Design and Synthesis of High Affinity Ligands for
Cyclophilins

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Declaration

This thesis is submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Edinburgh and is my own composition. Unless otherwise stated, the work has been carried out by myself and has not been previously submitted, in whole or in part, for any degree at this or any other university.

Colin John Dunsmore
University of Edinburgh
April 2005
Abstract

Cyclophilin A (CypA) is an ubiquitous intracellular protein that binds the immunosuppressant drug, cyclosporin A (CsA). CypA also plays important roles in protein folding and HIV-1 virus replication, whilst other cyclophilin isoforms have been implicated in nematode parasitic development. CsA inhibits these processes but is disadvantaged by its large molecular weight and immunosuppressive effects. Hence, there is a strong therapeutic driving force to design small, tight binding inhibitors, with which to probe cyclophilin functions.

In light of this, an in-house in silico database mining programme (LIDAEUS) was used to screen a library of small molecules for binding to CypA. This approach identified dimedone 33 as a lead which mimics two of the key interactions of CsA and was found to possess a $K_d = 22$ mM (cf. 30 nM for CsA). Several rounds of chemical derivatisation led to the synthesis of conformationally constrained ligands, the best of which 123 was found to bind CypA with a $K_d = 16$ μM, a greater than 1000-fold improvement on the original lead.

![Inhibitor 33 and 123](image)

Inhibitor 123 was tested in vivo against the nematode *C. elegans* to screen for antiparasitic effects. High concentrations (> 500 μM) of 123 were lethal resulting in rapid death of the worms. At lower concentrations the worms suffered growth defects and displayed cuticle shedding and severe gut development disorders, similar in phenotype to those observed in organisms treated with CsA.

Fluorescent tags were incorporated to investigate the uptake and localisation of the ligand within *C. elegans*. Sulfonamide 169a displayed similar effects to 123 and CsA in vivo and its fluorescence was detected throughout the worms’ gut lumen in areas where cyclophilins are thought to be involved in structural formation of the gut lining. These results demonstrate the potential of such ligands to act as chemical
probes to elucidate the role of cyclophilins at different stages of *C. elegans* development. Such a forward chemical genetics approach could in future provide valuable information concerning the biological mode of action of cyclophilins in nematode parasites and help to identify new targets for antiparasitic drugs.
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### Abbreviations

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<td>AAPF</td>
<td>Suc-Ala-Ala-Pro-Phe-pNA</td>
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<tr>
<td>Abu</td>
<td>L-α-aminobutyric acid</td>
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<tr>
<td>Ac</td>
<td>acetyl</td>
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<tr>
<td>ADME</td>
<td>absorption, distribution, metabolism, excretion</td>
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<tr>
<td>Ala</td>
<td>alanine</td>
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<td>Ar</td>
<td>aryl</td>
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<td>Arg</td>
<td>arginine</td>
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<tr>
<td>Asn</td>
<td>asparagine</td>
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<tr>
<td>Bmt</td>
<td>(4R)-4-[((E)-2-butenyl]-4-methyl-L-threonine</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
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<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
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<tr>
<td>CA</td>
<td>capsid</td>
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<td>CaM</td>
<td>calmodulin</td>
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<td>CaN</td>
<td>calcineurin</td>
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<tr>
<td>Cbz</td>
<td>benzyloxy carbonyl</td>
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<tr>
<td>CDI</td>
<td>1,1-carbonyldiimidazole</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
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<tr>
<td>CV</td>
<td>cone voltage</td>
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<tr>
<td>CypA</td>
<td>cyclophilin A</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMPU</td>
<td>N,N-dimethylpropyleneurea</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOS</td>
<td>diversity oriented synthesis</td>
</tr>
<tr>
<td>EDCI</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride</td>
</tr>
<tr>
<td>ee</td>
<td>enantiomeric excess</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionisation mass spectrometry</td>
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<td>Et</td>
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FAB  fast atom bombardment
FKBP  FK506 binding protein
Fmoc  9-fluorenlymethyloxycarbonyl
Gln  glutamine
Gly  glycine
His  histidine
HIV-1  human immunodeficiency virus type 1
HMPA hexamethyiphosphoric triamide
HOBr 1-hydroxybenzotriazole
HPLC high performance liquid chromatography
HTS high-throughput screening
Hz  Hertz
IL2 interleukin-2
Ile isoleucine
IPA  iso-propyl alcohol
IR  infrared
LC-MS liquid chromatography mass spectrometry
LDA lithium diisopropylamide
Leu leucine
LIDAEUS  Ligand Design At Edinburgh Uni versity
Lys lysine
Me methyl
MeLeu N-methylleucine
Met methionine
MeVal N-methylvaline
mp melting point
NBS  N-bromosuccinimide
NF-AT nuclear factor of activated T cells
NMO  N-methylmorpholine N-oxide
NMM  N-methylmorpholine
NMR nuclear magnetic resonance
Ph  phenyl
Phe  phenylalanine
pNA  p-nitroanilide
PPIase  peptidyl-prolyl isomerase
Pro  proline
PyBOP  benzotriazole-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate
Q-TOF  quadrupole time-of-flight
RAPA  rapamycin
RNA  ribonucleic acid
SAR  structure-activity relationships
Ser  serine
SFA  Sanglifehrin A
Suc  succinyl
TBS  tert-butyldimethylsilyl
TES  triethylsilyl
TFA  trifluoroacetic acid
TFE  2,2,2-trifluoroethanol
THF  tetrahydrofuran
TLC  thin layer chromatography
TMS  trimethylsilyl
TMSOTf  trimethylsilyl trifluoromethanesulfonate
Trp  tryptophan
UV  ultra violet
Val  valine
Xaa  any amino acid
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1 Introduction

This thesis describes the design and synthesis of high affinity ligands for cyclophilins, in particular human cyclophilin A (CypA). By way of introduction, a general review of drug discovery is given, with specific emphasis on new developments applicable to this project such as chemical genetics. The nature and functions of CypA are then described, followed by a review of the literature on CypA inhibitors. The chapter ends with an account of the ligand design approach that resulted in the discovery of the lead compound, dimedone.

1.1 Drug Discovery

1.1.1 Historical

The beginning of drug research as a discipline in its own right can be traced back to the early nineteenth century when researchers began to propose that the observed medicinal benefit of some plant extracts could be attributed to a single active ingredient. Support for this view grew when Frederick Sertürner successfully isolated the first active compound, morphine, from opium extract in 1815.\(^1\) It was not until the beginning of the twentieth century, however, that the next crucial breakthrough was made with the concept of 'receptors' as described by Paul Ehrlich.\(^2\) Ehrlich argued that certain chemoreceptors, as he classed them, would be different between species and could therefore be exploited therapeutically. This idea stemmed from observations that dyes (e.g. methylene blue), derived from coal-tar, showed affinity for biological tissues (e.g. nerve cells). Coal-tar, itself a by-product of the burgeoning chemical industries of the time, contained many organic derivatives, which would form the basis of early medicines and lead to the discovery of drugs such as aspirin 1 (Figure 1.1). The need to research, characterise, and develop these new substances led to the birth of the pharmaceutical industry.

The early pursuit of new drugs relied upon natural products isolated from plants, fungi, and bacteria. Often new discoveries were made in a serendipitous fashion, such as the discovery of penicillin 2 (Figure 1.1) by Fleming\(^3\) that led to the
development of antibiotics to treat bacterial infections. Once new lead compounds had been identified, their structures would be systematically altered using synthetic techniques with the hope of finding compounds with improved pharmacological profiles. Notable successes include the derivatives of the antibiotic, sulfanilamide 3 (Figure 1.1), which have given rise to effective diuretics and antihypertension drugs. However, much of this approach was still based on trial and error and the molecular basis of the observed effect was often poorly understood. This situation began to change with the characterisation of receptors and enzymes allowing chemists to design molecules for specific targets. Some of the early successes included the discovery of β-blockers, β-agonists and benzodiazepines.

Figure 1.1 Drug structures.

Despite the increase in the number of new classes of drugs reaching the market, traditional drug discovery was still heavily reliant on screening compound collections from pharmaceutical companies against newly discovered biological targets. These collections often suffered from a lack of chemical diversity; a result of being populated by analogues of a relatively small number of diverse lead compounds. Whilst natural products increased the number of structurally diverse compounds available, they routinely suffered from difficulties associated with their isolation and synthetic modification. Further challenges arose from advancements in biology, which gave rise to improved methods for protein production that enabled key proteins to be routinely produced in sufficient yield and purity for use in biological assays and structure determination. Similar progress in gene technology and DNA sequencing resulted in a huge increase in the number of hypothetical targets available to chemists; and finding ways to design and synthesise ligands for them all provided a unique challenge. To meet this challenge, the pharmaceutical
industries developed new technologies such as high-throughput screening and combinatorial chemistry.\(^5\)

### 1.1.2 High-Throughput Screening and Combinatorial Chemistry

The increasing availability of drug targets that could form the basis of *in vitro* assays, combined with advances in robotic technology, allowed pharmaceutical companies to automatically assess the activity of samples in their collections by screening them in multi-well microtitre plates. This process was referred to as high-throughput screening (HTS) and today it is capable of screening hundreds of thousands of compounds in a 24 hour period.\(^6\) To keep pace with HTS, new methods were developed to increase the number of compounds available for testing. Combinatorial chemistry involves the synthesis of compound libraries as opposed to single products. It has its origins in solid phase chemistry, as pioneered by Merrifield,\(^7\) since the immobilisation of molecules on resin allows for simple purification protocols and large excesses of reagents to drive the reactions to completion.\(^5\) Since much of the early work on solid phase was concerned with the synthesis of peptides, early uses of combinatorial chemistry involved the generation of peptide libraries. However, the full impact of combinatorial chemistry in this area was not fully realised until the publication of the split-pool technique (Figure 1.2).\(^5,8\)

**Figure 1.2** The split-pool synthetic scheme for the synthesis of a 27-member library.
Split-pool synthesis involves dividing the solid support into portions and reacting each portion with a single reagent (building block). The portions are pooled to provide a single batch of solid support containing a mixture of components. Repeating this divide-couple-recombine approach gives rise to a library where each resin bead contains one library member. An example of the power of this technique was given by Houghten and co-workers who synthesised over 34 million \(N\)-acetylated hexapeptides from 18 different amino acids.\(^9\)

Despite solid-phase techniques being tailor-made for peptide synthesis, such compounds are not ideal drug candidates due to their poor bioavailability. The combinatorial synthesis of “drug-like” (low molecular weight, organic molecules) compound libraries has received much attention. Although hampered by difficulties associated with transferring solution phase reactions to resin support, the area has given rise to some notable successes. For example, Ellman developed a solid phase route to benzodiazepines \(^9\) and used it to synthesis a 192-member library (Scheme 1.1).\(^10\) This seminal piece of work came to represent the potential of combinatorial synthesis to the pharmaceutical industry. The extent of the progress made since Ellman’s work was demonstrated by Schreiber and co-workers through their stereoselective synthesis of over two million natural-product like compounds using split-pool technology.\(^11\)

Combinatorial chemistry coupled with HTS has enabled vast numbers of compounds to be synthesised for testing against a range of biological targets. New technologies, such as analytical constructs to aid quantitation and isotope encoding strategies to allow mass spectrometry to identify hits, have led to further advances.\(^12\) However, doubts remain as to whether simply synthesising many more compounds will lead to a commensurate rise in the number of leads? It is true to say that whilst combinatorial chemistry and HTS have generated more hits than previous methods of drug discovery, turning these hits into viable drug candidates has proven to be much more difficult than was anticipated.\(^13\) Part of the reason for this may lie in figures that have estimated the total number of small organic molecules in the universe to be in the region of \(10^{200}\).\(^14\) It is obvious that only a fraction of this number could ever
realistically be synthesised and screened, thus an element of rational drug design must be considered when planning combinatorial libraries. This need is increasingly being met with *in silico* design methods such as molecular docking and virtual screening.

**Scheme 1.1**

1.1.3 Structure-Based Design

The growth of structural biology has seen an increasing number of protein structures solved by X-ray crystallography, NMR spectroscopy, or homology modelling. Knowledge of the 3D structure of proteins allows computer programmes to calculate the most favourable points of interaction between the protein and a ligand. The software achieves this by assessing the various binding points involving hydrogen bonding, electrostatic contacts, or Van der Waals interactions. This information can then be used to identify ligands with the required functional groups to interact at
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these key sites.\textsuperscript{15} This process is helped if an X-ray crystal structure of the protein-ligand complex is known, as is the case with CypA.\textsuperscript{16}

The challenge is to build virtual molecules into the protein binding site which are novel (\textit{de novo} design) yet chemically tractable. Docking algorithms are used to screen compound databases in order to predict which molecules will possess the best binding affinities for a particular target. The advantage of docking is that the process is fast and entire compound collections can be virtually screened. In addition, algorithms can now take into account key properties such as adsorption, distribution, metabolism and excretion (ADME), which can have a serious impact on the viability of a lead.\textsuperscript{17} Common criticisms of docking studies are that the suggested ligands show poor activity experimentally, or that they are synthetically challenging to make or modify.\textsuperscript{18} Whilst it is impossible for docking to guarantee the activity of all hits, ever more sophisticated algorithms should continue to minimise the number of false positives. The screening of databases of available compounds has helped to ensure that hits from virtual screening can be easily derivatised. In terms of our studies, we have exploited structure-based design to identify novel ligands for CypA, using an in-house molecular docking programme, LIDEAUS, to screen the Maybridge Fine Chemicals database for hits. This approach will be discussed in more detail in Section 1.5.

Computational studies play an increasingly important role in lead discovery and enable combinatorial chemistry to be tailored towards the synthesis of more focused libraries which stand a better chance of delivering compounds with optimised drug-like properties.\textsuperscript{19} For this approach to be successful, a detailed knowledge of the therapeutic target is necessary. Furthermore, an understanding of how a ligand interacts with its target on a molecular level is crucial for understanding its effect and designing analogues with improved pharmacological characteristics. The need to identify new drugable targets and understand their biological pathways is increasingly being met through the use of small molecules as probes for exploring protein functions. This field has been termed chemical genetics\textsuperscript{20} and is discussed below.
1.1.4 Chemical Genetics

Classical genetic screens in model organisms have proved incredibly useful in elucidating the genes that control important biological processes.\textsuperscript{21,22} Two complementary approaches are commonly employed: the first, termed forward genetics involves introducing random mutations into cells, screening the mutants for a phenotype (physiological effect) of interest, and then identifying the gene responsible; reverse genetics, on the other hand, entails introducing a mutation in a specific gene of interest and observing the phenotypic effect of this mutation in a cellular context. Despite the success of these methods, they do suffer from a number of limitations: (1) they are not suitable for investigations in mammals, whose size and slow rate of reproduction are prohibitive; (2) gene mutations cannot usually be turned on or off, restricting a thorough analysis of their effects; (3) mutations in important proteins are often lethal, preventing a study of their function.

Chemical genetics offers distinct advantages over the classical method due to the unique properties of small molecules: (1) their effect is often rapid and reversible (due to metabolism/clearing) enabling them to act as conditional alleles; (2) they can activate or deactivate their protein targets providing better information into the cause of phenotype changes. Chemical genetics should, however, be seen as a complement to the traditional approach, rather than a replacement, since the specificity of genetic mutations is unmatched by exogenous ligands at present.\textsuperscript{23} There is also the challenge of synthesising each small molecule, although this has become less of a problem with the development of combinatorial chemistry.

The power of chemical genetics lies in the ability of small molecules to perturb complex biological systems by binding to specific protein targets. With access to large numbers of compounds via combinatorial libraries, chemical genetic screens analogous to those of classical genetics can be used to identify protein targets (forward chemical genetics), or investigate the effect of ligands on a specific protein of interest (reverse chemical genetics). In the former, a library of diverse compounds is screened against an organism of interest, and those that cause a phenotypic
response selected and used to identify the protein target. In the latter, the library is screened against a specific purified protein to identify high affinity ligands, whose abilities to induce a phenotypic effect are then investigated. A classic example of forward chemical genetics is the discovery of the immunosuppressant molecule FK506 10 (Figure 1.3). The natural inhibitor of CypA, cyclosporin A (CsA) 11 (Figure 1.3), had until then been the sole treatment for preventing organ-transplant rejection. Kino et al. tested fermentation broth extracts from *Streptomyces* for their ability to mimic CsA's ability to block the production of interleukin-2. This phenotype-based screen identified FK506 10, which was found to be structurally unrelated to CsA 11. FK506 subsequently helped to elucidate the biological pathway that governs immunosuppression, thus providing a powerful endorsement of the forward chemical genetics approach.

**Figure 1.3** Structures of FK506 10 and CsA 11.

The scientific literature also contains examples of reverse chemical genetics that have yielded novel ligands for specific protein targets. For example, Komarov and co-workers identified a small molecule inhibitor of p53, whose effect was to reduce the damage to healthy tissues from anticancer treatments such as chemotherapy. In our work, we plan to explore the role of cyclophilins in the nematode parasite, *Caenorhabditis elegans*, by the use of novel small molecule inhibitors. It is hoped that inhibitors of CypA will have antiparasitic properties, but this relies on developing a clear understanding of the function cyclophilins perform in the organisms.
Chemical genetics, therefore, offers the potential to gain a greater understanding of biological pathways and uncover novel protein targets. In addition, since it relies on the use of small molecules, these can form the starting point for drug discovery programmes. In general, to be successful chemical genetics requires: (1) access to a diverse range of small molecules; (2) an appropriate HTS method; (3) accurate target identification and validation. These points are discussed below:

(1) The first requirement can be satisfied through the synthesis of focused combinatorial libraries. To ensure the structures are diverse it is often advantageous to employ structure-based design and virtual screening technologies as discussed previously. How the compounds are synthesised is decided through a retrosynthetic analysis of the suggested lead. In contrast, some groups have pursued a diversity-oriented synthesis (DOS) approach as a way to increase diversity into their library. DOS generally involves a combination of complexity forming reactions and split-pool technology to synthesise a vast array of structurally diverse small molecules for use as probes in biological systems.

(2) An efficient HTS method is essential to test the effects of library members on proteins of interest. In industry this is almost completely robotised, with assay volumes now decreased to the nanolitre level, enabling more compounds per hour to be screened at less cost. For the purposes of our research we have developed a novel \textit{in vitro} screen that uses electrospray mass spectrometry to provide a rapid indication of binding. The method can also be used to obtain a rank order for our synthetic ligands, which enables those of highest affinity to be selected for \textit{in vivo} testing. This technique and the results obtained will be discussed in depth in Chapter 4.

(3) Target identification and validation, traditionally a drawback of the chemical genetics process, have been greatly aided by the advent of new technologies such as DNA microarrays and expression profiling. The former involves monitoring the changes in gene expression of a cell, which has been modulated by a small molecule, to identify the effected gene and the subsequent target protein it encodes for. In the
latter, the pattern of gene expression changes that occurs when a ligand binds is used to produce an expression profile for the cell. Specific genes can be deleted and phenotype effects and expression profiles compared to identify whether the ligands bind specifically or promiscuously.

1.1.5 Conclusions

Drug discovery has made significant advances since the time when products derived from coal-tar or the natural world provided the only source of new medicines. Developments in molecular biology removed the traditional bottleneck of target identification and shifted the onus onto synthetic chemists involved in lead discovery. Their response was to invent new methodologies such as HTS and combinatorial chemistry. However, initially these techniques failed to live up to their promise of identifying many new lead compounds. This was largely due to a failed approach, where greater emphasis was place on quantity as opposed to quality. The need for a rational approach to drug design soon became apparent and molecular docking and virtual screening technologies played an important role in the planning of focused combinatorial libraries containing members with a high probability of interacting with protein targets. Computational methods rely heavily on pre-determined structural characteristics of the protein targets, but such information is not always readily available. In fact, it is often the case that the target itself has not been identified and/or its function remains unclear. The field of chemical genetics aims to solve these problems through the use of small molecules that can act as probes in biological systems. This approach has helped to elucidate complex biological pathways and identify previously unknown targets, with the added advantage that the structures of the small molecule modulators provide leads for drug discovery. With the sequencing of the human genome now complete, the number of potential drug targets looks set to rise exponentially. Chemical genetics (or genomics as it should now be referred) will play a valuable role in identifying the new protein targets and elucidating the possibilities for therapeutic intervention.
1.2 The Immunophilins

Cyclophilin A (CypA) and FK506 Binding Protein (FKBP) are ubiquitous intracellular proteins that have been given the generic name immunophilins on account of their ability to bind the immunosuppressive drugs, cyclosporin A (CsA) \textsuperscript{11} and FK506 \textsuperscript{10} respectively.\textsuperscript{30} CsA \textsuperscript{11} is specific for CypA, whereas FK506 \textsuperscript{10} only binds to FKBP. Despite possessing no sequence homology or apparent structural similarities both immunophilin/drug complexes target and inhibit the calcium/calmodulin dependent phosphatase calcineurin (CaN).\textsuperscript{31} Since CaN is a key regulatory species in the T-cell activation pathway, inhibition of this leads to immunosuppression. Another drug, rapamycin (RAPA) \textsuperscript{12} (Figure 1.4) was also found to complex FKBP and inhibit T-cell activation, however, unlike CsA \textsuperscript{11} or FK506 \textsuperscript{10}, its mechanism of immunosuppression does not involve the blocking of CaN.\textsuperscript{32}

\textbf{Figure 1.4} Structure of rapamycin \textsuperscript{12}.

The immunophilins are also enzymes which catalyse the \textit{cis-trans} isomerisation of prolyl amide bonds in peptide and protein substrates.\textsuperscript{33,34} This peptidyl-prolyl isomerase (PPIase) activity has important biological implications as it leads to protein folding, which in turn determines protein function. CsA \textsuperscript{11} or FK506 \textsuperscript{10}, when bound to their respective immunophilins, inhibit their PPIase activity and this was put forward as an early mechanism for immunosuppression.\textsuperscript{35} However, it was later shown that PPIase activity was not required for T-cell activation.\textsuperscript{36}
of PPIase, parvulin, was isolated from \textit{E. coli} in 1994 and is structurally unrelated to CypA or FKBP.\textsuperscript{37}

\subsection*{1.2.1 Cyclophilin A}

In 1984, Fischer isolated an 18 kDa protein from porcine kidney that was found to possess PPIase activity and, hence, became known as PPIase.\textsuperscript{38} In that same year, Handschumacher and fellow researchers purified a CsA binding protein from bovine spleen which they termed cyclophilin.\textsuperscript{39} At the time its amino acid sequence was thought unrelated to any known protein and it was not until 1989 that it was proved that PPIase and cyclophilin were identical.\textsuperscript{33} Over the past 18 years over 30 immunophilins belonging to the cyclophilin family have been discovered, with CypA the archetypal member.

\textbf{Figure 1.5} X-ray crystal structure of CypA bound to CsA 11.

The structure of CypA has been determined using NMR\textsuperscript{40} and X-ray crystallography.\textsuperscript{16} It consists of a \( \beta \)-barrel formed by eight antiparallel \( \beta \)-strands closed by two \( \alpha \)-helices at the top and bottom (Figure 1.5). Inside the barrel, six phenylalanines and a tyrosine residue orientate their side-chains together to create a very hydrophobic core. The binding site for CsA 11 is formed by two loops, which protrude out from the globular protein core, on top of two \( \beta \)-strands (Figure 1.5). It has also been confirmed as the PPIase active site.\textsuperscript{41}
1.2.2 Cyclosporin A

CsA 11 (Figure 1.3) is a cyclic undecapeptide of fungal origin that was discovered in 1973 and later shown to be a potent immunosuppressant (Sadimmune®, Neoral®).\(^ {42}\) CsA 11 binds to CypA with a dissociation constant \((K_d)\) of 46 nM\(^ {43}\) and is currently administered to organ transplant patients to prevent graft rejection. Aside from its clinical use, CsA 11 has allowed researchers investigating its mechanism of action to gain a much greater understanding of T-lymphocyte function and cellular signalling pathways.

Unlike CypA, whose structure in its free and bound states is essentially the same, the conformation of CsA 11 in solution and in the CypA/CsA 11 complex is very different.\(^ {44}\) In chloroform, CsA 11 consists of an antiparallel \(\beta\)-sheet with four intramolecular hydrogen bonds involving the four non-methylated NH groups. The amide bond between MeLeu-9 and MeLeu-10 is in the \textit{cis} conformation. However, in the bound form all four hydrogen bonds are disrupted and each amide bond is \textit{trans}.\(^ {45}\) A new intramolecular hydrogen bond between the hydroxyl group of the MeBmt-1 side-chain and the carbonyl oxygen of MeLeu-4 is also present. These unexpected discoveries gave fresh impetus to researchers’ attempts to design conformationally constrained analogues of CsA 11, in order to discover compounds with higher affinities.

1.2.3 Cyclophilin A – Cyclosporin A complex

Only CsA residues 9, 10, 11, 1, 2 and 3 make contact with CypA.\(^ {41}\) Thirteen key, highly conserved residues of CypA (Arg-55, Phe-60, Met-61, Gln-63, Gly-72, Ala-101, Asn-102, Ala-103, Gln-111, Phe-113, Trp-121, Leu-122 and His-126) are located within 4 Å of the bound ligand and define the active site of the protein. Of these, Phe-60, Met-61, Phe-113, Leu-122 and His-126 make up a deep hydrophobic pocket into which the MeVal-11 residue of CsA 11 is located. This pocket is also the binding site of the proline ring in peptide substrates. The six residues that interact with CypA are referred to as the binding domain of CsA 11. The remaining residues protrude out from the binding site and are involved in complexing to CaN.\(^ {46}\) These
residues make up the effector domain of CsA. There are five direct hydrogen bonds between CypA and CsA and five water molecules involved in mediating intermolecular interactions.

1.2.4 **Mechanism of Immunosuppression**

The mechanism by which CsA elicits its immunosuppressive effects has been extensively studied and reviewed. The CypA/CsA complex has a high affinity for the calmodulin-dependent serine/phosphatase calcineurin (CaN), exhibiting a dissociation constant of 33 nM. None of the individual components favour complexation with CaN indicating that it is the immunophilin/drug combination that is important.

**Figure 1.6** Schematic representing events in the immunosuppressive pathway.

T-cell activation begins with the stimulation of T-cell receptors, present on the membrane of a cell, by foreign antigens. This leads to an influx of calcium into the cell which results in calmodulin (CaM) binding to CaN (Figure 1.6). The CaM/CaN complex effects the dephosphorylation of nuclear factor of activated T-cells (NF-AT), a transcription factor that regulates the production of T-cell activation genes, such as interleukin-2 (IL2). Only the dephosphorylated form of NF-AT is capable of
passing into the nucleus and causing the transcription of the IL2 gene. Hence, inhibition of CaN by the immunophilin/drug complexes prevents NF-AT translocation and subsequent production of IL2.

Proof that inhibition of CypA's PPIase activity was not responsible for immunosuppression came from studies on a modified CsA derivative termed SDZ NIM 811 13 (Figure 1.7). The replacement of MeLeu-4 with Me-Ile resulted in improved binding to CypA and inhibition of its PPIase activity. The compound, however, was unable to interact with CaN and therefore did not give rise to immunosuppression.

Figure 1.7 Structure of SDZ NIM 811 13.

1.2.5 PPIase Mechanism

The majority of peptide bonds adopt the *trans* conformation due to unfavourable steric interactions between side chains when in the *cis* form. However, prolyl peptide bonds are an exception, with at least 10% found in the *cis* conformation at equilibrium. This is a consequence of the unique dialkyalted nature of proline, which reduces the energy difference between the two forms. The conformation prolylamides adopt is important in determining the structure and function of proteins. However, the rate of *cis-trans* isomerisation of prolylamides is slow on a 'biological' time scale and in some cases has been found to be rate determining. Nature overcomes this problem by using PPIases such as CypA to catalyse the *cis-trans* isomerisation of Xaa-Pro bonds (where Xaa = any amino acid). CypA is a highly
efficient enzyme and has broad substrate specificity. Structural studies have found that CypA prefers to bind substrates in the *cis* conformation despite the *trans* form dominating at equilibrium. It is thought that only *cis*-prolines can be accommodated into the hydrophobic pocket of CypA.

There have been many mechanisms proposed to explain the immunophilins' PPIase activity. However, the one now generally accepted involves catalysis by distortion. In this mechanism, the immunophilins bind the peptide substrate in a twisted amide conformation which results in a lower barrier to rotation due to a loss of amide resonance. It is thought that distortion of the amide bond is facilitated by hydrogen bonding of the prolyl nitrogen by the active site residue Arg-55 in CypA. Evidence in support of this mechanism came from the structure of CsA 11, where the MeBmt-1 side chain that is required for binding to CypA is located in approximately the same position in the active site as the amide carbonyl of a peptide substrate. Therefore, it has been suggested that the side-chain hydroxyl group may mimic the carbonyl of the peptide bond that undergoes isomerisation. In this way, CsA 11 could be binding as a transition state analogue. This view was strengthened by a similar study on FK506 10, where it was found that allylic \( A^{(1,3)} \) strain results in the ketone carbonyl lying in a plane orthogonal to the adjacent homoprolyl amide bond.

**Figure 1.8** Proposed mechanism of *cis/trans* isomerisation of prolyl peptides by CypA.
Figure 1.9 (A) Model of the transition state structure of the twisted peptidyl-prolyl amide bond. (B) Substructure of FK506 and (C) CsA that is proposed to mimic the twisted amide bond of a peptide substrate.

1.3 Other Functions of Cyclophilins

Due to their high abundance in a variety of cell types it is not surprising that cyclophilins, in particular CypA, have been implicated in a range of cellular processes besides immunosuppression. Some of the most important are discussed below.

1.3.1 Role in HIV-1

The Gag polyprotein of human immunodeficiency virus type 1 (HIV-1) is responsible for the assembly and packaging of new HIV-1 virions. Late in the
infectious cycle, Gag accumulates at the cell membrane and forms new virions that bud from the host cell. During the budding process Gag is cleaved by the viral protease to produce several proteins, including the matrix, capsid (CA) and nucleocapsid in a process called maturation. The matrix lines the viral membrane, whereas the CA protein forms a conical core structure which contains the viral RNA/nucleocapsid complex. On infecting a new host, the virion must disassemble to allow reverse transcription of the RNA genome. CA, therefore, plays a crucial role in HIV-1 replication and infectivity.

CypA has been reported to bind to Gag and become incorporated into the new virion, where it is thought to be important for maintaining the infectious nature of the disease. CypA from the host cell cytoplasm binds to the CA domain of Gag and subsequently becomes packaged into the budding virion. CsA 11 inhibits this process, which leads to virions devoid of CypA. Whilst this does not affect virion assembly or production, it has a drastic effect on viral infectivity. Although the function of CypA in viral replication is still poorly understood, it has been reported to act at an early stage of the retroviral life cycle, following receptor binding and membrane fusion but preceding reverse transcription. These findings suggest that CypA may play a role in viral CA core disassembly.

X-ray crystal structures of CypA in complex with portions of CA have been obtained and reveal a proline rich fragment of CA to bind to CypA. The Pro-90 residue of CA was found to bind in the hydrophobic pocket of CypA analogous to the proline of the tetrapeptide Suc-Ala-Ala-Pro-Phe-p-nitroanilide (AAPF), a commonly studied substrate for CypA. In addition, CA binds with a N- to C-terminal peptide chain direction, opposite to CsA 11, but the same as AAPF. These findings suggested a role for PPIase activity in HIV-1 infectivity. However, despite the similarities between the structures of bound CA and AAPF, significant differences remain. The most notable of these concerns the conformation of the prolyl amide bond: in CA it is \textit{\textit{trans}}, whereas in AAPF and all other known peptide substrates it is \textit{\textit{cis}}. Normally, unfavourable steric interactions involving the side chains of amino acids immediately preceding the proline prevent the peptides binding in the \textit{\textit{trans}} conformation.
However, glycine occupies this position in CA and its compact nature allows it to bind deeply in CypA’s hydrophobic pocket provided the *trans* conformation is adopted. The Gly-89-Pro-90 segment is highly conserved in all reported HIV-1 sequences suggesting it forms a critical recognition site for CypA. Mutation of either residue to Ala inhibits CypA packaging and blocks viral infectivity. In light of these findings it was thought unlikely that CypA’s PPIase activity was responsible for its role in HIV-1. Instead it was proposed that CypA probably acts as a molecular chaperone.\(^{52}\) However, Bosco *et al.* recently used NMR exchange spectroscopy to show that CypA is capable of catalysing the *cis/trans* isomerisation of the Gly-89-Pro-90 peptide bond, suggesting that PPIase activity may play a role after all.\(^{64}\)

There is, therefore, a need for further research in this area to ascertain the exact function of CypA in HIV-1 replication and infectivity.

Despite CsA\(^{11}\) inhibiting the effects of CypA it has been of little benefit as an anti-HIV drug. The main reason for this is that the dose required for inhibition of the CypA/CA complex is 10-100 fold higher than that required for immunosuppression.\(^{59}\) At such levels, CsA\(^{11}\) is toxic *in vivo*. There is, therefore, a strong therapeutic driving force to design inhibitors of CypA that might possess anti-HIV properties. The finding that non-immunosuppressive analogues such as SDZ NIM 811\(^{13}\) displayed potent anti-HIV activity against a number of cell lines helped to confirm that CsA’s antiviral activity was not related to its immunosuppressive action.\(^{65}\) Hence, new anti-HIV drugs do not have to be designed to interact with CaN. However, the HIV-1 virus is constantly evolving and studies have suggested that it may become insensitive to CsA\(^{11}\), similar to other primate immunodeficiency viruses which do not require CypA for replication.\(^{66}\) There may be, therefore, a limited window of opportunity to maximise the benefits of small molecule CypA inhibitors acting as anti-HIV drugs.

### 1.3.2 Antiparasitic Activity

Due to its potent immunosuppressive properties CsA\(^{11}\) is an important tool for investigating hosts’ immune response to parasitic infections. However, studies in
this area in the early eighties found CsA 11 to possess antiparasitic properties in schistosome and malaria. These unexpected findings prompted researchers to examine the nature of this effect and attempt to elucidate its mechanism of action. The antiparasitic activity of CsA 11 was later found to be widespread in both protozoa and helminth infections, although not all classes of parasite were susceptible and the observed effect was often unpredictable. For example, *Schistosoma mansomii* was found to be CsA 11 sensitive, whereas the filarial nematode, *Brugia malayi*, proved much less susceptible to CsA 11 mediated treatment. In addition, the immunosuppressive properties of CsA 11 can often complicate attempts to understand the host-parasite interaction. In the case of the protozoan *Trypanosoma cruzi*, CsA 11 actually exacerbated the infection, most likely as a result of suppressing the host's immune response.

How CsA 11 exerts its antiparasitic effects, and the reason behind the observed species specificity, has yet to be determined. However, its mechanism of action appears distinct from its immunosuppressive properties. Evidence in support of this view includes: 1) the doses required for antiparasitic effects are well below those needed to induce significant immunosuppression; 2) non-immunosuppressive analogues of CsA 11 have shown similar activity against organisms such as *S. mansomi*; 3) CsA 11 also shows antiparasitic action *in vitro*, suggesting that activity *in vivo* is not reliant on interactions with the host's immune system. Attention, therefore, turned to the intracellular targets of CsA 11, with parasitic cyclophilins the obvious candidates.

Cyclophilins have been described from a number of parasites including *Toxoplasma gondii*, *plasmodium falciparum*, *S. mansomi* and *B. malayi*. Cyclophilins of parasites that display sensitivity to CsA 11 (the first three listed) show strong similarities to human cyclophilins and possess a highly conserved region, capable of binding CsA 11. Furthermore, cyclophilins from CsA 11-sensitive parasites generally display PPIase activity, which is thought to be required for the correct folding of collagen, a crucial component of nematode cuticles. CsA 11's ability to block the PPIase activity of parasitic cyclophilins may, therefore, explain its
antiparasitic effects. CsA 11-insensitive parasites, like *B. malayi*, possess cyclophilins with significant structural differences compared to those isolated from CsA 11-sensitive species. Most notable are an eight amino acid divergent loop and a histidine residue in place of the Trp-121 in the active site. Since Trp-121 is a key recognition factor for CsA 11, its replacement may help explain *B. malayi*’s resistance to CsA 11.

The fact that multiple isoforms of cyclophilin exist in parasites suggests that they may have a range of different functions, some of which may be species specific. Understanding the role of such cyclophilins is important if new antiparasitic treatments are to be discovered. *Caenorhabditis elegans* is a free-living nematode that is genetically tractable, easily cultured and has a relatively fast reproductive cycle. It has also been found to contain no less than 11 cyclophilin isoforms, all of which possess PPIase activity. *C. elegans*, therefore, provides an ideal model for studying the function of the various cyclophilins and determining the action of CsA 11. This information will not only be of biological interest but will aid the design of new families of antiparasitic drugs.

### 1.4 Cyclophilin A Inhibitors

CsA 11 suffers from a number of serious side effects: it is toxic if administered in high doses; displays poor bioavailability due to its high molecular weight; and studying its effects are complicated by the presence of inhibitory and immunosuppressive functions. Hence, a great deal of research has been devoted over the last decade to the design of novel CsA 11 analogues which have improved binding and better pharmacological profiles.

#### 1.4.1 Modifications of CsA

Much of the work carried out into the design of CypA inhibitors has centred on the modification of CsA 11 residues, particularly those that comprise the drug’s binding domain. Kallen *et al.* investigated the structure-activity relationships (SAR) of eleven cyclosporin derivatives (Table 1.1). In general, they found that derivatives
at residues 1 or 2 exhibited diminished binding to CypA, whereas modifications of residue 4 did not significantly affect CypA binding but drastically decreased the complex’s immunosuppressive ability. These findings are in agreement with the observation that residues 1 and 2 belong to CsA’s binding domain, whereas residue 4 is associated with the drug’s effector region. In most cases, the conformation of CypA was conserved on binding to the ligand. Only in the case of Val substitution at position 2 was any change in the structure of the protein observed. This change was explained by increased bulk in the Abu-binding pocket pushing main-chain protein atoms closer together. One surprising finding in the study was the improved CypA binding observed for derivatives 209650, 209217, and 209825 respectively. The presence of a Val at position 2 had been shown to be detrimental to binding (33804), whilst the modifications at residue 3 were not shown to make any significant contacts with the protein. It was highlighted earlier that CypA is selective for the trans isomer of CsA 11, hence it may be the case that modification of certain residues results in a higher proportion of the preferred isomer being present at equilibrium. Further evidence that this may be a significant factor came from research carried out by Papageorgiou et al. which found that replacing the MeLeu-4 residue of CsA 11 with a sec-butyl group resulted in a four-fold increase in binding to CypA.78 X-ray analysis showed that the 3D structure of the ligand bound to CypA matches that of the CypA/CsA 11 complex, with the new side-chain not in contact with the protein but protruding out from the binding site into the solvent. Hence, the improved binding cannot be explained by structural differences.

Derivatives of residue 4 have been of interest to researchers as they offer a means of probing the interactions of the immunophilin/drug complex with CaN. The series of modifications of MeLeu-4 shown in Table 1.2 reveal that position 4 is very sensitive to structural changes, with branched β-carbon atoms 14 particularly disfavoured with respect to immunosuppression.79 These findings suggest that CaN has a very tight-binding pocket for MeLeu-4.
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**a)** IC₅₀ (derivative) / IC₅₀ CsA 11  
**b)** Measure of effect of suppression of IL2 production relative to CsA 11
Table 1.2

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<td>9.0</td>
</tr>
<tr>
<td>19</td>
<td>CH₂CF(CH₃)₂</td>
<td>2.1</td>
<td>7.0</td>
</tr>
<tr>
<td>20</td>
<td>CH₂C(OH)(CH₃)₂</td>
<td>0.9</td>
<td>110</td>
</tr>
</tbody>
</table>

a) IC₅₀ (derivative) / IC₅₀ CsA 11  
b) Measure of effect of suppression of IL2 production relative to CsA 11

Since the free and bound structures of CsA 11 differ significantly, Alberg and Schreiber used a structure-based design approach to design a tricyclic analogue 21 of CsA (Figure 1.10) that would more closely mimic the bound conformation. They achieved this by incorporating a bicyclic heterocycle between residues 6 and 9 of CsA 11. The tricyclic CsA derivative 21 showed a three-fold increase in affinity for CypA and was twice as effective at T-cell inhibition. The latter finding was most likely due to the conformational restraints of the derivative helping to minimise the entropy lost on binding CaN.

Figure 1.10 Structure of tricyclic CsA 21.
1.4.2 Novel Synthetic Inhibitors of CypA

Rather than attempt to modify the existing CsA 11 structure, a number of groups have turned their attention to the design of small molecules which mimic the important binding sites of peptide substrates. For example, Boros et al. used fluoroolefins 22, 23 (Figure 1.11) as mimics for the prolyl amide bond in the tetrapeptide substrate Suc-Ala-Gly-Pro-Phe-pNA.8

Figure 1.11 Fluoroolefins act as prolyl peptide mimics.

![Fluoroolefins](image)

The conformation of the Gly-Pro peptide bond was fixed by the fluoroolefin, with the (Z)-isomer equivalent to the trans configuration and the (E)-isomer corresponding to the cis configuration. CypA predominantly binds tetrapeptides in the cis form, hence, it was hoped that by conformationally constraining the ligands, improved binding to CypA would be observed. Preliminary results showed that the two (E)-isomers and one of the (Z)-isomers inhibited the PPIase activity of CypA.

Hart et al. have reported the synthesis of a (Z)-alkene cis-proline mimic by the route shown in Scheme 1.2.82 Analogous to the approach described above, the researchers conformationally locked the Suc-Ala-Ala-Pro-Phe-pNA peptide by introducing a (Z)-alkene to mimic the cis-prolyl amide bond favoured by CypA. Subsequent experiments showed that ligand 27 inhibited the PPIase activity of CypA with an IC$_{50}$ value of 6.5 ± 0.5 μM.
Wang et al. described the SAR of a number of bicyclic lactam molecules 28 (Table 1.3) that had been found to mimic the CypA bound conformation of cis-Gly-Pro. The study found that bicyclic lactams with hydrophobic groups adjacent to the lactam carbonyl 28e bound with highest affinity, whereas those with large N-alkylamide substituents 28a displayed the lowest affinity. In addition, the researchers attempted to design a transition state analogue that would mimic the twisted amide transition state of the natural substrate (Figure 1.12). It was envisaged that reducing the lactam carbonyl group to an amine functionality would afford a conformationally constrained ligand with a prolyl-like nitrogen atom that could interact with the Arg-55 residue in the active site of the protein. However, it was found that the transition state analogue 29b displayed lower affinity than its ground state equivalent 28b (Table 1.3). This disappointing result was probably due to the structure of the bound ligand failing to coincide with that of the natural substrate, preventing favourable interactions with active site residues such as Arg-55. Nevertheless, the results showed that it was possible to design small ligands with the ability to bind CypA and inhibit its PPIase activity.
Figure 1.12  Ground state and transition state analogues of prolyl peptide substrates. \(^{83}\)

![Diagram](image)

Table 1.3

<table>
<thead>
<tr>
<th>Derivative</th>
<th>(R_1)</th>
<th>(R_2)</th>
<th>(K_d) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28a</td>
<td>H</td>
<td>Bu'O</td>
<td>&gt;200</td>
</tr>
<tr>
<td>28b</td>
<td>2-CH(<em>2)C(</em>{10})H(_7)</td>
<td>Bu'O</td>
<td>17 ± 8</td>
</tr>
<tr>
<td>28c</td>
<td>CH(_2)Ph</td>
<td>BnO</td>
<td>124 ± 30</td>
</tr>
<tr>
<td>28d</td>
<td>2-CH(<em>2)C(</em>{10})H(_7)</td>
<td>BnO</td>
<td>4.7 ± 2</td>
</tr>
<tr>
<td>28e</td>
<td>2-CH(<em>2)C(</em>{10})H(_7)</td>
<td>Me</td>
<td>1.5 ± 0.05</td>
</tr>
<tr>
<td>28f</td>
<td>CH(_2)Ph</td>
<td>Bu'O</td>
<td>140 ± 32</td>
</tr>
<tr>
<td>28g</td>
<td>H</td>
<td>BnO</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Derivative</th>
<th>(R_1)</th>
<th>(K_d) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29a</td>
<td>H</td>
<td>&gt;200</td>
</tr>
<tr>
<td>29b</td>
<td>2-CH(<em>2)C(</em>{10})H(_7)</td>
<td>77 ± 18</td>
</tr>
</tbody>
</table>
Wu and co-workers employed a virtual screening approach to identify novel non-peptide inhibitors of CypA.\textsuperscript{84} They discovered the lead compound 30 (Figure 1.13), which was found to inhibit PPIase activity with an IC\textsubscript{50} value of 6 \textmu M. Various modifications of the aryl groups, central core and urea linkers were made in order to develop SAR data. Such studies uncovered a compound 31, with an IC\textsubscript{50} value ten-fold better than the original lead, which displayed neuroprotective/neurotrophic effects as a result of its interaction with cyclophilins in the brain.

**Figure 1.13** Novel non-peptidic inhibitors of CypA.

\begin{center}
\begin{tikzpicture}
\node at (0,0) {\includegraphics[width=\textwidth]{fig13.png}};
\end{tikzpicture}
\end{center}

### 1.4.3 Peptide Ligands

There have been numerous studies of CypA in complex with small proline containing peptides.\textsuperscript{85} These arose from researchers' desire to gain a greater understanding of CypA's PPIase mechanism. The CypA/peptide ligand structures have a number of common features:

1) They bind with an N- to C- chain direction, opposite to bound CsA 11.

2) The proline binds in a deep hydrophobic pocket in CypA's active site.

3) Hydrogen bonds exist between Asn-102 and the prolyl amide carbonyl and NH from the preceding amino acid. These interactions are key recognition factors.

4) A hydrogen bond exists between Arg-55 and the carbonyl oxygen on proline's C-terminal side. Arg-55 has also been postulated to form a hydrogen bond to the proline nitrogen during isomerisation.

5) The prolyl amide bonds are always found in the \textit{cis} conformation.
1.4.4. Sanglifehrins

Sanglier and co-workers screened microbial broth extracts for metabolites with affinities for CypA. They discovered an actinomycetes strain *Streptomyces* A92-308110 that produced novel macrolides, which they termed sanglifehrins. Sanglifehrin A (SFA) (Figure 1.14), the archetypal member, was found to exhibit a higher affinity for CypA than CsA but had no effect on the phosphatase activity of CaN. Nevertheless, SFA was found to block T-cell proliferation suggesting it has a different mode of action to CsA.87

![Figure 1.14 Structure of sanglifehrin A 32.](image)

1.5 Ligand Design

The previous sections have highlighted the significant role CypA and its analogues play in a number of important areas such as immunosuppression, protein folding, HIV-1 infectivity and parasitic development. Despite extensive research, however, the exact mode of action of cyclophilins in a number of processes remains to be elucidated. This field of research would benefit from the design and synthesis of novel ligands for CypA that could be used to probe its biological function and give insight into its mechanism of action. Most of the ligands designed to date, however, have been modified CsA derivatives, peptides, or complex natural products. These are not ideal candidates for *in vivo* studies due to their poor bioavailability and tendency to exert effects beyond those being studied (*i.e.* immunosuppression). In
addition, they are often challenging to synthesise. There is, therefore, a strong case for the use of small, non-immunosuppressive inhibitors of CypA that, in addition to acting as biological probes, could form the basis for the design of novel drugs to combat important diseases such as HIV-1 and parasitic infections.

Using the X-ray crystal structure of CypA, we have employed a structure-based design approach to identify novel ligands that can form strong interactions with CypA. To achieve this, an in-house in silico database mining programme LIDAEUS was used to select lead compounds, which were then modified synthetically to optimise protein-ligand interactions. The ligands were then screened using in vitro assays to determine their binding and the best inhibitors carried forward for in vivo testing. An overview of the entire process is given in Figure 1.15.

Figure 1.15 Overview of structure-based approach to ligand design.
1.5.1 Discovery of Lead Compound Dimedone

The programme LIDAEUS was used to select compounds from the Maybridge Fine Chemicals database.\textsuperscript{88} Using the X-ray crystal structure of CypA’s active site as a template, the programme docks ligands of appropriate type into the defined binding region. The generated fits are evaluated with scoring functions that account for Van der Waals, hydrophobic and H-bonding interactions. The ligand is then screened in a number of conformations to optimise favourable interactions and minimise any steric clashes. A least-squares energy minimisation is then carried out for each hit ligand in order to obtain a final ranked list of hits.

This process led to the identification of 5,5-dimethylcyclohexane-1,3-dione (dimedone) 33 (Figure 1.16) as a potential lead structure for CypA. Confirmation of dimedone 33’s viability as a lead was achieved by obtaining an X-ray crystal structure of it bound in the active site of CypA (Figure 1.17B). This revealed dimedone 33’s geminal dimethyl group binds in the hydrophobic pocket of the active site, mimicking the interactions of MeVal-11 in CsA 11 or the proline ring in peptide substrates. One of the carbonyl groups of dimedone 33 is close enough to form a H-bond with the Arg-55 residue of CypA, analogous to the MeLeu-10 residue of CsA 11 (Figure 1.17A). The dissociation constant ($K_d$) for dimedone 33 was 22 mM as determined by fluorescence binding assay (see Chapter 4). For comparison, the $K_d$ for CsA 11 was measured to be 36.8 nM by the same assay.\textsuperscript{89}

\textbf{Figure 1.16} Structure of dimedone 33.
Figure 1.17  (A) Dimedone 33 mimics two key residues of CsA 11 responsible for binding CypA; (B) X-ray crystal structure of dimedone 33 (green) and CsA 11 (highlighted) in complex with CypA.
2 Results and Discussion I

2.1 Aim

The overall aim of this project was to design and synthesise a new class of small molecule inhibitors for CypA that could be used to probe protein function and investigate protein-ligand interactions. The plan was to build upon the previous work that had identified dimedone 33 as a partial mimic of CsA, but attempt to overcome some of the drawbacks associated with the parent compound and its derivatives. Hence, conformationally constrained ligands 34 and 35 were proposed, based on the dimedone template (Figure 2.1). It was hoped that, compared to linear analogues, these would display greater affinities for CypA as a result of losing less entropy during binding. In addition, preliminary modelling studies indicated that incorporating an amide bond in the position shown would allow it to act as a mimic for the twisted-peptide bond of a bound peptide substrate. Thus, these molecules may bind as transition state analogues, which should improve their affinities.

Figure 2.1 Proposed lead compounds incorporating dimedone functionality within a conformationally rigid structure.

Since dimedone 33 is commercially available, inexpensive and easily functionalised at a number of positions it was decided to use this as the starting point for our synthetic approach. Using chiral α-amino acids as the basis for the construction of the second ring would allow for a diverse family of compounds to be synthesised. Two complementary synthetic routes were proposed. The first involved the regioselective alkylation of dimedone 33 at the 4-position using bromomethylketones.
36 derived from protected α-amino acids (Scheme 2.1). Deprotection of the amine followed by condensation with the carbonyl of dime done 33 would provide the molecules of interest 39. This route was attractive for a number of reasons: the regioselective alkylation of dime done is known in the literature; there are well-established methods for converting amino acids into their bromomethylketone derivatives; and the final condensation step should result in a favourable increase in rotational entropy.

**Scheme 2.1**

![Scheme 2.1](image)

**Scheme 2.2**

![Scheme 2.2](image)
The second approach (Scheme 2.2) involves condensation of dimedone \(33\) with an amino acid \(40\) (protected as its ester) to generate an enaminone derivative \(41\). The ester is then hydrolysed and reacted with diazomethane to generate a diazoketone \(43\). 

\(43\) can form a carbene species in the presence of a dirhodium catalyst, which can then insert into one of the adjacent C-H bonds on dimedone, thereby furnishing the final compound \(39\). Although this route is an elegant four-step synthesis to the target ligands, the uncertainty over the viability of the final step, given the other functionality present in the molecule, resulted in the first method taking precedence.

### 2.2 Alkylation of Dimedone

Berry et al. reported a procedure for the regioselective alkylation of dimedone \(33\) using a range of electrophiles (Scheme 2.3). This method involved the initial formation of a dianionic species \(44\) by treating dimedone \(33\) with two equivalents of LDA in the presence of an excess of hexamethylphosphoric triamide (HMPA). Subsequent treatment with various bromo-electrophiles led to alkylation exclusively at the 4-position. Such regioselectivity was a result of the differing reactivities of the two enolates: the enolate between the dicarbonyl being more stable, and hence less reactive, than the enolate generated by deprotonation at the 4-position.

**Scheme 2.3**

![Scheme 2.3](image)

**Table 2.1**

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Product</th>
<th>Yield/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PhCOCH(<em>2)</em>-</td>
<td>45a</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>MeO(_2)CCH(<em>2)</em>-</td>
<td>45b</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>CH(_2)CHCH(<em>2)</em>-</td>
<td>45c</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>Bu'O(_2)CCH(<em>2)</em>-</td>
<td>45d</td>
<td>52</td>
</tr>
</tbody>
</table>
Unfortunately, attempts to alkylate dimedone 33 with a range of electrophiles using Berry's conditions proved problematic. Initial attempts (Entries 1-3, Table 2.1) were poor to moderate yielding as a large number of by-products were obtained, which proved difficult to separate. These may have arisen from reaction at the 2-position or through O-acylation. In addition, the failure of the reaction with bromoacetophenone (Entry 1, Table 2.1) did not augur well for the use of other bromomethylketones as electrophiles. Berry had noted that the use of an excess of HMPA (3 eq) was critical to the reaction proceeding well. However, it was found that increasing the amount of HMPA employed in the alkylations had negligible effect on the yields obtained. In a further attempt to improve the reaction conditions, sodium iodide was added together with the electrophile in order to effect halogen exchange, thus generating a more reactive iodo-electrophile in situ. The addition of sodium iodide did not result in a significant improvement in the reaction, suggesting the reagents themselves might not be stable under the strongly basic conditions. This view was supported by the reaction involving the relatively base-stable tert-butyl bromoacetate, which afforded the desired alkylated derivative 45d in 52 % yield (Entry 4, Table 2.1). This yield was the best obtained to date and the reaction was free of many of the by-products that had complicated previous attempts. In fact, the only other compounds isolated after chromatography were unreacted starting materials. The relative success of this reaction lent credence to the argument that the forceful reaction conditions involving strong bases and hard dianion enolates may not be favoured by certain electrophiles with reactive functional groups. Hence, it was envisaged that using a softer enolate equivalent and less harsh conditions would allow for a greater variety of electrophiles to be used.

2.3 Silylation of Dimedone

2.3.1 Bis-Silyl Enol Ether of Dimedone

By synthesising the bis-silyl enol ether of dimedone 46 and carrying out alkylations using Mukaiyama-type chemistry it was felt that a greater degree of control could be exerted over the process. Direct conversion of dimedone 33 to the bis-silyl enol ether derivative 46 was attempted using a literature procedure which involved adding
dimedone 33 to a mixture of zinc chloride and triethylamine, followed by chlorotrimethylsilane (TMSCl), at 0 °C (Scheme 2.4). However, this failed to give any of the desired compound 46 and a majority of the starting material 33 was recovered. Changing the silylating reagent to trimethylsilyl trifluoromethanesulfonate (TMSOTf) failed to make a difference to the outcome of the reaction.

Scheme 2.4

\[
\begin{align*}
\text{Et}_3\text{N-ZnCl}_2, & \quad \text{TMSCl} \\
\text{or} & \\
\text{Et}_3\text{N} & \quad \text{TMSO} \\
\text{TMSOTf} & \quad \text{OTMS}
\end{align*}
\]

Torkelson and Ainsworth reported problems associated with self-condensation when trying to synthesise the bis-silyl enol ether 46 directly. Their solution was to proceed via the mono-silyl enol ether 47. This approach was followed and 47 was successfully synthesised in 95% yield by reacting dimedone 33 with hexamethyldisilazane as shown in Scheme 2.5. The mono-silyl enol ether 47 was carried forward to the next step without delay to guard against hydrolysis. 47 was dissolved in anhydrous THF and added to a solution of LDA at -78 °C via a cannula. The reaction was then quenched with TMSCl and worked-up. Distillation of the crude oil afforded the desired bis-silyl enol ether 46 in moderate yield (45%), which was stored at -20 °C under argon until required to prevent it hydrolysing back to the starting materials.

Scheme 2.5

\[
\begin{align*}
\text{33} & \quad \text{(Me}_3\text{Si)}_2\text{NH} & \quad \text{47} & \quad \text{46} \\
\text{(95\%)} & \quad \text{i) LDA} & \quad \text{(45\%)} & \quad \text{ii) TMSCl}
\end{align*}
\]
2.3.2 Reactions of the Bis-Silyl Enol Ether of Dimedone

A series of test reactions were devised to investigate the applicability of the bis-silyl enol ether 46 towards regioselective alkylations and hence, whether it could form the basis of the synthetic route to the target molecules (Scheme 2.6). The first electrophile chosen was benzaldehyde, as regioselective aldol reactions involving silyl enol ethers are very well known and studied since their development by Mukaiyama and co-workers in the seventies. The reaction proceeded in the presence of a titanium tetrachloride catalyst (TiCl₄) to afford the aldol product 48 in 17% yield. The TMS-groups were thought to have been hydrolysed during the work-up. From the NMR spectra it appeared that the reaction had occurred regioselectively, although the yield was disappointing.

Scheme 2.6

Attempts at forming the corresponding acylated product through the reaction with benzoyl chloride were unsuccessful. An examination of the NMR spectra of the major product revealed it to be the O-acylated derivative 49. There was no evidence that the 4-alkylated species had also formed. The lack of the expected product may have been a result of the bis-silyl enol ether 46 hydrolysing back to dimedone 33 during the course of the reaction. Acid chlorides are hard electrophilic centres and are likely to react preferentially with a hard nucleophilic centre, such as oxygen. A significant proportion of dimedone 33 exists in the enol form at equilibrium, thereby...
increasing the likelihood of reaction occurring at oxygen. However, alkylated derivatives such as 48 or 45d were found to be predominantly in the keto form, suggesting the ratios were very ligand sensitive. Changing the Lewis acid used (from TMSOTf to TiCl₄) had no impact on the course of the reaction.

The third and final reaction involved treating the TMS-protected dimedone 46 with methyl vinyl ketone. Silyl enol ethers are stable, neutral enolate equivalents and therefore favour conjugate addition. It was hoped that this reaction would provide a carbonyl containing alkyl chain at the 4-position that could undergo further derivatisation. However, the reaction did not proceed smoothly with a number of by-products detected by TLC. ESI-MS found the correct mass of the product 50, however a pure sample could not be isolated due to the number of other species present. In addition, the crude yield was low (14%).

In summary, while the reactions on the bis-silyl enol ether 46 showed some promise, the yields were poor and isolating pure products proved problematic. One of the main reasons for this is likely to be the lability of the TMS groups, which may undergo hydrolysis during the course of the reaction. To overcome this problem, more stable silyl-protecting groups could be used, such as triethylsilyl (TES) or tert-butyldimethylsilyl (TBS). However, the drawback of these would be the increase in steric bulk around the dimedone ring, which could hinder attack by electrophiles. In reality, alternative protecting groups were not investigated as the focus of the synthetic strategy changed due to progress made towards regioselective alkylations on dimedone analogues.

### 2.4 Alkylation of 3-Methoxy-5,5-dimethyl-cyclohex-2-enone

Whilst the regioselective alkylation of dimedone 33 proved difficult and low yielding, much greater success was achieved by alkylating a methoxy-enol ether analogue 51. 51 can be easily prepared by dissolving dimedone 33 in MeOH and treating with DIPEA and TMSCl (Scheme 2.7). The product was afforded in almost quantitative yield after work-up, without the need for further purification.
The methoxy-dimedone derivative 51 was first reacted with tert-butyl bromoacetate in a repeat of the most successful alkylation involving dimedone 33 (Scheme 2.8) (Entry 1, Table 2.2). Only one equivalent of LDA was now required due to the presence of the enol ether. The reaction proved successful (70 %) and higher yielding than the analogous one involving dimedone 33 (52 %). Optimisation of the conditions improved the yield to an excellent 90 % after column chromatography. The improved conditions involved: ensuring the reaction was carried out under scrupulously anhydrous conditions; replacing the toxic HMPA with N,N-dimethylpropyleneurea (DMPU), which could be bought anhydrous; and increasing the number of equivalents of electrophile used from 1.1 to 2. Under these conditions the kinetic enolate is favoured, ensuring alkylation always occurs adjacent to the carbonyl group. An X-ray crystal structure of the alkylated tert-butyl derivative 53a was obtained to prove this conclusively (Figure 2.2). No alkylated product arising from formation of the thermodynamic enolate was ever observed.
In the light of this promising result the reaction was repeated with a selection of electrophiles (Entries 2-4, Table 2.2). Yields were improved when compared to similar reactions involving dimedone 33 and the new conditions proved favourable to previously unreactive electrophiles (Entry 4). However, the reaction involving tert-butyl bromoacetate (Entry 1) was still the highest yielding suggesting its base stability was still an important factor. The poor yield (23 %) for the reaction involving bromoacetophenone (Entry 4) can be explained by the product 53d co-eluting with unreacted starting material, despite a range of solvents being tried. Alternative purification systems such as prep-HPLC could be used in future to acquire a greater yield for this product.

Table 2.3

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reactant</th>
<th>R</th>
<th>Product</th>
<th>Yield/%</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>53a</td>
<td>Bu'O_2CCH_2-</td>
<td>45d</td>
<td>76</td>
</tr>
<tr>
<td>2</td>
<td>53c</td>
<td>EtO_2CCH_2-</td>
<td>45e</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>53d</td>
<td>PhCOCH_2-</td>
<td>45a</td>
<td>69</td>
</tr>
</tbody>
</table>
A further advantage of using an enol ether derivative for the alkylation reaction is that it can be easily hydrolysed back to the diketone by treatment with aqueous acid (Scheme 2.9). To demonstrate this, compounds 53a, 53c and 53d were dissolved in acetone or THF and treated with 2M HCl (aq). After stirring overnight at room temperature and working-up, the three diketone derivatives 45a, 45d and 45e were obtained in good yield (Entries 1-3, Table 2.3). Importantly, the strong acidic conditions did not affect the side chains, which remained intact.

The development of a successful and robust method for the regioselective alkylation of a dimesdone analogue 51 provided the basis for an investigation into whether this method could work using amino acid derivatives as electrophiles. However, before this could be tested it was necessary to develop a route for converting amino acids into their bromomethylketone derivatives.

2.5 Synthesis and Reactions of Bromomethylketones

2.5.1 Monobromination of β-ketoesters

The most common route for synthesising bromomethylketones 36 from their amino acid equivalents 54 involves, first, activating the acid 54 as its acid chloride 55; followed by treatment with diazomethane to generate the diazoketone 56; and finally reacting this with HBr (Scheme 2.10). 

Scheme 2.10
However, a search of the literature uncovered another method, published by Hoffman et al., which was applicable to CBz-protected amino acids and avoided the use of diazomethane (Scheme 2.11). This approach was advantageous since diazomethane is toxic and potentially explosive resulting in the need for special glassware and extra safety precautions. In addition, the common diazomethane precursor, Diazald® (N-methyl-N-nitroso-p-toluenesulfonamide), was no longer commercially available and would have to be synthesised.

Scheme 2.11

Following Hoffman's procedure, CBz-L-alanine 54a was converted to the corresponding tert-butyl \( \beta \)-ketoester 57 by reaction with 1,1-carbonyldiimidazole (CDI) and subsequent treatment with the lithium enolate of tert-butyl acetate. The \( \beta \)-ketoester 57 was then dissolved in MeOH and \( N \)-bromosuccinimide added along with a catalytic amount of 2,6-lutidine as base. The result was a mixture of the mono-58 and dibrominated 59 species shown in Scheme 2.12. Surprisingly, storage of the mixture at \(-20^\circ C\) for 24 hours led to a predominance of the monobrominated material 58. The authors noted that the base was crucial for reaction to occur and postulated that it may be converting the monobrominated compound to its enolate, thereby shifting the equilibrium to the left. The crude monobrominated material was
decarboxylated by refluxing in a benzene/TFA mixture to afford pure bromomethylketone 36a after recrystallisation.

Scheme 2.12

2.5.2 Alkylations with N-CBz alanyl bromomethane

The enolate of the methoxy-dimedone derivative 51 was treated with N-CBz alanyl bromomethane 36a using the conditions described previously (Scheme 2.13). However, the reaction was unsuccessful, with only dimedone 33 and unreacted starting material being recovered after chromatography. A possible explanation for this could be that the enolate is simply abstracting a proton from the NH of the electrophile, thus reforming the starting material 51. Hence, the reaction was repeated using two equivalents of LDA in order to deprotonate the amine and form the enolate. Unfortunately, although the reaction led to a greater number of compounds by TLC, the desired product could not be isolated after chromatography. It was felt that the deprotonated amine may be undergoing side reactions leading to unwanted by-products. A way to overcome this would be to replace the CBz-protecting group with one that protects the nitrogen as a tertiary amine.

Scheme 2.13
2.5.3 Phthaloyl- and Dibenzyl-Protected Amino Acids

Phthaloyl and dibenzyl are two common bis-protecting groups for amines as they are easily attached, are unreactive to a range of reaction conditions, and can be easily removed by treating with hydrazine (phthaloyl) or hydrogenation (dibenzyl).

A two-step procedure was used to protect L-alanine 63 as its phthaloyl derivative 64 as shown in Scheme 2.14. The first step involved forming the reactive species 3-chloro-3-(dimethoxyphosphoryl)isobenzofuran-1(3H)-one 62 by treating phthaloyl dichloride 60 with trimethyl phosphite 61. Pure product 62 was obtained as a white solid after work-up, and this was reacted with L-alanine 63 in the presence of DIPEA to afford N-phthaloyl-L-alanine 64 in very good yield, without the need for further purification.

Scheme 2.14

A dibenzyl analogue was formed by refluxing L-alanine ethyl ester hydrochloride 40a with benzyl bromide in the presence of DIPEA overnight (Scheme 2.15). This reaction gave the dibenzyl ester 65 in 65% yield after chromatography, although it remained contaminated with a small amount of benzyl bromide starting material. An
improved synthesis was achieved by treating commercially available methyl-(L)-lactate 66 with triflic anhydride and 2,6-lutidine, followed by dibenzylamine (Scheme 2.16). This reaction gave the dibenzyl ester 67 in 85% yield exclusively as its (R)-enantiomer (stereoinversion occurs as a result of S_N2 attack by dibenzylamine).

Scheme 2.15

Scheme 2.16

2.5.4 Conversion of Protected Amino Acids to their Bromomethylketones

Attempts to convert N-phthaloyl-L-alanine 64 to its bromomethylketone derivative, via Hoffman’s method, proved largely unsuccessful with yields of less than 20% of final product obtained. Hence, attention was turned to the more classical diazomethane route described previously (Scheme 2.10). The diazomethane precursor, Diazald®, 69 was synthesised from the commercially available sulfonamide 68 as shown in Scheme 2.17. The product could be made successfully on a large scale and refrigerated for long periods without experiencing decomposition.

Scheme 2.17
Diazomethane was distilled as an ethereal solution by adding Diazald® 69, dissolved in ether, to a warmed mixture of potassium hydroxide solution and diethylene glycol ethyl ether. N-Phthaloyl-L-alanine 64 was activated as its acid chloride 70 and added to a cooled solution of diazomethane, which was then allowed to warm to room temperature (Scheme 2.18). After work-up, a mixture of diazoketone 71 and chloromethylketone was obtained. The latter arises from the diazoketone 71 reacting with HCl in situ. Using an excess of diazomethane should limit this problem but in practice some impurity always remained. Fortunately, the diazoketone 71 was found to be stable to chromatography, enabling a pure sample to be isolated. Treating this with HBr afforded the bromomethylketone 72 as a white crystalline solid.

Scheme 2.18

![Scheme 2.18](image)

An alternative recently published route was used to synthesise the corresponding dibenzyl-protected bromomethylketone 74. This route enabled the previously synthesised dibenzyl-ester 67 to be used directly, eliminating the need to convert it to its free acid. However, reacting the ester with dibromomethane, followed by methyllithium at −78 °C and quenching with aqueous ammonium chloride solution failed to give any of the desired bromomethylketone 74 (Scheme 2.19). Despite repeated attempts, the authors’ claims of high yields and minimum purification could
not be realised. Nevertheless, after a thorough investigation into the reaction conditions a modified method was developed which proved to be much more successful. It involved replacing the base with n-butyllithium (BuLi) and increasing the number of equivalents used from 1.8 to 4. The bromomethylketone derivative 74 was successfully formed and found to be 90 % pure by $^1$H NMR, with the only impurity identified as the aminoester starting material 67.

Scheme 2.19

2.5.5 Alkylations with Phthaloyl- and Dibenzyl-Protected Bromomethylketones

$\text{N-Phthaloylalanyl bromomethane 72 was reacted with the methoxy-dimedone}$ derivative 51 under standard conditions and an analysis of the crude product by ESI-MS found a base peak corresponding to the mass of the desired alkylated compound 75a (Scheme 2.20). However, TLC revealed a number of species present. Flash chromatography isolated a high running fraction whose $^1$H NMR spectrum contained peaks indicative of the alkylated product 75a. Nevertheless, the spectrum was not clean and included peaks which appeared to correspond to bromomethylketone starting material 72. Numerous solvent systems failed to reveal two distinct species by TLC and attempted mass-directed purification using a Waters ZMD 4000 instrument also proved unsuccessful.
Repeating the reaction using only one equivalent of the electrophile failed to aid attempts to isolate pure product. In addition, significant amounts of methoxy-dimedone starting material 51 were recovered suggesting that either it is being reformed from the enolate during reaction, or the electrophile 72 is relatively unreactive. In an effort to find conditions amenable to alkylation, reagents such as sodium iodide were added to try to form a more reactive halomethylketone in situ, but to little effect. Lewis acids, such as zinc chloride, have been known to enhance enolate alkylations but repeating the reaction with this reagent did not rectify the overall problems of numerous by-products, poor separation and low yields.

Analogous reactions with the dibenzyl-protected bromomethylketone 74 gave broadly similar results with over 50 % of methoxy-dimedone starting material 51 recovered after purification. In addition, product 75b was never detected by ESI-MS.

If the bromomethylketones were proving unreactive, this might be explained by the presence of a neopentyl centre adjacent to the site of alkylation. Whilst this did not appear to hinder the small chain ester or alkyl electrophiles, those involving bulkier groups may find the steric environment more restricting. To investigate if this was the case, the commercially available isomer of dimedone, 4,4-dimethyl-cyclohexane-1,3-dione 76, was converted to its enol ether derivatives 77 and 78 using the standard method (Scheme 2.21). The reaction was high yielding (81 %) but complicated by the fact that there were now two possible products, depending on which side the methoxy group adds to. However, it was possible to separate the isomers by flash chromatography with 3-methoxy-6,6-dimethyl-cyclohexane-2-enone 78 favoured 3:1
over its more sterically hindered neighbour 77. It should be noted that altering the position of the gem-dimethyl group was not expected to have a detrimental effect on binding, as molecular modelling studies had indicated that the hydrophobic binding pocket of CypA was flexible enough to accommodate the new structure.\textsuperscript{90}

**Scheme 2.21**

\[
\begin{array}{c}
\text{DIPEA} \\
\text{TMSCI} \\
\text{MeOH} \\
\text{MeOH} \\
\end{array} \\
\rightarrow \\
\begin{array}{c}
\text{76} \\
\text{O}
\end{array} \\
\begin{array}{c}
\text{O}
\end{array} \\
\begin{array}{c}
\text{MeOH} \\
\text{(81%)}
\end{array} \\
\begin{array}{c}
\text{77} \\
\text{Me} \\
\text{O}
\end{array} \\
\begin{array}{c}
\text{77} \\
\text{OMe}
\end{array} \\
\begin{array}{c}
\text{78}
\end{array}
\]

Repeating the alkylation reaction with the phthaloyl-protected bromomethylketone 72 and the enolate of the methoxy-isomer 77 did not afford isolable product (Scheme 2.22). Whilst the expected mass could be detected by ESI-MS, many by-products were again produced thus complicating attempts to purify. Mass directed purification on the ZMD 4000 appeared to separate fractions containing the mass of the product from those corresponding to starting material. However, the $^1$H NMR spectrum of the purified compound was complex and contained many unassignable peaks. This experiment would appear to rule out steric hindrance at the site of alkylation as the dominant factor for the failure of these reactions.

**Scheme 2.22**

\[
\begin{array}{c}
\text{OMe} \\
\text{i) LDA, DMPU} \\
\text{ii) PhthN, LBr} \\
\text{Me} \\
\end{array} \\
\rightarrow \\
\begin{array}{c}
\text{77} \\
\text{O}
\end{array} \\
\begin{array}{c}
\text{O}
\end{array} \\
\begin{array}{c}
\text{O}
\end{array} \\
\begin{array}{c}
\text{79}
\end{array}
\]

The failure of the reaction with bromomethylketones is in stark contrast to the success found when using alkyl halides or simple bromomethylesters as electrophiles. A possible explanation for this may lie with the acidity of the protons adjacent to the bromine atom of the electrophiles. In the case of bromomethylketones, these protons will be significantly more acidic than those
flanked by ester or alkyl group functionality. Hence, the enolate formed may act as a base and abstract a proton, thereby regenerating the starting material and forming a carbanion, which could decompose or undergo side reactions. The prospect of the enolate acting as a base would be less with electrophiles containing less acidic protons, suggesting why these reactions proceed in a much cleaner fashion. A possible solution would be to use an enolate equivalent such as an enamine that is only weakly basic.

2.5.6 Synthesis and Alkylation Reactions of Enaminone Derivatives

The enaminone derivative 80 can be easily formed in high yield by refluxing dimedone 33 and pyrrolidine in toluene in the presence of a Dean-Stark trap to remove water (Scheme 2.23).\textsuperscript{108} 80 can then be reacted with methyl iodide to form a quarternary ammonium salt 81. Attempts to transform this to an enamine 82 by treating with sodium hydride failed.

Scheme 2.23

Enaminone derivatives, such as 80, can be regioselectively alkylated in a similar manner to their enol ether analogues (Scheme 2.24).\textsuperscript{109,110} It was felt that the enaminone functionality might display different reactivity towards the bromomethylketones.
However, attempts to alkylate enaminone 80 with the phthaloyl-protected bromomethylketone 72 produced a mixture of compounds that could not be easily separated by flash chromatography. ESI-MS did detect the correct mass of the alkylated product 83 but an analysis by TLC appeared to indicate it ran very close to the baseline and was perhaps masked by unreacted enaminone starting material 80. Further studies using LC-MS seemed to confirm this and a solvent system was found to separate the fractions. This method was transferred to the ZMD 4000 platform, which was able to collect fractions with the correct mass, albeit, slightly contaminated with small amounts of starting material 80. However, after combining the relevant fractions and removing the solvent, an analysis of the product revealed it to be mixture of compounds. This mixture may have resulted from the instrument collecting other substances that do not ionise well in the mass spectrometer.

2.6 Summary

The problems associated with the regioselective alkylation of dimedone 33, such as poor yields, limited substrate range and a lack of reproducibility have been largely overcome by replacing dimedone 33 with its methoxy enol ether analogue 51. Bromomethylketone derivatives 72 and 74 were successfully synthesised from protected α-amino acids in an attempt to introduce varied functionality into the target molecules. However, the new optimised alkylation conditions failed with these compounds as electrophiles. Subsequent experiments have served to eliminate, or at least reduce, the notion that this was due to either steric hindrance at the alkylation site, or problems associated with free NH functionality in the amino acid derived electrophiles. The most plausible conclusion is that the protons adjacent to the
bromine group have a low enough $pK_a$ to enable the enolate to act as a base, thereby reforming the starting material and reducing product formation to a minimum. Attempts at using enaminones 80 as alternative reagents for alkylation were not successful.

Since the current synthetic strategy had not proved fruitful, despite thorough examination, attention turned to the alternative route described in Section 2.1 (Scheme 2.2). This approach proposed condensing aminoesters 40 with dimedone 33 to form enaminone derivatives 41, which could be subsequently hydrolysed and converted to their diazoketone equivalents 43. The diazoketones 43 offered scope for ring-closure via carbene mediated C-H insertion reactions. Despite reservations over the degree of control allowed by the final step, it was felt this route now merited further investigation.

2.7 Alternative Strategy Part I: Enaminones to Diazoketones

As described in Section 2.5.6, dimedone 33 readily reacts with amines under Dean-Stark conditions to form enaminone derivatives. The same is true when the amine is an amino acid. Hence, L-alanine ethyl ester hydrochloride 40a underwent a condensation with dimedone 33 to form the enaminone 41a in very good yield (Scheme 2.25). The ester group was hydrolysed by refluxing in saturated sodium bicarbonate solution.

Scheme 2.25
The knowledge gained from forming diazoketones from protected amino acids was now used to convert the acid derivative $42a$ to its diazoketone equivalent $43a$. However, this step did not prove straightforward with a number of compounds detected by TLC and no evidence of product $43a$ was found by ESI-MS. It was thought that acid chloride formation might have been hindered by side reactions involving other functional groups in the molecule. An alternative approach was to activate the acid as its mixed anhydride. This method involved treatment of a solution of the acid $42a$ with iso-butyl chloroformate in the presence of N-methylmorpholine (Scheme 2.26). The mixture was filtered and quickly added to the ethereal diazomethane solution. After work-up, a yellow oil was obtained that was shown to contain only two compounds by TLC. Flash chromatography separated the fractions as a colourless and viscous yellow oil respectively. The former, which was the major product, was found to have a mass of $m/z = 326$, with the latter having a mass of $m/z = 336$. Neither of these corresponded to the expected mass of the product ($m/z = 235$). Spectroscopic analysis revealed the presence of iso-butyl functionality in both fractions. It was proposed that this may have been caused by reaction of the amine with iso-butyl chloroformate to form a carbamate. Further NMR studies confirmed the formation of the carbamate and successfully identified the colourless oil as the $N$-protected methyl ester $84$ and the yellow oil as the $N$-protected diazoketone $85$.

**Scheme 2.26**

The formation of these derivatives was useful, not only for the progress made towards the development of a reliable route to the target compounds, but also as potential ligands for CypA in their own right. Previous work had shown
enaminone derivatives to be inhibitors of CypA so highly functionalised molecules such as 84 and 85 could contribute significantly to the understanding of protein-ligand interactions in this family.

The poor yield (13 %) of the diazoketone 85, however, was a problem. The poor yield can be explained by competition between the amino and hydroxyl groups for iso-butyl chloroformate, with any free acid present being readily methylated on exposure to diazomethane. Attempts to protect the nitrogen atom prior to the diazoketone step were not successful (Scheme 2.27). Despite trying a variety of functional groups (CBz, Boc, isobutylcarbonyl), and a range of conditions (Table 2.4), none of the desired products 86 were obtained.

**Scheme 2.27**

![Scheme 2.27](image)

**Table 2.4**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reagent</th>
<th>Base</th>
<th>Solvent</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bu'O₂CCl</td>
<td>NMM or NaHCO₃</td>
<td>H₂O/Dioxane or THF</td>
<td>Bu'O₂C-</td>
</tr>
<tr>
<td>2</td>
<td>CBz-Cl</td>
<td>NMM or NaH</td>
<td>THF or toluene</td>
<td>CBz-</td>
</tr>
<tr>
<td>3</td>
<td>(Boc)₂O</td>
<td>DMAP</td>
<td>MeCN</td>
<td>Boc-</td>
</tr>
</tbody>
</table>

The failure of these reactions was surprising, especially considering how rapidly the amine reacts under the acid activation conditions described in Scheme 2.26. It would imply that, rather than reacting with iso-butyl chloroformate directly, the amine prefers to attack the mixed anhydride formed in situ, thereby yielding the carbamate and regenerating the carboxylic acid. The conjugated nature of the amine renders it more like an amide in terms of reactivity than a secondary amine. The nitrogen’s lack of nucleophilicity would explain its failure to react directly with the range of protecting groups and suggests its reaction with the mixed anhydride must be driven by favourable transition state geometry in the molecule. Despite the problem of
chemoselectivity, the formation of the diazoketone moiety 85 was pleasing as it demonstrated the feasibility of the step. It was envisaged that using two equivalents of NMM and iso-butyl chloroformate would ensure complete conversion to the N-protected mixed anhydride, thereby eliminating the possibility of forming the methyl ester by-product 84. This reaction was tried and the yield of the diazoketone 85 did indeed increase (26 %), but not appreciably so.

An alternative approach to overcome the problem of N-alkylation would be to form enaminones containing a tertiary nitrogen atom. Standard N-protected amino acids are not suitable as the nitrogen is too deactivated to undergo condensation with dimedone 33. However, N-methyl amino acids offered greater potential. Commercially available N-methyl-L-alanine hydrochloride 87 was converted to its methyl ester 88 by treating with a mixture of thionyl chloride and methanol (Scheme 2.28). 88 was then reacted with dimedone 33 under the standard conditions to give the desired enaminone 89, but in poor yield (15 %).

Scheme 2.28

The most likely explanation for the low yield is that increased steric bulk around the nitrogen atom hinders attack on the dimedone ring. Since this was only the first step in the reaction it was not felt that the low yields could be tolerated, so attention reverted to the synthesised diazoketone 85, which could be used to test the validity of the ring-closing step.
2.8 Alternative Strategy Part II: Ring-Closure via Carbenoid-Induced C-H Insertion

2.8.1 Introduction

Carbenes 92 are important reactive intermediates that can be conveniently prepared from diazocarbonyl compounds 90 by thermal or photolytic loss of nitrogen gas (Scheme 2.29). Carbenes 92 undergo a variety of chemical reactions, such as cyclopropanation,\(^\text{113}\) C-H insertion,\(^\text{114}\) heteroatom-H insertion,\(^\text{114}\) ylide generation,\(^\text{115}\) and Wolff rearrangements.\(^\text{116}\) These processes, however, are often unselective and uncontrollable. By employing transition metal catalysts, the carbene can be trapped as a carbenoid species that exhibits much greater chemo-, regio-, and stereoselectivity.\(^\text{117}\) In general, those based on dirhodium complexes have proved the most effective catalysts (Figure 2.3).\(^\text{116}\)

Scheme 2.29

\[
\begin{array}{c}
\text{R} \quad \text{N}^+ \quad \text{N}^-
\end{array}
\]

\[
\begin{array}{c}
\text{O}
\end{array} \quad \text{H}
\]

\[
\begin{array}{c}
\text{R}
\end{array} \quad \text{N}^+ \quad \text{N}^-
\]

\[
\begin{array}{c}
\text{O}
\end{array} \quad \text{H}
\]

\[
\begin{array}{c}
\text{R}
\end{array} \quad \text{H}
\]

Figure 2.3 Structures of dirodium catalysts for carbenoid formation (axial ligands, L usually occupied by solvent).

Carbenoid-mediated C-H insertion reactions have been extensively investigated, with much of the work concentrating on intramolecular ring-closure in complex molecules.\(^\text{118}\) It has proved a powerful technique as it negates the need to activate
the site of ring-closure, an important consideration when many reactive functional
groups may be present in the molecule. Several groups have reported that five-
membered ring formation is favoured over larger or smaller ring sizes; and that the
order of reactivity of C-H sites to insertion is methine > methylene >> methyl.\textsuperscript{119,120}
However, steric factors can affect this selectivity and lead to the formation of four or
six-membered rings respectively. Often, simply changing the ligands around the
metal centre can drastically affect the chemoselectivity of the reaction. For example,
Padwa \textit{et al.} found that treating diazoketone 96 with Rh\textsubscript{2}(OAc)\textsubscript{4} \textsuperscript{93} gave
approximately a 1:1 mixture of C-H insertion 97 and cyclopropane 98 products
(Scheme 2.30)\textsuperscript{121} However, changing the catalyst to dirhodium perfluorobutyrate,
Rh\textsubscript{2}(pfb)\textsubscript{4} \textsuperscript{94}, resulted in exclusive formation of the insertion product 97. In contrast,
when the dirhodium carboxamide catalyst, Rh\textsubscript{2}(cap)\textsubscript{4} \textsuperscript{95}, was used only the
cyclopropanation pathway was followed. By including electron-withdrawing
fluorine atoms around the metal, the electrophilicity of the carbenoid was increased
resulting in a more reactive carbene species that was capable of inserting into a
tertiary C-H bond. Conversely, the introduction of electron-donating amide ligands
resulted in a less electrophilic carbenoid species, which preferentially reacted with
the nucleophilic alkene double bond.

\textbf{Scheme 2.30}

\begin{equation}
\begin{array}{ccc}
\text{96} & \xrightarrow{\text{Rh (II)}} & \text{97} + \text{98} \\
\text{Rh\textsubscript{2}(OAc)\textsubscript{4}} & 52 & : 48 \\
\text{Rh\textsubscript{2}(pfb)\textsubscript{4}} & 100 & : 0 \\
\text{Rh\textsubscript{2}(cap)\textsubscript{4}} & 0 & : 100 \\
\end{array}
\end{equation}

2.8.2 Ring-Closure of Diazoketone 85

An analysis of diazoketone 85 revealed that formation of a five-membered ring was
unlikely, raising the possibility that C-H insertion could be driven to create the
desired six-membered version exclusively. The presence of a double bond in the molecule was not considered a problem, as its involvement in a conjugated system should decrease the likelihood of it undergoing cyclopropanation reactions. The diazoketone 85 was dissolved in anhydrous DCM and added dropwise to a stirred solution of Rh₂(OAc)₄ 93 (2.5 mol %) in anhydrous DCM (Scheme 2.31). The resulting solution was stirred at room temperature overnight. A green residue was obtained after work-up, which on TLC analysis contained a mixture of compounds. ESI-MS found the correct mass for the product (m/z = 308), but the only fraction that could be isolated by chromatography that corresponded to this mass, did not give a $^1$H NMR spectrum consistent with the cyclised material. Furthermore, the spectrum was not clean, complicating attempts to identify the compound formed. The reaction was repeated with varying amounts of catalyst (from 1-5 mol %) and different reaction times but similar results were found.

Scheme 2.31

![Scheme 2.31](image)

(i) Rh₂(OAc)₄ 93, Rh₂(pfb) 94, or Rh₂(cap) 95, DCM or toluene, room temp. or ω (300W, 100 °C, 5mins)

Catalytic reactions can often be enhanced by the use of microwave radiation.¹²² Hence, diazoketone 85 was dissolved in anhydrous DCM in a microwave test-tube and Rh₂(OAc)₄ 93 added (Scheme 2.31). Bubbles of nitrogen gas were given off and the tube sealed and placed in a CEM microwave Discover unit and heated to 100 °C at 300 W for 5 mins. Flash chromatography of the crude material obtained after work-up isolated two main fractions: a yellow oil and a yellow solid. Spectroscopic analysis of the former gave inconclusive results but the correct mass (m/z = 308) was detected. Similar analysis of the yellow solid revealed the presence of iso-butyl
functionality and some peaks corresponding to dimedone signals, along with a mass of \( m/z = 250 \). These results suggested the yellow solid was a fragment of the starting material but the quality of the data was insufficient to prove this conclusively. In case DCM's poor ability to absorb microwave radiation was hindering the reaction, the solvent was replaced by toluene. Toluene was chosen as it is commonly used in the literature as an alternative to DCM for these type of reactions and, despite its relative lack of polarity, has been found to be effective at absorbing microwave radiation. However, the product obtained after work-up was found to be a similar mixture of compounds to that seen previously, suggesting the change of solvent had not made a significant difference to the course of the reaction.

Since the type of catalysts employed in these reactions can have a profound effect on the substrate's reactivity, it was decided to react the diazoketone 85 with Rh\(_2\)(pfb)\(_4\) 94 and Rh\(_2\)(cap)\(_4\) 95 respectively. Each reaction was run under standard conditions at room temperature using DCM as solvent. After work-up, both reactions gave products whose predominant mass was \( m/z = 326 \). A spot running close to the baseline using 1:1 hexane/ethyl acetate as eluent dominated both TLC's. The mass found could be explained by the substrate undergoing a Wolff rearrangement as shown in Scheme 2.32. However, an analysis of the \(^1\)H NMR spectra of both compounds did not indicate the presence of the acid derivative 102. It may be the case that some other nucleophile has quenched the ketene intermediate 101. One of the surprising features of these last two experiments is how two electronically distinct catalysts gave rise to a similar product, when the literature suggests they should have differing selectivities.

**Scheme 2.32**

![Scheme 2.32](image-url)
Since steric effects can have a significant impact on the course of carbenoid reactions, it was decided to synthesise the less sterically hindered diazoketone 106 by starting from the dimedone isomer 76. Hence, 4,4-dimethyl-cyclohexane-1,3-dione 76, was reacted with D/L-alanine ethyl ester hydrochloride 103, as shown in Scheme 2.33, to give the enamino 104. The reaction proceeded cleanly and did not give rise to any of the undesired isomer, presumably as a result of steric influences. The enamino was successfully hydrolysed to its carboxylic acid derivative 105, which was then converted to the diazoketone 106 upon activation and treatment with diazomethane. Two equivalents of iso-butyl chloroformate were again required to ensure yields were as high as possible, with the nitrogen again reacting to form the carbamate. The diazoketone 106 was dissolved in anhydrous DCM and added dropwise to a stirred solution of Rh$_2$(OAc)$_4$ 93 in anhydrous DCM. The resulting solution was stirred at room temperature overnight. After work-up, a brown solid was obtained which ESI-MS found to contain $m/z = 326$ as the base peak. No peak at $m/z = 308$ was detected. Analysis by TLC suggested the presence of a number of by-products. Repeating the reaction with Rh$_2$(pfb)$_4$ 94 gave similar results although this time the mass spectrum contained a small peak at $m/z = 308$. Attempts to purify these compounds by LC-MS were unsuccessful.

Scheme 2.33
Despite attempts to alter the selectivity of the reaction, either through changing the reaction conditions, the type of catalyst used, or the steric environment of the molecule, it appears that C-H insertion at the site required is disfavoured. The poor control over the site of C-H insertion may ultimately be due to the electronic nature of the methylene group. Ideally, the site of insertion should be able to stabilise the build-up of positive charge as the bond cleaves, however, the conjugated nature of the dimedone fragment does not allow for this. As it is both impractical and undesirable to significantly alter the structure of the molecule, it is difficult to prove this theory. It does appear, however, that the reaction is sensitive to a number of factors and controlling them is a considerable challenge.

2.9 Summary

The development of a second strategy towards the synthesis of the novel target compounds uncovered a number of important points:

- Dimedone 33 reacts readily with amino acids to form enaminone derivatives in good yields and high purity.
- The formation of the required diazoketones is achievable, albeit allowing for a side reaction involving the vinylogous amide. This side reaction in itself is synthetically interesting and warrants further investigation into the reaction mechanism.
- Since previous work had shown that dimedone-based enaminone derivatives were inhibitors of CypA, the synthesis of novel diazoketone derivatives offered scope for exploring the binding properties of this family.
- The use of carbenoid C-H insertion as a method for ring-closure was ultimately unsuccessful. Whilst the visible liberation of gas and detection of the correct product mass suggested the carbenoid intermediate had formed, its reactive nature ensured controlling the site of insertion was difficult.
- Changing the ligands around the rhodium catalyst or altering the reaction conditions did not improve the selectivity.
This chapter has served to highlight a range of important chemistry that has been developed during the search for a viable synthetic route to the target compounds. This work has led to the synthesis of a series of molecules based on dimedone 33, which contain a variety of functional groups. The plan is to test the most interesting of these against CypA.

The knowledge gained from the syntheses described in this chapter was used to design an improved synthetic strategy for the lead compounds. This approach built on the successful development of a regioselective alkylation procedure for methoxy-dimedone 51; coupled with the fact that dimedone 33 can undergo facile enaminone formation with amines or amino acids. With the chemistry in place to alkylate dimedone at the 4-position and ring-close at the 3-position, attention turned to developing substrates amenable to both processes, which would form the basis of the second ring. This research is discussed in Chapter 3.
3 Results and Discussion II

3.1 Development of Synthetic Route to Cyclised Derivatives

3.1.1 Synthesis of Acid Derivative

As described in the previous chapter, tert-butyl bromoacetate 108 was found to be the best electrophile for the alkylation of the methoxy-dimedone derivative 51. An additional advantage to the high yields and excellent purity of the alkylated compound 53a was its potential to be derivatised by hydrolysing the tert-butyl ester to the carboxylic acid 109. The hydrolysis was achieved by treating the ester with TFA in the presence of triethylsilane as a carbocation scavenger (Scheme 3.1).\(^{123}\) Importantly, the acid labile enol ether remained intact under these conditions, most likely as a result of the reaction being kept strictly anhydrous. Despite the reaction proceeding relatively cleanly, yields were lower than expected due to difficulties associated with removing excess TFA from the crude product. This problem was overcome by using zinc bromide as a replacement for TFA (Scheme 3.1).\(^{124}\) Although a large excess of the Lewis acid was required (5 eq), yields were excellent (87%) and the acid obtained after work-up required no further purification. The acid derivative 109 now provided a route for incorporating amines or amino acids into the ligands, as a means of increasing their structural diversity.

Scheme 3.1

![Scheme 3.1](image-url)
3.1.2 Incorporation of Amines

Allylamine 110 was chosen as a simple, readily available non-chiral amine with which to test the peptide coupling conditions. 110 was coupled to the acid derivative 109 using the standard EDCI-HOBt method (Scheme 3.2). Gratifyingly, this gave rise to the desired peptide 111 in good yield after chromatography. The enol ether functionality was found to be stable under these conditions, whereas the analogous diketone derivative would have been expected to undergo competing condensation reactions with the amine 110. Hence, the importance of preserving the enol ether during formation of the acid derivative became apparent.

Scheme 3.2

Now that the allyl derivative 111 had been successfully formed, the enol ether could be hydrolysed in standard fashion by treating with 2M HCl (aq) (Scheme 3.2). Two species were identified by ESI-MS after work-up: the diketone 112 and the cyclised enamimone 113. The formation of a five-membered ring provided a strong thermodynamic driving force, explaining why spontaneous cyclisation occurred during the hydrolysis reaction. However, diketone 112 remained the major product, suggesting the cyclisation process was slow. The reluctance of 112 to cyclise fully is likely to be due to the poor nucleophilicity of the amide nitrogen atom. Stirring a solution of the crude diketone 112/enaminone 113 mixture in the presence of
molecular sieves did not drive the cyclisation reaction towards completion. Increasing the reaction temperature also made little difference to the yield of cyclised material 113. It was discovered that more forcing conditions were required to ensure complete cyclisation. These conditions involved refluxing the mixture in toluene for two hours with a Dean-Stark trap to remove the water. The cyclised product 113 was obtained as a yellow solid in 29% overall yield from the enol ether 111. The structure of the cyclised product 113 was confirmed by X-ray crystallography (Figure 3.1). This compound now met the requirements of the proposed lead and had its binding to CypA tested using electrospray mass spectrometry (ESI-MS). This technique will be discussed in more depth in Chapter 4, but it is used primarily as a qualitative screen in order to identify the most promising ligands, which are then submitted for further testing. This method did not find the cyclised allyl derivative 113 to be a potent inhibitor of CypA. The failure of 113 to bind CypA was not surprising as dimedone 33 itself binds only very weakly to CypA (K_d = 22 mM) and the second ring of 113 is not very functionalised, reducing its ability to make additional contacts with the protein. However, the main aim of finding a route to the cyclised compounds had been fulfilled, and the challenge now was to develop and expand the chemistry to make it applicable to a wider range of substrates.

**Figure 3.1** X-ray crystal structure of 113.
3.1.3 Incorporation of α-Amino Acids

The incorporation of α-amino acids into the lead compounds would increase the functionality with which to probe CypA's active site. L-phenylalanine methyl ester hydrochloride 114 was used as the test compound due to it being readily available as a stock item. Reacting 114 with the acid 109 under the same conditions as before afforded the amide derivative 115 in 57 % yield as a mixture of diastereomers (Scheme 3.3).

Scheme 3.3

Fortunately, these could be separated by flash chromatography. It was not possible, however, to obtain an X-ray crystal structure of either diastereomer in order to determine their absolute stereochemistry. Both were subjected to the hydrolysis and cyclisation steps to form the desired cyclised compounds 116a and 116b in yields of approximately 10 %.

Scheme 3.4
Such low yields may have been due to cyclisation being incomplete after 2 hours. In hindsight, reflux times should have been extended to overcome the steric hindrance imposed by the bulky phenylalanine side-chain. However, the route provided enough of the compounds required for testing, and had been shown to be suitable for the incorporation of amino acids. In addition, the separation of the diastereomers 115a/b was encouraging, as the nature of the fused ring system should ensure they have significant conformational differences. It was hoped that CypA would show a preference for one diastereomer, thereby identifying a higher affinity ligand than if only the diastereomeric mixture had been screened. However, an initial screen by ESI-MS found no evidence of binding for either diastereomer. In order to understand this observation, preliminary molecular modelling studies were carried out using the known X-ray crystal structure of CypA in complex with a synthetic ligand, EM2/34 117 (Figure 3.2). This template was not ideal due to the lack of similarities between the structures of EM2/34 117 and 116. However, both contain the essential binding elements of the dimeredone moiety, whose location in the active site should be relatively conserved.

**Figure 3.2** Structure of EM2/34 117.

Hence, by overlaying the carbonyl and dimethyl functionalities of the ligands, it was possible to get an idea of where additional favourable contacts could be made. This process revealed the benzyl side-chain of ligand 116b to impart unfavourable steric bulk into the binding pocket (Figure 3.3A). The configuration of the other diastereomer 116a resulted in poor alignment of the key dimeredone residues in the active site. Interestingly, ESI-MS found the uncyclised versions to bind, indicating the importance of screening the intermediates for new lead compounds. It was not possible to accurately model these compounds, as the lack of available crystal data
on similar structures prevented their flexible side-chains from being orientated in the active site with any degree of certainty. Further binding studies on these ligands are discussed in Chapter 4.

**Figure 3.3** Molecular modelling studies on ligands 116b (A) and 113 (B).

**Figure 3.4** The use of molecular modelling to highlight structural modifications that would aid binding to CypA’s active site.

Ligand manually docked in the dimedone 33 binding pocket. Lysine and arginine side chains appear to be well placed to extend into the Abu pocket of CypA.
Modelling the allyl ligand **113** helped to confirm the hypothesis that the molecule's lack of functionality was responsible for its poor binding, as it is unable to make significant contacts with CypA's active site (Figure 3.3B). In addition to offering an insight into why the current ligands were not potent inhibitors, the modelling studies also provided options with which to improve the affinities of the molecules in question. It was proposed that incorporating lysine **118** or arginine **119** residues into the cyclised structures would improve binding by making contacts with the Abu pocket in CypA's active site (Figure 3.4).

### Scheme 3.5

![Scheme 3.5](image)

### Table 3.1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>R</th>
<th>Yield Step 1/%</th>
<th>Yield Steps 2/3 (THF)/%</th>
<th>Yield Steps 2/3 (acetone)/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Ac-NH(CH$_2$)$_4$-</td>
<td>76</td>
<td>7</td>
<td>N/A</td>
</tr>
<tr>
<td>b</td>
<td>O$_2$N-NHC(NH)NH(CH$_2$)$_3$-</td>
<td>85</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>c</td>
<td>CBz-NH(CH$_2$)$_4$-</td>
<td>97</td>
<td>Negligible</td>
<td>27</td>
</tr>
</tbody>
</table>
To test this theory, protected lysine and arginine amino acids 120a-c were successfully coupled to the acid derivative 109 (Scheme 3.5). These reactions were high yielding but it was not possible this time to separate the diastereomers obtained.

The diastereomeric mixtures 120a-c were carried through to the hydrolysis and ring-closing steps to furnish the cyclised compounds 122a-c. However, the yields for steps 2 and 3 were disappointing (Table 3.1). Changing the solvent from THF to acetone and monitoring the reactions to completion by TLC improved the yields of the hydrolysis step. This improvement was probably due to a solvent effect or a result of limiting the time the enol ethers 121a-c were exposed to acid. It was felt the diketone compounds formed during step 2 would be more hydrophilic than the starting enol ethers 121a-c resulting in product being lost in the aqueous phase during work-up. Hence, further attempts to improve the yield of the reaction involved basification and re-extraction of the aqueous phases. However, the amounts of product recovered were negligible.

Particular problems were encountered when attempting to cyclise the arginine derivative 121b. The crude diketone product from the hydrolysis step was found to be insoluble in a range of solvents, even at elevated temperatures. Such solubility issues posed particular problems for the cyclisation step, which required toluene as the solvent. The addition of a catalytic amount of acetic acid improved the solubility and allowed the cyclised product 122b to form. Yields were still low but sufficient to provide enough of 122b for testing. It was hoped that the more rigid conformation of the cyclised molecules would aid attempts to separate the diastereomers but this was not found to be the case. The exception to this was 122a, which appeared as a single diastereomer by NMR. It was assumed that problems associated with the purification of this compound had resulted in the loss of the other diastereomer.

Preliminary studies of the three new ligands 122a-c by ESI-MS revealed all three to bind CypA. These breakthrough results supported the crude molecular modelling data and justified the overall design of cyclised dimedone-based ligands as potential inhibitors of CypA. The ligands were submitted for further binding studies, which
revealed $122c$ to have the highest affinity with a dissociation constant of between 5-20 $\mu$M, as determined by fluorescence assay. This $K_d$ represented at least a 1000-fold improvement on the original lead dimedone $33$ and provided the impetus to examine the effect of $122c$ on parasitic organisms. These studies and the binding results of the other ligands are discussed in Chapter 4.

One of the problems that arose from initial testing of the cyclised ligands was their poor solubility in protic solvents, which hindered their analyses by biological assay. Although the nature of the protecting group could have a significant effect on the ligand’s ability to bind CypA, deprotection would generate a primary amine that should prove much more soluble in protic solvents. To test this theory, the CBz group of $122c$ was removed by treating a solution of the ligand in DCM with HBr in acetic acid (Scheme 3.6). Whilst ESI-MS found the correct mass for the deprotected material $123$, the $^1$H NMR spectrum was complex. The replacement of HBr with iodotrimethylsilane (TMSI) provided a better method for producing pure compound. 125 The new ligand $123$ was found to retain $122c$’s affinity for CypA whilst proving much more soluble in protic media. Therefore, $123$ was an ideal candidate for in vivo testing, which is discussed in Chapter 4.

**Scheme 3.6**

\[ \text{CBz} \text{N} \begin{array}{c} \text{H} \text{H} \text{H} \text{H} \\ \text{O} \text{N} \text{CO}_2 \text{Me} \end{array} \text{122c} \begin{array}{c} \text{HBr/AcOH} \\ \text{or} \\ \text{TMSI} \end{array} \text{H}_2 \text{N} \begin{array}{c} \text{H} \text{H} \text{H} \text{H} \\ \text{O} \text{N} \text{CO}_2 \text{Me} \end{array} \text{123} \]

### 3.1.4 Summary

A viable synthetic route to cyclised dimedone derivatives had now been established and the ability to incorporate amino acids into the structure of the lead compounds had been demonstrated. Molecular modelling was used to tailor the synthesis towards compounds that would stand a greater chance of forming complexes with CypA. Such studies led to the synthesis of novel lysine and arginine based ligands.
122a-c, which were the first molecules of their kind to show affinity for CypA. The CBz-lysine derivative 122c in particular was a potent inhibitor, with a dissociation constant 1000-fold less than dimedone 33. Removal of the protecting group from 122c did not affect the binding to CypA but did give rise to a ligand 123 with improved solubility in protic media. This was important for studies in vivo. It was now desirable to design a complementary series of ligands, which could probe what affect other structural features had on the binding to CypA.

3.2 Development of Second Ligand Series

3.2.1 Aim

One of the original driving forces behind the design of the lead compounds was that the amide carbonyl group might act as a mimic of the twisted peptide bond in prolyl substrates. It was of interest, therefore, to synthesise an analogous set of ligands, which were devoid of this carbonyl group and compare the binding of both sets. Such a comparison would help elucidate the role played by the amide carbonyl and lead to the development of structure-activity relationships (SAR).

The most straightforward route to such compounds would be to simply reduce the amide bonds of the cyclised ligands 122a-c respectively. However, the presence of a range of reactive functional groups in the molecules was suspected to have a detrimental effect on chemoselectivity. A better option was to generate the aldehyde and carry out a series of reductive aminations using amines or amino acids. This approach is discussed below.

3.2.2 Synthesis of Aldehyde Derivative

One option for obtaining the desired aldehyde 126 was to reduce the carboxylic acid derivative 109 to the alcohol 124 and then re-oxidise back to the aldehyde. However, the common reducing agents for the process, such as LiAlH₄ or borane, were unlikely to be compatible with the enol ether functionality. A recent report in the literature suggested forming an activated derivative using the peptide-coupling
agent, benzotriazol-1-yloxytrpyrrolidinophosphonium hexafluorophosphate (PyBOP), in the presence of DIPEA.\textsuperscript{126} It was reported that the HOBT ester formed \textit{in situ} could be easily reduced to the alcohol by treating with sodium borohydride (NaBH\textsubscript{4}). This reaction was attempted on the acid derivative 109, but no evidence of product 124 was found after work-up (Scheme 3.7). TLC analysis revealed the presence of a number of compounds, so the reaction was not taken any further.

\textbf{Scheme 3.7}

An alternative procedure was to start from an alkylated alkene derivative 53b, which could then be subjected to ozonolysis to form the aldehyde 126.\textsuperscript{127} This route was appealing as it built upon the familiar, robust chemistry that had been developed for the regioselective alkylation of methoxy-dimedone 51. The commercially available 3,3-dimethylallyl bromide 125 was chosen as a suitable electrophile as it had the advantage that the acetone by-product from the ozonolysis step would be volatile. Methoxy-dimedone 51 was successfully alkylated with this reagent under the standard conditions to give the product 53b in 81\% yield (Scheme 3.8).

\textbf{Scheme 3.8}

53b was then treated with ozone, in the presence of pyridine and Sudan red 7B as indicator for 8 hours (Scheme 3.9), after which time the red colour still persisted. Nevertheless, the mixture containing the ozonide was poured onto zinc dust and
acetic acid added. The aldehyde 126 was obtained in 11% yield after work-up and purification by flash chromatography.

Scheme 3.9

![Scheme 3.9](image)

The low yield can be partly explained by the recovery of a significant amount (28%) of the starting alkene 53b. Ozone generation appeared inefficient as represented by the long reaction time and failure of the indicator colour to pale completely. Since overcoming this problem proved difficult, an alternative oxidation protocol involving osmium tetroxide was pursued. This method involved treating the alkene derivative 53b with osmium tetroxide (as a solution in tert-butanol), in the presence of N-methylmorpholine N-oxide (NMO) for 2 hours (Scheme 3.10). After work-up, ESI-MS found the crude product to contain a peak matching the mass of the expected diol 127. 127 was reacted directly with sodium periodate (NaIO₄) to afford the aldehyde 126 in 41% overall yield, after work-up and purification by flash chromatography.

Scheme 3.10

![Scheme 3.10](image)
The yields were improved considerably by suspending the NaIO₄ on silica gel before adding a solution of the diol 127 in DCM dropwise. This method allowed the aldehyde 126 to be obtained in yields of up to 96% after purification by Kugelrohr distillation. A common problem with aldehydes is their tendency to degrade on storage, however, it was found that 126 could be refrigerated for weeks without any indication of degradation by NMR. The aim now was to investigate whether reductive aminations on the aldehyde 126 were possible.

3.2.3 Reductive Amination Reactions

The classical and most common procedure for forming secondary amines is reductive amination. Hence, it was the method of choice for incorporating amines into the dimedone derivatives. Allylamine 110 was again used to test the reaction conditions, which generally involved mixing the amine 110 and aldehyde 126 in the presence of catalytic acetic acid, followed by the addition of sodium cyanoborohydride (NaCNBH₃) (Scheme 3.11). However, despite repeated attempts, none of the desired product 129a was isolated. ESI-MS found peaks at m/z = 275 and 300 but it was not apparent what these corresponded to. The reaction often produced mixtures of products and the fractions isolated after chromatography had complicated and unassignable NMR spectra. A search of the literature revealed additives such as zinc chloride (ZnCl₂) or sodium acetate (NaOAc) could enhance reductive amination reactions but this was not found to be the case for our aldehyde 126. To check that the problem did not rest with allylamine 110, the reactions were repeated with benzylamine 128 (Scheme 3.11). However, similar problems, such as a lack of product formation and the presence of many by-products were encountered.

Scheme 3.11

\[
\text{R} = \begin{cases} 
\text{a) } \text{CH}_2, & \text{b) } \text{Ph} \\
\text{110} & \text{128}
\end{cases}
\]
These results forced an examination into whether imine formation was occurring. A stirred solution of the aldehyde 126 and allylamine 110 over molecular sieves was monitored by ESI-MS (Scheme 3.12). Mass spectrometry did not reveal the imine 130 to be present, but since most imines are relatively unstable, it may be that they are not preserved under the electrospray conditions.

Scheme 3.12

Alternatively, imine formation may require more forceful conditions; hence, allylamine 110, aldehyde 126 and catalytic acetic acid were refluxed in DCM for 3 hours (Scheme 3.13). Again, the imine 130 was not detected by ESI-MS, but subsequent reduction of the mixture with NaCNBH₃ produced some of the alcohol 124. NaCNBH₃ should not reduce aldehydes in preference to iminium ions, hence the presence of the alcohol 124 would suggest that imine formation is disfavoured under these conditions.

Scheme 3.13

The reaction was repeated with benzylamine 128, which, due to its higher boiling point, was used in conjunction with toluene as the solvent. A Dean-Stark trap was fitted to remove the water, but there was still no evidence of imine formation. After adding the reducing agent and working up, a brown oil was obtained, which did not
appear to contain any of the foreseen alcohol 124. However, there was again no trace of the desired product 129b and the material stuck to the baseline on TLC, complicating attempts to purify.

Reports in the literature suggested the use of titanium isopropoxide in combination with sodium borohydride as an effective means of reductive amination. The worry with using a stronger reducing agent was the tendency for the enol ether to react, but in light of the failures experienced so far it was felt worthwhile to try this method. The aldehyde 126 and benzylamine 128 were stirred in the presence of the Lewis acid overnight (Scheme 3.14), at which point a sample was taken for ESI-MS. Mass spectrometry detected a peak with the correct mass for the imine 131, the first time this had been observed. Sodium borohydride was added and the reaction stirred at room temperature for 8 hours.

Scheme 3.14

However, ESI-MS analysis of the crude product obtained after work-up failed to reveal a peak corresponding to the mass of the amine derivative 129b (m/z = 288). Instead, a peak at m/z = 272 was visible, which could be explained by the structure 133 shown in Scheme 3.15. 133 would arise from amine 129b undergoing intramolecular cyclisation to form the iminium species 132. In the presence of sodium borohydride, this would be reduced to the tertiary amine derivative 133.
Unfortunately, it was not possible to obtain a viable NMR spectrum to prove the existence of this compound. Attempts to purify the crude product were unsuccessful, mainly due to poor detection by TLC.

Scheme 3.15

The difficulties encountered with the reductive amination reactions were surprising, considering the relative simplicity of the reagents. From the experiments described above, it appears imine formation involving the aldehyde 126 is generally disfavoured. In the few cases where the presence of the imine was detected, the reaction tended to give rise to a number of by-products. The use of more forcing conditions in order to drive imine formation were also unsuccessful. It had previously been noted that the aldehyde 126 appeared stable over long periods and could even be subjected to column chromatography and emerge intact. Since aldehydes are generally reactive and unstable species, this suggests some feature of the molecule is conferring stability on the aldehyde fragment. The $\alpha,\beta$-unsaturated ketone of dimedone is in close proximity to the aldehyde functionality and a hydrogen bond between them would form a favoured six-membered ring. The oxygen atom would be particularly electron rich due its conjugation with the lone pair on the methoxide group, making this hypothesis more plausible. It could be imagined that this structure would confer a high degree of stability on the molecule and could hinder attack at the aldehyde by nucleophiles. In addition, attack at the
methoxide position on the ring may be favoured due to its greater electrophilic character. However, NMR and IR studies were not conclusive in proving the existence of a hydrogen bond between the aldehyde and ketone. Also, since the aldehyde 126 was a liquid at room temperature, it was not possible to obtain an X-ray crystal structure to reveal any hydrogen bonding present in the molecule.

To further investigate if hydrogen bonding between the aldehyde and ketone was the reason for the former's lack of reactivity, a second aldehyde derivative 136 was synthesised (Scheme 3.16). It was felt that the incorporation of an extra CH₂ group into the aldehyde chain might deter hydrogen bonding, as adopting a seven-membered ring conformation should be less stable. Alkylating methoxy-dimedone 51 with commercially available 5-bromo-2-methyl-2-pentene 134 gave rise to the desired alkene derivative 135 in 13% yield. The low yield can be explained by the use of only 1.1 equivalents (instead of 2) of the electrophile, which resulted in the recovery of methoxy-dimedone starting material 51 at the end of the reaction. Nevertheless, there was a sufficient quantity of the alkene 135 to take forward to the next step. Conversion to the aldehyde 136 was achieved by using the osmium tetroxide/NaIO₄ method, which gave the product 136 in 70% overall yield.

Scheme 3.16
The reductive amination of 136 with allylamine 110 and NaCNBH₃ (Scheme 3.17) afforded an orange oil, which contained a peak in the mass spectrum representative of the mass of the desired product 137. Evidence of the correct mass was encouraging, as the secondary amine product had never been detected using the other aldehyde 126. However, the crude product was found to run close to the baseline by TLC, even in polar solvent systems, which complicated attempts to purify. Flash chromatography isolated the main fraction as an off-white oil but the ¹H NMR spectrum contained many unassignable peaks and appeared to be a mixture of compounds. Due to progress made elsewhere and a lack of material, the reaction was not repeated. Nevertheless, it offered the first potential evidence of success in these reactions and hinted that the problems previously encountered were associated with the nature of the aldehyde 126.

Scheme 3.17

3.2.4 Methods to Overcome Problems Associated with Unreactive Aldehyde

Two alternative methods presented themselves as a means of overcoming the problems associated with the unreactive aldehyde. The first of these involved reducing the aldehyde 126 to the primary alcohol 124, and then carrying out a series of nucleophilic substitution reactions involving amines. The first step was achieved by treating a solution of the aldehyde 126 in anhydrous MeOH with solid-supported borohydride reagent (Scheme 3.18). Filtration and several resin washes afforded the alcohol 124 in 91 % yield after removal of the solvent. The ¹H NMR spectrum was clean and revealed no evidence of enol ether hydrolysis, implying that the system was tolerant of the stronger reducing agent. The alcohol was then activated as its mesylate derivative and treated with benzylamine 128 in the presence of a catalytic
amount of cesium carbonate (Scheme 3.18). An analysis of the crude product obtained after work-up revealed no presence of the desired secondary amine 129b. The major component of the crude was isolated and characterised as the furan derivative 138.

Scheme 3.18

In light of this, it was decided to convert the alcohol to its bromide equivalent to facilitate S_N2 reactions with the amine. Hence, the alcohol 124 was reacted with phosphorus tribromide at 0 °C (Scheme 3.19). This reaction did not give rise to any of the alkyl bromide 139, but instead produced the furan 138 as the major product. Whilst this is an interesting molecule to test against CypA, it appeared to be a major hindrance to using nucleophilic substitution as a means of forming the secondary amines.

Scheme 3.19

The second approach to resolving the issues related with the aldehyde 126, was to hydrolyse the enol ether back to the diketone, thereby eliminating a potential source...
of the poor reactivity. Hydrolysis was achieved in standard fashion by treating a solution of the aldehyde 126 in acetone with 2M HCl (aq) (Scheme 3.20). The correct mass for the diketone was detected after work-up, but the $^1$H NMR spectrum was not straightforward. Further analysis revealed the diketone had rearranged to the hemiacetal 140, which was present as the sole product. Exclusive formation of 140 was not surprising as the diketone exists predominantly as its enol tautomer and will therefore favour attack on the aldehyde. The formation of the hemiacetal was not a serious problem, however, as it was expected to be able to undergo reductive aminations.

Scheme 3.20

To test if this was the case, the hemiacetal 140 was reacted with benzylamine 128 in the presence of molecular sieves for 2 hours (Scheme 3.21). A sample of the reaction mixture was tested by ESI-MS and found to contain the imine 131. NaCNBH$_3$ was then added, the pH adjusted with acetic acid, and the mixture stirred for a further 3 hours at room temperature. The crude product obtained after work-up did not contain a peak in the mass spectrum for the expected secondary amine, but instead was shown to contain a mass which matched that of the fully cyclised compound 141. The presence of 141 was confirmed by NMR, after purification by flash chromatography. The successful synthesis of 141 was a significant result as not only had the problems associated with the reductive aminations been overcome, but a route had been found that led directly to the cyclised compounds of interest. On reflection, the tendency of the molecules to ring-close was not surprising, as they would be subject to a strong thermodynamic driving force once the secondary amine had formed. The reaction also demonstrated the increased nucleophilicity of the amine functionality compared to its amide equivalent.
3.2.5 Scope of Reductive Aminations on Hemiacetal

To test the robustness of the newly discovered reductive amination method, the reaction on the hemiacetal 140 was repeated with allylamine 110 (Scheme 3.22). The same conditions as before were used and the imine and cyclised compounds were again detected by ESI-MS. The correct product 142 was isolated by column chromatography but appeared contaminated by a small amount of unidentified impurity. Attempts to remove the impurity by re-columning using other solvent systems or by distillation were not successful. This problem was not encountered in the analogous reaction with benzylamine 128, so it is most likely associated with a side reaction involving allylamine 110. The reaction was repeated but purified by Kugelrohr distillation as opposed to chromatography. Distillation failed to isolate either the product 142 or impurity but gave a dark oil which was identified as the higher oxidation state product 143 by NMR.
143 may have formed under the high temperatures employed during purification or may have resulted from the enamine cyclising to form the conjugated product. Nevertheless, the synthesis of a new cyclised compound was pleasing as it increased the range of structures available for testing. Further attempts to obtain a pure sample of 142 were not made as the onus shifted on to testing the method with amino acids as this would lead into the fully functionalised derivatives.

Scheme 3.23

A solution of the hemiacetal 140 and Z-L-lysine methyl ester hydrochloride 120c in DCM were stirred at room temperature for 6 hours (Scheme 3.23), but no trace of the imine was detected by ESI-MS. It was presumed this was due to the amino acid being unreactive whilst present as its hydrochloride salt. Hence, DIPEA was added to generate the free amine and stirring was continued overnight. ESI-MS now showed the imine to be present, so NaCNBH₃ was added and the pH adjusted with acetic acid. After stirring for a further 24 hours, the reaction was worked up and the crude product shown to contain the cyclised derivative 144 by ESI-MS. Purification by flash chromatography afforded the required compound 144 in 56 % yield as a mixture of diastereomers. The success of this method with an amino acid was satisfying as it achieved the aim of synthesising an analogous ligand to the lead 122c.
that was devoid of amide functionality. The fact that the cyclised compound 144 was obtained in a single step (neglecting the synthesis of the hemiacetal) was particularly beneficial as it avoided the potentially problematic and low yielding ring-closing conditions. 144 could be deprotected with TMSI to afford the free amine 145 for comparison with the analogous amide containing ligand 123.

3.2.6 Summary

A new series of lead compounds had now been synthesised that contained a tertiary amine instead of an amide functional group. The route to such compounds involved the synthesis of an alkylated aldehyde derivative 126, obtained in excellent yield, which then rearranged to a hemiacetal 140 upon treatment with HCl. 140 was found to undergo a range of reductive amination reactions, which failed when started from the aldehyde 126 itself. Satisfyingly, these reactions gave rise to the cyclised products directly, avoiding the need for a separate ring-closing step. This method proved applicable to both amines and amino acids, and provided an interesting set of molecules for testing.

3.3 Use of Hydrazine to Demonstrate Six-Membered Ring Formation

In order to further examine which features of the cyclised lead compounds were crucial for binding CypA, it was necessary to increase their structural diversity. Since the original design had allowed for the incorporation of a second six-membered ring, it was worthwhile to see if the existing chemistry could be adapted for this purpose. Hydrazine was chosen as a simple, inexpensive and readily available molecule with which to perfect the chemistry. It was envisaged that substituted hydrazine derivatives could be used in future to synthesise more functionalised molecules.

Hydrazine is significantly more nucleophilic than other primary amines due to the contribution of the lone pair on the adjacent nitrogen atom, the so called \( \alpha \)-effect. Hence, standard peptide coupling conditions cannot usually be used, as di-addition
frequently occurs. It is more common to form hydrazides by reacting hydrazine with esters. The alkylated tert-butyl derivative 53a cannot be used for this purpose as the ester group is base stable and is therefore unreactive towards hydrazine. However, it was thought the equivalent ethyl ester derivative 53c would be more applicable. 53c had previously been synthesised in 61 % yield as a test ligand for the alkylation reaction (Entry 3, Table 2.2). Hydrazine was added dropwise to a solution of the ester 53c in ethanol and the reaction stirred at room temperature overnight (Scheme 3.24i). TLC indicated only starting material was present, so the reaction was heated under reflux for 18 hours (Scheme 3.24ii). However, this failed to produce any of the hydrazide 146, with the ester 53c recovered after work-up. More forceful conditions involving refluxing in toluene with a Dean-Stark trap were tried (Scheme 3.24iii), but again the ester 53c proved unreactive. Finally, microwave heating was employed, with ethanol as the solvent (Scheme 3.24iv). The reaction was held at a temperature of 140 °C for 15 minutes, but ESI-MS revealed only the presence of the ester 53c. TLC showed some baseline material but this produced a complicated NMR spectrum after purification and was most likely a by-product.

Scheme 3.24

\[
\begin{align*}
\text{Conditions: (i) EtOH, room temp; (ii) EtOH, reflux; (iii) toluene, reflux; (iv) 300W, 140 °C, 15 mins)}
\end{align*}
\]

The failure of the reaction with the ester derivative 53c led us to focus attention on the acid analogue 109 as an alternative reagent. The acid was activated in standard fashion with EDCI and HOBt (Scheme 3.25). Once TLC had shown the complete disappearance of the free acid, the solution containing the activated species was added dropwise to a solution of hydrazine in DCM at 0 °C. The high nucleophilicity of hydrazine was expected to facilitate a quick reaction, however, none of the hydrazide 146 was detected after work-up. Extending the reaction times also failed to give rise to the desired product 146. As an alternative activation protocol, the acid
derivative was converted to the acid chloride, which was then slowly added to hydrazine at 0 °C. This method gave a poor overall yield and a number of compounds by TLC. These findings suggested that hydrazine was undergoing side reactions such as di-addition, implying the reaction conditions had to more carefully controlled. To achieve this, the reaction with EDCI and HOBt was repeated but with the hydrazine solution cooled to −20 °C. The reaction was allowed to warm slowly to room temperature and then quenched with water. A yellow solid was obtained in good yield (58 %) after work-up, and was correctly identified as the hydrazide 146.

**Scheme 3.25**

146 was of sufficient purity to be used directly in the next step, hydrolysis of the enol ether (Scheme 3.26). It was feared that the presence of a primary amine may make this step problematic and as anticipated, a number of species were detected by TLC at the end of the reaction. Purification by flash chromatography isolated a single pure fraction as an off-white solid, but analysis by NMR did not reveal it to be the expected diketone derivative. The NMR spectra suggested that the compound had cyclised but the retention of a peak corresponding to the methoxy group was puzzling. In combination with ESI-MS (m/z = 208), the compound was eventually identified as the ring-closed structure 147.

It appeared that under acidic conditions, formation of the imine was favoured kinetically over the hydrolysis of the enol ether. It was envisaged that prolonged exposure to acid would result in 147 rearranging to the more stable enaminone derivative, but in practice this led to a number of by-products. This reaction requires further attention but it served to demonstrate that the synthetic route could be adapted to accommodate hydrazides and subsequent six-membered ring functionality. Future work in this area could involve the use of substituted hydrazides that could offer potential for further derivatisation.
Scheme 3.26

3.4 Route to Enantiomerically Pure Ligands

Chemistry had now been developed that allowed for the synthesis of a diverse range of cyclised ligands. It had proved reliable for the incorporation of amines or amino acids and had been tailored to meet the requirements for successful reductive amination reactions. The chemistry had also been shown to be flexible enough to manage the construction of six, as well as five-membered ring systems. However, a potentially serious drawback with the methods used to date was their lack of stereocontrol. The use of racemic carboxylic acid 109 or aldehyde 126 derivatives inevitably led to mixtures of diastereomers when reacted with chiral amine or amino acid starting materials. Whilst it was true that the diastereomers could sometimes be separated by chromatography, this was structure dependent and not very reliable. Human proteins are often very selective for a particular substrate conformation and can readily distinguish enantiomers. With one chiral centre present at a bridgehead carbon atom in the cyclised ligands, the two diastereomers were likely to have significant conformational differences. With these two points in mind, it was felt there was a strong possibility of CypA favouring one diastereomer over the other. Hence, a method was sought that would provide the lead compounds as single diastereomers.

3.4.1 Resolution of the Carboxylic Acid Derivative

A chemical resolution of the carboxylic acid derivative 109 appeared to offer the best opportunity for obtaining an enantiomerically pure synthetic route to the lead compounds. The acid derivative 109 had the advantage of being readily synthesised in large quantities and the potential to be resolved via chiral salt formation. Based on
similar work reported in the literature, enantiomerically pure \( \alpha \)-methyl benzylamine was chosen as the basic component.\(^{138}\) Both enantiomers of this are commercially available and relatively inexpensive. The racemic acid derivative \( 109 \) was dissolved in isopropyl alcohol (IPA) and one equivalent of \((R)-(+)\)-\( \alpha \)-methyl benzylamine \( 148a \) added (Scheme 3.27). After a few minutes a precipitate formed, which was filtered and recrystallised twice from IPA. The salt \( 149a \) was then hydrolysed back to the free acid by dissolving in EtOAc and washing with 1M HCl (aq). After drying the organic phase and removing the solvent, the acid derivative \( 150a \) was found to have an ee = 73 % by chiral HPLC. This result was encouraging as it appeared that the method was working, so the above process was repeated on the enantiomerically enriched acid and the material obtained at the end found to have an ee = 97 %. This value was acceptable for \( 150a \) to be used in the amino acid coupling step.

The above process was repeated using the other enantiomer of \( \alpha \)-methyl benzylamine \( 148b \). After two cycles this gave rise to the other acid enantiomer \( 150b \) with an ee = 95 %. Although the yields (\(~15\%) were moderate at best, the resolution could be carried out on a large enough scale to provide a sufficient amount of material for subsequent reactions. However, it was still necessary to identify the configuration of the chiral centre, hence crystals were grown of the salt \( 149a \) formed from the reaction with \((R)-(+)\)-\( \alpha \)-methyl benzylamine \( 148a \). Since the chirality of the amine was unambiguous, X-ray crystallography was used to identify the complexed acid as the \((R)\)-enantiomer \( 150a \) (Figure 3.4). It logically follows that \((S)-(+)\)-\( \alpha \)-methyl benzylamine \( 148b \) must be selective for the \((S)\)-enantiomer \( 150b \) of the acid. Both single enantiomers of the acid could now be coupled to chiral amino acids to obtain single diastereomers as products.
Scheme 3.27

\[
\begin{align*}
(R)-(+)-\alpha\text{-methylbenzylamine } 148a & \quad \text{IPA} \\
(\alpha)-(-)-\alpha\text{-methylbenzylamine } 148b & \quad \text{IPA}
\end{align*}
\]

Figure 3.4 X-ray crystal structure of salt formed between (R)-(+)-\alpha-methyl benzylamine 148a and acid derivative 109.
3.4.2 Synthesis of Enantiomerically Pure Cyclised Derivatives

Z-L-Lysine methyl ester hydrochloride 120c was chosen as the amino acid for the peptide-coupling step, as the cyclised ligand 122c based on this compound had been found to be a potent inhibitor of CypA. Any selectivity of one diastereomer in preference to the other by CypA should become evident from an analysis of the dissociation constants. The amino acid 120c was coupled to both (R)- and (S)-enantiomers 150a and 150b of the acid using the standard method (Scheme 3.28). After work-up and purification by flash chromatography, the peptide products were obtained as single diastereomers in yields of 69 % (S,R) (151a) and 78 % (S,S) (151b) respectively.

Scheme 3.28

These were then carried forward to the hydrolysis and cyclisation steps. However, the products isolated by chromatography after the final step were found to have undergone epimerisation at the bridgehead carbon. Whilst the ratios were better than 1:1, this was still a disappointing result. It was initially felt that the acid hydrolysis step had caused the epimerisation but repeating this reaction on 151b and isolating the products revealed the small amount of cyclised compound 153b present to be a single diastereomer (Scheme 3.29).
Scheme 3.29

\[
\begin{align*}
\text{CBz} & \text{N} - \text{CH} - \text{CO}_2\text{Me} \quad 151b \\
\text{H} & \text{CO}_2\text{Me} \quad 152b \\
\text{H} & \text{O} < > 2\text{M HCl (aq)} \\
\text{H} & \text{CO}_2\text{Me} \\
\text{CBz} & \text{N} - \text{CH} - \text{CO}_2\text{Me} \quad 153b
\end{align*}
\]

Hence, it became apparent that the epimerisation had occurred under the vigorous conditions employed during the final ring-closing step. As described in Section 3.1.2, refluxing in toluene had been the only successful method discovered for this process but more research may be needed in future to try and find an alternative means of ring-closure. Nevertheless, by isolating the cyclised ligands after the hydrolysis step, a sufficient quantity of each diastereomer 153a and 153b could be obtained for testing.

3.5 Conclusions

This chapter has served to highlight the development of a novel ligand series for human CypA. The key achievements were:

- The successful, large-scale synthesis of a methoxy-dimedone based carboxylic acid derivative 109 in excellent yield and high purity.
- The successful coupling of both amines and amino acids to the acid derivative 109, which increased the molecules’ structural diversity.
- The development of hydrolysis and ring-closing conditions that afforded the target cyclised ligands, albeit in low yields.
• The use of molecular modelling to alter the structure of the lead compounds to provide high affinity ligands for CypA, the first of their kind based on the cyclised structure.

• The synthesis of a complementary series of ligands, which were devoid of amide functionality, by a novel route that overcame problems encountered with reductive amination reactions.

• The incorporation of hydrazine into the cyclised structures to demonstrate six-membered ring formation was possible via this route.

• The modification of the synthetic route to afford enantiomerically pure ligands, by successfully resolving the acid derivative 109 using chiral amine salt formation.

3.6 Suggestions for Future Work

The synthetic route’s ring-closing step requires some development in order to improve yields and make it more applicable to a greater range of starting materials. In addition, the step’s current tendency to induce epimerisation would have to be overcome if any subsequent method is going to prove useful at synthesising single diastereomers.

Whilst the incorporation of hydrazine had demonstrated the route’s ability to incorporate a second six-membered ring into the lead compounds, the hydrazine derivative 147 did not leave much scope for further derivatisation. N-Stabilised carbanions, formed from species such as 154, could be used as alternative reagents for constructing the second ring system. It could be envisaged that nucleophilic attack by the carbanion on the activated acid species 155, followed by deprotection and cyclisation would generate a functionalised six-membered ring system 157 (Scheme 3.30).
The success of the resolution of the carboxylic acid derivative 109 offers a potential route towards the analogous enantiomerically pure aldehyde via a two-step reduction and oxidation protocol. Obviously, conditions would have to be carefully monitored to ensure that no racemisation occurs but if achievable, it would allow for the synthesis of the second ligand series as separate diastereomers.

Work carried out previously within our group has revealed that the presence of allyl functionality attached to dimedone 33 can confer high affinity for CypA. Hence, it would be interesting to observe what effect the incorporation of an allyl group adjacent to the dimethyl of the dimedone ring would have on the binding of the cyclised ligands. However, synthesising such a molecule is unlikely to be trivial. The easiest approach would be to carry out a regioselective alkylation with allyl bromide 158 on a single diastereomer i.e. 153b to provide 159 (Scheme 3.31). However, the strong basic conditions may not be tolerated by the other functional groups present in the molecule.
Scheme 3.31

An alternative strategy would be to synthesise the alkylated allyl derivative 160, hydrolyse back to the diketone 161, then reform the enol ether in the hope that a mixture of isomers 162 and 163 would be formed (Scheme 3.32). These could then be separated and the minor one 162 used as the basis for the synthetic route to the cyclised compound 159. Notable drawbacks of this method are: 1) the generation of a large number of diastereomers; 2) the need to carry out the reaction on a large scale due to the number of steps involved, with many of them likely to be low-yielding.

Scheme 3.32
4 Results and Discussion III

4.1 Analysis of Ligand Binding

The previous chapter detailed the successful development of a route for the synthesis of novel cyclised ligands based around the dimedone template. These ligands had their binding to CypA tested by a variety of techniques as described below. The protein production and assay work was carried out by Dr. Martin Wear and Dr. Brian McHugh at the Institute of Cell and Molecular Biology at Edinburgh University.

4.1.1 Protein Production

Human CypA was cloned into a pET vector and overexpressed in \textit{E. coli} BL21 cells. Protein purification protocols were developed in-house to give good yields of pure protein using affinity or ion exchange chromatography. Typical yields of \( \sim 7 \) mg of pure protein per litre of culture were routinely obtained.

4.1.2 Fluorescence Binding Assay

When CypA was isolated in 1984 and named after its affinity for the immunosuppressive drug CsA 11, a method was sought for determining the strength of the protein-drug interaction. Handshumacher \textit{et al.} used fluorescence spectroscopy to detect changes in the intrinsic fluorescence emitted from a single tryptophan residue (Trp-121) located in the active site of CypA.\textsuperscript{39} Upon binding CsA 11, they observed a two-fold enhancement in fluorescence intensity, which they attributed to an increase in hydrophobicity of the environment surrounding the tryptophan. The close contact between CsA 11 and Trp-121 and the associated fluorescence change enabled the researchers to calculate a dissociation constant \((K_d)\) for CsA 11 of 200 nM. Further studies refined this value to between 30 and 40 nM.\textsuperscript{43,89} The X-ray crystal structure of CsA 11 bound to CypA revealed a strong hydrogen bond interaction between Trp-121 and the carbonyl oxygen of MeLeu-9 in CsA 11.\textsuperscript{41} It is this contact that is believed to account for the significant changes observed in the fluorescence of Trp-121 upon complex formation. For the assay to be applicable to ligands other than CsA 11, it is necessary that the compounds bind...
close enough to Trp-121 (~ 5-6 Å) in order to perturb the microenvironment around the residue and cause a corresponding change in the observed fluorescence spectrum. Small molecular weight inhibitors do not always cause a change in the Trp-121 fluorescence as this residue is located at least 6 Å from the proline binding pocket where such molecules are thought to bind.

The fluorescence assay was performed using a PTI Quantmaster™ spectrofluorometer (Protein Technology International, Santa Clara, CA) on samples of CypA in the absence or presence of increasing amounts of ligand. Tryptophan fluorescence was excited at 295 nm and the emission scanned from 310 to 430 nm. Each spectrum was obtained from separate incubations of CypA/ligand and not from sequential additions of ligand to the same sample of CypA. Any change in the corrected fluorescence signal was assumed to be directly proportional to the concentration of the CypA/ligand complex. The observed fluorescence ($f_{obs}$) was buffer background subtracted and corrected for dilution and inner filter effects and the corrected fluorescence values plotted against ligand concentration. The data was least squares fit to Equation 4.1 to obtain a value for the $K_d$.

$$f_{corr} = f_t + (f_b - f_t) \times \{(K_d + [CypA] + [Lig]) - \sqrt{((K_d + [CypA] + [Lig])^2 - (4 \times [CypA] \times [Lig])) / 2 \times [Lig])}\}$$

Equation 4.1

where $f_{corr}$ = corrected fluorescence signal

$f_t$ = fluorescence of uncomplexed CypA

$f_b$ = fluorescence of CypA/ligand complex

$K_d$ = dissociation equilibrium constant

[CypA] = total CypA concentration

[Lig] = total ligand concentration

For example, Figure 4.1B shows the plot obtained for a CypA/CsA 11 complex. A least squares fit of this data to Equation 4.1 gave an apparent $K_d$ of 25 ± 6 nM for CsA 11. This value is in good agreement with those determined by other
researchers. Figure 4.1A displays the 2 to 3-fold enhancement that is observed from the tryptophan fluorescence of CypA when bound to CsA 11. As can be seen from the spectrum, this enhancement is also accompanied by an ~8 nm blue shift in the emission maxima from 350 nm to 342 nm.

**Figure 4.1** (A) Fluorescence spectrum of a) 20 μM CsA alone; b) 0.35 μM CypA alone; c) 0.35 μM CypA in the presence of 20 μM CsA. (B) Fluorescence enhancement at 342 nm of CypA plotted against concentration of CsA in μM. Each point is the mean from 3 separate measurements ± SE.

The above measurements for CsA 11 highlight the potential of the fluorescence assay for determining dissociation constants for ligands that bind CypA. However, as was stated previously, smaller, weaker binding ligands may not bind close enough to the Trp-121 to affect its fluorescence. This lack of perturbation can result in weak
inhibitors producing fluorescence spectra barely above the background, making it very difficult to accurately calculate the \( K_d \)'s. Whilst this problem can be overcome to a certain extent by increasing the protein concentration, this is not desirable as it rapidly depletes valuable protein stocks. Another common problem encountered concerns ligand solubility. In order to calculate an accurate \( K_d \) it is common to take readings over a wide range of ligand concentrations. If the ligand precipitates out of solution, this can cause scattering effects, which result in poor signal to noise ratios and a subsequent loss of sensitivity.

### 4.1.3 Peptidyl-Prolyl cis-trans Isomerase (PPIase) Assay

The ability of the synthetic ligands to inhibit CypA's PPIase activity was assessed using an \textit{in vitro} protease-coupled enzymatic assay (Figure 4.2).\textsuperscript{140} \( \alpha \)-Chymotrypsin selectively cleaves the C-terminal \( p \)-nitroanilide (\( p \)NA) bond of a commercially available peptide substrate (Suc-Ala-Ala-Pro-Phe-\( p \)NA) only when the prolyl amide bond is in the \textit{trans} configuration. CypA catalyses the conversion from \textit{cis} to \textit{trans}, and the subsequent release of the chromogenic \( p \)NA fragment can be monitored over time at 400 nm. The initial rate of substrate conversion by CypA combined with various concentrations of inhibitor is used to calculate an IC\textsubscript{50} value for each compound (\textit{i.e.} the amount of inhibitor required to reduce the enzymatic activity by half).

![Figure 4.2 Mechanism of PPIase assay.](image)
In proline containing peptides about 10% of the prolyl amide bonds are in the cis conformation. Such low amounts of active substrate can result in poor signal to noise ratios, which can obscure the results from the assay. This problem can be overcome by suspending the peptide substrate in a solution of LiCl/TFE, which converts most of it (~70%) to the cis form. CypA is incubated together with varying concentrations of ligand in buffer and transferred to a plastic cuvette. The reaction is initiated by addition of α-chymotrypsin, followed by peptide substrate (in LiCl/TFE), and immediately monitored spectrophotometrically at 4 °C. The trans-peptide present at equilibrium is cleaved within the deadtime, ensuring that any subsequent release of pNA can be attributed to isomerisation of the cis isomer. Potent inhibitors of CypA will retard this process and a plot of rate of chromophore release against the log of ligand concentration will allow an IC\textsubscript{50} value to be calculated for each ligand. The low temperature of the reaction is necessary to limit thermal isomerisation of the substrate, however, this caused problems with ligand solubility and in practice adjustments had to be made to ensure the ligand stayed in solution.

In addition to problems with ligand solubility, the assay has a number of other limitations:

1) The calculations assume that the rate of isomerisation is directly comparable to the rate of p-nitroanilide release.
2) Only the cis to trans and not the reverse trans to cis isomerisation can be investigated.
3) Ligands were initially dissolved in MeOH but problems arose due to increased absorbance at 400 nm. DMSO proved to be more effective (and improved solubility issues) but was hampered by fact it is a known, albeit weak, inhibitor of CypA.90
4) The assay is fairly laborious and time-consuming and several repetitions are often required to achieve reproducible results.
4.1.4 Electrospray Mass Spectrometry Assay

Whilst the *in vitro* assays described above are well-established and important methods for ascertaining binding and determining dissociation constants, they are hampered by restrictions placed on the type of ligands that can be screened, or by the length of time the assay takes to perform. Their use would be better served by concentrating on obtaining data for a small number of key ligands with a high probability of binding CypA. A method was therefore needed that could quickly identify promising ligands by the nature of their interaction with CypA.

Electrospray mass spectrometry (ESI-MS) has previously been used as a moderately high-throughput method for screening protein-ligand complexes to identify compounds with high affinities.\(^{141}\) The small amounts of sample required and the speed of the method make it an attractive technique for screening small compound libraries against a particular protein target. The soft-ionisation conditions are generally thought to preserve non-covalent interactions between the protein and ligand during transition from the solution to the gas phase. The versatility of the instrument can then allow a range of parameters including flow rate, pH, cone voltage and temperature to be varied in order to ensure the survival of the native complex. Another advantage of the electrospray process is that proteins often become multiply charged, giving characteristic charge-ion series within the operation range of common quadrupole analysers.

One of the first proteins to be studied by ESI-MS was FKBP, a member of the same immunophilin family as CypA.\(^{142,143}\) In addition to displaying a specific charge-ion series for FKBP, the method could detect the complexes formed between the protein and its known inhibitors, FK506 10 and RAPA 12. Furthermore, by measuring the relative peak intensities for free and complexed FKBP, and knowing the initial concentrations of protein and inhibitor, the researchers were able to determine dissociation constants for FK506 10 and RAPA 12 that were in good agreement with those found by other assays. These findings offered the potential to study
interactions between CypA and CsA in a similar way, and ultimately to extend this to complexes formed with the synthetic ligands.

In work carried about by Sally Shirran and continued by Hannah Florance, CypA was dialysed into 10 mM ammonium acetate at pH 6.8 (the physiological pH of CypA). A working protein concentration of 20 μM was used for analysis by ESI-MS. The experiment was conducted on a Micromass ZMD mass spectrometer and introduction of the sample was by direct infusion. The charge-ion series observed for CypA contains a narrow charge distribution over three charge states, the 8+ being the most dominant. This relatively low charge range suggests CypA is still in its folded, native form. Deconvolution of the spectrum reveals a mass of CypA of 18144 Da (Figure 4.3). This mass represents the documented mass of human CypA (18012 Da) plus a methionine residue that is involved in the transcription of the CypA gene.

Figure 4.3 Mass spectrum of CypA.

The successful ionisation and detection of CypA by ESI-MS was encouraging and focused attention onto whether the process could be repeated with CypA complexed
to CsA 11. Hence, a 1:1 mixture of CsA 11 and CypA (20 \( \mu \)M) in ammonium acetate buffer/10 % MeOH (the methanol was necessary to dissolve the ligand but its concentration was kept as low as possible so as not to disrupt the protein structure) was directly infused into the mass spectrometer. The deconvoluted mass spectrum clearly revealed a peak corresponding to the combined mass of CypA plus CsA (19348 Da) (Figure 4.4). The signal determines the exact stoichiometry for the complex, which as expected is 1:1. The experiment ably demonstrates the ability of ESI-MS to observe CypA complexes in the gas-phase.

**Figure 4.4** Mass spectrum of 1:1 CypA/CsA 11 complex.

4.2 Binding Results for the Synthetic Inhibitors

4.2.1 Results from ESI-MS Assay

Due to the speed of the method, new ligands were first tested using the ESI-MS assay in order to identify promising inhibitors, which could then be taken forward for further *in vitro* or *in vivo* testing. The ability of the method to use the relative peak intensities to determine dissociation constants was taken advantage of to generate a rank-order for the ligands. Table 4.1 shows the results for the key ligands tested to date.
Table 4.1 Results from ESI-MS assay.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Structure</th>
<th>ESI-MS $K_d$ (μM)$^*$</th>
<th>Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>122b</td>
<td><img src="image" alt="Structure 122b" /></td>
<td>31.9 ± 3.1</td>
<td>Nanomate®</td>
</tr>
<tr>
<td>121b</td>
<td><img src="image" alt="Structure 121b" /></td>
<td>61.5 ± 13.7</td>
<td>ZMD</td>
</tr>
<tr>
<td>123</td>
<td><img src="image" alt="Structure 123" /></td>
<td>72.6 ± 16.2</td>
<td>ZMD</td>
</tr>
<tr>
<td>122a</td>
<td><img src="image" alt="Structure 122a" /></td>
<td>85.1 ± 19.0</td>
<td>ZMD</td>
</tr>
<tr>
<td>115</td>
<td><img src="image" alt="Structure 115" /></td>
<td>91.9 ± 20.5</td>
<td>ZMD</td>
</tr>
<tr>
<td>153a</td>
<td><img src="image" alt="Structure 153a" /></td>
<td>120.6 ± 11.7</td>
<td>Nanomate®</td>
</tr>
<tr>
<td>122c</td>
<td><img src="image" alt="Structure 122c" /></td>
<td>128.1 ± 28.6</td>
<td>ZMD</td>
</tr>
<tr>
<td>153b</td>
<td><img src="image" alt="Structure 153b" /></td>
<td>159.4 ± 15.5</td>
<td>Nanomate®</td>
</tr>
<tr>
<td>109</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>181.6 ± 40.5</td>
<td>ZMD</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------</td>
<td>--------------</td>
<td>-----</td>
</tr>
<tr>
<td>121c</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>247.6 ± 55.2</td>
<td>ZMD</td>
</tr>
<tr>
<td>121a</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>376.9 ± 84.0</td>
<td>ZMD</td>
</tr>
<tr>
<td>144</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>Very weak binding</td>
<td>Nanomate®</td>
</tr>
<tr>
<td>145</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>No binding</td>
<td>Nanomate®</td>
</tr>
<tr>
<td>116</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>No binding</td>
<td>ZMD</td>
</tr>
<tr>
<td>113</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>No binding</td>
<td>ZMD</td>
</tr>
</tbody>
</table>

*All ligands were screened at 100 μM conc. against CypA (20 μM)*

The last column in the table details the instrument used to obtain the data. The ZMD was connected to a Waters 2700 HPLC pump to enable automated sample introduction. However, this approach proved problematic as in order to maintain adequate sensitivity, flow rates had to be kept to a minimum. Reduced flow rates resulted in increased run times and ensured only approximately 10 samples per hour could be tested. The ZMD also suffered from problems associated with sample carry over. These were largely overcome through the use of an elution gradient but this
had the downside of further increasing the sample run time. The Nanomate® employs nanospray ionisation, resulting in reduced sample volumes and flow rates. The unit is closed to the atmosphere, which prevents sample evaporation or contamination and helps the instrument maintain a steady back pressure. These conditions are important for ensuring high levels of reproducibility and throughput. When coupled to a sensitive analyser such as a Q-TOF micro (Quadrupole-Time of Flight) the Nanomate® was capable of screening 50 samples per hour with a high degree of accuracy. Unfortunately, the Nanomate® was located at Oxford University placing time constraints on its access.

From Table 4.1 it can be seen that both the lysine and arginine containing cyclised ligands bind strongly to CypA, with significantly higher affinities than the parent dimedone 33 ($K_d = 22$ mM by fluorescence – too weak to be observed by ESI-MS assay). In contrast, ligands 113 and 116 showed no binding to the protein and helped to corroborate the molecular modelling studies described in Section 3.1.3. The arginine derivative 122b proved to be the most potent binder by ESI-MS and the mass spectrum of its complex with CypA is shown in Figure 4.5.

**Figure 4.5** Mass spectrum of CypA/122b (1:5) complex.
The spectrum clearly shows a peak for the CypA/122b complex. However, the stoichiometry of ligand binding appears ambiguous due to the presence of peaks representing the binding of multiple ligands. It was felt that the extra peaks arose from ligand-ligand aggregation, a common problem in ESI-MS assays. On dilution into the aqueous environment favoured by the protein, small hydrophobic ligands can form strong interactions with each other. These interactions cause the ligands to cluster together to form micelle like structures. In the case of ligand 122b, it would appear that the micelles display affinity for CypA and could, in theory, be competing with one another for access to the active site. Such competition between different structures would obviously affect the validity of $K_d$ calculations. Reports in the literature suggest aggregation can be minimised by reducing the ligand concentration, implying that at high concentrations the ligands preferentially form clusters as opposed to complexes with the protein. A CypA/ligand (1:5) ratio was found to be optimum for detecting complexes using the ZMD, with poor sensitivity a factor at lower protein/ligand ratios. Spectrums run on the Nanomate®, however, have significantly improved signal to noise ratios for reasons described above, hence this was used to detect complexes at lower ligand concentrations. For example, Figure 4.6 shows the spectrum for a CypA/122b (1:2) complex.

**Figure 4.6** Mass spectrum of CypA/122b (1:2) complex.
As can be seen from the spectrum, aggregation has been considerably reduced suggesting the process is concentration dependent.

Aggregation could also be detected in CypA/CsA 11 complexes proving that even the strongest inhibitors of CypA exhibit this effect under certain electrospray conditions. Using the CypA/CsA 11 complex as a model system, tandem mass spectrometry (MS/MS) experiments were carried out to investigate the strength of cluster binding (Figure 4.7).

Figure 4.7 Spectra depicting the breakdown of the isolated peak 2308.8 [CypA + 2CsA + 9H]²⁺. As the voltage was increased a) 20.0 V; b) 30.8 V; c) 39.2 V the complex degraded in stages, reducing the charge state with each ligand loss. At high voltages, d) 50.3 V, both ligands were lost simultaneously.

This experiment involved selecting peaks corresponding to CypA + 1 CsA and CypA + 2 CsA respectively. The ligands were dissociated from the protein by accelerating them into a cell filled with argon gas at a pressure of 10⁻⁵ Torr. By increasing the acceleration voltage the strength of the protein to ligand binding could be assessed. This experiment found the second CsA 11 ligand to be much more weakly bound, with low acceleration voltages (20 V) able to dissociate it from CypA. The first ligand required significantly more energy to dissociate, implying that it is bound
much more tightly to CypA. In addition, at high voltages (50 V) both ligands were lost simultaneously but the detection of the 8+ charge state suggested the ligands may have been lost as a dimer, giving support that ligand-ligand aggregation is occurring as opposed to non-specific binding of two CsA's at separate sites on CypA.

The results from the previous experiment suggest that a single ligand is binding in CypA's active site and others are aggregating to it, especially at high concentrations. Therefore, it can be assumed that the calculated $K_d$ is a reliable measure of the affinity of a single ligand for CypA. Although the possibility of non-specific binding by ligand clusters cannot be completely ruled out, reducing the ligand concentration should help to minimise this. The $K_d$'s were calculated by comparing the intensity of the peaks due to protein/ligand complexes against those for protein alone. In general, it was found that the highest affinity ligands gave the most reliable $K_d$'s by this method. This was probably a result of their strong interactions with CypA helping to preserve the protein/ligand complex during desolvation. Weaker binding ligands would be more liable to dissociate from the protein under the electrospray conditions. Nevertheless, the primary function of this assay was to quickly identify tight-binding inhibitors for further investigation, and it has proved to be an excellent tool for this purpose.

Referring back to Table 4.1, a number of interesting trends come to light. For example, the uncyclised ligands 121a-c all show affinity for CypA with $K_d$'s 2 to 4-fold greater than their cyclised equivalents. This supports the argument that locking the molecules into a particular conformation improves their affinities as a result of reducing the entropic cost of binding. An exception to this was 115, which binds relatively strongly to CypA ($K_d = 91.9 \mu M$) in contrast to the cyclised version 116 which shows no binding to CypA by this assay. Section 3.1.3 described molecular modelling studies that suggested the steric bulk of the phenyl ring in 116 would be unfavourable for binding and this is borne out from the ESI-MS data. However, the success with the uncyclised ligand 115 implies that the structure's increased flexibility enables it to orientate itself in such a way as to minimise unfavourable
interactions. Whether the low \( K_d \) is due to additional contacts made by the side-chain or by the release of steric strain is, however, open to question.

As reported in the previous chapter, it was of interest to synthesise the cyclised ligands as individual diastereomers in order to investigate whether the protein had a preference for one particular configuration? From the results of the ESI-MS assay, it would appear that the \((S,R)\)-diastereomer 153a binds more strongly to the protein than the \((S,S)\)-diastereomer 153b. Whilst this conclusion must be viewed in light of the fact that the error in the method is relatively large, a difference in \( K_d \) of approximately 30 % is significant and worth investigating further. Since the preliminary data indicates that improvements in \( K_d \) could be achieved by screening individual enantiomers as opposed to the racemic mixture, future work should include the design of more methods for accessing the lead compounds in enantiomerically pure form. In addition to potentially leading to tighter binding compounds, this work should help to reveal more about the nature of CypA's active site, aiding subsequent design efforts.

The final interesting result obtained from the ESI-MS assay centres on the ligands 123 and 145. The former displays affinity for CypA, whereas the latter was found not to bind the protein. Since the only difference between the structures is the lack of an amide carbonyl group in 145, this raises the possibility that this functionality could be a key recognition site for CypA. In the original design of the cyclised ligands it was suggested that the amide carbonyl group might mimic the twisted amide bond of prolyl peptide substrates. To investigate this hypothesis, molecular modelling was used to manually dock the structure of 123 into CypA's active site and compare this to the known X-ray crystal structure of the bound dipeptide Ala-Pro (Figure 4.8).\(^\text{144}\) Asn-102 in CypA's active site is known to form a hydrogen bond with the oxygen atom of the prolyl amide in Ala-Pro. This interaction is highly conserved in most CypA complexes with linear peptides and, in the case of Ala-Pro, was measured to be 2.17 Å. In comparison, the amide oxygen atom of the docked ligand 123 was found to be 2.59 Å from Asn-102, thereby lending support to the view that this ligand could act as a transition state mimic. The lack of an amide bond
in 145 would explain its inability to bind CypA. An alternative hypothesis, however, is that the removal of the carbonyl group could change the \( pK_a \) and hybridisation state of the ring nitrogen thereby changing both the electronic and configurational properties of the molecule.

**Figure 4.8** Molecular modelling of 123 (red) using the known X-ray crystal structures of CypA (blue) bound to Ala-Pro (green) and CypA (black) bound to EM2/34 117 (not shown) as templates. The intermolecular distance between the amide oxygen atom of 123 and the Asn-102 residue is 2.59 Å. In the dipeptide the prolyl amide oxygen atom is 2.17 Å from Asn-102.

### 4.2.2 Results from In Vitro Assays

Following the primary screen by ESI-MS, a selection of the cyclised ligands had their binding determined by fluorescence and/or PPIase assay (Table 4.2). The best hit was ligand 123, which was shown to bind CypA with high affinity. The \( K_d \) of 16.1 ± 2.3 \( \mu \)M represents a greater than 1000-fold improvement compared to the parent compound dimedone 33; and the calculated IC\(_{50}\) of 6.8 ± 1.9 \( \mu \)M confirms 123 as a potent inhibitor of CypA’s PPIase activity. A plot of the corrected fluorescence against the concentration of 123 is shown in Figure 4.9. The apparent \( K_d \) was obtained from a least squares fit of the data to Equation 4.1 but with an extra linear term (lig. 123\(_{\text{fluor}} \times [\text{lig. 123}]\) added to take account of the concentration dependent increase in the fluorescence signal of 123 (in this case lig. 123\(_{\text{fluor}} = 3,800 \) fluorescence units \( \mu \)M\(^{-1}\)).
Table 4.2 Results from *in vitro* assays.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Structure</th>
<th>Fluorescence $K_d$ (µM)</th>
<th>PPIase $IC_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>123</td>
<td><img src="image1" alt="Structure" /></td>
<td>16.1 ± 2.3</td>
<td>6.8 ± 1.9</td>
</tr>
<tr>
<td>145</td>
<td><img src="image2" alt="Structure" /></td>
<td>Not tested</td>
<td>No inhibition</td>
</tr>
<tr>
<td>122c</td>
<td><img src="image3" alt="Structure" /></td>
<td>5.0-20.0*</td>
<td>No inhibition</td>
</tr>
<tr>
<td>122b</td>
<td><img src="image4" alt="Structure" /></td>
<td>5.0-30.0*</td>
<td>No inhibition</td>
</tr>
<tr>
<td>122a</td>
<td><img src="image5" alt="Structure" /></td>
<td>Not tested</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

* Evidence of binding but range quoted due to poor signal to noise ratios arising from scattering caused by poor ligand solubility.

Figure 4.9 Corrected intrinsic tryptophan enhancement at 348 nm of CypA plotted against concentration of 123. Each point is the mean from 3 separate measurements ± SE.
The small IC\textsubscript{50} value obtained for 123 by the PPIase assay is in stark contrast to the lack of inhibition found for 145. These findings support the earlier data from the ESI-MS assay and strengthen the view that the amide carbonyl group in 123 is required for the effective binding of CypA. As described above (Figure 4.8) this view offers the exciting possibility that 123 may bind as a transition state analogue. Such a result would be important for the design of new inhibitors and would confirm the logic of the original design process that proposed such structures. Efforts are currently focused towards obtaining an X-ray crystal structure of the CypA/123 complex, which would help elucidate the exact position of the amide carbonyl in the protein's active site.

In contrast to the excellent results obtained with 123, many of the other compounds highlighted by ESI-MS failed to inhibit the PPIase activity of CypA or gave poor signal to noise ratios in the fluorescence assay (Table 4.2). Whilst this may be a result of false hits generated by the original screen, it is more likely due to inherent problems concerning ligand solubility. As stated in the opening sections of this chapter, the \textit{in vitro} assays are reliant on the compound being completely soluble in the respective buffers used in the reactions. With most of the cyclised ligands shown in Table 4.2, this was not the case. The notable exceptions were ligands 123 and 145, where the free amine moieties significantly enhanced their solubility in polar solvents. Ligand solubility must therefore be taken into consideration during the design of such compounds, particularly in relation to studies in biological systems. DMSO is often the solvent of choice when issues concerning solubility arise but its use in the above assays was hindered by the fact that DMSO is a known, albeit weak, inhibitor of CypA,\textsuperscript{90} and could therefore distort the results.

In summary, the \textit{in vitro} assays support the findings from the ESI-MS assay and provide a further means of confirming and quantifying a particular ligand's affinity for CypA. However, the amount of information attainable is reliant on the ligands being compatible with the requirements of the assay, with solubility a major factor. Both \textit{in vitro} assays are time-consuming to perform so the value of using the ESI-MS assay as a fast, initial screen becomes apparent. The promising results obtained for
123 provide a strong case for this analytical approach, with this compound representing a completely new class of inhibitor of CypA. Furthermore, 123's excellent solubility in polar solvents makes it an attractive candidate for in vivo testing.

4.2.3 In Vivo Activity Screen

Caenorhabditis elegans, a free-living nematode worm, is an excellent model system for the study of nematode parasites. It is not only genetically tractable, but is easily and quickly cultured in liquid media and has a relatively rapid reproductive life cycle (of the order of 24 hours). C. elegans has been shown to express multiple isoforms of cyclophilin that appear to have important roles in reproduction and larval development, body-wall muscle formation and in the proper folding and processing of proteins involved in cuticle formation. However, this area is still poorly understood and little is known about the exact mode of action of cyclophilins and other folding proteins in C. elegans.

CsA 11 has been shown to cause biological effects such as cuticle shedding and gut structural defects in C. elegans, which have been attributed to the inhibition of cyclophilins PPIase activity. The large molecular weight, poor bioavailability and immunosuppressive functions of CsA 11 render it an unsuitable candidate for studying the effects of cyclophilin inhibition in C. elegans. However, chemical intervention by small molecule inhibitors could provide an important tool for probing the biological properties of parasitic cyclophilins in vivo.

A C. elegans in vivo screen has been developed to investigate the biological effect of the synthetic human CypA inhibitors. A fixed number of sexually mature worms were cultured in liquid media plus 2 % ethanol (to aid ligand solubility) in 24 well plates, exposed to varying doses of 123, and analysed over the course of five days (this allows for several reproductive cycles). Biological effects were then assessed by several parameters, including morphological and developmental abnormalities such as cuticle shedding defects and gut malformation, reduced motility, and reduced
High concentrations (> 500 μM) of 123 were lethal resulting in rapid death of the worms (Table 4.3). Compared to control conditions, worms exposed to concentrations < 500 μM had growth defects – the worms were shorter and “dumpier” in general and showed reduced motility (Table 4.4).

**Table 4.3** Observations after 24 hours of *C. elegans* treated with varying concentrations of 123.

<table>
<thead>
<tr>
<th>Conc. of Ligand 123</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM [Control: liquid media + 2 % EtOH]</td>
<td>Normal</td>
</tr>
<tr>
<td>2 mM</td>
<td>Instantly dead</td>
</tr>
<tr>
<td>1 mM</td>
<td>Instantly dead</td>
</tr>
<tr>
<td>500 μM</td>
<td>All dead</td>
</tr>
<tr>
<td>250 μM</td>
<td>Adults stunted and sluggish</td>
</tr>
<tr>
<td>100 μM</td>
<td>Adults stunted and sluggish</td>
</tr>
<tr>
<td>75 μM</td>
<td>Short but motile</td>
</tr>
<tr>
<td>50 μM</td>
<td>Short but motile</td>
</tr>
<tr>
<td>25 μM</td>
<td>Short but motile</td>
</tr>
<tr>
<td>10 μM</td>
<td>Short but motile</td>
</tr>
<tr>
<td>5 μM</td>
<td>Short but motile</td>
</tr>
</tbody>
</table>

**Table 4.4** Observations after 5 days of *C. elegans* treated with varying concentrations of 123.

<table>
<thead>
<tr>
<th>Conc. of Ligand 123</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM [Control: liquid media + 2 % EtOH]</td>
<td>Normal</td>
</tr>
<tr>
<td>2 mM</td>
<td>Dead</td>
</tr>
<tr>
<td>1 mM</td>
<td>Dead</td>
</tr>
<tr>
<td>500 μM</td>
<td>Low numbers of worms – sluggish with gut damage</td>
</tr>
<tr>
<td>250 μM</td>
<td>Stunted and sluggish with gut damage</td>
</tr>
<tr>
<td>100 μM</td>
<td>Stunted and sluggish with gut damage</td>
</tr>
<tr>
<td>75 μM</td>
<td>Stunted</td>
</tr>
<tr>
<td>50 μM</td>
<td>Normal</td>
</tr>
<tr>
<td>25 μM</td>
<td>Normal</td>
</tr>
<tr>
<td>10 μM</td>
<td>Normal</td>
</tr>
<tr>
<td>5 μM</td>
<td>Normal</td>
</tr>
</tbody>
</table>
The organisms treated with 123 also had cuticle shedding defects and severe gut development defects, similar in phenotype to those observed in organisms treated with CsA 11 (Figure 4.10). Furthermore, exposure to 123 markedly affected the reproductive abilities of the worms as reflected in large loss/reduction of larvae and embryo numbers.

Figure 4.10  Representative light-micrographs of worms grown in the absence or presence of either 123 (100 μM) or CsA 11 (25 μM) are shown in panels (A), (B) and (C), respectively. In (B) and (C), the cuticle shedding defect, absent in (A), is highlighted by red arrows. (D) Higher magnification light micrograph showing the abnormal gut tissue development present in worms exposed to 123 (100 μM) (red arrows).
The results with **123** were very encouraging and the data was used to determine an IC$_{50}$ value for its action against *C. elegans*. Figure 4.11 shows the dose-response curves for **123** after 48 hours exposure. Although the data is noisy, a specific trend exists and it is possible to calculate apparent IC$_{50}$ values by non-linear fitting the data to a standard model. An average IC$_{50}$ for **123** of $156 \pm 80$ $\mu$M was calculated by this method. For comparison, the IC$_{50}$ value for CsA **11** from a similar experiment was found to be $28 \pm 13$ $\mu$M. Hence, the biological effects of **123** compare well with those of CsA **11**, and the small molecule inhibitor possesses the additional advantages of ease of synthesis and better bioavailability compared to the large cyclic undecapeptide.

**Figure 4.11** Dose response curves for **123** based on (A) embryo numbers and (B) larvae numbers after 48 hours exposure.
Although it is possible that the biological effects of 123 are due to its toxicity \textit{in vivo}, the evidence suggests it is the ability of 123 to inhibit parasitic cyclophilins that is responsible for its mechanism of action. The fact that 123 has been shown to be a potent inhibitor of human CypA by \textit{in vitro} assay and to cause phenotypes similar to those found using CsA 11 provides a strong case for this conclusion.

In contrast to the results obtained with 123, the protected analogue 122c was found to possess no activity against \textit{C. elegans}, despite it being shown to inhibit CypA by fluorescence assay (Table 4.2). This finding again demonstrates the importance of good solubility and bioavailability, with the lipophilic protecting group of 122c rendering it much less soluble than the free amine containing inhibitor 123. The uptake of the former into \textit{C. elegans} would thus be limited, thereby explaining its lack of activity \textit{in vivo}. These results suggest that further improvements in activity could be achieved through modifications that increase the ligand's solubility in aqueous environments. For example, it could be envisaged that hydrolysis of the ester moiety of 123 would lead to the formation of a zwitterion that should further enhance its solubility in biological systems.

Ligands such as 123 offer great potential for the design of new antiparasitic drugs. Alternatively, these compounds could be employed as chemical probes to elucidate the role of cyclophilins in different stages of \textit{C. elegans} development. Such an aim could be realised by linking the observed phenotype with the biochemical mechanism of action of the small molecule inhibitors. This forward chemical genetics approach would provide valuable information about the biological actions of the cyclophilins in \textit{C. elegans} and hence, should identify new targets for antiparasitic drugs. For this approach to be successful, it will be necessary to investigate the uptake and localisation of ligands within \textit{C. elegans}. A convenient way to achieve this is to attach fluorescent labels to the compounds of interest to allow for their monitoring by fluorescence microscopy.
4.2.4 Synthesis of Fluorescently Labelled Derivatives

In order to incorporate fluorescent labels into the lead compounds, the commercially available dansyl lysine amino acid 165 was successfully converted to its methyl ester 166 by treating with thionyl chloride in methanol (Scheme 4.1). The ester 166 was then coupled to the racemic carboxylic acid derivative 109 to form the dansylated peptides 167a and 167b respectively. In this case it was possible to separate the diastereomers by column chromatography.

Scheme 4.1

After previously experiencing problems with epimerisation during the cyclisation of individual diastereomers (Section 3.4.2) it was decided to isolate the products after the hydrolysis of the enol ether moieties of 167a and 167b (Scheme 4.2). This approach allowed small quantities of the desired cyclised dansyl derivatives 169a and 169b (arising from intramolecular cyclisation of the diketone compounds 168a and 168b in situ) to be obtained for testing.
Unfortunately, the cyclised compounds 169a and 169b were not suitable candidates for crystallography hindering attempts to assign their absolute configurations. However, comparing their NMR spectra with those obtained for the enantiomerically pure ligands 153a and 153b enabled a tentative assignment to be made.

4.2.5 In Vitro Testing of Fluorescently Labelled Derivatives

Each diastereomer had its binding to CypA determined by ESI-MS, and then subsequently by in vitro assay (Table 4.5). The initial screen by ESI-MS revealed both compounds to bind CypA with the (S,R)-diastereomer 169a displaying slightly higher affinity for the protein than its (S,S)-analogue 169b. These results mirror
those found for ligands 153a and 153b (Table 4.1), thereby hinting at a possible preference for the ring proton to be in the R-configuration.

Table 4.5 Binding results for fluorescently labelled derivatives 169a and 169b.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Structure</th>
<th>ESI-MS $K_d$ (µM)</th>
<th>Fluorescence $K_d$ (µM)</th>
<th>PPIase IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>169a</td>
<td><img src="image" alt="Structure of 169a" /></td>
<td>113.2 ± 11.0</td>
<td>21.9 ± 3.5</td>
<td>207.0 ± 57.8</td>
</tr>
<tr>
<td>169b</td>
<td><img src="image" alt="Structure of 169b" /></td>
<td>133.2 ± 12.9</td>
<td>Not tested</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>
Comparing the $K_d$ for 169a with that obtained for 123 reveals that the incorporation of a dansyl tag does not significantly affect the binding to CypA. This was an important result, since the benefits afforded by the inclusion of a fluorescent label would be negated if the change in structure seriously affected the ligand’s ability to bind CypA. This finding also indicates that modifications to 123 involving coupling to the free amino group would not necessarily have a detrimental effect on binding. Hence, a range of analogues of 123 could be synthesised and used as probes to determine the nature of the interactions in the Abu pocket of CypA’s active site. However, the fact that the $K_d$ for 169a is approximately 30% weaker than that found for 123 implies that bulky groups such as dansyl might experience some unfavourable steric interactions upon binding to CypA. Having shown that 169a binds to CypA with high affinity, it was now tested in vivo in order to follow the passage of the ligand in C. elegans, thus helping to shed light on its molecular targets.

4.2.6 In Vivo Testing of Fluorescently Labelled Derivatives

C. elegans in liquid culture was treated with 169a and found to display cuticle shedding defects and gut malformation similar in phenotype to that found in organisms treated with 123 or CsA 11. In addition, the fluorescence of 169a was monitored using a fluorescence microscope fitted with a DAPI filter set. The results are shown in Figure 4.12. 169a was found to permeate throughout the gut lumen into areas where cyclophilins are thought to be present. There role here is proposed to involve the correct formation of the gut lining, which would correlate well with the structural defects observed after administration of the inhibitor 169a. The fact the ligand’s passage through the gut can be detected suggests its effect is not merely toxic, as one would expect the compound to kill the organism shortly after ingestion if this was the case. The results from the fluorescence study indicate that 169a locates and inhibits its particular target(s) thereby causing the effects described above. Whilst the evidence points to cyclophilins as the molecular targets, further work is necessary to rule out other potential candidates such as FKBPs. Whether the ligands are specific for a particular isoform has also to be determined.
Figure 4.12  Fluorescence microscopy studies of *C. elegans* after treatment with 169a. (A) Strong fluorescence detected throughout gut lumen in areas where cyclophilin proteins are thought to be present; (B) Fluorescence detected from gut lumen, which is malformed (red arrow). Small fluorescent vesicles are also visible (yellow bracket) but these are due to auto-fluorescence of the organism and not to the presence of 169a.
4.3 Conclusions

This chapter detailed the results obtained for the synthetic inhibitors in complex with CypA. The key results were:

- A novel ESI-MS binding assay has been developed to provide a quick means of assessing the ligands' affinity for CypA. This assay found a number of the cyclised compounds to bind to CypA and the method provided a rank-order to allow the most promising inhibitors to be identified for further testing.

- *In vitro* assays were used to quantitatively assess the strength of ligand binding and inhibition of CypA's PPIase activity. These confirmed ligand 123 as a potent inhibitor of CypA with an apparent *K*<sub>d</sub> = 16 µM and an IC<sub>50</sub> = 6.8 µM respectively. These figures represent a greater than 1000-fold improvement on the original lead dimedone 33.

- Evidence from ESI-MS and PPIase assays suggested the amide carbonyl of 123 plays a critical role in binding CypA. This view arose from studies on the de-oxy analogue 145, which showed no affinity for the protein. Molecular modelling studies indicated that the amide carbonyl of 123 might mimic the twisted amide transition state of a bound prolyl peptide. If confirmed, this would satisfy one of the original design requirements and provide support for the structure-based design approach.

- 123 was tested *in vivo* against the free-living nematode *C. elegans* and was found to cause cuticle shedding defects and severe gut development defects, similar in phenotype to those observed in organisms treated with CsA 11. In addition, 123 drastically reduced *C. elegans* reproductive capabilities. These findings imply that 123 is acting on cyclophilins present in *C. elegans*, which have been proposed to be involved in the correct folding of collagens required for cuticle formation.<sup>45,46</sup>

- Fluorescently labelled derivatives 169a and 169b were successfully synthesised by incorporating a dansyl containing lysine residue into the dimedone motif by the route described in Chapter 3.
• Dansyl derivative 169a was found to bind to CypA by ESI-MS and fluorescence assay with a $K_d = 20 \mu M$. It therefore appears that the bulky dansyl tag can be successfully incorporated into CypA's active site.

• 169a displayed similar effects to 123 and CsA 11 in vivo and its fluorescence was detected throughout the worms' gut lumen in areas where cyclophilins are thought to be involved in structural formation of the gut lining. The site-specific fluorescence provided strong evidence that the ligands were localising in cyclophilin dense regions and causing specific effects as a result of inhibiting these proteins.

4.4 Suggestions for Future Work

The success with 123 was due in no small measure to its excellent solubility in polar solvents. Whilst other cyclised ligands showed evidence of binding to CypA, their testing was hampered by poor solubility and/or bioavailability. Future work will therefore need to take into consideration methods to improve the solubility of the lead compounds. The hydrolysis of the ester moiety of 123 would yield a carboxylic acid derivative, which should enhance the ligand's solubility and would be of particular importance if additional groups were attached to the free amine.

Further work is required to elucidate the role of the amide carbonyl group in CypA's active site. X-ray crystallography could be used to solve the structure of the protein/ligand 123 complex and hence, determine the location of the amide carbonyl in the active site. Work is currently ongoing into obtaining crystals of 123 bound to CypA. A successful structure determination would also provide new possibilities for future ligand modifications by revealing which residues are in close contact with the molecule in the active site. Such studies could also reveal whether CypA has a preference for one diastereomer over the other?

This chapter highlighted the potential of using synthetic ligands, such as 123, for studying the role of parasitic cyclophilins in model organisms like *C. elegans*. An extension of this approach would be to study the effect of selectively inhibiting one
or more of the cyclophilin isoforms present in the nematode. A recently developed technique known as ‘proximity accelerated alkylation’ could be used for this purpose. Levitsky et al.,\textsuperscript{147} have recently reported the generation of specific cysteine mutants of human CypA (e.g. P105C) which retain wild-type PPIase activity but can be irreversibly inhibited in the presence of CsA analogues possessing a thiol specific electrophilic group such as the acrylamide derivative 170. Compound 170 is easily prepared from CsA, according to the sequence shown in Scheme 4.3, in which the MeBmt-1 side-chain is modified to provide an electrophilic acrylamide unit which is insufficiently reactive to alkylate endogenous thiols (e.g. glutathione) in a non-specific manner and can only conjugate to a protein after initial docking at the binding site. Crucially, modification at this region of CsA does not interfere with binding to cyclophilins.

**Scheme 4.3**

![Scheme 4.3](image)

**Figure 4.13** Proposed inhibitor 171 for proximity accelerated alkylation studies.

![Figure 4.13](image)

This approach provides a powerful way of using chemical probes to switch off one or more cyclophilins in an allele specific manner and could be extended to incorporate acrylamide units into the fluorescent inhibitors 169a and 169b. Inhibitors such as these would allow inactivated cyclophilin isoforms to be visually identified and localised in the worms. Modelling studies suggest that the dimedone portion of the
ligand needs to be \( \text{ca. } 8-12\text{Å} \) from the cysteine-105 residue suggesting that derivatives of type 171 would be suitable. Optimisation of both the length of spacer and also the specific residue on the cyclophilin to be modified could be carried out in order to fine tune the specificity of inhibition.
5 Experimental

5.1 General Procedures

$^1$H and $^{13}$C NMR spectra were recorded on Brüker AC250, DPX-360 or Varian Gemini-200 instruments. The following abbreviations are used: $\delta$, chemical shift; d, doublet; dd, doublet of doublets; dt, doublet of triplets; dq, doublet of quartets; $J$, coupling constant; m, multiplet; q, quartet; s, singlet; t, triplet. Chemical shifts ($\delta$) are reported in parts per million (ppm) and coupling constants ($J$) in Hz. Residual protic solvent, $\text{CHCl}_3$ ($\delta_H$ 7.26, s) was used as the internal standard in $^1$H NMR spectra, and $^{13}$C NMR shifts were referenced using $\text{CDCl}_3$ ($\delta_C$ 77.0, t) with broad band decoupling. Electrospray (ES) nominal mass spectra were recorded using a Micromass Platform II mass spectrometer (CV=35). Fast Atom Bombardment (FAB) high resolution mass spectra were recorded on a Kratos MS50TC instrument. LC-MS was performed on a C18 Luna Phenomenex column using an acetonitrile (0.1 % TFA)/water gradient over 30 minutes. Preparative LC-MS was performed on a Micromass ZMD 4000 with Gilson 215 liquid handler, eluting with a acetonitrile/water gradient over 30 minutes. Infrared absorption spectroscopy was performed on a Jasco-FT/IR-410 Spectrophotometer using standard techniques and $\nu_{\text{max}}$ values are quoted in cm$^{-1}$. Microwave reactions were carried out in a CEM Discover Microwave Synthesiser with Explorer Carousel. The reaction conditions were monitored using a pressure probe which was inserted through the septum of the reaction tube and black body irradiation measured to monitor the temperature of the sample. Normal phase high performance liquid chromatography (HPLC) was performed on a Waters 600 controller/pump utilising a 486 tunable absorbance detector and equipped with a Chiracel-ODH column with dimensions 25 x 0.46 cm. Samples were injected via a 20 µl loop with a flow rate of 1 ml/min, and eluted with an isocratic system of hexane/ethanol (9:1) with 0.1 % TFA as modifier. A wavelength of 254 nm was used for sample detection. CHN analysis was obtained for crystalline solids. A small sample was recrystallised three times then submitted for combustion analysis. CHN was not obtained for gummy solids or oils. Optical rotations were performed on an AA1000 polarimeter from Optical Activity Ltd.
(measurements made at the sodium D-line). Concentrations are given in g/100 ml. Melting points were obtained on a Gallenkamp melting point apparatus and are uncorrected. Analytical TLC was carried out on Merck aluminium-backed plates coated with silica gel 60 F254, 0.25 mm. Components were visualised using ultraviolet fluorescence (254 nm) and ammonium molybdate or permanganate dips. Flash chromatography was carried out using silica gel 60H (Merck 9385, 0.04-0.063 mm, 230-400 mesh). Diisopropylamine was distilled from solid sodium hydroxide and stored under dry nitrogen over 4Å molecular sieves. Anhydrous solvents were purchased from Aldrich or Baker and used as received. Other solvents and reagents were standard laboratory grade and used without further purification. Starting materials were purchased from Sigma-Aldrich, Acros or Novabiochem unless otherwise stated, and used as received.

5.2 Experimental Procedures

5.2.1 Alkylation of Dimedone

5.2.1.1 (2,2-Dimethyl-4,6-dioxo-cyclohexyl)-acetic acid tert-butyl ester 45d

\[
\text{O} \quad \text{O} \quad \text{O} \quad \text{O}
\]

A solution of lithium diisopropylamide (2.0 M, 7.13 ml, 14.26 mmol) in anhydrous THF (15 ml) was cooled to -78 °C under a dry nitrogen atmosphere. A solution of dimedone 33 (1.00 g, 7.13 mmol) and hexamethylphosphoric triamide (4.4 ml, 21.40 mmol) in anhydrous THF (7 ml) was then added dropwise over 5 min. After stirring for 1 hr at -78 °C, a solution of tert-butylbromoacetate 108 (1.05 ml, 7.13 mmol) in anhydrous THF (7 ml) was added and the mixture allowed to warm to room temperature overnight. The reaction was quenched with water (5 ml), diluted with ether (35 ml), and washed with 2M HCl (2 x 35 ml), then water (35 ml). The combined aqueous phase was extracted with ether (2 x 35 ml), and the total organic phase washed with brine (35 ml), dried (MgSO4), and rotary evaporated to give a
dark orange oil (1.84 g). Flash column chromatography on silica gel using DCM/EtOAc (20:1) yielded a yellow oil (0.94 g, 52 %): ν_max (CHCl_3) 1725.0, 1615.1; δ_H (250 MHz, CDCl_3) 0.67 (3H, s, C(C^A^H_3C^B^H_3)), 1.14 (3H, s, C(C^A^H_3C^B^H_3)), 1.43 (C(CH_3)_3), 2.29 (1H, dd, J 16.7, 3.8, CH^A^H^B^CO_2Bu^1), 2.44 (1H, dd, J 14.6, 2.5, CH^A^H^B^C(CH_3)_2), 2.66 (1H, dd, J 16.7, 9.4, CH^A^H^B^CO_2Bu^1), 2.73 (1H, d, J 14.6, CH^A^H^B^C(CH_3)_2), 3.13 (1H, dd, J 9.4, 3.7, CHCH_2CO_2Bu^1), 3.29 (1H, dd, J 16.5, 2.4, COCH^A^H^B^CO), 3.54 (1H, d, J 16.5, COCH^A^H^B^CO); δ_C (63 MHz, DEPT, CDCl_3) 22.0 (C(C^A^H_3C^B^H_3)), 27.9 (C(CH_3)_3), 28.3 (C(C^A^H_3C^B^H_3)), 29.9 (CH_2CO_2Bu^1), 33.4 (C(CH_3)_2), 55.7 (CHCH_2CO_2Bu^1), 56.0 (CH_2C(CH_3)_2), 58.2 (COCH_2CO), 80.9 (C(CH_3)_3), 171.8 (CO_2Bu^1), 202.7 (COCH_2CO), 202.9 (COCH_2CO); MS FAB (+ve) found m/z 255 (MH^+, 48 %), 181 (M^+-OBu^1, 89); HRMS FAB (+ve) found m/z 255.15972 (MH^+), C_{14}H_{23}O_4 requires 255.15963.

5.2.2 Silylation of Dimedone

5.2.2.1 5,5-Dimethyl-3-trimethylsilanyloxy-cyclohex-2-enone 47

Hexamethyldisilazane (2.00 ml, 9.42 mmol) was added to a solution of dimedone (33) (1.00 g, 7.13 mmol) in anhydrous DCM (20 ml). The solution was stirred under nitrogen at room temperature for 2 hrs. Excess hexamethyldisilazane was removed in vacuo to leave an orange liquid, which solidified to a white solid in the freezer (1.44 g, 95 %): mp 55-58 °C [lit.\textsuperscript{97} 56-58 °C]; δ_H (200 MHz, CDCl_3) 0.28 (9H, s, Si(CH_3)_3), 1.06 (6H, s, C(CH_3)_2), 2.17 (2H, s, CH_2C(CH_3)_2), 2.22 (2H, s, CH_2C(CH_3)_2), 5.37 (1H, s, CH=CO); MS ES (+ve) found m/z 213.0 (MH^+, 15 %).
Chapter 5

5.2.2.2 5,5-Dimethyl-1,3-bis-trimethylsilanyloxy-cyclohexa-1,3-diene 46

5,5-Dimethyl-3-trimethylsilanyloxy-cyclohex-2-enone 47 (6.30 g, 29.66 mmol) was dissolved in anhydrous THF (25 ml) and added dropwise via a cannula to a stirred solution of lithium diisopropylamide (2.0 M, 15.00 ml, 30.00 mmol) at —78 °C. The resulting solution was stirred at this temperature for 45 min, quenched with chlorotrimethylsilane (7.61 ml, 60.00 mmol), and allowed to warm to room temperature. The mixture was filtered, concentrated under reduced pressure to about 25 ml and filtered again. Further concentration produced a cloudy yellow liquid which was distilled under reduced pressure to give a colourless oil (3.80 g, 45 %): bp 57 °C (0.075 Torr) [lit. 94 112-113 °C (3 Torr)]; ν_{max} (neat) 1653.7 (C=C), 1604.0 (C=C); δ_{H} (250 MHz, CDCl3) 0.18 (9H, s, Si(CH3)3), 0.22 (9H, s, Si(CH3)3), 1.01 (6H, s, C(CH3)2), 2.04 (2H, d, J 1.1, CH2C(CH3)2), 4.39 (1H, m, CH=CO), 4.90 (1H, m, (OCCCH=CO); δ_{C} (63 MHz, DEPT, CDCl3) 0.13 (Si(CH3)3), 0.27 (Si(CH3)3), 28.9 (C(CH3)2), 32.6 (C(CH3)2), 44.61 (CH2C(CH3)2), 103.0 (CH=CO), 108.3 (COCH=CO), 147.0 (CO), 154.5 (CO); MS ES (+ve) found m/z 285.2 (MH^+, 25 %).

5.2.3 Alkylation Reactions on Bis-Silyl Enol Ether of Dimedone

5.2.3.1 4-(Hydroxy-phenyl-methyl)-5,5-dimethyl-cyclohexane-1,3-dione 48

A solution of benzaldehyde (0.074 g, 0.70 mmol) in anhydrous DCM (6 ml) was cooled to —78 °C and titanium tetrachloride (0.08 ml, 0.73 mmol) added. The resulting yellow mixture was stirred for 10 mins and a solution of 5,5-dimethyl-1,3-bis-trimethylsilanyloxy-cyclohexa-1,3-diene 46 (0.20 g, 0.70mmol) in anhydrous DCM (4 ml) added via a cannula. The mixture was stirred at —78 °C for 3 hrs, then allowed to warm to room temperature during which time the colour changed from
yellow to dark red. Saturated NaHCO₃ solution (5 ml) was added and after gas evolution had ceased, the mixture was extracted with ether (3 x 5 ml). The total organic phase was washed with water (5 ml), brine (5 ml), dried (MgSO₄), and rotary evaporated to give a yellow residue (0.14 g). Flash column chromatography on silica gel using EtOAc yielded an oily off-white solid (0.03 g, 17 %): νₘₚ (CHCl₃) 3683 (OH), 1728 (C=O), 1702 (C=O); δH (250 MHz, CDCl₃) 1.06 (3H, s, C(C₆H₃C₆H₃)), 1.22 (3H, s, C(C₆H₃C₆H₃)), 2.45 (1H, d, J 15.4, CH₃CH(CH₃)₃), 2.69 (1H, m, CHCH(OH)), 3.04 (1H, d, J 15.4, CH₃CH₂C(CH₃)₃), 3.36 (1H, d, J 16.8, COCH₃CH₂CO), 3.66 (1H, dd, J 16.8, 1.3, COCH₃CH₂CO), 5.29 (1H, d, J 2.7, CHCH(OH)Ph), 7.26-7.37 (5H, m, CH(OH)Ph); δC (63 MHz, DEPT, CDCl₃) 25.6 (C(C₆H₃C₆H₃)), 29.6 (C(C₆H₃C₆H₃)), 33.4 (C(CH₃)₃), 53.1 (CH₂C(CH₃)₃), 59.5 (OCCH₂CO), 65.7 (CHCH(OH)), 72.2 (CH(OH)Ph), [125.5 (CH), 127.8 (CH), 128.5 (CH), 5C, Ar-H], 143.0 (Ph), 204.4, (CO), 205.3 (CO); MS FAB (+ve) found m/z 247 (MH⁺, 75 %), 229 (MH⁺-H₂O, 100); HRMS FAB (+ve) found m/z 247.13313 (Iv₁IH) C₁₅H₁₉O₃ requires 247.13342.

5.2.3.2 Benzoic acid 5,5-dimethyl-3-oxo-cyclohex-1-enyl ester 49

![Diagram](attachment:image.png)

To a solution of 5,5-dimethyl-1,3-bis-trimethylsilyloxy-cyclohexa-1,3-diene 46 (0.20 g, 0.70 mmol) in anhydrous DCM (15 ml) was added benzoyl chloride (0.12 g, 0.85 mmol) and the resulting solution cooled to −78 °C. A solution of trimethylsilyl trifluoromethanesulfonate (0.02 ml, 0.10 mmol) in anhydrous DCM (3.5 ml) was then added via a cannula. The mixture was allowed to reach room temperature over 6 hrs, and was then stirred at this temperature over the weekend. Brine (5 ml) was added and the layers separated. The aqueous phase was extracted with ether (4 x 2.5 ml) and the total organic phase dried (MgSO₄) and rotary evaporated to give a brown oil (0.16 g). Flash column chromatography on silica gel using DCM/EtOAc (4:1) yielded a yellow liquid (0.08 g, 47 %): νₘₚ (neat) 1740.4 (ester), 1673.0 (α,β-
unsaturated ketone); \( \delta_H \) (250 MHz, CDCl\(_3\)) 1.15 (6H, s, C(CH\(_3\))\(_2\)), 2.33 (2H, s, CH\(_2\)C(CH\(_3\))\(_2\)), 2.53 (2H, d, \( J \) 1.2 CH\(_2\)C(CH\(_3\))\(_2\)), 6.06 (1H, m, COCH=COMe), 7.46-7.67 (3H, m, Ar-H), 8.06-8.12 (2H, m, Ar-H); \( \delta_C \) (63 MHz, DEPT, CDCl\(_3\)) 28.1 (C(CH\(_3\))\(_2\)), 33.2 (C(CH\(_3\))\(_2\)), 42.2 (PhO\(_2\)CCCH\(_2\)), 50.8 (COCH\(_2\)C(CH\(_3\))\(_2\)), 116.8, (COCH=CO), [128.7 (CH), 130.2 (CH), 134.1 (CH), 5C, Ar-H], 163.3 (OCOPh), 168.6 (CH=COCOPh), 199.6 (COCH=CO); MS ES (+ve) found \( m/z \) 245.2 (MH\(^+\), 28 %), 283.2 (MK\(^+\), 8); HRMS FAB (+ve) found \( m/z \) 245.11731 (M), \( C_{15}H_{17}O_3 \) requires 245.11777.

5.2.3.3 5,5-Dimethyl-4-(3-oxo-butyl)-cyclohexane-1,3-dione 50

![Structure of 5,5-Dimethyl-4-(3-oxo-butyl)-cyclohexane-1,3-dione](image)

A solution of methyl vinyl ketone (0.059 ml, 0.71 mmol) in anhydrous DCM (6 ml) was cooled to \(-78\) °C and titanium tetrachloride (0.077 ml, 0.71 mmol) added. The mixture was stirred for 10 mins and a solution of 5,5-dimethyl-1,3-bis-trimethylsilyl oxy-cyclohexa-1,3-diene 46 (0.20 g, 0.70 mmol) in anhydrous DCM (4 ml) added dropwise via a cannula. The mixture was stirred at \(-78\) °C for 3 hrs and then allowed to warm to room temperature. Saturated NaHCO\(_3\) solution (5 ml) was added and the layers separated. The aqueous phase was extracted with ether (3 x 5 ml) and the total organic phase dried (MgSO\(_4\)) and rotary evaporated to give a gummy yellow residue (0.08 g). Flash column chromatography on silica gel using DCM/EtOAc (4:1) as eluent isolated a yellow oil (0.02 g, 14 %). MS ES (+ve) found \( m/z \) 211.4 (MH\(^+\), 19 %), 233.4 (MNa\(^+\), 32), 141.4 (dimedoneH\(^+\), 58). However, \( ^1H \) NMR spectrum was complex and contained many unassignable peaks.
5.2.4 Synthesis and Alkylations of Methoxy-Dimedone Derivative

5.2.4.1 3-Methoxy-5,5-dimethyl-cyclohex-2-enone 51

![Structure of 3-Methoxy-5,5-dimethyl-cyclohex-2-enone 51]

Dimedone 33 (25.00 g, 178.32 mmol) was dissolved in anhydrous methanol (200 ml) and chlorotrimethylsilane (23.75 ml, 187.23 mmol) added. \( N,N \)-diisopropylethylamine (DIPEA) (65.23 ml, 356.63 mmol) was then added dropwise and the mixture stirred at room temperature overnight. The methanol was removed on the rotary evaporator and the residue dissolved in EtOAc (150 ml), washed with water (75 ml), saturated NaHCO₃ solution (75 ml), and then brine (75 ml). The organic phase was dried (MgSO₄) and rotary evaporated to give a pale yellow liquid (27.22 g, 99 %) which did not require further purification: \( \nu_{\text{max}} \) (neat) 1660.4 (\( \alpha,\beta \)-unsaturated ketone), 1609.3 (C=C); \( \delta_H \) (250 MHz, CDCl₃) 1.04 (6H, s, C(CH₃)₂), 2.18 (2H, s, \( CH_2C(CH_3)_2 \)), 2.24 (2H, s, \( CH_2C(CH_3)_2 \)), 3.66 (3H, s, OCH₃), 5.33 (1H, s, COCH=COCH₃); \( \delta_C \) (63 MHz, DEPT, CDCl₃) 28.1 (C(CH₃)₂), 32.4 (C(CH₃)₂), 42.5 (CH₂COCH₃), 50.6 (CH₂CO), 55.5 (OCH₃), 101.0 (COCH=CO), 176.8 (CH=COCH₃), 199.3 (COCH=CO); MS ES (+ve) found \( m/z \) 155.0 (MH⁺, 14 %), 177.1 (MNa⁺, 10).

5.2.4.2 (4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-ynyl)-acetic acid tert-butyl ester 53a

![Structure of (4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-ynyl)-acetic acid tert-butyl ester 53a]

\( n \)-Butyllithium (1.6 M, 22.29 ml, 35.67 mmol) was added dropwise to a solution of dry diisopropylamine (5.00 ml, 35.67 mmol) in anhydrous THF (12.5 ml) at 0 °C. After 30 min the mixture was cooled to –78 °C and a solution of 3-methoxy-5,5-dimethyl-cyclohex-2-enone 51 (5.00 g, 32.43 mmol) and dry \( N,N \)-dimethylpropyleneurea (DMPU) (12.5 ml) in anhydrous THF (12.5 ml) was added.
dropwise. After stirring for 1.5 hrs at -78 °C, a solution of tert-butyl bromoacetate 108 (9.45 ml, 64.86 mmol) and dry DMPU (2 ml) in anhydrous THF (6 ml) was added dropwise and the mixture allowed to reach room temperature overnight. The reaction was quenched with saturated ammonium chloride solution (20 ml), diluted with ether (50 ml) and washed with 2M KHSO₄ (20 ml), water (20 ml), and saturated NaHCO₃ solution (20 ml). The combined aqueous phase was extracted with ether (2 x 20 ml), and the total organic phase was washed with brine (30 ml), dried (MgSO₄) and rotary evaporated to give a viscous yellow oil (11.85 g). Flash column chromatography on silica gel using hexane/EtOAc (7:3-1:1) yielded a white solid (7.82 g, 90 %): mp 48-50 °C; ν max (nujol) 1732.7 (ester), 1664.3 (α,β-unsaturated ketone), 1620.8 (C=C); δ H (250 MHz, CDCl₃) 0.86 (3H, s, C(CH₃CHH₃)), 1.09 (3H, s, C(C₆H₃C₆H₃)), 1.46 (9H, s, OC(CH₃)₃), 2.13 (1H, d, J 17.4, CH²H₆C(CH₃)₂), 2.17 (1H, dd, J 16.0, 4.5, CH²H₆CO₂(CH₃)₃), 2.54 (1H, dd, J 17.4, 1.7, CH²H₆C(CH₃)₂), 2.63 (1H, dd, J 16.0, 8.2, CH²H₆CO₂(CH₃)₃), 2.79 (1H, dd, J 8.2, 4.5, CHCH₂CO₂(CH₃)₃), 3.67 (3H, s, OCH₃), 5.35 (1H, d, J 1.7, COCH=COCH₃); δ C (63 MHz, DEPT, CDCl₃) 21.2 (C(C₆H₃C₆H₃)), 27.9 (OC(CH₃)₃), 28.8 (C(C₆H₃C₆H₃)), 30.6 (CH₂CO₂(CH₃)₃), 35.5 (C(CH₃)₂), 44.2 (CH₂C(CH₃)₂), 52.8 (CHCH₂CO₂(CH₃)₃), 55.5 (OCH₃), 80.1 (OC(CH₃)₃), 100.7 (COCH=COMe), 172.6 (CO₂(CH₃)₃), 175.1 (COCH=COCH₃), 198.8 (COCH=COCH₃); MS FAB (+ve) found m/z 269 (MH⁺, 48 %), 291 (MNa⁺); HRMS FAB (+ve) found m/z 269.17579 (MH⁺), C₁₅H₂₅O₄ requires 269.17528.

5.2.4.3 3-Methoxy-5,5-dimethyl-6-(3-methyl-but-2-enyl)-cyclohex-2-enone 53b

\[
\text{\includegraphics[width=0.2\textwidth]{image}}
\]

n-Butyllithium (1.6 M, 22.29 ml, 35.67 mmol) was added dropwise to a solution of dry diisopropylamine (5.00 ml, 35.67 mmol) in anhydrous THF (12.5 ml) at 0 °C. After 30 min the mixture was cooled to -78 °C and a solution of 3-methoxy-5,5-dimethyl-cyclohex-2-enone 51 (5.00 g, 32.43 mmol) and dry DMPU (12.5 ml) in anhydrous THF (12.5 ml) was added dropwise. After stirring for 1.5 hrs at -78 °C, a
solution of 3,3-dimethylallyl bromide 125 (7.00 ml, 60.73 mmol) and dry DMPU (2 ml) in anhydrous THF (6 ml) was added dropwise and the mixture allowed to reach room temperature overnight. The reaction was quenched with saturated ammonium chloride solution (20 ml), diluted with ether (50 ml) and washed with 2M KHSO₄ (20 ml), water (20 ml), and saturated NaHCO₃ solution (20 ml). The combined aqueous phase was extracted with ether (2 x 20 ml), and the total organic phase washed with brine (30 ml), dried (MgSO₄) and rotary evaporated to give an orange oil (8.73 g). Flash column chromatography on silica gel using hexane/EtOAc (4:1-1:1) yielded a yellow oil (5.83 g, 81 %): νₚₛ (neat) 1656.6 (α,β-unsaturated ketone), 1616.1 (ring C=C); δ H (250 MHz, CDC₁₃) 0.96 (3H, s, C(CH₃CH₃)₃), 1.04 (3H, s, C(CH₃CH₃)₃), 1.56 (3H, s, CH=C(CH₃CH₃)₃), 1.64 (3H, d, J 1.2, CH=C(CH₃CH₃)₃), 1.97 (1H, dd, J 7.2, 5.9, COCHC(CH₃)₂), 2.16 (1H, d, J 17.5, CH₂C(CH₃)₂C(CH₃)₂), 2.19 (2H, m, CH₂CH=CMe₂), 2.31 (1H, d, J 17.6, CH₂C(CH₃)₂C(CH₃)₂), 3.64 (3H, s, OCH₃), 5.25 (1H, s, COCH=COCH₃); δ C (63 MHz, DEPT, CDC₁₃) 17.7 (CH=C(CH₃CH₃)₃), 24.4 (C(CH₃CH₃)₃), 24.9 (CH₂CH=CMe₂), 25.7 (CH=C(CH₃CH₃)₃), 28.7 (C(CH₃CH₃)₃), 35.1 (C(CH₃)₂), 41.6 (CH₂C(CH₃)₂), 55.4 (OCH₃), 57.5 (COCHC(CH₃)₂), 100.3 (COCH=COCH₃), 123.1 (CH₂CH=CMe₂), 131.6 (CH=CMe₂), 174.8 (COCH=COCH₃), 201.8 (COCH=COCH₃); MS FAB (+ve) found m/z 223 (M⁺H, 100 %), 207 (M⁺-CH₃, 50); HRMS FAB (+ve) found m/z 223.16977 (MH⁺), C₁₄H₂₃O₂ requires 223.16981.

5.2.4.4 (4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid ethyl ester

n-Butyllithium (1.6 M, 17.87 ml, 28.53 mmol) was added dropwise to a solution of dry diisopropylamine (4.00 ml, 28.53 mmol) in anhydrous THF (10 ml) at 0 °C. After 30 min the mixture was cooled to −78 °C and a solution of 3-methoxy-5,5-dimethyl-cyclohex-2-enone 51 (4.00 g, 25.94 mmol) and dry DMPU (10 ml) in anhydrous THF (10 ml) was added dropwise. After stirring for 1 hr at −78 °C, a
solution of ethyl bromoacetate (3.16 ml, 28.53 mmol) and dry DMPU (1.5 ml) in anhydrous THF (5 ml) was added dropwise and the mixture allowed to reach -45 °C and kept at this temperature for 1 hr. The reaction was quenched with saturated ammonium chloride solution (20 ml), diluted with ether (50 ml) and washed with 2M KHSO₄ (20 ml), water (20 ml) and saturated NaHCO₃ solution (20 ml). The combined aqueous phase was extracted with ether (2 x 20 ml), and the total organic phase washed with brine (30 ml), dried (MgSO₄), and rotary evaporated to give a viscous yellow oil (6.43 g). Flash column chromatography on silica gel using hexane/EtOAc (4:1) yielded a yellow oil (3.79 g, 61 %): vₘₐₓ (neat) 1733.7 (ester), 1651.5 (α,β-unsaturated ketone), 1617.0 (C=O); δH (250 MHz, CDCl₃) 0.84 (3H, s, CH₃), 1.07 (3H, s, CH₃), 1.24 (3H, t, J 7.1 OCH₂CH₃), 2.11 (1H, d, J 17.4, CH₃), 2.21 (1H, dd, J 16.0, 4.2, CH₂ HO₂CO₂Et), 2.53 (1H, dd, J 17.4, 1.2, CH₃), 2.67 (1H, dd, J 16.0, 8.6, CH₂ HO₂CO₂Et), 2.83 (1H, dd, J 8.6, 4.2, CH₂ CO₂Et), 3.64 (3H, s, OCH₃), 4.13 (2H, m, OCH₂CH₃), 5.32 (1H, d, J 1.7, COCH=COCH₃); δC (63 MHz, DEPT, CDCl₃) 14.1 (OCH₂CH₃), 21.1 (CH₂), 28.7 (CH₂), 29.4 (CH₂), 35.5 (CH₃), 44.2 (CH₂), 52.8 (CH₂), 55.6 (OCH₃), 60.4 (OCH₂CH₃), 100.6 (COCH=COCH₃), 173.4 (CO₂Et), 175.2 (COCH=COCH₃), 198.6 (COCH=COCH₃); MS FAB (+ve) found m/z 241 (M⁺, 52 %), 195 (M⁺-OEt, 100); HRMS FAB (+ve) found m/z 241.14349 (MH⁺), C₁₃H₂₁O₄ requires 241.14398.

5.2.4.5 3-Methoxy-5,5-dimethyl-6-(2-oxo-2-phenyl-ethyl)-cyclohex-2-enone 53d

\[
\text{\begin{tikzpicture}
    \draw[thick,->] (0,0) -- (1,0) node[midway,above] {Ph};
    \draw[thick,->] (1,0) -- (1.5,0) node[midway,above] {O};
    \draw[thick,->] (1.5,0) -- (2,0) node[midway,above] {OCH₂CH₃};
    \draw[thick,->] (2,0) -- (2.5,0) node[midway,above] {OCH₃};
    \draw[thick,->] (2.5,0) -- (3,0) node[midway,above] {OMe};
    \end{tikzpicture}}
\]

n-Butyllithium (1.6 M, 4.46 ml, 7.14 mmol) was added dropwise to a solution of dry diisopropylamine (1.01 ml, 7.14 mmol) in anhydrous THF (5.5 ml) at 0 °C. After 30 min the mixture was cooled to -78 °C and a solution of 3-methoxy-5,5-dimethyl-cyclohex-2-enone 51 (1.00 g, 6.49 mmol) and dry DMPU (1.68 ml, 19.50 mmol) in anhydrous THF (7.0 ml) was added dropwise. After stirring for 1 hr at -78 °C, a solution of 2-bromoacetophenone (1.29 g, 6.48 mmol) in anhydrous THF (1.5 ml)
was added dropwise and the mixture allowed to reach room temperature overnight. The reaction was quenched with water (2.5 ml), diluted with ether (25 ml), and washed with 2M HCl (3 x 25 ml), then water (25 ml). The combined aqueous phase was extracted with ether (2 x 25 ml), and the total organic phase washed with brine (25 ml), dried (MgSO₄) and rotary evaporated to give a brown oil (1.55 g). Flash column chromatography on silica gel using DCM/hexane (7:3) yielded a yellow oil (0.05 g, 23 %): v_max (neat) 1685.5 (PhC=O), 1653.7 (α,β-unsaturated ketone), 1616.1 (C=C); δ_H (250 MHz, CDCl₃) 0.93 (3H, s, C(CH₃)₂), 1.08 (3H, s, C(CH₂H₃C⁺H₃)), 2.17 (1H, d, J 17.3, CH⁺H⁺C(CH₃)₂), 2.68 (1H, dd, J 17.3, 1.3, CH⁺H⁺C(CH₃)₂), 2.70 (1H, dd, J 17.1, 4.1, CH⁺H⁺COPh), 3.26 (1H, dd, J 7.5, 4.1, CHCH₂COPh), 3.69 (1H, dd, J 17.1, 7.5, CH⁺H⁺COPh), 3.73 (3H, s, OCH₃), 5.37 (1H, d, J 1.8, COCH=COCH₃), 7.41-7.54 (3H, m, Ar-H), 7.99-8.03 (2H, m, Ar-H); δ_C (63 MHz, DEPT, CDCl₃) 21.3 (C(CH₃)₂), 29.0 (C(CH₃)₂), 33.0 (CH₂COPh), 357 (C(CH₃)₂), 44.4 (CH₂C(CH₃)₂), 52.0 (CHCH₂COPh), 55.6 (OCH₃), 100.7 (COCH=CO), [128.1 (CH), 128.4 (CH), 132.6 (CH), 5C, Ar-H], 137.3 (COPh), 175.3 (COCH=COCH₃), 198.9 (COPh), 199.2 (COCH=COCH₃); MS ES (+ve) found m/z 273 (MH⁺, 44 %); HRMS FAB (+ve) found m/z 273.14944 (MH⁺), C₁₇H₂₁O₃ requires 273.14907.

5.2.5 Enol Ether Hydrolysis

5.2.5.1 (2,2-Dimethyl-4,6-dioxo-cyclohexyl)-acetic acid ethyl ester 45e

![Chemical Structure](image)

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid ethyl ester (53c) (0.55 g, 2.29 mmol) was dissolved in THF (20 ml) and 2M HCl (20 ml) slowly added. The mixture was then stirred at room temperature overnight. The solvent was removed in vacuo and the residue taken up in EtOAc (50 ml) and washed with saturated NaHCO₃ solution (25 ml), then water (25 ml). The aqueous phase was extracted with EtOAc (2 x 25 ml) and the total organic phase washed with brine (30 ml), dried (MgSO₄) and rotary evaporated to give a yellow oil (0.54 g). Flash column
chromatography on silica gel using hexane/EtOAc (4:1-1:1) as eluent yielded a yellow oil (0.31 g, 60%): $\nu_{\text{max}}$ (CHCl$_3$) 1726.9, 1608.3; $\delta_H$ (360 MHz, CDCl$_3$) 0.71 (3H, s, C($A^3$H$_3$C$B^3$H$_3$)), 1.17 (3H, s, C($A^3$H$_3$C$B^3$H$_3$)), 1.27 (3H, t, J 7.1, CH$_3$CH$_2$O), 2.38 (1H, dd, J 16.18, 3.6, CH$_A^4$H$_3$C$_2$O$_2$Et), 2.48 (1H, dd, J 14.17, 2.5, CH$_A^4$H$_3$C(CH$_3$)$_2$), 2.77 (1H, dd, J 16.8, 9.5 CH$_A^4$H$_3$C$_2$O$_2$Et), 2.77 (1H, dd, J 14.17, 0.9 CH$_A^4$H$_3$C(CH$_3$)$_2$), 3.21 (1H, dd, J 9.5, 3.6, CH$_3$CH$_2$CO$_2$Et), 3.34 (1H, dd, J 16.5, 2.5, COCH$_A^4$H$_3$CO), 3.57 (1H, dd, J 16.5, 0.9 COCH$_A^4$H$_3$CO), 4.16 (2H, m, CH$_3$CH$_2$O); $\delta_C$ (63 MHz, DEPT, CDCl$_3$) 14.1 (C(CH$_3$)$_2$), 21.5 (C($A^3$H$_3$C$B^3$H$_3$)), 28.3 (C($A^3$H$_3$C$B^3$H$_3$)), 28.8 (CH$_2$CO$_2$Et), 33.4 (C(CH$_3$)$_2$), 55.6 (CH$_2$CO$_2$Et), 56.0 (CH$_3$C(CH$_3$)$_2$), 58.1 (COCH$_2$CO), 60.8 (CH$_3$CH$_2$O), 172.6 (CO$_2$Et), 202.6 (COCH$_2$CO), 202.8 (COCH$_2$CO); MS FAB (+ve) found $m/z$ 227 (MH$^+$, 87%), 249 (MNa$^+$, 66), (M$^+$-OEt, 84); HRMS FAB (+ve) found $m/z$ 227.12786 (MH$^+$), C$_{12}$H$_{19}$O$_4$ requires 227.12833.

5.2.5.2 5,5-Dimethyl-4-(2-oxo-2-phenyl-ethyl)-cyclohexane-1,3-dione 45a

3-Methoxy-5,5-dimethyl-6-(2-oxo-2-phenyl-ethyl)-cyclohex-2-enone 53d (0.20 g, 0.73 mmol) was dissolved in anhydrous THF (20 ml) and 2M HCl (20 ml) added. The resulting solution was stirred at room temperature overnight. The reaction mixture was diluted with DCM (50 ml) and the layers separated. The organic phase was washed with saturated NaHCO$_3$ solution (25 ml), water (25 ml) and brine (25 ml), then dried (MgSO$_4$) and rotary evaporated to give an orange oil (0.13 g, 69%): $\delta_H$ (360 MHz, CDCl$_3$) 0.78 (3H, s, C($A^3$H$_3$C$B^3$H$_3$)), 1.18 (3H, s, C($A^3$H$_3$C$B^3$H$_3$)), 2.51 (1H, dd, J 14.17, 2.5, CH$_A^4$H$_3$C(CH$_3$)$_2$), 2.84 (1H, d, J 14.17, CH$_A^4$H$_3$C(CH$_3$)$_2$), 2.84 (1H, dd, J 17.2, 2.7 CH$_A^4$H$_3$COPh), 3.34 (1H, dd, J 16.4, 2.5, COCH$_A^4$H$_3$CO), 3.57 (1H, dd, J 9.0, 2.5, CH$_3$CH$_2$COPh), 3.63 (1H, d, J 16.4, COCH$_A^4$H$_3$CO), 3.65 (1H, dd, J 17.2, 9.0, COCH$_A^4$H$_3$CO), 7.42-7.60 (3H, m, Ar-H), 7.99-8.02 (2H, m, Ar-H); $\delta_C$ (63 MHz, DEPT, CDCl$_3$) 21.9 (C($A^3$H$_3$C$B^3$H$_3$)), 28.6 (C($A^3$H$_3$C$B^3$H$_3$)), 32.5 (CH$_2$COPh), 33.5 (C(CH$_3$)$_2$), 54.7 (CH$_2$COPh), 56.2 (CH$_2$C(CH$_3$)$_2$), 58.3
Chapter 5

Experimental

(COCH₂CO), [128.0 (CH), 128.6 (CH), 133.2 (CH), 5C, Ar-H], 136.7 (COPh), 198.2 (COPh), 202.8 (COCH₂CO), 202.9 (COCH₂CO); MS FAB (+ve) found m/z 259 (MH⁺, 3.5 %), 281 (MNa⁺, 6 %); HRMS FAB (+ve) found m/z 259.13349 (MH⁺), C₁₆H₁₉O₃ requires 259.13342.

5.2.6 Synthesis of Bromomethylketones – Method 1

5.2.6.1 (S)-4-Benzoyloxycarbonylamino-3-oxo-pentanoic acid tert-butyl ester 57

1,1-Carboxyldiimidazole (2.30 g, 14.18 mmol) was added to a stirred solution of CBz-L-alanine 54a (3.00 g, 13.44 mmol) in anhydrous THF (45 ml), and the resulting mixture stirred at room temperature for 1 hr. Meanwhile, n-butyllithium (2.5 M, 16.90 ml, 42.25 mmol) was added dropwise to a stirred solution of dry diisopropylamine (5.96 ml, 42.25 mmol) in anhydrous THF (30 ml) at 0 °C. After 30 min this mixture was cooled to −78 °C and a solution of tert-butyl acetate (5.69 ml, 42.25 mmol) in anhydrous THF (10 ml) added dropwise. After stirring the lithium enolate solution for 1 hr at −78 °C, the imidazole solution was added dropwise and the resulting mixture stirred at −78 °C for 40 min. The reaction was quenched with 1M HCl (100 ml) and extracted with EtOAc (3 x 50 ml). The total organic phase was washed with brine (100 ml), dried (MgSO₄), passed through a short silica pad and rotary evaporated to give an orange oil (4.00 g). Flash column chromatography on silica gel using hexane/EtOAc (4:1) yielded an orange oil (2.43 g, 56 %): δH (250 MHz, CDCl₃) 1.60 (3H, d, J 7.2, CαCH₃), 1.69 (9H, s, OC(CH₃)₃), 3.66 (1H, d, J 15.8, COCH₂HBCO), 3.73 (1H, d, J 15.8 COCH₂HBCO), 4.70 (1H, m, Cα-H), 5.32 (2H, s, CH₂Ph), 5.89 (1H, d, J 7.4, NH), 7.57 (5H, s, Ar-H); δC (63 MHz, DEPT, CDCl₃) 17.0 (CαCH₃), 27.8 (OC(CH₃)₃), 47.0 (COCH₂CO), 55.6 (Cα), 66.8 (CH₂Ph), 82.2 (OC(CH₃)₃), [127.9 (CH), 128.1 (CH), 128.4 (CH), 5C, Ar-H], 136.1 (CH₂Ph), 155.6 (CONH), 165.9 (CO₂(CH₃)₃), 202.1 (NHC(CH₃)CO); MS FAB (+ve) found 322 (MH⁺, 17 %); HRMS FAB (+ve) found m/z 322.16553, C₁₇H₂₄NO₃ requires 322.16545.
5.2.6.2 (S)-(3-Bromo-1-methyl-2-oxo-propyl)-carbamic acid benzyl ester 36a

Solid N-bromosuccinimide (0.83 g, 4.66 mmol) was added to a solution of (S)-4-benzyloxycarbonylamino-3-oxo-pentanoic acid tert-butyl ester 57 (1.50 g, 4.66 mmol) and 2,6-lutidine (0.054 ml, 0.46 mmol) in anhydrous methanol (9.30 ml). The mixture was stirred at room temperature for 2.5 hrs, then stored in the freezer for 20 hrs. EtOAc (70 ml) was added and the mixture extracted with 50 % saturated brine (28 ml), and then saturated brine (28 ml). The total organic phase was dried (MgSO\(_4\)) and rotary evaporated to give a pale red oil (1.76 g). This was dissolved in benzene/TFA (8:1) (30 ml) and heated under reflux for 2 hrs. After cooling, the reaction was washed with water (2 x 14 ml), saturated NaHCO\(_3\) solution (2 x 14 ml) and brine (2 x 14 ml). The total organic phase was dried (MgSO\(_4\)) and rotary evaporated to give a pale orange solid (0.84 g). Recrystallisation from hexane/EtOAc yielded a white solid (0.5 g, 36 %): mp 82-84 °C [lit.\(^{101}\) 83-84 °C]; \(\delta_H\) (250 MHz, CDCl\(_3\)) 1.40 (3H, d, \(J\) 7.2, C\(_\alpha\)CH\(_3\)), 4.02 (1H, d, \(J\) 13.4, COCH\(^A\)H\(^B\)Br), 4.08 (1H, d, \(J\) 13.4 COCH\(^A\)H\(^B\)Br), 4.65 (1H, m, C\(_\alpha\)-H), 5.10 (2H, s, CH\(_2\)Ph), 5.52 (1H, d, \(J\) 5.5, NH), 7.34 (5H, s, Ar-H); \(\delta_C\) (63 MHz, DEPT, CDCl\(_3\)) 17.7 (C\(_\alpha\)CH\(_3\)), 31.4 (COCH\(_2\)Br), 53.5 (C\(_\alpha\)), 67.1 (CH\(_2\)Ph), [128.1 (CH), 128.2 (CH), 128.5 (CH), 5C, Ar-H], 136.0 (CH\(_2\)Ph), 155.6 (CONH), 201.0 (NHC(\(\text{CH}_3\))CO); MS FAB (+ve) found 300, 302 (MH\(^+\), 34 %), 256, 258 (MH\(^+\)-CO\(_2\), 7); HRMS FAB (+ve) found \(m/z\) 300.02389, 302.02129, C\(_{12}\)H\(_{15}\)NO\(_3\)Br requires 300.02353, 302.02161.

5.2.7 Phthaloylation of Amino Acids

5.2.7.1 3-Chloro-3-(dimethoxyphosphoryl)isobenzofuran-1(3H)-one 62

![](image-url)
Trimethyl phosphite 61 (8.14 ml, 69.01 mmol) was added dropwise to neat phthaloyl dichloride 60 (9.94 ml, 69.01 mmol) over 25 mins at such a rate that the temperature was kept below 50 °C. The mixture was then stirred at room temperature for 15 mins until gas evolution had ceased. Excess trimethyl phosphite was removed in vacuo and the pale yellow residue triturated with ether to afford a white crystalline solid (13.9 g, 73 %): mp 71-75 °C [lit. 78-80 °C]; δ_H (250 MHz, CDCl3) 3.71 (3H, d, J 10.6, OCH3), 4.05 (3H, d, J 10.6, OCH3), 7.68-7.96 (4H, m, Ar-H); MS ES (+ve) found m/z 299 (MNa+, 100 %).

5.2.7.2 (S)-N-Pthaloylalanine 64

3-Chloro-3-(dimethoxyphosphoryl)isobenzofuran-1(3H)-one 62 (12.16 g, 43.96 mmol) and L-alanine 63 (4.11 g, 46.17 mmol) were dissolved in MeCN/H2O (200 ml, 1:1), and DIPEA (30.63 ml, 175.84 mmol) added. The mixture was stirred at room temperature for 40 mins when TLC showed no trace of starting material. The mixture was concentrated in vacuo to a small volume, acidified to pH 1 with 2M HCl, and cooled to 0 °C for 30 mins. The white precipitate formed was filtered, washed with ice-cold 2M HCl and dried in vacuo over P2O5 to afford a white solid (8.10 g, 84 %): [α]D -23 (c 1.0, EtOH) [lit. 103 [α]D -22.5 (c 1.0, EtOH)]; mp 145-147 °C [lit.104 152-154 °C]; δ_H (250 MHz, CDCl3) 1.70 (3H, d, J 7.4, CH3), 5.02 (1H, q, J 7.4, Cα-H), 7.67-7.75 (2H, m, Ar-H), 7.81-7.88 (2H, m, Ar-H), 9.62 (1H, br, OH); (63 MHz, DEPT, CDCl3) 15.0 (CH3), 47.2 (CCH3), [123.5 (CH), 2C, Ar-H], 131.7 (ArCO), [134.2 (CH), 2C, Ar-H], 167.3 (CONCO), 175.4 (CO2H); MS ES (+ve) found m/z 218 (MH+, 100 %).
5.2.8 Dibenzylation of Amino Acids

5.2.8.1 (S)-2-Dibenzylamino-propionic acid ethyl ester 65

To a solution of L-alanine ethyl ester hydrochloride 40a (4.80 g, 31.25 mmol) and DIPEA (11.43 ml, 65.62 mmol) in anhydrous DCM (150 ml) was added benzyl bromide (7.81 ml, 65.62 mmol) dropwise. The solution was then heated under reflux overnight. The mixture was allowed to cool, then washed with water (50 ml), saturated NaHCO₃ solution (2 x 50 ml) and brine (50 ml). The total organic phase was dried (MgSO₄) and rotary evaporated to give an orange oil (8.60 g). Flash column chromatography on silica gel using hexane/EtOAc (4:1) as eluent yielded a yellow oil (6.00 g, 65 %) which was contaminated with some benzyl bromide starting material: vₘₐₓ (neat) 1729.8 (ester); δH (250 MHz, CDCl₃) 1.57 (3H, d, J 7.1, Cα-CH₃), 1.57 (3H, t, J 7.1, OCH₂CH₃), 3.74 (1H, q, J 7.1, Cα-H), 3.90 (2H, d, J 14.0, CH₂Ph), 4.10 (2H, d, J 14.0, CH₂Ph), 4.45 (2H, q, J 7.1 OCH₂CH₃), 7.44-7.66 (10H, m, Ar-H); δC (63 MHz, DEPT, CDCl₃) 14.5 (Cα-CH₃), 14.9 (OCH₂CH₃), 54.4 (CH₂Ph), 56.1 (Cα-H), 60.1 (OCH₂CH₃), [126.9 (CH), 128.2 (CH), 128.6 (CH), 10C, Ar-H], 139.9 (PhCH₂), 173.6 (CO₂Et); MS FAB (+ve) found m/z 298 (MH⁺, 98 %), 224 (M⁺-CO₂Et, 100).

5.2.8.2 (R)-2-Dibenzylamino-propionic acid methyl ester 67

To a stirred solution of methyl-(S)-lactate 66 (2.29 ml, 24.01 mmol) in anhydrous DCM (50 ml) at 0 °C was added triflic anhydride (4.44 ml, 26.41 mmol) followed by 2,6-lutidine (3.07 ml, 26.41 mmol). After stirring for 15 mins at 0 °C, a solution of dibenzylamine (14.31 ml, 74.44 mmol) in anhydrous DCM (25 ml) was added dropwise. The resulting mixture was stirred for 2 hrs at room temperature. The
mixture was concentrated in vacuo and the residue dissolved in hexane. This caused a yellowish solid to precipitate, which was filtered off and the filtrate passed through a short silica pad using ether as eluent. Evaporation of the ether yielded an orange oil (5.80 g, 85%): [\alpha]_D^\text{[a]} +98.2 (c 2.2, CHCl_3) [lit.\textsuperscript{105} [\alpha]_D^\text{[a]} +88.5 (c 2.2, CHCl_3)]; \nu_{\text{max}} (neat) 1734.2 (ester); \delta_H (250 MHz, CDCl_3) 1.53 (3H, d, J 7.1, C\alpha–CH_3), 3.71 (1H, q, J 7.1, C\alpha–H), 3.83 (2H, d, J 14.0, CH_2Ph), 3.93 (3H, s, OCH_3), 4.03 (2H, d, J 14.0, CH_2Ph), 7.42-7.60 (10H, m, Ar-H); \delta_C (63 MHz, DEPT, CDCl_3) 15.00 (C\alpha-CH_3), 51.2 (OCH_3), 54.4 (CH_2Ph), 56.1 (C\alpha-H), [126.9 (CH), 128.2 (CH), 128.6 (CH), 10C, Ar-H], 139.8 (PhCH_2), 174.2 (CO_2Me); MS ES (+ve) found m/z 284 (MH\textsuperscript{+}, 100%).

5.2.9 Synthesis of Bromomethylketones - Method 2

5.2.9.1 N-Methyl-N-nitroso-para-toluenesulfonamide 69 (Diazald®)

\[
\text{Me} - \begin{array}{c}
\text{O} \\
\text{N=O} \\
\text{S} \\
\text{O} \\
\text{N-Me}
\end{array}
\]

N-Methyl-para-toluenesulfonamide 68 (25.00 g, 134.95 mmol) was dissolved in acetic acid (250 ml) and the solution cooled to 0 °C. A solution of sodium nitrite (13.97 g, 202.43 mmol) in water (65 ml) was then added dropwise with vigorous stirring. The mixture was stirred at 0 °C for 20 mins, then diluted with water (500 ml). The yellow precipitate that formed was filtered off and dried in vacuo over P_2O_5 to provide a yellow crystalline solid (27.20 g, 94%): mp 60-62 °C [lit.\textsuperscript{148} 60.5-61.5 °C]; \nu_{\text{max}} (CHCl_3) 1504.7 (N=O), 1377.9 (SO_2 anti-sym), 1171.5 (SO_2 sym); \delta_H (250 MHz, CDCl_3) 2.44 (3H, s, CH_3-Ar), 3.11 (3H, s, N-CH_3), 7.37 (2H, d, J 8.6, Ar-H), 7.86 (2H, d, J 8.6, Ar-H); \delta_C (63 MHz, DEPT, CDCl_3) 21.7 (CH_3-Ar), 28.8 (N-CH_3), [127.9 (CH), 130.3 (CH), 4C, Ar-H], 134.0 (Ar-CH_3), 146.1 (Ar-SO_2); MS ES (+ve) found m/z 215 (MH\textsuperscript{+}, 4%), 237 (MNa\textsuperscript{+}, 7).
5.2.9.2 2-((S)-3-Diazo-1-methyl-2-oxo-propyl)-isoindole-1,3-dione 71

![Chemical Structure](image)

To a solution of N-phthaloyl-L-alanine 64 (1.00 g, 4.56 mmol) in anhydrous DCM (15 ml) at 0 °C was added oxalyl chloride (0.60 ml, 6.84 mmol) followed by 3 drops of anhydrous DMF. The resulting solution was stirred at 0 °C for 2.5 hrs and the volatiles removed in vacuo. Diazomethane was prepared by adding a solution of Diazald® 69 (5.00 g) in ether (45 ml) dropwise to a mixture of KOH (2.5 g), water (4 ml) and diethylene glycol mono-ethyl ether (14 ml) held at 50 °C. The distilled ethereal diazomethane solution (~ 50 ml, ~ 16.6 mmol) was collected at 0 °C and the crude acid chloride, dissolved in anhydrous DCM (15 ml), added dropwise to it. The mixture was stirred at room temperature for 2.5 hrs and excess diazomethane destroyed by a few drops of acetic acid. Saturated NaHCO₃ solution (25 ml) was added and the aqueous phase extracted with ether (2 x 15 ml). The total organic phase was washed with brine (25 ml), dried (MgSO₄) and rotary evaporated to give an orange oil (1.11 g). Flash column chromatography on silica gel using hexane/EtOAc (7:3) as eluent yielded a yellow oil (0.95 g, 86 %): [α]D -84 (c 0.5, CHCl₃) [lit.149 [α]D -88.5 (c 0.5, EtOAc)]; νmax (nujol) 2110.7 (N≡N), 1778.5 (lactam), 1723.1 (ketone), 1644.0 (C=N); δH (250 MHz, CDCl₃) 1.65 (3H, d, J 7.3, Cα-CH₃), 4.89 (1H, q, J 7.3, Cα-H), 5.41 (1H, s, CHN₂), 7.69-7.77 (2H, m, Ar-H), 7.81-7.86 (2H, m, Ar-H); δC (63 MHz, DEPT, CDCl₃) 14.5 (Cα-CH₃), 52.2 (C-H), 53.8 (C-H), [123.5 (CH), 2C, Ar-H], 131.7 (ArCO), [134.3 (CH), 2C, Ar-H], 167.5 (CONCO), 190.1 (COCHN₂); MS FAB (+ve) found m/z 244 (MH⁺, 47 %), 266 (MNa⁺, 14), 216 (MH⁺-N₂, 92), 174 (M⁺-COCHN₂, 100); HRMS FAB (+ve) found m/z 244.07200 (MH⁺), C₁₂H₁₀N₃O₃ requires 244.07222.
5.2.9.3 2-((S)-3-Bromo-1-methyl-2-oxo-propyl)-isoindole-1,3-dione 72

To a solution of 2-((S)-3-Diazo-1-methyl-2-oxo-propyl)-isoindole-1,3-dione 71 (1.62 g, 6.66 mmol) in DCM (80 ml) at 0 °C was added 48% HBr (1.50 ml, 13.32 mmol) dropwise. The reaction was stirred at room temperature overnight and saturated NaHCO₃ solution (80 ml) added. The aqueous phase was extracted with DCM (2 x 40 ml), and the total organic phase washed with brine (50 ml), dried (MgSO₄) and rotary evaporated to give an off-white solid (1.68 g, 85%): [α]D -41.5 (c 1.3, CHCl₃) [lit.¹⁵⁰ [α]D -36.61 (c 1.3, CHCl₃)]; mp 77-79 °C [lit.¹⁵⁰ 70-71 °C]; νmax (nujol) 1779.0 (lactam), 1713.4 (ketone); δH (250 MHz, CDCl₃) 1.64 (3H, d, J 7.2, CH₃), 3.96 (1H, d, J 12.8, CH₃Br), 4.01 (1H, d, J 12.8, CH₃Br), 5.18 (1H, q, J 7.2, Cα-H), 7.71-7.79 (2H, m, Ar-H), 7.84-8.08 (2H, m, Ar-H); δC (63 MHz, DEPT, CDCl₃) 14.5 (Cα-CH₃), 30.8 (CH₃Br), 51.6 (Cα-H), [123.7 (CH), 2C, Ar-H], 131.7 (ArCO), [134.5 (CH), 2C, Ar-H], 167.4 (CONCO), 197.5 (COCH₂Br); MS FAB (+ve) found m/z 296, 298 (MH⁺, 97%); HRMS FAB (+ve) found m/z 295.99261 (MH⁺), 297.99099 (MH⁺), C₁₂H₁₁BrNO₃ requires 295.99223, 297.99031.

5.2.10 Synthesis of Bromomethylketones - Method 3

5.2.10.1 (R)-1-Bromo-3-dibenzylamino-butan-2-one 74

A solution of (R)-2-dibenzylamino-propionic acid methyl ester 67 (0.50 g, 1.76 mmol) and dibromomethane (0.50 ml, 7.05 mmol) in anhydrous THF (20 ml) was cooled to −78 °C and n-butyl lithium (1.6 M, 4.41 ml, 7.05 mmol) added dropwise. The solution was then stirred at −78 °C for 30 mins. Saturated ammonium chloride solution (10 ml) was added and the reaction allowed to warm to room temperature.
The mixture was then extracted with ether (3 x 7 ml) and the total organic phase dried (MgSO₄) and rotary evaporated to give a gummy orange solid (0.71 g): δ_H (250 MHz, CDCl₃) 1.22 (3H, d, J 6.7, Cα–CH₃), 3.44 (2H, d, J 13.5, CH₂Ph), 3.64 (1H, q, J 6.7, Cα–H), 3.70, (2H, d, J 13.5, CH₂Ph), 4.15 (1H, d, J 13.2 CH²H²Br), 4.23 (1H, d, J 13.2 CH²H²Br), 7.30-7.37 (10H, m, Ar–H); MS FAB (+ve) found m/z 346, 348 (MH⁺, 23 %), 368 (MNa⁺, 100).

5.2.11 Synthesis of Enol Ether Derivatives from Dimedone Isomer

4,4-Dimethylcyclohexane-1,3-dione 76 (5.00 g, 35.70 mmol) was dissolved in anhydrous methanol (45 ml) and chlorotrimethylsilane (4.75 ml, 37.40 mmol) added. DIPEA (13.05 ml, 74.90 mmol) was then added dropwise and the mixture stirred at room temperature overnight. The methanol was removed in vacuo and the residue dissolved in EtOAc (50 ml), washed with water (25 ml), saturated NaHCO₃ solution (25 ml), and then brine (25 ml). The organic phase was dried (MgSO₄) and rotary evaporated to give a pale yellow liquid (4.77 g). Flash column chromatography on silica gel using hexane/EtOAc (4:1) as eluent yielded two yellow liquids:

5.2.11.1 3-Methoxy-4,4-dimethyl-cyclohex-2-enone 77

(1.22 g, 22 %): bp 90 °C (0.4 mBar) [lit. 151 46.8-48.9 °C (0.08 Torr)]; ν_max (neat) 1662.3 (α,β-unsaturated ketone), 1595.8 (C=C); δ_H (250 MHz, CDCl₃) 1.19 (6H, s, C(CH₃)₂), 1.81 (2H, t, J 6.4, CH₂C(CH₃)₂), 2.40 (2H, t, J 6.4, CH₂COMe), 3.66 (3H, s, OCH₃), 5.25 (1H, s, CH=COMe); δ_C (63 MHz, DEPT, CDCl₃) 25.6 (C(CH₃)₂), 33.8 (CH₂C(CH₃)₂), 35.7 (C(CH₃)₂), 36.3 (CH₂CO), 55.8 (OCH₃), 100.8 (CH=COMe), 183.9 (COMe), 199.6 (CO); MS ES (+ve) found m/z 177 (MNa⁺, 100 %).
5.2.11.2 3-Methoxy-6,6-dimethyl-cyclohex-2-enone 78

(3.21 g, 58 %): bp 85 °C (0.3 mBar) [lit. 151 45.1-46.1 °C (0.08 Torr)]; $\nu_{\text{max}}$ (neat) 1650.8 ($\alpha, \beta$-unsaturated ketone), 1613.2 (C=C); $\delta_H$ (250 MHz, CDCl$_3$) 1.09 (6H, s, C(CH$_3$)$_2$), 1.78 (2H, t, $J$ 6.4, CH$_2$C(CH$_3$)$_2$), 2.41 (2H, t, $J$ 6.4, CH$_2$COMe), 3.66 (3H, s, OCH$_3$), 5.25 (1H, s, CH=COMe); $\delta_C$ (63 MHz, DEPT, CDCl$_3$) 24.5 (C(CH$_3$)$_2$), 26.0 (CH$_2$C(CH$_3$)$_2$), 35.0 (CH$_2$COMe), 40.2 (C(CH$_3$)$_2$), 55.6 (OCH$_3$), 100.6 (CH=COMe), 176.4 (COMe), 204.3 (CO); MS ES (+ve) found $m/z$ 155 (MH$^+$, 80 %), 177 (MNa$^+$, 100).

5.2.12 Enaminone Synthesis

5.2.12.1 5,5-Dimethyl-3-pyrrolidin-1-yl-cyclohex-2-enone 80

Dimebdone 33 (5.00 g, 35.67 mmol) and pyrrolidine (2.98 ml, 35.67 mmol) were dissolved in toluene (150 ml) and the solution refluxed overnight using a Dean-Stark trap to remove the water. The toluene was removed in vacuo to give an orange solid (6.75 g, 98 %): mp 125-128 °C [lit. 152 129-132 °C]; $\nu_{\text{max}}$ (CHCl$_3$) 1599.7 ($\alpha, \beta$-unsaturated ketone), 1557.2 (C=C); $\delta_H$ (250 MHz, CDCl$_3$) 1.04 (6H, s, C(CH$_3$)$_2$), 1.92 (4H, m, NCH$_2$ CH$_2$CH$_2$CH$_2$), 2.17 (2H, s, CH$_2$CO), 2.25 (2H, s, CH$_2$CN), 3.18 (2H, m, NCH$_2$CH$_2$CH$_2$CH$_2$), 3.37 (2H, m, NCH$_2$CH$_2$CH$_2$CH$_2$), 4.99 (1H, s, CH=CN); $\delta_C$ (63 MHz, DEPT, CDCl$_3$) 24.6 (NCH$_2$CH$_2$CH$_2$CH$_2$), 25.2 (NCH$_2$CH$_2$CH$_2$CH$_2$), 28.6 (C(CH$_3$)$_2$), 32.6 (C(CH$_3$)$_2$), 41.7 (CH$_2$CN), 47.8 (NCH$_2$CH$_2$CH$_2$CH$_2$), 49.5 (CH$_2$CO), 97.0 (CH=CN), 161.8 (CH=CN), 195.5 (CO); MS ES (+ve) found $m/z$ 194 (MH$^+$, 100 %).
5.2.12.2 1-(3-Methoxy-5,5-dimethylcyclohex-2-enylidene)-pyrrolidine 81

5,5-Dimethyl-3-pyrrolidin-1-yl-cyclohex-2-enone 80 (1.00 g, 5.17 ml) was dissolved in methyl iodide (10 ml) and the solution gently refluxed overnight. Methyl iodide was removed in vacuo to give a bright yellow solid (1.70 g, 98 %): mp 121-125 °C [lit.\textsuperscript{108} 146-149.5 °C]; \(\nu_{\text{max}}\) (CHCl\(_3\)) 2438.6 (C=N), 1584.2 (C=C); \(\delta_H\) (250 MHz, DMSO-d\(_6\)) 1.25 (6H, s, C(CH\(_3\))\(_2\)), 2.21 (4H, m, NCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)), 2.62 (2H, s, CH\(_2\)), 2.92 (2H, s, CH\(_2\)), 4.06 (4H, m, NCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)), 4.16 (3H, s, OMe), 6.08 (1H, s, CH=COMe); \(\delta_C\) (63 MHz, DEPT, DMSO-d\(_6\)) 23.9 (NCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)), 24.1 (NCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)), 33.2 (C(CH\(_3\))\(_2\)), 31.6 (C(CH\(_3\))\(_2\)), 38.9 (CH\(_2\)), 41.6 (CH\(_2\)), 51.9 (NCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)), 58.0 (OCH\(_3\)), 94.0 (CH=COMe), 172.3 (COMe), 181.5 (C=N); MS ES (+ve) found m/z 208 (M\(^{+}\)-I, 100 %).

5.2.13 Alternative Synthetic Route: Enaminones to Diazoketones

5.2.13.1 (S)-2-(5,5-Dimethyl-3-oxo-cyclohex-1-enylamino)-propionic acid ethyl ester 41a

Dimedone 33 (4.56 g, 32.55 mmol) and L-alanine ethyl ester hydrochloride 40a (5.00 g, 32.55 mmol) were dissolved in toluene (150 ml) and acetic acid (5.5 ml) added. The solution was then refluxed overnight using a Dean-Stark trap to remove the water. The toluene was removed in vacuo and the residue taken up in ether (50 ml) and washed with water (25 ml), then saturated NaHCO\(_3\) solution (2 x 25 ml). The aqueous phase was extracted with ether (2 x 25 ml) and the total organic phase washed with brine (25 ml), dried (MgSO\(_4\)) and rotary evaporated to give a reddish oil (6.40 g, 82 %): \([\alpha]_D\) -35 (c 1.0, CHCl\(_3\)); \(\nu_{\text{max}}\) (CHCl\(_3\)) 3252.8 (N-H), 1739.0 (ester),
1584.7 ($\alpha,\beta$-unsaturated ketone), 1544.7 (C=C); $\delta_H$ (250 MHz, CDCl$_3$) 1.01 (3H, s, C(C$^\alpha$H$_3$C$^\beta$H$_3$)), 1.02 (3H, s, C(C$^\alpha$H$_3$C$^\beta$H$_3$)), 1.25 (3H, t, J 7.2, OCH$_2$CH$_3$), 1.40 (3H, d, J 7.0, C$_\alpha$-CH$_3$), 2.13 (2H, s, CH$_2$CNH), 2.18 (2H, s, CH$_2$CO), 4.05 (1H, q, J 7.0, C$_\alpha$-H), 4.17 (2H, q, J 7.2, OCH$_2$CH$_3$), 4.99 (1H, s, CH=CNH), 5.18 (1H, d, J 6.6, NH); $\delta_C$ (63 MHz, DEPT, CDCl$_3$) 14.0 (C$_\alpha$-CH$_3$), 17.8 (OCH$_2$CH$_3$), 28.1 (C(C$^\alpha$H$_3$C$^\beta$H$_3$)), 28.2 (C(C$^\alpha$H$_3$C$^\beta$H$_3$)), 32.8 (C(CH$_3$)$_2$), 43.3 (CH$_2$CNH), 50.2 (CH$_2$CO), 50.5 (C$_\alpha$-H), 61.7 (OCH$_2$CH$_3$), 96.4 (CH=CNH), 160.9 (CNH), 172.6 (CO$_2$Et), 197.0 (CO); MS ES (+ve) found m/z 239 (MH$^+$, 100%).

5.2.13.2 (S)-2-(5,5-Dimethyl-3-oxo-cyclohex-1-enylamino)-propionic acid 42a

(S)-2-(5,5-Dimethyl-3-oxo-cyclohex-1-enylamino)-propionic acid ethyl ester 41a (6.20 g, 25.90 mmol) was suspended in saturated NaHCO$_3$ solution (80 ml) and the suspension refluxed for 2.5 hrs after which time a homogeneous solution had formed. After allowing to cool, the solution was concentrated in vacuo and the residue acidified to pH 4 with 2M HCl. This caused a solid to precipitate which was filtered and dried over P$_2$O$_5$ to give an off-white solid (3.70 g, 68 %): mp 197-199 °C [lit.$^{112}$ 214-215 °C]; $[\alpha]_D$ –51.3 (c 1.5, H$_2$O) [lit.$^{112}$ $[\alpha]_D$ –128 (c 1.5, EtOH)]; $\nu_{max}$ (CHCl$_3$) 3341.1 (N-H), 1721.6 (acid), 1552.4 ($\alpha,\beta$-unsaturated ketone); $\delta_H$ (250 MHz, CD$_3$OD) 1.10 (6H, s, C(CH$_3$)$_2$), 1.52 (3H, d, J 7.1, C$_\alpha$-CH$_3$), 2.21 (2H, s, CH$_2$CNH), 2.40 (2H, s, CH$_2$CO), 4.14 (1H, q, J 7.1, C$_\alpha$-H); $\delta_C$ (63 MHz, DEPT, CD$_3$OD) 17.7 (C$_\alpha$-CH$_3$), 28.3 (C(CH$_3$)$_2$), 33.7 (C(CH$_3$)$_2$), 43.4 (CH$_2$CNH), 50.4 (CH$_2$CO), 52.3 (C$_\alpha$-H), 95.2 (CH=CNH), 167.8 (CNH), 175.4 (COOH), 199.3 (CO); MS ES (+ve) found m/z 212 (MH$^+$, 100 %), 234 (MNa$^+$, 89).
5.2.13.3  ((S)-3-Diazo-1-methyl-2-oxo-propyl)-(5,5-dimethyl-3-oxo-cyclohex-1-enyl) carbamic acid isobutyl ester 85

(S)-2-(5,5-Dimethyl-3-oxo-cyclohex-1-enylamino)-propionic acid 42a (1.00 g, 4.73 mmol) was suspended in anhydrous THF (20 ml) under dry nitrogen and the mixture cooled to -10 °C (ice/salt bath). N-Methylmorpholine (1.04 ml, 9.46 mmol) was added, followed by iso-butyl chloroformate (1.23 ml, 9.46 mmol) dropwise. The mixture was then stirred at -10 °C for 30 mins. The mixture was quickly filtered and the filtrate cooled to 0 °C. A concentrated ethereal solution of diazomethane [prepared as described in 5.2.9.2 using same quantities of reagents] was then added dropwise. The resulting yellow solution was allowed to warm to room temperature and stirred for 3 hrs. Excess diazomethane was destroyed by addition of a few drops of acetic acid. Saturated NaHCO$_3$ solution (25 ml) was then added and the aqueous phase extracted with ether (2 x 15 ml). The total organic phase was washed with brine (25 ml), dried (MgSO$_4$) and rotary evaporated to give a yellow oil (1.43 g). Flash column chromatography on silica gel using hexane/EtOAc (7:3) as eluent yielded a yellow oil (0.42 g, 26 %): $\delta$H (250 MHz, CDCl$_3$) 0.86 (6H, d, J 6.7, (CH$_3$)$_2$CH), 1.02 (3H, s, C(C$_5$H$_3$C$_3$H$_3$)), 1.03 (3H, s, C(C$_5$H$_3$C$_3$H$_3$)), 1.40 (3H, d, J 7.1, C$_\alpha$-CH$_3$), 1.86 (1H, m, (CH$_3$)$_2$CH), 2.20 (2H, s, CH$_2$CO), 2.36 (1H, dd, J 17.7, 0.8, C$_A$HC$_B$HCN), 2.60 (1H, dd, J 17.7, 1.0, C$_A$HC$_B$HCN), 3.87 (2H, d, J 6.6, CH$_2$OCO), 4.44 (1H, q, J 7.1, C$_\alpha$-H), 5.42 (1H, s, CH=CN), 5.81 (1H, s, CHN$_2$); $\delta$C (63 MHz, DEPT, CDCl$_3$) 14.7 (C$_\alpha$-CH$_3$), 18.9 ((CH$_3$)$_2$CH), 27.6 (CH$_3$)$_2$CH), 27.7 (C(C$_5$H$_3$C$_3$H$_3$), 27.9 (C(C$_5$H$_3$C$_3$H$_3$)), 33.6 (C(CH$_3$)$_2$), 44.2 (CH$_2$CN), 50.7 (CH$_2$CO), 53.4 (CHN$_2$), 61.5 (C$_\alpha$-H), 72.9 (CH$_2$OCO), 121.8 (CH=CN), 153.6 (OCON), 159.0 (CN), 191.7 (COCHN$_2$), 199.3 (CO); MS El (+ve) found m/z 336
Chapter 5

Experimental

(MH$^+$, 10 %), 266 (M$^+$-COCHN$_2$, 38), 206 (M$^+$-CO$_2$Bu$^+$-N$_2$, 38), 166 (MH$^+$-COCHN$_2$-CO$_2$Bu$^+$, 100); HRMS El (+ve) found $m/z$ 335.18451 (M$^+$), C$_{17}$H$_{25}$N$_3$O$_4$ requires 335.18398.

5.2.13.4 (S)-2-[(5,5-Dimethyl-3-oxo-cyclohex-1-enyl)-isobutoxycarbonyl-amino]- propionic acid methyl ester 86

By-product from reaction 5.2.13.3. Isolated as a colourless oil (0.06 g, 4 %) by flash chromatography on silica gel using hexane/EtOAc (7:3) as eluent: $v_{\text{max}}$ (CHCl$_3$) 1747.2 (ester), 1712.5 (carbamate), 1666.2 ($\alpha,\beta$-unsaturated ketone), 1621.8 (C=C); $\delta$$_H$ (250 MHz, CDCl$_3$) 0.88 (6H, d, $J$ 6.7, (CH$_3$)$_2$CH), 1.03 (3H, s, C(C$_{\alpha}$H$_3$C$_{\beta}$H$_3$)), 1.05 (3H, s, C(C$_{\alpha}$H$_3$C$_{\beta}$H$_3$)), 1.50 (3H, d, $J$ 7.2, C$_{\alpha}$-CH$_3$), 1.89 (1H, m, (CH$_3$)$_2$CH), 2.22 (2H, s, CH$_2$CO), 2.42 (1H, dd, $J$ 17.6, 0.7, C$_{\alpha}$HC$_{\beta}$HCN), 2.62 (1H, dd, $J$ 17.6, 1.0, C$_{\alpha}$HC$_{\beta}$HCN), 3.69 (3H, s, OCH$_3$), 3.89 (2H, d, $J$ 6.5, CH$_2$OCN), 4.42 (1H, q, $J$ 7.2, C$_{\alpha}$-H), 5.82 (1H, s, CH=CN); $\delta$$_C$ (63 MHz, DEPT, CDCl$_3$) 15.7 (C$_{\alpha}$-CH$_3$), 19.0 ((CH$_3$)$_2$CH), 27.7 (CH$_3$)$_2$CH), 28.0 (C(CH$_3$)$_2$), 33.6 (C(CH$_3$)$_2$), 44.1 (CH$_2$CN), 50.8 (CH$_2$CO), 52.5 (OCH$_3$), 58.2 (C$_{\alpha}$-H), 72.9 (CH$_2$OCO), 120.9 (CH=CN), 153.5 (OCON), 160.2 (CN), 171.2 (CO$_2$Me), 199.7 (CO); MS El (+ve) found $m/z$ 325 (M$^+$, 38 %), 266 (M$^+$-CO$_2$Me, 38), 224 (M$^+$-CO$_2$Bu$^+$, 38), 166 (MH$^+$-COCHN$_2$-CO$_2$Bu$^+$, 100); HRMS El (+ve) found $m/z$ 325.18892 (M$^+$), C$_{17}$H$_{27}$NO$_5$ requires 325.18945.
5.2.13.5 \textit{N-Methyl-L-alanine methyl ester} 88

\begin{center}
\includegraphics[width=0.2\textwidth]{structure_88}
\end{center}

Thionyl chloride (0.26 ml, 3.62 mmol) was slowly added to methanol (2 ml) cooled to -10 °C (ice/salt bath). \textit{N-Methyl-L-alanine hydrochloride} 87 (0.50 g, 3.58 mmol) was then added in portions and the resulting mixture heated under reflux for 1 hr. The volatiles were removed \textit{in vacuo} to give an orange oil (0.60 g, overweight): \(\delta_H\) (250 MHz, CDCl\(_3\)) 1.69 (3H, d, \(J_7.1\), \(C_\alpha-CH_3\)), 2.77 (3H, t, \(J 4.9\), NCH\(_3\)), 3.81 (3H, s, OCH\(_3\)), 3.96 (1H, m, \(C_\alpha-H\)); \(\delta_C\) (63 MHz, DEPT, CDCl\(_3\)) 14.2 (\(C_\alpha-CH_3\)), 30.8 (NCH\(_3\)), 53.2 (OCH\(_3\)), 56.0 (\(C_\alpha-H\)), 169.0 (CO\(_2\)Me); MS ES (+ve) found \textit{m/z} 118 (MH\(^+\), 100 %).

5.2.13.6 \textit{(S)-2-[(5,5-Dimethyl-3-oxo-cyclohex-1-enyl)-methyl-aminol-propionic acid methyl ester} 89

\begin{center}
\includegraphics[width=0.2\textwidth]{structure_89}
\end{center}

Dimedone 33 (0.48 g, 3.42 mmol) and crude \textit{N-Methyl-L-alanine methyl ester} 88 (0.40 g, 3.41 mmol) were dissolved in toluene (20 ml) and acetic acid (0.5 ml) added. The solution was then refluxed for 3 hrs using a Dean-Stark trap to remove the water. The toluene was removed \textit{in vacuo} and the residue taken up in DCM (30 ml) and washed with water (10 ml), then saturated NaHCO\(_3\) solution (2 x 10 ml). The aqueous phase was extracted with DCM (2 x 10 ml) and the total organic phase washed with brine (20 ml), dried (MgSO\(_4\)) and rotary evaporated to give a reddish oil (0.35 g). Flash column chromatography on silica gel using EtOAc as eluent yielded an orange oil (0.12 g, 15 %): [\(\alpha\)]\(_D\) -112 (c 1.0, CHCl\(_3\)); \(\nu_{\text{max}}\) (CHCl\(_3\)), 1743.8 (ester), 1612.2 (\(\alpha,\beta\)-unsaturated ketone), 1557.7 (C=C); \(\delta_H\) (250 MHz, CDCl\(_3\)) 1.00 (3H, s, C(\(C^AH_3C^BH_3\))), 1.01 (3H, s, C(\(C^AH_3C^BH_3\))), 1.39 (3H, d, \(J 7.1\), \(C_\alpha-CH_3\)), 2.09 (2H, s, CH\(_2\)CO), 2.22 (2H, d, \(J 3.3\) \ CH\(_2\)CNMe), 2.73 (3H, s, N-CH\(_3\)), 3.66 (3H, s, O-
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5.2.14 General Methods for Carbenoid-Mediated Ring-Closure

**Method A**

To a solution of Rh$_2$(OAc)$_4$ 93 (6.6 mg, 2.5 mol %) in anhydrous DCM (10 ml) was slowly added a solution of diazoketone 85 (0.20 g, 0.60 mmol) in anhydrous DCM (10 ml). The resulting mixture was stirred at room temperature overnight, then passed through a short silica pad. The filtrate was reduced *in vacuo* to give the crude product, which was purified by flash column chromatography on silica gel using hexane/EtOAc gradient as eluent.

**Method B**

As for Method A but reaction mixture refluxed for 1.5 hrs.

**Method C**

Diazoketone 85 (0.040 g, 0.12 mmol) was dissolved in anhydrous DCM (4 ml) in a microwave test-tube and Rh$_2$(OAc)$_4$ 93 (1.3 mg, 2.5 mol %) added. The test-tube was sealed and heated using microwave power (300 W) for 5 mins at 100 °C. The solvent was removed *in vacuo* to give the crude product which was purified as for Method A.

**Method D**

As for Method C but using anhydrous toluene as the solvent.
**Method E**

As for Method A but using Rh$_2$(cap)$_4$ 95 (1.3 mol %) as catalyst.

**Method F**

As for Method A but using Rh$_2$(pfb)$_4$ 94 (1 mol %) as catalyst.

5.2.15 **Summary of Results for Diazoketone 85**

Method A: Two main products isolated:

i) Yellow oil (0.02 g): $^1$H NMR complex and unassignable; MS ES (+ve) found $m/z$ 308 (MH$^+$, 73 %), 340 (100).

ii) Yellow solid (0.03 g): $\delta_H$ (250 MHz, CDCl$_3$) 1.00 (6H, d, $J$ 6.7), 1.09 (6H, s), 2.00 (1H, m), 2.13 (3H, s), 2.30 (2H, s), 2.57 (2H, s), 3.78 (2H, d, $J$ 6.7); $\delta_C$ (63 MHz, DEPT, CDCl$_3$) 19.3, 28.5, 35.5, 37.3, 52.7, 81.0, 111.9, 115.4, 138.1, 139.6, 192.3; MS ES (+ve) found $m/z$ 250 (100 %).

Method B: Three main products isolated:

i) and ii) as above.

iii) Yellow oil (0.02 g) possibly resulting from Wolff rearrangement 102: $\delta_H$ (250 MHz, CDCl$_3$) 0.93 (6H, d, $J$ 6.7, (CH$_3$)$_2$CH), 1.10 (3H, s, C(C$^A$H$_3$C$^B$H$_3$)), 1.13 (3H, s, C(C$^A$H$_3$C$^B$H$_3$)), 1.55 (3H, d, $J$ 7.0, C$_\alpha$-CH$_3$), 1.92 (1H, m, (CH$_3$)$_2$CH), 2.28 (2H, s, CH$_2$CO), 2.40 (1H, d, $J$ 17.5, C$^A$HC$^B$HCN), 2.75 (1H, dd, $J$ 17.5, 1.2, C$^A$HC$^B$HCN), 3.94 (2H, d, $J$ 6.6, CH$_2$OCN), 4.31 (1H, q, $J$ 7.0, C$_\alpha$-H), 4.38 (2H, m COCH$_2$), 5.83 (1H, s, CH=CN); $\delta_C$ (63 MHz, DEPT, CDCl$_3$) 14.3 (C$_\alpha$-CH$_3$), 19.1 ((CH$_3$)$_2$CH), 27.8 (CH$_3$)$_2$CH), 28.4 (C(CH$_3$)$_2$), 33.9 (C(CH$_3$)$_2$), 44.3 (CH$_2$CN), 50.7 (CH$_2$CO), 62.3 (C$_\alpha$-H), 65.8, 73.4 (CH$_2$OCO), 120.1 (CH=CN), 153.3 (OC=CN), 159.7 (CN), 199.3 (CO), 206.0 (ketone); MS ES (+ve) found $m/z$ 326 (MH$^+$, 100 %), 348 (MNa$^+$, 31).

Methods C and D gave similar results to above. Methods E and F gave predominantly compound with $m/z$ 326.
5.2.16 Synthesis of Diazoketone Isomer

5.2.16.1 2-(4,4-Dimethyl-3-oxo-cyclohex-1-enylamino)-propionic acid ethyl ester 104

4,4 Dimethyl-1,3-cyclohexanedione 76 (4.56 g, 32.55 mmol) and D/L-alanine ethyl ester hydrochloride 103 (5.00 g, 32.55 mmol) were dissolved in toluene (150 ml) and acetic acid (5.5 ml) added. The solution was then refluxed for 2.5 hrs using a Dean-Stark trap to remove the water. The toluene was removed in vacuo and the residue taken up in EtOAc (50 ml) and washed with water (25 ml), then saturated NaHCO₃ solution (2 x 25 ml). The aqueous phase was extracted with EtOAc (2 x 25 ml) and the total organic phase washed with brine (25 ml), dried (MgSO₄) and rotary evaporated to give a gummy yellow solid which was triturated with ether to produce a collectable pale yellow solid (5.74 g, 74 %): mp 107-108 °C; ν max (CHCl₃) 3290.9 (N-H), 1735.6 (ester), 1591.0 (α,β-unsaturated ketone), 1542.8 (C=C); δH (250 MHz, CDCl₃) 1.05 (3H, s, C(C₆H₃C₆H₃)), 1.07 (3H, s, C(C₆H₃C₆H₃)), 1.25 (3H, t, J 7.1, OCH₂CH₃), 1.39 (3H, d, J 7.0, C₆H₁₃H₂), 1.76 (2H, t, J 6.3 CH₂CMe₂), 2.36 (2H, t, J 6.3 CH₂CNH), 4.02 (1H, q, J 7.0, C₆H₁₃H₂), 4.14 (2H, q, J 7.1, OCH₂CH₃), 4.88 (1H, s, CH=CNH), 5.04 (1H, d, J 6.7, NH); δC (63 MHz, DEPT, CDCl₃) 14.0 (C₆H₁₃H₂), 17.9 (OCH₂CH₃), 24.9 (C(CH₃)₂), 26.4 (CH₂CNH), 35.5 (CH₂CMe₂), 39.5 (C(CH₃)₂), 50.4 (C₆H₁₃H₂), 61.7 (OCH₂CH₃), 96.2 (CH=CNH), 160.7 (CNH), 172.7 (CO₂Et), 202.5 (CO); MS FAB (+ve) found m/z 240 (MH⁺, 100 %), 166 (M⁺-CO₂Et, 34); HRMS FAB (+ve) found m/z 240.15993 (MH⁺), C₁₃H₂₂NO₃ requires 240.15997.
5.2.16.2 2-(4,4-Dimethyl-3-oxo-cyclohex-1-enylamino)-propionic acid 105

\[
\begin{align*}
\text{O} & \quad \text{OH} \\
\text{Me} & \quad \text{N} \quad \text{H}
\end{align*}
\]

2-(4,4-Dimethyl-3-oxo-cyclohex-1-enylamino)-propionic acid 104 (5.50 g, 22.98 mmol) was suspended in saturated NaHCO₃ solution (70 ml) and the suspension refluxed for 2.5 hrs, after which time a homogeneous solution had formed. After allowing to cool, the solution was concentrated in vacuo and the residue acidified to pH 1 with 2M HCl. This caused a solid to precipitate which was filtered and dried over P₂O₅ to give a white solid (2.86 g, 59 %): mp 199-200 °C; \( \nu_{\text{max}} \) (nujol), 1745.3 (acid), 1606.41 (\( \alpha,\beta \)-unsaturated ketone), 1556.3 (C=C); \( \delta_{\text{H}} \) (250 MHz, D₂O) 0.95 (6H, s, C(CH₃)₂), 1.31 (3H, d, J 7.2, C-CH₃), 1.63 (2H, t, J 6.5 CH₂CMe₂), 2.51 (2H, t, J 6.7 CH₂CNH), 4.29 (1H, m, C=H); \( \delta_{\text{C}} \) (63 MHz, DEPT, D₂O) 16.7 (C₃CH₃), 24.6 (C(CH₃)₂), 26.8 (CH₂CNH), 34.7 (CH₂CMe₂), 37.3 (C(CH₃)₂), 52.7 (C₃H), 174.7 (CNH), 176.1 (COOH), 198.2 (CO); MS FAB (+ve) found m/z 212 (MH⁺, 100 %), 166 (M⁺-CO₂H, 49); HRMS FAB (+ve) found m/z 212.12822 (MW) C₁₁H₁₈NO₃ requires 212.12867.

5.2.16.3 (3-Diazo-1-methyl-2-oxo-propyl)-(4,4-dimethyl-3-oxo-cyclohex-1-enyl) carbamic acid isobutyl ester 106

\[
\begin{align*}
\text{O} & \quad \text{N}^2 \quad \text{Me} \\
\text{N} & \quad \text{O} \quad \text{Me}
\end{align*}
\]

2-(4,4-Dimethyl-3-oxo-cyclohex-1-enylamino)-propionic acid 105 (1.00 g, 4.73 mmol) was suspended in anhydrous THF (20 ml) under dry nitrogen and the mixture cooled to -10 °C (ice/salt bath). N-Methylmorpholine (1.04 ml, 9.44 mmol) was
added, followed by isobutyl chloroformate (1.22 ml, 9.44 mmol) dropwise. The mixture was then stirred at -10 °C for 30 mins. The mixture was quickly filtered and the filtrate cooled to 0 °C. A concentrated ethereal solution of diazomethane [prepared as described in 5.2.9.2 using same quantities of reagents] was then added dropwise. The resulting yellow solution was allowed to warm to room temperature and stirred for 3 hrs. Excess diazomethane was destroyed by addition of a few drops of acetic acid. Saturated NaHCO₃ solution (25 ml) was then added and the aqueous phase extracted with ether (2 x 15 ml). The total organic phase was washed with brine (25 ml), dried (MgSO₄) and rotary evaporated to give a yellow oil (1.40 g). Flash column chromatography on silica gel using hexane/EtOAc (7:3) as eluent yielded a yellow oil (0.23 g, 14 %): \( \text{v}_{\text{max}} \) (CHCl₃), 2111.7 (N=N), 1706.7 (carbamate), 1662.3 (\( \alpha,\beta \)-unsaturated ketone); \( \delta \text{H} \) (250 MHz, CDCl₃) 0.88 (6H, d, J 6.7, (CH₃)₂CH), 1.08 (3H, s, C(AH₃B₃H₃)), 1.09 (3H, s, C(AH₃B₃H₃)), 1.40 (3H, d, J 7.2, CA-CH₃), 1.81 (2H, t, J 6.2 CH₂CMé₂), 1.83 (1H, m, (CH₃)₂CH), 2.51 (1H, m, CH₄H₃CN), 2.73 (1H, m, CH₄H₃CN), 3.87 (2H, d, J 6.5, CH₂OCON), 4.51 (1H, q, J 7.2, CA-H), 5.45 (1H, s, CH=CN), 5.76 (1H, s, CHN₂); \( \delta \text{C} \) (63 MHz, DEPT, CDCl₃) 14.8 (CA-CH₃), 19.0 ((CH₃)₂CH), 23.7 (CH₃₂CH), 23.8 (C(CH₃)₂), 27.8 (CH₂CN₃H), 35.7 (CH₂CMé₂), 40.4 (C(CH₃)₂), 53.6 (CHN₂), 60.7 (CA-H), 72.8 (CH₂OCO), 122.9 (CH=CN), 153.8 (OCON), 158.5 (CN), 192.0 (COCHN₂), 204.0 (CO); MS FAB (+ve) found \( m/z \) 336 (MH⁺, 11 %), 266 (M⁻COCHN₂, 20), 166 (MH⁺-COCHN₂-CO₂Bu⁻, 92); HRMS FAB (+ve) found \( m/z \) 336.19224 (MH⁺), \( C_{17}H_{26}N_{3}O_{4} \) requires 336.19233.

5.2.17 Synthesis of Acid Derivative

5.2.17.1 4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl-acetic acid 109
Method A

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid tert-butyl ester 53a (4.84 g, 18.03 mmol) was dissolved in anhydrous DCM (45 ml) and trifluoroacetic acid (15 ml) and triethylsilane (7.2 ml, 45.08 mmol) added. The mixture was then stirred at room temperature for 2 hrs. The volatiles were removed in vacuo to give a yellow oil (6.00 g), which was purified by flash column chromatography on silica gel using hexane/EtOAc (1:1) as eluent to give a white solid (2.95 g, 77 %): mp 120-122 °C (Found C, 62.3; H, 7.6; N, 0.08 %. C₁₁H₁₆O₄ requires C, 62.2; H, 7.6; N, 0.0 %); ν max (CHCl₃) 1709.6 (acid), 1652.7 (α,β-unsaturated ketone), 1615.1 (C=C); δ H (250 MHz, CDCl₃) 0.86 (3H, s, C(C₆H₃C₆H₃)), 1.11 (3H, s, C(C₆H₃C₆H₃)), 2.07 (1H, d, J 17.4, CH₃C₆H₃(CH₃)₂), 2.30 (1H, dd, J 15.5, 3.0, CH₃C₆H₃CO₂H), 2.55 (1H, dd, J 17.4, 1.1, CH₃C₆H₃(CH₃)₂), 2.73 (1H, dd, J 15.5, 8.8, CH₃C₆H₃CO₂H), 2.80 (1H, dd, J 8.8, 3.0, CH₂CH₂CO₂H), 3.68 (3H, s, OCH₃), 5.40 (1H, d, J 1.6, COCH=CO), 9.70 (1H, br, OH); δ C (63 MHz, DEPT, CDCl₃) 20.9 (C(C₆H₃C₆H₃)), 28.8 (C(C₆H₃C₆H₃)), 29.6 (CH₂CO₂H), 35.8 (C(CH₃)₂), 44.3 (CH₂C(CH₃)₂), 52.6 (CH₂CH₂CO₂H), 55.8 (OCH₃), 100.6 (COCH=CO), 176.1 (COCH=COCH₃), 178.2 (CO₂H), 199.4 (COCH=COCH₃); MS FAB (+ve) found m/z 213 (MH⁺, 100 %), 235 (MNa⁺, 89), 195 (M⁺-OH, 95); HRMS FAB (+ve) found m/z 213.11288 (MH⁺), C₁₁H₁₇O₄ requires 213.11268.

Method B

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid tert-butyl ester 53a (12.48 g, 46.51 mmol) was dissolved in anhydrous DCM (100 ml) and zinc bromide (52.37 g, 232.54 mmol) added. The mixture was stirred at room temperature overnight. Water (500 ml) was added and the mixture stirred for a further 3 hrs. The layers were separated and the aqueous phase extracted with DCM (3 x 250 ml). The total organic phase was washed with brine (200 ml), dried (MgSO₄), and rotary evaporated to give a sticky white solid, which was recrystallised from EtOAc/Hexane to give a white crystalline solid (5.74 g, 58 %). The filtrate was reduced in vacuo and the residue triturated to give a second crop of white solid (2.83
g, 29%). Both crops gave NMR spectra identical to those of the product from Method A.

5.2.18 Synthesis of Cyclised Ligands Incorporating Amines

General Coupling Method

To a solution of (4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (10 mmol) and amine/amino acid (10 mmol) in anhydrous DCM (80 ml) was added HOBt (15 mmol), EDCI (13 mmol), and DIPEA (20 mmol). The mixture was then stirred at room temperature overnight. The reaction was quenched with brine (40 ml) and extracted with DCM (3 x 20 ml). The total organic phase was dried (MgSO₄) and rotary evaporated to give the crude product.

5.2.18.1 N-Allyl-2-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetamide 111

Using the general method, allylamine 110 was coupled to (4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (0.50 g, 2.36 mmol) to give the crude product as a dark brown oil (1.14 g) after work-up. This was purified by flash column chromatography on silica gel using hexane/EtOAc (1:4) as eluent to give an orange solid (0.42 g, 71%): mp 64-66 °C; νmax (CHCl₃) 3320.8 (NH), 1653.7 (α,β-unsaturated ketone), 1615.1 (C=C); δH (360 MHz, CDCl₃) 0.83 (3H, s, C(CH₃)₂), 1.13 (3H, s, C(CH₃)₂), 2.12 (1H, d, J 17.4, CH₂H₂C(CH₃)₂), 2.18 (1H, dd, J 14.2, 2.6, CH₂H₂C(ONH)), 2.52 (1H, dd, J 17.4, 1.4, CH₂H₂C(CH₃)₂), 2.54 (1H, dd, J 14.2, 8.8, CH₂H₂C(ONH)), 2.70 (1H, dd, J 14.2, 8.8, CH₂H₂C(ONH)), 2.90 (1H, dd, J 17.4, 1.4, CH₂H₂C(ONH)), 3.66 (3H, s, OCH₃), 3.84 (2H, m, NHCH₂CH₃), 5.10 (2H, m, NHCH₂CH₂CH₂), 5.34 (1H, d, J 1.6, COCH=CO), 5.81 (1H, m, NHCH₂CH₂CH₂), 6.50 (1H, br, NH); δC (63 MHz, DEPT, CDCl₃) 21.0(C(CH₃)₂C(CH₃)₂), 28.8 (C(CH₃)₂C(CH₃)₂), 31.9 (CH₂CONH), 36.1 (C(CH₃)₂), 41.9 (NHCH₂), 44.2 (CH₂C(CH₃)₂), 53.5 (CH₂CONH), 55.7 (OCH₃),
100.7 (COCH=CO), 115.8 (NHCH2CHCH2), 134.3 (NHCH2CHCH2), 172.7
(CONH), 175.9 (COCH=COCH3), 200.4 (COCH=COCH3); MS FAB (+ve) found
m/z 252 (MH⁺, 25 %), 195 (M⁺-NHallyl, 100); HRMS FAB (+ve) found m/z
252.15921 (MH⁺), C14H22NO3 requires 252.15997.

5.2.18.2 1-Allyl-4,4-dimethyl-3,3a,4,5-tetrahydro-1H-indole-2,6-dione 113

\[
\text{N-Alllyl-2-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetamide 111 (0.20 g,}
\]
0.80 mmol) was dissolved in anhydrous THF (7 ml) and 2M HCl (7 ml) slowly
added. The mixture was then stirred at room temperature overnight. EtOAc (30 ml)
was added and the organic phase washed with water (10 ml) and saturated NaHCO3
solution (10 ml). The aqueous phase was back-extracted with EtOAc (2 x 15 ml) and
the total organic phase washed with brine (15 ml), dried (MgSO4) and rotary
evaporated to give a yellow oil (0.14 g). This was taken up in toluene (15 ml) and
acetic acid (0.5 ml) added. The mixture was then heated under reflux for 2 hrs using
a Dean-Stark trap to remove water. After allowing to cool, the toluene was removed
in vacuo and the residue dissolved in DCM (20 ml) and washed with water (10 ml),
than saturated NaHCO3 solution (10 ml). The aqueous phase was back-extracted
with DCM (2 x 10 ml) and the total organic phase washed with brine (15 ml), dried
(MgSO4) and rotary evaporated to give a yellow oil (0.10 g). This was purified by
flash column chromatography on silica gel using hexane/EtOAc (4:1:3:2) as eluent to
give an off-white solid (0.05 g, 29 %): mp 107-109 °C; δH (360 MHz, CDCl3) 0.88
(3H, s, C(CH3)2), 1.08 (3H, s, C(CH3)2), 2.31 (2H, s, CH2C(CH3)2), 2.37
(1H, dd, J 17.6, 8.5, CH2CON), 2.60 (1H, dd, J 17.6, 9.2, CON), 3.07
(1H, m, CHCH2CON), 4.10 (2H, m, NCH2), 5.14 (2H, m, NCH2CHCH2), 5.43 (1H,
dd, J 2.2, CH=CH), 5.66 (1H, m, NCH2CHCH2); δC (63 MHz, DEPT, CDCl3) 19.5
(C(CH3)2), 28.7 (C(CH3)2), 29.4 (CH2CON), 35.5 (CH3), 42.7
(NCH2), 44.6 (CH2CON), 52.9 (CH2C(CH3)2), 101.7 (C=CH), 118.4
5.2.19 Synthesis of Cyclised Ligands Incorporating Amino Acids

5.2.19.1 (S)-2-[2-(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-3-phenyl-propionic acid methyl ester 115a and 115b

Using the general method, L-phenylalanine methyl ester hydrochloride 114 was coupled to (4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (0.45 g, 2.12 mmol) to give a brown oil (1.60 g) after work-up. This was purified by flash column chromatography on silica gel using hexane/EtOAc (4:1-3:2) as eluent to give the diastereomers as (i) a colourless oil (0.24 g, 30\% ) and (ii) a yellow solid (0.21 g, 27\% ) respectively:

i) δ\textsubscript{H} (360 MHz, CDCl\textsubscript{3}) 0.83 (3H, s, C(CH\textsubscript{3}CH\textsubscript{3})), 1.09 (3H, s, C(CH\textsubscript{3}CH\textsubscript{3})), 2.12 (1H, d, J 17.4, CH\textsuperscript{A}H\textsuperscript{B}C(CH\textsubscript{3})), 2.13 (1H, m, CH\textsuperscript{A}H\textsuperscript{B}CONH), 2.50 (1H, dd, J 17.4, 1.4, CH\textsuperscript{A}H\textsuperscript{B}C(CH\textsubscript{3})), 2.56 (2H, m, CHCH\textsuperscript{A}H\textsuperscript{B}CONH), 3.06 (1H, dd, J 13.8, 6.2, PhCH\textsuperscript{A}H\textsuperscript{B}), 3.14 (1H, dd, J 13.8, 5.8, PhCH\textsuperscript{A}H\textsuperscript{B}), 3.70 (6H, s, OCH\textsubscript{3} and CO\textsubscript{2}CH\textsubscript{3}), 4.91 (1H, m, Cα-H), 5.38 (1H, d, J 1.6, COCH=COCH\textsubscript{3}), 6.63 (1H, br, NH), 7.20-7.47 (5H, m, Ar-H); δ\textsubscript{C} (63 MHz, DEPT, CDCl\textsubscript{3}) 20.8 (C(CH\textsubscript{3}CH\textsubscript{3})), 28.8 (C(CH\textsuperscript{A}H\textsuperscript{B}H\textsuperscript{B})), 31.4 (CH\textsubscript{2}CONH), 35.9 (C(CH\textsubscript{3})), 37.9 (PhCH\textsubscript{2}), 44.2 (CH\textsubscript{2}C(CH\textsubscript{3})), 52.1 (CO\textsubscript{2}CH\textsubscript{3}), 53.0 (Cα-H), 53.1 (CH\textsubscript{2}CH\textsubscript{2}CONH), 55.6 (OCH\textsubscript{3}), 100.7 (COCH=COCH\textsubscript{3}), [126.8 (CH), 128.4 (CH), 129.4 (CH), 5C, Ar-H], 136.1 (CH\textsubscript{2}Ph), 172.0 (CONH or CO\textsubscript{2}Me), 172.2 (CONH or CO\textsubscript{2}Me), 175.6 (COCH=COCH\textsubscript{3}), 199.9 (COCH=COCH\textsubscript{3}); MS FAB (+ve) found m/z 374 (MH\textsuperscript{+}, 87
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\textbf{Experimental}  \\

\%), 396 (MNa\textsuperscript{+}, 80), 195 (M\textsuperscript{+}-PheOMe, 100); HRMS FAB (+ve) found \textit{m/z} 374.19650 (MH\textsuperscript{+}), C\textsubscript{21}H\textsubscript{28}NO\textsubscript{5} requires 374.19675.

\textit{ii)} mp 131-134 °C; \(\delta\text{H} (360 \text{ MHz, CDCl}_3) 0.81 (3\text{H, s, C}(C^A\text{H}_3C^B\text{H}_3)), 1.10 (3\text{H, s, C}(C^A\text{H}_3C^B\text{H}_3)), 2.12 (1\text{H, d, J} 17.4, C^A\text{H}^B\text{C}(\text{CH}_3)_2), 2.15 (1\text{H, dd, J} 14.0, 2.4, C^A\text{H}^B\text{CONNH}), 2.53 (1\text{H, dd, J} 17.4, 1.3, C^A\text{H}^B\text{C}(\text{CH}_3)_2), 2.61 (1\text{H, dd, J} 14.0, 8.5, C^A\text{H}^B\text{CONNH}), 2.69 (1\text{H, dd, J} 8.5, 2.4, CHCH\textsubscript{2}\text{CONH}), 3.03 (1\text{H, dd, J} 13.8, 6.8, PhCH^A\text{H}^B), 3.15 (1\text{H, dd, J} 13.8, 5.7, PhCH^A\text{H}^B), 3.67 (3\text{H, s, CO}_2\text{CH}_3 \text{ or OCH}_3), 3.68 (3\text{H, s, CO}_2\text{CH}_3 \text{ or OCH}_3), 4.80 (1\text{H, m, C}_\alpha\text{-H}), 5.36 (1\text{H, d, J} 1.6, COCH=CO\text{CH}_3), 6.85 (1\text{H, br, NH}), 7.13-7.30 (5\text{H, m, Ar-H}); \delta\text{C} (63 \text{ MHz, DEPT, CDCl}_3) 20.9 (C(C^A\text{H}_3C^B\text{H}_3)), 29.0 (C(C^A\text{H}_3C^B\text{H}_3)), 31.5 (CH\text{CONH}), 36.1 (C(\text{CH}_3)_2), 37.8 (\text{PhCH}_2), 44.2 (\text{CH}_2\text{C}(\text{CH}_3)_2), 52.1 (\text{CO}_2\text{CH}_3), 53.1 (\text{CHCH}_2\text{CONH} \text{ or C}_\alpha\text{-H}), 53.4 (\text{CHCH}_2\text{CONH} \text{ or C}_\alpha\text{-H}), 55.6 (\text{OCH}_3), 100.8 (\text{COCH}=\text{CO}), [126.8 (\text{CH}), 128.4 (\text{CH}), 129.2 (\text{CH}), 5\text{C, Ar-H}, 136.2 (\text{CH}_2\text{Ph}), 172.0 (\text{CONH} \text{ or CO}_2\text{Me}), 172.6 (\text{CONH} \text{ or CO}_2\text{Me}), 175.7 (\text{COCH}=\text{CO}_3\text{H}), 199.9 (\text{COCH}=\text{CO}_3\text{H}); \text{MS FAB (+ve) found m/z} 374 (\text{MH}^+, 94 \%), 396 (\text{MNa}^+, 89), 195 (M^+-\text{PheOMe}, 100); \text{HRMS FAB (+ve) found m/z} 374.19635 (\text{MH}^+), C\textsubscript{21}H\textsubscript{28}NO\textsubscript{5} requires 374.19675.

\textit{5.2.19.2 (S)-1-(1-Benzyl-2-oxo-butyl)-4,4-dimethyl-3,3a,4,5-tetrahydro-1H-indole-2,6-dione 116a and 116b}

\(\begin{array}{c}
\text{(S)-2-[2-(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-3-phenyl-propionic acid methyl ester diastereomers 115a and 115b (0.22 g, 0.59 mmol; 0.17 g, 0.46 mmol) were dissolved separately in anhydrous THF (7 ml) and 2M HCl (7 ml) slowly added to both. The mixtures were then stirred at room temperature overnight. EtOAc (30 ml) was added and the organic phases washed with water (10 ml) and saturated NaHCO\textsubscript{3} solution (10 ml). The aqueous phases were back-extracted with}
\end{array}\)
EtOAc (2 x 15 ml) and the total organic phases washed with brine (15 ml), dried (MgSO\textsubscript{4}) and rotary evaporated to give two yellow oils (0.18, 0.13 g). These were taken up separately in toluene (15 ml) and acetic acid (0.2 ml) added to both. The mixtures were then heated under reflux for 2 hrs using a Dean-Stark trap to remove water. After allowing to cool, the toluene was removed in vacuo and the residues dissolved in DCM (20 ml) and washed with water (10 ml) and saturated NaHCO\textsubscript{3} solution (10 ml). The aqueous phases were back-extracted with DCM (2 x 10 ml) and the total organic phases washed with brine (15 ml), dried (MgSO\textsubscript{4}) and rotary evaporated to give two brown oils (0.081 g, 0.076 g). These were purified by flash column chromatography on silica gel using hexane/EtOAc (7:3) as eluent to give two yellow oils, (i) (0.018 g, 9\% ) and (ii) (0.011 g, 7\%):

\begin{align*}
n &\text{i) } \delta_\text{H} (250 \text{ MHz, CDCl}_3) 0.70 (3\text{H}, \text{s, C}(\text{C}^A\text{H}_3\text{C}^B\text{H}_3)), 1.03 (3\text{H}, \text{s, C}(\text{C}^A\text{H}_3\text{C}^B\text{H}_3)), 2.27 (1\text{H}, \text{dd, J} 17.6, 8.8, \text{CH}^A\text{H}^B\text{CON}), 2.27 (2\text{H}, \text{s, CH}_2\text{C}(\text{CH}_3)_2), 2.45 (1\text{H}, \text{dd, J} 17.6, 9.2, \text{CH}^A\text{H}^B\text{CON}), 2.88 (1\text{H}, \text{m, CHCH}_2\text{CON}), 3.29 (1\text{H}, \text{dd, J} 14.4, 11.6, \text{PhCH}^A\text{H}^B), 3.47 (1\text{H}, \text{dd, J} 14.4, 5.2, \text{PhCH}^A\text{H}^B), 3.77 (3\text{H}, \text{s, CO}_2\text{CH}_3), 4.94 (1\text{H}, \text{dd, J} 11.6, 5.2, \text{C}_\alpha-\text{H}), 5.25 (1\text{H}, \text{d, J} 2.0, \text{C} = \text{CHCO}), 7.08-7.28 (5\text{H}, \text{m, Ar-}\text{H}); \\
&\delta_\text{C} (90 \text{ MHz, DEPT, CDCl}_3) 19.3 (\text{C}(\text{C}^A\text{H}_3\text{C}^B\text{H}_3)), 28.5 (\text{C}(\text{C}^A\text{H}_3\text{C}^B\text{H}_3)), 29.1 (\text{CH}_2\text{CON}), 32.8 (\text{PhCH}_2), 35.3 (\text{C}(\text{CH}_3)_2), 44.5 (\text{CHCH}_2\text{CO}), 52.6 (\text{CH}_2\text{C}(\text{CH}_3)_2), 52.9 (\text{CO}_2\text{CH}_3), 54.9 (\text{C}_\alpha-\text{H}), 102.1 (\text{C} = \text{CHCO}), [127.1 (\text{CH}), 128.6 (\text{CH}), 128.7 (\text{CH}), 5\text{C, Ar-}\text{H}), 135.8 (\text{CH}_2\text{Ph}), 162.2 (\text{C} = \text{CHCO}), 168.2 (\text{CONH or CO}_2\text{Me}), 174.4 (\text{CONH or CO}_2\text{Me}), 196.5 (\text{C} = \text{CHCO}); \text{MS FAB (+ve) found } m/z 342 (\text{MH}^+, 100 \%), 364 (\text{MNa}^+, 54); \text{HRMS FAB (+ve) found } m/z 342.17040 (\text{MH}^+), \text{C}_{20}\text{H}_{24}\text{NO}_4 \text{ requires 342.17053}.

\text{ii) } \delta_\text{H} (250 \text{ MHz, CDCl}_3) 0.80 (3\text{H}, \text{s, C}(\text{C}^A\text{H}_3\text{C}^B\text{H}_3)), 1.02 (3\text{H}, \text{s, C}(\text{C}^A\text{H}_3\text{C}^B\text{H}_3)), 2.05 (1\text{H}, \text{dd, J} 17.5, 8.9, \text{CH}^A\text{H}^B\text{CON}), 2.23 (1\text{H}, \text{d, J} 16.9 \text{CH}^A\text{H}^B\text{C}(\text{CH}_3)_2), 2.32 (1\text{H}, \text{d, J} 16.9 \text{CH}^A\text{H}^B\text{C}(\text{CH}_3)_2), 2.44 (1\text{H}, \text{dd, J} 17.5, 9.1, \text{CH}^A\text{H}^B\text{CON}), 2.93 (1\text{H}, \text{m, CHCH}_2\text{CON}), 3.28 (1\text{H}, \text{dd, J} 14.3, 11.5 \text{PhCH}^A\text{H}^B), 3.48 (1\text{H}, \text{dd, J} 14.3, 5.5, \text{PhCH}^A\text{H}^B), 3.77 (3\text{H}, \text{s, CO}_2\text{CH}_3), 5.06 (1\text{H}, \text{dd, J} 11.3, 5.5, \text{C}_\alpha-\text{H}), 5.40 (1\text{H}, \text{d, J} 2.0, \text{C} = \text{CHCO}), 7.08-7.26 (5\text{H}, \text{m, Ar-}\text{H}); \delta_\text{C} (90 \text{ MHz, DEPT, CDCl}_3) 19.5
(C(AH3C8H3)), 28.7 (C(AH3C8H3)), 29.0 (CH2CON), 34.0 (PhCH2), 35.3
(C(CH3)2), 44.3 (CHCH2CO), 52.6 (CH2C(CH3)2), 53.1 (CO2CH3), 55.1 (Cα-H),
102.1 (C=CHCO), [127.2 (CH), 128.5 (CH), 129.0 (CH), 5C, Ar-H], 135.6 (CH2Ph),
162.5 (C=CHCO), 168.4 (CONH or CO2Me), 174.6 (CONH or CO2Me), 196.6
(C=CHCO); MS FAB (+ve) found m/z 342 (MH+, 100 %), 364 (MNa+, 36); HRMS
FAB (+ve) found m/z 342.17019 (MH+), C20H24N04 requires 342.17053.

5.2.19.3 (S)-6-Acetylamino-2-[2-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-
enyl)-acetylamino]-hexanoic acid methyl ester 121a

Using the general method, N-α-acetyl-L-lysine methyl ester hydrochloride 120a was
coupled to (4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (0.89 g,
4.20 mmol) to give an orange oil (7.00 g) after work-up. This was purified by flash
column chromatography on silica gel using EtOAc/5 % MeOH as eluent to give a
white foamy solid (1.29 g, 76 %) as a mixture of diastereomers: δH (250 MHz,
CDCl3) 0.81 (6H, s, C(AH3C8H3)), 1.09 (6H, s, C(AH3C8H3)), 1.41 (8H, m, CαH2
and CβH2), 1.71 (4H, m, CβH2), 1.97 (6H, s, CH3CONH) 2.11 (2H, d, J 17.5,
CHβHβC(CH3)2), 2.15 (2H, dd, J 14.2, 2.6, CHβHβCONH), 2.42 (2H, dd,
CHβHβCONH), 2.50 (2H, dd, CHβHβC(CH3)2), 2.71 (2H, m, CH2CH2CONH), 3.17
(4H, m, CαH), 3.64 (6H, s, OCH3), 3.66 (3H, s, OCH3), 3.67 (3H, s, OCH3), 4.46
(2H, m, CαH), 5.30 (2H, s, COCH=COCH3), 6.53 (2H, br, NH), 6.74 (1H, d, J 7.6,
NH), 6.89 (1H, d, J 7.4, NH); δC (63 MHz, DEPT, CDCl3) 21.0 + 21.1
(C(AH3C8H3)), 22.0 + 22.1 (CαH2), 22.8 (NHCCH3), 28.6 + 28.7 (C(AH3C8H3)),
28.8 (CβH2), 31.1 + 31.2 (CH2CONH), 31.7 + 31.9 (CβH2), 35.9 + 35.9 (CH(CH3)2),
38.3 + 38.6 (CγH2), 44.1 + 44.2 (CH2C(CH3)2), 52.1 (CHCH2CONH), 52.1
(CO2CH3), 53.2 + 53.3 (CαH), 55.7 (OCH3), 100.6 (COCH=COCH3), 170.2 + 170.4
(NHCCH3), 172.9 (CONH or CO2Me), 173.1 (CONH or CO2Me), 176.0
(COCH=COCH3), 200.5 (COCH=COCH3); MS ES (+ve) found m/z 397 (MH+, 100
%, 419 (MNa<sup>+</sup>, 41); HRMS FAB (+ve) found m/z 397.23389 (MH<sup>+</sup>), C<sub>20</sub>H<sub>32</sub>N<sub>2</sub>O<sub>6</sub> requires 397.23386.

5.2.19.4  (S)-6-Acetylamino-2-(4,4-dimethyl-2,6-dioxo-2,3,3a,4,5,6-hexahydro-
indol-1-yl)-hexanoic acid methyl ester 122a

(S)-6-Acetylamino-2-[2-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-
acetylamino]-hexanoic acid methyl ester 121a (1.00 g, 2.52 mmol) was dissolved in
anhydrous THF (25 ml) and 2M HCl (25 ml) slowly added. The mixture was then
stirred at room temperature overnight. EtOAc (50 ml) was added and the organic
phase washed with saturated NaHCO<sub>3</sub> solution (25 ml), then water (25 ml). The
aqueous phase was back-extracted with EtOAc (2 x 25 ml) and the total organic
phase washed with brine (30 ml), dried (MgSO<sub>4</sub>) and rotary evaporated to give a
yellow oil (0.20 g). This was taken up in toluene (20 ml) and acetic acid (0.5 ml)
added. The mixture was then heated under reflux for 3 hrs using a Dean-Stark trap to
remove water. After allowing to cool, the toluene was removed in vacuo and the
residue dissolved in DCM (20 ml) and washed with water (10 ml) and saturated
NaHCO<sub>3</sub> solution (10 ml). The aqueous phase was back-extracted with DCM (2 x 10
ml) and the total organic phase washed with brine (15 ml), dried (MgSO<sub>4</sub>) and rotary
evaporated to give a brown oil (0.10 g). This was purified by flash column
chromatography on silica gel using EtOAc/5 % MeOH as eluent to give a yellow oil
(0.062 g, 7 %): δ<sub>H</sub> (250 MHz, CDCl<sub>3</sub>) 0.88 (3H, s, C(CH<sub>3</sub>)<sub>3</sub>), 1.08 (3H, s,
C(CH<sub>3</sub>)<sub>3</sub>), 1.30 (2H, m, C<sub>4</sub>H<sub>2</sub>), 1.54 (2H, m, C<sub>6</sub>H<sub>2</sub>), 1.72 (2H, m, β-Lys), 2.00
(3H, s, CH<sub>3</sub>CONH), 2.33 (2H, s, CH<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>), 2.34 (1H, dd, J 17.5, 8.4,
CH<sub>2</sub>CH<sub>2</sub>CON), 2.58 (1H, ddd, J 17.5, 9.1, 1.2, CH<sub>H</sub>B<sub>2</sub>CON), 3.07 (1H, m, 
CHCH<sub>2</sub>CON), 3.46 (2H, m, C<sub>6</sub>H<sub>2</sub>), 3.71 (3H, s, OCH<sub>3</sub>), 4.53 (1H, m, C<sub>4</sub>H), 5.45 (1H, 
d, J 2.1, C=CHCO), 6.26 (1H, d, J 7.5, NH); δ<sub>C</sub> (63 MHz, DEPT, CDCl<sub>3</sub>) 19.5
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\[(\text{C}(\text{C}^\text{A}\text{H}_3\text{C}^\text{B}\text{H}_3)), 22.3 (\text{C}_7\text{H}_2), 23.0 (\text{NHCOCH}_3), 26.2 (\text{C}_6\text{H}_2), 28.7 (\text{C}(\text{C}^\text{A}\text{H}_3\text{C}^\text{B}\text{H}_3)), 29.5 (\text{CH}_2\text{CON}), 31.7 (\text{C}_9\text{H}_2), 35.5 (\text{C}(\text{CH}_3)_2), 40.0 (\text{C}_6\text{H}_2), 44.6 (\text{CHCH}_2\text{CON}), 51.8 (\text{C}_{\alpha-}\text{H}), 52.4 (\text{CO}_2\text{CH}_3), 52.9 (\text{CH}_2\text{C}(\text{CH}_3)_2), 101.0 (\text{C}=\text{CHCO}), 163.8 (\text{C}=\text{CHCO}), 169.8 (\text{CO}), 172.8 (\text{CO}), 175.2 (\text{CO}), 196.7 (\text{C}=\text{CHCO}); \text{MS ES (+ve) found } m/z 365 (\text{MH}^+, 17 \%), 387 (\text{MNa}^+, 100); \text{HRMS FAB (+ve) found } m/z 365.20766 (\text{MH}^+), \text{C}_{19}\text{H}_{29}\text{N}_2\text{O}_5 \text{ requires } 365.20765.\]

5.2.19.5 (S)-2-[2-(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-5-(\text{N'}-nitro-guanidino)-pentanoic acid methyl ester 121b

![Chemical Structure](image)

Using the general method, \(\text{N}'\)-nitro-L-arginine methyl ester hydrochloride 120b was coupled to (4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (2.00 g, 9.42 mmol) to give an orange solid (7.05 g) after work-up. This was purified by flash column chromatography on silica gel using EtOAc/5 \% MeOH as eluent to give an off-white solid (3.44 g, 85 \%) as a mixture of diastereomers: \(\delta_\text{H} (250 \text{ MHz, CDCl}_3) 0.83 (6\text{H, s, C}(\text{C}^\text{A}\text{H}_3\text{C}^\text{B}\text{H}_3)), 1.09 (6\text{H, s, C}(\text{C}^\text{A}\text{H}_3\text{C}^\text{B}\text{H}_3)), 1.67 (4\text{H, m, Arg-CH}_2), 1.89 (4\text{H, m, Arg-CH}_2), 2.12 (2\text{H, m, CH}^\text{A}\text{H}^\text{B}\text{C}(\text{CH}_3)_2), 2.25 (2\text{H, m, CH}^\text{A}\text{H}^\text{B}\text{CONH}), 2.49 (4\text{H, m, CH}^\text{A}\text{H}^\text{B}\text{C}(\text{CH}_3)_2 + \text{CH}^\text{A}\text{H}^\text{B}\text{CONH}), 2.70 (1\text{H, m, CHCH}_2\text{CONH}), 2.87 (1\text{H, m, CHCH}_2\text{CONH}), 3.26 (2\text{H, m, C}_8\text{H}_2), 3.42 (2\text{H, m, C}_8\text{H}_2), 3.65 (3\text{H, s, OCH}_3), 3.66 (3\text{H, s, OCH}_3), 3.69 (3\text{H, s, OCH}_3), 3.71 (3\text{H, s, CH}_3), 4.49 (1\text{H, m, C}_9\text{H}), 4.66 (1\text{H, m, C}_9\text{H}), 5.30 (1\text{H, s, COCH}=\text{COCH}_3), 5.33 (1\text{H, s, COCH}=\text{COCH}_3), 6.81 (1\text{H, br, NH}), 7.38 (2\text{H, m, NH}), 7.75 (4\text{H, m, NH}), 8.65 (1\text{H, br, NH}); \delta_\text{C} (63 \text{ MHz, DEPT, CDCl}_3) 20.9 (\text{CH}_3), 21.1 (\text{CH}_3), 28.7 (\text{CH}_3), 31.7 (\text{CH}_2), 35.6 (\text{quat}), 35.9 (\text{quat}), 40.4 (\text{CH}_2), 40.6 (\text{CH}_2), 43.9 (\text{CH}_2), 44.3 (\text{CH}_2), 52.5 (\text{CH}_3), 53.1 (\text{CH}), 55.8 (\text{CH}_3), 55.9 (\text{CH}_3), 100.4 (\text{CH}), 100.5 (\text{CH}), 159.3 (\text{quat}), 172.4 (\text{quat}), 176.4 (\text{quat}), 200.4 (\text{quat}); \text{MS ES (+ve) found } m/z 428 (\text{MH}^+, 54 \%), 450 (\text{MNa}^+, 60), 383 (\text{M}^+\text{-NO}_2, 98); \text{HRMS FAB (+ve) found } m/z 428.21447 (\text{MH}^+), \text{C}_{18}\text{H}_{30}\text{N}_5\text{O}_7 \text{ requires } 428.21452.
5.2.19.6 (S)-2-(4,4-Dimethyl-2,6-dioxo-2,3,3a,4,5,6-hexahydro-indol-1-yl)-5-
(N'-nitro-guanidino)-pentanoic acid methyl ester 122b

(S)-2-[2-(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-5-(N'-nitro-
guanidino)-pentanoic acid methyl ester 121b (0.50 g, 1.17 mmol) was dissolved in
acetone (10 ml) and 1M HCl (10 ml) slowly added. The mixture was then stirred at
room temperature overnight. The acetone was removed in vacuo and the residue
diluted with brine (10 ml) and extracted with EtOAc (3 x 20 ml). The total organic
phase was dried (MgSO₄), and rotary evaporated to give a yellow oil (0.40 g). This
was taken up in toluene (25 ml) and acetic acid (1.0 ml) added. The mixture was
then heated under reflux for 3 hrs using a Dean-Stark trap to remove water. After
allowing to cool, the toluene was removed in vacuo and the residue dissolved in
EtOAc (25 ml) and washed with water (10 ml) and saturated NaHCO₃ solution (10
ml). The aqueous phase was back-extracted with EtOAc (2 x 10 ml) and the total
organic phase washed with brine (15 ml), dried (MgSO₄) and rotary evaporated to
give a gummy solid. This was purified by flash column chromatography (dry-
loading) on silica gel using EtOAc/5 % MeOH as eluent to give an off-white solid
(0.040 g, 9 %) as a mixture of diastereomers: δH (360 MHz, CDCl₃) 0.90 (3H, s,
C(C₆H₅C₆H₅)), 0.91 (3H, s, C(C₆H₅C₆H₅)), 1.09 (6H, s, C(C₆H₅C₆H₅)), 1.58 (4H,
m, Arg-CH₂), 2.08 (4H, m, Arg-CH₂), 2.32 (2H, s, CH₃B₃C(CH₃)₂), 2.34 (2H, s,
CH₃B₃C(CH₃)₂), 2.43 (2H, m, CH₃B₃C(CH₃)₂), 2.69 (2H, m, CH₃B₃C(CH₃)₂), 3.21 (2H,
m, CH₃C(CH₃)₂), 3.30 (4H, m, C₆H₅H₂), 3.68 (3H, s, OCH₃), 3.69 (3H, s, OCH₃), 4.71
(1H, m, C₆H₅H), 4.92 (1H, m, C₆H₅H), 5.30 (1H, s, C=CHCO), 5.45 (1H, s, C=CHCO),
7.59 (1H, br, NH), 8.60 (1H, m, NH); δC (90 MHz, DEPT, CDCl₃); 19.5
(C(C₆H₅C₆H₅)), 23.8 (Arg-CH₂), 25.3 (Arg-CH₂), 28.6 (C(C₆H₅C₆H₅)), 29.3 + 29.4
(CH₂CON), 35.6 (C(CH₃)₂), 40.5 (C₆H₅H), 44.3 + 44.5 (CH₃C(CH₃)₂), 52.6
(CH₂C(CH₃)₂), 52.8 + 53.8 (C_=H), 52.9 + 53.0 (CO₂CH₃), 101.7 + 102.4
(C=CHCO), 159.2 (C=NH), 162.5 (C=CHCO), 168.7 (CO), 175.6 + 175.7 (CO),
197.3 (C=CHCO); MS ES (+ve) found m/z 396 (MH⁺, 84%), 418 (MNa⁺, 100); HRMS FAB (+ve) found m/z 396.18833 (MH⁺), C₁₇H₂₆N₅O₆ requires 396.18831.

5.2.19.7 (S)-6-Benzoxycarbonylamino-2-[2-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-hexanoic acid methyl ester 121c

Using the general method, N-e-CBz-L-lysine methyl ester hydrochloride 120c was coupled to (4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (2.00 g, 9.42 mmol) to give an orange oil (7.67 g) after work-up. This was purified by flash column chromatography on silica gel using hexane/EtOAc (1:1)-EtOAc as a gradient eluent to give an orange oil (4.47 g, 97%) as a mixture of diastereomers: δH (250 MHz, CDCl₃) 0.77 (6H, s, C(C₆H₃C₆H₃)), 1.04 (3H, s, C(C₆H₃C₆H₃)), 1.05 (3H, s, C(C₆H₃C₆H₃)), 1.40 (8H, m, C₇H₂ and C₈H₂), 1.71 (4H, m, C₉H₂), 2.09 (4H, m, CH₄H₃C(CH₃)₂ + CH₄H₃CONH), 2.43 (4H, m, CH₄H₃C(CH₃)₂ + CH₄H₃CONH), 2.62 (1H, m, CH₂CH₂CONH), 2.78 (1H, m, CH₂CH₂CONH), 3.11 (4H, m, C₉H₂), 3.36 (3H, s, OCH₃), 3.57 (3H, s, OCH₃), 3.63 (3H, s, CO₂CH₃), 3.65 (3H, s, CO₂CH₃), 4.45 (1H, m, C₉H₂), 4.58 (1H, m, C₉H₂), 5.01 (4H, m, CH₂Ph), 5.13 (1H, br, NH), 5.22 (1H, s, COCH=COCH₃), 5.30 (1H, s, COCH=COCH₃), 5.95 (1H, br, NH), 6.52 (1H, d, J 8.2, NH), 7.05 (1H, d, J 7.5, NH), 7.25 (10H, m, Ar-H); δC (63 MHz, DEPT, CDCl₃) 21.0 + 21.1 (C(C₆H₃C₆H₃)), 21.7 + 22.2 (C₇H₂), 28.7 (C(C₆H₃C₆H₃)), 29.1 (C₉H₂), 31.6 (CH₂CONH or C₉H₂), 31.7 (CH₂CONH or C₉H₂), 35.7 + 36.0 (C(CH₃)₂), 40.1 + 40.5 (C₆H₂), 44.0 + 44.2 (CH₂C(CH₃)₂), 51.6 + 51.9 (C₉H₂), 52.2 (CO₂CH₃), 53.2 + 53.3 (CH₂CH₂CONH), 55.4 + 55.6 (OCH₃), 66.1 + 66.4 (PhCH₂), 100.6 (COCH=COCH₃), [127.6 + 127.7 (CH), 127.9 (CH), 128.3 + 128.3 (CH), 10C, Ar-H], 136.5 + 136.9 (Ph), 156.4 + 156.7 (NHCOO), 172.4 + 172.7 (CONH or CO₂Me), 172.9 + 173.0 (CONH or CO₂Me), 176.0 (COCH=COCH₃), 200.4 (COCH=COCH₃); MS ES (+ve) found m/z 489 (MH⁺, 100
5.2.19.8 (S)-6-Benzoxycarbonylamino-2-(4,4-dimethyl-2,6-dioxo-2,3,3a,4,5,6-hexahydro-indol-1-yl)-hexanoic acid methyl ester 122c

(S)-6-Benzoxycarbonylamino-2-[2-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-hexanoic acid methyl ester 121c (2.26 g, 4.62 mmol) was dissolved in acetone (40 ml) and 2M HCl (20 ml) slowly added. The mixture was then stirred at room temperature overnight. The acetone was removed in vacuo and the residue diluted with brine (10 ml) and extracted with EtOAc (3 x 20 ml). The total organic phase was dried (MgSO₄), and rotary evaporated to give an orange oil (2.25 g). This was taken up in toluene (50 ml) and acetic acid (1.00 ml) added. The mixture was then heated under reflux for 3 hrs using a Dean-Stark trap to remove water. After allowing to cool, the toluene was removed in vacuo and the residue dissolved in EtOAc (50 ml) and washed with water (20 ml) and saturated NaHCO₃ solution (20 ml). The aqueous phase was back-extracted with EtOAc (2 x 20 ml) and the total organic phase washed with brine (30 ml), dried (MgSO₄) and rotary evaporated to give an orange oil (1.20 g). This was purified by flash column chromatography on silica gel using hexane/EtOAc (2:3) as eluent to give a yellow oil (0.58 g, 27 %) as a mixture of diastereomers: δH (360 MHz, CDCl₃) 0.83 (3H, s, C(C₆H₅C₆H₅)), 0.84 (3H, s, C(C₆H₅C₆H₅)), 1.00 (3H, s, C(C₆H₅C₆H₅)), 1.01 (3H, s, C(C₆H₅C₆H₅)), 1.17 (4H, m, C₆H₅C₆H₅), 1.42 (4H, m, C₆H₅C₆H₅), 1.96 (4H, m, C₆H₅C₆H₅), 2.26 (4H, s, CH₂C(CH₃)₂), 2.33 (2H, m, CH₄H₄C₄H₄), 2.56 (2H, s, CH₄H₄C₄H₄), 3.04 (6H, m, C₆H₅C₆H₅ + CH₄H₄C₄H₄), 3.62 (3H, s, OCH₃), 3.64 (3H, s, OCH₃), 4.64 (1H, m, C₆H₅C₆H₅), 4.79 (1H, m, C₆H₅C₆H₅), 4.99 (4H, m, CH₂Ph), 5.24 (1H, d, J 1.9, C=CHCO), 5.31 (1H, d, J 1.9, C=CHCO), 7.25 (10H, m, Ar-H); δC (63 MHz,
DEPT, CDCl₃ 19.3 (C(AH₃C₆H₃)), 23.0 + 23.2 (C₂H₂ or C₅H₂), 26.2 + 27.5 (C₄H₂
or C₅H₂), 28.5 (C(AH₃C₆H₃)), 29.0 (C₅H₂ or CH₂CON), 29.2 (C₅H₂ or CH₂CON),
35.3 + 35.4 (C(CH₃)), 40.4 (C₂H₂), 44.2 (CH₂CH₂CON), 52.5 (CH₂C(CH₃)), 52.6 +
52.7 (CO₂CH₃), 53.1 (C₆H₁), 66.3 (PhCH₂), 101.8 + 102.5 (C=CHCO), [127.9 (CH),
128.3 (CH), 10C, Ar-H], 136.4 (Ph), 156.2 (NHCOO), 161.7 + 162.6 (C=CHCO)
168.6 (CON or CO₂Me), 174.8 + 174.9 (CON or CO₂Me), 196.4 (C=CHCO); MS ES
(+ve) found m/z 457 (MH⁺, 100 %), 479 (MNa⁺, 45); HRMS FAB (+ve) found m/z
457.23332 (MH⁺), C₂₅H₃₃N₂O₆ requires 457.23386.

5.2.19.9 (S)-6-Amino-2-(4,4-dimethyl-2,6-dioxo-2,3,3a,4,5,6-hexahydro-indol-
1-yl)-hexanoic acid methyl ester 123

Method A

(S)-6-Benzylxocarbonylamino-2-(4,4-dimethyl-2,6-dioxo-2,3,3a,4,5,6-hexahydro-
indol-1-yl)-hexanoic acid methyl ester 122c (0.052 g, 0.11 mmol) was dissolved in
anhydrous DCM (3 ml) and iodontrimethylsilane (77.5 µl, 0.57 mmol) slowly added.
The mixture was then stirred at room temperature overnight. The mixture was
quenched with MeOH (100 µl) and stirred for 5 mins. The solvent was removed in
vacuo and the residue taken up in ether/30 % acetic acid (15 ml). The aqueous layer
was separated and extracted a further time with ether (7 ml) and then freeze-dried
overnight to give an orange foam (0.030 g, 83%): ν_max (CHCl₃) 3257.2 (NH₂),
1744.3 (ester), 1652.7 (α,β-unsaturated ketone), 1615.1 (C=C), 1558.2 (NH₂); δ_H
(250 MHz, DMSO) 0.83 (3H, s, C(AH₃C₆H₃)), 0.84 (3H, s, C(AH₃C₆H₃)), 1.03
(6H, s, C(AH₃C₆H₃)), 1.22 (4H, m, C₄H₂ or C₅H₂), 1.51 (4H, m, C₄H₂ or C₅H₂),
1.94 (4H, m, C₄H₂), 2.12 (2H, d, CH₄H₅C(CH₃)₂), 2.39 (2H, d, CH₄H₅C(CH₃)₂),
2.46-2.66 (4H, m, CH₂CON), 2.76 (4H, m, C₄H₂), 3.35 (2H, m, CH₄H₅C(CH₃)₂),
3.63 (6H, s, CO₂CH₃), 4.80 (1H, t, J 7.5, C₆H), 4.92 (1H, dd, J 10.3, 5.1, C₆H), 5.29 (1H,
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s, C=CHCO), 5.51 (1H, s, C=CHCO); δC (63 MHz, DEPT, DMSO) 20.2 (C(CH₃)₂C(CH₃)₂), 23.2 (C₇H₂ or C₈H₂), 27.0 (C₇H₂ or C₈H₂), 29.9 (C₉H₂), 28.9 (C(CH₃)₂C(CH₃)₂), 29.9 (CH₂CON), 36.3 (C(CH₃)₂), 39.6 (C₈H₂), 44.6 (CHCH₂CON), 53.3 (CO₂CH₃), 53.5 (CH₂C(CH₃)₂), 53.8 + 54.7 (C₆H), 101.3 + 101.8 (C=CHCO), 164.3 + 165.4 (C=CHCO), 170.1 (CON or CO₂Me), 176.3 + 176.5 (CON or CO₂Me), 196.9 + 197.0 (C=CHCO); MS FAB (+ve) found m/z 323 (MH⁺, 32%), 307 (MH⁺-NH₂, 43); HRMS FAB (+ve) found m/z 323.19702 (MW), C₁₇H₂₇N₂O₄ requires 323.19708.

**Method B**

(S)-6-Benzoxycarbonylamino-2-(4,4-dimethyl-2,6-dioxo-2,3,3a,4,5,6-hexahydroindol-1-yl)-hexanoic acid methyl ester 122c (0.25 g, 0.55 mmol) was dissolved in DCM (5 ml) and HBr in acetic acid (0.50 ml, 2.76 mmol) slowly added. The mixture was then stirred at room temperature for 2.5 hrs. The volatiles were removed in vacuo and the residue taken up in water (10 ml) and freeze-dried overnight to give a gummy bright orange solid. This was dissolved in acetone and a few drops of concentrated HCl added. The volatiles were removed in vacuo to give a foamy orange solid (0.15 g, 77%): MS ES (+ve) found m/z 323 (MH⁺, 100%), and ¹H NMR spectrum was representative of correct product but was not as clean as that obtained from Method A.

5.2.20 Synthesis of Aldehyde Derivative

5.2.20.1 (4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetaldehyde 126

**Method A**

3-Methoxy-5,5-dimethyl-6-(3-methyl-but-2-enyl)-cyclohex-2-enone 53b (5.00 g, 22.49 mmol) was dissolved in anhydrous DCM (180 ml) and pyridine (2.9 ml) and Sudan Red 7B (2.5 mg) added. The pink solution was cooled to -78 °C and ozone bubbled through for 8 hrs. Dry nitrogen was bubbled through the pale pink solution
for 15 mins to remove unreacted ozone and the solution then poured onto zinc dust (15 g). Acetic acid (20 ml) was added and the mixture allowed to warm to room temperature overnight. The mixture was passed through Celite and the filtrate washed with water (3 x 80 ml), 5 % NaOH solution (50 ml), and water (50 ml). The aqueous phase was back-extracted with DCM (100 ml) and the total organic phase washed with brine (50 ml), dried (MgSO₄) and rotary evaporated to give a brown liquid (2.50 g). This was purified by flash column chromatography on silica gel using hexane/EtOAc (7:3) as eluent to give a yellow oil (0.48 g, 11 %): $\nu_{max}$ (neat) 1722.1 (aldehyde), 1648.8 ($\alpha,\beta$-unsaturated ketone), 1614.1 (C=C); $\delta_H$ (250 MHz, CDCl₃) 0.86 (3H, s, C(C₅H₃C₆H₃)), 1.07 (3H, s, C(C₅H₃C₆H₃)), 2.15 (1H, d, $J = 17.4$, CH₃C(CH₃)₂), 2.23 (1H, dd, $J = 16.2, 3.2$, CH₃C(OH)), 2.54 (1H, dd, $J = 17.4, 1.2$, CH₃C(CH₃)₂), 2.81 (1H, dd, $J = 16.1, 9.2$, CH₃C(OH)), 2.93 (1H, dd, $J = 9.2, 3.2$, CH₃C(OH)), 3.66 (3H, s, OCH₃), 5.35 (1H, d, $J = 1.7$, COH=COC₃), 9.88 (1H, d, $J = 1.9$, COH); $\delta_C$ (63 MHz, DEPT, CDCl₃) 21.1 (C(C₅H₃C₆H₃)), 29.0 (C(C₅H₃C₆H₃)), 35.4 (C(CH₃)₂), 38.1 (CH₂C(OH)), 44.2 (CH₂C(CH₃)₂), 51.9 (CH₃C(OH)), 55.7 (OCH₃), 100.4 (COH=C(OH)), 175.9 (COH=COC₃), 198.5 (COH=COC₃), 201.2 (COH); MS ES (+ve) found $m/z$ 197 (MH⁺, 100 %), 195 (M⁺-COH, 46); HRMS FAB (+ve) found $m/z$ 197.11760 (MH⁺), C₁₁H₁₇O₃ requires 197.11777.

**Method B**

3-Methoxy-5,5-dimethyl-6-(3-methyl-but-2-enyl)-cyclohex-2-enone 53b (5.44 g, 24.46 mmol) was dissolved in acetone/water (4:1) (125 ml) and the solution cooled to 0 °C. N-methylmorpholine (5.73 g, 48.93 mmol), and osmium tetroxide (2.5 % in tert-butanol, 3.07 ml, 1 mol %) were then added and the mixture stirred at room temperature for 3.5 hrs. The mixture was partitioned between saturated sodium thiosulfate solution (50 ml) and EtOAc (50 ml) and the layers were separated. The aqueous phase was extracted with EtOAc (2 x 25 ml) and the total organic phase was washed with brine (50 ml), dried (MgSO₄) and rotary evaporated to give a dark brown oil (7.00 g): MS ES (+ve) found $m/z$ 257 (MH⁺ (diol), 100 %). This was carried through to the next step without purification.
To a vigorously stirred slurry of silica gel (48 g) and DCM (300 ml) was added a solution of sodium periodate (7.85 g, 36.68 mmol) in water (48 ml) dropwise. A solution of the diol (7.00 g) in DCM (50 ml) was then added dropwise. The mixture was stirred at room temperature for 1 hr, then filtered through a glass-sintered funnel. The silica was washed with DCM (2 x 100 ml) and the filtrate reduced in vacuo to give a brown liquid (5.12 g). This was purified by Kugelrohr distillation to give a yellow oil (4.61 g, 96 %): bp 195 °C (0.5 mBar); NMR spectra consistent with those from Method A.

5.2.21 Reductive Amination Reactions

Method A

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3- enyl)-acetaldehyde 126 (0.25 g, 1.27 mmol) and allylamine 110 (0.48 ml, 6.37 mmol) were dissolved in anhydrous MeOH (15 ml) and sodium cyanoborohydride (0.048 g, 0.77 mmol) added. The solution was reduced to pH 5-6 with acetic acid and the mixture stirred in the presence of 4 Å molecular sieves at room temperature overnight. The mixture was quenched with saturated NaHCO₃ solution (20 ml) and extracted with EtOAc (3 x 25 ml). The aqueous phase was back-extracted with EtOAc (2 x 15 ml) and the total organic phase washed with brine (25 ml), dried (MgSO₄) and rotary evaporated to give a dark brown oil: MS ES (+ve) found m/z 275 (56 %), 300 (100); ¹H NMR spectrum complex and unassignable with no indication of presence of required product.

Method B

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetaldehyde 126 (0.50 g, 2.55 mmol) was dissolved in anhydrous MeOH (8 ml) and allylamine 110 (0.76 ml, 10.19 mmol) added. A solution of sodium cyanoborohydride (0.16 g, 2.55 mmol) and zinc chloride (0.17 g, 1.27 mmol) in anhydrous MeOH (4 ml) was then added, and the mixture stirred at room temperature overnight. The mixture was quenched with 5 % NaOH solution and the volatiles removed in vacuo. EtOAc (50 ml) was added and the layers separated. The organic phase was washed with water (15 ml), brine (15 ml), then dried (MgSO₄) and rotary evaporated to give a bright orange oil (0.63 g):
MS ES (+ve) found \( m/z \) 275 (100 %), 300 (36); Flash column chromatography on silica gel using EtOAc/5 % MeOH isolated a colourless oil (0.25 g): MS ES (+ve) found \( m/z \) 300 (100 %); \(^1\)H NMR spectrum broad and unassignable

**Method C**

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetaldehyde 126 (0.30 g 1.52 mmol) and allylamine 110 (0.11 ml, 1.52 mmol) were dissolved in anhydrous MeOH (15 ml) and anhydrous sodium acetate (0.25 g, 3.06 mmol), followed by sodium cyanoborohydride (0.16 g, 2.55 mmol) were added. The mixture was then stirred in the presence of 4 Å molecular sieves at room temperature overnight. 1M HCl was used to bring the pH to pH 2, then saturated aqueous sodium carbonate was added to adjust to pH 10. The mixture was extracted with EtOAc (3 x 15 ml) and the total organic phase washed with brine (15 ml), dried (MgSO₄) and rotary evaporated to give a brown oil (0.26 g): MS ES (+ve) found \( m/z \) 261 (13 %), 275 (100), 300 (8); Purification by Kugelrohr distillation did not give rise to any discernible product.

**Method D**

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetaldehyde 126 (0.25 g 1.27 mmol) and benzylamine 128 (0.21 ml, 1.91 mmol) were dissolved in anhydrous MeOH (15 ml) and acetic acid (4 drops) added. The mixture was stirred at room temperature for 2 hrs and sodium cyanoborohydride (0.16 g, 2.55 mmol) added. Stirring was then continued overnight. The mixture was quenched with saturated NaHCO₃ solution (10 ml) and extracted with EtOAc (3 x 15 ml). The total organic phase washed with brine (15 ml), dried (MgSO₄) and rotary evaporated to give an orange oil (0.38 g): Flash column chromatography on silica gel using EtOAc as eluent failed to isolate any discernible product.

**Method E**

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetaldehyde 126 (0.25 g 1.27 mmol) and allylamine 110 (0.14 ml, 1.91 mmol) were dissolved in anhydrous DCM
(15 ml) and acetic acid (4 drops) added. The mixture was heated under reflux for 3 hrs, allowed to cool, and sodium cyanoborohydride (0.16 g, 2.55 mmol) added. The mixture was then stirred at room temperature overnight. The mixture was quenched with saturated NaHCO₃ solution (10 ml) and extracted with EtOAc (3 x 15 ml). The total organic phase was washed with brine (15 ml), dried (MgSO₄) and rotary evaporated to give a yellow oil (0.28 g): Flash column chromatography on silica gel using hexane/EtOAc (1:1) as eluent isolated a yellow oil (0.032 g, 13 %):

5.2.21.1 6-(2-Hydroxy-ethyl)-3-methoxy-5,5-dimethyl-cyclohex-2-enone 124

\[
\begin{align*}
\text{HO} & \quad \text{OMe} \\
\text{O} & \quad \text{Me}
\end{align*}
\]

\(\nu_{\text{max}}\) (neat) 3408.6 (OH), 1649.8 (\(\alpha,\beta\)-unsaturated ketone), 1613.2 (C=C); \(\delta_H\) (250 MHz, CDCl₃) 0.95 (3H, s, C(\(\text{CH₃C}_{\text{H₃}}\))), 1.24 (3H, s, C(\(\text{CH₃C}_{\text{H₃}}\))), 1.62 (2H, m, \(\text{CH}_{\text{2CH₂O}}\)), 2.20 (1H, dd, J 9.4, 3.6, \(\text{CH}_{\text{2CH₂O}}\)), 2.25 (1H, d, J 17.8, \(\text{CH}_{\text{A^H^B^C(CH₃)₂}}\)), 2.34 (1H, d, J 17.8, \(\text{CH}_{\text{A^H^B^C(CH₃)₂}}\)), 3.65 (2H, m, \(\text{CH}_{\text{₂CH₂O}}\)), 3.70 (3H, s, \(\text{OCH₃}\)), 4.98 (1H, d, J 1.9, COH); \(\delta_C\) (63 MHz, DEPT, CDCl₃) 23.3 (C(\(\text{CH₃C}_{\text{H₃}}\))), 28.2 (\(\text{CH}_{\text{₂CH₂O}}\)), 28.6 (C(\(\text{CH₃C}_{\text{H₃}}\))), 35.4 (C(\(\text{CH₃)₂}\)), 42.2 (\(\text{CH}_{\text{₂CH₂O}}\)), 54.8 (\(\text{CH}_{\text{₂CH₂O}}\)), 55.7 (\(\text{OCH₃}\)), 62.1 (\(\text{CH}_{\text{₂CH₂O}}\)), 100.5 (COCH=CO), 176.5 (COCH=COCH₃), 203.3 (COCH=COCH₃); MS FAB (+ve) found m/z 199 (MH⁺, 100 %), 221 (MNa⁺, 40) 181 (M⁺-OH, 80); HRMS FAB (+ve) found m/z 199.13344 (MH⁺), \(\text{C}_{11}\text{H}_{19}\text{O}_3\) requires 197.13342.

**Method F**

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetaldehyde 126 (0.25 g 1.27 mmol) and benzylamine 128 (0.126 ml, 1.16 mmol) were dissolved in anhydrous THF (10 ml) and titanium isopropoxide (0.68 ml, 2.54 mmol) added. The mixture was then stirred overnight at room temperature [MS ES (+ve) found m/z 286 (imineH⁺, 100 %)]. EtOH (3 ml) was added, followed by sodium borohydride (0.13 g, 3.47 mmol) and the mixture stirred for a further 8 hrs. The mixture was poured
into 2M aqueous ammonia solution (12 ml), filtered and washed with EtOAc (30 ml). The filtrate layers were separated and the aqueous extracted with EtOAc (20 ml). The total organic phase was washed with brine (20 ml), dried (Na₂SO₄) and rotary evaporated to give an orange oil (0.30 g): MS ES (+ve) found m/z 272 (100 %); very little trace by TLC.

5.2.22 Synthesis of Extended Aldehyde Derivative

5.2.22.1 3-Methoxy-5,5-dimethyl-6-(4-methyl-pent-3-enyl)-cyclohex-2-enone

Repeat of procedure 5.2.4.4 using 5-bromo-2-methyl-2-pentene 134 (5.00 ml, 37.59 mmol) as the electrophile. A yellow liquid (8.50 g) was obtained after work-up. This was purified by flash column chromatography on silica gel using hexane/EtOAc (4:1-1:1) gradient eluent to give a yellow oil (0.97 g, 13 %): ν<sub>max</sub> (neat) 1656.6 (α,β-unsaturated ketone), 1616.1 (ring C=C); δ<sub>H</sub> (250 MHz, CDCl₃) 0.95 (3H, s, C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H₃)), 1.04 (3H, s, C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H₃)), 1.48 (2H, m, CH₂CH₂CH=Me₂), 1.58 (3H, s, CH=C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H₃)), 1.66 (3H, s, CH=C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H₃)), 1.92 (1H, dd, J 8.4, 4.7, COCHC(CH₃)₂), 2.03 (2H, m, CH₂CH=CMMe₂), 2.14 (1H, d, J 17.7, CH<sup>A</sup>H<sup>B</sup>C(CH₃)₂), 2.31 (1H, d, J 17.7, CH<sup>A</sup>H<sup>B</sup>C(CH₃)₂), 3.65 (3H, s, OCH₃), 5.08 (1H, m, CH=CMMe₂), 5.25 (1H, s, COCH=COCH₃); δ<sub>C</sub> (63 MHz, DEPT, CDCl₃) 17.7 (CH=C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H₃)), 24.5 (C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H₃)), 25.6 (CH=C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H₃)), 26.3 (CH₃), 27.1 (CH₂), 28.5 (C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H₃)), 35.0 (CH(CH₃)₂), 41.3 (CH₂C(CH₃)₂), 55.4 (OCH₃), 56.5 (COCHC(CH₃)₂), 100.1 (COCH=COCH₃), 124.2 (CH₂CH₂CH=CMMe₂), 131.9 (CH=CMMe₂), 174.9 (COCH=COCH₃), 202.4 (COCH=COCH₃); MS FAB (+ve) found m/z 237 (MH⁺, 100 %); HRMS FAB (+ve) found m/z 237.18548 (MH⁺), C₁₅H₂₅O₂ requires 237.18546.
5.2.22.2 3-(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-propionaldehyde 136

Repeat of Method B (section 5.2.20.1) on 3-methoxy-5,5-dimethyl-6-(4-methyl-pent-3-enyl)-cyclohex-2-enone 135 (0.95 g, 4.02 mmol). A brown liquid (0.69 g) was obtained after work-up, which was purified by Kugelrohr distillation to give a yellow oil (0.60 g, 70 %): bp 150 °C (0.1 mBar); ν max (neat) 1721.2 (aldehyde), 1651.7 (α,β-unsaturated ketone), 1614.1 (C=C); δ H (250 MHz, CDCl3) 0.95 (3H, s, C(CH3)3), 1.06 (3H, s, C(CH3)3), 1.65 (1H, m, CHA-HβCH2COH), 1.85 (1H, m, CHA-HβCH2COH), 1.91 (1H, dd, J 10.9, 2.8, COCHC(CH3)2), 2.19 (1H, d, J 17.7, CHA-HβC(CH3)2), 2.29 (1H, d, J 17.7, CHA-HβC(CH3)2), 2.58 (2H, m, CH2CH2COH), 3.63 (3H, s, OCH3), 5.22 (1H, d, J 1.7, COCH=COCH3), 9.74 (1H, t, J 1.0, COH); δ C (63 MHz, DEPT, CDCl3) 18.1 (CH2CH2COH), 23.8 (C(CH3)3), 28.5 (C(CH3)3), 35.2 (CH2COH), 41.6 (CH2), 42.9 (CH2), 55.5 (OCH3), 55.7 (COCHC(CH3)2), 100.1 (COCH=CO), 175.3 (COCH=COCH3), 201.7 (CO), 202.2 (CO); MS FAB (+ve) found m/z 211 (MH+, 100 %), 233 (MNa+, 27); HRMS FAB (+ve) found 211.13346 (MH+), C12H19O3 requires 211.13349.

5.2.23 Reductive Amination on Extended Aldehyde

Repeat of Method A (section 5.2.21) using 3-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-propionaldehyde 136 (0.20 g, 0.951 mmol). An orange oil was obtained (0.19 g) after work-up: MS ES (+ve) found m/z 252 (MH+, 5 %), 259 (100); Flash column chromatography on silica gel using EtOAc/5 % MeOH as eluent isolated an off-white oil whose 1H NMR spectrum contained peaks representative of the desired product. However, spectrum was not clean and appeared to indicate the presence of a mixture of compounds.
5.2.24 Alternative Methods to Form Secondary Amines

5.2.24.1 6-(2-Hydroxy-ethyl)-3-methoxy-5,5-dimethyl-cyclohex-2-enone 124

![Chemical Structure](image)

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetaldehyde 126 (0.25 g, 1.26 mmol) was dissolved in anhydrous MeOH (5 ml) and solid-supported borohydride (0.37 g, 1.40 mmol) added. The mixture was then shaken at room temperature overnight. The MeOH layer was filtered and the resin washed with MeOH (5 x 5ml). The filtrate and washings were reduced in vacuo to give a brown oil (0.23 g, 91%). MS ES (+ve) found m/z 199 (MH⁺, 100%); ¹H NMR was consistent with that found for 5.2.21.1.

Nucleophilic Substitution Reaction with Benzylamine

6-(2-Hydroxy-ethyl)-3-methoxy-5,5-dimethyl-cyclohex-2-enone 124 (0.22 g, 1.11 mmol) was dissolved in anhydrous DCM (10 ml) and cooled to 0 °C. Anhydrous triethylamine (0.187 ml, 1.33 mmol), followed by methanesulfonyl chloride (0.103 ml, 1.33 mmol) were then added dropwise. The mixture was then allowed to warm to room temperature and stirred for 4 hrs. The solvent was removed in vacuo and the residue taken up in anhydrous DMF (10 ml). Benzylamine (0.139 ml, 1.11 mmol), followed by catalytic cesium carbonate (35 mg, 10 mol %) were then added, and the mixture stirred at room temperature overnight. Water (10 ml) was added and the reaction extracted with EtOAc (4 x 20 ml). The total organic phase was washed with brine (20 ml), dried (Na₂SO₄) and rotary evaporated to give an orange liquid (0.60 g). MS ES (+ve) and NMR failed to provide evidence of desired secondary amine product. Flash column chromatography on silica gel using hexane/EtOAc (7:3) as eluent isolated a yellow oil (0.062 g, 34%).
5.2.24.2 4,4-Dimethyl-3,3a,4,5-tetrahydro-2H-benzofuran-6-one 138

\[
\begin{align*}
\delta_H (250 \text{ MHz}, \text{CDCl}_3) & : 0.84 (3\text{H, s, } C(C^\text{A}H_3C^\text{B}H_3)), 1.05 (3\text{H, s, } C(C^\text{A}H_3C^\text{B}H_3)), 1.80 (1\text{H, dq, } J 12.4, 8.6, CH^\text{A}H^\text{B}CH_2O), 2.06 (1\text{H, m, } CH^\text{A}H^\text{B}CH_2O), 2.09 (1\text{H, d, } J 16.3, CH^\text{A}H^\text{B}C(CH_3)_2), 2.19 (1\text{H, d, } J 16.3, CH^\text{A}H^\text{B}C(CH_3)_2), 2.83 (1\text{H, ddd, } J 12.6, 7.8, 2.1, CHCH_2CH_2O), 4.15 (1\text{H, ddd, } J 11.8, 8.9, 5.5, CH_2CH^\text{A}H^\text{B}O), 4.46 (1\text{H, t, } J 8.7, CH_2CH^\text{A}H^\text{B}O), 5.32 (1\text{H, d, } J 1.8, C=CHCO), 9.88 (1\text{H, d, } J 1.9, COH); \\
\delta_C (63 \text{ MHz, DEPT, CDCl}_3) & : 19.7 (C(C^\text{A}H_3C^\text{B}H_3)), 24.1 (CH_2CH_2O), 29.2 (C(C^\text{A}H_3C^\text{B}H_3)), 34.9 (C(CH_3)_2), 50.2 (CHCH_2CH_2O), 52.0, (CH_2C(CH_3)_2), 72.7 (CH_2CH_2O), 99.4 (C=CHCO), 180.8 (C=CHCO), 198.6 (C=CHCO); MS ES (+ve) found m/z 167 (MH\textsuperscript{+}, 100%).
\end{align*}
\]

**Conversion to Alkyl Bromide**

6-(2-Hydroxy-ethyl)-3-methoxy-5,5-dimethyl-cyclohex-2-enone 124 (0.25 g, 1.26 mmol) was dissolved in anhydrous DCM (10 ml) and cooled to 0 °C. Phosphorus tribromide (75 \text{ ml}, 0.798 mmol) was slowly added and the mixture stirred at 0 °C for 15 mins, then at room temperature for 5 hrs. The mixture was poured onto ice and diluted with brine (20 ml). The aqueous phase was extracted with DCM (3 x 15 ml) and the total organic phase washed with brine (15 ml), dried (MgSO\textsubscript{4}) and rotary evaporated to give an orange liquid (0.17 g). This was purified by flash column chromatography on silica gel using hexane/EtOAc (7:3) as eluent to give an off-white solid (0.14 g, 67 %): MS ES (+ve) found m/z 167 (100 %); NMR spectra was consistent with that found for the furan derivative 138 (5.2.24.2).

5.2.25 Synthesis and Reductive Aminations of Hemi-Acetal

5.2.25.1 2-Hydroxy-4,4-dimethyl-3,3a,4,5-tetrahydro-2H-benzofuran-6-one 140

\[
\begin{align*}
\end{align*}
\]
(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetaldehyde 126 (2.00 g, 10.19 mmol) was dissolved in acetone (80 ml) and 2M HCl (40 ml) slowly added. The mixture was then stirred at room temperature overnight. The acetone was removed in vacuo and the mixture diluted with brine (30 ml) and extracted with EtOAc (3 x 50 ml). The total organic phase was dried (Na₂SO₄) and rotary evaporated to give an off-white solid (1.66 g). This was recrystallised from hexane/EtOAc to give a white solid (0.70 g, 38%). The filtrate was reduced in vacuo and the residue triturated with ether to afford a second crop of white solid (0.51 g, 27%): mp 111-113 °C; νₘₐₓ (CHCl₃) 3234.0 (OH), 1619.9 (C=O); δₜ (360 MHz, CDCl₃) 0.90 (3H, s, C(CH₃)₂), 1.10 (3H, s, C(CH₃)₂), 1.86 (1H, br, CH₂H₂COH), 2.11 (1H, br, CH₂H₂COH), 2.22 (1H, d, J 16.3, CH₂H₂C(CH₃)₂), 2.31 (1H, d, J 16.5, CH₂H₂C(CH₃)₂), 3.29 (1H, br, CH₂CHOH), 5.48 (1H, s, C=CHCO), 6.02 (1H, s, CH₂CHOH); δ (90 MHz, DEPT, CDCl₃) 19.9 (C(CH₃)₂), 31.1 (CH₂CHOH), 35.4 (C(CH₃)₂), 48.3 (CH₂CHOH), 51.9, (CH₂C(CH₃)₂), 99.6 (C=CHCO), 103.1 (CH₂CHOH), 181.9 (C=CHCO), 200.8 (C=CHCO); MS FAB (+ve) found m/z 183 (MH⁺, 100%); HRMS FAB (+ve) found m/z 183.10214 (MH⁺), C₁₀H₁₅O₃ requires 183.10212.

5.2.25.2 1-Benzyl-4,4-dimethyl-1,2,3,3a,4,5-hexahydro-indol-6-one 141

2-Hydroxy-4,4-dimethyl-3a,4,5-tetrahydro-2H-benzofuran-6-one 140 (0.20 g, 1.10 mmol) was dissolved in anhydrous DCM (10 ml) and benzylamine 128 (0.24 ml, 2.19 ml) added. The mixture was stirred in the presence of 4 Å molecular sieves at room temperature for 2 hrs. NaCNBH₃ (0.14 g, 2.19 mmol) was added and the solution adjusted to pH 6 with acetic acid. The mixture was then stirred at room temperature for a further 3 hrs. The solvent was removed in vacuo and the residue taken up in water (10 ml), basified with 5% NaOH solution to pH 10, and extracted with EtOAc (3 x 20 ml). The total organic phase was washed with brine (15 ml), dried (MgSO₄) and rotary evaporated to give a gummy colourless oil (0.26 g). This
was purified by flash column chromatography on silica gel using EtOAc/5 % MeOH as eluent to give a yellow oil (0.047 g, 17 %): \( \delta_H \) (360 MHz, CDCl\(_3\)) 0.90 (3H, s, C(\( ^A \)H\(_3\)C\( ^B \)H\(_3\))), 1.01 (3H, s, C(\( ^A \)H\(_3\)C\( ^B \)H\(_3\))), 1.71 (1H, m, CH\(^A\)H\(^B\)CH\(_2\)N), 1.95 (1H, m, CH\(^A\)H\(^B\)CH\(_2\)N), 2.12 (1H, d, \( J \) 16.4, CH\(^A\)H\(^B\)C(CH\(_3\)_2)), 2.24 (1H, d, \( J \) 16.4, CH\(^A\)H\(^B\)C(CH\(_3\)_2)), 2.83 (1H, ddd, \( J \) 12.1, 7.8, 1.2, CHCH\(_2\)CH\(_2\)N), 3.51 (2H, m, CH\(_2\)CH\(_2\)N), 4.32 (2H, s, PhCH\(_2\)), 5.13 (1H, s, C=CHCO), 7.12 (2H, m, Ar-H), 7.26 (3H, m, Ar-H); \( \delta_C \) (90 MHz, DEPT, CDCl\(_3\)) 19.8 (C(\( ^A \)H\(_3\)C\( ^B \)H\(_3\))), 22.1 (CH\(_2\)CH\(_2\)N), 28.9 (C(\( ^A \)H\(_3\)C\( ^B \)H\(_3\))), 34.6 (C(CH\(_3\)_2)), 49.8 (PhCH\(_2\)), 51.9 (CH\(_2\)C(CH\(_3\)_2) or CH\(_2\)CH\(_2\)N), 52.0 (CH\(_2\)C(CH\(_3\)_2) or CH\(_2\)CH\(_2\)N), 52.1 CHCH\(_2\)CH\(_2\)N), 91.4 (C=CHCO), 127.4 (CH), 127.9 (CH), 128.8 (CH) 5C, Ar-H], 135.3 (Ph), 168.5 (C=CHCO), 196.2 (C=CHCO); MS ES (+ve) found m/z 256 (MH\(^+\), 100 %); HRMS FAB (+ve) found m/z 256.16965 (MH\(^+\)), C\(_{17}\)H\(_{22}\)NO requires 256.17014.

5.2.25.3 1-Allyl-4,4-dimethyl-1,4,5,7-tetrahydro-indol-6-one 143

2-Hydroxy-4,4-dimethyl-3,3a,4,5-tetrahydro-2H-benzofuran-6-one 140 (0.54 g, 2.96 mmol) was dissolved in anhydrous DCM (25 ml) and allylamine 110 (0.24 ml, 3.26 mmol) added. The mixture was stirred in the presence of 4 Å molecular sieves at room temperature overnight. NaCNBH\(_3\) (0.14 g, 2.19 mmol) was added and the solution adjusted to pH 6 with acetic acid. The mixture was then stirred at room temperature for a further 24 hrs. The reaction was worked-up as described for 5.2.25.2 to give an off-white oil (0.43 g). This was purified by Kugelrohr distillation to give a dark brown oil (0.30 g, 50 %); bp 250 °C (0.5 mBar); \( \delta_H \) (250 MHz, CDCl\(_3\)) 1.25 (6H, s, C(CH\(_3\)_2)), 2.51 (CH\(_2\)C(CH\(_3\)_2)), 3.37 (2H, s, NCCH\(_2\)CO), 4.33 (2H, m, NCH\(_2\)CHCH\(_2\)), 4.97 (1H, m, NCH\(_2\)CHCH\(^A\)H\(^B\)), 5.18 (1H, m, NCH\(_2\)CHCH\(^A\)H\(^B\)), 5.91 (1H, m, NCH\(_2\)CHCH\(_2\)), 6.09 (1H, d, \( J \) 2.8, NCH=CH), 6.60 (1H, d, \( J \) 2.8, NCH=CH); \( \delta_C \) (63 MHz, DEPT, CDCl\(_3\)) 30.5 (C(CH\(_3\)_2)), 33.8 (C(CH\(_3\)_2), 37.4 (NCCH\(_2\)CO), 49.3 (NCH\(_2\)CHCH\(_2\)), 55.1 (CH\(_2\)C(CH\(_3\)_2)), 103.3 (NCH=CH), 117.1
(NCH<sub>2</sub>CHCH<sub>2</sub>), 120.7 (NCH=CH), 122.1 (CCH=CHN), 127.4 (NC<sub>2</sub>CO), 133.9 (NCH<sub>2</sub>CHCH<sub>2</sub>), 208.9 (NC<sub>2</sub>CO); MS ES (+ve) found m/z 204 (MH<sup>+</sup>, 65 %), 226 (MNa<sup>+</sup>, 15); HRMS FAB (+ve) found m/z 204.13897 (MH<sup>+</sup>), C<sub>13</sub>H<sub>18</sub>N<sub>0</sub> requires 204.13884.

5.2.25.4 (S)-6-Benzoxycarbonylamino-2-(4,4-dimethyl-6-oxo-2,3,3a,4,5,6-hexahydro-indol-1-yl)-hexanoic acid methyl ester 144

![Chemical Structure](image)

2-Hydroxy-4,4-dimethyl-3,3a,4,5-tetrahydro-2H-benzofuran-6-one 140 (0.80 g, 4.39 mmol) was dissolved in anhydrous DCM (40 ml) and N-ε-CBz-L-lysine methyl ester hydrochloride 120c (1.60 g, 4.83 mmol), followed by DIPEA (0.84 ml, 4.83 mmol) were added. The mixture was stirred at room temperature overnight. NaCNBH<sub>3</sub> (0.55 g, 8.79 mmol) was added and the solution adjusted to pH 6 with acetic acid. The mixture was then stirred at room temperature for a further 24 hrs. The reaction was worked-up as described for 5.2.25.2 to give a yellow oil (2.34 g). This was purified by flash column chromatography on silica gel using EtOAc/5 % MeOH as eluent to give a colourless oil (1.08 g, 56 %) as a mixture of diastereomers: \( \nu_{max} \) 1738.5 (ester), 1713.4 (carbamate), 1578.4 (\( \alpha, \beta \)-unsaturated ketone); \( \delta_H \) (360 MHz, CDCl<sub>3</sub>) 0.81 (3H, s, C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H<sub>3</sub>)), 0.83 (3H, s, C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H<sub>3</sub>)), 0.97 (6H, s, C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H<sub>3</sub>)), 1.24 (4H, m, Lys-CH<sub>2</sub>), 1.46 (4H, m, Lys-CH<sub>2</sub>), 1.84 (8H, m, C<sub>6</sub>H<sub>2</sub> + CH<sub>2</sub>CH<sub>2</sub>N), 2.06 (1H, d, \( J = 16.4 \), CH<sup>A</sup>H<sub>B</sub>C(CH<sub>3</sub>)), 2.11 (1H, d, \( J = 16.5 \), CH<sup>A</sup>H<sub>B</sub>C(CH<sub>3</sub>)), 2.18 (1H, d, \( J = 16.4 \), CH<sup>A</sup>H<sub>B</sub>C(CH<sub>3</sub>)), 2.23 (1H, d, \( J = 16.5 \), CH<sup>A</sup>H<sub>B</sub>C(CH<sub>3</sub>)), 2.79 (2H, m, CHCH<sub>2</sub>CH<sub>2</sub>N), 3.11 (4H, m, C<sub>6</sub>H<sub>2</sub>), 3.43 (4H, m, CH<sub>2</sub>CH<sub>2</sub>N), 3.63 (6H, s, OCH<sub>3</sub>), 4.07 (2H, m, C<sub>6</sub>H<sub>2</sub>), 4.93 (1H, s, C=CH<sub>CO</sub>), 4.97 (1H, s, C=CH<sub>CO</sub>), 5.00 (4H, m, CH<sub>2</sub>Ph), 5.13 (2H, br, NH), 7.25 (10H, m, Ar-H); \( \delta_C \) (63 MHz, DEPT, CDCl<sub>3</sub>) 19.6 + 19.7 (C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H<sub>3</sub>)), 22.0 (CH<sub>2</sub>CH<sub>2</sub>N), 22.9 + 23.3 (Lys-CH<sub>2</sub>), 28.3 (Lys-CH<sub>2</sub>), 28.8 + 28.9 (C(C<sup>A</sup>H<sub>3</sub>C<sup>B</sup>H<sub>3</sub>)), 29.2 (Lys-CH<sub>2</sub>), 34.4 + 34.6 (C(CH<sub>3</sub>)), 40.4 (C<sub>6</sub>H<sub>2</sub>), 47.8 + 48.5 (CH<sub>2</sub>C(CH<sub>3</sub>)), 51.5 + 52.0 (CHCH<sub>2</sub>CH<sub>2</sub>N),
52.1 \((\text{CH}_2\text{CH}_2\text{N})\), 52.3 + 52.4 \((\text{CO}_2\text{CH}_3)\), 56.8 + 57.0 \((\text{C}_n\text{H})\), 66.4 \((\text{PhCH}_2)\), 92.0 + 92.4 \((\text{C}^\equiv\text{CHCO})\), [127.9 \((\text{CH})\), 128.4 \((\text{CH})\), 10C, \(\text{Ar-H}\)], 136.4 \((\text{Ph})\), 156.4 \((\text{NHCOO})\), 169.2 \((\text{C}^≡\text{CHCO or CO}_2\text{Me})\), 170.6 + 170.7 \((\text{C}^≡\text{CHCO or CO}_2\text{Me})\), 196.6 \((\text{C}^≡\text{CHCO})\); MS FAB (+ve) found \text{m/z} 443 \((\text{MH}^+, 4\%\)), 465 \((\text{MNa}^+, 100\%)); HRMS FAB (+ve) found \text{m/z} 443.25401 \((\text{M}_{\text{T}^{'}})\), \(\text{C}_{23}\text{H}_{35}\text{N}_2\text{O}_5\) requires 443.25460.

5.2.25.5 \((\text{S})\)-6-Amino-2-(4,4-dimethyl-6-oxo-2,3,3a,4,5,6-hexahydro-indol-1-yl)-hexanoic acid methyl ester 145

\[
\begin{align*}
\text{H}_2\text{N} & \quad \begin{array}{c}
\text{N} \\
\end{array} \\
\text{CO}_2\text{Me} & \quad \begin{array}{c}
\text{O} \\
\end{array}
\end{align*}
\]

\((\text{S})\)-6-Benzylloxycarbonylamino-2-(4,4-dimethyl-6-oxo-2,3,3a,4,5,6-hexahydro-indol-1-yl)-hexanoic acid methyl ester 144 \((0.20 \text{ g}, 0.45 \text{ mmol})\) was dissolved in anhydrous DCM \((12 \text{ ml})\) and iodo(trimethyl)silane \((0.31 \text{ ml}, 2.26 \text{ mmol})\) slowly added. The mixture was then stirred at room temperature overnight. The mixture was quenched with MeOH \((0.4 \text{ ml})\) and stirred for 5 mins. The solvent was removed \textit{in vacuo} and the residue taken up in ether/30 \% acetic acid \((25 \text{ ml})\). The aqueous layer was separated and extracted a further time with ether \((10 \text{ ml})\) and then freeze-dried overnight to give an orange oil \((0.11 \text{ g}, 79\%)\): \text{v} \text{max} \(3415.3 \text{ (NH}_2\)), 1742.4 \text{ (ester)}, 1586.2 \((\alpha,\beta\text{-unsaturated ketone}), 1550.5 \text{ (NH}_2\); \(\delta_H\) \((360 \text{ MHz, DMSO}) 0.85 + 0.86 \text{ (6H, s, C(C}\text{H}_3\text{C}\text{H}_3)), 1.07 + 1.08 \text{ (6H, s, C(C}\text{H}_3\text{S}\text{H}_3)), 1.27 \text{ (4H, m, Lys-CH}_2\)), 1.56 \text{ (4H, m, Lys-CH}_2\), 1.77 \text{ (2H, m, CH}_4\text{H}\text{C}\text{H}_2\text{N}), 1.91 \text{ (4H, m, C}_\beta\text{H}_2\), 2.12 \text{ (2H, m, CH}_4\text{H}\text{C}\text{H}_2\text{N}), 2.23 \text{ (2H, m, CH}_4\text{H}\text{C}\text{H}_2\text{N}), 2.62 \text{ (2H, br-d, CH}_4\text{H}\text{C}\text{H}_2\text{N}), 2.81 \text{ (4H, m, C}\text{H}_2\text{H}_2\text{N}), 3.19 \text{ (1H, m, CHCH}_2\text{CH}_2\text{N}), 3.30 \text{ (1H, m, CHCH}_2\text{CH}_2\text{N}), 3.68 \text{ (4H, m, CH}_2\text{CH}_2\text{N}), 3.71 \text{ (6H, s, OCH}_3\)), 4.82 \text{ (2H, m, C}_\alpha\text{H}), 5.56 \text{ (1H, br-s, C=CHCO}), 5.62 \text{ (1H, br-s, C=CHCO}); \(\delta_C\) \((63 \text{ MHz, DEPT, DMSO}) 19.4 + 19.5 \text{ (C(C}\text{H}_3\text{C}\text{H}_3)), 20.5 \text{ (CH}_2\text{CH}_2\text{N}), 22.8 + 22.1 \text{ (Lys-CH}_2\)), 26.1 + 26.2 \text{ (Lys-CH}_2\), 26.9 \text{ (C}_\beta\text{H}_2\), 27.3 + 27.4 \text{ (C(C}\text{H}_3\text{C}\text{H}_3)), 34.4 + 34.7 \text{ (C(CH}_3)), 38.3 \text{ (C}_\alpha\text{H}), 46.1 + 46.4 \text{ (CH}_2\text{C(CH}_3)), 50.8 + 51.6 \text{ (CH}_2\text{CH}_2\text{N}), 52.1 + 52.4 \text{ (CHCH}_2\text{CH}_2\text{N}), 52.7 + 52.8 \text{ (CO}_2\text{CH}_3\), 57.9 + 58.3 \text{ (C}_\alpha\text{H}), 90.9 + 91.1 \text{ (C=CHCO)}, 168.4 + 171.8
(C=CHCO), 177.0 + 177.7 (CO₂Me), 189.3 + 190.0 (C=CHCO); MS FAB (+ve) found m/z 309 (MH⁺, 100 %); HRMS FAB (+ve) found m/z 309.21759 (MH⁺), C₁₁H₂₉N₂O₃ requires 309.21782.

5.2.26 Synthesis of Hydrazide Derivative

5.2.26.1 (4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid hydrazide 146

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (0.50 g, 2.36 mmol) was dissolved in anhydrous DCM (10 ml) and HOBt (0.38 g, 2.82 mmol) and EDCI (0.54 g, 2.82 mmol) added. The mixture was stirred at room temperature for 2 hrs then added dropwise to a solution of hydrazine hydrate (0.27 ml, 4.71 mmol) and cyclohexene (0.06 ml) in anhydrous DCM (5 ml) at -20 °C. After addition was complete, the reaction was quenched with water (5 ml) and extracted with EtOAc (2 x 10 ml). The organic phase was washed with saturated NaHCO₃ solution (10 ml) and brine (10 ml), then dried (MgSO₄) and rotary evaporated to give a gummy yellow solid (0.31 g, 58 %): νₘₐₓ (CHCl₃) 3329.5 (NH), 1652.7 (α,β-unsaturated ketone), 1615.1 (C=C); δH (250 MHz, CDCl₃) 0.82 (3H, s, C(CH₃CH₂H₃)), 1.11 (3H, s, C(CH₃CH₂H₃)), 2.11 (1H, d, J 17.4, CH²H³BC(CH₃)₂), 2.14 (1H, dd, J 14.4, 2.9, CH²H³CONH), 2.45 (1H, dd, J 14.4, 9.0, CH²H³CONH), 2.53 (1H, dd, J 17.4, 1.1, CH²H³C(CH₃)₂), 2.75 (1H, dd, J 9.0, 2.9, CHCH₂CONH), 3.65 (3H, s, OCH₃), 5.31 (1H, d, J 1.6, COCH=COCH₃), 7.78 (1H, br, NH); δC (63 MHz, DEPT, CDCl₃) 21.0 (C(CH₃CH₂H₃)), 28.8 (C(CH₃CH₂H₃)), 29.6 (CH₂CONH), 35.9 (C(CH₃)₂), 44.2 (CH₂C(CH₃)₂), 52.9 (CHCH₂CONH), 55.7 (OCH₃), 100.7 (COCH=CO), 173.8 (CONH or COCH=COCH₃), 175.8 (CONH or COCH=COCH₃), 200.0 (COCH=COCH₃); MS FAB (+ve) found m/z 227 (MH⁺, 80 %), 249 (MNa⁺, 43), 195 (M⁺-NHNH₂, 100); HRMS FAB (+ve) found m/z 227.13974 (MH⁺), C₁₁H₁₉N₂O₃ requires 227.13957.
5.2.26.2 7-Methoxy-5,5-dimethyl-4,4a,5,6-tetrahydro-2H-cinnolin-3-one 147

(4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid hydrazide 146 (0.20 g, 0.88 mmol) was dissolved in THF (7 ml) and 2M HCl (7 ml) slowly added. The mixture was then stirred at room temperature overnight. EtOAc (30 ml) was added and the organic phase washed with water (10 ml) and saturated NaHCO₃ solution (10 ml). The aqueous phase was back-extracted with EtOAc (2 x 15 ml) and the total organic phase washed with brine (15 ml), dried (MgSO₄) and rotary evaporated to give a brown oil (0.09 g). This was purified by flash chromatography on silica gel using hexane/EtOAc (3:2) as eluent to give a gummy white solid (0.01 g, 5 %): δ_H (250 MHz, CDCl₃) 0.94 (3H, s, C(CH₃)₂), 1.08 (3H, s, C(CH₃)₂), 2.03 (1H, d, J 16.5, CH(CH₃)₂), 2.25 (1H, t, J 15.3, CH(CH₃)CONH), 2.32 (1H, dd, J 16.5, 1.3, CH(CH₃)CONH), 2.45 (1H, dd, J 15.4, 6.3, CH₂CH₂CONH), 2.55 (1H, dd, J 15.3, 6.3, CH(CH₃)CONH), 3.67 (3H, s, OCH₃), 5.44 (1H, d, J 1.8, CH=COCH₃), 8.36 (1H, br, NH); δ_C (63 MHz, DEPT, CDCl₃) 21.1 (C(CH₃)₂), 27.9 (C(CH₃)₂), 28.0 (CH₂CONH), 34.9 (C(CH₃)₂), 41.7 (CH₂CH₂CONH), 43.9 (CH₂CH₂(CH₃)₂), 55.5 (OCH₃), 95.7 (CH=COCH₃), 153.2 (N=CCH), 164.8 (CH=COMe), 168.0 (CONH); MS FAB (+ve) found m/z 209 (MH⁺, 100 %), 231 (MNa⁺, 21); HRMS FAB (+ve) found m/z 209.12896 (MH⁺), C₁₁H₁₇N₂O₂ requires 209.12900.

5.2.27 Synthesis of Enantiomerically Pure Ligands

5.2.27.1 (R)-4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 150a

Racemic 4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (8.36 g, 39.38 mmol) was dissolved in a minimum volume of IPA and (R)-(+)α-methyl
benzylamine 148a (5.58 ml, 43.32 mmol) added. The resulting precipitate was filtered and recrystallised twice from IPA. The salt was dissolved in EtOAc (25 ml) and washed with 1M HCl (5 x 10 ml). The total acidic phase was extracted with EtOAc (2 x 10 ml) and the total organic phase washed with brine (15 ml), dried (MgSO₄) and rotary evaporated to give a white crystalline solid (1.68 g): 73 % ee by chiral HPLC (t<sub>R</sub> = 23 mins). The solid was purified by repeating the above procedure to afford a white crystalline solid (0.51 g, 12 %): 93 % ee by chiral HPLC (t<sub>R</sub> = 24 mins): [α]<sub>D</sub> = -36 (c 1.0, CHCl₃).

5.2.27.2 (S)-4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 150b

Racemic 4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (5.77 g, 27.18 mmol) was obtained from the mother liquors of the above reaction (5.2.27.1) and resolved using (S)-(−)-α-methyl benzylamine 149b (3.85 ml, 29.90 mmol) to give a white crystalline solid (0.50 g, 17 %): 98 % ee by chiral HPLC (t<sub>R</sub> = 10 mins): [α]<sub>D</sub> +37 (c 1.0, CHCl₃).

5.2.27.3 (S)-6-Benzoylcarbonylamino-2-[2-((R)-4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)acetylamino]-hexanoic acid methyl ester 151a

(R)-4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 150a (0.50 g, 2.36 mmol) was coupled to N-c-CBz-L-lysine methyl ester hydrochloride 120c (0.78 g, 2.36 mmol) using the general method (5.2.18) to give an orange oil (1.45 g) after work-up. This was purified by flash column chromatography on silica gel using hexane/EtOAc (1:1)-EtOAc as gradient eluent to give a yellow oil (0.80 g, 69 %):
\[ \nu_{\text{max}} \text{ (CHCl}_3\text{) } 3319.9 \text{ (NH), 1740.4 (ester), 1713.4 (carbamate), 1651.7 (}\alpha,\beta-\text{unsaturated ketone), 1614.1 (C=C); } \delta_\text{H} \text{ (360 MHz, CDCl}_3\text{) 0.83 (3H, s, C}^{A}\text{H}_3\text{C}^{B}\text{H}_3\text{), 1.11 (3H, s, C}^{A}\text{H}_3\text{C}^{B}\text{H}_3\text{)), 1.34 (2H, m, C}_2\text{H}_2\text{ or C}_2\text{H}_2\text{), 1.49 (2H, m, C}_2\text{H}_2\text{ or C}_2\text{H}_2\text{), 1.75 (2H, m, C}_3\text{H}_2\text{), 2.12 (1H, d, } J = 17.4, \text{CH}^{A}\text{H}_2\text{C(CH}_3\text{)_2}\text{), 2.19 (1H, d, } J = 11.9, \text{CH}^{A}\text{H}_2\text{CONH), 2.49 (1H, d, } J = 17.4, \text{CH}^{A}\text{H}_2\text{C(CH}_3\text{)_2}\text{), 2.65 (2H, m, CH}^{A}\text{H}_2\text{CONH + CHCH}_2\text{CONH), 3.16 (2H, m, C}_2\text{H}_2\text{), 3.62 (3H, s, OCH}_3\text{), 3.69 (3H, s, CO}_2\text{CH}_3\text{), 4.50 (1H, m, C}_2\text{H}_2\text{), 5.03 (2H, s, C}_2\text{H}_2\text{Ph), 5.16 (1H, br, NH), 5.36 (1H, s, COCH=COCH}_3\text{), 7.08 (1H, d, } J = 7.7, \text{NH), 7.30 (5H, m, Ar-H); } \delta_\text{C} \text{ (90 MHz, DEPT, CDCl}_3\text{) 20.9 (C}^{A}\text{H}_3\text{C}^{B}\text{H}_3\text{), 22.2 (C}_2\text{H}_2\text{ or C}_3\text{H}_2\text{), 28.7 (C}^{A}\text{H}_3\text{C}^{B}\text{H}_3\text{), 29.1 (C}_2\text{H}_2\text{ or C}_3\text{H}_2\text{), 31.6 (CH}_2\text{CONH or C}_2\text{H}_2\text{), 31.7 (CH}_2\text{CONH or C}_2\text{H}_2\text{), 36.1 (C(CH}_3\text{)_2}, \text{40.5 (C}_2\text{H}_2\text{), 44.1 (CH}_2\text{C(CH}_3\text{)_2}, \text{51.9 (C}_2\text{H}_2\text{), 52.2 (CO}_2\text{CH}_3\text{), 53.2 (CHCH}_2\text{CONH), 55.6 (OCH}_3\text{), 66.4 (PhCH}_3\text{), 100.7 (COCH=COCH}_3\text{), [127.6 (CH), 127.9 (CH), 128.4 (CH), 5C, Ar-H], 136.5 (Ph), 156.4 (NHCOCO), 172.7 (CONH or CO}_2\text{Me), 172.9 (CONH or CO}_2\text{Me), 175.9 (COCH=COCH}_3\text{), 200.3 (COCH=COCH}_3\text{); MS FAB (+ve) found m/z 489 (MH}^+, 58 \%, 511 (MNa}^+, 11); HRMS FAB (+ve) found m/z 489.26009 (MW), C}_2\text{6H}_37\text{N}_2\text{O}_7\text{ requires 489.26008.}

5.2.27.4 (S)-6-Benzylxycarbonylamino-2-((R)-4,4-dimethyl-2,6-dioxo-2,3,3a,4,5,6-hexahydro-indol-1-yl)-hexanoic acid methyl ester 153a

\[
\begin{align*}
\text{Ph} & \text{O} \text{N} \text{O} \\
\text{CO}_2\text{Me}
\end{align*}
\]

(S)-6-Benzylxycarbonylamino-2-[2-((R)-4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-hexanoic acid methyl ester 151b (0.32 g, 0.78 mmol) was dissolved in acetone (8 ml) and 2M HCl (8 ml) slowly added. The mixture was then stirred at room temperature overnight. The solvent was removed in vacuo and brine (5 ml) added. The mixture was extracted with EtOAc (3 x 10 ml), and the total organic phase washed with brine (8 ml), dried (MgSO}_4) and rotary evaporated to an orange oil (0.38 g). This was purified by flash column chromatography on silica gel
using hexane/EtOAc (2:3) as eluent to give a colourless oil (0.026 g, 9%): $\nu_{max}$

1743.3 (ester), 1720.2 (carbamate), 1619.0 (C=C); $\delta_H$ (360 MHz, CDCl$_3$) 0.90 (3H, s, C(C\(^\text{A}\)H\(^3\)C\(^\text{B}\)H\(^3\)))

1.08 (3H, s, C(C\(^\text{A}\)H\(^3\)C\(^\text{B}\)H\(^3\))), 1.23 (2H, m, C\(\gamma\)H\(^2\) or C\(\delta\)H\(^2\)), 1.49 (2H, m, C\(\gamma\)H\(^2\) or C\(\delta\)H\(^2\)), 2.10 (2H, m, C\(\delta\)H\(^2\)), 2.34 (2H, s, CH\(_2\)C(CH\(_3\))\(_2\)), 2.39 (1H, dd, J 17.8, 8.6, CH\(^\text{A}\)H\(^\text{B}\)CON), 2.62 (1H, dd, J 17.8, 9.2, CH\(^\text{A}\)H\(^\text{B}\)CON), 3.12 (3H, m, CH\(^2\) or C\(\text{H}\)_2), 3.71 (3H, s, OCH\(_3\)), 4.71 (1H, dd, J 10.1, 5.6, C\(\alpha\)H), 4.77 (1H, br, NH), 5.07 (2H, s, CH\(_2\)Ph), 5.38 (1H, d, J 2.0, C=CHCO), 7.34 (5H, m, Ar-H); $\delta_C$ (90 MHz, DEPT, CDCl$_3$) 19.5 (C(C\(\text{A}\)H\(^3\)C\(^\text{B}\)H\(^3\))), 23.2 (C\(\gamma\)H\(^2\) or C\(\delta\)H\(^2\)), 27.6 (C\(\gamma\)H\(^2\) or C\(\delta\)H\(^2\)), 28.7 (C(C\(^\text{A}\)H\(^3\)C\(^\text{B}\)H\(^3\))), 29.1 (C\(\beta\)H\(^2\) or CH\(_2\)CON), 29.2 (C\(\beta\)H\(^2\) or CH\(_2\)CON), 35.5 (C(CH\(_3\))\(_2\)), 40.5 (C\(\text{H}\)_2), 44.4 (CH\(_2\)CON), 52.7 (CH\(_2\)C(CH\(_3\))\(_2\)), 52.9 (CO\(_2\)CH\(_3\) ),

54.0 (C\(\alpha\)H), 66.6 (PhCH\(_2\)), 102.0 (C=CHCO), [128.1 (CH), 128.5 (CH), 5C, Ar-H], 136.5 (Ph), 156.3 (NHC\(_\text{O}\)), 162.7 (C=CHCO) 168.7 (CON or CO\(_2\)Me), 174.9 (CON or CO\(_2\)Me), 196.6 (C=CHCO); MS (+ve) found $m/z$ 457 (MH$^+$, 81%), 479 (MNa$^+$, 10); HRMS FAB (+ve) found $m/z$ 457.23382 (MH$^+$), C\(\text{C}\)_\(_3\)_\(_3\)N\(_2\)O\(_6\) requires 457.23386.

5.2.27.5  (S)-6-Benzoylcarbonylamino-2-[2-((S)-4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-hexanoic acid methyl ester 151b

(S)-4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 150b (0.50 g, 2.36 mmol) was coupled to N-\(\varepsilon\)-CBz-L-lysine methyl ester hydrochloride 120c (0.78 g, 2.36 mmol) using the general method (5.2.18) to give an orange oil (1.84 g) after work-up. This was purified by flash column chromatography on silica gel using hexane/EtOAc (1:1) as eluent to give a yellow oil (0.90 g, 78%): $\nu_{max}$ (CHCl$_3$) 3328.5 (NH), 1735.6 (ester), 1711.5 (carbamate), 1655.6 ($\alpha$/$\beta$-unsaturated ketone), 1614.1 (C=C); $\delta_H$ (250 MHz, CDCl$_3$) 0.82 (3H, s, C(C\(^\text{A}\)H\(^3\)C\(^\text{B}\)H\(^3\))), 1.09 (3H, s, C(C\(^\text{A}\)H\(^3\)C\(^\text{B}\)H\(^3\))), 1.44 (4H, m, C\(\gamma\)H\(^2\) + C\(\delta\)H\(^2\)), 1.77 (2H, m, C\(\beta\)H\(^2\)), 2.07 (1H, d, J 17.4,
5.2.27.6 (S)-6-Benzylxycarbonylamino-2-((S)-4,4-dimethyl-2,6-dioxo-2,3,3a,4,5,6-hexahydro-indol-1-yl)-hexanoic acid methyl ester 153b

(S)-6-Benzylxycarbonylamino-2-[2-((S)-4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-hexanoic acid methyl ester 151b (0.38 g, 0.78 mmol) was dissolved in acetone (8 ml) and 2M HCl (8 ml) slowly added. The mixture was then stirred at room temperature overnight. The solvent was removed in vacuo and brine (5 ml) added. The mixture was extracted with EtOAc (3 x 10 ml), and the total organic phase washed with brine (8 ml), dried (MgSO4) and rotary evaporated to an orange oil (0.38 g). This was purified by flash column chromatography on silica gel using hexane/EtOAc (2:3) as eluent to give a colourless oil (0.036 g, 10 %): νmax 1743.3 (ester), 1719.2 (carbamate), 1619.0 (C=C); δH (360 MHz, CDCl3) 0.91 (3H, s,
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\[ C(C^A H_3 C^B H_3) \], 1.09 (3H, s, C\((C^A H_3 C^B H_3)\)), 1.22 (2H, m, C\(_7\)H\(_2\) or C\(_8\)H\(_2\)), 1.51 (2H, m, C\(_7\)H\(_2\) or C\(_8\)H\(_2\)), 1.95 (1H, m, C\(_8\)H\(^A\)H\(^B\)), 2.11 (1H, m, C\(_8\)H\(^A\)H\(^B\)), 2.34 (2H, s, CH\(_2\)C(CH\(_3\))\(_2\)), 2.44 (1H, dd, \(J 17.8, 8.6\), CH\(^A\)H\(^B\)CON), 2.65 (1H, dd, \(J 17.8, 9.3\), CH\(^A\)H\(^B\)CON), 3.12 (3H, m, C\(_7\)H\(_2\) + CHCH\(_2\)CON), 3.69 (3H, s, OCH\(_3\)), 4.86 (2H, m, C\(_9\)H + NH), 5.07 (2H, s, CH\(_2\)Ph), 5.31 (1H, d, \(J 2.0\), C=CHCON), 7.33 (5H, m, Ar-H); \(\delta\)\(_C\) (90 MHz, DEPT, CDCl\(_3\)) 19.5 (C(C\(_7\)H\(_3\)C\(_8\)H\(_3\))), 23.3 (C\(_7\)H\(_2\) or C\(_8\)H\(_2\)), 26.3 (C\(_7\)H\(_2\) or C\(_8\)H\(_2\)), 28.7 (C(C\(_7\)H\(_3\)C\(_8\)H\(_3\))), 29.2 (C\(_8\)H\(_2\) or CH\(_2\)CON), 29.3 (C\(_8\)H\(_2\) or CH\(_2\)CON), 35.6 (C(CH\(_3\))\(_2\)), 40.5 (C\(_9\)H\(_2\)), 44.3 (CHCH\(_2\)CON), 52.6 (CH\(_2\)C(CH\(_3\))\(_2\)), 52.8 (CO\(_2\)CH\(_3\)), 53.2 (C\(_9\)H), 66.6 (PhCH\(_2\)), 102.6 (C=CHCO), [128.1 (CH), 128.5 (CH), 5C, Ar-H], 136.4 (Ph), 156.4 (NHCOO), 162.0 (C=CHCO) 168.7 (CON or CO\(_2\)Me), 175.0 (CON or CO\(_2\)Me), 196.8 (C=CHCO); MS ES (+ve) found \(m/z\) 457 (MH\(^+\), 100 %), 479 (MNa\(^+\), 12); HRMS FAB (+ve) found \(m/z\) 457.23388 (MH\(^+\)), C\(_{25}\)H\(_{33}\)N\(_2\)O\(_6\) requires 457.23386.

5.2.28 Synthesis of Fluorescently-Tagged Ligands

5.2.28.1 \((S)-2\text{-Amino-6-}(5\text{-dimethylamino-naphthalene-1-sulfonylamino})\text{-hexanoic acid methyl ester 166}

\[
\begin{align*}
\text{Me}_2\text{N-} & \quad \text{SO} \quad \text{N} \quad \text{NH}_2 \\
\text{H} & \quad \text{CO}_2\text{Me}
\end{align*}
\]

Commercially available \((S)-2\text{-amino-6-}(5\text{-dimethylamino-naphthalene-1-sulfonylamino})\text{-hexanoic acid 165}\) (1.00 g, 2.64 mmol) was suspended in anhydrous MeOH (15 ml) and the mixture cooled to 0 °C. Thionyl chloride (1.63 ml, 22.4 mmol) was then slowly added and the mixture stirred at room temperature overnight. The solvent was removed \textit{in vacuo} to give a foamy brown solid (1.20 g); \(\nu_{\text{max}}\) (KBr) 3433.6 (NH\(_2\)), 1746.2 (ester), 1320.0 (SO\(_2\)), 1141.7 (SO\(_2\)); \(\delta\)\(_H\) (360 MHz, CD\(_3\)OD) 1.49 (4H, m, C\(_7\)H\(_2\) + C\(_8\)H\(_2\)), 1.87 (2H, m, C\(_9\)H\(_2\)), 2.93 (2H, m, C\(_8\)H\(_2\)), 3.53 (6H, s, N(CH\(_3\))\(_2\)), 3.87 (3H, s, OCH\(_3\)), 4.03 (1H, m, C\(_9\)H), 7.93 (2H, m, Ar-H), 8.15 (1H, d, \(J 7.6, \text{Ar-H}\)), 8.42 (1H, d, \(J 7.2, \text{Ar-H}\)), 8.66 (1H, d, \(J 8.6, \text{Ar-H}\)), 8.96 (1H, d, \(J 8.7, \text{Ar-H}\))
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Experimental

Ar-H); $\delta$ C (90 MHz, DEPT, CD$_3$OD) 21.0 (CH$_3$), 28.3 (CH$_2$), 29.0 (CH$_2$), 41.5 (CH$_2$), 46.1 (N(CH$_3$)$_2$), 51.9 (C$_a$H + OCH$_3$), [118.9 (CH), 125.1 (CH), 2C Ar-H], 125.3 (quat), [126.1 (CH), 126.6 (CH), 126.9 (CH), 3C Ar-H], 128.7 (quat), [129.2 (CH), 1C Ar-H], 136.8 (quat), 138.7 (quat), 168.9 (CO$_2$CH$_3$); MS FAB (+ve) found $m/z$ 394 (MH$^+$, 44%); HRMS FAB (+ve) found $m/z$ 394.18083 (MH$^+$), C$_{19}$H$_{28}$N$_3$O$_4$S requires 394.18005.

5.2.28.2 (S)-6-(5-Dimethylamino-naphthalene-1-sulfonylamino)-2-[2-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-hexanoic acid methyl ester 167

4-Methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetic acid 109 (0.54 g, 2.54 mmol) was coupled to (S)-2-amino-6-(5-dimethylamino-naphthalene-1-sulfonylamino)-hexanoic acid methyl ester 166 (1.00 g, 2.54 mmol) using the general method (5.2.18) to give a brown oil (2.00 g) after work-up. This was purified by flash column chromatography on silica gel using hexane/EtOAc gradient eluent to give the individual diastereomers as two fluorescent foams: (i) (0.51 g, 34%) and (ii) (0.52 g, 35%).

i) $\nu$$_{max}$ (CHCl$_3$) 1738.5 (ester), 1639.2 ($\alpha_\beta$-unsaturated ketone), 1612.2 (C=C), 1319.1 (SO$_2$), 1143.6 (SO$_2$); $\delta$ H (250 MHz, CDCl$_3$) 0.89 (3H, s, C($^a$H$_3$C$^b$H$_3$)), 1.13 (3H, s, C($^a$H$_3$C$^b$H$_3$)), 1.33 (4H, m, C$_a$H$_2$ + C$_b$H$_2$), 1.60 (2H, m, C$_b$H$_2$), 2.13 (1H, d, J 17.4, C$^a$H$^b$C(CH$_3$)$_2$), 2.33 (2H, m, CH$_2$CONH), 2.55 (1H, d, J 17.4, C$^a$H$^b$C(CH$_3$)$_2$), 2.85 (6H, s, N(CH$_3$)$_2$), 2.90 (1H, m, C$\text{HCH}_2$CONH), 2.93 (2H, m, C$_a$H$_2$), 3.59 (3H, s, OCH$_3$), 3.67 (3H, s, CO$_2$CH$_3$), 4.60 (1H, m, C$_a$H), 5.67 (1H, d, J 1.3, COCH$\text{H}=\text{COCH}_3$), 6.45 (1H, d, J 8.5, NH$^+$), 6.82 (1H, t, J 5.8, NH$^+$), 7.14 (1H, d, J 7.0, Ar-H), 7.44 (1H, t, J 7.3, Ar-H), 7.48 (1H, t, J 7.3, Ar-H), 8.16 (1H, dd, J 7.3, 1.2, Ar-H), 8.33 (1H, d, J 8.7, Ar-H), 8.48 (1H, d, J 8.5, Ar-H); $\delta$ C (63 MHz, DEPT,
CDC13) 21.1 (C(C\textsubscript{A}H\textsubscript{3}C\textsubscript{B}H\textsubscript{3})), 21.3 (C\textsubscript{g}H\textsubscript{2} or C\textsubscript{b}H\textsubscript{2}), 28.3 (C\textsubscript{g}H\textsubscript{2} or C\textsubscript{b}H\textsubscript{2}), 28.8 (C(C\textsubscript{A}H\textsubscript{3}C\textsubscript{B}H\textsubscript{3})), 31.4 (C\textsubscript{p}H\textsubscript{2}), 32.1 (CH\textsubscript{2}CONH), 35.8 (C(CH\textsubscript{3})\textsubscript{2}), 42.1 (C\textsubscript{g}H\textsubscript{2}), 44.2 (CH\textsubscript{2}C(CH\textsubscript{3})\textsubscript{2}), 45.3 (N(CH\textsubscript{3})\textsubscript{2}), 51.5 (C\textsubscript{a}H), 52.2 (CO\textsubscript{2}CH\textsubscript{3}), 53.3 (CHCH\textsubscript{2}CONH), 55.9 (OCH\textsubscript{3}), 100.8 (COCH=COCH\textsubscript{3}), [115.0 (CH), 119.3 (CH), 123.1 (CH), 127.7 (CH), 128.9 (CH), 5C Ar-H], 129.6 (quat x 2), [129.7 (CH), 1C Ar-H], 135.6 (quat), 151.5 (quat), 172.6 (CONH or CO\textsubscript{2}Me), 172.8 (CONH or CO\textsubscript{2}Me), 176.7 (COCH=COCH\textsubscript{3}), 201.2 (COCH=COCH\textsubscript{3}); MS FAB (+ve) found m/z 588 (MH\textsuperscript{+}, 26 \%), 610 (MNa\textsuperscript{+}, 4); HRMS FAB (+ve) found m/z 588.27412 (MH\textsuperscript{+}), C\textsubscript{30}H\textsubscript{42}N\textsubscript{3}O\textsubscript{7}S requires 588.27435.

ii) \( \nu \text{max} \) (CHCl\textsubscript{3}) 3298.6 (NH), 1741.4 (ester), 1655.6 (\( \alpha,\beta \)-unsaturated ketone), 1613.2 (C=\text{-C}), 1317.1 (SO\textsubscript{2}), 1144.6 (SO\textsubscript{2}); \( \delta\text{H} \) (250 MHz, CDC\textsubscript{13}) 0.84 (3H, s, C(C\textsubscript{A}H\textsubscript{3}C\textsubscript{B}H\textsubscript{3})), 1.11 (3H, s, C(C\textsubscript{A}H\textsubscript{3}C\textsubscript{B}H\textsubscript{3})), 1.36 (4H, m, C\textsubscript{g}H\textsubscript{2} + C\textsubscript{b}H\textsubscript{2}), 1.60 (2H, m, C\textsubscript{p}H\textsubscript{2}), 2.13 (1H, d, \( J \) 17.5, CH\textsuperscript{A}H\textsuperscript{B}C(CH\textsubscript{3})\textsubscript{2}), 2.24 (1H, dd, \( J \) 14.0, 2.3, CH\textsuperscript{A}H\textsuperscript{B}CONH), 2.50 (1H, d, \( J \) 17.5, CH\textsuperscript{A}H\textsuperscript{B}C(CH\textsubscript{3})\textsubscript{2}), 2.61 (1H, dd, \( J \) 14.0, 8.9, CH\textsuperscript{A}H\textsuperscript{B}CONH), 2.70 (1H, dd, \( J \) 8.9, 2.3, CHCH\textsubscript{2}CONH), 2.85 (8H, brs, N(CH\textsubscript{3})\textsubscript{2} + C\textsubscript{g}H\textsubscript{2}), 3.64 (6H, s, OCH\textsubscript{3} + CO\textsubscript{2}CH\textsubscript{3}), 4.39 (1H, m, C\textsubscript{a}H), 5.45 (1H, d, \( J \) 1.3, COCH=COCH\textsubscript{3}), 5.73 (1H, t, \( J \) 6.0, NH), 7.05 (1H, d, \( J \) 7.7, NH), 7.14 (1H, d, \( J \) 7.1, Ar-H), 7.48 (2H, m, Ar-H), 8.19 (1H, dd, \( J \) 7.3, 1.2, Ar-H), 8.30 (1H, d, \( J \) 8.7, Ar-H), 8.49 (1H, d, \( J \) 8.5, Ar-H); \( \delta\text{C} \) (63 MHz, DEPT, CDC\textsubscript{13}) 21.2 (C(C\textsubscript{A}H\textsubscript{3}C\textsubscript{B}H\textsubscript{3})), 21.9 (C\textsubscript{g}H\textsubscript{2} or C\textsubscript{b}H\textsubscript{2}), 28.7 (C(C\textsubscript{A}H\textsubscript{3}C\textsubscript{B}H\textsubscript{3} + C\textsubscript{g}H\textsubscript{2} or C\textsubscript{b}H\textsubscript{2}), 31.2 (C\textsubscript{p}H\textsubscript{2}), 32.8 (CH\textsubscript{2}CONH), 36.0 (C(CH\textsubscript{3})\textsubscript{2}), 42.5 (C\textsubscript{c}H\textsubscript{2}), 43.9 (CH\textsubscript{2}C(CH\textsubscript{3})\textsubscript{2}), 45.3 (N(CH\textsubscript{3})\textsubscript{2}), 51.8 (C\textsubscript{a}H), 52.2 (CO\textsubscript{2}CH\textsubscript{3}), 53.1 (CHCH\textsubscript{2}CONH), 55.8 (OCH\textsubscript{3}), 100.6 (COCH=COCH\textsubscript{3}), [115.0 (CH), 118.9 (CH), 123.0 (CH), 128.0 (CH), 129.2 (CH), 5C Ar-H], 129.5 (quat), 129.7 (quat), [130.0 (CH), 1C Ar-H], 134.9 (quat), 151.7 (quat), 172.6 (CONH or CO\textsubscript{2}Me), 173.0 (CONH or CO\textsubscript{2}Me), 176.3 (COCH=COCH\textsubscript{3}), 200.7 (COCH=COCH\textsubscript{3}); MS FAB (+ve) found m/z 588 (MH\textsuperscript{+}, 18 \%), 610 (MNa\textsuperscript{+}, 4); HRMS FAB (+ve) found m/z 588.27438 (MH\textsuperscript{+}), C\textsubscript{30}H\textsubscript{42}N\textsubscript{3}O\textsubscript{7}S requires 588.27435.
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5.2.28.3  (S)-6-(5-Dimethylamino-naphthalene-1-sulfonylamino)-2-((S/R)-4,4-dimethyl-2,6-dioxo-2,3,3a,4,5,6-hexahydro-indol-1-yl)-4-methyl-hexanoic acid methyl ester 169

![Chemical Structure](image)

(S)-6-(5-Dimethylamino-naphthalene-1-sulfonylamino)-2-[2-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-hexanoic acid methyl ester 167a (0.40 g, 0.68 mmol) was dissolved in acetone (15 ml) and 2M HCl (15 ml) slowly added. The mixture was then stirred at room temperature overnight. The acetone was removed in vacuo and the residue diluted with brine (10 ml) and extracted with EtOAc (3 x 20 ml). The total organic phase was dried (MgSO₄) and rotary evaporated to give an orange oil (0.13 g). This was purified by flash column chromatography on silica gel using hexane/EtOAc (1:1) as eluent to give a bright yellow foam (0.015 g, 4 %): δH (360 MHz, CDCl₃) 0.92 (3H, s, C(CH₃)₃), 1.11 (3H, s, C(CH₃)₃C₆H₃), 1.22 (2H, m, C₇H₂ or C₈H₂), 1.42 (2H, m, C₇H₂ or C₈H₂), 1.92 (2H, m, C₈H₂), 2.36 (2H, s, CH₂C(CH₃)₂), 2.46 (1H, dd, J 17.7, 8.5, CH₄H₂CON), 2.71 (1H, dd, J 17.7, 9.2, CH₄H₂CON), 2.84 (1H, m, C₈H₂), 2.91 (6H, s, N(CH₃)₂), 3.15 (1H, ddd, J 9.2, 8.5, 2.2, CH₂CH₂CON), 3.69 (3H, s, CO₂CH₃), 4.79 (1H, m, C₆H₅), 4.88 (1H, t, J 6.2, NH), 5.30 (1H, d, J 2.2, C=CHCO), 7.21 (1H, d, J 7.5, Ar-H), 7.55 (2H, m, Ar-H), 8.22 (1H, dd, J 7.3, 1.3, Ar-H), 8.27 (1H, d, J 8.6, Ar-H), 8.56 (1H, d, J 8.6, Ar-H); δC (90 MHz, DEPT, CDCl₃) 19.6 (C(CH₃)₂), 22.8 (CH₂), 25.9 (CH₂), 28.5 (CH₂), 28.7 (C(CH₃)₂), 29.4 (CH₂), 35.6 (C(CH₃)₂), 42.7 (C₆H₅), 44.3 (CH₂CH₂CON), 45.4 (N(CH₃)₂), 52.6 (CH₂C(CH₃)₂), 52.8 (CO₂CH₃), 53.0 (C₆H₅), 102.6 (C=CHCO), [115.3 (CH), 118.7 (CH), 123.3 (CH), 128.4 (CH), 134.5 (quat x 2), [129.7 (CH), 130.4 (CH), 2C Ar-H], 134.5 (quat), 151.7 (quat), 162.2 (C=CHCO), 168.7 (CON or CO₂Me), 175.2 (CON or CO₂Me), 197.1 (C=CHCO); MS FAB (+ve) found m/z 556 (MH⁺, 100 %), 578
(MNa⁺, 26); HRMS FAB (+ve) found m/z 556.24859 (MH⁺), C₂₉H₃₈N₃O₆S requires 556.24813.

(S)-6-(5-Dimethylamino-naphthalene-1-sulfonylamino)-2-[2-(4-methoxy-6,6-dimethyl-2-oxo-cyclohex-3-enyl)-acetylamino]-hexanoic acid methyl ester 167b (0.40 g, 0.68 mmol) was dissolved in THF (15 ml) and 2M HCl (15 ml) slowly added. The mixture was then stirred at room temperature overnight. The THF was removed in vacuo and the residue diluted with brine (10 ml) and extracted with EtOAc (3 x 25 ml). The total organic phase was washed with saturated NaHCO₃ solution (10 ml), brine (10 ml), dried (MgSO₄) and rotary evaporated to give a yellow foamy solid (0.093 g). This was purified by flash column chromatography on silica gel using hexane/EtOAc (1:1) as eluent to give a bright yellow oil (0.033 g, 9 %): δ_H (360 MHz, CDCl₃) 0.93 (3H, s, C(C₃H₃C₆H₃)), 1.10 (3H, s, C(C₆H₃C₆H₃)), 1.19 (2H, m, C₆H₂ or C₈H₂), 1.41 (2H, m, C₆H₂ or C₈H₂), 1.95 (2H, m, C₆H₂), 2.35 (2H, s, C₆H₂(CH₃)₂), 2.44 (1H, dd, J 17.7, 8.6, CH₆H₂CON), 2.64 (1H, dd, J 17.7, 9.2, CH₆H₂CON), 2.83 (1H, m, C₆H₂), 2.89 (6H, s, N(CH₃)₂), 3.16 (1H, ddd, J 9.2, 8.6, 2.1, CH₂CON), 3.70 (3H, s, CO₂CH₃), 4.58 (1H, m, C₆H), 4.91 (1H, t, J 6.2, NH), 5.35 (1H, d, J 2.1, C=CHCO), 7.19 (1H, d, J 7.1, Ar-H), 7.54 (2H, m, Ar-H), 8.21 (1H, dd, J 7.3, 1.3, Ar-H), 8.26 (1H, d, J 8.6, Ar-H), 8.54 (1H, d, J 8.6, Ar-H); δ_C (90 MHz, DEPT, CDCl₃) 19.6 (C(C₆H₃C₆H₃)), 22.7 (CH₂), 27.2 (CH₂), 28.6 (CH₂), 28.7 (C(C₆H₃C₆H₃)), 29.2 (CH₂), 35.5 (C(CH₃)₂), 42.7 (C₆H), 44.4 (CH₂CON), 45.4 (N(CH₃)₂), 52.7 (CH₂C(CH₃)₂), 52.9 (CO₂CH₃), 53.8 (C₆H), 101.9 (C=CHCO), [115.2 (CH), 118.6 (CH), 123.2 (CH), 128.4 (CH), 4C Ar-H], 129.5 (quat), 129.6 (quat), [129.8 (CH), 130.4 (CH), 2C Ar-H], 134.5 (quat), 151.9 (quat), 162.9 (C=CHCO), 168.7 (CON or CO₂Me), 175.3 (CON or CO₂Me), 196.8 (C=CHCO); MS FAB (+ve) found m/z 556 (MH⁺, 45 %), 578 (MNa⁺, 23); HRMS FAB (+ve) found m/z 556.24782 (MH⁺), C₂₉H₃₈N₃O₆S requires 556.24813.
6 References

## Appendix A

Bond lengths [Å] and angles [deg] for 149a.

![Chemical Structure](image)

<table>
<thead>
<tr>
<th>Bond</th>
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### Chapter 7

#### Appendices

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Appendix B

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Appendix C

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