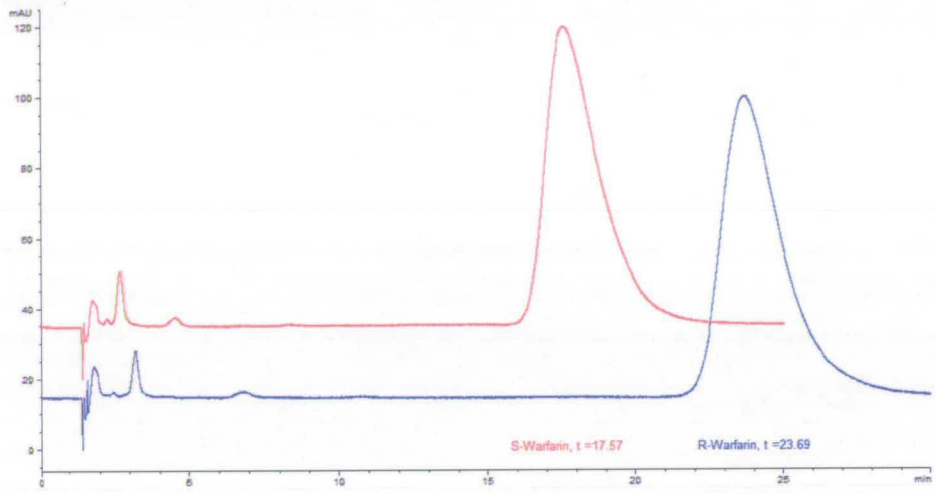


Figure 25 S- and R- Warfarin

Individual enantiomers of R and S-Warfarin are commercially available. However, the cost of these enantiomers was greater than £50 mg^{-1} and therefore a semi-preparative chiral separation method was utilised. Racemic Warfarin, was separated using ChiralCel-OD, a commercially available chiral column manufactured by Daicel (150 mm length by 4.6 mm i.d). Chiral separation was achieved with sequential injections of (RS)-Warfarin (~10 mg per injection) with a mobile phase composition of heptane/2-PrOH/acetic acid (80:20:1) and flow rate of 1.0 $mLmin^{-1}$, with samples collected using an HPLC integrated automated collector based on detection at 254 nm. The dried single enantiomers of (R)-Warfarin (>99.9% ee, determined by HPLC integration) and (S)-Warfarin (>99.9% ee) were then subsequently checked for optical purity with a ChiralCel-OD column.

Gradient screenings of Warfarin indicated that organic modifier (2-PrOH) concentrations of approximately 10% should be suitable for the subsequent isocratic screenings for all three protein CSPs. However gradient screening of Warfarin on both HSA-CSP and AGP-CSP gave better results in comparison to the previous isocratic screening runs, which gave much larger peak tailing.

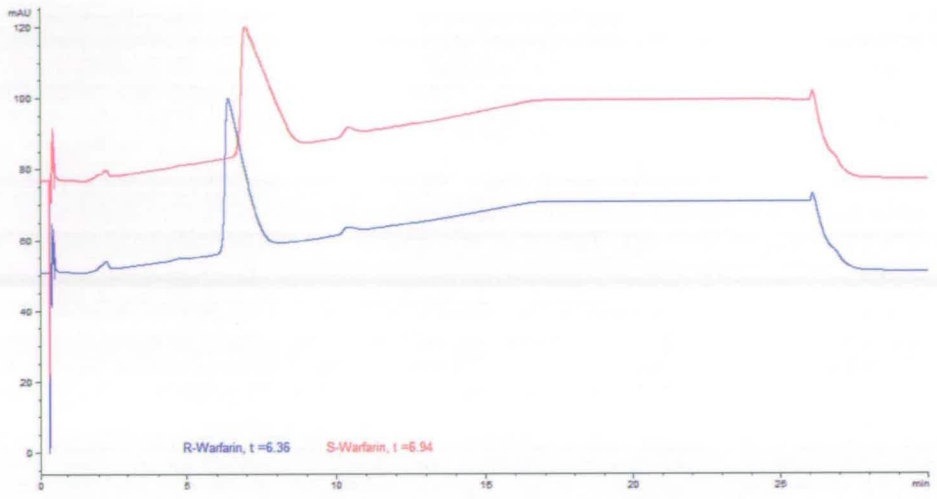
For BSA-CSP isocratic screenings (Figure 26) showed a clear difference in retention of sample with S-Warfarin eluted before the more strongly retained R-Warfarin.



*Mobile phase = Ammonium acetate buffer (pH 7.4)/2-PrOH (90:10), flow rate = 1.5 $mLmin^{-1}$, viewed at 280 nm

Figure 26 Screening of R and S-Warfarin on BSA-CSP.

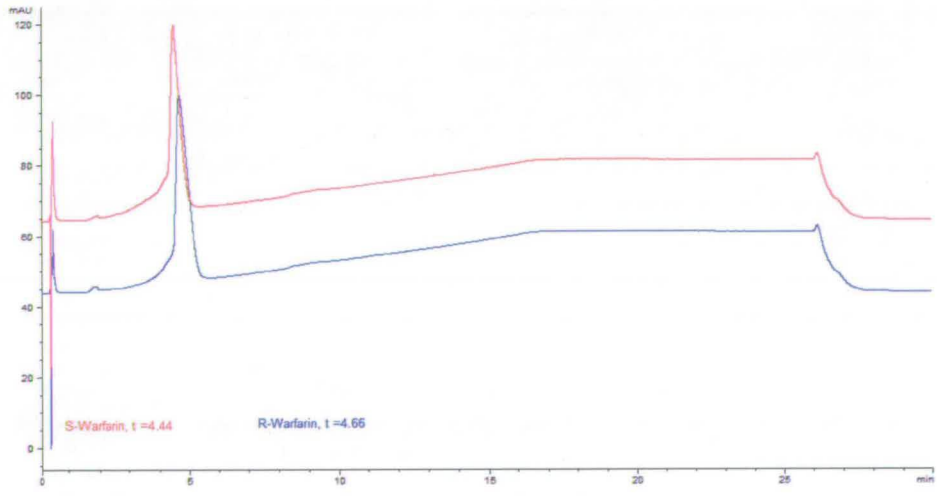
HSA-CSP was found to give an opposite elution order in comparison to BSA-CSP (Figure 27).



* Mobile phase = protein gradient method 2, flow rate = 1.0 $mLmin^{-1}$, viewed at 280 nm

Figure 27 Screening of R and S-Warfarin on HSA-CSP.

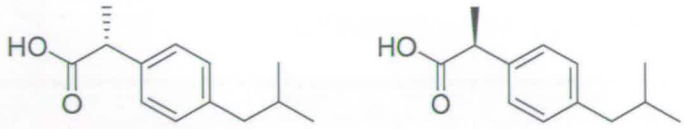
AGP-CSP gave the same order of enantiomer elution as BSA-CSP (S before R) (Figure 28).



* Mobile phase = protein gradient method 2, flow rate = 1.0 $mLmin^{-1}$, viewed at 280 nm

Figure 28 Screening of R and S-Warfarin on AGP-CSP

3.1.4.4 *Ibuprofen*

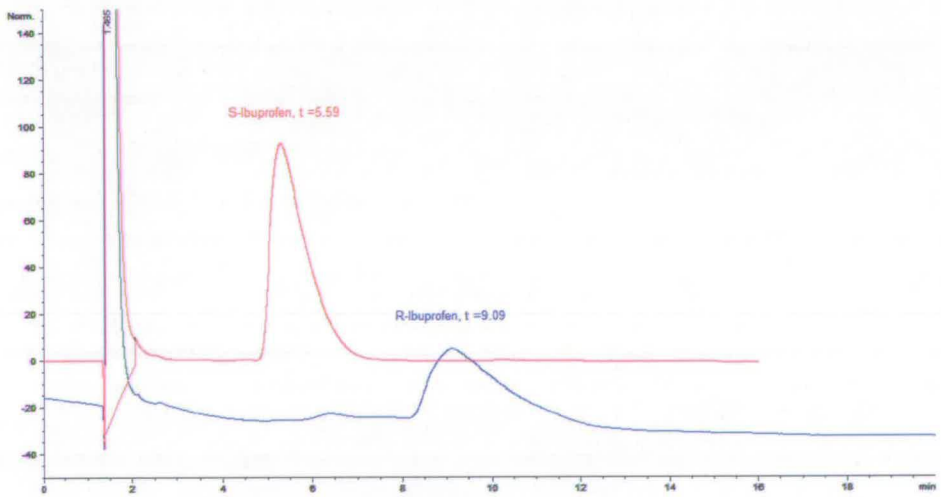


R-Ibuprofen S-Ibuprofen

Figure 29 R- and S-Ibuprofen

Unlike Warfarin, single enantiomers of Ibuprofen were commercially available and reasonably priced with good optical purity (>99% ee).

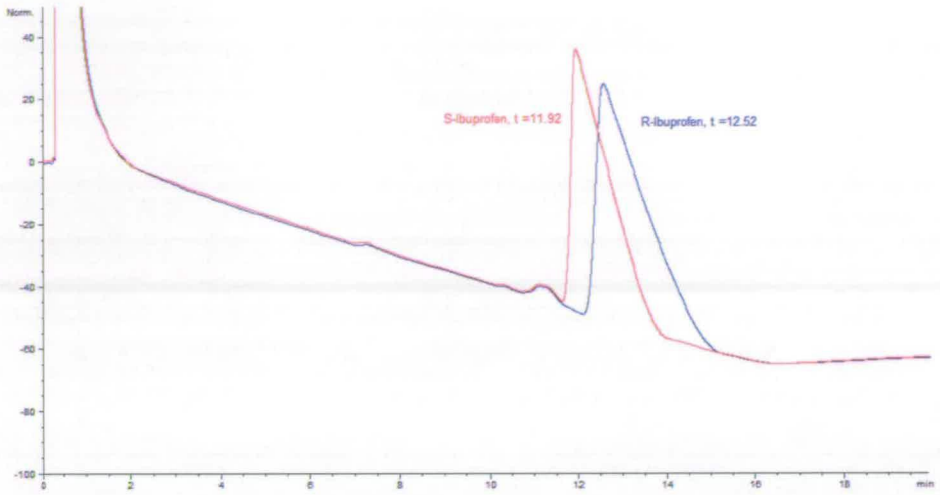
Results from preliminary gradient screenings of Ibuprofen indicated that relatively high concentrations of 2-PrOH modifier were necessary for elution. The screening of Ibuprofen on BSA (Figure 30) required the mobile phase to contain some 40% 2-PrOH.



* Mobile phase = Ammonium acetate buffer (pH 7.4)/2-PrOH (60:40), flow rate = 1.0 mLmin^{-1}, viewed at 230 nm

Figure 30 Screening of R and S-Ibuprofen on BSA-CSP

The screening of Ibuprofen on HSA-CSP (Figure 31) was better using the protein gradient method 2 (section 3.1.4.1). The elution order observed for R and S-enantiomers on both BSA and HSA-CSP was the same, with S-Ibuprofen eluting before R-Ibuprofen. No separation was observed when screened on AGP-CSP. This may be due to a much lower binding interaction between the acidic Ibuprofen and the acidic protein AGP. However, screenings of Ibuprofen on AGP-CSP in the literature indicate an elution order of R-Ibuprofen before S-Ibuprofen[248].



* Mobile phase = protein gradient method 2, flow rate = 1.0 $mLmin^{-1}$, viewed at 230 nm

Figure 31 Screening of R and S-Ibuprofen on HSA-CSP

3.1.4.5 *Tryptophan*

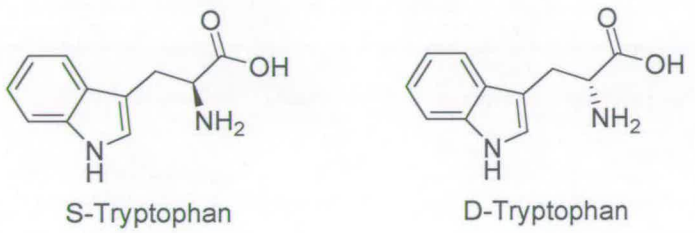
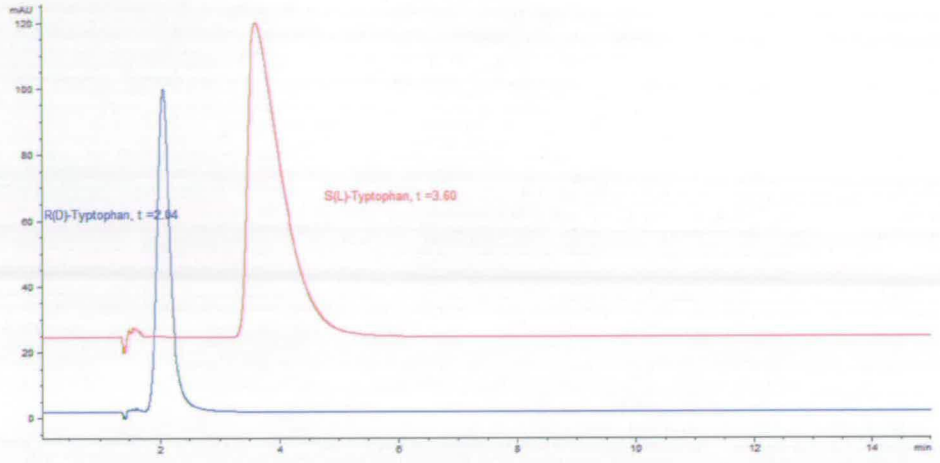


Figure 32 S- and D-Tryptophan

The final analyte for screening on the protein CSP was Tryptophan. Tryptophan is a common α-amino acid and is therefore readily available in both enantiomeric forms. Although it was not originally included as a possible screening analyte, its ease of availability gave a good opportunity to expand the screening data set. No organic modifier was necessary for any of the screenings. On BSA-CSP (Figure 33), Tryptophan was weakly retained, by the column. However, analysis was clearly enough to show that R(D)-Tryptophan was more strongly retained.

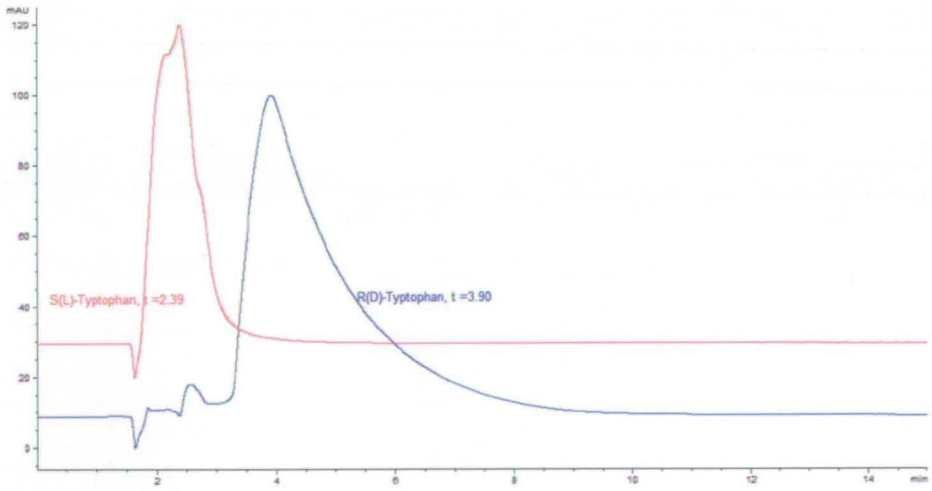


* Mobile phase = Ammonium acetate buffer (pH 7.4), flow rate = 1.0 $mLmin^{-1}$, viewed at 280 nm

Figure 33 Screening of R(D) and S(L)-Tryptophan on BSA-CSP

BSA-CSP (Figure 34), gave similar to results to HSA-CSP, and again gave low overall retention of Tryptophan on the column, but in this case R(D)-Tryptophan was better retained than the S(L)-Tryptophan enantiomer.

On the final protein CSP column, AGP-CSP, Tryptophan was found to bind so weakly that both enantiomers were eluted within 2 min, even with the flow rates as low as 0.2 $mLmin^{-1}$.



* Mobile phase = Ammonium acetate buffer (pH 7.4), flow rate = 1.0 $mLmin^{-1}$, viewed at 280 nm

Figure 34 Screening of R(D) and S(L)-Tryptophan on HSA-CSP

3.1.5 Summary of protein CSP screening results

Four analytes were screened on three separate protein CSP columns, using reverse phase HPLC. Chiral separation values for each separation have not been taken in this case. The nature of chiral separation means that there are numerous variable chromatographic factors resulting in an infinite number of specific conditions possible for each given separation and the screening results obtained. Thus Ibuprofen on HSA-CSP and Benzoin on BSA-CSP can not be compared directly by separation factor, because neither separation is fully optimised, nor are they optimised to the same degree. As well as the number of controllable chromatographic conditions possible, there are a number of uncontrollable factors such as the amount and condition of protein immobilised.

One resultant factor that should not change, within the same HPLC mode, is the order of elution for enantiomers of the analyte. The order of elution gives definitive information as to which enantiomer binds more strongly to the immobilised protein in the CSP column. The relative binding strength is opposite to that of the measured elution order and the overall results of the protein CSP screenings are shown (Table 9).

Protein CSP	Analyte	Screening elution order (e.g. X-Y = X before Y)
BSA	Benzoin	S-R
	Warfarin	S-R
	Ibuprofen	S-R
	Tryptophan	R-S
HSA	Benzoin	S-R
	Warfarin	R-S
	Ibuprofen	S-R
	Tryptophan	S-R
AGP	Benzoin	S-R
	Warfarin	S-R
	Ibuprofen	No data
	Tryptophan	No data

Table 9 Summary of analyte elution order screening results for protein CSP columns

3.2 Surface plasmon resonance biosensor

3.2.1 Introduction

SPR biosensors enable label-free detection of analytes binding to immobilised biomolecules and provide real-time quantitative information on binding interactions.

The sensor chip contains a gold layer, which creates the physical conditions required for surface plasmon resonance. Essentially, SPR detects mass changes in the aqueous layer close to the sensor chip surface by measuring changes in refractive index. Binding molecules increase the mass and the dissociation of bound molecules decrease mass, which allows continuous, real-time measurements via a sensorgram.

SPR biosensors can provide information on a number of factors: specificity, of the binding between two sets of molecules; concentration, of a particular active molecule; kinetics, the rates of association and dissociation of the binding partners; affinity, how strong the binding is between analyte and the immobilised partner molecule.

The screening of the same enantiomer analytes, as used for the previous HPLC protein CSP screening, on the SPR biosensor will show if there are useful relationships between the two different analytical technologies. A link in the results obtained from the SPR biosensor and chiral HPLC gives the possibility of utilising a modified SPR biosensor device as a high-throughput determinator of suitable CSP media for larger scale separation. The high-throughput possibility arises as some modern SPR biosensors can be used to screen large numbers of immobilised molecules simultaneously with the same analyte{Biacore, Accessed on 10/12/07 #219}.

The screening in this section was carried out using a BIAcore S51® SPR biosensor. This particular device can accommodate two different immobilised samples as well a blank sample for reference. The screening makes use of the SPR biosensor's ability to measure the affinity of binding interaction and screening was carried out using both individual single enantiomer analytes as well as the racemate analyte of the same material.

As the sensitivity of the equipment will be challenged, it possible that systematic and human error may affect these screening results. To reduce the amount of screening data affected by this, a nested design (Figure 35) was developed so that all the controllable factors are duplicated.

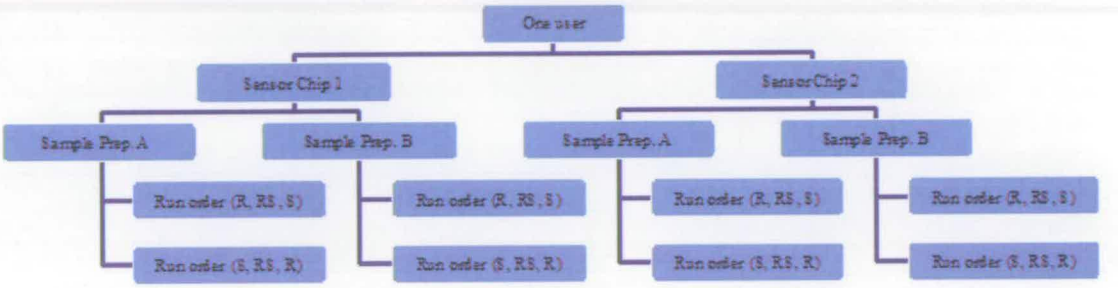
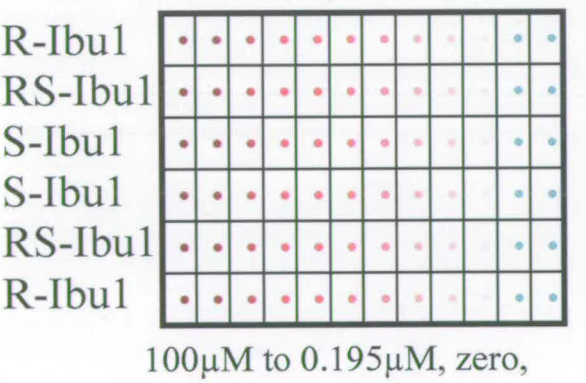


Figure 35 Nested design of SPR biosensor enantiomer screening structure.

The Biacore® S51 SPR biosensor uses a 96 well plate for analyte samples. The screening ran every compound (e.g. Warfarin) as both of its single enantiomers and as the racemate. Each analyte (e.g. R-Warfarin) was run over a large concentration range (from 0.195 M to 100 μM) as well as two zero concentration runs for each analyte. An example of a typical well plate is shown (Figure 36).



* 72 of the 96 wells are shown. The other (2 x12) wells were unused.

Figure 36 Typical layout of BIAcore SPR biosensor sample well plate.

3.2.2 SPR Biosensor chip preparation

Before screening of the enantiomers and racemates on the SPR biosensor, the protein binding partners of the analytes had to be immobilised onto the sensor chip (Series S - CM5).

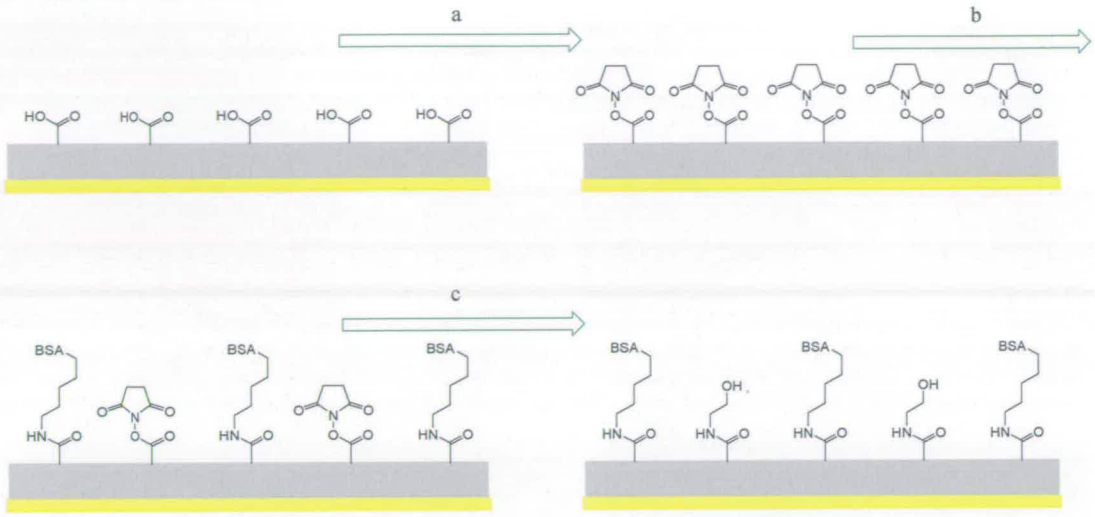
The sensor chip consists of a glass surface coated with a thin layer of gold covered with carboxymethylated dextran. Surface carboxy group are available for derivatisation, allowing the immobilisation of biomolecules.

The immobilisation of the proteins onto the sensor chip were achieved *in-situ*, with all reagents flowing over the sensor's surface, in the same way as screenings take place. The sensor chip has three separately addressable spots. The first spot will contain one immobilised protein (e.g. BSA sample 1), the second spot will also contain another immobilised protein (e.g. BSA sample 2 or HSA sample 1), and the third spot is left blank and is used as a reference spot for vital run calibration.

3.2.2.1 *Amine coupling immobilisation of BSA and HSA*

Both BSA and HSA were immobilised onto the sensor surface by forming covalent amide bonds with the carboxy derivatised surface. This was achieved using standard amine-coupling procedure in accordance with BIAcore® procedural handbook.

Initially a new sensor chip (S series CM5) was flushed with a steady flow of the running buffer (PBS (pH 7.4)/DMSO (99:1). The first step in immobilisation was activation of the sensor chip with EDC and NHS, to form active succinimide esters. Injected protein ligand (BSA or HSA) then flows over the sensor surface and the succinimide esters react with the amino groups or other nucleophilic groups on the protein to covalently link the protein ligand to the dextran. The BIAcore S51 biosensor automatically immobilises the sensor surface to approximately the requested concentration of protein, which is measured by the biosensor device in response units (RU, 1RU $\approx$ 1 pgmm^{-2} for most proteins, normal immobilisation range 4-10 kRU). This is achieved by an initial small injection of protein, which corresponds to a measured level of immobilisation. From this, the approximate amount of protein required is calculated and injected into the system for complete immobilisation. Once the protein (BSA or HSA) has been immobilised onto the sensor chip surface, any remaining active succinimide ester groups are reacted with ethanolamine, followed by 'cleaning' of the sensor chip using BIAdesorb solutions (sodium dodecyl sulphate and glycine), which removes any electrostatically bound ligand.

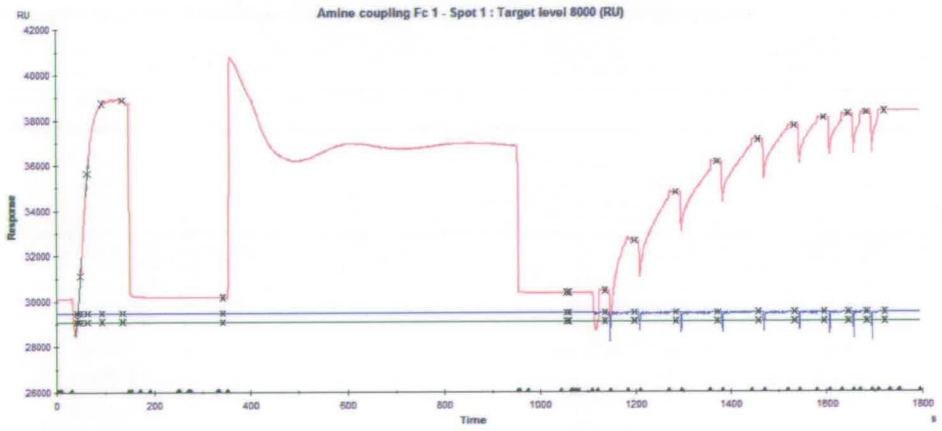


* Grey block represents the Dextran layer sitting on gold.

**Immobilisation uses a flow of reagent over the sensor surface.

(a) (i) Running buffer; PBS (pH 7.4)/DMSO (99:1),
(ii) N-Hydroxysuccinimide (NHS) (400 mM),
N-ethyl-N'-dimethylaminopropyl-carbodiimide (EDC) (400 mM),
(b) BSA (50 μgmL^{-1}) in NaOAc (pH 5.5, 10 mM) (c) (i) Ethanolamine-HCl (pH 8.5, 1.0 M), (ii) BIAdesorb 1; sodium dodecyl sulphate (0.5% w/v),
(iii) BIAdesorb 2; Glycine (pH 9.5, 50 mM),
(iv) Running buffer; PBS (pH 7.4)/DMSO (99:1)

Scheme 21 Loading of a BIAcore S series CM5 chip with BSA onto the surface.



* 30-150s = Injection of protein ligand solution (test concentration), 350-950s = NHS/EDC injected, 1100-1700s = sequential injection of protein ligand solution with subsequent immobilisation to sensor surface.

** Blocking of unreacted succinimide ester with Ethanolamine after immobilisation is not shown in sensorgram.

Figure 37 Response level during immobilisation of protein to chip sensor surface.

3.2.2.2 *Surface thiol coupling immobilisation of AGP*

The amine coupling immobilisation method used for BSA and HSA proteins could not be used for the immobilisation of AGP. Both BSA and HSA have a relatively high isoelectric point (pI = 4.7) and available amine groups, so amine coupling is the first choice for these ligands. AGP (pI = 2.7) is much more acidic therefore is not suitable for amine coupling, with the lack of free amine groups and electrostatic repulsion from the negatively charged dextran matrix on the sensor chip means than an alternative method was necessary.

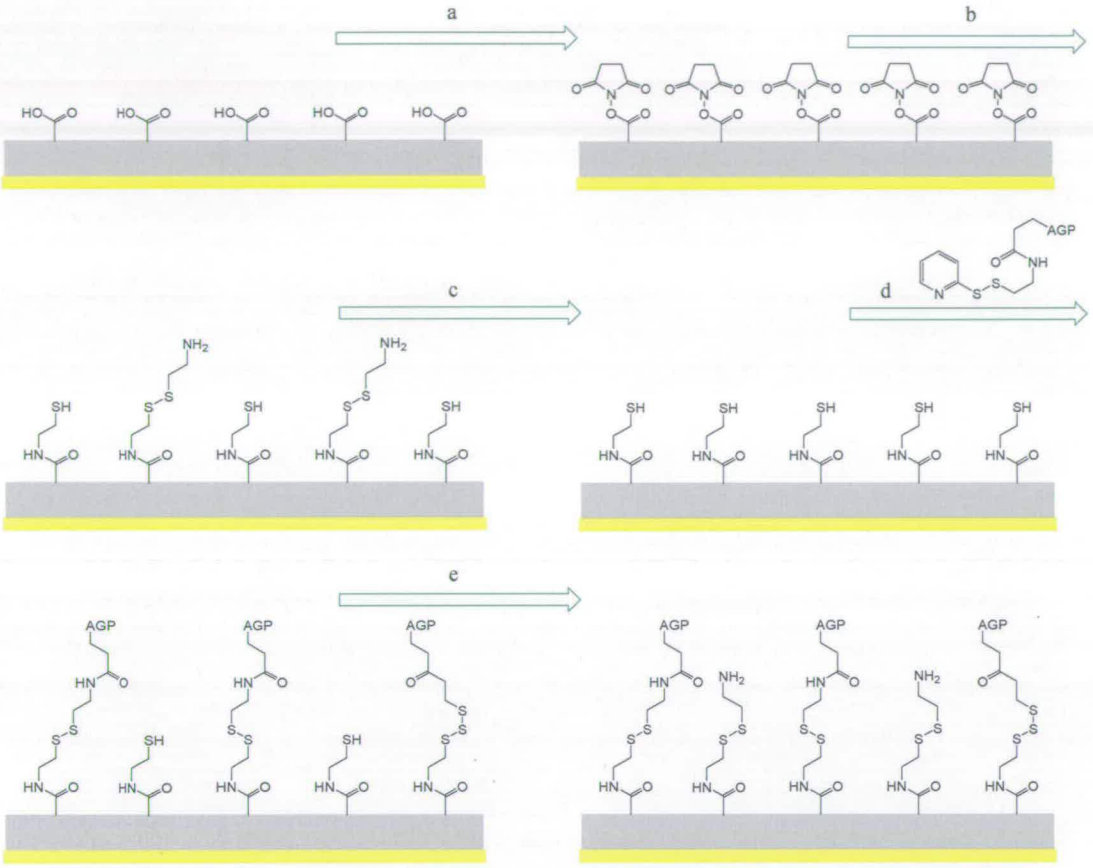
The thiol coupling chemistry is an alternative to the amine chemistry which can give fast and efficient immbolisation. For thiol coupling the protein ligand (AGP) has to be substituted with PDEA (2-(2-pyridinyldithio)ethaneamine), which raises the isoelectric point of the protein and improves the electrostatic interaction with the surface dextran matrix prior to coupling.

Initially the new sensor chip (S series CM5) was flushed with a steady flow of the running buffer (PBS (pH 7.4)/DMSO (99:1). The first step involves formation of the active succinimide ester on the surface with the addition of EDC and NHS. The surface is then derivatised by the addition of cystamine, leaving free thiol groups on sensor chip. This step may leave some unreactive disulphide groups on excess cystamine reacting with the surface thiol groups. To resolve this, a solution of DTE (Dithioerythritol) is flown across the sensor to reduce the disulphide groups back into their reduced thiol forms.

The PDEA-modified-AGP solution was prepared by dissolving human α-acid glycoprotein (AGP) and PDEA in MES buffer (pH 5.0, 0.1 M), followed by the addition of EDC (400 mM) and was then left to incubate for ten minutes at room temperature before changing the buffer to citrate buffer (pH 3.6, 10 mM) by size exclusion chromatography using an equilibrated NAP-10 column (prepacked sephadex G-25 column). The PDEA-modified-AGP was then further diluted with citrate buffer to 100 mM.

As before, this solution is then flowed across the thiol sensor chip surface and the reactive disulfide groups on the protein ligand react spontaneously with the immobilised thiols to covalently attach the protein to the surface. The immobilisation of AGP is completed by surface blocking of unreacted groups by

flowing solutions of PDEA and ethanolamine sequentially, followed by washing with BIAdesorb solutions.



* Grey block represents the Dextran layer sitting on gold.

**Immobilisation procedure uses flow of reagent sequentially over the sensor surface.

(a) (i) Running buffer; PBS (pH 7.4)/DMSO (99:1),
(ii) N-Hydroxysuccinimide (NHS) (400 mM),
N-ethyl-N'-dimethylaminopropyl-carbodiimide (EDC) (400 mM), (b) Cystamine (40 mM) in borate buffer (pH 8.0, 0.1 M),
(c) Dithioerythritol (DTE) (0.1 M) in borate buffer (pH 8.0, 0.1 M),
(d) PDEA-modified-AGP (~100 μgmL^{-1}) in citrate buffer (pH 3.6, 10 mM),
(e) (i) PDEA (20 mM), (ii) Ethanolamine-HCl (pH 8.5, 1.0 M),
(ii) BIAdesorb 1; sodium dodecyl sulphate (0.5% w/v),
(iii) BIAdesorb 2; glycine (pH 9.5, 50 mM),
(iv) Running buffer; PBS (pH 7.4)/DMSO (99:1)

Scheme 22 Loading of the BIAcore CM5 chip with AGP onto the surface using a thiol coupling method.

3.2.3 Screening on SPR Biosensor

3.2.3.1 *Introduction*

Once the protein has been immobilised onto the sensor surface, the SPR biosensor was ready for the screening of analytes. All analyte samples were taken from identical batches used in previous HPLC protein screening (section 3.1.3). Samples were firstly weighed using a calibrated high precision balance (±0.00000005 g or ±0.00005 mg). Samples were then dissolved in DMSO (ca. > 1000 μL) (±0.25 μL), followed by vortexing and sonication if necessary to give a fully dissolved 10 mM sample in DMSO. An aliquot (10 μL) of this solution was then taken up into PBS buffer (990 μL, pH 7.4) to give a 100 μM sample in PBS (pH 7.4)/DMSO (99:1).

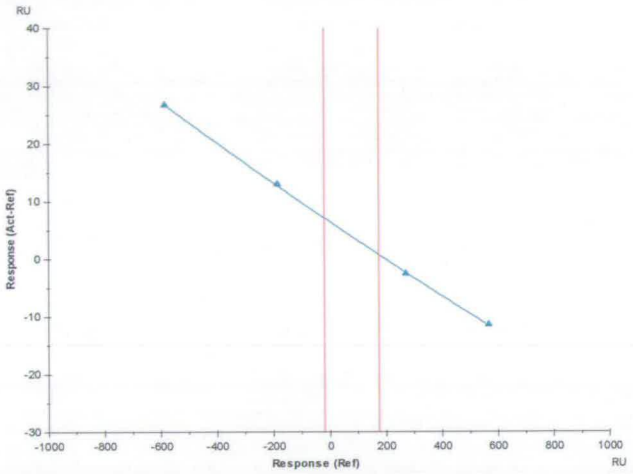
The 100 μM sample was used as the highest concentration sample of the SPR biosensor well plate. Serial dilutions of the 100 μM sample, with the running buffer of PBS (pH 7.4)/DMSO (99:1), across the subsequent nine wells, gave samples with concentrations of: 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.125 μM, 1.5625 μM, 0.78125 μM, 0.39063 μM and 0.19531 μM. Each of the samples are in a solution equal to the running buffer (PBS (pH 7.4)/DMSO (99:1)), with the final two wells as blanks, filled only with the running buffer.

For one 96 well plate used for screening on the SPR biosensor, each sample (e.g. R-Warfarin) had one row of wells (ten sample solutions at different concentration and two blanks). Six rows of wells on the plate were used per run; corresponding to six different samples. The BIAcore S51 screened each row sequentially. Firstly the two blank runs were screened, followed by the 0.19531 μM sample and then in order of increasing sample concentration, after which the next row of samples were screened.

3.2.3.2 *Warfarin*

The screening, data collection and data reprocessing was carried out in the same manner for all analytes. SPR biosensors measure the 'crude' sensorgram's response (RU) versus time after injection. Following this, the sensorgram data was then corrected by the BIAcore S51, for possible small errors between the composition of the running buffer (PBS (pH 7.4)/DMSO (99:1)) and the solution of the samples (also PBS (pH 7.4)/DMSO (99:1)). This is important as small variation in the bulk

solution flowing over the sensor spot, will result in a large difference in refractive index and therefore response measures. This is achieved by running further blank samples with slightly altered composition to cover the possible error in solution make-up. The response measured from the reference spot, with no immobilised protein, is compared to the simultaneous response measured from a protein immobilised spot (Figure 38).



* Red lines represents the range of the data measured on the reference spot (no protein immobilised)

Figure 38 Typical solvent correction on BIAcore S51

The software plots the average response of each sample (RU) versus sample concentration (μM) (e.g. Figure 39). At this point, all erroneous data points are removed. These are a fairly common occurrence in the BIAcore screening, which arise due to several factors which may affect the screening data. One likely cause is small solids in the running buffer (even after two filtration steps; in preparation and in the BIAcore device). These can cause partial blockages in the small channels that lead the flow over a sensor spot. It is therefore common that numerous consecutive sample runs or even a whole batch of run have erroneous results. A partial blockage can sometimes be unblocked spontaneously by the flow of running buffer, thus returning the system to normal. Erroneous results are generally far from the expected binding curve formed.

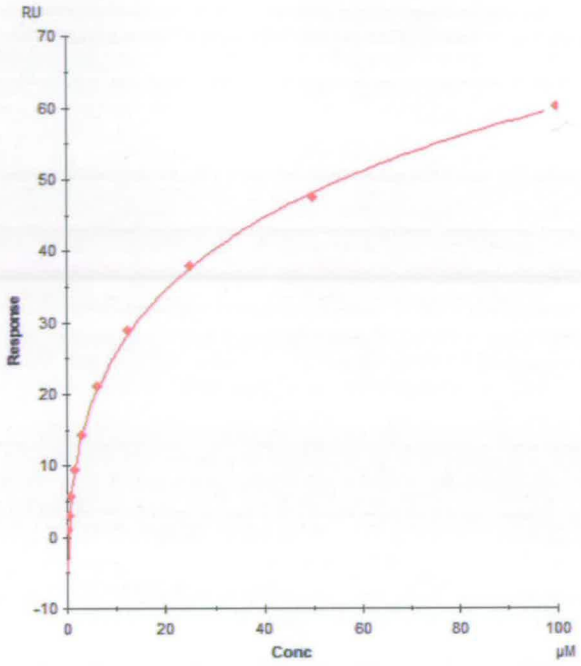
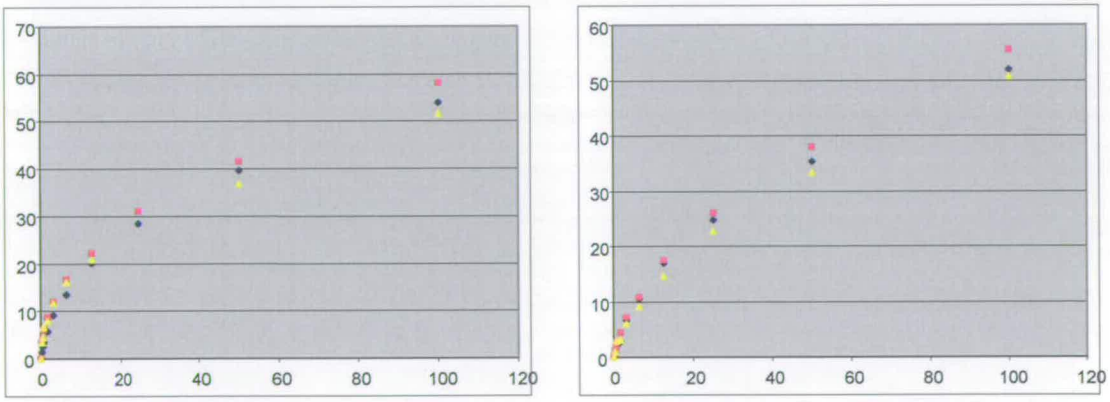


Figure 39 BIAcore screening of R-Warfarin on HSA

To compare the screenings of enantiomers, which were run separately to each other, the binding response values are extracted and comparative runs of enantiomers zeroed (so that all response data curves start at 0 RU) and then plotted on the same graph for comparison.



Response vs concentration/ µM Response vs concentration/ µM

Blue = R-Warfarin, Pink = RS-Warfarin, Yellow = S-Warfarin

Graph 12 Normalised results of BIAcore screening of Warfarin (screening order R, RS, S) on HSA (left) and BSA (right).

A comparison between the SPR biosensor binding strength results of the R and S enantiomers is shown in Graph 12. The results are compared for each given concentration, to determine which enantiomer has the strongest binding to the

immobilised protein. Data is also shown with other factors which may contribute towards systematic and human error, enantiomer run order (R or S screened first), sample preparation number and the age of the chip (after immobilisation of the protein).

Sample conc./μM	Strongest binding enantiomer (SPR)	Run Order (R or S first)	Sample Prep.	Chip Age (days)	Sample conc./μM	Strongest binding enantiomer (SPR)	Run Order (R or S first)	Sample Prep.	Chip Age (days)
0.19531	S	R	1	4	6.25	S	S	2	1
0.39063	S	R	1	4	12.5	S	S	2	1
0.78125	S	R	1	4	25	S	S	2	1
1.5625	S	R	1	4	50	S	S	2	1
3.25	S	R	1	4	100	S	S	2	1
6.25	S	R	1	4	0.19531	S	R	2	1
12.5	S	R	1	4	0.39063	S	R	2	1
25	S	R	1	4	0.78125	S	R	2	1
50	R	R	1	4	1.5625	S	R	2	1
100	R	R	1	4	3.25	S	R	2	1
0.19531	S	S	2	1	6.25	S	R	2	1
0.39063	S	S	2	1	12.5	S	R	2	1
0.78125	S	S	2	1	25	R	R	2	1
1.5625	S	S	2	1	50	R	R	2	1
3.25	S	S	2	1	100	R	R	2	1

Table 10 Results from BIAcore SPR screening of Warfarin on HSA

For the Warfarin analyte there were a total of six successful runs, three on HSA and three on BSA. This gave a total of thirty pairs of R and S data points for Warfarin on HSA and twenty eight pairs for Warfarin on BSA.

Sample conc./μM	Strongest binding enantiomer (SPR)	Run Order (R or S first)	Sample Prep.	Chip Age (days)	Sample conc./μM	Strongest binding enantiomer (SPR)	Run Order (R or S first)	Sample Prep.	Chip Age (days)
0.19531	S	R	1	4	12.5	R	S	2	1
0.78125	S	R	1	4	25	R	S	2	1
1.5625	S	R	1	4	50	R	S	2	1
3.25	S	R	1	4	100	R	S	2	1
6.25	R	R	1	4	0.19531	R	R	2	1
12.5	R	R	1	4	0.39063	R	R	2	1
25	R	R	1	4	0.78125	R	R	2	1
50	R	R	1	4	1.5625	R	R	2	1
100	R	R	1	4	3.25	R	R	2	1
0.19531	R	S	2	1	6.25	R	R	2	1
0.39063	S	S	2	1	12.5	S	R	2	1
0.78125	S	S	2	1	25	R	R	2	1
1.5625	R	S	2	1	50	R	R	2	1
6.25	R	S	2	1	100	R	R	2	1

Table 11 Results from BIAcore SPR screening of Warfarin on BSA

SPR biosensor screenings of Warfarin on AGP gave no usable data. The overall binding of Warfarin and AGP was less that 10% in magnitude compared with

values of Warfarin on HSA and BSA. This caused an increase of the noise to signal ratio and resulted in a larger number of erroneous results. The binding of Warfarin and AGP proved to be too weak, for the particular concentrations of immobilised AGP and Warfarin used, for reliable data.

All the data obtained from the screening of Warfarin on SPR biosensor was then collated with the data obtained from other SPR biosensor analyte screenings.

3.2.3.3 *Ibuprofen*

Screening and data analysis of Ibuprofen on the SPR biosensor was carried out using procedures analogous to those described for Warfarin. Larger numbers of Ibuprofen sample preparations and the overall number of times the analyte was screened was higher in comparison to the Warfarin screens, to increase the overall quantity of the data set for SPR screenings.

For the Ibuprofen analyte there were a total of fourteen successful runs, seven on HSA and seven on BSA. This gave a total of sixty six pairs of R and S data points for Ibuprofen on HSA and sixty-five pairs for Ibuprofen on BSA.

Sample conc/μM	Strongest binding enantiomer (SPR)	Run Order (R or S first)	Sample Prep.	Chip Age (days)	Sample conc/μM	Strongest binding enantiomer (SPR)	Run Order (R or S first)	Sample Prep.	Chip Age (days)
0.19531	S	R	1	1	6.25	R	S	4	0
0.39063	R	R	1	1	12.5	R	S	4	0
0.78125	R	R	1	1	25	R	S	4	0
1.5625	S	R	1	1	50	R	S	4	0
6.25	S	R	1	1	100	S	S	4	0
12.5	S	R	1	1	0.19531	R	R	4	0
25	S	R	1	1	0.39063	R	R	4	0
50	S	R	1	1	0.78125	R	R	4	0
100	S	R	1	1	1.5625	R	R	4	0
0.19531	R	R	2	3	3.25	R	R	4	0
0.39063	R	R	2	3	6.25	R	R	4	0
0.78125	R	R	2	3	12.5	R	R	4	0
1.5625	R	R	2	3	25	R	R	4	0
3.25	R	R	2	3	50	R	R	4	0
12.5	R	R	2	3	100	R	R	4	0
25	R	R	2	3	0.39063	R	R	5	2
50	R	R	2	3	0.78125	R	R	5	2
100	R	R	2	3	1.5625	R	R	5	2
0.19531	R	R	3	3	3.25	R	R	5	2
0.39063	R	R	3	3	6.25	R	R	5	2
0.78125	R	R	3	3	12.5	R	R	5	2
1.5625	R	R	3	3	25	R	R	5	2
3.25	R	R	3	3	50	R	R	5	2

6.25	R	R	3	3	100	R	R	5	2
12.5	R	R	3	3	0.39063	S	S	5	2
25	R	R	3	3	0.78125	R	S	5	2
50	R	R	3	3	1.5625	R	S	5	2
100	R	R	3	3	3.25	S	S	5	2
0.19531	R	S	4	0	6.25	S	S	5	2
0.39063	R	S	4	0	12.5	S	S	5	2
0.78125	R	S	4	0	25	S	S	5	2
1.5625	R	S	4	0	50	R	S	5	2
3.25	R	S	4	0	100	S	S	5	2

Table 12 Results from BIAcore SPR screening of Ibuprofen on HSA

Sample conc/μM	Strongest binding enantiomer (SPR)	Run Order (R or S first)	Sample Prep.	Chip Age (days)	Sample conc/μM	Strongest binding enantiomer (SPR)	Run Order (R or S first)	Sample Prep.	Chip Age (days)
0.19531	S	R	1	1	50	R	S	4	0
0.39063	R	R	1	1	100	R	S	4	0
0.78125	R	R	1	1	0.19531	R	R	4	0
1.5625	R	R	1	1	0.39063	R	R	4	0
6.25	R	R	1	1	0.78125	R	R	4	0
12.5	R	R	1	1	1.5625	R	R	4	0
25	R	R	1	1	3.25	R	R	4	0
50	R	R	1	1	6.25	R	R	4	0
100	R	R	1	1	12.5	R	R	4	0
0.19531	R	R	2	3	25	R	R	4	0
0.39063	S	R	2	3	50	R	R	4	0
0.78125	R	R	2	3	100	R	R	4	0
1.5625	R	R	2	3	0.19531	R	R	5	2
3.25	R	R	2	3	0.39063	R	R	5	2
100	S	R	2	3	0.78125	R	R	5	2
0.19531	R	R	3	3	1.5625	R	R	5	2
0.39063	R	R	3	3	3.25	R	R	5	2
0.78125	R	R	3	3	6.25	R	R	5	2
1.5625	R	R	3	3	12.5	R	R	5	2
3.25	R	R	3	3	25	R	R	5	2
6.25	R	R	3	3	50	R	R	5	2
12.5	R	R	3	3	100	R	R	5	2
25	R	R	3	3	0.19531	S	S	5	2
50	R	R	3	3	0.39063	S	S	5	2
100	R	R	3	3	0.78125	R	S	5	2
0.19531	R	S	4	0	1.5625	R	S	5	2
0.39063	R	S	4	0	3.25	R	S	5	2
0.78125	R	S	4	0	6.25	S	S	5	2
1.5625	R	S	4	0	12.5	R	S	5	2
3.25	R	S	4	0	25	R	S	5	2
6.25	R	S	4	0	50	R	S	5	2
12.5	R	S	4	0	100	R	S	5	2
25	R	S	4	0					

Table 13 Results from BIAcore SPR screening of Ibuprofen on BSA

However, as with Warfarin, the interaction of Ibuprofen with immobilised AGP failed to give any meaningful results. Again, the overall magnitude of binding response was too low between this pair both of which are acidic.

3.2.3.4 *Benzoin*

SPR biosensor screenings of Benzoin gave a lower magnitude of response in comparison to Warfarin and Ibuprofen. This indicates that both enantiomers of Benzoin have lower affinity to bind to the immobilised proteins than Warfarin and Ibuprofen analytes (this was also indicated by the short rentention times in the HPLC screenings of Benzoin). Therefore the noise to signal ratio was larger, reducing the reliability of accurate screened patterns between enantiomer binding differences.

The binding responses measured were approximately zero for all samples of concentration of 12.5 μM and below, leaving only three useful pairs of results per sample. For the Benzoin analyte there were a total of six completed runs, three on HSA and three on BSA. This gave only nine pairs of R and S data points for Benzoin on HSA and nine pairs for Benzoin on BSA. The results show that R-Benzoin binds more strong to BSA immobilised protein and conversely S-Benzoin more strongly to HSA, however there are limited data available to support definitive conclusions for this (these data will be collated with Warfarin and Ibuprofen SPR screening results for further analysis later in this chapter). All screening attempts involving Benzoin on AGP failed to give any useful data.

3.2.3.5 *Tryptophan*

These result displayed a similar binding response magnitude as seen in the SPR screening of Benzoin with very low binding levels making analysis with all but the highest concentration samples useless.

Tryptophan was run twice on HSA and twice on BSA; six sets of data obtained for each protein. Results indicated that S (or L)-Tryptophan enantiomer had stronger binding to both immobilised proteins, compared to R (or D)-Tryptophan. However, as with the Benzoin SPR screening the low number of data points obtained made it hard to give firm conclusions based exclusively on this set of data.

As with the data from the Benzoin screen, AGP screening with Tryptophan failed to give any usable results.

3.2.4 Summary of SPR biosensor screening results

Warfarin, Ibuprofen, Benzoin and Tryptophan enantiomer analytes were screened over a large sample concentration range on a BIAcore S51 SPR biosensor. The analytes were screened for their binding affinity to immobilised proteins (HSA, BSA and AGP). The comparative binding affinities of each analyte enantiomer were used to ascertain which enantiomer had the strongest binding affinity with each immobilised protein, at each sample concentration.

Warfarin and Ibuprofen were found to have a relatively strong binding affinity to HSA and BSA, which meant large quantities of data were obtained, not only on the relative binding strengths of the enantiomer, but also towards the robustness of the screening system [variables include sample preparation (up to five different preparations for Ibuprofen), the order of screening enantiomers (R or S first), and the age of the immobilised protein].

The SPR screenings of Warfarin and Ibuprofen were useful for comparison with HPLC screens of the same analytes. Information is garnered on the robustness and reliability of the system towards potentially determination of appropriate HPLC chiral stationary phase media.

The data obtained for Benzoin and Tryptophan analytes was much less extensive, but still give some insight into the HPLC-SPR biosensor relationship and how it changes when analytes with lower binding affinities are used.

All the SPR screenings with immobilised AGP gave very poor results, mainly due to very low overall binding affinity observed with all analytes. This was party due to a smaller quantity of protein successfully immobilised onto the sensor chip. The reasons for poor immobilisation of AGP onto the sensor are unknown and although the immobilisation process was repeated until what was thought to be a high enough immobilisation level was obtained, the binding affinity levels were still too low to obtain useful data.

3.3 Comparing SPR biosensor screening results with protein CSP screening data

3.3.1 Screening data correlation

Chiral HPLC screening of four analytes were carried out to obtain the relative order of elution for each pair of analyte enantiomers. The order of elution of an analyte was effectively a measure of the binding strength between individual enantiomer and the protein immobilised onto the packing materials in the HPLC column. The stronger binding enantiomer was retained longer in the column and thus will be eluted more slowly, in comparison to the weaker binding and faster eluting enatiomers.

SPR biosensor screenings measure continuous real-time binding interactions between analyte enantiomer samples and the protein immobilised onto the sensor chip. From these data the magnitude of each binding affinity was obtained and was compared with values for the corresponding opposite enantiomers. This gave SPR biosensor results showing which enantiomer bound more strongly.

The following section compares the results of HPLC and SPR to show that the screens give comparable data. Chiral HPLC is a very robust screening technique, with very little variation in sample retention times. Therefore the elution order of enantiomers on HPLC is always the same (under identical chromatographic conditions). SPR biosensor binding affinity measurements are less reliable, with a certain degree of variation between identical sample runs. HPLC results come from the sum of many thousands of interactions as the sample flows through the column, therefore creating a natural average. In comparison, SPR measures instantaneous real-time interactions between sample and immobilised protein, with these interactions averaged over the sample injection time (90 s).

However, the SPR biosensor does benefit from being faster, more automated and has the ability to supply more information simultaneously than HPLC. The use of multiple spots gives the possibility of samples being run on numerous immobilised materials. The direct comparison between HPLC and SPR biosensor screenings, showing all twelve combinations of immobilised protein and analyte are shown (Table 14). This summary of SPR biosensor data shows the most predominant

results from all of the pairs of data for a particular combination of analyte and immobilised protein.

Protein CSP	Analyte	HPLC Screening elution order (e.g. X-Y = X before Y)	SPR Screening Predominant binding strength (e.g. X-Y = Y stronger binding affinity)
BSA	Benzoin	S-R	S-R[a]
	Warfarin	S-R	S-R[b]
	Ibuprofen	S-R	S-R[c]
	Tryptophan	R-S	R-S[d]
HSA	Benzoin	S-R	S-R[e]
	Warfarin	R-S	R-S[f]
	Ibuprofen	S-R	S-R[g]
	Tryptophan	S-R	R-S[h]
AGP	Benzoin	S-R	No data
	Warfarin	S-R	No data
	Ibuprofen		No data
	Tryptophan		No data

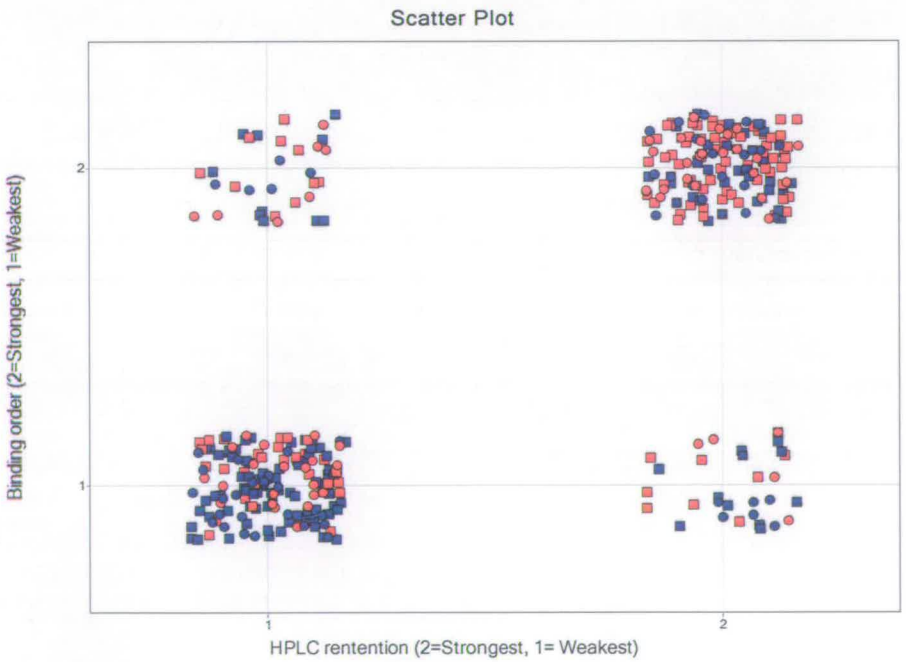
[a] Predominant relative binding strength from 9 pairs of data (8 = R strongest binder and 1 = S strongest binder), [b] 28 pairs of data (21 = S and 7 = R strongest), [c] 65 pairs of data (6 = R and 59 = S strongest), [d] 6 pairs of data (6 = S strongest), [e] 9 pairs of data (6 = R and 3 = S strongest), [f] 30 pairs of data (25 = R and 5 = S strongest), [g] 66 pairs of data (52 = R and 14 = S strongest), [h] 6 pairs of data (4 = S and 2 = R strongest).

Table 14 Comparison of screening results from HPLC and SPR biosensor.

Apart from AGP, which had no SPR biosensor data, all of the HPLC data correlated with the equivalent results from the SPR biosensor on first inspection. The exception to the trend was for Tryptophan on HSA, but this had a limited data set.

3.3.2 Statistical analysis

The correlated data from both the HPLC and SPR biosensor screens of Warfarin and Ibuprofen on HSA and BSA immobilised proteins are shown (Figure 40). Each mark represents a single SPR biosensor enantiomer screening, which is placed at the top or bottom depending on its relative binding strength (SPR). This point is correlated with the expected HPLC relative retention of enantiomer on the left or right scale. In short, areas at the top-right and bottom left quadrants are collections of points where the chiral HPLC and SPR biosensor data correlated well. Conversely, marks in the top-left and bottom-right quadrants are miss-matches between HPLC and SPR biosensor correlated data.



* Warfarin marks represented with circles, Ibuprofen represented with squares

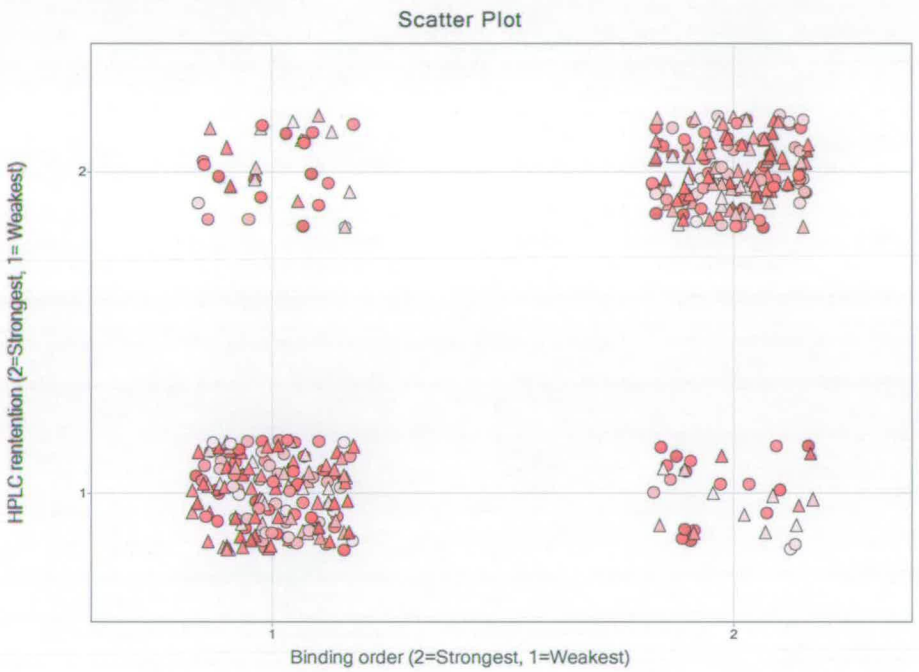
** Red marks are enantiomer ran first on SPR screenings, blue are enantiomer that followed.

Figure 40 Correlation of all data point between HPLC and SPR biosensor screenings of Warfarin and Ibuprofen analytes on HSA and BSA immobilised proteins.

The graph contains 378 marks (from 189 pairs of data). The total number of miss-matches is 64 marks (or 32 pairs of data). The cumulative binomial probability (CBP) of up to 32 failures out of 189 trials is 2.63×10^{-21}, using:

$$CBP = \sum_{x=0}^{x=z} \frac{n!}{x!(n-x)!} p^x (1-p)^{n-x}$$

Where z = 32 (the number of failed trials), n = 189 (the number of trials) and p = 0.5 (probability of failure for a two outcome system).



* Graduated colour of points, higher white saturation indicates lower sample concentration.

** Triangle marks represent screenings on immobilised BSA, circles on HSA.

Figure 41 Correlation of all data point between HPLC and SPR biosensor screenings of Warfarin and Ibuprofen analytes on HSA and BSA immobilised proteins.

SPR screening order of the enantiomers had no great affect on the results. Figure 40 shows red marks for the first screened enantiomer of the pair and blue for the latter screened enantiomer in the pair. Of the miss-matched pairs, 19 occur when what should be the weaker binding enantiomer (from HPLC) was screened first and 13 occur when the weaker binding enantiomer (from HPLC) was screened second. This slight bias can be explained by the presence of a slightly lower quantity of immobilised protein being present in the later run. The amount of protein immobilised will never be greater when the second enantiomer is screened over it,

but it may be slightly less, which in turn could contribute towards the reduced binding affinity recorded for the second enantiomer.

Concentration of sample used in the SPR biosensor screening is marked with a graduated colour scheme; Red (high concentration, 100 μM) to Clear/White (lowest concentration, 0.19531 μM). The miss-match points are a mixture of different concentrations; with slightly more being lower concentration samples. Therefore the sample concentration range used in SPR biosensor screening is suitable for these strong binding analytes (Warfarin and Ibuprofen).

3.4 Summary

Chiral HPLC and SPR biosensor screens were carried out on the enantiomers of four analytes; Warfarin, Ibuprofen, Benzoin and Tryptophan. These analytes were screened on three proteins; HSA, BSA and AGP, which were all immobilised onto either column packing materials or onto the surface of a sensor spot, for HPLC and SPR biosensor analysis respectively.

HPLC screens were carried out on commercially available protein chiral columns. Sample retention data were gathered for single enantiomers of each of the analytes. The analyte enantiomers were then compared for their relative retention times, which was effectively information on the relative binding strength of the enantiomers with the immobilised protein of the packing materials.

SPR biosensor screening focussed on the relative binding strength of the two enantiomers constituting an analyte. This was done by obtaining the binding affinity value, averaged over the sample injection, for a range of sample concentrations.

Results for the two screening methods were then compared. This was done by investigating the relative binding affinity of the enantiomers. If the SPR biosensor could correctly predict the relative binding strength of enantiomers in an analyte to match the corresponding data from chiral HPLC, then it could lead to the possibility of developing high-throughput SPR screenings which would then be scaled up for chiral HPLC.

Compared results were found to match very well, with the majority of SPR screening results agreeing with HPLC precedent. The results obtained for Warfarin

and Ibuprofen, which were strong binders to HSA and BSA proteins were very good. 157 out of 189 pairs of screening data for these analytes gave a matching order of elution between chiral HPLC and the SPR biosensor. Weaker binding analytes; Benzoin and Tryptophan gave less reliable results. Their lower over binding increased the noise to signal ratio therefore the comparative enantiomer binding affinities were more likely to switch over compared to the expected result. For these analytes results for the higher concentration samples which gave larger binding affinities were therefore used, reducing the error and giving more reliable results.

Drawbacks of the SPR biosensor system are the longer overall time required for accurate sample preparation, immobilisation of the protein, screening of multiple identical samples of different concentrations and the extensive data collection, correction and correlation required. As well as this the BIAcore S51 system suffers from numerous partial blockages in the flow lines due to their small size. This resulted in the loss of information for runs and sometimes whole plates of runs.

As this research represents only initial findings in this area, it is differcult to draw any definitive conclusions. However, with further development and the future improvements of SPR technology could make SPR biosensors a valuable tool for high-throughput determination of chiral stationary phase media and chiral chromatographic conditions for scaled up chiral HPLC.

Although the BIAcore S51 SPR biosensor has the ability to simultaneously analyse samples over two immobilised materials, other SPR biosensors have the ability to exceed this number and further future improvement will only increase the number of simultaneous analyses possible. Sensor chips with a large range of pre-immobilised protein can all be analysed simultaneously with the same sample to give information as to which protein can best differentiate between enantiomer binding strength. A larger array of immobilised materials would also improve the accuracy of analysis, by being able to immobilise the materials as duplicates or triplicates.

Further improvement of the SPR biosensing technology will also lead to enhancement in measured sensitivity. This gives the possibility of measuring interactions between small molecule enantiomeric samples and immobilised small

molecules. Therefore the SPR biosensor will be able to screen more potential chiral stationary phase media.

4 Reciprocal based CSPs

4.1 Introduction

This work was based on the premise that, if an immobilised enantiomer (+)-A as a CSP could separate a given racemate (+)-B and (-)-B, the counter argument should hold that an immobilised enantiomer of (+)-B as a CSP will be able to separate racemate (+)-A and (-)-A[210-212].

Following on from the work carried out in chapter 2, peptidic based materials were used to separate a range of racemate analytes, analogues based on the structure of anthryl ethanol **(34)**. This chapter investigated the chiral stationary phases based upon the use of one enantiomer of anthryl ethanol tethered to a silica support.

Following a successful synthesis, the CSP was screened against a small array of peptidic racemates and epimers, so as to reciprocate the results obtained in Chapter 2.

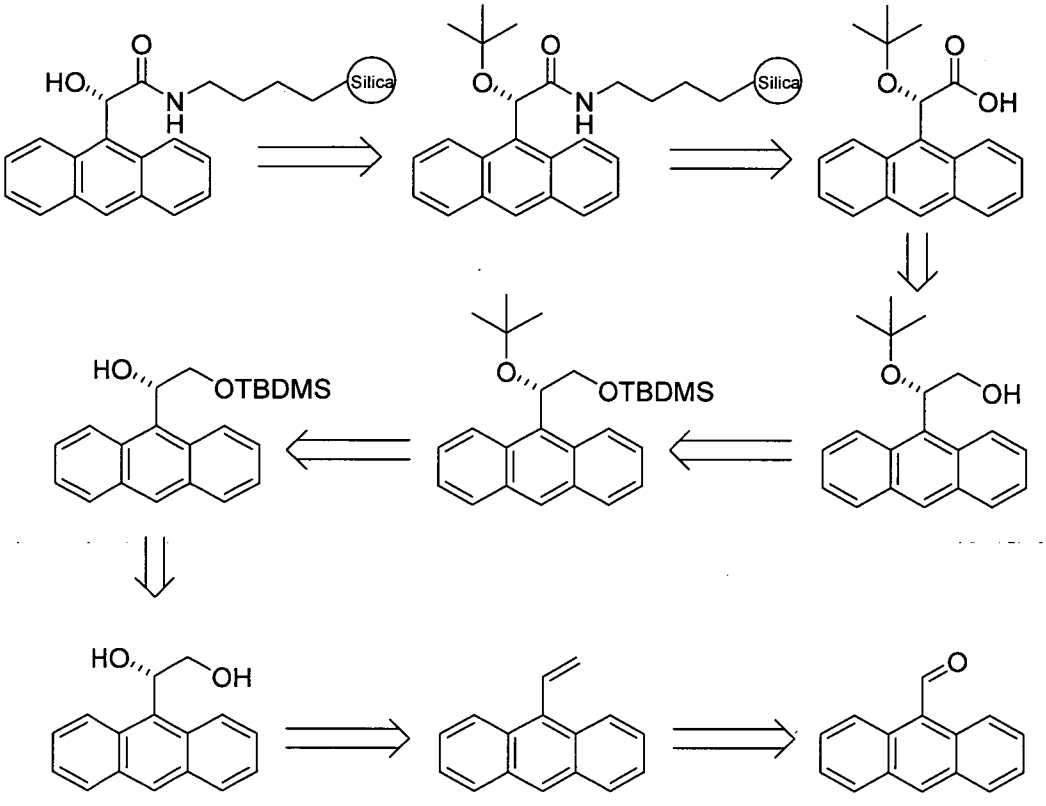
4.2 Synthesis of reciprocal CSPs

4.2.1 Retro-synthesis plan

Covalent attachment of an enantiomer of anthryl ethanol to the silica required modification of the anthryl ethanol structure. Aminopropyl silica was the support of choice, so the most appropriate solution was to incorporate a carboxylic acid moiety onto the anthryl ethanol. At the same time careful consideration had to be paid towards preserving as much of the anthryl ethanol structure as possible, so that the interactions between CSP and analyte were not drastically affected.

As the CSP had to be synthesised *en masse*, for packing into a HPLC column, the amount of optically pure small molecule required was in the region of 1.0 mmol. The most obvious point of attachment was the opposite side of the anthracene ring to the ethanol moiety, via a 9,10-disubstituted anthracene. Initial plans using this approach, started with 9,10-dibromoanthracene, however asymmetric monoderivatisation of 9,10-dibromoanthracene was only possible in very low yield.

An alternative point of attachement was via derivatisation of the ethanol moiety. This seemed to be a more appropriate choice as it could be achieved starting from, 9-Anthraldehyde. A retrosynthesis of this route is shown in Scheme 23.



Scheme 23 Planned retrosynthesis of Reciprocal CSP.

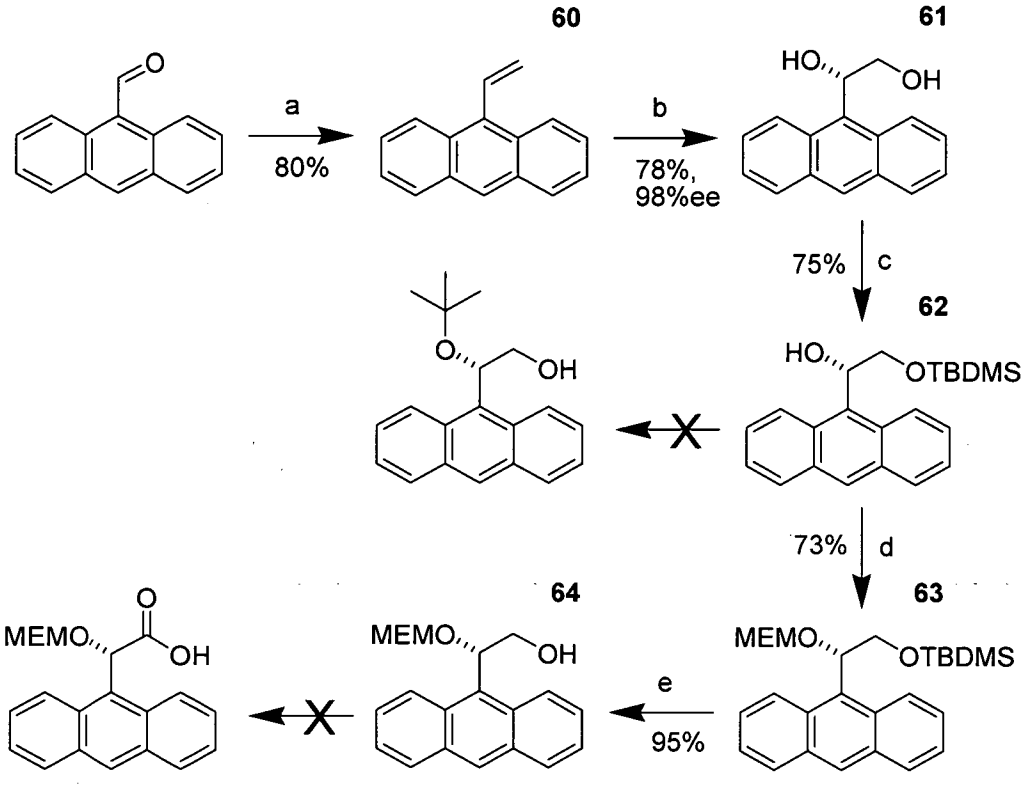
4.2.2 Forward synthesis of reciprocal CSP

9-Anthraldehyde underwent a Wittig reaction[250, 251] with methyltriphenylphosphonium bromide, using *n*-BuLi used as the base to form the reactive phosphorus ylide, to give 9-vinylanthracene **(60)**.

Next the molecule's optical character had to be incorporated. This was achieved using a Sharpless asymmetric dihydroxylation method[252 253], using AD-mix α reagent to form the desired single enantiomer diol, (S)-1-(9-anthracenyl) ethane-1,2-diol **(61)**, in good yield and excellent optical purity.

The next part of the synthesis involved both protection of the secondary alcohol and leaving the primary alcohol as a free hydroxyl group. This was achieved by selective protection of the primary hydroxyl group with TBDMS-Cl, using a bulky silyl protecting group (TBDMS), which was achieved in good yield and did not

lead to protection of the more sterically hindered secondary hydroxyl[254, 255], which gave (S)-1-(9-anthracenyl)-2-(tert-butyldimethylsilyloxy) ethanol **(62)**.



(a) Ph_3PCH_3Br, ^nBuLi, THF (b) AD-mix α, Water/^{t}BuOH (1:1),
(c) TBDMS-Cl, Imidazole, DMF, (d) MEM-Cl, DIPEA, DCM, (e) Bu_4NF, MeCN.

Scheme 24 Start of the synthesis of reciprocal CSP

The first choice of protecting group for the secondary alcohol was as a ^{t}butyl ether. This was due to the extensive use of ^{t}butyl protection in solid phase chemistry, and the belief that ^{t}butyl groups could be removed once attached to the solid phase. However, two common methods for introducing the ^{t}butyl ether functionality were unsuccessful. The use of liquid Isobutylene at -78 °C with sulfuric acid catalyst[256] or Amberlyst H-15 resin[257] and with ^{t}butyl alcohol and sulfuric acid[258] all gave no desired product.

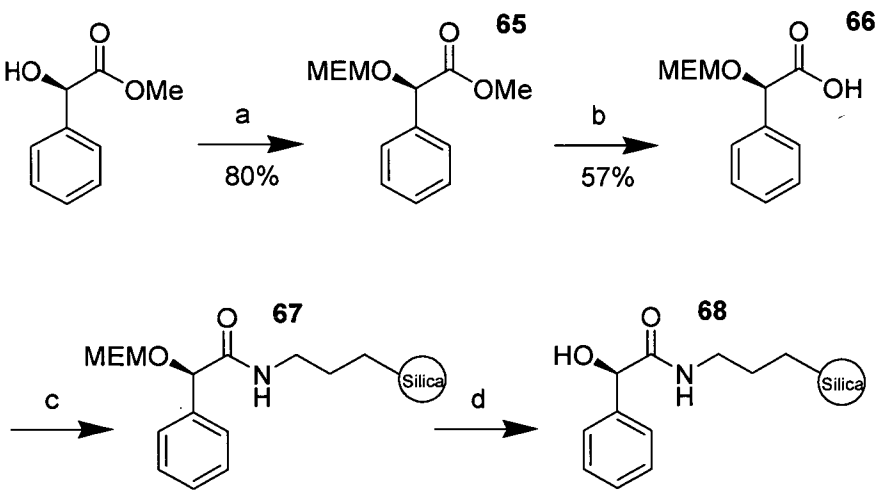
Other hydroxyl protecting groups were then considered instead. The desired protecting group had to fulfil a number of considerations for successful use, including; orthogonality to the TBDMS protecting group, deprotection not requiring strong base (to maintain silica integrity), protecting group incorporation should not use strong acid (due to potential deprotection of TBDMS) and deprotection should be possible under solid-phase synthesis conditions. As

alkoxyalkyl ethers fulfil all these requirements, the MEM protecting group was used. Protection of (S)-1-(9-anthracenyl)-2-(tert-butyldimethylsilyloxy) ethanol **(62)** was achieved by addition of MEM-Cl and DIPEA in DCM[259, 260] and purification gave the desired orthogonally protected diol **(63)**. This was followed by deprotection of the TBDMS group, using TBAF[259, 260], to give the primary alcohol **(64)** in good yield.

The next step required oxidation of the primary alcohol to give the carboxylic acid. A litrature procedure[254] that was successful for a similar compound was explored, which involved the use of combined oxidants $NaOCl_2$, NaOCl and TEMPO under pH controlled phosphate buffer conditions, however, this gave no desired product. Many more oxidative procedures were tried, including Jones reagent[261, 262] using different conditions; from which the only product was thought to be the unwanted ring oxidised anthraquinone[263]. Oxidation of the primary alcohol **(64)** to the required carboxylic acid was not possible under the conditions tried. Therefore, the structure of the planned reciprocal CSP was altered.

4.2.2.1 *MEM protecting group removal on solid phase methodology*

The final step of the synthesis involved the removal of the MEM protecting group to give the final CSP with a free hydroxyl group. However, because the reaction was using a synthetic method which is not well established on solid-phase, this reaction was first trialed by using a structurally similar, surrogate CSP.



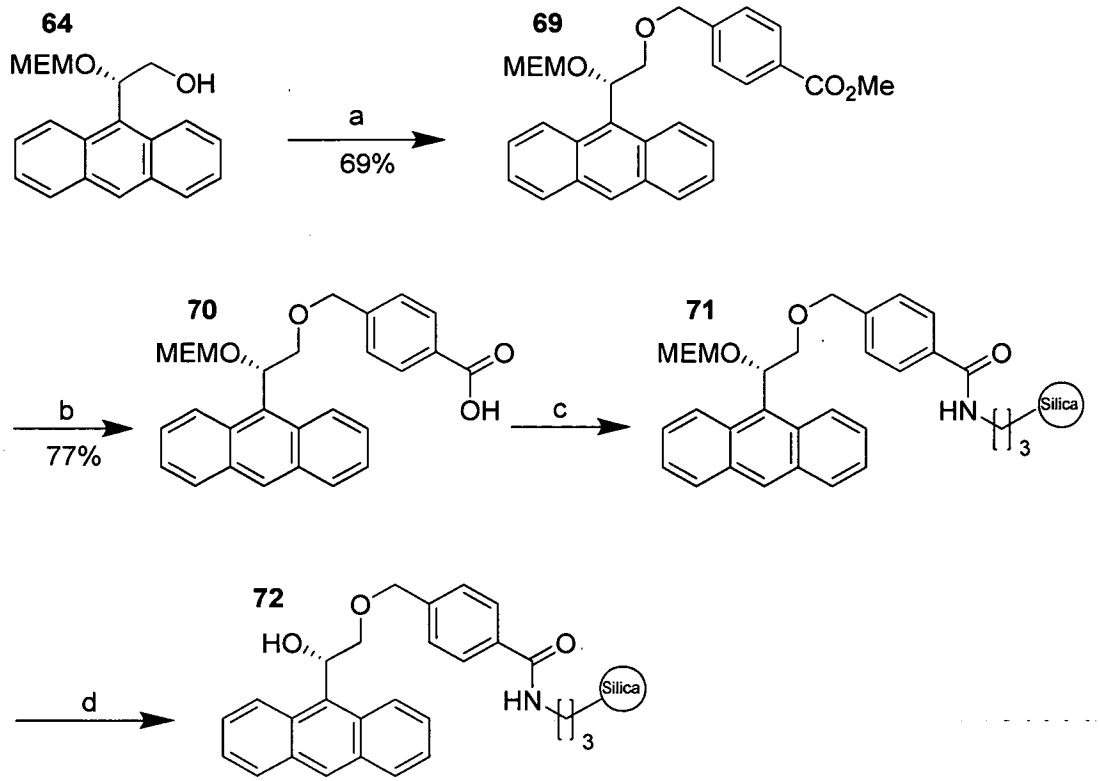
(a) MEM-Cl, DIPEA, DCM, (b) LiOH, Water, MeOH,
(c) Aminopropyl silica (12 μm), DIC, HOBt, DCM/DMF (4:1), (d) $ZnBr_2$, DCM.

*Scheme 25 Synthesis of CSP **(68)**.*

Firstly, (R)-(+)-methyl mandalate was protected with the MEM group using a procedure identical to that used for the synthesis of **(63)**, to give **(65)**. Saponification with LiOH gave the chiral carboxylic acid **(66)**, which was then coupled to aminopropyl silica using DIC and HOBt peptide coupling reagents. This gave the MEM protected silica **(67)**, which was subsequently deprotected by agitation as a slurry in DCM with $ZnBr_2$ for four days[264-266], to give the final CSP **(68)**. **(67)** and **(68)** were compared by elemental analysis to confirm MEM deprotection.

4.2.2.2 *Continuing synthesis of reciprocal CSP*

Synthesis of the reciprocal CSP was continued from primary alcohol **(64)**, by derivatising it with 4-(bromomethyl)methylbenzoate, to give the methyl ester **(69)**, which was subsequently saponified to give the carboxylic acid derivative **(70)**. Subsequently **(70)** was coupled to APS and MEM deprotection was achieved by utilising the same methodologies performed successfully in the synthesis of CSP **(68)**. Compound **(70)** was then coupled to APS using DIC and HOBt to give **(71)**, and finally the MEM preotecting group was then removed using $ZnBr_2$ yielding the free alcohol CSP **(72)**.



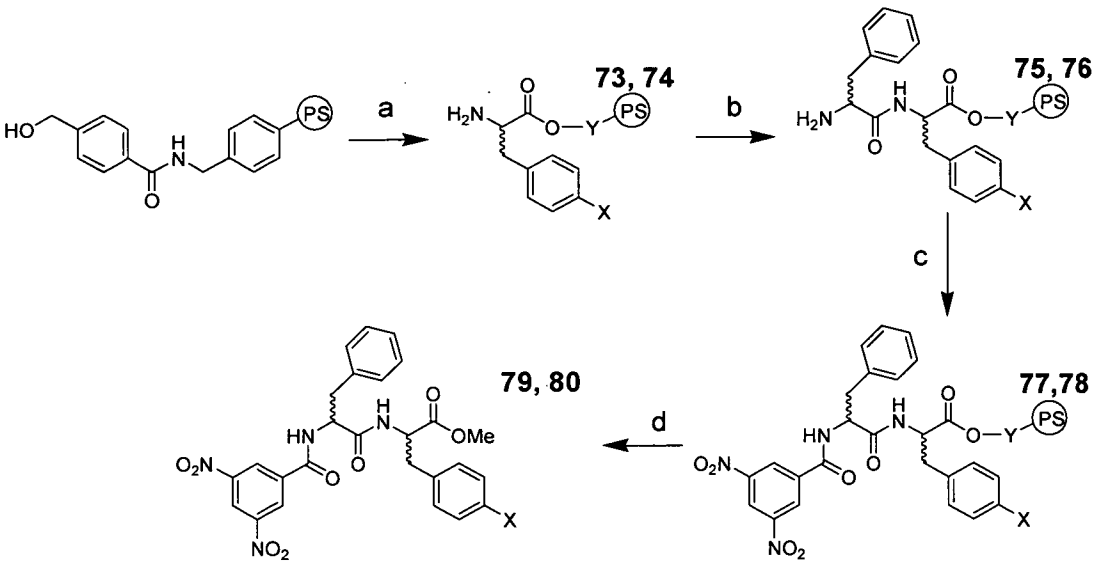
(a) (i) NaH, THF, (ii) 4-(bromomethyl) methylbenzoate, THF (b) NaOH, MeOH, (c) Aminopropyl silica (12 μm), DIC, HOBt, DCM/DMF (4:1), (d) $ZnBr_2$, DCM.

Scheme 26 Synthesis of reciprocal CSP (72).

4.3 Synthesis of small peptide screening library

To screen the reciprocal designed CSP **(72)**, a small array of peptides was synthesised, to be used as chiral HPLC analytes. Two of these analytes were dipeptides and were therefore a mixture of four stereoisomers. The synthesis of these (Scheme 27) was achieved using standard peptide chemistries. Starting with 4-hydroxymethylbenzoyl-aminomethyl polystyrene resin allowed cleavage of the peptide from the resin to leave an ester functionality. The resin was loaded with a racemic mixture of Fmoc-protected amino acids (Fmoc-D/L-Phe-OH or Fmoc-D/L-Tyr(O^{t}Bu)-OH), followed by Fmoc deprotection to give resins **(73)** and **(74)** respectively. A second racemic Fmoc amino acid was then coupled (Fmoc-D/L-Phe-OH), again followed by Fmoc deprotection to give the resins **(75)** and **(76)**. Both of these resins were then derivatised with 3,5-dinitrobenzoic acid, to give capped dipeptidic resins **(77)** and **(78)**. Cleavage from the resin was achieved using a mixture of DIPEA, DMF and MeOH giving the the methyl esters **(79)** and **(80)**.

Each peptide was a mixture of four stereoisomers; two enantiomers and two diastereoisomers.



(73), **(75)**, **(77)** and **(79)**, X = H, Y= 4-hydroxymethylbenzoyl-aminomethyl;
(a)(i) Fmoc-D/L-Phe-OH, DIC, HOBt, DMAP, DMF, (ii) piperidine/DMF (20:80);
(74), **(76)**, **(78)** and **(80)**, X = O^{t}Bu, Y= 4-hydroxymethylbenzoyl-aminomethyl
(a)(i) Fmoc-D/L- Tyr(tBu)-OH, DIC, HOBt, DMAP, DMF, (ii) piperidine/DMF (20:80);
(b)(i) Fmoc-D/L-Phe-OH, DIC, HOBt, DMF, (ii) piperidine/DMF (20:80),
(c) 3,5-dinitrobenzoic acid, DIC, HOBt, DMF, DCM, (d) DIPEA/DMF/MeOH (1:5:5).

Scheme 27 Synthesis of two dipeptidic analytes. Each is a mixture of four stereoisomers.

Four capped amino acids were also synthesised, through the use of rink amide resin, the derivatives containing methyl amide end fuctionalities instead of the methyl esters of **(79)** and **(80)**. The 3,5-dinitrobenzoyl amides of D and L-Phenylalanine and the 3,5-dinitrobenzoyl amides of D and L-Analine were synthesized using a rink linker[267, 268].

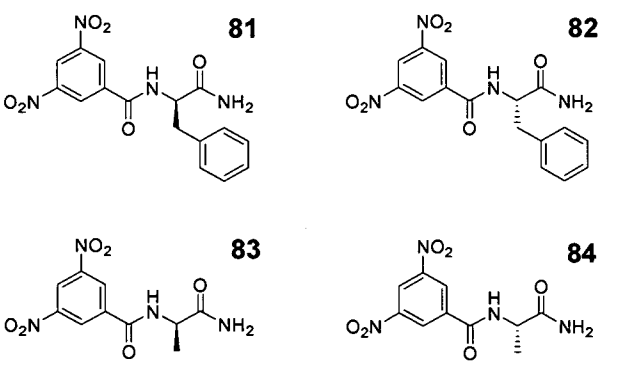


Figure 42 3,5-Dinitrobenzoyl caped amino acid enantiomers.

4.4 Screening of the reciprocal CSPs

CSP **(72)** was screened with the various different analytes, the first of which were the peptides **(79-84)**.

Dipeptides **(79)** and **(80)** both showed separation using moderate concentrations of organic modifier (~20%), concentrations lower than this did not elute the analyte fast enough. However, separation of both analytes was always into two peaks, rather than showing all four stereoisomers present in the analytes. Figure 43 shows the separation of **(80)** on CSP **(72)**, with the top HPLC trace showing two clear peaks when using 20% 2-PrOH in the mobile phase. It is presumed that each peak is two enantiomers and that the separation is only of diastereomer pairs. The bottom HPLC trace (Figure 43) again shows two peaks, but this time the most retained peak is close to showing 'internal' separation. Attempts to resolve the peaks to reveal more stereoisomers proved fruitless with the quality of chromatography on this CSP low, with poor peak shapes and a high degree of peak tailing, even with low analyte injection levels.

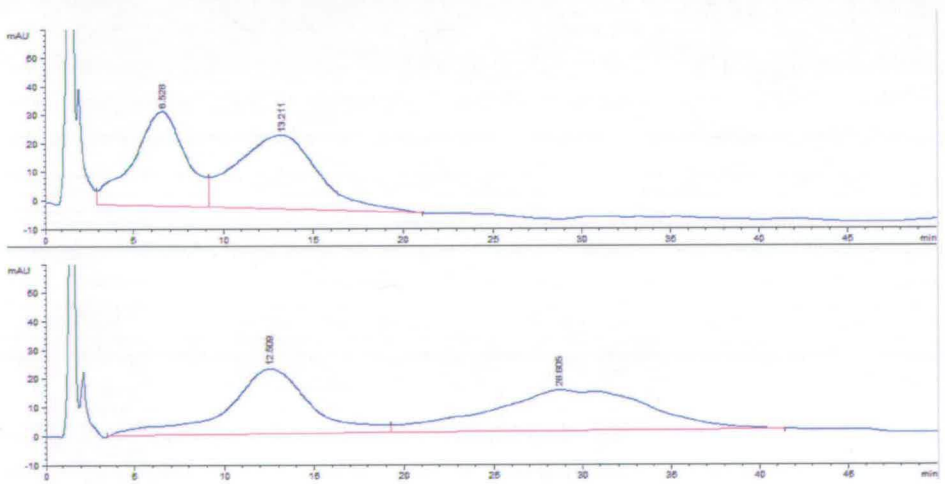


*Figure 43 Separation of **(80)** on CSP **(72)**; (Top) Heptane/2-PrOH (90:20), 0.3 mLmin^{-1}; (Bottom) Heptane/2-PrOH (90:10), 0.3 mLmin^{-1}.*

Capped amino acids **(81)-(84)** were then screened using similar chromatographic conditions. 3,5-Dinitrobenzoyl capped phenylalanine **(81)** and **(82)** were moderately separated by CSP **(72)** (Figure 44). 3,5-Dinitrobenzoyl capped alanine was also separated, but with a very small selectivity factor. The library of racemates **(29)-(31)** and **(35)-(39)** previously synthesised (section 2.2.2), was also screened on CSPs **(72)** but no separation of any racemate was observed.

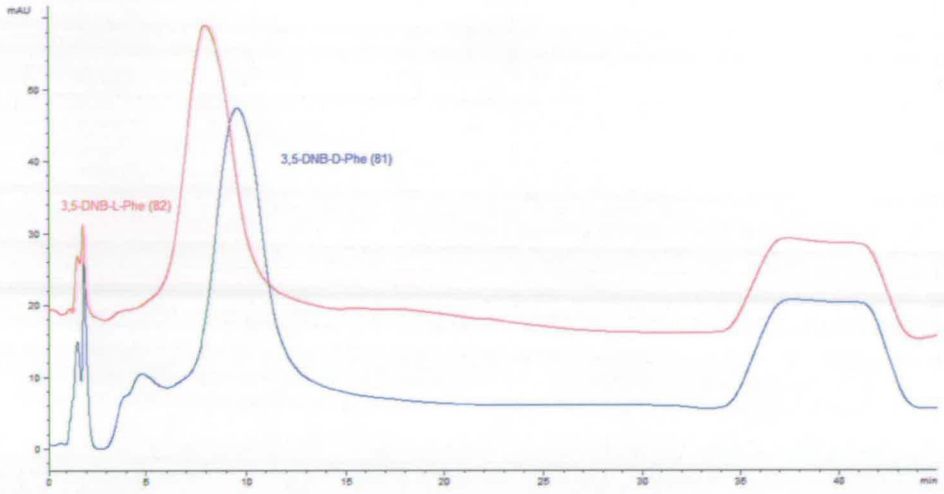
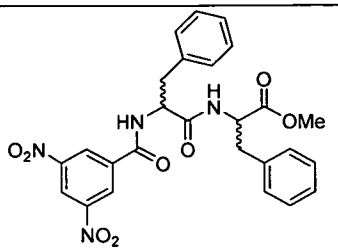
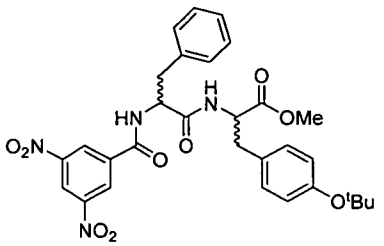
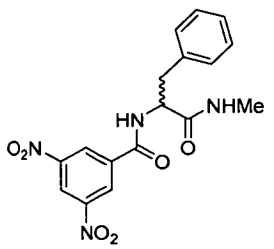
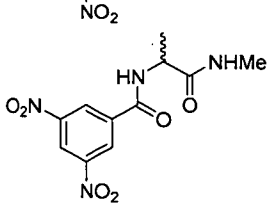


*Figure 44 Screening of enantiomers **(81)** and **(82)** on CSP **(72)**.*

The final library of racemate analytes was a selection of other commercially available pharmaceutical small molecules and amino acids: Bendroflumethiazide, Flavanone, Proglumide, Ibuprofen, Warfarin, Ephedrine, Flumequine, Indapamide, Chlorpheniramine, Benzoin, Phenylethanol, Benzyl-2-oxazolidinone, Tryptophan and Phenylalanine.

Due to the vast variety of different properties that the analytes posed, the screening was carried out using a gradient spanning a large polarity range (0-90% 2-PrOH). Three of the fourteen members of the racemate library were separated to some extent on CSP **(72)**, these were Ibuprofen, Proglumide and Chlorpheniramine.

The summary of results for analytes successfully separated on CSP **(72)** is shown in Table 15. CSP **(68)** was also screened using the same racemate analytes, but no separation was observed. This was expected as CSP **(68)** was not specifically designed for chiral separation, but to test synthetic methodologies used in the synthesis of CSP **(72)**.

Racemate analyte		CSP (72)	
		k'_1	α
	**(79)**	12.80	1.64*,[a]
	**(80)**	7.45	2.46*,[a]
	**(81)/(82)**	5.06	1.24[b]
	**(83)/(84)**	4.09	1.04[b]
	Ibuprofen	12.14	1.55[c]
	Proglumide	1.07	1.54[c]
	Chlorpheniramine	20.94	1.03[c]

** Separation is probably diastereomeric rather than enantiomeric*

[a] Mobile phase is heptane/2-propanol (90:10), flow rate of 0.3 mL/min.

[a] Mobile phase is heptane/2-propanol (70:30), flow rate of 0.3 mL/min.

[c] Gradient mobile phase heptane(A)/2-propanol(B) t = 0; 0% (B), t = 15; 10% (B), t = 25; 90% (B), t = 30; 90% (B), flow rate of 0.5 mL/min.

Table 15 Summary of successful separations on CSP (72).

4.5 Summary

Previously synthesised CSPs were capped dipeptidic structures attached to silica beads and were very successful in separating anthryl ethanol and other similar racemates. A reciprocal CSP **(72)** was synthesised based on an anthryl ethanol enantiomer attached to the silica bead. This was achieved starting from 9-anthraldehyde, the optical character of this CSP was inserted into the molecule using Sharpless asymmetric dihydroxylation. Some reciprocal racemates were synthesised as screening analytes, using solid-phase peptide chemistry.

The screening of reciprocal CSP **(72)** was carried out with a range of racemates, including a series commercially available small molecules and a previously synthesized library of carbinol racemates. Results obtained show some separation of the reciprocal peptide based analytes, although not as much as was hoped. Also separated were three members of another analyte library; Ibuprofen, Proglumide and Chlorphenirmaine.

5 3,5-Dinitrophenylalanine, non-proteinogenic amino acid based CSPs

5.1 Introduction

3,5-Dinitrobenzoyl was found to be the best CSP end group from a screened library of CSPs (section 2). Trying to augment chiral separation by using two 3,5-dinitrobenzoyl end groups, was found to give no improvement over the CSP with one end group.

The 3,5-dinitrobenzoyl group is a π-acceptor and as such interacts strongly with π-donating functionalities, such as most aryl groups. Other groups in common to CSPs, including all from section 2, are amino acids. They benefit from being synthetically useful through well known solid-phase amide bond formation and also posses natural chirality required for CSP's. Although the 3,5-dinitrobenzoyl groups has been used not only as an end group, it has not been used as an 'amino acid' in a CSP. Therefore we investigated using an amino acid equivalent of 3,5-dinitrobenzoyl, in a small number of simple peptidic CSPs.

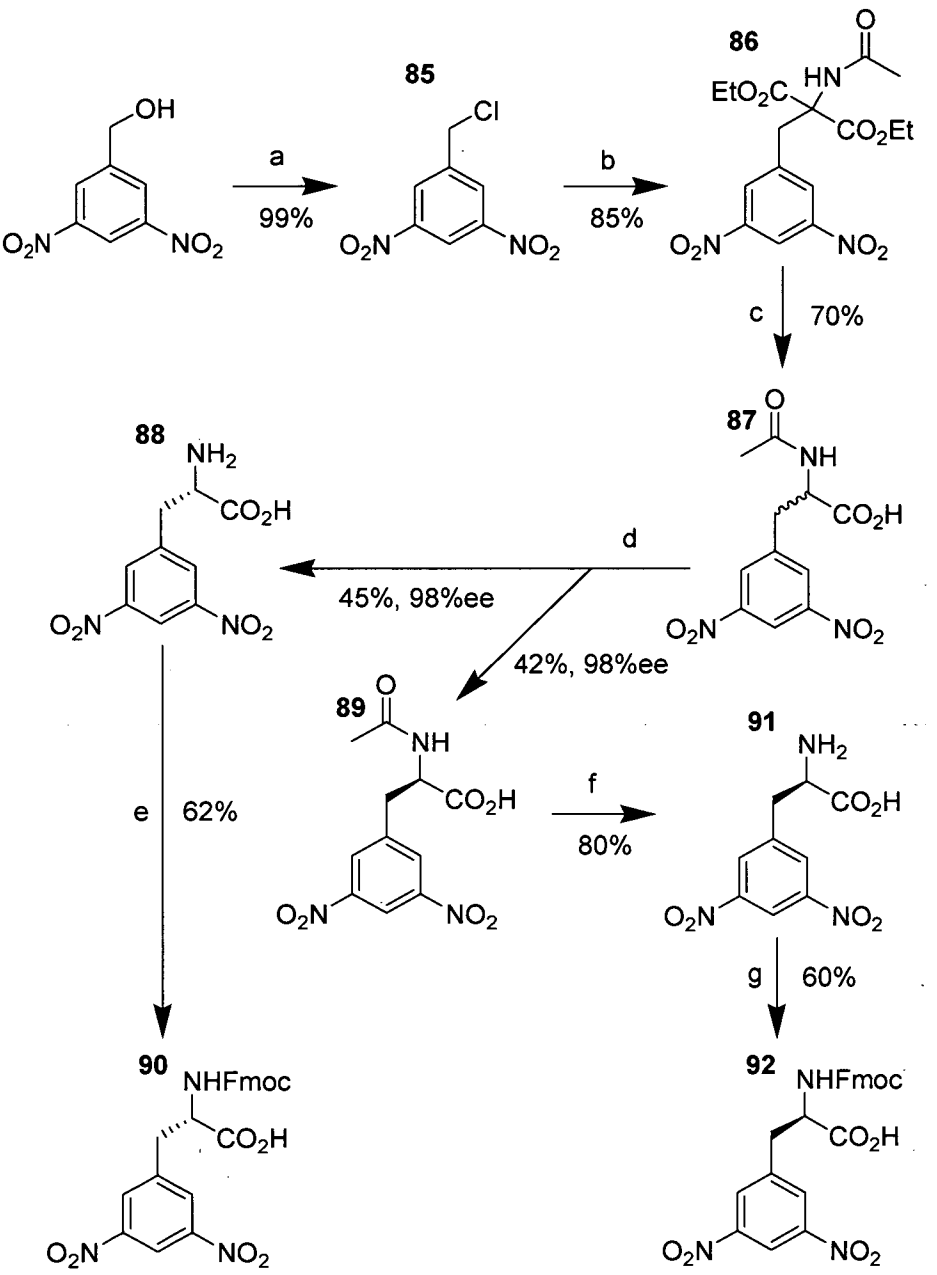
5.2 Synthesis of Fmoc-3,5-dinitrophenylalanine

3,5-Dinitrophenylalanine was chosen as the amino acid equivalent of 3,5-dinitrobenzoyl group. Inducing the chirality into the amino acid was achieved by resolution by deacetylation, which is a common method used in the synthesis of amino acids.

Synthesis was achieved starting with the commercially available 3,5-dinitrophenyl methanol. Chlorination using PCl_5[269] gave 1-(chloromethyl)-3,5-dinitrobenzene **(85)** in excellent yield. The next step incorporated the acid and amine functionalities. Diethyl acetamidomalonate was firstly deprotonated with $NaOEt$[231] which was then added to **(85)** and displaced the chloride to give diethyl 2-acetamido-2-(3,5-dinitrobenzyl) malonate **(86)** in good yield. Base hydrolysis of **(86)**, followed by careful reacidification gave the racemic N-acetyl-3,5-dinitrophenylalanine **(87)**.

Resolution of **(87)** was carried out after consideration of literature precedent[270-272]. Deacetylation was achieved using Acylase I (from *Aspergillus melleus*, 1.06 Umg^{-1}) with a catalytic amount of Colbalt (II) chloride hexahydrate in water, whilst carefully controlling the pH (7.0-7.2). This gave the deacetylated S-enantiomer L-3,5-dinitrophenylalaine **(88)** as well as the remaining optically resolved acylated D-enantiomer starting material, N-acetyl-D-3,5-dinitrophenylalanine **(89)**. L-3,5-Dinitrophenylalanine **(88)** was Fmoc protected using Fmoc-N-hydroxysuccinimide ester (Fmoc-OSu)[273] to give the desired final amino acid Fmoc-L-3,5-dinitrophenylalanine **(90)**.

The enantiomer **(90)** was also synthesised from the resolved product of N-acetyl-D-3,5-dinitrophenylalanine **(89)**, firstly by acid hydrolysis to give D-3,5-dinitrophenylalanine **(91)** and then by Fmoc protection, again using Fmoc-OSu, to give Fmoc-D-3,5-dinitrophenylalanine **(92)**.



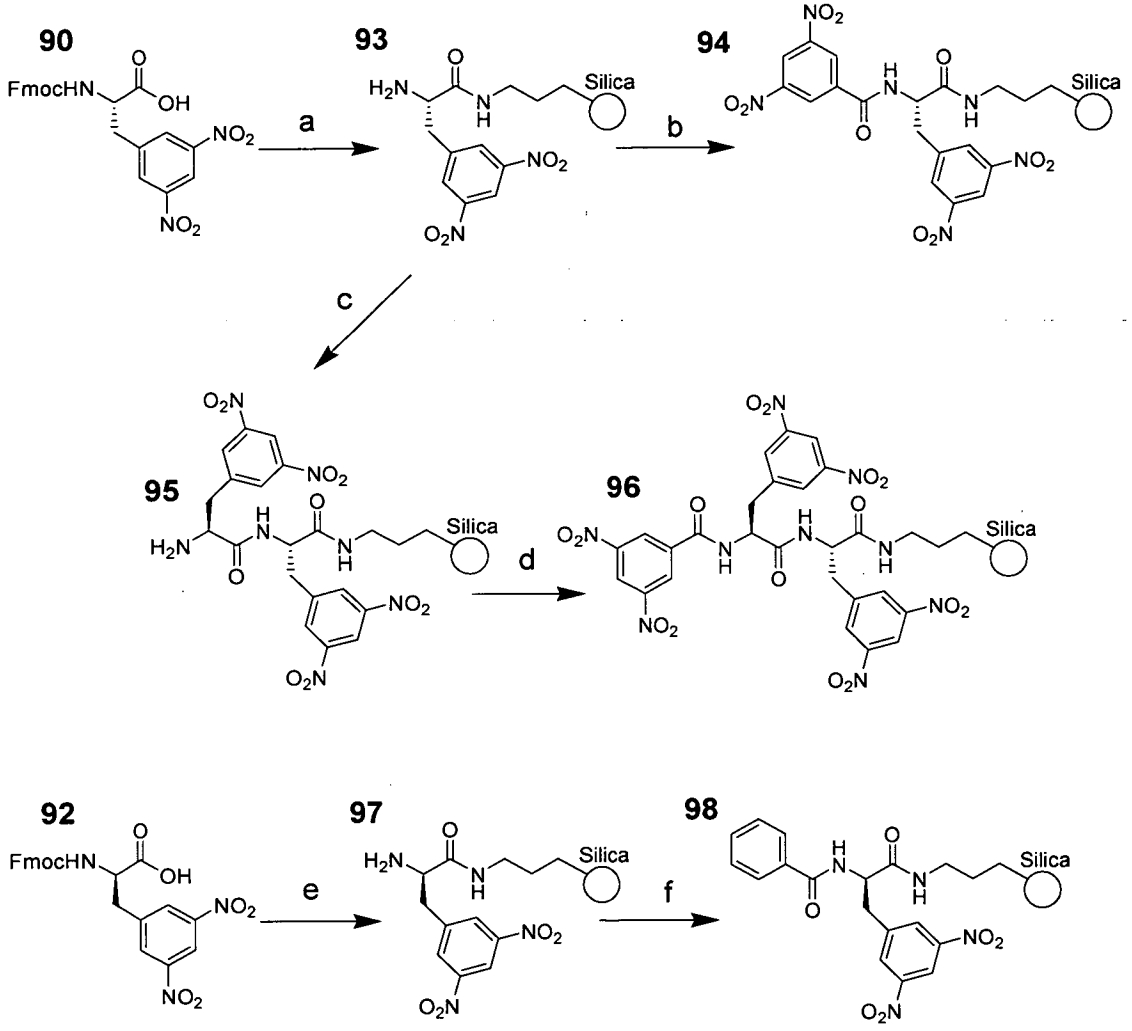
(a) PCl_5, $CHCl_3$, 50 °C, (b) Diethyl acetoamidomalonate, NaOEt, 50 °C,
(c) NaOH, EtOH, water,
(d) Acylase I (*Aspergillus melleus*, 1.06Ug^{-1}), $CoCl_2.6H_2O$, water, 37-40 °C,
(e) Fmoc-OSu, Na_2CO_3, water, dioxane, (f) HCl (0.2 M), 60 °C,
(g) Fmoc-OSu, Na_2CO_3, water, dioxane.

Scheme 28 Synthesis of 3,5-dinitrophenylalanine.

5.3 Synthesis of CSPs containing 3,5-dinitrophenylalanine

Three CSPs containing the amino acid 3,5-L-dinitrophenylalanine were synthesised using standard solid-phase peptide chemistry, where each coupling step was followed by Fmoc deprotection. 3,5-L-Dinitrophenylalanine **(90)** was coupled to

aminopropyl silica to give **(93)**. This in turn was capped with 3,5-dinitrobenzoic acid to give CSP **(94)**, which contains one 3,5-L-dinitrophenylalanine amino acid unit and one 3,5-dinitrobenzoyl end group. Another unit of 3,5-L-dinitrophenylalanine was coupled to **(93)**, to give silica support dipeptide **(95)**, which was subsequently capped with 3,5-dinitrobenzoic acid to give CSP **(96)**. This CSP contains two 3,5-L-dinitrophenylalanine units and one 3,5-dinitrobenzoyl end group.



(a)(i) Aminopropyl silica (12 μm), DIC, HOBt, DMF/DCM (1:4),
(ii) piperidine/DMF (5:95), (b) 3,5-dinitrobenzoic acid, DIC, HOBt, DMF/DCM (1:4),
(c)(i) **(90)**, DIC, HOBt, DMF/DCM (1:4), (ii) piperidine/DMF (5:95),
(d) 3,5-dinitrobenzoic acid, DIC, HOBt, DMF/DCM (1:4),
(e)(i) Aminopropyl silica (12 μm), DIC, HOBt, DMF/DCM (1:4),
(ii) piperidine/DMF (5:95), (f) benzoic acid, DIC, HOBt, DMF/DCM (1:4).

Scheme 29 Synthesis of three CSPs containing 3,5-dinitrophenylalanine.

The other 3,5-dinitrophenylalanine enantiomer was used for synthesis of a final CSP. 3,5-D-Dinitrophenylalanine was coupled to aminopropyl silica and

subsequently capped with benzoic acid giving CSP **(98)**, which is analogous to CSP **(94)**, but with the absence of nitro moieties in the end group.

5.4 Screening of 3,5-dinitrophenylalanine based CSPs

CSPs **(94)**, **(96)** and **(98)** were packed into empty HPLC columns and screened with a previously synthesised array of racemic analytes (section 2.2.2). The screenings were then compared to results for CSP **(5)**. Of particular interest was CSP **(96)**, which is analogous to CSP **(5)**, being dipeptidic with a 3,5-dinitrobenzoyl end group. The amino acids; Phe and Tyr(O^{t}Bu) of CSP **(5)**, have been replaced with stronger π-acceptor groups, using the amino acid 3,5-dinitrophenylalanine.

Screening results were very suprising, as CSPs **(94)** and **(96)** both showed zero chiral separation of any members of the the racemic analytes. Chromatographic conditions used were varied over the range found to be successful for similar CSP of section 2.2.3. Sample rentention values were similar to that of CSP **(5)**, but no separation was observed.

CSP **(98)** was almost as unsuccessful, but separated one member of the analytes. Racemate **(31)** was separated, but with only a small separation factor (k_1 = 4.23, α = 1.11; mobile phase = 2-PrOH/Heptane (3:97), flow rate = 0.3 mLmin^{-1}).

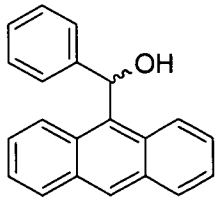


Figure 45 Racemate (31)

The negative results for CSPs **(94)** and **(96)** in particular indicate that the multiple 3,5-dinitrobenzoyl groups in the CSP structure actually negate the chiral separation properties rather than augment it. This could be because multiple similar groups will interact with the analyte with similar magnitude, meaning the analyte can interact with a variety of different areas of the CSP structure, rather than having a strong interaction in one specific site on the CSP. These startling results show the

importance of the CSP structure containing complimentary π-accepting and π-donating moieties, rather than exclusively using multiple groups of one character.

5.5 Summary

The non-proteinogenic α-amino acid, 3,5-dinitrophenylalanine was successfully synthesised. Chirality was introduced into the molecule by resolution using Acylase I (*Aspergillus melleus*) to deacetylate the L-enantiomer, whilst leaving the D-enantiomer acetylated. Both enantiomers were followed through to give the Fmoc protected amino acid optical isomers. These were used in the solid-phase synthesis of three novel CSPs and screened with a range of racemic analytes.

The results showed no chiral separation of any analytes for two CSPs and one low selectivity separation for the other CSP. This indicates that rather than augmenting chiral separation of peptidic and amino-acid based CSP by using the 3,5-dinitrophenylalanine amino acid, it actually made the CSP ineffectual.

This is possibly due the other π-accepting 3,5-dinitrophenylalanine groups interacting with analyte molecules in a similar way to that of the 3,5-dinitrobenzoyl end groups and therefore distracting the analyte from interacting with the CSP at one site.

6 Conclusions

The aims of this thesis were towards the improvement of chiral separation technology. For this several areas of chiral separation were explored by a number of experiments to establish a greater understanding for the resolution of a given enantiomer; by measuring changes in chiral separation through altering of chiral stationary phase structure, synthesis of peptide based libraries of chiral stationary phases towards fast and efficient development of novel chiral stationary phases, utilising high-throughput screening technology for faster determination of appropriate chiral stationary phase media, synthesis of specifically designed chiral stationary phases.

Chapter 2 involved the synthesis of a series of small libraries of Pirkle type chiral stationary phases. As Pirkle type chiral stationary phases rely heavily on π-π interactions for chiral resolution, the first library investigated the use of numerous head-groups for the CSP structure. The 3,5-dinitrobenzoyl end group which is utilised in many commercially available CSPs was found to be more successful in separating a range of racemates. The importance comes from its ability to interact strongly, mainly through π-π forces, with analyte enantiomers. This strong interaction enhances the scale of transient diastereomeric energies, thus improving the chances for chiral separation. Although alternative CSPs could not improve upon the separation ability of 3,5-dinitrobenzoyl, they did highlight important features of the end group. Such as, the removal or movement around the ring, of nitro moieties caused a large detrimental effect to the resolution observed. Other chiral stationary phase libraries helped determine that the second amino acid from the silica bead was found to have more influence over the overall chiral separation ability of the CSP, the incorporation two 3,5-dinitrobenzoyl groups into the CSP structure could also not improve the CSP's ability over that containing one 3,5-dinitrobenzoyl group. Also achiral spacers in CSP structure cause a detrimental effect on the chiral resolution, most notibily when placed next to the end group moiety.

Chapter 3 focused on probing the relationship between protein based chiral stationary phase separations of pharmaceutical compounds with the developing technology of surface plasmon resonance biosensors. Chiral HPLC and SPR

biosensor screens were carried out on the enantiomers of four analytes; Warfarin, Ibuprofen, Benzoin and Tryptophan. These analytes were screened on three proteins; HSA, BSA and AGP, which were all immobilised onto either column packing materials or onto the surface of a sensor spot, for HPLC and SPR biosensor analysis respectively. Using the resulting relative binding strength for enantiomers of each analyte in both HPLC and SPR gave a way of comparing these technologies. Results were found to match very well, with the majority of SPR screening results agreeing with HPLC precedent. The results obtained for Warfarin and Ibuprofen, which were strong binders to HSA and BSA proteins were very good. 157 out of 189 pairs of screening data for these analytes gave a matching order of elution between chiral HPLC and the SPR biosensor. Weaker binding analytes; Benzoin and Tryptophan gave less reliable results. Their lower over binding increased the noise to signal ratio therefore the comparative enantiomer binding affinities were more likely to switch compared to the expected result. Although there were some drawbacks with the SPR system, the advantages of the system and future potential improvements make it a valuable tool for high-throughput determination of chiral stationary phase media and chiral chromatographic conditions for scaled-up chiral HPLC.

Chapters 3 and 4 involved the synthesis of more structurally focused Pirkle type CSPs. In chapter 3 a chiral stationary phase based on an enantiomer of anthryl ethanol was developed in a 'reciprocal CSP' manner. However, the final CSP failed to convincingly show true CSP reciprocal ability in attempting to separate some peptide racemates; this may be due to the excessive structural changes necessary for the attachment of the chiral selector to silica stationary phase. Chapter 4 focused on developing CSP containing a non-proteinogenic amino acid 3,5-dinitrophenylalanine. This particular amino acid was chosen due to its structural similarity of the 3,5-dinitrobenzoyl group which is successfully employed as an end-group. However the resulting CSP gave very poor separation of a range of racemates, possibly due to too much structural similarity between the peptide chain and end-groups, therefore resulting in both groups competing for the same type of interaction with the analyte.

7 Experimental

7.1 General Information

NMR spectra were recorded on a Bruker ARX250, AV300, DPX360 and DPX 400 spectrometers operating at 250 MHz, 300 MHz, 360 MHz and 400 MHz for ^{1}H and 62.5 MHz, 75 MHz, 90 MHz and 100 MHz for ^{13}C spectra respectively. Chemical shifts (δ), referenced to the residual protonated solvent as an internal standard, are quoted in ppm, coupling constants (*J*) are reported in Hz. ESI mass spectra were recorded on a Fisons VG platform quadropole or a Waters ZMD single quadropole, equipped with an electronspray ionisation source, measuring monoisotopic masses. Electron ionization (EI) and Chemical ionization (CI) mass spectra were run on a ThermoQuest TraceMS gas chromatography mass spectrometer. Major peaks are reported with percentage intensities of the base peak. HRMS were run on a Bruker APEX3.

The Smith Synthesiser from Personal Chemsitry was used to perform microwave irradiations. Infra-red spectra were recorded on a BIORAD FTS 135 with a Golden Gate ATR access. All samples were run neat. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. UV-Vis spectra were recorded using an 8452 A diode array detector. Elemental analyses were performed by Medac Ltd or the service within the School of Chemistry at St. Andrews University. HPLC and chiral HPLC were carried out on HP Agilent systems.

Dry solvents were obtained by passing through an alumina column. Reagents were used as purchased from commercial sources unless otherwise specified. TLC analysis was carried out using foil-backed sheets coated with silica gel (0.25 mm) and containing the fluorescent indicator UV_{254}. Flash column chromatography was performed, on Sorbsil C60, 40-60 mesh silica.

Amino acids were of the L-configuration unless otherwise stated.

7.2 General solid-phase chemistry procedures

7.2.1 Colorimetric tests

7.2.1.1 *Quantitative Ninhydrin test*

A known mass of resin (ca. 5 mg) in a small test tube was treated with 3 drops of reagent A and 1 drop of reagent B and heated at 100 °C for 10 min. The test tube was cooled and 60 % aq EtOH (2 mL) added. The resin was removed by filtration through cotton wool and the deep blue filtrate collected in a 25 mL volumetric flask. The resin was washed with a solution of Et_4NCl (0.5 M in CH_2Cl_2, 2 x 0.5 mL) and the sample made up to 25 mL with 60 % aq EtOH. The absorbance at 570 nm was measured against a reagent blank. The level of amine present was calculated using the equation:

$$\text{Amount of amine present (mmol/g)} = [(A_{570} \times V)/(\varepsilon_{570} \times m)] \times 10^3$$

where A_{570} is the recorded absorbance at 570 nm, V is the final volume (mL), ε_{570} is an extinction coefficient suitable for most peptides (1.5×10^4 M^{-1} cm^{-1}) and m is the mass of the resin sample (mg).

Reagent A

- Solution 1: Phenol (40.0 g) was dissolved in EtOH (10 mL) with warming and then stirred over Amberlite mixed-bed resin MB-3 (4.0 g) for 45 min. The mixture was filtered.
- Solution 2: Potassium cyanide (65 mg) was dissolved in H_2O (100 mL). A 2 mL aliquot of this solution was diluted with pyridine (freshly distilled from ninhydrin) and stirred over Amberlite mixed-bed resin MB-3 (4.0 g). The solution was filtered and mixed with 'Solution 1' to give reagent A.

Reagent B

Ninhydrin (2.5 g) was dissolved in absolute EtOH (50 mL).

7.2.1.2 *Quantitative Fmoc test*

A known mass of resin (ca. 5 mg) was weighed into a 1 mL SPE syringe and treated with 20 % piperidine in DMF by percolating through the resin bed into a 25

mL volumetric flask. The filtrate was made up to volume with 20 % piperidine in DMF. The absorbance at 302 nm was measured against 20 % piperidine in DMF. The loading was calculated using the equation:

$$\text{Loading (mmol/g)} = [(A_{302} \times V)/(\varepsilon_{302} \times m)] \times 10^3$$

where A_{302} is the recorded absorbance at 302 nm, V is the final volume (mL), ε_{302} is the extinction coefficient of the piperidyl-fulvene adduct (7.8×10^3 M^{-1} cm^{-1}) and m is the mass of the resin sample (mg).

7.2.1.3 *Qualitative chloranil test*

To a small amount of resin (ca. 1-3 mg), one drop of 2 % acetaldehyde in DMF was added followed by one drop of 2 % *p*-chloranil in DMF and was allowed to stand for 5 min. Blue stained beads indicated the presence of secondary amines.

7.2.1.4 *Qualitative TNBS test (2,4,6-trinitrobenzenesulfonic acid)*

A few beads were suspended in 2 drops of a solution of 10% DIPEA in DMF and 2 drops of a solution 1% TNBS in DMF (commercially available). After a few minutes, the beads were washed with DMF to remove the red solution. A positive test was indicated by red beads and confirmed the presence of primary amines.

7.2.2 Silica purification procedures

7.2.2.1 *Filtration procedure to separate silica beads from reaction solution.*

Silica/reaction mixture slurries were poured into microfiltration tubes (Whatman®, 12 mL, 5 μm pore size), attached to a vacuum pump or a vacuum manifold. Mother liquor drained and the silica was washed normally washed with DMF (2 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL). The silica was then normally dried in vacuo.

7.2.2.2 *Centrifugation to separate silica beads from reaction solution.*

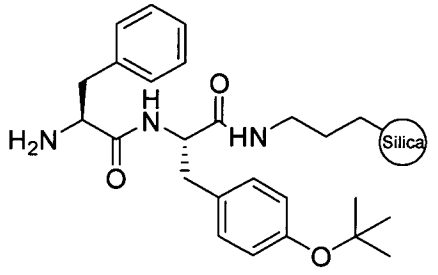
Silica/reaction mixture slurries were transferred from the reaction vessel to a centrifuge tube (50 mL), the solution was made up to 40 mL with DMF. The tube was centrifuged (8000 rpm, 10 min) and the supernatant is removed. The procedure

was repeated with further washings of DMF (2 x 40 mL) and Et_2O (2 x 40 mL). The silica was then normally dried in vacuo.

7.3 Experimental for Chapter 2

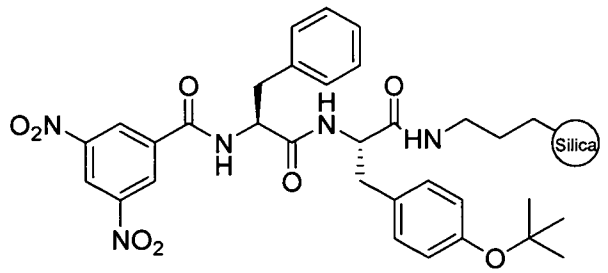
7.3.1 Synthesis of compounds

H-Phe-Tyr(^{t}Bu)-aminopropyl silica (2)



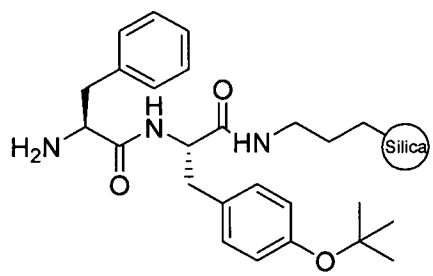
To a suspension of beaded aminopropyl silica (3, 10, 12 μm from ThermoHypersil and 4 μm from Capital HPLC, 0.45mmolg^{-1}, 1.0g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Tyr(^{t}Bu)-OH (10 eq., 4.5 mmol, 0.60 g) and HOBt (10 eq., 4.5 mmol, 0.6 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered (Whatman microfiltration device, 12 mL volume, 5 μm pore size) or centrifugated and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF (5% piperidine) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF (5% piperidine) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). The silica beads were then suspended in DCM/DMF (8:2, 10 mL) and Fmoc-Phe-OH coupled using the same procedure as described above. The beads were again filtered, washed and Fmoc deprotected, followed by filtration, washing and drying as above. Reaction completion was monitored by the TNBS test.

3,5-DNB-Phe-Tyr(^{t}Bu)-aminopropyl silica (3_3), (3_4), (3_{10}) and (3_{12})



To a suspension of H-Phe-Tyr(^{t}Bu)-aminopropyl silica **(2)** (1.0 g, 0.45 mmol) in DCM/DMF (8:2, 10 mL), was added a mixture of the 3,5-dinitrobenzoic acid (10 eq., 4.5 mmol, 0.60 g) and HOBt (10 eq., 4.5 mmol, 0.6 g) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3.2.1. H-Phe-Tyr(^{t}Bu)-aminopropyl silica (4)

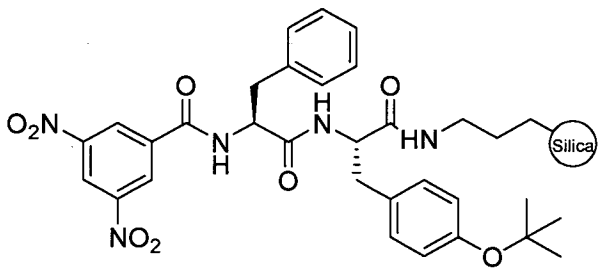


Procedure identical to H-Phe-Tyr(^{t}Bu)-aminopropyl silica **(2)**. H-Phe-Tyr(^{t}Bu)-aminopropyl silica **(4)** involved only the use of 10 μm aminopropyl silica from ThermoHypersil.

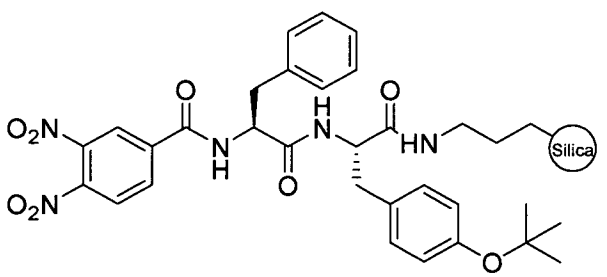
CSP-(5-23)

To a suspension of the H-Phe-Tyr(^{t}Bu)-aminopropyl silica **(4)** (1.0g, 0.45mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of the carboxylic acid **(X)** (10 eq., 4.5 mmol, 0.60 g) and HOBt (10 eq., 4.5 mmol, 0.6 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

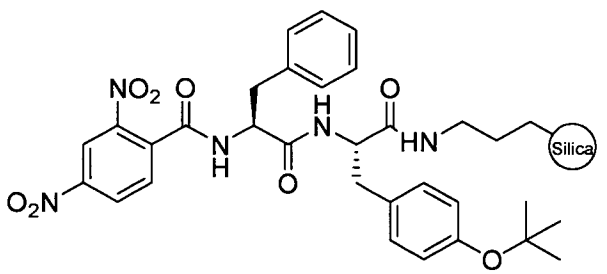
3,5-DNB-Phe-Tyr(^{t}Bu)-aminopropyl silica (5)



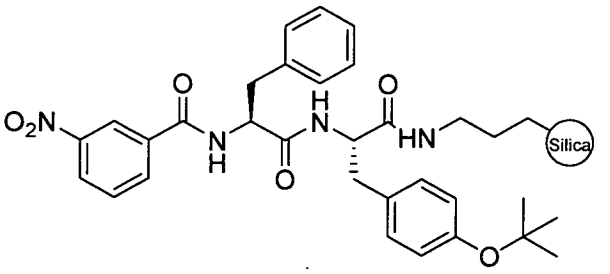
3,4-DNB-Phe-Tyr(^{t}Bu)-aminopropyl silica (6)



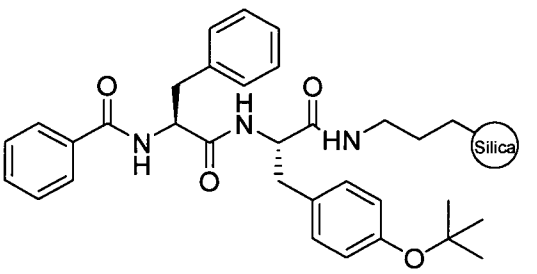
2,4-DNB-Phe-Tyr(^{t}Bu)-aminopropyl silica (7)



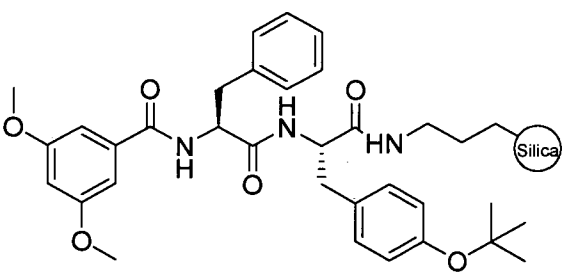
3-nitrobenzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (8)



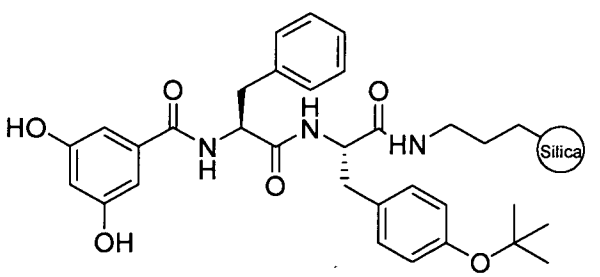
Benzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (9)



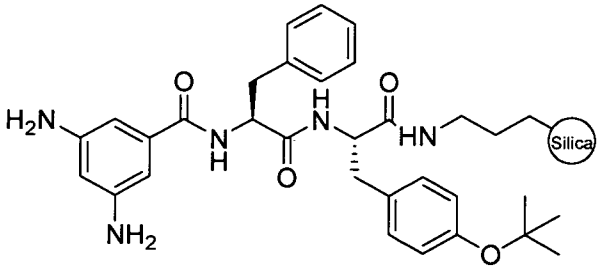
3,5-dimethoxybenzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (10)



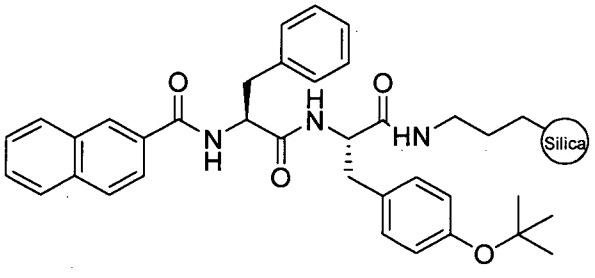
3,5-dihydroxybenzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (11)



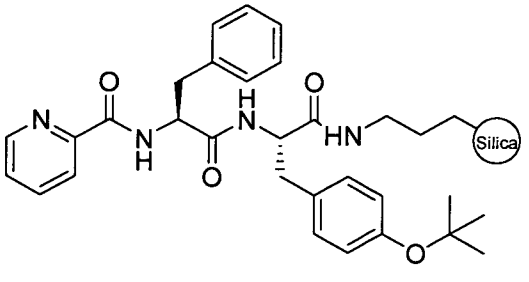
3,5-diaminobenzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (12)



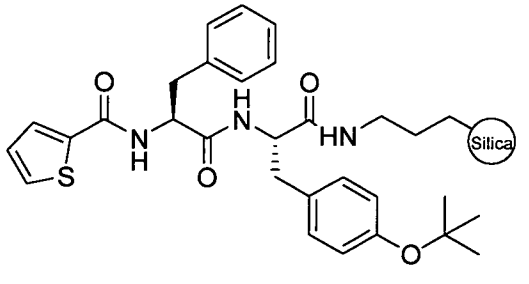
2-Naphthoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (13)



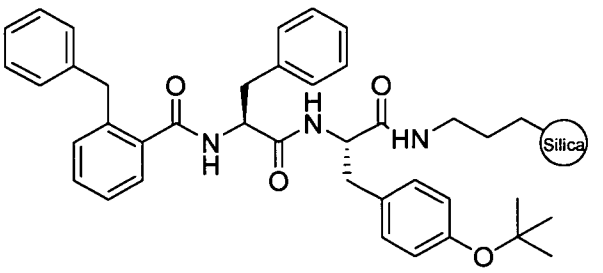
2-Pyridoyl -Phe-Tyr(^{t}Bu)-aminopropyl silica (14)



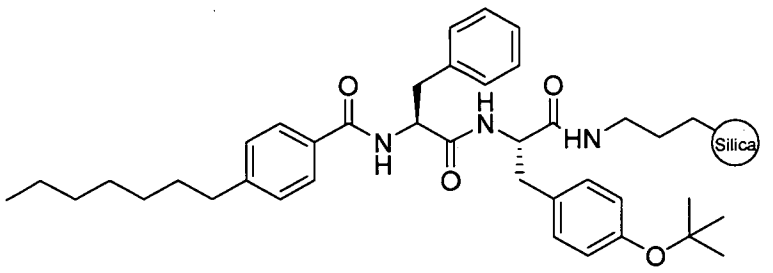
Thiophene-2-carboyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (15)



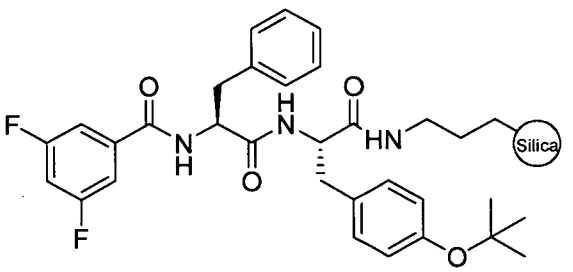
2-Benzylbenzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (16)



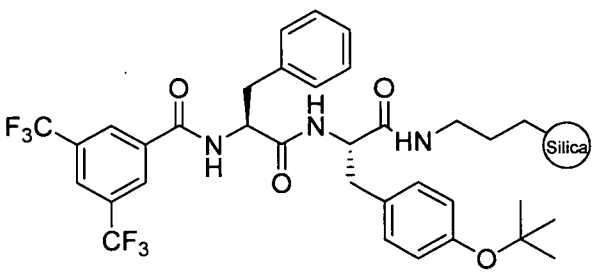
4-Heptylbenzooyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (17)



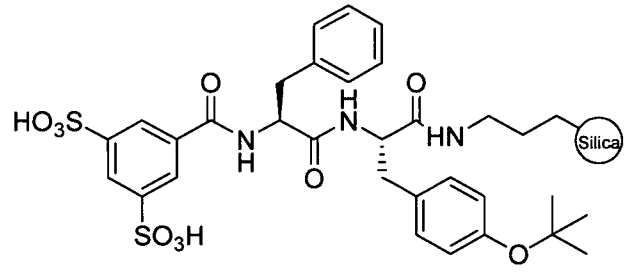
3,5-difluorobenzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (18)



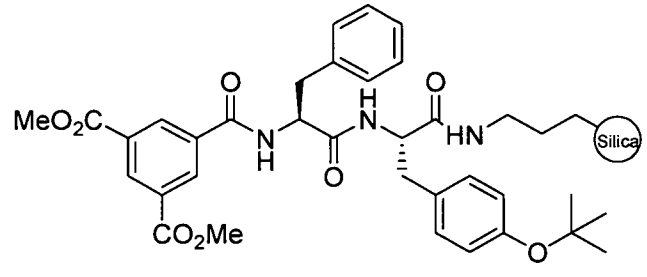
3,5-Bis(trifluromethyl)-Phe-Tyr(^{t}Bu)-aminopropyl silica (19)



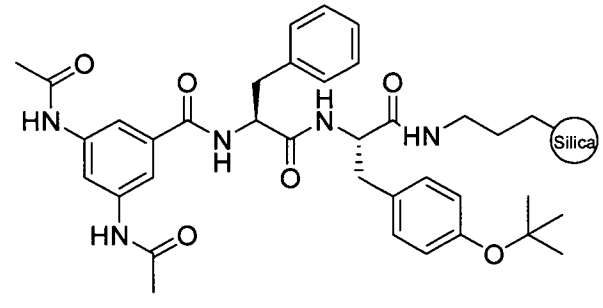
3,5-disulfobenzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (20)



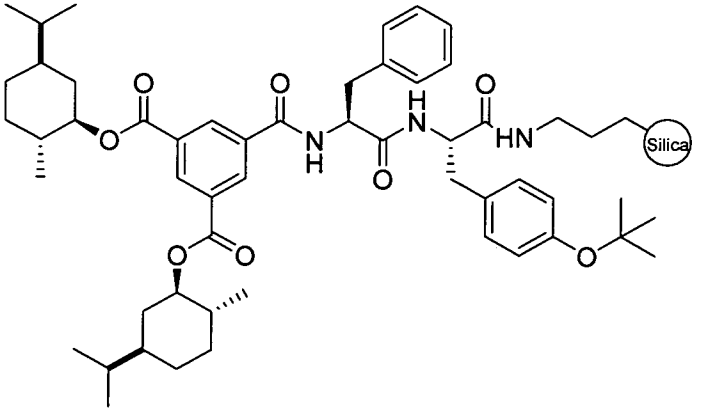
3,5-Bis(methoxcarbonyl)-Phe-Tyr(^{t}Bu)-aminopropyl silica (21)



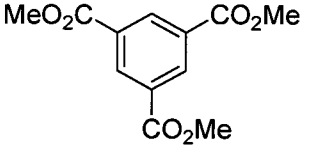
3,5-Bis(acetamido)benzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (22)



3,5-bis((1R,2R,5R)-mentholoxycarbonyl)benzoyl-Phe-Tyr(^{t}Bu)-aminopropyl silica (23)

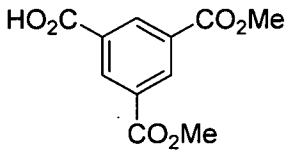


Benzene-1,3,5-tricarboxylic acid trimethyl ester (24)[274]



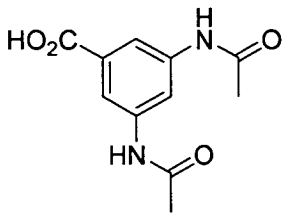
Following a procedure by Erickson et al[274]. 1,3,5-Benzenetricarboxylic acid (1.00 g, 1.0 eq.) was dissolved in DCM (20 mL), MeOH (20 mL) and conc. H_2SO_4 (200 μL). The reaction mixture was refluxed for 12h, cooled to room temperature and solvents removed *in vacuo*. The solid was dissolved in EtOAc (50 mL) and washed with sat. $NaHCO_3$ (100 mL) The organic layer was concentrated *in vacuo* to give a white solid, 1.15g (96%): m.p. 140°C (EtOH), literature 141-143°C[274]; ^{1}H NMR (d_6 -DMSO, 300MHz): 8.62 (s, 3H, 3 x ArH), 3.96 (s, 9H, 3 x ArC(O)OCH_3). ^{13}C NMR (d_6 -DMSO, 75MHz): 164.5 (C), 133.3 (CH), 130.9 (C), 52.7 (CH_3); *m/z* (EI): 252 $[M]^+$ (19%), 221 $[M-31(MeO)]^+$ (100%), 193 $[M-59(CO_2Me)]^+$ (32%).

Benzene-1,3,5-tricarboxylic acid dimethyl ester, (25)[274]



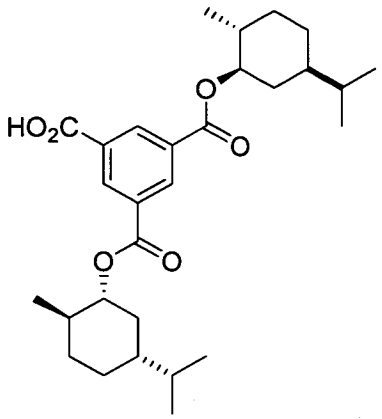
Following a procedure by Erickson et al[274]. Benzene-1,3,5-tricarboxylic acid trimethyl ester **(24)** (1.0 g, 1.0 eq.) was dissolved in MeOH (10 mL). NaOH (159 mg/1.0 eq.) was added to the reaction and stirred for 4 days. The reaction mixture was poured onto water (100 mL) and the solution washed with Et_2O (3 x 50 mL). The aqueous layer was acidified to pH 1 with HCl and extracted with Et_2O (3 x 100 mL), the combined organic layers were concentrated *in vacuo*, to give a cream solid 790mg (84%): m.p. 145°C (H_2O), literature 145-147°C (H_2O)[275]; ^{1}H NMR (d_6 -DMSO, 300MHz): 8.61 (s, 2H, 2 x ArH), 8.56 (s, 1H, 1 x ArH), 3.90 (s, 6H, 2 x ArC(O)OCH$_3$). ^{13}C NMR (d_6 -DMSO, 75MHz): 165.5 (C), 164.6 (C), 133.6 (CH), 133.0 (CH), 132.1 (C), 130.7 (C), 52.7 (CH_3); *m/z* (ES-) 236.9 $[M-H]^-$ (100%), 351.0 $[M+TFA-H]^-$ (10%).

3,5-Bis(acetylamino)benzoic acid (26)[276]



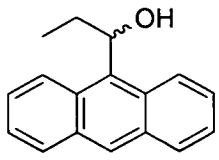
Following a procedure by Komatsu et al[277]. 3,5-Diaminobenzoic acid (1.0 g, 1 eq.) was slurried in Et_2O (100 mL) and treated with a solution of acetyl chloride (2.0 mL) in Et_2O (10 mL). To the reaction mixture was added a solution of pyridine (2.0 mL) in Et_2O (10 mL) over 1h, followed by stirring for 72h. EtOH (100 mL) was poured into the reaction and the solvents were removed *in vacuo* to give a dark oil. Water (100 mL) was added to oil to give a grey precipitate, which was collected by filtration, washed with water (100 mL) and 0.5 M HCl (50 mL). A wet grey precipitate was collected and dried *in vacuo* to give a grey solid, 1.30 g (84%): m.p. 300-305°C (H_2O), literature 299-300°C (H_2O)[276]; ^{1}H NMR (d_6 -DMSO, 300MHz): 10.11 (s, 2H, 2 x ArNHC(O)CH_3) 8.12 (s, 1H, ArH), 7.88 (s, 2H, 2 x ArH), 2.15 (s, 6H, 2 x ArNHC(O)CH_3). ^{13}C NMR (d_6 -DMSO, 75MHz): 168.5 (C), 167.1 (C), 139.7 (C), 131.4 (C), 114.6 (CH), 113.4 (C), 23.9 (CH_3); *m/z* (ES-) 236.9 $[M-H]^-$ (100%), 351.0 $[M+TFA-H]^-$ (10%).

Benzene-1,3,5-tricarboxylic acid bis((1R,2S,5S)-menthol) ester (27)



1,3,5-Benzenetricarbonyl chloride (1.00 g, 1 eq.) was dissolved in dry THF (20 mL), to this was added Et_3N (1.64 mL, 3.2 eq.) and the reaction mixture was cooled to 0°C in an ice bath. To the reaction was added a solution of (1R, 2S, 5R)-(-)-Menthol (3.2 eq.) in THF (20 mL), dropwise over 2h. The reaction was left to stir for a further 20h under a nitrogen atmosphere whilst being allowed to warm to room temperature. Water (50 mL) was added slowly to the reaction, which was left to stir for 2h. The reaction was extracted with Et_2O (4 x 50 mL) and the combined organic layers washed with water (50 mL), brine (50 mL), then dried with magnesium sulfate, filtered and concentrated *in vacuo*. The residue was crystalised from warm water to give a grey solid 960 mg (53%): m.p. 78-82°C (H_2O); ^1H NMR (d_6-DMSO, 250MHz): 8.86 (d, 2H, J = 2Hz, 2 x ArH), 8.78 (t, 1H, J = 2Hz, 1 x ArH), 5.10 (td, 2H, J = 10Hz, J = 4Hz), 2.70 (quintet, 2H, J = 2Hz), 2.20 (m, 2H), 2.05 (m, 2H), 1.88-1.73 (m, 8H), 1.40-1.36 (m, 4H), 1.08 (dd, 12H, J = 7Hz, J = 4Hz), 0.95 (m, 6H, J = 3Hz). ^{13}C NMR (d_6-DMSO, 62.5MHz): 166.5 (C), 164.4 (C), 146.9 (C), 140.0 (CH), 131.22 (C), 75.4 (CH), 46.9 (CH), 40.7 (CH_2), 34.0 (CH_2), 31.2 (CH), 26.6 (CH), 23.6 (CH), 22.2 (CH_3), 20.8 (CH_3), 16.8 (CH_3); *m/z* (ES-): 485 $[M-H]^-$ (100%); HR-*m/z* (FAB): $[M-H]^-$ ($C_{29}H_{42}O_6$) calculated: 485.2903, found: 485.2905 $[M-H]^-$.

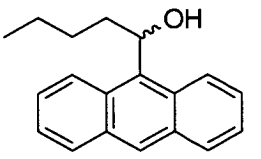
1-(9-Anthracenyl)-propan-1-ol (29)[278, 279]



Magnesium turnings (118mg, 10 eq.) and a magnetic stirrer bar were oven dried, a subaseal fixed in place and cooled to room temperature under a nitrogen atmosphere. THF (2 mL) was added and the reaction stirred for 5 min. Ethyl bromide (180 μL, 2.5 eq.) was added slowly to the reaction. The reaction was catalysed by the addition of a small amount of iodine (colourless to light brown). The reaction was stirred for 15 min (the flask became warm, light brown to colourless/grey/yellow), and further ethyl bromide (180 μL/2.5 eq.) was added slowly to the reaction (15 min) and the reaction stirred for a further 30 min. A 10 μL aliquot was taken from the reaction mixture and dissolved in water (1 mL). The basic solution was titrated against HCl (0.01 M), using phenolphthalene as an indicator, to give the concentration of the Grignard reagent *in-situ*.

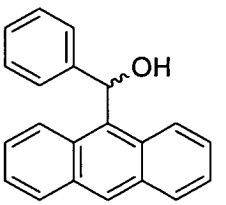
9-Anthraldehyde (100 mg/1.0 equiv.) under a nitrogen atmosphere was dissolved in freshly distilled THF (10 mL) and the reaction mixture cooled to 0°C in an ice bath. The prepared Grignard reagent (1.2 eq.) was added to 9-Anthraldehyde solution slowly over 15 min. The reaction was stirred at 0°C for a further 3h, then overnight. NH_4Cl 10% (w/v, 10 mL) was added slowly to the reaction and extracted with Et_2O (3 x 10 mL). The combined organic layers were washed with brine (10 mL), dried with $MgSO_4$, filtered and concentrated *in vacuo*. Purification was achieved by flash chromatography (Silica; Hexane: DCM 2:1 to 0:1), to give a yellow solid 92 mg (40%)[278, 279]: m.p. 110-112°C; ^{1}H NMR ($CDCl_3$, 400MHz): 8.40-8.50 (bs, 2H, 2 x Ar<u>H</u>), 8.17 (s, 1H, Ar<u>H</u>), 7.76-7.79 (m, 2H, 2 x Ar<u>H</u>), 7.19-7.32 (m, 4H, 4 x Ar<u>H</u>), 5.98 (dd, 1H, J = 8.5Hz, 6.5Hz, ArC<u>H</u>(OH)CH_2), 2.17-2.31 (m, 1H, ArCH(OH)C<u>H</u>H), 1.95-2.07 (m, 1H, ArCH(OH)CH<u>H</u>), 0.87 (t, 3H, J = 7Hz, $CH_2C\underline{H}_3$); *m/z* (EI) 236 $[M]^+$ (6%), 218 [M-18 $(H_2O)]^+$ (76%), 203 [M-18-15, E-15$(CH_3)]^+$ (100%).

1-(9-Anthracenyl)-pentan-1-ol (30)[280]



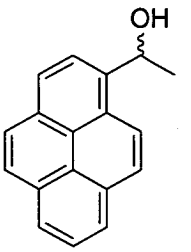
Following a procedure by Kanemasa et al[281]. 9-Anthraldehyde (500 mg, 1.0 eq.) is dissolved in dry THF (15 mL) and the reaction mixture is cooled to 0°C in an ice bath. ^{n}BuLi (1.165 mL, 2.5 M, 1.2 eq.) was added slowly over 20 min. The reaction was stirred for a further 3h at 0°C. To the reaction was added crushed ice and diluted with Et_2O (50 mL). The organic layer was extracted with Et_2O (3 x 30 mL), the combined organic layers were washed with brine (50 mL), then concentrated in vacuo. The crude yellow oil was purified by flash chromatography (silica; Hexane/DCM) to give a yellow solid, 535 mg (83%)[280]: m.p. 79-80°C, ^{1}H NMR ($CDCl_3$, 400MHz): 8.42-8.76 (bs, 2H, 2 x ArH), 8.30 (s, 1H, ArH), 7.91 (d, 2H, J = 9Hz, 2 x ArH), 7.33-7.42 (m, 4H, 4 x ArH), 6.18 (dd, 1H, J = 8.5Hz, 6.5Hz, ArCH(OH)CH_2), 2.32-2.43 (m, 1H, ArCH(OH)CHH), 2.01-2.11 (m, 1H, ArCH(OH)CHH), 1.49-1.63 (m, 2H, $CH_2CH_2CH_2$), 1.25-1.35 (septet, 2H, J = 7Hz, $CH_2CH_2CH_3$), 0.80 (t, 3H, J = 7Hz, CH_2CH_3); *m/z* (EI) 264 [M^+] (21%), 246 [M^+-18 (H_2O)] (9%), 207 (100%).

9-Anthracenyl(phenyl)methanol, (31)[282]



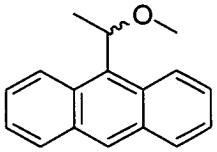
Following a procedure by Chern et al[283]. THF (10mL) was added to 9-Anthraldehyde (1.0 g/1.0 eq.) and this solution was cooled to 0°C using an ice bath. Phenylmagnesium bromide (5.33 mL/ 1.0 M, 1.2 eq.) was added slowly over 10 minutes. The reaction was stirred for a further 3h at 0°C. The reaction mixture was poured onto water (100mL) and extracted with Et_2O (3 x 100 mL). The combined organic layers were washed with brine, then concentrated in vacuo. The crude yellow oil was purified by flash chromatography (silica; Hexane/DCM) to give a yellow foam which is recrystalised from DCM/Hexane, to give cream crystals, 1.05 g (76%): m.p. 110-11°C (DCM/Hexane), literature 110-112°C[282]; ^{1}H NMR ($CDCl_3$, 300MHz): 8.52 (s, 1H, ArH), 8.37-8.42 (m, 2H, 2 x ArH), 8.04-8.08 (m, 2H, 2 x ArH), 7.39-7.50 (m, 7H, 7 x ArH), 7.21-7.34 (m, 3H, 2 x ArH, 1 x ArCH(OH)Ph), 2.72 (d, 1H, OH). ^{13}C NMR ($CDCl_3$, 75MHz): 143.8 (C), 134.0 (C), 131.8 (C), 129.9 (C), 129.3 (CH), 128.7 (CH), 128.3 (CH), 126.8 (CH), 126.1 (CH), 125.7 (CH), 125.0 (CH), 124.9 (CH), 70.3 (CH); *m/z* (EI) 284 $[M]^+$ (31%), 264 (100%).

1-(1-Pyrenyl)-ethanol, (33)[284]



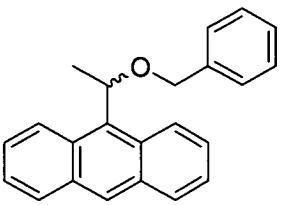
Following a procedure by Flowers et al[284]. 1-Pyrenecarboxaldehyde (500 mg/1.0 eq.) was dissolved in dry THF (15 mL), then cooled to 0°C in a ice bath. Methyl lithium (1.86 mL/1.4 M/1.2 eq.) was added to the reaction slowly over 10 min. The reaction was stirred at 0°C for 2h, then warmed to room temperature and stirred for a further 12h. The reaction was diluted with water (50 mL). The organic layer was extracted with Et_2O (3 x 50 mL). The combined organic layers were washed with brine and concentrated in vacuo. Purification was achieved by Flash Chromatography (DCM/Hexane, 1:1) to give a yellow oil/precipitate, which was recrystalised from benzene to give yellow crystals, 866 mg (81%): m.p. 112-113.5°C (Benzene), literature 113°C (Benzene)[284]; ^{1}H NMR ($CDCl_3$, 300MHz): 8.31-8.35 (d, 1H, J = 9.5Hz, ArH), 8.17-8.25 (m, 4H, 4 x ArH), 8.08-8.12 (d, 1H, J = 9.5Hz, ArH), 7.99-8.06 (m, 3H, 3 x ArH), 5.94-6.03 (q, 1H, J = 8.5Hz, ArCH(OH)CH_3), 2.22 (s, 1H, ArCH(OH)CH_3), 1.76-1.80 (d, 3H, J = 8.5Hz, ArCH(OH)CH_3). ^{13}C NMR ($CDCl_3$, 75MHz): 139.1 (C), 131.4 (C), 130.6 (C), 130.6 (C), 127.6 (CH), 127.6 (C), 127.5 (CH), 127.4 (C), 127.2 (C), 127.2 (CH), 125.9 (CH), 125.2 (CH), 125.1 (CH), 125.0 (CH), 124.8 (C), 122.5 (CH), 122.4 (CH), 67.3 (CH), 25.1 (CH_3); *m/z* (EI) 246 $[M]^+$ (55%), 202 (100%).

9-(1-Methoxy-ethyl)-anthracene (35)[285]



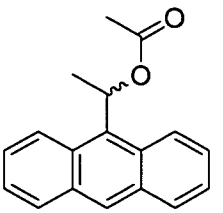
Following a procedure by Sanyal et al[285], THF (10 mL) was added to 1-(9-anthracenyl)-ethan-1-ol (500 mg/1.0 eq) and the reaction mixture cooled to 0°C in an ice bath. Dried NaH (64 mg/1.2 eq.) was suspended in a small quantity of dry THF (1 mL), which was added slowly, over 20 minutes, to the reaction mixture. After 1.5h stirring, methyl iodide (386 mg/1.2 eq.) was slowly added to the reaction, and stirred at room temperature for 5h. Crushed ice (approx. 500 mg) was added to the reaction mixture and diluted with Et_2O (50 mL). The organic layer was extracted with Et_2O (3 x 50 mL), combined, washed with brine (50 mL) and concentrated in vacuo. The crude yellow oil was purified by flash chromatography (silica; Hexane/DCM) then recrystallised from DCM/Hexane to give yellow crystals, 420 mg (79%): m.p. 93.5-94°C (DCM/Hexane), literature 94-95°C (DCM/Pentane)[285]; ^{1}H NMR ($CDCl_3$, 400MHz): 8.52-8.66 (bs, 2H, 2 x ArH), 8.3 (s, 1H, ArH), 7.89-7.94 (m, 2H, 2 x ArH), 7.34-7.42 (m, 4H, 4 x ArH), 5.84 (q, 1H, J = 7Hz, ArCH(OCH_3)CH_3), 3.12 (s, 3H, OCH_3), 1.77 (d, 3H, J = 7Hz, ArCH(OCH_3)CH_3). ^{13}C NMR ($CDCl_3$, 100MHz): 133.9 (C), 132.0 (C), 130.0 (C), 129.8 (CH), 129.7 (CH), 128.2 (CH), 126.0 (CH), 125.1 (CH), 77.0 (CH), 57.0 (CH_3), 23.0 (CH_3); *m/z* (EI) 236 $[M]^+$ (90%), 221 $[M\text{-}15(CH_3)]^+$ (100%).

9-(1-Benzyloxy-ethyl)-anthracene (36)[285]



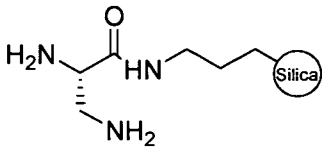
Following a procedure by Sanyal et al[285]. THF (10 mL) was added to 1-(9-Anthracenyl)-ethan-1-ol (500 mg/1.0 eq) and the reaction mixture cooled to 0°C on an ice bath. Dried NaH (64 mg/1.2 eq.) was suspended in a small quantity of dry THF (1 mL), which was added slowly, over 20 minutes, to the reaction mixture. After 1.5h stirring, benzyl bromide (460 mg/1.2 eq.) was slowly added to the reaction, and stirred at room temperature for 5h. Crushed ice (approx. 500 mg) was added to the reaction mixture and diluted with Et_2O (50 mL). The organic layer was extracted with Et_2O (3 x 50 mL), combined, washed with brine (50 mL) and concentrated in vacuo. The crude yellow oil was purified by flash chromatography (Hexane/DCM) to give a yellow oil, 502 mg (73%)[285]: ^1H NMR ($CDCl_3$, 300MHz): 8.61-8.91 (bs, 2H, 2 x ArH), 8.45 (s, 1H, ArH), 8.1 (m, 2H, 2 x ArH), 7.45-7.55 (m, 4H, 4 x ArH), 7.25-7.35 (m,5H, 5 x ArH), 6.14 (q, 1H, J = 7Hz, ArCH(OBn)CH_3), 4.40 (d, 1H, J = 12Hz, ArCH(CH_3)OCH(H)Ar'), 4.33 (d, 1H, J = 12Hz, ArCH(CH_3)OCH(H)Ar'), 1.90-1.96 (d, 3H, J = 7Hz, ArCH(OBn)CH_3). ^{13}C NMR ($CDCl_3$, 75MHz): 138.6 (C), 133.5 (C), 131.7 (C), 129.3 (C), 129.0 (CH), 128.8 (CH), 128.3 (CH), 128.0 (CH), 128.0 (CH), 127.5 (CH), 125.6 (CH), 124.8 (CH), 73.7 (CH), 70.7 (CH_2), 22.7 (CH_3); m/z (CI) 312 $[M]^+$ (12%), 205 (100%).

1-(anthracen-9-yl)ethyl acetate, (37) [285]



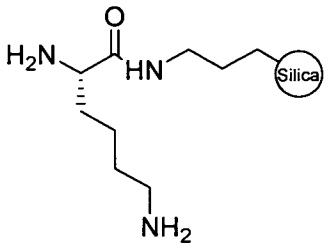
Following a procedure by Sanyal et al[285]. 1-(9-Anthracenyl)-ethan-1-ol (500 mg/1.0 eq.) and DMAP (27 mg/0.1 eq.) were dissolved in NEt_3 (5 mL). The solution was cooled to 0°C. Ac_2O (276 μL/1.2 eq.) added dropwise over 15 min. The reaction was stirred at 0°C whilst warming to room temperature overnight (15h). The reaction was diluted with Et_2O (2 mL), washed with 1M HCl (50 mL). The organic phase was washed with saturated Na_2CO_3 (50mL) and brine (50 mL), then concentrated in vacuo to give a yellow oil. Purification was achieved by Flash Chromatography (silica; Hexane/DCM, 2:1) to give yellow oil, which was crystalised with EtOH to give cream crystals 422mg (71%): m.p. 99.5-100.5°C (EtOH), literature 99-100°C (EtOH)[285]; ^{1}H NMR ($CDCl_3$, 300MHz): 8.62-8.68 (d, 2H, J = 9.5Hz, 2 x ArH), 8.45 (s, 1H, ArH), 8.01-8.06 (m, 2H, 2 x ArH), 7.44-7.60 (m, 5H, 4 x ArH, 1 x ArCH(OAc)CH_3), 2.12 (s, 3H, ArCH(CH_3)OC(O)CH_3), 1.96-2.00 (d, 3H, J = 7Hz, ArCH(OAc)CH_3). ^{13}C NMR ($CDCl_3$, 75MHz): 170.4 (C), 132.2 (C), 131.7 (C), 129.4 (CH), 129.1 (C), 128.5 (CH), 125.9 (CH), 124.8 (CH), 124.7 (CH), 68.9 (CH), 21.6 (CH_3), 21.2 (CH_3); m/z (EI) 264 $[M]^+$ (69%), 205 (100%).

H-Dap(H)-aminopropyl silica, (40)



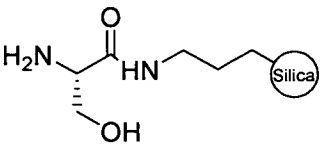
To a suspension of beaded aminopropyl silica (10 μm from ThermoHypersil, 0.45 $mmolg^{-1}$, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Boc-Dap(Boc)-OH (10 eq., 4.5 mmol, 0.60 g) and HOBt (10 eq., 4.5 mmol, 0.6 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered (Whatman microfiltration device, 12 mL volume, 5 μm pore size) and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Boc amide and ^{t}Butyl ether deprotection was achieved by shaking the fuctionalised silica in a solution of DCM/TFA/Water (50:50:1, 10 mL) for 10 min. Silica is filtered and washed with DCM (50 mL). Then skaking with DCM/TFA/Water (50:50:1, 10 mL) was repeated for 10 min. The silica was filtered, washed with DCM (50 mL) and dried.

H-Lys(H)-aminopropyl silica, (41)



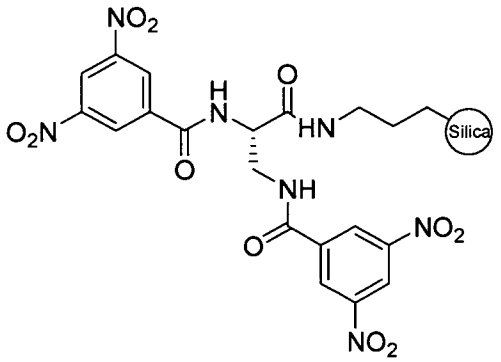
To a suspension of beaded aminopropyl silica (10 μm from ThermoHypersil, 0.45 $mmolg^{-1}$, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Lys(^{t}Bu)-OH (10 eq., 4.5 mmol, 0.60 g) and HOBt (10 eq., 4.5 mmol, 0.6 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered (Whatman microfiltration device, 12 mL volume, 5 μm pore size) and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF/piperidine (95:5, 10 mL) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF/piperidine (95:5, 10 mL) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL) and dried.

H-Ser(H)-aminopropyl silica, (42)



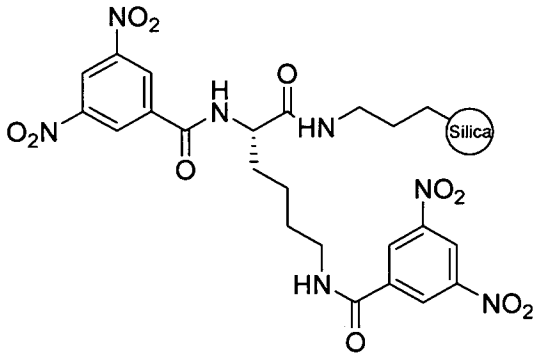
To a suspension of beaded aminopropyl silica (10 μm from ThermoHypersil, 0.45 $mmolg^{-1}$, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Ser(^{t}Bu)-OH (10 eq., 4.5 mmol, 0.60 g) and HOBt (10 eq., 4.5 mmol, 0.6 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered (Whatman microfiltration device, 12 mL volume, 5 μm pore size) and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF/piperidine (95:5, 10 mL) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF/piperidine (95:5, 10 mL) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). ^{t}Butyl ether deprotection was achieved by shaking the fuctionalised silica in a solution of DCM/TFA/Water (50:50:1, 10 mL) for 10 min. Silica is filtered an washed with DCM (50 mL). Then shaking with DCM/TFA/Water (50:50:1, 10 mL) was repeated for 10 min. The silica was filtered, washed with DCM (50 mL) and dried.

3,5-DNB-Dap(3,5-DNB)-aminopropyl silica (43)



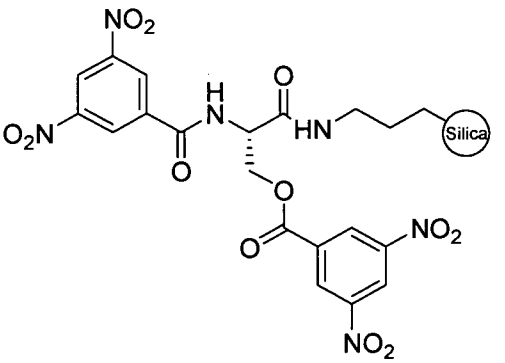
To a suspension of H-Dap(H)-aminopropyl silica **(40)** (1.0 g, 0.45 mmol) in DCM/DMF (8:2, 10 mL), was added a mixture of the 3,5-dinitrobenzoic acid (10 eq., 4.5 mmol, 0.60 g) and HOBt (10 eq., 4.5 mmol, 0.6 g) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3,5-DNB-Dap(3,5-DNB)-aminopropyl silica (44)



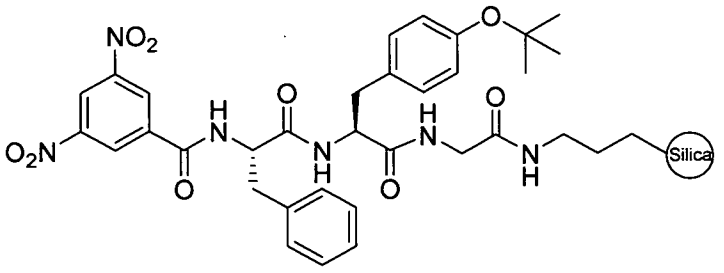
To a suspension of H-Lys(H)-aminopropyl silica **(41)** (1.0 g, 0.45 mmol) in DCM/DMF (8:2, 10 mL), was added a mixture of the 3,5-dinitrobenzoic acid (10 eq., 4.5 mmol, 0.60 g) and HOBt (10 eq., 4.5 mmol, 0.6 g) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3,5-DNB-Ser(3,5-DNB)-aminopropyl silica (45)



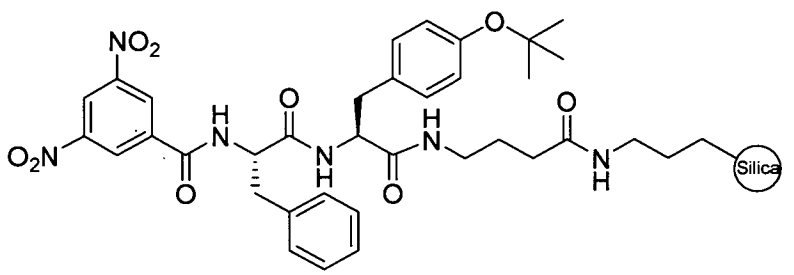
To a suspension of H-Ser(H)-aminopropyl silica **(42)** (1.0 g, 0.45 mmol) in DCM/DMF (8:2, 10 mL), was added a mixture of the 3,5-dinitrobenzoic acid (10 eq., 4.5 mmol, 0.60 g), HOBt (10 eq., 4.5 mmol, 0.6 g), DMAP (0.05 eq., 0.0225 mmol, 2.5 mg) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3,5-DNB-Phe-Tyr(^{t}Bu)-Gly-aminopropyl silica (46)



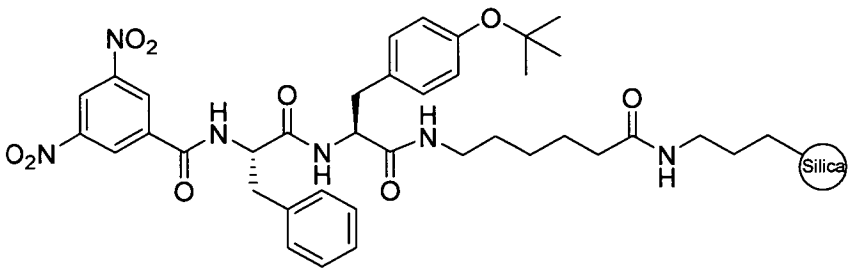
To a suspension of beaded aminopropyl silica (10, μm from ThermoHypersil, 0.45 mmolg^{-1}, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Gly-OH (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (5 eq., 2.25 mmol, 0.29 g, 0.35 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered (Whatman microfiltration device, 12 mL volume, 5 μm pore size) or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF (5% piperidine) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF (5% piperidine) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). The functionalised silica was then coupled with two further amino acids sequentially. These were Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Phe-OH and used the same coupling condition described above for Fmoc-Gly-OH. Each coupling was also followed by Fmoc deprotection. To a suspension of H-Phe-Tyr(^{t}Bu)-Gly-aminopropyl silica (1.0 g, 0.45 mmol) in DCM/DMF (8:2, 10 mL), was added a mixture of the 3,5-dinitrobenzoic acid (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3,5-DNB-Phe-Tyr(^{t}Bu)-Abu-aminopropyl silica (47)



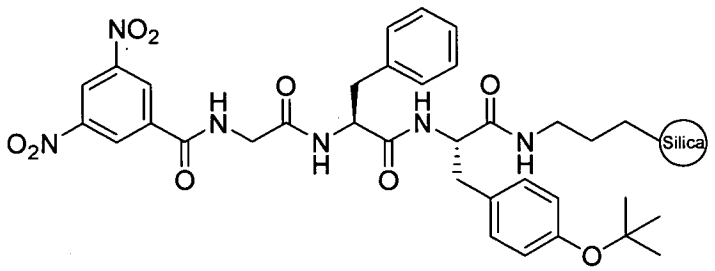
To a suspension of beaded aminopropyl silica (10, μm from ThermoHypersil, 0.45 mmolg^{-1}, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Abu-OH (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (5 eq., 2.25 mmol, 0.29 g, 0.35 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered (Whatman microfiltration device, 12 mL volume, 5 μm pore size) or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF (5% piperidine) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF (5% piperidine) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). The functionalised silica was then coupled with two further amino acids sequentially. These were Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Phe-OH and used the same coupling condition described above for Fmoc-Gly-OH. Each coupling was also followed by Fmoc deprotection. To a suspension of H-Phe-Tyr(^{t}Bu)-Abu-aminopropyl silica (1.0 g, 0.45 mmol) in DCM/DMF (8:2, 10 mL), was added a mixture of the 3,5-dinitrobenzoic acid (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3,5-DNB-Phe-Tyr(^{t}Bu)-Ahx-aminopropyl silica (48)



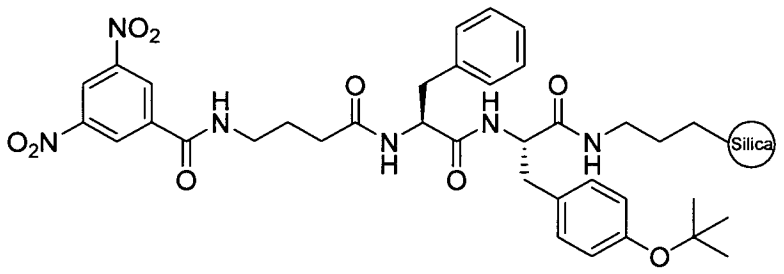
To a suspension of beaded aminopropyl silica (10, μm from ThermoHypersil, 0.45 mmolg^{-1}, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Ahx-OH (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (5 eq., 2.25 mmol, 0.29 g, 0.35 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered (Whatman microfiltration device, 12 mL volume, 5 μm pore size) or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF (5% piperidine) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF (5% piperidine) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). The functionalised silica was then coupled with two further amino acids sequentially. These were Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Phe-OH and used the same coupling condition described above for Fmoc-Gly-OH. Each coupling was also followed by Fmoc deprotection. To a suspension of H-Phe-Tyr(^{t}Bu)-Ahx-aminopropyl silica (1.0 g, 0.45 mmol) in DCM/DMF (8:2, 10 mL), was added a mixture of the 3,5-dinitrobenzoic acid (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered or centrifuged and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3,5-DNB-Gly-Phe-Tyr(^{t}Bu)-aminopropyl silica (49)



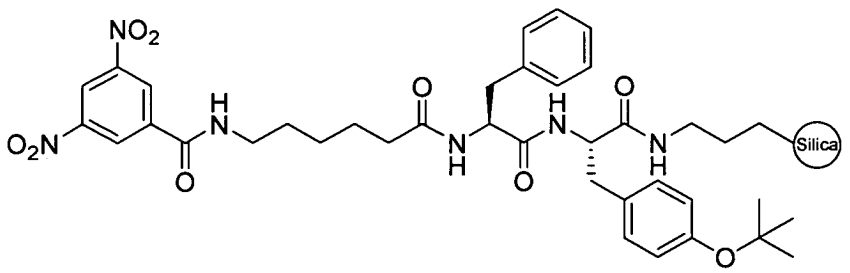
To a suspension of beaded H-Phe-Tyr(tBu)-aminopropyl silica **(4)** (10 μm, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Gly-OH (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (5 eq., 2.25 mmol, 0.29 g, 0.35 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF (5% piperidine) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF (5% piperidine) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). The functionalised silica was suspended with 3,5-dinitrobenzoic acid (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3,5-DNB-Abu-Phe-Tyr(^{t}Bu)-aminopropyl silica (50)



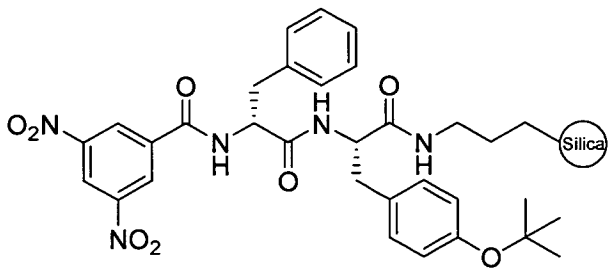
To a suspension of beaded H-Phe-Tyr(tBu)-aminopropyl silica **(4)** (10 μm, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Abu-OH (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (5 eq., 2.25 mmol, 0.29 g, 0.35 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF (5% piperidine) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF (5% piperidine) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). The functionalised silica was suspended with 3,5-dinitrobenzoic acid (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3,5-DNB-Ahx-Phe-Tyr(^{t}Bu)-aminopropyl silica (51)



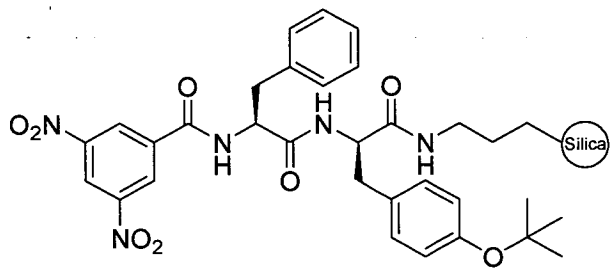
To a suspension of beaded H-Phe-Tyr(tBu)-aminopropyl silica **(4)** (10 μm, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Ahx-OH (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (5 eq., 2.25 mmol, 0.29 g, 0.35 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF (5% piperidine) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF (5% piperidine) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). The functionalised silica was suspended with 3,5-dinitrobenzoic acid (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

3,5-DNB-D-Phe-Tyr(ᵗBu)-aminopropyl silica (52)



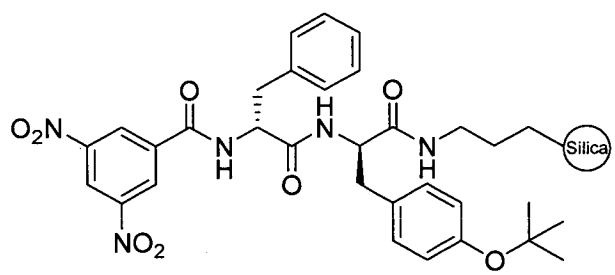
Synthesis identical to that of 3,5-DNB-Phe-Tyr(ᵗBu)-aminopropyl silica **(5)**, except Fmoc-D-Phe is used instead of Fmoc-Phe-OH.

3,5-DNB -Phe-D-Tyr(ᵗBu)-aminopropyl silica (53)



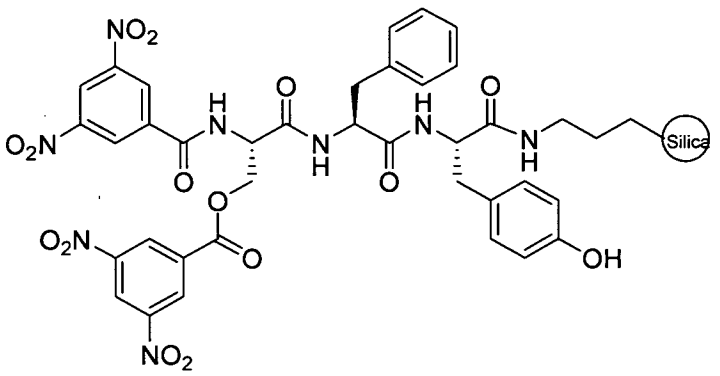
Synthesis identical to that of 3,5-DNB-Phe-Tyr(ᵗBu)-aminopropyl silica **(5)**, except Fmoc-D-Tyr(ᵗBu)-OH is used instead of Fmoc-Tyr(ᵗBu)-OH.

3,5-DNB-D-Phe-D-Tyr(ᵗBu)-aminopropyl silica (54)



Synthesis identical to that of 3,5-DNB-Phe-Tyr(ᵗBu)-aminopropyl silica **(5)**, except Fmoc-D-Tyr(ᵗBu)-OH and Fmoc-Phe-OH used instead of Fmoc-Tyr(ᵗBu)-OH and Fmoc-Phe-OH respectively.

3,5-DNB-Ser(3,5-DNB)-Phe-Tyr(H)-aminopropyl silica (56)



To a suspension of beaded H-Phe-Tyr(tBu)-aminopropyl silica **(4)** (10 μm, 1.0 g, 0.45 mmol) in a solution of DCM/DMF (8:2, 10 mL), was added a mixture of Fmoc-Ser(^{t}Bu)-OH (5 eq., 2.25 mmol, 0.30 g) and HOBt (5 eq., 2.25 mmol, 0.3 g) in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (5 eq., 2.25 mmol, 0.29 g, 0.35 mL) was added. The reaction mixture was then heated at reflux (50°C) for 3h. After cooling, the beads were filtered and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the functionalised silica in DMF (5% piperidine) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF (5% piperidine) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL). ^{t}Butyl ether deprotection was achieved by shaking the silica in a solution of DCM/TFA/Water (50:50:1, 10 mL) for 10 min. Silica was filtered and washed with DCM (50 mL). Then shaking with DCM/TFA/Water (50:50:1, 10 mL) was repeated for 10 min. The silica was filtered, washed with DCM (50 mL) and dried. The functionalised silica was suspended with 3,5-dinitrobenzoic acid (5 eq., 2.25 mmol, 0.30 g), HOBt (5 eq., 2.25 mmol, 0.3 g) and DMAP (0.05 eq., 0.0225 mmol, 2.5 mg) in DCM/DMF (8:2, 10mL). After gentle shaking of the resulting mixture for 10 min, DIC (10 eq., 4.5 mmol, 0.58 g, 0.7 mL) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered and washed with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

7.3.2 Solid-phase loading from Elemental analysis

Using:

$$\frac{\text{Loading (mmolg}^{-1}\text{) x m}_a\text{ (gmol}^{-1}\text{) x atoms in compound}}{1000} \times 100 = \text{Elemental composition (\%)}$$

CSP	Formula	C / %	H / %	N / %	C*	H*	N*	Average*
(5)	Silica-$C_{32}H_{36}N_5O_8$	14.22	1.34	2.66	0.37	0.37	0.38	0.37
(6)	Silica-$C_{32}H_{36}N_5O_8$	14.64	1.22	2.53	0.38	0.34	0.36	0.36
(7)	Silica-$C_{32}H_{36}N_5O_8$	13.58	1.44	2.42	0.35	0.40	0.35	0.37
(8)	Silica-$C_{32}H_{37}N_4O_6$	14.5	1.24	1.92	0.38	0.34	0.34	0.35
(9)	Silica-$C_{32}H_{38}N_3O_4$	12.89	1.11	1.51	0.34	0.29	0.36	0.33
(10)	Silica-$C_{34}H_{42}N3O_6$	14.02	1.17	1.43	0.34	0.28	0.34	0.32
(11)	Silica-$C_{32}H_{38}N_3O_6$	12.65	1.07	1.61	0.33	0.28	0.38	0.33
(12)	Silica-$C_{32}H_{40}N_5O_4$	13.34	1.24	2.34	0.35	0.31	0.33	0.33
(13)	Silica-$C_{36}H_{40}N_3O_4$	13.89	1.27	1.33	0.32	0.32	0.32	0.32
(14)	Silica-$C_{31}H_{37}N_4O_4$	12.34	1.19	1.98	0.33	0.32	0.35	0.34
(15)	Silica-$C_{30}H_{36}N_3O_4S$	10.03	1.23	1.33	0.28	0.34	0.32	0.31
(16)	Silica-$C_{39}H_{44}N_3O_4$	12.89	1.33	1.68	0.28	0.30	0.40	0.33
(17)	Silica-$C_{39}H_{52}N_3O_4$	13.43	1.55	1.45	0.29	0.30	0.35	0.31
(18)	Silica-$C_{32}H_{36}F_2N_3O_4$	12.94	1.45	1.52	0.34	0.40	0.36	0.37
(19)	Silica-$C_{34}H_{36}F_6N_3O_4$	12.88	1.33	1.32	0.32	0.37	0.31	0.33
(20)	Silica-$C_{32}H_{38}N_3O_{10}S_2$	11.99	1.33	1.63	0.31	0.35	0.39	0.35
(21)	Silica-$C_{36}H_{42}N_3O_8$	13.75	1.34	1.29	0.32	0.32	0.31	0.31
(22)	Silica-$C_{36}H_{44}N_5O_6$	14.54	1.55	2.09	0.34	0.35	0.30	0.33
(43)	Silica-$C_{36}H_{42}N_3O_8$	13.75	1.34	1.29	0.32	0.32	0.31	0.31

(53)	Silica-$C_{36}H_{44}N_5O_6$	14.54	1.55	2.09	0.34	0.35	0.30	0.33

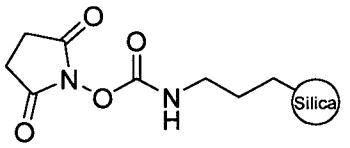
* = loading / $mmolg^{-1}$

Table 16 Elemental analysis and loading data for solid-phase compounds.

7.4 Experimental for Chapter 3

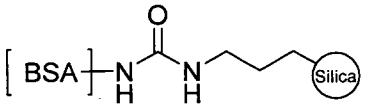
7.4.1 Synthesis of compounds

succinimidylcarbonate-aminopropyl silica (57)



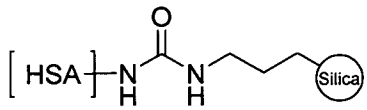
Using a procedure by Haginaka et al[128]. Aminopropyl silica (1.0 g, 0.45 mmolg^{-1}, 10 μm) was slurried in acetonitrile (20 mL) and reacted with *N,N*-disuccinimidyl carbonate (1.0 g) at 30 °C for 24 h. The silica was filtered and washed with acetonitrile (3 x 20 mL), water (2 x 20 mL) and methanol (2 x 20 mL) and then dried in vacuo.

BSA-urea-aminopropyl silica (58)



Using a procedure by Haginaka et al[128]. Succinimidylcarbonate-aminopropyl silica **(57)** (1.0 g, 0.45 mmolg^{-1}, 10 μm) was slurried in phosphate buffer (10 mL, 20 mM, pH 6.8). Bovine Serum Albumin (300 mg, Sigma-Aldrich, lyophilized powder, ≥95%) in phosphate buffer (5 mL, 20 mM, pH 6.8) was slowly added to the mixture at room temperature over 1 h. The reaction pH was monitored during the protein addition and corrected as necessary to keep it steady at 6.8. The reaction was stirred for a further 24 h at 30 °C. After cooling the reaction mixture was washed with water and reacted with; aminoethanol/water (1:50) adjusted to pH 6.8 with HCl, at room temperature for 1 h. The silica was filtered and washed with water (3 x 30 mL), water/ethanol (95:5, 3 x 20 mL).

HSA-urea-aminopropyl silica (59)

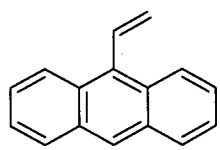


Procedure identical to BSA-urea-aminoproply silica **(58)**, except the use of Human Serum Albumin instead of Bovine Serum Albumin.

7.5 Experimental for Chapter 4

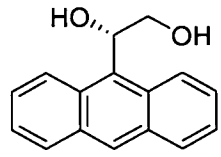
7.5.1 Synthesis of compounds

9-vinylanthracene (60)[286, 287]



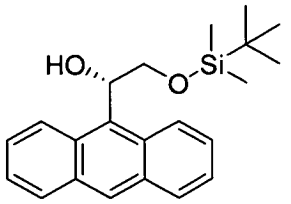
Using a procedure by Schurch et al[253], methyltriphenylphosphonium bromide (19.05 g, 53.34 mmol, 1.1 eq.) was added to THF (100 mL). The suspension was stirred at 0 °C and n-BuLi (1.0 M in THF, 50.91 mL, 50.91 mmol, 1.05 eq.) was added slowly. The mixture was warmed to room temperature and stirred for 30 min. 9-Anthraldehyde (10 g, 48.49 mmol, 1 eq.) in THF (100 mL) was added slowly to the reaction, which was stirred for a further 15 h, then MeOH (1 mL) added to the reaction. The slurry was diluted with petroleum ether (100 mL) and the supernatant solution was decanted and filtered through celite. The solids remaining in the mask were washed with petroleum ether (3 x 100 mL) and the supernatant solutions filtered through celite. The filtrate was concentrated in vacuo. Purification achieved by flash chromatography (silica; hexane/EtOAc) to give 9-vinylanthracene as a yellow solid, 9.32 g (80%): m.p. 58-59°C (DCM), literature: 60-63°C [286]; ^{1}H NMR ($CDCl_3$, 250MHz): 8.58 (s, 1H, 1 x ArH), 8.56-8.48 (m, 2H, 2 x ArH), 8.16-8.24 (m, 5H, 4 x ArH), 6.21 (dd, 2H, J = 12Hz, J = 4Hz), 5.83 (dd, 2H, J = 12Hz, J = 4Hz). ^{13}C NMR ($CDCl_3$, 62.5MHz): 134.0 (C), 133.9 (CH), 131.8 (C), 129.6 (C), 129.0 (CH), 126.7 (CH), 126.4 (CH), 125.7 (CH), 125.5 (CH), 123.3 (CH_2); *m/z* (EI): 204 $[M]^+$ (93%), 203 $[M\text{-}H]^+$ (100%). HR-*m/z* (EI): $[M]^+$ calculated: 204.0939, found: 204.0940.

(S)-1-(9-anthracenyl)ethane-1,2-diol (61)[253]



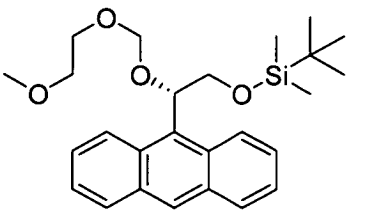
Using a procedure by Schurch et al[253], AD-mix α (50.0 g, 31.96 mmol, 1.1 eq.) in Water/^{t}BuOH (1:1, 500 mL) was cooled to 0 °C. 9-Vinylanthracene **(60)** (5.75 g, 28.14 mmol, 1.0 eq.) was added to the reaction which was stirred at 0 °C for 24 h. The mixture was quenched with Na_2SO_3 (38 g), warmed to room temperature, and stirred for 1 h. Organic layers was extracted with EtOAc (3 x 200 mL) and combined and washed with water (2 x 100 mL), dried with $MgSO_4$, filtered and concentrated in vacuo. Purification achieved by flash chromatography (silica; hexane/EtOAc), which gave (S)-1-(9-anthracenyl)ethane-1,2-diol as yellow crystals, 4.45 g (78%, 98%ee from Chiral-HPLC (Chiralcel-OD; Heptane/2-PrOH (90:10) screened versus opposite enantiomer)). m.p. 129-130 °C (DCM/Hexane); ^{1}H NMR ($CDCl_3$, 250MHz): 8.47 (d, H, J = 9 Hz, 2 x ArH), 8.24 (s, 1H, 1 x ArH), 7.87-7.78 (m, 2H, 2 x ArH), 7.37-7.24 (m, 4H, 4 x ArH), 6.19 (dd, 1H, J = 10Hz, J = 4Hz), 4.28 (dd, 1H, J = 12Hz, J = 10Hz), 3.73 (dd, 1H, J = 12Hz, J = 4Hz). ^{13}C NMR ($CDCl_3$, 62.5MHz): 132.3 (C), 130.4 (C), 130.1 (C), 129.2 (CH), 129.0 (CH), 126.1 (CH), 125.2 (CH), 125.0 (CH), 74.6 (CH), 69.5 (CH_2); *m/z* (ES+): 261 $[M+Na]^+$; HR-*m/z* (FAB): $[M]^+$ ($C_{16}H_{14}O_2$) calculated: 238.0994, found: 238.0999.

(S)-1-(9-anthracenyl)-2-(tert-butyldimethylsilyloxy) ethanol (62)



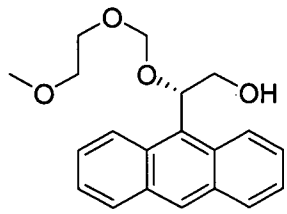
Using a procedure by Nieuwenhuis et al[255]. (S)-1-(9-anthracenyl)ethane-1,2-diol **(61)** (10.0 g, 42.00 mmol, 1.0 eq.) was dissolved in DMF (100 mL). Imidazole (2.86 g, 84.00 mmol, 2.0 eq.) and *tert*-butyldimethylsilyl chloride (6.33 g, 42.00 mmol, 1.0 eq.) were added at room temperature. After stirring for 24 h at room temperature, the solution was poured into water (50 mL) and extracted with Et_2O (3 x 50 mL). The combined organic layers were washed with brine (50 mL), dried with $MgSO_4$, filtered and concentrated in vacuo. Purification was achieved by flash chromatography (silica; hexane/EtOAc) to give (S)-1-(9-anthracenyl)-2-(tert-butyldimethylsilyloxy) ethanol as a clear/yellow oil, 11.09 g (75%); ^{1}H NMR ($CDCl_3$, 250MHz): 8.60 (d, 2H, J = 8 Hz, 2 x ArH), 8.32 (s, 1H, 1 x ArH), 7.92-7.87 (m, 2H, 2 x ArH), 7.44-7.32 (m, 4H, 4 x ArH), 6.24 (dd, 1H, J = 10 Hz, J = 4 Hz), 4.28 (dd, 1H, J = 12Hz, J = 10Hz), 3.82 (dd, 1H, J = 12Hz, J = 4Hz), 3.16 (s, 1H), 0.88 (s, 9H), 0.04 (s, 3H), 0.00 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 132.0 (C), 130.6 (C), 130.4 (C), 129.7 (CH), 129.0 (CH), 126.1 (CH), 125.3 (CH), 125.1 (CH), 72.6 (CH), 67.1 (CH_2), 26.4 (CH_3), 18.8 (C), -4.8 (CH_3); *m/z* (ES+): 335 $[M\text{-}OH]^+$ (100%), 375 $[M+Na]^+$ (20%), 407 $[M+Na+MeOH]^+$ (15%).

(S)-1-(9-anthracenyl)-1-((2-methoxyethoxy)methoxy)-2-(tert-butyldimethylsilyloxy) ethane (63)



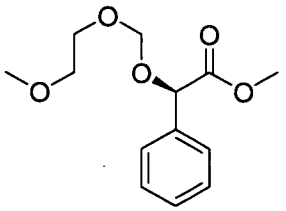
Using a procedure by Williams et al[266]. (S)-1-(9-anthracenyl)-2-(tert-butyldimethylsilyloxy) ethanol **(62)** (2.0 g, 5.672 mmol, 1.0 eq.) was dissolved in DCM (20 mL). DIPEA (1.48 mL, 8.308 mmol, 1.5 eq.) was added to the stirred reaction. Methoxyethoxymethyl chloride (1.06 mL, 8.308 mmol, 1.5 eq.) was added slowly to the reaction, which was stirred for a further 6 h. Then the solution was poured into water (20 mL) and extracted with Et_2O (3 x 50 mL). The combined organic layers were washed with brine (50 mL), dried with $MgSO_4$, filtered and concentrated in vacuo. Purification was achieved by flash chromatography (silica; hexane/EtOAc) to give (S)-1-(9-anthracenyl)-1-(methoxyethoxymethoxy)-2-(tert-butyldimethylsilyloxy) ethane as a clear oil, 1.83 g (73%). ^{1}H NMR ($CDCl_3$, 250MHz): 9.09 (s, 1H, 1 x ArH), 8.54 (s, 2H, 2 x ArH), 8.13-8.10 (m, 2H, 2 x ArH), 7.65-7.54 (m, 4H, 4 x ArH), 6.42 (dd, 1H, J = 8 Hz, J = 4 Hz), 5.02 (d, 1H, J = 7 Hz), 4.82 (d, 1H, J = 7 Hz), 4.64 (dd, 1H, J = 11 Hz, J = 8 Hz), 4.11 (dd, 1H, J = 11 Hz, J = 4 Hz), 3.62-3.48 (m, 2H), 3.42-3.35 (m, 4H), 0.93 (s, 9H), 0.06 (s, 3H), 0.01 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 132.2 (C), 131.6 (C), 130.5 (C), 129.5 (CH), 128.7 (CH), 127.3 (CH), 126.6 (CH), 125.2 (CH), 94.6 (CH_2), 67.1 (CH_2), 76.5 (CH), 72.0 (CH_2), 67.0 (2 x CH_2), 59.2 (CH_3), 26.2 (CH_3), 18.7 (C), -5.0 (CH_3); *m/z* (ES+): 463 $[M+Na]^+$ (100%). Elem. ($C_{26}H_{36}O_4Si$) calculated: C (70.80%), H (8.23%), found: C (70.66%), H (8.49%).

(S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy) ethanol (64)



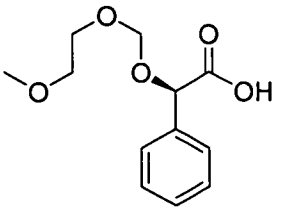
Using a procedure by Boger et al[238]. (S)-1-(9-anthracenyl)-1-(methoxyethoxymethoxy)-2-(tert-butyldimethylsilyloxy) ethane **(63)** (1.50 g, 3.404 mmol, 1.0 eq.) was dissolved in MeCN (15 mL). Bu_4NF (1 M in THF, 6.81 mL, 6.808 mmol, 2.0 eq.) was slowly added to the stirred reaction. The reaction was stirred for a further 1 h, then the solution was poured into water (20 mL) and extracted with Et_2O (3 x 50 mL). The combined organic layers were washed with brine (50 mL), dried with $MgSO_4$, filtered and concentrated in vacuo. Purification was achieved by flash chromatography (silica; hexane/EtOAc) to give (S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy) ethanol as a clear oil, 1.06 g (95%). ^1H NMR ($CDCl_3$, 250MHz): 8.72 (bs, 2H, 2 x ArH), 8.22 (s, 1H, 1 x ArH), 7.82-7.75 (m, 2H, 2 x ArH), 7.32-7.20 (m, 4H, 4 x ArH), 6.10 (dd, 1H, J = 8 Hz, J = 4 Hz), 4.61 (d, 1H, J = 7 Hz), 4.48 (d, 1H, J = 7 Hz), 4.32 (dd, 1H, J = 12 Hz, J = 8 Hz), 3.78-3.60 (m, 2H), 3.43-3.18 (m, 3H), 3.15 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 132.2 (C), 130.7 (C), 129.6 (CH), 129.3 (C), 129.1 (CH), 127.3 (CH), 126.6 (CH), 125.3 (CH), 95.0 (CH_2), 78.5 (CH), 72.2 (CH_2), 67.9 (CH_2), 66.2 (CH_2), 59.4 (CH_3); *m/z* (ES-): 325 $[M-H]^-$ (100%), 326 $[M(1 \times C^{13})-H]^-$ (30%).

(R)-methyl 2-((2-methoxyethoxy)methoxy)-2-phenylacetate (65)



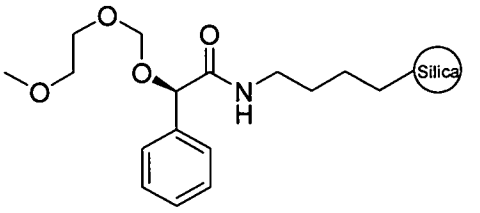
Using a procedure by Williams et al[266]. (S)-(+)-Methyl mandelate (1.0 g, 6.018 mmol, 1.0 eq.) was dissolved in DCM (20 mL). DIPEA (1.572 mL, 9.027 mmol, 1.5 eq.) was added to the stirred reaction. Methoxyethoxymethyl chloride (1.031 mL, 9.027 mmol, 1.5 eq.) added slowly to the reaction, which was stirred for a further 6 h. Then the solution was poured into water (10 mL) and extracted with Et_2O (3 x 25 mL). The combined organic layers were washed with brine (25 mL), dried with $MgSO_4$, filtered and concentrated in vacuo. Purification was achieved by flash chromatography (silica; hexane/EtOAc) to give (R)-methyl 2-((2-methoxyethoxy)methoxy)-2-phenylacetate as a clear oil, 1.22 g (80%). ^{1}H NMR ($CDCl_3$, 250MHz): 7.30-7.25 (m, 2H, 2 x ArH), 7.23-7.14 (m, 3H, 3 x ArH), 5.06 (s, 1H), 4.68 (d, 1H, J = 7 Hz), 4.60 (d, 1H, J = 7 Hz), 3.67-3.58 (m, 1H), 3.54 (s, 3H), 3.52-3.44 (m, 1H), 3.35-3.30 (m, 2H), 3.18 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 171.6 (C), 136.5 (C), 129.1 (CH), 129.0 (CH), 127.8 (CH), 94.4 (CH_2), 77.1 (CH), 72.0 (CH_2), 67.8 (CH_2), 59.3 (CH_3), 52.7 (CH_3); *m/z* (EI) 254 $[M]^+$ (100%).

(R)-2-((2-methoxyethoxy)methoxy)-2-phenylacetic acid (66)[288]



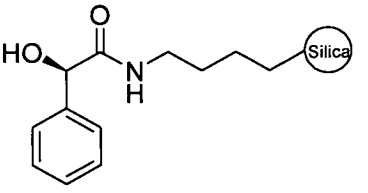
(R)-methyl 2-((2-methoxyethoxy)methoxy)-2-phenylacetate **(65)** (500 mg, 1.966 mmol, 1.0 eq.) was dissolved in MeOH (5 mL). To the stirred reaction was added a solution of LiOH (239 mL, 10.0 mmol) in water (5 mL) and the reaction was stirred for a further 6 h. Then the reaction mixture was extracted with Et_2O (3 x 25 mL). The combined organic layers were washed with brine (25 mL), dried with $MgSO_4$, filtered and concentrated in vacuo. Purification was achieved by flash chromatography (silica; hexane/EtOAc) to give ((R)-2-((2-methoxyethoxy)methoxy)-2-phenylacetic acid as a clear oil, 270 mg (57%). ^1H NMR ($CDCl_3$, 250MHz): 8.70-8.20 (bs, 1H), 7.30-7.22 (m, 2H, 2 x ArH), 7.20-7.13 (m, 3H, 3 x ArH), 5.06 (s, 1H), 4.65 (d, 1H, J = 7 Hz), 4.56 (d, 1H, J = 7 Hz), 3.67-3.58 (m, 1H), 3.52-3.44 (m, 1H), 3.35-3.30 (m, 2H), 3.17 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 175.1 (C), 135.9 (C), 129.3 (CH), 129.1 (CH), 127.9 (CH), 94.4 (CH_2), 76.9 (CH), 72.0 (CH_2), 68.2 (CH_2), 59.3 (CH_3); *m/z* (EI) 239 $[M-H]^+$ (100%).

(R)-2-((2-methoxyethoxy)methoxy)-2-phenylacetoyl aminopropyl silica (67)



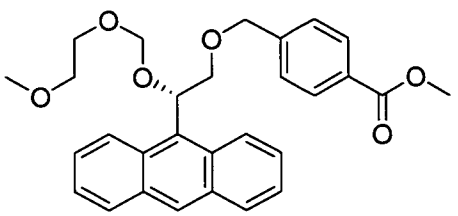
To a suspension of aminopropyl silica (10 μm, 0.45 $mmolg^{-1}$, 1.0 g, 0.45 mmol, 1.0 eq.) in DCM/DMF (8:2, 5 mL), was added a mixture of the ((S)-2-((2-methoxyethoxy)methoxy)-2-phenylacetic acid (203 mg, 0.9 mmol, 2.0 eq.) and HOBt (120 mg, 0.9 mmol, 2.0 eq.) in DCM/DMF (8:2, 5mL). After gentle shaking of the resulting mixture for 10 min, DIC (140 μL, 0.9 mmol, 2.0 eq.) was added. The reaction mixture was then heated to reflux (50°C) for 3h. After cooling the beads were filtered and washed with DMF (2 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

(R)-2-hydroxy-2-phenylacetoyl aminopropyl silica (68)



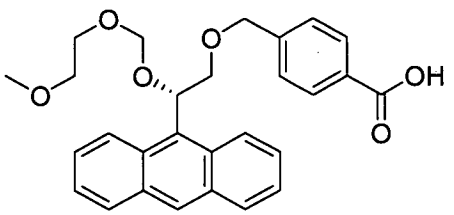
Using a procedure by Moreau et al[264]. (R)-2-((2-methoxyethoxy)methoxy)-2-phenylacetoyl aminopropyl silica **(67)** (700 mg, 0.3150 mmol, 1 eq.) was suspended in DCM (10 mL). $ZnBr_2$ (709 mg, 3.150 mmol, 10 eq.) was added to the reaction which was stirred at room temperature for 96 h. EtOH (made up to 40 mL) was added to the reaction and this was centrifuged (8000 rpm, 5 min), the supernatant was removed and the centrifuge procedure was repeated (3 x 40 mL). The silica was then dried in vacuo. The compound was then compared to **(67)** by elemental analysis (Section 7.5.2)

(S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy)ethoxymethyl-4-methylbenzoate (69)



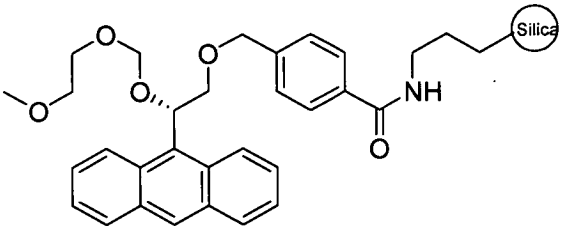
Using a procedure by Yamaguchi et al[289], to a stirred suspension of NaH (60% in mineral oil, 147 mg, 3.679 mmol, 1.5 eq.) in THF (10 mL) was added dropwise a solution of (S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy) ethanol **(64)** (800 mg, 2.453 mmol, 1.0 eq.) in THF (20 mL) over 1 h. The reaction was stirred for a further 1 h. Then methyl 4-(bromomethyl)benzoate (618 mg, 2.698 mmol, 1.1 eq.)was added and the reaction was stirred for a further 1 h, then water was added and the solvents removed in vacuo. The remaining suspension was diluted with water and EtOAc and acidified with HCl. Organic layers were extracted with EtOAc (3 x 50 mL), washed with brine (50 mL), dried with $MgSO_4$, filtered and concentrated in vacuo. Purification was achieved by flash chromatography (silica; hexane/EtOAc) to give (S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy)ethoxymethyl(4-methylbenzoate) as a yellow oil, 800 mg (69%). ^{1}H NMR ($CDCl_3$, 250MHz): 9.23 (bs, 1H, 1 x ArH), 8.67 (s, 2H, 2 x ArH), 8.27-8.18 (m, 4H, 4 x ArH), 7.80-7.68 (m, 4H, 4 x ArH), 7.60 (d, 2H, J = 8 Hz, 2 x ArH), 6.75 (dd, 1H, J = 9 Hz, J = 4 Hz), 5.12 (d, 1H, J = 7 Hz), 5.02-4.85 (m, 3H), 4.83-4.73 (m, 1H), 4.17 (s, 3H), 4.05-3.97 (m, 2H), 3.78-3.63 (m, 3H), 3.53 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 167.4 (C), 144.0 (C), 130.8 (CH), 130.0 (CH), 129.8 (C), 129.7 (2 x C), 129.6 (CH), 129.0 (CH), 127.3 (CH), 126.7 (CH), 125.3 (CH), 125.1 (CH), 94.3 (CH_2), 78.3 (CH), 74.2 (CH_2), 73.0 (CH_2), 72.0 (CH_2), 67.3 (CH_2), 59.3 (CH_3), 52.5 (CH_3); *m/z* (ES+): 497 $[M+Na]^+$ (100%).

(S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy)ethoxymethyl-4-benzoic acid (70)



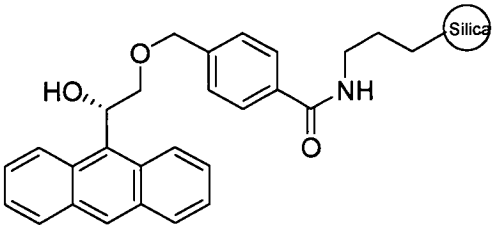
(S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy)ethoxymethyl-4-methylbenzoate **(69)** (750 mg, 1.582 mmol, 1.0 eq.) was dissolved in MeOH (20 mL). To the stirred reaction was added a solution of NaOH (632 mg, 10.0 mmol) in MeOH (20 mL), the reaction was heated to 50 °C and stirred for a further 96 h. After cooling the solvents were removed in vacuo. The residue was redissolved in EtOAc (25 mL) and water (25 mL). The aqueous layer was acidified with HCl and the organic layer was extracted with EtOAc (3 x 25 mL). The combined organic layers were washed with brine (25 mL), dried with $MgSO_4$, filtered and concentrated in vacuo. Purification was achieved by flash chromatography (silica; hexane/EtOAc) to give (S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy)ethoxymethyl-4-benzoic acid as an orange waxy oil/solid, 560 mg (77%). ^{1}H NMR ($CDCl_3$, 250MHz (Na salt)): 8.84 (bs, 1H, 1 x ArH), 8.33 (s, 2H, 2 x ArH), 7.96-7.87 (m, 4H, 4 x ArH), 7.45-7.32 (m, 4H, 4 x ArH), 7.27 (d, 2H, J = 8 Hz, 2 x ArH), 6.42 (dd, 1H, J = 9 Hz, J = 4 Hz), 4.80 (d, 1H, J = 7 Hz), 4.56-4.45 (m, 3H), 4.44-4.38 (m, 1H), 3.85-3.74 (m, 2H), 3.45-3.30 (m, 3H), 3.2 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 171.8 (C), 144.9 (C), 131.8 (C), 131.6 (C), 130.7 (CH), 129.8 (C), 129.7 (C), 129.6 (CH), 129.0 (CH), 127.4 (CH), 127.1 (CH), 125.8 (CH), 125.3 (CH), 94.3 (CH_2), 74.3 (CH), 74.2 (CH_2), 73.0 (CH_2), 72.0 (CH_2), 67.3 (CH_2), 59.3 (CH_3); *m/z* (ES+): 483 $[M+Na]^+$ (100%).

(S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy)ethoxymethyl-4-benzoyl aminopropyl silica (71)



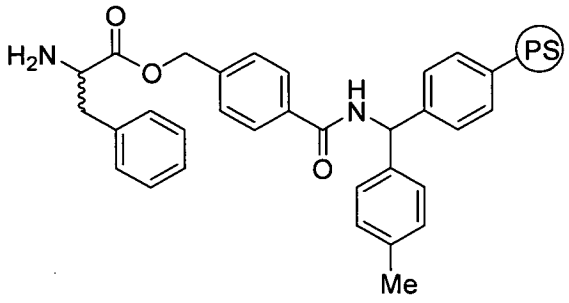
2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy)ethoxymethyl-4-benzoic acid (265 mg, 0.576 mmol, 1.28 eq.) and HOBt (78 mg, 0.576 mmol, 1.28 eq.) were dissolved in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (89 μL, 0.576 mmol, 1.28 eq.) was added. After a further 10 min shaking, the reaction was charged with aminopropyl silica (12 μm, 0.45 $mmolg^{-1}$, 1.0 g, 0.45 mmol, 1.0 eq.). The reaction mixture was stirred at room temperature for 24 h, then heated to reflux (50°C) for 3h. After cooling the beads were filtered and washed with DMF (2 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying under high vacuum. A negative TNBS indicated complete coupling.

(S)-2-(9-anthracenyl)-2-hydroxy-ethoxymethyl-4-benzoyl aminopropyl silica (72)



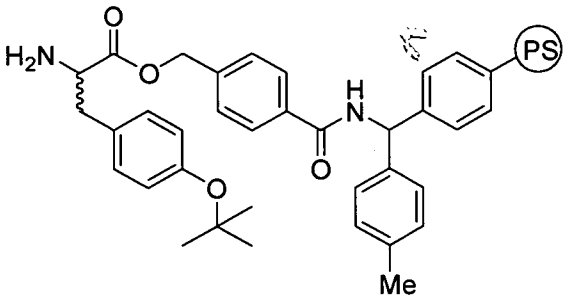
Using a procedure by Moreau et al[264], (S)-2-(9-anthracenyl)-2-((2-methoxyethoxy)methoxy)ethoxymethyl-4-benzoyl aminopropyl silica **(67)** (1199 mg, 0.45 mmol, 1 eq.) was suspended in DCM (20 mL). ZnBr (1013 mg, 4.5 mmol, 10 eq.) was added to the reaction which was stirred at room temperature for 96 h. EtOH (made up to 40 mL) was added to the reaction and this was centrifuged (8000 rpm, 5 min), the supernatant was removed and the centrifuge procedure was repeated (3 x 40 mL). The silica was then dried in vacuo.

H_2N-D/L-Phe-HMBA-aminomethyl polystyrene (73)



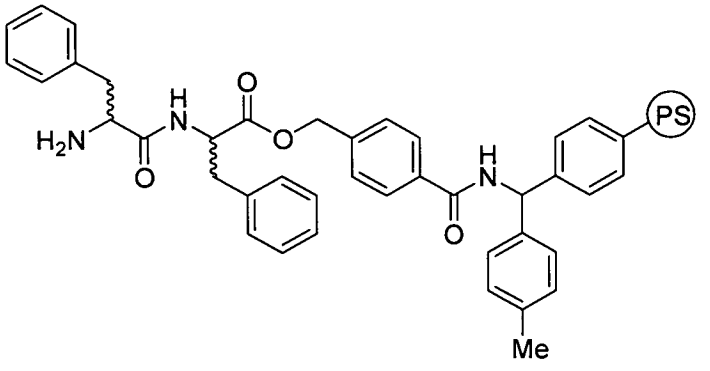
Based on a procedure by Novabiochem®[290], Fmoc-D/L-Phe-OH (496 mg, 1.28 mmol, 5 eq.) dissolved in DCM/DMF (1:1, 10 mL and HOBt (173 mg, 1.28 mmol, 5 eq.). After gentle shaking for 10 min, DIC (200 μL, 1.28 mmol, 5 eq.) was added to the mixture. This mixture was shaken for 15 min, then added to HMBA-AM polystyrene resin (0.8 $mmolg^{-1}$, 0.32 g, 0.256 mmol, 1 eq.) preswelled in DMF (20 mL). Excess DMF was removed after swelling. DMAP (3 mg, 0.0256 mmol, 0.1 eq.) was added to the reaction which was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL). The resin was then swelled in DMF and shaken in a solution of DMF/piperidine (80:20, 15 mL) for 15 min. The resin was then filtered and washed with DMF (2 x 20 mL), followed by shaking again in DMF/piperidine (80:20, 15 mL) for 15 min. The resin was filtered, washed with DMF (4 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

H_2N-D/L-Tyr(O^{t}Bu)-HMBA-aminomethyl polystyrene (74)



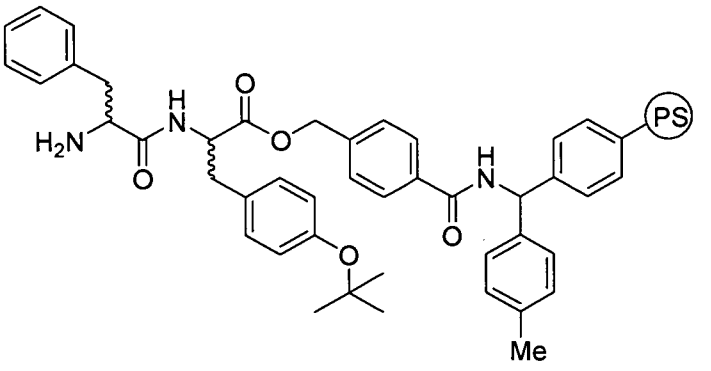
Based on a procedure by Novabiochem®[290], Fmoc-D/L-Tyr-OH (587 mg, 1.28 mmol, 5 eq.) was dissolved in DCM/DMF (1:1, 10 mL) and HOBt (173 mg, 1.28 mmol, 5 eq.). After gentle shaking for 10 min, DIC (200 μL, 1.28 mmol, 5 eq.) was added to the mixture. This mixture was shaken for 15 min, then added to HMBA-AM polystyrene resin (0.8 $mmolg^{-1}$, 0.32 g, 0.256 mmol, 1 eq.) preswelled in DMF (20 mL). Excess DMF was removed after swelling. DMAP (3 mg, 0.0256 mmol, 0.1 eq.) was added to the reaction which was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL). The resin was then swelled in DMF and shaken in a solution of DMF/piperidine (80:20, 15 mL) for 15 min. The resin was then filtered and washed with DMF (2 x 20 mL), followed by shaking again in DMF/piperidine (80:20, 15 mL) for 15 min. The resin was filtered, washed with DMF (4 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

H_2N-D/L-Phe-D/L-Phe-HMBA-aminomethyl polystyrene (75)



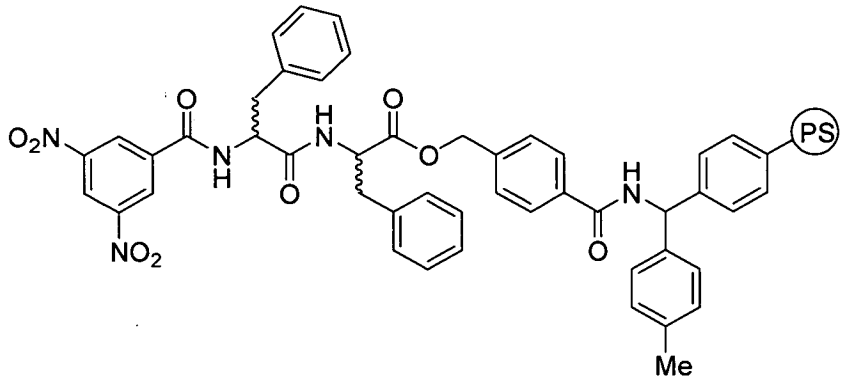
Fmoc-D/L-Phe-OH (496 mg, 1.28 mmol, 5 eq.) was dissolved in DCM/DMF (1:1, 10 mL), to this was added HOBt (173 mg, 1.28 mmol, 5 eq.). After gentle shaking for 10 min, DIC (200 µL, 1.28 mmol, 5 eq.) was added to the mixture. This mixture was shaken for 15 min, then added to H_2N-D/L-Phe-HMBA-aminomethyl polystyrene **(73)** (0.8 $mmolg^{-1}$, 0.32 g, 0.256 mmol, 1 eq.) preswelled in DMF (20 mL). Excess DMF was removed after swelling. The reaction was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL). The resin is then swelled in DMF and shaken in a solution of DMF/piperidine (80:20, 15 mL) for 15 min. The resin is then filtered and washed with DMF (2 x 20 mL), followed by shaking again in DMF/piperidine (80:20, 15 mL) for 15 min. The resin was filtered, washed with DMF (4 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

H_2N-D/L-Phe-D/L-Tyr(O^{t}Bu)-HMBA-aminomethyl polystyrene (76)



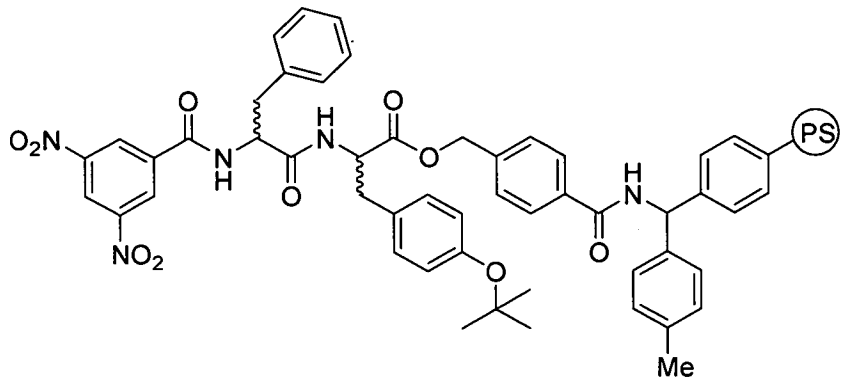
Fmoc-D/L-Phe-OH (496 mg, 1.28 mmol, 5 eq.) was dissolved in DCM/DMF (1:1, 10 mL), to this was added HOBt (173 mg, 1.28 mmol, 5 eq.). After gentle shaking for 10 min, DIC (200 μL, 1.28 mmol, 5 eq.) was added to the mixture. This mixture was shaken for 15 min, then added to H_2N-D/L-Tyr(O^{t}Bu)-HMBA-aminomethyl polystyrene **(74)** (0.8 mmolg^{-1}, 0.32 g, 0.256 mmol, 1 eq.) preswelled in DMF (20 mL). Excess DMF was removed after swelling. The reaction was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL). The resin was then swelled in DMF and shaken in a solution of DMF/piperidine (80:20, 15 mL) for 15 min. The resin was then filtered and washed with DMF (2 x 20 mL), followed by shaking again in DMF/piperidine (80:20, 15 mL) for 15 min. The resin was filtered, washed with DMF (4 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

3,5-DNB-D/L-Phe-D/L-Phe-HMBA-aminomethyl polystyrene (77)



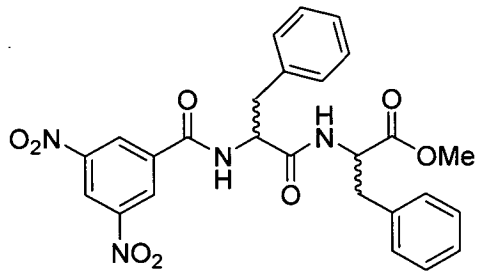
3,5-Dinitrobenzoic acid (272 mg, 1.28 mmol, 5 eq.) was dissolved in DCM/DMF (1:1, 10 mL), to this was added HOBt (173 mg, 1.28 mmol, 5 eq.). After gentle shaking for 10 min, DIC (200 μL, 1.28 mmol, 5 eq.) was added to the mixture. This mixture was shaken for 15 min, then added to H_2N-D/L-Phe-D/L-Phe-HMBA-aminomethyl polystyrene **(75)** (0.8 $mmolg^{-1}$, 0.32 g, 0.256 mmol, 1 eq.) preswelled in DMF (20 mL). Excess DMF was removed after swelling. The reaction was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

3,5-DNB-D/L-Phe-D/L-Tyr(O^{t}Bu)-HMBA-aminomethyl polystyrene (78)



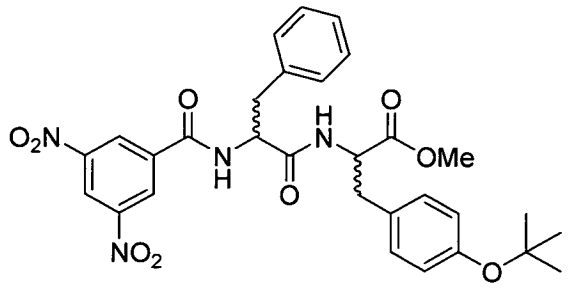
3,5-Dinitrobenzoic acid (272 mg, 1.28 mmol, 5 eq.) was dissolved in DCM/DMF (1:1, 10 mL), to this was added HOBt (173 mg, 1.28 mmol, 5 eq.). After gentle shaking for 10 min, DIC (200 μL, 1.28 mmol, 5 eq.) was added to the mixture. This mixture was shaken for 15 min, then added to H_2N-D/L-Phe-D/L-Tyr(O^{t}Bu)-HMBA-aminomethyl polystyrene **(76)** (0.8 mmolg^{-1}, 0.32 g, 0.256 mmol, 1 eq.) preswelled in DMF (20 mL). Excess DMF was removed after swelling. The reaction was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

3,5-DNB-D/L-Phe-D/L-Phe-OMe (79)



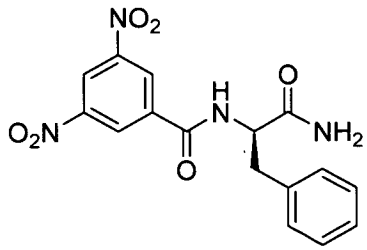
Based on a procedure by Novabiochem®[291], 3,5-DNB-D/L-Phe-D/L-Phe-HMBA-aminomethyl polystyrene **(77)** (0.32 g, 0.256 mmol, 1 eq.) in a solution of DMF/MeOH/DIPEA (5:5:1, 50 mL) was heated to 50 °C. This was stirred for 62 h and then cooled to room temperature. The resin was filtered and washed with DMF/MeOH (1:1, 3 x 20 mL). The collected solution was evaporated in vacuo and the residue precipitated in petroleum ether (20 mL). The collected solid was dried under high vacuo, 81 mg (61%). ^{1}H NMR ($CDCl_3$, 250MHz): 8.90-8.85 (m, 1H, 1 x ArH), 8.70-8.65 (m, 2H, 2 x ArH), 8.00-7.90 (m, 1H), 7.10-6.52 (m, 11H, 10 x ArH), 4.78-4.69 (m, 2H), 3.53 (s, 3H), 2.92-2.80 (m, 4H); *m/z* (ES+): 543 $[M+Na]^+$ (100%); (ES-): 519 $[M-H]^-$ (100%), 633 $[M-H+TFA]^-$ (58%).

3,5-DNB-D/L-Phe-D/L-Tyr(O^{t}Bu)-OMe (80)



Based on a procedure by Novabiochem®[291], 3,5-DNB-D/L-Phe-D/L-Tyr(O^{t}Bu)-HMBA-aminomethyl polystyrene **(78)** (0.32 g, 0.256 mmol, 1 eq.) in a solution of DMF/MeOH/DIPEA (5:5:1, 50 mL) was heated to 50 °C. This was stirred for 62 h and then cooled to room temperature. The resin was filtered and washed with DMF/MeOH (1:1, 3 x 20 mL). The collected solution was evaporated in vacuo and the residue precipitated in petroleum ether (20 mL). The collected solid was dried under high vacuo, 79 mg (52%). ^{1}H NMR ($CDCl_3$, 250MHz): 9.30-9.26 (m, 1H, 1 x ArH), 9.12-9.08 (m, 2H, 2 x ArH), 8.74-8.64 (m, 1H), 7.45-6.87 (m, 10H, 9 x ArH), 5.25-5.12 (m, 1H), 5.10-5.00 (m, 1H), 3.82 (s, 3H), 3.40-3.17 (m, 4H), 1.40 (s, 9H); *m/z* (ES+): 615 $[M+Na]^+$ (100%); (ES-): 591 $[M-H]^-$ (100%), 705 $[M-H+TFA]^-$ (62%).

3,5-DNB-D-Phe-NHMe (81)

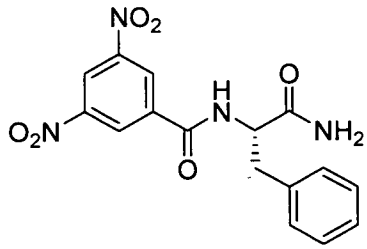


Based on a procedure by Novabiochem®[267, 268], Fmoc-D-Phe-OH (136 mg, 0.35 mmol, 5 eq.) and HOBt (47 mg, 0.35 mg, 5 eq.) were dissolved in DMF (5 mL). To this was added DIC (55 mg, 0.35 mmol, 5 eq.) and the mixture left to stand for 10 min. The mixture was then added to pre-swollen Rink amide resin (100 mg, 0.070 mmol, 0.7 $mmolg^{-1}$) and shaken for 3 h. The resin is collected by filtration and washed with DMF.). The resin was then swelled in DMF and shaken in a solution of DMF/piperidine (80:20, 15 mL) for 15 min. The resin was then filtered and washed with DMF (2 x 20 mL), followed by shaking again in DMF/piperidine (80:20, 15 mL) for 15 min. The resin was filtered, washed with DMF (4 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

3,5-Dinitrobenzoic acid (74 mg, 0.35 mmol, 5 eq.) was dissolved in DCM/DMF (1:1, 5 mL), to this is added DIC (55 µL, 0.35 mmol, 5 eq.) and HOBt (47 mg, 0.35 mmol, 5 eq.). This mixture was shaken for 15 min, then added to the preswelled resin. The reaction was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

The resin was then suspended in TFA/DCM (1:1, 5 mL) and left for 2 h. The resin was filtered and washed with DCM (3 x 20 mL). The combined filtrates were reduced in vacuo and dried to give the desired peptide 3,5-DNB-D-Phe-NHMe, 20 mg (80%). ^1H NMR (CD_3CN, 250MHz): 7.04 (t, 1H, J = 2 Hz, 1 x ArH), 6.84 (d, 2H, 2 x ArH), 5.38-5.22 (m, 5H, 5 x ArH), 2.82 (dd, 1H, J = 10 Hz, J = 5 Hz), 1.32 (dd, 1H, J = 14 Hz, J = 5 Hz), 1.06 (dd, 1H, J = 14 Hz, J = 10 Hz); *m/z* (ES+): 615 $[M+Na]^+$ (100%); *m/z* (ES-): 357 $[M-H]^-$ (100%).

3,5-DNB-L-Phe-NHMe (82)

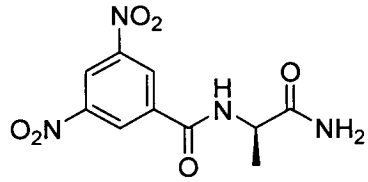


Based on a procedure by Novabiochem®[267, 268], Fmoc-L-Phe-OH (136 mg, 0.35 mmol, 5 eq.) and HOBt (47 mg, 0.35 mg, 5 eq.) were dissolved in DMF (5 mL). To this was added DIC (55 mg, 0.35 mmol, 5 eq.) and the mixture left to stand for 10 min. The mixture was then added to pre-swollen Rink amide resin (100 mg, 0.070 mmol, 0.7 $mmolg^{-1}$) and shaken for 3 h. The resin was collected by filtration and washed with DMF.). The resin was then swelled in DMF and shaken in a solution of DMF/piperidine (80:20, 15 mL) for 15 min. The resin was then filtered and washed with DMF (2 x 20 mL), followed by shaking again in DMF/piperidine (80:20, 15 mL) for 15 min. The resin was filtered, washed with DMF (4 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

3,5-Dinitrobenzoic acid (74 mg, 0.35 mmol, 5 eq.) was dissolved in DCM/DMF (1:1, 5 mL), to this was added DIC (55 µL, 0.35 mmol, 5 eq.) and HOBt (47 mg, 0.35 mmol, 5 eq.). This mixture was shaken for 15 min, then added to the preswelled resin. The reaction was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

The resin was then suspended in TFA/DCM (1:1, 5 mL) and left for 2 h. The resin was filtered and washed with DCM (3 x 20 mL). The combined filtrates were reduced in vacuo and dried to give the desired peptide 3,5-DNB-L-Phe-NHMe, 20 mg (80%). ^1H NMR (CD_3CN, 250MHz): 7.04 (t, 1H, J = 2 Hz, 1 x ArH), 6.84 (d, 2H, 2 x ArH), 5.38-5.22 (m, 5H, 5 x ArH), 2.82 (dd, 1H, J = 10 Hz, J = 5 Hz), 1.32 (dd, 1H, J = 14 Hz, J = 5 Hz), 1.06 (dd, 1H, J = 14 Hz, J = 10 Hz); *m/z* (ES-): 357 $[M-H]^-$ (100%).

3,5-DNB-D-Ala-NMe (83)

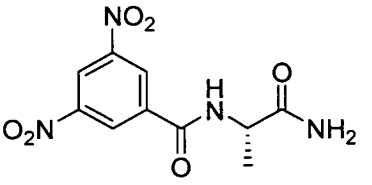


Based on a procedure by Novabiochem®[267, 268], Fmoc-D-Ala-OH (109 mg, 0.35 mmol, 5 eq.) and HOBt (47 mg, 0.35 mg, 5 eq.) were dissolved in DMF (5 mL). To this was added DIC (55 mg, 0.35 mmol, 5 eq.) and the mixture left to stand for 10 min. The mixture was then added to pre-swollen Rink amide resin (100 mg, 0.070 mmol, 0.7 $mmolg^{-1}$) and shaken for 3 h. The resin was collected by filtration and washed with DMF.). The resin was then swelled in DMF and shaken in a solution of DMF/piperidine (80:20, 15 mL) for 15 min. The resin was then filtered and washed with DMF (2 x 20 mL), followed by shaking again in DMF/piperidine (80:20, 15 mL) for 15 min. The resin was filtered, washed with DMF (4 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

3,5-Dinitrobenzoic acid (74 mg, 0.35 mmol, 5 eq.) dissolved in DCM/DMF (1:1, 5 mL), to this was added DIC (55 µL, 0.35 mmol, 5 eq.) and HOBt (47 mg, 0.35 mmol, 5 eq.). This mixture was shaken for 15 min, then added to the preswelled resin. The reaction was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

The resin was then suspended in TFA/DCM (1:1, 5 mL) and left for 2 h. The resin was filtered and washed with DCM (3 x 20 mL). The combined filtrates were reduced in vacuo and dried to give the desired peptide 3,5-DNB-D-Ala-NHMe, 12 mg (61%). ^1H NMR (CD_3CN, 250MHz): 7.12 (t, 1H, J = 2 Hz, 1 x ArH), 6.90 (d, 2H, 2 x ArH), 2.82 (q, 1H, J = 8 Hz), 1.02 (d, 3H, J = 8); *m/z* (ES-): 281 $[M-H]^-$ (100%).

3,5-DNB-L-Ala-NMe (84)



Based on a procedure by Novabiochem®[267, 268], Fmoc-L-Ala-OH (109 mg, 0.35 mmol, 5 eq.) and HOBt (47 mg, 0.35 mg, 5 eq.) were dissolved in DMF (5 mL). To this was added DIC (55 mg, 0.35 mmol, 5 eq.) and the mixture left to stand for 10 min. The mixture was then added to pre-swollen Rink amide resin (100 mg, 0.070 mmol, 0.7 $mmolg^{-1}$) and shaken for 3 h. The resin was collected by filtration and washed with DMF.). The resin was then swelled in DMF and shaken in a solution of DMF/piperidine (80:20, 15 mL) for 15 min. The resin was then filtered and washed with DMF (2 x 20 mL), followed by shaking again in DMF/piperidine (80:20, 15 mL) for 15 min. The resin was filtered, washed with DMF (4 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

3,5-Dinitrobenzoic acid (74 mg, 0.35 mmol, 5 eq.) dissolved in DCM/DMF (1:1, 5 mL), to this was added DIC (55 μL, 0.35 mmol, 5 eq.) and HOBt (47 mg, 0.35 mmol, 5 eq.). This mixture was shaken for 15 min, then added to the preswelled resin. The reaction was shaken at room temperature for 3 h. The resin was filtered and washed with DMF (3 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying in vacuo.

The resin was then suspended in TFA/DCM (1:1, 5 mL) and left for 2 h. The resin was filtered and washed with DCM (3 x 20 mL). The combined filtrates were reduced in vacuo and dried to give the desired peptide 3,5-DNB-L-Ala-NHMe, 12 mg (61%). ^1H NMR (CD_3CN, 250MHz): 7.12 (t, 1H, J = 2 Hz, 1 x ArH), 6.90 (d, 2H, 2 x ArH), 2.82 (q, 1H, J = 8 Hz), 1.02 (d, 3H, J = 8); *m/z* (ES-): 281 $[M-H]^-$ (100%).

7.5.2 Solid-phase loading from Elemental analysis

CSP	Formula	C / %	H / %	N / %	C*	H*	N*	Average*
(67)	Silica-$C_{15}H_{21}NO_4$	10.49	1.32	1.00	0.58	0.63	0.71	0.64
(68)	Silica-$C_{11}H_{13}NO_2$	9.88	1.18	0.97	0.75	0.91	0.69	0.78
(72)	Silica-$C_{26}H_{23}NO_4$	11.86	1.24	1.07	0.38	0.54	0.76	0.56

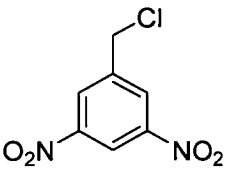
* = loading / $mmolg^{-1}$

Table 17 Elemental analysis and loading data for solid-phase compounds.

7.6 Experimental for Chapter 5

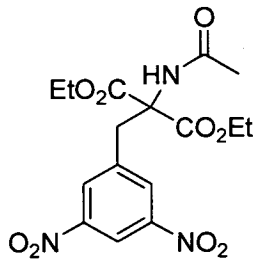
7.6.1 Synthesis of compounds

1-(chloromethyl)-3,5-dinitrobenzene (85)[292]



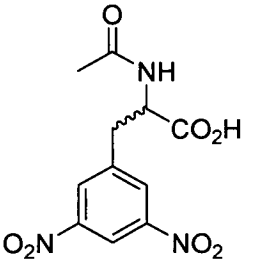
Based on a procedure by Lahiri et al[269], 3,5-dinitrobenzyl alcohol (9.20 g, 46.43 mmol, 1 eq.) was dissolved in chloroform (250 mL) and cooled to 0 °C. To the reaction PCl_5 (16.92 g, 81.260 mmol, 1.75 eq.) was added slowly over 10 min, and the reaction was stirred for a further 1 h. The reaction was cooled to room temperature and poured onto ice water (100 mL). The organic layer was extracted with DCM (100 mL), which was washed with water (5 x 50 mL), dried with $MgSO_4$, filtered and concentrated. The residue was redisolved in DCM (100 mL) and filtered through a silica bung, washed out with DCM, concentrated in vacuo, to give a yellow solid, 10.02 g (99%). m.p. 79-80°C (DCM), literature: 79.2-79.7°C (Chloroform/petroleum ether)[292]; ^{1}H NMR ($CDCl_3$, 250MHz): 9.20 (t, 1H, J = 2 Hz, 1 x ArH), 8.81 (d, 2H, J = 2 Hz, 2 x ArH), 4.96 (s, 2H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 149.1 (C), 142.0 (C), 129.0 (CH), 119.1 (CH), 43.9 (CH_2); *m/z* (FAB): 216 $[M(Cl^{35})]^+$, 218 $[M(Cl^{37})]$+ (100%).

Diethyl 2-acetamido-2-(3,5-dinitrobenzyl)malonate (86)[270, 293, 294]



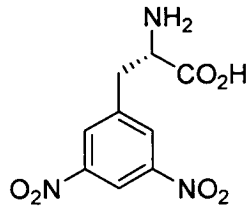
Based on a procedure by Santagada et al[231], diethyl 2-acetamidomalonate (9.88 g, 45.48, 1 eq.) dissolved in EtOH (50 mL) was added to a solution of NaOEt (3.42 g, 1.05 eq.) in EtOH (100 mL). The reaction was stirred for 15 min. 1-(Chloromethyl)-3,5-dinitrobenzene **(85)** (9.85 g, 45.48 mmol, 1 eq.) was added slowly to the reaction at 0 °C. Further EtOH (100 mL) was added to the reaction, which was then warmed to 50 °C and stirred for 2 h. The reaction was cooled to room temperature and the solvents removed in vacuo. The residue was redissolved in EtOH and filtered. The collected solid was washed with DCM (2 x 50 mL). The combined collected organic washings were concentrated in vacuo to give a white solid, 15.40 g (85%). ^{1}H NMR ($CDCl_3$, 250MHz): 9.11 (t, 1H, J = 2 Hz, 1 x ArH), 8.51 (d, 2H, J = 2 Hz, 2 x ArH), 6.77 (s, 1H), 4.48 (q, 4H, J = 8 Hz), 4.09 (s, 2H), 2.27 (s, 3H), 1.50 (t, 6H, J = 8 Hz); ^{13}C NMR ($CDCl_3$, 62.5MHz): 170.3 (C=O), 167.1 (C=O), 148.7 (C), 140.4 (C), 130.5 (CH), 118.2 (CH), 67.2 (CH_2), 63.9 (CH), 37.7 (CH), 23.4 (CH_3), 14.4 (CH_3); *m/z* (ES+): 420 $[M+Na]^+$ (100%), 398 $[M+H]^+$ (17%); (ES-): 432 $[M.HCl^{35}-H)]^-$ (100%), 396 $[M-H]^-$ (50%), 434 $[M.HCl^{37}-H)]^+$ (40%).

2-Acetamido-3-(3,5-dinitrophenyl)propanoic acid (87)[270, 293, 294]



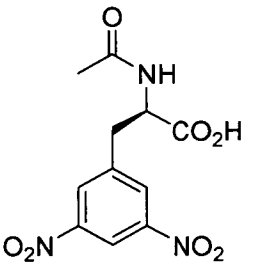
Based on a procedure by Santagada et al[231], diethyl 2-acetamido-2-(3,5-dinitrobenzyl)malonate **(86)** (5.70 g, 14.35 mmol, 1 eq.) was dissolved in EtOH (30 mL). To the reaction was added a solution of NaOH (2.30 g, 57.40 mmol, 4 eq.) in Water (15 mL). The reaction was stirred for 2 h at room temperature. The reaction was acidified to pH 2 with HCl (2 M). The solvents were removed in vacuo and the residue was redissolved in EtOH, filtered and concentrated in vacuo. The residue was purified by crystalisation from DCM to give yellow crystals, 2.93 g (70%). ^{1}H NMR (d_4- methanol, 250MHz): 8.89 (t, 1H, J = 2 Hz, 1 x ArH), 8.48 (d, 2H, J = 2 Hz, 2 x ArH), 4.79 (dd, 1H, J = 9 Hz, J = 5 Hz), 3.52 (dd, 1H, J = 14 Hz, J = 5 Hz,) 3.25 (dd, 1H, J = 14 Hz, J = 9 Hz), 1.94 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 173.7 (C=O), 173.3 (C=O), 149.9 (C), 143.6 (C), 131.0 (CH), 118.4 (CH), 54.5 (CH), 38.1 (CH_2), 22.4 (CH_3); *m/z* (ES-): 296.1 $[M-H]^-$ (100%), 593.2 $[2M-H]^-$ (58%).

(S)-2-Amino-3-(3,5-dinitrophenyl)propanoic acid (88)[270, 293, 294]



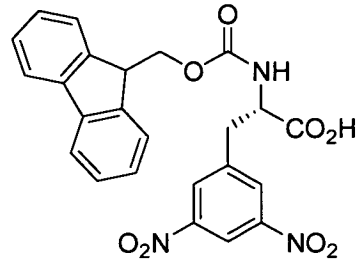
Based on procedures by Sisido et al[270], Whitesides et al[272] and Pirrung et al[271], 2-acetamido-3-(3,5-dinitrophenyl)propanoic acid **(87)** (1.00 g, 3.356 mmol, 1 eq.) was suspended in water (50 mL). Dil. NaOH was added to aid dissolving and then the pH readjusted (with dil. HCl) and maintained at 7.0-7.2. The solution was heated to 37 °C. Acylase I (from a*spergillus genus*) was dissolved in water (10 mL), $CoCl_2.6H_2O$ added to this solution which was then added to the reaction and stirred for 24 h. The reaction was adjusted to pH 5.0 with HCl (1.0 M), warmed to 40 °C with norit, and filtered. The filtrate was acidified to pH 1.5 with HCl (1.0 M) acid and extracted with ethyl acetate (3 x 25 mL). The aqueous was layer concentrated in vacuo, the residue was purified by crystallisation from MeOH to give (S)-2-Amino-3-(3,5-dinitrophenyl)propanoic acid **(88)** as a white powder, 386 mg (45%, 98%ee from Chiral-HPLC (Chiralcel-OD; Heptane/2-PrOH/AcOH (90:20:0.1) screened versus opposite enantiomer **(91)**). ^{1}H NMR (d_4- methanol, 250MHz): 8.95 (t, 1H, J = 2 Hz, 1 x ArH), 8.55 (d, 2H, J = 2 Hz, 2 x ArH), 4.85 (dd, 1H, J = 9 Hz, J = 5 Hz), 3.52 (dd, 1H, J = 14 Hz, J = 5 Hz), 3.25 (dd, 1H, J = 14 Hz, J = 9 Hz); ^{13}C NMR ($CDCl_3$, 62.5MHz): 174.8 (C=O), 149.8 (C), 144.3 (C), 133.2 (CH), 118.9 (CH), 55.5 (CH), 38.0 (CH_2), 22.4; *m/z* (ES-): 254.1 $[M-H]^-$ (100%).

(R)-2-Acetamido-3-(3,5-dinitrophenyl)propanoic acid (89)[270, 293, 294]



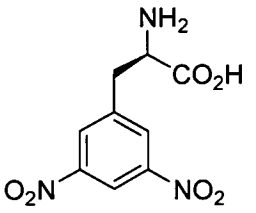
Based on procedures by Sisido et al[270], Whitesides et al[272] and Pirrung et al[271], 2-acetamido-3-(3,5-dinitrophenyl)propanoic acid **(87)** (1.00 g, 3.356 mmol, 1 eq.) was suspended in water (50 mL). Dil. NaOH was added to aid dissolving and then the pH readjusted (with dil. HCl) and maintained at 7.0-7.2. The solution was heated to 37 °C. Acylase I (from a*spergillus genus*) was dissolved in water (10 mL), $CoCl_2.6H_2O$ added to this solution which was then added to the reaction and stirred for 24 h. The reaction was adjusted to pH 5.0 with HCl (1.0 M), warmed to 40 °C with norit, and filtered through celite. The filtrate was acidified to pH 1.5 with HCl (1.0 M) acid and extracted with ethyl acetate (3 x 25 mL). The combined organic layers were washed with HCl (1.0 M), brine, dried with $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by crystallisation from water/methanol to give (R)-2-Acetamido-3-(3,5-dinitrophenyl)propanoic acid **(89)** as a white powder, 422 mg (42%, 98%ee from Chiral-HPLC of **(92)** (Chiralcel-OD; Heptane/2-PrOH (90:10) screened versus opposite enantiomer **(88)**). ^{1}H NMR (d_4- methanol, 250MHz): 8.74 (t, 1H, J = 2 Hz, 1 x ArH), 8.38 (d, 2H, J = 2 Hz, 2 x ArH), 4.66 (dd, 1H, J = 9 Hz, J = 5 Hz), 3.35 (dd, 1H, J =14 Hz, J = 5 Hz), 3.07 (dd, 1H, J = 14 Hz, J = 9 Hz), 1.80 (s, 3H); ^{13}C NMR ($CDCl_3$, 62.5MHz): 173.9 (C=O), 173.6 (C=O), 150.1 (C), 143.8 (C), 131.1 (CH), 118.5 (CH), 54.6 (CH), 38.3 (CH_2), 22.7 (CH_3); *m/z* (ES-): 296.1 $[M-H]^-$ (100%).

(S)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(3,5-dinitrophenyl)propanoic acid (90)[270, 293, 294]



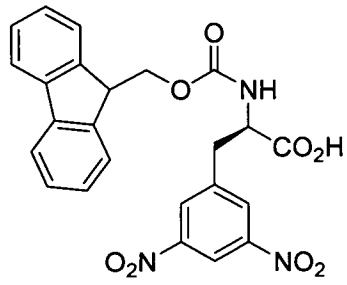
Based on a procedure by Novellino et al[273], (S)-2-amino-3-(3,5-dinitrophenyl)propanoic acid **(88)** (1.0 g, 2.095 mmol, 1 eq.) was suspended in 9% Na_2CO_3 (25 mL) and cooled to 0 °C. To this was added a solution of Fmoc-OSu (2.22 g, 6.285 mmol, 3 eq.) in dioxane (25 mL) and the reaction was stirred at room temperature for 6 h. Dioxane was removed in vacuo and the organic phase was extracted with ethyl acetate (3 x 25 mL). The combined organic layers were dried with $MgSO_4$, filtered and concentrated in vacuo. Purification was achieved by flash chromatography (silica; hexane/EtOAc) to give (S)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(3,5-dinitrophenyl)propanoic acid **(90)** as a white powder, 1.16 g (62%). ^{1}H NMR (d_4- methanol, 250MHz): 8.94 (t, 1H, J = 2 Hz, 1 x ArH), 8.65 (d, 2H, J = 2 Hz, 2 x ArH), 7.86 (dd, 2H, J = 8 Hz, J = 3 Hz, 2 x ArH), 7.65 (t, 2H, J = 8 Hz, 1 x ArH), 7.50-7.33 (m, 4H, 4 x ArH), 4.63 (dd, 1H, J = 9 Hz, J = 5 Hz), 4.50-4.32 (m, 3H) 3.60 (dd, 1H, J = 15 Hz, J = 4 Hz), 3.30 (dd, 1H, J = 15 Hz, J = 9 Hz); *m/z* (ES-): 477 $[M-H]^-$ (100%).

(R)-2-amino-3-(3,5-dinitrophenyl)propanoic acid (91)[270, 293, 294]



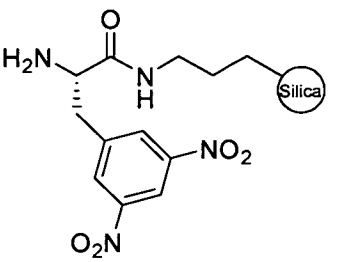
(R)-2-Acetamido-3-(3,5-dinitrophenyl)propanoic acid **(89)** (1.00 g, 3.356 mmol, 1 eq.) was stirred with HCl (0.2 M, 30 mL) for 3 h. To the reaction mixture ethyl acetate was added then extracted (3 x 25 mL). The aqueous layer was concentrated in vacuo, the residue was purified by crystallisation from MeOH to give (R)-2-Amino-3-(3,5-dinitrophenyl)propanoic acid **(91)** as a white powder, 687 mg (80%). ^{1}H NMR (d_4- methanol, 250MHz): 8.95 (t, 1H, J = 2 Hz, 1 x ArH), 8.55 (d, 2H, J = 2 Hz, 2 x ArH), 4.85 (dd, 1H, J = 9 Hz, J = 5 Hz), 3.52 (dd, 1H, J = 14 Hz, J = 5 Hz), 3.25 (dd, 1H, J = 14 Hz, J = 9 Hz); ^{13}C NMR ($CDCl_3$, 62.5MHz): 174.8 (C=O), 149.8 (C), 144.3 (C), 133.2 (CH), 118.9 (CH), 55.5 (CH), 38.0 (CH_2), 22.4; *m/z* (ES-): 254.1 $[M-H]^-$ (100%).

(R)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-(3,5-dinitrophenyl)propanoic acid (92)[270, 293, 294]



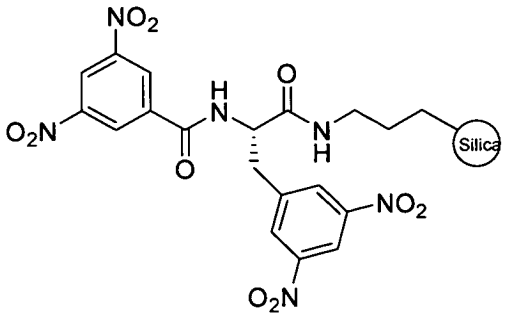
Based on a procedure by Novellino et al[273], (R)-2-amino-3-(3,5-dinitrophenyl)propanoic acid **(89)** (1.0 g, 2.095 mmol, 1 eq.) was suspended in 9% Na_2CO_3 (25 mL) and cooled to 0 °C. To this was added a solution of Fmoc-OSu (2.22 g, 6.285 mmol, 3 eq.) in dioxane (25 mL) and the reaction was stirred at room temperature for 6 h. Dioxane was removed in vacuo and the organic phase was extracted with ethyl acetate (3 x 25 mL). The combined organic layers were dried with $MgSO_4$, filtered and concentrated in vacuo. Purification was achieved by flash chromatography (silica; hexane/EtOAc) to give (R)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(3,5-dinitrophenyl)propanoic acid **(91)** as a white powder, 1.12 g (60%). ^{1}H NMR (d_4- methanol, 250MHz): 8.94 (t, 1H, J = 2 Hz, 1 x ArH), 8.65 (d, 2H, J = 2 Hz, 2 x ArH), 7.86 (dd, 2H, J = 8 Hz, J = 3 Hz, 2 x ArH), 7.65 (t, 2H, J = 8 Hz, 1 x ArH), 7.50-7.33 (m, 4H, 4 x ArH), 4.63 (dd, 1H, J = 9 Hz, J = 5 Hz), 4.50-4.32 (m, 3H) 3.60 (dd, 1H, J = 15 Hz, J = 4 Hz), 3.30 (dd, 1H, J = 15 Hz, J = 9 Hz); *m/z* (ES-): 477 $[M-H]^-$ (100%).

(S)-2-Amino-3-(3,5-dinitrophenyl)propanoylamidopropyl silcia (93)



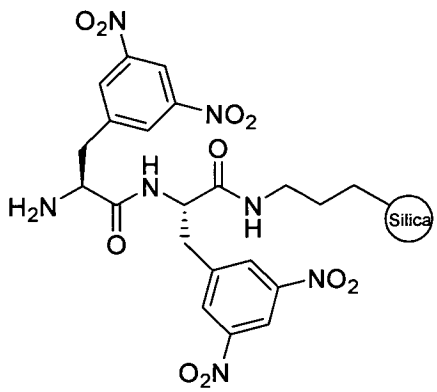
(S)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(3,5-dinitrophenyl)propanoic acid **(90)** (515 mg, 1.08 mmol, 1.5 eq.) and HOBt (146 mg, 1.08 mmol, 1.5 eq.) were dissolved in DCM/DMF (8:2, 20 mL). After gentle shaking of the resulting mixture for 10 min, DIC (167 μL, 1.08 mmol, 1.5 eq.) was added. After a further 10 min shaking, the reaction was charged with aminopropyl silica (12 μm, 0.45 $mmolg^{-1}$, 1.6 g, 0.72 mmol, 1.0 eq.). The reaction was heated to reflux (50°C) for 6h. After cooling the beads were filtered and washed with DMF (2 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying under high vacuum. A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF/piperidine (95:5, 10 mL) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF/piperidine (95:5, 10 mL) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL) to give (S)-2-Amino-3-(3,5-dinitrophenyl)propanoylamidopropyl silica **(93)**.

(S)-2-(3,5-Dinitrobenzoylamido)-3-(3,5-dinitrophenyl)propanoylamidopropyl silica (94)



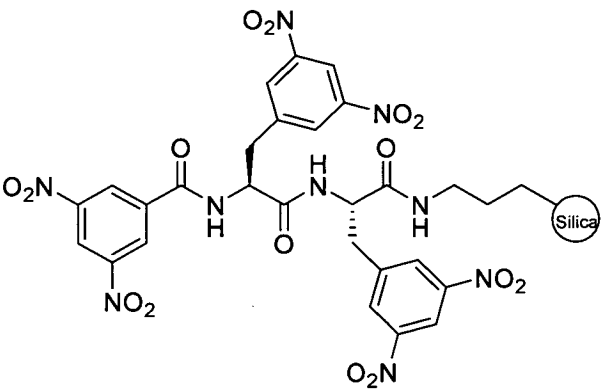
3,5-Dinitrobenzoic acid (0.24 g, 1.80 mmol, 5 eq.) and HOBt (0.24 g, 1.80 mmol, 5 eq.) were dissolved in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (350 μL, 1.80 mmol, 5 eq.) was added. After a further 10 min shaking, the reaction was charged with (S)-2-Amino-3-(3,5-dinitrophenyl)propanoylamidopropyl silcia **(93)** (0.8 g, 0.36 mmol, 1 eq.) and the reaction was heated to reflux (50°C) for 6h. After cooling the beads were filtered and washed with DMF (2 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying under high vacuum. A negative TNBS test indicated complete coupling giving (S)-2-(3,5-Dinitrobenzoylamido)-3-(3,5-dinitrophenyl)propanoylamidopropyl silica **(94)**.

(S,S)-2-(2-amino-3-(3,5-dinitrophenyl)propoylamido)-3-(3,5-dinitrophenyl)propanoylamidopropyl silica (95)



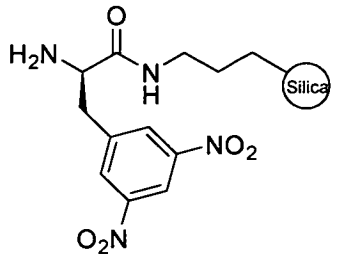
(S)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(3,5-dinitrophenyl)propanoic acid **(90)** (260 mg, 0.54 mmol, 1.5 eq.) and HOBt (73 mg, 0.54 mmol, 1.5 eq.) were dissolved in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (84 μL, 0.54 mmol, 1.5 eq.) was added. After a further 10 min shaking, the reaction was charged with (S)-2-Amino-3-(3,5-dinitrophenyl)propanoylamidopropyl silica **(93)** (0.8 g, 0.36 mmol, 1 eq.) and the reaction was heated to reflux (50°C) for 6h. After cooling the beads were filtered and washed with DMF (2 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying under high vacuum. A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the fuctionalised silica in DMF/piperidine (95:5, 10 mL) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF/piperidine (95:5, 10 mL) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL) to give (S,S)-2-(2-amino-3-(3,5-dinitrophenyl)propoylamido)-3-(3,5-dinitrophenyl)propanoylamidopropyl silica **(95)**.

(S,S)-2-(2-(3,5-Dinitrobenzoylamido)-3-(3,5-dinitrophenyl)propoylamido)-3-(3,5-dinitrophenyl)propanoylamidopropyl silica (96)



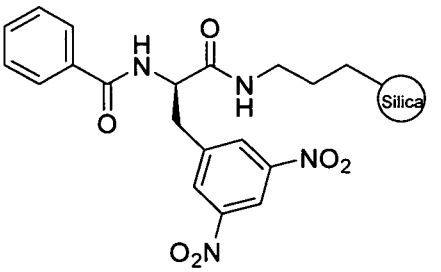
3,5-Dinitrobenzoic acid (0.24 g, 1.80 mmol, 5 eq.) and HOBt (0.24 g, 1.80 mmol, 5 eq.) were dissolved in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (350 µL, 1.80 mmol, 5 eq.) was added. After a further 10 min shaking, the reaction was charged with (S,S)-2-(2-amino-3-(3,5-dinitrophenyl)propoylamido)-3-(3,5-dinitrophenyl)propanoylamidopropyl silica **(95)** (0.8 g, 0.36 mmol, 1 eq.) and the reaction was heated to reflux (50°C) for 6h. After cooling the beads were filtered and washed with DMF (2 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying under high vacuum. A negative TNBS test indicated complete coupling giving (S,S)-2-(2-(3,5-Dinitrobenzoylamido)-3-(3,5-dinitrophenyl)propoylamido)-3-(3,5-dinitrophenyl)propanoylamidopropyl silica **(96)**.

(S)-2-Amino-3-(3,5-dinitrophenyl)propanoylamidopropyl silcia (97)



(R)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(3,5-dinitrophenyl)propanoic acid **(92)** (260 mg, 0.54 mmol, 1.5 eq.) and HOBt (73 mg, 0.54 mmol, 1.5 eq.) were dissolved in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (84 μL, 0.54 mmol, 1.5 eq.) was added. After a further 10 min shaking, the reaction was charged with aminopropyl silica (12 μm, 0.45 $mmolg^{-1}$, 0.8 g, 0.36 mmol, 1.0 eq.). The reaction was heated to reflux (50°C) for 6h. After cooling the beads were filtered and washed with DMF (2 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying under high vacuum. A negative TNBS test indicated complete coupling. Fmoc deprotection was achieved by shaking the functionalised silica in DMF/piperidine (95:5, 10 mL) for 15 min. The silica was filtered and washed with DMF (50 mL). Then shaking in DMF/piperidine (95:5, 10 mL) was repeated for 15 min, followed by filtration and washing with DMF (50 mL), DCM (50 mL) and Et_2O (50 mL) to give (S)-2-Amino-3-(3,5-dinitrophenyl)propanoylamidopropyl silica **(97)**.

(S)-2-(Benzoylamido)-3-(3,5-dinitrophenyl)propanoylamidopropyl silica (98)



Benzoic acid (0.218 g, 1.80 mmol, 5 eq.) and HOBt (0.24 g, 1.80 mmol, 5 eq.) were dissolved in DCM/DMF (8:2, 10 mL). After gentle shaking of the resulting mixture for 10 min, DIC (350 µL, 1.80 mmol, 5 eq.) was added. After a further 10 min shaking, the reaction was charged with (S)-2-Amino-3-(3,5-dinitrophenyl)propanoylamidopropyl silica **(97)**. (0.8 g, 0.36 mmol, 1 eq.) and the reaction was heated to reflux (50°C) for 6h. After cooling the beads were filtered and washed with DMF (2 x 20 mL), DCM (2 x 20 mL) and Et_2O (2 x 20 mL), followed by drying under high vacuum. A negative TNBS test indicated complete coupling giving (S)-2-(Benzoylamido)-3-(3,5-dinitrophenyl)propanoylamidopropyl silica **(98)**.

7.6.2 Solid-phase loading from Elemental analysis

CSP	Formula	C / %	H / %	N / %	C*	H*	N*	Average*
94	Silica-$C_{19}H_{17}N_6O_{10}$	8.79	0.84	2.94	0.39	0.49	0.35	0.41
96	Silica-$C_{28}H_{24}N_9O_{15}$	11.29	1.12	3.74	0.34	0.47	0.30	0.37
98	Silica-$C_{19}H_{19}N_4O_6$	10.51	0.94	2.09	0.46	0.49	0.37	0.44

* = loading / $mmolg^{-1}$

Table 18 Elemental analysis and loading data for solid-phase compounds.

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