SYNTHESIS OF NODULARIN ANALOGUES AS POTENTIAL PROTEIN PHOSPHATASES INHIBITORS

a thesis presented by
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to the UNIVERSITY OF ST ANDREWS in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY

St Andrews March 1998
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Date 2 4 th March . Signature of Supervisor ...............................
TO MY FAMILY

PAST, PRESENT AND

FUTURE ...
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ABSTRACT

Reversible phosphorylation of proteins on serine, threonine and tyrosine residues, is now widely accepted to be the principal mechanism for the control of intracellular events in eukaryotic and prokaryotic cells. The nodularins are known to be potent inhibitors of serine/threonine protein phosphatases, PP1c and PP2A, with sub-nanomolar inhibition constants. They have been shown to form covalent adducts with the enzymes and are known to be potent hepatotoxins and liver promoters.

Nodularin has the general structure: cyclo (((R)-erythro-β-methyl-Iso-Asp-(S)-X-Adda-(R)-iso-Glu-N-methyldehydrobutyric acid)), where (S)-X is a variable S-amino acid and Adda is the unique β-amino acid, (25,3S,85,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenylnonao-4,6-dienoic acid.

In order to investigate the mode of inhibition and also to probe the active-site binding interactions, we decided to synthesis new analogues of nodularin. Specific inhibitors for either PP1 or PP2A are not presently available, but would be useful biochemical tools in delineating the individual physiological roles of these enzymes.

We decided to synthesise, the potential inhibitor cyclo [βAla-(2R)-Glu-α-OMe-γ-Pro-(2R)-Asp-α-OMe-β-(2S)-Phe], a stripped-down nodularin macrocycle, and also an analogue which is suitable for synthetic elaboration at the "Adda position".

Using solution phase peptide synthesis (LPPS), four such nodularin analogues (both (25)- and (2R)-proline) were synthesised in seventeen steps. The cyclisation between the Phe and Asp residues were carried out using DIPEA under conditions of high dilution. NMR studies (TOCSY, ROESY) have elucidated the three dimensional structures which have been shown to be similar to the natural product, nodularin.

A shorter synthesis of these nodularin analogues was developed using solid phase peptide synthesis (SPPS). Two solid phase synthesis of the nodularin macrocycles, cyclo-[βAla-(2R)-Glu-α-OMe-γ-Pro-(2R)-Asp-β-(2S)-Phe]; one in which Fmoc-(2S)-Phe-βAla-(2R)-Glu-α-OMe-γ-Pro-(2R)-Asp(α-O-Wang Resin)-β-OAllyl is deprotected and then cyclised on the resin prior to cyclisation were found to be successful. Even though the resin-bound synthesis gave low yields for the cyclisation step, compared to the situation in solution, it offered advantages in the construction of the linear isopentapeptide precursor.

Initial studies have shown that the nodularin analogues 130 and 131 are moderate inhibitors (IC50 2.8 mmol) of PP1 when tested using the malachite green system.

Studies towards the synthesis of incorporating more suitable Adda functionalities, and the development of a radiolabelled protein phosphatase assay are currently being investigated within the group.
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<td>(2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-2((E)), 6((E))-dienoic acid</td>
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<td>adenosine 5'-triphosphate</td>
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<td>tertiary-butoxycarbonyl</td>
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<td>BSA</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>EDCI</td>
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<td>ethylenediaminetetra acetic acid</td>
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<td>isopropyl-β-D-thiogalactopyranoside</td>
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<td>IC₅₀</td>
<td>concentration required to reduce enzyme activity by 50%</td>
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<td>L-amp</td>
<td>L-ampicillin</td>
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<td>Masp</td>
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<td>N-Mdha</td>
<td>N-methyldehydroalanine</td>
</tr>
<tr>
<td>N-Mdhb</td>
<td>N-methyl dehydrobutyric acid</td>
</tr>
<tr>
<td>NMM</td>
<td>4-methylmorpholine</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>NOESY</td>
<td>nuclear Overhauser enhancement</td>
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<td>PFP</td>
<td>pentafluorophenyl</td>
</tr>
<tr>
<td>P₁</td>
<td>inorganic phosphate</td>
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<td>PKA</td>
<td>protein kinase A</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PKase</td>
<td>protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-nitrophenyl phosphate</td>
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<tr>
<td>PP1c</td>
<td>catalytic subunit of PP1</td>
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<td>PPIG</td>
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<td>PP1S</td>
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<td>protein phosphatase</td>
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<tr>
<td>PPx</td>
<td>protein phosphatase x</td>
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<tr>
<td>PyBOP</td>
<td>benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>SPPS</td>
<td>solid phase peptide synthesis</td>
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<tr>
<td>SV40</td>
<td>simian virus 40</td>
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<tr>
<td>TBTU</td>
<td>2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate</td>
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<tr>
<td>TES</td>
<td>triethyl silane</td>
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<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<td>TOCSY</td>
<td>phase sensitive 2-D total correlation spectroscopy</td>
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<td>Wang</td>
<td>p-benzyloxybenzyl alcohol</td>
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# Abbreviations for Amino Acids

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<th>Three-Letter Abbreviation</th>
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</tr>
<tr>
<td>Asparagine</td>
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<td>N</td>
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<tr>
<td>Aspartic Acid</td>
<td>Asp</td>
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<td>Cys</td>
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<td>Valine</td>
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1. Introduction

1.1 General Introduction

There are many hundreds of cell proteins containing phosphoserine and phosphothreonine residues. Their dephosphorylation is regulated by only four major classes of serine/threonine protein phosphatases - PP1, PP2A, PP2B and PP2C. The catalytic subunits of which show broad and overlapping substrate specificity in vitro.

Studies with inhibitors, such as okadaic acid and microcystin, have shown most of the measurable serine/threonine protein phosphatase activity in cell extracts is likely to be accounted for by PP1 and PP2A. Earlier research showed that these two phosphatases have significant regulatory roles in most of the major metabolic pathways: for example, glycolysis, glycogen synthesis, fatty acid synthesis, cholesterol biosynthesis, protein synthesis and lipolysis. More recent studies with phenotypes of genetic mutants in which these enzymes have been deleted have implicated PP1 and PP2A as essential in the regulation of processes as diverse as the transport of ions and nutrients into cells, gene transcription and the cell cycle. The involvement of PP1 and PP2A are expressed at micromolar concentrations in cells and share a 49% sequence homology with their catalytic core.

1.2 Phosphatases

Hundreds of metabolic pathways are controlled by a simple chemical event - the transfer of phosphoryl groups from one entity to another. In nature, these reactions are catalysed by two sets of enzymes - phosphatases and kinases. Phosphatases catalyse the hydrolysis (cleavage) reaction, whilst kinases operate in the synthetic direction (phosphorylation). This thesis reviews and compares, current knowledge about phosphatases, concentrating on protein phosphorylation in particular.
1.2.1 Categories of Phosphatases

Phosphatases can be classified into two major groups due to their substrate specificity and mechanism of action.\(^2\)

1.2.1.1 Substrate Specificity

There are a large variety of substrates for phosphatases ranging from small phosphorylated metabolites (such as glucose-6-phosphate), to secondary messengers (such as phosphoinositols), up to very large phosphorylated proteins.\(^6\)

There are three major groups of phosphatases:

- **Non-Specific** - these enzymes catalyse the hydrolysis of almost any phosphate;

- **Phosphoprotein Specific** - enzymes which use phosphoproteins or phosphopeptides as preferred substrates (protein phosphatases); and

- **Small-molecule Specific** - enzymes which hydrolyse one (or a group of structurally similar) substrate(s), for example, inositol monophosphatase (see Fig. 1.1).\(^5\)
1.2.1.2 Mechanism of Reaction

The secondary classification scheme is one according to the enzyme’s mechanism of reaction.

For example, some phosphatases use an active site nucleophile as the initial phosphoryl group acceptor “Ping-Pong”; others transfer it directly to a water molecule (see Scheme 1.1).^6

Scheme 1.1: Typical Phosphatase Reaction Mechanisms.

(a) Direct transfer to water, normally catalysed by active-site metal ions.

(b) Hydrolysis via a phosphoenzyme intermediate. Serine, cysteine and histidine are common nucleophiles used as the initial phosphoryl group acceptor.
This can be further sub-divided according to the phosphoryl group acceptor (cysteine, histidine or serine); and characteristically use a two-metal-ion dyad to bind phosphates and catalyse their subsequent hydrolysis, for example inositol monophosphatase.

### 1.3 Protein Phosphatases

Just over forty years ago it was first recognised that the enzymic phosphorylation and dephosphorylation of proteins (sometimes referred to as reversible protein phosphorylation) was a very dynamic process involved in the regulation of cellular functions. Prior to this, these proteins were primarily considered as proteins which had nutritional functions, such as providing a source of phosphorous for growing organisms. There was no understanding of how rapid turnover of protein-bound phosphate might occur, nor a knowledge of the mechanisms involved in the formation of phosphoproteins.

Reversible protein phosphorylation is now agreed to be the major mechanism for the control of a variety of biological processes, including metabolism, cell differentiation and proliferation, gene expression, transport, locomotion, development and memory. The activities of proteins controlled by phosphorylation are also varied and include enzymes, membrane receptors, transport proteins, ion pumps and proteins mediating DNA replication, transcription and translation.

During protein phosphorylation, phosphate moieties are transferred from ATP molecules by protein kinases, and are hydrolysed by protein phosphatase (see Fig. 1.2). In eukaryotic cells, reversible phosphorylation occurs predominantly on serine, threonine and tyrosine residues. Changes in the phosphorylation state of proteins are responsible for protein conformation which in turn cause functional changes. This process, together with its reversibility and scope for signal amplification, probably accounts for the importance of protein phosphorylation in signal transduction.
Protein phosphatases (PPases) are structurally and functionally diverse enzymes which can be represented by three distinct families:

- **Ser/Thr Phosphatases**,\(^{12,13}\) (which are also probably all two-metal-ion phosphatases);
- **Tyr phosphatases** (PTPases), and
- **Dual-Specificity Phosphatases**.

The Ser/Thr phosphatases (phosphatases which dephosphorylate phosphoserine and phosphothreonine residues in proteins) are discussed in more detail below.

### 1.4 Ser/Thr Protein Phosphatases

#### 1.4.1 General Classification

To date, over 200 enzymes can be counted in the Ser/Thr protein phosphatase class. Since 1983 the most widely adopted classification for Ser/Thr phosphatases, is that proposed by Ingebritsen and Cohen.\(^ {14,15}\) Because of their overlapping specificities, PPases cannot be classified by substrate alone, therefore, the action of specific inhibitors and activators is employed. Using these criteria, the
serine/threonine phosphatases in the cytosol of eukaryotic cells are divided into
two classes:

- **Type 1 (PPI)** - those inhibited by small thermostable acid-resistant proteins
termed inhibitor 1 (I-1), inhibitor 2 (I-2 or modulator) and DARPP-32, and
  which preferentially dephosphorylate the β-subunit of phosphorylase kinase;
  and

- **Type 2 (PP2)** inhibitor which are insensitive to inhibitors 1 and 2, and
  preferentially dephosphorylate the α-subunit of phosphorylase kinase.

Type 2 protein phosphatases are further sub-divided according to their requirement
for divalent cations: PP2A, like PP1, does not require cations, while PP2B (also
known as calcineurin) and PP2C show an absolute dependence for Ca²⁺ and Mg²⁺
respectively. The use of okadaic acid 1 (see p. 16), a specific phosphatase
inhibitor, further facilitates the discrimination between these classes.

Recent studies have shown Ser/Thr phosphatases PPI, PP2A and PP2C possess *in vitro*
phosphohistidine activity. Because phosphohistidine is unstable to heating
at low pH, a treatment which is used to analyse other phospho-
aminos acids, its
presence in many cellular proteins may have been missed in characterisation.

1.4.2 Gene Families

Although widely accepted, the protein phosphatase classification is flawed since it
does not reflect the actual phylogenetic relationship between the different Ser/Thr
protein phosphatases. Molecular cloning revealed PP2A was, in fact, much more
closely related to PP1 than PP2C. Moreover, in the past few years many novel
PPases have been identified which do not fit into the above classifications. Many
of these PPases are closely related to the existing classes, or are intermediates
between classes.
Recombinant DNA techniques have elucidated the primary structures of the phosphatases. The deduced protein sequences show there are two structurally distinct gene families; a "major" family which includes PP1, PP2A and PP2B, and a "smaller" family with a completely distinct primary structure which includes PP2C. The symbol PPP was chosen to designate the "major" family, and PPM (Mg\(^{2+}\) dependent protein phosphatases) for the "smaller" family.

PP1 and PP2A are similar in size and show 41% overall amino acid sequence identity. The extreme N and C termini are the most divergent regions, the identity rises to 49% if these regions are excluded. PP2B shows 39% identity to both PP1 and PP2A in the catalytic domain, with two small insertions in this region and a long C-terminal extension. The C-terminal extension possesses the calmodulin binding domain and regions which bind the regulatory subunit and suppress activity in the absence of Ca\(^{2+}\) and calmodulin (see Fig. 1.3). Within each family, the catalytic domains are highly conserved, suggesting similarities in tertiary structure and catalytic mechanisms. However, the most considerable structural and functional diversity of individual PPases is created as a result of a combination of associated regulatory domains and subunits.
1.4.3 Targetting Subunit Hypothesis

Many protein phosphatases (and kinases) have multiple substrates in vivo, which enables a diversity of responses to physiological stimuli. However, their broad specificities, including in vitro activity towards non-physiological protein substrates, suggests the need for mechanisms to restrict these enzymes in vivo. Protein phosphatases (and kinases) are regulated by regulatory (or “targetting”) subunits. This novel class of proteins, not only acts to restrict the location of the phosphatase (and kinase), but also modifies their catalytic and regulatory properties.

The targetting subunit (T_{sub}) is the part of a protein phosphatase (or kinase) which directs the catalytic subunit (C_{sub}) to the target locus (T_{loc}). The T_{sub} can be an organelle, a membrane, or a component in the soluble fraction of the cell. The T_{sub}
may function to position the $C_{ub}$ close to a particular substrate, or to sequestrate it from other substrates and ligands (e.g. inhibitors). The interaction between a $T_{ub}$ and a $C_{ub}$ may also alter the catalytic activity or specificity of the $C_{ub}$ for nearby substrates. Furthermore, if the binding equilibrium between $T_{ub}$ and either $T_{loc}$ or $C_{ub}$ is regulated, the altered properties of the $C_{ub}$ can be turned on and off reversibly.\textsuperscript{23,24}

1.4.4 Evolution of Protein Phosphatases

Molecular cloning of phosphatase subunits from different species indicates Ser/Thr protein phosphatases are among the most highly conserved proteins in evolution.\textsuperscript{25} This implies that protein phosphatases perform functions which are essential to all eukaryotic cells. Yet, Ser/Thr protein phosphatases share no structural homology with the Tyr protein phosphatases, suggesting that they evolved along separate evolutionary pathways.\textsuperscript{26}

Comparison of the catalytic subunits of the Ser/Thr protein phosphatases allows the generation of a phylogenetic tree. Such analysis reveals PP1\textsubscript{c} and PP2A\textsubscript{c} are 41\% homologous to each other.\textsuperscript{29} The data also shows these genes have been highly conserved through evolution. For example, PP1\textsubscript{c} from fungi, flies and humans are greater than 86\% identical in their primary structure. A similar argument can be put forward for PP2A\textsubscript{c}, which shows 93\% identity between flies and mammals.\textsuperscript{27} Thus considerable pressure must have been exerted during evolution to conserve the structure of PP1\textsubscript{c} and PP2A\textsubscript{c}.\textsuperscript{29} The conservation of PP2A\textsubscript{c} further supports an important function for the enzyme viability. This structural analysis indicates PP2B\textsubscript{c} is clearly more distantly related to PP1\textsubscript{c} and PP2A\textsubscript{c}, with PP2C\textsubscript{c} diverging very early in evolution, or even evolving from a different lineage.\textsuperscript{21}
1.4.5 Protein phosphatase 1 (PP1)

Type 1 protein phosphatases (PP1) comprises of widely distributed enzymes, found in all eukaryotic cells. A number of different forms of PP1 have been characterised. These forms differ in their substrate specificity which are measured in vitro using various phosphoproteins. They all contain an isoform of the same catalytic subunit, which has an extremely conserved primary structure, but differ in the targeting subunits which determine the activity, the substrate specificity, and the cellular location of the phosphatase. Furthermore, PP1 is inhibited by proteins, termed inhibitor 1 (I-1), inhibitor 2 (I-2 or modulator) and the phosphoprotein DARPP-32. These inhibitors can also be considered as regulatory subunits.

1.4.5.1 Structure of PP1c

The catalytic subunit of PP1, termed PP1c, a 37 kDa protein, is indistinguishable from the different forms of PP1, both in its structural and in its enzymic properties. Hence, the differences in the biochemical properties of the 'native' forms must be conferred by the non-catalytic components.

There are 10 deduced amino acid sequences available for proteins which could be classified as a putative type-1 protein phosphatase. Initially, two isoforms of PP1c were cloned from a rabbit skeletal muscle, and were termed α and β. However, in rat, cDNA cloning revealed the existence of at least four isoforms, termed α, γ1, γ2 and δ. Bacterially expressed PP1c (α, β and γ isoforms) differ in some aspects (sensitivity to phosphatase, binding to regulatory subunits, requirement for Mn²⁺ and specific activity) from native PP1c.
1.4.5.2 PP1 Holoenzyme Complexes

The holoenzymes are named according to their apparent subcellular location. For example, the glycogen-bound PP1 holoenzyme (PP1G) is a heterodimer of PP1 and a glycogen-binding subunit (G-subunit). PP1G is the major phosphatase acting upon phospholamban, a protein involved in Ca\textsuperscript{2+} uptake into the cardiac sarcoplasmic reticulum (SR), and in the regulation of the rate of cardiac muscle relaxation.

The G-subunit anchors the phosphatase to glycogen and increases its activity towards the glycogen-bound substrates: glycogen synthase and glycogen phosphorylase. The PP1 holoenzyme isolated from muscle SR has the same subunit as PP1G. It therefore appears that the G-subunit can target glycogen to the SR.

The PP1 bound to the myosin fraction in smooth muscle was purified as a trimer consisting of PP1 and polypeptides of 130 kDa and 20 kDa. The latter subunits are responsible for the enhanced activity towards heavy meromyosin or isolated myosin light chains. The myosin-bound form of PP1 in skeletal muscle (PP1M) was purified as a dimeric enzyme consisting of PP1 and a regulatory subunit which increased dephosphorylation of the myosin light chain, present in heavy meromyosin.

High levels of PP1 activity are also present in the nuclei of eukaryotic cells. Part of the nuclear PP1 is present in a latent form (termed PP1N\textsubscript{2}), and is composed of PP1 complexed to an inhibitory (NIPP-1) polypeptide.

A cytosolic form of PP1, termed PP1S, is composed of PP1 complexed to I-2 (or the modulator subunit). It has been suggested PP1S serves as a pool of inactive phosphatase, from which the catalytic subunit could be recruited if needed in other cellular compartments. I-2 could then function as a "chaperone" which folds the nascent PP1 into the required orientation.
1.4.5.3 Regulation of PP1

Many complex signals are integrated in the control of cellular PP1. The activity of PP1 in the different holoenzyme complexes is primarily mediated through the regulatory subunits. In addition, PP1-regulated enzymes demonstrate a characteristic inhibition by the thermostable proteins: I-1; I-2 and DARPP-32. A protein factor, termed deinhibitor, further modulates the actions of these inhibitor proteins. PP1 activity may also be regulated by the reversible phosphorylation of these protein components.

- **Inhibitor I & DARPP-32** - the related polypeptides, I-1 and DARPP-32, are cytosolic proteins which become strong inhibitors of PP1 after phosphorylation on Thr-35 of I-1 and Thr-34 of DARPP-32 by cyclic AMP- (cAMP-) dependent protein kinase (cAMP-PK). I-1, a 18.7 kDa protein, was discovered together with I-2. DARPP-32 was initially described as a phosphoprotein of 32 kDa in dopamine-innervated brain regions. Addition of dopamine or cAMP to sites of these brain regions increased the phosphorylation of DARPP-32, hence its name - dopamine and cAMP regulated phosphoprotein.

- **Inhibitor 2 or “Modulator”** - I-2 is a 22.9 kDa protein which has been isolated from rabbit skeletal muscle and rat adipose tissue. This protein resembles I-1 and DARPP-32 in that it has a highly symmetrical structure and a low content of amino acids, although there is no homology through cDNA sequencing.

I-2 blocks the activity of PP1 in two distinct ways:

i) by impeding the substrate binding (inhibition); and

ii) by inducing a conformational change of the catalytic subunit (inactivation).

I-2 is also required for the enzymic reactivation of inactive PP1. Due to these multiple effects it was proposed to replace the original name, I-2, with that of “Modulator.”
Deinhibitor - Deinhibitor is a thermostable polypeptide, first isolated from dog liver and shown to prevent the inactivation of PP1 by either I-1 or Modulator.\textsuperscript{48} The deinhibitor not only reduced inhibition of PP1 by I-1, but it also promoted dephosphorylation of I-1 by PP-1\textsubscript{c} in the absence of Mn\textsuperscript{2+}. Thus, the deinhibitor may act to antagonise the actions of I-1.

1.4.5.4 Physiological role of PP1

It is clear that PP1 is involved in many different cellular processes, as diverse as glycogen metabolism, calcium transport, muscle contraction and intracellular transport.\textsuperscript{12} In some cellular processes the substrates for PP1 (e.g. glycogen synthase and glycogen phosphorylase) have been identified. For other phosphoproteins, the contribution of PP1 to their dephosphorylation remains to be clarified.\textsuperscript{12}

Recent evidence from the study of cell cycle mutants has revealed that PP1 also plays an important role in the regulation of mitosis and chromosome segregation.\textsuperscript{39}

1.4.6 Protein Phosphatase 2A (PP2A)

1.4.6.1 Structure of PP2A

PP2A, also termed polycation-stimulated phosphatase, represents a substantial portion of Ser/Thr phosphatase activity in many cells.\textsuperscript{12} The enzyme has been isolated and characterised in several trimeric holoenzyme forms.\textsuperscript{50} The core structure consists of a 36 kDa catalytic subunit (PP2A\textsubscript{c}), complexed to two regulatory subunits; a 65 kDa subunit A (or PR65), and a variable regulatory subunit of 55 kDa (B or PR55), 54 kDa (B\textsuperscript{+}), 72 kDa (PR72), 74 kDa (B\textsuperscript{+}), or 130 kDa (PR130).\textsuperscript{51} \textit{In vivo}, PP2A is probably present as a trimer, although, the core dimer (PP2A\textsubscript{c};PR65) has been purified from many different tissues. This suggests the possibility of subunit rearrangements where the variable subunits are able to associate and dissociate from the core dimer (see Fig. 1.4).\textsuperscript{12}
The exact function of the regulatory subunits is not yet known, but it is considered they influence substrate specificity and/or subcellular localisation. The core dimer of PP2Ac and the PR65 regulatory subunit (A) with variable subunits of 55 kDa (PR55, or B subunit), 54 kDa (B' subunit), 72 kDa (PR72), 74 kDa (B" subunit) or 130 kDa (PR130). PR55, PR72 and PR130 refer to cloned sequences; the 54 and 74 kDa subunits have not been cloned. In addition, the core dimer can interact with PTPA and associates with SV40 and polyoma small-t and middle-T antigens. Molecular cloning has revealed the existence of several isoforms of each subunit. Two forms of the catalytic subunit (α, β), two forms of PR65 (α, β), three isoforms of PR66 (α, β, γ) and two forms of PR72 exist. The α and β isoforms of PP2Ac from mammalian tissues show 97% amino acid identity. However, they are distinct gene products since they possess unrelated nucleotide sequences.
1.4.6.2 Regulation of PP2A

- **Tyrosine phosphatase activity** - although PP2A was originally purified as a Ser/Thr protein phosphatase it can, under certain conditions, dephosphorylate Tyr residues. The tyrosine phosphatase activity of PP2A towards exogenous substrates is increased in the presence of ATP. Furthermore, a 37 kDa protein, termed PTPA, was isolated and characterised which, in the presence of ATP and Mg\(^{2+}\) activates the Tyr phosphatase activity of PP2A. Whilst the mechanism has yet to be established, it is known that the PTPA-induced tyrosine phosphatase activity of PP2A is stabilised by small-t or middle-T antigens.

- **Interaction with regulatory subunits** - interaction of PP2A with the regulatory subunits PR65, PR55, PR72 and others, influence its substrate activity. The PP2A trimer containing PR72 preferentially dephosphorylates Ser-120 and Ser-123 in simian virus 40 (SV40) large-T antigen, whereas dephosphorylation of Thr-124 is only efficiently catalysed by the PP2A trimer containing PR55.

1.4.6.3 Physiological role of PP2A

PP2A has high activity *in vitro* towards the regulatory enzymes of glycolysis/gluconeogenesis, fatty acid synthesis, amino acid breakdown, lipolysis, and catecholamine synthesis suggesting it may be the major phosphatase acting to regulate these pathways *in vivo*.

The tumour promoter okadaic acid 1, microcystin-LR 2 and the related class of inhibitors of PP2A and PP1, are proving to be valuable tools in identifying or substantiating their physiological roles (see Fig. 1.5). It is possible to specifically inhibit PP2A activity in cell-free extracts because PP2A requires lower concentrations of okadaic acid (OA) for inhibition than does PP1. The okadaic acid class of compounds will be discussed in more detail in Section 1.5.2.1.
1.4.7 Protein phosphatase 2B

PP2B, or calcineurin, was first identified as a major calmodulin-binding protein from the brain, where it accounts for up to 1% of the total protein. PP2B is a heterodimer of calcineurin A (60 kDa), and calcineurin B (19 kDa). Calcineurin A is the catalytic subunit which also binds to calmodulin. Calcineurin B is the regulatory, Ca$^{2+}$-binding subunit.

The A subunit has four functional domains: (i) catalytic; (ii) B-subunit binding; (iii) calmodulin-binding, and (iv) autoinhibitory.
Functionally distinct, subunit B contains four Ca\(^{2+}\)-binding loops, and shows 35% homology to calmodulin.\(^{12}\)

Three PP2B A-subunits (CNA\(_{\alpha}\), CNA\(_{\beta}\) and CNA\(_{\gamma}\)) have been encoded by three separate genes. The CNA\(_{\alpha}\) gene gives rise to two transcripts (\(\alpha1\) and \(\alpha2\)), and the CNA\(_{\beta}\) gene gives rise to three transcripts (\(\beta1\), \(\beta2\) and \(\beta3\)).

Whilst this would appear less complicated for the B-subunit, recent studies have shown two genes encode for this subunit.\(^{12}\)

1.4.7.1 Regulation of PP2B

PP2B is dependent upon Ca\(^{2+}\) for activity and is stimulated by calmodulin, but the purified enzyme also requires divalent metal cations, such as Mn\(^{2+}\), Mg\(^{2+}\) or Ni\(^{2+}\).

Partial proteolysis of calcineurin converts it to a form which no longer requires Ca\(^{2+}\) for activity. This is due to destruction of the C-terminus of the catalytic subunit, which contains an autoinhibitory domain (see Fig. 1.3, P 8).\(^{56}\) Binding of calmodulin to a nearby site and binding of Ca\(^{2+}\) to the B-subunit allows the enzyme to overcome this auto-inhibition.\(^{12}\)

Inhibition of calcineurin activity towards model substrate peptides by the immunosuppressant/immunophilin complex (FK506/FKBP) is mediated by both calcineurin subunits.\(^{57}\) Association of calcineurin with FK506/FKBP is dependent on Ca\(^{2+}\) unless the auto-inhibitory domain on the catalytic subunit is removed. Recent studies have indicated FK506/FKBP interacts with the latch region of the CNB subunit. However, the association of the CNB with CNA is required for the former to adopt the correct conformation to permit interaction with drug/immunophilin complexes.\(^{12}\)

PP2B has a rather restricted substrate specificity. The \(\alpha\)-subunit of phosphorylase kinase, I-1, DARPP-32 and the type II regulatory subunit of cAMP-PK are excellent \textit{in vitro} substrates.
1.4.7.2 Physiological role of PP2B

T-cell activation, in response to the interaction of the T-cell receptor with antigen, is mediated by induction of interleukin-2 gene expression. Activation of at least two signalling pathways are needed for this induction. One pathway can be activated with Ca$^{2+}$ ionophores, and the other with phorbol esters. At the level of the promoter, induction of the interleukin-2 gene expression requires co-operative interactions of several transcription factors. The Ca$^{2+}$-dependent pathway is mediated via activation of calcineurin. This leads to dephosphorylation of the cytosolic subunit of the transcription factor, NF-AT. The dephosphorylated subunit then migrates to the nucleus where it combines with nuclear factors to form the transcription factor NF-AT. NF-AT binds to, and then activates, the interleukin-2 promoter.

1.4.8 Protein Phosphatase 2C

PP2C was originally identified as a Mg$^{2+}$-dependent protein phosphatase, which is structurally different from all other Ser/Thr protein phosphatases. The enzyme is a member of a multigene family, is monomeric (with a molecular mass of 43-48 kDa) and has demonstrated a relatively broad substrate specificity. Two isoforms (α 42 kDa, β 44 kDa) of PP2C have been cloned. Further analysis has revealed the existence of subtypes for both isoforms. Three α subtypes have been identified, with α1 being the most dominant species, whereas only two subtypes of the β-isoform have been found. Whereas PP2C shares no structural homology with PP1, PP2A or PP2B, some sequence homology was observed with the mitochondrial enzyme pyruvate dehydrogenase phosphatase.

In vitro studies show PP2C has unusually high activity towards the enzymes of cholesterol metabolism. However, in vivo studies designed to examine the role of
PP2C in this metabolism pathway have not been carried out, and thus, the physiological significance of PP2C remains to be established.\textsuperscript{12}

1.4.9 Novel protein phosphatases

Ser/Thr protein phosphatases which differ in their biochemical properties from PP1, 2A, 2B and 2C have been isolated from their cDNAs. These all belong to the PPP class of protein phosphatases.

1.4.9.1 Protein phosphatase-3 (PP3)

PP3, an okadaic acid-sensitive phosphatase, has been extracted from bovine brain and is found to be active in the absence of divalent cations.\textsuperscript{59} This putative, membrane-bound enzyme demonstrates substrate specificity similar to PP1, yet it is not inhibited by I-2. PP3 has a molecular mass of 36 kDa, which has a conserved six amino acid region with PP1 and PP2A. The remainder of PP3 has no additional identity with PP1, but has 50% identity with PP2A. The physiological function of PP3 is unknown, although current evidence suggests a role in cell proliferation.\textsuperscript{59}

1.4.9.2 Protein phosphatase-4 (PP4)

PP4, which was originally termed PPX, is isolated from rabbit liver.\textsuperscript{60} PP4 is a 35 kDa protein which shows 65% primary sequence homology with PP2A, and 45% identity to PP1. As with PP2A, PP4 preferentially dephosphorylates the $\alpha$ subunit-rather than the $\beta$ subunit of phosphorylase kinase, and is unaffected by I-1 and I-2.\textsuperscript{61} The sensitivity of PP4 to OA is similar to PP2A. PP4 is localised mainly in the centrosomes, and is thought to play a role in their function, possibly in the control of microtubule assembly.
1.4.9.3 Protein phosphatase-5 (PP5)

A novel human Ser/Thr protein phosphatase, PP5, and a structurally related phosphatase, PPT1, have been identified.\(^6^2\) Their predicted molecular mass is 58 kDa and they comprise a C-terminal phosphatase catalytic domain, which shows homology with the phosphatase domain of other members of this family, and an N-terminal domain. PP5 is related to PP1, PP2A and PP2B, but has an N-terminal extension of 200 amino acids. This extension contains four tetraterptide repeats (TPR) i.e. four repeats of 34 amino acids, three of which are tandemly arranged. These repeats are found in proteins which are involved in the regulation of RNA biogenesis and mitosis.\(^6^2\)

1.4.9.4 Protein phosphatase-6 (PP6)

A novel Ser/Thr protein phosphatase has been identified by cDNA, designated PP6.\(^6^3\) The predicted amino acid sequence indicates a 35 kDa protein showing 57\% homology to PP2A. In human cells, three forms of PP6 mRNA have been found, with the highest levels of expression in human body in testis, heart and skeletal muscle. It is thought PP6 may be involved in cell cycle regulation.\(^6^3\)

1.4.10 Structure and mechanism of Ser/Thr phosphatases

Despite a wealth of information about the biological processes involving Ser/Thr protein phosphatases, chemical and structural information about the enzymes is sparse and little is known about how they catalyse phosphate hydrolysis.
1.4.10.1 Structure and catalytic mechanism of the PPP family

Most of the mechanistic information on these proteins stem from mutagenesis studies of bacteriophage λ phosphatase and purple acid phosphatase, phosphatases with substantial homology to the catalytic domains of PP1, PP2A and PP2B.

- **Catalytic site** - solution of the X-ray crystal structures of PP1, the PP2B holoenzyme and the PP2B complex with FK506/FKBP12 provide an understanding of some of the fundamental properties of these proteins, namely molecular mechanisms of catalysis, substrate recognition and regulation.

The first co-crystallisation of the catalytic subunit of PP1 complexed to microcystin 2 was reported in 1994 by Barford et al. The crystals were reported to be orthorhombic at a resolution of 2.8 Å. Later, the crystal structure of the mammalian protein phosphatase-1, PP1, complexed with the hepatotoxin, microcystin, at 2.1 Å resolution, was delineated by Goldberg et al. In the same year, the catalytic subunit of human PP1γ and its complex with tungstate at 2.5 Å was reported by Egloff et al.

Recently, Egloff et al. reported the crystal structure of PP1 with the M-subunit at 3.0 Å.

The structure of PP2B was determined by Griffith et al. at 2.5 Å as a complex containing both, FK506 and the FK506 binding protein (FKBP). The same complex was also described by Kissinger et al. who also reported the crystal structure of human PP2B heterodimer at 2.1 Å.

The catalytic domains of PP1 and PP2B adopt an identical core structure based on a central distorted β-sandwich of 11 β-strands surrounded on one side by seven α-helices and a three-stranded β-sheet (see Fig. 1.6).
**Figure 1.6: View of the catalytic subunit of PP1γ onto the catalytic-site channel**

Metal ions are shown as green spheres within a binuclear metal centre to which a tungstate ion (an analogue of phosphate) is co-ordinated. Regions of the polypeptide which correspond to the three invariant sequence motifs present in the PPP family of phosphatases and other metallophosphoesterases, such as λ bacteriophage phosphatase and kidney bean purple acid phosphatase, are shown in red. The Cα positions of residues which co-ordinate the divalent metal ions and tungstate ion are indicated as blue and red spheres.
The interface of the two β-sheets at the top of the β-sandwich creates a shallow channel. Three parallel β-strands of sheet 1 constitute a mononucleotide-binding domain with the secondary structure organisation β-α-β-α-β. The three invariant sequence motifs form the loops connecting with the carboxy terminus of the β-strand with α-helices (see Fig. 1.7). These loops, together with those emanating from the carboxy terminus of two β-strands of the opposite β-sheet, provide the catalytic residues. This scaffolding contains the Ser/Thr protein phosphatases signature motif identified during studies with bacteriophage λ phosphatase.\textsuperscript{11}

Ser/Thr protein phosphatases are metalloenzymes\textsuperscript{67,71,72} and two divalent metal ions (Mn\textsuperscript{2+} and Fe\textsuperscript{2+} in PP1, Zn\textsuperscript{2+} and Fe\textsuperscript{2+} in PP2B) at the centre of the catalytic site are co-ordinated by Asp64, Asp92, Asn124 and His66 of the mononucleotide-binding motif and His173 and His248 of sheet 2 [Note: Residue numbers refer to PP1].
**Figure 1.7** (a) Detailed view of the catalytic site of PPI in the presence of a tungstate ion, an analogue of phosphate. One of the two water molecules shown co-ordinated to the metal ions is proposed to act as a nucleophile during the catalytic reaction.

(b) Schematic of the reaction mechanism catalysed by PPPs.

The side chain of Asp92 and a water molecule bridge both metal ions to form a binuclear metal centre. A second metal-bound water molecule is co-ordinated to the Fe^{2+} ion. The catalytic sites of two of the phosphatase crystal structures contain bound oxyanions, tungstate in PPIγ1 and phosphate in one of the PP2B structures.

**Mechanism** - a catalytic mechanism, suggested by the X-ray structures, is entirely consistent with site-directed mutagensis performed on PPI and the λ bacteriophage phosphatase. A metal-bound water molecule acts as a nucleophile to attack the phosphorous atom of a phosphate group in an S_N2 mechanism.
Metals assist in this catalysis in two ways:

- as Lewis acids to enhance the nucleophilicity of the metal-bound water; and
- by enhancing the electrophilicity of the phosphorous atom.

The sidechain of His125 probably donates a proton to the leaving-group oxygen of a Ser or Thr sidechain. Such a role for His125 is consistent with the loss of catalytic activity of PP1 His125 mutants and mutations of the equivalent residue of λ-phosphatase. Other mutational studies have shown replacement of any of the metal-co-ordinating residues in PP1 and λ PPase leads to either severe loss in activity or production of insoluble proteins during over-production in *E. coli*. The proposal for a single-step dephosphorylation reaction is consistent with earlier data suggesting PP2B cannot catalyse transphosphorylation reactions and the fact phosphoryl enzyme intermediates do not appear to form during the reaction catalysed by PPPs.

1.4.10.2 Structural mechanism of regulation

Most available evidence suggests the carboxy-terminal regions of PPP catalytic domains are crucially important for communicating regulatory signals to the catalytic site.

The mechanisms of inhibition of PP1c by microcystin-LR 2, and PP2B both by its natural inhibitor the auto-inhibitory (AI) domain, and by the FKBP12-FK506 complex, have been defined by the structure of the PP1-microcystin complex and from two PP2B structures.

- **Inhibition of PP1c by microcystin** - microcystin-LR 2 is a complex cyclic heptapeptide which interacts with three distinct regions on the surface of PP1 (see Fig. 1.8).
Figure 1.8: Protein Phosphatase 1a complexed to microcystin-LR

Microcystin interacts with the hydrophobic groove, the metal sites and to Cys273.

One of these involves a carboxylate and carbonyl group of the toxin which interact with two of the metal-bound water molecules, hence blocking substrate binding directly. Other sites consist of a protein hydrophobic groove and the β12/β13 loop where the Sγ atom of Cys273 of PP1e forms a covalent bond to one of the side-chains of the toxin, and the side-chain of Tyr272 packs against a leucine residue of the toxin. Interestingly, the conformation of microcystin-LR does not change from its solution structure when in complex with PP1 whereas the β12/β13 loop if PP1 undergoes a conformational change which avoids steric conflict between Tyr276 of PP1 and the Mdha sidechain of microcystin-LR. The solution structure of another PP1/PP2A toxin, a pentapeptide, motuporin 4 which
resembles microcystin-LR suggests a similar model of interaction between PP1 and this inhibitor.\textsuperscript{11}

**Resistance to toxins** - the role of the $\beta_{12}/\beta_{13}$ loop as a site of interaction with toxins was anticipated earlier from the finding the substitution of Cys269 of PP2A to Gly within the $\beta_{12}/\beta_{13}$ loop causes resistance to okadaic acid. The equivalent residue in PP1 is a Phe, and replacing this with a Cys enhances okadaic acid binding activity.\textsuperscript{77} Other studies showed mutation of Tyr272 of the $\beta_{12}/\beta_{13}$ loop causes a dramatic loss of potency of okadaic acid, calyculin A, and tautomycin as well as microcystin and motuporin.\textsuperscript{78} These findings suggest toxins of PP1 and PP2A, despite their dissimilar structures interact with their target through the $\beta_{12}/\beta_{13}$ loop. Significantly, the equivalent loop appears to play a role in interactions of PP2B with immunosuppressant/immunophilin complexes.\textsuperscript{11}

In the structure of the full-length PP2B holoenzyme, the A1-domain lies over the substrate-binding channel of the catalytic domain such which a Glu sidechain hydrogen bonds with two of the metal-bound water molecules. This interaction sterically hinders substrate and is reminiscent of PP1 inhibition by microcystin.\textsuperscript{66}

**FKBP12 and FK506 binding** - neither FKBP12 or FK506 individually can associate with PP2B; their interaction with PP2B requires a composite recognition surface of the binary complex.\textsuperscript{11} The quaternary complex of the PP2B holoenzyme with FKBP12 and FK506 indicates the conformation of FKBP12 is nearly identical to the form in the binary complex of FKBP12 and FK506 with minor flexibility's observed in the His87 and Ile90 loop of FKBP12.\textsuperscript{69,70} The major site of interaction of PP2B is the base of the B-subunit-binding helix of the A subunit, and the B-subunit with a minor interaction with the $\beta_{12}/\beta_{13}$ loop of the catalytic site.\textsuperscript{11} FK506 is situated 25 Å from the catalytic site and cannot participate directly in phosphatase inhibition. FKBP12 sterically blocks access to the catalytic site for large macromolecules, although since its closest approach to the catalytic site is 10 Å, it does not prevent dephosphorylation of small molecule substrates such
$p$-nitrophenol phosphate ($p$NPP). The region of FKBP12 forms most of the interactions with PP2B, suggesting PP2B interacts with its anchoring protein and with FKBP12 by similar mechanisms.\(^7\)

1.4.10.3 Structure and catalytic mechanism of the PPM family

The sequences of protein phosphatases of the PPM family share no similarity with those of the PPP family; however, the structures of these two families are strikingly similar. Recently, Barford et al., published the crystal structure of PP2C at 2 Å resolution (see Fig. 1.9).\(^8\)
Figure 1.9: Crystal Structure of PP2C

(A) Ribbon representation of PP2C.

(B) Detailed view of the catalytic site of PP2C. The two Mn$^{2+}$ ions are shown as green spheres co-ordinated by invariant aspartate residues, and water molecules are shown as gold spheres.

Mammalian PP2C consists of two domains:

- an amino-terminal catalytic domain with six $\alpha$-helices and 11 $\beta$-strands, common to all members of the PP2C family; and

- a 90-residue carboxy-terminal domain of three $\alpha$-helices.

The catalytic domain is dominated by a central buried $\beta$-sandwich, formed by the association of two anti-parallel $\beta$-sheets, both of which are flanked by a pair of anti-parallel $\alpha$-helices, which are inserted between the two central $\beta$-strands. The carboxy-terminal domain is formed from three anti-parallel $\alpha$-helices which are
remote from the catalytic site, suggesting a role in defining substrate specificity rather than catalysis.\textsuperscript{80}

At the catalytic site of PP2C, two Mn\textsuperscript{2+} ions within a binuclear metal centre are co-ordinated by four invariant aspartate residues and a non-conserved glutamate residue. These residues are situated at the top of a central β-sandwich. Six water molecules co-ordinate the two metal ions. One of these water molecules bridges the two metal ions and four form hydrogen bonds to a phosphate ion at the catalytic site. Dephosphorylation is probably catalysed by metal-activated water molecules which act as nucleophiles and general acids in a similar mechanism proposed for the PPP family.\textsuperscript{11}
1.5 Serine/Threonine Protein Phosphatase Inhibitors

1.5.1 Introduction

Inhibitors of eukaryotic protein kinases and phosphatases are a chemically diverse array of natural and synthetic compounds.

These include:

- Medicines (immunosuppressants, tumour suppressants and anti-inflammatory agents);
- Potions (a purported aphrodisiac which doubles as a wart remover!); and
- Poisons (diarrhetic toxins, liver toxins, tumour promoters, an insect defence chemical and herbicides).\textsuperscript{81}

These substances are valuable pharmacological probes and affinity ligands for enhancing our knowledge of cellular effects of the signalling pathways. More broadly, this basic research is also leading to the development of drugs to control specific cellular responses, and enzyme based assays to detect toxins in food and water.

There are two functional classes of natural inhibitors:

- \textit{Endogenous Proteins and Peptide Inhibitors} - which regulate particular protein phosphatases within eukaryotic cells [for example inhibitors 1 and 2, which are specific for PP1 (see section 1.4.5.3, P 12)], and

- \textit{Secondary Metabolites} - produced by bacteria, fungi, plants, dinoflagellates and insects, whose natural roles may be ecological - in signalling, attack and defence interactions among organisms.\textsuperscript{82,83}
1.5.2 Type 1 and 2A Serine/Threonine Phosphatase Inhibitors

1.5.2.1 Okadaic Acid Class of Compounds

There are over 30 compounds in the okadaic acid class of inhibitors which all apparently bind to the same site on PP1 and PP2A and inhibit their activities. These include the four structurally diverse different classes; okadaic acid, calyculin, tautomycin and microcystin. Since okadaic acid was the first member of this group to be isolated, they are referred to the Okadaic Acid Class of Compounds.\(^7\)

Okadaic acid is 50-100 times more effective an inhibitor for PP2A than for PP1. This is not the case for the other compounds in this class, which are equally effective against PP1 and PP2A (see Table 1.1).\(^9\)

Table 1.1: Inhibition of Ser/Thr Protein Phosphatases by the Okadaic Acid Class of Compounds

<table>
<thead>
<tr>
<th></th>
<th>PP1 (IC(_{50}) nM)</th>
<th>PP2A (IC(_{50}) nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okadaic Acid</td>
<td>3.4</td>
<td>0.07</td>
</tr>
<tr>
<td>Calyculin A</td>
<td>0.3</td>
<td>0.13</td>
</tr>
<tr>
<td>Tautomycin</td>
<td>0.7</td>
<td>0.65</td>
</tr>
<tr>
<td>Microcystin-LR</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

1.5.2.2 The Okadaic Acid Pathway of Tumour Promotion

Human carcinogenesis occurs in multi-stages, which include initiation, promotion and progression, and later metastasis associated with genetic changes, such as mutation and deletion (see Fig. 1.10).
Cancer development in humans takes a long period of time; for example, the preneoplastic changes may be already present in the cells many years earlier than the appearance of cancers. Thus, occurrence of cancer is often associated with ageing.

Initiation and tumour promotion, are usually defined thus:

- **Initiation** - is the mutation of a protooncogene in a normal cell, and

- **Tumour promotion** - is the clonal growth of initiated cells.

![Cancerogenesis Diagram](image)

**Figure 1.10: Cancerogenesis**

Compared with the long period of tumour development in humans, the tumour promotion stage in a two-stage carcinogenesis experiment on mouse skin takes only about ten weeks. Thus, mouse skin is ideally suited for biochemical and molecular studies of tumour promotion with various tumour promoters.

Tumour promoters can be classified into two groups:

- **TPA-type tumour promoters**, and

- **non-TPA-type tumour promoters**.

TPA (12-O-tetradecanoylphorbol-13-acetate) was the first tumour promoter to be isolated, and indeed was only known tumour promoter until the late 1970s.
Classically, all TPA-type promoters are defined as those which bind to phorbol ester receptors in cell membranes, activating Ca\(^{2+}\)-activated phospholipid-dependent kinase C (PKC) \textit{in vitro}. Therefore, all tumour promoters of the TPA-type induce the same biochemical and biological activities. In contrast, the non-TPA type tumour promoters do not bind to the phorbol esters or activate PKC. As for the mechanisms of action of non-TPA type tumour promoters, each class may act upon cells through different pathways.

In 1988, okadaic acid and dinophysistoxin-1 were reported to induce tumour promotion in two-stage carcinogenesis experiments, on mouse skin initiated with 7, 12-dimethylbenz[a]anthracene (DMBA) \textsuperscript{16}. Later, it was also found okadaic acid induced tumour promotion in rat glandular stomach initiated with \(N\)-methyl-\(N\)-nitro-\(N\)-nitrosoguanidine (MNNG), \textsuperscript{17}, (see Fig. 1.11).

These experiments established okadaic acid had a different mode of action to TPA. Hence, okadaic acid belongs to the non-TPA type tumour promoters.
Okadaic acid interacts with receptors (identified as PP1 and PP2A) in cells and inhibits their action. The biochemical consequence of inhibition is an increase of phosphorylation, causing hyperphosphorylation, which results in discordant regulation of both protein kinases and protein phosphatases. This mechanism is now generally accepted as the Okadaic Acid Mechanism of Tumour Promotion (see Fig. 1.12).

Figure 1.12: Hyperphosphorylation

Two stage carcinogenesis experiments revealed microcystin-LR was a new tumour promoter in rat liver, initiated with diethylnitrosamine (DEN). Nodularin was also thought to be a liver tumour promoter, because both nodularin and microcystin-LR inhibited PP1 and PP2A with the same specific activity. Nodularin turned out to be a more potent tumour promoter than microcystin-LR, and in addition, to have a slightly lower initiating activity. The results suggested that nodularin is a new environmental carcinogen rather than simply a tumour promoter.

Okadaic acid binds to PP1 and PP2A in particulate (membrane) and cytosolic fractions of various tissues. Since the microcystins and nodularins are also liver tumour promoters which inhibit PP1 and PP2A, experiments were carried out to see if these compounds bind to the same receptors. Inhibition of specific $[^3]H$-okadaic acid binding to particulate and cytosolic fractions of mouse liver was first studied. Microcystin and nodularin inhibited the specific binding of specific $[^3]H$-okadaic acid dose dependently, hence have the same mode of action and so
belong to the Okadaic Acid Class of Inhibitors. Moreover, additional receptor binding testing with $^3$H-okadaic acid, has identified over thirty compound in the okadaic acid class, including calyculin A and tautomycin. Microcystin-LR is the most potent liver tumour promoter to date.\textsuperscript{97}

1.5.2.2.1 Okadaic Acid

Okadaic acid is a C$_{38}$ polyether fatty acid with a molecular backbone spanning six tetrapyran rings and one tetrafururan ring (see Fig. 1.13).\textsuperscript{99} It was first isolated from the common black marine sponges Halichondria okadai and Halichondria melanodocia.\textsuperscript{100} The structure is very similar to acanthifolicin 7 isolated from the sponge Pandoras acanathafolium, which was the first polyether carboxylic acid to be reported from marine sources.\textsuperscript{101} Okadaic acid has subsequently shown to be produced by several types of dinoflagellates (marine plankton) including Prorocentrum lima and Dinophysis sp.\textsuperscript{102}

![Figure 1.13: Okadaic Acid and Analogues](image)

Okadaic acid is the causative agent for diarrhetic shellfish poisoning (DSP), regulates mitosis in the cell cycle and specifically inhibits PP1 and PP2A resulting in the accumulation of phosphorlylated proteins in cells.\textsuperscript{103}
Diarrhetic shellfish toxin was first isolated from the digestive glands of mussels and named dinophysistoxin 1 (DTX1) after the genus of the causative dinoflagellate. By spectral comparison with okadaic acid, DTX1 was identified to be 35-(R)-methylolokadiac acid. A series of congeners substituted with various fatty acids, 7-O-acyl-35-(R)-methylolokadiac acids (DTX3), were isolated as the toxic component of poisonous scallops from North-eastern Japan. Recently 31-dimethyl-35-methylolokadaic acid (DTX2), [okadaic acid tetramethyl ether] has been isolated from Irish mussels.

The total synthesis of okadaic acid was reported in 1987 by Isobe et al. The X-ray crystal structure of the O-bromobenzyl ester of okadaic acid was reported in 1981. A full NMR study of the solution structure okadaic acid revealed that it was identical to the crystal structure. The fact the solution and solid state conformations are comparable suggests the biologically active conformation of okadaic acid is likely to be similar. With this information available, okadaic acid is a good template for the generation of a pharmacophore model of the okadaic class of compounds.

Okadaic acid is found to inhibit PP2A at the lowest concentration ($K_i$ of 30 pM), PP1 at the next lowest concentration ($K_i$ of 150 nM), and PP2B at the highest concentration ($K_i$ of 5 µM); it shows no effect on PP2C.

1.5.2.2 Calyculin A

Calyculin A 5A was first isolated in 1986 from a marine sponge, Discodermia calyx, collected in the Gulf of Sagami in Japan. It is a strong inhibitor of starfish development and a strong toxic compound against leukaemia cells. It was later found to belong to the okadaic acid class of inhibitors, having subnanomolar inhibitor activity towards PP1 and PP2A.

Structurally, calyculin A differs markedly from okadaic acid, in spite of their overlapping binding properties (see Fig. 1.14). Calyculin A has a variety of
interesting functional groups. It contains an octamethyl polyhydroxylated C_{28} fatty acid which is linked to two \( \gamma \)-amino acids and esterified by phosphoric acid. The phosphate monoester is highly resistant to acid or enzymic hydrolysis, and is thought to play a central role in the interaction of calyculin A with its binding sites, although this remains to be demonstrated.\(^{109}\)

\[
\text{Calyculin A} \quad \text{Calyculin B} \quad \text{Calyculin C} \quad \text{Calyculin D} \\
\text{Calyculin E} \quad \text{Calyculin F} \quad \text{Calyculin G} \quad \text{Calyculin H}
\]

**Figure 1.14: Structural Calyculins**

Seven other calyculins have since been isolated from *Discodermia calyx*, (calyculin B-H).\(^{112,113}\) The absolute stereochemistry was elucidated in 1991,\(^{114}\) and found that calyculins E - H are geometrical isomers (at C-2 and C-6) of calyculins A-D. The stereochemistry of calyculin A was shown to be enantiomeric to the original report.\(^{109}\)

Earlier this year the dephosphorylated derivative of calyculin A was reported. This was isolated from *D. calyx* by Fusetani *et al.*, and was found to be a potent inhibitor of PP1 and PP2A with IC\(_{50}\) values of 3.0 and 8.2 nM, respectively.\(^{115}\)

There have been several attempted syntheses of calyculin A, including a total synthesis reported in 1992 by Evans *et al.*\(^{116}\) This turned out to be the enantiomer of calyculin A ([+]-calyculin A).
Recently the total synthesis of the correct enantiomer \((-\)-calyculin A\) was reported by Masamune et al.\textsuperscript{117}

1.5.2.2.3 Tautomycin

In 1987, Isono and co-workers isolated an antifungal antibiotic named tautomycin \textsuperscript{6} from the culture of \textit{Streptomyces spiroverticillatus},\textsuperscript{118} and later determined its structure.\textsuperscript{119}

\[ \text{Tautomycin (6)} \]

It was found that tautomycin specifically inhibits PP1 and PP2A with an IC\textsubscript{50} of 20 nM, and binds to the same site as okadaic acid.\textsuperscript{120} This antibiotic also causes morphological change of human leukaemia cells.\textsuperscript{121}

Although the total synthesis of tautomycin has not been reported, there have been several partial syntheses.\textsuperscript{122}

1.5.2.2.4 Microcystins and Nodularins

1.5.2.2.4.1 Introduction

Cyanobacterial blooms (or blue-green algae blooms) occur on 70 per cent of reservoirs world-wide, and have a wide range of social, economic and environmental impacts.\textsuperscript{123,124} Not only does their high biomass contribute to aesthetic problems such as surface scums and unpleasant odour, but of particular concern for animal and human health is the production of bioactive secondary metabolites which have highly toxic properties.\textsuperscript{125} These secondary metabolites include both cytotoxins and biotoxins,\textsuperscript{126} but only the biotoxins are a primary
concern for animal and human health. The most common biotoxins include the potent neurotoxic alkaloids [anatoxin-a (11), anatoxin-a(s) (12), saxitoxins (13) and related paralytic shellfish poisons], plus the more commonly occurring hepatotoxic cyclic peptides [microcystins and nodularin (14)] and the hepatotoxic alkaloid [cylindrospermopsin (15)]. High doses of some of these toxins, the hepatotoxic cyclic peptides (microcystins and nodularins) in particular, severely damage the liver and can be fatal. Recent research has also highlighted the risk that low doses may promote human liver tumours.

![Cyanobacterial Toxins](image)

**Figure 1.15: Cyanobacterial Toxins**

The hepatotoxic cyclic peptides (microcystins and nodularins) are discussed in more detail below.

1.5.2.2.4.2 Chemical Structures and Their Characteristic Features

The microcystins are produced by both filamentous (*Anabaena flos-aquae, Oscillatoria agardhii, Nostoc sp, Hapalosiphon*) and colonial (*Microcystis aeruginosa* and *Microcystis viridis*) cyanobacteria [see Appendix 1]. The nodularins are produced by *Nodularia spumigena*, a filamentous brackish water cyanobacterium.  

128-142
Microcystin was first isolated in 1959 by Bishop et al. *Microcystis aeruginosa* is also the most common bloom-forming cyanobacterium present in cases of human and animal poisoning.\(^{143}\)

Nodularin was the first blue-green algae implicated in hepatotoxic animal poisonings. Although, *Nodularia spumigena* was isolated in 1878,\(^{144}\) the total structure of nodularin was not delineated for over a century.\(^{147}\)

The gross structures of the hepatotoxins contained in *M. Aeruginosa* were elucidated by Botes *et al.*\(^ {145,146}\) as cyclic peptides in 1985, and Rinehart *et al.*\(^ {147}\) reported the total structure three years later. The general structure is cyclo[\((R)-\text{Ala}^1-(S)-\text{X}^2-(R)-\text{βMeAsp}^3-(S)-\text{Y}^4-\text{ADDA}^5-(R)-\text{isoGlu}^6-\text{Mdha}^7\]. The structure uniformly is composed of the (R)-amino acid, alanine (Ala); β-linked erythro-β-methylaspartic acid (β-Me-Asp); γ-linked glutamic acid (Glu); two variable (S)-amino acids (X and Y) with combinations known to include, for example, leucine and alanine (LA), leucine and arginine (LR), tyrosine and arginine (YR), tyrosine and alanine (YA), and tryosine and methionine (YM); and the two unusual amino acids, N-methyldehydroalanine (Mdha) and 3-amino-9-methoxy-10-phenyl-2, 6, 8,-trimethyldeca-4, 6-dienoic acid (Adda). The stereochemistry of Adda has recently been determined and assigned as 2S, 3S, 8S, 9S, thus completing the absolute structures of microcystins.\(^ {147}\) The two variable (S)-amino acids provide the basis of the nomenclature for the microcystins.

Once the basic structure was determined, many reports on microcystins quickly followed. More than 50 microcystins have been isolated to date [see Appendix 1].\(^ {143}\) The structural differences among microcystins depend on mainly on two variable (S)-amino acids (X and Y), and secondly, on the presence or absence of methyl groups on β-Me-Asp and/or Mdha.\(^ {148}\) Several components possessing modified structures have also been isolated which have a 9-O-acetyl-Adda or a 9-O-demethyl-Adda moiety; isopropyl or methyl monoester
of the α-carboxyl group on the Glu moiety; and the N-methyl serine or serine instead of Mdha.

One of the characteristic structural features in microcystins is the presence of a β-amino acid, Adda; this amino acid is also conserved in nodularins. The geometric isomer at C-7 in the Adda moiety having 4(£), 6(£) is considered to be essential for biological activity. This amino acid increases the hydrophobicity of the whole microcystin molecule. Microcystins possess another unusual unsaturated amino acid, Mdha, which serves as a Michael addition acceptor. Indeed, microcystins react smoothly with glutathione and cysteine to give their adducts.

Although nodularin is a pentapeptide, its structure is closely related to microcystins. Nodularin is composed of Adda and (R)-erythro-β-methylaspartic acid (β-MeAsp) as well as 2-(methylamino)-2-dehydrobutyric acid (Mdhb) instead of N-methyldehydroalanine (Mdha) of microcystins, however, nodularin lacks the variable (S)-amino acid (X) and the (R)-Ala residue (see Fig. 1.15).

Motuporin ([(S)-Val²]-nodularin) is a new member of the microcystins/nodularin family. It is closely related to nodularin where the (S)-Arg at the variable (X) position is replaced with (S)-Val in the case of nodularin. Motuporin was isolated from a sponge, *Theonella swinhoei*, collected from Papua New Guinea, and is shown to be a potent inhibitor PP1 and PP2A.¹⁴⁹

### 1.5.2.2.4.3 Relationship Between Structure and Toxicity

As mentioned above, over 50 microcystins have been observed thus far. Clinical signs of hepatotoxicosis have been observed in both wild and domestic animals. The finding that microcystin activates phosphorylase a is consistent with microcystin and nodularin being potent inhibitors of PP1 and PP2A. This finding is important because inhibition of protein phosphatases implicates microcystins as potent tumour promoters and carcinogens.
There are several modifications at different sites of the microcystin molecule: two variable (S)-amino acids; methyl groups Mdha and/or β-Me-Asp; Adda; Glu; and Mdha. The toxicity is classified roughly into three groups, depending upon the replacement of the two variable (S)-amino acids: strong: -LR, -LA and -YR; medium, -WR; or weak, -RR and -M(0)R. Desmethylated components on Mdha and β-Me-Asp show medium hepatotoxicity.

Adda appears to be of great importance in the hepatotoxicity. Removal or saturation of Adda greatly reduces the toxicity of microcystin-LR. Additionally, geometrical isomers at C-7 of Adda in microcystin-LR and -RR were essentially non-toxic. Replacement of the methoxy group at the C-9 in Adda by acetoxyl and hydroxy groups does not reduce the toxicity. The toxicity of microcystin-LR was substantially reduced by the (C₃H₇O) unit in the Glu residue, suggesting a free carboxylic acid on the Glu unit may be important in toxicity. Reduction of the double bond and addition of glutathione in Mdha do not significantly reduce the toxicity of microcystin-LR. These accumulated results, suggest the Adda and Glu residues of the microcystin-LR play highly important roles in the hepatotoxicity of microcystins.

1.5.2.2.4.4 Toxic mechanism

The cellular uptake mechanisms of microcystin have been studied using radiolabelled dihydromicrocystin. The uptake of radiolabelled microcystins were shown to be specific for rat hepatocytes.

First, the membrane penetrating capacity of microcystin was analysed, but found to be low. Therefore, there was no support for the assumption that microcystin could penetrate cell membranes by simple diffusion. This conclusion indicated that microcystin required an active uptake mechanism.

The hepatocytic membrane contains carriers responsible for the active uptake of bile acids. These carriers are often collectively referred to as the bile acid transport
system, which acts in a multi-specific manner, that is many organic substances can be transported as bile salts.

Because complete inhibition of the hepatocellular uptake is achieved with microcystin in the presence of bile acid transport inhibitors and bile acid, suggests the bile acid transport system is a carrier for microcystin.

The consequence of acute liver intoxication, is a rapid and progressive alteration of the cells microfilaments which leads to aggregation of microfilaments near the centre of the cell. As a result of this loss of cellular support, the hepatocytes shrink. These shrunken cells separate from one another and from the sinusoids. The cells of the sinusoids separate as well, causing blood to spill into the liver tissue. Collectively the signs of poisoning in these animals include weakness, vomiting, cold extremities and diarrhoea. Death most usually results from intrahepatic haemorrhage and hypervolemic shock. This bleeding swiftly leads to death.

1.5.2.2.4.5 Biosynthesis

There have been two reports published on the biosynthesis of microcystins and nodularins.

Moore et al. investigated the biosynthesis of microcystin-LR from M. Aeruginosa PCC-7820. (2S)-Methionine, (2S)-phenylalanine, glutamic acid, acetate and pyruvate were found to be the precursors.

Converted products were analysed by $^{13}$C NMR spectra. The biosynthetic labelling pattern of these precursors, as determined by $^{13}$C NMR spectroscopy, for microcystin-LR is shown below (see Fig. 1.16).
Adda is biosynthesised from the carbon skeleton of phenylalanine and four molecules of acetate.

The Me groups on C6 and C8 of Adda are methionine-derived, whereas the C2 of Adda appears to arise from two sources one of which was methionine.

The (R)-Glu residue is derived from (S)-glutamic acid, but β-Me-Asp does not arise from rearrangement of glutamic acid, instead it was synthesised from acetyl-CoA and pyruvic acid; and

(S)-Glutamic acid and acetate are incorporated into the Arg residue.

Difficulties were encountered when studying the biosynthesis of nodularin due to the low yield of nodularin from the producing organism. Nevertheless, studies by Rinehart et al.\textsuperscript{161} reported similar findings to Moore.
1.5.2.2.4.6 Linear Peptides Relating to Microcystins

Rinehart et al.\textsuperscript{161} reported the isolation of a linear peptide, Adda-(\(R\))-Glu-Mdha-(\(R\))-\(\beta\)-Me-Asp-(\(S\))-Arg-OH, from cultured \textit{Nodularia spumigena}, and three linear peptides;

1) Adda-(\(R\))-Glu-Mdha-(\(R\))-(\(S\))-Leu-(\(R\))-\(\beta\)-Me-Asp-(\(S\))-Arg-OH,

2) (\(S\))-Leu-(\(R\))-\(\beta\)-Me-Asp-(\(S\))-Arg-Adda-(\(R\))-Glu-Mdha-(\(R\))-(\(S\))-Ala-OH, and

3) (\(S\))-Phe-(\(R\))-\(\beta\)-Me-Asp-(\(S\))-Arg-Adda-(\(R\))-Glu-Mdha-(\(R\))-Ala-OH

from a water bloom of \(M.\ sp\). The peptides obtained did not show apparent toxicity, and are considered to be biogenetic precursors of nodularins and microcystins.

1.5.2.2.4.7 Bacterial Degradation of Microcystin-LR

Recently it was reported degradation of microcystin-LR was mediated by at least three intracellular hydrolytic enzymes.\textsuperscript{162}

Microcystin-LR was cleaved between the Adda and Arg peptide bond by an enzyme designated, \textit{microcystinase} to yield an identical linear peptide, as first identified by Rinehart \textit{et al.}\textsuperscript{161} (see above). The linearised microcystin-LR was then cleaved between Ala and Leu with a second proteinase to give a tetrapeptide, Adda-(\(R\))-Glu-Mdha-(\(S\))-Ala-OH. The third enzyme gave rise to undetected smaller peptides and amino acids.

1.5.2.2.4.8 Synthesis of Microcystins/Nodularins

Several groups have reported the synthesis of the C\textsubscript{20} amino acid,\textsuperscript{163} Adda, the first in 1989 by Carmichael \textit{et al.}\textsuperscript{152} In this synthesis, the Adda residue was retro-synthetically divided into an amino acid part (C\textsubscript{1} to C\textsubscript{8}) and an aromatic part (C\textsubscript{7}-C\textsubscript{10}).
Using a combination of solid phase peptide synthesis and β-elimination in solution, Zetterström et al. synthesized the tetrapeptide fragment from microcystin containing the N-methyldehydroalanine (N-Mdha) amino acid. This was achieved by synthesis of the tetrapeptide, Ac-R-γ-Glu-[N-S-diMeCys]-R-Ala-Leu on MBHA resin and subsequent removal, followed by simple reduction of the diMeCys fragment.

In 1995, Shreiber and co-workers, published the first total synthesis of the natural product, motuporin. More recently, another total synthesis was reported by Chamberlin and co-workers, who synthesised Microcystin-LA. A solid phase synthesis of the microcystin macrocycle has also been published, using MBHA resin.

1.5.2.2.5 Molecular Modelling and NMR Studies

1.5.2.2.5.1 Molecular Modelling

Before the co-crystallised structure of human PP1 with microcystin (elucidated by Barford et al.), insight into the possible receptor-bound conformation of the okadaic acid class of compounds had to be gained from the free ligand information.

Partial structure elucidation of microcystin-LR was published by Rinehart in 1988. The configuration of the novel amino acid, Adda, was determined by NMR spectroscopy. These NMR spectroscopy studies did not reveal the complete solution structure of microcystin-LR. This provided the basis for Quinn and co-workers to use molecular modelling to determine the low energy conformation of the okadaic acid class of compounds with their receptors. The fact that microcystin-LR is conformationally restrained, due to its cyclic nature, and that it displays high affinity for the receptor, allowed it to be a useful tool for pharmacophore elucidation.
During the development of the pharmacophore model, a report describing the superimposition of microcystin-LR and nodularin was published by Lanaras et al.\textsuperscript{169} This work proposed that the Adda group in both microcystin-LR and nodularin were fitted together, the peptide rings of microcystin-LR and nodularin formed an angle of 90°.

Rigid superimposition of microcystin-LR and nodularin using ‘equivalent’ atoms of the two Adda residues, the arginine residues and the (R)-isoglutamic acid showed both the peptide rings aligned in the same plane as did the Adda amino residues.\textsuperscript{170} Contrary to the findings of Lanaras et al.,\textsuperscript{169} these results show that microcystin-LR and nodularin inhibit PP1 and PP2A with almost the same activity.

Quinn et al. then extended the model to include okadaic acid and calyculin A.\textsuperscript{171} In this study, okadaic acid was found to assume a cyclic conformation forming a cavity held together by an intramolecular hydrogen bond between the C1 carboxyl, attached to the first tetrahydropyran ring and the C24 hydroxyl group attached on the fourth tetrapyrany ring.\textsuperscript{169}

The minimum energy conformation of calyculin A, was shown to have a number of hydrogen bonds within the molecule forming a cavity similar to that formed in okadaic acid.\textsuperscript{169}

Molecular modelling allowed common regions of okadaic acid, calyculin A and microcystin-LR to be recognised and a pharmacophore model was developed.\textsuperscript{171} The Conserved Acid Binding Domain Pharmacophore Model, consists of a central core containing one conserved acid group and two potential hydrogen-binding sites (I and II), and a non-polar side-chain (see Fig. 1.17).
Figure 1.17: The conserved acid binding site of okadaic acid, calyculin A and microcystin-LR showing the potential hydrogen bonding areas I and II, and the hydrophobic side chains.

1.5.2.2.5.2 NMR Studies

Two three-dimensional structures of microcystins (microcystin-LR and -LY) were compared by Rudolph-Böhner et al.\textsuperscript{172} using 2D NMR spectroscopy in DMSO, and distance geometric calculations.

For microcystin-LY a single family of highly convergent structures was obtained.
This family is characterised by a relatively compact boat-like ring structure with the large side chain of the Adda residue protruding from the concave side, in close proximity to the Tyr side chain. Conversely, for the microcystin-LR the calculations result in three conformational families characterised by an even more compact ring structure. The Adda and Arg side chains protrude from the ring distal from one another caused by the access of the Adda side chain, an essential residue for activity, which results from the close proximity of the aromatic Tyr residue. A significant enthalpic cost would be expected for disturbance of this hydrophobic collapse and corresponding lower binding affinity to receptor molecules would be predicted. From the structures of the two related microcystins, and homology with other known toxins, a working hypothesis was proposed whereby, the Adda side-chain interacts with a hydrophobic groove of the phosphatases while the rest of the microcystin acts as a scaffold to help stabilise the interdigitation of the Adda with additional intermolecular interactions.

More recently, Bagu and co-workers extended this hypothesis to include motuporin. Microcystin-LR and motuporin solution structures were determined by 2D NMR spectroscopy in water. Once again, both structures consisted of a boat-like ring or saddle-shaped motif.
Furthermore, the solution structure of nodularin in water was published by Annila *et al.*\(^{174}\). The three-dimensional solution structure was studied by NMR spectroscopy and molecular dynamics simulations. From these studies, it was shown the cyclic backbone adopts a well-defined conformation, but the remote parts of the side chains of arginine as well as the Adda, have large spatial dispersion. This resembles very closely that of microcystin-LR in the chemically equivalent segment.

Instead of analysing the solution structures of the microcystin-LR in water\(^{173}\) or DMSO\(^{172}\) alone, Trogen *et al.*\(^{175}\) used a mixture of DMSO and water in order to determine the three-dimensional structures.

The receptors for these molecules are most likely to be composed of both hydrophilic and hydrophobic potions and therefore, DMSO will presumably better mimic these properties than can aqueous solutions. Furthermore, in order to mimic the viscosity in cells a DMSO/H\(_2\)O (80/20, v/v) cryoprotective mixture was chosen.

The conformational studies revealed only one conformational family in each solvent. Once again, the peptide ring has a saddled-shaped form, and the side-chains of Arg and the remote part of Adda are quite flexible without any well-defined structure. Structural differences are found in the residues Mdhb, Ala and Leu for the two solvents. The DMSO/water structure resembles more closely the structure of microcystin-LR complexed to PP-1.\(^{56}\) However, these structural differences do not affect the toxicity, since this part of the molecule is absent by Mdhb in the equally toxic nodularin.

**1.5.2.5.3 Molecular Models for Bound Motuporin, Microcystins, Okadaic Acid and Calyculin A**

Predictions for the bound conformations of the okadaic class of inhibitors have used a combination of solution structures from NMR studies, molecular modelling,
and X-ray structures of the bound inhibitors to PP1 and the free ligands (see Fig. 1.19).

![Figure 1.19: Superimposition of the three-dimensional structures of okadaic acid, calyculin A and microcystin-LR.](image)

The free NMR solution structure of microcystin-LR matches its crystal structure when bound to PP1c (see Fig. 1.20). The rigid saddle-shaped backbone provides a proper framework for the hydrophobic, ionic and covalent interactions between microcystin-LR and the phosphatase. These interactions involve the Adda and Masp/Glu residues, which are essential for inhibition, and the Mdha which is required for the secondary covalent linkage. The hydrophobic, Adda, group located behind the rigid saddle of microcystin-LR, is flexible in solution and therefore, able to adjust to fit into the hydrophobic groove of PP1c. Because hydrophilicity may be the initial driving force behind the binding of microcystin-LR into PP1c, the Adda residue could be responsible for anchoring the cyclic backbone ring into its bound position. The other contributing residues for inhibition, Masp and Glu, have orientated, negatively charged carboxyl groups, underneath the saddle, to interact with positively charged Arg-96 of PP1. Finally, the secondary covalent linkage that forms after inhibition is dependent on the modified amino acid Mdha being located at the front and top of the toxin saddle to link covalently to Cys-273.
Accurate docking of the unbound structures of microcystin-LL, motuporin, okadaic acid and calyculin A relies on the assumption that, like microcystin-LR, each of the free structures of these toxins would not change dramatically upon binding. It is also dependent on the assumption that PPlc itself does not differ greatly in conformation when microcystin-LR or the other inhibitors are bound. The latter assumption is supported by X-ray crystallographic determination of free PPlc (i.e. without microcystin-LR)\textsuperscript{67} which was found to be in overall agreement with the previously reported microcystin-LR-PPlc bound complex.\textsuperscript{66} This indicates that the ligand does not have a significant impact on the overall tertiary structure of PPlc when bound to the enzyme. Because microcystin-LL and motuporin are similar to microcystin-LR in terms of their structure and function, they were superimposed onto the bound crystal structure of microcystin-LR as a starting point for docking onto PPlc. A similar approach was employed for
okadaic acid and calyculin A, whose free crystal structures are very similar to the tertiary structure of microcystin-LR.

The absence of a covalent linkage between motuporin and PP1c is the only functional difference between bound motuporin and microcystin-LR. Docking of motuporin solution structure onto the crystal structure resulted in only a minor displacement from the starting point (see Fig. 1.21). Comparison of motuporin docked onto PP1c with the crystal structure complex between microcystin-LR and PP1c shows how the Mdhb residue in motuporin is not only surface accessible, but highly exposed to solvent, which is not true for the covalently bounded residue Mdha in microcystin-LR. The inability of motuporin and nodularin to form a covalent link to PP1c and PP2Ac may ultimately explain why the nodularins may be able to function as carcinogens. Since these toxins form no covalent linkages to PPases, their electrophilic Mdhb residues may be free to form direct adducts with nucleophilic groups on other informational macromolecules.

Figure 1.21: Superimposition of the free averaged solution structure of motuporin onto the bound crystal structure of microcystin-LR.

Motuporin and its labelled residues are in yellow. The blue structures and labelled residues belong to microcystin-LR. The white labelled residues are common between the two structures.
The model for the docked okadaic acid has distinct similarities to the bound microcystin-LR. This would explain how these structurally diverse toxins are able to inhibit PP1. The model of calyculin A, like okadaic acid, shares many features with bound microcystin-LR. In calyculin A, the unusual phosphate group is predicted to contribute to binding, possibly interacting with Arg96 and/or Arg221.

1.5.2.3 Other PP1 and PP2A Inhibitors

1.5.2.3.1 Cantharidin and Endothall

Cantharidin (exo, exo-2,3-dimethyl-7-oxobicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride), 19 is a naturally occurring toxin present in over 1500 different species of the Chinese blister beetle (*Mylabris phalerata*), (see Fig. 1.22). The toxin has also been isolated from the Spanish fly (*Cathar is vesicatoria*). The dried body of the Chinese beetle was first used by the people of China as a traditional medicine over 2000 years ago. The toxin was isolated in Europe during the 1800’s and used in the treatment of warts, as an aphrodisiac and as an abortifacient. By the early 1900’s cantharidin was determined to be too toxic for the use as an internal medicine.\(^{178}\)

![Figure 1.22: Cantharidin (19) and Endothall (20)](image)

In 1992, Li and Casida reported cantharidin, and a series of cantharidin analogues, including its herbicidal analogue, endothall 20, were mM inhibitors of PP1 and PP2A.\(^{179}\)
In 1991, Miao and Anderson reported the isolation of rubrolides A to H (21 A to H), a new family of tunicate metabolites, from *Ritterella nibra* (see Figure 1.23). The rubrolides are potent in vitro antibiotics and they show moderate, but selective, inhibition of PP1 and PP2A. They also represent a new family of biologically active tunicate metabolites which do not contain nitrogen, and two members of the family (B and H) are chlorinated which is unique to this type of metabolites.

**Figure 1.23: Rubrolides A - H**

![Rubrolides A - H](image-url)
1.5.2.3.3 Isopalinurin

A weak protein phosphatase inhibitor (IC₅₀ 2.5 mmol), was isolated from an Australian marine sponge, *Dysidea* sp. Isopalinurin 22, a sesterterpene tetronic acid was identified as an agent for antibiotic activity and also as a protein phosphatase inhibitor. This latter activity was not sufficient to warrant further testing of the compound, although related compounds and structural analogues are subject to ongoing investigations.

Isopalinurin (22)

1.5.3 Type 2B Serine/Threonine Phosphatase Inhibitors

1.5.3.1 Cyclosporin A, FK506 and Rapamycin

Cyclosporin A 23, FK506 3 and rapamycin 24 are naturally occurring secondary metabolites which inhibit PP2B (see Fig. 1.24).
Cyclosporin A is a lipid soluble, cyclic undecapeptide produced by *Tolypocladium inflatum*, a fungus isolated from a Norwegian soil sample. Cyclosporin A is valuable clinically as an immunosuppressant drug for organ transplant patients.

FK506 and rapamycin structurally related to each other, but not to cyclosporin A, are lipid soluble macrocyclic lactones. FK506 is produced by *Streptomyces tsukabaensis*, a soil bacterium from northern Japan. Rapamycin is produced from *Streptomyces hygroscopicus*, a bacterium isolated from a soil sample collected in Easter Island. Although FK506 and rapamycin are both immunosuppressants, they are still under evaluation as therapeutic agents.

On entering eukaryotic cells, cyclosporin A, FK506 and rapamycin bind to a distinct protein before binding to PP2B; cyclosporin A binds to the protein,
cyclophilin, whereas FK506 and rapamycin bind to the FK506-binding protein (FKBP).\textsuperscript{184,185}

Both these protein-drug complexes (cyclosporin A-cyclophilin and FK506-FKBP) specifically inhibit PP2B with nanomolar affinities.

Inhibition of PP2B prevents T-cell activation and hence, has revolutionised organ transplantation through its widespread use in the prevention of graft rejection.

1.5.3.2 Pyrethroid Insecticides

The pyrethroid insecticides contain two distinct classes; Type I and Type II class. A recent report by Enan and Matsumura have shown Type II pyrethroids [cypermethrin 25, deltamethrin 26, permethrin 27 and fenvalerate 28] are extremely potent inhibitors of PP2B with IC\textsubscript{50} values ranging from 0.01 to 1 nM (see Fig. 1.25).\textsuperscript{86}

![Pyrethroid Insecticides](image)

**Figure 1.25: Pyrethroid Insecticides**
1.6 Serine/Threonine Protein Phosphatase Substrates

Ser/Thr protein phosphatases can be differentiated by their ability to dephosphorylate small phosphopeptides.\(^{187}\)

1.6.1 Substrates for Protein Phosphatase 1

The substrate specificity of PP1 appears to be principally mediated by targeting subunits which associate with the catalytic subunit of PP1 and direct it to the target locus, thus increasing its activity toward specific substrates.\(^{188}\) PP1 is the protein phosphatase most reluctant to dephosphorylate phosphopeptide substrates, even if they are quite long, suggesting that a higher order of structure in the protein substrate is needed to optimise PP1 catalytic activity.

Like PP2A and PP2C, PP1 by far prefers Thr-P over Ser-P. Moreover, a prolyl residue immediately C-terminal to the phosphoamino acid is a strong negative determinant.\(^{189}\) Unlike PP2C and PP2B, however, PP1 activity towards peptides is not abolished by an acidic cluster C terminal to the phosphorylated residue,\(^{199}\) and seems to be substantially increased by multiple basic residues on the N-terminal side.\(^{191}\)

Recently, a very basic 25- amino acid corresponding to the phosphorylation site domain of the MARCKS protein, which contains several residues phosphorylated by PKC, has been reported to be dephosphorylated by PP1 as rapidly as phosphorylase-a, although not as efficiently as the intact MARCKS protein.\(^{92}\)

1.6.2 Substrates for Protein Phosphatase 2A

In contrast to PP1, PP2A is able to dephosphorylate a number of short phosphopeptides as efficiently as protein substrates, or even faster. This property of PP2A has been assessed with a variety of peptides.
Once again with PP2A it was found that short phosphothreonyl peptides were invariably dephosphorylated much more efficiently than their phosphoserine counterparts. Two such peptides in particular, RRATpVA and RRREEETpEEE(AA), proved far better substrates than phosphorylase itself.

1.6.3 Substrates for Protein Phosphatase 2B

Unlike PP2A and PP2C, PP2B is unable to dephosphorylate short peptide substrates (10 residues or less). Two phosphorylated nonadecapeptides, however, one corresponding to residues 20-38 of DARPP-32 were almost as good substrates as the parent protein. This suggests an extended stretch of N-terminal amino acids, possibly in the conformation of an amphipathic β-sheet, is required for efficient dephosphorylation.

1.6.4 Substrates for Protein Phosphatase 2C

The capacity of PP2C to dephosphorylate short phosphopeptides is reminiscent of PP2A. Also reminiscent of PP2A is the marked preference for phosphothreonyl peptides over their phosphoserine homologues. Consequently, the phosphothreonyl hexapeptide, RRATpVA is an excellent substrate for both PP2C and PP2A. In contrast to PP2A, however, PP2C is inactive towards phosphopeptides bearing acidic residue(s) adjacent to the C-terminal side of the phosphorylated residue.

1.6.5 Conclusions

The classes of substrates of Ser/Thr protein phosphatases fall into two categories:

- those whose activity toward short peptides is negligible as compared to protein substrates, namely PP1 and PP2B, and
- those which dephosphorylate a number of short phosphopeptides as readily as their protein substrates, namely PP2A and PP2C.
The two former phosphatases presumably require structural determinants outside the immediate vicinity of the phosphorylation site, for efficient dephosphorylation, while the latter are able to recognise such features which are included in the primary structure around the phosphorylated residue.

The principal phosphopeptide used for the sensitive and specific monitoring of Ser/Thr protein phosphatases are summarised below (see Table 1.2).

**Table 1.2: Principal phosphopeptides used for Assays**

<table>
<thead>
<tr>
<th>PHOSPHOPEPTIDES</th>
<th>USAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>KKKKRFSpFKKSpFKLSGFSpFKKNKK (MARCKS peptide)</td>
<td>assay of PP1</td>
</tr>
<tr>
<td>RRATpVA</td>
<td>sensitive assay of PP2A and PP2C</td>
</tr>
<tr>
<td>RREEETpEEE</td>
<td>sensitive assay of PP2A</td>
</tr>
<tr>
<td>LDPRQVEMIRRRRPTpPAML (DARPP-32 peptide)</td>
<td>assay of PP2B</td>
</tr>
<tr>
<td>DLDVIPGRFDVpVAAE</td>
<td>(also dephosphorylated by PP2A)</td>
</tr>
</tbody>
</table>
2. Results and Discussion

2.1 Introduction

The design and synthesis of specific inhibitors for either PP1 or PP2A is of vital importance, because they can provide valuable tools for the study and identification of cellular processes which are mediated by these PPases. Intervention in the signalling mechanisms of specific cell types could offer therapeutic opportunities in several areas of medicine, including cancer, host defence, arthritis and atheroma. At present there are no specific inhibitors for either enzyme. Once specific inhibitors are synthesised detailed SAR study can be carried out.
2.2 Design of Nodularin Analogues

The hepatotoxins, nodularins and microcystins, are cyclic isopeptides (see Fig. 2.1). Although they are of different size, they are very potent inhibitors of PP1 and PP2A (0.1 nM). Recent analysis has shown that the key functional groups on both systems could occupy similar enzyme bound positions in space, simultaneously. Hence, the similar groups in microcystins and nodularins can be assumed to be functionally equivalent.

![Diagram of Microcystin-LR and Nodularin](image_url)

Figure 2.1: Microcystin-LR and Nodularin

The cyclic toxins contain two free carboxylic acid groups, an N-methyl dehydroamino acid moiety and an unusual, C$_{20}$ lipophilic moiety (Adda).

The two α-carboxylic acids groups, (2R)-Glu and (2R, 3S)-3-methylaspartic acid residues, are known to be important since the ester derivatives of active toxins were inactive. Thus, both of these would have to be retained in the cyclic target. Several variants of microcystin, which retain biological activity, do not have the 3-methyl group of (2R, 3S)-3-methylaspartic acid.

The Adda residue, (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, is highly conserved in both families of the toxin. However, it has been reported that the stereochemistry of the double bond at C-6 of the Adda residue in both microcystin-LR and microcystin-RR is not crucial for biological activity and that the 6Z-isomer is only 100-fold less active against PP2A.
(ED$_{50}$ = 80 nM for both isomers) than the more common 6E-isomer (ED$_{50}$ = 0.28 M for microcystin-LR and 0.78 nM for microcystin-RR). Unsurprisingly, the Adda residue itself is totally inactive. A microcystin variant possessing a C-9 hydroxyl group in place of the methoxy group in the Adda side-chain was fully active. However, the catalytic hydrogenation product of microcystin-LR showed little or no activity as a hepatotoxin. Since, the hydrogenation reaction reduced not only the diene, but presumably also the dehydroamino acid residue this would indicate that either a rigid lipophilic Adda side-chain, or a Michael-type acceptor is required for biological activity, or possibly both.

Dr AP Mehrotra tested the importance of the dehydroamino acid, which is thought to serve as a Michael adduct with Cys-273 of PP1$_{\alpha}$, by the reduction of microcystin-LR with sodium borohydride. The resulting mixture of diastereoisomers (ca. ~5:1) were found to be as potent inhibitors of PP2A as microcystin-LR (Scheme 2.1). This result was also confirmed by the group of Mackintosh et al. who replaced the Cys-273 by alanine and found no significant detriment to activity which suggests that covalent bond formation is a secondary event and occurs reversibly.

![Scheme 2.1: NaBH$_4$ Reduction](image-url)
Therefore, designing a simpler macrocycle target for synthesis has to incorporate four of the five principal conserved features to retain biological activity:

- the cyclic isopeptide;
- the two carboxylic acids; and
- the rigid lipophilic amino acid.

The basic structure of nodularin was initially chosen to form potential protein phosphatase inhibitors, as it contains two fewer residues than microcystin without reduced potency.

Although the Adda moiety has been shown to be essential for inhibition, the first analogues chosen contained β-alanine as a simple replacement (see Fig. 2.2).[^15]

It was reasoned, on the basis of computer aided design, that the variable (2S)-Arg residue could be replaced by the less polar hydrophobic amino acid [(2S)-Phe]. This has the added advantage that it contains an UV chromophore which would aid chromatographic separation of intermediates during the synthesis of the macrocycle. The stereochemistry around the C-α centre of the N-methylalanine residue in the dihydromicrocystin samples had no affect on biological activity, therefore, it was first chosen to use glycine and later sarcosine as a replacement in the stripped-down macrocycle. Finally, the two α-carboxylic acids were retained in the analogues, but the (2R, 3S)-3-methylaspartic acid was replaced with simply (2R)-aspartic acid (see introduction).
2.3 Solution Phase Peptide Synthesis

Peptide synthesis originated in the work of Emil Fischer, whose goal was to prepare "albumoses," or proteins. This early work led to the formation of the peptide bond between α-amino acids, and established the importance of optical purity in synthetic products. In the 1930s attempts to synthesise a variety of small peptide substrates for the recently discovered proteolytic enzymes led to the development of the first reversible protecting group. The discovery of naturally occurring biologically active peptides stimulated an explosion of synthetic activity in the 1950s and 1960s, which laid the foundation for modern day synthesis.

Today, there are numerous methods for synthesising peptides in the solution phase, including the use of carbodiimides and active esters. The method chosen for the majority of the peptide synthesis in this work, was the mixed anhydride method of peptide coupling. This method was chosen for its advantages of high yield, speed and relative purity of products. The mixed anhydride method has been studied in detail to minimise the possibility of racemization and increase yield. The reaction involves a reaction of a protected amino or peptide acid with an alkyl chloroformate in the presence of a tertiary amine base, to form an anhydride. The anhydride is then reacted with an amine to form the peptide linkage.
It has been found that racemization at the amino acid peptide centres can be minimised depending on the steric bulk of the base and choice of solvent. It was found that, although methylamine reacts the fastest, it causes maximum racemization. The best results were achieved when a combination of NMM, iso-butyl chloroformate and dry THF were used. A minimum volume of dry DMF is often added when there is a problem with the solubility with one or more of the starting materials.

2.4 Original Design and Strategy

The preliminary target, replacing the Adda residue by β-alanine was thus identified as cyclo[-β-Ala-(2R)-Glu-α-OMe-γ-Sar-(2R)-Asp-α-OMe-β-(2S)-Phe-] 30. Disconnection of the cyclic nodularin analogue gives the linear pentapeptide H2N-(2R)-Asp-α-OMe-β-(2S)-Phe-β-Ala-(2R)-Glu-α-OMe-γ-Gly-OH 31. Subsequent disconnection of the linear pentapeptide gave two peptide fragments; AspPhe dipeptide 32, and SarGluGly tripeptide 33, (Scheme 2.2).

![Scheme 2.2: Disconnection of the nodularin based analogue](image-url)
Initial work, by Dr AP Mehrotra, made use of orthogonal protecting groups on the N- and C-terminal (N-benzyloxycarbonyl and benzyl ester respectively) of the pentapeptide 31.195

All attempts at cyclising the in situ pentapeptide failed. Although it was difficult to understand why the model cyclisation did not occur, it was conceivable that the NH moiety of the glycine residue, which is replaced by an NMe moiety in the natural toxins, was stabilising an unreactive conformation through transannular H-bond formation. Accordingly the synthesis was repeated using sarcosine in the place of glycine.

At the same time, the strategy for the use of protecting groups was altered so that the N-terminal and C-terminal functionality's could be unmasked separately in order to take advantage of a wider range of activated ester cyclisation protocols, including the pentafluorophenyl ester method. Hence, the linear pentapeptide, Boc-(2R)-Asp-α-OMe-β-(2S)-Phe-β-Ala-(2R)-Glu-α-OMe-γ-Sar-OBn 34 was chosen as our first target compound.

2.4.1 Synthesis of Linear Pentapeptide 34: Boc-(2R)-Asp-α-OMe-β-(2S)-Phe-β-Ala-(2R)-Glu-α-OMe-γ-Sar-OBn

(2R)-γ-Benzyl-N-butoxycarbonylglutamate 35 was treated with ethereal diazomethane to give the methyl ester 36 as a white amorphous solid (see Scheme 2.3). Catalytic hydrogenation of the benzyl ester followed by activation of the γ-carboxy group of compound 37 by using N-methyl morpholine (NMM) and isobutylchloroformate (IBCF) to give the mixed anhydride, which was then treated with benzyl sarcosinate p-toluene sulfonate 38 to afford the required amide 39 in 94% overall yield (mp 76-7 °C; [α]D +13.4 (MeOH)). The N-Boc protection was then removed using hydrogen chloride gas in ethyl acetate to give the amine salt 40 in 85% yield. Treatment with N-Boc-βAla then gave the tripeptide ester 41 in 82% yield (mp 78-9 °C; [α]D +26 (MeOH)). The 1H NMR spectrum showed two sets of signals, one set for each of the cis- and trans- rotomeric forms of the γ-Glu-Sar amide bond. Subsequent removal of the N-Boc protecting group using
hydrogen chloride gas in ethyl acetate afforded the hydrochloride salt 42 in 85% yield as an amorphous hydrosopic solid (see Scheme 2.3).

Scheme 2.3: Synthesis of βAlaGluSar Tripeptide 42

The required N-terminal β-aspartyl phenylalanyl dipeptide fragment 48 was prepared starting from (2R)-N-{butoxycarbonyl-β-benzyl aspartate 43, which was treated with ethereal diazomethane to give the methyl ester 44 as a white solid. Catalytic hydrogenation of the benzyl ester group followed by activation of the γ-carboxy group of compound 45 using NMM and IBCF to give the mixed anhydride which was then treated with benzyl phenylalanne p-toluenesulphonate 46 to give the dipeptide 47 in 72% yield (mp 132-3 °C; [α]D -4.4 (MeOH)). The benzyl ester group was removed with catalytic hydrogenation to give the required dipeptide 48 in quantitative yield.

The resulting free acid 48 was then activated by using NMM and IBCF and the resulting mixed anhydride was treated with the tripeptide hydrochloride 42 to afford the fully protected pentapeptide 34 in 72% yield (mp 82-3 °C; [α]D +6.22 (MeOH)).
In order to effect cyclisation, the pentafluorophenyl ester method was first considered. Thus, pentafluorophenyl ester 49 was prepared in three steps through catalytic hydrogenation of the benzyl ester group of pentapeptide 34, activation of the unmasked carboxylic acid group with EDCI, and finally, reaction of the activated ester with pentafluorophenol. The required PFP ester 49 was obtained in 63% yield after chromatographic purification (see Scheme 2.4).

Scheme 2.4: Synthesis of Linear Pentapeptide 49

However, after several attempts, none of the expected cyclic peptide 50 was obtained (as judged by $^1$H-NMR and mass spectrometry) after the N-protection was removed with TFA, and the amine salt treated with DIPEA (see Scheme 2.5).
This was in concordance with Dr. AP Mehrotra's findings when glycine was used instead of sarcosine. It is difficult to offer an explanation as to why this reaction repeatedly failed to work.

In order to offer an explanation as to why this reaction did not work, modelling of the amine salt was carried out by Dr. John Wilkie, using Discover by Biosym (a molecular mechanics program using the AMBER forcefield). This revealed, through dynamics run for 0.5 ns, that the lowest energy conformations in which the amine sat approached the pentafluorophenyl ester was not in the required orientation. The amine approached the ester at an angle of 68.1° which did not interact favourably with the electron density surrounding the carbonyl group (see Fig. 2.3).
In view of the fact that we were unable to effect macrolactamisation via this disconnection, a new disconnection (between the Phe and Asp, see Scheme 2.2) was examined which gave the linear pentapeptide precursor $\text{H}_2\text{N}-(2S)-\text{Phe}-\beta-\text{Ala}-(2R)-\text{Glu-}\alpha\text{-OMe-}\gamma-\text{Sar}-(2R)-\text{Asp-}\alpha\text{-OMe-}\beta-\text{Opfp}$ 51.

Once again, this peptide was modelled using the same dynamics as before (see above). The modelling revealed that the amine could this time approach the ester in an orientation which should affect cyclisation. The angle was favourable with the electron density surrounding the carbonyl group (90.3°), and the distance between the two ends of the linear pentapeptide was 3.53 Å which is close enough for cyclisation (see Fig. 2.4).
Indeed when Dr AP Mehrotra attempted the cyclisation of 51, the desired cyclic peptide 29 was obtained in 24% overall yield.\textsuperscript{201}
2.4.2 Synthesis of Restrained Proline-containing Cyclic Pentapeptides;

cyclo[β-Ala-(2R)-Glu-α-OMe-γ-Pro-(2R)-Asp-α-OMe-β-(2S)-Phe] 52

The NMR spectral data for the sarcosine-containing cyclic pentapeptide, prepared by Dr AP Mehrotra, showed the existence of three stable populated conformations. These were assigned using TOCSY, HSQC and COSY NMR experiments. However, it was not possible to assign specific NMR signals to the conformational structures for the macrocycle, as the sarcosine residue allows free movement about the Cα-N bond and possesses no N-H proton which can report on the torsional angle about that bond. Therefore, we decided to consider the syntheses of restrained analogue containing (2S) and (2R)-Pro in the place of Sar in order to circumvent these problems.

Disconnection of the proline containing pentapeptide 52, gives the linear pentapeptide precursor H2N-(2S)-Phe-β-Ala-(2R)-Glu-α-OMe-γ-Pro-(2R)-Asp-α-OMe-β-OH 53. Subsequent disconnection gives two peptide fragments; PheβAla dipeptide 54, and GluProAsp tripeptide 55 (see Scheme 2.6).
We decided to embark on the synthesis of the (2S)-proline containing constrained macrocycle.

In order to construct the GluProAsp tripeptide 55, two alternative approaches were considered: one starting from the C-terminal (see Scheme 2.7) and the other starting from the N-terminal (see Scheme 2.8).

The synthesis of the N-terminal tripeptide started with (2S)-proline which was reacted with p-toluene sulfonic acid and benzyl alcohol under Dean-Stark conditions to give benzyl prolinate p-toluene sulfonate 56A as a yellow oil, which was contaminated with benzyl alcohol (see Scheme 2.7). The crude benzyl ester was then coupled with Boc-(2R)-Glu-α-OMe 37 to give the dipeptide which was subsequently deprotected and reacted with (2R)-Asp-αOMe-βOBzl 58 to give the
tripeptide 59A. However, due to the difficulty encountered with the purification of the intermediates (due to benzyl alcohol contamination), this route was abandoned.

Scheme 2.7: Synthesis of Tripeptide (61A)

The synthesis of the C-terminal, on the other hand, started with β-benzyl (2R)-N-(tert-butoxycarbonyl)aspartate 63. Treatment of the benzyl ester with ethereal diazomethane followed by N-Boc deprotection using dry HCl gas in ethyl acetate gave the amine salt 59 as a colourless oil in 91% yield. Reaction of the amine salt 59 with the mixed anhydride of N-Boc-(2S)-proline, afforded the dipeptide 64A as a clear oil in 78% yield, ([Found: C, 61.1; H, 7.3; N 6.2. requires C_{22}H_{30}N_{2}O_7: C, 60.8; H, 7.0; N, 6.4%]; [α]_D -5.41 (MeOH)).  N-terminal deprotection of the dipeptide 64A with dry HCl, and subsequent mixed anhydride coupling to N-Boc-(2R)-Glu-αOMe 37, gave the tripeptide 66A in 82% yield (mp 92-3 °C; [α]_D -19.2 (MeOH)). Finally, the tripeptide was N-terminal deprotected with dry HCl gas, to give the hydrochloride salt 67A in 84% yield (see Scheme 2.8).
2.4.2.1 Synthesis of the N-terminal Dipeptide

The required N-terminal phenylalanyl β-alanyl dipeptide fragment of target 68A was prepared by first activation of N-Boc-(2S)-phenylalanine 69 by IBCF and NMM, to give the mixed anhydride, followed by reaction with benzyl β-alaninate p-toluene sulfonate 70 to yield the dipeptide 71 in 85% (mp 92-3 °C; [α]D +0.63 (MeOH). Deprotection of the benzyl ester group by catalytic hydrogenation then gave the free acid 72 in quantitative yield (see Scheme 2.9).
Reagents and conditions: i, NMM, t-BuOCOCl, β-Ala-OBn,p-TsOH 70, THF, DMF, -15 °C->r.t., 12 h, 87%; ii, H₂, 10% Pd/C, EtOH, r.t. 12 h, 99%; iii, NMM, t-BuOCOCl, 67A, THF, DMF, -15 °C->r.t., 12 h, 68%; iv, H₂, 10% Pd/C, EtOH, r.t. 12 h, 99%; v, EDCI, C₆H₄OH, CH₂Cl₂, 0 °C->r.t., 12 h, 68%; vi, TFA, CH₂Cl₂, r.t., 45 min, quant.; vii, DIPEA, CH₂Cl₂, r.t., 7 days, 54%.

Scheme 2.9: Synthesis of Cyclic Pentapeptide (52A)

The dipeptide 72, was activated using NMM and IBCF, and the resulting mixed anhydride treated with the tripeptide hydrochloride 67A and NMM to afford the required fully protected pentapeptide 68A in 68% yield, after recrystallisation from ethyl acetate and hexane (mp 118-9 °C; [α]₀ +31.67 (MeOH)).

In order to activate the peptide for cyclisation, the C-terminal benzyl ester of 68A was removed through catalytic hydrogenation and was replaced by a pentafluorophenyl ester group, using EDCI and pentafluorophenol in dichloromethane, to give the ester 74A in 68% yield, after chromatographic purification. Deprotection of the N-Boc pentapeptide PFP ester 74A with TFA in dichloromethane gave the amine salt which was dried under high vacuum. The
amine TFA salt was treated with DIPEA in dichloromethane under conditions of very high dilution.

The cyclisation reaction was followed by TLC (see experimental section), and after seven days at room temperature, the appearance of a new less polar band was judged to be complete. Immediately, after concentration of the reaction mixture under reduced pressure, the residue was purified by flash chromatography and recrystallised from acetone/diethyl ether to afford the desired cyclic isopeptide 52A in 52% yield. The macrocycle (mp >220 °C (decomp.)), was found to be insoluble in most organic solvents, but was soluble in DMSO.

The epimer 52B, containing the (2R)-Pro residue instead of the (2S)-Pro was prepared in exactly the same manner, and was obtained in 16% overall yield.

Both macrocycles gave satisfactory analytical data. The TOCSY spectrum revealed that each macrocycles existed as a mixture of the trans- and cis-γ-Glu-Pro amide rotomers (see Fig. 2.5 and Fig. 2.6).

In each case the replacement of the Sar residue by Pro allowed us to deduce the 3-D structures of each conformer to be deduced as required.

Figure 2.5: TOCSY Spectrum 1 of Macrocycle
The three-dimensional structure for the major isomer (trans isomer) in the (2S)-proline macrocycle was deduced by restrained simulated annealing, using twenty H-H distance restraints generated from a ROESY experiment. Each ROE
interaction was defined as strong, medium or weak, and energy penalties were applied when the corresponding H-H distances lay outside the range: 0.18-0.27 nm; 0.18-0.33 nm; and 0.18-0.5 nm, respectively (see Appendix 2). Nine of the ten initial models converged to a consensus structure. The lowest energy structure had a low ROE forcing potential (6.9 kcal/mol), indicating that it was consistent with experimental data, and showed close structural similarity with the corresponding portion with microcystin-LR (see Fig. 2.7).

![Three-Dimensional Structures of Macrocycle](image)

**Figure 2.7: Three-Dimensional Structures of Macrocycle**

### 2.5 Solid Phase Synthesis for Nodularin Analogues

While the synthetic route to the nodularin macrocycle was proving to be fairly efficient, the time required to prepare such compounds was too long to employ for the preparation of the hundreds of structural variants needed for a full SAR study.
One of the most time-consuming steps was the purification of intermediates since this usually required a different procedure for each step. Solid phase peptide synthesis, on the other hand, unifies the purification of intermediates to simple washing procedures.

Two solid phase routes were, therefore, considered in which:

- the entire isopeptide would be prepared and cyclised on the resin; and
- the solid-phase assembled isopentapeptide would be removed from the resin prior to cyclisation.

### 2.5.1 Solid Phase Peptide Synthesis

The idea of solid phase peptide synthesis (SPPS), which uses the attachment of one of the reactants of a reaction to an insoluble, solid support with subsequent purification by filtration, was first conceived by Merrifield in 1959. In this method the carboxy terminal amino acid is covalently adhered to an insoluble polymer via a linker, and the peptide is grown one residue at a time, on this polymer. Solutions containing the appropriate reagents are allowed to contact the polymer with shaking. At the conclusion of each step, the polymer containing the peptide is simply filtered away from the solution which contains soluble by-products and impurities. The completed peptide is then removed from the polymer resin by a simple cleavage step (see Scheme 2.10). The advantage of this method is the ease with which the peptide is separated from soluble by-products of the reaction.

Merrifield's original approach was based on a step-wise strategy starting with the C-terminal residue.\(^{202}\) About the same time, Letsinger and Kornet recommended a similar technique in which the N-terminal residue was protected by a polymeric form of the benzyloxy-carbonyl group, and the chain was lengthened stepwise.\(^{203}\) However, because of the inherent problem of racemization in the elongation of the peptide chain from the N-terminal residue, the Merrifield approach remains the method of choice.
The solid phase peptide synthesis method developed by Merrifield utilises a Boc-amino blocking group.\textsuperscript{204} The repeated acidolysis required for Boc deprotection could lead to alteration of sensitive peptide bonds as well as, acid catalysed side reactions, therefore the development of other protecting groups became important - the base labile N-\(\alpha\)-protecting group, 9-fluorenylmethoxy-carbonyl (Fmoc) group is one such group.\textsuperscript{205,206}

The Fmoc group is rapidly removed by primary (ethanolamine) and some secondary amines (piperidine), whilst more slowly by tertiary amines. Removal is
also more rapid in polar media (DMF). The Fmoc group is most often deprotected by 20% piperidine/DMF, although other amines have also been used.

The electron withdrawing fluorene ring of the Fmoc group renders the lone hydrogen on the β-carbon very acidic, and therefore susceptible to removal by weak bases. After β-elimination, the Fmoc group proceeds through a carbanion intermediate to form dibenzofulvene (see Scheme 2.11).

\[ \text{Scheme 2.11: Deprotection of the base-labile Fmoc Group} \]

The type of resin used for the synthesis is also of vital importance. There are several resins offering use with Fmoc-based strategies. We chose to use Wang resin which is the most successful and widely used polystyrene-based resin known to date.\(^{207}\)

\[ \text{Wang (4-Alkoxybenzyl) Resin 75} \]

The resin consists of a polystyrene bead onto which an acid-labile linker has been attached.
2.5.2 Synthesis of the Linear Pentapeptide; β-Allyl (2S)-N-(9-fluorenylmethoxycarbonyl)phenylalanyl-β-alanyl-(2R)-α-methylglutamyl-γ-(2R)-prolyl-(2R)-aspartate 76

The disconnection of the cyclic pentapeptide gives the linear precursor (2S)-phenylalanyl-β-alanyl-(2R)-glutamyl-α-OMe-γ-prolyl-(2R)-aspartate 53, as before. This was synthesised sequentially from the C-terminus using Wang resin and Fmoc protecting strategy for the N-terminal amino acids.

However, in order to cyclise the linear pentapeptide, a suitable protecting strategy for the α-carboxylic acids on aspartic and glutamic acids was required.

The general methodology for SPPS is based on the use of independent classes of protecting groups which can be removed by a specific chemical treatment that does not affect the protecting groups of other classes. By definition, such classes of protecting groups are said to be orthogonal.

The concept of orthogonality has been applied to this synthesis on solid support by the use of Fmoc and Me protecting groups for the protection of the N-terminus and the side-chains of the growing peptide, respectively. Selective cleavage of the allyl group, using a palladium catalyst, for the respective protection of the β-carboxylic acid on the aspartic acid allows for a third dimension of orthogonality.

2.5.2.1 Synthesis of α-Methyl-(2R)-N-(9-fluorenylmethoxycarbonyl) glutamate 77

In order to synthesise the α-methyl protected Fmoc-glutamic acid 77 (the starting material for one of the intermediates) two methods were tried. The first employed protection of the γ-carboxyl group as a benzyl ester, which could be removed after Fmoc protection by catalytic hydrogenation (see Scheme 2.12). Allyl ester
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Protection of the γ-carboxyl group of glutamic acid was the second method to be considered (see Scheme 2.13).

γ-Benzyl-9-fluorenylmethoxycarbonyl (2R)-glutamate 79 was prepared from γ-benzyl-(2R)-glutamate 78 and fluorenylmethoxycarbonyl chloride under basic conditions to give the required compound as a white solid in 72% yield (mp 137-8 °C; [α]₀ -12.9 (MeOH)). The resulting compound was treated with ethereal diazomethane to give the methyl ester 80. Subsequent catalytic hydrogenation, to remove the benzyl ester, failed to give high yields of the required acid. This was due to instability of the Fmoc group under such conditions, even after addition of a small amount of TFA to the reaction mixture, the yield was still very poor.

Reagents and conditions: i, Fmoc-Cl, KCO₃, dioxane, water, 82%; ii, CH₂N₂, EtOAc, Et₂O, quant.; iii, H₂, 5% Pd/C, EtOH, r.t., 12 h, 20%.

Scheme 2.12: Synthesis of glutamate (77) using γ-benzyl ester protection

Thus, the strategy with allyl ester protection seemed more attractive at this stage (see Scheme 2.13).
The allyl ester group was added to the γ-carboxy group of (2R)-glutamic acid 81, under acidic conditions, with allyl alcohol to give the hydrochloride salt in 72% yield. The N-terminal was treated with fluorenylmethoxycarbonyl chloride, and then the α-carboxyl group protected with diazomethane, to yield the fully protected diester 84 in 82% overall yield (mp 84-5 °C, [α]D + 22.14 (MeOH)).

\[
\begin{align*}
\text{HO}_2\text{C} & \quad \text{H} \\
\text{H}_2\text{N} & \quad \text{O H} & \quad \text{I I}
\end{align*}
\]

Reagents and conditions: i, TMS-Cl, Allyl-OH, 72%; ii, Fmoc-Cl, KCO₃, dioxane, water, 82%; iii, CH₂N₂, BtOAc, Et₂O, quant.; iv, Pd(PPh₃)₄, PhSiH₃, DCM (dry), 76%.

Scheme 2.13: Synthesis of glutamate (77) using γ-allyl ester protection

In 1950 the allyloxycarbonyl group (Aloc) was introduced by Stevens and Watanabe for the protection of amine and alcohol functions. Cleavage of the Aloc group was accomplished by catalytic hydrogenolysis using platinum or palladium catalysts, the use of metallic sodium in liquid ammonia, or the use of phosphonium iodide in glacial acetic acid. Unfortunately, these methods for removal of the Aloc group were not straightforward, which limited its use in organic synthesis. With the palladium catalysed allyl removal reactions developed by Tsuji and Trost, the greater potential of the allyl protecting groups was recognised.
Allyl carboxylates and allyl carbonates are rapidly cleaved in the presence of soluble palladium catalyst [typically Pd(PPh₃)₄] and a nucleophile acting as an allyl scavenger, to regenerate the free unprotected function. Such catalytic methodology is of special interest for peptide synthesis because the deprotection conditions are usually mild enough to be compatible with the presence of the acid labile butyryl, Boc protections and the base labile Fmoc protections, provided, in the latter case, that a nucleophilic allyl group acceptor of sufficiently low basicity is used.

A variety of nucleophilic species has been used for intercepting the intermediate π-allyl complexes, which includes carbon nucleophiles, heterophiles (amines, thiols and carboxylates), and hydride donors (HCO₂H, Bu₃SnH, NaBH₄).

A variety of these nucleophiles (pyrrolidine, dimedone) were tried with our amino acid 83, however, on work up, the yields were typically very low (5-25%). On analysis of the NMR spectra (¹H & ¹³C), it appeared that the Fmoc group was being removed, as well as the allyl ester.

In 1995, Dessolin et al.²¹¹ reported the use of a new allyl group scavenger, the hydride donor phenyltrihydrosilane (PhSiH₃). This cleaves allyl carboxylates to silyl derivatives within 5 to 15 min. [In dichloromethane at room temperature in the presence of Pd(PPh₃)₄ (0.02 molar equivalent) and PhSiH₃ (1.2 - 2 molar equivalents)]. The silyl derivatives may, in turn, be instantaneously hydrolysed upon exposure to water.
The phenyltrihydrosilane reagent was successful in our hands, and gave the desired ester 77 \( \text{mp 130-1 } ^\circ \text{C; } [\alpha]_D +19.5 \) \( \text{(MeOH)} \) in 76% after purification by column chromatography.

2.5.2.2 Synthesis of \( \beta\)-Allyl (2\( R\))-N-(9-fluorenylmethoxycarbonyl)-aspartyl-Wang resin 85

Using the same procedure as for the Fmoc-Glu-\( \gamma \)-allyl ester 83, the Fmoc-Asp-\( \beta \)-allyl ester 88 \( \text{mp 110 } ^\circ \text{C, } [\alpha]_D +3.04 \) \( \text{(MeOH)} \) was prepared in 86% overall yield and then attached to Wang resin, using a method developed by Seiber (see Scheme 2.15). The \( \alpha \)-carboxyl group was activated as its 2,6-dichlorobenzoic anhydride, using 2,6-dichlorobenzyol chloride, and then reacted with the 4-hydroxymethyl group of Wang resin to give the immobilised diester. The remaining hydroxyl groups on the resin were then benzoylated with benzoyl chloride and pyridine.
Scheme 2.15: Addition of the First Residue (Asp) onto Wang Resin

The loading was checked using a method from the technical notes from Novabiochem\(^{213}\) (see Appendix 4)

Using the above procedure, loadings between 70-80% were achieved

2.5.2.3 Solid Phase Synthesis of Peptide Sequence 76

The SPPS of the linear pentapeptide was carried out on a Rainin PS3 automated peptide synthesiser.

The Fmoc group was removed by a 20% piperidine/DMF solution. A 5% NMM/DMF solution was used for the activation of the Fmoc-(2S)-Pro 89 by PyBOP, which was coupled to the resin bound amino acid. Once again, removal of the Fmoc group followed by reaction with PyBOP-activated Fmoc-(2R)-Glu-α-OMe 77 gave the immobilised Fmoc-tripeptide triester.
Removal of the Fmoc group followed by reaction with PyBOP activated Fmoc-β-Ala 90 gave the tetrapeptide triester which was deprotected and reacted with PyBOP-activated Fmoc-(25)-Phe 91 to give the Fmoc-pentapeptide triester 76 (see Scheme 2.16).

At this stage, a small amount of peptide (30 mg) was cleaved from the resin using a mixture of DCM/TFA/TEA/water (53:40:2.5), and subsequently precipitated with ether to yield the required pentapeptide 76A in almost quantitative yield (mp 115-6 °C; [α]o -13.0 (MeOH)).

### 2.5.3 Isopeptide Cyclised On Wang Resin using PyBOP

With the successful synthesis of the linear pentapeptide on the resin, the allyl ester was removed, under nearly neutral conditions, with Pd(PPh3)4 in DMSO/THF/0.5 M HCl (aq.)/morpholine (2:2:1:0.1). The 1H NMR spectrum of a sample cleaved from the resin by TFA at this stage showed that the allyl group had been completely removed. The N-terminal Fmoc group was removed using 20% piperidine/DMF to give the free N-amino acid group [monitored by the Kaiser test (see Appendix 4)], (see Scheme 2.16).

Once again a small amount of the cleaved peptide displayed the expected NMR and mass spectral data.

Cyclisation was carried out using PyBOP and HOBt in the presence of DIPEA in DMF. A negative Kaiser test (see Appendix 4) showed that no free acid groups were present after 7 days. Cleavage from the resin using TFA and subsequent ether precipitation gave the crude macrocyclic monoester 93 in 78% overall yield. Reverse-phase HPLC analysis of the material 93 (ca. 50% of the total) displayed one major band and several close running bands which were subsequently removed by HPLC (see Fig. 2.8). The pure material (obtained in 30% yield) eluted as a single band and gave the expected ES mass spectrum (574 Da, M+).
Removal of the Fmoc group followed by reaction with PyBOP activated Fmoc-β-Ala 90 gave the tetrapeptide triester which was deprotected and reacted with PyBOP-activated Fmoc-(2S)-Phe 91 to give the Fmoc-pentapeptide triester 76 (see Scheme 2.16).

At this stage, a small amount of resin (30 mg) was cleaved from the resin using a mixture of DCM/TFA/TEA/water (53:40:2:5), and subsequently precipitated with ether to yield the desired pentapeptide 76A in almost quantitative yield (mp 115-6 °C; [α]D -13.0 (MeOH)).

2.5.3 Isopeptide Cyclised On Wang Resin using PyBOP

With the successful synthesis of the linear pentapeptide on the resin, the allyl ester was removed, under nearly neutral conditions, with Pd(PPh3)4 in DMSO/THF/0.5 M HCl (aq.)/morpholine (2:2:1:0.1).214 The 1H NMR spectrum of a sample cleaved from the resin by TFA at this stage showed that the allyl group had been completely removed. The N-terminal Fmoc group was removed using 20% piperidine/DMF to give the free N-amino acid group [monitored by the Kaiser test (see Appendix 4)], (see Scheme 2.16).

Once again a small amount of the cleaved peptide displayed the expected NMR and mass spectral data.

Cyclisation was carried out using PyBOP and HOBT in the presence of DIPEA in DMF.214 A negative Kaiser test (see Appendix 4) showed that no free acid groups were present after 7 days. Cleavage from the resin using TFA and subsequent ether precipitation gave the crude macrocyclic monoester 93 in 78% overall yield. Reverse-phase HPLC analysis of the material 93 (ca. 50% of the total) displayed one major band and several close running bands which were subsequently removed by HPLC (see Fig. 2.8). The pure material (obtained in 30% yield) eluted as a single band and gave the expected ES mass spectrum (574 Da, M+).
Scheme 2.16: Cyclisation to give 93 Achieved on Wang Resin
Figure 2.8: HPLC of macrocycle, before and after purification (dashed line)

$^1$H and $^{13}$C NMR spectra recorded in DMSO, and subsequently analysis using COSY, TOCSY and HSQC experiments, revealed the presence of two major conformers, as expected, corresponding to the \textit{trans}- and \textit{cis}- Glu-$\gamma$-Pro rotomers. Saponification of the monoester gave a diacid 94A which displayed identical ES mass spectrum (560 Da, M$^+$) and HPLC band to that derived from the solution phase synthesis.

Treatment of a sample of the crude material with diazomethane gave the crude diester 52A which displayed several NMR spectral signals coincident to those for
the pure solution-phase synthesised material, and further analysis indicated that the crude macrocycle was ca. 50% pure, in keeping with the HPLC analysis of the precursors.

2.5.4 Isopeptide Cyclised On Wang Resin using pentafluorophenyl activation

In an attempt to improve the overall yield of 30% for the macrocycle 93 it was decided to cyclise the linear pentapeptide using pentafluorophenyl activation (see Scheme 2.17).
The allyl protecting group on the linear pentapeptide 76, was removed as before, and converted to the pfp ester 95 using pentafluorophenol and EDCI. The pfp ester was treated with 20% piperidine in DMF, to remove the Fmoc protection, to give the diester 96. A small amount of this material was cleaved from the resin using TFA to give the desired compound which gave the expected NMR ($^1$H, $^{13}$C and $^{19}$F) data. Treatment of the diester 96 with DIPEA in DMF gave the resin-
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bound macrocycle. Removal of the resin then gave crude macrocycle 93 which was of similar purity to the material prepared using PyBOP-activation on analysis by reverse-phase HPLC (see Fig. 2.9).

![HPLC plots of Cyclisation using DIPEA and PyBOP](image)

Figure 2.9: HPLC plots of Cyclisation using DIPEA and PyBOP

2.5.5 Linear Isopentapeptide Prepared on Wang-Resin, But Removed From Resin Prior To Cyclisation

In order to discover the basis of the low purity of the cyclised materials on the resin, the linear pentapeptide 76 was removed from the resin, and the free Asp α-carboxy group was methylated with diazomethane. The crude product 97 was
> 95% pure as judged by NMR and HPLC, and gave the expected analytical and spectroscopic data {mp 120-1 °C; [\(\alpha\)]D -17.8 (MeOH)}.

The allyl group and Fmoc groups were removed sequentially (see Scheme 2.18) to give the amino acid 99 which was then cyclised, through activation of the carboxy group with PyBOP to afford the crude macrocycle 52A in almost quantitative recovery (see Scheme 2.18).
Scheme 2.18: SPPS of Linear Isopeptide (97) and then Off-resin Cyclisation

Reagents and conditions: i, DCM, TFA, H₂O, TES (50:42:5:3), quant.; ii, CH₂N₂, DCM, Et₂O quant.; iii, Pd(PPh₃)₄, PhSiH₃, DCM, 70%; iv, Piperidine, DMF, 97%; v, PyBOP, DIPEA, DMF, 78%; vi, 1 M NaOH, MeOH.
NMR analysis indicated that this material was at least 85% pure and identical to the material obtained from the solution-phase synthesis (see Fig. 2.10 and Scheme 2.8).

Figure 2.10: NMR of 52A from solution phase and solid/solution phase

Although the presence of the resin appears to inhibit the macrolactamation step compared to the situation in solution, this methodology offers significant advantages in the construction of the linear isopentapeptide precursor. Therefore,
a combination of solid and solution phase synthesis offers the best strategy to the construction of cyclic isopeptides.

The synthesis can be shortened further by designing the linear isopentapeptide to be attached to the Wang resin at the β-carboxy position instead of the α-carboxy position. This will allow cyclisation to occur immediately after cleavage from the resin (see Scheme 2.19).
Reagents and Conditions: i, 20% Piperidine, DMF; ii, DCM, TFA, H2O, TES (50:42:5:3), quant.; iii, PyBOP, DIPEA, DMF, 78%; iv, 1 M NaOH, MeOH.

Scheme 2.19: Synthesis of Linear Pentapeptide Attached to the Resin on the β-Carboxylic Acid, then Cyclisation off Resin

2.5.5.1 Synthesis of α-Methoxy (2R)-N-(9-fluorenylmethoxycarbonyl) aspartyl-Wang resin 104

Using the same procedure as for the Fmoc-Glu-α-OMe ester 77, the
Fmoc-Asp-α-OMe ester 103 {mp 124-5 °C; [α]D +19.05 (MeOH)} was synthesised (see Scheme 2.13). Attachment to Wang resin then gave the immobilised diester 104. Loadings were comparable to the aspartic acid attached at the α-carboxy position (82%) (see Scheme 2.20).

Reagents and conditions: i, CH3N2, EtOAc, Et2O, quant.; ii, Pd(PPh3)4, PhSiH3, DCM (dry), 78%; iii, Wang Resin (0.84 mmol g⁻¹ substitution), pyridine, 2,6-dichlorobenzoylchloride, DMF, then benzoyl chloride, pyridine, DMF, 82% loading.

Scheme 2.20: First Residue Attached to Wang Resin on β-carboxylic acid

The resin attached Fmoc-Asp-α-OMe is now ready to be used in the peptide synthesiser for the synthesis of the linear isopentapeptide. Removal of the Fmoc group would then allow cyclisation using PyBOP.

2.6 Introduction of Adda Moiety

Whilst the construction of the cyclic peptide using solid-phase peptide synthesis was being carried out, it was important to examine functional groups which could be utilised for the incorporation of a rigid lipophilic moiety at the Adda position and hence give biological activity.
Either the Adda moiety could be added before cyclisation or after cyclisation.

2.6.1 Introduction of Adda Moiety After Cyclisation

2.6.1.1 Previous Studies - Diene

Dr A P Mehrotra designed a diene fragment which could be incorporated into the macrocycle via a hydroxymethyl analogue, which could undergo subsequent oxidation and a Wittig reaction with an appropriate ylide (see Scheme 2.21).

![Scheme 2.21: Synthesis of Diene Fragment](image-url)

Reagents and conditions: i. LiBF₄, NMM, THF, -30 °C, 30 mins; ii. NaBH₄, THF, -15 °C to r.t., 82%; iii. Dess Martin periodinane, CH₂Cl₂, 15 h, r.l.90%; iv. NaBH₄, EtOH, 40 min, 79%; v. PBr₃, pyridine, hexane, 0 °C to r.t., 85%; vi. PPh₃, toluene, 6 h, a; vii. BuLi in hexane, THF, -78 °C to r.t. 30%.

In order to transfer the chemistry from the model synthesis to the macrocycle, the β-alanine was replaced by the hydroxymethyl analogue. The macrocycle containing sarcosine was prepared using the same chemistry as for the β-alanine macrocycles 52, however, the desired product was not only found to be unstable, but was also obtained in a very low yield.

We attempted to prepare the (2S)- and (2R)-proline hydroxymethyl macrocycles using solution phase synthesis, however, the linear pentapeptides appeared to be unstable and gave a variety of unidentified breakdown products. On completion of the synthesis, the macrocycles were obtained in approximately 10% yield, and appeared to be as unstable as their linear counterparts.
We attempted the same strategy using solid phase synthesis, however, the transfer of the hydroxymethyl compound for use on the solid phase was also problematic. The synthesis of \((2R)-N-(9-\text{Fluorenylmethoxycarbonyl})-4\text{-hydroxy-1-butyrate}\) (the starting material for the hydroxymethyl intermediate for the solid phase), was attempted from the Fmoc-\(\beta\)-allyl-(2\(R\))-aspartate 88 which was converted to the mixed carbonic anhydride and then reduced with sodium borohydride in THF to give the alcohol 113 in 84% yield \(\{\text{mp } 124-5 \degree \text{C}; [\alpha]_{D} +8.14 \text{ (MeOH)}\}\). Removal of the allyl ester using Pd(PPh₃)₄ and PhSiH₃, however, led to the formation of a \(\gamma\)-lactone ring 114 exclusively in 90% yield (HRMS: found \([M + H]^+ 324.1239. C_{19}H_{18}NO_4 \text{ requires } 324.1236\), (see Scheme 2.22).

\[
\begin{align*}
\text{Reagents and conditions:} & \text{ i, IBCF, NMM, THF,} -30 \degree \text{C, 30 mins; ii, NaBH}_4, \text{THF,} -15 \degree \text{C to r.t., 84%; iii, Pd(PPh}_3)_4, \text{PhSiH}_3, \text{DCM, 90%}.}
\end{align*}
\]

Scheme 2.22: Preparation of Hydroxymethyl Aspartate

In light of the instability of the alcohol pentapeptides, and the problems encountered synthesising the hydroxymethyl amino acid for the solid phase strategy, a new approach for the synthesis of precursors for the addition of the Adda moiety was considered.
2.6.2 Benzyl Ester Functionality

We proposed, that if a β-alanine was replaced by α-carboxy protected aspartate residue, this could be unmasked once cyclisation had taken place and subsequent reactions could be achieved on the free carboxylic acid to yield potential inhibitors. Benzyl ester protection was chosen as it is relatively stable to acidic and basic conditions. Whilst Dr A Maude concentrated on the solution phase synthesis, we decided to carry out the corresponding synthesis using solid phase chemistry.

The synthesis of the benzyl protected precursor 116 which was required for the solid phase synthesis started with β-allyl-9-fluorenylmethoxycarbonyl (2R)-aspartate 88. Reaction of the caesium salt of ester 88 with benzyl bromide gave the benzyl ester in 96% yield (mp 82-83 °C; [α]_D + 8.7 (MeOH)). Allyl ester deprotection using Pd(PPh₃)₄ and PhSiH₃ then gave desired compound 116 in 76% yield (mp 96-7 °C; [α]_D -9.17 (MeOH)) (see Scheme 2.23).

![Scheme 2.23: Synthesis of Benzyl Protected Precursor (16) for Solid Phase Synthesis](image)
This precursor was then used in the peptide synthesiser using the same strategy as before (see Section 2.5.2.3) to yield the required pentapeptide 117 in almost quantitative yield (mp 102-4 °C; (Found: C, 64.6; H, 5.7; N 6.9. requires C_{53}H_{57}N_{5}O_{14}: C, 64.4; H, 5.8; N, 7.1%; [α]_D + 17.8 (MeOH)).

After synthesis of the linear pentapeptide 117, once again the allyl ester was removed, under nearly neutral conditions, with Pd(PPh_3)_4 (see Section 2.5.3). The N-terminal Fmoc group was removed to give the free N-amino acid group 118. Cyclisation was carried out using PyBOP and HOBt in the presence of DIPEA in DMF. A negative Kaiser (ninhydrin) test (see Appendix 4) showed that no free amino groups were present after 7 days. Cleavage from the resin using TFA and subsequent ether precipitation gave a mixture of products which did not correspond to the required macrocycle 119. HPLC analysis showed that the major peak, according to mass spectroscopy was a result of the loss of the benzyl ester group from the macrocycle. One possible explanation for this is the position of the benzyl ester upon cyclisation. On cleavage the benzyl ester is exposed to the TFA solvent conditions, whereas before cyclisation the benzyl ester was masked by the linear pentapeptide. A variety of cleavage conditions were tried, however, all seemed to give rise to the loss of the benzyl ester.

The parallel synthesis carried out by Dr A Maude on the solution phase only gave rise to 44% yield of the required benzyl ester macrocycle from the final coupling step.

Therefore, on the basis of both the above results, the possibility of synthesising analogues without Adda functionality before cyclisation was deemed unsuitable.

**2.6.3 Addition of Adda Functionality Before Cyclisation**

Whilst the above work was in progress, both Shreiber and Chamberlin reported the synthesis of the natural products, motuporin and microcystin-LA respectively, with introduction of the Adda moiety before cyclisation. Solid phase peptide synthesis is very suitable for addition of the moiety prior to cyclisation, and opens up opportunities for combinatorial chemistry.
A secondary amine was chosen instead of β-alanine for the synthesis because molecular modelling revealed that the secondary amine should fit into the hydrophobic pocket on the enzymes PP1 and PP2A. 4-Benzylpiperidine was chosen to couple onto the α-carboxyl moiety which could be used in the peptide synthesiser.

The α-benzylpiperindo γ-allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartate 120 was prepared from the reaction of the pre-formed mixed anhydride of Fmoc-β-allyl-(2R)-aspartate 88 using NMM and IBCh to give the mixed anhydride which was then reacted with the 4-benzylpiperidine. The resulting product showed presence of Fmoc-OH, presumably due to the proton on the β-carbon of the Fmoc group being very acidic, and therefore susceptible to removal by weak bases such as 4-benzylpiperidine. Therefore, the resulting ester was immediately deprotected using Pd(Ph3)4 and PhSiH3 to yield the free acid 121 in 54% overall yield (mp 79-81 °C; [α]D + 27.3 (MeOH)).
Scheme 2.24: Synthesis of 
\( \alpha \)-benzylpiperindo-(2R)-N-9-fluorenylmethoxycarbonyl-aspartate

Using the same methodology as above, two other precursors for use in the peptide synthesiser were prepared with benzylamine and benzylpiperazine moieties: \( \alpha \)-benzylamido \((2R)-N-(9\text{-fluorenylmethoxycarbonyl})\)aspartate \(124\) (mp 141-2 °C; \([\alpha]_D + 13.84 \text{ (MeOH)}\)) and \(\alpha\)-benzylpiperizido \((2R)-N-(9\text{-fluorenylmethoxycarbonyl})\)aspartate \(125\) (mp 74-5 °C; \([\alpha]_D + 9.05 \text{ (MeOH)}\)), were prepared in 62% and 56% overall yield, respectively.
Dr J Wilkie has modelled the cyclic isopeptide 126 interaction with the PP1e using INSIGHT-II, and found that the 4-benzylpiperidine moiety, which is approximately the same length as Adda, fits into the hydrophobic pocket on the enzyme leaving the acidic moieties to interact with the metals via water.

Thus, the cyclic peptide 126 containing the 4-benzyl piperidine moiety was synthesised on the resin using the same methodology as before (see Section 2.5.3). Once again the linear pentapeptide 127 was obtained in almost quantitative yield and did not require further purification \( \{134-6 \, ^\circ\mathrm{C}; [\alpha]_D + 30 \, \text{(MeOH)}\} \).
C- and N- terminals were deprotected, and cyclisation was achieved in 7 days. The HPLC trace showed three major products, one of which gave rise to the desired cyclic pentapeptide 126. The recovered yield was very poor (> 20%). This synthesis has subsequently been repeated by Dr A Maude, using the combination of solution and solid phase (see Section 2.5.5) and higher yields have been obtained.

2.7 Isolation and Purification of Protein Phosphatase 1 (PP1)

The gene which encodes for PP1, incorporating a 6-His tag (see Section 2.7.1), was cloned into pET21a™ expression vector by Mrs J Sullivan (Zeneca Pharmaceuticals). Single colonies were selected and stored as a glycerol stock at -78 °C [for cDNA sequence see Appendix 3].

PP1 was expressed from the glycerol stock. The enzyme was purified using the Novagen His•Bind Buffer Kit, and dialysed overnight.

An SDS-PAGE mini-gel was performed using the lysate, cell paste and the purified enzyme against Perfect Protein™ markers. The gel was stained for protein with Coomassie blue and silver stain.

![Figure 2.13: SDS-PAGE mini-gel of PP1](image-url)
There are several conclusions which can be drawn from the gel:

- approximately 80% of the enzyme appears in the cell paste, which suggests the enzyme is insoluble on purification, and would need refolding in order in increase the yield; and

- the band from the lysate, at 37 kDa, which correlates to PP1 was judged to be 90% pure.

Protein determinations were performed using the Biorad reagent (based on the Bradford assay) using BSA as standard. The quantity of PP1 purified from 40 cm² cultures, following correction for purity, was 140 μg. This equates to 3.5 mg/litre culture (previously published by Zhang et al. as 10 mg/litre^2^).

2.7.1 Nickel chelation theory

Genes which are cloned into pET21a™ vectors carry a His•Tag sequence, stretch of either 6 or 10 consecutive histidine residues that can be expressed at the N-terminal or C-terminal of the target protein. The His•Tag sequence binds to divalent cation (Ni²⁺) immobilised on the His•Bind metal chelation resin. After unbound proteins are washed away, the target protein is recovered by elution with imidazole. Columns can be regenerated and reused many times. The versatile system provides a convenient, economical means of purification without the need to develop new protocols for each protein. It also allows the purification of target proteins under gentle, native conditions necessary for maintaining activity of soluble proteins.

2.7.2 Biochemical Characteristics of Recombinant PP1

Work carried out by Mrs J Sullivan showed that the activity of recombinant PP1 against pNPP is dependent on the inclusion of Mn²⁺ during the induction period. Removal of Mn²⁺ resulted in a 50% loss of activity, this was previously reported. To date, the enzyme preparations have retained full activity (12 weeks maximum).
2.8 Assay of Ser/Thr Protein Phosphatases

2.8.1 Methods Available

The two methods used most commonly to measure protein phosphatase activity are:

- a continuous or single time-point spectrophotometric assay of hydrolysis of a non-proteinaceous compound such as p-nitrophenylphosphate (pNPP): and
- a single-time point assay of inorganic phosphate released from a phosphorylated substrate (either a phosphorylated protein or a synthetic peptide).

2.8.2 Measurement of Protein Phosphatase Activity using p-Nitrophenylphosphate

Phosphatases are often assayed with the non-specific substrate, p-nitrophenolphosphate (pNPP), however, pNPP is not applicable to all phosphate releasing enzymes – indeed in our system there was no activity with native enzyme.\textsuperscript{224} pNPP cannot be employed when one wishes to explore the phosphoprotein substrate selectivity of a phosphatase. It, however, does have advantages in that it is a freely available substrate and can be used in a continuous spectrophotometric assay.\textsuperscript{224}

2.8.3 Measurement of Protein Phosphatase Activity using Phosphorylated Synthetic Peptides

Pimma and co-workers have used peptides as model substrates for Ser/Thr protein phosphatases.\textsuperscript{218,219} There studies show that PP1 is almost inactive towards all small phosphopeptides tested so far. PP2A, PP2B and PP2C, however, are able to use small phosphopeptide substrates, although there are differences in specificity.
In general, PP2B requires longer peptides of around 20 residues, whereas this is not the case for PP2A or PP2C. Some of these peptides are unrelated to any known physiological substrate. Other observations that have been extrapolated thus far include; PP1, PP2A and PP2C, by far prefer p-Thr over p-Ser\(^{219}\) and unlike PP2 and PP2B, PP1 activity towards peptides is not abolished by an acid cluster C-terminal to the phosphorylated residue, and seems to be substantially increased by multiple basic residues on the N-terminal\(^{218}\).

Two synthetic phosphorylated substrates were tested and characterised on both recombinant PP1 (purified at Zeneca), and native PP1 (purified from rabbit skeletal muscle, and bought from TCS Biologicals):

- RRATpVA (128); and
- KRTpIRR (129)

In order to carry out any meaningful studies on either recombinant or native PP1, for example, phosphoprotein selectivity of a phosphatase, and characterisation of the enzyme itself, it was important to develop an accurate and sensitive assay for the dephosphorylation reaction carried out by PP1.

The \(^{32}\)P labelled substrate, phosphorylase, has been used as the substrate of choice in most purifications and kinetic studies. However, this, not only, has the major disadvantage that it has a short half life (14 days), but relies on costly and hazardous radiolabelled substrates. A colourimetric assay, on the other hand, offers similar sensitivity to that achieved with a radiolabelled assay, but without the hazards and disposal liabilities\(^{220}\). The colourimetric assay which was employed was the Malachite green assay\(^{221,222}\).

The synthetic phosphopeptide RRATpVA 128 has been found to be the most effective substrate for PP2C so far identified. As it is also a common synthetic substrate for PP2A it appeared as an ideal substrate to try with our recombinant and native PP1.

The hexapeptide, KRTpIRR 129, has been implicated as a substrate for Ser/Thr protein phosphatases by TCS Biologicals. However, the kinetic parameters for
PP1 have not been published, therefore, this seemed a suitable substrate for testing with both the recombinant and native PP1.

2.8.4 Summary of Substrate Selectivity with Recombinant PP1 and Native

All assays were carried out under steady state conditions. A summary of the results are documented below (see Table 2.3).

Table 2.3: Summary of Substrate Selectivity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>RECOMBINANT PP1</th>
<th>NATIVE PP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_m$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>$pNPP$</td>
<td>21 °C 37.56</td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>25 °C 53.04</td>
<td>2.78</td>
</tr>
<tr>
<td>$RRATpVA$</td>
<td>21 °C 1.04 6.58</td>
<td>6299</td>
</tr>
<tr>
<td></td>
<td>25 °C 0.42 6.97</td>
<td>16787</td>
</tr>
<tr>
<td>$KRTpIRR$</td>
<td>21 °C 0.17 3.87</td>
<td>23081</td>
</tr>
<tr>
<td></td>
<td>25 °C 0.09 4.12</td>
<td>45382</td>
</tr>
</tbody>
</table>

Because $KRTpIRR$ 129 was the better substrate with both Native PP1 and recombinant enzymes, it was decided to use this as a substrate with any subsequent assays.
2.8.5 Inhibition of Native and Recombinant PP1 with Nodularin and Okadaic Acid

Having established the kinetic parameters of both enzymes with the hexapeptide substrate 129, it was important to test inhibitors in order to ensure that our assay was reliable and accurate.

Nodularin and okadaic acid, are both tight binding inhibitors that have been well characterised with PP1.

Using Native PP1 and KRTpIRR as a substrate under steady state conditions, both of these inhibitors gave IC$_{50}$ values which were comparable to literature (see Table 2.4).

Table 2.4: Enzyme inhibition using KRTpIRR as substrate. Assays carried out at 25 °C with 20 min incubation time

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$ (nM)</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodularin</td>
<td>0.1</td>
<td>0.14</td>
</tr>
<tr>
<td>Okadaic Acid</td>
<td>10-50</td>
<td>22.4</td>
</tr>
</tbody>
</table>

Because nodularin showed tight binding characteristics, we were able to establish the purity and activity of the native enzyme (bought from TCS).

It was found, using Grafit, that each assay contained 1.97 nM native PP1, which correlates to 2.5% pure/active protein to the published data. To check this result, an SDS-PAGE mini-gel was performed using the native PP1 against the recombinant PP1 and Perfect Protein™ markers. No protein, at the expected band of 37 kDa, was observed on the gel, and hence, this finding reinforced the original result.
From this result, the $k_{cat}$ and specificity constants were calculated for the substrates RRATpVA and KRTpIRR respectively.

When testing the recombinant enzyme under steady state conditions, it was obvious that it had different characteristics to the native PP1. Nodularin was not a tight binding inhibitor, instead it seem to possess biphasic kinetics (see Table 2.5).

**Table 2.5: Recombinant PP1 with Inhibitor - Nodularin**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ref. (nM)</th>
<th>$K_i 1$ (nM)</th>
<th>$K_i 2$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodularin</td>
<td>0.1</td>
<td>86.34</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>135.43</td>
<td>1.4</td>
</tr>
</tbody>
</table>

It is difficult to offer an explanation as to why the recombinant enzyme is so different from the native enzyme, however, there is some precedent for recombinant forms of PP1 exhibiting altered activity towards inhibitors.\textsuperscript{226} It is believed that the recombinant PP1 does not fold correctly, and hence has altered sensitivity towards inhibitors.

In our case, the recombinant PP1 seems to have two different isoforms of the same enzyme, one which displays a $K_i$ value which is comparable to the literature result ($K_i 2$), and one which has decreased sensitivity towards inhibitors ($K_i 1$).

PP1 has been shown to be refolded in the presence of inhibitor-2,\textsuperscript{226} which acts like a chaperone, to yield the correctly folded PP1. This is currently under investigation at Zeneca Pharmaceuticals.

### 2.8.6 Synthetic Inhibitors

Due to the problems encountered with the recombinant PP1, all dose response assays were carried out using the native PP1 under steady state conditions, using
KRTpIRR as substrate at 25 °C.

The synthetic inhibitors tested with native PP1 are shown in Figure 2.14.

![Synthetic Inhibitors](image)

**Figure 2.14: Synthetic Inhibitors**

These were all prepared by the hydrolysis (1 M NaOH for 1 h at rt) of the methyl esters.

A summary of the results are shown in Table 2.6.

**Table 2.6: IC₅₀ Values of Synthetic Inhibitors**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>94A</td>
<td>Undetectable</td>
</tr>
<tr>
<td>94B</td>
<td>Undetectable</td>
</tr>
<tr>
<td>130</td>
<td>2.89</td>
</tr>
<tr>
<td>131</td>
<td>2.72</td>
</tr>
</tbody>
</table>
Although, the above results are disappointing, it is clear that a hydrophobic residue at the Adda position is of vital importance for a compound to be an effective inhibitor. This was not an unexpected result, as crystallographic studies have indicated that the hydrophobic interaction of the Adda residue with PP1 is the first interaction.

Subsequent modelling, has suggested the amide bond between the 4-benzyl piperidine and the carboxylic acid may be at the wrong angle to adopt the correct conformation to fulfil binding in the hydrophobic pocket.

2.9 Conclusions and Future Work

2.9.1 Inhibitors

The aim of this study was to synthesise cyclic isopeptides based on the natural product, nodularin, as potential inhibitors of PP1 and/or PP2A. Initial work concentrated on the synthesis of the cyclic isopeptide portion of the inhibitor so that the basic chemistry could be sorted out without the added complication of the Adda residue.

We began by disconnecting the cyclic isopeptide between the sarcosine and aspartic acid residues, however, the linear pentapeptide did not undergo cyclisation to give the desired compound. Molecular modelling suggested that the linear pentapeptide did not adopt the correct conformation to achieve cyclisation.

However, cyclisation was successfully accomplished when the isopeptide was disconnected between the aspartic acid and phenylalanine residues, pentafluorophenyl activation was used and the synthesis of the linear peptide was carried out using solution phase peptide synthesis.

Whilst acceptable yields were obtained using standard solution phase synthesis, the synthesis was very time consuming. Therefore, in an effort to shorten the synthesis solid phase peptide synthesis, using PyBOP activation and Fmoc and allyl protection was considered.
Although we had hoped to carry out both the synthesis of the linear isopeptide and its subsequent cyclisation on the solid phase, yields were low for the cyclisation step.

To overcome this problem, the linear pentapeptide was synthesised on the resin, and then subsequently removed from the resin. The free carboxyl group was protected, and the protecting groups on the C- and N- terminal were removed prior to cyclisation. Subsequently, the synthesis was further shortened by attachment of the Wang resin to the β-carboxylic acid of aspartic acid instead of the original α-carboxylic acid.

Although cyclisation was initially achieved using PyBOP, problems encountered with the removal of by-products has now led to the use of BOP-Cl instead.

Introduction of a hydrophobic moiety was achieved using the synthesis outlined previously (see Section 2.5.5). The synthesis of inhibitors that incorporate more elaborate hydrophobic moieties are currently being synthesised by Dr M O'Donnell in order to carry out a detailed SAR study with both PP1 and PP2A enzymes.

2.9.2 Assays

Although a successful assay procedure for PP1 has been developed, there are still problems associated with the availability of the substrate and enzyme.

Because KRTpIRR is a very good substrate with PP1, the synthesis of this hexapeptide would prove to be useful. This, coupled with RRATpVA, the excellent substrate for PP2A which has been synthesised previously, would enable studies to be carried out on both PP1 and PP2A in order to find a selective inhibitor.

Once the folding problems with the recombinant enzyme has been overcome, this enzyme could become a reliable and inexpensive source of PP1. This would then allow enzyme assays and kinetic studies to be carried out in house.
3. Experimental

Elemental microanalyses were performed in the departmental microanalytical laboratory.

NMR spectra were recorded on a Bruker AM-300 spectrometer ($^1$H, 300 MHz; $^{13}$C, 75.4 MHz), a Varian Gemini 200 ($^1$H, 200 MHz; $^{13}$C, 50.3 MHz), a Varian Gemini-300BB ($^1$H, 300 MHz; $^{13}$C, 75.4 MHz) and a Varian Unity Plus 500 spectrometer ($^1$H, 500 MHz; $^{13}$C, 125.6 MHz). Chemical shifts are described in parts per million downfield shift from SiMe$_4$ and are reported consecutively as position ($\delta_H$ or $\delta_C$), relative integral, multiplicity (s -singlet, d -doublet, t -triplet, q -quartet, quin -quinet, dd -doublet of doublets, sep -septet, m -multiplet, and br -broad), coupling constant (J/Hz) and assignment (numbering according to the IUPAC nomenclature for the compound). $^1$H-NMR spectra were referenced internally on $^2$H$_2$O ($\delta$ 4.68), C$^6$H$_5$Cl (6 7.27) or $^2$(H$_3$C)$_2$SO (6 2.47). $^{13}$C-NMR were referenced on C$^6$H$_5$Cl (6 77.5) or $^2$(H$_3$C)$_2$SO (6 39.70).

Pyrrolidine ring carbon and hydrogen's are assigned in NMR spectra as $\alpha$, $\beta$, $\gamma$, $\delta$, going anticlockwise from the ring nitrogen, according to normal convention. Where more than one conformational isomer can be detected in the NMR spectrum due to the presence of a tertiary amide bond, these are assigned as $c$ (cis) or $t$ (trans), according to the isomeric state of the amide bond.

IR spectra were recorded on a Perkin-Elmer 1710 FT IR spectrometer. The samples were prepared as Nujol mulls or thin films between sodium chloride discs. The frequencies ($\nu$) as absorption maxima are given in wavenumbers (cm$^{-1}$) relative to a polystyrene standard. Mass spectra and accurate mass measurements were recorded on a VG 70-250 SE. Major fragments were given as percentages of the base peak intensity (100%). UV spectra were recorded on Pye-Unicam SP8-500 or SP8-100 spectrophotometers.

Flash chromatography was performed according to the method of Still et al.$^{227}$ using Sorbisil C 60 (40-60 μm mesh) silica gel. Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates.
chromatography was carried out on 0.25 mm precoated silica gel plates (Macherey-Nagel SIL g/UV254) and compounds were visualised using UV fluorescence, iodine vapour, ethanolic phosphomolybdic acid, or ninhydrin.

Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured at 23 °C on an Optical Activity AA-1000 polarimeter using 10 or 20 cm path length cells and are given in 10⁻¹ deg cm² g⁻¹.

The solvents used were either distilled or of analar quality and light petroleum refers to that portion boiling between 40 and 60 °C. Solvents were dried according to literature procedures. Ethanol and methanol were dried using magnesium turnings. Propan-2-ol, DMF, toluene, CH₂Cl₂, acetonitrile, diisopropylethylamine, pyridine and triethylamine were distilled over CaH₂. THF and diethyl ether were dried over sodium/benzophenone and distilled under nitrogen. Thionyl chloride was distilled over sulfur and the initial fractions were always discarded. N-Methyl morpholine was distilled over ninhydrin.

SDS-PAGE was performed following the procedure of Laemmli on a discontinuous medium. A Mini-Protean II Dual Slab Cell apparatus was used.

Centrifugations were carried out on a CENTRIKON C-124 centrifuge. Pooled fractions from various steps were concentrated by ultrafiltration using an AMICON apparatus (43 or 76 mm diameter), through YM10 or a YM30 membrane, under N₂.

Protein purifications were determined by the method of Lowry et al. Approximately diluted samples of the protein solution were mixed with Coomasie blue dye reagent (100 mg of Coomasie brilliant blue dissolved in 50 cm³ of 95% ethanol to which 100 cm³ of 85% of phosphoric acid has been added and the whole diluted to 1 l with water). After 5 min the absorbance was measured at 595 nm. Standard curves were prepared by using bovine serum albumin.
Diazomethane:

Potassium hydroxide (6.0 g) was dissolved in water (10 cm$^3$), isopropanol (35 cm$^3$) and diethyl ether were added. The solution was heated and maintained at 50 °C using a water bath. Diazald (21.4 g), dissolved in diethyl ether (130 cm$^3$), was added to the solution over 45 min. The yellow distillate was collected in a flask immersed in an ice bath and the whole system was fitted with a drying tube. An additional amount of ether (100 cm$^3$) was added until the ether distilling over was colourless. The ethereal solution in the receiver (~200 cm$^3$) contained approximately 4 g of diazomethane.

General Cyclisation Procedure using DIPEA and pfp Activation

To a stirred solution of the pentapeptide activated ester (75 mg, 0.087 mmol) in DCM (3 cm$^3$), was added trifluoroacetic acid (3 cm$^3$). The reaction was allowed to stir at room temperature for 1 h., after which time no starting material was observed by TLC. The reaction mixture was concentrated under reduced pressure to yield a colourless oil.

The residue was dissolved in DCM (200 cm$^3$), treated with DIPEA (210 mm$^3$), and stirred at room temperature for 7 days. The reaction mixture was concentrated under reduced pressure, redissolved in ethyl acetate (30 cm$^3$), and washed with 1M HCl (3 x 20 cm$^3$), 5% NaHCO$_3$ (2 x 20 cm$^3$), water (2 x 20 cm$^3$) and brine (1 x 20 cm$^3$). The organic phase was dried (MgSO$_4$) and the solvent removed under reduced pressure to give a colourless oil.
General Solid Phase Synthesis and Peptide Removal from Wang Resin

Solid phase synthesis of the pentapeptides described below were carried out using the Rainin PPS automated peptide synthesizer. The synthesis employed Fmoc chemistry and the C-terminal end amino acids were linked to β-allyl (2R)-N-(9-fluorenylethoxycarbonyl)aspartyl-Wang resin 85. Amino acids and the activating agent PyBOP were purchased from Novabiochem chemicals, solvents DMF, piperidine and N-methylmorpholine from Sigma-Aldrich. A four fold excess of the amino acid was used for each coupling procedure. The N-α-Fmoc group was deprotected using a 20% piperidine/DMF solution and the activation was achieved using a 5% NMM/DMF solution.

The peptides were cleaved from the resin using a mixture of TFA/TEA/H₂O/DCM.
**α-Methyl γ-benzyl (2R)-N-(tert-butoxycarbonyl)glutamate diester 36**

To a stirred solution of the γ-benzyl (2R)-N-(tert-butoxycarbonyl)glutamate (1.00 g, 2.96 mmol) in diethyl ether (15 cm³) at 0 °C was added diazomethane (10 cm³) dropwise. After 30 min, the solution was purged with nitrogen to remove excess diazomethane and was then concentrated under reduced pressure to give the ester as a colourless oil. Upon storage the oil solidified to give the diester as a white amorphous solid in almost quantitative recovery, mp 33-34 °C; [α]D +10.46 (c 1 in MeOH) (lit., -18.0 (c 5.2 in MeOH) (for the S-isomer)); υ max(CH₃Cl₂)/cm⁻¹ 3373 (NH), 2879 (CH), 1758 (CO, urethane) and 1715 (CO, ester); δH(400 MHz; CDCl₃) 1.15 [9 H, s, (CH₃)₃], 1.85-2.30 (2 H, m, β-CH₂), 2.40-2.55 (2 H, m, γ-CH₂), 3.75 (3 H, s, CH₃), 4.37 (1 H, q, J 5.1 α-H), 5.10-5.20 (3 H, m, PhCH₂ and NH) and 7.35 (5 H, s, Ph); δC(100 MHz; CDCl₃) 28.73 (PhCH₂), 28.89 [(CH₃)₃], 32.52 (γ-CH₂), 52.83 (α-C), 53.47 (CH₃), 67.48 (PhCH₂), 80.35 [(CH₃)₃], 128.60 and 129.04 (Ar-CH), 135.69 (Ar-C quaternary), 135.69 (CO, urethane) and 170.41 and 173.33 (CO, esters); m/z (EI) 292 (10%, [M - CO₂CH₃]⁺), 236 (16, [M - C₆H₅ - CO₂CH₃ + H⁺]), 192 (47, [M - Boc - CO₂CH₃ + H⁺]), 108 (12, PhCH₂OH⁺) and 91 (100, PhCH₂).

**α-Methyl (2R)-N-(tert-butoxycarbonyl)glutamate 37**

To a stirred solution of benzyl ester 36 (1.02 g, 2.9 mmol) in ethanol (30 cm³) was added 10% palladium on carbon (100 mg) and the mixture was stirred under an atmosphere of hydrogen for 3 h. The catalyst was removed by filtration through a pre-washed celite pad and the filtrate was concentrated under reduced pressure to give a colourless oil in quantitative recovery which was refractory to crystallisation, [α]D +27.14 (c 5.0 in MeOH) (lit., -27.7 (c 4.8 in MeOH) (for the S-isomer)); υ max(CH₃Cl₂)/cm⁻¹ 3373 (NH), 1720 (CO, urethane) and 1700 (CO, ester); δH(300 MHz; CDCl₃) 1.15 [9 H, s, (CH₃)₃], 1.82-2.23 (2 H, m, β-CH₂), 2.38-2.57 (2 H,
m, γ-CH₂), 3.78 (3 H, s, CH₃), 4.38 (1 H, q, J 5.3, α-H), 5.20 (1 H, d, J 8, urethane) and 6.69-7.31 (1 H, br s, CO₂H); δc(50.3 MHz; C²HCl₃) 27.78 (β-CH₂), 28.64 [(CH₃)₂], 30.47 (γ-CH₂), 52.89 (α-C), 53.47 (CH₃), 80.62 [C(CH₃)₃], 156.13 (CO, urethane), 176.74 (CO, ester) and 176.84 (CO, acid); m/z (Cl) 262 (4%, [M + H]⁺), 206 (100, [M + 2H - C₄H₉ + H]²⁺), 162 (49, [M + H C₅H₉O₂ + H]²⁺) and 144 (64, C₆H₁₀NO₃⁻).

**Benzyl sarcosinate p-toluenesulfonate 38**

To a stirred suspension of sarcosine (9.98 g, 110 mmol) in toluene (200 cm³) was added benzyl alcohol (120 cm³, 55.6 mmol) followed by p-toluene sulfonic acid (25.61 g, 120 mmol). The mixture was refluxed under Dean-Stark conditions for 6 h. On cooling white crystals formed which were filtered at the pump, washed with ether and recrystallised using methanol/ether to give the benzyl ester 38 as white crystals (30 g, 80%) mp 91-92 °C, νₙₐₓ(CH₂Cl₂)/cm⁻¹ 3436 (NH), 1751 (CO, ester) and 1660 (NH, amide); δH(200 MHz (²H₃C)SO) 2.32 (3 H, s, CH₃), 2.65 (3 H, s, N-CH₃), 4.07 (2 H, s, CH₂), 5.27 (2 H, br, PhCH₂), 7.10-7.60 (9 H, m, Ph) and 9.00 (3 H, s, NH₃); δc(50.3 MHz (²H₃C)SO) 21.09 (CH₃), 32.98 (CH₂), 48.43 (N-CH₃), 67.18 (PhCH₂), 125.80, 126.14, 128.34, 128.54, 128.70 and 128.76 (Ar-CH), 135.35 (Ar-C quaternary) and 166.99 (CO, ester); m/z (Cl) 180 (100%, [M + H - TsOH]⁺) and 90 (62, [C₅H₆NO₂]⁻).
Benzy | [(2R)-(tert-butoxycarbonyl)-α-methyl-glutamyl]-γ-sarcosinate diester 39

To a stirred solution of the α-methyl (2R)-(tert-butoxycarbonyl)glutamate 37 (1.45 g, 6.22 mmol) in dry THF (25 cm³) at -15 °C was added N-methylmorpholine (678 mm³, 6.22 mmol). Isobutylchloroformate (843 mm³, 6.22 mmol) was added and the suspension was stirred at -15 °C for a further 5 min. A mixture of benzyl sarcosinate p-toluenesulfonate 38 (2.09 g, 6.22 mmol) and N-methylmorpholine (678 mm³, 6.22 mmol) in dry DMF (10 cm³) and THF (25 cm³) was added and was left to stir overnight. The hydrochloride salts were removed by filtration and the solution concentrated under reduced pressure to give a pale yellow oil which was redissolved in ethyl acetate (15 cm³), washed successively with water (10 cm³), 5% aqueous NaHCO₃ (15 cm³), 10% citric acid (15 cm³) and brine (15 cm³), and then dried (MgSO₄), and concentrated under reduced pressure to give a colourless oil which crystallised on standing to give the required compound (2.21 g, 84%), mp 76-77 °C (lit., 75-77 °C) (Found: C 59.6; H, 7.1; N, 6.5. C₂₁H₃₀N₂O₇ requires C, 59.6; H, 7.2; N, 6.6%); [α]₀ +13.4 (c 1.51 in MeOH) (lit., +13.4 (c 1.5 in MeOH)); ʋ_max(CH₂Cl₂)/cm⁻¹ 3341 (NH), 2975 (CH), 1747 (CO, urethane), 1713 (CO, esters) and 1651 (CO, amide); δH(200 MHz; CDCl₃, mixture of rotomers) 1.40 [9 H, s, (CH₃)₂], 1.90-2.35 (2 H, m, β-CH₂), 2.45-2.80 (2 H, m, γ-CH₂), 3.03 and 3.10 (3 H, s, N-CH₃), 3.80 (3 H, s, CH₂O), 4.00-4.60 (3 H, m, CH₂ (Sar) and α-H), 5.15 (2 H, br, PhCH₂), 5.35 (1 H, s, NH), 6.65 (1 H, br, NH) and 7.30-7.40 (5 H, m, Ph); δC(50.3 MHz; CDCl₃, mixture of rotomers) 28.12 (β-CH₂), 28.79 [CH₂]₁, 29.75 (γ-CH₂), 35.57 and 36.94 (N-CH₃), 50.06 [CH₂ (Sar)] 52.89 (CH₂), 53.54 (α-C), 67.44 (PhCH₂), 78.21 [C(CH₃)₃], 128.83, 128.94, 129.12 and 129.22 (Ar-CH), 135.83 (Ar-C
Benzyl [α-methyl (2R)-glutamyl]-γ-sarcosinate hydrochloride 40

Hydrogen chloride gas was bubble into dry ethyl acetate (50 cm³) at 0 °C and stirred for 1 h. To this was added a solution of the glutamyl-sarcosyl dipeptide 39 (2.21 g, 5.23 mmol) dissolved in ethyl acetate (20 cm³) and the mixture was stirred for 1.5 h at room temperature. The solution was concentrated under reduced pressure to give the required salt as an essentially pure hydroscopic solid (1.78 g, 95%) (HRMS: found [M + H - HCl]⁺, 323.1615. C₁₆H₂₂NO₅ requires 323.1607); [α]₀ +13.50 (c 0.64 in MeOH); νₘₐₓ(CH₂Cl₂)/cm⁻¹ 3323 (NH), 1759 (CO, ester) and 1633 (CO, amide); δH(200 MHz; ²H₂O, mixture of rotomers) 2.02-2.20 (2 H, m, β-CH₂), 2.38-2.69 (2 H, m, γ-CH₂), 2.85 and 3.02 (3 H, 2 x s, N-CH₃), 3.73 (3 H, s, CH₂), 4.02-4.18 [3 H, m, CH₂ (Sar) and α-H], 5.11 (2 H, s, PhCH₂) and 7.35 (5 H, s, Ph); δC(50.3 MHz; ²H₂O) 27.74 (β-CH₂), 31.25 (γ-CH₂), 39.51 (N-CH₃), 53.03 [CH₂ (Sar)], 54.97 (CH₃), 56.43 (α-C), 70.32 (PhCH₂), 131.12, 131.2, 131.53 and 131.63 (Ar-CH), 137.97 (Ar-C quaternary), 172.97, 173.83 and 176.95 (CO, amide and esters); m/z (Cl) 323 (13%, [M + H - HCl]⁺), 263 (31, [M - CO₂Me]⁺), 91 (94, PhCH₂⁺), 84 (100, CH₂CHCO₂Me⁺) and 56 (52, CH₂CH₂CO⁺).
Benzyl $N$-(tert-butoxycarbonyl)-$\beta$-alanyl-[\(\alpha\)-methyl (2R)-glutamyl]$\gamma$-sarcosinate diester 41

This compound was prepared in a manner identical to that described for glutamyl-sarcosyl diester 39 using $N$-(tert-butoxycarbonyl)-$\beta$-alanine (554 mg, 2.93 mmol), and the hydrochloride 40 (1.05 g, 2.93 mmol), to give the required product as an oil, which upon trituration with diethyl ether afforded a white solid (1.19 g, 82%), mp 78-79 °C (Found: C, 58.2; H, 7.0; N, 8.5. C$_{23}$H$_{33}$N$_2$O$_7$ requires C, 58.4; H, 7.2; N, 8.5%); $\left[\alpha\right]_D^{o} +26.0$ (c 0.25 in MeOH); $\omega_{\text{max}}$(CH$_2$Cl$_2$)/cm$^{-1}$ 2928 (CH), 1746 (CO, urethane) and 1655 and 1634 (CO, amides); $\delta_{\text{H}}$(200 MHz; C$_6$H$_6$Cl$_6$, mixture of rotomers) 1.40 [9 H, s, (CH$_3$)$_3$], 2.01-2.23 [2 H, m, $\beta$-CH$_2$ (Glu)], 2.39-2.40 [2 H, m, $\gamma$-CH$_2$ (Glu)], 2.49-2.56 [2 H, m, $\beta$-CH$_2$ (\(\beta\)-Ala)], 2.97 and 3.07 (3 H, 2 x s, N-CH$_3$), 3.39 [2 H, q, J 5.8, $\alpha$-CH$_2$ (\(\beta\)-Ala)], 3.7 (3 H, s, CH$_3$), 4.19 [2 H, dd, J 12.1 and 5.3, $\alpha$-H (Glu)], 5.10-5.35 [3 H, m, PhCH$_2$ and NH (urethane)], 6.90 [1 H, d, J 7.3, NH (amide)] and 7.25-7.40 [5 H, m, Ph]; $\delta_{\text{C}}$(50.3 MHz; C$_6$H$_6$Cl$_6$, mixture of rotomers) 28.73 [$\beta$-CH$_2$ (Glu)], 28.89 [(CH$_3$)$_3$], 32.52 [$\gamma$-CH$_2$ (Glu)], 36.20 and 37.32 [2 x CH$_2$ (\(\beta\)-Ala)], 41.81 (N-CH$_3$), 48.53 [CH$_2$ (Sar)], 52.83 [$\alpha$-C (Glu)], 53.47 (CH$_3$), 67.48 (PhCH$_2$), 80.35 [C(CH$_3$)$_3$], 128.50, 128.58 and 129.09 (Ar-CH), 135.69 (Ar-C, quaternary), 155.69 (CO, urethane), 170.10 and 172.89 (CO, amides) and 173.25 and 173.56 (CO, esters); $m/z$ (CI) 494 (25%, [M + H]$^+$), 393 (34, [M + H - C$_6$H$_5$O$_2$]$^+$), 91 (56, PhCH$_2^+$) and 59 (100, CO$_2$Me$^+$).
Benzyl p-alanyl-[α-methyl (2R)-glutamyl]-γ-sarcosinate hydrochloride 42

This compound was prepared in a manner identical to that described for the hydrochloride 40 using the tripeptide 41 (0.98 g, 1.99 mmol) to give the required product as a white hydroscopic solid (821 mg, 96%), \( [\alpha]_D^0 +8.53 \) (c 0.95 in MeOH); \( \nu_{\text{max}}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1} 3327 \) (NH), 2972 (CH), 1752 (CO, ester) and 1653 (CO, amides); \( \delta_H(200 \text{ MHz}; ^2\text{H}_2\text{O}, \text{mixture of rotomers}) 1.72-2.14 \) [2 H, m, γ-CH\(_2\) (Glu)], 2.39-2.52 [2 H, m, β-CH\(_2\) (Glu)], 2.53-2.68 [2 H, m, α-CH\(_2\) (β-Ala)], 2.82 and 2.98 (3 H, 2 x s, N-CH\(_3\)), 3.07-3.18 [2 H, m, β-CH\(_2\) (β-Ala)], 3.62 (3 H, s, CH\(_3\)), 4.18-4.38 [3 H, m, δ-CH\(_2\) (Glu) and CH\(_2\) (Sar)], 5.11 (2 H, s, PhCH\(_2\)) and 7.32 (5 H, s, Ph); \( \delta_C(50.3 \text{ MHz}; ^2\text{H}_2\text{O}, \text{mixture of rotomers}) 28.48 \) [PH\(_2\) (Glu)], 31.58 [γ-CH\(_2\) (Glu)], 34.41 and 38.26 [2 x CH\(_2\) (β-Ala)], 39.66 (N-CH\(_3\)), 52.81 [CH\(_2\) (Sar)], 53.22 [α-C (Glu)], 55.02 (CH\(_3\)), 70.43 (PhCH\(_2\)), 130.35, 131.57 and 131.82 (Ar-CH) and 173.93 and 178.09 (CO, ester and amide); \( m/z \) (CI) 394 (45%, \( [\text{M} + \text{H} - \text{HCl}]^+ \)), 318 (97, \( [\text{M} + \text{H} - \text{HCl} - \text{PhCH}_2 + \text{H}]^+ \)), and 180 (100, \( [\text{M} + \text{H} - \text{HCl} - \text{C}_9\text{H}_1\text{N}_2\text{O}_4]^- \)).

α-Methyl β-benzyl (2R)-N-(tert-butoxycarbonyl)aspartate 44

This compound was prepared in a manner identical to α-methyl γ-benzyl (2R)-N-(tert-butoxycarbonyl)glutamate 36 using β-benzyl (2R)-N-(tert-butoxycarbonyl)aspartate 43, (1 g, 3.09 mmol) to give the required compound as a white crystalline solid in quantitative yield, which did not require further purification, mp 65-66 °C (lit.,\(^{222}\) 67-68 °C (for the (2S)-isomer)); \( [\alpha]_D^0 +8.13 \) (c 1.04 in MeOH) (lit.,\(^{222}\) -7.1 (c 1.0 in acetone) (for the (2S)-isomer)); \( \nu_{\text{max}}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1} 3401 \) (NH), 2977 (CH), 1737 (CO, urethane) and 1500 (CO, amide); \( \delta_H(200 \text{ MHz}; ^2\text{H}_2\text{Cl}_2) 1.44 \) [9 H, s, (CH\(_3\))\(_3\)], 2.82-3.12 (2 H, m, β-CH\(_2\)), 3.71 (3 H, s, CH\(_3\)), 4.55-4.62 (1 H, m, α-CH\(_3\)), 5.13 (2 H, s, PhCH\(_2\)), 5.49 (1 H, br,
NH) and 6.94-7.75 (5 H, m, Ph); δ(50.3 MHz; 6CHCl3) 28.78 [(CH3)3], 38.39 (β-CH2), 50.3 (α-C), 53.81 (CH3), 68.90 (PhCH2), 80.51 [C(CH3)3], 128.12, 128.56 and 129.09 (Ar-CH), 136.2 (Ar-C quaternary), 156.31 (CO, ester) and 171.29 and 171.98 (CO, esters); m/z (Cl) 338 (4%, [M + H], 282 (22, [M + H - C6H9 + H]), 238 (8, [M + H - C3H5O2 + H]), 91 (100, PhCH2) and 57 (90, C4H9+).

α-Methyl (2R)-N-(tert-butoxycarbonyl)aspartate ester 45

This compound was prepared in a manner identical to α-methyl (2R)-N-(tert-butoxycarbonyl) glutamate 37, using α-methyl β-benzyl (2R)-N-(tert-butoxycarbonyl)aspartate 44 (1 g, 3.07 mmol) to give the required compound as a white crystalline solid in quantitative yield, which did not require further purification, mp 98-99 °C νmax(CH2Cl2)/cm⁻¹ 3510-2840 br (OH and NH) 2927 (CH), 1745 (CO, urethane), 1662 (CO, amide) and 1611 (CO, amide); δ(300 MHz; 6CHCl3) 1.44 [9 H, s, (CH3)3], 2.86 (1 H, dd, J 17.7 and 4.1, 1 H of β-CH2), 3.04 (1 H, dd, J 17.1 and 4.1, 1 H of β-CH2), 3.74 (3 H, s, CH3), 4.58 (1 H, t, J 4.2, α-H), 5.55 (1 H, d, J 8.3, NH) and 8.30 (1 H, br, OH); δ(75.4 MHz; 6CHCl3) 28.35 [(CH3)3], 36.73 (β-CH2), 49.78 (α-C), 53.92 (CH3), 89.54 [C(CH3)3], 155.62 (CO, urethane), 171.66 (CO, ester) and 176.24 (CO, acid); m/z (EI) 247 (1%, M+), 188 (30, [M - COOCH3]+) and 57 (100, C4H9+).
Benzyl [(2R)-N-(tert-butoxycarbonyl)-α-methyl-aspartyl]-β-(2S)-phenylalaninate diester 47

This compound was prepared in a manner identical to that described for glutamyl-sarcosyl diester 39 using α-methyl-(2R)-N-(tert-butoxycarbonyl) aspartate 45 (910 mg, 3.68 mmol) and benzyl (2S)-phenylalaninate p-toluenesulfonate 46 (1.52 g, 3.68 mmol), to give the required product as an oil which upon trituration with diethyl ether afforded a white solid, which was not purified further (1.94 g, 99%), mp 132-133 °C (Found: C, 64.8; H, 6.5; N 5.5. C_{26}H_{32}N_{2}O_{7} requires C, 64.5; H, 6.6; N, 5.8%); (HRMS: found: [M + H]^+, 485.2282. C_{26}H_{33}N_{2}O_{7} requires 485.2293); [α]_D -4.4 (c 1.1 in MeOH); υ_{max} (CH_{2}Cl_{2})/cm^{-1} 3350 (NH), 2983 (CH), 1746 (CO, urethane), 1720 (CO, esters) and 1675 (CO, amide); δ_{H}(300 MHz; C_{6}D_{6}) 1.49 [9 H, s, (CH_{3})_{3}], 2.66 [1 H, dd, J 15.9 and 4.6, 1 H of β-CH_{2} (Asp)], 2.91 [1 H, dd, J 17.1 and 16.1, 1 H of β-CH_{2} (Asp)], 3.03-3.16 [2 H, m, β-CH_{2} (Phe)], 3.73 (3 H, s, CH_{3}), 4.49 [1 H, q, J 4.2, α-H (Asp)], 4.88 [1 H, q, J 5.7, α-H, (Phe)], 5.14 (2 H, AB, J 12.3 and 6, PhCH_{2}), 5.62 [1 H, d, J 6, NH (urethane)], 6.04 [1 H, d, J 7.8, NH (amide)] and 6.96-7.42 (10 H, m, Ph); δ_{C}(75.4 MHz; C_{6}D_{6}) 28.42 [(CH_{3})_{3}], 37.93 [β-CH_{2} (Asp and Phe)], 50.32 [α-C (Phe)], 52.74 [α-C (Asp)], 53.29 (CH_{3}), 67.49 (PhCH_{2}), 80.1 [C(CH_{3})_{3}], 127.26, 128.71, 128.77 and 129.41 (Ar-CH), 135.05 and 135.62 (Ar-C quaternary), 155.74 (CO, urethane) and 169.52, 171.27 and 171.91 (CO, esters); m/z (Cl) 485 (12%, [M + H]^+), 429 (30, [M + H - C_{4}H_{9} + H]^+), 385 (38, [M + H - C_{4}H_{9}O_{2} + H]^+), 356 (100, [M - C_{3}H_{7}NO_{3}]^+), 256 (22, C_{16}H_{18}NO_{2}^+) and 91 (20, PhCH_{2}^+) and 57 (32, C_{4}H_{9}^+).
[(2R)-N-(tert-Butoxycarbonyl)-α-methyl-aspartyl]-β-(2S)-phenylalanine 48

This compound was prepared, through catalytic hydrogenolysis, in a manner identical to that described for α-methyl (2R)-N-(tert-butoxycarbonyl) glutamate 37 using benzyl [α-methyl (2R)-N-(tert-butoxycarbonyl)aspartyl]-β-(2S)-phenylalaninate diester 47 (1 g, 2.14 mmol) to give a white solid in quantitative recovery (810 mg), mp 64-65 °C (HRMS: found: [M + H]⁺, 395.1814. C₁₉H₂₇N₂O₇ requires 395.1822); [α]₀ +21.25 (c 1.2 in MeOH); v_max(CH₂Cl₂)/cm⁻¹ 3333 (NH), 2926 (CH), 1749 (CO, urethane), 1710 br (CO, ester and acid) and 1664 (CO, amide); δ_H(300 MHz; C⁶D₆) 1.37 [9 H, s, (CH₃)₃], 2.61-3.60 (4 H, m, 2 x β-CH₂), 3.60 (3 H, s, CH₃), 4.37 [1 H, br, α-H (Phe)], 4.71 [1 H, br, α-CH, (Asp)], 5.62 [1 H, d, J 8.3, NH (urethane)], 6.74 [1 H, br, NH (amide)] and 7.06-7.17 (5 H, m, Ph); δ_C(50.3 MHz; C⁶D₆) 28.33 [(CH₃)₃], 38.01 [β-CH₂ (Asp) and β-CH₂ (Phe)], 50.81 [α-C (Asp)], 52.99 (CH₃), 53.86 [α-C (Phe)], 80.56 [C(CH₃)₂], 127.32, 128.82 and 129.81 (Ar-CH), 136.71 (Ar-C quaternary), 156.35 (CO, urethane), 170.43 and 172.60 (CO, ester and amide) and 174.10 (CO, acid); m/z (Cl) 485 (5%, [M + H]⁺), 339 (15, [M + H - C₄H₉ + H]⁺), 295 (17, [M + H - C₅H₇O₂ + H]⁺), 266 (100, [M - C₅H₇NO₃]⁻) and 57 (14, C₄H₉⁻).
Benzyl [(2R)-N-[(tert-butoxycarbonyl)-α-methyl-aspartyl]-β-(2S)-phenylalanyl-β-alanyl [α-methyl(2R)-glutamyl]-γ-sarcosinate triester 34

This compound was prepared in a manner identical to that described for glutamyl-sarcosyl diester 39, using [α-methyl (2R)-N-[(tert-butoxycarbonyl)aspartyl]
-β-(2S)-phenylalanine 48, (583 mg, 1.36 mmol) and benzyl β-alanyl-[α-methyl (2R)-glutamyl]-γ-sarcosinate hydrochloride 42 (513 mg, 1.36 mmol) to give the required compound as a white crystalline solid. Purification by silica chromatography (94% CH₂Cl₂-MeOH) gave compound 34 as a white crystalline solid (750 mg, 72%), mp 82-83 °C (Found: C, 59.2; H, 6.5; N 8.9. C₃₈H₃₅N₇O₁₂ requires: C, 59.3; H, 6.7; N, 9.1%); [α]D +6.22 (c 1.24 in 1.45 MeOH); νmax(CH₂Cl₂)/cm⁻¹ 3316 (NH), 2955 (CH), 1756 (CO, urethane) and 1658 (CO, amides); δH (500 MHz; CCl₄, mixture of rotomers) 1.37 and 1.39 [9 H, 2 x s, (CH₃)₃], 1.96-2.03 [1 H, m, 1 H of γ-CH₂ (Glu)], 2.11-2.53 [6 H, m, 1 H of β-CH₂ (Glu), γ-CH₂ (Glu) and 1 H of β-CH₂ (Asp) and CH₂ (β-Ala)], 2.75-3.04 [3 H, m, 1 H of β-CH₂ (Asp) and β-CH₂ (Phe)], 3.04 and 3.06 (3 H, 2 x s, N-CH₃), 3.35-3.48 [2 H, m, CH₂ (β-Ala)], 3.63, 3.65, 3.67 and 3.69 [6 H, 4 x s, CH₂ (Asp and Glu)], 3.97-4.12 [2 H, m, CH₂ (Sar)], 4.10-4.44 [2 H, m, 2 x α-H (Glu and Asp) 4.57 (1 H, br, α-H (Phe)); 5.08 and 5.13 (2 H, 2 x s, PhCH₂), 5.66 [1 H, d, J 8.36, NH (urethane)], 6.61-6.91 [3 H, m, 3 x NH (amide)] and 7.11-7.30 (10 H, m, Ph); δC (75.4 MHz; CCl₄, mixture of rotomers) 27.98 [β-CH₂ (Glu)], 28.32 [(CH₃)₂], 29.46 [γ-CH₂ (Glu)], 35.24 (N-CH₃), 35.52 and 35.92 [2 x CH₂ (β-Ala)], 36.67 (N-CH₃), 37.60 [β-CH₂ (Asp)], 38.22 [β-CH₂ (Phe)], 49.77 [α-C
(Asp)], 50.38 [CH$_2$ (Sar)], 51.62 [α-C (Glu)], 52.46 and 52.55 (2 x CH$_3$), 54.53 [α-C (Phe)], 67.09 (PhCH$_2$), 79.88 [C(CH$_3$)$_2$], 126.91, 128.36, 128.57, 128.73, 128.84 and 129.39 (Ar-CH), 135.41 and 136.94 (Ar-C quaternary) and 155.72 (CO, urethane), 169.22, 170.21, 171.21, 171.83, 172.47 and 173.28 (CO, amides and esters); m/z (FAB) 792 (5%, [M + Na]$^+$), 770 (1, M$^-$), and 120 (100, [C$_8$H$_5$N + H]$^+$).  

[(2R)-N-(tert-Butyloxy carbonyl)-α-methyl-aspartyl]-β-(2S)-phenylalanyl-β-alanyl-[α-methyl(2R)-glutamyl]-γ-sarcosinate diester 48

This compound was prepared in a manner identical to α-methyl (2R)-N-(tert-butoxycarbonyl)glutamate 37, using triester 34 (250 mg, 0.325 mmol) to give the required compound as a white crystalline solid in quantitative recovery, which was not purified further, mp 55-56 °C (HRMS: found [M + Na]$^+$, 702.2975. C$_{37}$H$_{44}$N$_5$O$_{12}$Na requires 702.2962); $\nu_{\text{max}}$(CH$_2$Cl$_2$)/cm$^{-1}$ 3296 (NH), 1745 (CO, ester) and 1649 (CO, amides); $\delta_{\text{H}}$(300 MHz; C$_5$D$_5$N, mixture of rotomers) 1.35 [9 H, s, (CH$_3$)$_3$], 1.91-2.14 [2 H, m, γ-CH$_2$ (Glu)], 2.21-2.59 [6 H, m, β-CH$_2$ (Glu), β-CH$_2$ (Asp) and α-CH$_2$ (β-Ala)], 2.76-2.82 [2 H, m, β-CH$_2$ (Phe)], 2.82 and 2.99 (3 H, 2 x s, N-CH$_3$), 2.37 [2 H, br, β-CH$_2$ (β-Ala)], 3.60, 3.61, 3.62 and 3.63 [6 H, 4 x s, CH$_3$ (Asp and Glu)], 3.93-4.18 [2 H, m, CH$_2$ (Sar)], 4.40 [2 H, br, 2 x α-H (Glu and Asp)] 4.57 [1 H, br, α-H (Phe)], 5.71 [1 H, d, J 7, NH (urethane)] and 7.03-7.58 [8 H, m, Ph and 3 x NH (amides)]; $\delta_{\text{C}}$(75.4 MHz; C$_5$D$_5$N, mixture of rotomers) 26.19 [β-CH$_2$ (Glu)], 28.36 [C(CH$_3$)$_2$], 29.58 [$\gamma$-CH$_2$ (Glu)], 35.29 (N-CH$_3$), 35.96 and 36.83 [2 x CH$_2$ (β-Ala)], 37.64 [β-CH$_2$ (Asp)], 38.26 [β-CH$_2$ (Phe)], 50.08 [α-C (Asp)] 50.38 [CH$_2$ (Sar)], 52.52 [α-C (Glu)], 52.58 and 54.62 (2 x CH$_3$), 57.96 [α-C (Phe)], 80.00 [(CH$_3$)$_3$], 126.85, 127.12, 128.51, 128.58, 129.31 and 129.38 (Ar-CH), 136.66 (Ar-C quaternary), 155.81 (CO urethane), 170.41, 171.50, 171.67, 171.99, 172.40 and 172.57 (CO, esters and amides) and 173.50 (CO, acid); m/z (FAB) 702 (40%, [M + Na]$^+$), 680 (12, [M + H]$^-$), 580 (45, [M + H - C$_3$H$_5$O$_2$ + H]$^+$) and 120 (100, [C$_8$H$_5$N + H]$^+$).
β-Pentafluorophenyl [(2R)-N-(2R)-tert-butoxycarbonyl]-α-methyl-aspartyl]-β-
(2S)-phenylalanyl-β-alanyl-[α-methyl (2R)-glutamyl]-γ- sarcosinate triester 49

To a stirred solution of the pentapeptide carboxylic acid 48 (160 mg, 0.22 mmol) in
CH\textsubscript{2}Cl\textsubscript{2} (20 cm\textsuperscript{3}) at 0 °C was added pentafluorophenol (122 mg, 6.63 mmol)
followed by EDCI (99 mg, 0.33 mmol). The reaction mixture was allowed to warm
to room temperature and stirred over night. The solution was concentrated under
reduced pressure to yield a colourless oil which was purified by flash chromatography on silica eluting with 95% CH\textsubscript{2}Cl\textsubscript{2}-MeOH to give triester 49 as a
white crystalline solid (119 mg, 63%), mp 109-110 °C; \nu\text{max}(CH\textsubscript{2}Cl\textsubscript{2})/cm\textsuperscript{-1} 3322
(NH), 2969 (CH), 1757 (CO, urethane) and 1656 (CO, amides); \delta\textsubscript{H}(300 MHz;
C\textsubscript{6}HCl\textsubscript{3}, mixture of rotomers) 1.41 [9 H, s, (CH\textsubscript{3})\textsubscript{3}], 2.02-2.61 [8 H, m, γ-CH\textsubscript{2}
(Glu), β-CH\textsubscript{2} (Glu), β-CH\textsubscript{2} (Asp) and CH\textsubscript{2} (β-Ala)], 2.79-3.04 [2 H, m, β-CH\textsubscript{2}
(Phe)], 3.12 (3 H, s, N-CH\textsubscript{3}), 3.14-3.59 [2 H, m, CH\textsubscript{2} (β-Ala)], 3.67 and 3.68 [6 H,
2 x s, CH\textsubscript{3} (Asp and Glu)], 4.09-4.62 [5 H, m, CH\textsubscript{2} (Sar), 2 x α-H (Glu and Asp)
and α-H (Phe)], 5.78 [1 H, d, J 7.2, NH (urethane)], 6.58, 6.75 and 6.90 [3 H, 3 x
br, NH (amides)] and 7.08-7.34 (5 H, m, Ar-H); \delta\textsubscript{C}(75.4 MHz; C\textsubscript{6}HCl\textsubscript{3}, mixture of
rotomers) 26.77 [β-CH\textsubscript{2} (Glu)], 28.75 [(CH\textsubscript{3})\textsubscript{3}], 29.92 [γ-CH\textsubscript{2} (Glu)], 35.97
(N-CH\textsubscript{3}), 36.20 and 37.08 [2 x CH\textsubscript{2} (β-Ala)], 38.01 [β-CH\textsubscript{2} (Asp)], 38.59 [β-CH\textsubscript{2}
(Phe)], 50.07 [α-C (Asp)], 50.71 [CH\textsubscript{2} (Sar)], 51.87 [α-C (Glu)], 52.11 and 52.75
(2 x CH\textsubscript{3}), 54.78 [α-CH (Phe)], 80.30 [C(CH\textsubscript{3})\textsubscript{3}], 127.30, 128.99 and 129.69
(Ar-CH), 137.20 (Ar-C quaternary), 156.01 (CO, urethane), 171.28, 171.98 and
172.73 (CO, esters and amides) and 173.61 (CO, acid); \textit{m/z} (ES) 884 (2%, [M +
K\textsuperscript{+}]), 868 (4, [M + Na\textsuperscript{+}]), 846 (7, [M + H\textsuperscript{+}]), 213 (100, C\textsubscript{7}H\textsubscript{2}O\textsubscript{2}F\textsubscript{5})
, 157 (57, C\textsubscript{6}H\textsubscript{7}NO\textsubscript{4}\textsuperscript{+}) and 101 (25, C\textsubscript{5}H\textsubscript{9}O\textsubscript{2}^-).
Benzyl [(2R)-N-(tert-butoxycarbonyl)-\(\alpha\)-methyl-glycyl]-(2S)-prolinate diester 58A

This compound was prepared in a manner identical to that described for glutamyl-sarcosyl diester 39 using \(\alpha\)-methyl (2R)-N-(tert-butoxycarbonyl)glutamate 37, (2.03 g, 5.78 mmol) and benzyl (2S)-prolinate \(p\)-toluenesulfonate (2.09 g, 6.22 mmol), to give the required compound as colourless oil, which was contaminated with 50% benzyl alcohol (2.0 g, 77%), (HRMS: found [M + H]\(^+\), 449.2279, \(C_{23}H_{33}N_2O_7\) requires 449.2288); \(\nu_{\text{max}}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1}\) 3362 (NH), 2976 (CH), 1744 (CO, urethane) and 1720 and 1699 (CO, esters); \(\delta_1H(300\text{ MHz; } \text{CDCl}_3,\text{ mixture of isomers})\) 1.40 [9 H, s, (CH\textsubscript{3})\textsubscript{3}], 1.81-2.54 [8 H, m, \(\gamma\)-CH\textsubscript{2} and \(\beta\)-CH\textsubscript{2} (Pro and Glu)], 3.36-3.51 [2 H, m, \(\delta\)-CH\textsubscript{2} (Pro)], 3.67 and 3.69 (3 H, s, CH\textsubscript{3}), 4.05-4.58 (2 H, m, 2 x \(\alpha\)-H), 5.04-5.19 (2 H, AB, J 12, PhCH\textsubscript{2}), 5.45 [1 H, d, J 8.1, NH (urethane)] and 7.20-7.30 (5 H, m, Ph); \(\delta_1C(75.4\text{ MHz; } \text{CDCl}_3)\) 22.51 [c, \(\gamma\)-CH\textsubscript{2} (Pro)], 24.64 [t, \(\gamma\)-CH\textsubscript{2} (Pro)], 27.06 [t, \(\beta\)-CH\textsubscript{2} (Glu)], 27.56 [c, \(\beta\)-CH\textsubscript{2} (Glu)], 28.26 [t, (CH\textsubscript{3})\textsubscript{3}], 29.11 [c, (CH\textsubscript{3})\textsubscript{3}], 29.99 27.06 [\(\gamma\)-CH\textsubscript{2} (Glu)], 30.62 [t, \(\beta\)-CH\textsubscript{2} (Pro)], 31.29 [c, \(\beta\)-CH\textsubscript{2} (Pro)], 46.57 [c, \(\delta\)-CH\textsubscript{2} (Pro)], 46.99 [t, \(\delta\)-CH\textsubscript{2} (Pro)], 52.31 (t, CH\textsubscript{3}), 52.54 (t, CH\textsubscript{3}), 53.40 [t, \(\alpha\)-C (Glu)], 53.52 [c, \(\alpha\)-C (Glu)], 58.89 [t, \(\alpha\)-C (Pro)], 59.50 [c, \(\alpha\)-C (Pro)], 66.85 (t, PhCH\textsubscript{2}), 67.34 (c, PhCH\textsubscript{2}), 79.88 [C(CH\textsubscript{3})\textsubscript{3}], 127.76, 127.76, 127.91 and 128.86 (Ar-CH), 135.86 (Ar-C quaternary), 155.86 (CO, urethane) and 171.23, 172.28 and 173.07 (CO, esters); \(m/z\) (CI) 449 (55%, [M + H]\(^+\)), 349 (20, [M + H - C\textsubscript{7}H\textsubscript{5}O\textsubscript{2} + H]\(^+\)) and 206 (100 [M + 2H - C\textsubscript{11}H\textsubscript{16}NO\textsubscript{3}]\(^+\)).
α-Methyl β-benzyl (2R)-aspartate diester hydrochloride 59

This compound was prepared in a manner identical to that described for the hydrochloride 40 using α-methyl β-benzyl (2R)-N-(tert-butoxycarbonyl)aspartate diester 62 (1.0 g, 3.07 mmol), to yield a white hygroscopic solid which was not purified any further (820 mg, 98%), mp 139-140 °C (lit. 137.8-138 °C (for the S-isomer)); [α]D +17.9 (c 2.0 in MeOH), (lit. +19.2 (c 4.2 in water) (for the S-isomer)); νmax(CH2Cl2)/cm⁻¹ 3405 (NH), 2967 (CH), 1739 (CO, ester) and 1634 (CO, amide); δH(200 MHz; CDCl3) 3.35 (2 H, br, p-CH₂), 3.65 (3 H, s, CH₃), 4.65 (1 H, br, α-H), 5.14 (2 H, s, PhCH₂), 7.32 (5 H, br, Ph) and 8.86 (1 H, br, OH); δC (50.3 MHz; CDCl₃) 34.82 (β-CH₂), 50.01 (α-C), 54.12 (CH₃), 67.97 (PhCH₂), 128.54, 128.96 and 129.03 (Ar-CH), 137.96 (Ar-C quaternary) and 168.55 and 170.67 (CO, esters); m/z (Cl) 238 (100%, [M - H - Cl]⁺).

β-Benzyl [(2S)-N-(tert-butoxycarbonyl)prolyl]-α-methyl (2R)-aspartate diester 64A

This compound was prepared in a manner identical to that described for glutamyl-sarcosyl diester 39, using N-(tert-butoxycarbonyl)-(2S)-proline, (215 mg, 1 mmol) and α-methyl β-benzyl (2R)-aspartyl diester hydrochloride 59 (273 mg, 1 mmol), to give the required compound as colourless oil which was refractory to crystallisation (343 mg, 78%) (Found: C, 61.1; H, 7.3; N 6.2. C₂₂H₂₉NO₇ requires: C, 60.8; H, 7.0; N, 6.4%) (HRMS: found [M + H]⁺, 435.2124. C₂₂H₂₉NO₇ requires 435.2131); [α]D -5.41 (c 1.21 in MeOH); νmax(CH₂Cl₂)/cm⁻¹ 3323 (NH), 2976 (CH), 1739 (CO, urethane) and 1699 (CO, esters); δH(300 MHz; CDCl₃) 1.43 [9
H, s, (CH₃)₃, 1.82-2.22 [4 H, m, γ-CH₂ and β-CH₂ (Pro)], 2.85 [1 H, dd, J 17.1 and 4.8, 1 H of β-CH₂ (Asp)], 3.06 [1 H, dd, J 14.1 and 4.7, 1 H of β-CH₂ (Asp)],
3.09-3.45 [2 H, m, δ-CH₂ (Pro)], 3.66 (3 H, s, CH₃), 4.18-4.37 [1 H, m, α-H (Pro)], 4.87 [1 H, q, J 4.5, α-H (Asp)], 5.09 (2 H, s, PhCH₂), 7.28-7.34 (5 H, m, Ph) and 7.37 (1 H, br, NH); δ_C(75.4 MHz; C^HCl;) 24.14 [c, γ-CH₂ (Pro)], 24.27 [t, γ-CH₂ (Pro)], 28.40 [t, (CH₃)₃], 28.75 [c, (CH₃)₃], 29.08 [c, β-CH₂ (Pro)], 36.78 [β-CH₂ (Asp)], 47.25 [t, δ-CH₂ (Pro)], 48.49 [c, δ-CH₂ (Pro)], 48.68 [α-C (Asp)], 53.13 (CH₃), 60.65 [c, α-C (Pro)], 60.74 [t, α-C (Pro)], 67.29 (PhCH₂), 80.81 [C(CH₃)₃], 128.61, 128.81 and 129.04 (Ar-CH), 136.20 (Ar-C quaternary), 156.04 (CO, urethane) and 171.06, 171.11 and 171.38 (CO, esters); m/z (Cl) 435 (100%, [M + H]^+), 379 (55, [M + H - C₅H₅O₂]^+ + H)^+ and 335 (97, [M + H - C₅H₅O₂ + H]^+).

β-Benzyl [(2R)-N-(tert-butoxycarbonyl)-prolyl]-α-methyl (2R)-aspartate diester 64B

This compound was prepared in a manner identical to that described for glutamyl-sarcosyl diester 39, using N-(tert-butoxycarbonyl)-(2R)-proline (215 mg, 1 mmol) and α-methyl β-benzyl (2R)-aspartyl diester hydrochloride 59 (273 mg, 1 mmol), to give the required compound as colourless oil which was refractory to crystallisation (321 mg, 74%), (HRMS: found [M + H]^+ 435.2124. C₂₂H₃₁N₂O₇ requires 435.2131); [α]_D +40.6 (c 1.4 in MeOH); υ_max(CH₂Cl₂)/cm⁻¹ 3296 (NH), 2974 (CH), 1756 (CO, urethane), 1712 (CO, esters) and 1527 (CO, amides); δ_H(300 MHz; C^HCl;) 1.20 [9 H, s, (CH₃)₃], 1.77-2.02 [4 H, m, γ-CH₂ and β-CH₂ (Pro)], 2.87 [1 H, dd, J 17.1 and 4.2, 1 H of β-CH₂ (Asp)], 3.03 [1 H, dd, J 17.1 and 4.2, 1 H of β-CH₂ (Asp)], 3.36-3.49 [2 H, m, δ-CH₂ (Pro)], 3.68 (3 H, s, CH₃), 4.08 [1 H, br, α-H (Pro)], 4.83 [1 H, q, J 4.5, α-H (Asp)], 5.09 (2 H, s, PhCH₂), 6.93 (1 H, br, NH amide) and 7.35 (5 H, s, Ph); δ_C(75.4 MHz; C^HCl;) 21.05 [c, γ-CH₂ (Pro)], 23.7 [t, γ-CH₂ (Pro)], 28.33 [(CH₃)₃], 31.01 [t and c, β-CH₂ (Pro)], 36.49 [β-CH₂ (Asp)] 46.99 [t and c, δ-CH₂ (Pro)], 48.62 [α-C (Asp)], 52.72 (CH₃),...
60.24 [t and c, \( \alpha-C \) (Pro)], 66.85 (Ph\( \text{CH}_2 \)), 80.58 \([\text{C}(\text{CH}_3)_2]\), 128.4, 128.71 and 129.03 (Ar-\( \text{CH} \)), 135.55 (Ar-C quaternary) and 170.62 (CO, esters and amides); \( m/z \) (Cl) 435 (100%, \([\text{M} + \text{H}]^+\)), 379 (14, \([\text{M} + 2 \text{H} - \text{C}_4\text{H}_9]^+\)) and 335 (23, \([\text{M} + 2 \text{H} - \text{C}_2\text{H}_5\text{O}_2]^+\)).

\[\beta\text{-Benzyl [(2S)-prolyl]-}\alpha\text{-methyl (2R)-aspartate diester hydrochloride 65A}\]

This compound was prepared in a manner identical to that described for the hydrochloride 40 using the prolyl-aspartyl diester 64A, (750 mg, 1.73 mmol), to yield a white hydroscopic solid which was not purified any further (590 mg, 93%), mp 101-102 °C (Found: C, 55.0; H, 6.3; N, 7.4%). requires \( \text{C}_{17}\text{H}_{32}\text{N}_2\text{O}_5\text{Cl} \cdot 0.2 \text{H}_2\text{O}: \) C, 54.7; H, 6.1; N, 7.5%). (HRMS: found \([\text{M} + \text{H} - \text{HCl}]^+\), 335.1600. \( \text{C}_{17}\text{H}_{32}\text{N}_2\text{O}_5 \) requires 335.1607); \([\alpha]_D^0\) -28.28 (c 1.32 in MeOH); \( \nu_{\text{max}}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1}\) 3189 (NH), 2959 (CH), 1739 (CO, ester) and 1684 (CO, amide); \( \delta_H(300 \text{ MHz}; \text{CDCl}_3) \) 1.89-1.99 [3 H, \( \gamma\)-\( \text{CH}_2 \), 1 H of \( \beta\text{-CH}_2 \) (Pro)], 2.38-2.58 [1 H, m, 1 H of \( \beta\text{-CH}_2 \) (Pro)], 2.94 [2 H, d, \( J = 5.7 \), \( \beta\text{-CH}_2 \) (Asp)], 3.43 [2 H, br, \( \delta\text{-CH}_2 \) (Pro)], 3.6 (3 H, s, \( \text{CH}_3 \)), 4.62-4.86 [2 H, m, \( \alpha\text{-H} \) (Pro) and \( \alpha\text{-H} \) (Asp)], 5.07 [2 H, q, \( J = 12.3 \), \( \text{CH}_2\text{Ph} \)], 7.27 (5 H, s, Ph), 7.84 (1 H, br, NH) and 9.10 (1 H, d, \( J = 7.5 \), NH'); \( \delta_C(75.4 \text{ MHz}; \text{CDCl}_3) \) 24.26 [\( \gamma\text{-CH}_2 \) (Pro)], 30.39 [\( \beta\text{-CH}_2 \) (Pro)], 36.02 [\( \beta\text{-CH}_2 \) (Asp)], 46.74 [\( \delta\text{-CH}_2 \) (Pro)], 49.45 [\( \alpha\text{-CH} \) (Asp)], 52.84 (CH3), 59.70 [\( \alpha\text{-C} \) (Pro)], 66.86 (Ph\( \text{CH}_2 \)), 128.17, 128.3, 128.35 and 128.49 (Ar-\( \text{CH} \)), 135.45 (Ar-C quaternary) and 169.04, 170.11 and 170.86 (3 x CO, esters and amides); \( m/z \) (Cl) 335 (39%, \([\text{M} + \text{H} - \text{HCl}]^+\)), 303 (82, \([\text{M} - \text{HCl} - \text{CH}_3]^+\)) and 213 (100, \( \text{C}_{10}\text{H}_{12}\text{NO}_4 \)).

\[\beta\text{-Benzyl [(2R)-prolyl]-}\alpha\text{-methyl (2R)-aspartate diester hydrochloride 65B}\]

This compound was prepared in a manner identical to that described for the hydrochloride 40 using the prolyl-aspartyl diester 64B (200 mg, 0.46 mmol), to give the required compound a colourless oil which was refractory to crystallisation.
(155 mg, 91%) (HRMS: found [M + H - HCl]^+ 335.1612. C_{17}H_{22}N_{2}O_{3} requires 335.1607); [α]_D +24.65 (c 0.43 in MeOH); ν_{max}(CH_{2}Cl_{2})/cm^{-1} 2955 (CH), 1740 (CO, esters) and 1683 (CO, amide); δ_{H}(300 MHz; C_{6}D_{6}) 1.83-2.12 [3 H, m, γ-CH_{2} and 1 H of β-CH_{2} (Pro)], 2.41-2.57 [1 H, m, 1 H of β-CH_{2} (Pro)], 2.95 [2 H, m, β-CH_{2} (Asp)], 3.41 [2 H, d, J 7.2, δ-CH_{2} (Pro)], 3.56 (3 H, s, CH_{3}), 4.70 [1 H, br, α-H (Pro)], 4.83 [1 H, q, J 4.5, α-H, (Asp)], 5.07 (2 H, s, PhCH_{2}), 7.27 (5 H, s, Ph), 8.00 (1 H, br, NH) and 8.71 (1 H, d, J 7.8, NH); δ_{C} (50.3 MHz; C_{6}D_{6}) 24.85 [γ-CH_{2} (Pro)], 30.80 [β-CH_{2} (Pro)], 36.23 [β-CH_{2} (Asp)], 47.20 [δ-CH_{2} (Pro)], 49.74 [α-C (Asp)], 53.34 (CH_{3}), 60.06 [α-C (Pro)], 67.59 (PhCH_{2}), 128.91 and 129.08 (Ar-CH), 135.80 (Ar-C quaternary) and 169.27, 170.98 and 171.09 (3 x CO, esters and amide); m/z (CI) 335 (100%, [M + H - HCl]^+), 303 (11, [M - HCl - CH_{3}]^+); 144 (32, C_{6}H_{10}NO_{3}).

β-Benzyl [α-methyl (2R)-N-(tert-butoxycarbonyl)glutamyl]-γ-(2S)-prolyl-
- [α-methyl (2R)-aspartate] triester 66A

This compound was prepared in a manner identical to that described for the glutamyl-sarcosyl diester 39, using α-methyl (2R)-N-(tert-butoxycarbonyl) glutamate 37, (277 mg, 1.19 mmol) and the hydrochloride triester 65A, (440 mg, 1.19 mmol) to give the required compound as a colourless oil which was recrystallised using ethyl acetate/hexane to give a white solid (566 mg, 82%), mp 92-93 °C (Found: C, 57.9; H, 6.8; N 7.2. C_{28}H_{39}N_{3}O_{10} requires: C, 58.2; H, 6.8; N, 7.3%); (HRMS: found [M + H]^+ 578.2702. C_{28}H_{40}N_{3}O_{10} requires 578.2714); [α]_D
-19.2 (c 1.25 in MeOH); $\nu_{\text{max}}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1}$ 3354 (NH), 2956 (CH) and 1742 (CO, esters); $\delta_H(300 \text{ MHz}; \text{C}^6\text{DCl}_3)$ 1.46 [9 H, s, (CH$_3$)$_3$], 1.64-2.69 [8 H, m, $\gamma$-CH$_2$ and $\beta$-CH$_2$ (Pro and Glu)], 2.87-2.98 [2 H, m, $\beta$-CH$_2$ (Asp)], 3.37-3.40 [8-CH$_2$ (Pro)], 3.52 and 3.58 [6 H, s, 2 x CH$_3$], 4.08 [1 H, br, $\alpha$-H (Pro)], 4.28-4.38 [1 H, br, $\alpha$-H (Glu)], 4.83 [1 H, q, $J = 4.5$, $\alpha$-H, (Asp)], 5.13 (2 H, s, PhCH$_2$), 5.10 [1 H, d, $J = 5.7$ NH (urethane)], 7.29 (5 H, s, Ph) and 7.68 (1 H, d, $J = 5.7$, NH); $\delta_C(75.4$ MHz; $\text{C}^6\text{DCl}_3$) 22.60 [c, $\gamma$-CH$_2$ (Pro)], 24.56 [t, $\gamma$-CH$_2$ (Pro)], 28.03 [8-CH$_2$ (Glu)], 28.35 [(CH$_3$)$_3$], 28.97 [8-CH$_2$ (Glu)], 30.17 [t, $\beta$-CH$_2$ (Pro)], 32.13 [c, $\beta$-CH$_2$ (Pro)], 36.87 [$\beta$-CH$_2$ (Asp)], 46.94 [t, 8-CH$_2$ (Pro)], 47.39 [c, $\delta$-CH$_2$ (Pro)], 48.71 [$\alpha$-C (Asp)], 52.56 [$\alpha$-C (Glu)], 52.76 [CH$_3$ (Asp)], 52.77 [CH$_3$ (Glu)], 60.23 [$\alpha$-C (Pro)], 66.74 (PhCH$_2$), 80.8 [C(CH$_3$)$_3$], 128.31, 128.38, 128.59 and 128.69 (Ar-CH), 135.80 (Ar-C quaternary), 135.91 (CO, urethane) and 170.31, 170.66, 171.27, 171.31 and 171.62 (CO, esters and amides); $m/z$ (CI) 578 (76%, [M + H]$^+$) and 478 (100, [M + H - C$_3$H$_5$O$_2$ + H]$^+$).

**$\beta$-Benzyl [α-methyl (2R)-N-(tert-butoxycarbonyl)glutamyl]-γ-(2R)-prolyl-
-α-methyl (2R)aspartate triester 66B**

This compound was prepared in a manner identical to that described for glutamyl-
sarcosyl diester 39, using α-methyl (2R)-N-(tert-butoxycarbonyl)glutamate ester
37, (144 mg, 0.62 mmol) and the hydrochloride triester 65B, (229 mg, 0.62 mmol)
to give the required compound as a colourless oil which was refractory to
crystallisation (250 mg, 70%), (Found: C, 58.4; H, 7.1; N 7.3. C$_{28}$H$_{39}$N$_3$O$_{10}$
requires: C, 58.2; H, 6.8; N, 7.3%); (HRMS: found [M + H]$^+$, 578.2707.
C$_{28}$H$_{39}$N$_3$O$_{10}$ requires 578.2714); [$\alpha$]$_D$ +46.04 (c 1.56 in MeOH); $\nu_{\text{max}}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1}$
3324 (NH), 2956 (CH) and 1742 (CO, esters); $\delta_H(300 \text{ MHz}; \text{C}^6\text{DCl}_3)$ 1.40 [9 H, s,
(CH$_3$)$_3$], 1.83-2.59 [8 H, m, $\gamma$-CH$_2$ and $\beta$-CH$_2$ (Pro and Glu)], 2.87 [1 H, dd, $J$
17.1 and 4.2, 1 H of $\beta$-CH$_2$ (Asp)], 3.01 [1 H, dd, $J = 17.1$ and 4.2, 1 H of $\beta$-CH$_2$
(Asp)], 3.38-3.59 [2 H, m, (8-CH$_2$ Pro)], 3.64-3.70 (6 H, m, 2 x CH$_3$), 4.30 [1 H,
br, $\alpha$-H (Pro)], 4.51 [1 H, d, $J = 5.1$, $\alpha$-H (Glu)], 4.82 [1 H, q, $J = 4.8$, $\alpha$-H (Asp)],
5.11 (2 H, s, PhCH₂), 5.34 [1 H, br, NH (urethane)], 7.29 (5 H, s, Ph) and 7.43 [1 H, d J 5, NH (amide)]; δc(74.4 MHz; C²HCl₃) 24.01 [c, γ–CH₂ (Pro)], 24.95 [t, γ–CH₂ (Pro)], 27.48 [c, β–CH₂ (Glu)], 27.93 [t, β–CH₂ (Glu)], 28.05 [c, (CH₃)₃], 28.40 [t, (CH₂)₃], 30.46 [t, β–CH₂ (Pro)], 32.07 [c, β–CH₂ (Pro)], 36.11 [γ–CH₂ (Glu)], 36.48 [β–CH₂ (Asp)], 46.94 [t, δ–CH₂ (Pro)], 47.39 [c, δ–CH₂ (Pro)], 48.76 [c, α–C (Asp)], 48.83 [t, α–C (Asp)], 52.43 [α–C (Glu)], 52.74 [CH₃ (Asp)], 52.81 [CH₃ (Glu)], 60.48 [t, α–C (Pro)], 61.33 [c, α–C (Pro)], 66.91 [t, PhCH₂], 67.11 (c, PhCH₂), 80.81 [C(CH₃)₃], 128.47, 128.77 and 128.67 (Ar-CH), 135.59 (Ar-C quaternary), 135.73 (CO, urethane) and 170.63, 171.11, 171.23, 171.38, 171.90 and 173.04 (CO, esters and amides); m/z (Cl) 579 (14%, [M + 2 H]⁺), 478 (22, [M + 2 H – C₅H₉N₂O₂]⁺ and 435 (100, [M + H – C₆H₅NO₂]⁺).

β-Benzyl [α-methyl (2R)-glutamyl]-γ-(2S)-prolyl-[α-methyl (2R)-aspartate] triester hydrochloride 67A

This compound was prepared in a manner identical to that described for the hydrochloride 40 using the glutamyl-prolyl-aspartyl triester 66A, (310 mg, 0.54 mmol) as a starting material, to yield a white hydroscopic solid which was not further purified (262 mg, 95%), mp 84–85 °C (HRMS: found [M + H – HCl]⁺, 478.2182. C₂₉H₃₂N₃O₈ requires 478.2189; [α]D -41.07 (c 1.34 in MeOH); υmax(CH₂Cl₂)/cm⁻¹ 3316 (NH), 2977 (CH) and 1685 (CO, amides); δH(300 MHz; C²HCl₃) 1.80-2.80 [8 H, m, γ–CH₂ and β–CH₂ (Pro and Glu)], 2.86-3.04 [2 H, m, β–CH₂ (Asp)], 3.4-3.59 [δ–CH₂ (Pro)], 3.64, 3.67 and 3.78 [c and t, 6 H, s, 2 x CH₃]), 4.26 [1 H, br, α–H (Pro)], 4.7 [1 H, br, α–H (Glu)], 4.86 [1 H, q, J 6, α–H (Pro)], 5.11 (2 H, s, PhCH₂), 7.33 (5 H, s, Ph), 8.15 (1 H, d J 7.8, NH) and 8.65 (1 H, br, NH₃); δc(75.4 MHz; C²HCl₃) 24.76 [γ–CH₂ (Pro)], 24.91 [β–CH₂ (Glu)], 29.29 [γ–CH₂ (Glu)], 30.53 [β–CH₂ (Pro)], 36.37 [β–CH₂ (Asp)], 47.89 [δ–CH₂ (Pro)], 48.94 [α–C (Asp)], 52.71 [α–C (Glu)], 52.92 [CH₃ (Asp)], 53.53 [CH₃ (Glu)], 60.36 [α–C (Pro)], 67.02 (PhCH₂), 128.43, 128.49 and 128.66 (Ar-CH), 135.67 (Ar-C quaternary) and 169.91, 170.67, 171.61, 172.01 and 172.06 (CO,
esters, amides and acid); \(m/z\) (Cl) 478 (42%, [M + H - HCl]^-), 460 (88, [M - 2 H - Me - HCl]^-) and 144 (100, C₆H₁₀NO₃⁻).

\[\beta\text{-Benzyl }[\alpha\text{-methyl }(2R)\text{-glutamyl}]-\gamma-(2R)\text{-prolyl-}[\alpha\text{-methyl }(2R)\text{-aspartate}]\text{ triester hydrochloride 67B}\]

This compound was prepared in a manner identical to that described for the hydrochloride 40 using the glutamyl-prolyl-aspartyl triester 66B, (210 mg, 0.54 mmol) as a starting material, to yield a white hydroscopic solid which was not further purified (164 mg, 88%) mp 87-88 °C (HRMS: found [M + H - HCl]^-, 478.2198. C₂₂H₃₂N₃O₈ requires 478.2189); \([\alpha]_D^1 \text{+16.4} \text{ (c 0.7 in MeOH)};\]

\(\nu_{\text{max}}(\text{cm}^{-1})\): 2966 (CH), 1750 (CO, esters) and 1636 (CO, amide); \(\delta_h(300 \text{ MHz; } C^2\text{HCl})\): 1.78-3.11 [10 H, m, \(\gamma\)-CH₂ and \(\beta\)-CH₂ (Pro and Glu), \(\beta\)-CH₂ (Asp)], 3.39-3.50 [\(\delta\)-CH₂ (Pro)], 3.63 [3 H, s, CH₃ (Asp)], 3.77 [3 H, s, CH₃ (Glu)], 4.31 [1 H, br, \(\alpha\)-H (Pro)], 4.49 [1 H, br, \(\alpha\)-H (Glu)], 4.85 [1 H, br, \(\alpha\)-H (Asp)], 5.1 (2 H, s, PhCH₂), 7.32 (5 H, s, Ph), 7.99 (1 H, br, NH) and 8.42 (1 H, br, NH₃); \(\delta_c(75.4 \text{ MHz; } C^2\text{HCl})\): 24.64 [\(\gamma\)-CH₂ (Pro)], 29.53 [\(\beta\)-CH₂ (Glu)], 29.79 [\(\gamma\)-CH₂ (Glu)], 30.89 [\(\beta\)-CH₂ (Pro)], 36.47 [\(\beta\)-CH₂ (Asp)], 47.86 [\(\delta\)-CH₂(Pro), 49.04 [\(\alpha\)-C (Asp)], 52.86 [\(\alpha\)-C (Glu)], 53.07 [CH₃ (Asp)], 53.66 [CH₃ (Glu)], 60.83 [\(\alpha\)-C (Pro)], 66.88 (PhCH₂), 128.39, 128.43, 128.43 and 128.66 (Ar-CH), 135.75 (Ar-C quaternary) and 169.88, 170.96, 171.13, 171.6 and 172.13 (CO, esters, amides and acid); \(m/z\) (Cl) 478 (14%, [M + H - HCl]^-), 460 (100, [M - 2 H - Me - HCl]^-) and 287 (96, C₁₁H₁₄N₂O₇⁻).
Benzyl (2S)-N-(tert-butoxycarbonyl)phenylalanyl-β-alaninate ester 71

This compound was prepared in a manner identical to that described for glutamyl-sarcosyl diester 39, using N-(tert-butoxycarbonyl)-(2S)-phenylalanine 69 (2.65 g, 10 mmol) and benzyl β-alaninate p-toluenesulfonate salt 70 (3.51 g, 10 mmol) to give a white solid. Recrystallisation from ethyl acetate/hexane gave the required pure dipeptide as a white crystalline solid (3.64 g, 85%), mp 92-93 °C (Found: C, 67.3; H, 7.1; N, 6.6. C_{24}H_{30}N_{2}O_{5} requires: C, 67.6; H, 7.1; N, 6.6%) (HRMS: found [M + H]^+, 427.2227. C_{24}H_{31}N_{2}O_{5} requires 427.2233); [α]_D^0 +0.63 (c 0.71 in 1.45 MeOH); ν_max(CH_2Cl_2)/cm^-1: 3325 (NH), 2984 (CH), 1746 (CO, urethane) and 1673 (CO, ester and amide); δ_H(300 MHz; CDCl_3) 1.40 [9 H, s, (CH_3)_3], 2.40-2.48 [2 H, m, α-CH_2 (β-Ala)], 2.99 [2 H, br, β-CH_2 (Phe)], 3.38-3.50 [2 H, m, β-CH_2 (β-Ala)], 4.26 [1 H, m, α-H (Phe)], 5.08 [2 H, s, PhCH_2], 6.28 [1 H, br, NH (urethane)] and 7.16-7.39 (10 H, m, Ph); δ_C(75.4 MHz; CDCl_3) 28.41 [(CH_3)_3], 33.96 [α-CH_2 (β-Ala)], 34.83 [β-CH_2 (Phe)], 38.97 [β-CH_2 (β-Ala)], 56.13 [α-C (Phe)], 66.64 (PhCH_2), 80.27 [C(CH_3)_3], 127.03, 128.39, 128.55 128.77 and 129.39 (Ar-CH), 135.71 (Ar-C quaternary), 155.4 (CO, urethane) and 171.24 and 172.11 (CO, amide and ester); m/z (CI) 427 (100%, [M + H]^+), 371 (63, [M + H - C_4H_9 + H]^+) and 327 (21, [M + H - C_3H_5O_2 + H]^+).
(2S)-N-(tert-Butoxycarbonyl)phenylalanyl-β-alanine 72

This compound was prepared in a manner identical to α-methyl (2R)-N-(tert-butoxycarbonyl) glutamate (37), using (71) (2.13 g, 5 mmol) to give the required compound as a white crystalline solid in quantitative yield, which was not purified further, mp 89-90 °C [α]D +1 (c 0.65 in MeOH); νmax(CH2Cl2)/cm–1 3316 (NH), 3004 br (OH), 1727 (CO, urethane) and 1669 (CO, amide); δH(200 MHz; CDCl3) 1.39 [9 H, s, (CH3)3], 2.31-2.49 [2 H, m, α–CH2, (β–Ala)], 2.91-3.12 [2 H, m, β–CH2 (Phe)], 3.32-3.55 [2 H, m, β–CH2 (β–Ala)], 4.28 [1 H, br, α–H (Phe)], 5.09 [1 H, d, J 7.6, NH (urethane)], 6.28 [1 H, br, NH (amide)] and 7.12–7.37 (5 H, m, Ph); δC(75.4 MHz; CDCl3) 28.35 [(CH3)3], 33.61 [α–CH2 (β–Ala)], 34.65 [β–CH2 (Phe)], 39.24 [β–CH2 (β–Ala)], 55.73 [α–C (Phe)], 80.72 [C(CH3)3], 127.04, 128.65 and 129.58 (Ar–CH), 136.71 (Ar–C quaternary), 156.02 (CO, urethane), 171.94 (CO, amide) and 175.58 (CO, acid); m/z (Cl) 337 (30%, [M + H]+), 281 (100, [M + H – C4H6 + H]+) and 237 (39, [M + H – C5H9O2 + H]+).

β-Benzyl (2S)-N-(tert-butoxycarbonyl)phenylalanyl-β-alanyl-[α-methyl (2R)-glutamyl]-γ-(2S)-prolyl-[α-methyl (2R)-aspartate] triester 68A

This compound was prepared in a manner identical to that described for the glutamyl-sarcosyl diester 39, using the phenylalanyl-β-alanyl dipeptide 72 (246 mg,
0.73 mmol) and tripeptide 67A (376 mg, 0.73 mmol) to give the required compound as a colourless oil which was recrystallised using ethyl acetate/hexane (390 mg, 62%), mp 118-119 °C (Found: C, 59.7; H, 6.7; N 8.4. C_{40}H_{53}N_{5}O_{12}. 0.5 H_{2}O requires: C, 59.7; H, 6.6; N, 8.7%); [α]_{D}^{21} +31.67 (c 1.2 in MeOH); \nu_{\text{max}}(\text{CH}_{2}\text{Cl}_{2})/\text{cm}^{-1} 3307 (\text{NH}), 2955 (\text{CH}), 1740 (\text{CO, urethane}) and 1655 (\text{CO, esters and amides}); \delta_{t} (500 \text{ MHz}; \text{C}_{2}\text{H}_{5}\text{Cl}) 1.34 [9 \text{ H, s, } (\text{CH}_{3})_{3}], 1.81-2.42 [10 \text{ H, m, } \gamma-\text{CH}_2 \text{ and } \beta-\text{CH}_2 \text{ (Pro and Glu) and } \alpha-\text{CH}_2 \text{ (}\beta-\text{Ala})], 2.92-3.13 [4 \text{ H, m, } \beta-\text{CH}_2 \text{ (Asp) and } \beta-\text{CH}_2 \text{ (Phe)}], 3.37-3.43 [4 \text{ H, m, } \delta-\text{CH}_2 \text{ (Pro) and } \beta-\text{CH}_2 \text{ (}\beta-\text{Ala})], 5.14 (2 \text{ H, s, PhCH}_2), 5.22 [1 \text{ H, d } J 5.6, \text{ NH (urethane)}], 6.88 [2 \text{ H, br, 2 x NH (amide)}], 7.18-7.38 (10 \text{ H, m, } \text{Ph}) and 7.76 (1 \text{ H, d } J 5.4, \text{ NH}); \delta_{c}(75.4 \text{ MHz; C}_{2}\text{H}_{5}\text{Cl}) 22.76 [c, \gamma-\text{CH}_2 \text{ (Pro)}], 24.71 [t, \gamma-\text{CH}_2 \text{ (Pro)}], 27.08 [\beta-\text{CH}_2 \text{ (Glu)}], 28.36 [(\text{CH}_{3})_{3}], 28.87 [\gamma-\text{CH}_2 \text{ (Glu)}], 30.48 [t, \beta-\text{CH}_2 \text{ (Pro)}], 31.63 [c, \beta-\text{CH}_2 \text{ (Pro)}], 32.18 [\alpha-\text{CH}_2 \text{ (}\beta-\text{Ala})], 35.48 [c, \beta-\text{CH}_2 \text{ (Asp)}], 35.88 [c, \beta-\text{CH}_2 \text{ (Asp)}], 36.49 [\beta-\text{CH}_2 \text{ (}\beta-\text{Ala})], 39.18 [\beta-\text{CH}_2 \text{ (Phe)}], 46.97 [t, \delta-\text{CH}_2 \text{ (Pro)}], 47.51 [c, \delta-\text{CH}_2 \text{ (Pro)}], 48.84 [c, \alpha-\text{C (Asp)}], 49.31 [t, \alpha-\text{C (Asp)}], 52.02 [\alpha-\text{C (Glu)}], 52.63 [\text{CH}_3 \text{ (Asp)}], 52.82 [\text{CH}_3 \text{ (Glu)}], 55.82 [\alpha-\text{C (Phe)}], 60.11 [t, \alpha-\text{C (Pro)}], 61.37 [c, \alpha-\text{C (Pro)}], 66.89 (\text{PhCH}_2), 80.03 [(\text{CH}_{3})_{3}], 126.83, 128.44, 128.55, 128.67, 128.73 and 129.52 (\text{Ar-C}), 135.46, 135.63 and 135.75 (\text{Ar-C quaternary}), 155.45 (\text{CO, urethane}) and 170.53, 170.82, 171.02, 171.51, 171.65, 171.78, 171.85, 172.15 and 172.62 (c and t, \text{CO esters and amides}); m/z (FAB) 818 (72%, [M + Na]^{+}), 718 (54, [M - \text{C}_2\text{H}_5]^{+}, 696 (73, [M + H - \text{C}_3\text{H}_6\text{O}_2 + H]^{+}) and 133 (100).
β-Benzyl (2S)-N-(tert-butoxycarbonyl)phenylalanyl-β-alanyl-[α-methyl (2R)-glutamyl]-γ-(2R)-prolyl-[α-methyl (2R)-aspartate] triester 68B

This compound was prepared in a manner identical to that described for the glutamyl-sarcosyl diester 39, using the phenylalanyl-β-alanyl dipeptide 72 (246 mg, 0.73 mmol) and the tripeptide 67B (376 mg, 0.73 mmol) to give the required compound a colourless oil which was recrystallised using ethyl acetate/hexane (400 mg, 68%), mp 118-119 °C (Found: C, 60.6; H, 6.8; N 8.5. C_{40}H_{32}N_{5}O_{12} requires: C, 60.4; H, 6.7; N, 8.8%); [α]_D +15.0 (c 0.9 in MeOH); v_{max}(CH_2Cl_2)/cm^{-1} 3325 (NH), 1751 (CO, ester) and 1668 (CO, esters and amides); δ_H(300 MHz; CDCl_3) 1.34 [9 H, s, (CH_3)_2], 1.81-2.42 [10 H, m, γ-CH_2, β-CH_2 (Pro and Glu) and α-CH_2 (β-Ala)], 2.92-3.17 [4 H, m, β-CH_2 (Asp) and β-CH_2 (Phe)], 3.33-3.59 [4 H, m, δ-CH_2 (Pro) and β-CH_2 (β-Ala)], 3.63, 3.65, 3.67 and 3.69 (c and t, 6 H, s, 2 x CH_3), 4.28 [1 H, br, α-H (Pro)], 4.42-4.59 [2 H, m, α-H, (Glu and Pro)], 4.82 [1 H, br, α-CH (Asp)], 5.14 (2 H, s, PhCH_2), 5.22 [1 H, br, NH (urethane)], 6.68 (1 H, br, NH), 3.92 (1 H, br, NH) and 7.18-7.39 (10 H, m, Ph); δ_C(75.4 MHz; CDCl_3) 22.37 [c, γ-CH_2 (Pro)], 24.59 [t, γ-CH_2 (Pro)], 25.46 [t, β-CH_2 (Glu)], 25.74 [c, β-CH_2 (Glu)], 28.09 [(CH_3)_2], 30.16 [γ-CH_2 (Glu)], 30.51 [t, β-CH_2 (Pro)], 31.86 [c, β-CH_2 (Pro)], 35.38 [α-CH_2 (β-Ala)], 35.48 [t, β-CH_2 (Asp)], 35.74 [c, β-CH_2 (Asp)], 36.06 [β-CH_2 (β-Ala)], 38.71 [β-CH_2 (Phe)], 46.88 [t, δ-CH_2 (Pro)], 47.26 [c, δ-CH_2 (Pro)], 48.58 [α-C (Asp)], 52.12 [α-C (Glu)], 52.31 [CH_3 (Asp)], 52.55 [CH_3 (Glu)], 55.64 [α-C (Phe)], 59.86 [α-C (Pro)], 66.69 (PhCH_2), 79.62 [C(CH_3)_2], 126.58, 128.11, 128.26, 128.31, 128.43 and 129.24 (Ar-CH), 135.21 and 135.8 (Ar-C quaternary), 155.12 (CO, urethane) and 170.5, 170.83, 171.1, 171.25, 171.36, 171.6, 171.85 and 172.29 (CO, esters and amides); m/z (Cl) 796 (7%, [M + H]^+), 696 (77, [M + H - C_5H_9NO_4]^+) and 625 (100, [M - C_5H_9NO_4]^-).
(2S)-N-(tert-Butoxycarbonyl)phenylalanyl-β-alanyl-[α-methyl (2R)-glutamyl]-γ-(2S)-proyl-[α-methyl (2R)-aspartate] diester 73A

This compound was prepared in a manner identical to α-methyl-(2R)-N-(tert-butoxycarbonyl)glutamate 37, using the triester 68A (130 mg, 0.165 mmol) to give the required compound as a white crystalline solid in quantitative yield, which was not purified further, mp 86-87 °C (Found: C, 54.7; H, 6.5; N 9.3. C33H42N6O12.H2O requires: C, 54.8; H, 6.8; N, 9.6%); [α]D +31.67 (c 1.2 in MeOH); δH(300 MHz; C6D6Cl) 1.29 [9 H, s, (CH3)2], 1.78-2.48 [10 H, m, γ-CH2 and β-CH2 (Pro and Glu) and α-CH2 (β-Ala)], 2.65-3.17 [4 H, m, β-CH2 (Asp) and β-CH2 (Phe)], 3.32-3.58 [4 H, m, δ-CH2 (Pro) and β-CH2 (β-Ala)], 3.63, 3.65 and 3.68 (c and t, 6 H, s, 2 x CH3), 4.21-4.4 [2 H, m, α-H (Phe and Pro)], 4.42-4.58 [1 H, m, α-H, (Glu)], 4.78 [1 H, br, α-H (Asp)], 5.22 [2 H, br, NH (urethane and OH)], 7.08-7.28 (5 H, m, Ph) and 7.35-7.79 (2 H, m, NH); δC(75.4 MHz; C6D6Cl) 22.66 [c, γ-CH2 (Pro)], 24.65 [t, γ-CH2 (Pro)], 26.64 [β-CH2 (Glu)], 28.30 [(CH3)2], 28.99 [γ-CH2 (Glu)], 30.06 [t, β-CH2 (Pro)], 31.41 [c, β-CH2 (Pro)], 32.16 [α-CH2 (β-Ala)], 35.65 [β-CH2 (Asp)], 35.97 [β-CH2 (β-Ala)], 38.85 [β-CH2 (Phe)], 47.18 [t, δ-CH2 (Pro)], 47.50 [c, δ-CH2 (Pro)], 48.69 [c, α-C (Asp)], 49.10 [t, α-C (Asp)], 52.06 [α-C (Glu)], 52.62 [CH3 (Asp)], 52.74 [CH3 (Glu)], 55.91 [α-C (Phe)], 60.41 [t, α-C (Pro)], 61.39 [c, α-C (Pro)], 80.04 [C(CH3)2], 126.82, 128.54 and 129.44 (Ar-CH), 137.01 (Ar-C quaternary), 155.78 (CO, urethane) and 171.19, 171.58, 171.67, 171.95, 172.17, 172.34, 172.56, 172.72, 173.17 and 173.55 (c and t, CO); m/z (ES) 728 (5%, [M + Na]+), 706 (6, [M + H]+), 157 (95, C7H11NO4+ ) and 140 (100, C7H11NO5+ ).
(2S)-N-(tert-Butoxycarbonyl)phenylalanyl-β-alanyl-[α-methyl (2R)-glutamyl] -γ-(2R)-proyl-[α-methyl (2R)-aspartate] diester 73A

This compound was prepared in a manner identical to α-methyl (2R)-N-(tert-butoxycarbonyl)glutamate 37, using the triester 68B (130 mg, 0.165 mmol) to give the required compound as a white crystalline solid in quantitative yield, which was not purified further, mp 86-87 °C (Found: C, 54.8; H, 6.8; N 9.4. C_{33}H_{47}N_{5}O_{12}.H_{2}O requires: C, 54.8; H, 6.8; N, 9.6%); [α]_D +31.67 (c 1.2 in MeOH); ν_{max}(CH_{2}Cl_{2})/cm^{-1} 3322 (NH), 2954 (CH), 1739 (CO, urethane) and 1655 (CO, esters and amides); δ_{H}(300 MHz; C_{2}HCl_{3}) 1.30 [9 H, s, (CH_{3})_{3}], 1.82-2.38 [10 H, m, γ-CH_{2} and β-CH_{2} (Pro and Glu) and α-CH_{2} (β-Ala)], 2.74-3.05 [4 H, m, β-CH_{2} (Asp) and β-CH_{2} (Phe)], 3.37-3.62 [4 H, m, δ-CH_{2} (Pro) and β-CH_{2} (β-Ala)], 3.64 and 3.67 (6 H, s, 2 x CH_{3}), 4.35-4.39 [2 H, m, α-H (Phe and Pro)], 4.52 [1 H, br, α-H, (Glu)], 4.77 [1 H, br, α-H (Asp)], 5.42 [2 H, br, NH (urethane)], 7.15-7.22 (5 H, m, Ph) and 7.24-7.74 (3 H, m, NH); δ_{C}(75.4 MHz; C_{2}HCl_{3}) 22.71 [c, γ-CH_{2} (Pro)], 24.91 [t, γ-CH_{2} (Pro)], 25.93 [t, β-CH_{2} (Glu)], 26.36 [c, β-CH_{2} (Glu)], 28.31 [(CH_{3})_{3}], 30.84 [γ-CH_{2} (Glu)], 31.64 [t, β-CH_{2} (Pro)], 32.21 [c, β-CH_{2} (Pro)], 35.56 [α-CH_{2} (β-Ala)], 36.01 [β-CH_{2} (Asp)], 36.27 [β-CH_{2} (β-Ala)] 38.74 [β-CH_{2} (Phe)] 47.27 [δ-CH_{2} (Pro)] 47.64 [t, δ-CH_{2} (Pro)], 48.81 [t, α-C (Asp)], 49.16 [c, α-C (Asp)], 52.43 [α-C (Glu)], 52.73 [CH_{3} (Asp)], 52.9 [CH_{3} (Glu)], 55.96 [α-C (Phe)], 59.95 [t, α-C (Pro)], 60.65 [c, α-C (Pro)], 79.94 [C(CH_{3})_{3}], 126.81, 128.53 and 129.45 (Ar-CH), 137.05 (Ar-C quaternary), 155.7 (CO, urethane) and 171.5, 171.82, 172.08, 172.44, 172.61 and 173.39 (c and t, CO, esters and amides), m/z (ES) 744 (2%, [M + K]^+), 728 (8, [M + Na]^+), 706 (38, [M + H]^+), 606 (15, [M + H - C_{4}H_{7}O_{2} + H]^+), 157 (95, C_{7}H_{11}NO_{2}^+) and 140 (100, C_{10}H_{11}NO_{2}^+).
β-Pentafluorophenyl (2S)-N-(tert-butoxycarbonyl)phenylalanyl-β alanyl-
-α-methyl (2R)-glutamyl-γ-(2S)-prolyl-α-methyl (2R)-aspartate] triester 74A

To a stirred solution of the pentapeptide carboxylic acid diester 73A (130 mg, 0.18 mmol) in CH₂Cl₂ (20 cm³) at 0 °C was added pentafluorophenol (102 mg, 0.55 mmol) followed by EDCI (82 mg, 0.28 mmol). The reaction mixture was allowed to warm to room temperature and stirred over night. The solution was concentrated under reduced pressure to yield a colourless oil which was purified by flash chromatography on silica eluting with 95% CH₂Cl₂/MeOH to give triester 74A as a white crystalline solid (100 mg, 62%), mp 79-81 °C (HRMS: found [M + H + Na]+, 894.2978. C₃₉H₄₇N₅O₁₄F₃Na requires 894.2961); \( \nu_{\text{max}}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1} \) 3311 (NH), 2957 (CH), 1742 (CO, urethane) and 1655 (CO, esters and amides); \( \delta_\text{H}(300 \text{ MHz}; \text{CDCl}_3) \) 1.33 [9 H, s, (CH₃)], 1.80-2.44 [10 H, m, γ-CH₂ and β-CH₂ (Pro and Glu) and α-CH₂ (β-Ala)], 2.84-3.57 [8 H, m, β-CH₂ (Asp), β-CH₂ (Phe), δ-CH₂ (Pro) and β-CH₂ (β-Ala)], 3.69 and 3.72 (6 H, s, 2 x CH₃), 4.27-4.38 [2 H, m, α-H (Phe and Pro)], 4.62 [1 H, br, α-H (Glu)], 4.99 [1 H, q, \( J = 5.7 \), α-CH (Asp)], 5.27 [1 H, d, \( J = 8.1 \), NH (urethane)], 6.82-6.96 (2 H, m 2 x NH), 7.12-7.26 (5 H, m, Ph) and 7.88 (1 H, d, \( J = 7.8 \), NH); \( \delta_\text{C}(75.4 \text{ MHz}; \text{CDCl}_3) \) 22.73 [c, γ-CH₂ (Pro)], 24.71 [t, γ-CH₂ (Pro)], 27.21 [β-CH₂ (Glu)], 28.30 [(CH₃)₃], 28.97 [γ-CH₂ (Glu)], 30.36 [t, β-CH₂ (Pro)], 31.67 [c, β-CH₂ (Pro)], 32.17 [α-CH₂ (β-Ala)], 35.50 [β-CH₂ (Asp)], 35.59 [β-CH₂ (β-Ala)], 39.04 [β-CH₂ (Phe)], 46.90 [c, δ-CH₂ (Pro)], 47.54 [t, δ-CH₂ (Pro)], 48.82 [t, α-C (Asp)], 49.48 [c, α-C (Asp)], 51.82 [α-C (Glu)], 52.63 [CH₃ (Asp)], 53.08 [CH₃ (Glu)], 55.89 [α-C (Phe)], 60.10 [t, α-C (Pro)], 61.35 [c, α-C (Pro)], 79.95 [C(CH₃)₃], 126.84, 128.54, 128.90 and 129.44 (Ar-CH), 137.04 (Ar-C quaternary), 136.40, 138.10, 139.57, 141.60 and 142.8 (C-F, pfp), 155.51 (CO, urethane), 166.69 (CO, pfp) and 170.78, 171.74, 171.79, 172.88 and 172.60 (c and t, CO, esters and amides), m/z (ES) 895 (34%, [M + Na]+), 873 (41, [M + H]+, 196 (27, C₅H₁₀NO₄⁻), 158 (36, C₅H₆NO₃⁻) and 101 (100, C₅H₆O₂⁻).
β-Pentafluorophenyl (2S)-N-(tert-butoxycarbonyl)phenylalanyl-β-alanyl-[
α-methyl (2R)-glutamyl]-γ-(2R)-prolyl-[α-methyl (2R)-aspartate] triester 74B

To a stirred solution of the pentapeptide carboxylic acid diester 73B (120 mg, 0.17 mmol) in CH₂Cl₂ (20 cm³) at 0 °C was added pentafluorophenol (94 mg, 0.51 mmol) followed by EDCI (76 mg, 0.26 mmol). The reaction mixture was allowed to warm to room temperature and stirred over night. The solution was concentrated under reduced pressure to yield a colourless oil which was purified by flash chromatography on silica eluting with 95% CH₂Cl₂/MeOH to give triester 74B as a white crystalline solid (100 mg, 67%), mp 80-81 °C (HRMS: found [M + H + Na]⁺, 894.2923. C₃₉H₄₆N₃O₁₂F₃Na requires 894.2961); νₓₓₓ(CH₂Cl₂)/cm⁻¹ 3300 (NH), 2956 (CH), 1743 (CO, urethane) and 1652 (CO, esters and amides); δ_H(300 MHz; C⁶DCl; 1.35 [9 H, s, (CH₃)₃], 1.86-2.44 [10 H, m, γ-CH₂ and β-CH₂ (Pro and Glu) and α-CH₂ (β-Ala)], 2.84-3.62 [8 H, m, β-CH₂ (Asp), β-CH₂ (Phe), δ-CH₂ (Pro) and β-CH₂ (β-Ala)], 3.73 and 3.77 (6 H, s, 2 x CH₃), 4.20-4.58 [3 H, m, α-H (Phe, Pro and Glu)], 4.92 [1 H, br, α-H (Asp)], 5.18 [1 H, br, NH (urethane)], 6.84-6.99 (2 H, m, 2 x NH), 7.04-7.38 (5 H, m, Ph) and 7.45 (1 H, br, NH); δ_C(75.4 MHz; C⁶DCl) 24.44 [γ-CH₂ (Pro)], 27.00 [β-CH₂ (Glu)], 30.74 [(CH₃)₃], 30.84 [γ-CH₂ (Glu)], 31.23 [β-CH₂ (Pro)], 32.74 [α-CH₂ (β-Ala)], 34.19 [β-CH₂ (Asp)], 35.65 [β-CH₂ (β-Ala)], 37.83 [β-CH₂ (Phe)], 49.65 [δ-CH₂ (Pro)], 48.82 [t, α-C (Asp)], 50.97 [c, α-C (Asp)], 52.64 [α-C (Glu)], 52.82 [CH₃ (Asp)], 53.23 [CH₃ (Glu)], 56.84 [α-C (Phe)], 62.10 [α-C (Pro)], 82.17 [C(CH₃)₂], 125.22, 129.05, 130.69 and 131.57 (Ar-CH), 137.77 (Ar-C quaternary), 139.17 (C-F, pfp), 157.61 (urethane, CO) and 171.71, 171.82, 172.93 and 173.71 (c and t, CO, esters and amides); m/z (ES) 895 (11%, [M + Na]⁺), 873 (32, [M + H]⁺, 212 (100) and 196 (47, C₆H₁₀NO₄⁺).
To a stirred solution of the N-((tert-butoxycarbonyl)-protected pentafluorophenyl ester 74A (78 mg, 0.09 mmol) in CH₂Cl₂ (10 cm³) was added trifluoroacetic acid (10 cm³). The reaction mixture was stirred for 1 h when the reaction was judged to be complete by TLC. The solution was concentrated under reduced pressure, tritrated with diethyl ether (10 cm³), and the precipitate was collected and thoroughly dried under high vacuum for 6 h. The residue was dissolved in CH₂Cl₂ (500 cm³), treated with DIPEA (316 mm³) and then left to stir under Ar for eight days. The reaction mixture was concentrated under reduced pressure and immediately purified by flash chromatography on silica eluting with 94% CH₂Cl₂-MeOH to give a white crystalline solid which was recrystallised from acetone/diethyl ether (28 mg, 52%) mp >220 °C (decomp.) (Found: C, 55.8; H, 6.4; N 11.3. C₂₈H₂₇N₃O₉.H₂O requires: C, 55.8; H, 6.1; N, 11.6%) (HRMS: found [M + H]⁺, 588.2685. C₂₈H₂₇N₃O₉ requires 588.2683); ν_max (Nujol)/cm⁻¹ 3360 (NH) and 1719 (CO, esters and amides); δ[H (500 MHz, (CD₃)₂SO)] 1.79-1.90 [5 H, m, 1 H of β-CH₃ (c, Glu), β-CH₃ (t, Glu) and γ-CH₃ (t, Pro)], 1.92-2.07 [6 H, m, 1 H of β-CH₂ (c, Glu), γ-CH₂ (c, Pro), β-CH₂ (t, Pro) and 1 H of β-CH₂ (c, Pro)], 2.11-2.27 [6 H, m, 1 H of β-CH₂ (c, Pro), 1 H of γ-CH₂ (c, Glu), γ-CH₂ (t, Pro) and 1 H of α-CH₂ (c and t, α-Ala)], 2.34-2.61 [5 H, m, 1 H of γ-CH₂ (c, Glu), 1 H of β-CH₂ (c and t, Asp)], 2.68-2.82 [3 H, m,
1 H of β-CH₂ (c and t, Asp), 1 H of β-CH₂ (t, Phe)], 2.90-3.03 [2 H, m, 1 H of β-CH₂ (c and t, Phe)], 3.08-3.25 [3 H, m, 1 H of β-CH₂ (c, Phe) and 1 H of α-CH₂ (c and t, β-Ala)], 3.40-3.59 [6 H, m, δ-CH₂ (c and t, Pro) and 1 H of β-CH₂ (c and t, β-Ala)], 3.62, 3.64, 3.66, and 3.71 (12 H, s, 2 × CH₂, c and t), 4.13-4.25 [3 H, m, α-H (c, Phe), α-H (t, Glu) and α-H (c, Asp)], 4.28 [1 H, dd, J 8.3 and 2.7, α-H (c, Pro)], 4.38-4.44 [2 H, m, α-H (c, Glu) and α-H (t, Pro)], 4.47 [1 H, sep, J 3.1, α-H (t, Asp)], 4.56 [1 H, q, sep, J 5.5, α-H (t, Phe)], 7.21-7.34 (10 H, m, c and t, Ph), 7.78 [1 H, t, J 5.5, NH (c, β-Ala)], 7.88 [1 H, t, J 5.5, NH (t, β-Ala)], 8.11 [1 H, d, J 6.7, NH (c, Asp)], 8.20 [1 H, d, J 9.2, NH (t, Phe)], 8.21 [1 H, d, J 7.9, NH (t, Glu)], 8.30 [1 H, d, J 7.3, NH (c, Phe)], 8.32 [1 H, d, J 7.9, NH (c, Glu)], and 8.38 [1 H, d, J 7.3, NH (t, Asp)]; δ(C¹H₃)SO) 21.88 [c, γ-CH₂ (Pro)], 23.92 [t, γ-CH₂ (Pro)], 24.02 [c, β-CH₂ (Glu)], 26.66 [t, β-CH₂ (Glu)], 28.78 [t, γ-CH₂ (Glu)], 29.31 [c, γ-CH₂ (Glu)], 30.53 [t, β-CH₂ (Pro)], 31.46 [c, β-CH₂ (Pro)], 34.49 [α-CH₂ (β-Ala)], 34.78 [c, β-CH₂ (Asp)], 34.93 [c, β-CH₂ (Asp)], 35.57 [β-CH₂ (β-Ala)], 37.77 [c, β-CH₂ (Phe)], 39.18 [t, β-CH₂ (Phe)], 46.31 [t, δ-CH₂ (Pro)], 46.84 [c, δ-CH₂ (Pro)], 49.4 [c, α-C (Asp)], 50.1 [t, α-C (Asp)], 50.8 [α-C (Glu)], 51.79, 51.9, 52.05 and 52.14 (c + t, CH₃), 53.71 [t, α-C (Phe)], 55.52 [c, α-C (Phe)], 59.65 [t, α-C (Pro)], 59.71 [c, α-C (Pro)], 126.12, 127.91, 128.03, 128.92 and 129.03 (Ar-CH), 137.82 and 137.86 (Ar-C quaternary) and 168.88, 170.13, 170.98, 171.08, 171.14, 171.3, 171.42, 171.96, 172.18 and 172.6 (CO, ester and amides); m/z (Cl) 588 (8%, M⁺), 556 (25, [M – CH₃]⁺) and 391 (100, [M – C₂H₅N₂O₂]⁺).

Cyclo-[β-Ala-(2R)-Glu-α-OMe-γ-(2R)-Pro-(2R)-Asp-α-OMe-β-(2S)-Phe-] 52B

This compound was prepared in a manner identical to that described for the macrocycle 52A using the N-(tert-butoxycarbonyl)-protected pentafluorophenyl ester 74B (89 mg, 0.1 mmol) to give the required compound as a colourless oil, which was immediately purified by flash chromatography on silica eluting with 94% CH₂Cl₂/MeOH. This gave a crystalline solid which was recrystallised from
acetone/diethyl ether to yield a white crystalline solid, mp >222 °C (decomp.)
(Found: C, 55.9; H, 6.2; N 11.3. C_{28}H_{37}N_{5}O_{6}.H_{2}O requires: C, 55.8; H, 6.1;
N, 11.6%) (HRMS: found [M + H]^{+}, 588.2654. C_{28}H_{37}N_{5}O_{6} requires 588.2670);
υ_{max}(CH_{2}Cl_{2})/cm^{-1} 3395 (NH), 1743, 1724 and 1660 (CO, esters and amides);
δ_{H}(500 MHz; (C_{2}H_{5})_{2}SO) 1.76-2.03 [12 H, m, 1 H of β–CH_{2} (c and t, Pro), 1 H of
β–CH_{2} (c, Pro), γ–CH_{2} (c and t, Pro), β–CH_{2} (c and t, Glu) and 1 H of γ–CH_{2}
(t, Glu)], 2.18-2.67 [12 H, m, 1 H of γ–CH_{2} (c, Glu), 1 H of γ–CH_{2} (c and t, Glu),
1 H of β–CH_{2} (t, Pro), α–CH_{2} (c and t, βAla) and β–CH_{2} (c and t, Asp)],
2.77-3.15 [6 H, m, 1 H of β–CH_{2} (c and t, β-Ala) and β–CH_{2} (c and t, Phe)], 3.38-
3.48 [5 H, m, 1 H of β–CH_{2} (c and t, β-Ala) and δ–CH_{2} (c and t, Pro)], 3.68, 3.72,
3.74, and 3.78 [12 H, s, 2 x CH_{3}, c and t], 4.29-4.5 [8 H, m, α–H (c and t, Phe),
α–H (c and t, Glu), α–H (c and t, Asp) and α–H (c and t, Pro)], 7.23-7.42 (10 H,
m, c and t, Ph), 7.9-7.93 [2 H, m, NH (c, β-Ala), NH (t, Glu)], 7.99-8.03 [2 H, m,
(c, Asp), NH (t, β-Ala)] and 8.2-8.29 [4 H, m, NH (c and t, Phe) NH (c, Glu) and
NH (t, Asp)]; δ_{C}(75.4 MHz; (C_{2}H_{5})_{2}SO) 22.62 [c, γ–CH_{2} (Pro)], 25.06 [t, γ–CH_{2}
(Pro)], 25.73 [t, β–CH_{2} (Glu)], 26.04 [c, β–CH_{2} (Glu)], 28.98 [c and t, γ–CH_{2}
(Glu)], 30.23 [t, β–CH_{2} (Pro)], 31.97 [c, β–CH_{2} (Pro)], 35.46 [α–CH_{2} (β-Ala)],
35.71 [t, β–CH_{2} (Asp)], 36.09 [c, β–CH_{2} (Asp)], 36.60 [c, β–CH_{2} (β-Ala)], 37.03
[t, β–CH_{2} (β-Ala)], 38.03 [c, β–CH_{2} (Phe)], 38.74 [t, β–CH_{2} (Phe)], 47.01
[c, δ–CH_{2} (Pro)], 47.77 [t, δ–CH_{2} (Pro)], 50.05 [α–C (Asp)], 51.58 [α–C (Glu)],
52.69, 53.13 and 53.23 [c and t, CH_{3}], 54.61 [α–C (Phe)], 60.47 [t, α–C (Pro)],
61.41 [c, α–C (Pro)], 126.89, 127.16, 128.68, 129.16 and 129.3 (Ar-CH), 136.22
and 137.32 (Ar-C quaternary) and 170.35, 171.45, 171.52, 172.04 and 172.68
(CO, esters and amides); m/z 588 (9%, M^{+}), 556 (27, [M – CH_{3}]^{+}) and 391 (100,
[M – C_{12}H_{16}N_{2}O_{2}]^{+}).
γ-Benzyl (2R)-N-(9-fluorenylmethoxycarbonyl)glutamate 79

To an ice-cold stirred suspension of γ-benzyl (2R)-glutamate 78 (1 g, 4.22 mmol) in water (30 cm³) was added potassium carbonate (945 mg, 6.75 mmol). A pre-stirred solution of 9-fluorenylmethoxycarbonyl chloride (1.21 g, 4.64 mmol) in dioxane (30 cm³) was added. The resulting solution was warmed to room temperature and stirred for 4 hours, then poured into water (20 cm³) and the dioxane was removed under reduced pressure. The resulting solution was washed with diethyl ether (2 x 50 cm³) and acidified to pH 2 at 0 °C with 6 mol dm⁻³ HCl. The resulting solution was then extracted with diethyl ether (3 x 50 cm³). The ethereal solutions were combined, dried (MgSO₄) and the solvent removed under reduced pressure to yield a colourless oil. Crystallisation with ether/light petroleum gave a white solid (1.24 g, 72%) mp 137-8 °C (lit., 136-8 °C (for the S-isomer)) (Found: C, 70.3; H, 5.4; N 3. Calc. C₂₇H₃₂NO₆: C, 70.6; H, 5.5; N, 3%) (HRMS: found [M + H]+, 460.1766. C₂₇H₃₂NO₆ requires 460.1760); [α]D -12.9 (c 1.51 in MeOH) (lit., [α]D +13.8 (c 1.5 in MeOH (for the S-isomer))

νOH (300 MHz; CDCl₃) 1.95-2.53 (4 H, m, γ-CH₂ and β-CH₂), 4.09-4.61 (6 H, m, fluorenyl CH₂O, fluorenyl CH and α-H), 5.12 (2 H, s, PhCH₂), 5.48 [1 H, d, J 8.1, NH (urethane)] and 7.26-7.76 (13 H, m, Ph); δC(75.4 MHz; CDCl₃) 27.39 (β-CH₂), 30.48 (γ-CH₂), 47.26 (fluorenyl CH), 53.32 (α-C), 66.78 and 67.38 (2 x CH₂O), 120.13, 125.17, 127.23, 127.89, 128.44, 128.51 and 127.83 (Ar-CH), 141.46, and 143.73 (Ar-C quaternary) 156.13 (CO, urethane) and 172.5 (CO, ester); m/z (Cl) 460 (8%, [M + H]+), 179 (100, C₁₄H₁₁⁺) and 130 (64, C₅H₄NO₃⁻).
**α-Methyl γ-benzyl (2R)-N-(9-fluorenylmethoxycarbonyl)glutamate 80**

This compound was prepared in a manner identical to α-methyl γ-benzyl (2R)-N-((tert-butoxycarbonyl)glutamate diester 36, using ester 79 (1.04 g, 2.54 mmol) to give the required compound as a white crystalline solid in quantitative yield, which did not require further purification, mp 97-98 °C (Found: C, 71.0; H, 5.7; N, 3.0. C_{28}H_{27}NO_{6} requires: C, 71.0; H, 5.8; N, 3.0%) (HRMS: found [M + H]^+ 474.1913. C_{28}H_{28}NO requires 474.1917); [α]_{D} +16.7 (c 0.45 in MeOH); v_{max}(CH_{2}Cl_{2})/cm^{-1} 3375 (NH), 2955 (CH), 1736 (CO, urethane) and 1536 (CO, esters); δ_{H}(300 MHz; CDCl_{3}) 1.95-2.58 (4 H, m, γ-CH2 and β-CH2), 3.75 (3 H, s, CH3), 4.09-4.58 (4 H, m, fluorenlyl CH2O, fluorenlyl CH and γ-H), 5.13 (2 H, s, PhCH2), 5.57 [1 H, d, J 7.5 NH (urethane)] and 7.23-7.83 (13 H, m, Ph); δ_{C}(50.3 MHz; CDCl_{3}) 27.69 (p-CHj), 30.37 (γ-CH3), 47.32 (fluorenlyl CH), 52.69 (CH3), 53.48 (α-C), 66.71 and 67.19 (2 x CH2O), 120.16, 125.24, 127.26, 127.89, 128.45 and 128.76 (Ar-CH), 135.91, 141.48, 143.89 and 144.07 (Ar-C quaternary) 156.15 (CO, urethane) and 172.50 and 172.66 (CO, esters); m/z (Cl) 474 (32%, [M + H]^+), 252 (100, C_{13}H_{18}NO_{4}^+), 179 (91, C_{6}H_{10}NO_{3}^+) and 144 (57, C_{6}H_{10}NO_{3}^-).

**γ-Allyl (2R)-glutamate hydrochloride 82**

To a stirred suspension of (2R)-glutamic acid 81 (2.21 g, 15 mmol) in dry allyl alcohol (70 cm^3) under N_{2}, was added dropwise chlorotrimethylsilane (4.75 cm^3, 47.5 mmol) dropwise. The resulting solution was stirred at RT for 18 h., after which diethyl ether (200 cm^3) was added at 0 °C to give a white precipitate which was collected by filtration, washed with diethyl ether and dried under vacuum (2.18
g, 65%) mp 131-132 °C (lit.,235 130-132 °C (for the (2S)-isomer), [α]D - 22.1 (c 1.1 in MeOH) (lit., [α]D +22.5 (c 1 in MeOH), (for the (2S) isomer)); υmax(CH2Cl2)/cm⁻¹ 2863 br (OH) and 1729 (CO, ester); δH(300 MHz; 2H2O) 2.08-2.21 (2 H, sep, J 7.2, β-CH2), 2.55 (2 H, t, J 6.0, γ-CH2), 3.98 (1 H, t, J 6.9, α-H), 4.52 (2 H, d, J 5.7, CH3O), 5.21 (2 H, dd, J 15.9, 1.5 CH2=CH) and 5.84 (1 H, m, CH2=CH); δc(75.4 MHz; 2H2O) 25.00 (β-CH2), 29.75 (γ-CH2), 52.31 (α-C), 66.28 (CH2O), 171.79 (CO, ester) and 174.23 (CO, acid); m/z (Cl) 188 (84%, [M – Cl]⁻) and 130 (100, C3H8NO2⁻).

γ-Allyl (2R)-N-(9-fluorenylmethoxycarbonyl)glutamate 83

This compound was prepared in a manner identical to γ-benzyl (2R)-N-(9-fluorenylmethoxycarbonyl)glutamate 79, using γ-allyl (2R)-glutamate hydrochloride 82, (943 mg, 4.22 mmol) to give the required compound as a white crystalline solid in quantitative recovery, which was not purified further, mp 113-4 °C (HRMS: found [M + H]⁺, 410.1595. C23H24NO6 requires 410.1603; [α]D +30.4 (c 1.3 in MeOH); υmax(CH2Cl2)/cm⁻¹ 3364 (NH), 2965 (CH) and 1746 (CO, urethane); δH(300 MHz; C6H5Cl) 2.01-2.58 (4 H, m, γ-CH2 and β-CH2), 4.18-4.62 (6 H, m, 2 x CH2O, fluorenyl CH and α-H), 5.21-5.58 (2 H, m, CH2=CH) 5.58 [1 H, d, J 7.8, NH (urethane)], 5.82-5.98 (1 H, m, CH2=CH) and 7.23-7.79 (8 H, m, Ph); δc(75.4 MHz; C6H5Cl) 27.42 (β-CH2), 30.40 (γ-CH2), 47.24 (fluorenyl CH), 53.39 (α-C), 65.67 and 67.34 (2 x CH2O), 118.68 (CH2=CH), 120.13, 125.23, 127.25 and 127.89 (Ar-CH), 132.03 (CH2=CH), 141.46, 143.76 and 143.97 (Ar-C quaternary) 156.42 (CO, urethane) and 172.5 and 175.66 (CO, ester and acid); m/z (Cl) 424 (17%, [M + 2 H + Na]⁻), 410 (64, [M – CO2]⁻), 181 (100, C14H15⁻) and 130 (48, C3H8NO2⁻).
α-Methyl γ-allyl(2R)-N-(9-fluorenylmethoxycarbonyl)glutamate 84

This compound was prepared in a manner identical to diester 36, using ester 83 (1.04 g, 2.54 mmol) to give the required compound as a white crystalline solid in quantitative yield, which did not require further purification mp 84-85 °C (Found: C, 67.9; H, 5.8; N 3.5. C_{24}H_{25}NO_{6} requires: C, 68.1; H, 5.9; N, 3.3%) (HRMS: found [M + H]^+, 424.1766. C_{24}H_{26}NO_{6} requires 424.1760); [α]_D +22.14 (c 0.56 in MeOH); ν_{max}(CH_{2}Cl_{2}) cm^{-1} 3364 (NH), 2965 (CH) and 1751 (CO, urethane); δ_{H}(300 MHz, C_{6}D_{5}Cl) 1.95-2.53 (4 H, m, γ-CH$_2$ and β-CH$_2$), 3.75 (3 H, s, CH$_3$), 4.09-4.61 (6 H, m, 2 x CH$_2$O, fluorenyl CH and α-H), 5.21-5.38 (2 H, m, CH$_2$=CH), 5.56 [1 H, d, J 8.1, NH (urethane)], 5.84-5.97 (1 H, m, CH$_2$=CH) and 7.26-7.77 (8 H, m, Ph); δ_{C}(75.4 MHz, C_{6}D_{5}Cl) 27.04 (β-CH$_2$), 30.29 (γ-CH$_2$), 47.29 (fluorenyl CH), 52.70 (CH$_3$), 53.48 (α-C), 65.54 and 67.21 (2 x CH$_2$O), 118.61 (CH$_2$=CH), 120.14, 125.22, 127.23 and 127.87 (Ar-CH), 132.14 (CH$_2$=CH), 141.46, 143.85 and 144.03 (Ar-C quaternary) 156.13 (CO, urethane) and 172.50 (CO, ester); m/z (Cl) 424 (45%, [M + H]^+), 179 (94, C$_{14}$H$_{11}$^+), 144 (78, C$_{8}$H$_{10}$NO$_3$^+) and 57 (100, C$_{4}$H$_{9}$^+).

α-Methyl (2R)-N-(9-fluorenylmethoxycarbonyl)glutamate ester 77

![Chemical structure](image)

To a stirred solution of diester 84 (423 mg, 1 mmol) and phenylsilane (247 mm$_3$, 2 mmol) in dry DCM (30 mm$_3$) under Ar, was added tetrakis (triphenylphosphine) Pd (0) (23 mg, 0.02 mmol). The reaction mixture was allowed to stir at room
temperature for 2 h (after which time no starting material was observed by TLC) and then concentrated under reduced pressure. The crude material was purified using silica chromatography (60% ethyl acetate/hexane) to give a white solid (310 mg, 81%) mp 130-131 °C (Found: C, 64.6; H, 5.4; N 3.7%). C_{21}H_{21}NO_6 requires: C, 64.8; H, 5.5; N, 3.7%) (HRMS: found [M + H]^+ 384.1443. C_{21}H_{21}NO_6 requires 384.1447); [α]_D +19.5 (c 0.63 in MeOH); ν_{max}(CH_2Cl_2) cm^{-1} 3355 (NH), 2965 (CH) and 1732 (CO, urethane); δ_{H}(300 MHz; CDCl_3) 1.92-2.57 (4 H, m, γ-CH₂ and β-CH₂), 3.75 (3 H, s, CH₃), 4.18-4.56 (4 H, m, CH₂O, fluorenyl CH and α-H), 5.45 [1 H, d, J 7.2, NH (urethane)] and 7.25-7.77 (8 H, m, Ph); δ_{C}(75.4 MHz; CDCl₃) 27.57 (P-CH₂), 29.89 (γ-CH₂), 47.28 (fluorenyl CH), 52.74 (CH₃), 53.29 (α-C), 67.24 (CH₂O), 120.14, 125.19, 127.23 and 127.88 (Ar-CH), 141.47 and 143.81 (Ar-C quaternary), 156.15 (CO, urethane) and 177.54 (CO, acid); m/z (Cl) 384 (7%, [M + H]^+) 179 (31, C_{14}H_{11}^+) and 144 (100, C_{6}H_{10}NO_{2}^+).

β-Allyl (2R)-aspartate hydrochloride 87

This compound was prepared in a manner identical to γ-allyl (2R)-glutamate hydrochloride 82, using (2R)-aspartic acid, (5.33 g, 40 mmol) to give the required compound as a white crystalline solid, which did not require further purification (7.3 g, 87%), mp 184-185 °C (lit., 234 185-6 °C (for the (2S)-isomer)); [α]_D +7.7 (c 8.0 in AcOH) (lit., 234 -9.5 (c 8.0 in AcOH) (for the (2S)-isomer)); ν_{max}(CH_2Cl_2) cm^{-1} 3364 (NH), 2965 (CH), 1751 (CO, ester) and 1536 (amide); δ_{H}(300 MHz; D_2O) 3.03-3.06 (2 H, m, β-CH₂), 4.27 (1 H, t, J 4.8, α-H), 4.60 (2 H, d, J 6, CH₂O), 4.65 (3 H, s, NH₃⁺), 5.15-5.27 (2 H, m, CH_2=CH) and 5.77-5.90 (1 H, m, CH_2=CH); δ_{C}(75.4 MHz; D_2O) 34.05 (β-CH₂), 49.37 (α-C), 66.78 (CH₂O), 119.00 (CH₂=CH), 131.47 (CH_2=CH), 170.94 (CO, ester) and 171.19 (CO, acid); m/z (Cl) 174 (100%, [M + H–HCl]^+).
β-Allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartate 88

This compound was prepared in a manner identical to γ-benzyl (2R)-N-(9-fluorenylmethoxycarbonyl)glutamate 83, using γ-allyl (2R)-aspartate hydrochloride 87, (530 mg, 2.5 mmol) to give the required compound as a white crystalline solid in quantitative yield, which was not purified further (930 mg, 94%), mp 110-111 °C; (Found: C, 66.3; H, 5.4; N 3.6. C_{22}H_{22}NO_{6} requires: C, 66.1; H, 5.4; N, 3.5%) (HRMS: found [M + H]^+ , 396.1450. C_{22}H_{22}NO_{6} requires 396.1453); [α]_D +3.04 (c 0.46 in MeOH); ν_{max}(CH_{2}Cl_{2})/cm^{-1} 3363 (NH), 2956 (CH) and 1741 (CO, urethane); δ_{H}(300 MHz; C_{6}DCl_{3}) 2.94 (1 H, dd, J = 18 and 3.4, 1 H of β–CH₂), 3.13 (1 H, dd, J = 18 and 3.45, 1 H of β–CH₂), 4.20-4.79 (6 H, m, 2 x CH₂O, fluorenyl CH and α–H), 5.21-5.38 (2 H, m, CH_{2}=CH), 5.82-6.21 [2 H, m, CH_{2}=CH and NH (urethane)], 7.25-7.82 (8 H, m, Ph) and 9.01 (1 H, br, OH); δ_{C}(75.4 MHz; C_{6}DCl_{3}) 36.54 (β–CH₂), 47.20 (fluorenyl CH), 50.51 (α–C), 66.07 and 67.60 (2 x CH₂O), 119.05 (CH_{2}=CH), 120.51, 125.28, 127.26, 127.53, 127.90 and 128.41 (Ar-CH), 131.98 (CH_{2}=CH), 141.44, 143.78 and 143.92 (Ar-C quaternary) 156.35 (CO, urethane), 170.96 (CO, ester) and 175.46 (CO, acid); m/z (Cl) 396 (12%, [M + H]^+) and 179 (100, C_{14}H_{11}+).
β-Allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartyl-Wang resin 85

A suspension of Wang resin (500 mg, 0.84 mmol g⁻¹ OH substitution) and β-allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartate 88 (660 mg, 1.67 mmol) were stirred in dry DMF (2.5 cm³) at RT under N₂ for 15 min. Pyridine (100 mm³, 2.77 mmol) and 2,6-dichlorobenzoyl chloride (105 mm³, 1.67 mmol) were added and the suspension stirred at room temperature for 20 h, then filtered and washed with DMF (25 cm³), DCM (25 cm³) and methanol (25 cm³). The percentage loading was checked determined to be 70% (see Appendix 4). The remaining hydroxyl groups of the resin were benzoylated with benzoyl chloride and pyridine in DCM for 2 h., washed with DMF (25 cm³), DCM (25 cm³) and methanol (25 cm³), and dried under vacuum and checked once again for percentage loading (772 mg, 70% loading).
β-Allyl (2S)-N-(9-fluorenylmethoxycarbonyl)phenylalanyl-β-alanyl-[α-methyl (2R)-glutamyl]-γ-(2R)-prolyl-[(2R)-aspartate] diester 76A

β-Allyl (2S)-N-(9-fluorenylmethoxycarbonyl)phenylalanyl-β-alanyl-[(2R)-α-methyl-glutamyl]-γ-(2R)-prolyl-[(2R)-aspartate-Wang resin] diester 76 (2.1 g, 426 mg petidyl content, 0.05 mmol) was synthesised on the peptide synthesiser and treated with cleavage mixture TFA/TEA/H2O/DCM (40:2:5:53) at room temperature for 1 h. The resin was filtered off and the solvent concentrated under reduced pressure (4-5 cm²). The peptide was then precipitated with excess diethyl ether to give a white solid in quantitative yield (426 mg), mp 115-116 °C (Found: C, 59.6; H, 6.6; N 7.9. C₃₅H₅₁N₅O₁₂ requires: 3 H₂O: C, 59.5; H, 6.9; N, 7.7%); [α]D-13.0 (c 1.3 in MeOH); νmax(CH₂Cl₂)/cm⁻¹ 3276 (NH), 2977 (CH) and 1727 br (CO, esters and amides); δH(300 MHz; C⁶HCl₃) 1.76-2.54 [10 H, m, γ-CH₃ and β-CH₃ (Pro and Glu) and α-CH₃ (p-Ala)], 2.82-3.14 [4 H, m, β-CH₂ (Asp) and β-CH₂ (Phe)], 3.32-3.47 [4 H, m, δ-CH₂ (Pro) and β-CH₂ (β-Ala)], 3.77 (3 H, s, CH₃), 4.16-4.88 [9 H, m, 4 x α-H, 2 x CH₂O and fluorenyl CH], 4.97 [1 H, br, NH (urethane)], 5.14-5.36 (2 H, m, CH₂=CH), 5.76-5.94 (1 H, m, CH₂=CH), 6.18 (2 H, br, NH) and 7.00-7.23 (15 H, m, Ph, 2 x NH); δC(75.4 MHz; C⁶HCl₃) 24.53 [γ-CH₂ (Pro)], 26.86 [β-CH₂ (Glu)], 28.84 [γ-CH₂ (Glu)], 29.77 [β-CH₂ (Pro)], 34.97 [α-CH₂ (β-Ala)], 35.36 [β-CH₂ (Asp)], 36.34 [β-CH₂ (β-Ala)], 38.71 [β-CH₂ (Phe)], 46.90 [δ-CH₂ (Pro)], 47.42 [α-C (Asp)], 51.55 [α-C (Glu)], 52.51 [CH₃ (Asp)], 56.02 [α-C (Phe)], 60.00 [α-C (Pro)], 65.56 and 67.31 (2 x
CH₂O), 118.45 (CH₂=CH), 119.96, 125.14, 126.87, 127.1, 127.75, 128.49, 128.64, 129.41, 130.13, 131.83 and 133.37 (Ar-CH), 136.67 (CH₂=CH), 141.29 and 141.74 (Ar-C quaternary), 156.55 (CO, urethane) and 170.61, 171.94 and 172.7 (CO esters and amides); m/z (ES) 892 (20%, [M + K]⁺), (42, [M + Na]⁺), 855 (28, [M + 2H]⁺), 718 (49, [M + H – C₁₅H₁₁O₂]⁻) and 102 (100).

(2S)-Phenylalanyl-β-alanyl-[α-methyl (2R)-glutamyl]-γ-(2R)-prolyl-[[(2R)-aspartate] ester 92A

To a stirred solution of resin 76 (495 mg, 0.3 mmol) in a stirred mixture of DMSO/THF/0.5 M HCl/NMM (2:2:1:0.1), (25 cm³) under Ar, was added tetrakis (triphenyl phosphine) Pd (0) (104 mg, 9 x 10⁻³ mol). The resin was stirred slowly at room temperature for 3 h, after which time the resin was filtered and washed with THF (25 cm³), DCM (25 cm³), and methanol (25 cm³).

The resin was then treated with 20% piperidine/DMF (10 cm³) for 30 min [reaction monitored by the Ninhydrin Test (see Appendix 4)], after which time the resin was filtered and washed with DMF, DCM and methanol respectively. This was then treated with cleavage mixture TFA/TES/H₂O/DCM (40:2:5:53) at room temperature for 1 h. The resin was filtered off and the solvent concentrated under reduced pressure (4-5 cm³). The peptide was then precipitated with excess diethyl ether to give a white solid in quantitative yield (177 mg), νmax(CH₂Cl₂)/cm⁻¹ 3200 br (NH and OH) and 1729 br (CO, esters and amides); δH(300 MHz; ²H₂O) 1.44-2.39
[10 H, m, γ-CH₂, β-CH₂ (Pro and Glu) and α-CH₂ (β-Ala)], 2.64-3.04 [4 H, m, β-CH₂ (Asp) and β-CH₂ (Phe)], 3.17-3.4 [4 H, m, δ-CH₂ (Pro) and β-CH₂ (β-Ala)], 3.57 (t, 3 H, s, CH₃), 3.59 (c, 3 H, s, CH₃), 4 (1 H, t, J 7.4 α-H), 4.08-4.38 (2 H, m, 2 x α-H), 4.58-4.61 (1 H, m, α-H) and 7.02-7.38 (5 H, m, Ph); δC(75.4 MHz; CCl₄) 22.13 [c, γ-CH₂ (Pro)], 25.05 [t, γ-CH₂ (Pro)], 29.66 [γ-CH₂ (Glu)], 29.77 [t, β-CH₂ (Pro)], 31.74 [t, β-CH₂ (Pro)], 34.23 [α-CH₂ (β-Ala)], 35.26 [β-CH₂ (Asp)], 35.45 [β-CH₂ (β-Ala)], 36.88 [β-CH₂ (Phe)], 47.93 [c, δ-CH₂ (Pro)], 47.86 [t, δ-CH₂ (Pro)], 48.98 [t, α-C (Asp)], 49.19 [c, α-C (Asp)], 52.39 [α-C (Glu)], 52.94 [CH₂ (Asp)], 54.44 [α-C (Phe)], 60.25 [t, α-C (Pro)], 60.8 [c, α-C (Pro)], 128.06, 129.23, 129.44, 130.63, 133.75 and 133.9 (Ar-CH), 135.89 (Ar-C quaternary) and 173.43, 173.64, 173.72, 173.89, 173.97, 174.27 and 174.4 (CO esters and amides); m/z (ES) 620 (5%, [M + K]⁺), 614 (10, [M + Na]⁺) and 592 (100, [M + H]⁺).

Cyclo-[β-Ala-(2R)-Glu-α-OMe-γ-(2R)-Pro-(2R)-Asp-β-(2S)-Phe-] 93A

To a stirred suspension of resin 92 (100 mg, 0.06 mmol) in DMF (5 cm³) and DIPEA (102 mm³, 0.6 mmol) under Ar, was added PyBOP (157 mg, 0.3 mmol) and HOBt (41 mg, 0.3 mmol). The resin was stirred slowly at room temperature for 7 days, (reaction monitored by the Ninhydrin Test), filtered and washed with THF (25 cm³), DCM (25 cm³) and methanol (25 cm³). The resulting resin was treated with cleavage mixture TFA/TES/H₂O/DCM (40:2:5:53) at room temperature for 1 h.
The resin was filtered off and the filtrate concentrated under reduced pressure (4-5 cm$^3$). The peptide was then precipitated with excess diethyl ether to give a white solid which was purified by reverse phase HPLC (10 mg, 30%) mp >180 °C (decomp.) (HRMS: found [M + H − H$_2$O]$^-$, 556.2420. C$_{27}$H$_{44}$N$_3$O$_7$ requires 556.2407); $\nu$$_{\text{max}}$ (CH$_2$Cl$_2$)/cm$^{-1}$ 3302 (NH) and 1735 and 1670 (CO, esters and amides); $\delta$$_H$ [500 MHz; (C$_2$H$_5$)$_2$SO] 1.72-2.02 [11 H, m, 1 H of $\beta$-CH$_2$ (c and t, Pro), 1 H of $\beta$-CH$_2$ (c, Pro), $\gamma$-CH$_2$ (c and t, Pro) and $\beta$-CH$_2$ (c and t, Glu)], 2.12-2.55 [15 H, m, $\gamma$-CH$_2$ (c and t, Glu), 1 H of $\gamma$-CH$_2$ (c and t, Glu), 1 H of $\beta$-CH$_2$ (c, Pro), 1 H of $\beta$-CH$_2$ (c and t, Asp) and $\alpha$-CH$_2$ (c and t, $\beta$-Ala)], 2.66-3.2 [8 H, m, 1 H of $\beta$-CH$_2$ (c and t, Asp), $\beta$-CH$_2$ (c and t, Phe) and 1 H of $\beta$-CH$_2$ (c and t, Asp)], 3.4-3.49 [6 H, m, 1 H of $\beta$-CH$_2$ (c and t, $\beta$-Ala) and $\delta$-CH$_2$ (c and t, Pro)], 3.55, 3.67, 3.78, and 3.79 [12 H, s, 2 x CH$_3$, c and t], 4.08-4.48 [8 H, m, $\alpha$-H (c and t, Phe), $\alpha$-H (c and t, Glu), $\alpha$-H (c and t, Asp) and $\alpha$-H (c and t, Pro)], 7.21-7.39 (10 H, m, Ph, c and t), 7.75-7.97 [3 H, m, NH (c and t, $\beta$-Ala), NH (c, Asp)], 8.14-8.29 [5 H, m, NH (t, Asp), NH (c and t, Phe) and NH (c and t, Glu)]; $\delta$$_C$(75.4 MHz; C$_2$H$_5$O$_2$H$^-$) 23.89 [$\gamma$-CH$_2$ (Pro)], 26.18 [$\beta$-CH$_2$ (Glu)], 29.25 [$\gamma$-CH$_2$ (Glu)], 31.61 [$\beta$-CH$_2$ (Pro)], 34.39 [$\alpha$-CH$_2$ (Ala)], 35.11 [$\beta$-CH$_2$ (Asp)], 37.03 [$\beta$-CH$_2$ (Ala)], 38.76 [$\beta$-CH$_2$ (Phe)], 46.68 [$\delta$-CH$_2$ (Pro)], 46.91 [$\alpha$-C (Asp)], 51.53 [$\alpha$-C (Glu)], 51.73 (CH$_3$), 56.53 [$\alpha$-C (Phe)], 59.5 [$\alpha$-C (Pro)], 126.11, 127.93, 128.45, 128.96 and 129.34 (Ar-CH), 135.25 (Ar-C quaternary) and 169.12, 170.28, 170.71, 171.18 and 172.28 (CO ester, amides and acid); m/z (ES) 596 (4, [M + Na]$^+$), 574 (16, [M + H]$^+$) and 212 (100).
β-Allyl (2S)-N-(9-fluorenylmethoxycarbonyl)phenylalanoyl-β-alanyl-[α-methyl (2R)-glutamyl]-γ-(2R)-prolyl-[α-methyl (2R)-aspartate] triester 97

This compound was prepared in a manner identical to diester 36, using diester 76, (853 mg, 1 mmol) to give the required compound as a white crystalline solid in quantitative yield, which did not require further purification mp 120-121 °C

(Found: C, 61.9; H, 6.3; N 7.7. C₆₆H₅₃N₅O₁₂ requires: 1.5 H₂O: C, 61.7; H, 6.3; N, 7.8%; [α]D -17.8 (c 0.25 in MeOH); vmax(CH₂Cl₂)/cm⁻¹ 3301 (NH), 2953 (CH), and 1739 and 1652 (CO, esters and amides); δH (300 MHz; CDCl₃) 1.82-2.52 [10 H, m, γ-CH₂, β-CH₂ (Pro and Glu) and α-CH₂ (β-Ala)], 2.79-3.19 [4 H, m, β-CH₂ (Asp) and β-CH₂ (Phe)], 3.25-3.8 [4 H, m, δ-CH₃ (Pro) and β-CH₂ (β-Ala)], 3.51, 3.6, 3.68 and 3.72 (c and t, 6 H, 4 x s, CH₃), 4.08-4.97 [9 H, m, 4 x α-H, 2 x CH₂O and fluorenyl CH], 5.09-5.37 (2 H, m, CH₂=CH), 5.68 [1 H, d, J 7.9, NH, (urethane)], 5.79-5.86 (1 H, m, CH₂=CH), 6.90 (1 H, d, J 7.7, NH), 7.03 (1 H, br, NH) and 7.18-7.76 (15 H, m, Ph, 2 x NH); δC (75.4 MHz; CDCl₃) 24.63 [γ-CH₂ (Pro)], 27.07 [β-CH₂ (Glu)], 29.14 [γ-CH₂ (Glu)], 30.36 [β-CH₂ (Pro)], 35.28 [α-CH₂ (β-Ala)], 35.73 [β-CH₂ (Asp)], 36.33 [β-CH₂ (β-Ala)], 39.07 [β-CH₂ (Phe)], 47.14 [8-CH₂ (Pro)], 47.5 [α-C (Asp)], 51.90 [α-C (Glu)], 52.59 [CH₃ (Asp)], 52.86 [CH₃ (Glu)], 56.10 [α-C (Phe)], 60.09 [α-C (Pro)], 65.73 and 67.00 (2 x CH₂O), 118.60 (CH₂=CH), 120.05, 125.19, 126.90, 127.15, 127.79, 128.55, 129.48 and 131.86 (Ar-CH), 136.97 (CH₂=CH), 141.33 and 143.93 (Ar-C quaternary), 156.12 (CO, urethane) and 170.41, 171.71, 171.87 and 172.66 (CO,
esters and amides); m/z (ES) 891 (12%, [M + Na]⁺), 869 (50, [M + H]⁺), 646 (28, [M + H - C₁₅H₁₉O₂]⁻ and 111 (100).

(2S)-N-(Fluorenlymethoxycarbonyl)phenylalanyl-β-alanyl-[α-methyl (2S)-glutamyl]-γ-(2R)-prolyl-[α-methyl (2R)-aspartic acid] 98

This compound was prepared in a manner identical to ester 77, using the triester 97, (220 mg, 0.25 mmol) to give the required compound as a white crystalline solid (150 mg, 72%) mp >124 °C (decomp.); [α]₀ -13.5 (c 1 in MeOH); ν(max)(CH₂Cl₂)/cm⁻¹ 3300 (NH), 3229 br (OH), and 1801 and 1721 (CO, esters and amides); δH(300 MHz; CH₃OH) 1.87-2.45 [8 H, m, γ-CH₂, β-CH₂ (Pro and Glu) and α-CH₂ (β-Ala)], 2.74-3.14 [4 H, m, β-CH₂ (Asp) and β-CH₂ (Phe)], 3.28-3.52 [4 H, m, δ-CH₂ (Pro) and β-CH₂ (β-Ala)], 3.58, 3.59, 3.65 and 3.66 (c and t, 6 H, 4 x s, CH₃), 4.09-4.85 [7 H, m, 4 x α-H, CH₂O, fluorenyl CH], and 7.18-7.77 (13 H, m, Ph); δc(75.4 MHz; CDCl₃) 24.12 [γ-CH₂ (Pro)], 26.53 [β-CH₂ (Glu)], 29.5 [γ-CH₂ (Glu)], 31.85 [β-CH₂ (Pro)], 34.84 [α-CH₂ (β-Ala)], 35.53 [β-CH₂ (Asp)], 37.63 [β-CH₂ (β-Ala)], 37.92 [β-CH₂ (Phe)], 47.23 [δ-CH₂ (Pro)], 47.67 [α-C (Asp)], 49.60 [α-C (Glu)], 51.97 [CH₃ (Asp)], 52.05 [CH₃ (Glu)], 56.74 [α-C (Phe)], 60.41 [α-C (Pro)], 66.72 (CH₂O), 119.66, 124.92, 125.07, 126.5, 126.92, 127.54, 128.21 and 129.13 (Ar-CH), 137.39, 141.25 and 143.90 (Ar-C quaternary), 156.87 (CO, urethane) and 171.98, 172.32, 172.66, 172.94, 173.12 and 176.14 (CO, esters, amides and acid); m/z (ES) 839 (1%, M⁺) and 113 (100).
To a stirred solution of the diester 98 (100 mg, 0.12 mmol) in dry DMF (3 cm³), was added piperidine (17 mm³, 0.18 mmol). The reaction mixture was allowed to stir at room temperature for 45 min, and then concentrated under reduced pressure and redissolved in water (10 cm³) and acidified with TFA. The resulting solution was washed with DCM and the acidic layer concentrated under reduced pressure to yield the required compound as a white hydroscopic solid (60 mg, 83%) mp 67-69 °C (HRMS: found [M + H]^+ 793.3748. C_{22}H_{24}NO_{3} requires 793.3772); [α]D +14.5 (c 1.1 in MeOH); ω_{max}(CH_{2}Cl_{2})/cm⁻¹ 3286 (NH), 2955 br (OH), and 1746 and 1670 (CO, esters and amides); δ_{H}(300 MHz, ²H₂O) 1.45-2.18 [10 H, m, γ-CH₂, β-CH₂ (Pro and Glu) and α-CH₂ (β-Ala)], 2.64-3.04 [4 H, m, β-CH₂ (Asp) and β-CH₂ (Phe)], 3.15-3.36 [4 H, m, δ-CH₂ (Pro) and β-CH₂ (β-Ala)], 3.57 and 3.60 (6 H, 2 x s, 2 x CH₂), 3.97-4.59 [4 H, m, 4 x α-H] and 7.07-7.38 (5 H, m, Ph); δ_{C}(75.4 MHz; C²H₂O²H) 24.07 (γ-CH₂ (Pro)), 26.20 [β-CH₂ (Glu)], 29.48 [γ-CH₂ (Glu)], 31.41 [β-CH₂ (Pro)], 34.88 [α-CH₂ (β-Ala)], 35.34 [β-CH₂ (Asp)], 36.42 [β-CH₂ (β-Ala)], 40.15 [β-CH₂ (Phe)], 46.84 [δ-CH₂ (Pro)], 46.90 [α-C (Asp)], 49.86 [α-C (Glu)], 51.58 and 52.40 (2 x CH₃), 56.06 [α-C (Phe)], 60.53 [α-C (Pro)], 125.11, 126.73, 126.92, 128.42, 128.59 and 129.19 (Ar-CH), 136.02 (Ar-C quaternary) and 170.55 and 172.39 (CO ester, amides and acid); m/z (FAB) 793 (4%, [M + H]^+) and 115 (100, C₆H₅O₄^-).
Cyclo-[β-Ala-(2R)-Glu-α-OMe-γ-(2S)-Pro-(2R)-Asp-α-OMe-β-(2S)-Phe-] 52A

To a stirred solution of the pentapeptide 99, (60 mg, 9.92 x 10⁻³ mol) in DMF (80 cm³), was added PyBOP (28.4 mg, 0.11 mmol) and DIPEA (102 mm³, 0.99 mmol). The resulting solution was stirred at room temperature for 7 days and then concentrated under reduced pressure and immediately purified by flash chromatography on silica eluting with 94% CH₂Cl₂/MeOH to give a white solid which contained PyBOP as a contaminant. Further purification by preparative HPLC on a Poros 10 R2 reverse-phase column [using isocratic reverse-phased conditions, eluting with 18% acetonitrile-82% water as eluent at a flow rate of 2 cm³ min⁻¹, with the detector set at 220 nm] gave the required compound which possessed identical analytical data to the compound prepared previously from the pentafluorophenyl ester (see page 152).
α-Methyl γ-allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartate 102

This compound was prepared in a manner identical to diester 36, using γ-allyl (2R)-(9-fluorenylmethoxycarbonyl)aspartate 88, (1.00 g, 2.54 mmol) to give the required compound as a white crystalline solid in quantitative recovery, which did not require further purification (1.04 g) mp 96-97 °C (Found: C, 67.4; H, 5.7; N, 3.5. C_{23}H_{24}NO_{6} requires: C, 67.5; H, 5.6; N, 3.4\%) (HRMS: found [M + H]+, 410.1609 C_{23}H_{24}NO_{6} requires 410.1603); [α]_{D} -15 (c 0.3 in MeOH); $\nu_{\text{max}}$(CH_{2}Cl_{2})/cm^{-1} 3374 (NH), 2965 (CH) and 1751 (CO, urethane); δ_{H}(300 MHz; C_{2}HCl_{6}) 2.91 (1 H, dd, J 17.4 and 4.1, 1 H of β-CH$_2$), 3.09 (1 H, dd, J 16.8 and 4.5, 1 H of β-CH$_2$), 3.77 (3 H, s, CH$_3$), 4.20-4.74 (6 H, m, 2 × CH$_2$O, fluorenyl CH and α–H), 5.21-5.38 (2 H, m, CH$_2$=CH), 5.82-6.21 [2 H, m, CH$_2$=CH and NH (urethane)] and 7.23-7.79 (8 H, m, Ph); δ_{C}(75.4 MHz; C_{2}HCl_{6}) 36.75 (β-CH$_2$), 47.23 (fluorenyl CH), 50.53 (α–C), 53.01 (CH$_3$), 65.89 and 67.4 (2 × CH$_2$O), 118.92 (CH$_2$=CH), 120.15, 125.28, 127.23 and 127.89, 127.9 (Ar-CH), 131.76 (CH$_2$=CH), 141.44, 143.86 and 143.98 (Ar-C quaternary) 156.12 (CO, urethane) and 170.76 and 171.32 (CO, esters); m/z (CI) 410 (57%, [M + H]+) and 179 (100, C$_{13}$H$_{11}$).
α-Methyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartate 103

This compound was prepared in a manner identical to α-methyl (2R)-N-(9-fluorenylmethoxycarbonyl)glutamate 77, using diester 102 (242 mg, 0.50 mmol) to give the required compound as a white crystalline solid (134 mg, 60%) mp 124-125 °C, (Found: C, 64.8; H, 5.2; N 3.6. C_{20}H_{19}NO_6 requires: C, 65.0; H, 5.2; N, 3.8%); (HRMS: found [M + H]^+ 370.1298. C_{20}H_{20}NO_6 requires 370.1291); [α]_D +19.05 (c 0.21 in MeOH); ν_{max}(CH_2Cl_2) cm^{-1} 3335 (NH), 3100 br (OH) and 1727 (CO, ester); δ_{H}(300 MHz; C^1H^1) 2.82 (1 H, m, p-CH), 3.71 (3 H, s, CH), 4.08-4.62 (4 H, m, CH_2O, fluorenyl CH and α-H) and 7.24-7.82 (13 H, s, CH). δ_{C}(75.4 MHz; C^1H^1) 35.76 (β-CH2), 47.03 (fluorenyl CH), 50.69 (α-C), 51.71 (CH3), 66.85 (CH_2O), 119.64, 124.96, 126.88 and 127.50 (Ar-CH), 134.33, 141.28, 143.88 and 143.93 (Ar-C quaternary) 157.06 (CO, urethane), 171.91 (CO, ester) and 172.57 (CO, acid); m/z (Cl) 370 (99%, [M + H]^+), 192 (64, [M + 2H - C_4H_11]^+), 179 (100, C_4H_11^+) and 148 (85, C_9H_6NO_4^+).
α-Methyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartyl-Wang resin 104

This was prepared in a manner identical to that described for β-allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartyl-Wang resin 85, using ester 103, (1 g, 2.71 mmol) and the loading was determined to be 84% (see Appendix 4).

β-Allyl (2R)-N-(9-fluorenylmethoxycarbonyl)-4-hydroxy-1-butyrate 112

To a stirred solution of β-allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartate 88 (530 mg, 1.34 mmol) in dry THF (15 cm³) at -30 °C, was added N-methylmorpholine (147 mm³, 1.34 mmol). Isobutylchloroformate (182 mm³, 1.34 mmol) was added and the suspension was stirred at -30 °C for 30 min, and then filtered directly into a stirred solution of sodium borohydride (31 mg, 0.80 mmol) in dry THF (10 cm³). The reaction mixture was stirred at -30 °C for 15 mins and was then allowed to warm up to room temperature. After 3 h, water (10 cm³) was carefully added and the resulting solution was concentrated under reduced pressure until the THF had been removed. The remaining aqueous solution was extracted with ethyl acetate (3 × 25 cm³) and the combined organic extracts were
washed with brine (20 cm³), then dried (MgSO₄) and then concentrated under reduced pressure. The crude crystalline material was recrystallised from ethyl acetate/hexane to yield a white solid (430 mg, 84%) mp 124-125 °C (Found: C, 69.3; H, 6.2; N 3.7. C₂₂H₂₃NO₅ requires: C, 69.3; H, 6.1; N, 3.7%) (HRMS: found [M + H]⁺ 382.1662. C₂₂H₂₄NO₅ requires 382.1658); [α]₀⁺ 8.14 (c 0.43 in MeOH); δmax(CH₂Cl₂)/cm⁻¹ 3335 (NH), 1746 (CO, urethane) and 1707 (CO, ester); 8H(300 MHz; CDCl₃) 2.47 (1 H, br, OH), 2.68 (2 H, d, J4.48, β-CH₂), 3.73 (2 H, d, J2, CH₂OH), 4.06-4.60 (6 H, m, 2 x CH₂O, fluorenyl CH and α-H), 5.22-5.41 (2 H, m, CH₂=CH), 5.59 [1 H, d, J 5.7, NH, (urethane)], 5.22-5.97 (1 H, m, CH₂=CH) and 7.26-7.78 (8 H, m, Ph); δC(75.4 MHz; CDCl₃) 35.95 (β-CH₂), 47.33 (fluorenyl CH), 49.87 (α-C), 64.35 (CH₂OH), 65.7 and 67.02 (2 x CH₂O), 118.89 (CH=CH), 120.13, 125.18, 127.21 and 127.86 (Ar-CH), 131.86 (CH₂=CH), 141.45 and 143.93 (Ar-C quaternary) 156.37 (CO, urethane) and 171.55 (CO, ester); m/z (Cl) 382 (6%, [M + H]⁺), 179 (100, C₁₄H₁₁⁺) and 102 (74, C₇H₁₀O₂⁻).

α-Benzyl β-allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartate
diester 115

β-Allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartate 88 (395 mg, 1 mmol) was dissolved in MeOH (20 cm³) and the pH adjusted to 7 with 20% CsCO₃. The solvent was then removed under reduced pressure, redissolved in DMF (20 cm³) and benzyl bromide (131 mm³, 1.1 mmol) added. The resulting mixture was stirred at rt for 6 h and then the solvent removed under reduced pressure to give a white solid which was taken up in ethyl acetate (30 cm³) and washed with water (2 x 30
The organic extracts were dried (MgSO\textsubscript{4}) and concentrated under reduced pressure to give a colourless oil which was crystallised using ethyl acetate/hexane to yield a white solid (418 mg, 86%) mp 82-83 °C (Found: C, 70.8; H, 5.6; N 3.0. C\textsubscript{29}H\textsubscript{27}NO\textsubscript{6} requires: 0.25 H\textsubscript{2}O: C, 71.1; H, 5.6; N, 2.9%); (HRMS: found [M + 2H]\textsuperscript{+}, 487.2002. C\textsubscript{29}H\textsubscript{29}NO\textsubscript{6} requires 487.1995); [\alpha]\textsubscript{D} +8.7 (c 0.5 in MeOH); \nu\textsubscript{max}(CH\textsubscript{2}Cl\textsubscript{2})/cm\textsuperscript{-1} 3351 (NH) and 1739 (CO, urethane); \delta\textsubscript{H}(300 MHz; C\textsubscript{6}H\textsubscript{6}) 2.9 (1 H, dd, J 12.6 and 4.4, 1 H of \textbeta-CH\textsubscript{2}), 3.1 (1 H, dd, J 12.6 and 4.5, 1 H of \textbeta-CH\textsubscript{2}), 4.19-4.76 (6 H, m, 2 x CH\textsubscript{2}O, fluorenyl CH and \textalpha-H), 5.13-5.36 [5 H, m, CH\textsubscript{2}=CH, PhCH\textsubscript{2} and NH (urethane)], 5.78-5.98 (1 H, m, CH\textsubscript{2}=CH) and 7.26-7.82 (13 H, m, Ph); \delta\textsubscript{C}(75.4 MHz; C\textsubscript{6}H\textsubscript{6}) 36.75 (\textbeta-CH\textsubscript{2}), 47.23 (fluorenyl CH), 50.64 (\textalpha-C), 65.87, 67.44 and 67.77 (3 x CH\textsubscript{2}O), 118.97 (CH\textsubscript{2}=CH), 120.13, 125.27, 127.23, 127.87, 128.43, 128.64, 128.75, 128.94, and 129.17 (Ar-CH), 131.73 (CH\textsubscript{2}=CH), 135.28, 141.34, 143.84 and 143.99 (Ar-C quaternary), 156.11 (CO, urethane) and 170.66 (CO, ester); m/z (Cl) 487 (41%, [M + 2H]\textsuperscript{+}), 179 (47, C\textsubscript{14}H\textsubscript{11}+) and 57 (100, C\textsubscript{4}H\textsubscript{5}).

\textalpha-Benzyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartate 116

This compound was prepared in a manner identical to \textalpha-methyl (2R)-N-(9-fluorenylmethoxycarbonyl)glutamate 77, using diester 115 (242 mg, 0.50 mmol) to give the required compound as a white crystalline solid (134 mg, 60%), mp 96-97 °C (Found: C, 69.7; H, 5.1; N 3.0. C\textsubscript{26}H\textsubscript{24}NO\textsubscript{6} requires. 0.20 H\textsubscript{2}O: C, 69.6; H, 5.2; N, 3.1%); (HRMS: found [M + H]\textsuperscript{+}, 446.1596. C\textsubscript{26}H\textsubscript{24}NO\textsubscript{6} requires 446.1604); [\alpha]\textsubscript{D}-9.17 (c 0.3 in MeOH); \nu\textsubscript{max}(CH\textsubscript{2}Cl\textsubscript{2})/cm\textsuperscript{-1} 2993 br (OH) and 1719 (CO, ester); \delta\textsubscript{H}(300 MHz; C\textsubscript{6}H\textsubscript{6}) 2.92 (1 H, dd, J 12.6 and 4.4, 1 H of \textbeta-CH\textsubscript{2}), 3.11 (1 H, dd, J 12.6 and 4.5, 1 H of \textbeta-CH\textsubscript{2}), 4.18-4.78 (4 H, m, CH\textsubscript{2}O, fluorenyl CH and \textalpha-H), 5.21 (2 H, m, PhCH\textsubscript{2}), 5.81 [1 H, s, NH (urethane)] and 7.26-7.77 (13 H, m, Ph); \delta\textsubscript{C}(75.4 MHz; C\textsubscript{6}H\textsubscript{6}) 36.42 (\textbeta-CH\textsubscript{2}), 47.20 (fluorenyl CH), 50.41 (\textalpha-C), 67.52 and 67.9 (2 x CH\textsubscript{2}O), 120.15, 125.24, 127.25, 127.9, 128.42, 128.69 and 128.77 (Ar-CH), 135.14, 141.44 and 143.77 (Ar-C quaternary) 156.15 (CO,
urethane), 170.54 (CO, ester) and 175.39 (CO, acid); m/z (Cl) 446 (7%, [M + H]^+), 224 (27, C_{14}H_{10}CO_2^+), 179 (100, C_{12}H_{11}^+) and 91 (5, PhCH_2^-).

β-allyl (2S)-N-(9-fluorenylmethoxycarbonyl)phenylalanyl-α-benzyl (2R)-aspartyl]-α-methyl (2R)-glutamyl]-γ-(2R)-prolyl-(2R)-aspartic acid 117

This compound was prepared in a manner identical to that for the diester 76A starting from β-allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartyl-Wang resin 85 to give the required pentapeptide as a white solid in quantitative yield (296 mg, 0.3 mmol), which did not require further purification, mp 102-104 °C (Found: C, 64.6; H, 5.7; N 6.9. C_{35}H_{37}N_5O_{14} requires: C, 64.4; H, 5.8; N, 7.1%); [α]_D + 17.8 (c 0.2 in MeOH); δ_{max}(CH_2Cl_2)/cm^{-1} 3311 (NH), 2950 (CH), and 1733 (CO, esters and amides); δ_{H}(300 MHz; C^2HCl_3) 1.81-2.58 [10 H, m, γ-CH_2 and β-CH_2 (Pro and Glu) and α-CH_2 (β-Ala)], 2.84-3.37 [6 H, m, 2 x β-CH_2 (Asp) and β-CH_2 (Phe)], 3.42-3.71 [4 H, m, δ-CH_2 (Pro) and β-CH_2 (β-Ala)], 3.72 (t, 3 H, s, CH_3), 3.8 (t, 3 H, s, CH_3), 4.09-5.00 [10 H, m, 5 x α-H, 2 x CH_2O and fluorenyl CH], 5.01-5.37 (4 H, m, CH_2=CH, CH_2Ph), 5.65 [1 H, d, J 8.8, urethane]), 5.75 [c, 1 H, d, J 8.4, NH (urethane)], 5.83-5.94 (1 H, m, CH_2=CH), 6.31 (c, 1 H, d, J 7.9, NH), 6.39 (t, 1 H, d, J 7.8, NH) and 7.11-7.77 (21 H, m, Ph and 2 x NH); δ_{C}(75.4 MHz; C^2HCl_3) 24.51 [γ-CH_2 (Pro)], 25.53 [β-CH_2 (Glu)], 28.71 [γ-CH_2 (Glu)], 29.66 [β-CH_2 (Pro)], 35.34 [α-CH_2 (β-Ala)], 35.43 and 35.67 [β-CH_2 (Asp)], 36.67 [β-CH_2 (Asp)], 42.5 [γ-CH_2 (Pro)] and 175.39 (CO, acid); m/z (Cl) 446 (7%, [M + H]^+), 224 (27, C_{14}H_{10}CO_2^+), 179 (100, C_{12}H_{11}^+) and 91 (5, PhCH_2^-).
This compound was prepared in a manner identical to ester 92 to give the required pentapeptide in quantitative yield, which did not require further purification, $\nu_{\text{max}}$(CH$_2$Cl$_2$)/cm$^{-1}$ 3311 br (OH), 2936 (CH), and 1751 (CO, esters and amides); $\delta_H$(300 MHz; $^2$H$_2$O) 1.54-2.88 [10 H, m, $\gamma$-CH$_2$, $\beta$-CH$_2$ (Pro and Glu) and $\beta$-CH$_2$ ($\beta$-Ala)], 2.88-3.12 [6 H, m, 2 x $\beta$-CH$_2$ (Asp) and $\beta$-CH$_2$ (Phe)], 3.29-3.62 [4 H, m, $\delta$-CH$_2$ (Pro) and $\alpha$-CH$_2$ ($\beta$-Ala)], 3.59 (c, 3 H, s, CH$_3$), 3.61 (t, 3 H, s, CH$_3$), 4.08-4.75 [5 H, m, 5 x $\alpha$-H], 5.02-5.05 (2 H, m, CH$_2$Ph) and 7.01-7.79 (10 H, m, Ph); $\delta_C$(75.4 MHz; C$_2$DCl$_3$) 24.00 [$\gamma$-CH$_2$ (Pro)], 26.20 [$\beta$-CH$_2$ (Glu)], 29.64 [$\gamma$-CH$_2$ (Glu)], 31.75 [$\beta$-CH$_2$ (Pro)], 34.32 [$\beta$-CH$_2$ ($\beta$-Ala)], 35.08 and 35.46
Experimental

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$[\beta-\text{CH}_2 \text{(Asp)}], \ 36.66 \ [\alpha-\text{CH}_2 \text{(\beta-Ala)}], \ 39.27 \ [\beta-\text{CH}_2 \text{(Phe)}], \ 44.49 \ [\delta-\text{CH}_2 \text{(Pro)}], \ 47.31 \text{ and } 47.84 \ [\alpha-\text{C} \text{(Asp)}], \ 50.02 \ [\alpha-\text{C} \text{(Glu)}], \ 52.96 \ [\text{CH}_3 \text{(Asp)}], \ 56.02 \ [\alpha-\text{C} \text{(Phe)}], \ 60.37 \ [\alpha-\text{C} \text{(Pro)}], \ 66.12 \ \text{(CH}_2\text{O}), \ 127.77, \ 128.13, \ 128.82, \ 129.19, \ 129.69, \ 130.35, \ 130.61, \ 133.75 \text{ and } 133.9 \ \text{(Ar-CH)}, \ 134.47 \text{ and } 135.9 \ \text{(Ar-C quaternary)} \text{ and } 169.41, \ 169.86, \ 170.78, \ 173.72, \ 174.32, \ 174.62 \text{ and } 176.57 \ \text{(CO, esters, amides and acids);} \ \text{m/z (ES) } 711 \ (4, \ [\text{M}+2\text{H}]^+) \text{ and } 277 \ (100).$

$(2\text{R})-N-(9\text{-Fluorenylmethoxycarbonyl})\text{aspartyl \alpha-4-benzylpiperidinamide 121}$

To a stirred solution of ester 88, (1 g, 2.53 mmol) in dry THF (25 cm$^3$) at -15 °C was added N-methylmorpholine (276 mm$^3$, 2.53 mmol). Isobutylchloroformate (343 mm$^3$, 2.53 mmol) was added and the suspension was stirred at -15 °C for a further 5 min. A solution of 4-benzylpiperidine (356 mg, 2.53 mmol) and N-methylmorpholine (276 mm$^3$, 2.53 mmol) in THF (25 cm$^3$) was added and the mixture left to stir overnight. The hydrochloride salts were removed by filtration and the filtrate concentrated under reduced pressure to give a pale yellow oil which was redissolved in ethyl acetate (15 cm$^3$), washed successively with water (10 cm$^3$), 5% aqueous NaHCO$_3$ solution (15 cm$^3$), 10% citric acid solution (15 cm$^3$) and then brine (15 cm$^3$). The organic phase was dried (MgSO$_4$) and concentrated under reduced pressure to give the required diester 120 which was immediately used in the next reaction.

To a stirred solution of the above diester and phenylsilane (624 mm$^3$, 5.06 mmol) in dry DCM (30 cm$^3$) under Ar, was added tetrakis (triphenylphosphine) Pd (0) (58
mg, 0.05 mmol). The reaction was allowed to stir at room temperature for 18 h, and then concentrated under reduced pressure. The crude material was immediately purified using silica chromatography (60% ethyl acetate/hexane) to give a white solid (700 mg, 54%) mp 79-81 °C (Found: C, 71.3; H, 6.2; N 5.1. C31H32N2O3. 0.5 H2O requires: C, 71.4; H, 6.3; N, 5.4%); [α]D +27.3 (c 0.3 in MeOH); vmax(CH2Cl2)/cm⁻¹ 3279 (NH), 3100 br (OH) and 1718 (CO, ester); δH(300 MHz; C6D6) 1.13-1.28 (5 H, m, CHCH2Ph and 2 x CH2-CH2NH), 1.70 (4 H, br, 2 x CH2-CH2NH), 2.46-3.04 [4 H, m, PhCH2 and βCH2 (Asp)], 3.98-4.59 (4 H, m, CH2O, fluorenyl CH and α-H), 5.09 (1 H, br, OH), 6.18 [1 H, dd J 21 and 9, NH (urethane)] and 7.04-7.77 (13 H, m, Ph); δC(75.4 MHz; C6D6) 31.42 (2 x CH2-CH2NH), 37.47 (βCH2), 37.95 (2 x CHCH2Ph), 42.94 (CH2-CH2NH), 46.19 (PhCH2), 46.94 (α-C), 47.49 (fluorenyl CH), 67.09 (CH2O), 119.84, 125.03, 125.93, 126.96, 127.58, 128.16 and 128.60 (Ar-CH), 139.59, 141.13, 143.53 and 143.59 (Ar-C quaternary) 155.68 (CO, urethane), 169.88 (CO, ester) and 173.66 (CO, acid); m/z (Cl) 513 (1%, [M + H]+) and 179 (100, C14H11+).

β-Allyl (2R)-N-(9-fluorenylmethoxycarbonyl)aspartyl α-benzylamide 122

This compound was prepared in a manner identical to that described for glutamyl-sarcosyl diester 39, using the ester 88, (790 mg, 2 mmol) and benzylamine (218 mm³, 2 mmol) to give the required compound as a white crystalline solid (715 mg, 74%) mp 141-142 °C, (Found: C, 71.3; H, 5.9; N 6.0. C29H30N2O5 requires C, 71.2; H, 5.8; N, 5.7%) (HRMS: found [M + 2H]+, 486.2169. C29H30N2O5 requires 486.2155); [α]D +13.84 (c 0.43 in MeOH); vmax(CH2Cl2)/cm⁻¹
3296 (NH) and 1756 and 1742 (CO, esters and amide); δ$_{H}$(300 MHz; C$_{2}$HCl$_{2}$) 1.91 (2 H, s, PhCH$_{2}$), 2.59 (1 H, dd, $J$ 10.4 and 6.4, 1 H of β–CH$_{2}$), 2.72 (1 H, dd, $J$ 10.4 and 6.2, 1 H of β–CH$_{2}$), 4.16-4.78 (4 H, m, CH$_{2}$O, fluorenyl CH and α–H), 5.21-5.38 (2 H, m, CH$_{2}$=CH), 5.82-5.91 [2 H, m, CH$_{2}$=CH, NH (urethane)], 6.81 (1 H, br, NH) and 7.22-7.81 (13 H, m, Ph). δ$_{C}$(75.4 MHz; C$_{2}$HCl$_{2}$) 36.21 (β–CH$_{2}$), 43.71 (PhCH$_{2}$), 57.23 (fluorenyl CH), 51.19 (α–C), 65.91 and 67.28 (2 x CH$_{2}$O), 118.97 (CH$_{2}$=CH), 120.18, 125.08, 127.22, 127.65, 127.93 and 128.83 (Ar–CH), 131.66 (CH$_{2}$=CH), 141.46 and 143.74 (Ar-C quaternary) 152.89 (CO, urethane) 168.02 (CO, amide) and 170.25 (CO, ester); m/z (Cl) 486 (92%, [M + 2H]$^+$), 263 (100, [M + 2H – C$_{14}$H$_{11}$CO$_{2}$]$^+$) and 179 (60, C$_{14}$H$_{11}$). 

(2R)-N-(9-Fluorenylmethoxycarbonyl)aspartyll α-benzylamide 124

This compound was prepared in a manner identical to ester 77, using the diester 122 (575 mg, 1.19 mmol) to give the required compound as a white crystalline solid (462 mg, 87%), mp 118-119 °C (HRMS: found [M + H]$^+$, 445.1769. C$_{26}$H$_{32}$N$_2$O$_5$ requires 445.1763); [α]$_D$+6.88 (c 0.32 in MeOH); ν$_{max}$ (Nujol)/cm$^{-1}$ 3335 (NH) and 1742 and 1712 (CO, esters and amide); δ$_{H}$(300 MHz; C$_{2}$H$_2$O$_2$H) 1.98 (2 H, s, PhCH$_{2}$), 2.77 (1 H, dd, $J$ 11.1 and 6.5, 1 H of β–CH$_{2}$), 2.91 (1 H, dd, $J$ 10.7 and 6.3, 1 H of β–CH$_{2}$), 4.07-4.68 (4 H, m, CH$_{2}$O, fluorenyl CH and α–H), 4.99 (1 H, br, OH) and 7.18-7.86 (13 H, m, Ph). δ$_{C}$(75.4 MHz; C$_{2}$H$_2$O$_2$H) 35.87 (β–CH$_{2}$), 42.84 (PhCH$_{2}$), 47.03 (fluorenyl CH), 51.85 (α–C), 65.62 and 66.89 (2 x CH$_{2}$O), 119.69, 125.0, 126.93, 127.09, 127.55 and 128.23 (Ar–CH), 138.46, 141.28 and 143.93 (Ar-C quaternary) 157.07 (CO, urethane) and 170.15 and 172.81 (CO, amide and acid); m/z (Cl) 445 (6%, [M + H]$^+$) and 179 (100, C$_{14}$H$_{11}$).
\( \beta\text{-Allyl} \ (2R)\text{-N-(9-fluorenylmethoxycarbonyl)aspartyl} \ \alpha\text{-benzylpiperizinamide} \)

This compound was prepared in a manner identical to that described for glutamyl-sarcosyl diester 39, using of ester 88, (790 mg, 2 mmol) and benzylpiperizine (348 mm\(^3\), 2 mmol) to give the required compound as a white waxy solid (763 mg, 81%), (HRMS: found \([M + H]^+\), 554.2667. \( \text{C}_{33}\text{H}_{36}\text{N}_3\text{O}_6 \) requires 554.2655); \([\alpha]_D^0 +4.19 \ (c 0.31 \text{ in MeOH}); \nu_{\text{max}}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1} \ 3290 \ (\text{NH}), \ 2963 \ (\text{CH}) \) and 1732 and 1647 (CO, esters and amide); \( \delta_{\text{H}}(300 \text{ MHz; } \text{C}_3\text{HCl}_3) \) 2.43 (2 H, br, PhCH\(_2\)), 2.67 (1 H, dd, \( J 16.2 \) and 5.7, 1 H of \( \beta\text{-CH}_2\)), 2.87 (1 H, dd, \( J 15.9 \) and 6.6, 1 H of \( \beta\text{-CH}_2\)), 3.43-3.76 [8 H, m, 4 x CH\(_2\) (Piz)], 4.17-4.64 (3 H, m, CH\(_2\text{O and fluorenyl CH}\)), 5.09 (1 H, q, \( J 6.6, \alpha\text{-H} \)), 5.21-5.35 (2 H, m, CH\(_2\text{=CH}\)), 5.84-5.97 (1 H, m, CH\(_2\text{=CH}\)), 6.06 [1 H, d, \( J 9.6, \text{NH (urethane)} \) and 7.26-7.77 (13 H, m, Ph); \( \delta_{\text{C}}(75.4 \text{ MHz; } \text{C}_3\text{HCl}_3) \) 37.70 (\( \beta\text{-CH}_2\)), 42.45 (PhCH\(_2\)), 47.23 (fluorenyl CH), 47.54 (Piz CH\(_2\)), 52.62 (\( \alpha\text{-C} \)), 65.7 and 67.23 (2 x CH\(_2\text{O} \)), 118.67 (CH\(_2\text{=CH}\)), 120.15, 125.26, 127.23, 127.53, 127.127.9, 128.5 and 129.31 (Ar-CH), 132.03 (CH\(_2\text{=CH}\)), 137.43, 141.44 and 143.86 (Ar-C quaternary) 155.74 (CO, urethane) and 168.86 and 170.46 (CO, amide and ester); \( m/\zeta \) (CI) 554 (100%, \( [M + H]^+ \)), 358 (14, \( [M - \text{C}_{12}\text{H}_{11}\text{O}]^+ \)), 332 (15, \( [M - \text{C}_{13}\text{H}_{11}\text{O}_2]^- \)), 179 (24, \( \text{C}_{14}\text{H}_{11}^- \)) and 57 (57, \( \text{C}_3\text{H}_5\text{O}^- \)).
(2R)-N-(9-Fluorenylmethoxycarbonyl)aspartyl β-benzylpiperizinamidamide 125

This compound was prepared in a manner identical to α-methyl (2R)-N-(9-fluorenylmethoxycarbonyl)glutamate 39, using α-benzylpiperizido γ-allyl-(2R)-N-(9-fluorenylmethoxycarbonyl)aspartamide 123, (575 mg, 1.04 mmol) to give the required compound as a white hydroscopic solid which still showed signs of impurity (763 mg, 69%) mp 74-75 °C, ν_{max}(CH2Cl2)/cm^{-1} 3314 (NH), 3064 br (OH) and 1718 and 1635 (CO, esters and amide); δ_{H}(300 MHz; CDCl3) 2.01 (2 H, s, PhCH2), 2.57-3.98 (10 H, m, β-CH2, CH2 x 4 Piz), 4.04-4.73 (3 H, m, CH2O, fluorenyl CH), 4.98-5.04 (1 H, m, α-H) and 6.83-7.99 (13 H, m, Ph); δ_{C}(75.4 MHz; CDCl3) 37.47 (P-CH2), 37.95 (PhCH2), 42.01 (Piz CH2), 46.94 (fluorenyl CH), 47.49 (α-C), 67.24 (2 x CH2O), 119.84, 124.59, 125.03, 125.93, 127.58, 128.16 and 128.60 (Ar-CH), 139.59, 141.13, 143.53 and 143.59 (Ar-C quaternary) 155.68 (CO, urethane) and 168.88 and 173.66 (CO, amide and ester); m/z (Cl) 511 (1%, [M + H]+) and 179 (100, C14H11O).

N-(9-Fluorenylmethoxycarbonyl)(2S)-phenylalanyl
-α-4-benzylpiperidinylamido (2R)-aspartyl]-α-methyl (2R)-glutamyl]-γ-(2R)-prolyl-β-allyl-(2R)-aspartic acid 127

This compound was prepared in a manner identical to diester 76 to give the required pentapeptide as a white solid in quantitative yield, which did not require
further purification, mp 134-136 °C (HRMS: found [M + H + Na]⁺, 1077.4556. C₃₆H₆₅N₆O₁₂Na requires 1077.4586); [α]D +30 (c 1 in MeOH); νₘₐₓ(CH₂Cl₂)/cm⁻¹ 3302 (NH), 2952 (CH), and 1739 (CO, esters and amides); δₑₐₚ(300 MHz; C₂HCl₃) 1.18-1.43 (4 H, m, 2 x CH₂-CH₂NH), 1.7 (4 H, br, CH₂-CH₂NH), 2.46-3.04 [5 H, m, CHCH₂Ph, CHCH₂Ph and βCH₂ (Asp)], 1.81-2.58 [10 H, m, γ-CH₂, β-CH₂ (Pro and Glu) and α-CH₂ (β-Ala)], 2.84-3.37 [6 H, m, 2 x β-CH₂ (Asp) and β-CH₂ (Phe)], 3.42-3.71 [4 H, m, δ-CH₂ (Pro) and β-CH₂ (β-Ala)], 3.72 (c, 3 H, s, CH₃), 3.8 (t, 3 H, s, CH₃), 4.09-5.00 [10 H, m, 5 x α-H, 2 x CH₂O and fluorenyl CH], 5.01-5.37 (4 H, m, CH₂=CH and CH₂Ph), 5.65 [t, 1 H, d, J 8.8, NH (urethane)], 5.75 [c, 1 H, d, J 8.4, NH (urethane)], 5.83-5.94 (1 H, m, CH₂=CH), 6.31 (c, 1 H, d, J 7.9, NH), 6.39 (t, 1 H, d, J 7.8, NH) and 7.11-7.77 (21 H, m, Ph and 2 x NH); δ_C(75.4 MHz; C₂HCl₃) 24.51 [γ-CH₂ (Pro)], 25.53 [β-CH₂ (Glu)], 28.71 [β-CH₂ (Glu)], 29.66 [β-CH₂ (Pro)], 35.34 [α-CH₂ (β-Ala)], 35.43 and 35.67 [β-CH₂ (Asp)], 36.67 [β-CH₂ (β-Ala)], 39.28 [β-CH₂ (Phe)], 43.48 [δ-CH₂ (Pro)], 46.90 and 47.17 [α-C (Asp)], 51.04 [α-C (Glu)], 52.81 [CH₃ (Asp)], 55.69 [α-C (Phe)], 60.68 [α-C (Pro)], 65.54, 66.95 and 67.75 (3 x CH₂O), 118.46 (CH₂=CH), 120.13, 123.71, 125.22, 127.05, 127.26, 127.57, 127.93, 128.36, 128.57, 128.88, 129.62 and 132.12 (Ar-CH), 136.44 (CH₂=CH), 135.22, 136.43, 138.24, 141.41, 142.24 and 141.74 (Ar-C quaternary), 156.65 (CO, urethane) and 170.45, 170.95, 171.38, 171.84 and 173.33 (CO esters, amides and acid); m/z (ES) 1011 (1%, [M + Na]⁺), 989 (2, [M + 2H]²⁺), 211 (100), 195 (57, [C₁₂H₁₁O]³⁻) and 157 (78, [C₇H₅O₂]⁺).
Cyclo\{(2S)-Phe-[(2R)-α-4-benzylpiperidinylamido-Asp]-(2R)-α-OMe-Glu-γ-(2S)-Pro-β-(2R)-Asp\} 126

This compound was prepared in a manner identical to 93 using the diester, 127, (316 mg, 0.3 mmol) which was selectively deprotected [see 92] and cyclised to give the required compound after purification using preparative HPLC on a C-18 column [using isocratic reverse-phased conditions, eluting with 35% acetonitrile-65% water as eluent at a flow rate of 4.5 cm$^3$ min$^{-1}$, with the detector set at 220 nm] (40 mg, 17%) mp >167 °C (decomp.), $\nu_{\text{max}}$(CH$_2$Cl$_2$)/cm$^{-1}$ 3301 (NH), 2939 (CH), and 1732 and 1672 (CO, esters and amides); $\delta_H$(300 MHz; C$_6$H$_5$Cl$_3$) 1.18-1.43 (4 H, m, 2 x CH$_2$-CH$_2$NH), 1.7 (4 H, br, 2 x CH$_2$-CH$_2$NH), 2.46-3.04 [5 H, m, CH/CH$_2$Ph, CH/CH$_2$Ph and β/CH$_2$ (Asp)], 1.81-2.58 [10 H, m, γ-CH$_2$, β-CH$_2$ (Pro and Glu) and α-CH$_2$ (β-Ala)], 2.84-3.37 [6 H, m, 2 x β-CH$_2$ (Asp) and β-CH$_2$ (Phe)], 3.42-3.71 [4 H, m, δ-CH$_2$ (Pro) and β-CH$_2$ (β-Ala)], 3.72 (c, 3 H, s, CH$_3$), 3.80 (t, 3 H, s, CH$_3$), 4.09-5.00 [10 H, m, 5 x α-H, 2 x CH$_2$O and fluorenyl CH], 5.01-5.37 (4 H, m, CH$_2$=CH and CH$_2$Ph), 5.83-5.94 (1 H, m, CH$_2$=CH), 6.31 (c, 1 H, d, J 7.9, NH), 6.39 (t, 1 H, d, J 7.8, NH) and 7.11-7.77 (13 H, m, Ph, 3 x NH); $m/z$ (ES) 1011 (1%, [M + Na$^+$]), 989 (2, [M + 2H$^+$]), 211 (100), 195 (57, C$_{14}$H$_{11}$O$^+$) and 157 (78, C$_7$H$_9$O$_4$-).
General Hydrolysis Procedure for the Cyclo-Esters to Yield the Bioactive Materials

To a stirred solution of the cyclic pentapeptide (0.03 mmol) in methanol (2 cm$^3$) and water (2 cm$^3$) was added NaOH (0.065 mmol). The reaction was allowed to stir at room temperature for 2 h, after which time the methanol then was removed under reduced pressure. The aqueous layer was acidified using trifluoroacetic acid and the cyclic peptide extracted using DCM (2 cm$^3$). The organic phase was dried (MgSO$_4$) and the solvent removed under reduced pressure.

**Cyclo-[β-Ala-(2R)-Glu-γ-(2S)-Pro-(2R)-Asp-β-(2S)-Phe]- 94A**

This compound was prepared as the described general procedure above, $m/z$ (ES) 582 (3%, [M + Na]$^+$), 561 (7, [M + 2H]$^+$); >95% HPLC purity on a Poros 10 R2/H reverse phase column [using isocratic reverse-phased conditions eluting with 24% acetonitrile-76% water as eluent at a flow rate of 5 cm$^3$ min$^{-1}$ with the detector set at 220 nm].

**Cyclo-[β-Ala-(2R)-Glu-γ-(2S)-Pro-(2R)-Asp-β-(2S)-Phe]- 94B**

This compound was prepared as the described general procedure above, $m/z$ (MALDITOF) 582 (10%, [M + Na]$^+$), 560 (5, [M + H]$^+$); >95% HPLC purity on a Poros 10 R2/H reverse phase column [using isocratic reverse-phased conditions eluting with 24% acetonitrile-76% water as eluent at a flow rate of 5 cm$^3$ min$^{-1}$ with the detector set at 220 nm].

**Cyclo{[(2S)-Phe-[(2R)-α-4-benzylpiperidinylamido-Asp]-(2R)-Glu-γ-(2S)-Pro-β-(2R)-Asp]} 130**

This compound was prepared as the described general procedure above, $m/z$ (MALDITOF) 784 (8%, [M + H + Na]$^+$), 762 (3, [M + 2H]$^+$); >95% HPLC purity on a C-18 reverse phase column [using isocratic reverse-phased conditions eluting with 51% acetonitrile-49% water as eluent at a flow rate of 1 cm$^3$ min$^{-1}$ with the detector set at 220 nm].
3.1 Enzymic Studies

3.1.1 Materials

The *Escherichia coli* expression vector plasmid pET21a, together with the HIS•bind resin, were purchased from Novagen and used according to the manufacturers instructions. Native PPl, isolated from rabbit muscle, was purchased from TCS Biologicals Ltd. The phosphorylated peptides RRATpVA and KRTpIRR were purchased from Immune Systems (Bristol, UK). All other reagents were purchased from Sigma unless otherwise indicated.

3.1.2 Expression of PPl

The gene which encodes for PPl was cloned into a pET21a™ expression vector by Mrs J Sullivan (Zeneca Pharmaceuticals). Single colonies were selected and stored as glycerol stock.

5 cm³ L-amp broth was inoculated using the glycerol stock and grown up at 37 °C overnight. This was then used to inoculate 120 cm³ fresh L-amp broth and the cells grown to an OD of 0.4 (~3 h) in the presence of 1 mM MnCl₂. IPTG was then added to a final concentration of 0.1 mM and the culture grown at 25 °C overnight. The cells were harvested by centrifugation and sonicated in 4 cm³ culture of the ice cold binding buffer (5 mM imidazole, 200 mM NaCl, 20 mM Tris-HCl pH 7.9). The particulate matter was removed by centrifugation at 1300 rpm for 5 min and the supernatant purified on a Novagen His•Bind column.

The protein was eluted off the column in the standard elution buffer (1 M imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, 2 mM MnCl₂, 5% glycerol and 0.03% Brij 35 pH 7.9). The sample was then dialysed at 4 °C for 3 h against 25 mM triethanolamine-HCl pH 7.5, 1 mM MnCl₂, 0.1 mM EGTA, 0.2 M NaCl, 0.03% Brij 35, 0.1% 2-mercaptoethanol and 50% glycerol. The dialysis was repeated overnight and the enzyme stored at -20 °C.
3.1.3 Assays of PP1

PP1 was diluted prior to use with 50 mM Tris (pH 7.4), 0.01 mM EDTA, 1% β-mercaptoethanol and BSA (1 mg/cm³) at 4 °C. All kinetic constants were calculated using Grafit analysis under steady state conditions.

3.1.3.1 pNPP as a substrate

The activity of PP1 with pNPP as substrate was assayed at room temperature and 25 °C. Reaction mixtures contained 80 mM Tris/HCl, 40 mM KCl, 60 mM MgCl₂, 10 mM dithiothreitol and 2 M MnCl₂ (pH 8.1). To start the pNPP phosphatase activities, reaction was started by addition of enzyme and the initial rate of liberation of p-nitrophenol was measured by recording the change in absorbance at 405 nm on a 96 well-plate.

A standard curve was constructed using a nitrophenol standard, and the specific activity was calculated using a nitrophenol standard.

![Graph 3.1: pNPP Standard Curve](image)

where the gradient of the graph is 0.465.
3.1.3.2 Recombinant PP1

The assay was performed over 60 min at 25 °C, under steady state conditions, using 19 nM enzyme concentration and varying the substrate concentration (0.06-80 mM). The results were plotted using the unweighted non-linear regression fit in *Grafit*.

![Graph 3.2: pNPP with Recombinant PP1 after 60 min](image)

The assay at 21 °C gave very similar results. Using the pNPP calibration curve (see Graph 3.1) \( k_{\text{cat}} \), \( K_m \) and the specificity constant were calculated (see Table 3.7)
Table 3.7: pNPP Kinetic Measurements with Recombinant PPI

<table>
<thead>
<tr>
<th>TEMPERATURE</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 °C</td>
<td>37.56</td>
<td>1.68</td>
<td>45.33</td>
</tr>
<tr>
<td>25 °C</td>
<td>53.04</td>
<td>2.78</td>
<td>52.41</td>
</tr>
</tbody>
</table>

3.1.3.3 Phosphopeptides as substrates

The activity of PPI with peptide substrates was assayed at room temperature and 25 °C in a reaction containing 50 mM Tris (pH 7.5), 0.1 mM EDTA and 0.5 mM dithiothreitol.

Phosphate released was detected by addition of malachite green reagent (25 mm$^3$) to a sample (100 cm$^3$) containing phosphate which formed a green solution. After the colour had been allowed to develop for 20 min, the adsorption in the visible spectrum at 660 nm was measured in a 96 well plate.

A standard curve was set up by measuring the adsorption of known concentrations of phosphate, and then plotting the absorption as a function of phosphate concentration. This was expressed over a region of 0-8 nmol phosphate (see Graph 3.3).
This curve fits the following equation:

$$x = \frac{\ln(1-y)}{0.01633}$$

Equation 1

where $y = OD$ reading

**Colour Reagent**

Concentrated sulfuric acid ($60 \text{ cm}^3$, $d = 1.84 \text{ g/l}$) was slowly added to $300 \text{ cm}^3$ of water. The solution was then cooled to room temperature and $440 \text{ mg}$ of malachite green added. The resulting orange solution was stable for at least a year at rt. On the day of use (~20 min before use), $2.5 \text{ cm}^3$ of $7.5\%$ ammonium molybdate was added to $10 \text{ cm}^3$ of the dye solution, followed by $0.2 \text{ cm}^3$ of $11\%$ Tween 20.
3.1.3.4 RRATpVA as Substrate

Recombinant PP1

In order to check that we were using steady state conditions, the first experiment varied the enzyme concentration at a fixed substrate concentration (0.2 mM).

![Graph 3.4: Varying concentrations of enzyme over time period at fixed substrate concentration](image)

From the graph it was evident that either 4.7 nM or 9.5 nM enzyme concentration was suitable for assays. Because the OD change was quite significant with 9.5 nM after 20 min, this was chosen for all enzyme assays using recombinant enzyme.

The equilibrium constant \((K_a)\), the catalytic constant \((k_{cat})\) and the specificity constant \((k_{cat}/K_m)\) were determined by varying the substrate concentration, assuming the enzyme is 100% pure and 100% active (assumed from Gel and Protein Determination Assay). The reaction was performed at 21 °C and 25 °C. As the
substrate concentration required was varied (0.005 mM - 4.7 mM), and the results were fitted by unweighted non-linear regression using Grafit.

Graph 3.5: RRApVA with Recombinant Enzyme

From the graph above (see Graph 3.5) and the malachite green standard curve (see Graph 3.3), $k_{cat}$, $K_m$ and the specificity constant were calculated. The results are tabulated below:

<table>
<thead>
<tr>
<th>TEMPERATURE</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$k_{cat}/K_m$ ($M^{-1}s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 °C</td>
<td>1.04</td>
<td>6.58</td>
<td>6299</td>
</tr>
<tr>
<td>25 °C</td>
<td>0.42</td>
<td>6.97</td>
<td>16787</td>
</tr>
</tbody>
</table>
Native PP1 Enzyme

From initial assays with the native enzyme, it was obvious that \textit{RRATpVA} was a very poor substrate with the native enzyme. Thus, obtaining an accurate \( V_{\text{max}} \) and \( K_m \) value would use up too much of the valuable Native PP1 enzyme, we decided to only determine the specificity constant.

For this experiment only very low concentrations of substrate (> \( K_m \)) are used, so that we are still on the linear part of Graph 3.2. This was checked at a variety of substrate concentrations (0.065 mM – 0.8 mM), at a fixed enzyme concentration (6.75 nM), (see Graph 3.5) and at 25 °C. The results are plotted below:

![Graph 3.5: Vary Substrate Concentration with Native Enzyme](image)

Because the line is linear, a time course was taken when the substrate concentration was at 0.153 mM.
The rate of the above line gave the specificity constant \( \frac{k_{\text{cat}}}{K_m} \), only. This is because at low substrate concentrations \( \nu = [E_t][S]k_{\text{cat}}/K_m \), where \( k_{\text{cat}}/K_m \) is the apparent second order rate constant.

Hence, using the equation:

\[
\nu = [E_t][S]k_{\text{cat}}/K_m
\]

The rate, \( \nu \), was determined using the slope of Graph 3.6, which correlates to 1.14 nM PO\(_4^{3-}\)/s\(^{-1}\). Therefore, the specificity constant, \( k_{\text{cat}}/K_m \) is 1.102 M\(^{-1}\)s\(^{-1}\).

This implies that RRATpVA is a 10-fold better substrate for the recombinant enzyme, than for the native enzyme.
3.1.3.5 KRTpIRR (129) as Substrate

Recombinant PP1

Once again, assays were performed over 20 min at 21 °C and 25 °C, under steady state conditions.

Having established, once again, that 9.5 nM was a suitable enzyme concentration, the substrate concentration was varied (0.002 – 2 mM). The results plotted using the unweighted non-linear regression fit in Grafit are shown below.

Graph 3.7: KRTpIRR as Substrate with Recombinant PP1

From the graph above and the malachite green standard curve, k_{cat}, K_m and the specificity constant were determined, see Table 3.9.
Table 3.9: KRTpIRR Kinetic Measurements with Recombinant PP1

<table>
<thead>
<tr>
<th>TEMPERATURE</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 °C</td>
<td>0.17</td>
<td>3.87</td>
<td>23 081</td>
</tr>
<tr>
<td>25 °C</td>
<td>0.09</td>
<td>4.12</td>
<td>45 382</td>
</tr>
</tbody>
</table>

Native PP1 Enzyme

The assay was performed over 20 min at 25 °C, under steady state conditions, using 3.2 nM enzyme concentration and varying the substrate concentration (0.002 – 2 mM). The results plotted using the unweighted non-linear regression fit in Grafit are shown in Graph 3.8.
Graph 3.8: KRTpIRR with Native Enzyme

From the above graph $K_m$ 0.55 mM, $k_{cat}$ 8.18 s$^{-1}$ and the specificity constant is 14871 M$^{-1}$s$^{-1}$. This implies that KRTpIRR 129 is a 2-fold better substrate for the recombinant enzyme. It is, however, 13-fold better than RRATpVA 128.
APPENDIX 1: MICROCYSTIN ANALOGUES

![Chemical structure of Microcystin-LR (2)]

<table>
<thead>
<tr>
<th>Cyclic-Microcystin Analogues</th>
<th>1</th>
<th>2 (X)</th>
<th>3</th>
<th>4 (Y)</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcystin-LR (^{143})</td>
<td>R-Ala</td>
<td>S-Leu</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-RR (^{167})</td>
<td>R-Ala</td>
<td>S-Arg</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-YR (^{144})</td>
<td>R-Ala</td>
<td>S-Tyr</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-FR (^{171})</td>
<td>R-Ala</td>
<td>S-Phe</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-AR (^{165})</td>
<td>R-Ala</td>
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<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-WR (^{171})</td>
<td>R-Ala</td>
<td>S-Trp</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-LA (^{148})</td>
<td>R-Ala</td>
<td>S-Leu</td>
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<td>S-Ala</td>
<td>Adda</td>
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<td>S-Tyr</td>
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<td>S-Ala</td>
<td>Adda</td>
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<td>N-Mdha</td>
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<tr>
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<td>Adda</td>
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<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-LY (^{141})</td>
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<tr>
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<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-LV&lt;sup&gt;141&lt;/sup&gt;</td>
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<td>S-Val</td>
<td>Adda</td>
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<td>N-Mdha</td>
</tr>
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<tr>
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<td>S-Leu</td>
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<td>S-Met</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-LE&lt;sup&gt;141&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Leu</td>
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<td>S-Phe</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>Microcystin-MOIR&lt;sup&gt;129&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Met(0)</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
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<td>N-Mdha</td>
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<td>Masp</td>
<td>Ala</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
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<td>Adda</td>
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<td>[S-Ser] Microcystin-LR&lt;sup&gt;142&lt;/sup&gt;</td>
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<td>S-Leu</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>S-Ser</td>
</tr>
<tr>
<td>[S-Ser] Microcystin-RR&lt;sup&gt;129&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Arg</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>S-Ser</td>
</tr>
<tr>
<td>[S-Ser] Microcystin-HtyR&lt;sup&gt;129&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Hty</td>
<td>Masp</td>
<td>S-Arg</td>
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</tr>
<tr>
<td>[IBZ]-Adda Microcystin-LR&lt;sup&gt;116&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Leu</td>
<td>Masp</td>
<td>S-Arg</td>
<td>(IBZ)-Adda</td>
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<td>N-Mdha</td>
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<tr>
<td>[IBZ]-Adda Microcystin-RR&lt;sup&gt;229&lt;/sup&gt;</td>
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<td>S-Arg</td>
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<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>[DMAdda] Microcystin-LR&lt;sup&gt;151&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Leu</td>
<td>Masp</td>
<td>S-Arg</td>
<td>DMAdda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>[ADMAAdda] Microcystin-LR&lt;sup&gt;151&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Leu</td>
<td>Masp</td>
<td>S-Arg</td>
<td>ADMAAdda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
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<td>S-Leu</td>
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<tr>
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<td>R-Ala</td>
<td>S-Leu</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>ADMAAdda</td>
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<td>S-Arg</td>
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<td>N-Mdha</td>
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<tr>
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<td>R-Ala</td>
<td>S-Leu</td>
<td>R-Asp</td>
<td>S-Har</td>
<td>ADMAAdda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
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<tr>
<td>[R-Glu(OC&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;)] Microcystin-LR&lt;sup&gt;129&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Leu</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>(OC&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;) ester</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>[R-Glu(OC&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;OH)] Microcystin-LR&lt;sup&gt;129&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Leu</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>(OC&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;OH) ester</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>[R-Glu(OC&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;)] Microcystin-LR&lt;sup&gt;129&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Leu</td>
<td>Masp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>(OC&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;) ester</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>[R-Asp] Microcystin-LR&lt;sup&gt;129&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Leu</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
</tr>
<tr>
<td>[R-Asp] Microcystin-RR&lt;sup&gt;151&lt;/sup&gt;</td>
<td>R-Ala</td>
<td>S-Arg</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdha</td>
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<tr>
<td>[R-Asp] Microcystin-YR¹⁰⁰</td>
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<td>S-Tyr</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdhb</td>
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<td>S-Lau</td>
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<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdhb</td>
</tr>
<tr>
<td>[R-Asp, Ser] Microcystin-LR¹⁰⁰</td>
<td>R-Ala</td>
<td>S-Lau</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>S-Ser</td>
</tr>
<tr>
<td>[R-Asp, Dha] Microcystin-RR¹⁰⁰</td>
<td>R-Ala</td>
<td>S-Dha</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>Dha</td>
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<tr>
<td>[R-Asp, Dha] Microcystin-HtyR¹⁰⁰</td>
<td>R-Ala</td>
<td>S-Dha</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>Dha</td>
</tr>
<tr>
<td>[Oha] Microcystin-LR¹⁰⁰</td>
<td>R-Ala</td>
<td>S-Dha</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>Oha</td>
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<td>[Mea] Microcystin-LR¹⁰⁰</td>
<td>R-Ala</td>
<td>S-Dha</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mea</td>
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<tr>
<td>Nodularin¹⁰⁰</td>
<td>R-Ala</td>
<td>S-Dha</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdhb</td>
</tr>
<tr>
<td>Motuporin²⁴⁴</td>
<td>R-Ala</td>
<td>S-Dha</td>
<td>R-Asp</td>
<td>S-Arg</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdhb</td>
</tr>
<tr>
<td>[DAAda] Nodularin²⁴⁴</td>
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<td>S-Dha</td>
<td>R-Asp</td>
<td>DMAAdda</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdhb</td>
</tr>
<tr>
<td>[6Z]-Adda] Nodularin²⁴⁴</td>
<td>R-Ala</td>
<td>S-Dha</td>
<td>R-Asp</td>
<td>[6Z]-Adda</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdhb</td>
</tr>
<tr>
<td>[R-Asp] Nodularin²⁴⁴</td>
<td>R-Ala</td>
<td>S-Dha</td>
<td>R-Asp</td>
<td>Adda</td>
<td>R-Glu</td>
<td>N-Mdhb</td>
<td></td>
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</table>
APPENDIX 2: ROE INTERACTIONS IN MAJOR ISOMER OF CYCLIC PEPTIDE (52A)

ROE INTERACTIONS

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Type</th>
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<tbody>
<tr>
<td>Asp HN : Asp H(\alpha)</td>
<td>Strong</td>
</tr>
<tr>
<td>Asp HN : Asp H(\beta)</td>
<td>Weak</td>
</tr>
<tr>
<td>Asp HN : Asp H(\beta')</td>
<td>Weak</td>
</tr>
<tr>
<td>Asp HN : Pro H(\alpha)</td>
<td>Medium</td>
</tr>
<tr>
<td>Asp HN : Pro H(\beta)</td>
<td>Weak</td>
</tr>
<tr>
<td>Asp HN : Pro H(\beta')</td>
<td>Weak</td>
</tr>
<tr>
<td>Phe HN : Asp H(\beta)</td>
<td>Strong</td>
</tr>
<tr>
<td>Phe HN : Asp H(\beta')</td>
<td>Strong</td>
</tr>
<tr>
<td>Ala H(\beta) : Glu HN</td>
<td>Weak</td>
</tr>
<tr>
<td>Phe HN : Phe H(\alpha)</td>
<td>Strong</td>
</tr>
<tr>
<td>Phe HN : Phe H(\beta')</td>
<td>Weak</td>
</tr>
<tr>
<td>Phe HN : Phe H(\beta)</td>
<td>Medium</td>
</tr>
<tr>
<td>Phe HN : Phe H(\epsilon)</td>
<td>Weak</td>
</tr>
</tbody>
</table>

Restraints applied as symmetrical energy well, with force constant 30 kcal/Å\(^2\) and a maximum potential of 1000 kcal/mol per constant. No energy penalty was applied when H-H distances lay outside the range 1.8-2.7 Å (strong), 1.8-3.3 Å (medium) and 1.8-5 Å (weak), respectively.
<table>
<thead>
<tr>
<th>Residue 1</th>
<th>Residue 2</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe HN</td>
<td>Phe H5</td>
<td>Medium</td>
</tr>
<tr>
<td>Phe HN</td>
<td>Ala HN</td>
<td>Medium</td>
</tr>
<tr>
<td>Phe Hα</td>
<td>Phe H5</td>
<td>Weak</td>
</tr>
<tr>
<td>Phe Hα</td>
<td>Phe H5</td>
<td>Medium</td>
</tr>
<tr>
<td>Phe Hα</td>
<td>Ala HN</td>
<td>Strong</td>
</tr>
<tr>
<td>Phe Hβ'</td>
<td>Phe H5</td>
<td>Weak</td>
</tr>
<tr>
<td>Phe Hβ</td>
<td>Phe H5</td>
<td>Medium</td>
</tr>
<tr>
<td>Phe Hβ'</td>
<td>Ala HN</td>
<td>Medium</td>
</tr>
<tr>
<td>Phe Hβ'</td>
<td>Phe H5</td>
<td>Weak</td>
</tr>
<tr>
<td>Phe Hβ</td>
<td>Phe H5</td>
<td>Medium</td>
</tr>
<tr>
<td>Ala HN</td>
<td>Ala Hα</td>
<td>Medium</td>
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<tr>
<td>Ala HN</td>
<td>Ala Hα'</td>
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<td>Ala HN</td>
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<td>Ala HN</td>
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<td>Ala Hα</td>
<td>Glu HN</td>
<td>Weak</td>
</tr>
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<td>Ala Hα'</td>
<td>Glu HN</td>
<td>Weak</td>
</tr>
<tr>
<td>Ala Hβ</td>
<td>Glu HN</td>
<td>Weak</td>
</tr>
<tr>
<td>Glu HN</td>
<td>Glu Hα</td>
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<td>Glu HN</td>
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<tr>
<td>Glu H5</td>
<td>Glu Hα</td>
<td>Weak</td>
</tr>
<tr>
<td>Glu H5</td>
<td>Glu Hα'</td>
<td>Weak</td>
</tr>
<tr>
<td>Glu Hγ</td>
<td>Pro H5</td>
<td>Medium</td>
</tr>
<tr>
<td>Glu Hγ'</td>
<td>Pro H5</td>
<td>Medium</td>
</tr>
</tbody>
</table>
APPENDIX 3: RECOMBINANT PP1 (BOLD) AND NATIVE DNA SEQUENCES

Martinez/Needleman-Wunsch DNA Alignment

Minimum Match: 9; Gap Penalty: 1.10; Gap Length Penalty: 0.33
CTTTGCCAAGCGGCAGCTGGTGACACTTTTCTCAGCTCCCAACTACTGTGG CGAGTTTGACAATGCTGGC

GCCATGATGAGTGTGGACGAGACCCTCATGTGCTCTTTCCAGATCCTCAA GCCCGCCGACAAGAACAAGG

GGAAGTACGGGCAGTTCAGTGGCCTGAACCCTGGAGGCCGACCCATCACCC CACCCCGCAATTCCGCCAA

AGCCAAGAAA------------------------TAG
APPENDIX 4: ESTIMATION OF LEVEL OF FIRST RESIDUE ATTACHMENT - FROM NOVABIOCHEM\textsuperscript{\textregistered} CATALOGUE

Dry Fmoc amino acid-resin (2 mg) was placed into two x 10 mm matched silica UV cells, to this was added 20% piperidine in DMF (3 cm\textsuperscript{3}), and the resin agitated with the aid of a Pasteur pipette for 2-3 min.

The cells were placed in a spectrophotometer using a third matched cell with 20% piperidine in DMF (3 cm\textsuperscript{3}) as a reference (no resin). Positions 2 and 3 held the resin samples.

The absorbance at 290 nm was read and an estimate of first residue attachment was taken from the correlation graph (see Graph 4.1). The average of these two values were used to calculate the percentage loading.

Graph 4.1: Correlation Graph for Percentage Loading for First Residue Attachment; Based on 2 mg Resin
Kaiser (Ninhydrin) Test

The most widely used qualitative test for the presence or absence of free amino groups was devised by Kaiser. Free amino groups show a dark blue colour.

The following solutions were prepared:

- 5 g of ninhydrin was dissolved in 100 cm³ ethanol
- 80 g of liquefied phenol was dissolved in 20 cm³ ethanol
- 2 cm³ of 0.001 M Potassium Cyanide solution was added to 98 cm³ pyridine

A few resin beads, which had been washed several times with ethanol, were transferred to a small ampule and 2 drops of each of the solutions above were added. This was mixed well, and heated to 120 °C for 4-6 mins. A positive test is indicated by blue resin beads.
APPENDIX 5: DETERMINATION OF KINETIC PARAMETERS

Introduction

For many enzymes, the rate of catalysis, v, varies with the substrate concentration, [S], in a manner shown in Graph 4.1.

Graph 4.1: A plot of the reaction velocity, V, as a function of the substrate concentration, [S], for an enzyme that obeys Michaelis-Menten kinetics ($V_{max}$ is maximal velocity and $K_m$ is the Michaelis constant)

At a fixed concentration of enzyme, v is almost linearly proportional to [S] when [S] is small. At high [S] v is nearly independent of [S].

In 1913, Michaelis and Menten proposed a simple model to account for these kinetic characteristics (see Scheme 4.1).
Scheme 4.1: Simple Michaelis-Menten Equation

The critical feature in their treatment is that a specific enzyme-substrate (ES) complex is a necessary intermediate in catalysis. The enzyme-substrate reaction is divided into two processes. First the substrate ($S$) binds rapidly and reversibly to the enzyme ($E$) to form an ES complex. The reaction then occurs in a slow second step giving the product, $P$, and regenerating the enzyme.

Because the breakdown of ES complex to $E$ and $S$ is faster than the breakdown of ES complex to $E$ and $P$, the rate, $v$, is defined by:

$$v = k_{cat}[ES] \quad \text{Equation 2}$$

$[ES]$ can also be expressed by:

$$ES = k_1[E][S] \quad \text{Equation 3: Rate of Formation}$$

$$ES = (k_2 + k_{cat})[ES] \quad \text{Equation 4: Rate of Breakdown}$$

Assays are carried out under steady-state conditions. In a steady state, the concentrations of enzyme-bound intermediates stay the same while the concentrations of starting materials and products are changing. This occurs when the rate of formation and breakdown of the ES complex are equal.

$$k_1[E][S] = (k_2 + k_{cat})[ES] \quad \text{Equation 5}$$
Equation 5 can be rearranged:

$$[ES] = \frac{[E][S]}{(k_1 + k_{cat}) / k_1} \quad \text{Equation 6}$$

Equation 7 can be simplified by defining a new constant, $K_m$, called the *Michaelis constant*:

$$K_m = \frac{k_1 + k_{cat}}{k_1} \quad \text{Equation 7}$$

and substituting it into equation 6, which then becomes:

$$[ES] = \frac{[E][S]}{K_m} \quad \text{Equation 8}$$

The concentration of uncombined substrate, $[S]$, is very nearly equal to the concentration to the total substrate concentration, provided that the concentration of enzyme is much lower than that of the substrate. The concentration of uncombined enzyme, $[E]$, is equal to the total enzyme concentration, $[E_t]$, minus the concentration of the $ES$ complex.

$$[E] = [E_t] - [ES] \quad \text{Equation 9}$$

Substituting this expression for $[E]$ in equation 9,

$$[ES] = \frac{([E_t] - [ES])[S]}{K_m} \quad \text{Equation 10}$$

Solving equation for $[ES]$ gives

$$[ES] = [E_t] \frac{[S]}{[S] + K_m} \quad \text{Equation 11}$$
By substituting this expression for $[ES]$ into equation 3, we get

$$v = \frac{[S]}{[S] + K_m} \quad \text{Equation 12}$$

The maximal rate, $V_{max}$, is attained when the enzyme sites are saturated with substrate—that is, when $[S]$ is much greater than $K_m$—so that $[S]/([S] + K_m)$ approaches 1. Thus,

$$V_{max} = k_{cat}[E_i] \quad \text{Equation 13}$$

Substituting equation 14 into equation 13 yields the Michaelis-Menten equation:

$$v = \frac{[S]}{[S] + K_m} \quad \text{Equation 14}$$

Significance of Michaelis-Menten Parameters

$k_{cat}$ – Catalytic Constant

In simple Michaelis-Menten mechanisms, in which there is only one substrate-enzyme complex and all binding steps are very fast, $k_{cat}$ is simply a first order rate constant for the chemical conversion of the $ES$ complex to the $EP$ complex.

Constant $k_{cat}$ is often called the turnover number of the enzyme because it represents the maximum number of substrate molecules converted to products per unit of time, or the number of times the enzyme ‘turns-over’ per unit time.
$K_m$ - Real and Apparent Equilibrium Constants

Although it is only for the simple Michaelis-Menten mechanism or in similar cases that $K_m = K_i$, the true dissociation constant of the enzyme-substrate complex, $K_m$, may be treated for some purposes as an apparent dissociation constant. For example, the concentration of free enzyme in solution may be calculated from the relationship:

$$K_m = \frac{[E][S]}{\sum [E[S]]} \quad \text{Equation 15}$$

Where $\sum [E[S]]$ is the sum of all the bound enzyme species.

$K_m$ is the substrate concentration at which

$$v = \frac{V_{\text{max}}}{2} \quad \text{Equation 16}$$

Specificity Constant: $k_{\text{cat}}/K_m$

$k_{\text{cat}}/K_m$ determines the specificity for competing substrates, and is called the Specificity Constant.

It is an apparent second-order rate constant that refers to the properties and the reactions of the free enzyme and free substrate.

Graphical Representation of Data

The Michaelis-Menten equation is often transformed into a linear form for analysing data graphically and detecting deviations from ideal behaviour. One of the best known methods is the double-reciprocal or Lineweaver-Burk plot. Inverting both sides of equation 15 gives the Lineweaver-Burk plot.

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}[S]} \quad \text{Equation 17}$$
Plotting $1/v$ against $1/[S]$ gives an intercept of $1/V_{\text{max}}$ on the $y$ axis as $1/[S]$ tends toward zero, and of $1/[S] = -1/K_m$ on the $x$ axis. The slope of the line is $K_m/V_{\text{max}}$.

The Lineweaver-Burk plot has the disadvantage of compressing the data points at high substrate concentrations into a small region and emphasising the points at lower concentrations. Hence, data collected in our study were fitted to unweighted non-linear regression Michaelis-Menten fit, using Grafit, which avoids the overemphasis of small values of $v$ and $S$, thus yielding the parameters $K_m$ and $k_{cat}$. 
4. References


1421-1430.


83 R. E. Honkanen, H. Zwiller, R. E. Moore, B. S. Khatra, M. Dukelow and A. L.


159 W. W. Carmichael, *Scientific American*, 1994, **64-72**.


