SYNTHESIS OF CONFORMATIONALLY

RESTRAINED PEPTIDES

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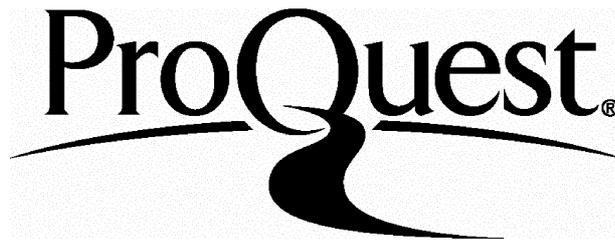
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This thesis is dedicated to the memory of George Andrews and Gordon MacKenzie.

ACKNOWLEDGEMENTS

ABSTRACT

The synthesis of an artificial amino acid residue, bearing two α-amino acid centres, is detailed. The residue has been designed to act as a conformational restraint when incorporated into peptides. The intended target structural motif is the α-helix, and the restraint takes the form of a macrocycle in a central position in the peptide chain, which is intended to nucleate helix formation. The synthesis has been achieved by the use of two different asymmetric methodologies.

Details of the final synthetic route to the residue are included, as well as details of several other synthetic routes which proved unsuccessful. The final route involves the use of an octanoic acid derivative. This is initially reacted with a chiral lithiated pyrazine cyanocuprate complex to generate the R-chiral centre, followed by the introduction of the S-chiral centre using an asymmetric azidation methodology. These reactions have been employed in sequence to give optimum yield and efficiency. The sequence of reaction followed also simplifies the differentiation of the two chiral centres, giving the molecule in a form suitable for solid phase peptide synthesis.

The attempted syntheses of peptides bearing this residue is also detailed. This process has been performed by standard Fmoc methodology, using the triply orthogonal allyl based protecting group, cleaved by palladium catalysis, to allow selective reaction to form the macrocycle. This loop is arranged in an i-$(i+4)$ substitution pattern, suggested in the literature to be the most effective spacing for performing this task.

Other sections of this thesis describe the general background to helical structure stabilisation, the asymmetric synthesis of amino acids and the solid phase synthesis of peptides.

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ABBREVIATIONS

n-BuLi	n-Butyl lithium (solution in hexane)
C.D.	Circular dichroism
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DEAD	Diethylazodicarboxylate
DIEA	Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMPU	1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyramidinone
DMSO	Dimethyl sulphoxide
EI	Electronic Ionisation
FAB	Fast Atom Bombardment
HATU	O-(1H-7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HBTU	O-(1H-7-Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HMDS	Hexamethyldisilylazane
HMPA	Hexamethylphosphoramide
HMPT	Hexamethylphosphorus triamide
hGRF	Human growth hormone releasing factor
HOAc	Acetic acid
HPLC	High pressure liquid chromatography
IR	Infra red
KHMDS	Potassium hexamethyldisilylazide
MALDI-TOF	Matrix assisted laser desorption ionisation time of flight
NBS	N-Bromosuccinamide
n.m.r.	nuclear magnetic resonance
NMM	N-Methylmorpholine
TBAF	Tetra-n-butyl ammonium fluoride
TBDMS	*tert*-Butyldimethylsilyl
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
tlc	Thin layer chromatography
Ts, Tosyl	*para*-Toluenesulphonyl
UV	Ultraviolet

CHAPTER ONE

INTRODUCTION

1.1   SYNTHETIC RESIDUE AND TARGET PEPTIDE

This thesis is concerned with the synthesis of an artificial amino acid (fig. 1.2), and the attempted incorporation of this residue into two peptides. The residue has been designed to stabilise α-helical secondary structure. Both of the target sequences are drawn from the biologically active portion of the peptide hormone, human Growth Hormone Releasing Factor (hGRF), a 44 residue peptide which mediates the release of growth hormone by action on a receptor in the pituitary gland.[1]

hGRF (1-44)   Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ser-Ala-
              Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-
              Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH$_2$

hGRF (1-29)   Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ser-Ala-
              Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-NH$_2$

Figure 1.1: Sequences of hGRF and Active Portion

The biologically active section has been found to be the 29 *N*-terminal residues, by activity studies.[2] Studies on the structure of this peptide by n.m.r. found that in water-trifluoroethanol solution it exists in a mainly α-helical structure, divided into two portions by a disordered section.[3] Trifluoroethanol has been found to increase helical structure in peptides such as hGRF,[4] and is thought in some cases to mimic the effect of peptide association with a membrane, hence allowing the stabilisation of amphiphilic sequences. Considerable structural detail concerning hGRF has been published, making the sequence a suitable target for determining the effects of incorporation of the artificial amino acid residue.
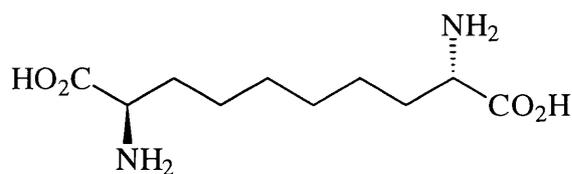
Figure 1.2: Designed Synthetic Target

Computer modelling[5] suggested that linking two α-amino acid centres could easily be achieved using an aliphatic chain. Modelling also suggested that this linkage have the lowest energy cost by employing different chirality at each α-centre.[6] This allows the use of the shortest length of linker. This requirement was dictated by the desire for strongest promotion of helical structure. The use of a longer linker would be less effective, due to the increased degrees of freedom it would allow in the peptide sequence.[7] A representation of the peptide sequence incorporating this residue has been included (fig. 1.4).

hGRF (1-29); Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Xaa-Tyr-Arg-Lys-Xaa-Leu-Ser-Ala-
Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-

Figure 1.3: Modified hGRF Sequence

The designed residue has been synthesised using two asymmetric methods, introducing each chiral centre sequentially. Enantioselective processes were required because of the natural chirality of amino acids. The processes to be utilised were also required to deliver the amino acid in a protected form suitable for solid phase peptide synthesis. This required differentiation of the two chiral centres by chemoselective methods, most easily achieved during the synthesis of the residue. Sequential unmasking and protection of the amine and carboxylic acid was employed during the course of the reaction, to avoid difficulties foreseen in selective protection at the end of the synthesis.

The synthesis of cyclic peptides is well documented,[8] but chain growth is not generally

continued after cyclisation.[9] Cyclisation in these cases is generally the terminating step in the synthetic route. The incorporation of the artificial residue into the target peptides was attempted by the use of a mutually orthogonal protecting group strategy. This methodology has been reported in the literature, allowing the deprotection of the second α-amino acid centre at the correct stage in the synthesis without other side chain deprotection. Synthesis of the C-terminal amide required the use of particular linker systems. Particular resin loading conditions were also required to prevent possible cross reaction at stages in the synthesis.

Analysis of structure is possible by the use of C.D. and n.m.r. methods. These determine the level of organised helical structure by different physical characteristics. If the modelling is proved correct, helicity could be induced in the target peptides at lower trifluoroethanol concentrations, or perhaps under purely aqueous conditions. The proportion of the peptide which is folded in solution would be shown by C.D. analysis.

1.2   APPLICATIONS TO PROTEIN STRUCTURE STUDIES

Large protein structures are renowned for their stability, but sections of proteins known to form particular structures *in situ* tend to lose structure when taken out of context.[10] The cooperative nature of the folding, as well as the complexity of proteins, makes the study of folding a complicated process. In many cases, data is very difficult to obtain in the absence of crystal structures. The complexity arises from the nature of the translation of the simple, one dimensional sequence of amino acids into three dimensional structure.

The "programs" that direct folding are still not clearly understood, but attempts to use data available for the prediction of structure have been made.[11] However, there is, at present, no definite method of deducing structure from amino acid sequence, and so information concerning stabilising interactions could help in this context.

The use of shorter peptide sequences would therefore simplify this process, as they allow spectroscopic techniques ineffective for large systems to be employed. It is the energetic contribution of particular local interactions to stability that requires
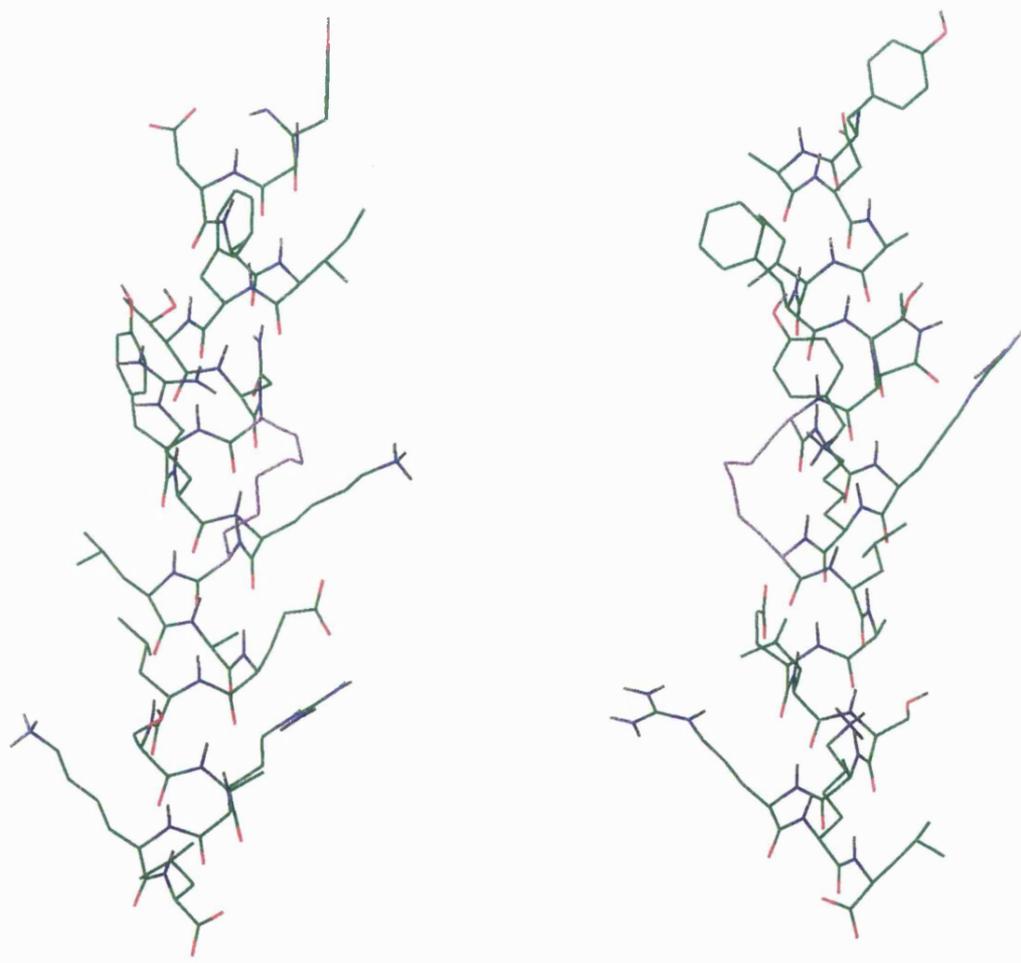
Figure 1.4: hGRF Containing Designed Synthetic Residue

assessment. This information could then lead to a clearer understanding of the stabilising forces prevalent in proteins. The problem in this approach is, as mentioned above, that the absence of stable structure from short sequences makes this type of study difficult. The process of folding for the majority of proteins is very fast, these molecules folding on the milli- and even microsecond timescale.[12] The process does not involve sampling of possible conformations, as it could then take the lifespan of the universe to find the "correct" form.[13]

It would, therefore, be desirable to create sequences of organised structure in short peptides to enable closer study of other interactions. A number of stabilising interactions have been found, experimentally and by analysis of protein structure databases, and these have been used in the study of short sequence helix stability. As full study of these interactions is not complete, alternative methods of stabilising helices, such as the inclusion of novel residues, could be of benefit.

The advantage that the residue could have in such studies is that the direction is not by a rigid group, and hence unlikely to overestimate any stabilising effect. The residue has been designed to promote formation of the first turn of a helix. The flexible nature of the linker should allow helix formation only when energetically favourable, allowing other interactions to be more clearly evaluated. A related disease therapy approach is the use of short, ordered peptides as enzyme inhibitors. Instead of blocking enzyme active sites, these peptides can act to prevent the binding of cofactors, or the formation of enzyme complexes.[14]

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CHAPTER TWO

PEPTIDE HELICAL STRUCTURE

2.1 PROTEIN STRUCTURE

Proteins are made up of chains of amino acids, drawn from the twenty proteinogenic amino acids available to mammals, along with many others known to have few sources. With the relatively few residues commonly known, however, the one dimensional sequence of amino acids translates to complex three dimensional structures. The description of protein structure is then divided into four levels, of increasing complexity.

At the first level, or the primary structure, is the amino acid sequence which makes up the protein or peptide. This is data which can be easily obtained by means of protein sequencing.[1] The peptide chain can then fold into a regular ordered local structural pattern, giving what is termed secondary structure. This can take the form either of repeating angles between residues, or of what is termed "coil" structure, which follows no deducible repeat. Although irregular in comparison to repeating structures, these coil lengths are replicated regularly between like proteins. The arrangement of local structures in relation to each other is described as tertiary structure, the level of monomeric protein structure. The final level is quaternary structure, which describes the interactions between different proteins, for example in the cases of dimeric, trimeric and quadrimeric complexes which are frequently found in nature.

However in these systems, shortfalls in the methods of description can arise. In considering primary structure, ordering of amino acid residues in a sequence may be unclear, especially in longer protein chains. At the level of secondary structure, the precise initiation and termination points of a type of local structure often are dependent on the definitions used. When the tertiary and quaternary levels are considered, precise information regarding interactions is generally scarce. The use of short peptide sequences from these larger units would therefore appear to be one way of deducing the local structure of portions of the protein, allowing a picture to be created of the

overall protein structure. The study of protein structure is a complicated field, and the employment of techniques that simplify effects may be useful.

Because of the cooperative nature of the folding of proteins, small portions rarely take up the structure in isolation that they do *in situ*. In these examples, any organised structure seen is liable to be transient, existing for a short time before unwinding. Larger proteins may be folded and unfolded several times by chemical methods,[2] each time giving the same structure. The process of folding for the majority of proteins is very fast, and these large molecules can fold on the milli- and even microsecond timescale.[3] The precise reasons for the speed of folding are not fully understood. It has been deduced that, if the process of folding was performed by random sampling of possible conformations, it could take the lifespan of the universe to find the "correct" form.[4]

Hence, the folding of proteins is known to be a process of complex interactions between different portions of the molecule. However, the folding times make following the process by spectroscopic techniques a complex undertaking.[5] The use of shorter peptide systems could, therefore, give information relating to the initial folding mechanisms of secondary structural motifs, and perhaps some insight into the effects that separate pieces of secondary structure have on each other.

The motifs that make up the majority of ordered structure are the α-helix and the β-sheet. Although 65% of organised structures are α-helices,[2] other types are found, including the 3_{10}-helix, which is frequently found at the terminus of α-helices. As the intended target for incorporation was an α-helical peptide, this review will concentrate on describing α-helical structure and characteristics.
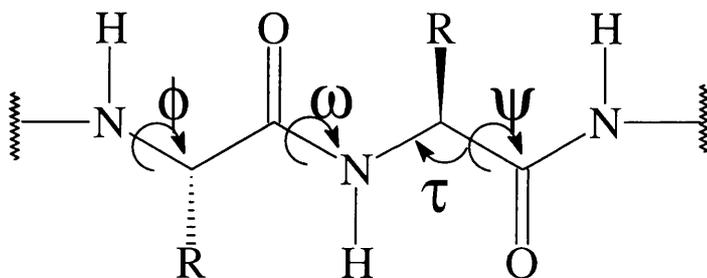
Figure 2.1: Angles Describing the Peptide Bond

A system capable of describing the various torsion angles present in a chiral amino acid has been devised. The major angles are denoted ϕ,ψ, and they describe the torsional angle between groups on the helix backbone, as shown on fig. 2.1. These angle are complimented by ω, which describes the generally planar amide bond torsion angle. However, this angle is usually fixed as either 180° or 0°, due to the electronic nature of the bond. As may be expected, the regular repeat of the helix along with the regular general form of amino acids means that certain angles along the helix backbone are replicated at each residue. This allows the values of ϕ and ψ to be plotted against each other in a graph termed a Ramachandran plot.[6] This plot describes areas of space associated with each form of secondary structure, and so experimental values of ϕ, ψ can be quickly compared. This forms the basis for the deduction of initiation and termination of structure in one method of statistical analysis.[7]

2.2 α-HELICAL STRUCTURES
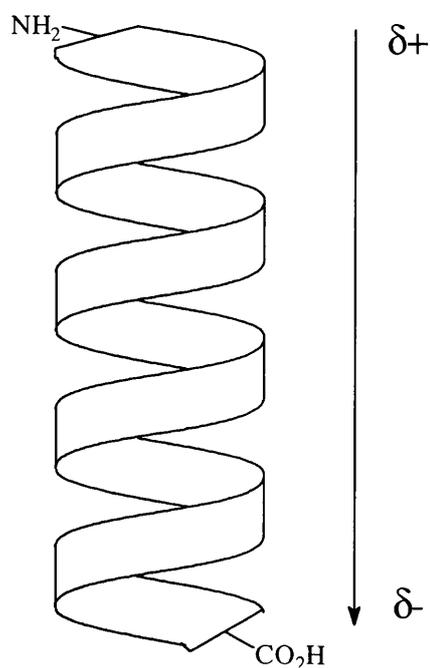
Figure 2.2: Ribbon Representation of Helix Backbone

Helices are formed of amino acids, coiled into a tubular system, stabilised by hydrogen

bonding between residues above and below each other. Carbonyl groups and amide protons are orientated in opposing directions, meaning that the hydrogen bonds run in the same direction along the surface of the helix. This creates hydrogen bonds between the i-$(i+4)$ residues, giving a series of interlinked loops. α-Helices found in nature are right-handed, due to the chirality of the amino acids. A regular repeat of 3.6 residues *per* complete turn allows the i-$(i+4)$ hydrogen bond interaction that has been described. The helix rises 5.4Å *per* complete turn, or 1.5Å *per* single residue, the hydrogen bonds repeating as the helix rises. *L*-Chiral amino acids are not capable of stacking in a left-handed form, due to the close and destabilising interaction that would occur between the i residue β-carbon, and the $i+4$ residue carbonyl oxygen. However, more conformationally constrained disubstituted residues have been found by crystallography to prefer the tighter 3_{10}- helical form.[8]

Peptide bonds in helices are all aligned in the same direction. As each amide bond has an associated dipole, an overall macrodipole is created that runs along the axis of the helix.[9] This macrodipole means that even in a longer sequence, an effective charge resides at the ends of the helix. The terminal amino group of a sequence is termed the *N*-cap, and is slightly positively charged in character, whilst the carboxylic acid is termed the *C*-cap, and is slightly negatively charged. The effect arises from the charges associated with these functional groups in monomeric amino acids. The overall effect is not additive, but is stronger over longer sequences, and can have important consequences in terms of the stability of helices.

Hydrophilic surface
R= Asp, Glu, Arg,
Asn, Gln, etc.

Hydrophobic surface
R= Leu, Ile, Phe
Tyr, Val, etc.
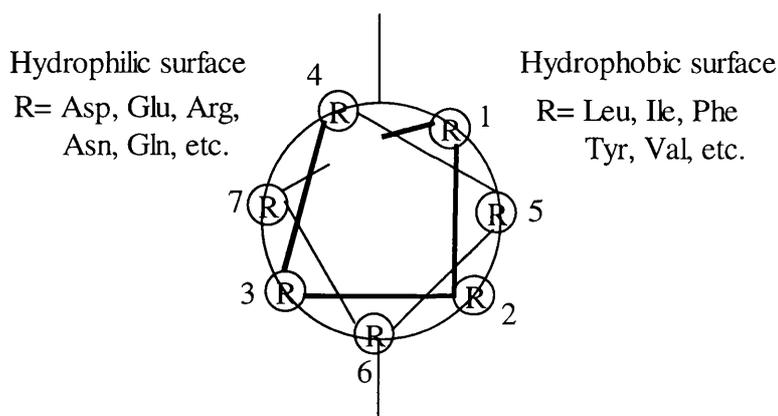
Figure 2.3: Amphiphilic Helix Character

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The twenty different amino acids have widely differing properties, leading to other characteristics in helices. When residues are incorporated into helices, the cylindrical structure allows residues separated in the sequence to be close in space. In certain sequences, this can have the effect of grouping hydrophobic and hydrophilic groups on opposing sides of the helix. This dual character can give rise to particular stabilising effects as groups associate in order to reduce contact with water.

2.3 STABILITY OF HELICES

The study of helices in short peptides is difficult, due to their transient existence. However, the study of large proteins is in practise difficult due to their rapid folding times and greater stability. It is therefore necessary to develop systems that can allow the different factors influencing the stability of helices to be assessed. Above all, the formation of a helix is an enthalpically driven process, the formation of a large number of hydrogen bonds giving a decrease in overall free energy at the expense of decrease in entropy. Although significant, in short peptide systems the stabilisation gained by helix formation can be affected by a number of factors. These include the solvent, as hydrogen bonding solvents can compete for bonds with the amide groups, meaning that folding to a helix is less likely than if an apolar solvent is used. This has been shown by studies of the stability of peptide helices in water-alcohol systems, which found that mixed systems were more effective in stabilising ordered helical structure than single solvent systems.[10] However, the precise mechanism of these interactions is still unclear. Other studies have been performed to find the effect of altering electrolyte concentrations and temperature on the stability of helical systems.

The energy values observed for the formation of peptide helices have been used along with statistical studies using protein structure databases, to propose mathematical models capable of determining the probability of a residue in a peptide being part of a helix. There are two major systems, the first by Zimm and Bragg,[11] and the second described by Lifson and Roig.[7] These models are based on certain assumptions, including the absence of side chain interactions, which have been shown to be invalid experimentally. Adaption is also required to take into account factors not included

initially such as end capping, which will be described later.[12] Both models predict that peptides of less that 50 residues should not give any significant level of helicity. This has not been supported by experimental observations, where far shorter sequences have been found to contain a significant proportion of peptide helix. Also, both models employ a "two state" function, whereby the peptide is either totally folded or totally unfolded. Again, observations by means of C.D. and n.m.r. have shown that the process of folding is reversible, and that significant fraying at both ends of a helix is very common, negating this simple model.

These models do, however, show helix folding as a two stage process. The first of these is the formation of the first turn, described as the initiation step, which is the statistically unlikely event. This is given the symbol σ, which usually has a value of the order 10^{-5}. This requires the organisation of four residues in a chain into a conformation capable of closing a loop by means of a hydrogen bond. This is not greatly favoured by the energetics of the system, as a significant loss of entropy must occur for the enthalpically favourable hydrogen bond to form. However, the next stage of the process is an equilibrium, with the next residue in the sequence joining the forming helix with an equilibrium constant s, which is usually approximately 1. This is possible as only a small amount of entropy will be lost in terms of freedom, whilst the energy gained by the stabilising hydrogen bond formation will be significantly higher. The value of s is different for each amino acid, and the value of s for a particular residue can be taken as an indication of the helix forming tendency of that residue. However, residues with low s values are still found in helices.[13]

2.4   EFFECTS OF AMINO ACID SUBSTITUTIONS

The sequence of amino acids occurring in a peptide contains the information required for the folding of that peptide. As the amino acids differ only in terms of side chain functionalities, it seems reasonable to assume that the major directing force comes from these side chains. Hence, altering the composition of a peptide should alter its folding pattern and three dimensional structure.

$$\text{Ac-Ala-Xaa}\left[\text{-Ala-Ala-Ala-Xaa-}\right]_n\text{Ala-NH}_2$$

Xaa= Lys, Glu, Gln
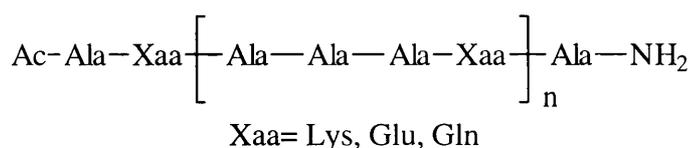
Figure 2.4: Representative Baldwin Sequence

Studies performed on several alanine based peptides have revealed that a high level of organised, helical structure can occur in peptides only 13-16 residues in length.[14] These structures were stable, and, although significant fraying was seen by n.m.r. and C.D. measurements, a large proportion of the peptides contained a helix of some length. This result shows helix formation in sequences significantly shorter than those suggested by the mathematical models described earlier, in turn casting doubt on the value of such modelling. In particular, speculation on the value of s for alanine has given widely differing alternatives.[14,15] Whilst the precise value of s for a residue cannot be accurately predicted outside of the context of a particular peptide, significant evidence of the helix forming propensity of alanine does exist. The analysis of protein structure databases shows alanine as the most commonly found residue in the internal positions of helices.[16] This seems to suggest that a good method of stabilising the helical structure in a peptide is to "dope" the sequence with alanine.

The helix favouring tendency of alanine is thought to be due to the nature of the side chain methyl group. This is comparatively small and not β-branched, so interactions between the side chain and the helix backbone are reduced. As the methyl group is only mildly hydrophobic, contact with water in the helical form is only mildly destabilising. However, the side chain also contributes to the stability of the hydrogen bonding structure by partly shielding the structure from solvent interactions.[17]
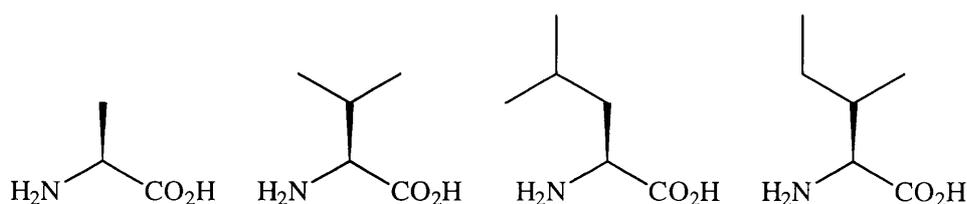
Figure 2.5: Alanine, Valine, Leucine, Isoleucine

Other β-branched residues such as valine and leucine have preferences for different structures, shown by their lower values for *s*. This is due to the enthalpic cost of accommodating the bulkier side chains counting against the energetic stabilisation provided by helix formation. The occurrence of β-branched side chain residues in a sequence does not mean that the sequence will not form an α-helix. Indeed, the additional hydrophobic effects of the aliphatic side chain of leucine gives rise to the "leucine zipper" dimeric species[18].

The substitution of more sterically bulky or constrained residues into a sequence generally has a negative effect on the likelihood of helix formation. However, the precise effect is dependent on the position of the residue in the sequence. For example, proline is rarely found in the central positions of a helix, and causes a kink in the structure where it does occur.[19]
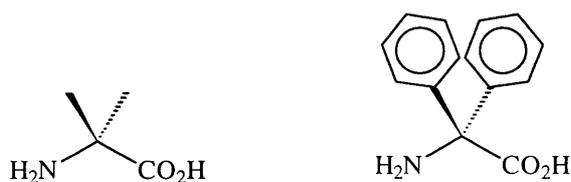
Figure 2.6: Aminoisobutyric acid, Diphenylglycine

The effects of incorporation of α,α-disubstituted residues into peptides have also been studied. These achiral molecules have been found to show a preference for 3_{10}- helical structure in crystallised peptide sequences, as side chain/main chain interactions are minimised in this form.[20] Whereas *L*-chiral amino acids form right-handed helices, these achiral molecules have no overall preference, forming both left- and right-handed forms. The conformational preference of these residues is dependent on the precise sequence in which they occur. The level of substitution of dialkyl residues will affect the preference of the sequence, with greater levels of *L*-chiral amino acids directing folding to the right-handed form. Evidence for this has come from crystallography.[21]

Whilst these groups have effects due the their inherent constraints, more simple systems also have helix-breaking tendencies due to their greater conformational

freedom. Glycine has no side chain, and so the entropic cost for this residue of folding is higher than other, more constrained residues. This greater cost in turn means that initiating or joining a helix is less likely for glycine, or other amino acids such as β-alanine.

2.5   CAPPING BOX EFFECTS

As has been stated, proline has a negative effect on helix stability in short sequences when incorporated in an internal position, but can be effective at nucleating helices when found in a terminal position. Indeed, proline is frequently found in the first turn of a helix, where the additional rigidity makes the formation of the first turn a more likely process. This is termed a capping effect. As certain residues are preferred at internal positions, so there is a preference for certain residues in terminal positions. In the mathematical models produced, this is a factor that was not at first addressed, as all positions were treated as being equivalent. Adaptations of the theory have since allowed the incorporation of these effects.[12]

The mathematical theories postulated that the formation of the first turn of the helix was the most unlikely part of the folding process. As has been stated previously, in the Zimm-Bragg equation, the probability of this conformation being adopted is given the term σ, which has a low value. However, this assumed that no stabilising forces would drive the formation of the first turn, and so all residues could be equally effective in this position. This is not reflected in the analysis of the protein databases,[22] nor in the experimental results available. Indeed, as the closure of the first turn by one hydrogen bond is only marginally thermodynamically stable, further interactions would make the structure considerably more stable, and hence long lived. This in turn makes the value of σ significantly higher than those put forward, allowing the formation of helices in much shorter systems.

The premise by which these systems work is the stabilisation of the first turn of a helix, by the formation of a hydrogen bond between the main chain of the peptide and the side chain of a residue in the first turn. The most effective residues for this task are those with short, polar side chains. Both serine and threonine have been found to
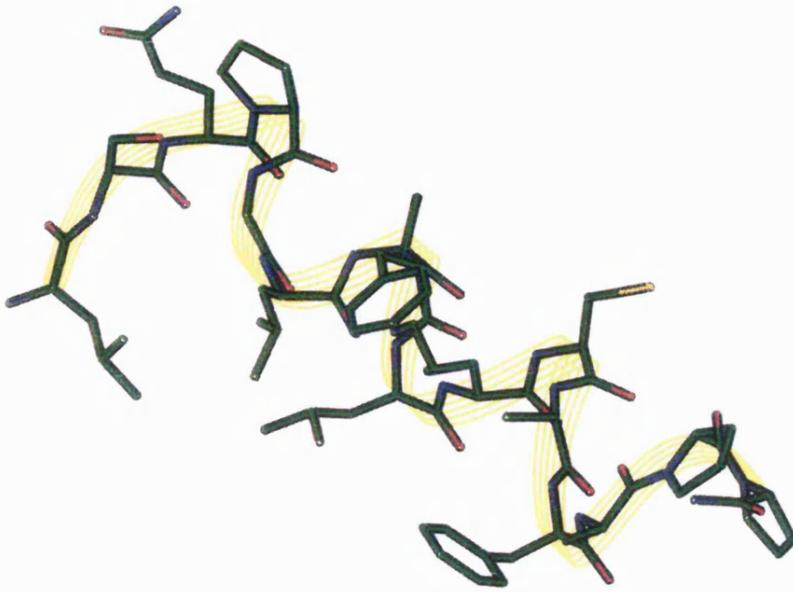
Figure 2.7: Proline Capping Box
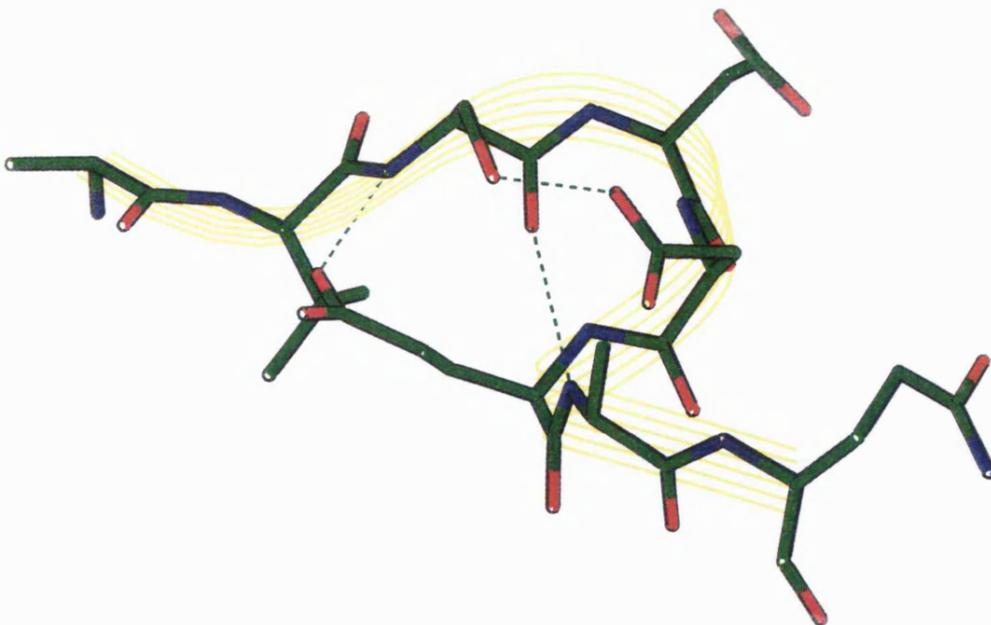
Figure 2.8: Serine Capping Box

perform this task well, leading to the theory that a regular "capping box" can be found at the start of a large number of helices.[23,24] Aspartic acid has also been found to be effective in this role. The proline capping system works by a different method, but with similar results.[25] In this case, the conformational lock provided by the pyrrole ring means that the entropy loss on arrangement to the first turn is lower, and hence the free energy stabilisation is greater.

Results in the literature point to the N-cap, or terminus, of the helix as being the more important in terms of stabilising effects. It has been found that specific stabilising interactions are more likely to exist at this terminus,[26] or are more easily deduced. The larger number of interactions at the C-terminus appear to be "stop" signals, with the final turn of the helix becoming more 3_{10}- than α- in character.

The use of methods such as these to stabilise short *de novo* peptides is difficult, as the majority of effects have been detected from crystallography, and hence from large proteins. The design of capping box systems has been achieved, with the synthesis of a template reporter molecule.[27] The synthesis of other, "open chain" systems is clearly possible, but in short peptides the greater conformational freedom may interfere with both their formation and action.

2.6   SIDE CHAIN - MAIN CHAIN INTERACTIONS

As mentioned previously, the regular repeat of amide bonds in a helical peptide leads to an electronic effect termed a macrodipole. This gives the termini of the helix effective charges, slightly positive at the N-cap, slightly negative at the C-cap, which can be employed in attempts to stabilise helical structures. This has been achieved by positioning oppositely charged residues at the termini, stabilising the charge.[28] In one example, Ala_{20} was fused with either Lys_{20} or Glu_{20}, at either terminus of the alanine block. It was found that altering the pH to allow the polar side chains to become charged resulted in the stabilisation of the helical structure when the charge was at the "correct" terminus, and destabilising when at the other. It was found that the helix was propagated further into the lysine or glutamic acid blocks under the appropriate pH conditions.

$$H_2N-(Ala)_{20}-(Lys)_{20}-CO_2H$$

$$H_2N-(Glu)_{20}-(Ala)_{20}-CO_2H$$
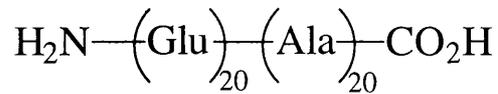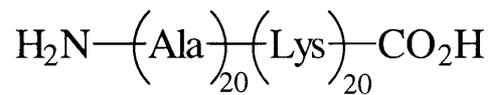
Figure 2.9: Sequences Stabilised by pH Conditions

Similar results have also been obtained by consideration of alanine based short peptide systems.[29] In this study, polar residues under suitable pH conditions were placed at various positions in the sequence to examine the variability of the stabilising effect. The results concurred with those set out above, with positive charges stabilising the C-cap, and negative charges the N-cap. These results are also reflected in nature, with statistical studies on protein databases showing this type of relationship to be common.[13] More specific work has been done on the effect of incorporation of charged histidine residues at the C-terminus of peptides.[30] This has also been found to be stabilising, depending on the precise positioning of the residue in the sequence.
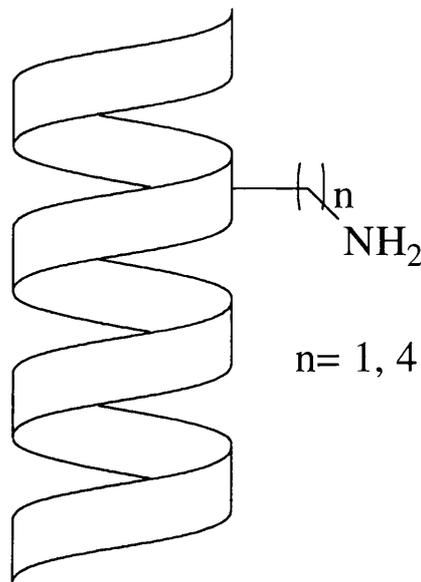
Figure 2.10: Inclusion of Charged Groups at Variable Distances to the Helix Backbone

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The effect of altering the spacing group between a charged side chain terminus and the helix backbone has also been investigated. This has been done by the incorporation of basic residues with alkyl side chains of varying length. These have included lysine and ornithine, plus shorter side chain residues.[31] It was found that longer side chain residues such as lysine have higher helix propensities, whilst the shorter side chains were found to be more destabilising. It is thought that the major destabilising force is the side chain amine competing in hydrogen bonding with backbone amide groups. The effect of bringing the amine group closer in space to the helix backbone could then increase this competition.

A similar effect has also been noted with the incorporation of aspartic and glutamic acids. It has been found that glutamic acid has a generally higher helix propensity than aspartic acid, which increases under high pH conditions. The helix forming propensity of aspartic acid does not increase on becoming charged, but the residue does "improve" in terms of capping effects.[32]

The investigation of these effects has required the development of modelling systems capable of incorporating side chain effects as well as capping effects in the calculation of helix propensities.[33]

2.7   SIDE CHAIN - SIDE CHAIN BONDING

Additional interactions may stabilise the α-helical form, as described above: greater enthalpic gain counts against the entropy lost on folding. Several different types of interaction are possible, involving both natural and unnatural amino acids.

2.7.1   Salt Bridge Formation

The formation of loops by salt bridges gives stability to the first turn of the helix. These interactions can occur either *via* simple hydrogen bonding or by the chelation of metal ions.
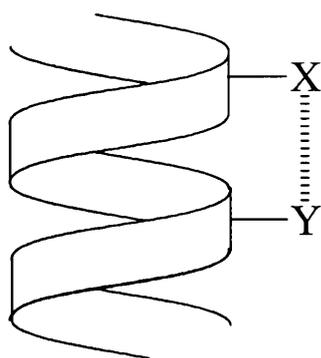
Figure 2.11: General Stabilising Interactions

The interaction of basic and acidic residues is well studied, and these interactions are known to stabilise several different sequences. Aspartic and glutamic acids are known to interact with lysine, arginine and histidine to create additional side chain hydrogen bonds. These interactions can be deduced from crystallography, despite the problems of locating hydrogen positions by this technique.[34] However, the use of similar interactions to stabilise helices in short peptide sequences has also proven to be effective. Peptides bearing both lysine and glutamic acid have been used to calculate values of k for guest amino acids in host-guest studies.[35] These have incorporated varying numbers of residues capable of side chain hydrogen bonding, and are usually alanine based.[29]
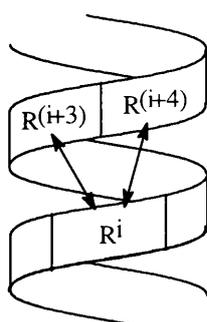
Figure 2.12: Idealised Intrahelix Interactions

The study of isolated portions of longer peptides first indicated this effect. It was found

that a pH dependent transition from coil to helix could be induced, showing the importance of the protonated states of two residues. This work was corroborated by further substitution studies,[36] and the position dependence of the interaction was also found. This work showed that an *i-(i+4)* spacing of the residues was the most effective at promoting helix stability.[37] It was also found that the ordering of the two substituents was not important, but that the interaction was most likely singly charged Glu^0-$Lys(NH_3^+)$. These effects could be affected by the pH, and also by screening by means of increasing salt concentration.
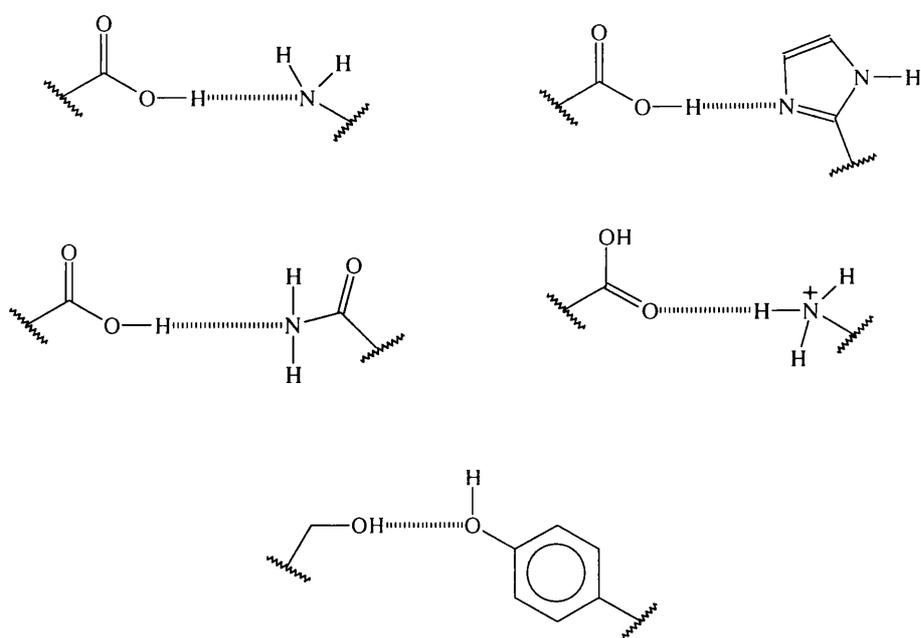
Figure 2.13: Possible Hydrogen Bonding Interactions Between Amino Acids

Whilst these studies have focused on acid-base interactions, there are other side chains capable of hydrogen bonding. These include the amide side chains of glutamine and asparagine, as well as serine and tyrosine which can, in some cases, take part in ion pair interactions.[35] Using *i-(i+4)* separation of residues, it has been found that aspartic acid under neutral or basic pH conditions gave a strong interaction when glutamine was positioned closer to the *N*-terminus.[38] However, this interaction was position dependent, the opposite ordering giving no effect. This interaction has also been found in several proteins.

2.7.2 Other Charged Interactions

It is also possible to generate salt bridge interactions using methods other than hydrogen bonds. Transition metals are frequently found in enzyme active sites, bound using basic, acid or in some cases sulphur-bearing side chains. These types of interactions can also be used in the stabilisation of helical peptides, by the binding of metals by appropriate residues.
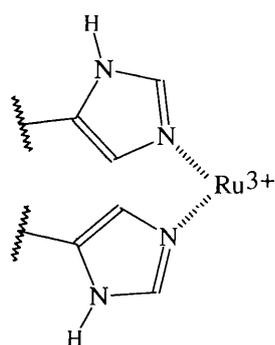
Figure 2.14: Binding of Ru(III) by Histidine

One example of this effect was the formation of a stable helix (up to 80% helical content) in a 16 residue peptide.[39] This sequence contains two histidines, in an *i-i+4* arrangement. The peptide, on treatment with a ruthenium salt, was found to have greatly increased helical content, regardless of the position of the histidines in the peptide. The helical content was determined by C.D. measurements, and the interactions of the histidines and the ruthenium were determined by means of n.m.r. and absorption spectroscopy. Thermal denaturation studies showed that the metal gave a further 1kcal mol^{-1} of stabilisation to the helix. This is a significant value, in view of the marginal stability of helices in short peptides.
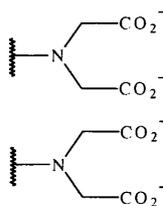
Figure 2.15 Aminodiacetic Acid Side Chain Groups

It is also possible to use unnatural residues to give better metal binding properties. The synthesis of four amino acid analogues bearing the metal binding aminodiacetic acid groups has been performed.[40] These residues have been incorporated into peptides of varying lengths, with different separation gaps, i-$(i+4)$ and i-$(i+3)$. Preliminary results have shown that several peptides are significantly stabilised by the addition of Cd^{2+} ions. In one case, this resulted in an increase from 0% to 82% helical content at $4^{\circ}C$ and pH 7.9.
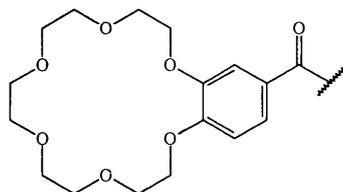
Figure 2.16: Crown Ether Acyl Group

Another charged interaction that has been utilised is the stabilisation of a charged species by a crown ether. This has been used in an attempt to stabilise helical structure by the interaction between lysine and a modified lysine, acylated with a crown ether.[41] Several different separations were tried, including the i-$(i+4)$ regime successful with other methods. In these cases, however, the methodology gave no extra stability to peptides in several different solvents, including less polar solvents. The greatest stabilisation was found to be for β-sheet formation between two strands. However, these peptides were very short (7 residues), and also N-terminal BOC protection was still in place. Both of these factors would weigh against the formation of helices, regardless of the stabilisation acquired by the interaction. The results do not make it impossible that this interaction could stabilise longer peptide sequences.
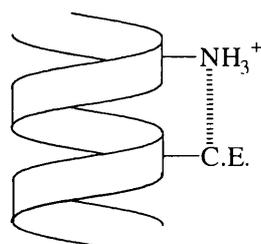
Figure 2.17: Crown Ether (C.E.)-Charged Lysine Interaction

2.7.3 Uncharged Interactions

As may be expected from the differing properties of proteinogenic amino acids, several other interactions that stabilise secondary structure are known. These include hydrophobic interactions, which are difficult to quantify, as well as specific interactions between certain residues. It has also been found to be possible to modify the properties of other amino acids.

An example of this latter approach has used an 18-member sequence containing four methionine residues.[42] This sequence has been found to give an amphiphilic helix, stabilised by aggregation between hydrophobic faces at higher concentrations. The high helicity was reduced by oxidation of the methionine residues, which removed the amphiphilic character. This effect shows the stability that reduction of solvent contact with hydrophobic residues can give in proteins. However, this work also showed that significant helicity was present at low peptide concentrations (4μmol), where such structure could be stabilised by the reduction of solvent contacts in the folded form.
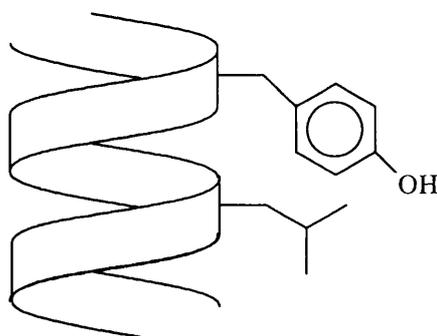
Figure 2.18: Tyrosine-Leucine Interaction

Specific interactions have been found between several different residues. Hydrophobic interactions between tyrosine and either valine or leucine have been found and characterised.[43] This work has shown that both possible interactions are most stabilising when the spacing between residues is i-(i+4), with the tyrosine towards the N-terminus. However, in the case of the Tyr-Leu interaction the order is less critical. In this case, the greater size and flexibility of the leucine side chain may contribute to this effect. The stabilisation is thought to arise from the reduction of water contact between

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the hydrophobic residues. This result is significant, when it is noted that the k values of the residues involved show all are helix breakers. This is partly borne out by results that show that the interaction is less stabilising in internal positions in the sequence, where the helix breaking effects would be stronger.
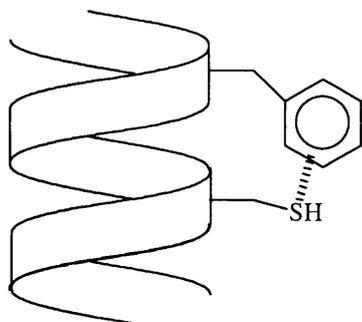
Figure 2.19: Phenylalanine-Sulphur Interaction

Another interaction of this type is that between phenylalanine and sulphur bearing residues.[44] This type of interaction is commonly found in proteins, and in this study was found to contribute significantly to helix stability. The most effective separation was found to be i-$(i+4)$ with both cysteine and methionine in the C-terminal position. The interaction is thought to arise between the electronegative sulphur atom with the slightly electropositive aromatic protons. There is also some mutual shielding for the two hydrophobic residues. The substitution of only one of the pair of residues gave a decrease in helical content in comparison to a model alanine based system. Although helicity was higher overall in the alanine based model peptide, the helicity was found to be higher with both substituents present than when only one substitution was made.
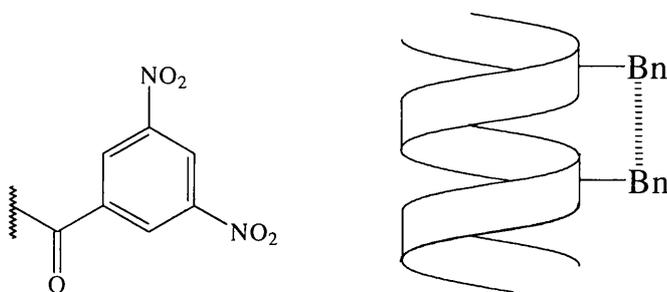
Figure 2.20: Nitrobenzyl (Bn) Side Chain Capping Groups and Interactions

The incorporation of artificial residues capable of acting by π-stacking has also been examined.[45] These have been created by the modification of lysine, *via* acylation of the ε-amino group using dinitrobenzoyl. These residues were employed in pairs, with varied spacing. In order to determine the effect of hydrophobic interactions between lysine side chains, different acylating groups were used. The overall results showed again that the *i-(i+4)* spacing was the most effective stabilising arrangement. This was checked by increasing the percentage of water, which increased the helical content up to 90% water. It was found that the effect from the lysine side chain aliphatic portion was negligible in comparison.

2.7.4 Cyclic Disulphide Formation

It is also possible to stabilise helical structures by means of formal covalent bonds between residues separated in the sequence. The commonly employed natural method of performing this task is to use disulphide bonds. This is illustrated in the example of charybdotoxin,[46] where the tertiary structure of the protein is locked using disulphide bonds. In nature, however, this method is not usually employed to stabilise single helices.
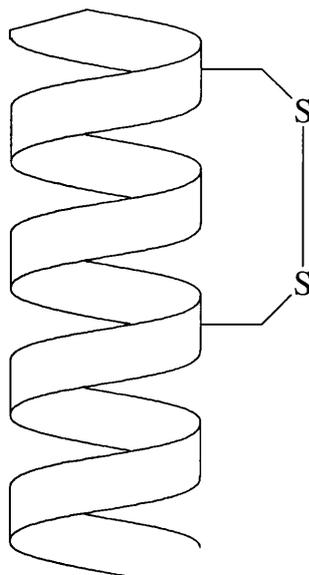
Figure 2.21: Disulphide *i-(i+7)* Bonds

The synthesis of a short, alanine based peptide containing two designed cysteine homologues has been performed, giving rise to helices stable at 60°C after disulphide bond formation.[47] The homologues, 2-amino-6-mercaptohexanoic acid derivatives were synthesised from lysine, and incorporated in an *i-(i+7)* fashion into four peptides of varying length. Analysis was then performed in the Acm- protected, deprotected and oxidised states, under conditions of varying temperature. The results showed that although reasonably helical as the Acm- protected form (expected due to the high alanine content), the disulphide linked peptides were almost completely helical at 0°C, and still retained significant (>48%) helicity at higher temperatures.

Disulphide cross links have also been used to stabilise helices indirectly by bundle formation. In one example, a synthetic protein has been created,[48] initially by the segment condensation of four sections sequentially using thioether links. The design of these peptides is most likely to give amphiphilic helices; after the formation of the tetramer, folding to a four helix bundle of a type commonly found in nature is most likely. The closure of a disulphide bond between the two "uncapped" termini, possible using differential protection, gives the final "cyclic" protein. Not surprisingly, this bundle is highly stable, retaining 80% of the helicity present at room temperature when heated to 80°C.

The non covalent interactions described above make the formation of the first turn of a helix statistically more likely. The additional stability that these interactions give to the energetically marginal first turn formation is reinforced by the formation of formal bonds. The macrocyclic structures created reduce the number of conformations possible. This in turn means that the loss of entropy is much lower, and so the first turn is more energetically stable.

2.7.5 Cyclic Amide Formation

It is also possible to form rigid structures using different types of covalent bonds. This has been achieved several times by means of amide bond formation between lysine and either aspartic or glutamic acids. One method involves the synthesis of Lys-Glu *i-(i+4)* bonds, in seven residue peptides.[49]
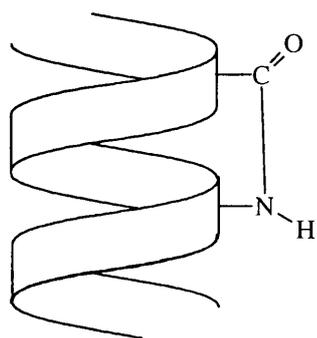
Figure 2.22: Amide *i-(i+4)* Bonds

These have been assembled in a segment condensation fashion to form larger units. A trimeric species of this type was found to be slightly helical in aqueous solution, but at higher levels when in 50% trifluoroethanol/water solution. The authors suggest that the stabilising effect of these bridges is small, when considered against the similar results for uncyclised model peptides. This is possibly due to the size of the macrocycle defined by amide bond formation. Given the separation in space of the two residues in a helix, the formation of an eight membered chain between them is unlikely to give sufficient conformational direction to form the first turn of the helix.

In addition to this work, studies on the stabilising effect of Asp-Lys amide links have been undertaken.[50] In these cases, *i-(i+4)* amide bridges have again been used, with both possible orientations employed. In this case, it was found that there was a slight increase in the helical content of the peptide, dependent on the positioning of the amide bridge, and the ordering of the bridging residues. The analogue containing the Asp-Lys bridge was found to have only low helix content even under helix promoting (TFE rich) conditions. The most stabilised variant of the system was found to have the amide bridge situated towards the *N*-terminus. Again, this reflects the theory described above that the N-terminus is the more significant in helix formation.

The closure of other possible amide macrocycles has also been examined in a systematic manner, to deduce the most stable organisation. This has been done by the closure of macrocycles in short peptides bearing acid and amine side chain residues of various lengths, in an *i-(i+3)* fashion.[51] The model peptides were of the form Ac-

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amine-Pro-Gly-acid-NH_2 and were found to give mainly turn structures under the conditions used. This is unsurprising given the short length of each peptide.
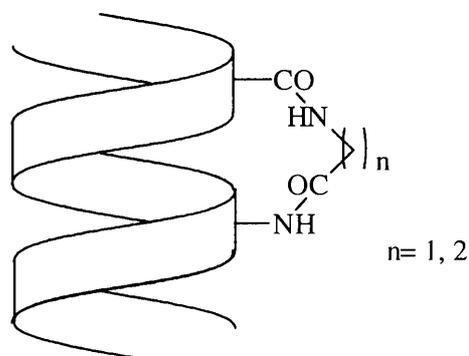
Figure 2.23: Amide *i-(i+4)* Bonds, with Varying Linker Lengths

The closure of macrocycles has also been used to generate helical peptides based on hGRF (1-29) sequence.[52] One of these sequences, involving an Asp-Lys *i-(i+4)* amide bond has been found to retain significant helicity by modelling studies. This helicity has also been correlated with the biological activity of this system. Other work on hGRF has also demonstrated that amide bond formation can give increased helical stability.[53] This has lead to the synthesis of a large number of analogues, both mono- and dicyclised forms. Again, the form cyclised between residues *i-(i+4)* has proved to be the most biologically active, which suggests that it is the most helical. The ideal ring size has also been deduced by incorporating a linker, *e.g.* Gly, β–Ala, between the acid and amine bearing residues. This has shown that in terms of biological activity, the ideal macrocycle ring size is 22 atoms. The difference between activities for even small changes in the ring size is significant.

2.8   TEMPLATE EFFECTS

The macrocycle created by the joining of side chain groups is flexible in nature, and requires the organisation of the peptide chain to give the first hydrogen bonds. Template reporter groups[54] give the effect of preorganisation of the amide bonds due to their more rigid, fused ring natures. Conformational equilibrium then alters the

organisation of hydrogen bond acceptors present on the template until an optimal form for helix formation is reached. After this, the formation of the helix should be a simple equilibrium, as described by the folding theories. With the use of these groups, deduction of the value of s for a particular residue should become simpler.[55] The first of these groups to be developed was an N-terminal template, derived from proline cross linked using a thioether bridge, with three carbonyl groups orientated to act as hydrogen bond acceptors. This system required that a particular conformation was again taken up by the template, as only one conformation could initiate the folding process. The major difference in possible structures concerns the two possible conformations of the thioether, and determination of the relative amounts of each conformer can be achieved by simple n.m.r. analysis of proton couplings.
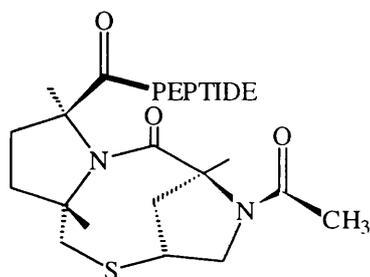
Figure 2.24: Kemp Helix Template Molecule

These groups have resulted in the determination of different values of s, and different assignments of stabilising effects. The effect of the incorporation of solubilising lysine residues in alanine based peptides has also been called into question.[56] In this work, it is suggested that the interaction of lysine with the helix of alanine rich peptides is more critical to the stability than the helix forming tendency of alanine itself.

Other helix inducing templates that have been developed include a modification of the system described above using three α-methylproline residues, cyclised by the use of a mercaptoacetic acid group.[57] Also, the synthesis of a group capable of capping the C-terminus of a peptide has been performed, involving the synthesis from a tripeptide of a moiety bearing a stable positive charge, capable of stabilising the macrodipole.[58] No results concerning the efficiency of these groups have been reported as yet.

The use of larger groups responsible in part for the folding of peptides has also been described. This approach, termed TASP (Template Assembled Synthetic Protein), has been devised to allow the use of amphiphilic sequences to promote helix formation.[59] The TASP molecule is formed of a macrocycle, made up of two Lys-Gly-Lys sequences linked by two naphthalene derivatives. Differential protection of the lysine side chains allows incorporation of different peptide sequences at different times, and the close proximity provided by the template means that any helix formation will be stabilised by association of hydrophobic faces. The results of a C.D. study show that the TASP-bound peptides contain a significantly higher degree of helicity in comparison to the free peptides. Other results have shown that other peptide tertiary structures can also be stabilised by this method.[60]

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CHAPTER THREE

ASYMMETRIC SYNTHESIS OF α-AMINO ACIDS

3.1   INTRODUCTION

To attempt to summarise all methods making up the field of the asymmetric synthesis of amino acids would be an enormous task. Fortunately, several good reviews[1] and books[2] exist which deal with the subject in detail. As the strategies employed tend to be general, discussion will be limited to the more significant methods in each section.

These have been subdivided into different categories: chiral alkylation reactions, generally employing anionic glycine equivalents; chiral amination or azidation reactions, usually of alkyl substituted acids; chiral carboxylation reactions, achieved by the use of carboxyl precursors as intermediates prior to hydrolysis; the expanding field of asymmetric hydrogenation, using a variety of catalysts; the elaboration of side chains of chiral amino acids; the use of enzymatic reactions; and finally, the use of the apparently more simplistic method of resolution of racemic mixtures.

3.2   CHIRAL ALKYLATION REACTIONS

As the only amino acid lacking a side chain, and hence a chiral centre, glycine is a natural target for elaboration *via* the attachment of different functional groups to the α-carbon. Glycine may be considered to be simply a substituted carboxylic acid, which allows that it may be activated *via* its deprotonated enolate form. However, in order to permit this type of activation, the regioselective action of bases is required. Hence, any methodology must involve the protection of the carboxylic acid and amine groups.

An additional problem is that the planarity of an enolate ion gives rise to problems of face selectivity of electrophilic attack. This means that if a simple enolate is used, a racemic mixture will be produced. If preference of one enantiomer over another is required, then some means of directing the approach of the electrophile must be included. This is termed the use of a chiral auxiliary.
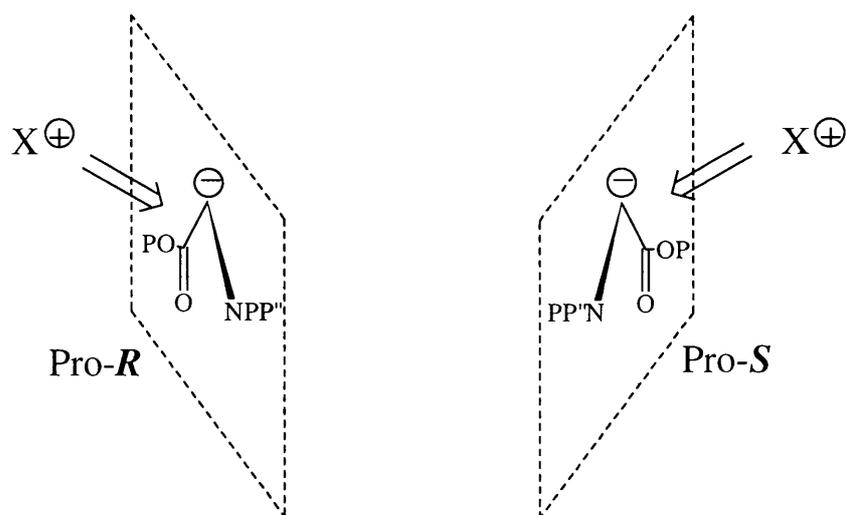
Figure 3.1: Enantioselective Alkylation of Glycine Enolates

The majority of methods in this section, which forms by far the bulk of literature methods, use glycine as a source of amino and carboxylic acid functionality, avoiding the problems set out above using protecting groups, with the incorporation of a group to direct alkylation. The most effective methods perform these three tasks in simple molecules. The incorporation of glycine into ring structures gives protection of both termini, while enforcing a rigid geometry. Hence, a directing group as part of this ring will profoundly influence the face selectivity of alkylation.
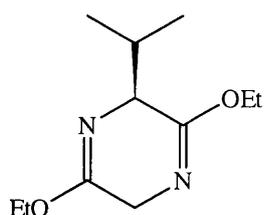
Figure 3.2: 6-(*S*)-Isopropyl-2,5-diethoxy-3,6-dihydropyrazine

The chiral *bis*-lactim ether template molecule was first introduced by Schöllkopf in 1979.[3] The molecule is a simple dihydropyrazine, formed by the condensation between an *L*-chiral amino acid and glycine, the resulting lactam being ethylated or methylated to prevent amide cross reaction. On treatment with strong base, this ring is

deprotonated to give an anionic species which is then treated with an electrophile to give rise to a new chiral centre. Initially, this route employed haloalkanes,[4] but has been expanded to include aldol type chemistry[5] and Michael type chemistry[6] as well.
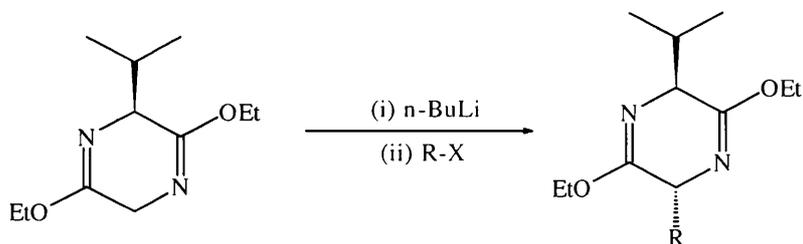
Figure 3.3: General Alkylation Scheme

The process is directed by the chirality of the S-valine auxiliary. After deprotonation, the ring takes up a virtually flat form, the charge being delocalised into the two imines present in the ring. This flatness forces the side chain to jut out from one side of the ring, hence making approach from that face of the ring by the electrophile quite unfavourable. Several different amino acids have been employed as the auxiliary in this type of system, including most of the β-substituted proteinogenic amino acids.[7] The most effective auxiliary for this type of molecule is tert-leucine,[8] bearing the sterically bulky tert-butyl side chain. This residue is not readily accessible, so it has been found that valine,[4] carrying an isopropyl side chain, provides sufficient steric bulk to direct the attack, with no effect on chiral induction.
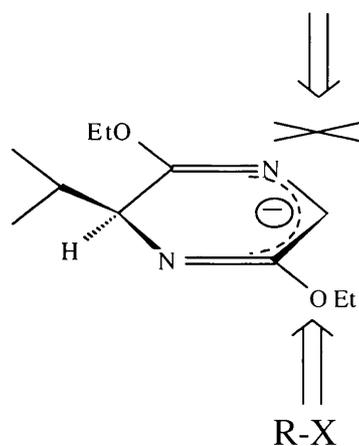
Figure 3.4: Alkylation of Lithiated Pyrazine

This also allows easy access to unsaturated side chain amino acids *via* dehydrative methods. In the case of Michael addition using unsaturated esters and ketones, chiral induction at the β-centre has been found to be high. This is thought to be due to complexation of the carbonyl group of the substrate with the lithium salt of the pyrazine, giving preference to attack through one face of the substrate. The product amino acids in their esterified form, along with esterified valine, are liberated by a simple acidic cleavage method.
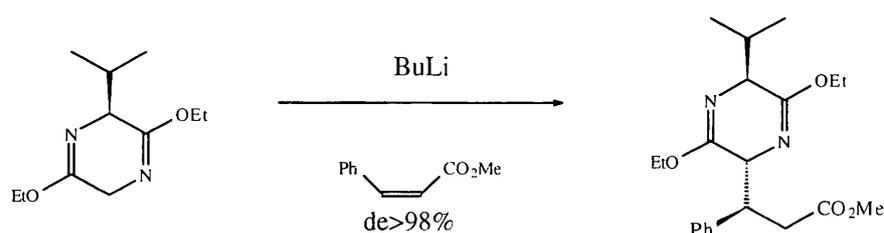
Figure 3.5: Michael Addition Scheme

With aldol type reactions, a β-chiral centre is also generated. However, this process is generally not well directed by this reagent.
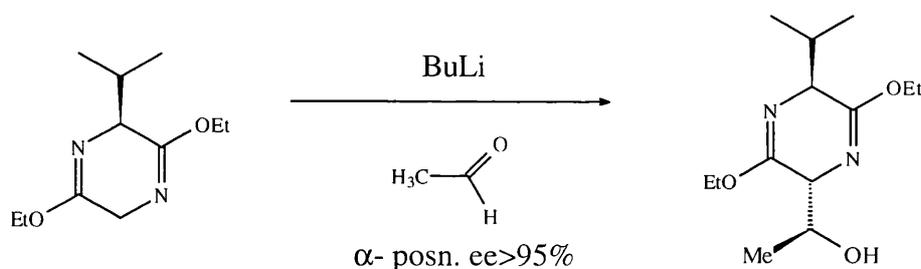
Figure 3.6: Aldol Reaction Scheme

The use of these molecules is, however, limited by their highly basic nature. More complex substrates are known to give poor yields or e.e.s or, in some cases, cross reaction.[9] New methods of employment have, therefore, been developed in order to limit their reactivity. In their total synthesis of the amino acid anticapsin,[10] Baldwin and co-workers found that the lithiated pyrazine could give rise to 1,4- elimination of HI from their conjugated alkylating reagent. They found that by using the higher order

cyanocuprate generated from the lithiated pyrazine, they could get alkylation at a much higher yield, with limited cross reaction.
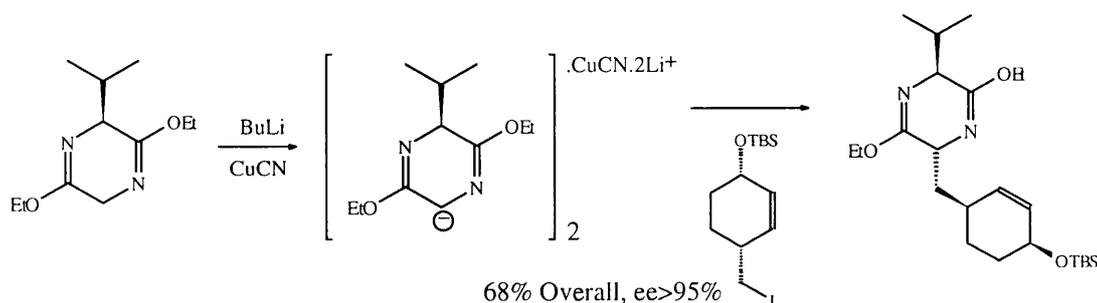
Figure 3.7: Alkylation of Pyrazine Cyanocuprate Complex

Pyrazine molecules have also been used to produce α,α-disubstituted amino acids, for example by substituting alanine in the place of glycine.[11] This has become one of the larger areas of application of this methodology, giving easy access to a large number of systems by a known and reliable method. In this case, the larger steric bulk of the side chain of valine appears to direct the deprotonation to the less hindered site. It is, therefore, reasonable to assume that similar directing forces apply in the case of glycine, although there will be some stabilisation as a secondary as opposed to a tertiary carbanion will be created.

The methods described above use S-chiral auxiliaries to generate R-chiral products. This can be very useful if unnatural products are required, but of limited value otherwise. In other cases, the auxiliary needed would be of the unnatural R-chirality, making the synthesis of the pyrazine antipode an expensive process. Fortunately, there are several other routes giving similar high e.e.s that are currently available.

The amines in these types of syntheses are generally required to be diprotected. Although more usually employed as a form of mild acid labile linker, the imine group fulfils this criterion by being unreactive to the bases employed. In the methodologies shown above, it is possible to see that the formation of two imines protect the nitrogen groups present in the ring. The imine group has also been used to protect amines in other systems, allowing the enolisation and functionalisation of glycine. The simplest of these routes couple esterified glycine to a chiral aldehyde,[12] which can then be reacted

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with an electrophile following activation to give the α-substituted product. Variations on this general scheme include the incorporation of chiral esters.[13] In accompaniment to these acyclic systems, several cyclic systems containing an imine have been described. These have included β-lactams[14] and benzodiazepinones,[15] with direction of alkylation being provided by chirality present in the ring.
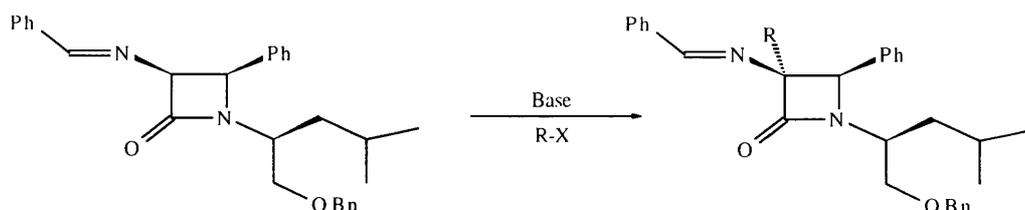
Figure 3.8: Alkylation of β-Lactam
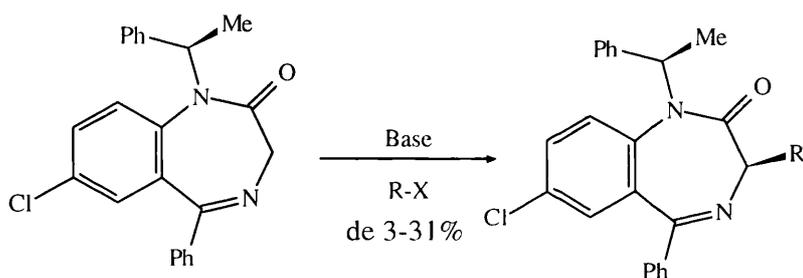
Figure 3.9: Alkylation of Benzodiazepinone

An alternative method of chiral alkylation of the glycine enolate form has been described by Seebach.[16] This route employs either an oxazolidinone or imidazolidinone as the template, these being the five membered aminal rings formed by condensation of pivaldehyde and glycine, in the presence or absence, respectively, of an alkyl amine, followed by further protection of any amino group present. The conformation of the ring is determined by the bulky *tert*-butyl group, which is in a pseudoequatorial position. This, in conjunction with the pseudoaxial conformation of the two heteroatom lone pairs, directs the approach of the incoming electrophile to the bottom face of the enolate. This process has been termed the "self-reproduction of chirality".

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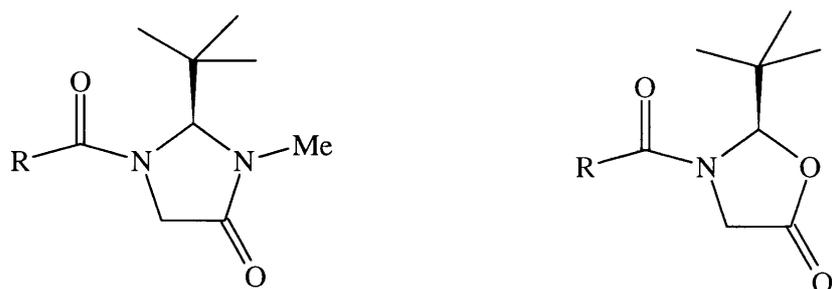
Figure 3.10: Seebach Chiral Auxiliaries

As the components for the assembly of the ring systems are all achiral, this method is highly versatile, able to give both *R*- and *S*-products depending on the ring form used. These ring systems are also reactive with a range of electrophiles, in a fashion similar to that described for the pyrazine molecule.[17] However, the achiral starting materials make the synthesis of the ring systems a complex process, requiring a resolution step using mandelic acid. The system is more usually formed incorporating a chiral residue, as this not only allows further functionalisation, but can direct the formation of the ring system, eliminating the need for resolution. An example of this approach is the use of methionine to give a route to α-ethyl-*allo*-threonine.[17] For the more stable imidazolidinones, more sensitive products are beyond the scope of the method, due to the harsh acidic conditions required for unmasking of the products.[18]
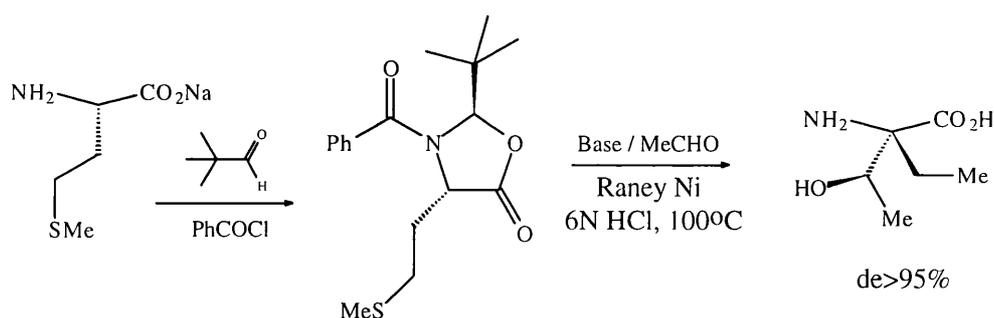
Figure 3.11: Synthesis of *Allo*-isothreonine

The last major auxiliary group of this type is the chiral oxazinone series, developed by Williams.[19] These reagents are formed from benzoin, which is initially converted to the

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amino alcohol, and the racemate formed resolved using glutamate salts. The enantiomerically pure compound is converted to the ester using bromoacetic acid, followed by closure of the 6-membered ring using acid catalysis. Final protection of the amide gives the reagent in a form ready for activation. The use of benzoin gives a ready supply of starting material, and whilst the procedure does contain a resolution step, this gives the process easy access to both enantiomers of the required oxazinone.
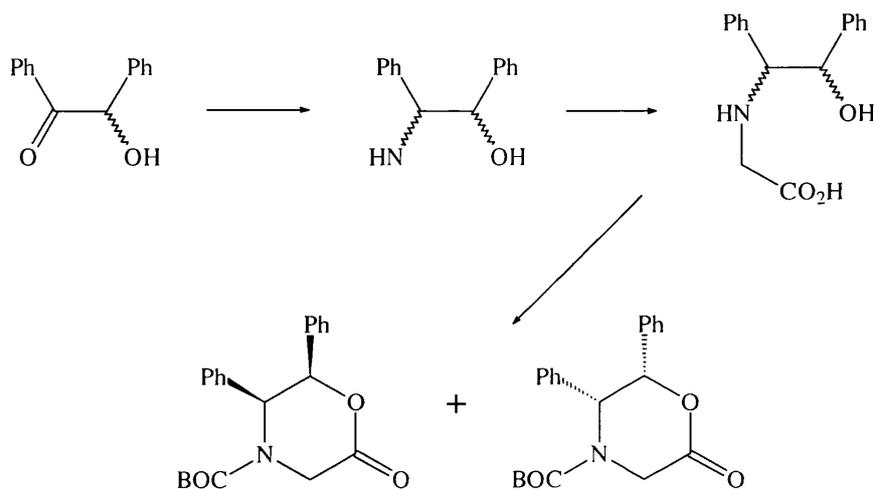
Figure 3.12: Synthesis of Williams Chiral Oxazinone

This molecule can also be functionalised in two different ways. Firstly, it is possible to effect enolisation using strong base, with the two phenyl rings providing steric bulk and hence direction to the approach of the electrophile.[20] Studies have shown this reaction to work in reasonable yield with good e.e..[19]

It is also possible to functionalise this residue using a glycine cation pathway. Reaction of the oxazinone with N-bromosuccinamide gives rise to the chiral bromo-compound in excellent yield, which is subsequently reacted with a nucleophile in the presence of a Lewis acid to give the alkylated product. The most frequently used Lewis acid is zinc (II) chloride, which is thought to promote formation of the iminium ion in activating the ring.[21] Results show that alkylation occurs with retention of chirality from the bromooxazinone. Yields reported vary from reasonable to good, with excellent accompanying e.e. values. This route also has the advantage that it allows access to protected amino acids, depending on the type of amide protection used, and on the

ring cleavage conditions. These are generally hydrogenation, or reducing metal conditions.
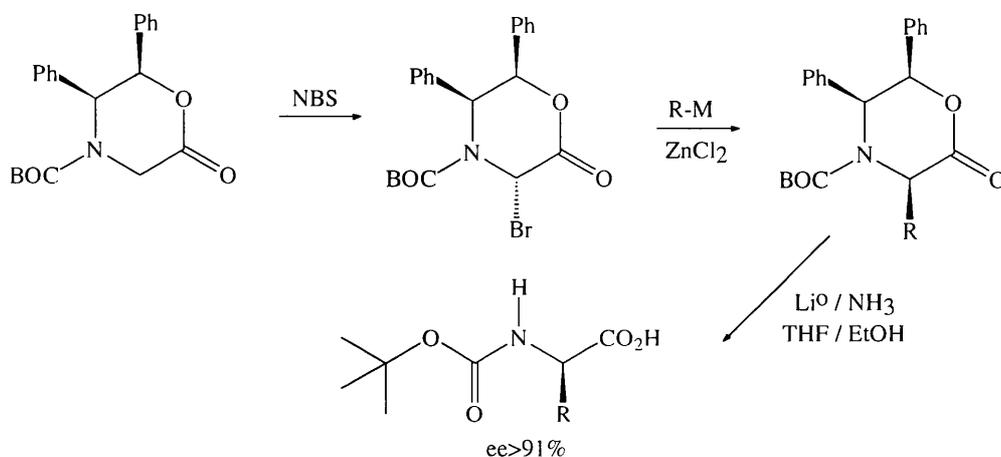
Figure 3.13: Alkylation of Pyrazine *via* Bromide

As with previous reagents, reactions have been carried out with several different types of nucleophiles, in the presence of different Lewis acids. Varying the strength of the nucleophile, Lewis acid and solvent polarity independently has shown that the reaction may be tuned to give either the *syn-* or *anti*-adduct as the major product.[22]
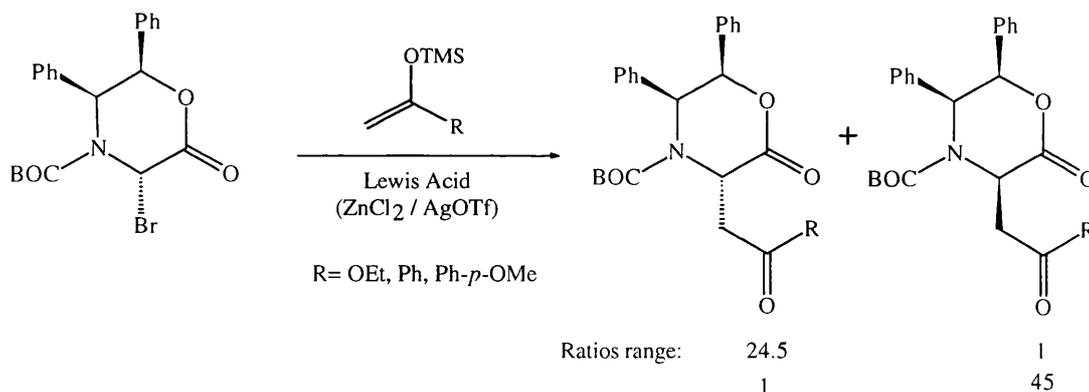
Figure 3.14: Alkylation of Oxazinone *via* Bromide

The Schöllkopf pyrazine molecule has also been functionalised by this method. After initial deprotonation, the anion is reacted with hexachloroethane, giving the chloropyrazine product with mainly *cis*-selectivity. This then allows reaction with

nucleophilic reagents, such as malonate ions,[23] as shown in the scheme below. However, this reagent does not give retained stereochemistry in the final products.
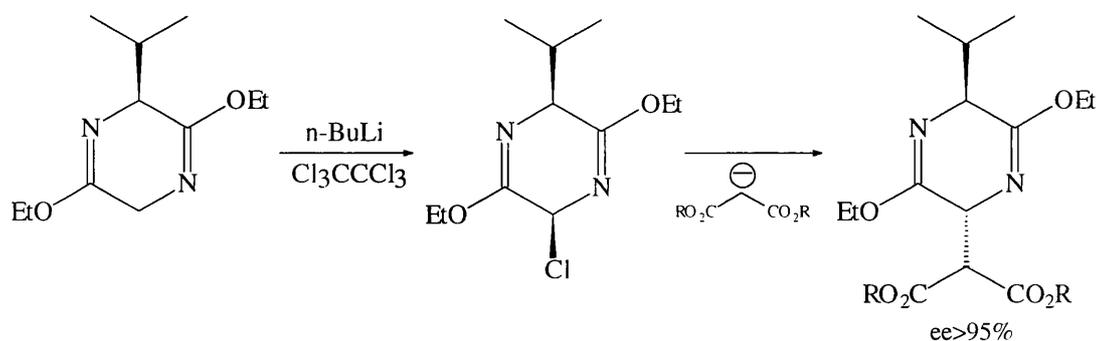
Figure 3.15: Alkylation of Pyrazine by Malonate Anion

3.3 CHIRAL AMINATION REACTIONS

The approaches taken to promote chiral amination do not differ strongly from those involved with alkylation reaction, with the use of chiral auxiliaries predominating. Differences arise as alkylations generally employ a chiral reagent, whereas aminations, and the related azidation reactions, can more easily employ auxiliaries that can be reclaimed after use.

Two of the more commonly used auxiliaries are the oxazolidinones developed by Evans,[24] and the chiral sulphonamide derived from isobornene developed by Oppolzer.[25]
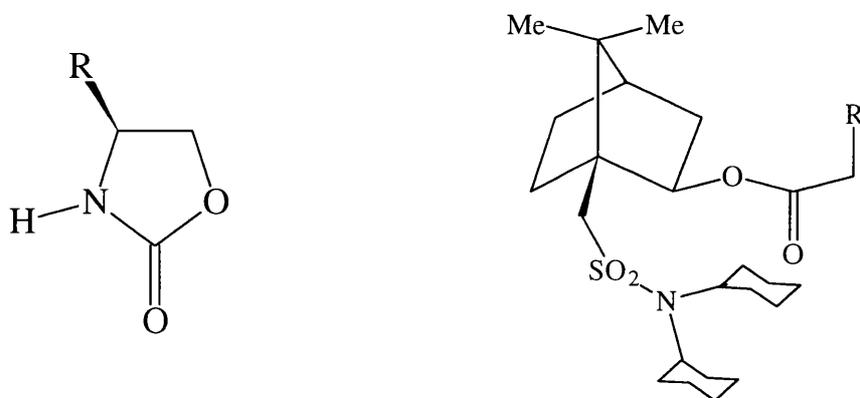
Figure 3.16: Evans and Oppolzer Auxiliaries

The oxazolinone series utilises the chiral pool, in the form of the natural amino acids, to give easy access to chiral auxiliaries. The favoured residues for this process have β-branched side chains, to give steric bulk. In most cases, valine is employed, as the isopropyl side chain is short and conformationally restricted, giving a rigid structure when incorporated into the ring. As the reactions involving this system are generally high yielding and enantioselective, they have become widely used. As with the Schöllkopf series, several different starting materials may be employed and so a large number of routes to these comparatively simple molecules exist.

The group is employed by acylation with an achiral acid, bearing the functionality desired on the amino acid side chain. This can then be deprotonated to give an enolate ion. Further reaction with a different Lewis acid to give either Z- or E-isomers of the enolate may be employed, followed by addition of an electrophile to give the amine equivalent. Unmasking of the acid is easily achieved by use of lithium hydroperoxide, after which the auxiliary can be reclaimed, and the amine revealed by processes dependant on the amine equivalent used.
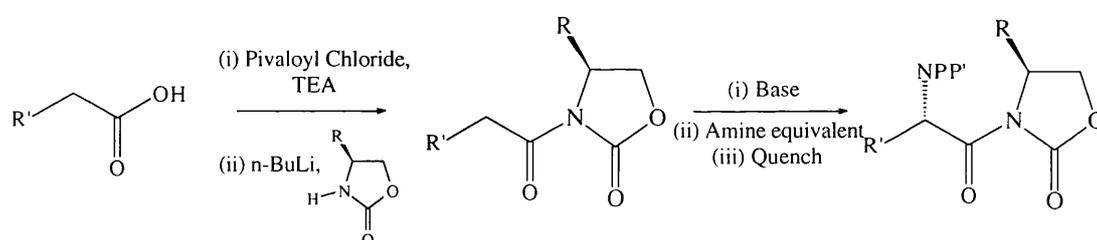
Figure 3.17: General Scheme for Electrophilic Amination using Evans Auxiliary

The most commonly employed electrophiles are diazodicarboxylates, or organic azide donors. As chiral induction is directed by the asymmetry of the auxiliary, the most effective electrophiles are those with the greatest constraints on their approach due to their steric bulk. Hence, di-tert-butyl azidodicarboxylate[24] and triisopropylsulphonyl azide[26] are the donors of choice. In both these cases, high diastereoselectivity is accompanied by good yield. These methods allow access to amino acids otherwise difficult to obtain, because of difficulty in performing alkylation as described above. After removal of the oxazolidinone, the amine is revealed in the first case by the action

of acid hydrolysis followed by hydrogenation using Raney nickel, or in the second case by reduction by a number of different methods, including Lindlar catalysis[27] and Staudinger reaction.[28]

The advantage of this type of reaction is its simplicity, with easy introduction and cleavage of the directing group. The Evans' oxazolidinone route has been used in the synthesis of several complex peptidic antibiotics,[26] with stereochemistry introduced to relatively complex side chains with little or no cross reaction.
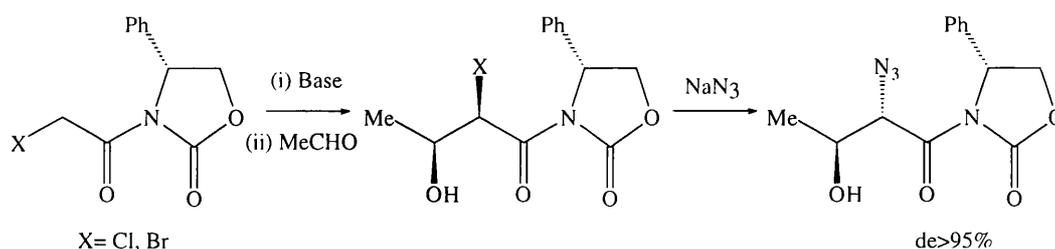
Figure 3.18: General Scheme for Nucleophilic Amination using Evans Auxiliary

The system has also been used in reactions to give nucleophilic amination, which can be useful if opposing chirality is required. In this type of process, chirality is introduced during the attachment of a side chain to an α-haloacetyl oxazolidinone, which can then impart chirality to the product at the α-position. After this, the halogen can be replaced by an azide, with inversion of stereochemistry at this point. This type of reaction has been used in conjunction with aldol chemistry to give products with not just α-stereocontrol, but β-control as well. This is postulated to occur because of the particular transition state the enolate ion-substrate complex takes up.
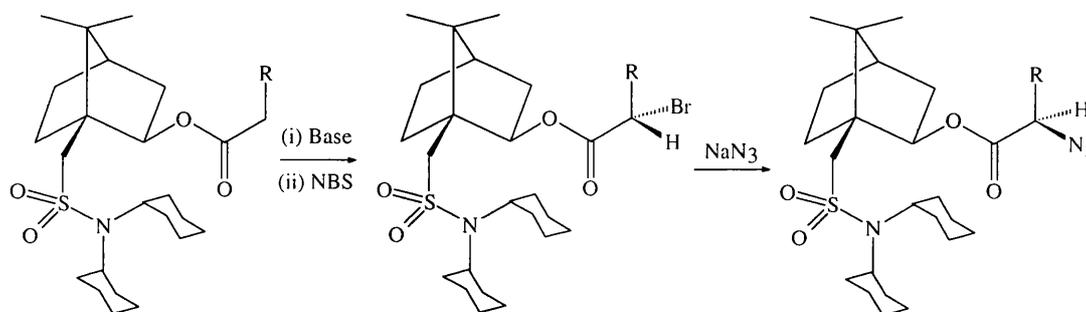
Figure 3.19: General Scheme for Nucleophilic Amination Using Oppolzer Auxiliary

The Oppolzer auxiliary has also been used to perform this type of reaction. The form of the reagent means that the silyl enol ether can be generated, then quenched using NBS, or its chlorinated equivalent. The halide produced is chiral, and the stereochemistry can once again be inverted by exchange with azide. Cleavage of the auxiliary gives a product with stereochemistry inverted from the halide. This reaction has also been performed with control at the β-carbon in the synthesis of *L-allo*-isoleucine.[29] In this case, crotonate attached to the auxiliary was treated with diethyl copper, giving chiral Michael addition to the double bond, prior to bromination at the α-position. After S_N2 exchange to the azide with accompanying inversion, the amine was obtained *via* reduction.
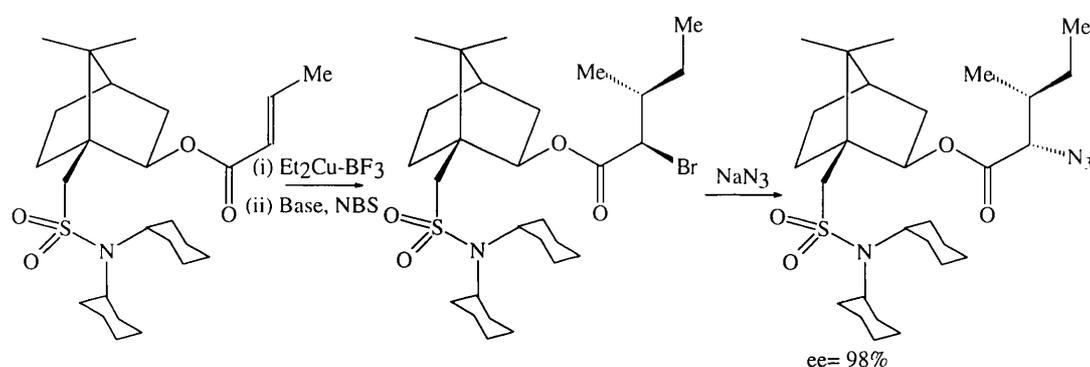
Figure 3.20: Synthesis of *Allo*-isoleucine

3.4 CHIRAL CARBONYLATION REACTIONS

Introduction of the carboxylic acid functionality is the last method to be considered. To date, relatively few methods of performing this stereoselectively have been published, and only two form the backbone of the majority of syntheses performed in this fashion.

These two reactions are the Strecker synthesis[30] and the Ugi reaction[31]. In both cases, nucleophilic attack is performed on a functionalised imine, generating products which can then be unmasked by acid hydrolysis to reveal the new carboxylic acid functionality. Both reactions are, however, non-stereoselective if an auxiliary is not used. Fortunately, the generation of chiral complexes capable of undergoing these types of reactions is a well known and relatively trivial process.

The Strecker synthesis is quite simple in its approach, requiring the initial condensation of an aldehyde with an amine to give the imine. Stereoselectivity can be introduced at this point by the use of a chiral amine. Introduction of a stereocentre next to the imine means that one face of the planar species is more effectively blocked, and hence attack from the opposite face will be favoured. The nucleophile used is cyanide and after the attachment of the nitrile group, hydrolysis to the acid is performed using concentrated hydrochloric acid. Cleavage of the chiral auxiliary is most regularly achieved by hydrogenation, but this does result, due to the removal of the amine to the product, in the destruction of the auxiliary. Both yields and e.e.s are variable, depending on the nature of the directing group, and on the starting aldehyde. In the example below, e.e.s of greater than 98% were achieved by the use of either enantiomer of α–methyl benzylamine in conjunction with simple, straight chain aldehydes.[32] The HCN was introduced to an ethanolic solution of the imine as a gas.
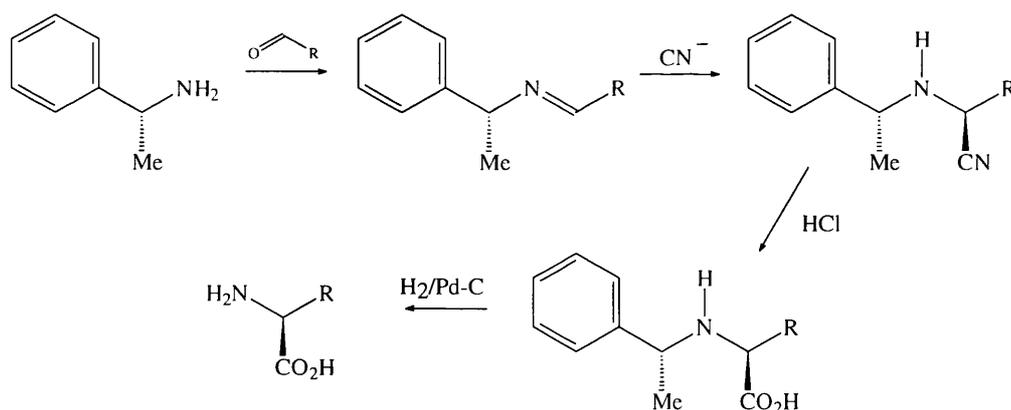
Figure 3.21: General Scheme of Strecker Reaction

A modification of this system can give rise to α,α-disubstituted amino acids by the use of a ketone in the place of the aldehyde. The imine created is susceptible to attack as directed by the chiral centre already present. In one example, phenylacetone is used along with α- methylbenzylamine, to give an imine that is reacted with sodium cyanide in ethanol to give the nitrile.[33] Acid hydrolysis, followed by hydrogenation to remove the auxiliary gives the α-methyl, α-substituted amino acid in good yield with e.e.s reported as above 98%.

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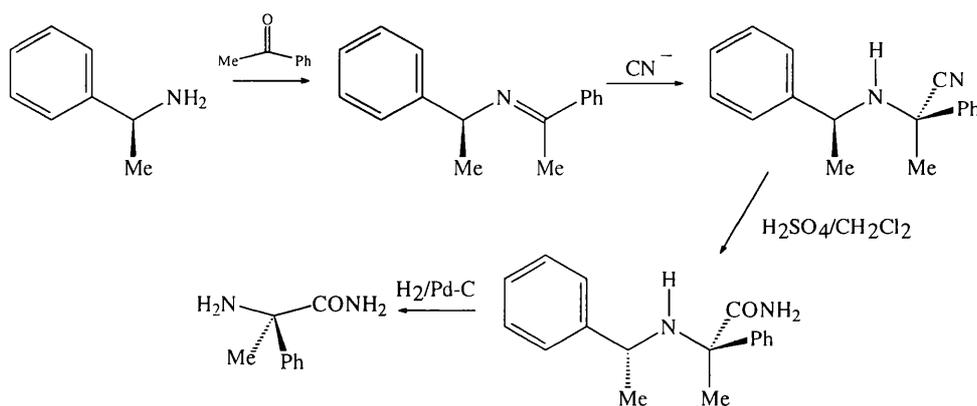
Figure 3.22: Synthesis of α-Methylphenylalanine

The use of several different chiral amines used as auxiliaries has also been reported. These have included amino dioxane derivatives[34] and protected glycosides converted to amines at the anomeric position.[35] Both of these methods tend to provide good yields and good e.e.s, dependent again on side chain bulk in the case of the dioxanes, and on the use of a Lewis acid such as zinc chloride to suppress anomerisation during nitrile attack in the carbohydrate example.
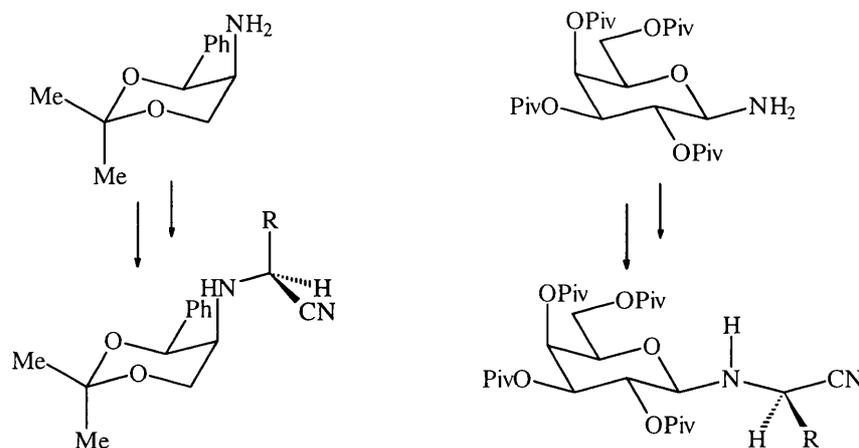
Figure 3.23: Strecker Reactions Directed by Carbohydrate Auxiliaries

The major drawback with this type of reaction is the toxicity of the reaction mixture, which would require special containment. This problem can be partly overcome by the use of solid sodium cyanide. Another possible problem is the cleavage regime, which

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requires a strong catalyst, in some cases under high pressure. This could lead to problems of cross reactivity with certain aldehyde or ketone side chains.

The alternative methodology is the use of the Ugi reaction, which employs an isonitrile in the place of the cyanide. In one example, an imine created from a substituted aldehyde and pivalyl-protected galactose, aminated in the anomeric position, is treated with a substituted isonitrile, this time in the presence of formic acid.[36] The resulting adduct is attacked by the acid, giving an amide and a carbonylated amine. The product can be freed from the auxiliary by methanolic HCl, and the amide cleaved by aqueous HCl. This route has been used to give phenylglycine, which is prone to racemisation, in good yield and e.e..
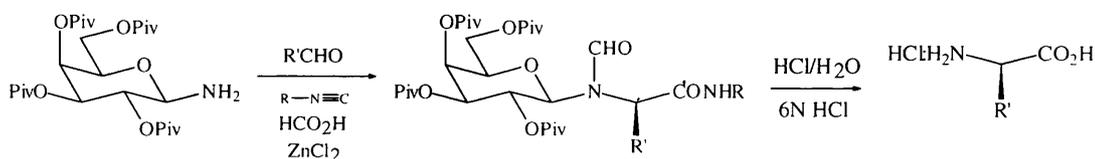
Figure 3.24: Asymmetric Ugi Reaction Directed by Carbohydrate Auxiliary

Continuing the use of carbohydrates as auxiliaries, 1-amino glucose has been condensed with isobutylaldehyde, and then reacted with *tert*-butyl isonitrile, to give rise to the charged adduct.[37] This is then reacted with the trifluoroacetic acid salt of glycine, which through action as an acid creates the dipeptide *tert*-butyl amide, glycylvaline, in high d.e. (98%) whilst still attached to the auxiliary. Simple treatment with water then gives deglycosylation, to reveal the dipeptide product. This method could be used to generate various dipeptides, but glycine is most easily used as no side chain protection is required.

This route has advantages of milder conditions than those required for the Strecker synthesis, but has some disadvantages in terms of ease of access to the desired isonitriles.

3.5 ASYMMETRIC HYDROGENATION

The final method of generation of a new chiral centre is the use of asymmetric hydrogenation. It has been known for some time that it is possible to selectively hydrogenate one face of a molecule by heterogeneous catalysts, directed by an auxiliary.[38] More recently, the development of chiral ligands, such as BINAP,[39] has given rise to more efficient homogenous catalytic systems.

One example of the use of an auxiliary is that shown below, where the condensation of an N-aminoindoline with a α-keto ester gives rise to a hydrazonolactone.[40] The imine present in the new heterocycle may be reduced out using aluminium amalgam with water, which after hydrogenation over palladium gives the new amino acid, along with recovery of the indoline auxiliary. In this example, yields are moderate to good, with c.e.s of over 92% in each case tried.
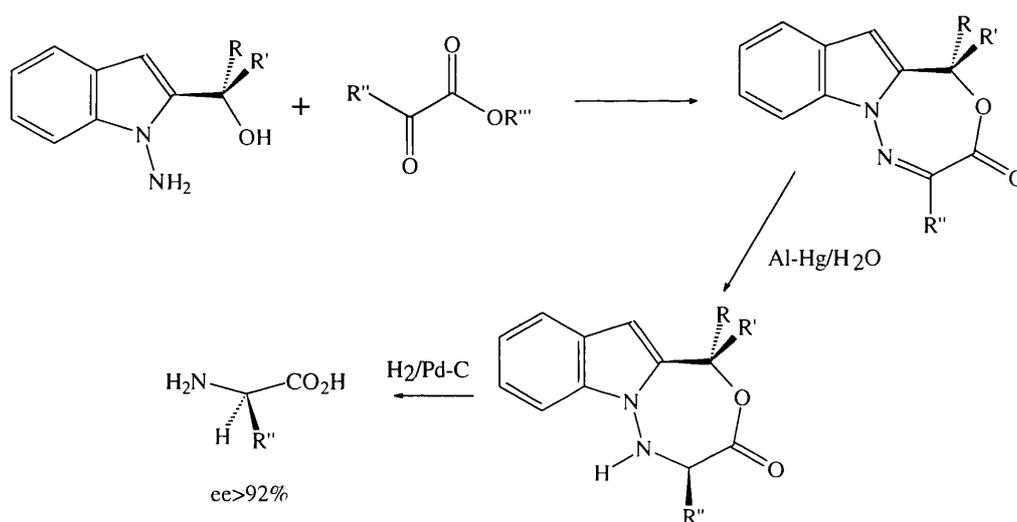
Figure 3.25: Asymmetric Hydrogenation using Palladium Catalysis

A molecule similar to that used in the chiral alkylation method of Schöllkopf has also been employed. A di-acylated piperazinedione has been treated with aldehyde in the presence of base, to give an unsaturated derivative.[41] After removal of the acyl protecting groups, the unsaturation is reduced using H_2-palladium, directed by the stereochemistry of the other side chain. Again, acid hydrolysis reveals the product

amino acid, in variable yield and e.e.. One possible reason for this could be the poor separation of product from auxiliary.
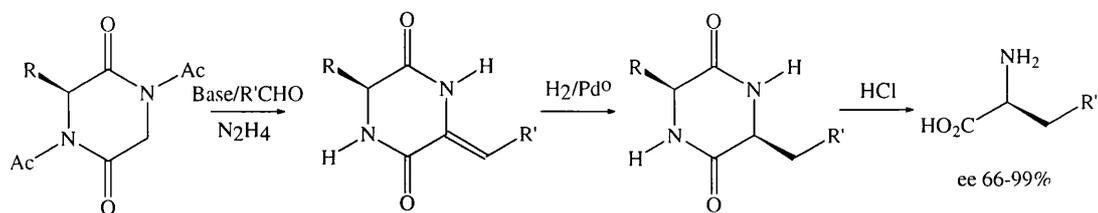
Figure 3.26: Asymmetric Hydrogenation of Piperazinediones by Palladium Catalysis

Another peptide-based method tried has been the use of tripeptides containing a dehydroalanine in a central position.[42] Other chiral residues are positioned either side of the dehydro-residue, and the trimer is then treated with H_2-palladium charcoal. Yields are variable, dependent not only on side chain bulk for the directing groups, but on position of the residue in the sequence. It has been found that the C-terminal residue had the major directing effect. β-Lactam derivatives have also been used. These are generally furnished by the reaction of an imine bearing a directing group and a ketene.[43] The strained ring then provides direction for further elaboration before the rings are opened by hydrogenation to reveal the final products.

In the cases above, chirality generally introduced by reactions prior to hydrogenation is used to direct the chirality of the final products. In the second method of reaction, the substrate is an achiral material, induction occurring because of chirality present in the ligands bound to the catalyst. This in theory allows a very high turnover of product for the quantity of catalyst used.
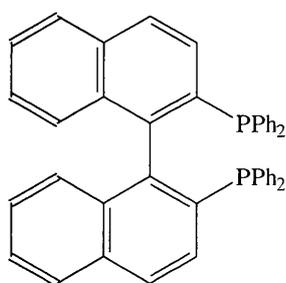
Figure 3.27: BINAP Chiral Ligand

The most widely known of these ligands is the *bis*-(naphthyldiphenylphosphine) system, generally termed BINAP.[39] The two naphthyl groups of this molecule cannot lie planar to each other, so the ligand has two distinct rotameric forms when bound to a metal ion. This molecule is available as either rotamer, the two forms giving different chirality products, and in conjunction with rhodium as a catalyst has given results up to 98% e.e.. It is now used in industrial processes, along with several other variants which essentially act in a similar way. The most effective catalysts, from a standpoint of e.e., appear to be those that create a rigid cycle around the metal centre, with few or no bonds free to rotate. However, the results are in general very impressive regardless of the system considered. The substrates for these types of reactions are generally dehydroalanine derivatives, which only needs minimal protection. In many cases, simple acetylation of the amino function will suffice.[44] As stated, different chiralities will be produced dependent on the ligand used. While (*S*)-BINAP will give *R*-chiral products, its complementary form will provide *S*-amino acids. However, yields have been observed to fall if the substrate is β-disubstituted.[45]

These catalysts now change the problem from one of ensuring good e.e. values, to one of providing suitable substrates for the reaction. The generation of suitable dehydroalanine derivatives is less difficult in theory, as the residue contains no chiral centres, so any process that generates a chiral centre in the form of a racemate is perfectly acceptable, as this chirality will be removed prior to hydrogenation. One of the most commonly used methods is the dehydrohalogenation of β-chloro-substrates using diazobicycloundecene (DBU).[46]
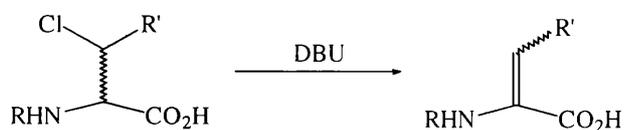
Figure 3.28: Dehydrohalogenation of β-Chloroalanine

An alternative method involves the generation of azalactones *via* a dehydration mechanism, followed by reaction with either an amino acid to give chain extension, or base in alcohol to provide a terminal ester. In both cases, studies have been performed

to determine the effects on hydrogenation of alternative chiral centres being present.[47]
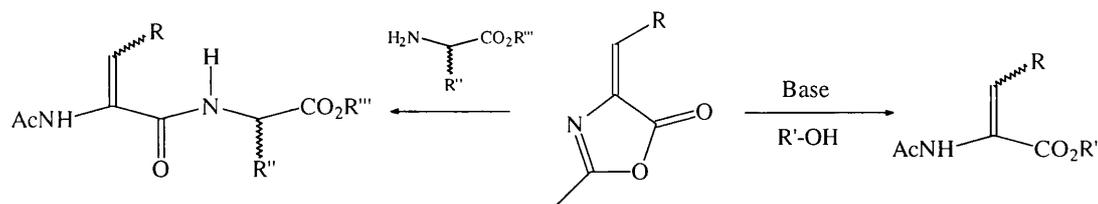
Figure 3.29: Homologation of Azalactones

Some results of hydrogenation studies point to the position of the dehydroalanine being important, with better induction if the residue is placed near the C-terminus. This seems to indicate that some competing directing force exists in the case of polypeptides.

These methods provide the final possibilities of creating a new α-chiral centre. The use of heterogeneous catalysts require the use of auxiliaries, directing the approach of the substrate to a metal surface. In this case, use of two dehydro-residues can give poor induction due to the distance from the auxiliary. The use of homogenous catalysis can give superior results, dependent on the substrate. Again, this induction is dependent on the sequence in which the dehydro-residue appears, and its position in that sequence. However, as the best results are achieved with minimally protected amino acids, it would appear that these type of residues are most suitable for treatment. The major drawback with this method is the significant cost of the chiral ligands, which reduces its general application.

3.6   SIDE CHAIN ELABORATION METHODS

As the chiral pool contains a great number of amino acids, the creation of new chiral centres is in many cases unnecessary. The functionality present in the side chains of natural amino acids allow modification to give different properties, and incorporation of more sensitive substrates than previously mentioned methodologies will allow. This principal has also been developed to give rise to a series of reagents that are chiral, and can be homologated from the β-position with preservation of asymmetry. This type of

route is termed the β-synthon approach, and utilises conditions which result in the maintenance of chiral integrity. These reagents tend to employ serine, which has a reactive and easily modified side chain as a starting material. Modification of the hydroxyl group can give rise to a carbon activated to either electrophilic or nucleophilic attack. The cases below both involve activated serine analogues.

The structure of serine allows the formation of a β-lactone between the carboxylic acid and the side chain hydroxy function. The synthesis and use of this type of reagent has been pioneered by Vederas.[48] The highly strained ring can be opened by the attack of Grignard reagents, by cyanocuprate complexes,[49] and also by other nucleophiles, including activated thiols. This type of reaction has been achieved by use of caesium carbonate catalysis, most recently in the case of a synthesis of a lanthionine derivative formed by reaction of a β-lactone and penicillamine.[50]
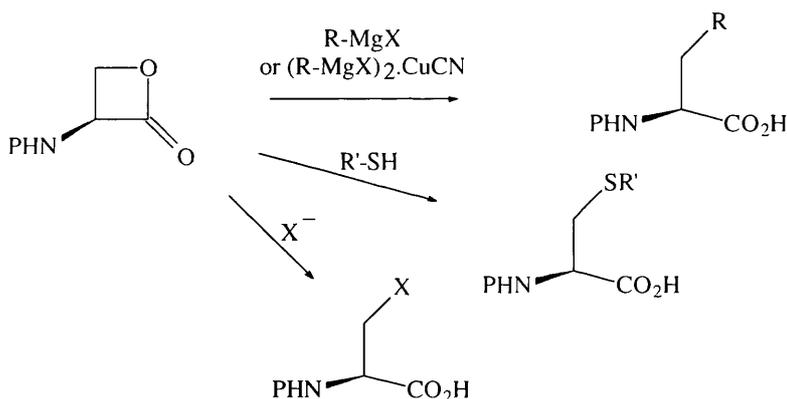
Figure 3.30: General Scheme of Serine β-Lactone Ring

A related approach developed by Jackson uses the modification of serine to iodoalanine, followed by activation with zinc dust under conditions of sonication. This gives rise to a molecule activated to electrophilic attack. Palladium catalysis conditions are generally employed to give good reactions with acid chlorides or aryl halides.[51] Further developments of this work have included the use of iodoalanine reagents activated as zinc/copper couples, derived from copper (I) cyanide/lithium chloride complexes.[52] In these cases, it was found that either tosylates or chlorides were the best leaving groups on the electrophiles. This process has also been developed to give

side chain unsaturated amino acids, by the use of propargyl and vinylic systems. However, the reaction is dependent on the use of an activated electrophile, and yields can be only moderate, even in these systems.
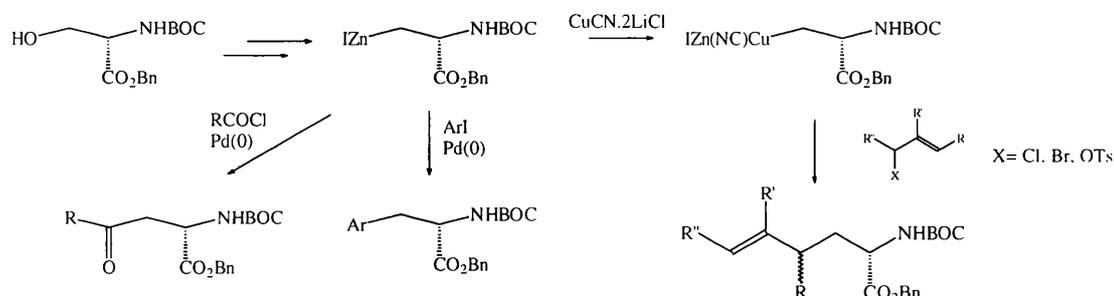
Figure 3.31: General Scheme for use of Zinc-Iodoalanine Complex

Another method of side chain extension has been the use of Stille couplings.[53] This type of reaction, along with Heck-type reactions, have been used to generate allyl glycine derivatives.[54] The reaction involves the activation of a propargyl glycine with tributyltin hydride, which inserts across the triple bond, and allows further reaction with an unsaturated substrate *via* a palladium catalysed process.
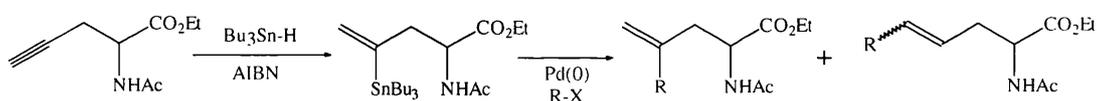
Figure 3.32: General Scheme for Side Chain Homologation using

Palladium Catalysed Coupling Reactions

3.7   USE OF ENZYMATIC METHODS

There are two basic ways in which enzymes can be manipulated in synthesis. The first is the use of systems that nature uses in the generation of amino acids. The second is to employ the inherent stereoselectivity of enzymes to give selectivity between amino acids on the basis of chirality.

The production of amino acids by bacteria allows access to a large number of preparative methods. Species responsible for particular active enzymes can be isolated

and then utilised, either in whole cell fermentations, or as purified or immobilised enzyme preparations.

The main synthetic enzymatic route involves transamination, a process whereby the amino group of one amino acid is donated to a substrate. It has also been shown to be possible to use ammonia as the amine source,[55] and in certain cases this is the only suitable donor. More usually, however, either aspartic or glutamic acid are required.

The prochiral substrates used in these reactions are usually α-keto acids, with the required side chain in place. An example is the industrial synthesis of S-phenylalanine from phenylpyruvic acid using an aspartate transaminase.[56] In this reaction, aspartic acid is the amine donor, the oxaloacetic acetic acid produced being decarboxylated to give pyruvic acid. This process has pyridoxal phosphate as a cofactor. The pyruvic acid generated may also be used in the generation of more phenylpyruvate to continue the reaction.
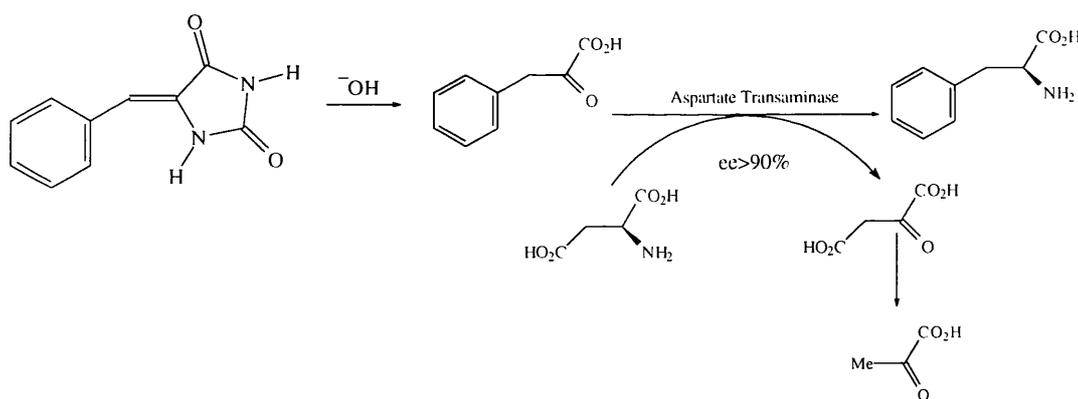
Figure 3.33: Formation of Phenylalanine by Transamination Reaction

Although the use of amino acid donors is widespread in nature, the use of ammonia as a precursor would have obvious commercial advantages. One synthetic route to S-tryptophan has been shown to use indole with pyruvic acid and ammonia, which in immobilised E. Coli cells has given the desired product, recycling the excess ammonia and acid.[57] This process has been performed in a continuous reactor system.

70
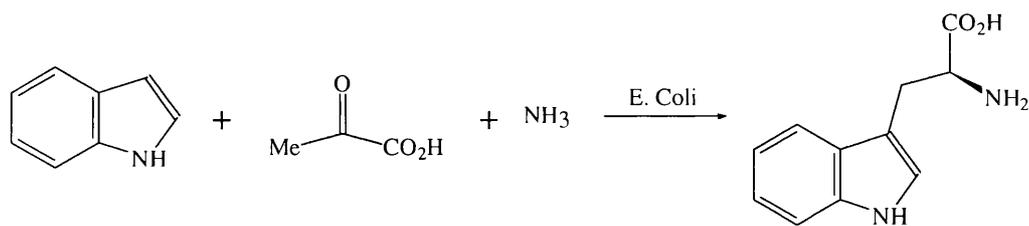
Figure 3.34: Tryptophan Synthesis by Ammonia Insertion

It is also possible to perform aminations on unsaturated substrates to give chiral products by the addition of ammonia across a double bond. One example of this type of process is the use of aspartase from *E. coli* which, when mixed with fumaric acid in the presence of ammonia, gives *S*-aspartic acid at high conversion.[58] This preparation has also proved to be useful in the synthesis of alanine, as use of an *S*-aspartate β-decarboxylase system gives access to *S*-alanine.[58] The ammonia-lyase enzymes have also been shown to be effective in these type of reactions, one example being the phenylalanine specific enzyme.[59] This reaction also appears to work by the simple insertion of ammonia into the α-centre of a prochiral substrate with selectivity.

3.8   RESOLUTION OF RACEMIC MIXTURES

The second, and more commonly applied, use of enzymes is in the field of chiral resolutions. The inherent selectivity of enzymes for one enantiomer over the other means that manipulation of protecting groups can allow differentiation. However, there are an increasing number of chemical methods to perform this task by kinetic resolution, examples of which will also be described.

Several different classes of enzymes can be use to perform resolutions, the majority acting in only two possible ways. The first method of resolution acts by cleavage at the amino group, the second by cleavage at the carboxylic acid, both performed with stereocontrol.

Amidase and lipase enzymes act to cleave either amide or ester groups from the acid terminus respectively. As the product from several synthetic reactions can be a

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racemic, or relatively poorly enriched, mixture, the use of an enzyme gives rise to an easy method of chiral resolution. Most methods follow the general pathway of treatment of the protected substrate using an enzyme to free only one enantiomer. After this, the mixture can be treated chemically to give products with different properties, or selectively extracted, as the solubilities of protected and unprotected residues are very likely to be different.

One example of the use of this type of system is the application of an aminopeptidase from *Pseudomonas putida* to the resolution of the carboxyamide product of a Strecker synthesis.[55] In this case, phenylglycine amide was made in racemic form, then after selective cleavage of the S-chiral amide, the mixture was treated with benzaldehyde to give the Schiff base of each isomer. The R-product was insoluble in the aqueous solvent, and could be removed by simple filtration to give almost enantiopure product after simple acid hydrolysis.
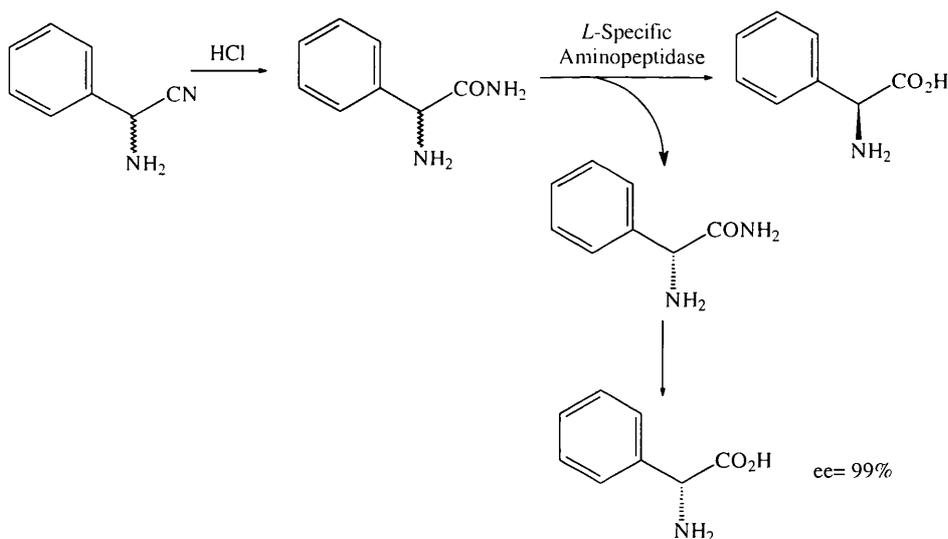
Figure 3.35: Resolution of Strecker Product

Lipase enzymes act by cleavage of esters in the place of amides. One reported case is the use of an alkaline protease from a bacteria which has been found to give very rapid hydrolysis of benzyl esters.[60] The purity of the product is dependent on the particular enzyme and reaction conditions used. Most generally, the majority of enzymes selectively cleave S-chiral substrates, meaning that R-chiral products are obtained in

the highest yield. This arises as an artefact of purification, as usually the S-product will remain in aqueous solvent, along with any incorrectly reacted R-isomer, whilst the unreactive R-isomer will be collected without contamination.

The second type of enzymes are the acylases, which act by the removal of N-protecting groups under similar constraints. One example of this type of reaction involves the resolution of a Strecker reaction product.[61] This is acylated, after hydrolysis of the nitrile, to give the N-acyl product which is then treated with "Amano" acylase to give the free S-chiral product, which can be extracted. The R-isomer is available in this case by fractional crystallisation, followed by acid hydrolysis of the acyl group. Both enantiomers can be obtained in high e.e., up to 98%.
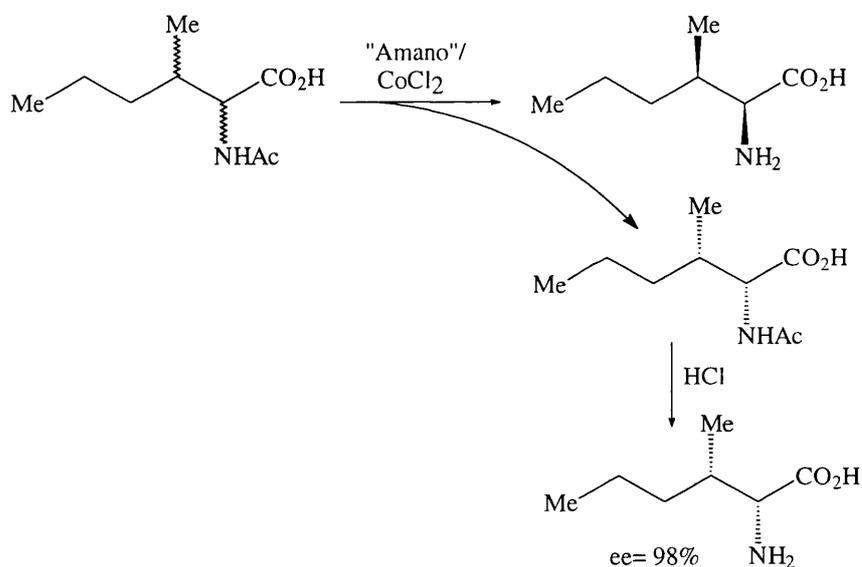
Figure 3.36: Resolution by Selective Deacylation

The final group of enzymes perform ring opening on hydantoins, five membered rings formed from amino acids. A phenylglycine derivative is prepared by the action of

R-hydantoinase from *Bacillus brevis* on the condensation product between phenol, urea and glyoxylic acid under acid conditions, the product being a racemate.[55] Use of R-hydantoinase opens the ring to give only the desired enantiomer as an N-carbamoyl amino acid, the protecting group being cleaved by chemical methods. In this case, spontaneous racemisation of the remaining S-hydantoin can give rise to almost 100% conversion with the expected excellent e.e..

Spontaneous
Racemisation

D-Hydantoinase

CO_2H

NH_2

$NaNO_2/$
HCl

CO_2H

NH_2
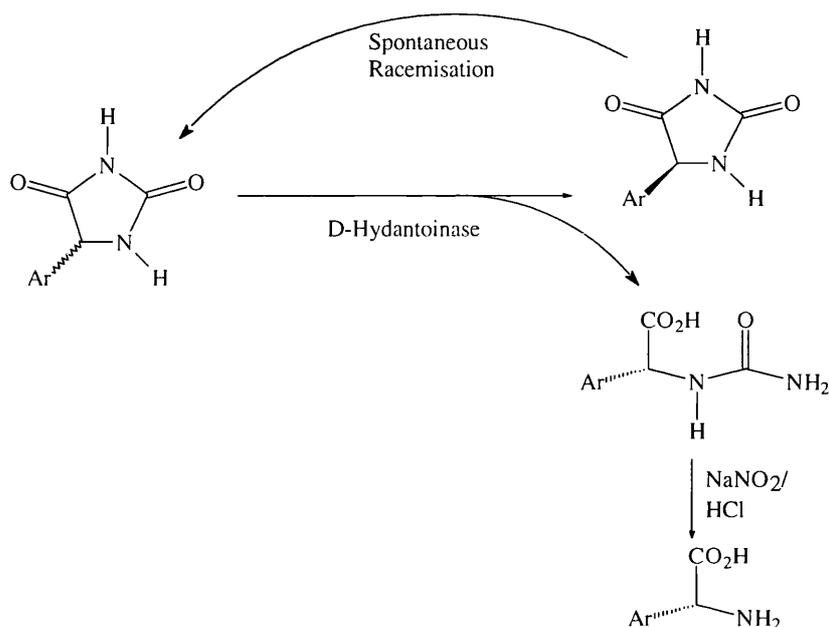
Figure 3.37: Amino Acid Synthesis by Hydantoin Opening

It is also possible to perform similar types of resolutions without the use of enzymes, *via* enantioselective reactions. This type of selection is termed kinetic resolution, and employs reactions which will give far faster reaction with only one of the isomers present.

In the system described below, the proton α- to the ketone, ester and amine functionalities is highly labile, giving a racemic mixture. This has been chemically resolved using a BINAP-system with a ruthenium catalyst, which reduces the ketone to an alcohol stereoselectively.[62] The α-proton of the hydroxy product of this reaction is far less labile (*cf.* serine and threonine), and so this process, which also directs to a

large extent the stereochemistry of the α-centre, gives resolution over two centres. This has been achieved with almost 100% conversion of starting material, with e.e.s of 94%.
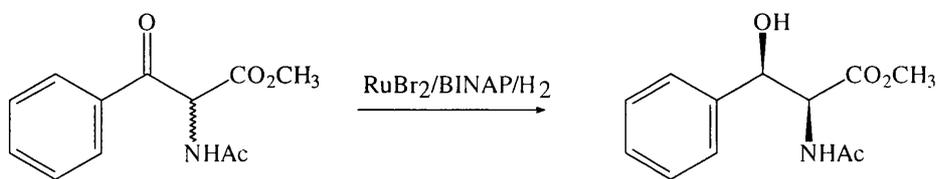
Figure 3.38: Kinetic Resolution using BINAP Hydrogenation

The asymmetric epoxidation of Sharpless has also been employed for this purpose. The resolution of N-tosyl amino acids from N-tosyl furfurylamines has been achieved by the use of a selective epoxidation and ring expansion of only the undesired product.[63] Using L-(+)-diisopropyl tartrate as directing group, only the R-isomer of the racemic mixture reacted, allowing reclamation of the desired S-furfurylamine. This can then be unmasked to give the amino acid by action of ozone, or by other oxidative methods. Yields were up to 50%, as expected for a resolution with no method of racemisation present, and with good e.e. (90-98%).
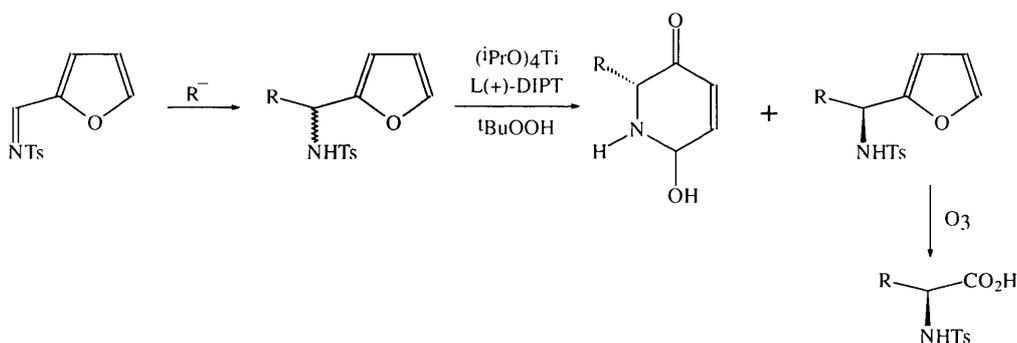
Figure 3.39: Resolution of Furan Species by Sharpless Epoxidation

The routes described above are an overview of the most general reaction types. Descriptions have been limited to general examples. Several methods particular to specific amino acid targets have been left out completely, as these are not applicable to

the majority of targets.

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CHAPTER FOUR

SOLID PHASE PEPTIDE SYNTHESIS

4.1 INTRODUCTION

In 1962, Merrifield pioneered a new method for the synthesis of peptides.[1] Prior to this, peptide sequences were synthesised using protected amino acids in conjunction with coupling reagents in solution. This involved stepwise assembly of a peptide sequence, with purification and characterisation after each reaction. The lengthy and elaborate procedure greatly limited the quantity of peptide available by synthesis.

In solid phase synthesis, the first amino acid of a desired sequence is attached to an inactive solid support, in the initial case a polystyrene resin. After this, the next *N*-protected amino acid is reacted with the free amine of the growing peptide, followed by removal of the *N*-terminal protecting group. This process is repeated until the desired peptide sequence is generated.[2] The principal advantage of this system is the ease of workup. Previously, aqueous workup conditions were required, which proved difficult with certain insoluble products. The retention of the product on solid support means that by-products and excess reagents can be simply and completely removed by simple filtration.

P————OC—NHP
(i) Deprotect
(ii) Couple Residue 2

P————OC—NH—OC—NHP
(i) Deprotect
(ii) Couple Residue 3

P————OC—NH—OC—NH—OC—NHP
(i) Deprotect
(ii) Cleavage from Resin

HO$_2$C—NH—OC—NH—OC—NH$_2$
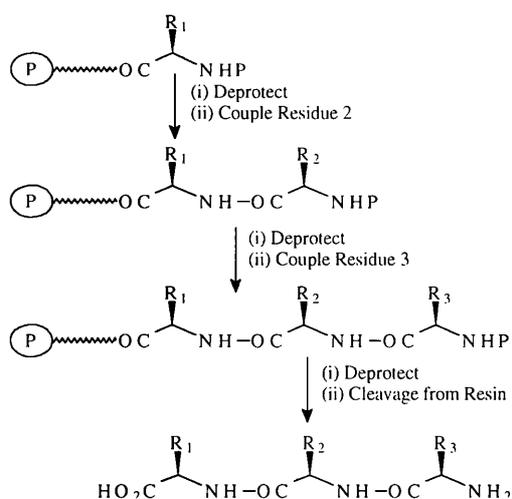
Figure 4.1: General Scheme for Peptide Synthesis

In classical synthesis, starting materials are generally employed in a 1:1 ratio in order to make purification easier. This restriction does not apply to the solid phase methodology due to the ease of workup. In most cases, a three fold excess of reagents and materials is used to drive reactions to completion.

The synthesis of peptides by solid phase methods has been the subject of a large number of literature reports,[3] and several different methodologies are known for this purpose. Although different in vital respects, these are linked by common requirements. These are that the solid support be inert under chain extension conditions; that the N-terminal protection should be easily cleaved; that cross reaction at side chain functional groups should not occur; and that the product should be easily recovered in good yield at the end of the synthesis.

These requirements have given rise to "orthogonal" protecting group strategies, designed to prevent reaction at side chain sites whilst allowing easy exposure of the N-terminal amine group for chain extension. Also, several different "linker" groups - handles for the attachment of amino acids to solid phase supports- have been designed which allow cleavage of the peptide product only under specific conditions. The design of syntheses has also evolved to give the cleavage of side chain protection under the same conditions as required for peptide cleavage from the linker.

4.2 SOLID SUPPORT RESIN TYPES

The selection of solid support type is dependent on the strategy for synthesis being employed. This is due to the possible chemical reactivity of the polymer support under the conditions for coupling, deprotection and cleavage that must be used. A support must be inert under the conditions used in order to prevent cross reaction, but must be active enough to be easily functionalised, allowing for the attachment of linkers. The physical characteristics of a polymer also play an important role. These include the solubility of polymers, as well as their porosity and their "swelling" characteristics. These properties are important, as they determine how well reagents and monomers penetrate the resin to internal active sites.

Polystyrene (PS) was the polymer initially employed for solid phase synthesis,[1] cross-

linked with divinyl benzene. This resin was used as it was easily accessible, and could be simply functionalised. Chloromethylation of the pendant phenyl groups gave a site for attachment of the amino acid monomers as phenyl esters.
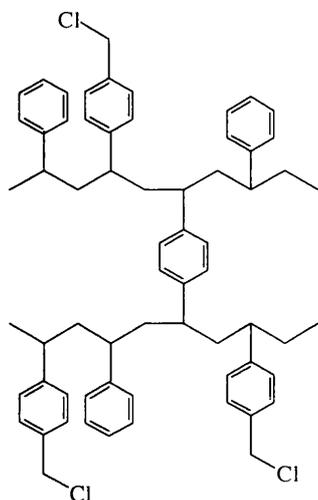
Figure 4.2: Chloromethylated Polystyrene Resin

This type of structure is well penetrated by non-polar solvents such as dichloromethane (DCM), but is less effective if the more polar dimethylformamide (DMF) is used. The ester linkage created during incorporation of the amino acid monomer is cleavable by strong acid conditions, but not by mild conditions. This selectivity was used in the initial studies of the solid phase approach and this type of resin is still widely used for the synthesis of short peptides today.

The use of these resins is limited, however, by the poor solvation of larger peptides by DCM. This is because the growth of the peptide product changes the physical character of the bead from mainly hydrophobic to mainly hydrophilic. At this point, the swelling characteristics (the ease of penetration to internal sites by reagents) change and so the use of hydrophobic DCM becomes less effective.[4]

The next major class of polymers used are the polyamide bearing resins. These are formed from polyacrylamide, generally crosslinked with bis-acryloylethylenediamine. In one example,[5] active sites are incorporated by copolymerisation of small amounts of acryloylsarcosine or other reactive materials.
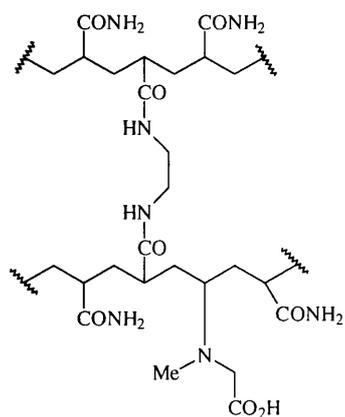
Figure 4.3: Sarcosine Pendant Polyacrylamide Resin

The inert nature of the aliphatic chains, and the pendant amide groups mean that cross reaction is unlikely. It is possible to control the physical characteristics of the resin by altering the quantity or nature of the cross-linking agent. This type of resin is well swollen by a large range of solvents, including DCM and DMF.

As peptide structures are held together by hydrogen bonding, the amide backbone of the polymer is capable of bonding to the guest peptide. This can lead to problems in the synthesis of peptides, as free amino groups may be stabilised by H-bonding, making them less susceptible to attack by electrophiles. The H-bonding may also give rise to steric problems due to the close proximity of resin and peptide. However, it is more usual that such effects occur intra- and interstrand in peptides being synthesised.[4]
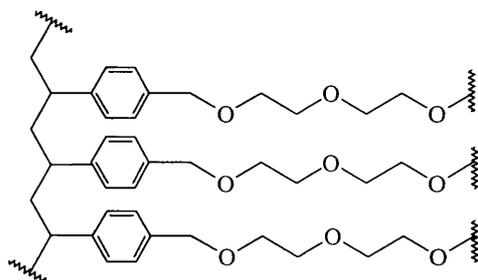
Figure 4.4: Polyethyleneglycol-Polystyrene Graft Copolymer

One modification of the polystyrene resin system is the use of a block copolymer with

polyethylene glycol, as shown in fig. 4.4.[6] The polystyrene backbone is crosslinked to give rigidity, and the resin created is reacted with polyethylene glycol spacers prior to activation with a suitable linker. The system has different swelling properties to the PS system described above, as the less hydrophobic nature of the polyether allows better solvation of the peptide. As this polyether is not capable of H-bonding it is unlikely to interact with the peptide product.

Another possible combination of monomers is the use of polyacrylamide/polystyrene composite mixtures, commercially known as polyhipe.[7] These resins differ mainly in terms of physical properties.
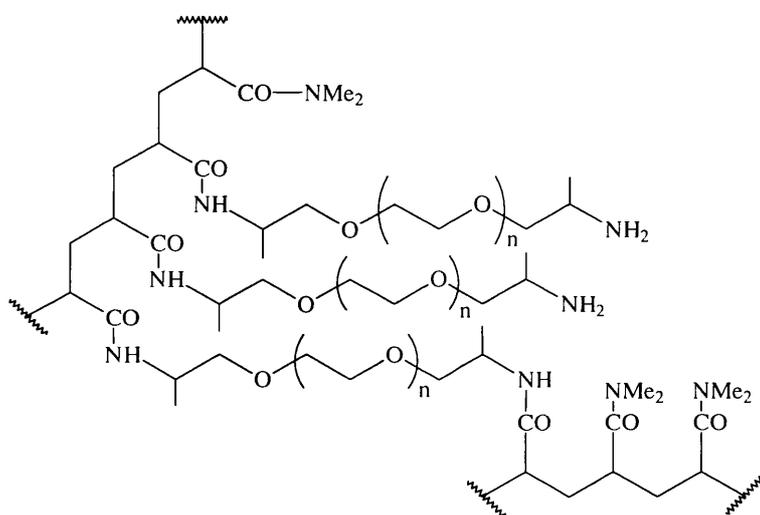
Figure 4.5: PEGA Resin

PEGA resin is formed by the grafting of polyethylene glycol onto a polyacrylamide backbone[8], crosslinking with *bis*-2-acylamidoprop-1-yl polyethylene glycol. These supports are highly hydrophilic, and are swelled well by H-bonding solvents. Again, the backbone is not affected by the standard synthesis conditions, and the highly permeable nature of the polymer means that it is effective even when very large substrates are used. It has also been found that these supports can be used in conjunction with enzymes. One example of this has been the use of resin bound compounds to find substrates for subtilisin Carlsberg.[9]

4.3   SOLID PHASE SYNTHESIS LINKERS

The synthesis of peptides on solid support requires that the product be easily collected at the end of the synthesis. This is most effectively achieved by the use of a connecting group, or linker. The chemical requirements for a linker are that it should remain intact during normal synthesis, and should be cleaved only when treated at the end of the synthesis. The groups initially used by Merrifield were benzyl ester derivatives,[1] as this was the method used to functionalise the polymer resin. However, the advent of new synthetic methodologies has allowed the development of linkers with milder cleavage properties.

4.3.1   Strong Acid Labile Linkers

These types of linker are most commonly used for BOC methodology, and are unaffected by the mild acid conditions used for *N*-terminal amine deprotection. The linkers are usually benzyl ester derivatives, ring substituted to give different cleavage properties by stabilisation of the cation produced.[10]
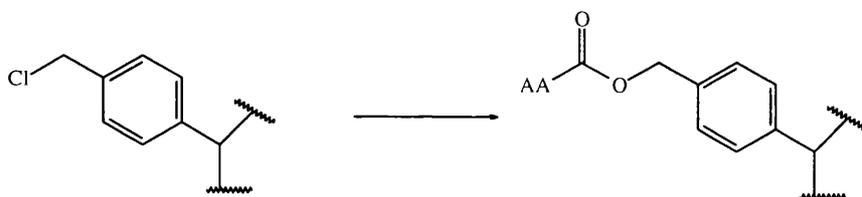
Figure 4.6: Functionalisation of PS Resin

The Merrifield technique employs a simple benzyl ester group,[11] created by the reaction of a carboxylic acid with the chloromethylbenzyl group formed from the backbone. This gives the product as its free acid after deprotection and cleavage steps.[1] A modification of this system is the PAM linker, which incorporates a further phenylacetamidomethyl group.[12]

This group has been shown to give less peptide cleavage during the routine acid BOC cleavage. The ester functionality is stabilised by electron withdrawal, making the linker

less susceptible to hydrolysis.
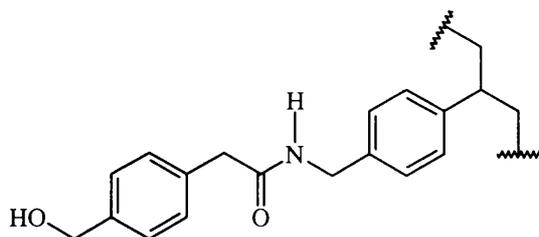
Figure 4.7: 4-Hydroxymethylacetic Acid Linker Group

4.3.2 Mild Acid Labile Linkers

A milder acid cleavage regime allows the use of more labile linkers. These have included the use of dibenzyl systems,[13] and 4-hydroxymethyl-3-methoxyphenoxyacetic acid (HMPA),[14] both of which give highly stabilised cations under cleavage conditions. It is this stability that allows their use under such mild conditions, and also provides some of the driving force for the cleavage reaction. Another system, based on a protecting group, that gives stable cations on cleavage is the trityl residue.[15]
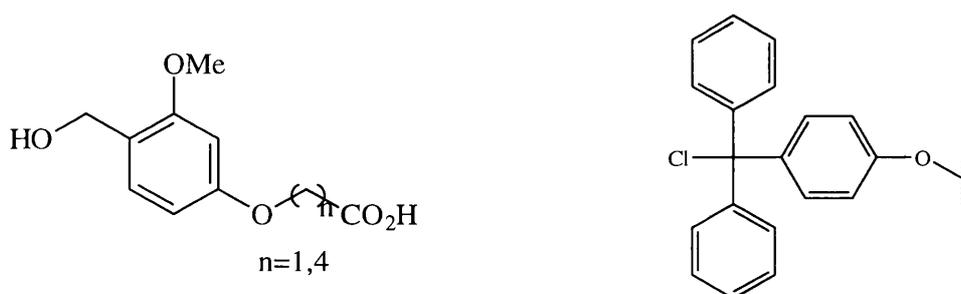
Figure 4.8: Mild Acid Labile HMPA and Trityl Linkers

Hydroxymethylmethoxyphenyl derivatives, attached to either acetic or butanoic acid, can be attached to the resin by standard methods, and then directly functionalised with the starting monomer. The electron donating substituent groups on the benzene ring give stability to the cation, and hence increase acid lability. The butyric acid derivative

has been found to show higher acid lability than the acetic acid form.[16]

The high acid lability of the trityl group has also been utilised. Attachment to the resin is achieved *via* the *para*-position of one of the phenyl rings. The amino acid is then attached by the methyl position. As with the HMPA- based systems, the acid lability of this group can be tuned by the use of substituents on the ring. Groups capable of donation of electrons to the ring system have been found to give stabilising effects.[15]
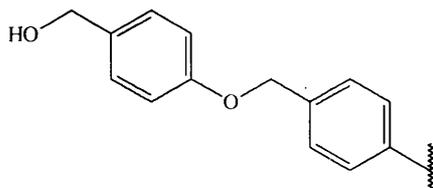
Figure 4.9: Wang 4-Hydroxymethylphenol Linker

The use of linked diphenyl systems is also possible. The most commonly used of these is the system devised by Wang.[17] In this case it is again 4-hydroxymethylphenol that is use, this time attached directly to the resin *via* an ether link. After ether formation, the linker can be loaded and synthesis can be performed by the standard Fmoc procedure. Cleavage of the final products is again possible using mild acid conditions.

4.3.3 *C*-Terminal Amide Linkers

A large number of peptides are found in nature as *C*-terminal amides, and so there is interest in the synthesis of sequences carrying a *C*-terminal amide group. Such groups are difficult to put in place following final deprotection and cleavage, due to problems of regioselectivity. However, certain linkers allow the synthesis of *C*-terminal amides directly, with the amide nitrogen being donated by the linker itself. Derivatives suitable for both synthesis protocols have been developed.

While several different linkers exist for use under strong acid cleavage conditions, the most widely used linker is the methylbenzylamine derivative (MBHA).[18] This linker is generated by reaction of methylbenzoyl chloride with polystyrene resin, giving a ketone which is then converted to the amine prior to use. In this case, the linker is labile to HF

conditions, and so can be easily cleaved at the end of the synthesis.

Figure 4.10: MBHA Linker

The cation produced on cleavage is stabilised by delocalisation into the two benzene rings. A forerunner of this system is the benzhydylamine system (BHA), which is less easily cleaved than the MBHA system, especially when certain amino acids are used as the terminal residue.

Several linkers are available for the synthesis of C-terminal amides by Fmoc methodology. One of the first to become available was the design of Rink,[13] which is similar to that set out above for the BOC methodology. The additional methoxy substituents increase the lability of the system by electron donation, and so the group is cleaved by mild acid.
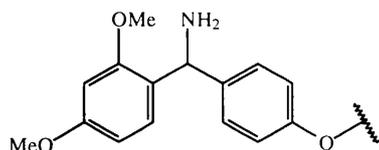
Figure 4.11: Rink-Type Linker

A different structure is employed in the xanthenyl system devised by Sieber.[19] Cleavage from this linker gives the peptide amide, and the production of a cation stabilised by delocalisation across the ring system. In this case, the delocalisation is particularly strong due to the creation of a π-system in the central ring, lowering the energy of the system overall.
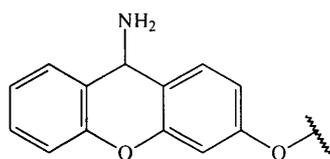
Figure 4.12: Sieber Linker Group

Another linker is that put forward by Ramage,[20] based on the stability of the carbocation produced by the dibenzocyclohepta-1,4-diene system. This molecule is useful in the generation of terminal amides, but less so in the synthesis of terminal acids, due to the lability of the ester linkage to mildly acidic coupling reagents such as hydroxybenzotriazole. The more stable terminal groups are amines, both primary and secondary having been used successfully, and hydrazines.
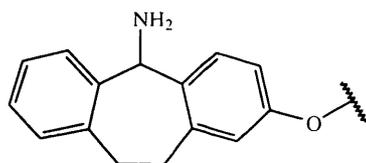
Figure 4.13: Ramage Linker Group

In both the Sieber and Ramage systems, attachment to the resin is via an ether linkage at an appropriate substitution position. This, in addition to acting as a tether, also stabilises the cation produced on cleavage of the peptide product under acidic conditions.

4.4   PROTECTING GROUP STRATEGIES

The synthesis of peptides requires the use of protecting groups to direct synthesis. The functionality of peptide side chains creates problems of chemoselectivity, where reaction only at the N-terminus of the main chain is desired. It is, therefore, necessary to direct reaction to only the N-terminal amino group, preventing reaction between monomers and with side chain groups. Thus, the α-amino protecting group must be

cleavable under different conditions to the side chain protecting group. Several different methodologies exist, defined by the main α-amino protecting groups. The two major strategies, Fmoc and BOC, will be described.

The protecting group initially used by Merrifield was the benzyloxycarbonyl (Cbz-) group, which can be cleaved by hydrogenation.[1] However, this group is not suitable for solid phase synthesis, due to the need for a catalyst for cleavage by hydrogenation. The poor penetration of solid phase support by a solid catalyst resulted in incomplete cleavage, showing the requirement for complete and preferably solution phase deprotection.

4.4.1 *tert*-Butoxycarbonyl Methodology

One of the first groups to be used was the *t*-butoxycarbonyl (BOC) group.[21] This group is stable to basic conditions, but is readily cleaved by mild acidic conditions.[22] This process is very rapid, allowing complete deprotection in a short time. These kinetic factors make this process amenable to automation on a larger scale.

α-BOC-Aspartic acid-γ-benzyl ester

α-BOC-Serine-β-benzyl ether

α-BOC-Lysine-N ε-Cbz
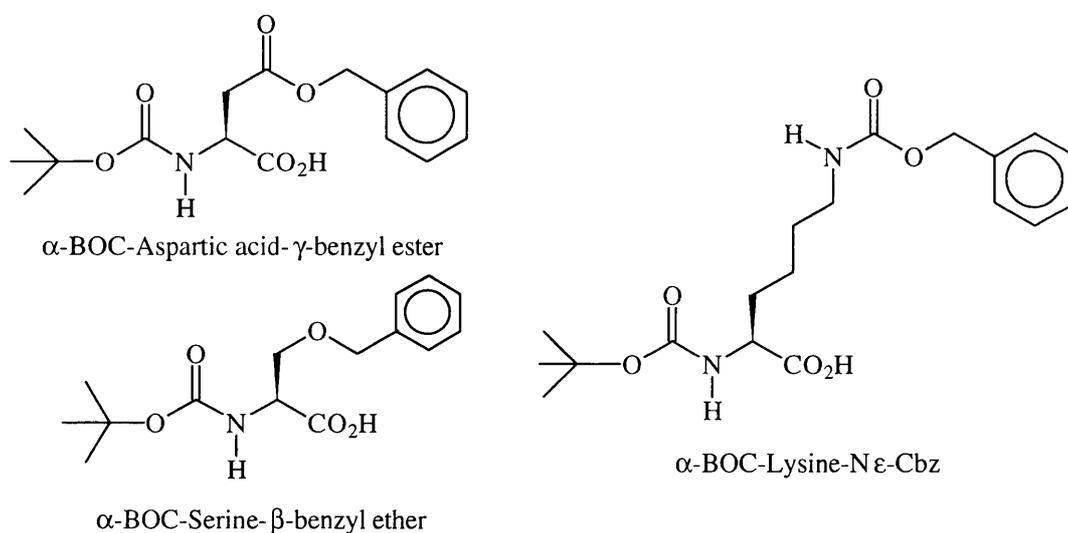
Figure 4.14: Side Chain Protecting Groups for BOC Strategy

The BOC group is unstable to acid, but is stable to the standard conditions used for peptide coupling, and the by-products. A procedure using trifluoroacetic acid is

employed for main chain deprotection, requiring that side chain protecting groups stable to mild acid are used. The synthesis and purification of BOC protected amino acids is also readily achieved.

Several different groups stable to mild acid conditions are known, and can be employed as side chain protection. Benzyl based systems are known to be labile to strong acid conditions, and so these groups are frequently employed.[10] Side chain amino groups are usually protected as benzyloxycarbamates, whilst carboxylic acids and hydroxyl functions are protected as benzyl esters and ethers respectively. This methodology is generally employed in conjunction with a polystyrene resin and a strong acid labile linker (see later), and is termed the Merrifield technique.[23]

4.4.2 Fluorenylmethoxycarbonyl Methodology

An alternative protection methodology is to use a base cleaved main chain protecting group, which then allows the use of mild acid cleavable side chain protection and linkers. This system gives the advantage of milder conditions throughout the synthesis and deprotection steps, which will reduce racemisation and cross reaction. The most widely used group of this type is fluorenylmethoxycarbonyl (Fmoc), which can be cleaved extremely swiftly by the use of mild bases.[24] This is due to the highly stabilised nature of the anion produced. The synthesis of Fmoc protected residues is a simple procedure which gives solid products, which can be easily purified.

α-Fmoc-Aspartic acid-γ-*tert*-butyl ester

α-Fmoc-Serine β-*tert*-butyl ether

α-Fmoc-Lysine-N ε-BOC
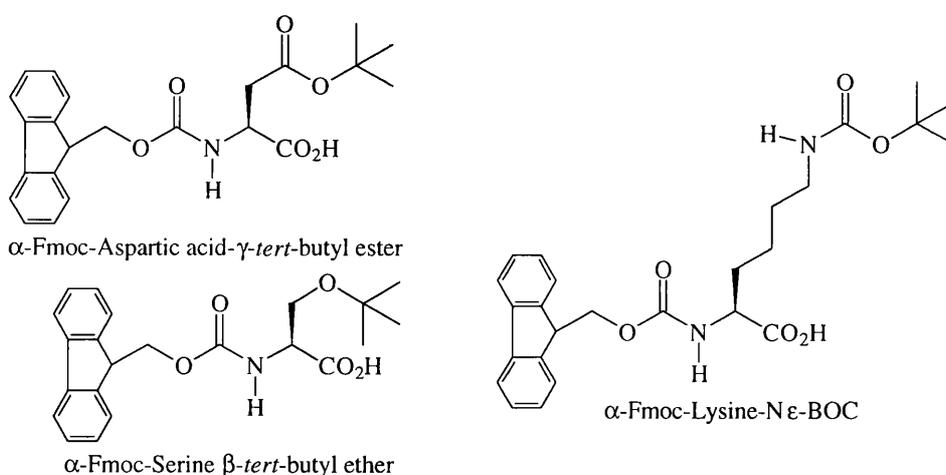
Figure 4.15: Side Chain Protecting Groups for Fmoc Strategy

The synthesis of peptides is performed using similar coupling agents, as the Fmoc group is stable to such mildly acidic conditions. After coupling, the Fmoc group can be rapidly removed by use of a 20% piperidine solution.[25] An advantage to this route is that the deprotection by-products are detectable by U.V. or conductivity analysis, which allows the course of the deprotection to be followed. As well as giving information on the process of deprotection, this can also give information into the level of coupling being achieved during each step.

Several different groups are known to fulfil the criteria of base stability, mild acid lability required by this route. Amongst these are the trityl group,[26] and the main α-amino protection used previously, BOC.[22] When this methodology is used in conjunction with mild acid sensitive linkers (see later), this methodology allows the use of milder conditions for the synthesis of peptide sequences. In consequence, purification of final products should be simplified. However, it is necessary to include "scavenger" molecules in deprotection steps in order to prevent cross reaction by active cations released in cleavage reactions.[27] These cations are particularly reactive with sulphur bearing side chains, and the inclusion of thiol-bearing and other reactive molecules can help to prevent reaction with the peptide product.[28]

4.4.3 Orthogonal Protecting Groups

In the synthesis of more complex peptide systems, it is occasionally necessary to obtain reaction at sites other than the N-terminal amine. In order to achieve this, removal of one protecting group selectively must be accomplished, necessitating the use of a third type of protection. It must also be possible to manipulate this group in isolation from the other functionalities.
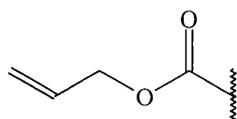
Figure 4.16: Allyl Based Protecting Groups

One of the groups that can be used in this way is the allyl system,[29] which is stable to mild acid and base. It can be used to protect either amines as the carbamate, or acids as the ester. The group can be removed by the use of a palladium catalyst, which complexes to the double bond of the allyl group. This allows easy cleavage of the protection by a number of different reagents including acetic acid[30] and silyl amines,[31] as well as tin hydride reagents.[32]

As the most simple systems for this purpose involve the use of reagents that the remainder of the protected peptide is inert to, non-carbon based systems are also usable. It is possible, for example, to use the silyl ether protection methods for the side chain protection of residues, including non-proteinogenic residues. The synthesis of a carbohydrate-side chain bearing residue, and subsequent incorporation into a peptide using Fmoc- methodology has been described.[33] The side chain in this case is protected as TMS-ethers, which are deprotected under the mild acid peptide cleavage conditions.

Figure 4.17: TMS-Protected Carbohydrate Side Chain Amino Acid

The use of photolytic systems has also been proposed. Nitrobenzyl based systems[34] have been used to protect both amines and carboxylic acids, and can be removed by irradiation. This process should leave all other groups unaffected.

Figure 4.18: Photocleavable *o*-Nitrobenzyl Group

It is also possible to use methods such as hydrogenation of benzyl derivatives.[35] The BOC groups employed as side chain protection in the Fmoc methodology are known to be stable to these conditions, but problems of resin penetration exist with heterogeneous catalysts, and so deprotection can be problematic.

Nitrobenzene systems have also been used to give groups cleavable under specific conditions. One such group is the 4-acetyl-2-nitrophenylethyl group, which can be used in conjunction with the BOC methodology.[36] This group has been found to be easily cleaved using TBAF solution, but stable to the acidic conditions required for BOC deprotection. TBAF has also been used for the cleavage of other ester groups, including 4-nitrobenzyl, 2,2,2-trichloroethyl and phenacyl esters.[37] These studies were again performed using BOC methodology.

4.5   COUPLING REAGENTS

The process of formation of an amide bond is a condensation reaction, evolving water as the by-product. A large number of peptide coupling reagents now exist which drive this reaction by removal of water. Several different ways of activating the carboxylic acid function have been used. In general, solid phase synthesis "grows" peptides from their *C*-terminus to the *N*-terminus. This is the opposite direction to the growth of proteins at ribosomes in nature.

This is in part due to the mild conditions that nature uses to synthesise proteins. Under the harsher conditions required for chemical synthesis, activation of a carboxylic acid in the presence of an amide group can lead to a cyclisation process which can racemise the amino acid, and hence render the product stereochemically impure. The cyclisation

process involves the formation of an oxazolone, catalysed by base, followed by the further action of base at the now acidic α-proton to give an achiral product.[38]

X= Activating Group
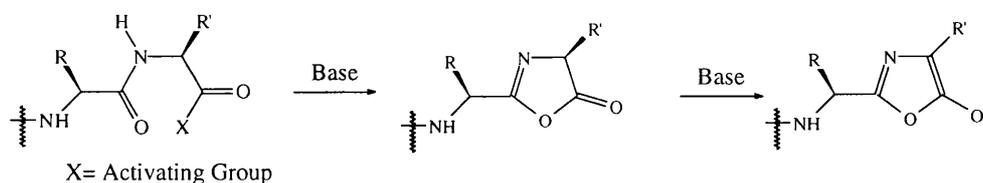
Figure 4.19: Oxazolone Formation During Peptide Formation

However, this ring system can be opened by amines, to give the same product as would be created by normal reaction. The opening of the ring reinserts the chiral centre, but with no stereocontrol, so the final product is a mixture of stereoisomers.

Due to this effect, the majority of syntheses are carried out under conditions of C-terminal activation of urethane protected monomers. These are less susceptible to cyclisation than peptides themselves. Activation of monomers as acid chlorides is also avoided, as these groups are known to generate N-carboxyanhydrides, which may give cross reaction. However, these molecules are reactive with amines giving amide products, so chain extension is possible.
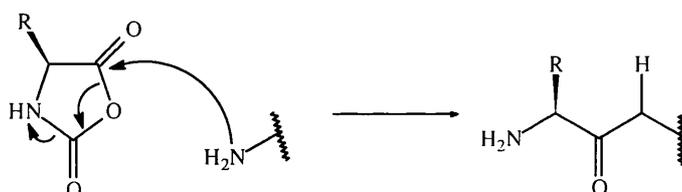
Figure 4.20: Peptide Synthesis using N-Carboxyanhydride Activation

A large number of the reagents used for coupling act by taking up the "water" produced in a stabilised form. These reagents act with high chemoselectivity, suppressing the formation of oxazolones whilst still activating the carboxyl function to nucleophilic attack. It has been found that one of the most effective dehydrating and activating groups is the carbodiimide series, specifically the dicyclohexyl- (DCC)[39] and

diisopropyl- (DCCI)[40] variants. These groups act by forming an activated ester with the carboxylic acid, which is then susceptible to nucleophilic attack by the amine, giving the amide and a dialkyl urea as a by-product.
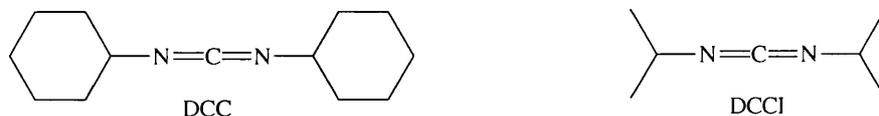
Figure 4.21: Dialkylcarbodiimide Reagents

These reagents are highly effective, and are particularly useful if large quantities are to be coupled, but have drawbacks. The first of these is that racemisation of certain more labile amino acids, such as phenylalanine, can occur as a result of the increased acidity of the α-proton. Secondly, the urea by-products are relatively insoluble, and therefore can be difficult to remove when using solid phase synthesis.

To increase the scope of these reagents, it is possible to use them in conjunction with other activating agents. The main reagent to be employed is hydroxybenzotriazole,[41] (HOBt) although it is also possible to use the pyridyl version,[42] termed HOAt. Both of these reagents give active esters which are highly reactive, but less likely to racemise than the DCC esters. The use of these mixtures is now common in solid phase peptide synthesis. In both cases these reagents appear to work *via* a catalytic system, being regenerated after each coupling.

Figure 4.22: Benzotriazolyl-Based Peptide Synthesis Reagents

However, as has been stated previously, the urea by-products are insoluble in certain solvents, and this may lead to practical problems in solid phase synthesis. Several other systems have, therefore, been devised to allow the use of the activating HOBt or HOAt

groups but avoiding these problems. The first of these was the benzotriazolyloxy-trisdimethylaminophosphonium hexafluorophosphonate (BOP) reagent developed by Castro.[43] This reagent has been augmented by several different variants, with the hydroxybenzotriazole function retained. Various different leaving groups have been incorporated into molecules, allowing the easy removal of the water from the condensation and the hydroxybenzotriazole by-product. Versions of this molecule have been termed HATU,[42] TBTU[44] and HBTU,[45] dependent on the leaving group and counterion used. The counterion most commonly used is the hexafluorophosphate ion, or the tetrafluoroborate ion, both of which are used because of their high solubility in organic solvents.
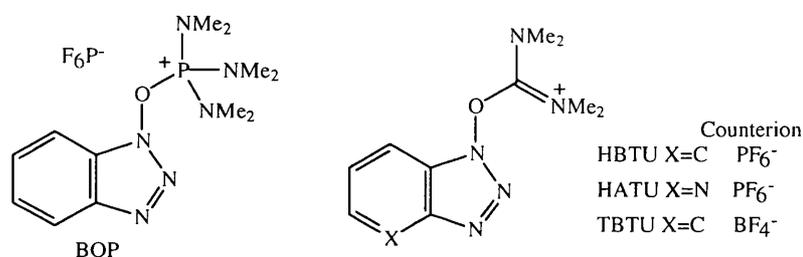
Figure 4.23: Peptide Coupling Reagent

It is also possible to activate amino acids by generating the symmetrical or unsymmetrical anhydrides.[46] A drawback of the use of symmetrical anhydrides is that only half of the amino acid can be coupled, meaning that expensive residues are not economically used by this method. More effective is the use of unsymmetrical anhydrides, especially the use of carbonate type derivatives. These are useful as reaction is directed towards the amino acid carbonyl due to the effect of the electron donating alkoxy-group on the carbonate carbonyl. This process can also be directed by steric effects due to the alkyl group. These groups are not stable over a long time and require to be formed immediately prior to use. This stability problem gives rise to problems with their use, especially in automated synthesis procedures.
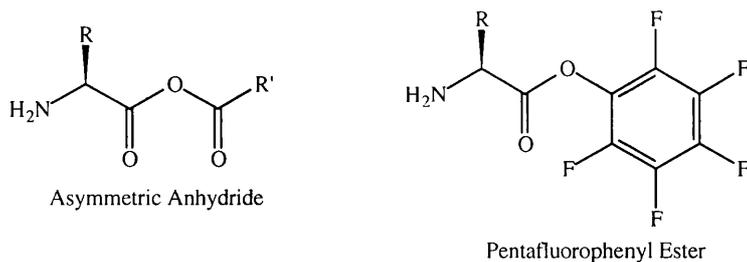
Figure 4.24: Anhydride and Ester Activating Groups

An alternative method which gives more stable reagents involves the use of phenyl esters, with various electron withdrawing groups promoting aminolysis. Especially useful is the pentafluorophenyl ester,[47] which is highly activated towards nucleophilic attack by the amino group, but is also stable over longer periods of time.

However, problems in syntheses still occur with both of these strategies. While reactions can be forced to high yields, it is very rare for coupling efficiency to reach 100%. This in turn can lead to deletion sequences, where a single residue has been omitted from the desired sequence. Certain arrangements of amino acids are also known as "difficult sequences", and the inclusion of these arrangements in a peptide tend to lead to poor incorporation, and hence reduced yields. These effects are generally due to poor steric effects caused by the ordering of the local physical structure of the peptide when bound to the resin. Whilst the occurrence of deletions does reduce yield, a more serious problem that arises is in the purification of the products. In the synthesis of peptides of medium size, deletions can occur at random positions in the sequence. This makes the removal of impurities difficult, as the removal of one residue will only make a small difference to the characteristics of the sequence. These effects are artefacts of the process of chain extension, as, by speeding up the process, attachment to solid phase makes purification of the intermediates impossible. The purification of intermediates would require the cleavage of the peptide from the support after each step, which would negate the advantages of the solid phase method in terms of its overall time efficiency.

The number of repeated steps also leads to problems with the synthesis of longer sequences. Although the coupling efficiency in each step may approach 100%, a yield

97

of 99% for each addition would still give a maximum possible yield for a 30 residue peptide of 74%. This figure falls still further as the chain is extended further, imposing a ceiling on the length of peptides obtainable by this route. Although the length of peptides available may be increased by the use of the segment condensation approach,[3] this becomes experimentally difficult with greater size. As the regular methods of synthesis involve the cleavage of the side chain protection at the same time as cleavage of the product from the resin, the chemoselectivity of methods for joining two backbone groups becomes crucial. This is particularly true as longer and more functionalised peptides are considered, as they may bear a high degree of side chain functionality which may give cross reaction.

4.6   CYCLIC PEPTIDES

Cyclic peptides are commonly found in nature, performing several different functions, from the antibiotic vancomycin[48] to the hormone oxytocin.[49] As such, there is interest in the synthesis of these systems and their analogues. There also exists a large body of literature describing their structure and syntheses.

Nature has achieved cyclisation in several ways. These have included the formation of thioether, disulphide and amide bond formation, either in a "head-to-tail" fashion, or between side chain functional groups. Other macrocyclic structures include cyclic peptides closed by tyrosine oxidations.[50] Advantages of cyclic structures have been found to include increased efficacy and slower biological degradation.[51] The interest in the synthesis of these molecules also stems from the small amounts available in nature.

The synthesis of cyclic peptides can be performed in two distinct manners, either by cyclisation of a linear sequence in solution, or by ring closure between two groups whilst the peptide is grown on a resin. Achieving regioselectivity in ring closure is the main problem in macrocyclisation.

4.6.1   Synthesis of Cyclic Peptides "Off-Resin"

This approach is typified by the synthesis of a linear precursor before cyclisation to the

final product. An example of this approach is the synthesis of an analogue of oxytocin, a neurohormone which contains a single disulphide bond.[49] This analogue contains an additional amide bond between the side chains of glutamic acid and lysine incorporated in the sequence. After the assembly of the sequence on solid support, the cleaved peptide is treated with potassium ferricyanide in ammonia to give the disulphide ring closed product. Closure of the amide macrocycle is then performed using diphenylphosphorylazide activation. These processes are facile, as under conditions of high dilution, no dimeric product is likely. Cross reaction is also unlikely as there are no other reactive side chains in this peptide. However, this is not the case in more complex systems.

$$\text{H—Cys—Tyr—Ile—Glu—Asn—Cys—Pro—Lys—Gly—NH}_2$$

$$\beta\text{-Mpa}^1, \text{Glu}^4, \text{Cys}^6, \text{Lys}^8\text{-Oxytocin}$$
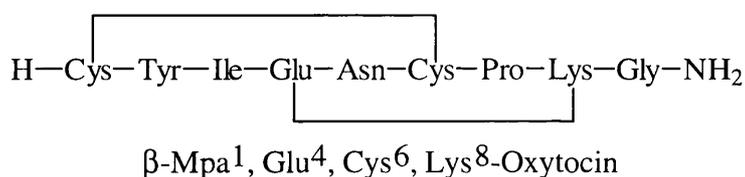
Figure 4.25: Bicyclooxytocin

The cyclisation of peptides off resin has many problems of regioselectivity, especially if fully deprotected fragments are used. These may bear several groups that are capable of reacting, which can result in highly complex product mixtures. This problem can be prevented by the use of orthogonal protecting groups, under conditions where cross reaction is unlikely.

One example of this process is the synthesis of insulin, which contains three disulphide bonds and is formed of two individual peptide strands. If this system was treated under conditions as simple as those above, a number of different products would be obtained. The synthesis of this molecule has, however, been achieved by the use of orthogonal protecting group strategy.[52] In this case, the use of three different protecting groups, trityl, acetamidomethyl and tert-butylthioether, has allowed the deprotection of only one sulphur on each strand at a time, giving only the desired product.

(A)

(B)

Cys6:Cys7———Cys11————————————Cys20—

Cys7————————————————————Cys19
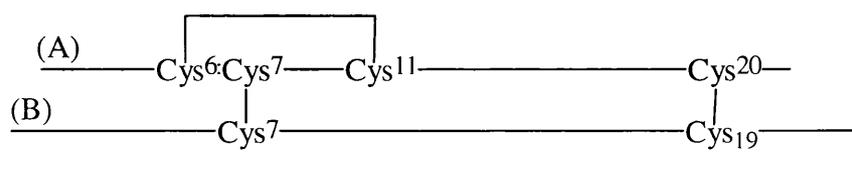
Figure 4.26: Disulphide Bonds Present in Insulin

It is also possible to synthesise lactam closed cycles by similar routes. A CCK analogue containing two lysine residues has been cyclised by the use of a succinic acid linker.[53] The synthesis of this system was performed by solution methods, growing the two lysine containing portions of the peptide before the linking of the two side chains. This was performed by initial reaction of succinic anhydride with one lysine section. The free acid of the succinic acid was then activated, and the second lysine section was introduced. The ring was closed by initial deprotection of the required amine and carboxylic acid groups, and use of BOP as a coupling reagent.

$$
\underset{\text{Tyr}—\text{Lys}—\text{Gly}—\text{Trp}—\text{Lys}—\text{Asp}—\text{Phe}—\text{NH}_2}{\overset{\overset{\text{O}}{\overset{\|}{\text{C}}}—\text{CH}_2\text{CH}_2—\overset{\text{O}}{\overset{\|}{\text{C}}}}{\underset{\text{NH}}{\big|}\qquad\underset{\text{NH}}{\big|}}}
$$
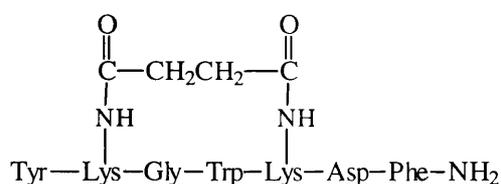
Figure 4.27: Succinate Crosslinked Cholecystokinin Analogue

The immunosuppressant cyclosporin series of cyclic peptides contain 11 amino acids, including seven N-methylated residues. These are known to depress coupling yields due to steric effects,[54] thereby complicating the synthesis of systems bearing such residues. The synthesis of a series of analogues of cyclosporin has been performed, varying residues in only two positions.[54] After formation of the retained core of the structures, the next two amino acids were put in place using diphenylphosphinic mixed anhydride activation with C-terminal tert-butyl protection. After treatment with acid, final closure of the cycle was achieved using BOP. The yields for this process were generally low, due to the poor reactivity of the N-methyl residues.
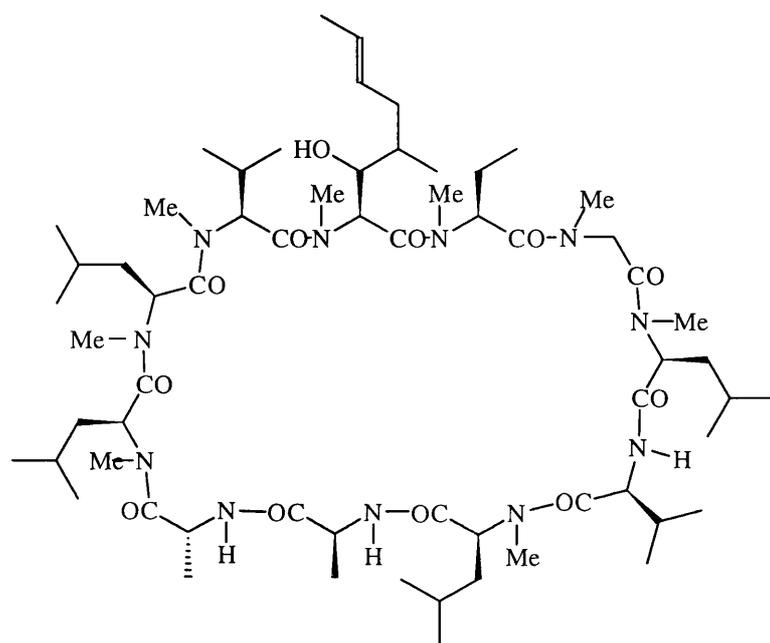
Figure 4.28: Cyclosporin A

The synthesis of a series of cyclic peptides using solid phase methods, followed by a low/high acid selective cleavage *versus* deprotection scheme has also been reported.[55] In this scheme, the linear peptides were synthesised by standard methods, using a HMBA linker. After assembly was complete, the solid phase was treated with 1% TFA in dichloromethane to give the free, but still protected, peptides. These were then cyclised using BOP, before side chain deprotection using 95% TFA.

The synthesis of smaller peptide macrocycles is also possible by side chain ring closure. A series of angiotensin inhibitors have been found including K-13 and OF4949-III, which are formed of tyrosine tripeptides. These are closed by aryl ether linkages termed isodityrosine units. Two separate syntheses of these residues have been reported that perform the synthesis in different ways. The first of these used an Ullman biaryl ether synthesis between two cinnamic acid derivatives, followed by introduction of amine functionality by asymmetric synthetic methods.[56] Synthesis of the peptide cycle was then achieved by standard solution methods. An alternative method employs the synthesis of a tripeptide prior to the formation of the ether linkage.[57] After the construction of the backbone, with the incorporation of a 4-nitro-3-fluorophenylalanine

derivative, cyclisation is performed under mildly basic conditions to give the macrocycle in good yield.
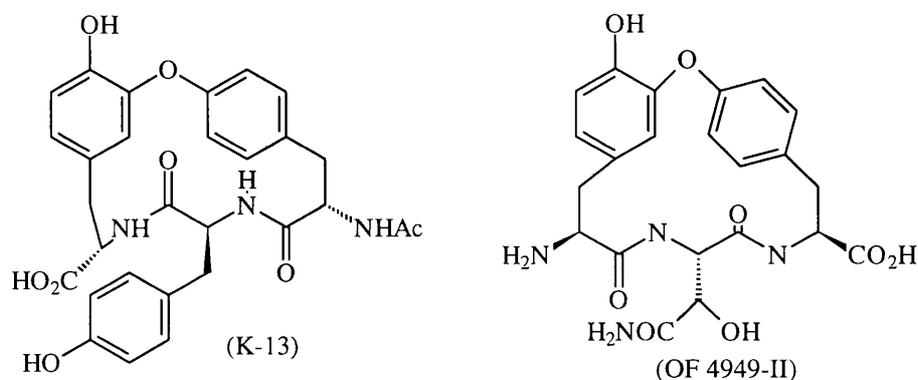
Figure 4.29: Isodityrosine Based Peptides

4.6.2 Synthesis of Cyclic Peptides "On-Resin"

The synthesis of cyclic peptides on solid support is similarly constrained by the need for regioselective reaction. This is easily achieved by the use of a triply orthogonal protecting group strategy, allowing the process to occur completely on the solid support. An additional problem is caused by the method of attachment of the peptide to the solid support. This is normally achieved *via* the α-amino acid centre, which would preclude the formation of "head to tail" cyclic structure without cleavage or rearrangement.

Methods have been devised to give cyclic peptides closed "head to tail" in an "on resin" method. One of these has involved the initial functionalisation of the resin using the side chain carboxylic acid groups of Asp or Glu.[30] In these cases, the α-amino acid functions are protected as the Fmoc carbamate and allyl ester respectively. The allyl group is not labile to the standard peptide synthesis conditions, which allows the easy formation of the linear precursor. The allyl group can then be removed by a palladium catalysed process, and the cyclisation performed using BOP. Several different anchor points in the peptide were used, and no difference in the product based on position used was found. As with syntheses performed by normal coupling, the use of different linkers could provide amidated termini. In this case, attachment through the side chain

would give rise to different residues, *e.g.* glutamine from glutamic acid. However, this serves to increase the number of possible anchor points.
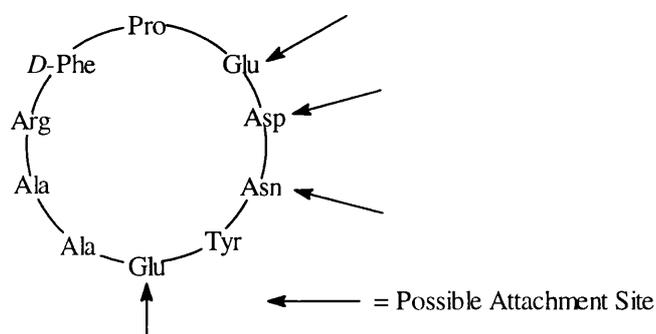
Figure 4.30: Model "Head to Tail" Peptide

Similarly, the synthesis of a cyclic seven residue tachykinin antagonist peptide has been performed.[58] The attachment of the initial residue, aspartic acid, was *via* the γ-carboxylic acid, with the α-carboxylic acid blocked as the allyl ester. Chain extension was then performed using standard methods, and then cyclisation performed using PyBOP after palladium catalysed deprotection. Subsequent cleavage from the resin followed by side chain deprotection gave the required product.
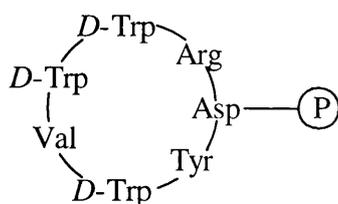
Figure 4.31: Tachykinin

The synthesis of "head to tail" cyclic peptides has also been performed using the BOC methodology.[59] In this case, an Asp- residue, α-protected as the Fmoc ester, was attached to the resin and the next six residues attached using standard methodology. After base cleavage of the Fmoc- ester, cyclisation was performed using BOP. Cleavage and side chain deprotection by standard HF treatment gave the desired cyclic product.

$$\text{BOC—Tyr—}D\text{ Trp-Val—}D\text{ Trp—}D\text{ Trp—Asp—OFm}$$

$$\begin{array}{c} D\text{-Trp} \\ \diagup \quad \searrow \\ D\text{-Trp} \qquad \text{Arg} \\ | \qquad\qquad \text{Asp} \\ \text{Val} \qquad\qquad \diagup \\ \diagdown \quad \text{Tyr} \\ D\text{-Trp} \end{array}$$
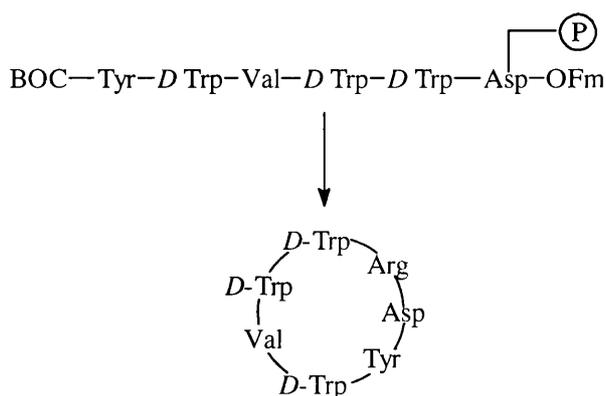
Figure 4.32: Tachykinin

Another method of synthesis of cyclic peptides "on resin" is by the crosslinking of side chains. One example of this is the synthesis of several hGRF analogues using the BOC methodology.[51] In this study, several cyclic analogues were synthesised using glycine as a linker. The Fmoc- group was used as the side chain orthogonal protecting group. These cycles were created then by the addition of Fmoc-glycine to the side chain of a basic residue, followed by BOC deprotection of the main chain and extension with an Fmoc side chain protected acidic residue. After Fmoc deprotection, the cycle could be closed using BOP as a coupling agent. Multicyclic variants were also produced by the same methods, the rings created being separate on the same strand.

The synthesis of an endothelin analogue has been performed using a residue bearing two α-amino acid centres.[60] In this procedure, the second N-terminus to be introduced was protected as the benzyl carbamate, and the acid as the CAM ester, both groups being stable to the anhydrous Fmoc cleavage conditions. After chain extension, the CAM ester was removed using aqueous sodium hydroxide and the cyclisation performed using TBTU. The resulting peptide was deprotected and cleaved by standard methods prior to the removal of the Cbz by hydrogenation. This method does not, however, allow further chain extension after closure of the cycle.
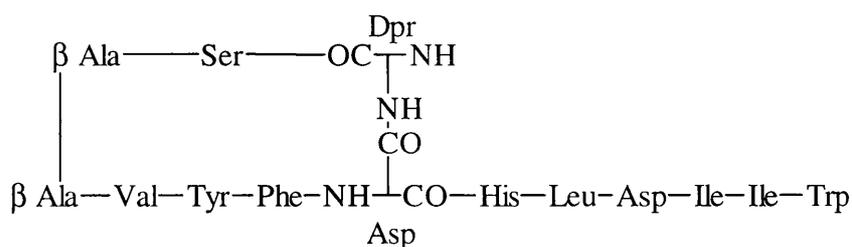
Figure 4.33: Synthetic Endothelin Analogue

A study of the most effective coupling reagents has been performed in the synthesis of side chain cyclised peptides.[61] The results of this study are that in the case used, the more modern HBTU and HATU reagents were the most effective coupling agents, giving the highest yield with the minimum of epimerisation.

4.7 USE OF ARTIFICIAL RESIDUES DESIGNED AS STRUCTURAL CONSTRAINTS

A number of artificial residues have been reported, and their inclusion into peptide sequences studied. These have included modified character proteinogenic amino acids, disubstituted residues, conformationally restrained monomers, and complex template molecules designed to initiate structure.

The incorporation of certain turn mimic residues has also been studied by solid phase synthesis. One example is the incorporation of a Gly-Pro type-IV turn mimic into a sequence using Fmoc methodology.[62] The fused bicyclic residue was derived from allylproline, giving a diastereomeric mixture by functionalisation using diazo-compounds following enolisation. The residue was introduced using HBTU, and by monitoring was found to give incorporation of greater than 99%. The peptide sequence then required no special treatment for further extension.

The synthesis of a hydroxyproline-aspartic acid piperazinedione has also been detailed, along with the synthesis of a cyclic peptide incorporating this residue.[63] The fused bicyclic system includes free amine and carboxylic acid functionalities, along with a second carboxylic acid which acts as a tether to the solid support.
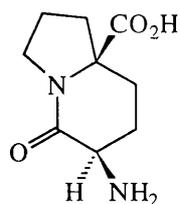
Figure 4.34: Reverse Turn Analogue

The use of a triply orthogonal protecting group system has allowed the construction of the peptide and its subsequent cyclisation.
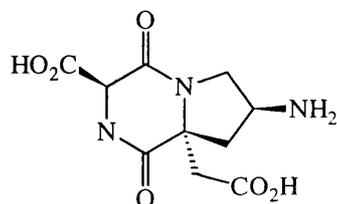
Figure 4.35: Peptide Turn Analogue

The partial solid phase synthesis of a di-(α-amino acid) containing peptide has been described,[64] the peptide backbone without the complex residue being assembled prior to cleavage from the resin. Incorporation of the two termini was performed stepwise, using HOBt/DCCI as a coupling mixture, in tandem with a triply orthogonal protecting group strategy. This protocol allowed the assembly of the linear precursor, which was cyclised by initial conversion to the acid hydrazide. This was followed by activation as the azide using isoamyl nitrite, to give an active carboxylic acid terminus for reaction with the free amino group.
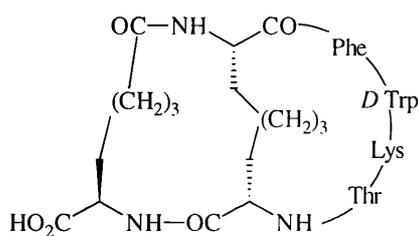
Figure 4.36: Bicyclic Somatostatin Analogue

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CHAPTER FIVE

SYNTHESIS RESULTS

5.1   INTRODUCTION

Retrosynthetic analysis of the target molecule showed two possible routes that could be developed. The first of these, set out below, shows the formation of the R-chiral centre, followed by the introduction of a β-synthon containing the S-chiral centre. Several methods exist for the introduction of the former,[1] whilst two major routes have been described to achieve the latter.[2,3] It was decided that the Schöllkopf *bis*-lactim ether reagent would be employed for the introduction of the R-chiral centre,[4] followed by use of the β-lactone derived from serine[2] to introduce the S-stereocentre.
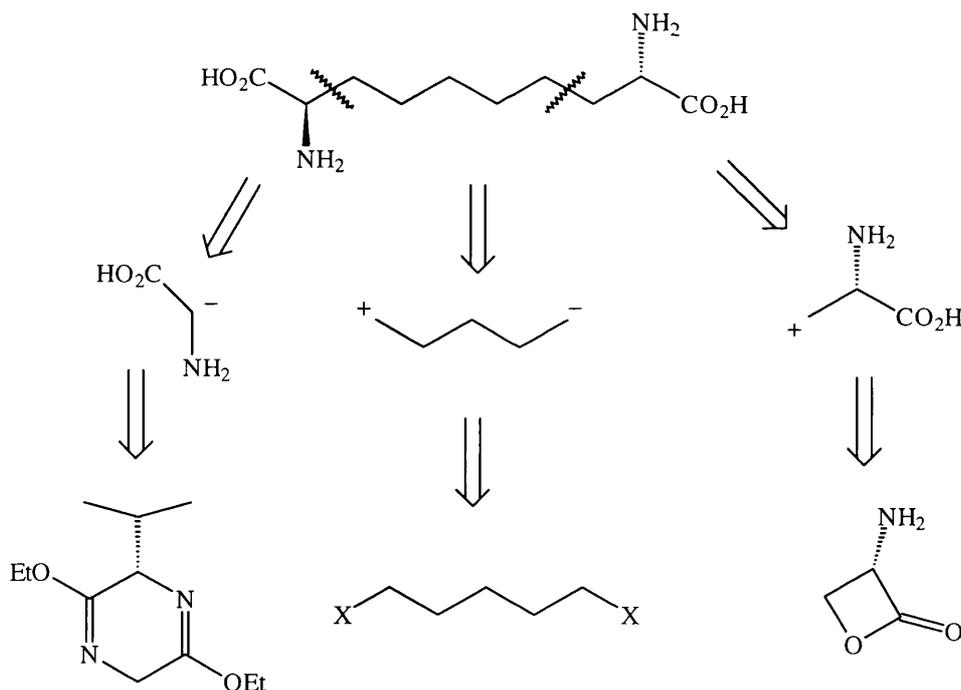
Figure 5.1: Retrosynthetic Analysis I

An alternative approach uses a disconnection of the R-amino function, using an acid synthon, and then reaction with an amine equivalent. Again, the R-chiral centre requires introduction, and this is most easily achieved by the use the previously

110

mentioned Schöllkopf reagent. In order to introduce the S-amine functionality, it is possible to use the chiral azidation methodology developed by Evans.[5]
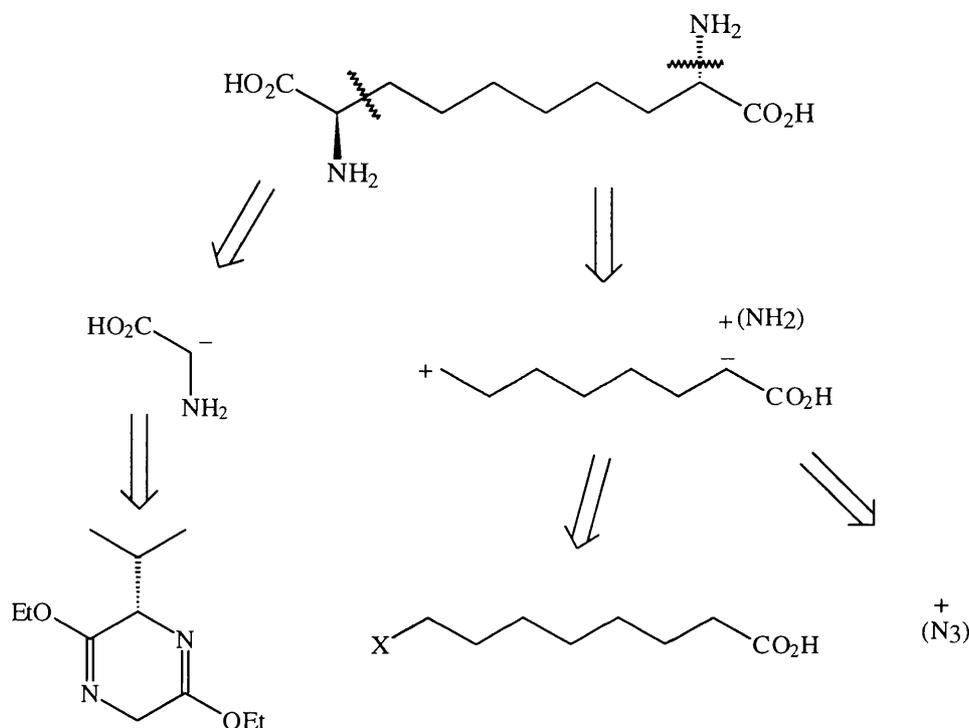
Figure 5.2: Retrosynthetic Analysis II

In both cases, the same method has been used for the creation of the R-chiral centre, using different substrates. Therefore, the results for this methodology will be treated together. However, major differences lie in the introduction of the S-chiral centre. As the two attempted routes are fundamentally different, they have been discussed separately. Finally, the route used to synthesise the target molecule and the rationale behind the reaction sequence used will be described.

5.2 DEVELOPMENT OF A ROUTE TO THE R-CHIRAL CENTRE

The majority of literature reports describing the use of Schöllkopf bis-lactim ether reagents contain details only for substrates with little or no side chain functionality, as previously described in chapter 2. The subsequent section will detail the attempted alkylation reactions under normal and modified conditions.

5.2.1 Synthesis of the Schöllkopf Pyrazine Reagent

The synthesis of the *bis*-lactim ether was achieved by a literature route,[4] with little modification.
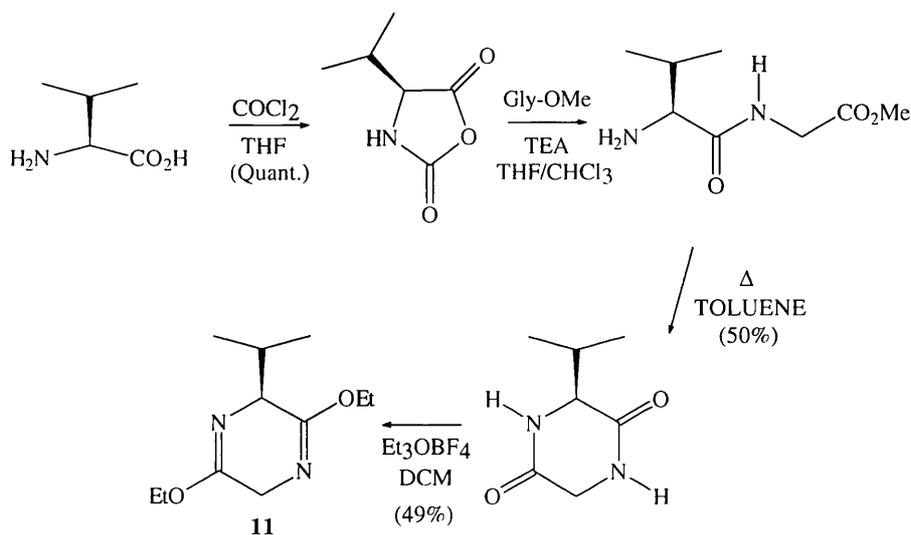
Figure 5.3: Synthesis of 2-(*S*)-isopropyl-3,6-diethoxy-2,5-dihydropyrazine

In the original preparative method, gaseous phosgene was employed to give the activated-protected *S*-valine-*N*-carboxyanhydride (**4**), but the use of this particular form of the reagent was deemed unwise, and a solution in toluene was used instead. This reaction was found to be most successful when performed overnight, and the use of an increased amount of THF solvent gave the clear solution described in the literature. The course of the reaction was not easily followed, the product being unstable to tlc, and heat sensitive. However, after the complete dissolution of the starting material, the desired product was isolated in high yield. Reaction of this material with glycine methyl ester hydrochloride in the presence of TEA gave the dipeptide product as the methyl ester, which was then cyclised to the *bis*-lactam by simple reflux in toluene. The product was obtained as a stable white solid, which could be stored at room temperature until required.

The *bis*-lactam was then converted to the pyrazine by alkylation of the amide carbonyls. In the literature, this was achieved by methylation, although it was also stated that ethyl protection was equally effective.[4] On the basis of this observation, the

slower reacting trimethyloxonium tetrafluoroborate was substituted for the triethyl salt. This reagent is known to be more reactive than the methyl equivalent, and is also soluble in the reaction solvent dichloromethane, hence improving the reaction rate. The synthesis of **11** was achieved in reasonable yield (25%) over 4 steps, with little difficulty.

The normal methods[4] for the use of the pyrazine molecule involve its deprotonation using n-BuLi, followed by stirring with the desired electrophile for 5 hours. In the cases quoted, yields were good to excellent, with accompanying high e.e. values. The bromide was selected as this was the electrophilic species most commonly used.
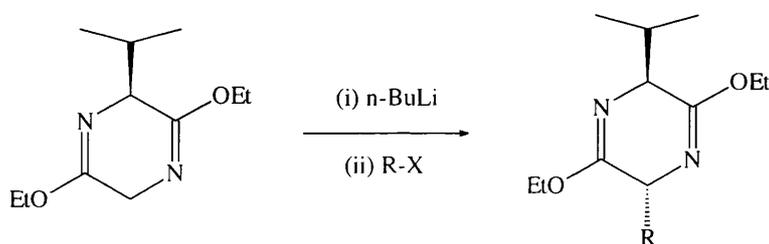
Figure 5.4 General Scheme of Alkylation Reaction

5.2.2 Use of Pentanediol Derivatives

We required a difunctional substrate molecule, which would undergo regioselective alkylation, as further elaboration would be needed after introduction of the R-chiral centre. Reports showed that alkylation yields tended to fall as larger, less activated substrates were employed, and especially if longer chain n-haloalkanes were used.[4] As both of the possible routes to the target molecule required the use of such substrates, this was a problem that had to be addressed.

The first route was envisaged to proceed *via* the use of a bromopentane alcohol derivative. This was to be initially coupled to the pyrazine, generating the R-chiral centre, followed by deprotection of the alcohol function and conversion to the halide. This product could then be converted to a Grignard or cyanocuprate reagent, followed by reaction with a β-lactone to give the S-chiral centre.

113

HO⁀⁀⁀OH   (i) NaH
          ————→   HO⁀⁀⁀O—TBS
          (ii) TBS-Cl
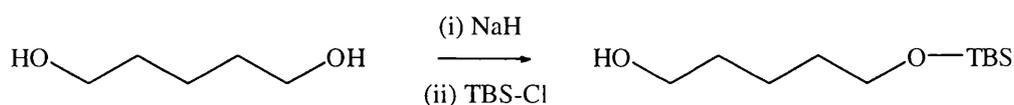
Figure 5.5: Monoprotection of Diol

It was thought that manipulation of 1,5-pentanediol could give the desired protected alkylating reagent. Monoprotection of pentan-1,5-diol was performed by formation of the *tert*-butyldimethylsilyl ether.[6] Initial monodeprotonation of the diol using one equivalent of sodium hydride, giving a white precipitate. This was followed by the addition of a solution of *tert*-butyldimethylchlorosilane in THF. Conversion of the remaining alcohol to the bromide was then attempted.

Several methods of direct halogenation were attempted, with little quantifiable success.

HO⁀⁀⁀O—TBS   ————→   Br⁀⁀⁀O—TBS
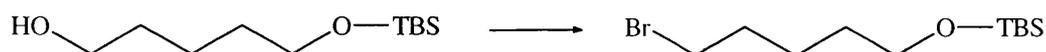
REAGENT	YIELD
SOBr$_2$	X
PBr$_3$	X
Br$_2$/PPh$_3$	X
CBr$_4$/PPh$_3$	X

Figure 5.6: Bromination of Alcohol

The triphenylphosphine based methodologies should generate leaving groups from the alcohol *in situ*, in the form of the phosphine oxide, with concomitant bromine insertion. Thionyl bromide is thought to act by a similar mechanism. In all cases, it was difficult to show that any reaction had occurred, due to the apparent instability of the product molecule to conditions of mass spectrometry. The lability of the bromine atom gave rise to a characteristic breakdown pattern, based on the ejection of ethene and propene. This phenomenon has also been encountered with other terminally halogenated *n*-chain hydrocarbons. In certain cases, the absence of desired product is not particularly surprising, as conditions were employed that could lead to deprotection of the ether group, and/or to cross reaction.

The failure of direct bromination methods suggested that employment of an activated intermediate could give better results. To this end, the monoprotected diol was tosylated.[7] An exchange reaction was then performed using sodium bromide in DMF, under anhydrous conditions.[8] This method gave rise to acceptable yields of the desired product, which was isolated and purified. The possibility of converting the starting diol into the ditosylate, to be followed by monohalogenation, was also investigated. However, the reaction to perform the halogenation was cumbersome, and required the use of a 3-fold excess of sodium bromide. As this made selective reaction unlikely, this route was not pursued further.

HO O—TBS —Ts-Cl→ TsO O—TBS

NaBr

Br O—TBS
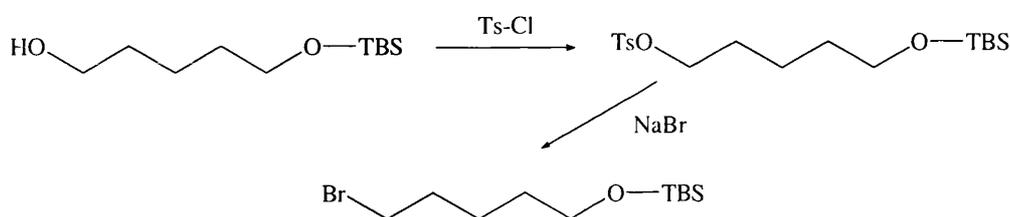
Figure 5.7: Bromination *via* Tosylate Intermediate

5.2.3 Use of 8-Bromooctanoic Acid Equivalents

The second possible disconnection was then investigated. The new scheme now required that the pyrazine reagent is reacted with an octanoic acid derivative.

The system initially described involved the reaction of a simple bromide with the lithiated pyrazine, following the conditions employed by Schöllkopf. In this system, however, similar results were not obtained. Our substrate is larger, and less active than those used in the studies of Schöllkopf and others.[9]

The initial reaction using unprotected acid returned only starting material, probably due to quenching of the pyrazine anion, so some form of protection was required to be incorporated. The most convergent choice was to use the oxazolidinone chiral auxiliary required in a later reaction (see below), as this would reduce the overall number of steps required. During the coupling with this species, it was found that starting material was again being reclaimed in good yield. This was thought to arise from enolisation of the oxazolidinone protected acid by the lithiated pyrazine.

Figure 5.8: Alkylation of Pyrazine Reagent

X	Y	YIELD
H	H	—
(oxazolidinone acyl)	H	—
(oxazolidinone acyl)	N3	20%
(tert-butyl ester)	H	—
(O—TBS ester)	H	—

The enolate formed would then be stable enough to remain intact throughout the reaction, to be quenched on workup. To prevent this from occurring, it was decided to also try using the azidated product as a substrate, as the azido group should prevent enolisation at the α-carbon. Several other functionalised substrates were also tried; the *tert*-butyl ester was used to determine the effect of steric bulk, whilst the silyl ether of octanol was employed to determine the effect of the carbonyl group, which could act in competition with the halide as an electrophile. The yields in all cases were, however, disappointing.

Details of another synthesis of a complex amino acid using the Schöllkopf methodology were then found in the literature. This employed an iodinated substrate to good effect,[10] along with the addition of highly polar co-solvents. It was therefore decided to attempt the coupling by this route, converting the brominated materials to the iodo-equivalents *via* the Finkelstein reaction,[11] using sodium iodide in acetone

solvent.

Figure 5.9: Iodination by Finkelstein Reaction

The alkylation reactions attempted were performed by the same method as described above, by deprotonation using n-BuLi in THF at -78°C, followed by stirring with the electrophile for five hours. Several different iodinated derivatives were tried in this synthesis, along with different co-solvents in the alkylation reaction, namely DMPU and HMPA. These were proposed to act as polarising agents, increasing the electrophilic character of the substrates and so promoting the reaction. The results for this series of trial reactions are set out below.
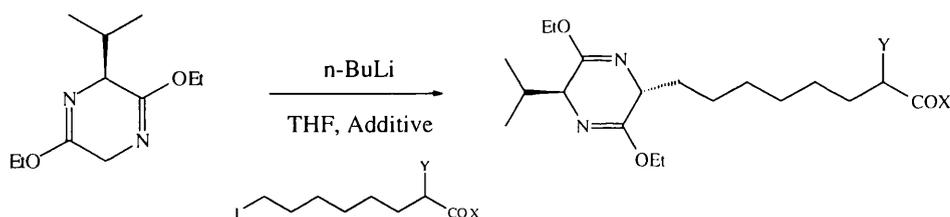
| X	Y	Additive	Yield
	H	HMPA	——
"	H	DMPU	——
"	N3	DMPU	——
	H	DMPU	48%

Figure 5.10: Pyrazine Alkylation Reactions using Different Co-Solvents

In all cases, results remained disappointing, giving results that were not significantly improved over the bromo-substrates. At no stage did either of the additives used give

product, except in the case of the *tert*-butyl ester, which was not found to be amenable to scaling-up.

5.2.4 Activation of the Deprotonated Pyrazine *via* Cuprate Formation

A further modification of the Schöllkopf methodology has recently been published.[12] In this, the deprotonated pyrazine is first converted to the higher order cyanocuprate before reaction with an electrophile to give the alkylated product. The labile substrate employed in that work was not structurally similar to octanoic acid, but it was felt that this method could be applied to our substrate. In all cases, the iodo-derivative was employed, as this was thought to be the best leaving group.
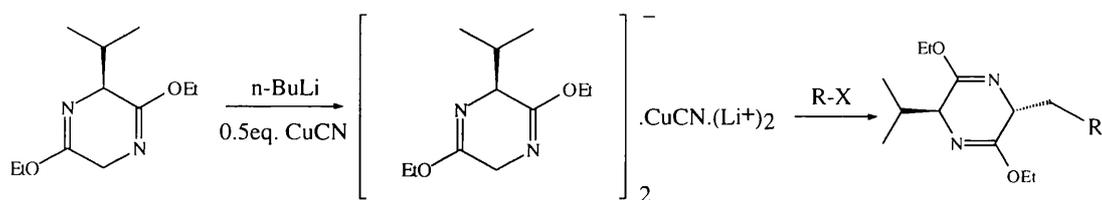
Figure 5.11: Alkylation of Pyrazine *via* Cyanocuprate Complex

Whilst the literature described a very short time for the formation of the cyanocuprate species, it was found that a longer contact time (*ca.* 2-3 minutes) was required in some cases for complete dissolution of the solid copper (I) cyanide. There were found to be no adverse effects on the yield of the reaction as a result of this extended reaction time at 0°C. In contrast to the original reaction scheme, it was found that no coupling occurred if the temperature was maintained at -78°C throughout the reaction. However, performing the reaction at -23°C gave excellent results. Reaction at this temperature also seemed to have no effect on the enantioselectivity of the reaction, as determined by chiral HPLC. These factors point to the cyanocuprate formed being a stable species relative to the simple lithiated pyrazine. This in turn explains the higher yield of alkylated product, as more of the anionic species reacts in the desired fashion, rather than quenching either by the formation of an enolate with the substrate, or by other processes.

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The attempt to form the fully masked amino acid product (**23**) directly by using the azidated acyl oxazolidinone as the alkylating agent was unsuccessful, even under these modified conditions. It is believed that this was due to the effect of incorporating a transition metal into the reaction. The alkylating agent in this case includes two carbonyl groups, and the dipolar azide group, which could result in complexation with the cyanocuprate, away from the electrophilic centre. If this was the case, then formation of the desired product would require the closure of an eleven membered ring, an entropically unlikely process.

| X	Y	Yield
	H	—
''	N_3	20%
	H	95%
O—Me	H	78%
O O TMS	H	—
O—Si	H	90%
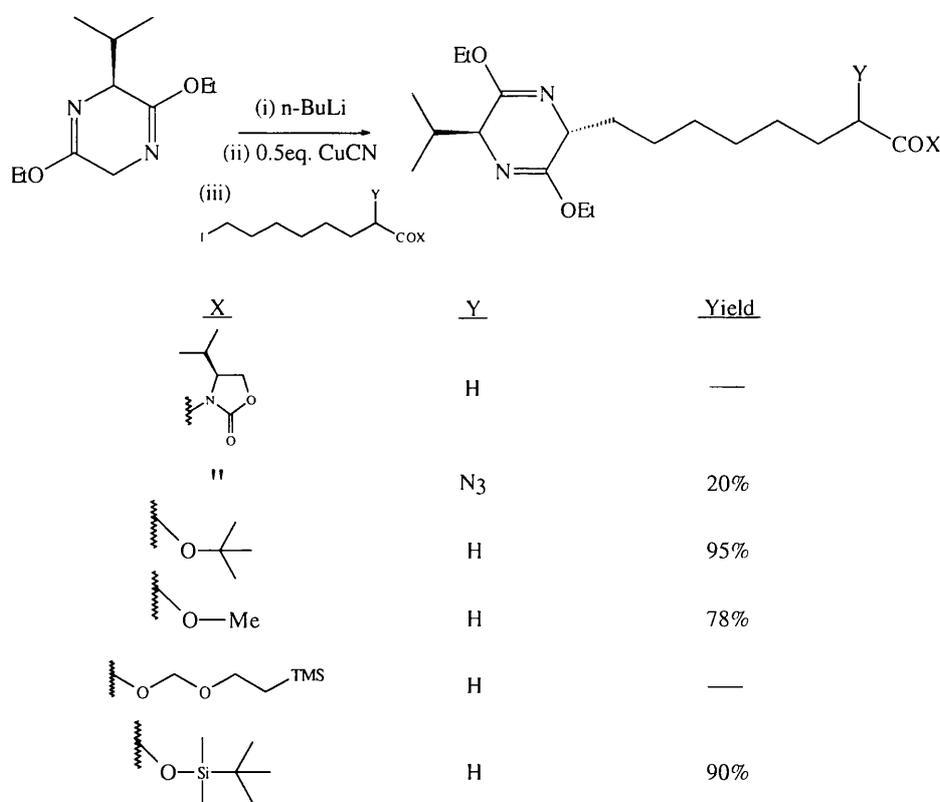
Figure 5.12: Alkylation of Pyrazine Cyanocuprate Reagent

However, the process did give very good results with certain ester derivatives. In particular, the *tert*-butyl ester initially used as a test case gave excellent results with the new methodology, as did the *tert*-butyldimethylsilyl ester. This latter protecting group was used, as the protecting group was required to be easily removed. The more hydrolytically stable SEM group was also tried, but the results obtained were very

poor, possibly as a result of cross reaction at the methoxy group present in the ester. None of the desired product was ever isolated, and nor were any identifiable fragments. The possibility of cross reaction was partially confirmed by use of the methyl ester with the cyanocuprate system, a reaction which gave the desired product in good yield, without optimisation. Also, no cross reaction was ever observed with the tert-butyldimethylsilyl ester, or the ether.[12] These results seem to indicate that although the bulk of the ester group plays a small role in shielding the carbonyl, the nature of the protecting group is more crucial. The presence of the methoxy group in SEM protection provides a source of acidic protons which may quench the reactive pyrazine, and give rise to a new reactive species. This in turn may cause the unidentifiable mixture of products. Simple desilylation during the aqueous workup procedure was discounted as this would have given rise to the ethoxymethyl ester, which is not only identifiable by spectroscopy, but would have been silica labile, giving the known hydrolysed product. Overall, the results indicate the compatibility of the tert-butyldimethylsilyl ester group with the method described.

5.2.5 Deprotection of Alkylated Pyrazine Octanoate Esters

The results above, whilst showing that direct synthesis of the pyrazine alkylated with the azidated acyl oxazolidinone was not possible, did give two possible substrates for the synthesis of the pyrazine octanoic acid, via different ester groups. Both of these groups, the tert-butyl ester and the tert-butyldimethylsilyl ester (TBDMS), were available in excellent yield from the previous step, but required cleavage of the ester function prior to further functionalisation.

The standard conditions for the cleavage of a tert-butyl ester involve the use of aqueous acid. The desire to retain the pyrazine ring system, which is opened by acid catalysed hydrolysis, meant that these conditions could not be used. However, it is also possible to remove the ester by the use of an anhydrous acid, such as dry trifluoroacetic acid.[13] Initial experiments in this area suggested that this route was viable, but on scaling-up significant problems with cross reaction were encountered, and no identifiable product was obtained. It was thought that this poor result was due

to the presence of TFA anhydride in the system generated during the drying process, but replacement of the reagent with fresh, undistilled reagent gave no improvement in results. Alternative methods were also tried, including reflux with p-toluenesulphonic acid in benzene and treatment with dried ion exchange resin, but these were also found to be ineffective. In the light of the failure to effect this deprotection with the methods mentioned, no further alternatives were tried.

| X	Conditions	Yield
O—	TFA	—
O—Me	LiOH	Quant.
O—Si—	TBAF	Quant.
O—	TsOH	—
O—	Amberlite	—
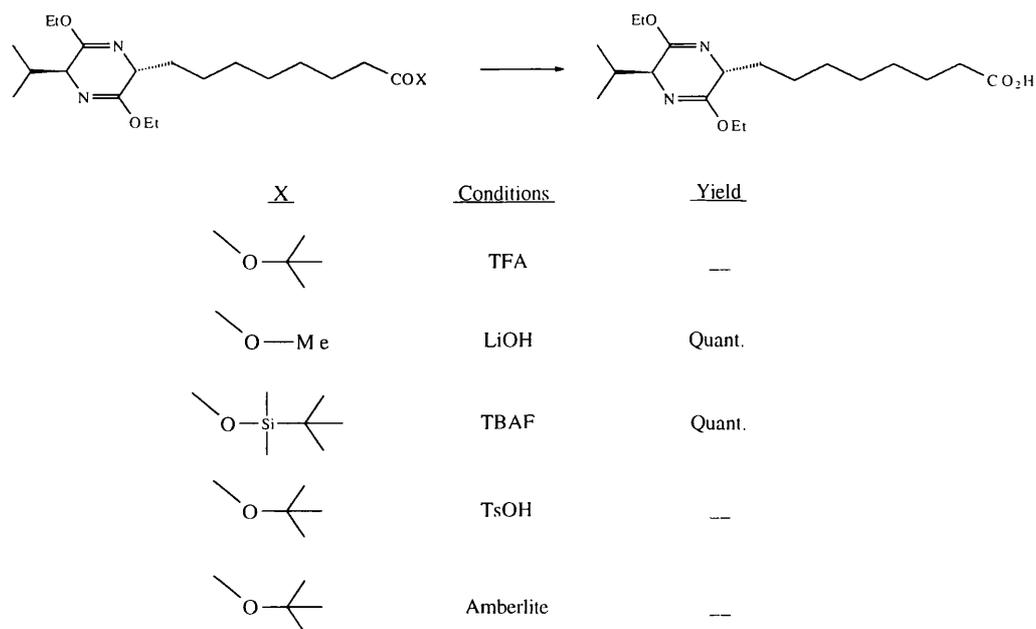
Figure 5.13: Ester Cleavage Reaction Results

The cleavage of silyl esters is generally a simple hydrolytic procedure, the less hindered TMS esters being unstable to aqueous workup conditions. In the TBDMS ester system employed, the most common conditions used for unmasking are the use of a fluoride donor, such as tetra N-butylammonium fluoride in THF solution,[14] to give the product acid after aqueous workup. In this synthesis, it was felt that the alkaline buffer solution used in the workup of the cyanocuprate species was likely to cause partial deprotection, so minimal treatment would be required to remove the remaining silyl ester present. The ester deprotection step was therefore included as part of the alkylation procedure. The product was treated with two equivalents of TBAF in THF solution, and stirred for one hour before acidification and extraction into ether. The yield quoted for the synthesis of the pyrazine octanoic acid is therefore over two steps.

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Although only performed once, the pyrazine octanoic acid was also synthesised from the methyl ester derivative, by standard lithium hydroxide in THF/water solution conditions. This process was also high-yielding, and so afforded an alternative route. In this case, the use of an aqueous base was successful, as the imines present in the pyrazine are not labile to these conditions.

5.3   INTRODUCTION OF S-CHIRAL CENTRE

As can be seen from the initial retrosynthetic schemes (figs. 5.1, 5.2), there were two proposed methods for introduction of the S-chiral centre. Attention was focused initially on the use of the β-lactone, which was well detailed in the literature.[15] The alternative route involved the amination of an enolate by an amine equivalent, the stereochemistry of the product being directed by a chiral auxiliary.

5.3.1   Use of the L-Serine β-Lactone Reagent

The synthesis and use of this material has been the subject of several papers,[16] and it was considered to give access to the S-stereochemistry we required at the opposing end of our dimeric amino acid target. The synthesis of this reactive molecule from S-serine had been described, via a Mitsunobu-type process,[17] closing the lactone between the serine hydroxy-and carboxylic acid functionalities. The substrate for this reaction was a di-protected serine derivative. This compound was prepared by the described methods. However, the yields obtained over the two steps were very low, making this route less viable.
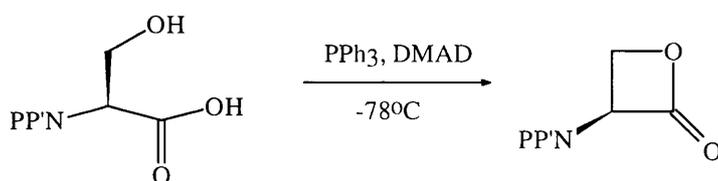

Figure 5.14: β-Lactone Formation Reaction

Serine was first benzylated, followed by amine diprotection using the Cbz group. The viscous oily product of this reaction was purified by reverse phase chromatography, using a water based solvent system. However, treatment of a solution of this material in THF with a triphenylphosphine/dimethylazodicarboxylate (DMAD) adduct at -78°C gave none of the desired product. It is believed that, in this case, the substrate, due to its physical properties, cannot be completely dried, and the trapped water then interferes with the Mitsunobu reaction. This would explain the generation of triphenylphosphine oxide which was isolated after the reaction.
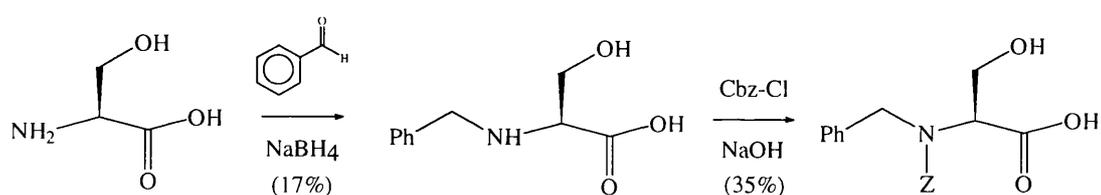

Figure 5.15: Synthesis of Diprotected Serine I

As the major problem with the approach to the protected substrate arose from the water present in the chromatography solvent, the use of different protecting groups was attempted. As literature pointed to the need for diprotection of the amine,[2] methods for achieving this in one step were tried. The first of these was the use of the phthalimido- group, introduced by use of N-ethoxycarbonyl phthalimide, as set out below.[18] The protecting group is introduced by exchange in aqueous carbonate solution, and the product is collected after acidification. However, no solid product was obtained on acidification, and extraction into ethyl acetate was required; once again, purification was required, in order remove phthalimide impurities. This proved extremely difficult, and so this method was abandoned.
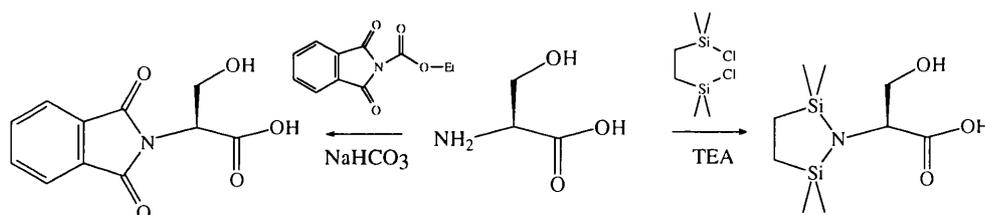

Figure 5.16: Synthesis of Diprotected Serine II

An alternative method attempted involved the use of a silyl-based protecting group, (bis-dimethylsilyl)ethane (STABASE).[19] The free amino acid was then reacted with the dichlorosilane precursor in the presence of TEA under anhydrous conditions. The product was collected as an oil after removal of solvent. However, the product proved to be highly water sensitive, and again purification proved impossible.

After the completion of this work, a paper describing the synthesis of a similar substrate was published, detailing the use of a monoprotected serine derivative.[16] The method described would have avoided the purification problems encountered. As the synthesis of a suitably protected and purified serine derivative proved impossible, the strategy was abandoned.

5.3.2 Use of the Evans Chiral Azidation Methodology

The alternative disconnection of the target shows that the S-chiral centre can be created via the reaction of an amine equivalent with an enolate ion.[5] In the method mentioned, the amine equivalent used is an organic sulphonyl azide, which is reacted with an enolate generated using the potassium form of the base hexamethyldisilylazide (KHMDS). The reaction gives diastereoselectivity by discrimination against approach from one face, blocked by the presence of a chiral auxiliary.
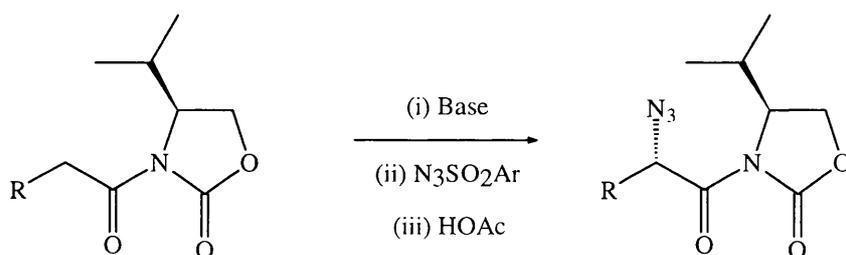

Figure 5.17: General Chiral Azidation Reaction

The reaction scheme followed involved the use of octanoic acid derivatives coupled to the oxazolidinone derived from S-valine. The azide donor used was triisopropylsulphonyl azide (trisyl azide). The generation of the auxiliary was achieved in two steps by reduction of S-valine using sodium borohydride/iodine in refluxing

THF,[20] followed by closure of the ring using phosgene solution as a carbonyl donor.[21] The resulting product was highly crystalline, and could be easily purified to give a good yield over two steps.


NaBH4/ I2
(69%)

COCl2
(69%)
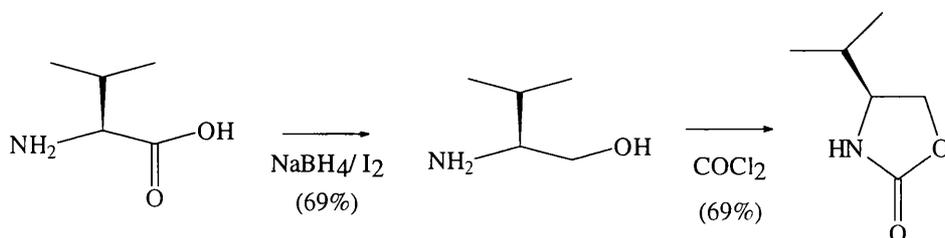
Figure 5.18: Scheme of Formation of Oxazolidinone

Two substrates were employed for these syntheses; the 8-bromoctanoic acid and the 8-(pyrazine) octanoic acid. Use of the acid labile pyrazine moiety requires that certain steps in this route are modified, to avoid premature deprotection. This in turn ensured that the number of protection/deprotection steps are kept to a minimum.

Derivatisation of the octanoic acid derivatives was achieved by activation of the carboxylic acid function as a mixed anhydride using isopropyl chloroformate, followed by addition of a solution of the lithiated anion of the auxiliary.[5] The reactions were performed at -78°C, and the products isolated after quenching in 1M sodium hydrogen sulphate solution and removal of organic solvent prior to extraction.

TEA

(i) n-BuLi,   (ii) Aq. Quench

(84%)
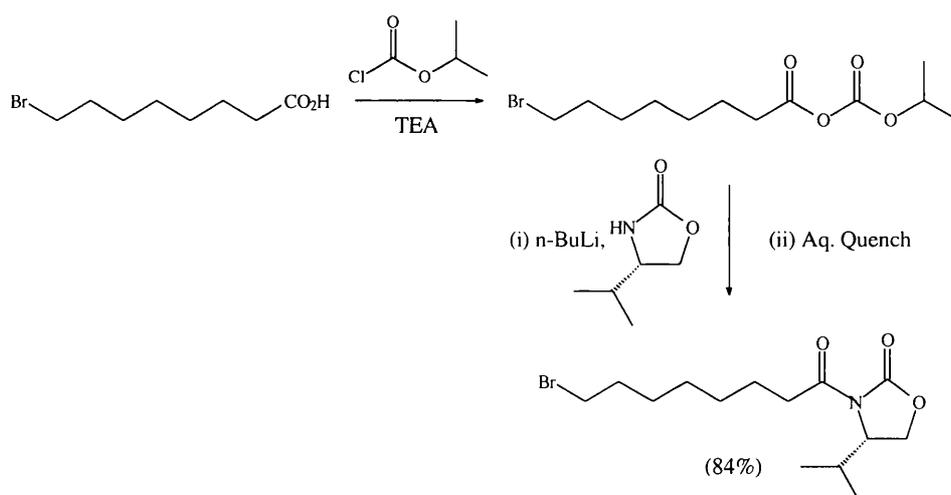
Figure 5.19: Acylation of Oxazolidinone

However, in the case of pyrazine bearing molecules, the acid sensitive substrates required alternative methods, and here it was found that 1% sodium hydrogen carbonate was equally effective as a quench.

After addition of the auxiliary, enolisation of the substrate was performed by use of KHMDS at -78°C, followed by the addition of the azide donor in THF solution. This gave rise to a hydrazido- adduct, which after a short contact time (*ca.* 1-2 minutes) was quenched by the addition of glacial acetic acid, which hydrolysed the adduct to give the azidated product.

| Quench Reagent	Product
HOAc	Pyrazine Ring-Opening
HOAc/KOAc	-N$_3$
KOAc	No Reaction
Phophate Buffer	Cross Reaction
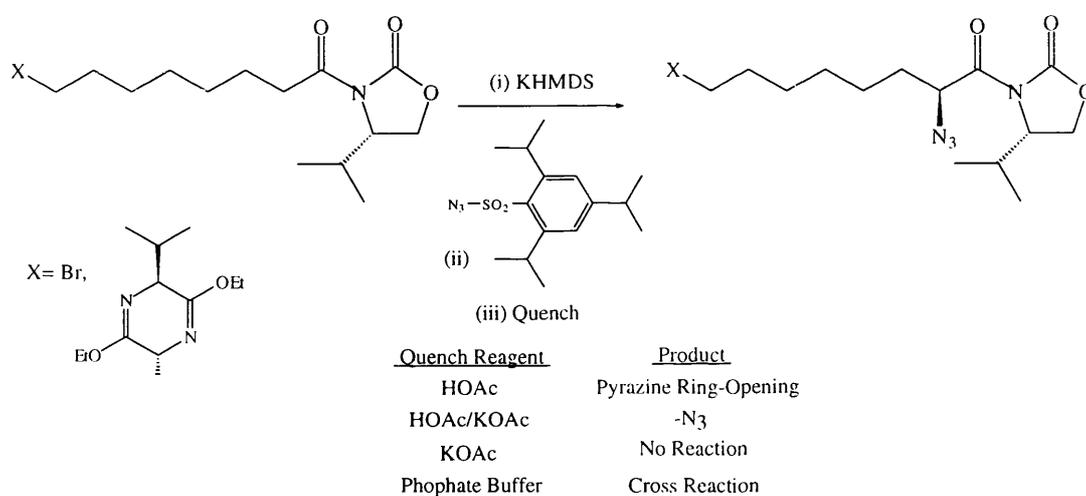
Figure 5.20: Azidation of Acyl Oxazolidinone

The acid sensitive substrate was not, however, stable to these conditions, and so alternatives were tried. In the literature, it had been found that potassium acetate was the most effective quench, giving better yields of desired product than acetic acid. This was suggested to be due to the role of the potassium salt in the hydrolysis of the adduct.[5] In this system, similar results were not obtained; this may have been due to the more complex nature of the acid side chain of X compared to the mainly alkyl side chains described. The use of phosphate buffer (pH 7.4) was also tried, but again in this case no product was obtained. Evans had noted that this agent had given rise to diazo-formation and not azido transfer,[22] and so this process may have been competing. The best conditions found were the use of a buffer system of 1 part glacial acetic acid to

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3.5 parts saturated aqueous potassium acetate. The use of this system gave reproducibly good yield with high e.e. as determined by chiral HPLC.

5.4 ASSEMBLY OF THE "MASKED" TARGET MOLECULE

As has been described above, the retrosynthetic schemes showed two possible methods for assembly of the target molecule, but the failure of one route resulted in only one strategy being followed. However, the precise order of introduction of functionality remained important.
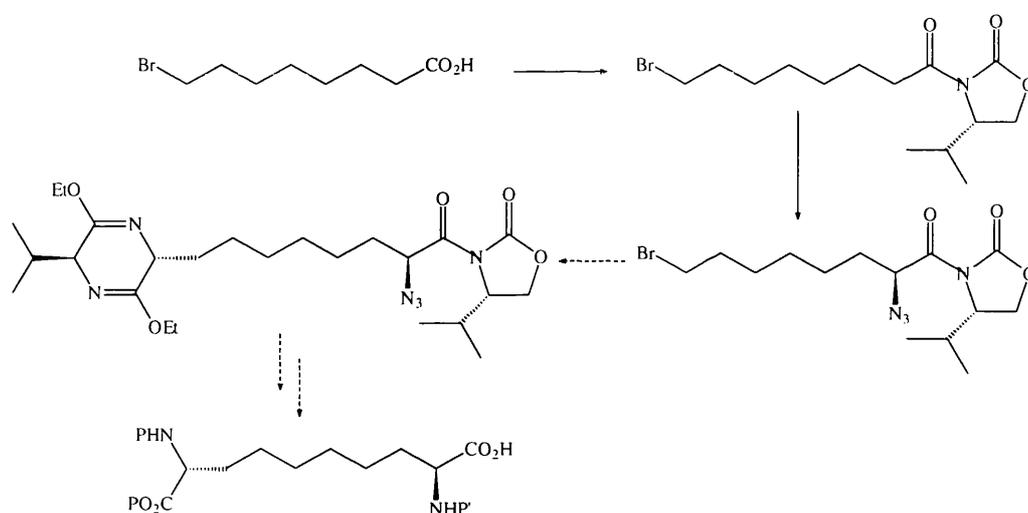

Figure 5.21: Synthetic Scheme I

As has been detailed previously, although the correctly acylated oxazolidinone was readily available, alkylation of the pyrazine ring proved to be difficult with this material as substrate. The application of different conditions to the synthesis set out above did not affect the results significantly. As this route described the most "efficient" method of synthesis, having the fewest steps, these results were disappointing. However, in the course of test reactions, several ester substrates had been found to give good results for the problematic alkylation reaction. Hence an alternative method was adopted.

This route is simply an adaption of the previous route, with the incorporation of the chiral auxiliary at a later stage in the synthesis. After azidation as described above, the

masked residue was obtained in reasonable yield.
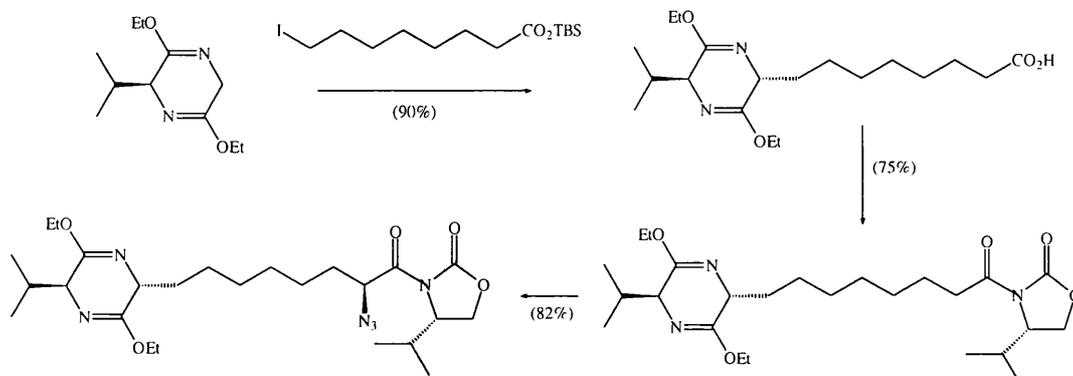
Figure 5.22: Synthetic Scheme II

5.5   UNMASKING OF THE AMINO ACID RESIDUE

The free amino acid was revealed by standard literature deprotection steps.[4.22] The optimum sequence for these steps to ensure maximum differentiation was found to be initial removal of the oxazolidinone auxiliary using lithium perhydroxide, followed by cleavage of the pyrazine ring by mild aqueous acid/THF solution.


Yield (three steps) = 92%
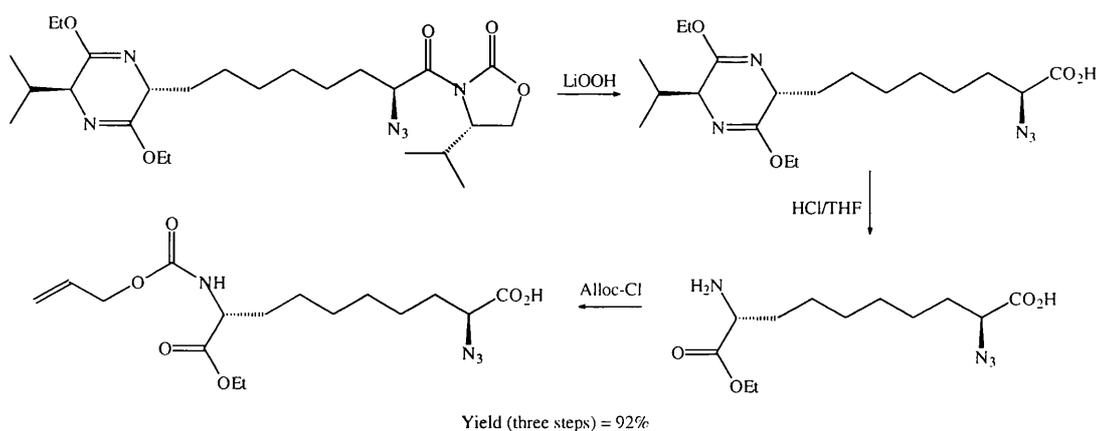
Figure 5.23: Scheme of Cleavage of Auxiliaries and Amine Protection

This order was decided upon by the lability of the chiral centres to these conditions; if

the pyrazine was cleaved first, the ester group could be cleaved by the basic conditions present in the second step.As this would have given two carboxylic acid groups, distinguishable only by stereochemistry, this alternative route was rejected.

Purification was simplified by protection of the new α-amino centre as its allyloxycarbonyl derivative. This process gave the required product in excellent yield over three steps, with recovery of the oxazolidinone auxiliary, and with all functionality in forms that allowed chemoselective methods to be applied.

5.6 SELECTIVE TRANSESTERIFICATION REACTIONS

It is necessary to differentiate between two carboxylic acid functionalites at this time. This is possible due to the presence of one of the carboxylic acid groups in an esterified form. Several methods have been described for transesterification, but few that specifically perform transesterification in the presence of free acids.[23]
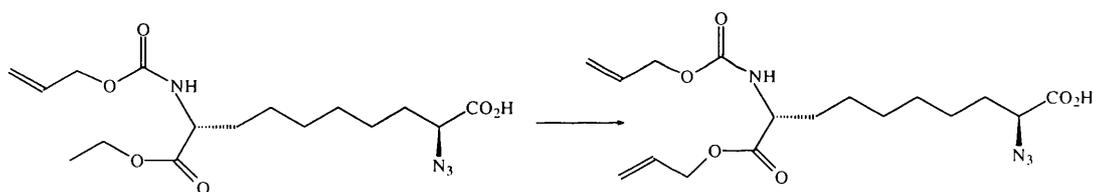
| Reagent	Yield
Al(O^{i}Pr)$_3$	——
AlCl$_3$/ Allyl Alcohol	——
Ion Exchange resin/ Allyl Alcohol	——
DBU/ LiBr/ Allyl Alcohol	54%

Figure 5.24: Transesterification Reactions

The use of organometallic reagents is well known, specifically the use of titanium and aluminium alkoxy salts. However, a more detailed study of the literature showed that the particular reaction under consideration was not catalysed by titanium tetraallyloxide, and so this reaction was not attempted. The use of aluminium isopropoxide in allyl alcohol was also attempted, but found to be ineffective, even if

the reactive material was added to alcohol and stirred prior to the addition of the substrate. The failure of these reagents was unsurprising, as most could be employed to generate esters from free acids.

A recently reported approach involving the use of diazabicycloundecene (DBU) in conjunction with lithium bromide in allyl alcohol was then attempted.[24] This reaction involves the creation of an equilibrium between the base and solvent, and the lithium bromide and substrate. In the previous cases, 0.5 equivalents of DBU had been used to transesterify amino acid esters. As the substrate bore a free carboxylic acid functionality, the quantity of DBU used had to be modified. It was felt that the use of 1.5 equivalents would be most effective, as 1 equivalent would act to neutralise the acid. In this case, the deprotonated acid was thought to make a poor electrophile to the reactive allyl species. In the literature study, it had been found that the use of either reagent in isolation gave no results. Lithium iodide has, however, been used in other systems to promote ester exchange, giving some insight into the role of the bromide salt.
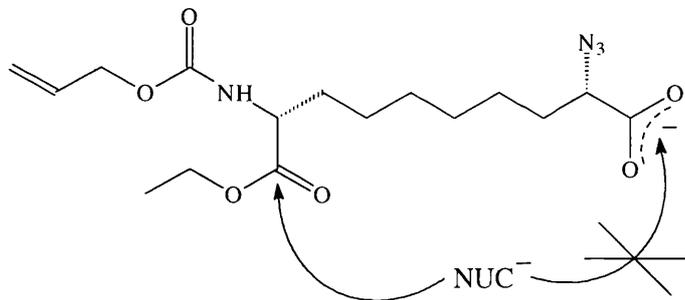

Figure 5.25: Scheme of Nucleophilic Transesterification

This reaction was found to give acceptable yields of the allyl ester, with no deprotection of the N-alloc group, or diester formation seen. Some racemisation has been reported in the transesterification of amino acids in the literature, but none was observed with this substrate.

5.7 REDUCTION OF AZIDE GROUP AND PROTECTION OF AMINE

Several methods for the reduction of azides exist, many using heterogeneous catalysts to affect hydrogenation of the substrate. However, other, milder approaches also exist which utilise hydrogen donors in a stoichiometric fashion to give the amino product.


Reagent	Yield
Lindlar/ H_2	—
PPh_3/ H_2O	(70%)
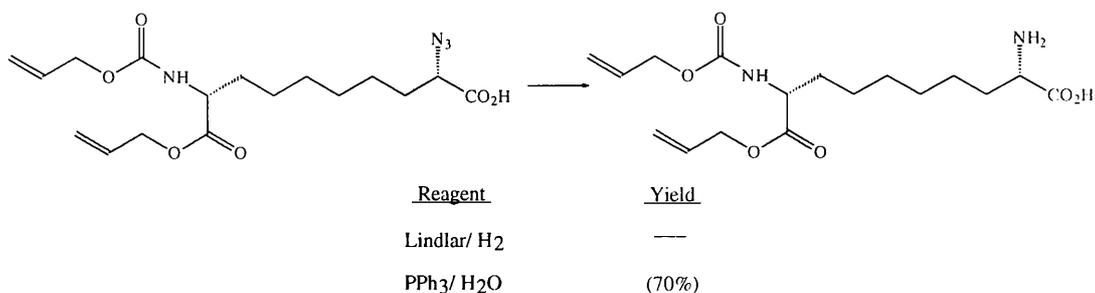
Figure 5.26: Methods for Reduction of the Azide Group

The catalytic methods are limited by the reactivity of the molecule, which bears allyl functionality which may be reduced itself. Hydrogenation of the allyl groups would remove the "orthogonal" nature of the selected protecting group, and so the use of stronger catalysts is not possible. The Lindlar catalyst, palladium on calcium carbonate, is known to reduce azides, whilst leaving double bond systems intact.[25] The use of this system gave inconclusive results. Whilst the reaction was performed several times, the results varied between attempts, possibly due to poisoning of the catalyst.
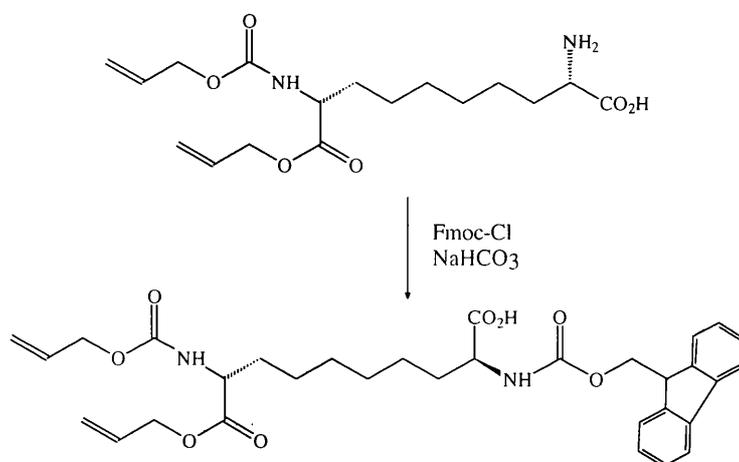

Figure 5.27: Protection of Free Amine

The Staudinger reaction was also attempted, involving the use of triphenylphosphine and water.[26] In this case, the stirring of a solution of triphenylphosphine in wet THF gave good results. The reaction solvents were removed and the residue treated with fluorenylmethyl chloroformate in dioxane/water solution to give the target protected amino acid in acceptable yield. Purification of the material gave the monomer in a form suitable for solid phase peptide synthesis.

5.8   SYNTHESIS OF THE TARGET PEPTIDES

The overall target of the synthesis was the incorporation of the monomer into peptide sequences. This was attempted by automated peptide synthesis, with two peptides as the synthetic objectives. The first of these was a short sequence, a hexamer formed of the portion of hGRF that surrounds the substitution site, and the longer sequence of (1-29) hGRF.

5.8.1   Synthesis of the Short Model Peptide

The designed peptide sequence was Asn-Xaa-Tyr-Arg-Lys-Xaa-Leu-NH$_2$, where Xaa represents the two α-termini of our monomer. Synthesis was performed using a Rink-type resin,[27] using HBTU mediated coupling, and 1 hour coupling and deprotection times were employed. The progress of deprotection was followed by means of UV monitoring. After the incorporation of the first leucine residue, the synthesised monomer was incorporated with a coupling time of 1 hour. The inclusion of the next three residues was then performed by standard Fmoc- methodology. After this time, a small quantity of the peptide was cleaved and analysed by mass spectrometry. However, no positive results were obtained, with no peak corresponding to the side chain protected fragment found. A signal at 1183 Daltons was found, 87 Daltons higher than calculated, but could not be assigned.

The removal of the Alloc-protecting groups was performed by literature method, the employment of *tetrakis*(triphenylphosphine) palladium in a DCM/NMP/HOAc solvent[28] under nitrogen atmosphere. After removal of the catalyst and by-products,

and washing, the cyclisation was attempted using HBTU as a coupling reagent.[29] This process was performed manually, before return of the support to the synthesiser for attachment of the final residue. Cleavage of product from the resin, along with side chain deprotection, was performed by standard methods, without the presence of scavengers.

Four peaks were observed by HPLC of the crude mixture, which gave only a small yield of solid. Purification of this mixture was attempted using preparative HPLC, giving four fractions. However, none of the desired product was seen by mass spectroscopy. The only clearly attributable mass was that of the deletion sequence, Asn-Tyr-Arg-Lys-Leu-NH_2. Again, a peak was found at 1183 Daltons, but this could not be positively identified. The very low quantity of this material made further analysis very difficult.

Only one attempt was made at the synthesis of this product, meaning that optimum conditions for the incorporation of this residue were not known. Hence, coupling of the residue, which was known by continuous monitoring to have been irregular, and cyclisation may have required additional time, or alternative activation procedures. Lack of time, and material, prevented the synthesis of this peptide being investigated further.

5.8.2 Synthesis of the hGRF Analogue

The full peptide sequence has been described previously (see chapter 1). Our intention was to synthesise an analogue, with monomer residue incorporated between the positions 9 and 13 of the sequence. The full length peptide is 29 residues long, with an amphiphilic character in the C-terminal portion. The intended site of incorporation is above this portion, at the C-terminal end of the projected first helical section of the natural peptide.

The synthesis was carried out by Fmoc- methodology, using a PAL-PEG-PS resin with 0.12mmolg^{-1} substitution level and the previously described HBTU coupling reagent. The sequence was extended until incorporation of the artificial residue was required. With reference to the uncertain level of incorporation seen in the previous example, it

was decided to change the conditions for coupling. This was performed manually, using HATU as a coupling reagent, with mixing for 1.5 days. Capping of unreacted amino groups was then performed using acetic anhydride, prior to cleavage of the N-terminal Fmoc- group using piperidine. The next three residues were then introduced on an automatic synthesiser, followed by the deprotection of the N-terminus. After this, the resin was transferred to a manual synthesiser and Alloc- deprotection performed using palladium catalysis, with the solvent system described above, for 2 hours. The cleavage mixture was then removed and the resin thoroughly washed, followed by cyclisation of the N-terminus to the side chain carboxylic acid using HATU. This reagent has been suggested in the literature to be the most effective method of performing such cyclisations.[30]

The resin was then returned to an automatic synthesiser, for chain extension. This was followed by means of conductivity monitoring, which indictated that whilst the next 8 residues were incorporated, the couplings were not complete, and were irregular. This indicates that the incorporation of the last residues are difficult processes, perhaps caused by preorganisation of peptide product still bound to the resin. This could have made access of the reagents to the free N-terminus difficult, in turn giving the low coupling levels observed.

The final peptide product was cleaved using 95% TFA solution, with an ethanedithiol/thioanisole/phenol scavenger cocktail.[31] After lyophilisation, analysis by HPLC showed five compounds were present. These were separated by preparative HPLC, but none of the desired product could be found by mass spectroscopy. Again, the presence of protected fragments was suspected, but no fully deprotected peptide was found. It has been noted that difficulties can occur in the deprotection of peptides which have partially folded.

The inconclusive mass spectrometry results for both of these systems point to the desired products not being formed in these reactions. However, further analysis is still being performed on the full hGRF peptide analogues. Again, the small quantity of sample available makes analysis difficult. Also, if partially protected groups are present, any decomposition of these groups over time will further complicate the procedure.

5.9 FURTHER WORK

At this time, the scheme for the synthesis of the designed diamino diacid (fig. 1.2) is complete, and is almost fully optimised. However, improvements remain to be made with the purification of certain products, specifically the transesterification reaction product, and the final reduction/protection steps product. It may also prove that the results obtained in this work allow the synthesis of alkene-linked diamino diacids in shorter time.

The major bulk of work examining the incorporation of this residue and its structural effects on peptides remains to be done. It may be the case that solution synthesis of the short peptide may provide the best system for optimisation of the cyclisation reaction. These conditions would be the most advantageous, as they would allow the reaction to be followed by techniques such as n.m.r.. In the short model peptide, this may allow the identification of difficult steps.

The problematic deprotection and cleavage steps also require more thorough investigation. This could also be achieved *via* the short model peptide, as the deprotection could be easily followed over time using n.m.r. and mass spectroscopy. In this way, problem protecting groups could be identified and avoided.

Identification of the structure of the short model peptide may also help in understanding of the difficulties experienced in attempted chain growth of the hGRF analogue peptide. It is felt that if this molecule creates a turn whilst still polymer-bound, this may cause steric hindrance to incoming amino acids. This type of structure could be easily identified in the short model peptide. Investigation of the helix forming capability of the group, in both short model peptide and in hGRF(1-29), could also be investigated.

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CHAPTER SIX

EXPERIMENTAL

6.1 CHARACTERISATION PROCEDURES

^{1}H n.m.r. spectra were recorded at 60MHz (JEOL PMX-60), 200MHz (Bruker WP-200 and Varian XL-200), 250MHz (Bruker WP250), 400MHz (Varian XL-400), using CDCl$_3$, CD$_3$OD or d$_6$-DMSO as solvents. ^{13}C spectra were recorded at 100MHz (Varian XL-400) using CDCl$_3$ as solvent. All chemical shifts are given in ppm, relative to tetramethylsilane.

Mass Spectra were recorded using FAB or EI ionisation on a VG ZAB SE machine, using matrices for FAB.

Optical rotations were recorded on a Perkin-Elmer 141 polarimeter or a JASCO J-600 polarimeter in CHCl$_3$ solutions, standardised using sucrose solution.

Infra red spectra were recorded on a Perkin-Elmer FT-IR 1605 spectrometer, using solution (KBr cell, CHCl$_3$ solution), or thin film (KBr disks).

Peptides were synthesised using Fmoc- methodology, using either an ABI "synergy" 432A automated synthesiser, or an ABI 431A automated synthesiser. Amino acids, coupling reagents and appropriate resins were supplied by Perceptive Biosystems.

HPLC was performed (normal phase) on Kromasil (0.5 x 25cm) analytical column, using either refractive index or UV monitoring, Waters associate pump or Gilson 305 pump systems. Reverse phase was performed using Hichrom KR100-5C18, same pump systems. Chiral HPLC was performed using Daicel OD column (0.46mm x 25cm),UV monitoring, Gilson 305 pump system. Peptides were analysed and purified using Vydac ODS 218TP columns (analytical, 0.46 x 25cm; preparatory, 2.2 x 25cm), UV monitoring, Shimadzu LC-9A pump

Chromatography (tlc) was performed using silica 60 F$_{254}$ plates (E. Merck+Co.), or on reverse phase RP-18 F$_{254S}$ (E. Merck+Co.). Column chromatography was performed using silica 60, supplied by BDH, or reverse phase silica 100-C18, RP (Fluka). Solvent

systems are described for each separation.

THF solvent was dried by refluxing from sodium/benzophenone under dry N_2 atmosphere. Alcohol solvents were dried by refluxing from magnesium turnings, activated by iodine, under dry N_2 atmosphere. DCM was dried by refluxing from calcium hydride or phosphorus pentoxide. All other materials were used as supplied, or purified by literature method.[1]

Reactions using phosgene solution were made safe by purging using a stream of dry N_2 in a fume hood. The gaseous effluent was passed through a concentrated ammonia solution, evolving white fumes whilst phosgene was still present. After no further fumes were seen, the reaction was safe to be continued outside of the fume hood. All waste ammonia solution was stored overnight, prior to disposal by dilution and committed to the drain.

Reactions involving copper cyanide were worked up using a basic buffer solution, as described later. The cyanide containing aqueous layers were collected, and treated with bleach solution, with the pH maintained above 7. All contaminated equipment was placed in this solution in a fume hood overnight, prior to disposal by the drain and washing with copious quantities of water.[2]

6.2   EXPERIMENTAL PROCEDURES

1-(*tert*-Butyldimethylsilyloxy) pentan-1,5-diol (1)[3]
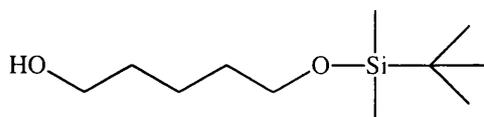
To a slurry of sodium hydride (60% dispersion in oil, 0.29g, 9.6mmol) in THF (12ml) was added 1,5-pentanediol (0.83g, 8.0mmol) dropwise, the reaction evolving gas and producing a thick, white precipitate. After stirring for 1 hour at room temperature, a solution of TBDMS-Cl (1.45g, 9.62mmol) in THF (6ml) was added dropwise over 5 minutes.

The resulting solution was stirred for 1 hour, during which time the precipitate

dissolved slowly. After this time, the solvents were removed *in vacuo*, and the residue suspended in ether. The organic layer was washed with water, then brine. The organic layers were dried ($MgSO_4$), and the solvent removed *in vacuo* to give the product as a clear oil.

Purification was performed by distillation (95°C @ 0.5mmHg) or by flash chromatography (35% EtOAc/ Hexane, R_f=0.26), giving a yield of the product as a clear oil, 0.63g, 2.88mmol, 30%.

^{1}H n.m.r. (200MHz, $CDCl_3$); δ=0.02 (6H, s, Si-(CH_3)2), δ=0.87 (9H, s, Si-C(CH_3)$_3$); δ=1.3-1.4 (6H, m, alkyl chain); δ=3.6 (4H, t,t, (J=6.4Hz, 6.4Hz), O-CH_2-CH_2)

IR; ν=3370cm^{-1} (-OH); ν=2930cm^{-1} (C-H); ν=1254cm-^{1}, 836cm^{-1} (Si-(CH_3)$_2$); ν=1101cm^{-1} (Si-O)

Mass Spec. (FAB) 219 (M+1)$^+$

Glycine methyl ester hydrochloride (2)[4]

$$ClH:H_2N\diagup\diagdown CO_2Me$$
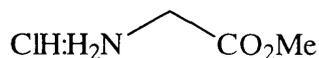
To a cooled (water bath) suspension of glycine (12.0g, 0.16mol) in methanol (400ml) was added thionyl chloride (24.0ml, 0.32mol, 2eq.) dropwise, with care taken to keep the temperature of the reaction mixture under 40°C. The resulting greenish solution was stirred at room temperature for 12 hours, then worked up by removal of solvent to give a solid, which was purified by recrystallisation from ethanol. The product was dried over solid sodium hydroxide, giving a quantitative yield of fine white crystals .

Melting point; 175°C (lit. 175°C (dec.))[4]

N-Benzyl-(*S*)-serine (3)[5]

To a solution of *S*-serine (1.01g, 9.62mmol) in 2N sodium hydroxide solution (10ml) at 6°C was added benzaldehyde (0.95ml, 9.34mmol), and the resulting solution was stirred for 30 minutes. Sodium borohydride (0.102g, 2.68mmol) was then added in
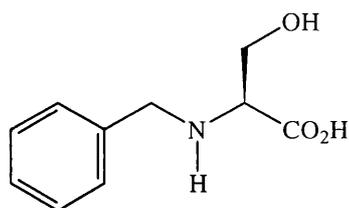
portions, with the reaction temperature maintained at 10°C. When completed, the mixture was stirred for 1 hour, and then further quantities of benzaldehyde (0.95ml, 9.34mmol) and sodium borohydride (0.102g, 2.68mmol) were added. After this, the reaction was stirred at room temperature for 2 hours, after which the reaction was washed with ether. The aqueous layer was then acidified, and the product was then extracted into ethyl acetate.

The ethyl acetate layers were then dried (Na_2SO_4), followed by removal of solvent *in vacuo*. This gave the product as a white solid, which could be recrystallised from water to give the title compound, 0.360g, 17% yield.

^{1}H n.m.r. (200MHz, CD_3OD), ; δ=4.0 (3H, m, -CH-CH$_2$-); δ=4.36 (2H, s, Ph-CH$_2$-); δ=5.0 (broad s, -NH-, -OH, -CO$_2$H); δ=7.58 (5H, m, Aryl protons)

IR; ν=3235cm^{-1} (-OH, -NH-); ν=2669cm^{-1} (-CO$_2$H); ν=1640cm^{-1}, 1590cm^{-1} (C=O); ν=754cm^{-1}, 694cm^{-1} (aryl)

4-(S)-Isopropyloxazolidin-2,5-dione (4)[6]
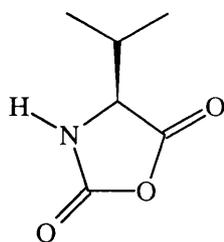
To a stirred suspension of S-valine (6.0g, 0.051mol) in THF (84ml) under Ar atmosphere was added phosgene in toluene solution (2.32M, 66ml, 3eq.) dropwise over 10 minutes. After this time, the mixture was left to stir for 18 hours, or until all solid had dissolved. Excess phosgene was then purged using N_2, scrubbed through concentrated ammonia. After white fumes were no longer evolved, solvent was

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removed *in vacuo* on a cold water bath to give a white solid.

Due to the reactive nature of the substance, it was used immediately without further purification.

S-Valinylglycine methyl ester (5)[6]
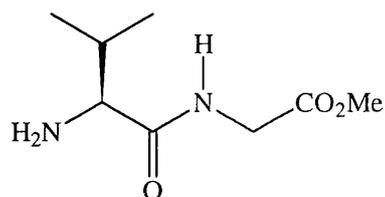
Glycine methyl ester hydrochloride (6.53g, 0.052mol) was dissolved in chloroform (64.2ml), and the solution cooled to -78°C with vigorous stirring. To this was added TEA (16.0ml, 0.114mol) in one portion, giving a white slurry. To this slurry at -78°C was added 4-(*S*)-isopropyloxazolidin-2,5-dione (0.051mol) in THF (51ml) solution dropwise over 30 minutes. After addition was complete, the mixture was stirred for 30 minutes at -78°C, then 1.5 hours at room temperature. The reaction mixture was filtered, and the solvents removed *in vacuo* to give the product as a thick oil. This was passed immediately into the next step.

6-(*S*)-Isopropylpiperazine-2,5-dione (6)[6]
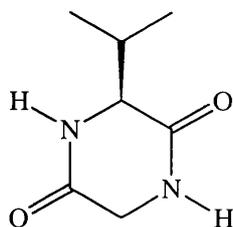
S-Valinylglycine methyl ester (0.051mol) was taken up in toluene (216ml), and the solution heated at reflux for 12 hours. After this time, a pulpy solid had formed, which was collected by filtration, and dried for 3 days at 100°C under high vacuum to yield the brownish crude product. This could be purified by recrystallisation from water.

^{1}H n.m.r.; δ=0.83, 0.94 (6H, d (J=6.8Hz), d (J=7.0Hz), CH-(C$\underline{H}_3$)$_2$); δ=2.1 (1H, d of

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septets (J=6.9, 4.0Hz), CH-C$\underline{\text{H}}$-(CH$_3$)$_2$); δ=3.52 (1H, t, (J=3.4Hz), CH-C$\underline{\text{H}}$-NH); δ=3.72 (2H, d of d (J=17.8, 3.1Hz), -NH-C$\underline{\text{H}}_2$-CO); δ=8.2 (s, -N$\underline{\text{H}}$-), 8.0 (s, -N$\underline{\text{H}}$-)

IR; ν=3190cm^{-1} (-NH-); ν=1668cm^{-1} (C=O)

Mass spec.; 157 (M+1)

N-Ethoxycarbonyl phthalimide (7)[7]
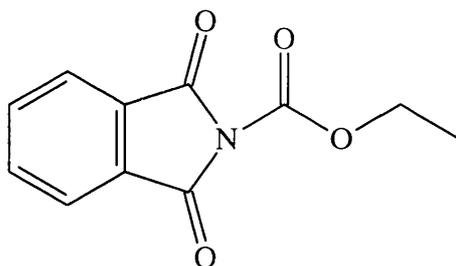
To a cooled (5°C) solution of phthalimide (1.0g, 6.80mmol) in DMF (3.5ml) and TEA (1ml, 7.19mmol, 1.05eq.) was added ethyl chloroformate (0.69ml, 16.1mmol) over 45 minutes with stirring. After this time, the reaction was allowed to warm to room temperature, and stirred for a further 1 hour. The reaction was then quenched by addition to water (21ml), evolving a thick white precipitate. The solid was collected by filtration, and dried over P$_2$O$_5$. The product was recrystallised from ethanol, after treatment with decolourising charcoal. This gave the product as white crystals, yield 0.84g, 3.83mmol, 56%.

^{1}H n.m.r. (200MHz, CDCL$_3$); δ=1.43 (3H, t (J=7.2Hz), CH$_2$-C$\underline{\text{H}}_3$); δ=4.47 (2H, q (J=7.0Hz), C$\underline{\text{H}}_2$-CH$_3$); δ=7.90 (4H, d of m, Aryl protons)

IR; ν=3200cm^{-1} (-NH-); ν=1769cm^{-1} (C=O); ν=718cm^{-1} (aryl)

Mass Spec.; 220 (M+1)

Melting point; 81-83°C (lit. 80°C)

N-Phthaloyl-*S*-serine (8)[7]
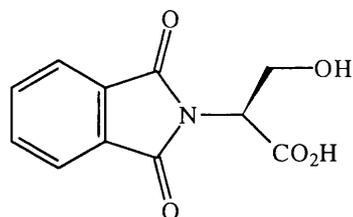
To a solution of *S*-serine (2.1g, 20.0mmol) and sodium bicarbonate (2.13g, 25.0mmol, 1.25eq.) in water (15ml) stirred at room temperature was added *N*-ethoxycarbonyl phthalimide (4.5g, 20.5mmol, 1.03eq.) in one portion. The reaction was followed by the dissolution of the solid *N*-ethoxycarbonyl phthalimide. When this was nearly complete (*ca.* 20 minutes), the reaction mixture was acidified using concentrated hydrochloric acid, and the product extracted using ethyl acetate (3x100ml).

The organic layers were dried (Na$_2$SO$_4$), then the solvent removed *in vacuo* to give the product as a viscous oil. The purification of this material proved impossible, as recrystallisation and chromatography with several different systems failed.

N-Benzyl-*N*-Benzyloxycarbonyl-*S*-serine (9)[8]
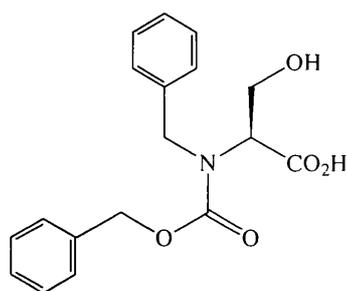
N-Benzyl-*S*-serine (2.00g, 10.0mmol) was dissolved in 2N sodium hydroxide solution (5ml) and THF (1.7ml) and cooled to 5°C. To this mixture was added benzyl chloroformate (2.3ml, 16.1mmol) over 30 minutes, the reaction temperature maintained below 10°C and pH>8. After this time, a white slurry had been generated. After 30 minutes, the mixture was acidified to pH 2 using concentrated hydrochloric acid, evolving a voluminous white precipitate. The mixture was then extracted using ethyl acetate (3x100ml), and the organic layers were dried (Na$_2$SO$_4$), followed by the

removal of solvent *in vacuo*. Purification was then performed by reverse phase chromatography (70% MeCN/H$_2$O, R$_f$=0.40), giving the product as a clear, thick oil, yield 1.15g, 3.50mmol, 35%.

B.Pt.; 100°C @ 0.3mmHg (dec.)

^{1}H n.m.r.; δ=4.10 (2H, broad d, Ph-CH$_2$-N-); δ=4.60 (2H, m, HO-CH$_2$-CH-); δ=5.16 (2H, s, Ph-CH$_2$-O); δ=6.14 (s, -OH, -CO$_2$H); δ=7.37 (10H, m, C$_6$H$_5$-,C$_6$H$_5$-)

Mass Spec.; 330 (M+1)

N-Benzyl-*N*-benzyloxycarbonyl-(*S*)-serine-β-lactone (10)[9]
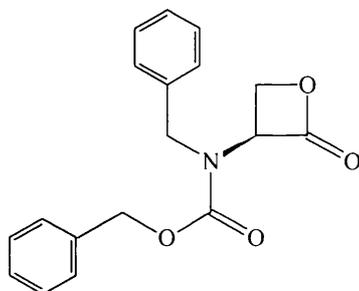
To a cooled (-78°C) solution of triphenyl phosphine (1.96g, 7.46mmol) in THF (30ml), was added diethylazodicarboxylate (1.16ml, 7.46mmol) over 15 minutes and the mixture stirred for 1 hour. To the white slurry generated was added a solution of **9** (2.46g, 7.48mmol) in THF (30ml) over 10 minutes, and the mixture stirred for 2 hours. After this time, the reaction was allowed to warm to room temperature, and the solvent removed *in vacuo* to give a thick white residue. Purification using chromatography, followed by analysis, showed none of the desired product.

6-(*S*)-Isopropyl-3,6-dihydro-2,5-diethoxypyrazine (11)[6]
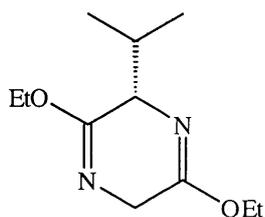
To a solution of 6-(S)-isopropylpiperazine-2,5-dione (dried *in vacuo* at 100°C for three days) (2.133g, 13.7mmol) in DCM (45ml) was added triethyloxonium tetrafluoroborate (7.81g, 41.1mmol, 3eq.) in one portion. The mixture was then stirred for 12 hours, giving a black solution. The reaction was then quenched by addition to a phosphate buffer solution (NaH_2PO_4 6.41g, 41.1mmol, Na_2HPO_4 19.5g, 137mmol, H_2O 117ml), and extraction of the aqueous layer using DCM (3x80ml). The organic layer was then dried ($MgSO_4$), and the solvent removed to give the crude product as a brown oil. Purification was performed by distillation (B.Pt. 80-85°C @ 0.1mmHg), or by flash chromatography (8% EtOAc/hexane system, R_f=0.44). The product was obtained as a light, clear oil, yield 1.43g, 6.74mmol, 49%.

^{1}H n.m.r.; δ=0.71, 0.98 (6H, d (J=7.0Hz), d (J=7.0Hz), CH-(C$\underline{H}_3$)$_2$); δ=1.23 (6H, t (J=5.4Hz), O-CH$_2$-C$\underline{H}_3$); δ=2.25 (1H, d of septets (J=3.9, 7.1Hz), CH-C$\underline{H}$-(CH$_3$)$_2$); δ=3.92 (2H, d (J=1.3Hz), N-C$\underline{H}_2$-C-); δ=4.1 (5H, complex m, C$\underline{H}$-α, O-C$\underline{H}_2$-CH$_3$)

IR; ν=1696cm-1 (C=N); ν=1239cm-1, 1034cm-1 (=C-O-C-)

Mass Spec.; 213 (M+1)

5-Bromo-1-(*tert*-butyldimethylsilyloxy) pentane (12)
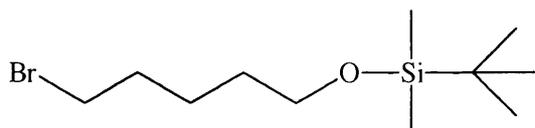
(i)[10] To a solution of triphenylphosphine (0.64g, 2.44mmol) in THF (5ml) was added NBS (0.44g, 2.47mmol) in THF (5ml), giving a pinkish-white solid adduct. To this was added 1-(*tert*-butyldimethylsilyloxy) pentan-1,5-diol (1.0g, 4.59mmol) in THF (6ml), and the reaction was stirred for 12 hours. Removal of solvents *in vacuo* gave a brownish oil that was suspended in water, followed by extraction into ether. No product was identified by tlc, n.m.r., or mass spectrometry.

(ii)[11] To a solution of 1-(*tert*-butyldimethylsilyloxy) pentan-1,5-diol (0.50g, 2.28mmol) and triphenylphosphine (0.64g, 2.44mmol) in DMF (2.5ml) was added bromine dropwise, until colour remained in solution. During the addition of bromine, the

temperature was maintained below 55°C. After the addition was complete, the reaction was quenched by the addition of water; followed by extraction with ether. After removal of solvent *in vacuo*, no product was observed by mass spectrometry.

(iii)[12] To a cooled (0°C) solution of 1-(*tert*-butyldimethylsilyloxy) pentan-1,5-diol (0.99g, 5.59mmol) and triphenylphosphine (1.34g, 5.10mmol) in DCM (6ml) was added a solution of carbon tetrabromide (2.30g, 6.92mmol) in DCM (3.5ml) in one portion. The resulting solution was stirred for 12 hours at room temperature. Removal of solvent *in vacuo* gave a brown oil, which showed no product by mass spectrometry.

(iv) To a solution of 1-(*tert*-butyldimethylsilyloxy) pentan-1,5-diol (0.50g, 2.29mmol) and carbon tetrabromide (1.33g, 4.00mmol) in ether (4ml) was added HMPT (0.42ml, 2.29mmol) in ether solution (1ml). The grey-black suspension produced was then stirred overnight, followed by quenching with methanol, and removal of solvent *in vacuo*. No product was observed by mass spectrometry.

(v)[13] To a solution of 1-(*tert*-butyldimethylsilyloxy) pentan-1,5-diol (0.50g, 2.29mmol) in chloroform (3ml) was added TEA (0.39ml) and the mixture cooled to 0°C. Thionyl bromide (0.21ml, 2.75mmol) in chloroform (1ml) was then added over 15 minutes, followed by stirring for 12 hours. The reaction was then quenched using ethanol, and water added. The aqueous layer was washed with ether (2x100ml), and the collected organic layers dried (MgSO$_4$). After removal of the solvents *in vacuo*, no product was observed by mass spectrometry.

(vi)[14] To a cooled (0°C) solution of phosphorus tribromide (0.45g, 1.65mmol) in benzene (0.25ml) and pyridine (0.07ml) was added 1-(*tert*-butyldimethylsilyloxy) pentan-1,5-diol (0.96g, 4.56mmol) dropwise. The mixture was then stirred for 12 hours, followed by quenching with water, and washing with ether (3x80ml). The organic layers were dried (MgSO$_4$), and the solvents removed *in vacuo* to give a brownish oil. No product was observed by mass spectrometry.

(vii)[15] To a solution of sodium bromide (2.77g, 26.9mmol, 10eq.) in DMF (40ml) with powdered molecular sieves (5.0g) was added 5-tosyl-1-(*tert*-butyldimethylsilyloxy)-pentan-1,5-diol (1.00g, 2.69mmol) in one portion. The reaction mixture was stirred at 80°C, until tlc indicated no starting material remained. After this time, the reaction was

quenched with water, then extracted using ether. The organic layer was dried (MgSO$_4$), and the solvents removed *in vacuo* to give a brownish oil. The product could be purified by chromatography (45% ether/hexane, R$_f$=0.45), to give a clear oil, yield 0.45g, 1.60mmol, 59%.

^{1}H n.m.r.; δ=0.05 (6H, s, Si-(CH$_3$)$_2$); δ=0.87 (9H, s, Si-C(CH$_3$)$_3$); δ=1.52, 1.85 (6H, m, Alkyl Chain); δ=3.48 (2H, t, (J=6.7Hz), CH$_2$-C<u>H</u>$_2$-O); δ=3.61 (2H, t, (J=6.6Hz), CH$_2$-C<u>H</u>$_2$-Br)

1-Tosyl-5-(*tert*-butyldimethylsilyloxy) pentane (13)
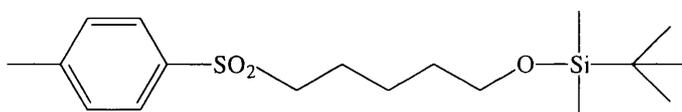
To a solution of tosyl chloride (1.15g, 6.00mmol) in pyridine (10ml) was added 1-(*tert*-butyldimethylsilyloxy) pentan-1,5-diol (1.0g, 4.59mmol) dropwise. The resulting mixture was stirred for 12 hours, then added to ether (100ml). This mixture was then washed with 10% potassium carbonate, and the organic layer dried (MgSO$_4$). Removal of the solvent *in vacuo* gave a low melting solid, which could be recrystallised from DCM and ether or hexane, on cooling (-23°C). This could also be purified by chromatography (30% EtOAc/hexane, R$_f$=0.72).

^{1}H n.m.r.; δ=-0.03 (6H, s, Si-(C<u>H</u>$_3$)$_2$); δ=0.82 (9H, s, Si-C(C<u>H</u>$_3$)$_3$); δ=1.35, 1.6 (6H, br. m, Alkyl Chain); δ=2.40 (3H, s, Ts-C<u>H</u>$_3$); δ=3.50 (2H, t, (J=6.0Hz), O-C<u>H</u>$_2$-CH$_2$); δ=3.96 (2H, t, (J=6.0Hz), CH$_2$-C<u>H</u>$_2$-O); δ=7.30 (2H, d, (J=8.5Hz), Aryl); δ=7.72 (2H, d, (J=8.4Hz), Aryl)

^{13}C n.m.r.; δ=70.3; δ=62.4; δ=31.7; δ=28.3; δ=25.6; δ=21.5; δ=21.3; δ=18.0; δ=-5.0

IR; ν=1423cm^{-1} 1217cm^{-1} (-SO$_3$-); ν=1011cm^{-1} (Si-O); ν=836 (Si-(CH$_3$)$_2$)

Mass Spec.; 373 (M+1); 315 (M-*tert* butyl); 201 (Tol-SO$_3$)

## 1,5-(Ditosyl) pentandiol (14)

TsO⌒⌒⌒⌒OTs
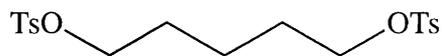
To a solution of tosyl chloride (3.82g, 20.0mmol) in pyridine (20ml) was added pentan-1,5-diol, and the mixture was stirred for 12 hours at room temperature. The reaction was then added to ether, followed by washing with 10% potassium carbonate. The organic layer was collected and dried ($MgSO_4$), and removal of solvents *in vacuo* gave the product as a yellowish solid.

As this route was subsequently abandoned, this material was not purified further.

^{1}H n.m.r.; δ=1.3, 1.5 (6H, br.m, Alkyl chain); δ=2.29 (6H, s, Ts-C$\underline{H}_3$); δ=3.35 (4H, t, (J=6.5Hz), O-C$\underline{H}_2$-CH$_2$); δ=7.10 (4H, d, (J=7.8Hz), Aryl protons), d=7.69 (4H, d, (J=7.9Hz), Aryl protons)

(*S*)-Serine benzyl ester (15)
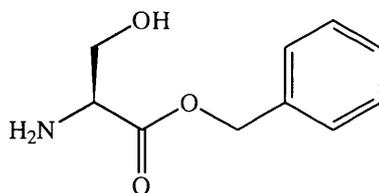
(i) Benzyl alcohol (2.0ml) was treated with trimethylsilyl chloride (0.23ml, 2.45mmol) and stirred for 30 minutes. This was then added to a slurry of *S*-serine (0.50g, 4.76mmol) in THF (1.0ml) with stirring at room temperature. The reaction was then stirred for 12 hours, after which time it was poured into ether, and washed with sat. sodium bicarbonate. Analysis by tlc showed no ninhydrin, u.v. active spot, indicating the reaction was unsuccessful.

(ii) To a solution of DCC (1.01g, 4.90mmol) and DMAP (0.06g, 0.48mmol) in benzyl alcohol (2.0ml) was added *S*-serine (0.50g, 4.76mmol), and the resulting suspension was stirred overnight at room temperature. The reaction mixture was added to ether, followed by washing with saturated sodium bicarbonate. The organic layer was dried and the solvents removed. On analysis, no product was found.

STABASE-*S*-serine (16)
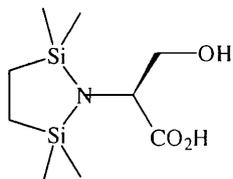
To a stirred suspension of *S*-serine (0.84g, 8.0mmol) in DCM (5ml)and TEA (2.23ml, 16.0mmol) was added *bis*-(dimethychlorosilyl) ethane (1.72g, 8.0mmol) in DCM (3ml), forming a thick white precipitate. This reaction was stirred for 1 hour, then the solids were removed by filtration. The title compound was obtained as a brownish oil, which proved impossible to purify or characterise.

Dimethoxydicarbonylhydrazine (17)[16]
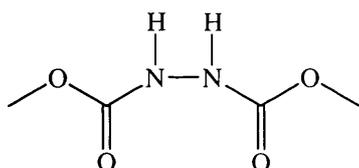
A solution of hydrazine hydrate (5.00g, 0.10mol) in ethanol (25ml) was cooled to 0°C, and methyl chloroformate (18.9g, 0.20mol) was added dropwise, the temperature being maintained below 20°C. After addition of approximately half of the methyl chloroformate, a solution of sodium carbonate (10.6g, 0.13mol) in water (40ml) was added dropwise along with the remainder of the chloroformate. The reaction was stirred for 1 hour, then the precipitate produced was removed by filtration, followed by removal of the organics from the filtrate, and subsequent cooling on an ice bath. Additional precipitate was collected, and added to the first crop, followed by extraction of this residue using warm (50°C) acetone. After this process, any undissolved solid was removed by filtration, then the solvent removed from the filtrate to give a white solid. Yield 11.8g, 0.08mol, 80%

[1]H n.m.r.; δ=4.12 (s) -C$\underline{H}_3$; δ=5.36 (s) -N$\underline{H}$-

Dimethylazodicarboxylate (18)
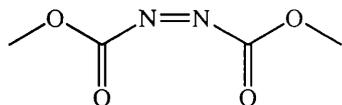
Bis-(methoxycarbonyl)hydrazine (2.00g, 13.5mmol) was suspended in DCM (45ml) and pyridine (0.98g, 12.2mmol) in a separating funnel. NBS (2.18g, 12.2mmol) was added in one portion, and the mixture gently shaken, resulting in an orange solution. The mixture was then left to stand for 45 minutes, with occasional shaking.

After this time, the reaction mixture was washed with water, and the organic layer was dried (Na_2SO_4). Solvents were then removed *in vacuo* to give a deep orange oil, yield 1.46g, 10.0mmol, 74%.

(*S*)-Valinol (19)
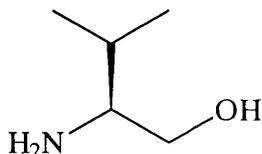
(i)[16] To a cooled (0°C) suspension of *S*-valine (1.0g, 8.54mmol) in THF (8.5ml) was added lithium aluminium hydride (0.32g, 8.54mmol) as a slurry in THF (8.5ml). After the initial evolution of gas was complete, the mixture was heated to reflux for 1.5 hours. The reaction was then worked up by quenching with methanol, followed by addition of 2N sodium hydroxide solution (25ml). The aqueous mixture was then extracted using EtOAc (3x50ml), and the organic layers dried (Na_2SO_4). Removal of the solvents yielded a small quantity of yellowish oil.

(ii)[17] To a stirred suspension of *S*-valine (10.0g, 85.5mmol) and sodium borohydride (7.48g, 214mmol) in THF (224ml) was added a solution of iodine (21.7g,85.5mmol) in THF (56ml) dropwise with cooling (ice bath). After addition was complete and effervescence had ceased, the resulting white solution was heated at reflux for 12 hours.

On completion, the reaction was allowed to cool to room temperature, and was then

quenched by the addition of methanol, dropwise. When the vigorous reaction had subsided, the solvent was removed *in vacuo* to give a white slurry. This was then treated with 20% potassium hydroxide, and the product extracted using ethyl acetate. This product was not purified further, and was used directly in the next step. The product was obtained as a clear oil, yield 6.07g, 58.9mmol, 69%.

^{1}H n.m.r.; δ=0.76 (6H, d, (J=8Hz), -CH-(C$\underline{H}_3$)$_2$); δ=1.44 (1H, m, (J=6Hz) -CH-C$\underline{H}$-(CH$_3$)$_2$); δ=2.54 (br. s, -N$\underline{H}_2$,-O$\underline{H}$); δ=2.42 (1H, m, NH$_2$-C$\underline{H}$(CH)-CH$_2$-); δ=3.16 (1H, d of d, (J=8,10Hz) -CH-C$\underline{H}^a$H^b-OH); δ=3.44 (1H, d of d, (J=4, 12Hz) -CH-CH$^a\underline{H}^b$-OH)

Mass Spec.(E.I.); 102 (M-1); 72 (M-31, -CH$_2$-OH)

4-(S)-Isopropyloxazolidin-2-one (20)[18]
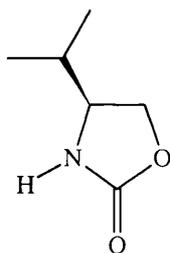
S-Valinol (1.0g, 9.71mmol) was dissolved in toluene (18.9ml) and to this was added 12.5% potassium hydroxide solution (25.2ml). The biphasic mixture was then cooled (0°C), on an ice bath, and phosgene in toluene solution (15.6ml, 30.2mmol, 3eq.) was added dropwise with vigorous stirring over 15 minutes.

On completion, the mixture was stirred for a further 15 minutes at 0°C, then the layers separated. The organic layer was washed once with concentrated ammonia solution, then dried (MgSO$_4$). On removal of solvent *in vacuo*, a white solid was obtained, which was recrystallised from ethyl acetate/hexane to give the title compound. Further purification could be performed by chromatography (40% EtOAc/hexane, R$_f$=0.20). The product was obtained as white crystals, yield 0.87g, 6.74mmol, 69.4%.

M.Pt.; 68-70°C (lit. 70-72°C)

^{1}H n.m.r.; δ=0.86, 0.96 (6H, d of d, (J=6.7, 13.2Hz), -CH-(C$\underline{H}_3$)$_2$); δ=1.75 (1H,d of

152

sept., (J=6Hz), -CH-C<u>H</u>-(CH₃)₂); δ=3.58 (1H, q, (J=8Hz), -NH-C<u>H</u>(CH)-CH₂);

δ=4.80 (1H, d of d, (J=6.3, 8.6Hz) -CH-CH^a<u>H</u>^b-O-); δ=4.42 (1H, t, (J=8.3, 8.8Hz) - CH-C<u>H</u>^aH^b-O-); δ=6.70 (br. s, -N<u>H</u>-)

Mass Spec.; 130 (M+1)

## *4-(S)-Isopropyloxazolidin-2-one*-N-*(8'-bromooctanoate)* (21)
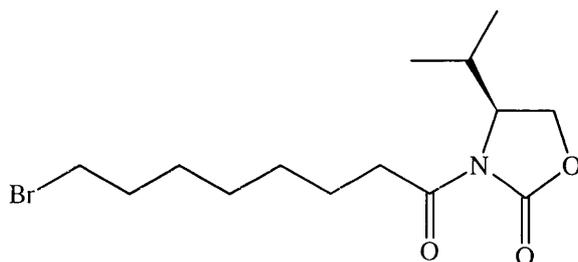
To a solution of 8-bromooctanoic acid (0.40g, 1.79mmol) in THF (33.5ml) at -78°C was added TEA (0.37ml, 2.68mmol, 1.5eq.) and then isopropyl chloroformate (2.15ml, 1M solution in toluene, 2.15mmol, 1.2eq.) dropwise. After stirring for 15 minutes, the mixture was warmed to room temperature for 45 minutes, then recooled to -78°C.

To this was then added by cannula a suspension of **20** (0.46g, 3.58mmol, 2eq.) in THF, pretreated with n-BuLi (1.36ml, 2.5N solution in hexane, 3.40mmol, 0.95eq.) for 15 minutes, at -78°C. After this addition was complete, the mixture was then stirred for 30 minutes at -78°C, then allowed to warm to room temperature. The reaction was then quenched by addition to 1N sodium hydrogen sulphate (150ml), followed by removal of organic solvents *in vacuo*. Extraction of the aqueous layer was performed using ethyl acetate (3x100ml), and the combined ethyl acetate washes were dried (Na₂SO₄) The solvent was removed *in vacuo* to give the product as a clear oil, which was purified by chromatography (30% EtOAc/hexane, R_f=0.50); yield 0.50g, 1.49mmol, 84%.

¹H n.m.r.; δ=0.9 (6H, d, d, (J=7.4, 8.6Hz), CH-(C<u>H</u>₃)₂); δ=1.3 (br.s), 1.6 (br.t), 1.8 (br.t), (Alkyl Chain); δ=2.3 (1H, d of sept., (J=4Hz), CH-C<u>H</u>-(CH₃)₂); δ=2.9 (2H, m,

CH$_2$-C$\underline{H}_2$-CO-); δ=3.4 (2H, t, (J=6.7Hz) Br-C$\underline{H}_2$-CH$_2$-); δ=4.17 (d of d) -CH-CH^a$\underline{H}^b$-O; δ=4.23 (t) -CH-C$\underline{H}^a$H^b-O; δ=4.42 (d of t) NH-C$\underline{H}$(CH)-CH$_2$

^{13}C n.m.r.; δc=173 2; δc=154; δ=63.3; δ=58.3; δ=35.4; δ=33.9; δ=32.7; δ=28.8; δ=28.4; δ=28.3; δ=27.9; δ=24.3; δ=17.9; δ=14.6

IR; ν=1779cm^{-1} (C=O); ν=1702cm^{-1} (C=O); ν=914cm^{-1} (C=O)

Mass Spec.; 334, 336 (M+1)$^+$; 130; 55

tert-Butyl 8-bromooctanoate (22)
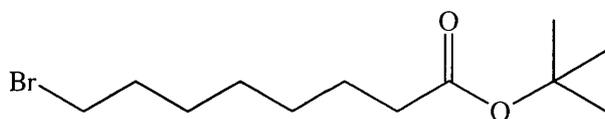
(i)[4] 8-Bromooctanoic acid (0.50g, 2.24mmol) was dissolved in *tert*-butyl alcohol, and the resulting solution was cooled to 0°C (ice bath). To this was added freshly distilled thionyl chloride (0.33ml, 4.48mmol) dropwise with stirring over 15 minutes. The reaction was then stirred overnight, then added to DCM (25ml). Saturated sodium bicarbonate was added until no further gas was evolved. After separation of the layers, the aqueous layer was then washed with DCM (2x25ml), and the collected organics were dried (MgSO$_4$). On removal of solvent in vacuo, the crude product was purified by chromatography (20% ether/hexane, R$_f$=0.24) to give a clear oil, yield (typical) 20-30%.

(ii) 8-Bromooctanoic acid (1.0g, 4.48mmol) was dissolved in thionyl chloride (2.0ml) and the resulting clear solution was stirred for 1 hour, followed by removal of the excess thionyl chloride. The liquid residue was then suspended in THF (30ml) and to this mixture was added a slurry of potassium *tert*-butoxide (0.50g, 4.50mmol) in THF (30ml) over 30 minutes with cooling (ice bath).

The product was isolated by removal of the solvent, and purified by chromatography (20% ether/hexane, R$_f$=0.24) yield 0.36g, 33%

(iii)[19] 8-Bromooctanoic acid (0.20g, 8.97mmol) was dissolved in *tert*-butyl acetate (13.4ml), and to this was added concentrated sulphuric acid (0.09ml, 1.1eq.), in one

portion. The resulting mixture was then stirred for 48 hours, then poured into ethyl acetate (50ml) and washed with water (20ml) and saturated sodium bicarbonate (20ml). The organic layer was then dried (MgSO₄), and the solvent removed to give the product as a clear oil. Purification was by column chromatography (20% ether/hexane, R$_f$=0.24), yield 53%

^{1}H n.m.r.; δ=1.2 (6H, br.s, Alkyl Chain); δ=1.4 (9H, s, C-(C$\underline{H}_3$)₃); δ=1.5, 1.7 (4H, br.m, Alkyl Chain); δ=2.18 (2H, t, (J=8.0Hz) -CH₂-C$\underline{H}_2$-CO); δ=3.45 (2H, t, (J=8.0Hz) Br-C$\underline{H}_2$-CH₂

IR; ν=2934cm^{-1} (C-H); ν=1717cm^{-1} (C=O); ν=750cm^{-1} (C-Br)

Mass Spec.(FAB); 278, 280 (M$^+$)

5-(S)-Isopropyl-2,5-dihydro-3,6-diethoxypyrazine-2-(4-(S)-isopropyloxazolidin-2-one-3-(N)-) octanoate (23)
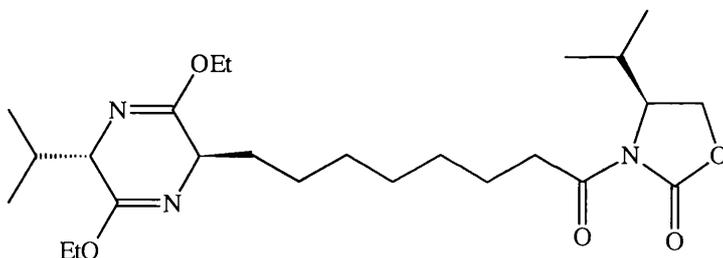
(i) To a cooled (-78°C) solution of 6-(S)-isopropyl-2,5-diethoxy-3,6-dihydropyrazine (0.20g, 0.94mmol) in THF (2.3ml) was added n-BuLi (0.38ml, 0.94mmol) in one portion, and stirring continued for 10 minutes. After this time, a solution of 4-(S)-isopropyloxazolidin-2-one-N-(8-bromooctanoate) (0.31g, 0.94mmol) in THF was added *via* syringe dropwise, with the temperature maintained at -78°C. Stirring was continued for a further 5 hours at this temperature, then the reaction was worked up by removal of solvent, followed by chromatography (40% EtOAc/hexane). No product was isolated.

(ii) To a cooled (-78°C) solution of 5-(S)-isopropyl-2,5-dihydro-3,6-diethoxypyrazine-2-(R)-octanoic-10-acid (0.626g, 1.77mmol) in THF (31ml)/TEA (0.37ml, 2.65mmol)

was added isopropyl chloroformate (2.12ml, 1N solution) dropwise. The resulting solution was stirred at -78°C for 30 minutes, then warmed to room temperature for 1 hour before being recooled to -78°C.

To this was then added a solution of 4-(S)-isopropyloxazolidin-2-one (0.250g, 1.94mmol, 1.1eq.) in THF (10ml), pretreated with n-BuLi (0.78ml, 2.5N solution, 1.94mmol), at -78°C *via* a cannula. Stirring was continued at this temperature for 30 minutes, and the reaction was then quenched by addition to a 1% sodium bicarbonate solution. After removal of the THF solvent *in vacuo,* the product was extracted using ethyl acetate (3x100ml).

After drying (Na$_2$SO$_4$), removal of solvent gave the crude oily product, which was purified by column chromatography (20% EtOAc/hexane, R$_f$=0.46), 0.763g, 93% yield.

^{1}H n.m.r.; δ=0.66 (d, (J=6.8Hz), 3H, CH-C$\underline{H}$$_3$); δ=1.0 (d, (J=6.9Hz), 3H, CH-C$\underline{H}$$_3$); δ=0.83 (d, (J=7.0Hz), 3H, CH-C$\underline{H}$$_3$); δ=0.88 (d, (J=6.9Hz), 3H, CH-C$\underline{H}$$_3$); δ=1.24 (t, (J=7.1Hz), 6H, O-CH$_2$-C$\underline{H}$$_3$); δ=1.25 (br.s, 6H, Alkyl Chain); δ=1.6, 1.7 (br.m, 4H, Alkyl Chain); δ=2.24 (d of sept, (J=3.2Hz), 1H, CH-C$\underline{H}$-(CH$_3$)$_2$); δ=2.34 (d of sept, (J=3.9Hz), 1H, CH-C$\underline{H}$-(CH$_3$)$_2$); δ=2.8, 2.9 (d of d of d, (J=8.0, 14.0, 24.0Hz), 2H, -CH$_2$-CO); δ=3.8 (t, (J=3.4Hz), 1H, Val-α$\underline{H}$); δ=3.95 (d of d, (J=4.3, 10.0Hz) C$\underline{H}$-CH$_2$-); δ=4.0-4.3 (Complex m., 6H, O-C$\underline{H}$$_2$-CH$_3$, -CH-C$\underline{H}$$_2$-O; δ=4.4 (d of t, (J=3.5, 8.4Hz), 1H, CH-C$\underline{H}$-CH$_2$)

^{13}C n.m.r.; δ=173; δ=163; δ=162; δ=154; δ=63.3; δ=60.6; δ=60.4; δ=60.3; δ=60.3; δ=58.3; δ=55.4; δ=35.5; δ=34.1; δ=31.7; δ=29.4; δ=29.3; δ=29.0; δ=28.3; δ=24.4; δ=19.1; δ=17.9; δ=16.6; δ=14.6; δ=14.4; δ=14.3

Mass Spec.; 466 (M+1)$^+$; 422; 337; 211; 169

Accurate Mass; C$_{25}$H$_{44}$N$_3$O$_5$ calculated 446.3290, found 446.3281

Optical Rotation; [α]$_D$= +35.3 (2.721mgml^{-1}, CHCl$_3$)

# Triisopropylbenzenesulphonyl azide (24)[22]
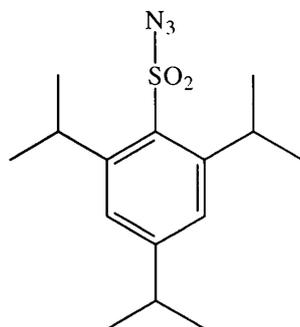
To a cooled (0°C) solution of sodium azide (1.29g, 19.9mmol) in 30% water/acetone (20ml) was added triisopropylbenzenesulphonyl chloride (2.00g, 6.65mmol) in acetone solution (6.0ml) in portions over 30 minutes. After completion of the addition, the mixture was stirred for 1 hour and gradually warmed to room temperature. The reaction was then added to ice water (20ml), giving a thick white precipitate. The slurry was extracted using ether, and the collected organic layers were dried (Na_2SO_4). Removal of the solvent *in vacuo* gave a white solid, which could be purified by chromatography (5% ether/hexane, R_f=0.12) or recrystallisation (ether/hexane, cooled to -23°C).

^{1}H n.m.r.; δ=1.3 (18H, d,d, (J=4.0, 8.0Hz) CH-(C$\underline{H}_3$)$_2$); δ=2.9 (1H, sept., (J=8.0Hz) *p*-C$\underline{H}$-(CH$_3$)$_2$); δ=4.0 (1H, sept., (J=8.0Hz) *o*-C$\underline{H}$-(CH$_3$)$_2$); δ=4.2 (1H, sept., (J=8.0Hz) *o*-C$\underline{H}$-(CH$_3$)$_2$; δ=7.2 (s,s) (Aryl, 2H)

IR; ν=2125cm^{-1} (-N$_3$); ν=1217cm^{-1} (-SO$_3$-)

Mass Spec.; 308 (M-1), 267 (M-42)

8-Bromooctan-1-ol (25)[18]
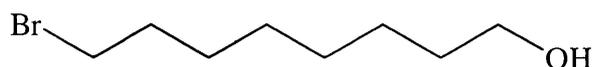
To a stirred suspension of lithium aluminium hydride (0.053g, 1.34mmol) in ether (1.2ml) was added 8-bromoctanoic acid (0.50g, 2.24mmol) in ether (0.3ml) dropwise. After this process was complete (*ca.* 30 minutes), the reaction was quenched using

water, followed by addition of 10% sulphuric acid. The aqueous layer was then washed using ether (50ml), and the organics dried ($MgSO_4$). The solvent was then removed *in vacuo* to give the title compound as an oil, 0.388g, 83% yield.

**8-Bromooctan-1-ol (1-*tert*-butyldimethylsilyl ether) (26)[20]**

Br⟍⟋⟍⟋⟍⟋⟍⟋⟍⟋⟍⟍O—TBDMS
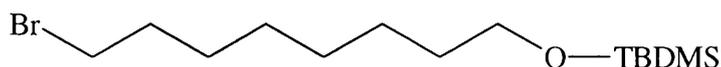
Imidazole (0.25g, 3.74mmol, 2eq.) and 8-bromooctan-1-ol (0.39g, 1.87mmol) were suspended in DMF (1.4ml) and to this solution was added *tert*-butyldimethylchlorosilane (0.43g, 2.80mmol). The resulting solution was stirred at room temperature overnight.

After this time, the solvent was removed *in vacuo,* and the residue was suspended in water. The aqueous layer was then washed with ether, and the organic layers dried ($MgSO_4$). Removal of the solvent gave the crude product as a clear oil. Purification was performed by chromatography (20% EtOAc/hexane, R_f=0.9), yield 0.27g, 0.84mmol, 45%.

1H n.m.r.; δ=0.02 (9H, s, -Si-(CH_3)$_2$); δ=0.87 (6H, s, -Si-C(CH_3)$_3$); δ=1.28 (8H, br.s, Alkyl Chain); δ=1.45 (2H, br.m, Alkyl Chain), 1.83 (2H, br.m, Alkyl Chain); δ=3.38 (2H, t, (J=7.9Hz), -CH_2-O); δ=3.58 (2H, t, (J=7.9Hz), -CH_2-Br)

*(6-(S)-Isopropyl-2,5-dihydro-3,6-diethoxypyrazine-3-(R))-octyl-1-*tert-*butyldimethylsilyl ether (27)*
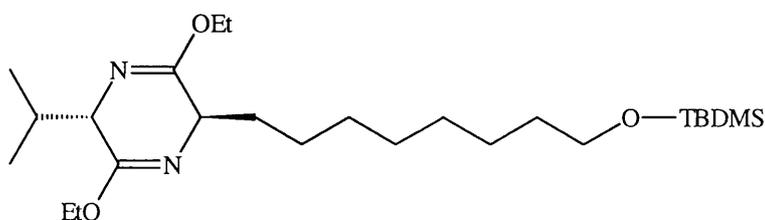
To a cooled (-78°C) solution of 6-(S)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine (0.065g, 0.309mmol) in THF (0.85ml) was added n-BuLi (0.12ml, 2.5N solution,

1eq.), and stirring continued for 10 minutes. After this time, 1-(*tert*-butyldimethylsilyloxy) 8-bromooctane (0.10g, 0.308mmol) in THF (0.74ml) was added by syringe, and stirring was continued for 5 hours at -78°C. After this time, the solvents were removed *in vacuo*, and the residue suspended in ether (40ml). The ether layer was then washed with water (20ml), and dried (Na$_2$SO$_4$). Removal of the solvent in vacuo gave an oil, which was found after chromatography (10% EtOAc/hexane) to contain only recovered starting materials.

tert-*Butyl (6-(S)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine-3-(R)) octanoate* (28)
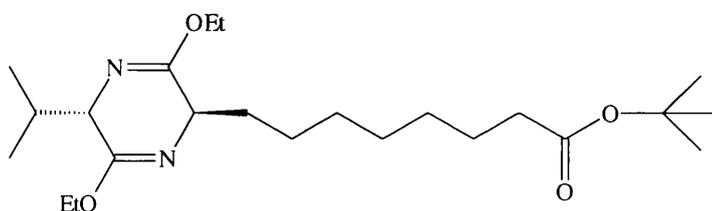
(i) To a cooled (-78°C) solution of 6-(*S*)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine (0.10g, 047mmol) in THF (1.1ml) was added n-BuLi (0.19ml, 2.5N solution), and stirring continued for 10 minutes. After this time, a solution of *tert*-butyl 8-bromooctanoate (0.13g, 0.47mmol) in THF (1.0ml) was added *via* syringe dropwise, with the temperature maintained at -78°C. Stirring was continued for 5 hours, then the solvent was removed *in vacuo* followed by chromatography (20% EtOAc/hexane, R$_f$=0.46), 0.01g, 6%.

(ii) To a cooled (-78°C) solution of 6-(*S*)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine (0.076g, 0.36mmol) in THF (1.0ml) was added n-BuLi (0.19ml, 2.5N solution), and stirring continued for 10 minutes. After this time, a solution of *tert*-butyl 8-iodooctanoate (0.095g, 0.29mmol) in THF (1.0ml) was added *via* syringe dropwise, with the temperature maintained at -78°C. Stirring was continued for 5 hours, then the solvent was removed *in vacuo*, followed by chromatography (10% EtOAc/hexane, R$_f$=0.36), yield 15%.

(iii) To a cooled (-78°C) solution of 6-(*S*)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine (0.050g, 0.24mmol) in THF (0.60ml) was added n-BuLi (0.13ml, 1.87N solution) and

stirring continued for 10 minutes. After this, a solution of *tert*-butyl 8-iodooctanoate (0.15g, 0.47mmol) in DMPU (0.20ml) and THF (0.50ml) was added *via* syringe, and the mixture stirred for 5 hours. The solvents were then removed *in vacuo* and the residue suspended in ether, followed by washing with 2N HCl. The organic layer was then dried ($MgSO_4$), and the solvent removed *in vacuo*, and purification performed by chromatography (20% ether/hexane, $R_f=0.62$), yield 0.047g, 0.11mmol, 48%.

(iv) To a cooled (-78°C) solution of 6-(*S*)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine (1.0g, 4.72mmol) in THF (8ml) was added n-BuLi (1.9ml, 2.5N solution) and stirring continued for 10 minutes. After this time, the lithiated pyrazine was added *via* a teflon cannula to a cooled (0°C) slurry of copper (I) cyanide (0.21g, 2.36mmol) in THF (8ml). Stirring was continued until all solid had dissolved. The reaction was then recooled to -78°C, and a precooled solution of *tert*-butyl 8-iodooctanoate (1.15g, 3.54mmol) in THF (8ml) was added by cannula. This mixture was then warmed to -23°C, and stirring was continued for 5 hours, followed by standing at this temperature overnight.

The reaction was then quenched using a concentrated ammonia/saturated ammonium chloride solution (43ml, 1:9 ammonia:ammonium chloride), followed by the addition of ether (300ml). After removal of the aqueous layer, the organics were washed with ammonia/ammonium chloride buffer solution (3x216ml), and dried (Na_2SO_4). Removal of solvents *in vacuo* gave the title product as a clear oil, purified by chromatography (5% EtOAc/hexane, $R_f=0.18$). Yield 0.90g, 2.19mmol, 93% (typically 90-95%).

^{1}H n.m.r.; $\delta=0.68$, 1.02 (6H, d, d, (J=6.8, 6.8Hz), CH-(CH$_3$)$_2$); $\delta=1.25$ (6H, t, (J=7.1 Hz), CH$_2$-CH$_3$); $\delta=1.27$ (6H, br. s, Alkyl Chain); $\delta=1.42$ (9H, s, -C(CH$_3$)$_3$); $\delta=1.53$ (2H, br.m, Alkyl Chain), 1.70 (2H, br.m, Alkyl Chain); $\delta=2.17$ (2H, t, (J=8Hz), -CH$_2$-CO); $\delta=2.18$ (1H, d of sept, (J=4Hz), -CH-(CH$_3$)$_2$); $\delta=3.84$ (1H, t, (J=1.1Hz), val α-H); $\delta=3.94$ (1H, t, (J=4Hz), α-H); $\delta=4.08$ (4H, m, O-CH$_2$-CH$_3$)

Mass Spec.; 411 (M+1); 353 (M-57, *tert*-butyl); 309 (M-44(101), CO_2)

4-(S)-Isopropyl-oxazolidin-2-one-3-(N)-8-Bromo-2-(S)-azido-octanoate **(29)**
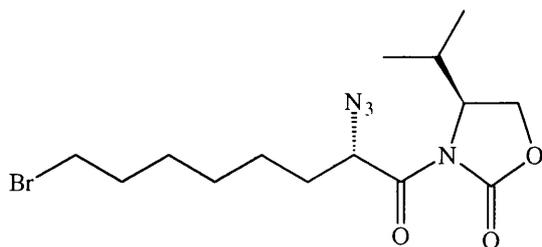

To a cooled solution of 4-(*S*)-isopropyl-oxazolidin-2-one-*N*-(8-bromooctanoate) (0.30g, 0.90mmol) in THF (3.0ml) was added potassium HMDS solution (4.9ml, 0.2N solution) dropwise over 5 minutes. The mixture was then stirred for 30 minutes, and a precooled solution of trisyl azide (0.33g, 1.08mmol) in THF (3.6ml) was added *via* cannula. The mixture was stirred for 1-2 minutes, then quenched by the addition of of glacial acetic acid (0.26ml, 4.5eq.), followed by warming to room temperature. Stirring was continued overnight, and the product was then collected by removal of solvent and chromatography (35% EtOAc/hexane, R_f=0.15), yield 0.28g, 0.75mmol, 83%.

^{1}H n.m.r.; δ=0.89 (6H, d,d, (J=6Hz, 8Hz) -CH-(C$\underline{H}_3$)$_2$; δ=1.39 (6H, br.m, Alkyl Chain); δ=1.81 (2H, br.t, Alkyl Chain); δ=2.38 (1H, d of sept., (J=4.0Hz), CH-C$\underline{H}$-(CH$_3$)$_2$); δ=3.38 (2H, t, (J=6.0Hz), Br-C$\underline{H}_2$-); δ=4.07 (1H, q, (J=8.0Hz), -CH-C$\underline{H}^a$H^b-); δ=4.24 (1H, d of d, (J=8.0,12.0Hz) -CH-CH$^a\underline{H}^b$-); δ=4.39 (m) -CH-C$\underline{H}$-CH^{a}H^b-; δ=4.92 (1H, q, (J=6.0, 8.0Hz, -C$\underline{H}$-N$_3$)

IR; ν=1779cm^{-1} (C=O); ν=1702cm^{-1} (O-(C=O)-N); ν=2114cm^{-1} (-N$_3$)

Mass Spec.; 334 (M-42, N$_3$)

## *tert*-Butyl 8-iodooctanoate (30)[21]
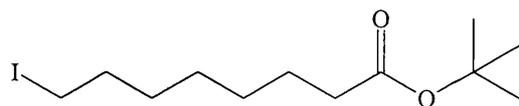
To a solution of *tert*-butyl 8-bromooctanoate (0.10g, 0.36mmol) in acetone (1.0ml) was added sodium iodide (0.16g, 1.07mmol) and the mixture stirred for 2 hours at room temperature. After this time, a white precipitate had been generated. The solvent

was removed and residue taken up in water (20ml), followed by washing with ether (30 ml). The organic layer was collected and dried (MgSO₄), and removal of the solvent gave the product as a clear oil. Purification was achieved by either chromatography (10% EtOAc/hexane) or distillation (130-135°C @ 0.1mmHg), yield 82%.

¹H n.m.r.; δ=1.32 (6H, br.s, Alkyl Chain); δ=1.43 (9H, s, -C(C$\underline{H}$₃)₃); δ=1.59 (2H, br.m, Alkyl chain), 1.75 (2H, br.m, Alkyl Chain); δ=2.18 (2H, t, (J=7.0Hz), -C$\underline{H}$₂-CO-); δ=3.51 (2H, t, (J=7.0Hz), -C$\underline{H}$₂-I)

Mass Spec.; 327 (M+1)

4-(S)-Isopropyloxazolidin-2-one-3-(N)-8-iodo-2-(S)-azido-octanoate (31)
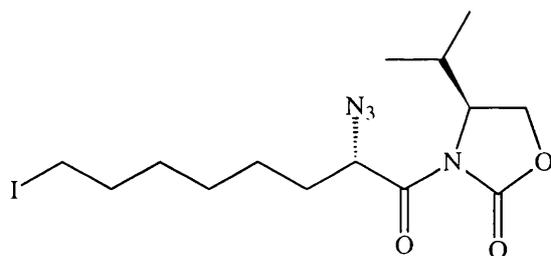
To a solution of 4-(*S*)-isopropyloxazolidin-2-one-3-(*N*)-8-bromo-2-(*S*)-azido-octanoate (1.45g, 3.87mmol) in acetone (10.0ml) was added sodium iodide (1.74g, 11.6mmol, 3eq.), and the mixture stirred for 2 hours. After this time, a white precipitate had been generated. The solvent was removed and residue taken up in water (50ml) and ether (150ml). The organic layer was collected and dried (Na₂SO₄). Removal of the solvent *in vacuo* gave the product as a clear oil. Purification was performed by chromatography (25% EtOAc/hexane, R$_f$=0.63) yield 1.31g, 3.10mmol, 80%.

¹H n.m.r.; δ=0.6, 0.8 (6H, d,d, (J=6.9,6.9Hz), CH-(C$\underline{H}$₃)₂); δ=1.33 (6H, br.s, Alkyl chain); δ=1.6 (2H, br.m, Alkyl chain); δ=1.75 (2H, br.t, Alkyl chain); δ=2.35 (1H, d of sept., (J=3.9Hz), CH-C$\underline{H}$-(CH₃)₂); δ=3.15 (2H, t, (J=6.9Hz), I-C$\underline{H}$₂-CH₂-); δ=4.2 (3H, complex m., -C$\underline{H}$-C$\underline{H}$ᵃHᵇ-); δ=4.96 (1H, q, (J=4.7,9.1Hz), -CH₂-C$\underline{H}$-N₃)

IR; ν=2113cm⁻¹ (-N₃); ν=1780cm⁻¹, 1701cm⁻¹ (C=O)

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Mass Spec.; 382 (M-40?)

8-(R)-(6-(S)-Isopropyl-2,5-diethoxy-3,6-dihydropyrazine)-2-(S)-azidooctanoyl-3'-(N)-(4'-(S)-isopropyloxazolidin-2'-one) (32)
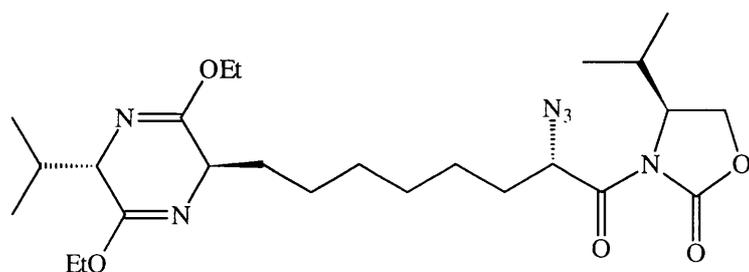
(i) To a cooled (-78°C) solution of 6-(S)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine (0.023g, 0.11mmol) in THF (0.27ml) was added n-BuLi (0.044ml, 2.5N solution), and stirring continued for 10 minutes. After this time, a solution of 4-(S)-isopropyl-oxazolidin-2-one-3-(N)-8-bromo-2-(S)-azido-octanoate (0.041g, 0.11mmol) in THF (0.23ml) was added via syringe dropwise, with the temperature maintained at -78°C. Stirring was continued for a further 5 hours at this temperature, then the solvent was removed, followed by chromatography (10% EtOAc/hexane, R$_f$=0.26), isolated yield 0.012g, 0.024mmol, 22%.

(ii) To a cooled (-78°C) solution of 6-(S)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine (0.050g, 0.24mmol) in THF (0.60ml) was added n-BuLi (0.10ml, 2.5N solution, and stirring continued for 10 minutes. After this time, a solution of 4-(S)-isopropyl-oxazolidin-2-one-3(N)-8-iodo-2(S)-azido octanoate (0.10g, 0.24mmol) in DMPU (0.25ml) and THF (0.25ml) was added via syringe, and the reaction then stirred for 5 hours. After removal of the solvent, the residue was suspended in ether, followed by washing with 2N HCl. After drying (MgSO₄) and removal of solvent, purification was attempted by chromatography (30% EtOAc/hexane), but no product was isolated.

(iii) To a solution of 6-(S)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine (0.050g, 0.24mmol) in THF (0.60ml) was added n-BuLi (0.12ml, 1.9N solution) in one portion. After deprotonation, a solution of 4-(S)-isopropyloxazolidin-2-one-3-(N)-8-iodo-2-(S)-azido-octanoate (0.10g, 0.24mmol) in HMPA (0.085ml) and THF (0.6ml) was added

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via syringe, and the reaction then stirred for 5 hours at -78°C. Removal of THFwas followed by chromatography (30% EtOAc/ hexane), but no product was found. No starting material was recovered.

(iv) To a cooled (-78°C) solution of 5-(S)-isopropyl-2,5-dihydro-3,6-diethoxypyrazine-2-(4-(S)-Isopropyloxazolidin-2-one-3-(N)-)-octanoate (1.0g, 2.15mmol) in THF (7ml) was added KHMDS solution (11.8ml, 2.37mmol, 1.1eq.), and stirring continued for 30 minutes. After this time, a cooled solution of trisyl azide (0.79g, 2.58mmol, 1.2eq.) in THF (8.6ml) was added *via* cannula. The reaction was allowed to proceed for 2-3 minutes before quenching using a saturated potassium acetate/glacial acetic acid buffer (3.5:1), (4.5eq., 0.58ml).

The resulting suspension was stirred for 2 hours at room temperature before removal of solvent, and purification by chromatography (30% EtOAc/hexane, R_f=0.19) gave the product as a clear/yellow oil, 0.90g, 1.78mmol, 83% yield.

^{1}H n.m.r.; δ=0.67 (d, (J=6.7Hz), 3H, -CH-C$\underline{H}_3$); δ=1.0 (d, (J=6.7Hz), 3H, -CH-C$\underline{H}_3$); δ=0.8 (d, (J=7.0Hz), 3H, -CH-C$\underline{H}_3$); δ=0.9 (d, (J=7.0Hz), 3H, -CH-C$\underline{H}_3$); δ=1.25 (t, (J=7.0Hz), 6H, -CH$_2$-C$\underline{H}_3$); δ=1.25,1.35 (br.s, 4H, Alkyl chain); δ=1.6-1.8 (br.m, 4H, Alkyl chain); δ=2.20 (d of sept., (J=3.1Hz), 1H, CH-C$\underline{H}$-(CH$_3$)$_2$); δ=2.40 (d of sept., (J=3.7Hz), 1H, CH-C$\underline{H}$-(CH$_3$)$_2$); δ=3.80 (t, (J=3.4Hz), 1H, Val α-$\underline{H}$); δ=3.90 (d,d, (J=4.2, 9.8Hz), 1H, C$\underline{H}$-CH2); δ=4.0, 4.3 (complex m, 7H, O-C$\underline{H}_2$-CH$_3$, -CH-C$\underline{H}$-C$\underline{H}_2$-; δ=4.9 (d of d, (J=4.8, 9.0Hz), 1H, -CH$_2$-C$\underline{H}$-N$_3$)

^{13}C n.m.r.; δ=171.0; δ=163.3; δ=163.0;δ=63.6; δ=60.6; δ=60.5; δ=60.4; δ=60.3; δ=58.7; δ=55.4; δ=34.1; δ=31.7; δ=31.1; δ=29.3; δ=28.9; δ=28.1; δ=26.1; δ=24.3; δ=19.1; δ=17.9; δ=16.6; δ=14.5; δ=14.4; δ=14.3

IR; ν=2108cm^{-1} (-N$_3$); ν=1782cm^{-1} (C=O), ν=1690cm^{-1} (C=N); ν=1224cm^{-1}, 1035cm^{-1} (=C-O-CH$_2$-)

Mass Spec.; 507 (M+1); 466 (M-42)

Accurate Mass; C$_{25}$H$_{42}$N$_6$O$_5$ calculated 507.3290, found 507.3295

Optical Rotation; [α]$_D$= +60.5° (1.621mgml^{-1}, CHCl$_3$)

6-(S)-Isopropyl-3,6-dihydro-2,5-diethoxypyrazine-2-(R)-octanoic-10-acid (33)
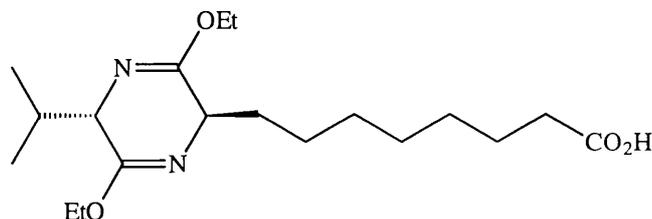
(i)   *tert*-Butyl   (6-(*S*)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine-3-(*R*))-octanoate (0.078g, 0.19mmol) was dissolved in dry trifluoroacetic acid (1.0ml), and the mixture stirred at room temperature for 1 hour under Ar atmosphere. After this time, the reaction mixture was added to water, and then extracted with ether. The organic layer was then washed with saturated sodium bicarbonate, and dried (Na$_2$SO$_4$). Purification was achieved by use of chromatography (50% ether/hexane, acidified with 0.1% formic acid, R$_f$=0.30) to give a clear oil, yield 0.054g, 0.15mmol, 80%.

(ii) Crude *tert*-butyldimethylsilyl (6-(*S*)-isopropyl-2,5-diethoxy-3,6-dihydropyrazine-2-(*R*))-octanoate (2.04mmol, assumed) was placed under an Ar atmosphere, and TBAF in THF solution (6.12ml, 1M, 2eq. w.r.t. *tert*-butyldimethylsilyl 8-iodooctanoate) was added, and the mixture stirred for 1 hour. After this time, the mixture was diluted with saturated ammonium chloride solution (25ml), and washed with ether (200ml). The organic layer was collected and dried (Mg$_2$SO$_4$). Removal of the solvent gave a clear oil, which was purified by chromatography (20% EtOAc/hexane, + 0.1% formic acid, R$_f$=0.25), 0.626g, 1.77mmol, 87% yield.

(iii) To a solution of methyl-3-(*R*)-(6-(*S*)-isopropyl-2,5-diethoxy-3,6-dihydropyrazine) octanoate in THF/water, cooled on an ice bath, was added lithium hydroxide in one portion. The reaction was then stirred for 1 hour, followed by removal of the organic solvent *in vacuo*. The aqueous layer was acidified, then extracted using ether. The ether layers were dried (MgSO$_4$) and removal of the solvent *in vacuo* gave the product as a clear oil. Purification was performed by chromatography (50% ether/hexane + 0.1% formic acid, R$_f$=0.30), yield 90%.

^{1}H n.m.r.; δ=0.67 (d, (J=6.8Hz), 3H, -CH-CH$_3$); δ=1.0 (d, (J=6.9), 3H, -CH-(CH$_3$); δ=1.24 (t, (J=7.1Hz) -CH$_2$-CH$_3$); δ=1.27 (br.s, 6H, Alkyl chain); δ=1.59 (br.pent., 2H,

Alkyl chain); δ=1.70 (br.m, 2H, Alkyl chain); δ=2.25 (d. of sept., (J=3.2Hz), 1H, -C$\underline{H}$-(CH$_3$)$_2$); δ=2.3 (t, (J=7.6Hz), 2H, -C$\underline{H}_2$-CO-); δ=3.87 (t, (J=3.5Hz), 1H, Val-α$\underline{H}$); δ=3.9 (d,d, (J=2.0, 4.2Hz), 1H, C$\underline{H}$-CH$_2$); δ=4.1 (m, 6H, O-C$\underline{H}_2$-CH$_3$, CH-C$\underline{H}_2$-CH$_2$)

^{13}C n.m.r.; δ=179.1; δ=163.5; δ=163.1; δ=60.6; δ=60.6; δ=60.5; δ=55.3; δ=34.0; δ=33.9; δ=31.7; δ=29.3; δ=29.1; δ=29.0; δ=24.7; δ=24.3; δ=19.0; δ=16.6; δ=14.3; δ=14.3

Mass Spec.; 355 (M+1); 211

Accurate Mass; C$_{19}$H$_{35}$N$_2$O$_4$ calculated 355.2590, found 355.2597

Optical Rotation; [a]$_D$= -24.7 (2.833mgml^{-1}, CHCl$_3$)

1-Ethyl-2-(R)-amino-1,10-decanoic diacid (34)
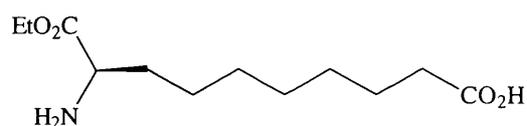
6-(*S*)-Isopropyl-3,6-dihydro-2,5-diethoxypyrazine-2-(*R*)-octanoic-10-acid (0.22g, 0.54 mmol) was suspended in 0.25N HCl/THF (6eq., 13ml), and the resulting solution was stirred for 2 hours. During this time, the reaction was monitored by tlc, developed using ninhydrin. After this indictated that no further starting material remained, the reaction was stripped of solvent, and the product hydrochloride was passed directly into the next reaction.

1-Ethyl-2-(R)-(allyloxycarbonylamino)-1,10-decanoic diacid (35)
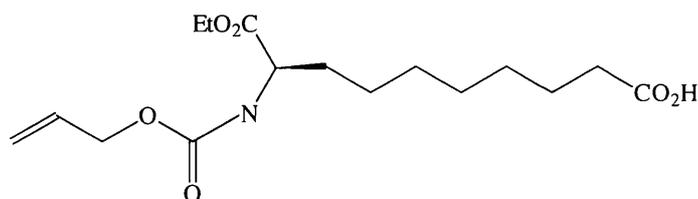
A mixture of 1-ethyl-2-(*R*)-aminodecanoic-1,10-diacid and Val-OEt were dissolved in a solution of dioxane/water, and to this was added sodium bicarbonate in one portion.

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The mixture was then cooled on an ice bath, and allyl chloroformate was added. The reaction was then stirred at 0°C for 12 hours, then the solvent was removed *in vacuo*. Purification was performed by chromatography (20% EtOAc/hexane, R_f=0.51).

^{1}H n.m.r.; δ=1.25 (3H, t, (J=7.1Hz), -CH$_2$-C$\underline{H}_3$); δ=1.26 (6H, br.s, Alkyl chain); δ=1.60 (4H, br.m, Alkyl chain); δ=2.24 (2H, t, (J=8Hz), -C$\underline{H}_2$-CO); δ=4.16 (2H, q, (J=6Hz), -C$\underline{H}_2$-CH$_3$); δ=4.24(2H, br.m, -CH-C$\underline{H}_2$-CH$_2$-); δ=4.54 (2H, d, (J=5.2Hz), =CH-C$\underline{H}_2$-O); δ=5.22 (2H, d of d, (J=10.0, 18.0Hz), C$\underline{H}_2$=CH-); δ=5.90 (1H, m, CH2=C$\underline{H}$-CH$_2$)

Mass Spec.; 329 (M$^+$); 330 (M+1)

Accurate Mass; C$_{16}$H$_{28}$NO$_6$ calculated 330.1910, found 330.1917

**(Trimethylsilyl)ethoxymethyl 8-bromooctanoate (36)^{22}**
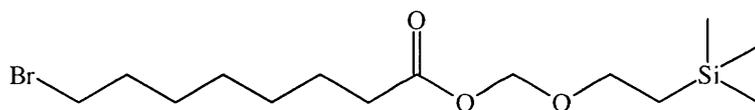
To a cooled (0°C) solution of 8-bromooctanoic acid (0.10g, 0.45mol) in THF (1.5ml) was added TEA (0.072ml, 0.52mmol) with stirring. To this was then added trimethylsilylethoxymethyl chloride (0.083ml, 0.47mmol) dropwise, and the reaction was stirred for 1 hour, before filtration of the mixture and removal of the solvents to give the product as a clear oil. A sample was reserved for analysis, and the remainder passed immediately into the next step.

^{1}H n.m.r.; δ=0.0 (9H, s, -Si-(C$\underline{H}_3$)$_3$); δ=0.9 (2H, t, (J=7.3Hz), -CH$_2$-C$\underline{H}_2$-Si); δ=1.3 (6H, br.s, Alkyl chain); δ=1.58 (2H, br.m, Alkyl chain); δ=1.78 (2H, br.pent., Alkyl chain); δ=2.28 (2H, t, (J=7.0Hz), -O-C$\underline{H}_2$-CH$_2$-); δ=3.34 (2H, t, (J=6.8Hz), -C$\underline{H}_2$-CO-); δ=3.64 (2H, t, (J=7.3Hz), Br-C$\underline{H}_2$-CH$_2$-); δ=5.22 (2H, s, O-C$\underline{H}_2$-O)

^{13}C n.m.r.; δ=173.3; δ=88.7; δ=67.7; δ=34.3; δ=33.8; δ=32.7; δ=28.9; δ=28.4; δ=27.9; δ=24.6; δ=18.0; δ=-1.4

(Trimethylsilyl)ethoxymethyl 8-iodooctanoate (37)[21]
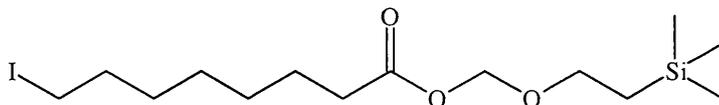
To (trimethylsilyl)ethoxymethyl 8-bromooctanoate (0.45mmol) under Ar was added a solution of sodium iodide (0.20g, 1.34mmol, 3eq.) in dry acetone (1ml). Stirring was continued for 1.5 hrs, before the addition of ether (5ml) and subsequent removal of the inorganic solids by filtration. Removal of the solvents *in vacuo* then gave the product as a clear oil. Purification was performed by chromatography (8% EtOAc/hexane, R_f=0.31), 0.12g, 67%.

^{1}H n.m.r.; δ=0.01 (9H, s, -Si-(C$\underline{H}_3$)$_3$); δ=0.95 (2H, d, (J=8.1Hz), -CH$_2$-C$\underline{H}_2$-Si-); δ=1.33 (6H, br.s, Alkyl chain); δ=1.60 (2H, br.m, Alkyl chain,); δ=1.80 (2H, br.m, Alkyl chain); δ=2.3 (2H, t, (J=7.5Hz), -CH$_2$-C$\underline{H}_2$-CO-); δ=3.1 (2H, t, (J=7.0Hz), I-C$\underline{H}_2$-CH$_2$-); δ=3.60 (2H, d, (J=7.1Hz), O-C$\underline{H}_2$-CH$_2$); δ=5.20 (2H, s, O-C$\underline{H}_2$-O)

(Trimethylsilyl)ethoxymethyl (6-(S)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine)-2-(R)-octanoate (38)
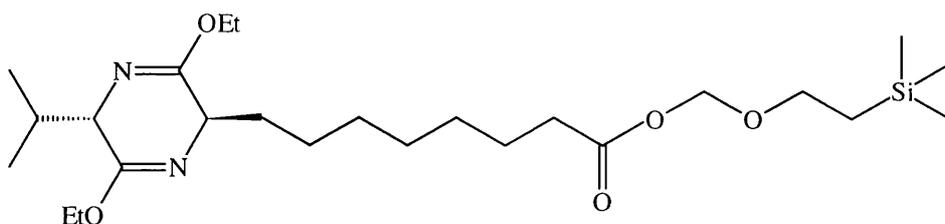

To a cooled (-78°C) solution of 6-(*S*)-isopropyl-3,6-dihydro-2,5-diethoxypyrazine (1.0g, 4.72mmol) in THF (8ml) was added n-BuLi (1.9ml, 2.5N solution, 1eq.) in one portion, and stirring continued for 10 minutes. After this time, the lithiated pyrazine was added *via* a teflon cannula to a cooled (0°C), stirred slurry of copper (I) cyanide (0.21g, 2.36mmol, 0.5eq.) in THF (8ml). Stirring of the mixture was continued until all solid had dissolved. The reaction was then recooled to -78°C, and a precooled solution of (trimethylsilyl)ethoxymethyl 8-iodooctanoate (1.15g, 3.54mmol) in THF (8ml) was added by cannula. This mixture was then warmed to -23°C, and stirring continued for

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5 hours, followed by standing at this temperature overnight.

The reaction was quenched by addition of a concentrated ammonia/saturated ammonium chloride solution (43ml, 1:9 ammonia/ammonium chloride), followed by the addition of ether (300ml). After separation of the aqueous layer, the organic layers were then washed with further ammonia/ammonium chloride buffer solution (3x216ml), and dried (Na$_2$SO$_4$). Removal of solvents *in vacuo* gave a clear oil, but no identifiable products were isolated.

8-Iodooctanoic acid (39)

To a solution of 8-bromooctanoic acid (2.00g, 8.97mmol) in dry acetone (15ml) at room temperature was added sodium iodide (4.03g, 26.9mmol, 3eq.). This mixture was stirred for 2 hours, then the solvents removed *in vacuo*. The residue was then taken up in water (20ml), and extracted using ether (70ml). The organic layer was then washed with saturated. thiosulphate solution, dried (MgSO$_4$) and the solvent removed *in vacuo* to give a white solid. The product was purified by column chromatography (38% ether/hexane + 0.1% formic acid, R$_f$=0.63), 2.30g, 8.52mmol, 95% yield.

^{1}H n.m.r.; δ=1.3 (6H, br.s, Alkyl chain); δ=1.6 (2H, br.pent., Alkyl chain); δ=1.8 (2H, br.pent., Alkyl chain); δ=2.3 (2H, t, (J=6.9Hz), -C$\underline{H}_2$-CO-); δ=3.2 (2H, t, (J=7.0Hz), I-C$\underline{H}_2$-)

IR; ν=1708cm^{-1}; (I-CH$_2$ stretch masked)

Mass Spec.; 271 (M+1)

tert-Butyldimethylsilyl 8-iodooctanoate (40)
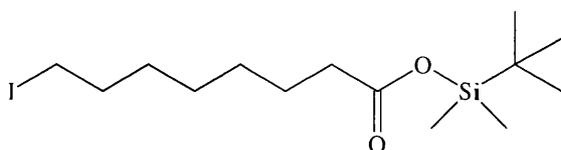
To a cooled (0°C) solution of 8-iodooctanoic acid (8.96mmol) was in THF (25ml) was added TEA (1.50ml, 10.7mmol) with stirring. To this was added TBDMS-chloride (1.41g, 9.41mmol, 1.05eq.) in THF (5ml) dropwise over 10 minutes. This mixture was then stirred for 1 hour, followed by removal of TEA:hydrochloride by filtration. Removal of the solvents *in vacuo* gave the product as a clear oil. Purification was performed by distillation, 2.68g, 6.99mmol, 78% yield.

B.Pt.; 160°C @ 0.1mmHg

^{1}H n.m.r.; δ=0.2 (9H, s, Si-(C$\underline{H}_3$)$_2$); δ=0.9 (6H, s, Si-(C$\underline{H}_3$)$_3$); δ=1.3 (6H, br.s, Alkyl chain); δ=1.58 (2H, br.m, Alkyl chain); δ=1.78 (2H, br.m, Alkyl chain); δ=2.3 (2H, t, (J=7.4Hz), -C$\underline{H}_2$-CO-); δ=3.2 (2H, t, (J=7.0Hz), -C$\underline{H}_2$-I)

^{13}C n.m.r.; δ=174.0; δ=35.9; δ=44.4; δ=30.3; δ=28.8; δ=25.5; δ=24.9; δ=17.6; δ=7.1

Mass Spectrum; 385 (M+1); 327 (M-58); 253 (M-132)

Accurate Mass; $C_{14}H_{30}IO_2Si$ calculated 385.1050, found 385.1060

tert-*Butyldimethylsilyl (6-(S)-Isopropyl-2,5-diethoxy-3,6-dihydropyrazine-3-(R))-octanoate* (41)
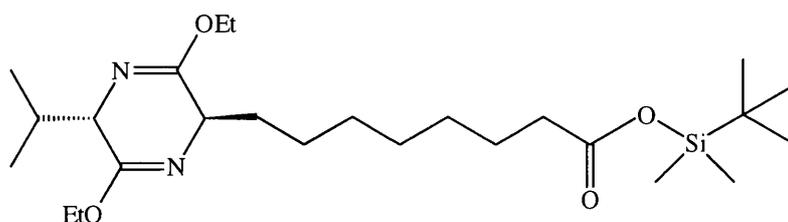

To a cooled (-78°C) solution of 6-(*S*)-isopropyl-2,5-diethoxy-3,6-dihydropyrazine (0.866g, 4.08mmol) in THF (6ml), was added n-BuLi (1.63ml, 1eq.), and stirring continued for 10 minutes. The mixture was then added *via* teflon cannula to a cooled (0°C) suspension of copper (I) cyanide (0.181g, 2.04mmol) in THF (6ml). Stirring was continued until all the solid had dissolved.

The mixture was then recooled to -78°C, and a precooled solution of *tert*-butyldimethylsilyl 8-iodooctanoate (1.175g, 3.06mmol) in THF (6ml) was added *via*

cannula. The reaction was then warmed to -23°C and stirring continued for 5 hours. The reaction was then stored overnight at this temperature.

The reaction was quenched using a concentrated ammonia/saturated ammonium chloride solution (1:9) (37ml), to which was added ether (440ml). After removal of the aqueous layer, the organic layer was washed with buffer solution (3x300ml) and dried (Na_2SO_4). The solvents were removed *in vacuo* to give the crude product, which was then used directly in the next reaction.

Methyl 8-bromooctanoate (42)
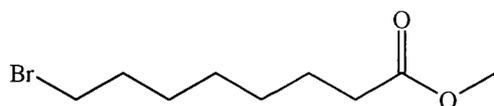
8-Bromooctanoic acid (0.20g, 0.897mmol) was dissolved in methanol (20ml), and to this solution was added thionyl chloride (0.13ml, 1.79mmol, 2eq.), and the resulting solution was stirred at reflux for one hour. After this time, the solution was cooled, and the solvents were removed *in vacuo*. The residue was then purified by chromatography (40% Et_2O/Hexane, R_f=0.63), 0 0.18g, 0.764mmol, 85%.

^{1}H n.m.r.; δ=1.32 (br.s, 6H, Alkyl chain); δ=1.62 (br.pentet, 2H, Alkyl chain); δ=1.84 (br. pentet, 2H, Alkyl chain); δ=2.30 (t, (J=7.4Hz), 2H, -C$\underline{H}_2$-CO-); δ=3.40 (t, (J=7.0Hz), 2H, Br-C$\underline{H}_2$-); δ=3.66 (s, 3H, O-C$\underline{H}_3$)

IR; ν(max)= 1738cm^{-1} (C=O); ν(max)= 1172cm^{-1} (C-O)

Mass Spec.; (FAB); 237, 239 (M$^+$); 205, 207; 157; 137

Accurate mass; $C_9H_{17}O_2Br$ calculated 237.0480, found 237.0490

Methyl 8-Iodooctanoate (43)
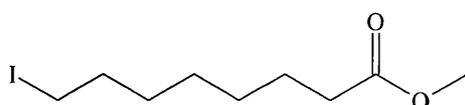
To a solution of methyl 8-bromooctanoate (0.11g, 0.45mmol) in acetone (1ml) was added sodium iodide (0.20g, 1.34mmol, 3eq.), and the resulting solution was stirred for 1 hour. During this time a white precipitate was evolved. The reaction was then suspended in water (10ml), and extracted with ether (20ml). After drying of the organic layer (MgSO$_4$) and removal of the solvent *in vacuo*, the residue was purified by chromatography (40% Et$_2$O/Hexane, R$_f$=0.70), yield 0.10g, 0.37mmol, 83%.

^{1}H n.m.r.; δ=1.29 (br.s, 6H, Alkyl chain); δ=1.58 (br.t, 2H, Alkyl chain); δ=1.77 (pentet, (J=7.3Hz), 2H, Alkyl chain); δ=2.27 (t, (J=7.4Hz), 2H, -CH$_2$-CO-); δ=3.14 (t, (J=7.0Hz), 2H, -CH$_2$-Br); δ=3.63 (s, 3H, O-CH$_3$)

IR; ν(max)= 1732cm^{-1} (C=O); ν(max)= 1176cm^{-1} (C-O)

Mass spec.; (FAB); 285 (M+1)(100%); 253; 237,239; 157; 125

Accurate mass; C$_9$H$_{17}$O$_2$I calculated 285.0340, found 285.0352

Methyl-3-(R)-(6-(S)-isopropyl-2,5-diethoxy-3,6-dihydropyrazine) octanoate (44)
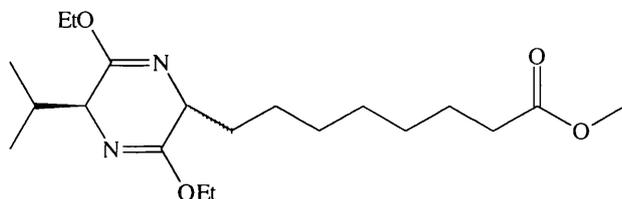
To a cooled (-78°C) solution of 6-(S)-isopropyl-2,5-diethoxy-3,6-dihydropyrazine (0.100g, 0.472mmol) in THF (1.2ml), was added n-BuLi solution (0.30ml, 1.6M, 1eq.), and stirring continued for 10 minutes. The mixture was then added *via* teflon cannula to a cooled (0°C) suspension of copper (I) cyanide (0.020g, 0.236mmol) in THF (1.2ml). Stirring was continued until all the solid had dissolved.

The mixture was then recooled to -78°C, and a precooled solution of methyl 8-iodooctanoate (0.105g, 0.370mmol) in THF (1.2ml) was added *via* cannula. The reaction was then warmed to -23°C and stirring continued for 5 hours. The reaction was then stored overnight at this temperature.

The reaction was quenched using a concentrated ammonia/saturated ammonium

chloride solution (1:9) (4.3ml), to which was added ether (47ml). After removal of the aqueous layer, the organic layer was washed with buffer solution (3x35ml) and dried (Na_2SO_4). The solvents were removed *in vacuo* to give the crude product, which was then purified by chromatography (20% Et_2O/Hexane, R_f=0.28), yield 0.067g, 0.184mmol, 78%.

^{1}H n.m.r.; δ=0.7 (d of d, (J=6.9, 14.3Hz), 3H, -CH-C$\underline{H}_3$); δ=1.0 (d of d, (J=2.4, 6.9Hz), 3H, -CH-C$\underline{H}_3$); δ=1.25 (t, (J=7.1Hz), 6H, -CH$_2$-C$\underline{H}_3$); δ=1.26 (br.s, 6H, Alkyl chain); δ=1.57-1.70 (br.m, 4H, Alkyl chain); δ=2.23 (m, 1H, -CH-(CH$_3$)$_2$); δ=2.27 (t, (J=7.7Hz), 2H, -C$\underline{H}_2$-CO-); δ=3.64 (s, 3H, O-C$\underline{H}_3$); δ=3.85 (t, (J=3.2Hz), 1H, Val-α$\underline{H}$); δ=3.95 (d,d, (J=3.2, 5.0Hz), 1H, -C$\underline{H}$-CH$_2$-); δ=4.07 (m, 6H, O-C$\underline{H}_2$-CH$_3$, CH-C$\underline{H}_2$-CH$_2$-)

Mass Spec.; 369 (M+1); 211

## 8-(R)-(6-(S)-Isopropyl-2,5-diethoxy-3,6-dihydropyrazine)-2-(S)-azidooctanoic acid (45)
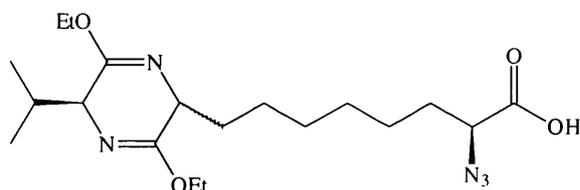
To a cooled (0°C) solution of 8-(R)-(6-(S)-isopropyl-2,5-diethoxy-3,6-dihydro-pyrazine)-2-(S)-azidooctanoyl-3'-(N)-(4'-(S)-isopropyloxazolidin-2'-one) (0.07g, 0.138mmol) in THF (2.1ml) and water (0.69ml) was added hydrogen peroxide (0.019ml, 5.52mmol, 4eq.), followed by lithium hydroxide monohydrate (0.012g, 0.276mmol, 2eq.). The resulting solution was stirred at this temperature for 30 minutes, then the organics were removed *in vacuo* and the residue was suspended in ether (20ml). The layers were then separated, and the organic layer was washed with sat. ammonium chloride solution (5ml), followed by drying (Na_2SO_4). Purification was performed by chromatography (35% Et_2O/Hexane+ 0.1% HCO_2H, Rf=0.39), yield 0.018g, 4.55x10^{-5}mol, 33%.

^{1}H n.m.r.; δ=0.7 (d, (J=6.4Hz), 3H, CH-C$\underline{H}_3$); δ=1.0 (d, (J=6.4Hz), 3H, CH-C$\underline{H}_3$); δ=1.26 (t, (J=7.0Hz), 6H, CH$_2$-C$\underline{H}_3$); δ=1.36 (br.s, 6H, Alkyl chain); δ=1.74 (br.m, 4H, Alkyl chain); δ=2.26 (d of sept., (J=3.2Hz), 1H, -C$\underline{H}$-(CH$_3$)$_2$); δ=3.78 (d,d, (J=6.0, 8.0Hz), 1H, -C$\underline{H}$-CH$_2$-); δ=3.94 (t, (J=4.0Hz), 1H, -C$\underline{H}$-(CH$_3$)$_2$); δ=4.06 (m, 5H, O-C$\underline{H}_2$-CH$_3$, CH$_2$-C$\underline{H}$N$_3$)

IR; ν(max)=2108cm^{-1} (-N$_3$); ν(max)=1691cm^{-1} (C=N, C=O); ν(max)=1239, 1035cm^{-1} (-O-C=)

Mass Spec.; 396 (M+1); 211

(10-Ethyl ester) 9-(R)-allyloxycarbonylamino-2-(S)-azidodecanoic-1,10-diacid (46)
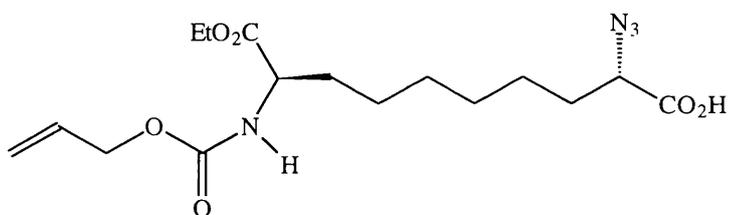
To a cooled (0°C) solution of 8-(*R*)-(6-(*S*)-isopropyl-2,5-diethoxy-3,6-dihydro-pyrazine)-2-(*S*)-azidooctanoyl-3'-(*N*)-(4'-(*S*)-isopropyloxazolidin-2'-one (0.49g, 0.963 mmol) in THF (14.4ml)/water (4.8ml) solution was added hydrogen peroxide (0.13ml, 100 vol. solution, 4eq.), and lithium hydroxide (0.081g, 1.93mmol, 2eq.) in one portion. This mixture was stirred for 30 minutes, then acidified to pH=4 and the aqueous layer extracted using ethyl acetate.

The organic layer was then stripped of solvent and the oily residue was treated with hydrochloric acid/ THF solution (23.1ml, 0.25M solution, 6eq.) at room temperature for 2 hours. After this time, the solvents were removed and the residue was taken up in dioxane (6.3ml) and water (3.1ml). To this was added sodium bicarbonate (0.81g, 9.63mmol), and the solution cooled to 0°C. Allyl chloroformate (3eq., 0.33ml, 2.89mmol) was then added in one portion added, and stirring continued for 12 hours. The organic solvents were then removed *in vacuo*, and the aqueous solution acidified followed by extraction using ethyl acetate. After drying (Na$_2$SO$_4$), the ethyl acetate

layers were reduced *in vacuo* to give the product as a clear oil.

Purification was performed by chromatography (40% EtOAc/hexane + 0.1% formic acid, R_f=0.20), 0.330g, 0.895mmol, 93%.

^{1}H n.m.r.; δ=1.26 (t, (J=7.2Hz), 3H, -CH$_2$-C$\underline{H}_3$); δ=1.30 (br.s, 4H, Alkyl chain); δ=1.60 (br.pent., 2H, Alkyl chain); δ=1.75 (br.m, 2H, Alkyl chain); δ=3.85 (d,d, (J=5.3, 8.1Hz), 1H, CH$_2$-C$\underline{H}$-NH-); δ=4.18 (q, (J=7.0Hz), 2H, O-C$\underline{H}_2$-CH$_3$); δ=4.32 (d,d, (J=7.5, 13.0Hz), 1H, -CH$_2$-C$\underline{H}$-N$_3$); δ=4.54 (d, (J=5.2Hz), 2H, =CH-C$\underline{H}_2$-O); δ=5.25 (d, (J=10.4Hz), t, (J=9.9Hz), 2H, C$\underline{H}_2$=CH-); δ=5.89 (octet, (J=5.9Hz), 1H, CH$_2$=C$\underline{H}$-CH$_2$-); δ=6.05 (br.s, ~2H, -N$\underline{H}$-, -CO$_2\underline{H}$)

^{13}C n.m.r.; δ=174.7; δ=172.7; δ=132.5; δ=65.8; δ=61.6; δ=61.5; δ=53.7; δ=32.6; δ=31.1; δ=28.8; δ=28.7; δ=28.6; δ=25.4; δ=24.8; δ=14.2

IR; ν=2109cm^{-1} (-N$_3$); ν=1720cm^{-1} (C=O); ν=912cm^{-1} (-CH=CH$_2$)

Mass Spec.; 371 (M+1), 393 (M+Na$^+$), 297

Accurate Mass; C$_{16}$H$_{27}$N$_4$O$_6$ calculated 371.1920, found 371.1931

Optical Rotation; [α]$_D$= -14.7 (0.614mgml^{-1}, CHCl$_3$)

*(10-Allyl ester) 9-(**R**)-allyloxycarbonylamino-2-(**S**)-azidodecanoic-1,10-diacid* (**47**)
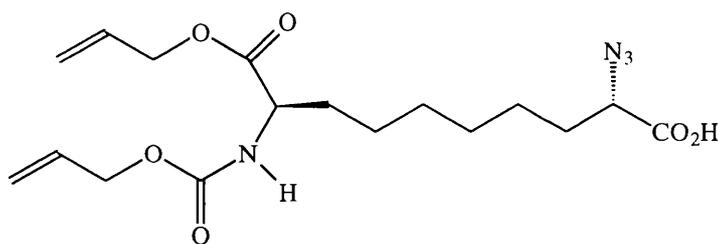
(1-Ethyl ester) 2-(R)-allyloxycarbonylamino-9-(S)-azidodecanoic-1,10-diacid (0.330g, 0.892mmol) and lithium bromide (5eq., 0.388g, 4.46mmol) were dissolved in dry, distilled allyl alcohol (6.4ml), and the resulting solution was cooled to 0°C (ice bath). To this was added freshly distilled DBU (1.5eq., 0.20ml, 1.34mmol) in one portion. The mixture was then stirred at 0°C for 12 hours, and after this time the solvent was removed. The residue was suspended in saturated ammonium chloride solution (20ml),

acidified with 2N HCl. The aqueous layer was then extracted using ethyl acetate (3x60ml), and the collected organics dried (Na_2SO_4). Removal of the solvent gave a clear oil, purified by chromatography (40% EtOAc/hexane + 0.1% formic acid, R_f=0.18), 0.145g, 43%.

^{1}H n.m.r.; δ=1.30 (br.s, 8H, Alkyl chain); δ=1.64 (br.m, 2H, Alkyl chain); δ=1.83 (br.m, 2H, Alkyl chain); δ=3.84 (d,d, (J=5.1, 81Hz), 1H, NH-C$\underline{H}$-CH$_2$); δ=4.35 (d,d, (J=7.9, 13.3Hz), 1H, N_3-C$\underline{H}$-CH$_2$); δ=4.54 (d, (J=5.3Hz), 2H, =CH-C$\underline{H}_2$-O); δ=4.60 (d, (J=4.9Hz), 2H, =CH-C$\underline{H}_2$-O); δ=5.25 (complex m, 4H, C$\underline{H}_2$=CH-); δ=5.87 (octet, (J=6.0Hz), 2H, CH$_2$=C$\underline{H}$-CH$_2$-): δ=9 (br.s, ~2H, -CO$_2\underline{H}$, -N$\underline{H}$-)

^{13}C n.m.r.; δ=175.1; δ=172.4; δ=172.4; δ=132.4; δ=131.4; δ=118.8; δ=117.8; δ=65.9; δ=61.6; δ=53.7; δ=32.5; δ=31.1; δ=29.6; δ=28.7; δ=28.6; δ=25.4; δ=24.9

I.R.; ν=2109cm-1 (-N^3); ν=1736cm^{-1} (C=O); ν=1206cm^{-1} (-C-O-CO-); ν=909cm^{-1} (-CH=CH$_2$)

Mass Spec.; 383 (M+1)$^+$, 357 (M-), 297 (M-)

Accurate Mass; $C_{17}H_{27}N_4O_6$ calculated 383.1920, found 383.1931

Optical Rotation; [α]$_D$= -11.1 (1.261mgml^{-1}, CHCl$_3$)

(1-Allyl ester) 2-(R)-allyloxycarbonylamino-9-(S)-(9-fluorenylmethoxycarbonyl) aminodecanoic-1,10-diacid (48)
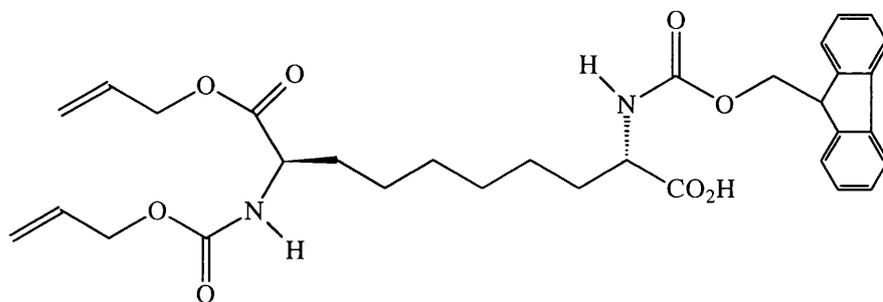
To a solution of (1-allyl ester) 2-(R)-allyloxycarbonylamino-9-(S)-azidodecanoic-1,10-diacid (0.380mmol) in THF (3.4ml) and water (0.2ml) was added triphenylphosphine (0.109g, 0.418mmol, 1.1eq.), and the resulting mixture was stirred for 24 hrs. After

this time, the solvents were removed *in vacuo*, and the white residue was taken up in dioxane (1.5ml). Sodium bicarbonate (0.096g, 1.14mmol, 3eq.) in water (3.2ml) was added. This mixture was cooled (0°C), and a solution of 9-fluorenylmethyl chloroformate (0.147g, 0.570mmol, 1.5eq.) in dioxane (1ml) was added dropwise. The mixture was stirred for 4 hours, followed by removal of the organic solvents *in vacuo*. The aqueous layer was then acidified, and extracted using ethyl acetate. After drying (Na_2SO_4), ethyl acetate was removed to give a white solid. Purification was performed by chromatography (20% EtOAc/hexane + 0.1% formic acid, R_f=0.16), or by HPLC (normal phase, 10% EtOAc/hexane) yield 0.128g, 0.222mmol, 58%.

^{1}H n.m.r.; δ=1.30 (br.s, 8H, Alkyl chain); δ=1.66 (br.s, 2H, Alkyl chain); δ=1.84 (br.m, 2H, Alkyl chain); δ=4.10 (t, (J=5.8Hz), 1H, Fmoc O-CH_2-C$\underline{H}$); δ=4.36 (1H, m, α-H); δ=4.42 (1H, m, α-H); δ=4.40 (d, (J=7.0Hz), 2H, O-C$\underline{H}_2$-CH); δ=4.57 (d, (J=5.1Hz), 2H, =CH-C$\underline{H}_2$-O); δ=4.63 (d, (J=5.2Hz), 2H, =CH-C$\underline{H}_2$-O); δ=5.30 (m, 4H, C$\underline{H}_2$=CH-); δ=5.90 (complex m, 2H, CH_2=C$\underline{H}$-CH_2); δ=7.3-7.4 (complex q., 8H, Aryl protons)

^{13}C n.m.r.;δ=172.3; δ=171.9; δ=155.8; δ=141.3; δ=132.5; δ=132.0; δ=131.9; δ=131.5; δ=128.0; δ=127.7; δ=127.2; δ=127.1; δ=126.9; δ=125.0; δ=124.9; δ=120.2; δ=120.0; δ=118.9; δ=117.8; δ=76.4; δ=65.9; δ=65.8; δ=53.7; δ=53.6; δ=32.5; δ=32.5; δ=32.2; δ=28.7; δ=28.6; δ=28.5; δ=25.0; δ=24.9

IR; ν(max)= 2932cm^{-1} (-CO_2H); ν(max)= 1715cm^{-1} (C=O); ν(max)= 1508cm^{-1} (N-H);

Mass Spec.; 578 (M$^+$), 601 (M+Na$^+$), 279 (M-299)

Accurate mass; $C_{32}H_{38}N_2O_8$ calculated 578.3088, found 578.2850

Optical Rotation; [α]$_D$= -6.8° (3.1mgml^{-1})

Short Model Peptide

Synthesis was performed on a Rink MBHA resin, (0.49mmolg^{-1} substitution level), using HBTU (0.5M) as coupling reagent, in solution with DIEA (0.5M).[23] Deprotection of Fmoc was performed using 20% piperidine in DMF. Coupling times

were 1 hour in all cases, including cyclisation. Allyl deprotection was performed using *tetrakis*-triphenylphosphine palladium(0) in a (10:1:2) DMF/NMM/HOAc solution, followed by washing with DIEA (0.5%) and sodium diethyldithiocarbamate (0.5%) in DMF solutions.[24] Cleavage and final deprotection was performed by means of 95% TFA/water solution, and the peptide triturated with ether. Following collection and lyophilisation from acetic acid, purification was attempted by HPLC (gradient, 10% acetonitrile-60% in water, + 0.1% TFA, 40 minutes). However, no product could be unambiguously found.

hGRF Analogue

Synthesis was performed on a PAL-PEG-PS resin (0.12mmolg^{-1} substitution level), using HBTU (0.5M) as coupling reagent, in solution with DIEA (0.5M).[23] Deprotection of Fmoc was performed using 20% piperidine solution in DMF. Coupling times in this stage of the synthesis were all 1 hour. After deprotection of the *N*-terminus, the resin was transferred to a manual synthesiser, and thoroughly washed with DMF. The resin was then suspended in a solution of HATU (0.5M) with DIEA (0.5M) in DMF, and to this was added the synthesised monomer in DMF (0.33M, mmol).[25] The reaction mixture was then agitated using argon for 1.5 days. After this time, the resin was washed thoroughly, and then returned to the synthesiser for the inclusion of the central three amino acids. This was performed using HBTU as described above.

The resin was then put into the manual synthesiser, and Alloc deprotection performed using *tetrakis*-triphenylphosphine palladium(0) in a solution of DMF/NMM/HOAc (10:1:2) for 1 hour.[24] After removal of the cleavage mixture, and thorough washing with sodium diethyldithiocarbamate (0.5%) and DIEA (0.5%) solutions to remove palladium by-products. Cyclisation of the monmer side chain was then attempted using HATU (0.5M) and DIEA (0.5M) and DMF, agitated for 1.5 days. After washing, the extension of the peptide was performed by automated synthesis using HBTU to perform couplings.

After completion and cleavage of the terminal Fmoc group, the peptide was

deprotected and cleaved from the resin using 95% TFA/water (20ml) with ethanedithiol (800μl) /thioanisole (200μl) /phenol (300mg) scavengers.[29] The peptide was then precipitated using ether, and lyophilised from acetic acid. Purification was performed by HPLC (gradient, 10% acetonitrile-60% in water, + 0.1% TFA, 30 minutes), giving five different products. However, none could be unambiguously assigned as the desired product.

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