SYNTHESES OF PHOSPHOPEPTIDE SUBSTRATES FOR USE IN PROTEIN PHOSPHATASE ASSAYS

a thesis presented by

Pantea Hormozdiari

to the

University of St. Andrews

in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY

St. Andrews September 1996

Copyright

Declaration

I, Pantea Hormozdiari, hereby certify that this thesis, which is approximately 51150 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date: 26 Sept 1996 Signature of candidate

I was admitted as a research student in October 1992 and as a candidate for the degree of Ph.D. in August 1993; the higher study for which this is a record was carried out in the university of St. Andrews between 1992 and 1996.

Date: 26 Sept 1996 Signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date: 26 Sept 1996 Signature of supervisor

بنام خدا

DEDICATED TO MY FAMILY

I would like to dedicate this work to my ever-loving and supporting family; my mother, father and sister. I thank them for their patience, understanding and never-ending love. I thank my mother and father for everything that I am and have achieved now, and will, in the years to come.

یوسف گم گشته باز آید بکنعان غم مخور
کلبهٔ احزان شود روزی گلستان غم مخور

این دل غمدیده حالش به شود دل بدمکن
وین سر شوریده باز آید بسامان غم مخور

گر بهار عمر باشد باز بر تخت چمن
چتر گل بر سر کشی ای مرغ خوشخوان غم مخور

دور گردون گر دو روزی بر مراد ما نگشت
دائما یکسان نباشد حال دوران غم مخور

هان مشو نومید چون واقف نه ای از سر غیب
باشد اندر پرده بازیهای پنهان غم مخور

ای دل ار سیل فنا بنیاد هستی برکند
چون ترا نوح است کشتی بان ز طوفان غم مخور

در بیابان گر بشوق کعبه خواهی زد قدم
سرزنشها گر کند خار مغیلان غم مخور

گرچه منزل بس خطرناک است و مقصد ناپدید
هیچ راهی نیست کانرا نیست پایان غم مخور

حال ما در فرقت جانان و ابرام رقیب
جمله میداند خدای حال گردان غم مخور

حافظا در کنج فقر و خلوت شبهای تار
تا بود وردت دعا و درس قرآن غم مخور

نسیم باد صبا دوشم آگهی آورد
که روز محنت و غم رو بکوتهی آورد

بمطربان صبوحی دهیم جامهٔ چاک
بدین نوید که باد سحر گهی آورد

بیا بیا که تو حور بهشت را رضوان
درین جهان ز برای دل رهی آورد

همی رویم بشیراز با عنایت بخت
زهی رفیق که بختم بهر رهی آورد

بجبر خاطر ما کوش کاین کلاه نمد
بسا شکست که با افسر شهی آورد

چه نالها که رسید از دلم بخرمن ماه
چو یاد عارض آن ماه خرگهی آورد

رسانداریت منصور بر فلک حافظ
چو التجا بجناب شهنشهی آورد

حافظ شیرازی

Acknowledgements

I would like to thank Professor David Gani for his help and supervision. I would also like to thank Dr Walter Ward for his help and enthusiasm in the project, despite the lack of enzymology in the work carried out. Thanks to Geoff in the enzymology lab, the members of the peptide lab, especially Dr Richard Luke, Rick, Mike and Jim, Dr John Harding in the radiochemistry lab, and Dr Richard Shute for their help during my stay at Zeneca Pharmaceuticals.

A very special and warm thank you goes to Dr Mahmoud Akhtar and Mrs Stacey Wilkie. I thank Akks for the chats (scientific and non-scientific), the proof-readings, the lifts, his friendship and for his patience. I thank Stacey for her cherished friendship throughout my time in St. Andrews. To my labmates Drs Andrew G. Cole (George) and David A. Perrey (Merrence) for introducing me to the world of organic chemistry, for making the transition from pharmacy to bioorganic chemistry as easy as realistically possible and for making our lab the best ever. To Dr Amit P. Mehrotra, Mahmoud and Kerri L. Webster for having provided what must be the second bestest lab ever; especially, for the most amusing and thought-provoking conversations one could ever hope to be party to. Thank you to my friends, in and out of St. Andrews, Leila, Angelina, Sougand, Tom, Kerri, Tracy Neal, Roger Pybus, Andrew, Matthew J. Jones, Rachel Keith, Jane R. Binks, Emmanuelle Beushausen and Kathryn. I would like to thank: Mrs Melanja Smith for her friendly face, the chats and for always being there when an nmr needed to be done; Dr Trevor Rutherford for the chats and the nmr's; Dr John Wilkie for the molecular modelling; Stacey and Trevor for being fellow 'cat-lovers'!; Dr Kathryn Morris for the dinners and concerts; Roger, Kerri and Tracy for "do you want to have lunch at the West Port?"; Dr Jurgen Schulz (Jerry) for being the most amusing German I have met to date; Amit for his interesting conversations on drinks, movies, the X-files and for introducing a whole new dictionary of words to the lab!; Arwel Lewis for having the most expressive face I have ever seen! and Kenny, thank you... And a general thanks to those who I have forgotten to mention.

Finally, I would like to thank the EPSRC and Zeneca Pharmaceuticals for financial support.

ABSTRACT

Phosphorylation and dephosphorylation of regulated proteins carried out by protein kinases and phosphatases respectively, is now generally recognised as the major intracellular control mechanism in both eukaryotic and prokaryotic cells. Investigation of the molecular basis of the control induced by phosphorylation, is entirely dependent on the availability, in sufficient quantities, of phosphopeptides and phosphoproteins. The development of general methods for the large scale preparation of synthetic phosphopeptides of any length and sequence is therefore highly desirable.

The sequence RRAT(P)VA has been shown to be a good substrate for PP2A and PP2C. Attempts were therefore made to synthesise the phosphopeptide, RRAT(P)VA, traditionally prepared by phosphorylations using protein kinases, *via* a synthetic strategy. Initial attempts at the solution phase synthesis of the hexapeptide sequence were unsuccessful. The sequence was therefore synthesised using solid phase peptide chemistry in near quantitative yields.

Solid phase phosphorylation of the peptide using various N,N-diisopropyl dialkyl/ aryl phosphoramidites, phosphorochloridates and salicylchlorophosphite was carried out. Repeated phosphorylations using diphenylphosphorochloridate afforded the diphenyl protected phosphate triester, Ac-RRAT(OP(OPh)$_2$)VA-OH, in 80% yield. However, attempted deprotection of the phenyl protecting groups was unsuccessful. A more reactive phosphorochloridate was, therefore, sought in the form of dipentafluorophenyl phosphorochloridate. A single treatment of the hexapeptide, Ac-RRATVA-OH, with dipentafluorophenylphosphorochloridate in the presence of base proceeded smoothly and yielded the required phosphopeptide, Ac-RRAT(P)VA-OH, in 60% overall yield. The phosphopeptide prepared was used in nmr spectroscopic assays with the protein phosphatases PPλ and PP2A. The nmr spectroscopic studies indicated that Ac-RRAT(P)VA-OH was indeed a substrate for the enzymes.

The protocol developed for solid phase phosphorylations using dipentafluorophenyl phosphorochloridate was shown to be applicable to serine residues. The serine analogue of Ac-RRAT(P)VA-OH, Ac-RRAS(P)VA-OH, was prepared in a similar manner. It is hoped that this phosphorylating agent provides an alternative efficient method for the preparation of phosphopeptides and phosphoproteins.

Contents

List of Figures

List of Schemes

List of Tables

List of Experimental Procedures

Abbreviations

Adda	(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-2(E),6(E)-dienoic acid
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
cAMP	adenosine 3',5'-cyclic monophosphate
Boc	tert-butoxycarbonyl
Cbz (Z)	benzyloxycarbonyl
CK-2	casein kinase-2
*m*CPBA	*m*-chloroperoxybenzoic acid
DCM	dichloromethane
DEN	diethylnitrosamine
DIPEA	diisoprpopylethylamine
DMAP	4-dimethyamino pyridine
DMBA	7,12-dimethylbenz[a]anthracene
DMF	N,N-dimethylformamide
DMS	dimethylsulfide
DMSO	dimethylsulfoxide
DSP	diarrhetic shellfish poisoning
DTT	dithiothreitol
DTX-1	dinophysistoxin-1
EDT	1,2-ethanedithiol
E/S	electrospray
FAB	fast atom bombardment
Fmoc	9-fluorenyl-methoxycarbonyl
IBCF	isobutylchloroformate
IC_{50}	concentration required to reduce enzyme activity by 50%
I-1	inhibitor-1
I-2	inhibitor-2 (modulator)
IR	infrared
Measp	(2*R*,3*S*)-3-methylaspartic acid
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
N-Mdha	N-methyldehydroalanine
N-Mdhb	N-methyldehydrobutyric acid
NMM	N-methylmorpholine
nmr	nuclear magnetic resonance
P_i	inorganic orthophosphate

PK	protein kinase
PKA	protein kinase A
PKC	protein kinase C
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
PPx	protein phosphatase x (where x=1, 2A, 2B, 2C, 3, 4, *etc.*)
$PP1_c$	catalytic subnit of PP1
PyBOP	benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate
Reagent K	crystalline phenol/ 1,2-ethanedithiol/ thioanisole/ water/ TFA (0.75 g/ 0.25 cm^3/ 0.5 cm^3/ 0.5 cm^3/ 10 cm^3)
SPPS	solid phase peptide synthesis
TES	triethylsilane
TFA	trifluoroacetic acid
TFE	trifluoroethanol
THF	tetrahydrofuran
TLC	thin layer chromatography
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
UV	ultraviolet
Wang	p-benzyloxybenzyl alcohol

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CHAPTER ONE

INTRODUCTION

1.0 INTRODUCTION

1.1 General Introduction

Thousands of proteins are expressed in a typical mammalian cell, of which a third are now believed to contain a covalently bound phosphoryl group.[1] While in many cases the significance of this bound phosphoryl group is unclear, it is nevertheless now obvious that the covalent modification of regulatory proteins coordinates diverse processes within a cell to provide tight control of the cell's physiology.

Most extracellular agonists exert their effects on cells by activating or inhibiting transmembrane signalling systems that control the production of second messengers *e.g.* cyclic AMP, Ca^{2+} and diacylglycerol. These second messengers then mediate the actions of agonists by modulating the activities of protein kinases and protein phosphatases.[2] Phosphorylation and dephosphorylation (Fig. 1.1) of serine, threonine and tyrosine residues, carried out by protein kinases and protein phosphatases respectively, trigger conformational changes in regulated proteins.[2] These conformational changes may be local to, or remote from the site of phosphorylation, and the changes can alter the surface properties of the protein resulting in changes in self-association or recognition by other proteins. These changes in the properties of regulated proteins, lead to the physiological responses that are evoked by particular agonists.[2]

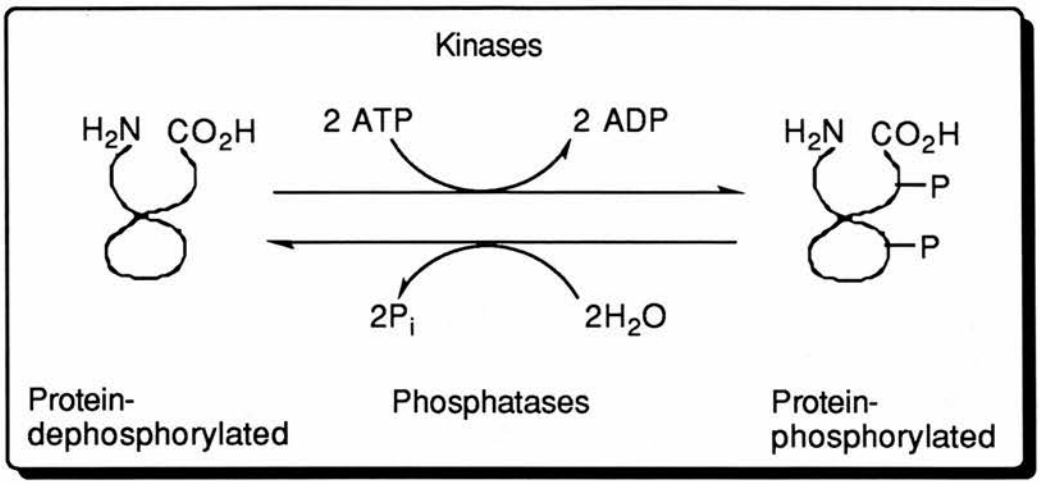


Figure 1.1: *Schematic representation of reversible phosphorylation.*

Protein phosphorylation is the most ubiquitous intracellular control mechanism and is known to occur in both eukaryotic and prokaryotic cells. In eukaryotic cells, it regulates metabolic pathways as diverse as gene transcription and translation, membrane transport, muscle contractility, cell division and growth, photosynthesis, learning and memory.[3,4] The protein phosphatases that dephosphorylate serine or threonine residues in proteins are considered below in more detail.

1.2 Protein Serine/ Threonine Phosphatases

1.2.1 General Classification

Protein phosphatases have broad and overlapping substrate specificities *in vitro*. Therefore, they cannot be classified by substrate alone and the action of specific inhibitors and activators is used as a criterium for their classification. Based on biochemical parameters, the Ser/ Thr protein phosphatases were initially divided into two classes.[3] Type-1 protein phosphatases (PP1) are inhibited by two small heat- and acid-stable proteins, termed inhibitor-1 (I-1) and inhibitor-2 (I-2), and preferentially dephosphorylate the β-subunit of phosphorylase kinase. Type-2 phosphatases (PP2 members) are insensitive to I-1 and I-2 and preferentially dephosphorylate the α-subunit of

phosphorylase kinase. Type-2 phosphatases can be further subdivided into three distinct enzymes based on their perceived requirement for divalent cations. PP2A is active towards most substrates in the absence of divalent cations, whereas PP2B is a Ca^{2+}-dependent, calmodulin-stimulated enzyme and PP2C is dependent on Mg^{2+}.[3,4] The use of the tumour promoter okadaic acid and liver toxin microcystin, further facilitate the discrimination between different classes.[5] Although still widely in use, this classification does not reflect the actual phylogenetic relationship between the different Ser/ Thr phosphatases. Molecular cloning has revealed PP2A to be more closely related to PP1 than to PP2C.[6] Moreover, many novel protein phosphatases have been identified that do not fit into this classification. Many of these proteins are closely related to the existing classes or are intermediates between classes. From a phylogenetic point of view, it might be more reasonable to group PP1, PP2A and PP2B in one family and PP2C in a separate family.[6]

1.3 Protein Phosphatase-1 (PP1)

1.3.1 Structure of PP1

The native structure of PP1 is a 1:1 complex between the catalytic subunit ($PP1_c$, molecular mass 37 kDa) and a number of regulatory subunits (also known as 'targetting subunits').[1] Two isoforms of $PP1_c$, α (330 amino acids) and β (311 amino acids) have been identified in rabbit skeletal muscle.[7] They differ by virtue of their distinct N-terminal sequence. From residue 34 in $PP1_c\alpha$, the nucleotide sequence for both isoforms is identical, indicating that $PP1_c\alpha$ and $PP1_c\beta$ are generated from the same gene by differential transcription and/ or splicing of the mRNA.[7] In rat, cDNA cloning has revealed at least four isoforms, termed α, γ1, γ2 and δ. Bacterially expressed $PP1_c$ (α, β and γ isoforms) differs from the native form in some aspects *e.g.* sensitivity to phosphatase inhibitors, binding to regulatory subunits and specific activity. The recombinant $PP1_c$ isoforms regain their native characteristics following incubation with I-2 and subsequent phosphorylation of I-2 by glycogen synthase kinase 3 (GSK3).[6,8] A study of deletion mutants of $PP1_c$

indicated that removal of the C-terminus of the protein (up to 33 reisdues) has no effect on the activity of the enzyme, nor on the binding of either okadaic acid or I-2.[9]

1.3.2 Evolutionary Conservation and Homology

The primary structure of $PP1_c$ shows an extreme phylogenetic conservation. PP1α from *Drosophila* and rabbit are 92% identical[10] and this agrees with the remarkable similarity in enzymatic and regulatory properties of $PP1_c$ in both groups. Homologues of $PP1_c$ in yeast and *Aspergillus* are more than 70% identical to rabbit PP1α. The primary structure of $PP1_c$ is strikingly homologous to $PP2A_c$ and $PP2B_c$ but is not related to that of PP2C.[11] The homology between different protein phosphatases is especially pronounced in the region corresponding to residues 60 to 130 of PP1α. This domain is therefore likely to contain the catalytic site and is probably not involved in the interaction with the specific effectors of $PP1_c$, like I-1 and I-2 (also known as modulator).[8] However, some other regions of $PP1_c$ are also extremely conserved and may be essential for activity *e.g.* a single amino acid mutation (Arg245 to Gln) in yeast $PP1_c$ proved fatal.[8] This Arg residue is conserved in all species of $PP1_c$, $PP2A_c$ and $PP2B_c$. Proteins that show a high degree of evolutionary conservation are generally considered to be those that are crucial to cellular function and the protein phosphatases are among the most highly conserved proteins known.[3]

1.3.3 The Targetting Subunit Hypothesis

Many protein kinases and phosphatases act upon more than one substrate *in vivo*, which enables a range of diverse responses to physiological stimuli. However, their broad substrate specificities, including *in vitro* activity towards non-physiological protein substrates, suggests the need for mechanisms to restrict the actions of these enzymes *in vivo*. Studies have shown that some of the major protein kinases and phosphatases are regulated by 'targetting subunits'.[1] This novel class of proteins not only acts to restrict the location of kinase and phosphatase catalytic subunits, but also modifies their catalytic and

regulatory properties, thereby playing a key role in ensuring the fidelity of protein phosphorylation.[1]

The part of the phosphatase that directs the catalytic subunit to the target locus is the targetting subunit.[1] The target locus can be an organelle, a membrane or a component in the soluble fraction of the cell. The targetting subunit may function to position the catalytic subunit close to a particular substrate, or to sequestrate it from other substrates and ligands (*e.g.* inhibitors).[1] The interaction between a targetting subunit and a catalytic subunit may also alter (allosterically) the catalytic activity or specificity of the catalytic subunit for nearby substrates.

As mentioned, $PP1_c$ can form a complex with a number of regulatory, targetting subunits.[1] It can also associate with proteins that inhibit its activity, *i.e.* I-1 and I-2, both of which can also be considered as regulatory subunits. The holoenzymes are named according to their apparent subcellular location.[8] For instance, the glycogen-bound PP1 holoenzyme (PP1G) is a heterodimer of $PP1_c$ and a 124 kDa[12] glycogen-binding subunit (G-subunit).[13] The G-subunit anchors the phosphatase to glycogen and increases its activity towards the glycogen-bound substrates: glycogen synthase and glycogen phosphorylase.[14]

The PP1 holoenzyme isolated from muscle sarcoplasmic reticulum (SR) has the same subunit composition as PP1G. It therefore appears that the G-subunit can target $PP1_c$ to either glycogen or the SR.[15] The G-subunit has a region of 32 hydrophobic residues at its C-terminus, which is probably responsible for the interaction of the G-subunit with membranes.[12] Sarcoplasmic reticulum-located PP1G is the major phosphatase that acts upon phospholamban,[16] a protein involved in Ca^{2+} uptake into the cardiac SR and in the regulation of the rate of cardiac muscle relaxation.

The myosin-bound form of PP1 (PP1M) can be found in skeletal and smooth muscle. The form in smooth muscle is a heterotrimer consisting of $PP1_c$ and polypeptide subunits of

130 and 20 kDa. These latter subunits are responsible for enhanced activity towards isolated myosin light chains.[17] The PP1M form in skeletal muscle is a dimeric enzyme consisting of $PP1_c$ and a regulatory subunit that also increases the dephosphorylation of myosin light chains.[18]

High levels of PP1 activity are also present in the nuclei of eukaryotic cells,[3] where it is reported to co-localise with chromosomes at mitosis.[19] Part of the nuclear PP1 is present in a latent form (termed $PP1N_\alpha$), and is composed of $PP1_c$ complexed to an inhibitory polypeptide (NIPP-1).[20] Two closely related forms of NIPP-1 (termed a and b) have been purified form bovine thymus and are proteins of 18 and 16 kDa respectively.

1.3.4 Heat-Stable Inhibitors of PP1

Inhibitor-1 is a 18.7 kDa protein which is activated as an inhibitor of PP1 after phosphorylation on Thr35 through the action of cyclic AMP (cAMP)-dependent protein kinase. Phosphorylation of I-1 occurs *in vivo* in skeletal muscle after adrenaline injection and also in rabbit liver after glucagon administration.[3]

DARPP-32 (dopamine and cAMP-regulated phosphoprotein), a 22.6 kDa protein, is an isoform of I-1 that inhibits PP1 with the same potency.[6] Although the expression pattern of DARPP-32 differs between species, in most species it is typically expressed in brain regions with dopaminergic innervation and striatonigral neurons. However, it is also found in renal tubule cells, adrenal medulla, pineal gland and brown adipose tissue. The N-terminal region of I-1 and DARPP-32, which is the most conserved region, also contains the inhibitory domain.[8]

Inhibitor-2 is a 22.8 kDa protein that differs in sequence to I-1. The inhibition of $PP1_c$ by I-2 is clearly different from that of I-1, since much higher I-2 concentrations are required to cause inhibition. Moreover, the action of I-2 is reversed upon its digestion by trypsin.[8] Modulator blocks the activity of PP1 in two distinct ways, namely, by impeding the

substrate binding (inhibition) and by inducing a conformational change of the catalytic subunit (inactivation). Inactivation of $PP1_c$ is observed at equimolar concentrations of phosphatase and modulator, whereas, as mentioned, higher concentrations of modulator are required for inhibition. This indicates that $PP1_c$ possesses two separate binding sites for I-2, *i.e.*, a low-affinity 'inhibition site' and a high-affinity 'inactivation site'.[8]

1.3.5 Deinhibitor

Deinhibitor is a thermostable protein, of molecular mass 9 kDa, which prevents the inactivation of $PP1_c$ by I-1 and I-2. Deinhibitor is itself inactivated *in vitro* by cAMP-dependent protein kinase[21] and can be reactivated by dephosphorylation by high molecular weight native forms of PP2A. This may indicate an example of a cascade of protein phosphatase activation, whereby PP2A reactivates deinhibitor, in turn reducing the inhibition of PP1 by I-1 and I-2 and promoting PP1 activity. A more detailed characterisation of the biochemical properties of deinhibitor and its phosphorylation in intact cells is not yet known.

1.3.6 Regulation of PP1

PP1 is a highly regulated protein phosphatase whose function in the different holoenzyme complexes is primarily mediated through the regulatory subunits.[1] In addition, as discussed, the activity of PP1 is modulated by the heat-stable inhibitors and the deinhibitor protein.

1.3.6.1 Regulation of the Glycogen-Bound PP1

Phosphorylation of the G-subunit by the cAMP-dependent protein kinase results in the release of $PP1_c$.[22] At physiological salt concentrations, the free catalytic subunit ($PP1_c$) is about 5-fold less active than the holoenzyme, in dephosphorylating glycogen synthase and glycogen phosphorylase[14] and will presumably be further inhibited by I-1. Phosphorylation of the G-subunit occurs *in vivo* in response to adrenaline (acting *via*

cAMP) and the resulting dissociation of PP1G contributes significantly to the inactivation of glycogen synthase. Conversely, dephosphorylation of the G-subunit, *in vitro*, is catalysed by PP2A, PP2B and PP2C.[22]

1.3.6.2 Regulation of Nuclear PP1

Latent PP1Nα can be activated *in vitro* through phosphorylation of NIPP-1 by cAMP-dependent protein kinase or casein kinase-2.[20] PP2A can reverse this activation. It remains to be determined whether PP2A exerts the same effect *in vivo* but if so, PP2A could control PP1 activity in the nucleus. The regulation of nuclear phosphatases is poorly understood and further work in this area is required.

1.3.7 Physiological Roles of PP1

Through the interaction of PP1 with its different targetting subunits, it is clear that this enzyme is involved in many different processes, as diverse as glycogen metabolism, calcium transport, muscle contraction, protein synthesis and intracellular transport.[3,8,23]

Recent evidence from the study of cell cycle mutants has revealed that PP1 plays an important role in the regulation of mitosis and also in chromosome segregation. The temperature-sensitive Bim G11 mutant of *Aspergillus nidulans* carries a mutation in the $PP1_c$ gene. At the restrictive temperature, mutants are unable to complete anaphase of mitosis successfully.[24] Further work is however necessary to define the role of PP1 during cell cycle progression.

1.4 Protein Phosphatase-2A (PP2A)

1.4.1 Structure of PP2A

Several trimeric holoenzyme forms of PP2A exist and have been characterised.[3] The core of these structures consists of a 36 kDa catalytic subunit ($PP2A_c$) complexed with a

regulatory subunit of 65 kDa (PR65 or the A subunit). This core dimer associates with variable regulatory subunits of 55 kDa (PR55 or the B subunit), 54 kDa (the B' subunit), 72 kDa (PR72) or 74 kDa (the B" subunit) (Fig. 1.2).[6] *In vivo*, PP2A is probably only present as a trimer, but this possibility remains a controversial issue as the core dimer has been purified from many different tissues. These findings suggest the occurence of subunit rearrangements where the variable subunits are able to associate and dissociate from the core dimer.[6]

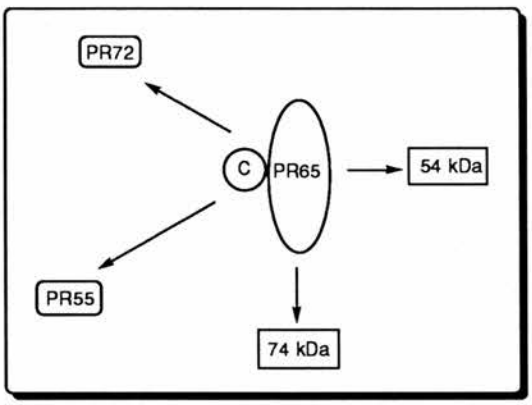


Figure 1.2: *Trimeric holoenzyme forms of PP2A.*

The exact function of the regulatory subunits is not yet completely resolved, but they probably influence substrate specificity and/ or subcellular localisation. The regulatory subunit PR72, for instance, contains a potential nuclear localisation signal in its primary sequence,[25] possibly explaining the presence of PP2A in the nucleus.

Molecular cloning has revealed the existence of several isoforms of each subunit.[26] Two isoforms of the catalytic subunit (α, β), two isoforms of PR65 (α, β), three isoforms of PR55 (α, β, γ) and two isoforms of PR72 exist. Most PP2A subunits appear to be ubiquitously expressed. In *Drosophila melanogaster* the situation is less complex, since every subunit is encoded by a single gene.[6] The *Drosophila* PP2A catalytic subunit is 94% identical to the mammalian enzyme, perhaps reflecting one of the most remarkable degrees of evolutionary conservation in any known protein.

1.4.2 Regulation of PP2A

1.4.2.1 Regulation by Polycations

PP2A is markedly activated by polycations, such as polylysine and protamine, which have been widely used to characterise this enzyme.[23] The exact mechanism of activation is unclear but is considered to occur primarily *via* the interaction of these polycations with the catalytic subunit and does not involve dissociation of the enzyme complex. The effect of the polycations varies with the substrate used, indicating that substrate interactions may also occur.[23] The differing effects of protamine on the dephosphorylation of synthetic phosphopeptides which differed by a single amino acid, is a clear example of this selectivity.

1.4.2.2 Regulation by Inhibition

The catalytic subunit of PP2A can be phosphorylated *in vitro* by various tyrosine kinases. Phosphorylation occurs on Tyr307 at the C-terminus of the protein and leads to 90% loss in activity; dephosphorylation reactivates the phosphatase. Studies by Chen *et al.*, have suggested this reactivation to be consistent with an autodephosphorylation reaction.[28] The inclusion of the phosphatase inhibitor okadaic acid in the assays, resulted in more rapid phosphorylation of the Thr residue.[28] The *in vivo* phosphorylation of the catalytic subunit has not been correlated with activity changes. Nevertheless, it has been suggested that activation of growth factor receptors leads to transient inactivation (Tyr phosphorylation) of PP2A, thereby amplifying and prolonging the activation of protein kinases.[28] These data suggest that the activity of PP2A can be modulated by extracellular signals, placing PP2A in a pivotal position to modulate signal transduction pathways.

In vitro phosphorylation of the catalytic subunit on threonine residues leading to a loss of activity has also been reported.[29] This reaction is catalysed by an autophosphorylation-activated protein kinase, and leads to about 80% inactivation of PP2A.[29] This has the elegance of amplifying the signal propagated by the kinase. Interestingly, the kinase itself

is dephosphorylated and inactivated by PP2A. Mechanistically, however, these data are difficult to rationalise.

1.4.2.3 Tyrosine Phosphatase Activity of PP2A

Although PP2A was originally purified as a Ser/ Thr protein phosphatase, it can, under certain conditions, dephosphorylate phosphotyrosine residues. The tyrosine phosphatase activity of PP2A towards exogenous substrates is increased in the presence of ATP or PP_i, tubulin and MAP-2.[30] Furthermore, a 37 kDa protein (termed PTPA) was isolated and characterised which, in the presence of ATP and Mg^{2+}, activates the tyrosine phosphatase activity of PP2A. This effect is limited to the dimeric form of PP2A, and to a lesser extent to the isolated catalytic subunit.[31,32] The tyrosine phosphatase activity of PP2A after PTPA stimulation is of the same order of magnitude as its Ser/ Thr phosphatase activity. The mechanism by which PTPA stimulates the tyrosine phosphatase activity of PP2A remains to be established.

1.4.2.4 Other Methods of Regulation

The mechanisms responsible for regulating PP2A activity in normal cells are still poorly understood. As well as the methods already mentioned, PP2A activity can also be regulated by ATP. $PP2A_c$ is more sensitive to inhibition than $PP1_c$.[23] Studies suggest that physiological concentrations of ATP might influence the substrate specificity of PP2A.

The interaction of $PP2A_c$ with the regulatory subunits PR65, PR55, PR72 and others influence its substrate specificity.[6] Analysis of yeast and *Drosophila* mutants lacking one particular PP2A subunit confirms the importance of regulatory subunits. For instance, yeast carrying a mutation in the TPD3 gene (encoding a PR65 homologue) shows defects in tRNA gene transcription resulting in the cells becoming mutinucleated.[33]

1.4.3 Physiological Roles of PP2A

The assignment of a functional role for PP2A has relied on the identification of good *in vitro* substrates. However, through the use of okadaic acid (**1**) (Fig. 1.3) and related inhibitors, it is possible to specifically inhibit PP2A activity in cell-free extracts.[6]

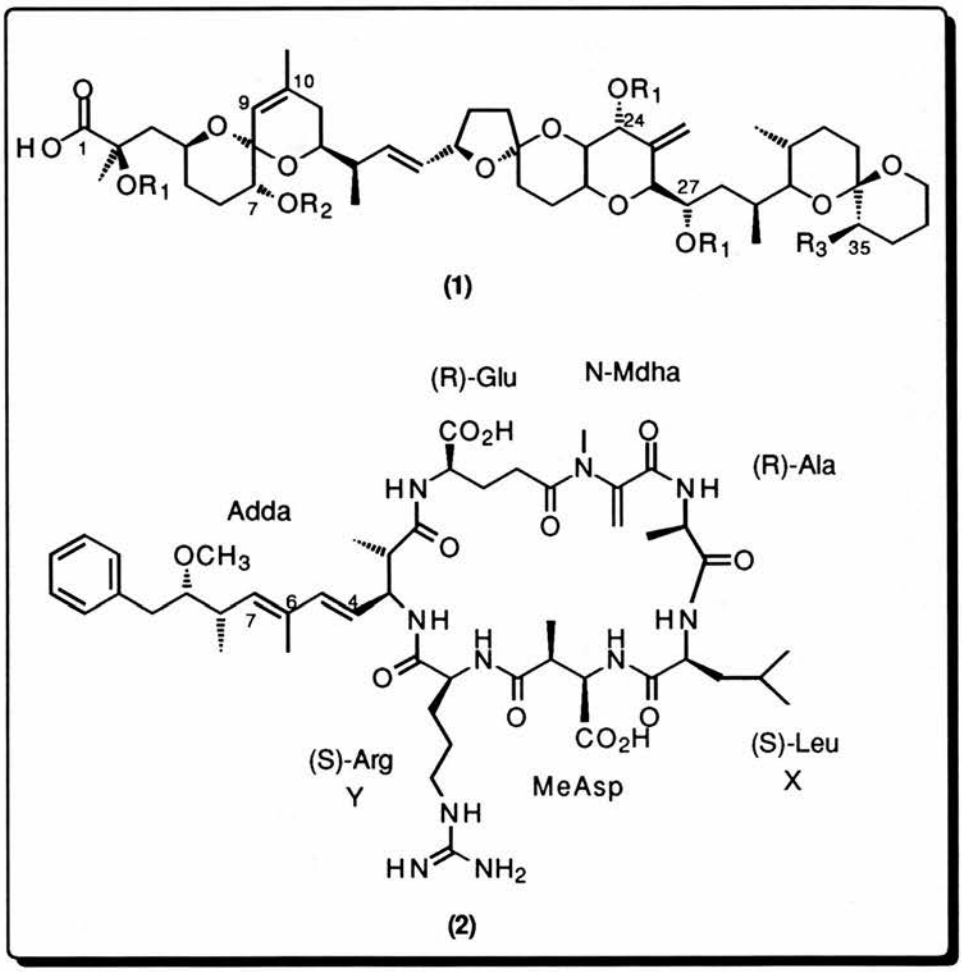


Figure 1.3: *Protein phosphatase inhibitors Okadaic acid (1) and Microcystin-LR (2).*

Although one cannot exclude the possibility that PP2A-like enzymes are affected, this approach, often in combination with purified PP2A preparations, has been used to identify potential PP2A substrates in a wide variety of cellular processes. Several regulatory enzymes involved in glycolysis/ gluconeogenesis, fatty acid synthesis, amino acid breakdown, lipolysis and catecholamine synthesis are excellent substrates for PP2A suggesting that it may be the major phosphatase acting to regulate these pathways *in*

vivo.[3,23] Other physiological roles for PP2A include modulation of muscle contraction, synaptic transmission, signal transduction, RNA splicing and cell cycle progression.[3,23]

PP2A isoforms are the major phosphatases acting on intact smooth muscle myosin and causing muscle relaxation.[23] PP2A has been found to dephosphorylate proteins that are themselves involved in signal transduction pathways. For instance, PP2A is the only phosphatase that significantly dephosphorylates the deinhibitor protein,[21] thus potentially regulating PP1 activity in cells. A role for PP2A in regulating protein kinases has also been suggested.[23] Several high molecular weight forms of PP2A rapidly dephosphorylate protein kinase C autophosphorylated *in vitro*.

1.5 Protein Phosphatase-2B (PP2B)

1.5.1 Structure of PP2B

PP2B or calcineurin was first identified as a major calmodulin-binding protein in the brain, where it accounts for up to 1% of the total protein. Later, PP2B was shown to display Ser/Thr protein phosphatase activity. PP2B is a heterodimer of calcineurin A (molecular mass 60 kDa) and calcineurin B (molecular mass 19 kDa) (Fig. 1.4). Calcineurin A is the catalytic subunit and also binds to calmodulin, whereas calcineurin B is the regulatory, Ca^{2+}-binding subunit.[3]

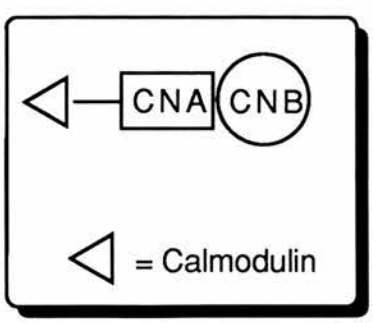


Figure 1.4: *Schematic representation of PP2B.*

Calcineurin B is a member of the 'EF hand' family of Ca^{2+}-binding proteins. It contains four Ca^{2+}-binding loops and shows 35% sequence identity to calmodulin.[6] The N-terminus of the B subunit is myristoylated, which might account for the association of PP2B with particulate fractions in the brain.[6] Myristoylation, the covalent addition of myristic acid to a protein, is thought to be important in attaching some proteins to cell membranes.

The use of monoclonal antibodies revealed the presence of different isoforms of PP2B catalytic subunit in the brain compared with other tissues. The catalytic subunit is encoded by three separate genes (CNAα, CNAβ and CNAγ). The B subunit is encoded by two genes. The CNBα gene gives rise to an isoform that is highly expressed in the brain (CNBα1), but is also present in most other tissues. The second gene (CNBβ) is specifically expressed in the testis.[6]

1.5.2 Regulation of PP2B

PP2B is dependent on Ca^{2+} for activity and is stimulated by calmodulin, moreover, the purified enzyme also requires divalent metal ions such as Mn^{2+}, Mg^{2+} or Ni^{2+}. PP2B binds calmodulin with high affinity (k_D 1 nM) at physiological concentrations of Ca^{2+} (700 nM). Calmodulin binding to the isolated A subunit has a lower affinity (k_D 30 nM). Studies have shown the B subunit to be essential for maximal enzyme activity and it also enhances the interaction of calmodulin with the A subunit.[34] Partial proteolysis of calcineurin converts it to a form which no longer requires Ca^{2+} for activity. This is due to the removal of the C-terminus of the catalytic subunit, which contains an autoinhibitory domain.[35] Binding of calmodulin to a nearby site and binding of Ca^{2+} to the B subunit allows the enzyme to bypass this autoinhibition.

PP2B is the major cellular target of immunosuppressive drugs that elicit their pharmacological actions by inhibiting phosphatase activity. The drugs, cyclosporin A [CsA, (**3**)] and FK506 (**4**) (Fig. 1.5) bind to immunophillins, the cellular levels of which

greatly exceed the concentration of drug required for immunosuppression. PP2B catalytic subunit is the primary target of this drug-immunophillin complex.[36] This has identified a major role for PP2B in T-cell activation.

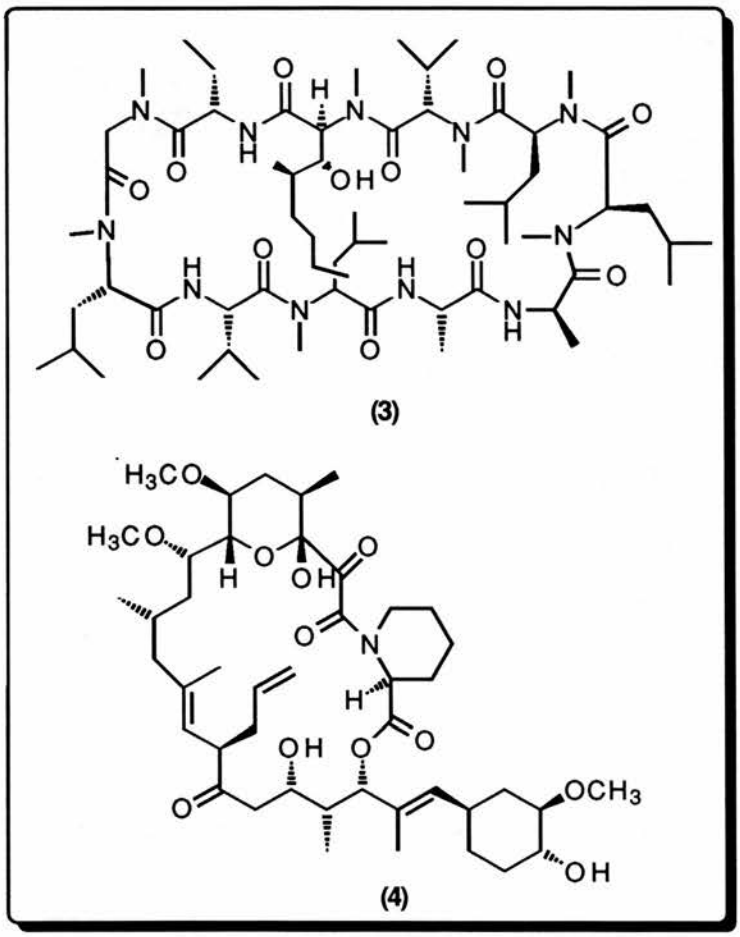
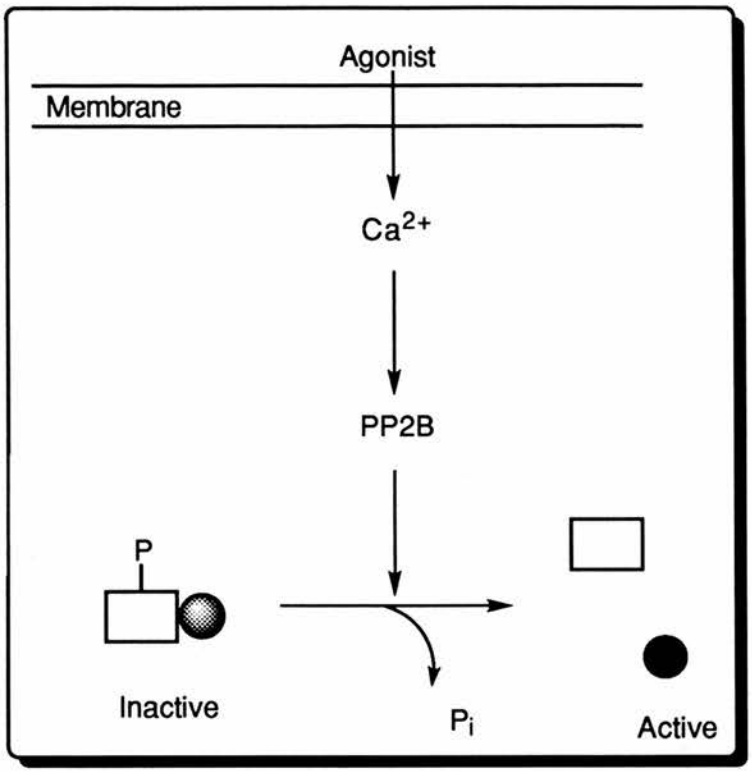


Figure 1.5: *Protein phosphatase inhibitors Cyclosporin A (3) and FK506 (4).*

Calcineurin has a rather restricted substrate specificity. The α subunit of phosphorylase kinase, I-1, DARPP-32 and the type II regulatory subunit of cAMP-PK (RII) are excellent *in vitro* substrates.[6] Interestingly, all of these proteins are regulators of either protein kinases or protein phosphatases. It appears likely that calcineurin functions in a cascade system and is the key enzyme controlling the activity state of other enzymes with much broader substrate specificity (Scheme 1.1).



Scheme 1.1: *Schematic representation of a cascade system. The white square denotes I-1; the grey circle, an inactive PP1 complexed with I-1 and the black circle, an active PP1.*

1.5.3 Physiological Roles of PP2B

PP2B is found in highest concentrations in the nervous system. It is localised to neurons suggesting that the enzyme plays a role in neuronal functions. Studies in the budding yeast, *Saccharomyces cerevisiae*, a good model for the study of PP2B function, have shown that PP2B is responsible for the stimulation of Na^+ export.[37] It seems that PP2B may be involved in the regulation of ion channels in both neuronal and non-neuronal cells.

PP2B has been isolated from bovine cardiac tissue, where it dephosphorylates substrates for cAMP-dependent protein kinase in sarcolemma.[23] In the mouse exocrine pancreas, PP2B has been identified and could participate in control of hormonal secretion.[38]

The demonstration of the *in vitro* actions of PP2B and its specific association with particulate fractions supports a role for this protein phosphatase in the control of ion channels, receptors and cytoskeletal structures involved in such diverse processes as neuronal function, cell motility and exocytosis.[3,6,23] Many of these studies have evaluated the dephosphorylation of the phosphoproteins *in vitro* and thus a physiological role of PP2B in the regulation of these potential target proteins *in vivo* remains to be established.

1.6 Protein Phosphatase-2C (PP2C)

PP2C is a Mg^{2+}-dependent Ser/ Thr protein phosphatase. The enzyme shows half-maximal activity at 1 mM Mg^{2+} and has been isolated from heart, brain, liver and skeletal muscle.[6] The enzyme is monomeric with a molecular mass of 43-48 kDa depending on the source.[3] Two major isoforms (α and β) of PP2C have been cloned.[3] Peptide mapping of the two isoforms revealed only 49 identities in a common sequence of 62 amino acids and this provided clear evidence for distinct gene products.[23] Other amino acid sequencing studies have shown that PP2C has little structural homology with PP1, 2A and 2B and that PP2C enzymes are products of a distinct gene family. The properties of a protein phosphatase isolated from archaebacteria resemble those of PP2C and this might suggest that PP2C-like phosphatases arose earlier in evolution than the other Ser/ Thr phosphatases.[39]

In vitro studies have shown that PP2C has unusually high activity towards the enzymes of cholesterol synthesis, especially the rate-limiting enzyme, hydroxymethylglutaryl CoA reductase.[39,23] However, *in vivo* studies designed to examine the role of PP2C in this metabolic pathway have not been carried out. The insensitivity of PP2C to okadaic acid permits the convenient and accurate quantitation of the activity of the enzyme. The treatment of intact cells with okadaic acid could help in elucidating the physiological role of this enzyme *in vivo*.

1.7 Other Protein Phosphatases

Other novel protein phosphatases belonging to the PP1/ PP2A/ PP2B family have been identified from their cDNAs and are considered below.

1.7.1 Protein Phosphatase-3 (PP3)

A Ser/ Thr protein phosphatase, designated as type 3, was isolated and purified to homogeneity from bovine brain.[40] PP3, although similar to PP1 and PP2A, was found to be distinct from these enzymes, at the biochemical level. PP3 has a molecular mass of 36 kDa, preferentially dephosphorylates the β-subunit of phosphorylase kinase and does not require divalent cations for activity.[40] However, unlike PP1, PP3 is stimulated rather than inhibited by picomolar to micromolar concentrations of I-2. It is also inhibited by both okadaic acid (**1**) (IC_{50} of 5.2 nM) and microcystin-LR (**2**) (Fig. 1.3) (IC_{50} of 0.27 nM).[40] Amino acid sequencing of peptides derived from PP3 indicate a conserved six amino acid region with PP1 and 2A. The remainder of the PP3 peptide has no additional identities with PP1 but has 50% identity with PP2A. The low degree of homology in the amino acid sequences of PP1, PP2A and PP3 indicated that PP3 was not an isoform of any known Ser/ Thr phosphatase and could represent a novel protein phosphatase. The physiological functions of PP3 remain to be identified.[40]

1.7.2 Protein Phosphatase-4 (PP4)

PP4, initially termed PPX, was identified from a rabbit liver cDNA sequence. Its deduced amino acid sequence[41,42] was found to be 65% identical to PP2A and 45% identical to PP1 isoforms. PP4 has a molecular mass of 35 kDa and preferentially dephosphorylates the α-subunit of phosphorylase kinase. It is not inhibited by I-2 and is active in the absence of divalent cations. Its sensitivity to the inhibitors, okadaic acid and microcystin, is similar but not identical to that displayed by the catalytic subunit of PP2A. These findings place PP4 in the 'PP2A-like' class and distinguish it from PP1, 2B and 2C.[41] The PP2A:PP4

activity ratio, however, varies from 1:1 to 13:1 with different substrates indicating that the specificities of the two enzymes are distinct. Despite amino acid sequence similarity of PP4 and 2A catalytic subunits, PP4 does not bind the 65 kDa regulatory subunit of PP2A. PP4, therefore, either exists as a free catalytic polypeptide *in vivo*, or interacts with its own regulatory subunit(s). These differences as well as <70% identity to PP2A places PP4 as a novel protein phosphatase and not as an isoform of PP2A.[41]

Further experiments by Brewis *et al.* demonstrated that although PP4 was present in the cytoplasm and more strongly in the nucleus of human cells, it localised intensely to the centrosomes during mitosis. This suggests that PP4 may play a role in cell division and microtubule nucleation.[41]

1.7.3 Protein Phosphatase-5 (PP5)

Human PP5 and a structurally related phosphatase in *Saccharomyces cerevisiae*, PPT1, were identified from their cDNA and gene respectively.[42,43] The predicted molecular mass of PP5 and PPT1 is 58 kDa and they comprise a C-terminal catalytic domain and an N-terminal domain, which has four repeats of 34 amino acids. The C-terminal half of PP5 contains all of the regions that are conserved among the members of the PP1/ 2A/ 2B gene family, although it shows <40% identity to these phosphatases.[43] On the other hand, the presence of sequence identities in PP5 and PPT1 throughout the C- and N-terminal domains suggests that PPT1 is likely to be a yeast homologue of human PP5. Therefore, human PP5 and yeast PPT1 comprise a new subfamily of Ser/ Thr phosphatases.[43] Bacterially expressed PP5 is able to dephosphorylate phosphoserine residues in proteins. It is not inhibited by I-1 or I-2 and is more sensitive than PP1, but not PP2A, to okadaic acid.

The four repeats of 34 amino acids in the N-terminal domain of PP5, are similar to the tetratricopeptide repeat (TPR) motifs which have been found in several proteins that are required for mitosis, transcription and RNA splicing.[44] Investigation of the intracellular

distribution of PP5, shows a localisation predominantly in the nucleus. This localisation suggests that like other nuclear TPR-containing proteins, PP5 may play a role in the regulation of RNA biogenesis and/ or mitosis.[43]

1.7.4 Protein Phosphatase-λ (PPλ)

The N-terminal halves of the proteins encoded by the open reading frame, *orf* 221, in bacteriophage λ and the homologous gene of π80 show 35% identity to PP1 and 2A.[45,46] The *orf* 221 gene has been cloned and expressed in bacteria, and the recombinant protein shown to possess Ser/ Thr phosphatase activity. Characterisation of the enzyme has revealed that it is resistant to okadaic acid, I-1 and I-2 and absolutely required Mn^{2+} for activity.[45]

1.7.5 Protein Phosphatase-Z (PPZ)

The *Saccharomyces cerevisiae* enzyme PPZ exists in two isoforms (PPZ1 and PPZ2) that show about 60% identity to yeast PP1 and 40% identity to PP2A.[47,48] Both phosphatases contain a catalytic domain that is preceded by a long N-terminal domain, rich in serine and asparagine residues in PPZ1 and serine and arginine residues in PPZ2. Although the N-terminal domains are only 43% identical to each other, the catalytic domains are 93% identical, indicating that these two phosphatases are likely to have similar or overlapping functions.[42] Both isoforms are involved in the maintenance of osmotic stability in yeast.[49]

1.7.6 Protein Phosphatase-Q1 (PPQ1)

PPQ1 is a 61 kDa *Saccharomyces cerevisiae* Ser/ Thr protein phosphatase that contains a C-terminal domain which is 60% similar to PP1, and a serine/ aspartic acid-rich N-terminal domain. Deletion of the gene encoding PPQ1 results in a reduced rate of protein synthesis.[50]

1.7.7 Protein Phosphatase-G (PPG)

PPG from *Saccharomyces cerevisiae* is related to PP2A and is characterised by an extension of 50 amino acids at the C-terminus of PP2A. Disruption of the gene showed that it is not essential for growth, but plays a role in glycogen accumulation.[51]

1.8 Crystal Structures

Mammalian purple acid phosphatases (PAPs) are enzymes of molecular mass ~37 kDa that contain an oxide-bridged dinuclear iron active site. These enzymes and the protein phosphatases possess phosphoserine/ phosphothreonine phosphatase activity and are able to hydrolyze the substrate *p*-nitrophenylphosphate. Given these similarities, Vincent *et al.* compared the amino acid compositions of the human placental PAP and rabbit skeletal muscle PP2A and found a region of ~30% absolute homology.[52] A high percentage of the conserved residues, postulated as being possible metal-binding ligands were tyrosine, histidine, glutamic and aspartic acid residues. The homology between the sequences was thought to reflect the presence of a common or at least related metal-containing active site in both classes of proteins.[52] The structural and mechanistic implications are discussed below.

1.8.1 Crystal Structure of PP1

Barford *et al.* reported on the first co-crystallisation of the catalytic subunit of PP1 in complex with microcystin-LR.[53] The crystals determined at 2.8 Å were reported to be orthorhombic.

Goldberg *et al.* later described the crystal structure of mammalian PP1 complexed with microcystin-LR and resolved to 2.1 Å.[54] This crystal structure revealed that PP1 is a metalloenzyme unrelated in architecture to the tyrosine phosphatases. Tyrosine phosphatases do not require metal ions and use a cysteine residue as a nucleophile to effect catalysis.

The catalytic domain of PP1 can be divided conceptually into two tightly linked sub-structures.[54] The N-terminal region (the 'N-subdomain', residues 7 to 182) includes a β-α-β-α-β metal-coordinating unit and forms a compact structure in which the two metal ions are embedded (Fig. 1.6 a). The C-terminal region (the 'C-subdomain', residues 183 to 300) forms an irregular structure that sits on top of the N-subdomain and provides likely binding sites for substrates and inhibitors.[54] The N- and C-subdomain division is suggested by sequence comparison, which indicates that the N-subdomain is conserved in the λ-phosphatase, but that the C-terminal region of that enzyme may be unrelated.

The published crystallographic data show that PP1 contains two metal ions which lie close to each other near the C-termini of the β-strands of the β-α-β-α-β unit (Fig. 1.6 b).[54] The authors suggest that the metal ions are located at the active site. This conclusion is based on the location of the metal ions at the surface of the enzyme, as well as the strict conservation of the metal coordinating residues in PP1, 2A and 2B and the severe effect on catalysis caused by mutation of these residues. The role of the metals in PP1 does not appear to be primarily structural: the metal ion-binding site is constructed from regions of the protein core that appear to present the metal ions at the surface in a manner suitable for interaction with substrates. The authors were unable to identify the metal ions in the structure conclusively. Although the presence of metal ions Mg, Ni, Cu and Zn in the crystal structure was ruled out, the crystallographic method used could not discriminate between the presence of Fe, Mn or Co. Each of the metal ions in PP1 is coordinated by five ligands, in square-pyramidal (M1) and distorted trigonal bipyramidal (M2) arrangements (Fig. 1.6 c). The distance between the two metal ions is 3.3 Å, this close approach being facilitated by a carboxylate oxygen of Asp92 and a water molecule or hydroxyl ion (W2), each of which forms a bridge between the two metals. The six protein residues that coordinate the metals in PP1 (Asp64-His66-Asp92-Asn124-His173-His248) are strictly conserved in the eukaryotic Ser/ Thr phosphatases and the five that are in the N-subdomain are also conserved in the bacteriophage λ phosphatase (Appendix 1).

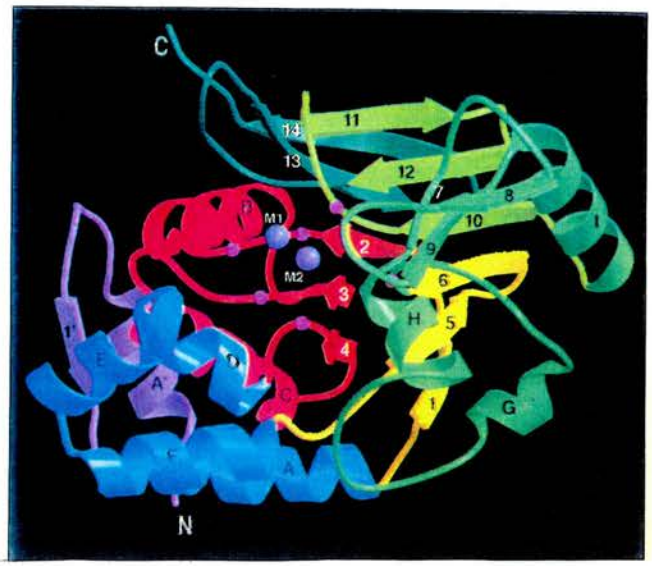


Figure 1.6 (a): *Ribbon diagram of PP1 showing the β-α-β-α-β metal binding motif (red), active site metals (violet spheres) and metal ligands (violet circles).*

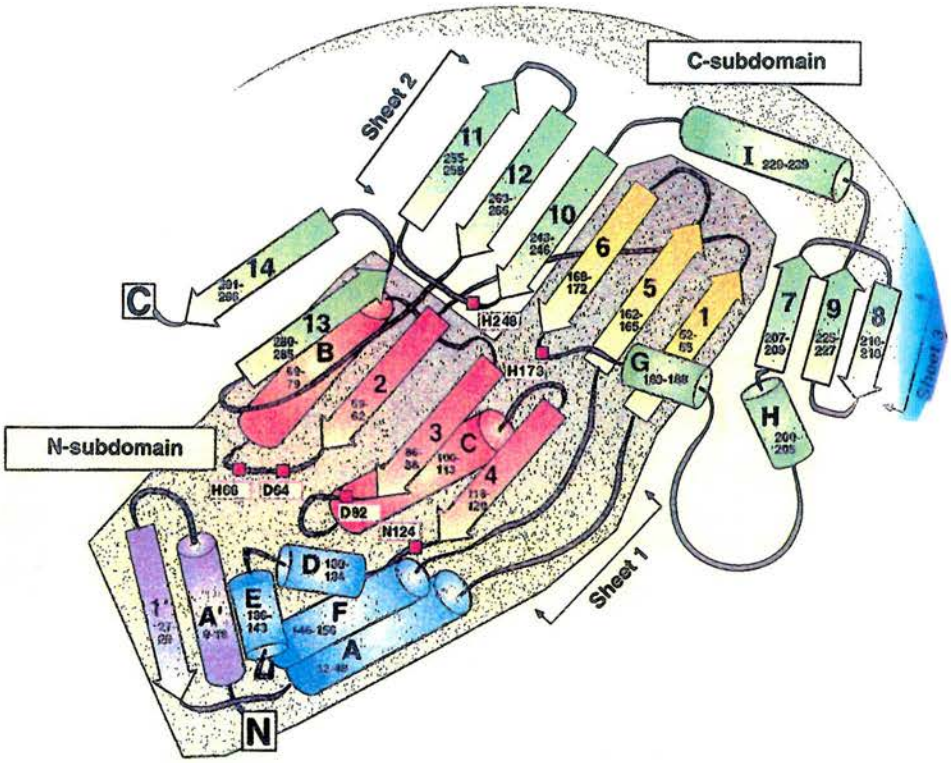


Figure 1.6 (b): *Schematic representation of the secondary structure of PP1. α-Helices are shown as cylinders and β-strands as arrows. The β-α-β-α-β scaffolding that binds the two metal ions is coloured red and the metal ligands indicated with red squares. Regions of the structure coloured green make up the C-subdomain while the rest constitute the N-subdomain. Both diagrams were reprinted with permission from Nature [54], Copyright (1995) Macmillan Magazines Limited.*

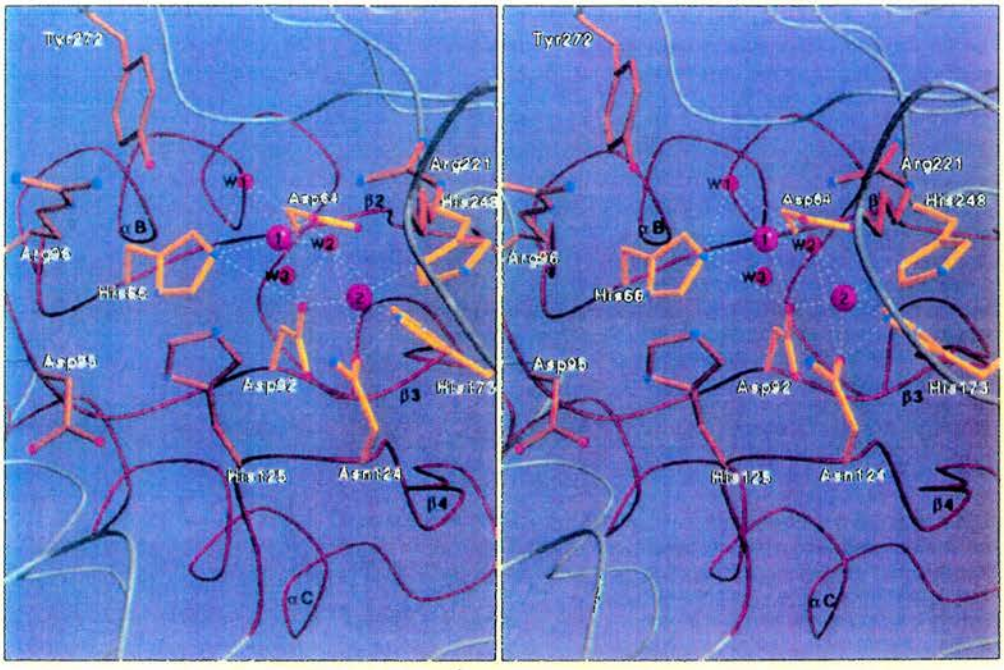


Figure 1.6 (c): *Stereo view of the active site of PP1. Metal ions 1 and 2 (purple) and waters W1, W2 and W3 (red) are drawn as spheres. Side chains of amino acids that are likely to be involved in catalysis are in brown.*

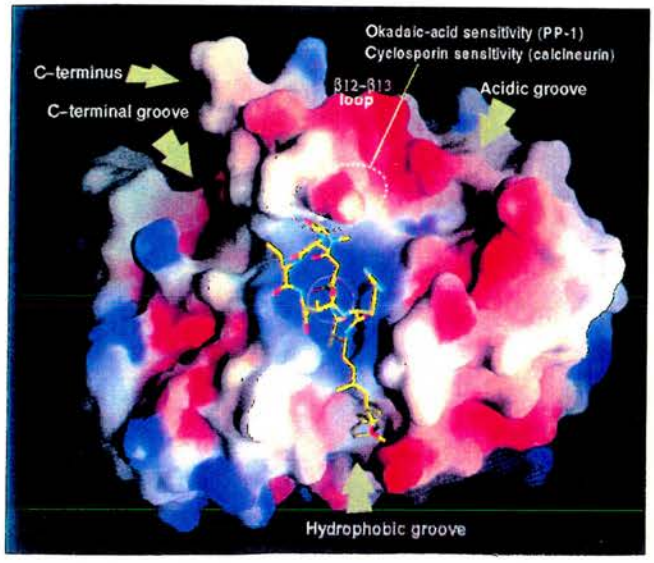


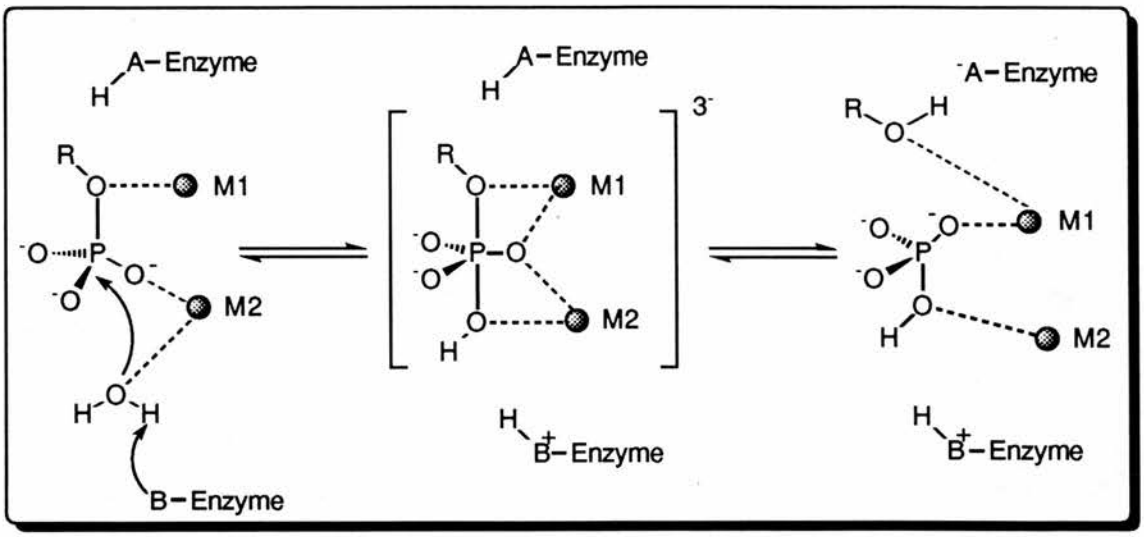
Figure 1.6 (d): *Electrostatic surface potentials of PP1. Both diagrams were reprinted with permission from Nature [54], Copyright (1995) Macmillan Magazines Limited.*

The crystal structure of the catalytic subunit of $PP1_{\gamma 1}$ and its complex with tungstate determined at 2.5 Å resolution,[55] confirmed the findings of Goldberg *et al.* and identified the two metal ions as Mn^{2+} and Fe^{2+}.

1.8.1.1 Metal-Assisted Catalysis

The two metal ions and two conserved arginine residues (Arg96 and Arg221) in PP1, together create a region of positive electrostatic potential at the active site that is the only such region on the surface of the enzyme (Fig. 1.6 d). A model of a substrate phosphate bound to the active site, in which the phosphate oxygens are coordinated by the metal ions and a number of active-site residues at reasonable interaction distances is shown in Fig. 1.6 e. An intimate interaction of this type in which the metal ions impart a strong electrostatic effect probably underlies their major catalytic role in PP1. Metal ions, by stabilising negative charges, can make a phosphate ester more susceptible to nucleophilic attack and can also stabilise the additional charge that develops on the phosphate during the attack. Metal ions can also promote reaction by positioning the nucleophile correctly for attack on the P-atom. This enhances the electrophilicity of the P-atom through Lewis acid coordination of the metal ion to one of the O-atoms. The binding of another metal ion to the O-atom of the leaving group, provides a further advantage.

The Ser/ Thr phosphatases therefore, presumably use their active site metal ions to bind substrate, activate a nucleophile, and/ or stabilize the transition state(s) of the phosphate-ester hydrolysis. A plausible mechanism for two-metal-ion catalysis of this reaction is shown in Scheme 1.2. The nature of the nucleophile is presently unknown, although a metal-bound water molecule or hydroxyl group is a reasonable candidate. Alternatively, the imidazole ring of the conserved His125 (coupled with the carboxylate of Asp95) could act as the nucleophile or perhaps as a general acid to promote the departure of the leaving serine or threonine residue.[56]



Scheme 1.2: *Two-metal ion catalysis of phosphate ester hydrolysis.*

1.8.1.2 Surface Grooves and Inhibitor Binding

The surface topology of PP1 is characterised by three distinct grooves that radiate from the central, shallow active site: a 'hydrophobic groove' containing a patch of hydrophobic side chains conserved in PP2A and calcineurin, an 'acidic groove' formed entirely within the C subdomain and containing a number of acidic side chains and a 'carboxy-terminal groove'. Microcystin (**2**) binds to PP1 in such a way as to interact with three distinct regions of the surface: the metal ion-binding site, the hydrophobic groove and the edge of the C-terminal groove near the active site (Fig. 1.6 f).[54] Indirect coordination of microcystin to the metal ions occurs *via* two of the metal-liganded water molecules, and involves the carboxylate group of the γ-linked (2*R*)-Glu and the adjacent carbonyl oxygen of the toxin. The long hydrophobic Adda side chain packs into the hydrophobic groove, where it is accomodated snugly. Interactions between microcystin and the C-terminal groove occur at the loop connecting β12 to β13, where the (2*S*)-Leu side chain of microcystin packs close to the side chain of Tyr272. Microcystin, therefore, inhibits PP1 by embedding itself in the hydrophobic groove and overlapping the active site.

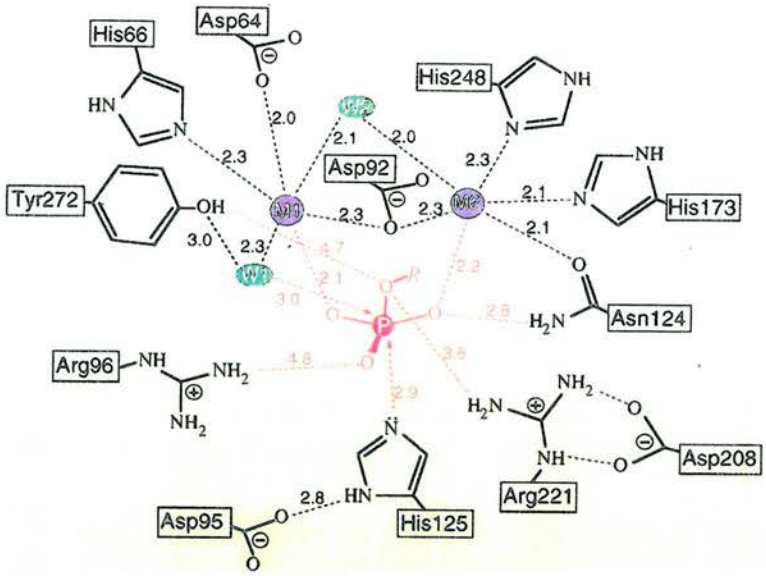


Figure 1.6 (e): *Diagram of the active site of PP1. Metal ions (M1 and M2) are coloured purple and water molecules (W1 and W2) are green. Water molecule W3 is not drawn: in its place, a possible binding mode for a modelled substrate phosphate group is shown in red.*

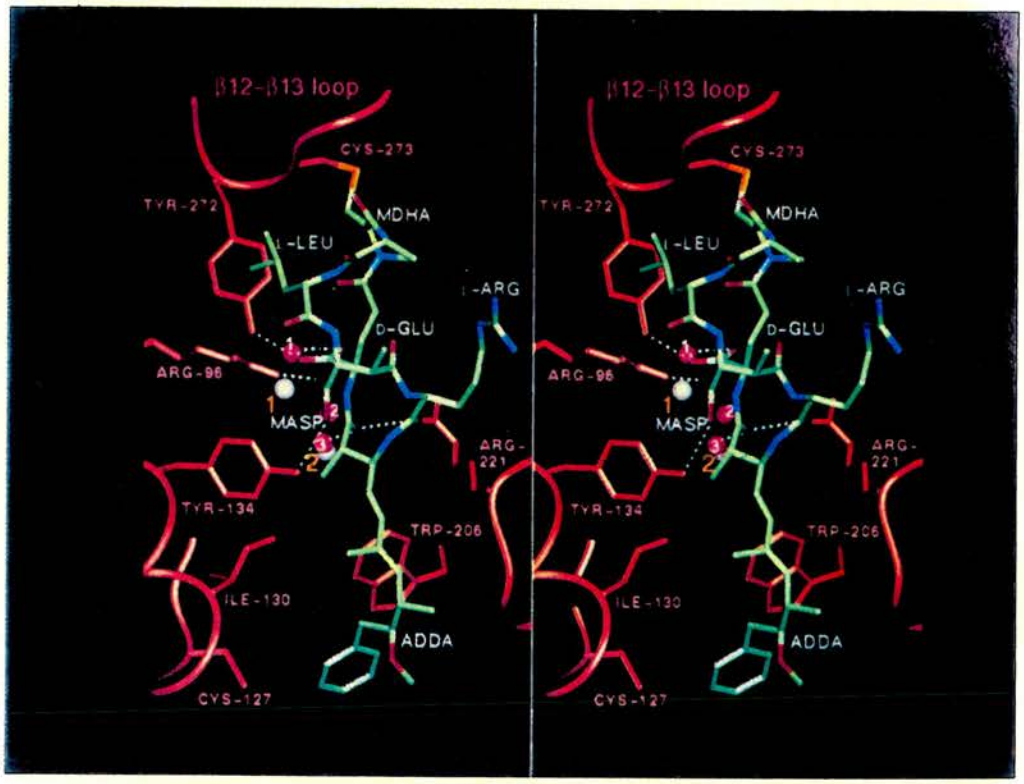


Figure 1.6 (f): *Stereo view of microcystin-LR at the active site of PP1. Atoms are colour-coded: carbon, green; nitrogen, blue and oxygen, red. Main chain and selected side chain protein atoms are in brown; metal ions M1 and M2 (white) and waters W1, W2 and W3 (red) are drawn as spheres. Both diagrams were reprinted with permission from Nature [54], Copyright (1995) Macmillan Magazines Limited.*

The interaction of I-1 and DARPP-32 with PP1 is preceded by the phosphorylation of a threonine residue in these inhibitors. The phosphate group of the phosphothreonine is thought to bind to the active site of PP1. The sequence surrounding this threonine in DARPP-32 (-RRRRP-pT-PAMLFR-, where pT is phosphothreonine) or I-1 (-RRRRP-pT-PATLVLT-) suggests that the acidic residues around the edge of the acidic groove (Asp210, Asp212, Asp220, Asp253, Glu252, Glu256, Glu275) might interact with the arginine residues preceding the phosphothreonine. Furthermore, the hydrophobic residues that follow the phosphothreonine could interact with side chains in the hydrophobic groove.

1.8.2 Crystal Structure of Calcineurin

The crystal structure of calcineurin (CaN), a heterodimer composed of an A subunit (CaNA) and a B subunit (CaNB) has now been determined at 2.1 Å resolution.[57] CaNA comprises of four distinct functional domains: a catalytic domain, a CaNB-binding domain, a calmodulin-binding domain and an autoinhibitory (AI) domain. CaNA contains a globular catalytic domain (residues 14-342) plus an extended, mostly α-helical region (residues 343-373) which forms the CaNB-binding domain (Fig. 1.7 a). CaNB, is composed of two lobes with two calcium ions bound by EF-hand motifs in each lobe. An 18-residue segment of the C-terminal region of CaNA (Ser469- Arg486) lies over the apparent substrate-binding cleft in the catalytic domain, comprising the bound portion of the AI domain (Fig. 1.7 a). This AI segment consists of two short α-helical regions plus five residues in an extended conformation. The residues that participate in binding interactions with the catalytic domain are the most highly conserved residues within the AI segment. Residues on the C-terminal portion of the AI segment (Glu481-Arg-Met-Pro484) make the most extensive contacts with the substrate-binding cleft. Glu481 hydrogen bonds to water molecules bound to the active site metal ions. Although the helical regions of the AI segment contact the catalytic domain, their conformations preclude extensive interactions with the substrate-binding cleft. The C-terminal lobe, containing the higher-affinity Ca^{2+} sites, is the most intimately associated with CaNA. This lobe may serve to anchor the

subunits and transduce the effects of Ca^{2+} binding at the lower-affinity (micromolar) sites on the N-terminal lobe to the catalytic domain.

Two metal ions, Fe^{2+} and Zn^{2+}, have been assigned to the active site. The dimetal active site is similar to the zinc-iron site in the structure of kidney bean purple acid phosphatase (KBPAP).[58] There are three metal-bound water molecules at the active site that could potentially represent the attacking nucleophile (Fig. 1.7 b). Two of these, a metal-bridging water molecule (W1) and a water molecule bound by the iron (W2), occupy positions similar to those of water molecules observed at the dimetal active site of PP1.[54] A third water molecule (W3) is bound by the iron and hydrogen bonded to His151 (Fig. 1.7 b). The ability of a water molecule to bind at this position in the PP1 structure appears to be sterically precluded when the inhibitor, microcystin, is complexed. The His151 side chain forms a hydrogen bond with the side chain of Asp121 (Fig. 1.7 b) and the imidazole side chain is a potential proton acceptor for W3. Modelling experiments have indicated that an activated water molecule in this position could be the attacking nucleophile. An Fe^{2+}-bound hydroxide ion has also been proposed to serve as the attacking nucleophile in KBPAP.[58]

The crystal structure of FKBP12-FK506-CaN complex has also been determined at 3.5 Å resolution.[57] Neither CaN nor FKBP12 underwent structural changes that were detectable at 3.5 Å resolution. Unlike the native CaN crystal structure, the AI segment in the complex is not seen at the active site nor elsewhere in the crystal structure. The FKBP12-FK506 complex contacts two distinct areas on CaN (Fig. 1.7 c). At the large site of interaction (primary recognition site), the composite surface formed by FK506 and surrounding residues of FKBP12 contacts residues on the exposed side of the CaNB-binding helix of CaNA and surrounding residues from both lobes of CaNB (Fig. 1.7 c). Additional interactions are seen between FKBP12 and the catalytic domain of CaNA near one side of the substrate binding cleft (secondary recognition site). FKBP12-FK506 exhibits non-competitive inhibition of CaN. This implies the formation of a catalytically inactive

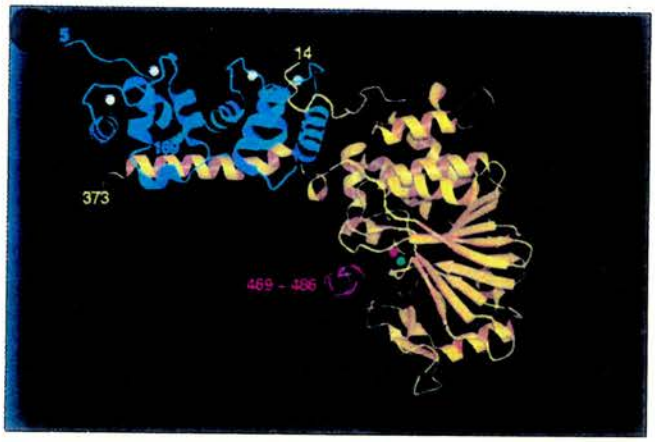


Figure 1.7 (a): *Ribbon representation of the human CaN heterodimer showing secondary structure elements. The CaNA subunit is shown in yellow, CaNB subunit in cyan and the AI segment in red. Fe (red) and Zn (green) ions are bound in a cleft in the catalytic domain. Four calcium ions (white) are bound to CaNB.*

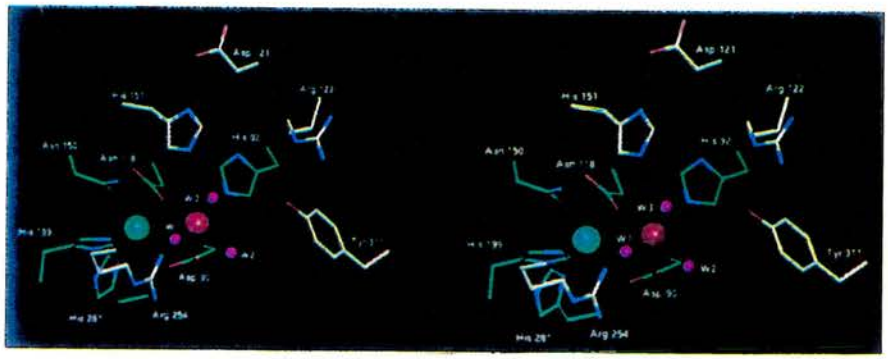


Figure 1.7 (b): *Stereo view of the CaN active site. Metal ligating side chains (C, green; N, blue and O, red) and conserved active site side chains (C, white; N, blue and O, red) are shown. The Zn (green) and Fe (red) are 3.14 Å apart. Both diagrams were reprinted with permission from Nature [57], Copyright (1995) Macmillan Magazines Limited.*

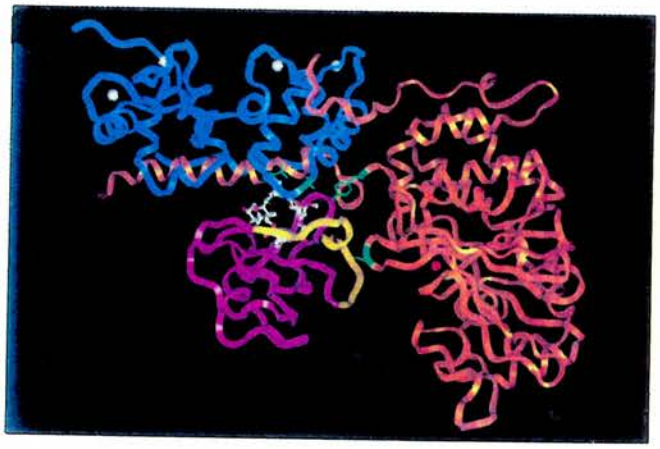


Figure 1.7 (c): *Ribbon representation of the FKBP12-FK506-CaN complex as viewed in Fig. 1.7 (a). CaNA is shown in orange and CaNB in blue. CaNA and CaNB residues implicated in cyclosporin A-cyclophilin binding are shown in green with side chains displayed. FKBP12 is shown in purple. FK506 is shown in ball-and-stick representation (C, white; N, blue and O, red). Fe (red) and Zn (green) in the CaNA active site are shown as spheres. Reprinted with permission from Nature [57], Copyright (1995) Macmillan Magazines Limited.*

enzyme-substrate-inhibitor complex as observed with CaN where the binding of FKBP12-FK506 does not directly obstruct the active site or substrate-binding cleft. Comparison of the structures of native CaN and the FKBP12-FK506-CaN complex indicate that large conformational changes are not responsible for inhibition by FKBP12-FK506 (Fig. 1.7 a, c). However, the authors postulate that contact by FKBP12-FK506, particularly at the secondary recognition site (residues 310-314 and 159 of CaNA), could produce subtle changes in active site geometry, not detectable at the resolution of the experiment, that result in inhibition.

1.9 Dual-Specificity Phosphatases

The protein tyrosine phosphatases (PTPs) consist of a collection of over 40 enzymes. They are often subcategorized into three groups: receptor-like PTPs, intracellular PTPs and dual specificity PTPs.[59] The dual specificity phosphatases constitute a special class of intracellular PTPs and are unique among the PTPs in their ability to utilise phosphoserine and phosphothreonine as substrates in addition to phosphotyrosine. Their substrate specificity is extremely limited and except for the presence of a similar phosphate binding loop, these enzymes appear to be structurally very different from the tyrosine-restricted phosphatases. Another interesting feature of the dual specificity PTPs is their preference for doubly phosphorylated substrates.[59]

The dual specificity PTPs share the greatest sequence similarity with the product of *vaccinia* virus open reading frame H1 (VH1).[60] The purified protein encoded by the VH1 open reading frame expressed in bacteria hydrolyses substrates containing phosphoserine and phosphotyrosine.[60] Without exception, all PTPs contain an active site signature motif, HCXXGXXR (where X is any amino acid), which harbours the catalytic cysteinyl residue involved in formation of a phosphoenzyme intermediate. Mutagenesis of the cysteine residue in VH1 abolishes catalytic activity towards both phosphoserine and phosphotyrosine substrates, suggesting that hydrolysis proceeds by a common

mechanism.[60] Other dual specificity PTPs include PAC1 (phosphatase of activated cells), a mitogen-induced nuclear PTP[61] and cdc25 isoforms.[62]

1.10 Protein Serine/ Threonine Phosphatase Inhibitors

1.10.1 Introduction

In 1878 George Francis published the first written report of animal poisoning by a cyanobacterium (blue-green alga).[63] However, a significant amount of information regarding the symptoms and pathology produced by these cyanobacteria was not available until the 1960's.[64] Although cyanobacteria are found in almost any environment ranging from hot springs to Antarctic soils, known toxic members are mostly planktonic. Published accounts of field poisonings are the result of consumption of water soluble and temperature stable toxins. These toxins are either released by the cyanobacterial cells or loosely bound so that changes in cell permeability or age allow their release into the environment. Lethal and sub-lethal doses of these toxins become available to the animals during periods of heavy cell growth termed "waterblooms". The increasing eutrophication of water supplies from urban and agricultural sources which raises mineral nutrient levels, has increased the occurence and intensity of these annual blooms, posing an ever-increasing risk to livestock, bird, fish and adverse effects on human health.[65]

The cyanobacteria are known to produce two chemical groups of potent biotoxins, neurotoxic alkaloids belonging to the species *Anabaena* (anatoxins) and *Aphanizomenon* (aphantoxins), and a family of related cyclic peptides.[66,67] These cyclic peptides belong to a class of structurally diverse compounds represented by okadaic acid, calyculin A, tautomycin and microcystin-LR.

The Okadaic Acid Class of Compounds

1.10.2 Okadaic Acid

Okadaic acid (**1**) (Fig. 1.8) is a polyether derivative of a C_{38} fatty acid, isolated from two sponges, *Halichondria okadai*, a black sponge, commonly found along the Pacific coast of Japan, and *H. melanodocia*, a Carribean sponge collected in the Florida Keys.[68] Okadaic acid and 35-methylokadaic acid [dinophysistoxin-1, DTX-1, (**5**), Fig. 1.8] were first isolated as causative agents of diarrhetic shellfish poisoning (DSP) in Japan.[69] They were shown to be produced by several types of dinoflagellates (marine planktons) and to accumulate in the midgut glands of bivalves feeding on the dinoflagellates *Dinophysis fortii* and *D. ocuminata*. Similar cases of DSP were then reported in countries such as Mexico, Spain, The Netherlands, Scandinavia, France and South America. The high morbidity rate and worldwide distribution of DSP posed a serious threat to shellfish industries and to public health. Various other derivatives of okadaic acid were then either isolated from *H. okadai* as minor components or semi-synthesised from okadaic acid. Acanthifolicin (**6**) (Fig. 1.8), structurally an episulfide derivative of okadaic acid was isolated from the marine sponge *Pandoras acanthifolium* .[70] The total synthesis of okadaic acid has also been reported.[71]

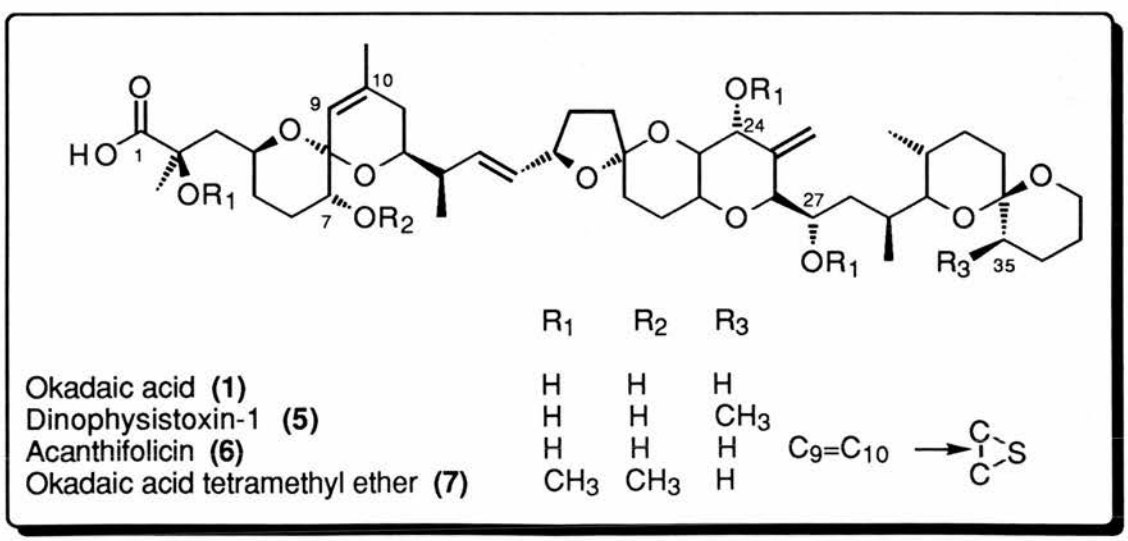


Figure 1.8: *Okadaic acid and derivatives.*

Tumour promotion is an important topic in chemical carcinogenesis. Tumour promoters are divided into two general groups of TPA-type and non-TPA-type, (TPA, 12-O-tetra-decanoyl-phorbol-13-acetate). The TPA-type are defined as those promoters that bind to phorbol ester receptors in cell membranes, activating the calcium-activated, phospholipid-dependent protein kinase C (PKC) *in vitro* .[72] These include TPA (**8**), teleocidin A (**9i**) and B (**9ii**) and aplysiatoxin (**10**) (Fig. 1.9). Although the mechanism of action of the TPA-type tumour promoters was well investigated, its pathway was not generally accepted as the pathway of tumour promotion in various organs.[73]

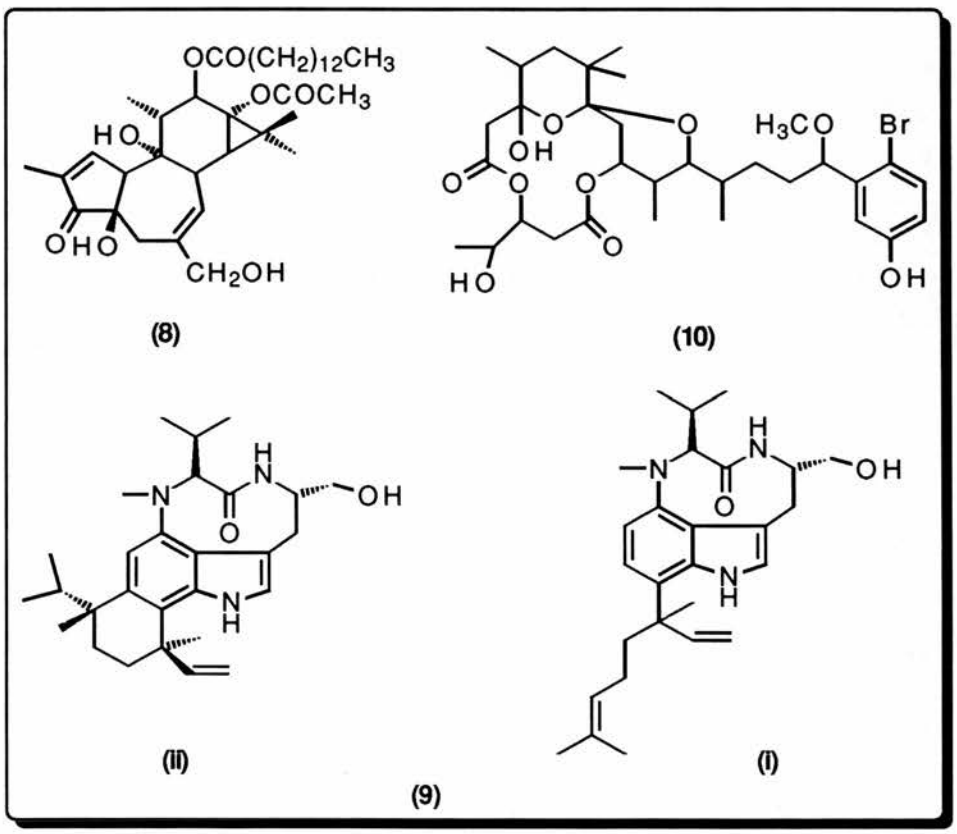


Figure 1.9: *TPA-type tumour promoters.*

Okadaic acid was shown to induce tumour promotion in two-stage carcinogenesis experiments, on mouse skin initiated with 7,12-dimethylbenz[a]anthracene (DMBA, **11**)[74] as well as in rat glandular stomach initiated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, **12**) (Fig. 1.10).[73,75]

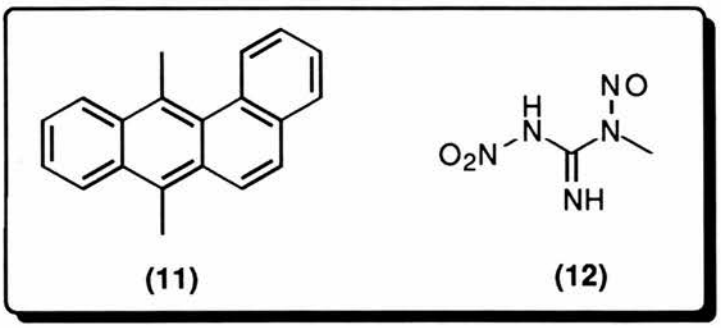


Figure 1.10: *Tumour initiators.*

Investigation of okadaic acid and its derivatives as possible tumour promoters unravelled a new mode of tumour promotion distinct from the TPA-type.[74] These compounds were found to bind to specific receptors in the particulate and cytosolic fractions of mouse skin and various other organs and inhibit the activity within the fractions. These receptors were later identified as protein phosphatases 1 and 2A.[76] Okadaic acid but not okadaic acid tetramethyl ether (**7**) (Fig. 1.8), an inactive compound, inhibited the specific [^{3}H] okadaic acid binding to the cytosolic and particulate fractions of mouse skin and other mouse tissues.[77,78] However, the total amount of specific binding to the receptors was greater in the cytosolic fractions than the particulate fractions. Okadaic acid was found to be 50-100 times more effective as an inhibitor for PP2A than for PP1.

Other compounds which inhibit PP1 and 2A[79-86] in a similar manner to okadaic acid belong to four structurally diverse group of compounds, represented by okadaic acid, calyculin, microcystin and tautomycin. Since okadaic acid was the first tumour promoter of this class, they are referred to as the okadaic acid class of compounds. Their mechanism of inhibition, now recognised as the general mechanism of tumour promotion in various organs,[87] is called the okadaic acid pathway of tumour promotion. Unlike okadaic acid, other compounds of this class are equally effective against PP1 and 2A. The order of potency for inhibition of the catalytic subunit of PP1 and 2A is microcystin > calyculin A > tautomycin, and the IC_{50} values for the compounds range from 0.1 to 0.7 nM.[88] The okadaic acid class of compounds are much less potent inhibitors of PP2B and have no

effect on PP2C, alkaline phosphatases, acid phosphatases and inositol 1,4,5-trisphosphate phosphatase.[3,81,89,90] It is important to note that none of the okadaic acid class of compounds inhibits protein tyrosine phosphatase 1 (PTP1) at concentrations up to 10 μM. Moreover, these compounds exhibit selective inhibition of the Ser/ Thr protein phosphatase family (Table 1.1).[88]

Compounds	PP1	PP2A	PTP
Okadaic acid	3.4	0.07	>10000
Calyculin A	0.3	0.13	>10000
Microcystin-LR	0.1	0.1	>10000
Tautomycin	0.7	0.65	>10000
Na vanadate	1900	>1000	0.9

Table 1.1: *Inhibition of the Ser/ Thr protein phosphatases by the okadaic class of compounds. Entries are IC_{50} values in nM.*

1.10.3 Calyculins

Calyculin A **(13a)** (Fig. 1.11) was isolated from a marine sponge, *Discodermia calyx*, and found to be a strong toxic compound against L1210 leukaemia cells.[91] Its structure contains an octamethyl-polyhydroxylated C_{28} fatty acid that is linked to two γ-amino acids and esterified by phosphoric acid. Seven calyculins, B to H **(13b-13h)** (Fig. 1.11), were later isolated from the same sponge, their structures[92,93] and their absolute stereochemistries were elucidated.[94] The synthesis of the spiroketal subunit of calyculin A [95] and also its total syntheses have been reported.[96,97]

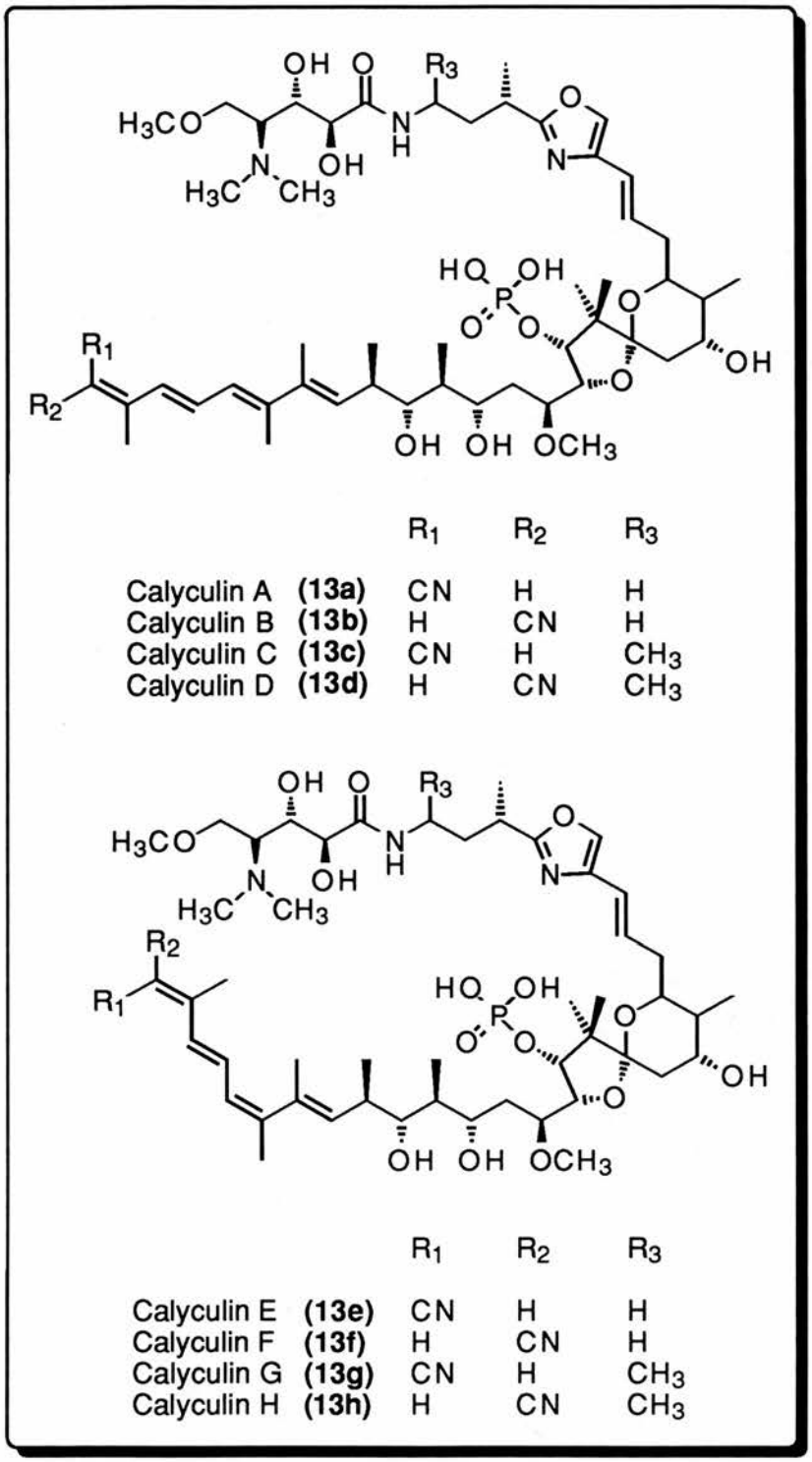


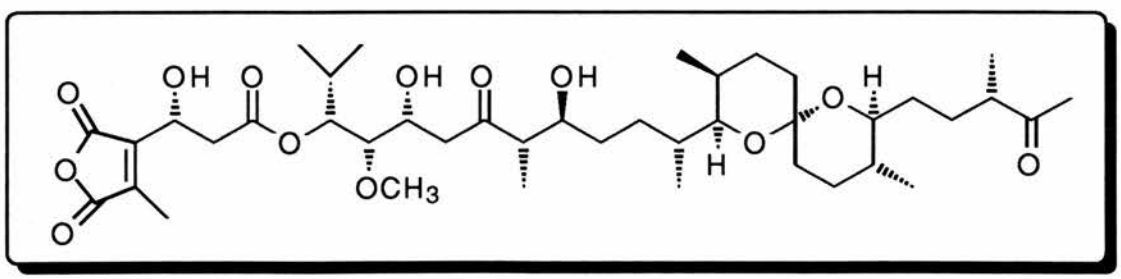
Figure 1.11: *The Calyculins.*

Calyculin A was also found to be a potent inhibitor of PP1 and 2A, but with a profile distinct from that of okadaic acid. Indeed, calyculin A was found equally effective against PP1 and PP2A.[84,88,98] The calyculins A to H were found to possess similar potencies

towards PP1 (IC_{50} values of 0.6 to 0.75 nM) and PP2A (IC_{50} values of 2.6 to 14.0 nM) in the cytosolic fraction of mouse brain. It was shown that calyculin A inhibited the specific [^{3}H] okadaic acid binding to particulate and cytosolic fractions of mouse skin in a dose-dependent manner. Compared with okadaic acid, calyculin A, showed a 10 times stronger binding affinity to the particulate fraction but the same affinity to the cytosolic fraction as okadaic acid.[84] The binding affinities to the receptors in the cytosolic fractions were well correlated with tumour-promoting activities in two-stage carcinogenesis experiments in mouse skin and other tissues. However, this was not the case with binding affinities to the particulate fractions.[84] The tumour promoting acitvity of calyculin A was found to be as strong as okadaic acid. It was concluded that if the binding affinity to the particulate fraction reflected the binding to PP1 and that to the cytosolic fraction reflected binding to PP2A, based on the relative potencies of okadaic acid and calyculin A against PP1 and PP2A, the inhibition of PP2A in the cytosolic fraction rather than that of PP1 in the particulate fraction seemed to be an essential biochemical reaction for tumour promotion.[73,84]

1.10.4 Tautomycin

In 1987, Isono *et al.* isolated an antifungal antibiotic, tautomycin (**14**), from the culture of *Streptomyces spiroverticillatus*[99] and later identified its structure.[100]



Tautomycin (**14**).

Besides antifungal activity, it was found that tautomycin induced a morphological change (bleb formation) in human leukaemia cells K562 which correlated with protein

phosphorylation.[101] A partial and total synthesis of tautomycin[102-105] and the determination of its absolute configuration have been reported.[106]

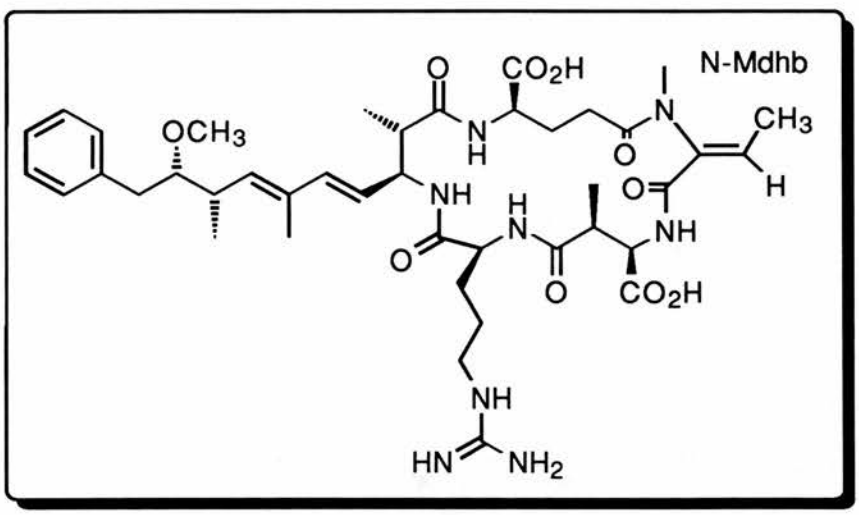
Tautomycin **(14)** was classified as a member of the okadaic acid class. The compound inhibits the activity of the catalytic subunits of PP1 and 2A with IC_{50} values of 0.7 and 0.65 nM respectively.[83,88,107] When used in two-stage carcinogenesis experiments on mouse skin, it failed to exhibit tumour-producing activity. Since inhibition of PP1/ 2A and tumour promoting activity were well correlated, absence of tumour promotion was attributed to the chemical nature of tautomycin, *i.e.* polarity of its structure, and its probable difficulty in penetrating the basal cell layer of mouse skin. Recently, Sugiyama *et al.* established that tautomycin exists in two forms, the acid anhydride and the diacid, and the diacid was shown to be the real active form of the compound.[108]

1.10.5 Microcystins and Nodularins

Toxin-producing genera of fresh and brackish water cyanobacteria are now known to include species of filamentous *Nodularia*, *Nostoc* and *Oscillatoria,* and the most studied genus, the coccoid *Microcystis*. The toxins that have been isolated, 53 known so far,[109-127] belong to a family of closely related cyclic hepatotoxic heptaisopeptides (termed microcystins) and pentaisopeptides (termed nodularins). These compounds have molecular weights in the range of 824 to 1044 Da. The most well-known and studied toxins, microcystin-LR **(2)** (Fig. 1.3) and nodularin **(15)**, were first isolated from *Microcystis aeruginosa* and *Nodularia spumigena* respectively.[128,129] The general structure of microcystins is cyclo(-*R*-Ala^1-*S*-X^2-*R*-MeAsp^3-*S*-Y^4-ADDA^5-*R*-isoGlu^6-Mdha^7), where X and Y are variable (*S*)-amino acids, *R*-MeAsp is *R-erythro*-β-methyl-aspartic acid and Mdha is N-methyldehydroalanine.[130] The two variable (*S*)-amino acids form the basis of the nomenclature for the microcystins. Demethylation can occur on amino acid number 3 and/ or 7[66,109-116,119,121,122]; X= leucine, arginine, tyrosine and Y= arginine, alanine and methionine. A (*R*)-Ser containing variant (in place of the (*R*)-Ala residue), originally

thought to be an invariable amino acid of the microcystins, has been isolated from *Nostoc* sp. 152.[119]

Nodularin **(15)** lacks the variable L-amino acid X and the (*R*)-Ala residue, while the N-methyldehydroalanine of the microcystins is replaced by a methyl analogue, N-methyldehydroaminobutyric acid (Mdhb).



Nodularin **(15)**.

Motuporin, [(*S*)-Val[2]]-nodularin, was isolated from a marine sponge, *Theonella swinhoei* Gray, found in Papua New Guinea.[131] The enantiospecific total synthesis of motuporin has been the subject of a recent publication.[132] Like other members of the okadaic acid class of compounds, motuporin inhibits the protein phosphatases. As well as its tumour promoting activity, motuporin was found to exhibit considerable cytotoxicity *in vitro* against various cancer cell lines.[131] The cytotoxicity of motuporin was thought to be a function of the non-polar amino acid valine replacing the polar arginine in nodularin. To test this hypothesis, a dimethylpyrimidinyl (DMP) analogue of nodularin was semi-synthesised; the DMP group being far less polar than arginine. This analogue was also found to be cytotoxic to L1210 leukaemia cell line whereas nodularin is not (personal communication of B. W. Choi to authors of *J. Applied Phycology*, 1994, **6**, 159-176.). Other nodularins have also been reported; two, [DMAdda[3]]-nodularin and [(6Z)-Adda[3]]-

nodularin were obtained from a bloom sample from Lake Ellesmere, New Zealand and one, [D-Asp[1]]-nodularin was isolated from cultured cells of *N. spumigena* .[133]

(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-(4E, 6E)-dienoic acid, Adda, an unusual C_{20} β amino acid, is a remarkable common structural feature of these toxins.[128,134] Adda seems to be important for hepatotoxicity, since ozonolysis or hydrogenation of the diene system yields inactive compounds.[135,136] Geometrical isomers at C-6 of the Adda residue are also inactive (Fig. 1.12).[135,136] In contrast, borohydride reduction of the dehydroamino acid unit or the conjugate addition of glutathione to this unit, yields derivatives that are nearly as toxic as the parent compound.[137,138] The absolute configuration and the total synthesis of Adda have been reported, and Adda itself shown not to be toxic.[128,139-144] Two microcystins with modified Adda units that retain toxicity have been reported.[114,118,119]

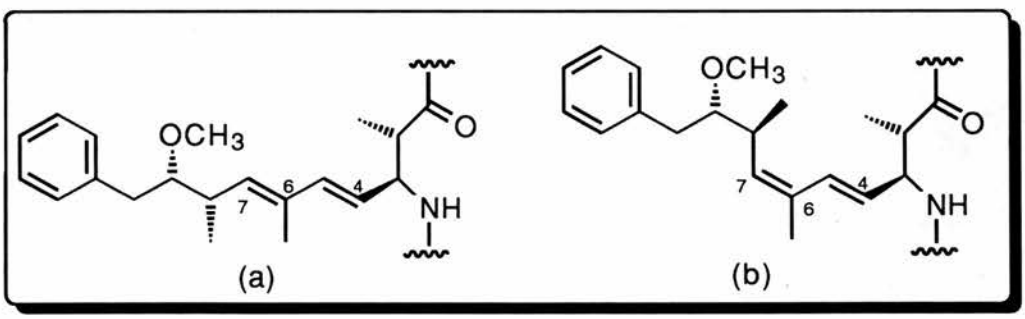


Figure 1.12: *Geometrical isomers of Adda: (a) (4E, 6E)-Adda and (b) (4E, 6Z)-Adda.*

Early reports of the pathology of the microcystins in laboratory and domestic animals,[64] describe abnormalities in the blood supply to the peripheral tissues with an accumulation of blood in the liver. Later it was established that these toxins have a unique organotrophy in the liver, with acute lethal intraperitoneal and oral administrations of microcystin-LR to mice or rats causing massive intrahepatic hemorrhages and cellular necrosis within a few hours.[145,146] On an enzymic basis, the microcystins and nodularins have been established as inhibiting protein phosphatases 1 and 2A and this inhibition is presumably the mechanism by which they exert their hepatotoxicity.[81,83,85,147,148] The well-known

hepatotoxic compounds α-amanitin and phalloidin do not show any inhibitory effects on the protein phosphatases similar to the microcystins and nodularin, lending support for the notion that a different mode of action operates for the cyclic peptides.

1.10.5.1 Related Linear Peptides

A linear peptide, Adda-(*R*)-isoGlu-Mdhb-(*R*)-MeAsp-(*S*)-Arg-OH **(16)** (Fig. 1.13), was isolated from cultured cells of *Nodularia spumigena.* The ratios of this linear peptide to its cyclic counterpart, nodularin, were determined from cultivation studies and the linear peptide was found to be the biogenetic precursor of the cyclic compound.[149] Similarly, three linear peptides were isolated from a water bloom of *Microcystis sp.* collected from Homer Lake, Illinois. These were (*S*)-Leu-(*R*)-MeAsp-(*S*)-Arg-Adda-(*R*)-Glu-Mdha-(*R*)-Ala-OH, (*S*)-Phe-(*R*)-MeAsp-(*S*)-Arg-Adda-(*R*)-Glu-Mdha-(*R*)-Ala-OH and Adda-(*R*)-isoGlu-Mdha-(*R*)-Ala-(*S*)-Leu-(*R*)-MeAsp-(*S*)-Arg-OH **(17)** (Fig. 1.13). The latter linear peptide, **(17)**, is thought to be the biogenetic precursor of microcystin-LR while the other two linear peptides are considered degradation products of the hepatotoxin.[149]

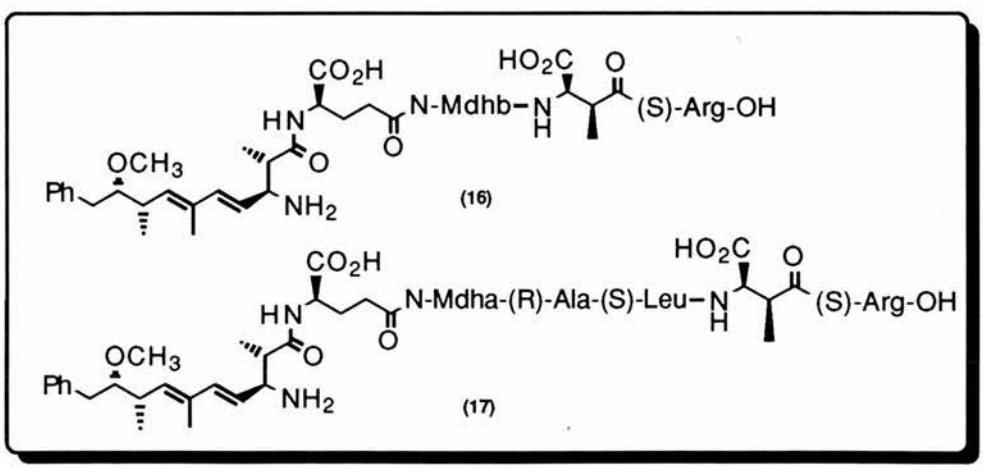


Figure 1.13: *Linear biogenetic precursors.*

The linear peptides did not show apparent toxicity to mice (i.p.) at 1.0 and 1.1 mg/ kg, for the pentapeptide and heptapeptide respectively,[149] which confirms the cyclic structure of the nodularins and microcystins to be essential for toxicity.

1.10.5.2 Biosynthesis of Microcystins and Nodularins

Origins of Adda and MeAsp Units

The presence of the two unusual amino acids, Adda and (2*R*,3*S*)-3-methylaspartic acid (MeAsp), in the structures of microcystin and nodularin, makes these cyclic peptides of particular biosynthetic interest. Moore *et al.*[150] studied the biosynthesis of these units using feeding experiments with isotopically labelled precursors. From these experiments, they concluded that the Adda unit was a mixed polyketide made of phenylalanine (as phenylacetate), and the methyls on C-2, C-6 and C-8 were methionine-derived.[150] The C-3 to C-8 segment of Adda appeared to be derived from acetate (Fig. 1.14).

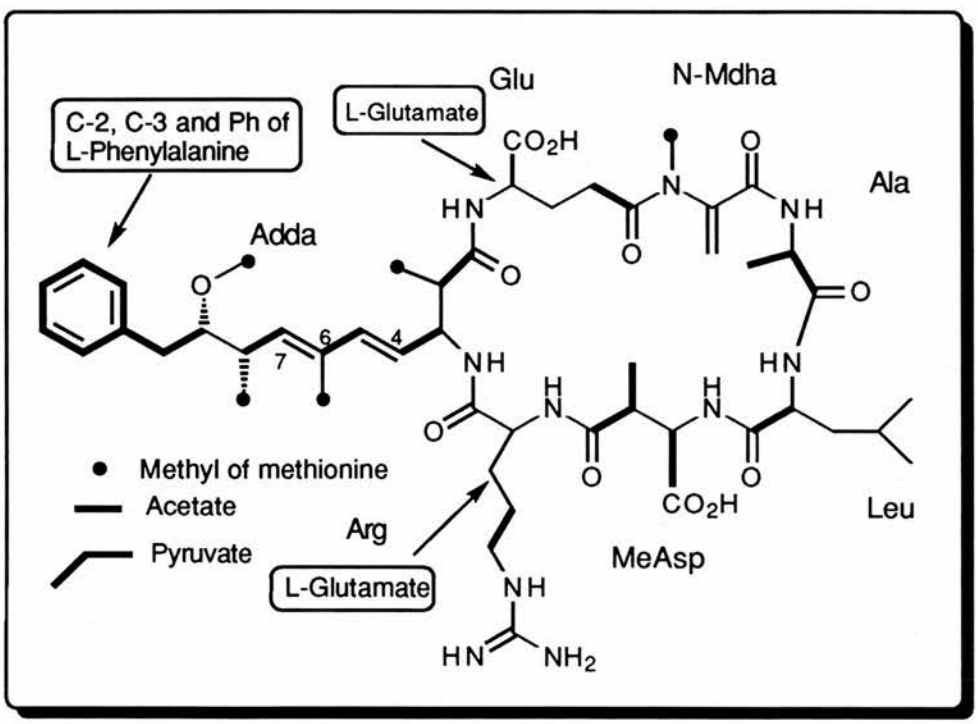
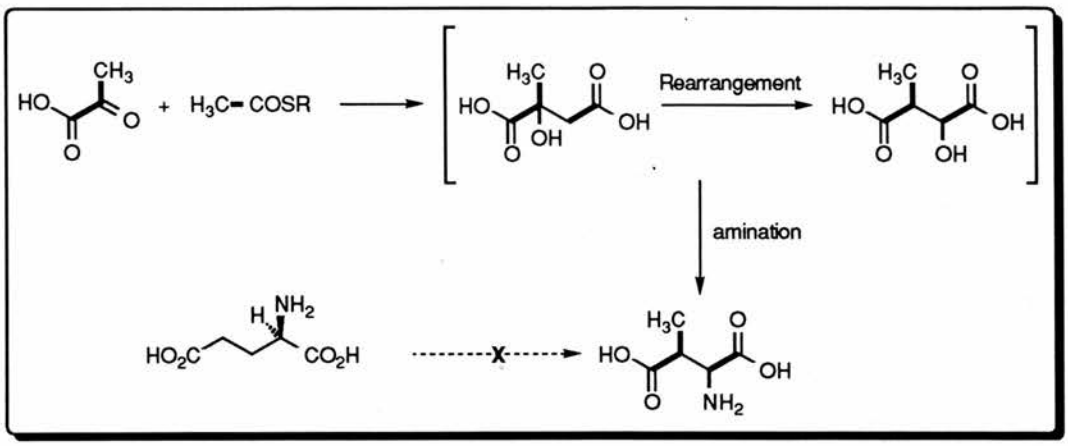


Figure 1.14: *Origin of carbons in microcystin-LR.*

Moore *et al.*[150] concluded that the MeAsp unit was not derived from the known pathway, *i.e.* the rearrangement of glutamic acid, as is the case in anaerobic bacteria.[151] This residue appeared to be formed by the condensation of pyruvate and acetate followed by rearrangement and amination of 2-hydroxy-2-methylsuccinic acid (Scheme 1.3).



Scheme 1.3: *Proposed biosynthetic pathway for the MeAsp residue.*

Biosynthetic studies on nodularin using ^{13}C-labelled precursors in feeding experiments gave similar results. The origins of the carbons were as for that in microcystin-LR and the incorporation of a ^{14}C-labelled threonine residue was an indication of the expected route to dehydrobutyrine.[152]

1.10.5.3 Microcystin Binding to PP1 and 2A

It has been proposed that the okadaic acid class of compounds interact with a common binding site on protein serine/ threonine phosphatases. It has also been shown that amino acid substitutions at the C-termini of PP1 and PP2A decrease the sensitivity of these enzymes to the toxins of the okadaic acid class.[153] The sequence SAPNYC, including Cys273, is conserved in virtually all known members of the PP1/ 2A family. Incubation of [^{125}I]microcystin with PP1 results in the formation of an adduct that is stable to 1% SDS or 70% formic acid treatment.[154] This is indicative of a covalent interaction. Site-directed mutagenesis of Cys273 residue in the C-terminus of PP1 to Ala, Ser or Leu abolished covalent binding to microcystin and increased the IC_{50} values by 5-20 fold.[154] The same result was obtained from the reduction of the Mdha residue of microcystin with ethanethiol. It is therefore suggested that Cys273 of PP1 binds covalently to the Mdha residue of microcystin *via* a Michael-type addition. Although, the Mdha residue is not necessary for inhibition of phosphatase activity, it is likely that following the initial recognition and

inhibitory interaction of the toxin with the protein, the Mdha group is free to react with Cys273 in PP1.[154] Similar experiments using [^{125}I]microcystin have confirmed these findings and identified the position for covalent binding in PP2A to be Cys266.[155]

1.10.5.4 Structure-Activity Relationships

Structure-activity relationship studies, based upon PP1 and 2A inhibition, have shown that okadaic acid (**1**), DTX-1 (**5**) and acanthifolicin (**6**) are equally effective as inhibitors.[76] Therefore, the presence of one extra carbon on the G-ring of okadaic acid, as in the case of DTX-1, or replacement of the olefin in the B ring with an episulfide, as in the case of acanthifolicin, does not affect activity. Semi-synthetic derivatives including okadaic acid methyl ester, acanthifolicin methyl ester, okadaic acid tetramethyl ether and okadaic acid methyl ester tetramethyl ether were all found to be totally inactive. These results indicate that the ionic nature of the carboxylate group as well as the four hydroxyl groups at C-2, C-7, C-24 and C-27 of okadaic acid are important for activity.[76]

Studies on the microcystins and nodularins have shown that the Adda unit is essential for activity.[76] The less common (6Z)-Adda microcystins are 100 fold weaker as inhibitors than the parent (6E)-Adda microcystins.[156] However, the methoxyl group at the C-9 position of Adda does not seem to affect activity as microcystins with an acetoxyl group at this position retain hepatotoxicity.[114,115] The free carboxylate group of the invariant (*R*)-Glu is also essential for activity since the monopropyl ester derivative of microcystin-LR has been shown to be inactive.[115]

1.10.5.5 Molecular Modelling and NMR Spectroscopy

Given that the three structurally different compounds, okadaic acid, microcystin-LR and calyculin A all bind to the same site of the protein phosphatases *i.e.* they all interact at the same modulatory site of the protein phosphatases,[73,78,81,85] they might possess a common

pharmacophore.[157] This so called common pharmacophore was the subject of a number of molecullar modelling studies.

Lanaras *et al.* suggested a computer model of the 3-dimensional structures of microcystin-LR and nodularin, as having planar and rectangular shaped peptide rings and of optimized Adda forming a U-shape. In this model, when the Adda moieties of the two hepatotoxins were overlaid, the peptide rings of microcystin-LR and nodularin formed an angle of 90° to each other.[158] No intramolecular hydrogen bonding was found in these hepatotoxins. Since complementarity between ligand and receptor is necessary for binding, ligands binding a common receptor must have common features. The specific interaction of microcystin-LR and nodularin with a common binding site on the protein phosphatases put forward the argument that the models proposed by Lanaras *et al.* did not represent the conformations of the molecules which bind to the same receptor. Also lack of hydrogen bonding contribution to production of stable conformations in compounds that have a number of hydrogen bonding acceptors and donors did not seem reasonable. Alternative computer generated minimised 3-dimensional structures of microcystin-LR and nodularin, showed the same orientation of Adda with respect to the planar peptide rings and the carboxylate groups.[159] In this computer model, the arginine residues of microcystin-LR and nodularin showed a common spatial alignment. However, this residue does not seem to be necessary for activity as comparison of the enzyme inhibition and receptor binding of microcystin-LR and the naturally occuring microcystin-LA showed similar specific activity.[160] Another compelling piece of evidence for this, is the isolation of motuporin,[131] where the arginine residue of nodularin is replaced by valine without loss of activity. Quinn *et al.* then extended this computer model to include okadaic acid and calyculin A as well as microcystin-LR (Fig. 1.15).[157] In this study, okadaic acid was found to assume a cyclic conformation forming a cavity held together by intramolecular hydrogen bonding. The crystal structure of calyculin A also showed hydrogen bonding within the molecule forming a cavity similar to that formed in okadaic acid.[91] Therefore, the computer generated minimum energy conformations of these compounds indicated a common

pharmacophore consisting of a central core containing one conserved acidic group, two potential hydrogen bonding sites and a non-polar side chain. This model seemed consistent with the structure-activity relationships available.

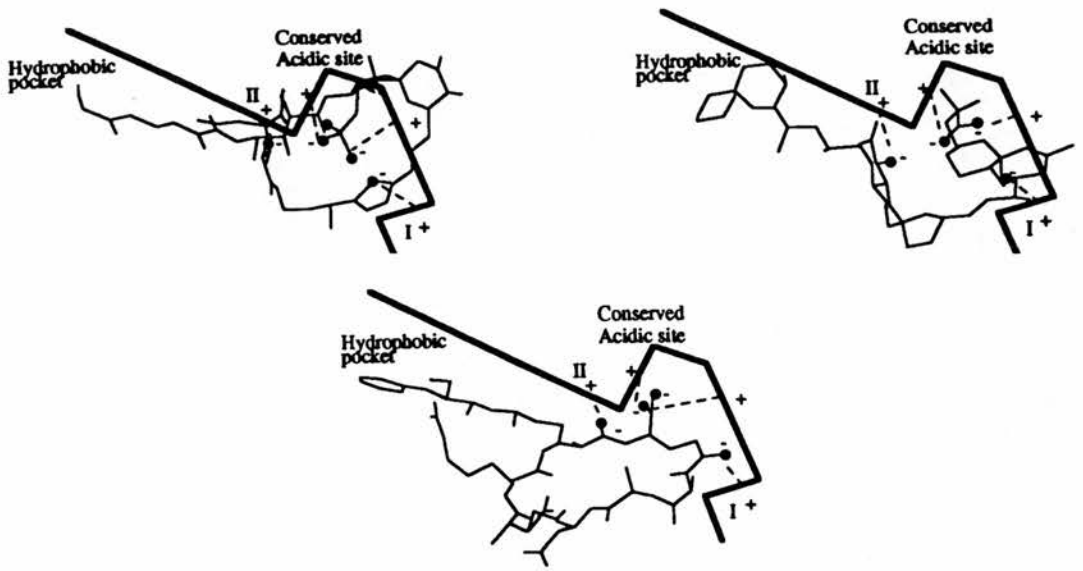


Figure 1.15: *Schematic representation of the common pharmacophore of calyculin A, okadaic acid and microcystin-LR. Areas I and II represent the potential hydrogen bonding sites.*

Rudolph-Bohner *et al.* compared the 3-dimensional structures of two microcystins, microcystin-LR and -LY using 2D NMR and distance geometry calculations.[161] For microcystin-LY, a single family of highly convergent structures was obtained. These were characterised by a compact boat-like shape, with the large side chain of the Adda residue protruding from the concave side, in close proximity of the tyrosine residue (Fig. 1.16). This restricted access of the Adda residue, resulting from the close proximity of the aromatic tyrosine residue is thought to account for the lower toxicity of microcystin-LY. The additional enthalpic energy required to disturb the hydrophobic collapse, would lower binding affinities to the receptor molecules.

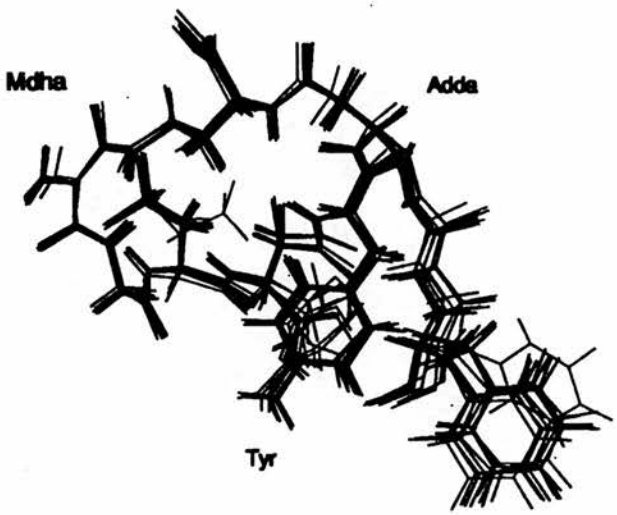


Figure 1.16: *Superimposition of 10 microcystin-LY structures with the smallest distance violations.*

In microcystin-LR, the Adda side chain was found protruding above the main plane, distant from the sequentially adjacent arginine residue, probably due to repulsion between the guanidino function of arginine and the hydrophobic Adda. The resulting family of structures was an even more compact ring structure (Fig. 1.17). From these structures and homology of other microcystins, Rudolph-Bohner *et al.* proposed a hypothesis involving the Adda side chain interacting with a hydrophobic pore of the phosphatases. The rest of the microcystin acts as a scaffold to help stabilise the interdigitation of the Adda with additional intermolecular interactions.

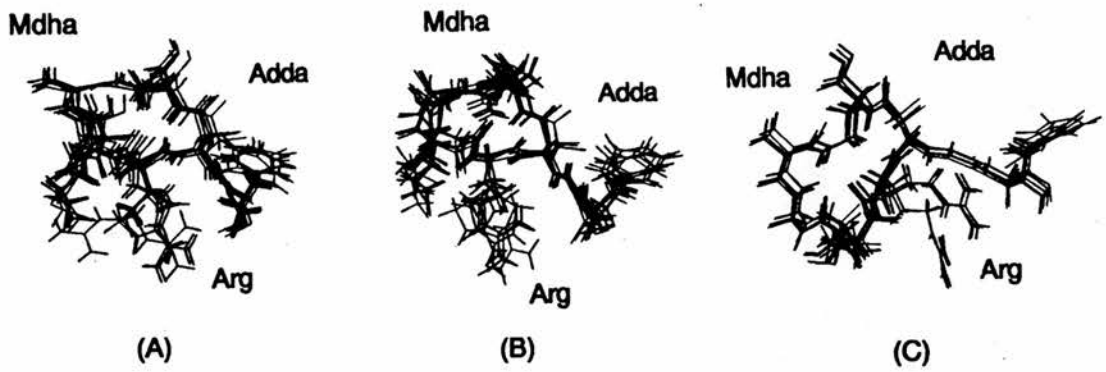


Figure 1.17: *Superimposition of the most convergent microcystin-LR structures subdivided in three conformational families A, B and C.*

More recently, Mierke *et al.* have extended this working hypothesis to include nodularin (Fig. 1.18) and okadaic acid (Fig. 1.19).[162] Nodularin was found to display a structure similar to microcystin-LR. The (*R*)-Ala-Leu dipeptide in microcystin-LR, not shared by nodularin, is expelled out of the plane of the ring like a conformational exon and this allows maximum overlapping of the rest of the molecule with nodularin.

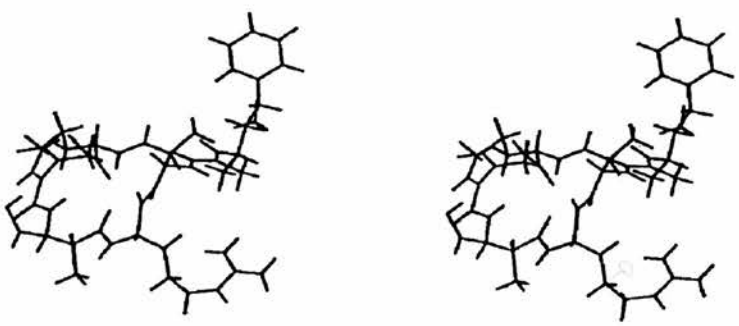


Figure 1.18: *Stereo view of the lowest energy conformation of nodularin.*

Energy minimisation studies on okadaic acid were found to adopt a chain reversal in the C-terminal portion of the molecule compared with the studies of Quinn *et al.* The data obtained from this study, envisage an enzyme-inhibitor complex involving the insertion of the hydrophobic tail of the compounds into a protein cavity of defined dimensions and the carboxyl function involved in decisive hydrogen bondings or electrostatic interactions.

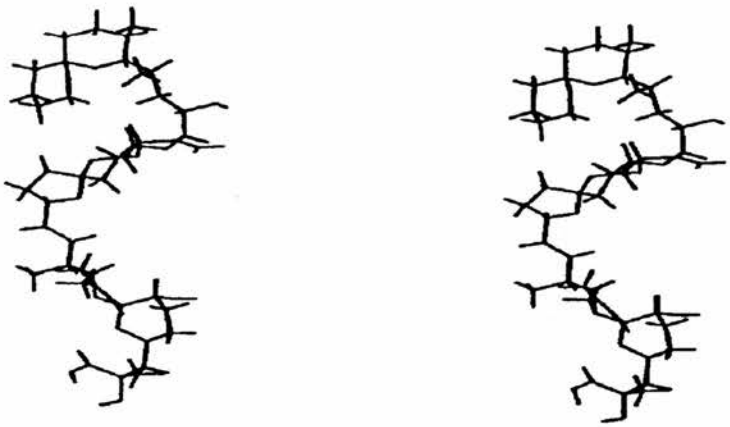


Figure 1.19: *Stereo view of the lowest energy conformation of okadaic acid.*

1.10.6 Reason for Toxin Production

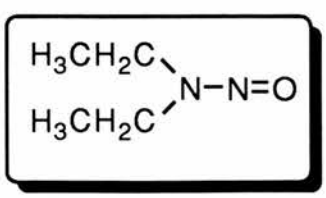
Since, livestock have never been the primary predators of the cyanobacteria, the reason for production of toxins that are capable of killing livestock and large animals remains intriguing. It has been reported that cyanobacterial neurotoxins and hepatotoxins can also be very harmful to minute zooplanktons that feed on the cyanobacteria.[163] The toxins produced may therefore be directly lethal (especially the neurotoxins), or they may reduce the number and size of offspring produced by the zooplanktons. Toxin production may therefore have a protective effect. Another possibility is that the toxins might once have had some critical function that they have since lost or that is still to be discovered.

In fact, the isolation of a type-1 protein phosphatase from the unicellular marine dinoflagellate, *Prorocentrum lima*,[164] an established producer of okadaic acid, is evidence for the latter speculation. The biochemical profile of the PP1 isolated from *P. lima* was identical to the catalytic subunit of PP1 from rabbit skeletal muscle. *P. lima* PP1 (apparent molecular mass 37.5 kDa) was highly sensitive to inhibition by mammalian I-1 and I-2, and to nanomolar concentrations of okadaic acid itself. *P. lima* PP1 was also sensitive to inhibition by microcystin-LR, although the concentrations employed (1.7 nM) suggest that this enzyme is not as potently affected as the mammalian form of the enzyme. No type-2A protein phosphatase activity was detected in *P. lima*. Production of okadaic acid by

different *P. lima* isolates was found to vary significantly, depending on the sea water used as a nutrient source. A 6-7 fold increase in okadaic acid production by *P. lima* , when grown under controlled conditions, correlated with an up to 300 fold increase in PP1 activity.[164] If *P. lima* PP1 represents an intracellular target for okadaic acid, an obvious potential role for okadaic acid in inhibiting PP1 *in vivo* might be to regulate behavioural and physiological responses in this flagellate which are controlled by reversible phosphorylation. The regulation of PP1 by okadaic acid in this way could be an example of a primitive signalling pathway involving reversible phosphorylation in this early evolved marine eukaryote.

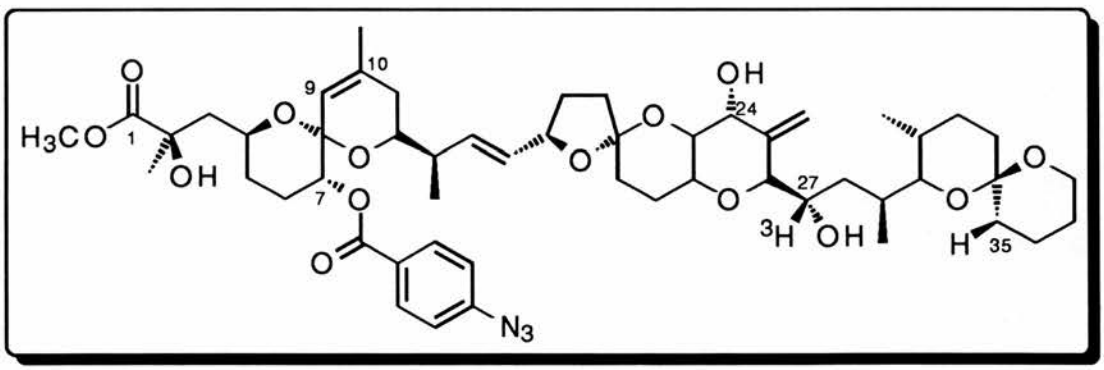
1.10.7 The Okadaic Acid Pathway of Tumour Promotion

Tumour promoters are agents that induce tumour development from initiated cells. This involves two-stage carcinogenesis experiments in cells initiated with either DMBA (**11**), MNNG (**12**), or in the case of microcystin-LR, diethylnitrosamine [DEN, (**18**)].[165] Experiments of this kind with the okadaic acid class of compounds have established a mechanism, now generally accepted as the pathway of tumour promotion in various organs.[73] This pathway is also thought to be the biochemical pathway followed in the process of tumour promotion in human cancer development.[166] It is of interest to note that the toxins of the okadaic acid class do not seem to initiate a cell's progression towards becoming cancerous; however, once some other agent has triggered early changes, the toxins then promote the development of further carcinogenic alterations.



Diethylnitrosamine (**18**).

The tumour promoting activities of the okadaic acid class of compounds are comparable to that of the TPA-type compounds which bind to specific receptors, the phorbol ester receptors. It was therefore assumed that the okadaic acid class might also interact with specific receptors within cells. These receptors were later identified to be the protein phosphatases 1 and 2A.[76] How okadaic acid might interact with the protein receptors PP1 and 2A was the subject of a series of experiments using an okadaic acid photoaffinity probe, methyl-7-O-(4-azidobenzoyl)-[27-^{3}H]okadaate (**19**), and purified PP2A. The results showed the formation of an ^{3}H-adduct with the 37 kDa catalytic subunit of PP2A.[167] Given that other members of the okadaic acid class interact with the protein phosphatases in a similar manner, the binding site of okadaic acid to the catalytic subunit of PP2A was considered to be utilised for binding by all of the okadaic acid class of compounds.



Methyl-7-O-(4-azidobenzoyl)-[27-^{3}H]okadaate (**19**).

The main target organ of the okadaic acid pathway is the liver. Microcystin-LR is the strongest liver tumour promoter known at the present time.[148] In experiments using the intraperitoneal administration of [^{3}H]-okadaic acid and [^{3}H]-dihydromicrocystin-LR into mice, microcystin-LR was found to possess a higher liver specificity over okadaic acid.[168] The results of these experiments showed the specific incorporation of [^{3}H]-dihydromicrocystin-LR in the liver, whereas [^{3}H]-okadaic acid was found incorporated in the small and large intestines.[168] This could be the reason for the acute toxicity of okadaic acid as a causative agent in DSP. The specific toxicity of the

microcystins to the liver is due to the bile acid transporter systems which carry them into hepatocytes, the functional cells of the liver.[169] There, the microcystins cause distortion of the hepatocytes by acting on the cytoskeleton, the gridwork of protein strands that among other functions, give shape to the cells. The cytoskeletal structures most affected by the toxins, the intermediate filaments, become hyperphosphorylated, causing the hepatocytes to shrink.[163,170] As the cytoskeleton shrinks, the fingerlike projections through which the hepatocytes interact with neighbouring cells withdraw, breaking the cell's contact with other hepatocytes and with sinusoidal capillaries. The blood carried by these capillaries then seeps into the liver tissue, accumulating there and causing local tissue damage which often results in death by haemorrhagic shock. Studies on primary cultured rat hepatocytes treated with microcystin-LR (1 μM) have shown the induced phosphorylation of various proteins. Amongst these, two proteins, cytokeratin 8 (55 kDa) and cytokeratin 18 (49 kDa), were found to be phosphorylated at rates three fold higher than other proteins. The cytokeratins are a type of intermediate filaments. This hyperphosphorylation was accompanied by morphological changes from the normal round form of the hepatocytes to a bleb form, indicating that hyperphosphorylation of cytokeratins is a significant biochemical process associated with liver tumour promotion.[171]

The okadaic acid pathway can be summarized as follows: okadaic acid and other compounds in this class bind to PP1/ PP2A and become incorporated into the cells where they inhibit the activities of the phosphatases. This results in the hyperphosphorylation of various proteins (Fig. 1.20), such as the intermediate filaments. These filaments seem to be significant target proteins for the okadaic acid pathway. Another important effect of the okadaic acid class of compounds in the nuclei, is the sustained activation of gene expression, as well as hyperphosphorylation of the suppressor gene products.[166]

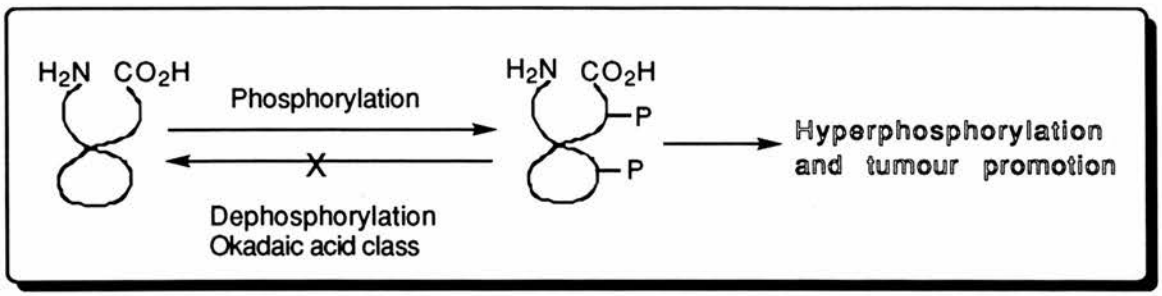


Figure 1.20: *The okadaic acid pathway.*

Liver tumour promotion by microcystins should also be considered possible in humans. A study in Qidong County, north of Shanghai, China, where people drink pond and ditch water contaminated with high levels of blue-green algae, reported an incidence of primary liver cancer eight times higher than in populations drinking well water.[172] This study strongly suggests that drinking water contaminated with microcystins might induce human liver cancer. The levels of these toxins in drinking water should therefore be monitored and regulated to prevent high risk exposure.

1.11 Other Bioactive Compounds Isolated from the *Microcystis* sp.

Microalgae have received much attention as rich sources of novel bioactive compounds.[173-176] Blue-green algae especially have been shown to be excellent sources of cytotoxins and fungicides.[177] Although the emphasis here is on other biologically active compounds isolated from the *Microcystis* sp., other bioactive compounds have also been isolated from other blue-green algae. These include puwainaphycins A-E[173,178] isolated from a terrestrial blue-green alga *Anabaena* sp. BQ-16-1 and oscillariolide, a macrolide isolated from a marine blue-green alga *Oscillatoria* sp.[179]

1.11.1 Micropeptins A and B

Micropeptins A and B were isolated from *Microcystis aeruginosa* NIES-100. The structures of these two cyclic depsipeptides were elucidated on the basis of 2D NMR and chemical degradation.[180] Micropeptin A **(20a)** (Fig. 1.21) was found to be OA-Glu-cyclo-(Thr-Val-N-MeTyr-Leu-Ahp-Lys), where OA is octanoic acid and Ahp is 3-amino-6-hydroxypiperidone. It was found to inhibit plasmin and trypsin with IC_{50} values of 0.026 and 0.071 μg/ cm^3, but did not inhibit thrombin, chymotrypsin and elastase. The structure of micropeptin B is as micropeptin A except for the octanoic acid moiety being replaced by hexanoic acid. Micropeptin B **(20b)** (Fig. 1.21) was found to inhibit plasmin and trypsin with IC_{50} values of 0.035 and 0.25 μg/ cm^3, but did not inhibit other serine proteases.

1.11.2 Microcystilide A

Microcystilide A **(21)** (Fig. 1.21) was isolated from *Microcystis aeruginosa* NO-15-1840. It is a non-Adda-containing cyclic depsipeptide, with an amino acid composition of: cyclo-{(*R*)-*p*-OH-PLac-(*S*)-Gln-(*S*)-Thr-(*S*)-Ile-(*S*)-MeTyr-(*S*)-Leu-Ahp-(*S*)-Tyr), where (*R*)-*p*-OH-PLac is (*R*)-parahydroxyphenyllactate and Ahp is 3-amino-6-hydroxypiperidone.[181] The molecule seems to have an elongated shape constricted in the middle by possible hydrogen bonding between isoleucine NH hydrogen and the axial hydrogen group of Ahp. Microcystilide A was first recognised as a component which causes convulsions or spasms in mice on intraperitoneal injections. It was only weakly cytotoxic against some cancer cell lines, but found to be active in the cell differentiation assay using HL-60 cells at a concentration of 0.5 mg/ cm^3.[181]

1.11.3 Microviridin

Microviridin **(22)** (Fig. 1.21), a tetradecapeptide, was isolated from an axenical clonal strain of the cyanobacterium *Microcystis viridis* from a bloom on Kasumigaura Lake.[182] Along with microviridin, two microcystins-LR and -YR were also isolated from the same bloom. The amino acid sequence of microviridin was found to be: Ac-Tyr-Gly-Gly-Thr-

Phe-Lys-Tyr-Pro-Ser-Asp-Trp-Glu-Glu-Tyr-OH. All the amino acids were found to be of L form. This peptide seemed to be decomposed rapidly by other bacteria and cells of the above strain incubated with unsterilized waters of the lakes did not survive for 6 hours. This could be a reason for this tetradecapeptide not being detected in 'field' samples of *M. viridis*. Microviridin was found to strongly inhibit tyrosinase activity to form melanin from tyrosine at 3.3×10^{-4} M.[182]

1.11.4 Microginin

Microginin **(23)** (Fig. 1.21) was isolated from cultured cells of *Microcystis aeruginosa.* Its structure, elucidated on the basis of 2D NMR data and chemical degradation, was found to be Ahda-(*S*)-Ala-(*S*)-Val-(*S*)-MeTyr-(*S*)-Tyr. Ahda was found to be a new β-amino acid, 3-amino-2-hydroxydecanoic acid, its stereochemistry at C-2 being R.[183] The asymmetric synthesis of Ahda[184] and the total synthesis of microginin[185] have been reported. Microginin was found to inhibit the angiotensin-converting enzyme with an IC_{50} value of 7.0 μg/ cm^3, but did not inhibit papain, trypsin, chymotrypsin or elastase at 100 μg/ cm^3. Identification of microginin as an angiotensin-converting enzyme (ACE) inhibitor has very important implications in the field of cardiology for the treatment of hypertension.

1.11.5 Aeruginosin 298-A

Aeruginosin 298-A **(24)** (Fig. 1.21) was isolated from the freshwater blue-green alga *Microcystis aeruginosa* and its structure elucidated by 2D NMR.[186] The structure of this linear peptide was found to be Hpla-Leu-Choi-Argol, where Hpla is 4-hydroxyphenyllactic acid, Choi is a new imino acid 2-carboxy-6-hydroxyoctahydroindole and argol is a guanidine-containing, arginine-like residue. Aeruginosin 298-A was found to inhibit thrombin and trypsin with IC_{50} values of 0.3 and 1.0 μg/ cm^3 respectively, but not papain, chymotrypsin, elastase and plasmin.[186]

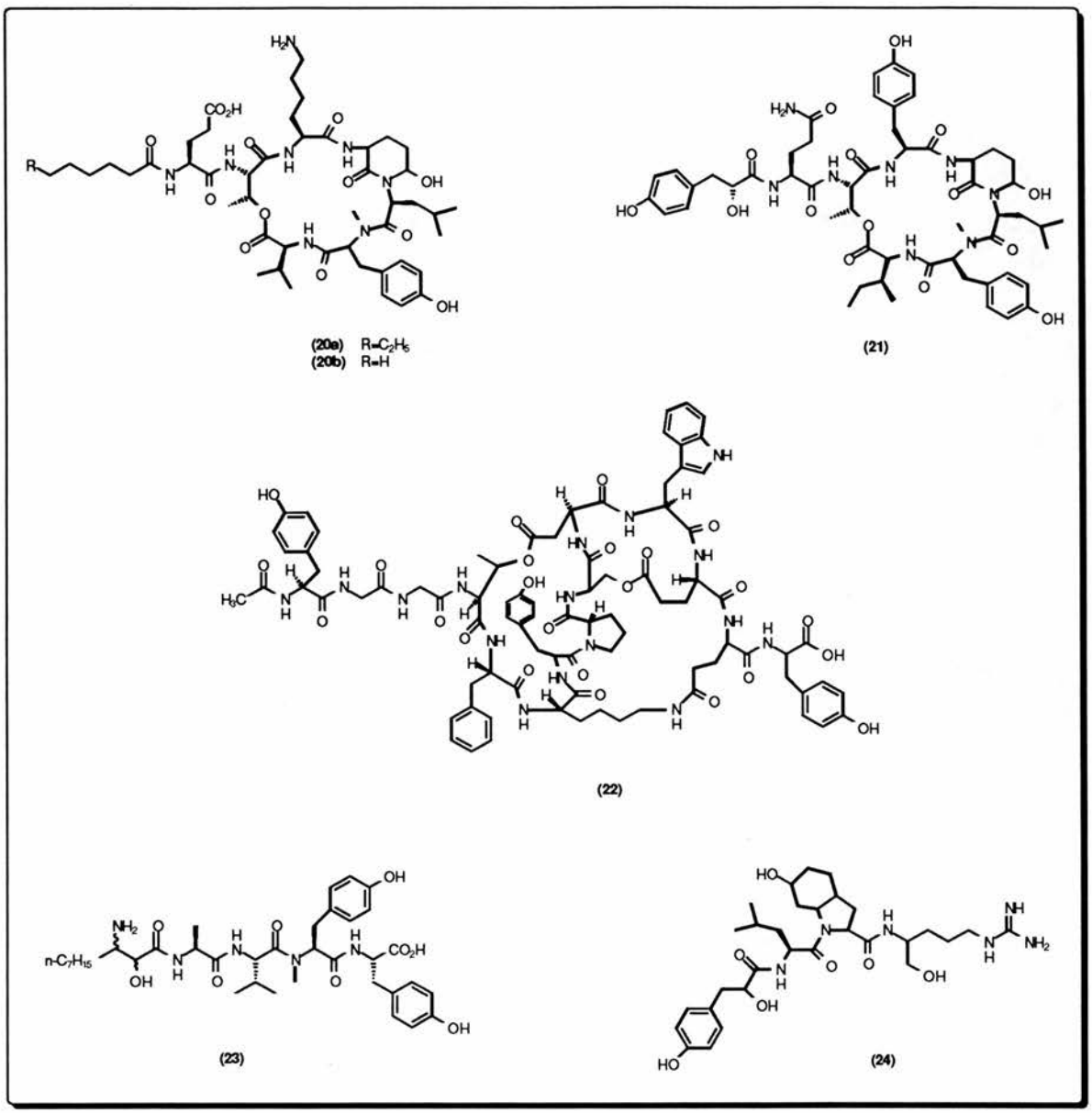


Figure 1.21: *Bioactive compounds isolated from the* Microcystis *sp.*

1.12 Other Protein Phosphatase Inhibitors

These include a structurally diverse group of compounds. Many possess other biological properties as well as phosphatase inhibitory activity.

1.12.1 Cantharidin

Exo,exo-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride, commonly known as cantharidin (**25**) (Fig. 1.22), is the natural toxicant of blister beetles. The total syntheses of cantharidin have been reported.[187,188] The toxic properties of cantharidin and its herbicidal analogue, endothall (**26**) (Fig. 1.22), are well characterized. Studies into the cellular mechanisms underlying the toxicty of these compounds, found them to be strong inhibitors of the serine/ threonine protein phosphatases 1 and 2A.[189] Like okadaic acid, cantharidin inhibited the activity of the purified catalytic subunit of PP2A (IC_{50} = 0.16 μM) at a lower concentration than PP1 (IC_{50} = 1.7 μM) and only inhibited the activity of PP2B at high concentrations.[190,191] Cantharidin and its analogues are additional pharmacological probes to investigate cellular processes that are regulated by reversible protein phosphorylation.

1.12.2 Cylindrospermopsin

In 1979, an outbreak of hepatoenteritis on Palm Island in northern Queensland, Australia, was traced back to the cyanobacterium, *Cylindrospermopsis raciborskii*, a species that had not previously been described as toxic.[192] The structure of the hepatotoxin isolated, cylindrospermopsin (**27**) (Fig. 1.22), was elucidated and it was found to belong to a new class of alkaloids possessing a cyclic guanidine group.[193,194]

1.12.3 Isopalinurin

5-[13-(Furan-3-yl)-2,6,10-trimethyltrideca-2,4,10-trienyl]-4-hydroxy-3-methylfuran-2(5H)-one, trivially known as isopalinurin (**28**) (Fig. 1.22), a sesterterpene tetronic acid, was isolated from a Southern Australian marine sponge, *Dysidea* sp., collected in Bass Strait. It was shown to have mild antibiotic activity against a *Micrococcus* sp. as well as weak protein phosphatase inhibitory activity (active to 2.5 mM).[195]

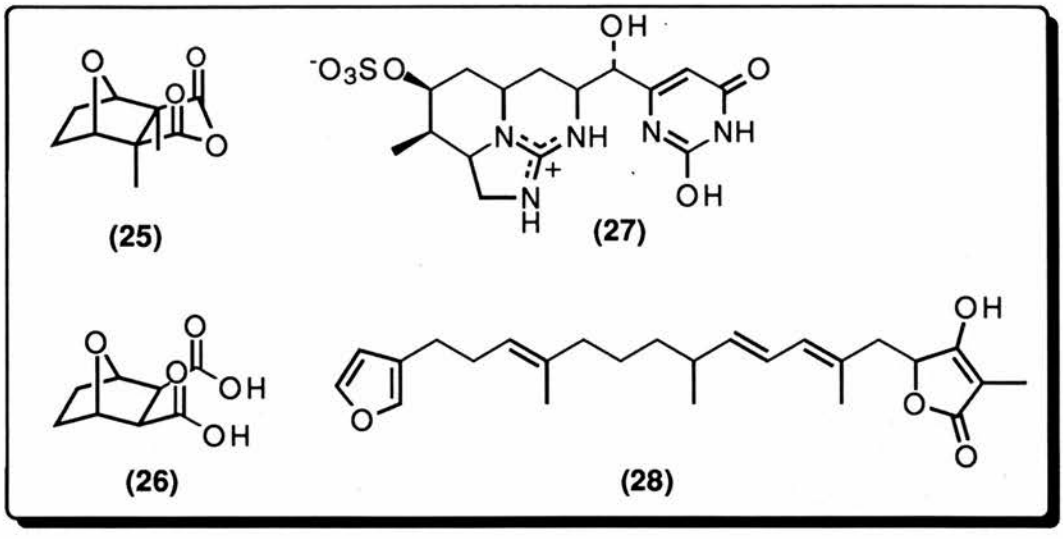


Figure 1.22: *Other protein phosphatase inhibitors.*

1.12.4 Rubrolides

Rubrolides A to H (**29a-h**) (Fig. 1.23), a family of biologically active tunicate metabolites, were isolated from a Northeastern Pacific marine invertebrate, *Ritterella rubra*.[196] They were shown to have potent *in vitro* antibacterial activity and a moderate but selective PP1 and PP2A inhibitory activity. The most active phosphatase inhibitor in this family of compounds was found to be rubrolide B, with IC_{50} values of 155 and 60 μM, against PP1 and 2A respectively.[197]

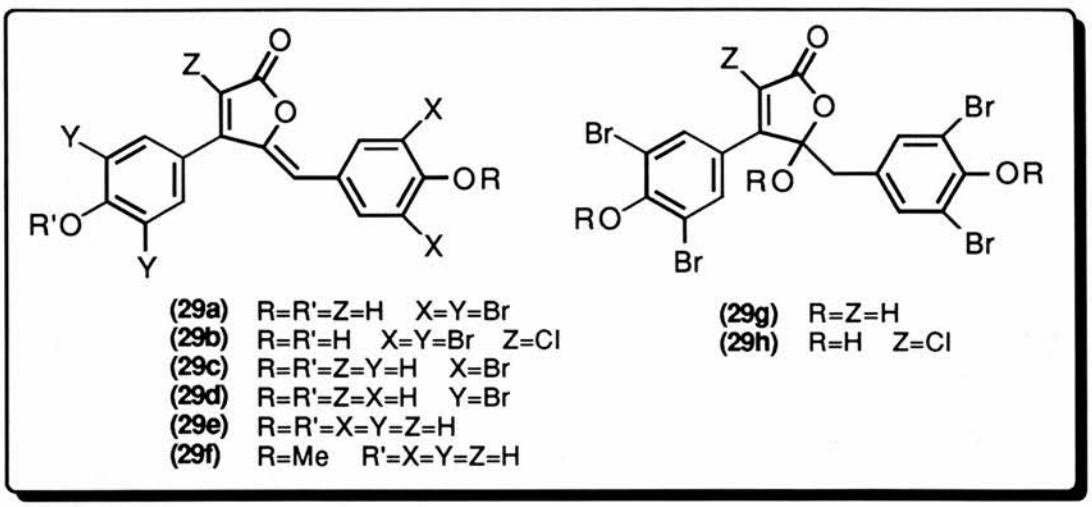


Figure 1.23: *Rubrolides A-H.*

1.12.5 Cyclosporin A and FK506

Cyclosporin A [CsA, (**3**)] and FK506 (**4**) (Fig. 1.5) are naturally occuring secondary metabolites. CsA, a lipid-soluble cyclic undecapeptide of molecular mass 1203, was isolated from the fungus *Tolypocladium inflatum*. FK506, a lipid-soluble macrocyclic lactone of molecular mass 822, was isolated from a soil bacterium *Streptomyces tsukubaensis* in Japan.[198,199] These immunosuppressant drugs bind a soluble intracellular protein (immunophillin), which then mediates their action by interacting with another target molecule. The CsA-binding protein is cyclophillin and the receptor for FK506 is FKBP (FK506-binding protein). Cyclophillins and FKBPs comprise two families of ubiquitous and often abundant proteins conserved from prokaryotes to eukaryotes.[198] To date, the only characterized target of immunophillin-ligand complexes is calcineurin (PP2B).[200,201] Calcineurin binds to both cyclophillin-CsA and FKBP-FK506, but not the free ligands or proteins; despite no obvious similarity in their three-dimensional structures, this binding is mutually exclusive.[201] While cyclophillins or FKBPs alone might not be involved in signal transduction, complexes of the immunophillins with their natural ligands have profound consequences. Both the cyclophillin-CsA and FKBP-FK506 complexes were shown to inhibit a previously unrecognized step in a family of Ca^{2+}-dependent signal transduction pathways that emanate from the multichain immune receptors. In summary,

CsA and FK506 inhibit intracellular signal transduction pathways that regulate early events in the cell cycle. By inhibiting these pathways in T cells, the natural products can suppress the immune system in living animals.

1.12.6 Pyrethroid Insecticides

Pyrethroids are very potent insecticides which are now widely used. Among them highly modified type II pyrethroids are particularly known for their chemical and biological stability and the unusual *in vivo* symptoms they cause. One of the most recognizable effects of the type II compounds is the profound stimulation of neurotransmitter release at the synapse.[202] It has now been established that the type II pyrethroids (cypermethrin, deltamethrin and fenvalerate) are potent inhibitors of isolated calcineurin from bovine brain with IC_{50} values ranging from 0.01 to 1 nM.[203] It is of interest to note that the sensitivity of calcineurin toward type II pyrethroids was limited only to those optical or geometrical isomers that are known to be insecticidal. Non-insecticidal chiral isomers of these pyrethroids, neuroactive type I pyrethroids and neuroactive chlorinated hydrocarbon insecticides did not show comparable potencies against this enzyme.[203]

The toxicological meaning of this inhibitory activity is realised by the vast roles of phosphatases, which participate in many diverse cellular functions, including signal transduction pathways.[3] While there could be some other minor and specific phosphatases in the synapse, calcineurin is the most dominant and wide spectrum Ca^{2+}-calmodulin stimulated phosphatase.[3] It is known to be the main force in bringing the transmission-induced changes back to the resting state. Therefore, the expected consequence of inhibition of calcineurin is sustained neurotransmitter release and eventual blockage. Failure to return phosphoproteins to their dephosphorylated state (*i.e.* resting) renders them unresponsive to the next stimulus.

1.12.7 Tumour Necrosis Factor-α

Tumour necrosis factor-α (TNF-α) was found to be an endogenous tumour promoter.[204] It was found that human TNF-α induces phosphorylation of the same proteins *in vitro* as does okadaic acid, although TNF-α directly activates various protein kinases and okadaic acid inhibits protein phosphatases. It is therefore postulated that tumour promoters such as TPA, teleocidin and okadaic acid are TNF-α inducers, and that TNF-α acts as an endogenous tumour promoter and a central mediator of tumour promotion.[204]

1.13 Synthetic Peptides as Substrates

A large number of biologically important proteins in mammalian cells namely, enzymes, growth factor receptors, oncogenic proteins, *etc.*, contain a covalently bound phosphate group and exist as phosphoproteins. Despite the importance of protein phosphorylation as a general mechanism for the control of cellular functions in eukaryotic cells, the molecular basis for control is not clearly understood.

Despite its general occurrence, protein phosphorylation is a selective process involving a minority of the potentially available Ser, Thr and Tyr residues in proteins. This selectivity results from several factors, among which the primary structure surrounding the phosphorylated amino acid has been shown, in many instances, to play a crucial role.[205,206] It has been shown that protein kinases recognise short amino acid sequences that fulfill certain structural requirements. Therefore, although the use of synthetic peptides has established the specificity determinants for phosphorylation by the kinases, such determinants for dephosphorylation by the protein phosphatases have not been fully investigated. Several studies have tried to redress this issue by using synthetic peptides corresponding to short sequences of physiologically important *in vivo* phosphoproteins. These peptides are usually phosphorylated enzymatically by [^{32}P]-ATP and casein kinase 2 (CK-2) or c-AMP dependent protein kinase (PK-A).

Agostinis *et al.* have shown that the different forms of PP2A display striking preference for phosphothreonyl peptides over phosphoseryl derivatives.[205] The synthetic peptide RRAS(^{32}P)VA, taken from the phosphorylated site of pyruvate kinase, and its Thr(^{32}P) analogue were shown to have dephosphorylation rates of 32 and 1484 pmol/ min/ cm^{-3} respectively.[206] The authors also note a higher specificity of action for the holoenzymes compared with the catalytic subunits.[205] Phosphothreonine therefore is a powerful positive determinant for enzymatic dephosphorylation by PP2A. Similar observations were also reported with PP2C using the above synthetic peptides phosphorylated by PK-A.[207] The PP2A preference for phosphothreonyl relative to phosphoseryl residues is intriguing, since the pThr content in proteins is only about 10% that of pSer. It is conceivable that the rapid dephosphorylation of pThr residues by PP2A and 2C, as well as the obvious preference of protein kinases for Ser residues over Thr residues might contribute to this. However, the broad phosphothreonyl phosphatase specificity must allow for a mechanism to spare the pThr sites that are physiologically significant. This occurs through the inclusion of negative determinants that hamper pThr dephosphorylation. A structural modification that greatly impairs the dephosphorylation efficiency of both PP2A and PP2C is the presence of proline residues around the pThr.[205,207] In the sequence RRAT(P)VA, replacement of the Val residue immediately downstrream to the phosphothreonine residue with Pro abolishes dephosphorylation, whereas substitution of the alanine N-terminal to the phosphothreonine residue with proline has almost no effect.[207] This is evident from dephosphorylation rates of 1870 and 7 pmol/ min/ cm^{-3} for the phosphopeptides RRPT(P)VA and RRAT(P)PA respectively compared with 4760 pmol/ min/ cm^{-3} for RRAT(P)VA.[205] This negative effect on dephosphorylation can be observed in the Thr-Pro motif of some proteins *e.g.* I-1, I-2 and DARPP32, where the phosphothreonyl residue is protected from premature dephosphorylation by PP2A and 2C.

The slow dephosphorylation of pSer residues can be enhanced by the presence of multiple N-terminal basic residues (residues possessing positively charged side chains) *e.g.* Arg residues. These multiple basic resiudes can act as positive determinants if they are situated

in the relative positions including 3 to 6, and not too close to the pSer. Seemingly, other positions can be occupied by a variety of other amino acids without great changes in dephosphorylation efficiency. For instance, the dephosphorylation rate of peptides RRRRAS(P)VA and RRRRAAS(P)VA (relative to phosphorylase a) is 1.061 and 2.467 respectively compared with 0.095 for RRAS(P)VA. Basic residues at positions 3 to 6 are also positive determinants for several protein kinases including those dependent on c-AMP and c-GMP. This structural feature could therefore allow PP2A to reverse the phosphorylation by these enzymes. The requirement for multiple N-terminal basic residues for efficient dephosphorylation might be the reason why three or more basic groups are often found in phosphorylation sites where these residues are not strictly required for phosphorylation.[208] Generally, the negative effect of Pro is more evident with pThr peptides, whereas the N-terminal basic residues act as a positive determinant for pSer peptides and are not required for efficient dephosphorylation of pThr residues.

Despite the similarities in specificity between PP2A and PP2C, some differences are also observed.[207] Unlike PP2A, additional basic arginine residues fail to improve the dephosphorylation rate of RRAS(P)VA by PP2C.[207] The peptide RRREEET(P)EEEAA, which is an excellent substrate for PP2A, is not dephosphorylated by PP2C at all. This is most likely due to the presence of acidic residues surrounding the pThr, since in RRS(P)T(P)VA, substitution of the Val with Glu drastically reduces the dephosphorylation rate by PP2C. In contrast, PP2A was not affected by the Val to Glu substitution. The specificity of PP2C towards phosphopeptides appears to be narrower than PP2A and can be clearly distinguished from that of PP1, which is almost inactive towards all the peptides that have been tested for PP2A and 2C.[205,209] Whether the specificity of PP2C for phosphopeptides *in vitro* also reflects its activity *in vivo* towards endogenous substrates remains to be further investigated.

While PP2A and PP2C are active on short phosphopeptides, an extended N-terminal stretch appears to be a necessary, albeit not sufficient, condition for an optimal

dephosphorylation by calcineurin.[210] This positive effect is mediated by higher order structural features and does not depend on a given consensus sequence. However, a number of shorter peptides are also dephosphorylated by calcineurin, their efficiency as substrates depending on local structural features. All the peptides that are appreciably dephosphorylated by PP2B contain basic residue(s) on the N-terminal side.[210] A basic residue at position 3 relative to the phosphorylated residue plays a particularly positive role in determining the dephosphorylation of short peptides. Acidic residue(s) immediately downstream to the phosphoamino acid are powerful negative determinants, preventing dephosphorylation of otherwise suitable substrates. This characteristic is reminiscent of PP2C.[207] Unlike PP2A and PP2C, calcineurin displays an only moderate preference for phosphothreonyl peptides, and does not perceive the motif Ser/Thr-Pro as a strong negative determinant. It is therefore possible that phosphoacceptor sites of I-1 and DARPP32 are preferentially dephosphorylated by calcineurin.

The results obtained from the studies carried out so far, are not as clear cut as for the protein kinases. An obvious relationship between the structural features responsible for the dephosphorylation of peptide substrates and those occuring at the phosphorylated sites of physiological protein substrates has not been established. Despite this, studies with phosphorylated synthetic peptides[211] have shown that some structural features determining the site specificity of many protein kinases, namely the nature of the phosphoacceptor residue and the presence of charged side chains surrounding it, can also deeply influence the dephosphorylation process catalysed by some protein phosphatases. Also, the responsiveness to local determinants differs with the nature of the protein phosphatase. To what extent these peptide substrate specificities reflect a 'site specificity' towards the native protein substrate and how these manifest themselves in target recognition *in vivo* remain to be investigated. Nevertheless, the availability of phosphopeptides creates the possibility of using these artificial substrates for the specific detection and assay of the different classes of protein phosphatases. For instance, the phosphothreonyl peptide RRAT(P)VA can prove to be very useful for the assay of PP2A and PP2C. In effect, this peptide is a better

substrate for PP2A and 2C than phosphorylase *a* and phosphocasein, protein substrates that are routinely used in assays of these enzymes.

At present, there is a considerable lack of kinetic and mechanistic information on the protein phosphatases. The use of substrates in assays of the protein phosphatases is dependent on the enzymic phosphorylation of specified sequences and the use of ^{32}P-labelled substrates. The synthesis of phosphopeptides and their use in establishing a more reliable assay system therefore becomes apparent.

CHAPTER TWO

RESULTS and DISCUSSION

2.0 RESULTS AND DISCUSSION

2.1 Introduction

Protein phosphorylation is now generally recognized as a major regulatory process mediated by protein kinases and phosphatases. There is evidence that protein phosphorylation is a regulatory element in carcinogenesis and that it is also associated with certain diseases. Investigation of the molecular basis of this regulation induced by phosphorylation, is entirely dependent on the availability, in sufficient quantities, of phosphopeptides and phosphoproteins. Phosphorylated peptides and proteins are only produced in small quantities within cells and their instability to extremes of pH makes isolation difficult. Therefore, development of general methods for the synthesis of phosphopeptides of any length and sequence is highly desirable. The use of synthetic phosphopeptides can shed light on the primary site requirements of the different protein phosphatases. Such an approach will also provide useful information on the importance of the molecular integrity of the physiological substrates as well as the significance of the subunit structure of the phosphatases. A more detailed understanding of the role and mechanism of the protein phosphatases will offer therapeutic opportunities in several areas of medicine, including cancer, host defence, arthritis and atheroma.

2.2 Design of a Protein Phosphatase Substrate

Although protein kinases have been subjected to a more detailed study with respect to specificity determinants governing the phosphorylation process, some assumptions can also be made for the protein phosphatases. It is conceivable that local structural features surrounding the phosphorylated amino acid are perceived as either positive or negative determinants by the protein phosphatases, thereby influencing the efficiency and the specificity of the dephosphorylation process. A review of the literature available on the phosphopeptides used for determining the substrate specificities of the protein

phosphatases 1, 2A, 2B and 2C has revealed some common concensus among some members of this important class of enzymes.

Using PP2A, studies on phosphopeptides with truncated N-terminal ends have shown that at least two residues must be located on the N-terminal side of the phosphorylated residue in order to obtain detectable dephosphorylation of the peptide.[206] This is particularly true for phosphoserine- (pSer) compared with phosphothreonine- (pThr) containing peptides. The nature of these residues is an important determinant for the rate and efficiency of dephosphorylation. Multiple N-terminal basic residues, situated between 3 to 6 residues upstream of the phosphorylated amino acid, improves the dephosphorylation of peptides, particularly phosphoseryl ones. The presence of at least two arginine residues on the N-terminal side of the pSer/ pThr has been found to be the general concensus (Table 2.1).

Peptides [(a)]	Dephosphorylation rate [(b)]
pSSSEE	n.d.
LpSSSEE	0.004
SLpSSSEE	0.037
ESLpSSSEE	0.069
RRLpSSLRA	0.434
RRApSVA	0.095
RRRRApSVA	1.061
RRRRAApSVA	2.467
RRRRAAApSVA	1.164

Table 2.1: *Dephosphorylation rate of phosphoseryl peptides by PP2A.*[206] (a) *Peptides phosphorylated with either PK-A, CK-2 or PK-C. pS= phosphoserine residue. Basic residues are underlined.* (b) *Dephosphorylation rates are relative to phosphorylase-a. n.d.= relative dephosphorylation rate < 0.001.*

A negative determinant for dephosphorylation is the presence of a proline residue immediately downstream of the pThr residue. This feature is also shared by PP2C where a

dramatic decrease in dephosphorylation activity is observed. A characteristic of PP2C, not shared by PP2A, is the negative effect of acidic residues surrounding the pThr on the dephosphorylation rate (Table 2.2).

Peptides	PP2A	PP2C
RRApTVA	320	98.3
RRPpTVA	316	106
RRApTPA	0	0
RRpSpTVA	120	81.6
RRpSpTEA	130	7
RRREEEpTEEEAA	200	0

Table 2.2: *Differences in substrate specificity between PP2A and PP2C. Activities are expresssed as pmol/ min/ cm^{-3}.* [207]

It appears that dephosphorylation of phosphothreonyl peptides is not strictly dependent on the positive determinants required for the efficient dephosphorylation of phosphoserine whereas negative determinants *e.g.* a C-terminal proline play a more important role with phosphothreonine. Irrespective of the primary structure of the phosphopeptide substrates, PP2A and 2C invariably display a striking preference for pThr over pSer peptides (Table 2.3).

Peptides	Dephosphorylation rate (pmol/ min/ cm^{-3})
RRApTVA	1484
RRApSVA	32
RRREEEpTEEEAA	1417
RRREEEpSEEEAA	126
pTEEEEE	159
pSEEEEE	<1

Table 2.3: *Preferential dephosphorylation of phosphothreonyl peptides by PP2A.*[206] *Phosphoserines substituted for phosphothreonines are underlined.*

From the observations made in these studies, it was apparent that the sequence for the phosphopeptide substrate must contain certain determinants for efficient dephosphorylation by the protein phosphatases 2A or 2C. These determinants included:

(i) a threonine rather than a serine residue as the potential phosphorylatable amino acid,

(ii) at least two arginine residues at the N-terminal side of pThr and in the relative position 3 from the phosphorylated amino acid, although these play a less important role for pThr compared with pSer, and

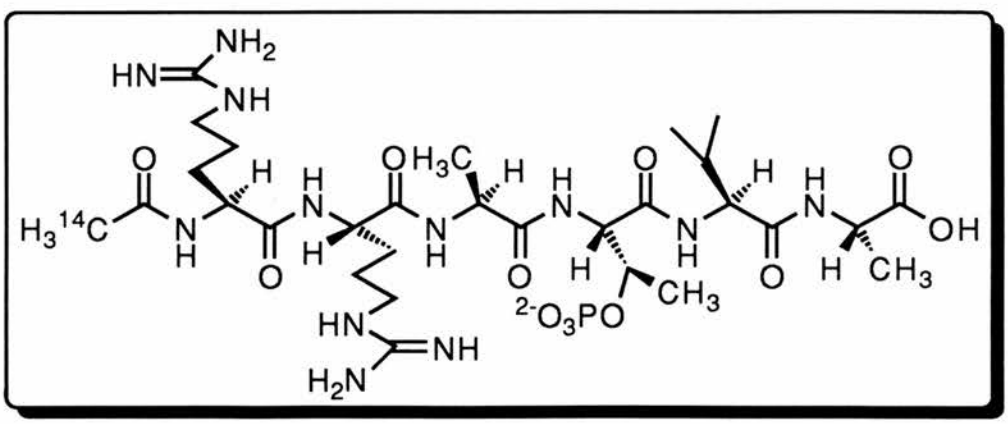
(iii) a featureless C-terminus devoid of negative determinants.

The substrate sequence that best fitted these criteria was found to be RRApTVA. This phosphopeptide possessed the minimum requirements for efficient dephosphorylation and had been shown to be an effective substrate for both PP2A and PP2C,[205,207,208] hence the choice of the substrate sequence.

Traditionally, the preparation of phosphopeptides for use in assays of protein phosphatases has involved the use of protein kinases *e.g.* PK-A, CK-2 or protein kinase C for phosphorylation of the peptide sequence with [^{32}P]-ATP as the source of the phosphate group. This method of phosphopeptide synthesis is inherently flawed for two reasons. Firstly, enzymic phosphorylation although attractive, is limited in its use due to the sequence specificity of the protein kinases. It is therefore only applicable to very specific peptide sequences and does not allow for a proper assessment of substrate specificity and the construction of structure-activity relationships for the protein phosphatases. Unlike protein phosphatases, protein kinases display a preference for pSer residues over pThr ones and therefore, the extent of phosphorylation of peptide sequences containing threonine is quite low. For instance, the sequence RRApTVA, chosen to be the most effective for use in assays of PP2A and 2C, is only phosphorylated 10-40% by PK-A.[205,208] Therefore, multiple incubations have to be set up to obtain appreciable quantities of the phosphopeptide required.

Secondly, use of $^{32}P_i$ liberated in the phosphatase assays for determining the rate and extent of dephosphorylation is not entirely desirable. ^{32}P has a short half life of 14.8 days. For kinetic data obtained from the use of a ^{32}P-labelled phosphopeptide to be meaningful, these should start from the same or similar starting points. Due to the short half life of ^{32}P, loss of activity over a period of time would not enable an accurate assessment of the phosphatase kinetics. Therefore, reliable data can only be obtained with freshly prepared samples of the phosphopeptide and this would involve repeated batch preparations of the compound.

In view of the above, we decided, at the outset of our work, to adopt a synthetic approach to the phosphorylation of our substrate sequence and to replace the need for ^{32}P, as a means for quantification of the dephosphorylation rates, with a more stable labelled material. Introduction of a ^{14}C-label into the substrate in the form of an N-terminal acetyl group would provide a handle for detecting and quantifying phosphatase activity. The ^{14}C-radiolabel offers considerable advantages over the use of ^{32}P because of its longer half life of 5730 years. We therefore chose compound **(30)** as our potential protein phosphatase substrate.



Potential protein phosphatase substrate ***(30)***.

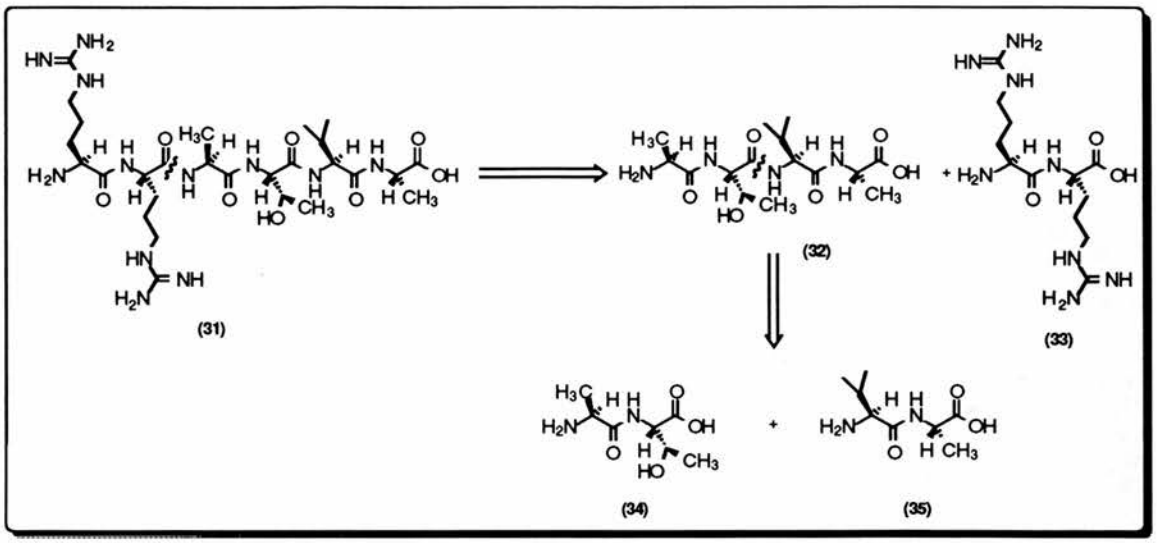
2.3 Solution Phase Peptide Synthesis

The mixed anhydride method of peptide coupling is one of the many methods for synthesising peptides. Others include the use of acid chlorides and activated esters including carbodiimide adducts.[212] The former method is used for its advantages in speed, yield and relative purity of products. It was therefore chosen as the method of choice for the peptide couplings.

The mixed anhydride method has been studied in detail and the optimum conditions for peptide coupling (yield and minimisation of racemisation) have been established.[213,214] The best results have been obtained using a combination of NMM as the tertiary base, IBCF as the alkyl chloroformate and dry THF or dry DMF (depending on the solubility of the amino acid) as the solvents for the reaction.[214] The reaction involves the attack of the tertiary base on the alkyl chloroformate to form a quaternary ammonium species, which in turn reacts with the carboxylate moiety of a suitably N-protected amino acid. The anhydride formed in this manner, then reacts with the amine moiety of a suitably C-protected amino acid to give the peptide linkage.

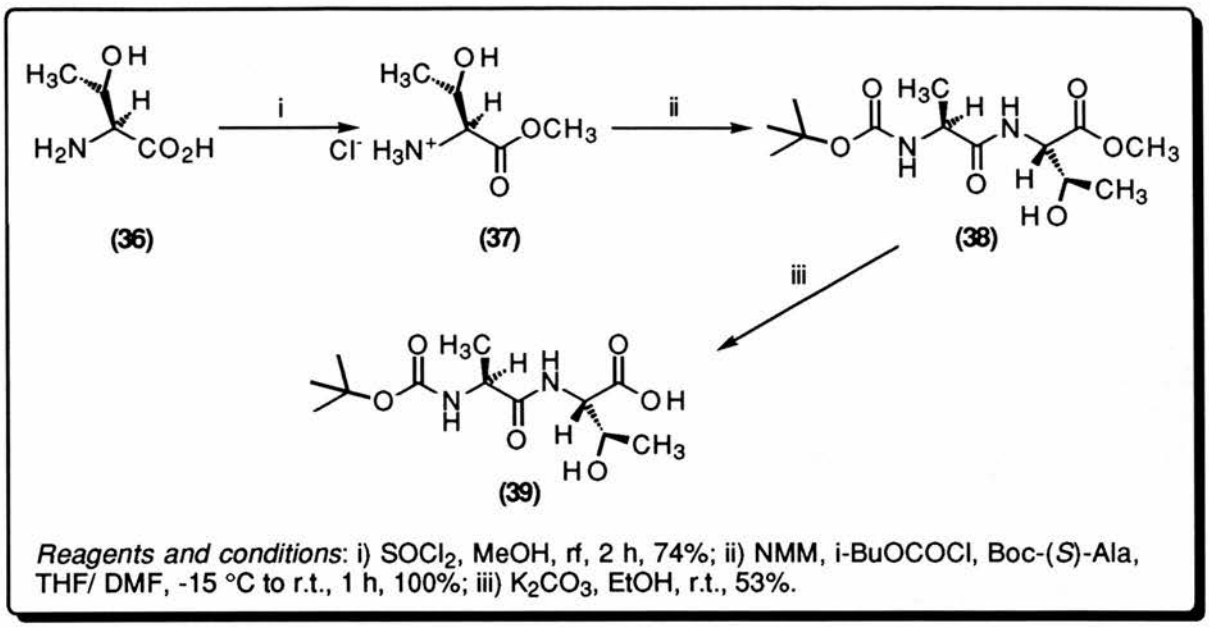
2.4 Solution Phase Synthesis of the Peptide Sequence

The synthesis of the substrate was carried out *via* a fragment-based approach. Disconnection of the hexapeptide sequence **(31)** gave a tetrapeptide **(32)** and an Arg-Arg dipeptide **(33)**. Disconnection of the tetrapeptide in turn, gave two synthetically manageable dipeptides, an Ala-Thr dipeptide **(34)** and a Val-Ala dipeptide **(35)** (Scheme 2.1).



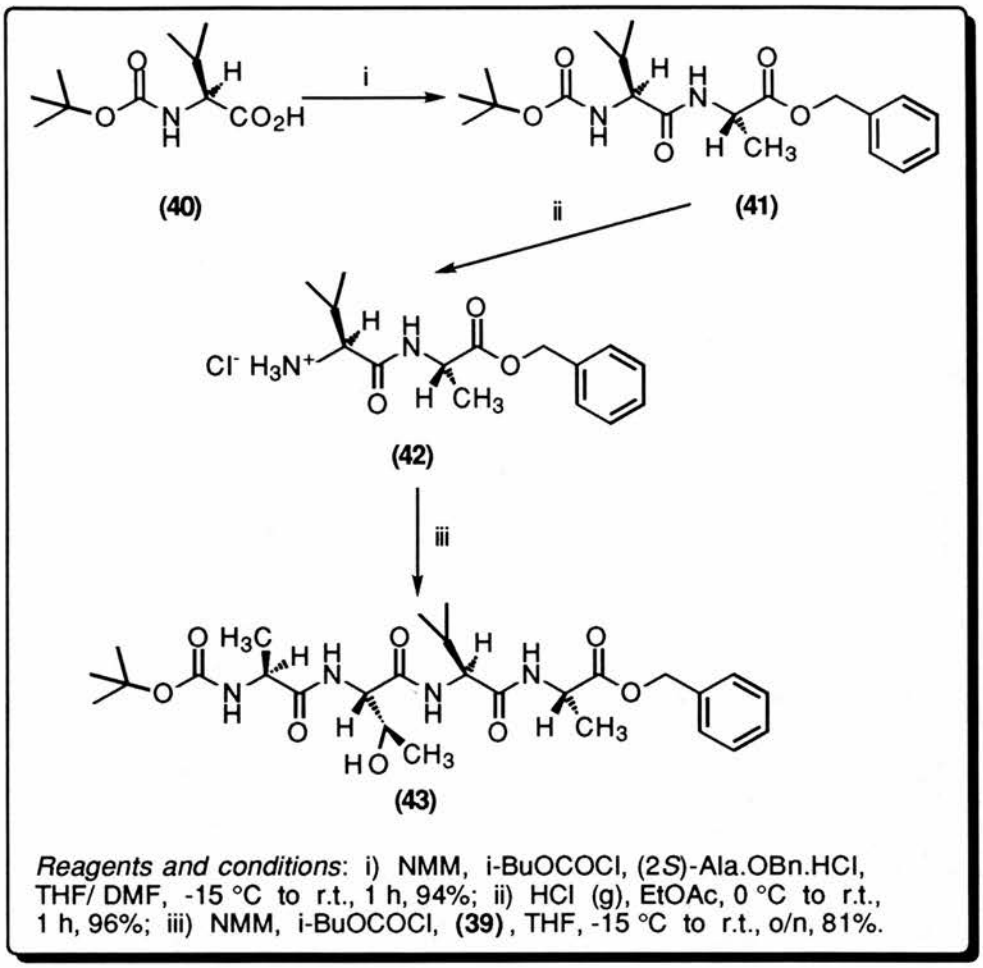
Scheme 2.1: *Disconnection of the hexapeptide substrate sequence.*

(2*S*)-Threonine methyl ester hydrochloride salt (**37**) was prepared by refluxing (2*S*)-threonine (**36**) in dry methanol with thionyl chloride to afford the product as an oil in 74% yield. This was then coupled to Boc-(2S)-Ala to give the target dipeptide (**38**) as a solid, in near quantitative yield {m.p. 103-104 °C (lit.,[215] 104.5-105 °C); *m/z* (Found: $[M+H]^+$, 305.1709. Calc. for $C_{13}H_{25}N_2O_6$: *m/z*, 305.1712)}. Mild base catalysed hydrolysis of the ester (**38**) using ethanolic potassium carbonate afforded the N-protected peptide (**39**) as a white solid {m.p. 77-80 °C} (Scheme 2.2).



Scheme 2.2: *Synthesis of the N-α-Boc protected dipeptide (39).*

Mixed anhydride mediated coupling of Boc-(2*S*)-Val (**40**) and (2*S*)-Ala-OBn.HCl afforded the dipeptide (**41**) in 94% yield {m.p. 85 °C (lit.,[216] 82-83 °C); *m/z* (Found: [M + H]$^+$, 379.2233. Calc. for $C_{20}H_{31}N_2O_5$: *m/z*, 379.2233)}. Deprotection of the Boc group from dipeptide (**41**) using HCl gas gave the amine salt (**42**), which was refractory to crystallisation, in 96% yield. Peptide bond formation between the deprotected dipeptides (**39**) and (**42**) in the usual manner gave the fully protected tetrapeptide (**43**), the initial target, in 81% yield {m.p. 166-168 °C; *m/z* (Found: [M + Na]$^+$, 573.2903. $C_{27}H_{42}N_4O_8Na$ requires: *m/z*, 573.3000)} (Scheme 2.3).



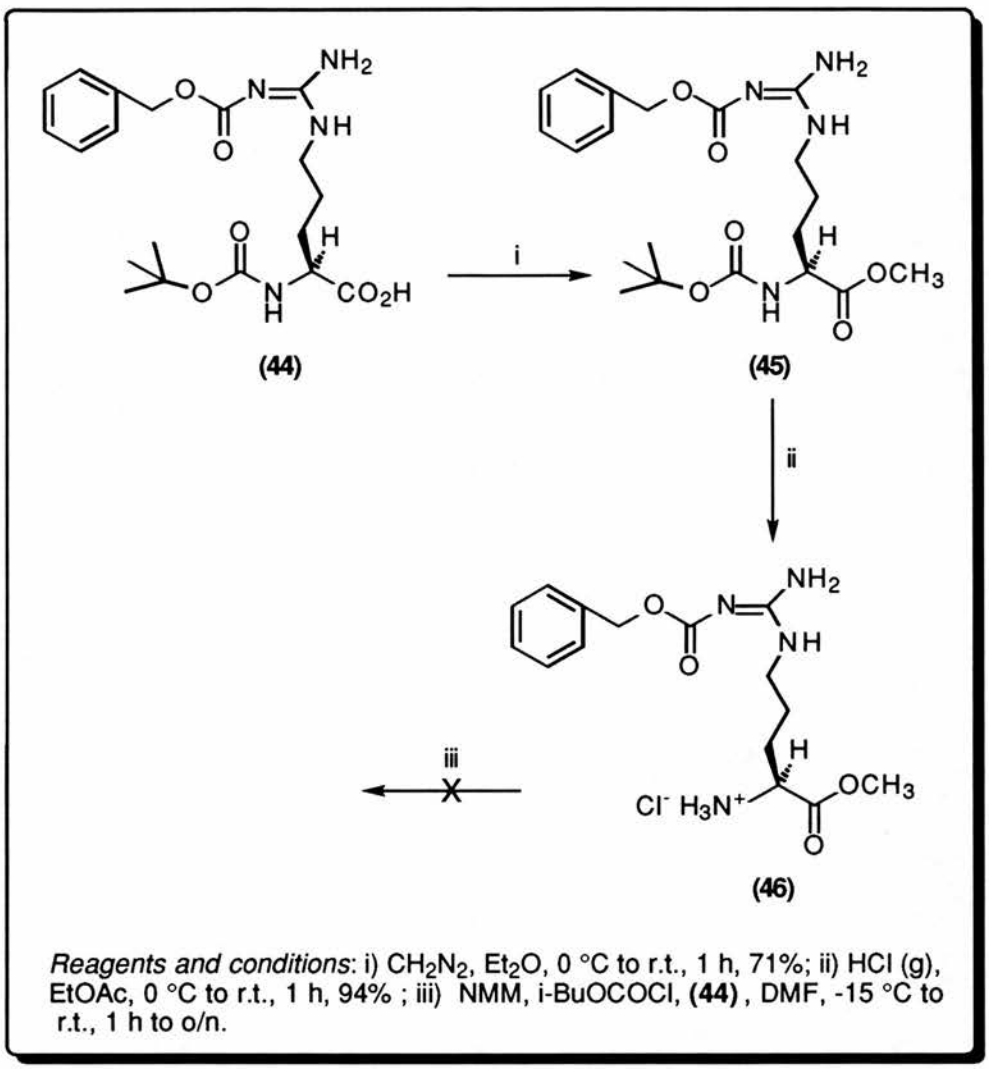
Reagents and conditions: i) NMM, i-BuOCOCl, (2*S*)-Ala.OBn.HCl, THF/ DMF, -15 °C to r.t., 1 h, 94%; ii) HCl (g), EtOAc, 0 °C to r.t., 1 h, 96%; iii) NMM, i-BuOCOCl, **(39)**, THF, -15 °C to r.t., o/n, 81%.

Scheme 2.3: *Synthesis of the fully protected tetrapeptide (43).*

The synthesis of the dipeptide Arg-Arg however, proved to be problematic. The use of arginine residues in peptide synthesis raises the issue of the manner of protection of the side chain guanidino group and its inherent basicity.[217] Indeed, the incorporation of the highly basic arginine into synthetic peptides has posed more problems than have been encountered with any other protein-derived amino acid. The successful coupling of an arginine residue with another amino acid in a peptide linkage requires the basicity and nucleophilicity of the side chain to be adequately and appropriately masked prior to the peptide-forming step. Strategies designed to overcome these difficulties in the preparation of arginyl peptides have been the subject of much study for many years.[218-223] Although protonation is thought to provide sufficient protection and salts (mostly hydrochlorides) of N-α-protected arginine derivatives have been incorporated in peptide chains, the use of an

arginine derivative with a suitable substituent on the guanidine is preferred. This arises largely because intermediates possessing guanidinium ions in arginine side chains are highly insoluble in organic solvents. We therefore, decided to make use of suitably protected arginine residues rather than rely on protonation.

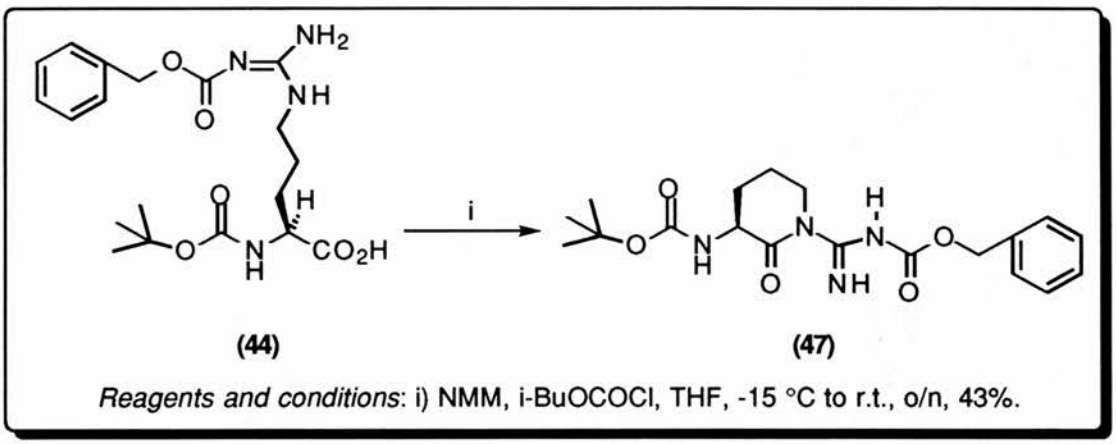
The initial hexapeptide substrate was designed with an N-α-Boc protection and a benzyl ester at the C-terminal end, in mind. This would allow the selective acid catalysed deprotection of the N-terminal for the subsequent introduction of the acetyl moiety. To this end, we initially chose Cbz groups for guanidino-protection, in order to allow for the simultaneous deprotection of the C-terminus and the arginine side chains. Treatment of Boc-(2*S*)-Arg(Cbz).OH (**44**) with diazomethane at 0 °C gave the ester (**45**) as a white solid in 71% yield {m.p. 75-77 °C; *m/z* (Found: $[M + H]^+$, 423.2244. $C_{20}H_{31}N_4O_6$ requires: *m/z*, 423.2247)}. Boc deprotection of (**45**) using HCl gas afforded the amine salt (**46**) as an oil in 94% yield (Scheme 2.4). The insolubility of the arginine derivatives used in attempted mixed anhydride coupling reactions to give Arg-Arg peptides however, was the source of some problems. Although (**44**) was soluble in dry THF and (**46**) in dry DMF, addition of the salt (**46**) in DMF to a solution containing the mixed anhydride of (**44**) in THF resulted in "oiling out". Apparently no coupling took place and the solvent for both (**44**) and (**46**) in the reaction was therefore changed to dry DMF. Several attempts to identify the conditions for the synthesis of the Arg-Arg dipeptide were pursued, including choice of solvent, activating agents and the base used, and each failed to give the desired product.



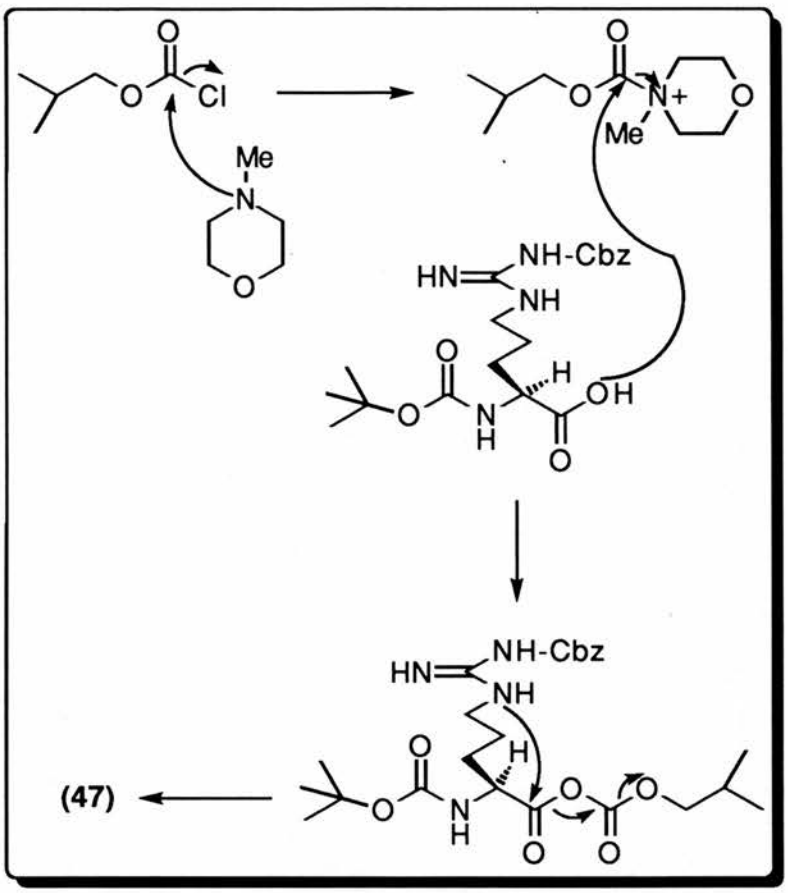
Scheme 2.4: *Attempted synthesis of the Arg(Cbz)-Arg(Cbz) dipeptide.*

The ^{1}H nmr spectrum of the compound obtained from some of these reactions did not contain the signals for the methyl ester at 3.74 ppm. An experiment was therefore carried out to determine the extent of mixed anhydride formation in the reaction. This involved reacting **(44)** in dry THF with NMM and IBCF at -15 °C for 5 min. and then allowing the mixture to warm up to room temperature overnight. Subsequent work-up and ^{1}H nmr spectrum of the crude product suggested the formation of a cyclic compound **(47)**, the structure of which was confirmed to be piperidone-related by mass spectrometry {*m/z* (Found: $[M + H]^+$, 391.1975. $C_{19}H_{27}N_4O_5$ requires: *m/z*, 391.1981)} (Scheme 2.5). It is reasonable to assume that the mechanism of the reaction involves the preliminary activation

of the carboxyl group by the activating agent (the chloroformate) followed by an intramolecular cyclisation to give an anhydroarginine derivative (Scheme 2.6). This intramolecular cyclisation is ascribed to the fact that the basicity of the guanidino moiety is not completely masked. We therefore decided to use an arginine derivative with a nitro group on the guanidine.

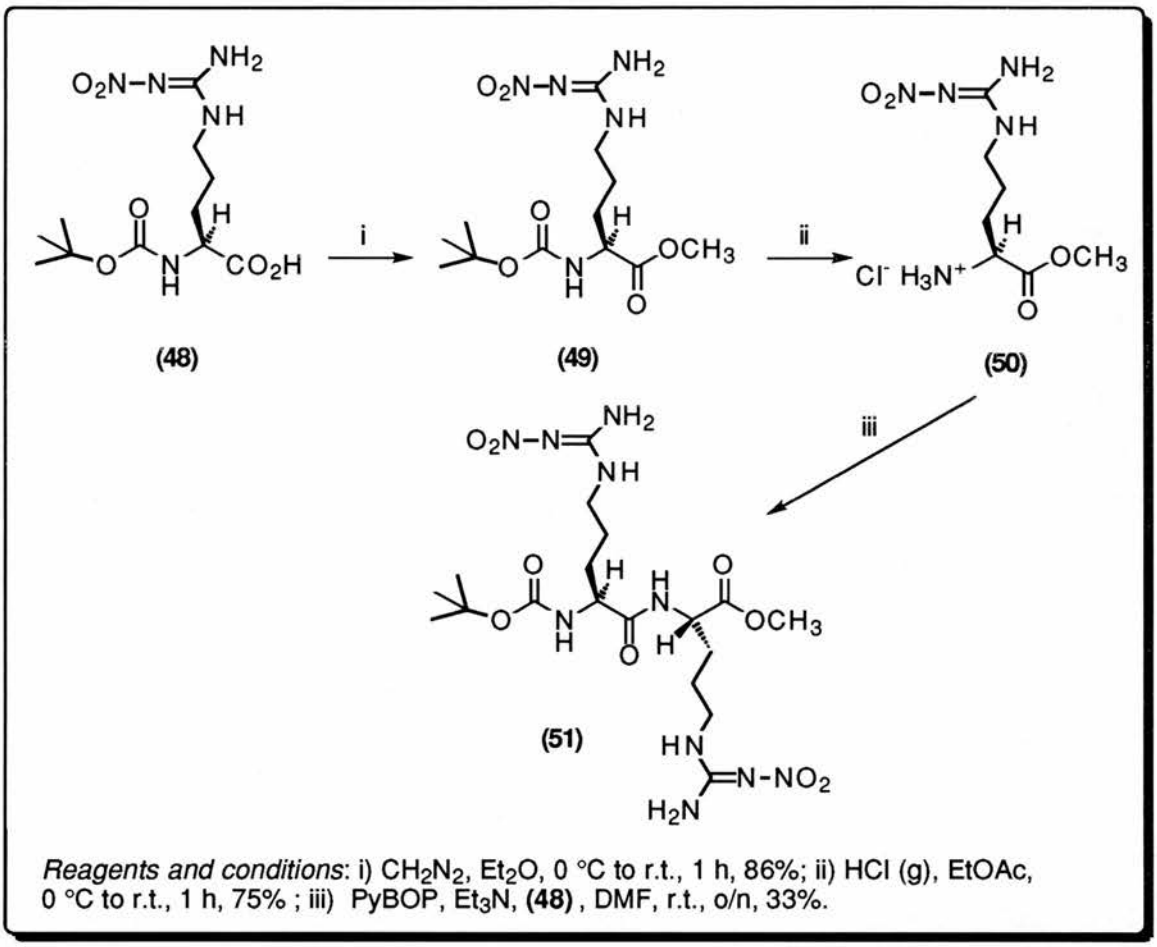


Scheme 2.5: *Formation of the cyclic compound* ***(47)***.



Scheme 2.6: *Proposed mechanism for the formation of* ***(47)****.*

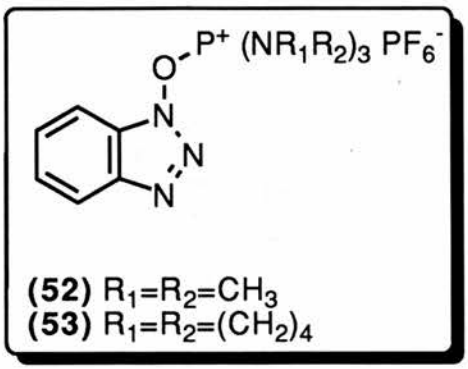
Derivatives Boc-(2*S*)-Arg(NO_2).OMe **(49)** {*m/z* (Found: $[M + H]^+$, 334.1733. $C_{12}H_{24}N_5O_6$ requires: *m/z*, 334.1727)} and (2*S*)-Arg(NO_2).OMe.HCl **(50)** were synthesised in similar ways to those described for the ester **(45)** and the salt **(46)**, in 86 and 75% yield respectively (Scheme 2.7).



Scheme 2.7: *Synthesis of the Arg(NO_2)-Arg(NO_2) dipeptide (**51**).*

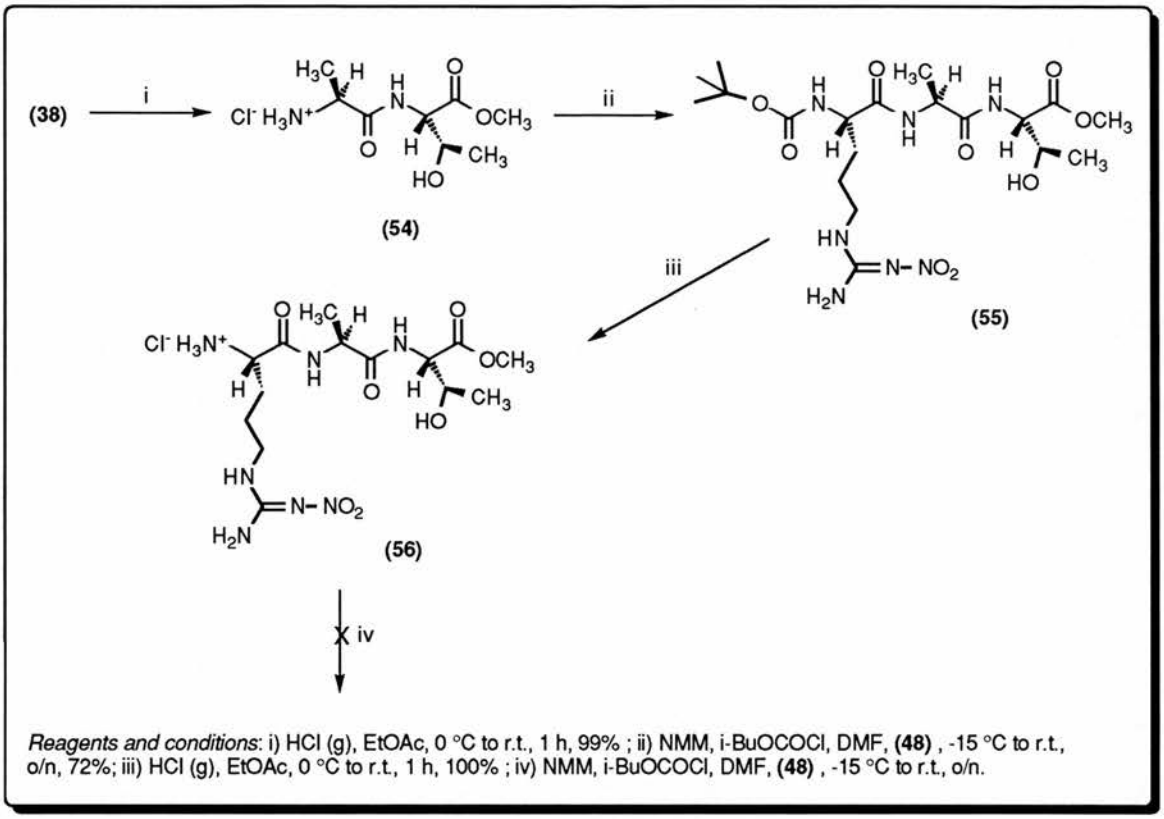
The mixed anhydride method of coupling failed to give the desired Arg(NO_2)-Arg(NO_2) dipeptide as before, and therefore alternative activating agents were investigated. Benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate (PyBOP) was used for the coupling reaction of **(48)** and **(50)**. PyBOP **(53)**[224] is an analogue of BOP **(52)** where dimethylamino groups in BOP are replaced by pyrrolidino groups. It has been found to yield coupling rates as good as, if not better than, those observed with BOP. It is also preferable to BOP because of the lack of formation of the carcinogenic hexamethylphosphoric triamide (HMPA) involved in the manufacture and use of BOP. Use of this activating agent afforded the desired product **(51)** in low yields as an orangy-brown viscous oil that was refractory to crystallisation or purification (Scheme 2.7). The synthesis of an Arg-Arg dipeptide therefore, appeared to be rather elusive and not

desirable. We therefore turned our attention to incorporating the arginine residues sequentially.



Activating agents: BOP (52); PyBOP (53).

Boc deprotection of the fully protected dipeptide (**38**) using HCl gas gave the methyl ester (**54**) in 99% yield. Coupling of this to the acid (**48**) afforded the fully protected tripeptide (**55**) in 72% yield. Boc deprotection of (**55**) and subsequent coupling to a second molecule of (**48**) failed to give the desired tetrapeptide (Scheme 2.8).



Reagents and conditions: i) HCl (g), EtOAc, 0 °C to r.t., 1 h, 99% ; ii) NMM, i-BuOCOCl, DMF, **(48)** , -15 °C to r.t., o/n, 72%; iii) HCl (g), EtOAc, 0 °C to r.t., 1 h, 100% ; iv) NMM, i-BuOCOCl, DMF, **(48)** , -15 °C to r.t., o/n.

Scheme 2.8: *Attempted sequential incorporation of the Arg(NO_2) residues.*

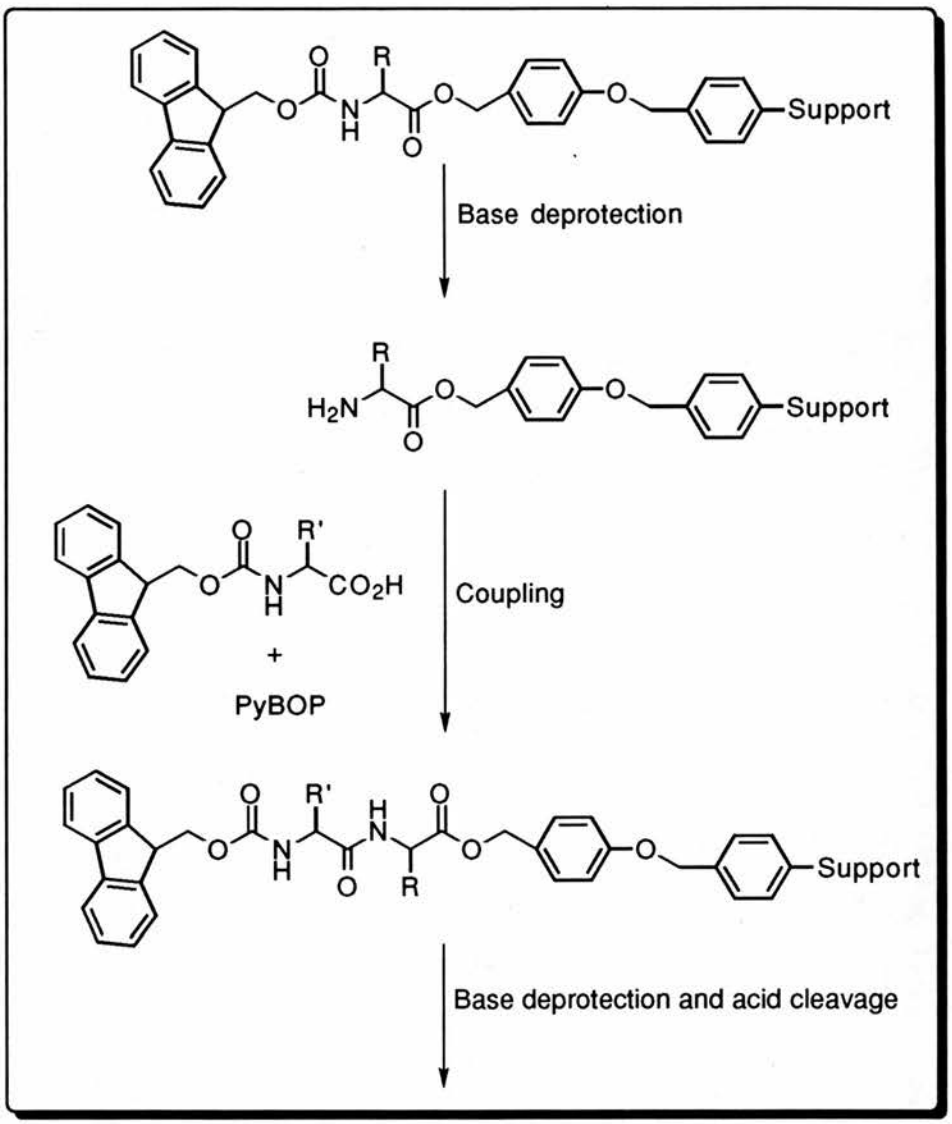
At this point, due to the difficulties facing the solution phase synthesis of the hexapeptide sequence, alternative methods of peptide assembly were considered.

2.5 Solid Phase Peptide Synthesis

In solid phase peptide synthesis (SPPS), an N-α-derivatised amino acid is attached to an insoluble (solid) support *via* a linker. The amino acid may be attached directly to the linker-support or first attached to the linker with subsequent attachment of the amino acid-linker to the support. The N-α-blocking group is then removed (deprotected) and the amino acid-linker-support is thoroughly washed with solvent. The next amino acid, N-α-protected, is then coupled to the amino acid-linker-support as either a preactivated species (*i.e.* symmetrical anhydride, active ester) or directly (*in situ*) in the presence of the activator. Coupling may also be carried out using peptides instead of single amino acids, where the peptide is N-α and side chain protected. Protected peptide coupling is referred to as

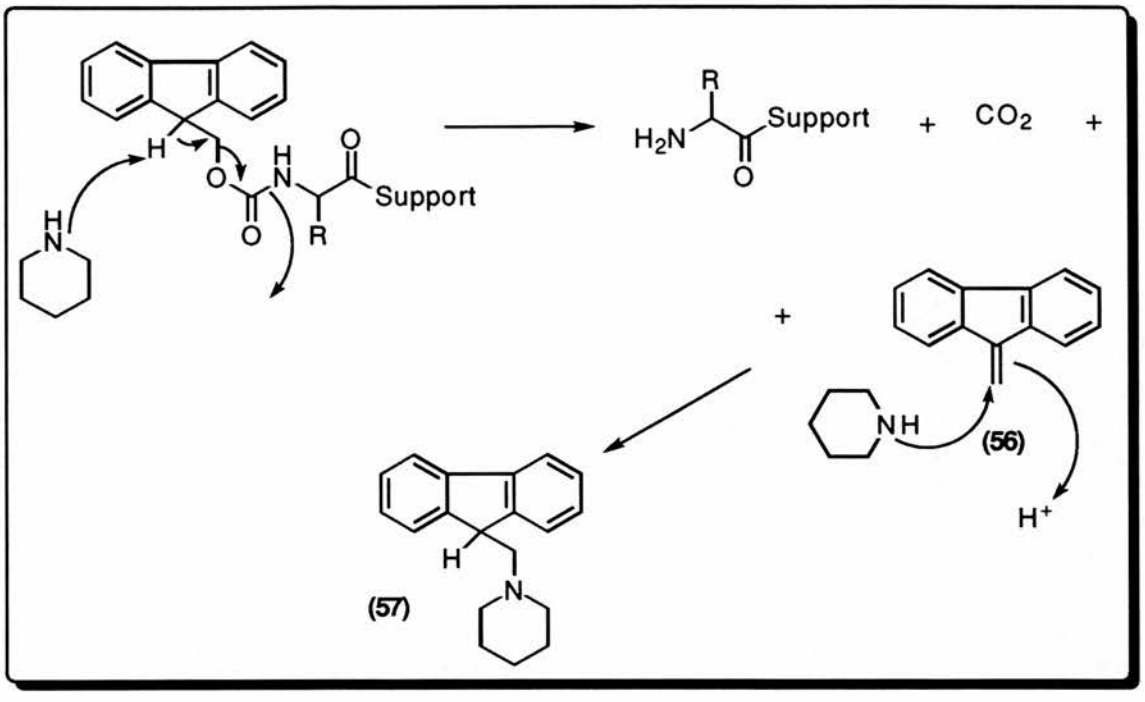
fragment (or segment) condensation. After this reaction is complete, the N-α-dipeptide (or oligopeptide)-linker-support is washed with solvent. The deprotection/ coupling cycle is repeated until the desired sequence of amino acids is generated. The peptide-linker-support is cleaved to obtain the peptide as a free acid or amide, depending on the chemical nature of the linker. Ideally, the cleavage reagent also removes the amino acid side chain protecting groups, which are stable to the deprotection reagent. These steps may be carried out as either a batch process, where the support is filtered between each step, or as a continuous flow process, where the support is always solvated during reagent exchange.[225]

The solid phase peptide synthesis method developed by Merrifield,[226] utilises a Boc-amino blocking group. The repeated acidolysis required for Boc deprotection (which results in side reactions), prompted the development of other protecting groups. Among these, the base-labile N-α-protecting group, 9-fluorenylmethoxycarbonyl (Fmoc) group has been used in SPPS since 1978 (Scheme 2.9).[227,228] The Fmoc group is, in general, rapidly removed by primary (cyclohexylamine, ethanolamine) and some secondary (piperidine, piperazine) amines, and slowly by tertiary (triethylamine, N,N-diisopropylethylamine) amines. Removal is also more rapid in polar media (DMF) than non-polar ones (DCM). The Fmoc group is most often deprotected in solid phase peptide synthesis by 20-50% piperidine/ DMF, although other amines have also been used.



Scheme 2.9: *General principle of Fmoc solid phase peptide synthesis.*

The electron withdrawing fluorene ring system 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 (**56**). The dibenzofulvene thus formed is susceptible to attack by piperidine to form a stable fulvene-piperidine adduct (**57**) (Scheme 2.10).



Scheme 2.10: *Deprotection of the base-labile Fmoc group.*

Fmoc amino acid (or fragment) acylation reactions must be highly efficient to ensure homogeneous peptide products. Obtaining optimal conditions is highly dependent on the nature of the acylation (activating) agent and amino acid activated species, solvation of the resin-bound growing peptide chain and potential side reactions.

Coupling of amino acids in SPPS can be carried out by means of a preformed symmetrical anhydride, a preformed mixed anhydride, an acid chloride, an active ester or *in situ.* The latter is the direct addition of the activating agent and the N-α-protected amino acid to the resin and is the method employed for the synthesis of peptides in our work. The solvation of the peptide-resin is perhaps the most crucial condition for peptide synthesis. Efficient solvation of cross-linked polystyrene leaves the linear peptide chains as accessible as if free in solution. The ability of the peptide-resin to swell increases with increasing peptide length due to a net decrease in free energy from solvation of the linear peptide chains. Therefore, swelling of the peptide-resin should theoretically never limit amino acid couplings, provided that proper solvation conditions exist. Polar solvents, such as DMF

and trifluoroethanol (TFE), have been shown to enhance peptide-resin swelling and thus improve coupling efficiency.

Fmoc SPPS is primarily designed for acidolysis of the peptide-linker. Since non-acidic conditions are utilised for N-α-amino group deprotection, weak acids can be used for cleavage and side chain deprotection. The majority of linkers and side chain protecting groups for Fmoc SPPS have therefore been designed with this in mind. Strong acid cleavage is also compatible with Fmoc amino acid side chain protecting groups, however, weak acid cleavage (TFA) was the method of choice in our reactions. General side chain protection by ^{t}Bu (for Asp, Glu, Ser, Thr and Tyr), Boc (for Arg, His, Lys), Pmc (for Arg) and others permit for simultaneous cleavage and deprotection. This however, gives rise to carbocations which are capable of side reactions and must therefore be scavenged. The use of the appropriate scavenger is dependent on the side chain protecting groups within the peptide sequence.

Although the use of Fmoc SPPS has been greatly advanced since its initial usage in 1978, certain problems still exist, such as Arg side chain protection and peptide-resin solvation.[225]

2.6 Solid Phase Synthesis of the Peptide Sequence

The SPPS of the substrate sequence was carried out on a Rainin PS3 automated peptide synthesizer. Fmoc methodology was utilised (see experimental section) and peptide couplings were carried out *in situ* as discussed previously. The resin used in all cases was the Wang resin (Fig. 2.1) which is a weak acid (TFA)-labile resin. The Arg residues were initially NO_2- and later Pmc-protected, and the Thr residue was introduced without side chain protection. Double couplings of the amino acids Arg, Thr and Val were carried out to ensure maximum coupling efficiency. The stereochemistry of all of the amino acids is (*S*).

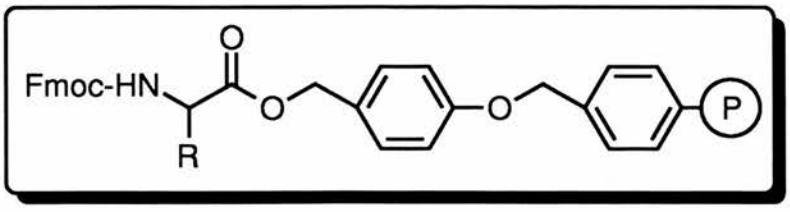
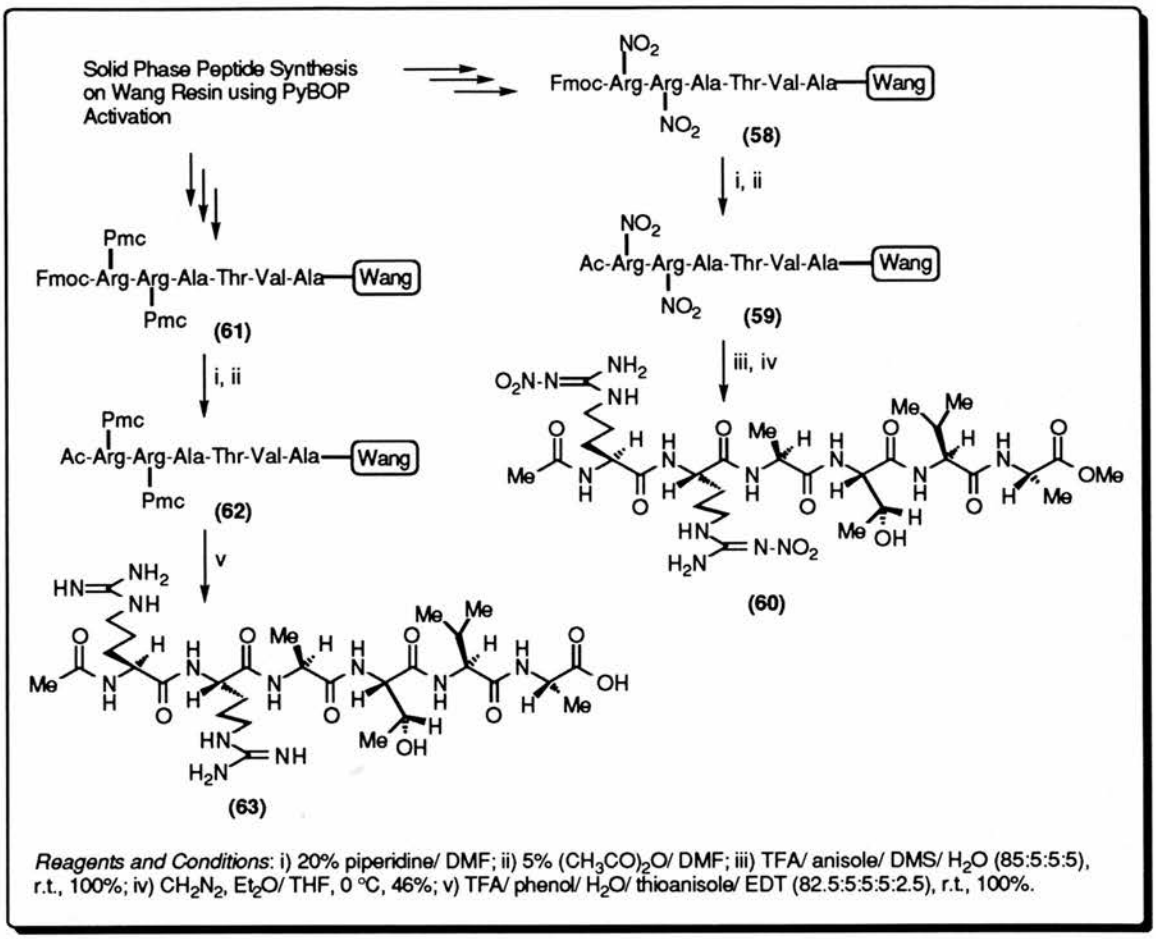


Figure 2.1: *Structure of a pre-loaded Wang resin.*

The N-α-Fmoc group was removed by a 20% piperidine/ DMF solution and a 5% NMM/ DMF solution was used for activation of the peptides by PyBOP. The cleavage mixture used initially, when the Arg residues were NO_2 protected, was TFA/ anisole/ DMS/ water (85:5:5:5). This was later changed to a mixture of crystalline phenol/ 1,2-ethanedithiol/ thioanisole/ water/ TFA (5:2.5:5:5:82.5), also known as reagent K,[229] when the Arg residues were Pmc protected. The Pmc group is more TFA-labile than other protecting groups of Arg[230-232] and is therefore preferred, especially in sequences containing multiple Arg residues. A soft nucleophile (thioanisole) has been shown to accelerate Arg(Pmc) deprotection by TFA and water seems to be an essential scavenger.

Fmoc-R(NO_2)R(NO_2)ATVA-Wang resin **(58)** was synthesised as described and the N-terminal Fmoc group was removed using 20% piperidine/ DMF (Scheme 2.11). To assess the feasibility of introducing an acetyl moiety at this stage and obtaining optimum conditions for this reaction, a cold solution of acetic anhydride (5% $(CH_3CO)_2O$/ DMF) was employed to protect the N-terminal. The peptide **(59)** was then cleaved from the resin using the cleavage mixture TFA/ anisole/ DMS/ water (85:5:5:5) to yield the free acid in near quantitative yield {m.p. 240 °C (decomp.)}. Methyl ester derivatisation of the free acid using diazomethane followed by lyophilisation gave the hexapeptide **(60)** as a solid {m.p. 260 °C (decomp.)}. The fully protected peptide **(60)** was now ready for phosphorylation (Scheme 2.11).



Scheme 2.11: *Synthesis of the resin-bound peptides.*

The synthesis of Fmoc-R(Pmc)R(Pmc)ATVA-Wang resin **(61)** was carried out in a similar manner. Fmoc deprotection, N-α-acetyl protection, cleavage of the resin and the Pmc protecting groups using reagent K and lyophilisation afforded Ac-RRATVA-OH **(63)** (Fig. 2.2) as a white solid {m.p. 174 °C; *m/z* (FAB) 714 (13.51%, M^+), 656 (14.86, $[M - Ac]^+$) and 133 (100, $[C_4H_6NO_3 + NH_3]^+$)} (Scheme 2.11).

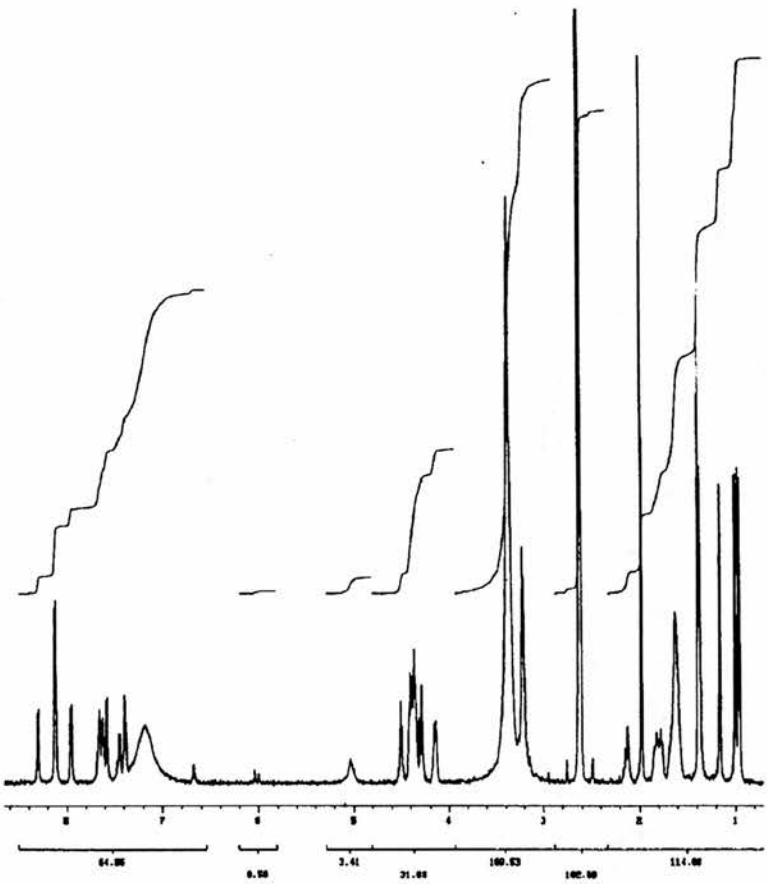
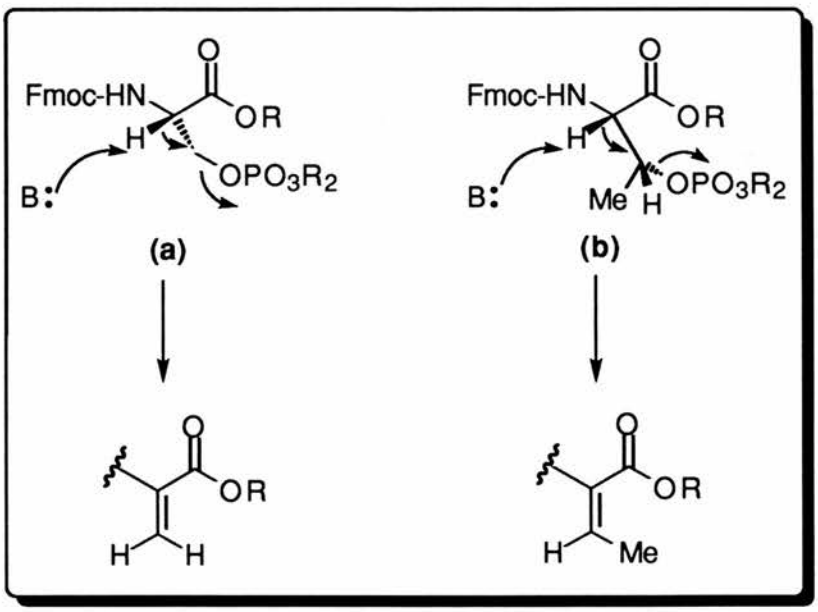


Figure 2.2: *^1H nmr spectrum of* ***(63)***.

2.7 Solution Phase Phosphorylation

Solution phase phosphorylation is applicable to the synthesis of small phosphopeptides and has been carried out successfully for the synthesis of a number of phosphopeptides. Solution phase phosphorylation provides two alternative possibilities: (i) preparation of the fully protected peptide, followed by phosphorylation of the hydroxyl function of serine/ threonine[233] or alternatively, (ii) incorporation of the phosphate group as a suitably protected phosphoamino acid. This involves the solution phase synthesis of protected serine/ threonine amino acids,[234,235] followed by subsequent incorporation of this in the peptide chain. Successful application of the latter procedure involves the use of a suitable N-terminal protection group. For instance, the base-labile N-α-Fmoc protecting group cannot be employed for the synthesis of phosphoserine/ phosphothreonine amino acids. Conditions necessary for the removal of the Fmoc group will lead to β-elimination in these

amino acids (Scheme 2.12). However, it can be used for the synthesis of protected phosphotyrosine residues, since there is no danger of β-elimination in the tyrosine residue upon removal of the Fmoc group.



Scheme 2.12: *β-elimination in serine- (**a**) and threonine-containing (**b**) peptides.*

Commonly used phosphorylating agents fall into two main categories of phosphorohalidates and phosphoramidites. The former group consists of the phosphorochloridates, $(RO)_2POCl$ **(64)** (Fig. 2.3); the most commonly used reagents in this group are benzyl or phenyl esters [*i.e.* in **(64)**, R=Bn or Ph]. Reviews of the recent literature[235-237] on phosphorylation procedures express concern over the use of the phosphorochloridates. Their use as phosphorylating agents for peptide substrates has been reported to give incomplete phosphorylation (for R=Bn), partial deprotection on hydrogenation (for R=Ph)[235] and formation of unknown byproducts.[238]

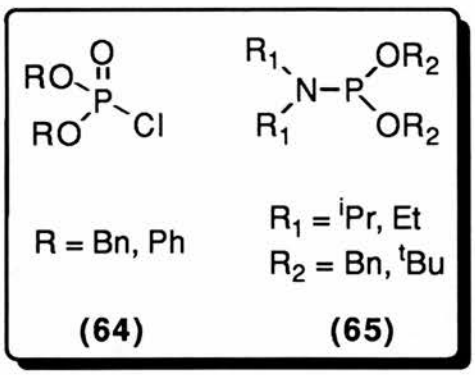
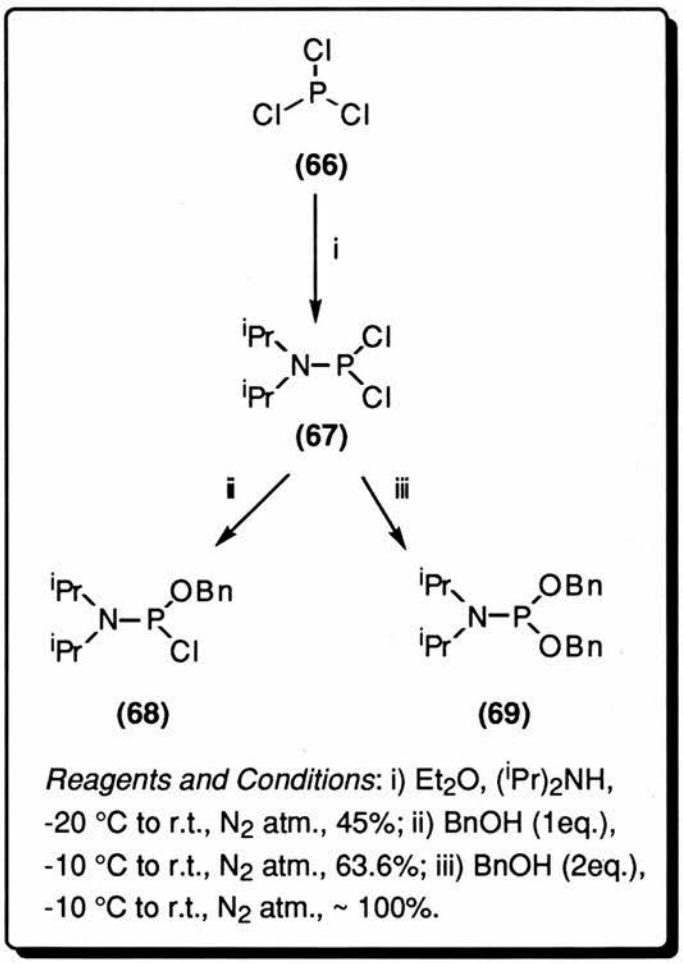


Figure 2.3: *General structure of phosphorochloridates (**64**) and phosphoramidites (**65**).*

Phosphoramidites **(65)** (Fig. 2.3), on the other hand, are thought to be stable and highly reactive reagents for the 'phosphite triester' phosphitylation of simple alcohols. The use of phosphoramidites, $(R_1)_2NP(OR_2)_2$, for the preparation of protected phosphoamino acids and phosphopeptides and also their application in oligosaccharide and oligodeoxynucleotide chemistry has been reported quite extensively.[233-237,239-245] These reagents have also been used for the preparation of protected hydroxyamino acid phosphoramidites[246] and ribonucleoside phosphoramidites and subsequently in the synthesis of phosphate diesters and oligoribonucleotides.[247,248] N,N-Diisopropyl- or N,N-diethyl- dibenzyl and di-^{t}butyl phosphoramidites are among the most commonly used reagents in this group.

We therefore chose to employ a phosphoramidite for our phosphitylating reactions. We initially chose N,N-diisopropyl dibenzylphosphoramidite, not only because of its regular use as a phosphitylating agent in the literature but also due to considerable experience with this reagent within the group.[249] N,N-Diisopropyl dichlorophosphoramidite **(67)** was prepared from the reaction of phosphorous trichloride **(66)** and dry diisopropylamine under a nitrogen atmosphere, followed by fractional distillation of the residue to give the product as a colourless oil in 45% yield {bp. 80-81 °C/ 8 mmHg (lit.,[249] 72-74 °C/ 7 mmHg}; δ_P (121.5 MHz, C^2HCl_3) 169.84 ppm}. The oil solidifies at -10 to -20 °C and is stable for months at this temperature. N,N-diisopropyl dichlorophosphoramidite can be

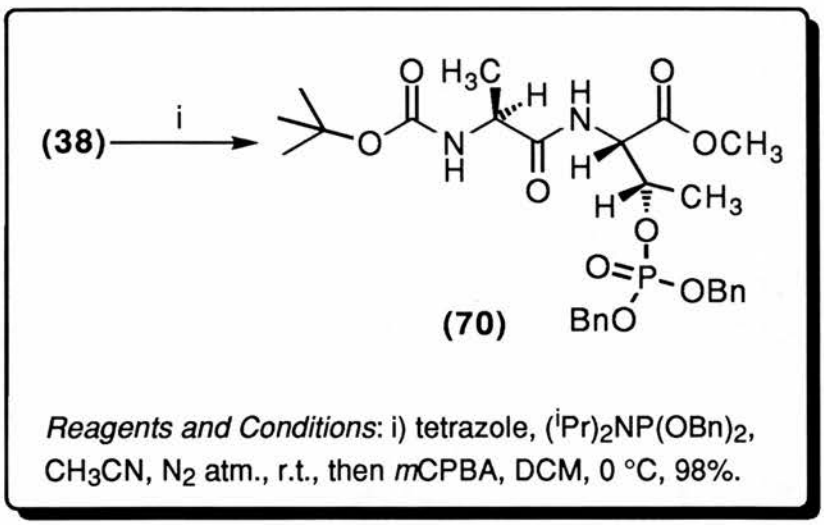
used as a precursor for the synthesis of a wide range of N,N-diisopropyl dialkylphosphoramidites. The reaction of **(67)** with benzyl alcohol under a nitrogen atmosphere yielded N,N-diisopropyldibenzyl phosphoramidite **(69)** as a yellow oil in near quantitative yield {δ_P (121.5 MHz, C^2HCl_3) 148.1 ppm} (Scheme 2.13).



Scheme 2.13: *Preparation of the phosphoramidites (68) and (69).*

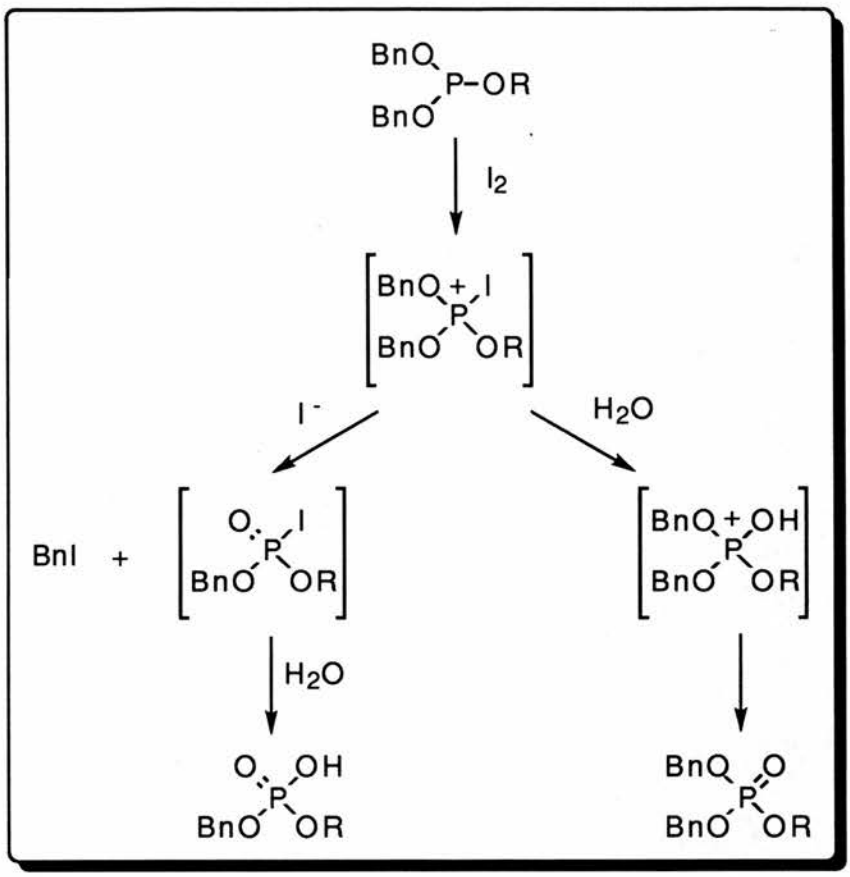
To assess the validity of the phosphitylating reaction of the peptide substrate, initial reactions were carried out on the fully protected dipeptide **(38)**. Treatment of **(38)** with N,N-diisopropyl dibenzylphosphoramidite and 1H-tetrazole under a nitrogen atmosphere afforded the phosphite triester of **(38)** which was oxidised, without isolation, under non-nucleophilic conditions using *m*-chloroperoxybenzoic acid (*m*CPBA) at 0 °C to give **(70)**

as a pale yellow oil in 98% yield {δ_P (121.5 MHz, C^2HCl_3) -0.75ppm; *m/z* (Found: [M + H]$^+$, 565.2311. Calc. for $C_{27}H_{38}N_2O_9P$: *m/z*, 565.2314)} (Scheme 2.14).



Scheme 2.14: *Synthesis of the phosphorylated triester (70).*

*m*CPBA is the oxidant of choice on account of its ready commercial availability, ease of handling and high reactivity. Other oxidation procedures have also been reported. However, the use of ^{t}butyl hydroperoxide has been reported to be associated with the formation of side products making purification difficult,[239] and the use of iodine/ water is thought to be unsuitable for the oxidation of benzyl and ^{t}butyl phosphites.[236] This latter oxidant is said to cause extensive benzyl and ^{t}butyl cleavage. The formation of the iodo phosphonium iodide activates the benzyl group to iodide-mediated debenzylation and this process competes with the addition of water (Scheme 2.15).



Scheme 2.15: *Iodide-mediated debenzylation.*

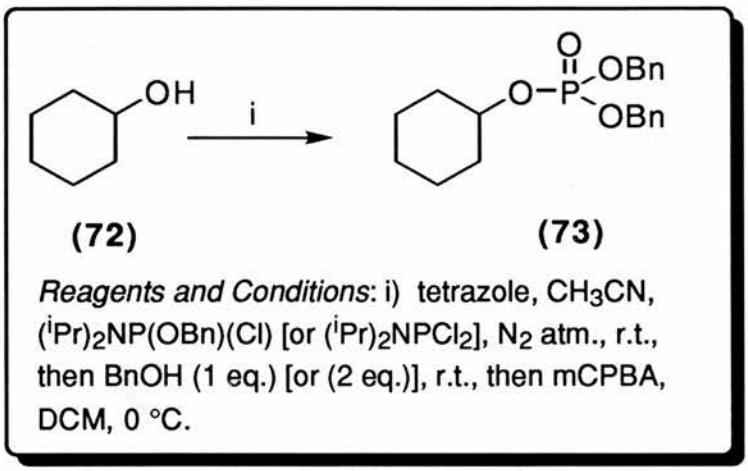
The phosphorylation of the hydroxyl function of the threonine residue in **(70)** was determined by ^{1}H and ^{13}C nmr spectroscopy. The ^{1}H nmr spectrum clearly shows the downfield shift of the β CH from 4.3 ppm in the unphosphorylated dipeptide **(38)** to 4.7 ppm in **(70)** and the ^{13}C spectrum shows a phosphorus-coupled doublet signal for the threonyl β CH at 53.0 (J_{PC} 11.8). Attempted Boc deprotection of the dipeptide **(70)**, for the possible incorporation of the N-deprotected dipeptide into the target sequence, met with failure resulting in partial loss of the benzyl ester protecting groups.

The above reaction established the use of N,N-diisopropyl dibenzylphosphoramidite as a feasible procedure for the solution phase phosphitylation of the substrate. The next step of the reaction, *i.e.* the oxidation step, however required further investigation. Therefore, trial reactions using Fmoc-(S)-Thr.OMe **(71)** and either *m*CPBA or ^{t}BuOOH as the oxidising

agent were carried out. Unlike the observations of Yu *et al.*, our reactions showed the oxidising agents to be of comparable utility as judged by nmr spectroscopy. Both reaction mixtures required purification by flash column chromatography. We therefore decided to use a combination of N,N-diisopropyl dibenzylphosphoramidite and *m*CPBA to phosphorylate our peptide sequence. This however proved difficult; attempted phosphorylations of Fmoc-R(NO_2)R(NO_2)-ATVA.OMe {*m/z* (Found: $[M + H]^+$, 999.4616. Calc. for $C_{43}H_{63}N_{14}O_{14}$: *m/z*, 999.4648)} and the hexapeptide (**60**) failed to give the desired product and instead yielded compounds that could not be identified. No definite or conclusive reasons can be given for the results obtained from these reactions, however, steric hindrance could play a role in determining the course of the reaction. The presence of two arginine residues with their long extended side chain and nitro protection N-terminal to the threonine as well as a valine immediately C-terminal to it, coupled with the inherent bulkiness of the benzyl groups in N,N-diisopropyl dibenzylphosphoramidite, might prevent interaction between the phosphitylating agent and the peptide. The problems of aggregation and β-sheet formation have been well documented in solution phase peptide synthesis.[250-253] The changes in physico-chemical properties of a peptide chain in solution are induced by conformational transitions of the peptide. The conformational transition that affects the solubility and reactivity of a peptide chain most is the β-structure. Hydrophobic residues such as Ala, Val and Ile have a strong tendency to form β-structures. β-Sheet formation occurs primarily at chain lengths of less than 20 residues. The critical chain length to form intermolecularly hydrogen-bonded conformations in solution has been determined to be n= 6-7.[254] β-Sheet aggregation in the hexapeptide sequence could therefore be a contributory factor to the phosphorylation reaction being unsuccessful. The reactivity of N,N-diisopropyl dibenzylphosphoramidite was also questioned and it was postulated that the monobenzyl or the precursor dichloro phosphoramidites would be more reactive and efficient phosphitylating agents.

N,N-Diisopropyl monobenzylchlorophosphoramidite (**68**){δ_P (121.5 MHz, C^2HCl_3) 7.40 ppm; *m/z* (CI) 256 (13%, $[M - Cl + NH_4]^+$), 238 (3, $[M - Cl]^+$), 148 (6, [M - Cl - CH_2Ph

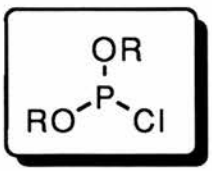
+ H]$^+$), 102 (100, [iPr_2NH + H]$^+$) and 91 (9, CH_2Ph^+)} was prepared in a similar manner to the N,N-diisopropyl dibenzyl derivative using only one equivalent of benzyl alcohol. This reagent however was not as clean as the other phosphoramidites that were prepared. Trial reactions on cyclohexanol (**72**) using either the dichloro, monobenzyl or dibenzyl phosphoramidites were carried out as described previously. In the case of the monobenzyl or dichloro derivatives, after the formation of the phosphite, the reaction mixtures were stirred with one or two equivalents of benzyl alcohol respectively and then oxidised *in situ* as outlined previously (Scheme 2.16).



Scheme 2.16: *Synthesis of cyclohexyl dibenzyl phosphate (73).*

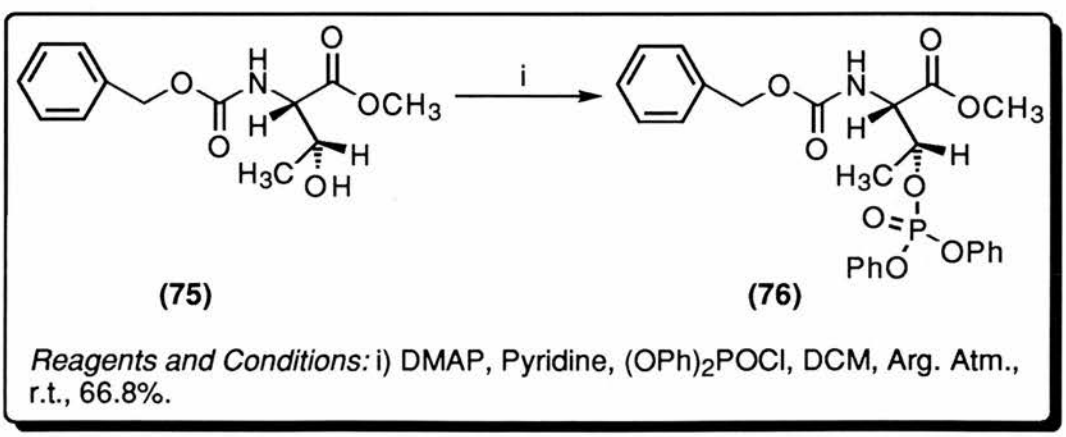
Although the dichloro phosphoramidite was slightly more reactive than the other two reagents, this did not exert a profound effect on the rate or extent of reaction. All of the reaction mixtures that were obtained required purification by flash column chromatography. Changing the solvent (THF or CH_3CN) or base (Et_3N or DIPEA) did not seem to alter the results of the reactions. We therefore concluded that a change in the phosphoramidite used would not improve the phosphorylation reaction of the substrate peptide.

In our hands, the use of phosphoramidites had met with little success and we therefore decided to try an alternative group of reagents. Two alternatives were available, phosphorochloridites [$(RO)_2PCl$] (**74**) or phosphorochloridates [$(RO)_2POCl$] (**64**).



General structure of phosphorochloridites (74).

The phosphorochloridites, although more reactive, require an oxidative step for the conversion of the formed phosphite to phosphate. Phosphorochloridates, however, do not require an oxidative step, their synthesis and use being well documented.[235,255-261] Dibenzyl- and diphenyl-phosphorochloridates are two of the most widely used reagents in this group, reacting with amines and alcohols to give aminophosphonates and phosphoric esters. The benzyl and phenyl protecting groups of the products can be conveniently removed by hydrogenolysis. The commercially available diphenylphosphorochloridate was therefore used in trial reactions using a range of secondary alcohols, Fmoc-(*S*)-Thr.OMe (**71**), Z-(*S*)-Thr.OMe (**75**) and cyclohexanol (**72**). In all cases, reaction of the alcohol in the presence of DMAP and either dry pyridine or Et_3N as the base, under an argon atmosphere gave the desired product (Scheme 2.17).



Scheme 2.17: *Preparation of the diphenyl phosphate triester (76).*

The reaction mixtures obtained with (**71**) and (**72**) required purification by flash column chromatography to give the respective phosphate triester products as oils, (**77**) {δ_P (121.5

MHz, C^2HCl_3) -12.52 ppm; *m/z* (Found: $[M + H]^+$, 588.1778. Calc. for $C_{32}H_{31}NO_8P$: *m/z*, 588.1787)} and **(78)** {δ_P (121.41 MHz, C^2HCl_3) -12.06 ppm; *m/z* (Found: $[M + H]^+$, 333.1249. Calc. for $C_{18}H_{22}PO_4$: *m/z*, 333.1255)} (Fig. 2.4), whereas the reaction of **(75)** yielded the pure phosphate triester **(76)** without further purification {δ_P (121.5 MHz, C^2HCl_3) -12.25 ppm; *m/z* (Found: $[M + H]^+$, 500.1478. Calc. for $C_{25}H_{27}NO_8P$: *m/z*, 500.1474)}. Diphenylphosphorochloridate therefore seemed like a suitable reagent for our purposes. However, attempted phosphorylations of the hexapeptide **(60)** failed to give the desired product, probably due to the same reasons as for the phosphoramidites. Synthesis of the phosphopeptide remained very elusive and it was concluded that solution phase phosphorylation of the substrate sequence should be abandoned in favour of alternative methods of phosphorylation.

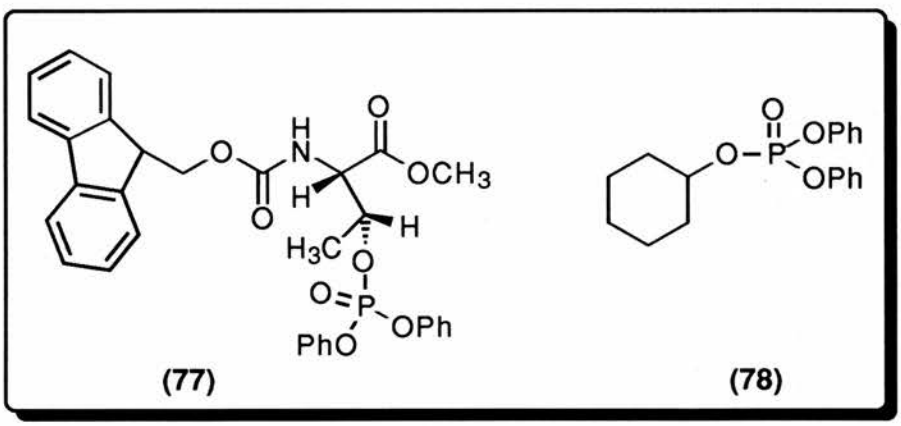


Figure 2.4: *Diphenyl phosphate triester of Fmoc-(S)-Thr.OMe (77) and cyclohexanol (78).*

2.8 Enzymic Phosphorylation

Among the factors responsible for the specificity of protein kinases, the primary structure around the phosphorylation site has been shown to play a relevant role. Evidence has emerged that different protein kinases are able to recognise residues of protein substrates characterised by distinct and definite amino acid sequences. The primary structure requirements of the cyclic AMP-dependent protein kinase have been extensively

investigated and the sequence RRATVA shown to be a substrate, although not as good a substrate as its serine homologue. We therefore decided to investigate the possibility of phosphorylating the sequence by a kinase.

Cyclic AMP-dependent protein kinase (from Sigma) and ATP were used in incubations of the substrate Ac-RRATVA-OH at 37 °C (pH 7.5) according to Meggio *et al.* (Appendix 2, procedure 1).[262] Aliquots were removed at 15, 30 and 45 min, the reactions terminated with 0.25 M HCl and the pH adjusted to 9. The mixtures were then applied to an AG1 x 2 anion exchanger (acetate form) and eluted with a degassed solution of 30% acetic acid.[263] The fractions were tested for the presence of the phosphopeptide by LDMS. No peaks corresponding to the phosphorylated peptide could be observed in any of the eluted fractions. Two possible reasons for this could be envisaged: (i) the above sequence was not a good substrate for the kinase, or alternatively (ii) some component in the incubation mixture was either preventing the phosphorylation of the substrate, or removing the phosphate incorporated by the kinase. Since a Thr residue is not a strong determinant for phosphorylation by kinases but at the same time a positive determinant for dephosphorylation by the phosphatases, presence of a phosphatase in the incubation mixture would remove all traces of any possible phosphopeptide formed. To test this hypothesis, sodium vanadate, a general inhibitor of the protein phosphatases, was added to the incubation mixtures in a final volume of 1 mM and the experiments repeated. Although not in appreciable quantities, some phosphopeptide was detected confirming the contamination of the cAMP-dependent protein kinase with a protein phosphatase. The mixtures were however mainly starting material, so we decided to employ a different kinase in the incubations in the hope of improving the yield of the phosphopeptide. Therefore, the catalytic subunit of protein kinase from bovine heart (from Boehringer Mannheim) (10 μg, equivalent to 61 mU) was incubated with the substrate at 37 °C (pH 7.5) as described previously and again no phosphopeptide was observed. A review of the amount of enzyme used in the literature (10 U)[208] brought us to the conclusion that the absence of phosphopeptide is most probably due to insufficient enzyme in the incubation mixture.

Due to the high cost of the catalytic subunit of the protein kinase, the use of this enzyme did not seem viable and further incubations were carried out with the cAMP-dependent kinase.

More incubations of the cAMP-dependent kinase and the substrate were carried out according to Agostinis *et al.*[208] at 30 °C (pH 6.5) (Appendix 2, procedure 2). This, as well as other experiments involving addition of more enzyme to the incubation mixtures or prolonged incubation times ranging from 3-6 hrs to overnight failed to improve the outcome. In all cases, although some phosphopeptide could be observed by LDMS, the amounts were not appreciable. The enzymic method of phosphorylation was, therefore, considered unsuitable for the preparation of large quantities of phosphopeptide.

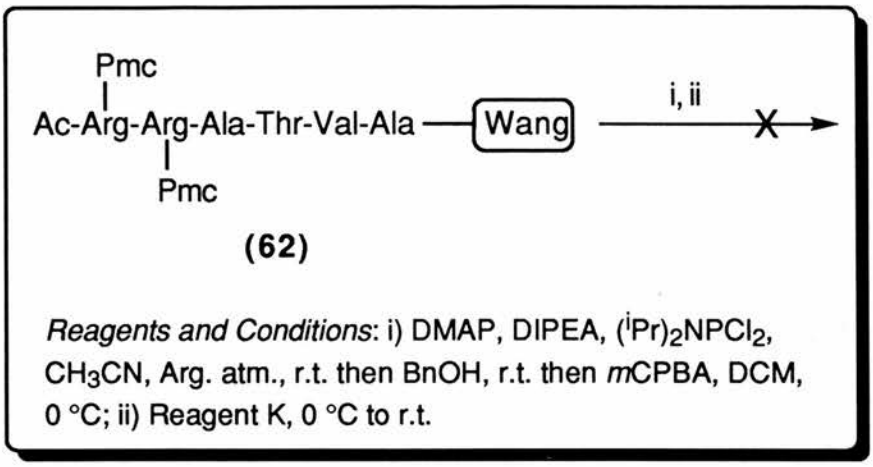
2.9 Solid Phase Phosphorylation

As in the case of solution phase, synthesis of phosphopeptides attached to a solid support provides two alternative strategies. The synthesis of a protected phosphoamino acid building block that can be introduced in the course of SPPS, or preparation of a peptide with an unprotected hydroxyl function followed by phosphorylation on the resin, also known as 'global phosphorylation'. A disadvantage of the first method is that the widely used Fmoc chemistry cannot be employed for peptides containing phosphotriesters of Ser/ Thr residues. The latter method was therefore chosen as the most favourable option for the synthesis of our phosphopeptide sequence. More recently, serine and threonine phosphodiester building blocks have been used for the synthesis of Ser/ Thr-containing phosphopeptides.[264] All of the following attempts at global phosphorylation on the solid phase were carried out on the sequence Ac-Arg(Pmc)-Arg(Pmc)-Ala-Thr(OH)-Val-Ala-Wang resin (**62**). The peptide-resins were all suspended in DMF prior to the phosphorylations to allow sufficient swelling of the resins. After completion of the reaction, the Wang resin and the Pmc groups were removed simultaneously by treatment with a mixture of TFA and appropriate scavengers.

It is interesting to note that most reports of phosphopeptide synthesis in the literature are for phosphotyrosine and phosphoserine peptides. Reports of peptides containing phosphorylated threonine are not only scarce but also, very often the yields for the synthesis of these peptides are much lower than the phosphotyrosine or phosphoserine containing peptides. This could be due to the fact that phosphorylation of a secondary alcohol is somewhat more difficult that an aromatic or primary alcohol.

2.9.1 Phosphoramidite Phosphorylations

The main bulk of the literature on the synthesis of phosphopeptides, either *via* the protected phosphoamino acid approach or the global phosphorylation method, reports the use of phosphoramidites as the phosphitylating agents.[265-272] These are considered to be the reagents of choice for the 'phosphite-triester' formation of alcohols, followed by subsequent oxidation of the phosphite to the desired phosphate. The choice of the best oxidising agent however, meets with some inconsistency. Each of the three main reagents generally employed for this purpose, namely *m*CPBA, ^{t}BuOOH or a solution of I_2, appear to be favoured by different authors. Our comparative studies on the use of either *m*CPBA or ^{t}BuOOH however, showed these two reagents to be similar and of comparative reactivity. On the basis of the information presented in the literature, we decided to use an appropriate phosphoramidite for phosphitylation and *m*CPBA/ ^{t}BuOOH for oxidation of our peptide. To ensure complete phosphorylation of the peptide, each step of the reaction was repeated at least once. From our earlier studies, we had established N,N-diisopropyl dichlorophosphoramidite to be slightly more reactive than the mono- and di-benzylated species. This reagent was therefore used in the phosphitylation reactions. Treatment of **(62)** with N,N-diisopropyl dichlorophosphoramidite (10 eq.) in the presence of DMAP/ dry DIPEA (10 eq.) under an argon atmosphere for 3 h followed by the addition of dry benzyl alcohol and oxidation by *m*CPBA (10 eq.) at 0 °C for 1 h afforded the resin-bound peptide. Deprotection of the peptide-resin using reagent K^{229} for 2 h, followed by precipitaion of the peptide with dry diethyl ether and lyophilisation failed to give the desired phosphopeptide (Scheme 2.18).



Scheme 2.18: *Attempted phosphorylation of* ***(62)*** *using a phosphoramidite.*

The ^{1}H nmr spectrum showed the isolated product to be Ac-RRATVA-OH **(63)**. This was confirmed by the absence of phosphorus-coupled doublet signals for the β-C of Thr in the ^{13}C nmr spectrum. It was, however, not clear whether the phosphorylation reaction had not proceeded at all or that the phosphate moiety had been removed during cleavage of the resin. Perich *et al.* report a similar case where the crude mixture of their phosphotyrosine containing peptide, obtained using Fmoc-Tyr(PO_3Bn_2).OH, only contained 10% of the desired product.[268] The authors remained uncertain as to the manner of loss of the phosphate group. In the case of our peptide sequence, if the phosphorylation reaction failed to take place, one possible explanation for this could be steric constraints. Steric constraints, especially due to secondary structure formation as a result of peptide chain association and aggregation, can hamper the phosphitylation reaction. Polar solvents such as DMF are usually sufficient to disrupt the peptide chain aggregates responsible for β-sheet formation. The solvents of choice for phosphorylation reactions are usually dry DCM/ CH_3CN. It therefore appears that although the petide-resins were suspended in DMF prior to the reactions, the ratio of the polar solvent to the non-polar solvents required for phosphorylation, might not have been sufficient to prevent secondary structure formation. The problem could be overcome by increasing the volume of polar solvent used

or suspending the peptide-resin in DMF for a longer period of time before initiating the phosphitylation reaction.

We therefore decided to try the phosphitylation reaction with a different phosphoramidite and to this end chose N,N-diisopropyl di-^{t}butylphosphoramidite. The ^{t}butyl group is an ideal phosphate protecting group for use in solid phase phosphorylation reactions. The acidic conditions required for the removal of the peptide from the resin will also remove the ^{t}butyl protecting groups within 1-2 h. Initial reactions using this phosphoramidite (5 eq.) in the same manner as described previously, followed by oxidation with *m*CPBA and deprotection using TFA/ H_2O/ TES (85:12:3) did not afford the desired product. The phosphitylation reaction was repeated using the di-^{t}butyl phosphoramidite (20 eq.). Treatment of the resin-bound peptide **(62)** with N,N-diisopropyl di-^{t}butylphosphoramidite in the presence of tetrazole (50 eq.) under an argon atmosphere, followed by oxidation with ^{t}BuOOH (20 eq.) afforded the peptide-resin which on deprotection using reagent K^{229} for 2 h gave a solid after lyophilisation (Scheme 2.19). The ^{1}H nmr of the compound showed some interesting features including the presence of two signals at 5.62 and 7.76 ppm (Fig. 2.5). This splitting pattern is reminiscent of a phosphonate, the J_{PH} of 641 Hz being typical for this type of compound. The major peak in the ^{31}P nmr spectrum at 5.21 ppm, also pointed to the formation of a phosphonate species **(79)**.

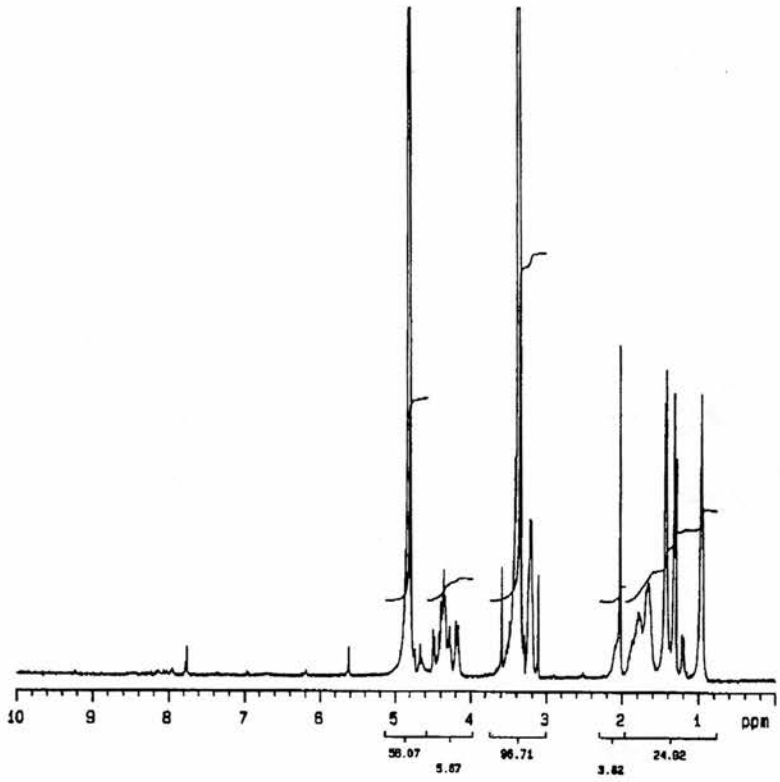
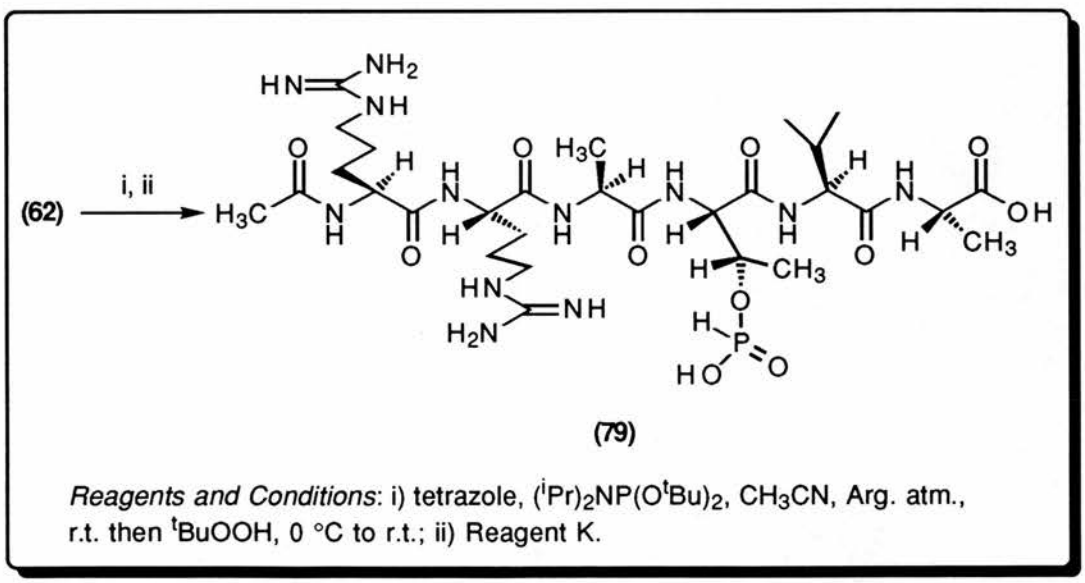


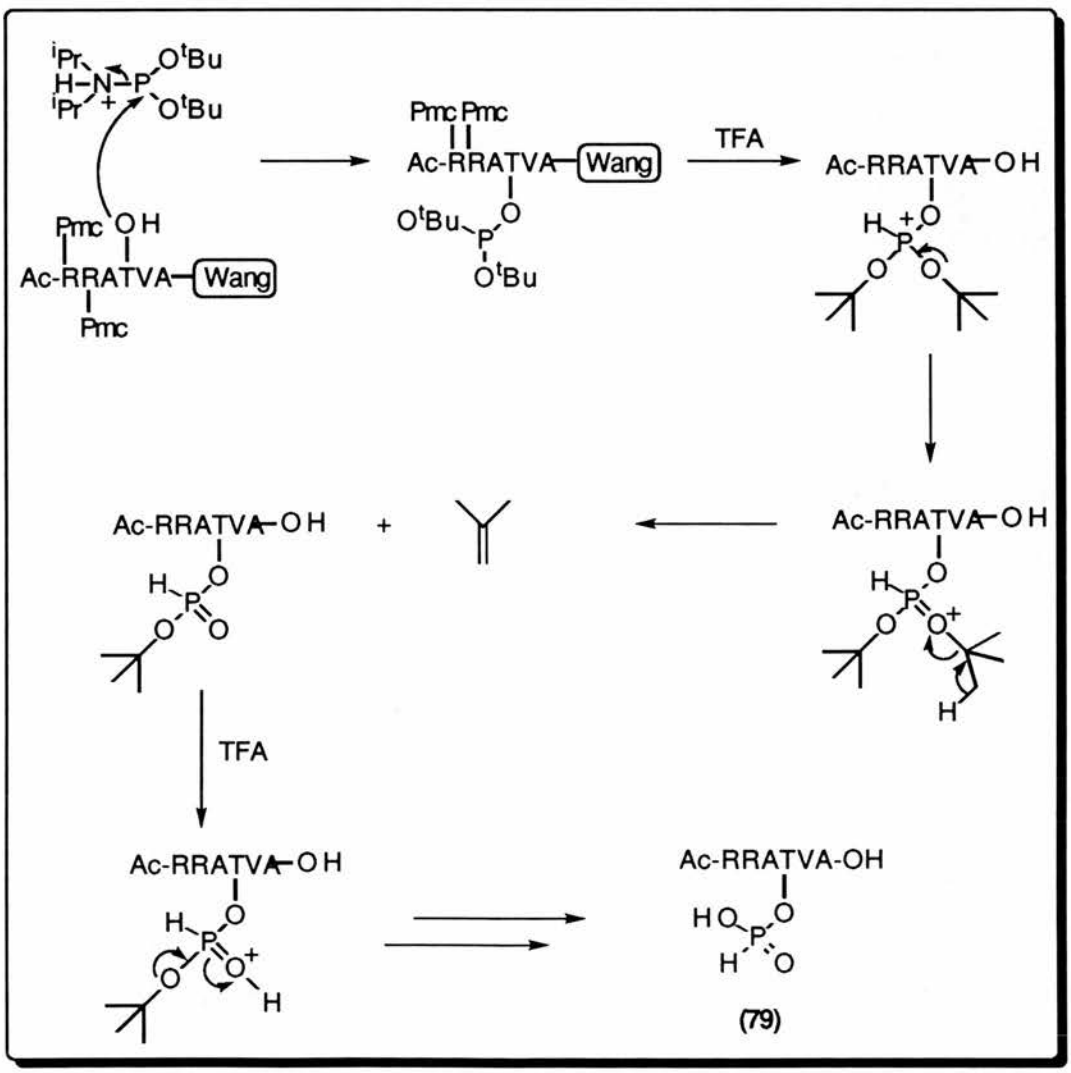
Figure 2.5: *^1H nmr spectrum of (79) showing the P-H splitting pattern.*



Scheme 2.19: *Formation of the H-phosphonate by-product (79).*

This was a very unexpected result and one that is not referred to in the mainstream literature. So far, only one other case of such a side reaction has been reported.[273]

Ottinger *et al.* report the formation of an H-phosphonate peptide by-product in their attempts to phosphorylate a tyrosine residue. Interestingly, the phosphitylating and oxidising agents used by Ottinger *et al.* were N,N-diethyl di-^{t}butylphosphoramidite and ^{t}BuOOH, the same reagents and conditions employed by us. Further evidence for the assignment of the by-product as an H-phosphonate peptide, was obtained from experiments where the oxidation stage was omitted. The major product from this experiment was found to be the H-phosphonate peptide.[273] The formation of this H-phosphonate can be attributed to incomplete oxidation of the phosphitylated intermediate followed by conversion to the phosphonate by the cleavage mixture (Scheme 2.20).



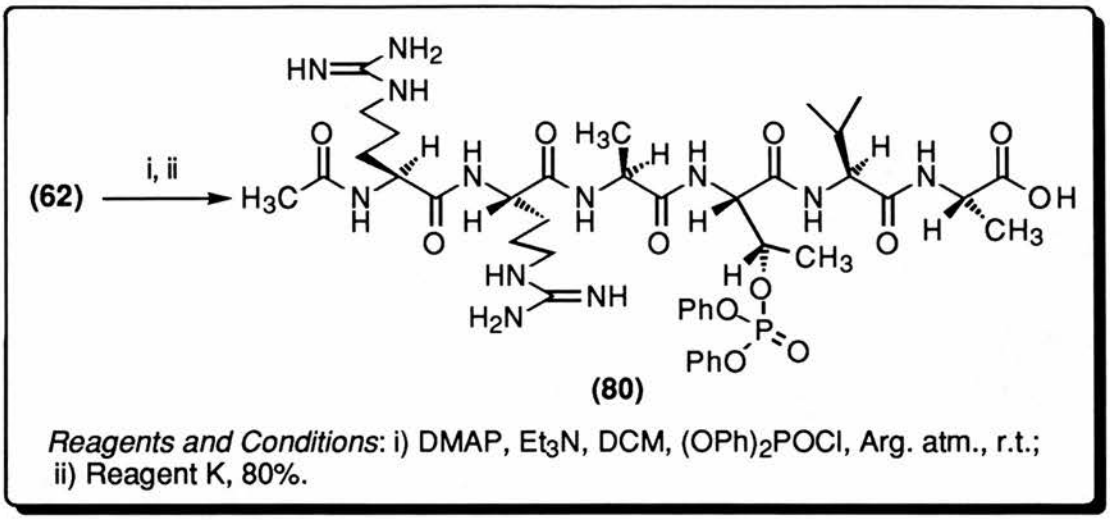
Scheme 2.20: *Possible mechanism for the formation of the phosphonate* ***(79)****.*

A change in the oxidation conditions however did not eliminate this by-product formation. At this point, phosphorylation of the peptide using a phosphoramidite did not seem plausible and alternative methods were sought.

2.9.2 Phosphorochloridate Phosphorylations

Although not a popular choice, phosphorochloridates have been employed in solid phase phosphorylations of peptides.[238,274,275] The ease with which our trial reactions in solution phase using diphenylphosphorochloridate had afforded the required phosphorylated species, combined with the fact that there was no need for an oxidative process using a phosphorochloridate, prompted us to investigate the efficiency of this reagent in solid phase chemistry.

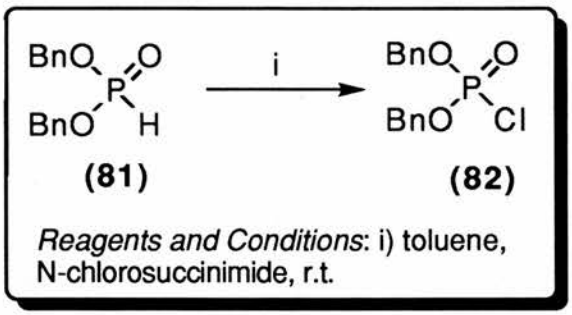
As in the case of the phosphoramidites, repeated phosphorylation reactions were employed with the use of the phosphorochloridates to ensure complete reaction. Treatment of the resin-bound peptide **(62)** with a five fold excess of diphenylphosphorochloridate in the presence of DMAP and dry Et_3N under an argon atmosphere for 6 h, followed by removal of the resin and lyophilisation gave a white fluffy solid. The ^1H nmr spectrum was not conclusive as to whether phosphorylation had taken place or not, however the ^{31}P nmr spectrum showed the presence of a small peak (after 34859 scans!) that could possibly correspond to the phosphorylated compound. The reaction was therefore repeated with a 20 fold excess of reagents (Scheme 2.21). The white solid that was obtained after lyophilisation in 80% yield was the pure diphenyl-protected phosphopeptide **(80)** {δ_P (121.42 MHz; ^2H_2O) -11.44 to -11.75 ppm}.



Scheme 2.21: *Synthesis of the diphenyl phosphate triester (80).*

The use of diphenylphosphorochloridate as a phosphorylating agent and the subsequent deprotection of the phenyl protecting groups have been reported.[275-287] The removal of the phenyl protection involves hydrogenolysis using platinum dioxide (Adams catalyst) or platinum/ activated carbon. Treatment of the phosphate triester **(80)** with Adams catalyst (1.0 eq./ phenyl group) under a hydrogen atmosphere at room temperature for 4-5 h, followed by removal of the catalyst by filtration and workup afforded an oil. ^{1}H Nmr spectroscopic analysis of the compound showed that removal of the phenyl protecting groups was incomplete. The reaction was therefore repeated and left to proceed for 24 h. Analysis of the products were again indicative of partial hydrogenolysis. A change of solvent from 1,4-dioxane to 40% TFA/ AcOH failed to yield the required deprotected product. Reports in the literature state complete removal of phenyl protection can be effected within 30 min to 3 h.[275-287] We did not find this to be the case and decided to attempt hydrogenolysis by a different catalyst, platinum/ activated carbon. The phosphate triester **(80)** was left to stir at room temperature with Pt/ C (10%)/ H_2 overnight, but these conditions again did not achieve full deprotection of the phosphate ester. The products of all of the above attempted deprotections displayed a ^{31}P spectrum that was almost identical to the starting material **(80)**, with a δ_P shift of -11.76 ppm. However, ^{1}H nmr spectra showed integrals corresponding to partial loss of the phenyl groups.

The successful synthesis of the phosphopeptide triester using diphenylphosphorochloridate prompted us to try the reaction with an alternate phosphorochloridate, one where the full deprotection of the phosphate protecting groups would be effected in a more facile and effective manner. To this end dibenzylphosphorochloridate was chosen, although we knew that phosphorylation would be less facile. The reaction of dibenzyl phosphite (dibenzyl phosphonate, **81**) with N-chlorosuccinimide[288] in dry toluene at room temperature for 2 h afforded dibenzylphosphorochloridate **(82)** {δ_P (121.41 MHz; d_8-toluene) main peak at 4.997 ppm} (Scheme 2.22). This compound decomposed on attempted distillation but can be used for phosphorylation reactions as a solution in toluene.

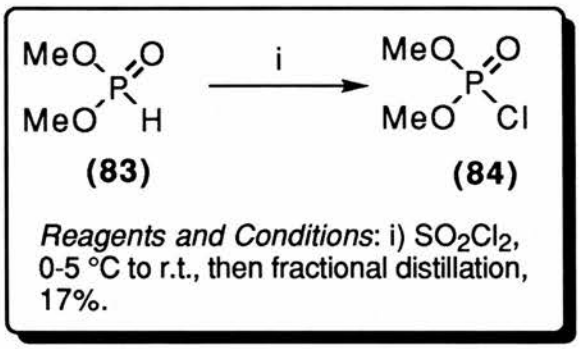


Scheme 2.22: *Preparation of dibenzylphosphorochloridate (82).*

Treatment of the resin-bound peptide **(62)** with dibenzylphosphorochloridate in toluene (20 eq.) overnight as described previously afforded the phosphate triester. Deprotection and cleavage using reagent K[229] gave a mixture of compounds as judged by ^{31}P nmr spectroscopy. The ^1H nmr spectrum showed that starting material was the major component. The phosphorylation reaction was repeated and again a mixture of compounds was obtained. The deprotection procedure had failed to remove the benzyl protection and the results obtained did not seem to be consistent. Dibenzylphosphorochloridate is unstable to distillation and can not be obtained as a single compound. The starting material used in its synthesis, dibenzyl phosphite, is also of technical grade and can not be purified. Reactions using the solution of dibenzylphosphorochloridate in toluene are, therefore, prone to produce a mixture of compounds. This situation presents the problem of extensive purification after deprotection and cleavage of the resin. The ^1H nmr spectrum of

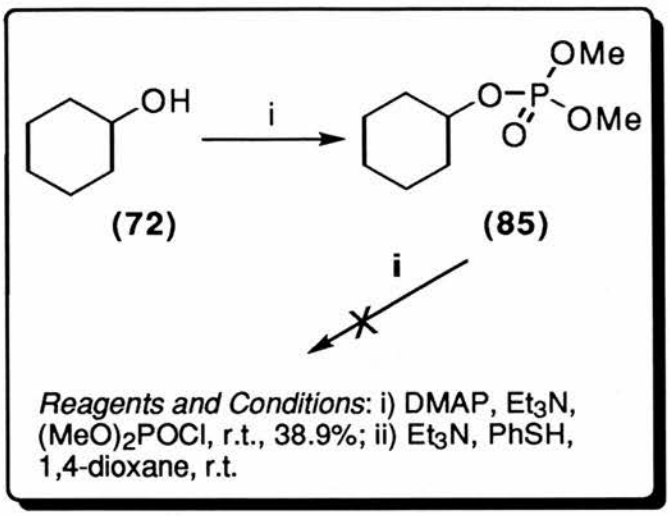
the products also indicated that the phosphorylation reaction using dibenzylphosphorochloridate did not go to completion and, therefore, the reagent did not seem to be viable in the synthesis of our phosphopeptide.

Dimethylphosphorochloridate (**84**) was prepared from the reaction of sulfuryl chloride and dimethyl phosphite (**83**)[289] at 0-5 °C and fractionally distilled to give the product as a clear oil in 17% (b.p. 75-80 °C/ 0.6 mmHg) {δ_P (121.41 MHz; C^2HCl_3) main peak at 7.73 ppm} (Scheme 2.23).



Scheme 2.23: *Preparation of dimethylphosphorochloridate (84).*

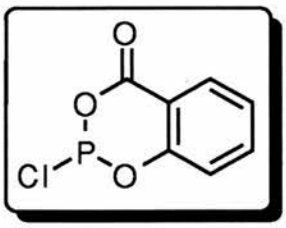
A trial reaction using cyclohexanol and dimethylphosphorochloridate in the presence of DMAP/ Et_3N afforded the triester (**85**), albeit in a low yield of 39% as an oil {*m/z* (Found: $[M + H]^+$, 209.0947. Calc. for $C_8H_{18}PO_4$: *m/z*, 209.0943); δ_P (121.41 MHz; C^2HCl_3) main peak at 0.37 ppm}. Attempted deprotection of (**85**) using a mixture of thiophenol, Et_3N and 1,4-dioxane at room temperature[290] failed to give the desired product (Scheme 2.24). The use of dimethylphosphorochloridate was, therefore, not considered further for the solid phase phosphorylation of the peptide.



Scheme 2.24: *Attempted deprotection of the dimethyl protecting groups.*

2.9.3 Phosphonate Phosphorylations

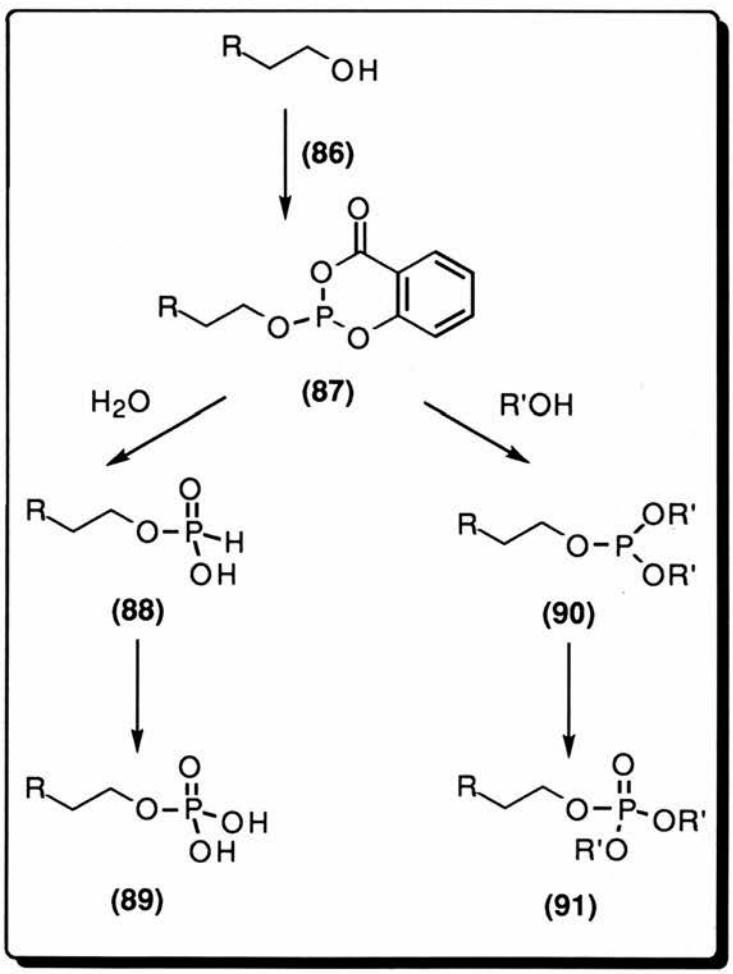
The use of phosphonate chemistry in the synthesis of phosphite intermediates and phosphates has mainly occurred in the field of carbohydrate chemistry.[291-297] There has only been one account of the use of phosphonates for the synthesis of phosphopeptides.[298] The most frequently used reagent for the preparation of phosphonates is 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one, also known as salicylchlorophosphite **(86)**.



Salicylchlorophosphite (86).

The easily accessible and crystalline reagent salicylchlorophosphite, was synthesised over a century ago by Anschütz *et al.*,[299] and its structure and fundamental chemistry established further by Young.[300] The general reaction of phosphonate formation involves the treatment of the hydroxyl function of the alcohol with the reagent **(86)** in the presence of a

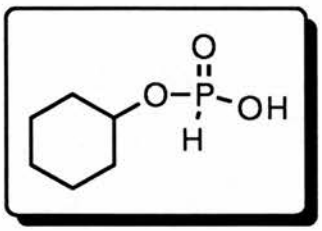
base at room temperature. Upon formation of the cyclic intermediate (**87**), water is added to the reaction mixture, which upon further work-up and purification, yields the phosphonate (**88**). The so formed phosphonate can then be oxidised to a phosphate (**89**). The cyclic intermediate can also be reacted with another alcohol to yield a phosphite intermediate (**90**) (Scheme 2.25). We therefore decided to establish the reactivity of salicylchlorophosphite in trial reactions using cyclohexanol as the secondary alcohol, with the possibility of further applications of this reagent in solid phase phosphorylations of our peptide.



Scheme 2.25: *General reaction of phosphonate formation.*

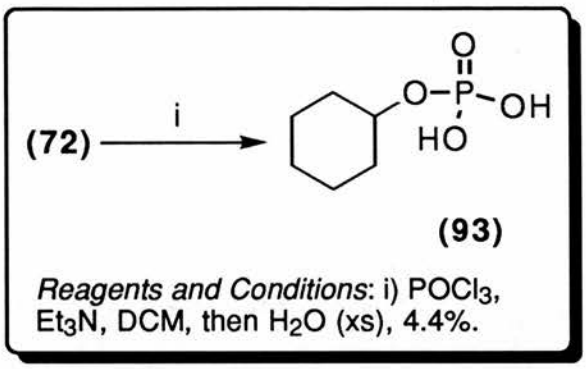
Treatment of cyclohexanol (**72**) and dry Et_3N in dry 1,4-dioxane with salicylchlorophosphite,[293] followed by the addition of water yielded a mixture of products that on

purification by silica column chromatography gave the phosphonate (**92**) in 35% yield {δ_P (121.41 MHz; C^2HCl_3) 4.47 ppm; *m/z* (Found: $[M + H]^+$, 165.0683. Calc. for $C_6H_{14}PO_3$: *m/z*, 165.0681)}.



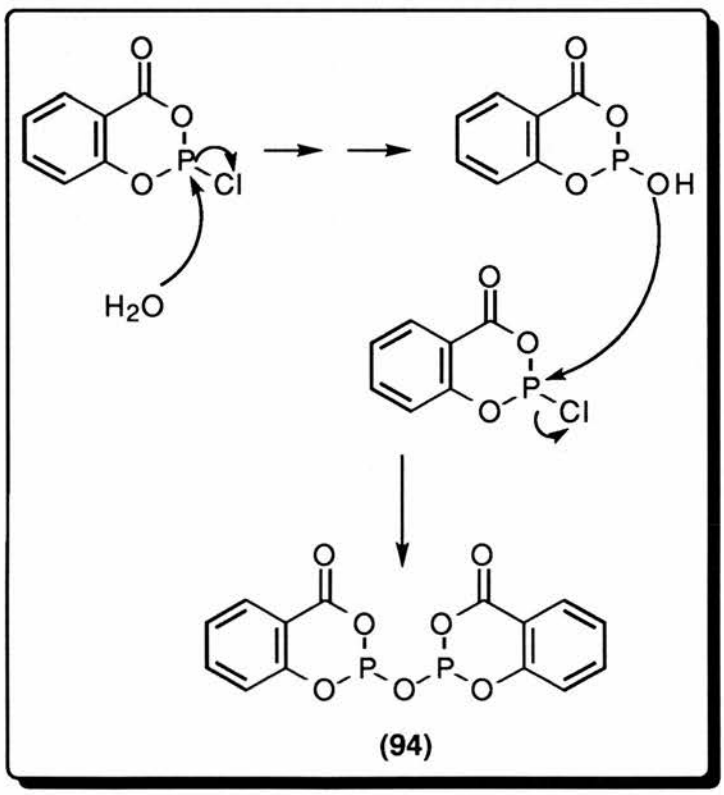
*Cyclohexylphosphonate (**92**).*

The characteristic feature of the phosphonate are the peaks at 5.1 and 8.54 ppm in the ^1H nmr spectrum. The coupling constant of 688 Hz is indicative of a P-H bond. Because the reaction of salicylchlorophosphite with the alcohol and subsequent work-up seemed rather straightforward, attempts were made to prevent side-product formation during the reaction and therefore improve the yield. Repeating the reaction under the same conditions gave a mixture of compounds that was subjected to silica column chromatography. Analysis of the product obtained after the purification appeared to indicate that the conversion of the phosphonate to a phosphate occurred on the silica column. The phosphate obtained was characterised by the absence of the P-H signals in ^1H nmr and in ^{13}C nmr spectra by the downfield shift of the CHOP signal from 74.79 ppm ($J_{P,C}$ 5.89) to 76.6 ppm ($J_{P,C}$ 6.29 Hz). There was also a change in the CH_2CHOP signals in ^{13}C nmr spectrum from a set of phosphorus coupled doublets in the phosphonate to a single doublet in the phosphate. The distinct shift in the ^{31}P signal from 4.47 ppm to -2.73 ppm was another diagnostic feature of this compound. The phosphate obtained in this manner was identical to a sample of cyclohexylphosphate (**93**) prepared from the reaction of cyclohexanol and phosphorus oxychloride in 4.4% yield {*m/z* (Found: $[M + H]^+$, 181.0634. Calc. for $C_6H_{14}PO_4$: *m/z*, 181.0630); δ_P (121.41 MHz; C^2HCl_3) -0.19 ppm} (Scheme 2.26). The purification of the phosphonate therefore did not appear to give consistent results and optimising reaction conditions to forego the need for column chromatography became important.



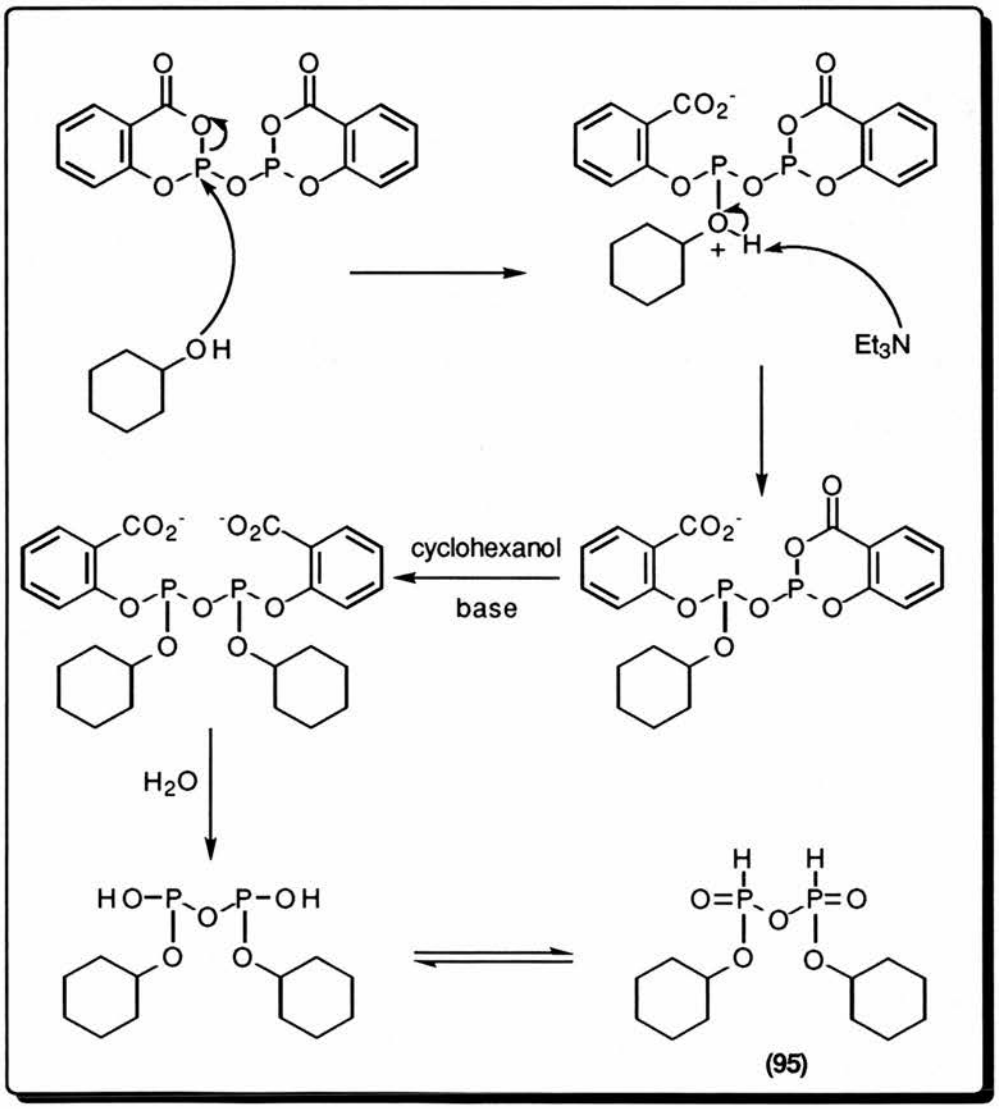
Scheme 2.26: *Synthesis of cyclohexylphosphate* ***(93)***.

The reagent salicylchlorophosphite is moisture-sensitive and prone to hydrolysis. It was therefore reasoned that the prolonged reaction times might be responsible for the breakdown of the reagent during reaction and subsequent formation of side-products from the hydrolysis compounds (Scheme 2.27).



Scheme 2.27: *Possible hydrolysis product* ***(94)*** *of salicylchlorophosphite.*

A time-course reaction using cyclohexanol was carried out where aliquots of the reaction mixture were removed at specified time intervals. The reaction of salicylchlorophosphite with the alcohol was shown to be complete within 10 min and the formation of two distinct products was observed by ^{1}H and ^{13}C nmr spectroscopy. An analysis of the ^{13}C nmr spectrum showed the presence of a set of phosphorus coupled doublets for the CH_2CHOP of the phosphonate and only a single phosphorus coupled doublet for the side product. This indicated a symmetrical compound and the possibility of the formation of a pyrophosphonate derivative **(95)** was considered likely (Scheme 2.28).

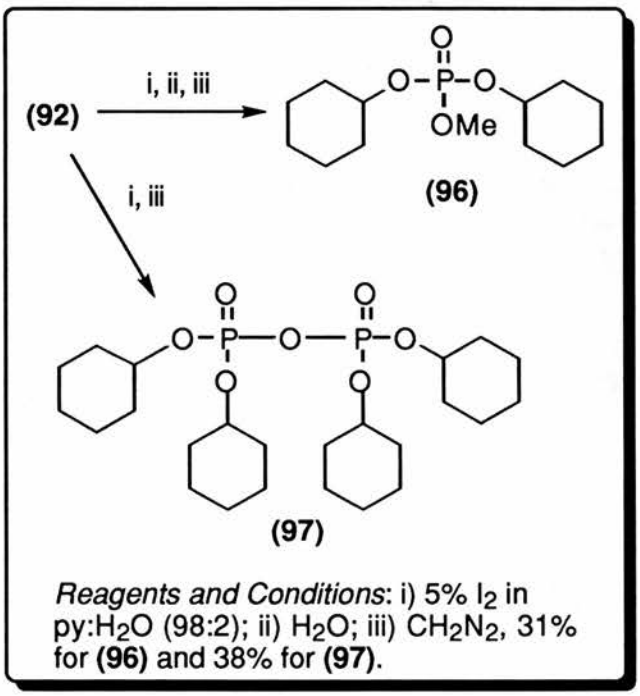


Scheme 2.28: *Possible formation of the pyrophosphonate (95) side product.*

The presence of a side product after just 10 min, prompted us to investigate the effect of increasing the number of equivalents of salicylchlorophosphite used on the formation of the side product. Increasing the salicylchlorophosphite 2-5 fold did not appear to have any significant effect. Although a change of base from Et_3N to pyridine seemed to give a higher proportion of the phosphonate (**92**), the improvement in the yield of the reaction was small. Further permutations on the reaction conditions (varying the number of equivalents of base and phosphorylating agent) failed to improve the overall reaction or the yield and attention was, therefore, concentrated on the oxidation step.

The oxidation of phosphonates to their respective phosphates has been the subject of a study by Garegg *et al.*[301] The most efficient oxidation procedure was found to be the treatment of the phosphonate with iodine in pyridine-water. This procedure has also been used by others[291-293,295,296,298,302] in the preparation of phosphates from phosphonates. Treatment of the purified cyclohexylphosphonate (**92**) with iodine (1-5%; w/ v) in pyridine-water (98:2; v/ v) gave conflicting results. These varied from no reaction at all to a reaction where a mixture of compounds was obtained. ^{31}P Nmr spectroscopic analysis of the mixture revealed that a contaminant was the major product {δ_P (121.41 MHz; C^2HCl_3) -14.77 ppm} and that the required phosphate and some unreacted phosphonate were minor components. A trial reaction was carried out to identify the side product formed in the oxidation process. Cyclohexylphosphonate (**92**) was treated with I_2/ pyridine:water as outlined previously and the reaction mixture was divided into two portions. One half was worked up as before and the other half was poured into water. In both cases, a mixture of compounds was obtained as judged by ^1H and ^{31}P nmr spectroscopy. Both mixtures were then treated with ethereal diazomethane. The reaction that was poured into water, was found to contain a compound with nmr signals corresponding to the presence of a methyl ester group. The structure of this compound was elucidated with the aid of mass spectrometry and was found to be dicyclohexyl methyl phosphate (**96**) {*m/z* (Found: $[M + H]^+$, 277.1572. Calc. for $C_{13}H_{26}PO_4$: *m/z*, 277.1569); δ_P (121.41 MHz; C^2HCl_3) main peak at -0.995 ppm} (Scheme 2.29). The

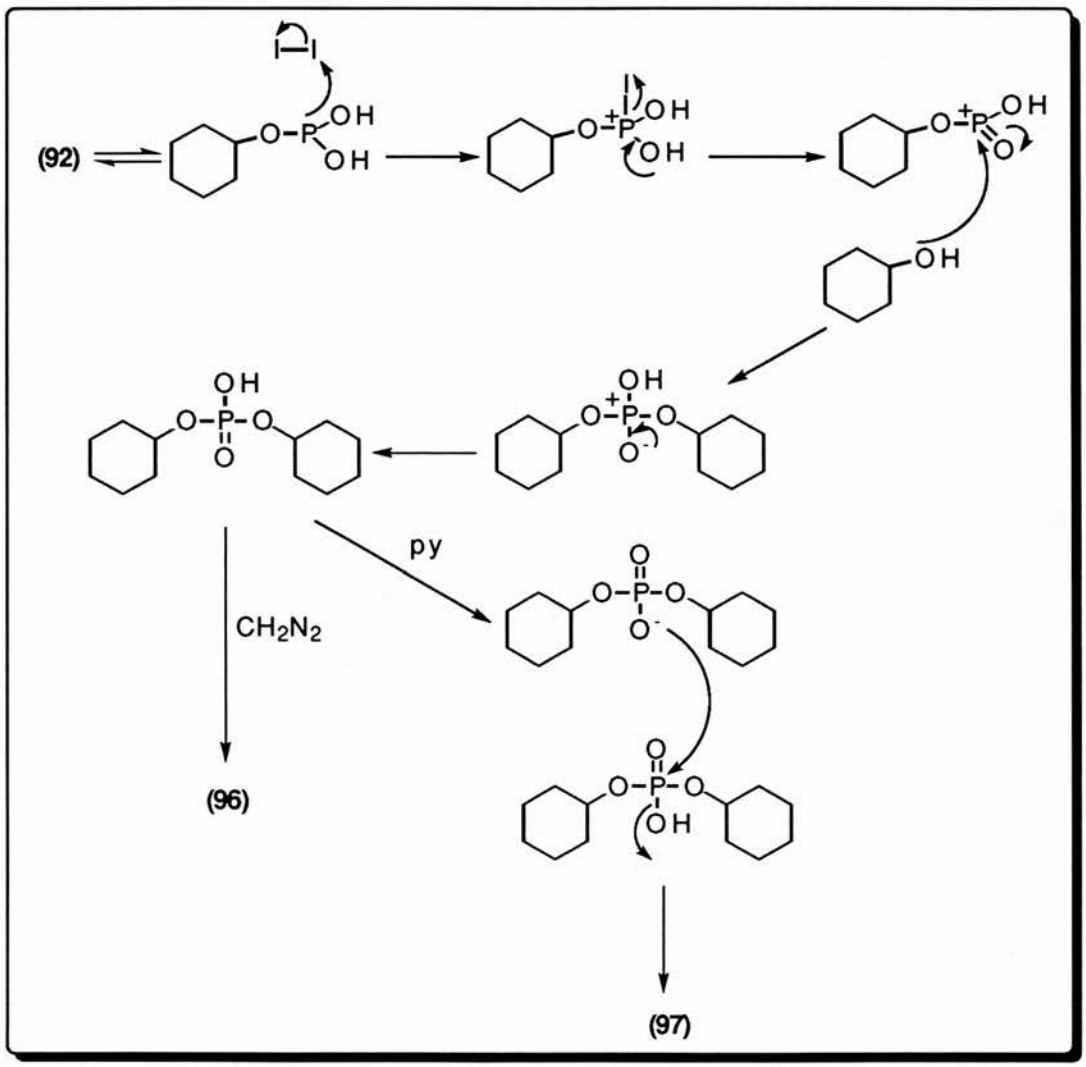
structure of the compound obtained from the other reaction half was also examined by mass spectrometry and found to be tetracyclohexyl pyrophosphate **(97)** {*m/z* (Found: [M + H]$^+$, 507.2651. Calc. for $C_{24}H_{45}P_2O_7$: *m/z*, 507.2641); δ_P (121.41 MHz; C^2HCl_3) main peak at -14.73 ppm} (Scheme 2.29). Comparison of ^1H and ^{31}P nmr spectra of **(97)** before and after treatment with diazomethane showed no change at all. In view of this, the side product formed in the oxidation process was identified to be a pyrophosphate (Scheme 2.30).



Scheme 2.29: *Formation of the pyrophosphate (97).*

Attempts were then made to investigate an alternative method of oxidation. Thus, the cyclic intermediate formed from the reaction of cyclohexanol with salicylchlorophosphite was treated with 10% hydrogen peroxide in THF. The reaction mixture obtained from this oxidation was different to that obtained from the iodine oxidation as judged by ^1H and ^{31}P nmr spectroscopy. However, as with the iodine oxidation, a mixture of compounds was obtained and the peak corresponding to the desired phosphate (as compared with the sample of cyclohexylphosphate prepared) did not constitute the major product. Analysis of the ^{31}P nmr spectrum did not show the presence of a pyrophosphate species as observed

previously, however other unidentified peaks were observed. These results indicated the oxidation procedures using either iodine or hydrogen peroxide would not be satisfactory. This result together with the problems encountered in the synthesis of the phosphonate itself and the low yield of the reaction led us to investigate an alternative method of phosphorylation and to abandon the phosphonate method altogether.



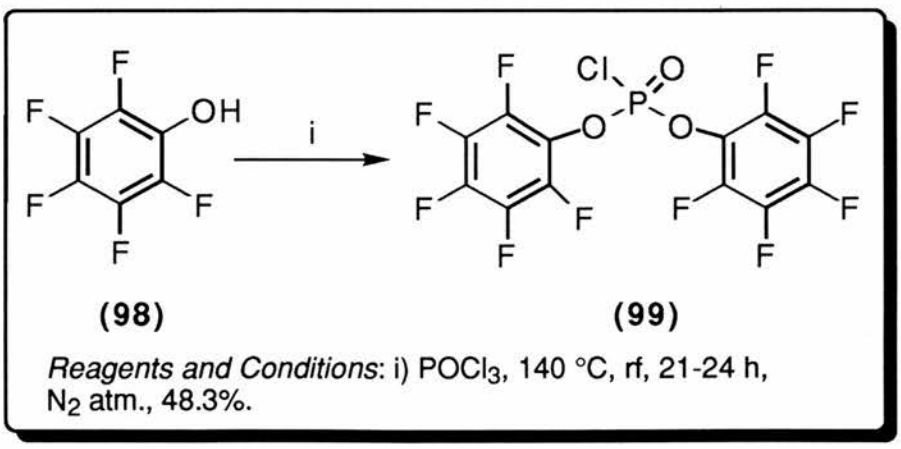
Scheme 2.30: *Possible mechanism of formation of* ***(96)*** *and* ***(97)*****.**

2.9.4 Dipentafluorophenylphosphorochloridate Phosphorylations

In our attempts to phosphorylate the hexapeptide sequence, the best results obtained were with the use of phosphorochloridates *i.e.* diphenylphosphorochloridate. It was therefore

reasonable to pursue further phosphorylations using this class of phosphorylating agents. However, a more reactive reagent with considerably more labile protecting groups was required. We therefore set to design such a compound. We reasoned that the use of electron-withdrawing substituents on the aromatic phenyl ring would increase the electrophilicity of the phosphorus centre and hence the reactivity of the phosphorylating agent. To this end, the synthesis of dipentafluorophenylphosphorochloridate (**99**) was considered. To our knowledge, this reagent has not been used in phosphorylation reactions in either peptide or carbohydrate chemistry or in solid phase reactions. The preparation and use of this reagent, therefore, would provide an excellent opportunity to contribute to a growing field in chemistry and possibly also combinatorial chemistry. We hope the reagent would provide an alternative method for the preparation of phosphopeptides and thus their use in probing one of the most exciting, important and expanding groups of enzymes, the protein phosphatases.

Using a modification of a general procedure,[303] dipentafluorophenylphosphorochloridate (**99**) was prepared by treating phosphorus oxychloride with 1.8 equivalents of pentafluorophenol (**98**) at 140 °C under a nitrogen atmosphere for 21-24 h (Scheme 2.31).



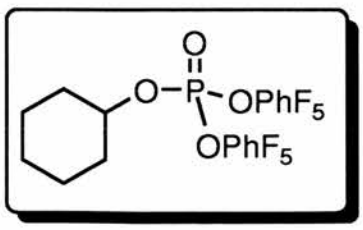
Scheme 2.31: *Synthesis of dipentafluorophenylphosphorochloridate (**99**).*

Initial reactions for the preparation of the reagent involved refluxing for 4-5 h, however a higher yield of the required phosphorylating agent was obtained with prolonged reaction

times of 21-24 h. The reagent was then purified by removing the unreacted starting materials and the monopentafluorophenyl derivatised species by fractional distillation. This gave the product in 48.3% yield as a brown oil that solidifies and was stable on storage at 0 °C {δ_P (121.41 MHz; C^2HCl_3) main peak at -15.05 ppm; *m/z* (Found: $[M]^+$, 447.9118. Calc. for $C_{12}PO_3F_{10}Cl$: *m/z*, 447.9114)}. The phosphorylating agent so obtained was 85-90% pure as judged by ^{19}F and ^{31}P nmr spectroscopy.

At the point of preparation of this compound, we were not aware of its synthesis and use previously. Pentafluorophenyl phosphorochloridates were prepared by R. H. Boschan *et al.* as described in a patent in 1967.[304] The mono- and di- pentafluorophenyl phosphorochloridates prepared were then reacted with BuOH to give dibutyl pentafluorophenyl phosphates. The authors report the use of these patented reagents as base stocks for hydraulic fluids, as heat transfer media and as lubricants in aircraft systems.[304-305]

Trial reactions using cyclohexanol were first carried out. Treatment of cyclohexanol with dipentafluorophenylphosphorochloridate in the presence of a base under an argon atmosphere gave a mixture that on purification by silica column chromatography yielded the cyclohexyl phosphate triester (**100**) as an oil in 17.9% yield {δ_P (121.41 MHz; C^2HCl_3) -11.225 ppm; *m/z* (Found: $[M]^+$, 512.0228. Calc. for $C_{18}H_{11}PO_4F_{10}$: *m/z*, 512.0235)}. The downfield shift of the CHOP signal from 3.6 ppm in cyclohexanol to 4.8 ppm, and from 70.79 to 82.65 ppm ($J_{P,C}$ 4.88) in the ^1H and ^{13}C nmr spectra respectively, was indicative of the formation of the phosphate triester.



Cyclohexyl dipentafluorophenyl phosphate (***100***).

In the model reactions dipentafluorophenylphosphorochloridate reacted approximately 30-fold more rapidly with cyclohexanol than diphenylphosphorochloridate to yield the required phosphate triester. The increased reactivity of the dipentafluorophenyl reagent, prompted us to improve the yield of the reaction. Repetition of the reaction under the same conditions failed to improve the yield. A time-course experiment revealed the reaction to be complete within 15 min and to consist of a mixture of mono- and dipentafluorophenyl phosphate esters. The mono pentafluorophenyl species was reasoned to arise from two possible sources: either

(i) small amounts of monopentafluorophenylphosphorochloridate were present in the phosphorylating agent used in the reaction, or

(ii) the mono species was the hydrolysis product of the dipentafluorophenyl derivative.

A change of base from Et_3N to pyridine or use of catalytic quantities of DMAP failed to improve reaction conditions. Later, work carried out by M. Hillier in our laboratories showed the order of addition of the reagents to play an important role. Earlier reactions involved the addition of the phosphorylating agent in dry DCM to a stirred solution of cyclohexanol and base in dry DCM. It was found that a reverse order of the above *i.e.* the addition of cyclohexanol and base to a stirred solution of dipentafluorophenyl-phosphorochloridate yields the required phosphate triester in essentially quantitative yield.

The hydrolysis of the pentafluorophenyl groups was the next objective. Treatment of the phosphate triester with water for 24 h failed to hydrolyse the protecting groups. A prolonged reaction time of 7 days resulted in the hydrolysis of one of the pentafluorophenyl groups as judged by the upfield shift of CHOP to 4.3 ppm in ^1H and to 80.09 ppm in ^{13}C nmr spectra. It was also characterised by ^{31}P nmr spectroscopy and mass spectrometry {*m/z* (Found: $[M]^+$, 346.0390. $C_{18}H_{11}PO_4F_{10}$ requires: *m/z*, 346.0393); δ_P (121.41 MHz; C^2HCl_3) main peak at -5.18 ppm}. Mass spectrometry also showed the presence of the starting material, the dipentafluorophenyl protected phosphate. Although the complete hydrolysis of the protecting groups was not obtained by treatment with distilled water, the

results were nevertheless encouraging and the use of a base to accelerate the rate of hydrolysis was considered. The phosphate triester (**100**) was treated with a weak base, 5% $NaHCO_3$, at room temperature for 12 h. ^{19}F and ^{1}H Nmr spectroscopy of the reaction mixture indicated partial hydrolysis of the protecting groups to give the monopentafluorophenyl protected phosphate diester. Mass spectrometry showed that the mixture contained some of the required cyclohexylphosphate, so it was considered that complete hydrolysis could be achieved by treatment with increased number of equivalents of base. Treatment of the phosphate triester (**100**) with an excess of base, however, failed to achieve the complete hydrolysis of the pentafluorophenyl groups. The complete hydrolysis of the groups was then attempted using an ethanolic solution of KOH and an organic base, pyridine/ H_2O mixture, to no avail. The acid lability of these esters has been noted previously.[306-307] Attention was therefore focussed on the possible acid catalysed hydrolysis of the pentafluorophenyl groups and its application to solid phase peptide synthesis.

Treatment of the resin-bound Ac-R(Pmc)R(Pmc)ATVA-Wang resin (**62**) with 10 equivalents of dipentafluorophenylphosphorochloridate under an argon atmosphere gave the resin-bound phosphate triester (**101**). The deprotection of (**101**) was investigated using two different cleavage mixtures, namely reagent K[229] and a TFA/ H_2O/ TES (90: 5: 5) mixture and the results were found to be similar. The simultaneous deprotection of the Pmc and pentafluorophenyl groups and cleavage from the resin yielded a white solid after lyophilisation. This was purified by ion exchange chromatography using the strongly basic anion exchanger Dowex 1 x 2-200 (chloride form) to give the pure phosphopeptide (**102**) (Fig. 2.6) in 60% overall yield, compared with the unphosphorylated compound, (over 14 solid phase steps) {m.p. 190 °C (decomp.); δ_P (121.41 MHz; $^{2}H_2O$) -0.616 ppm; *m/z* (E/S) 795 (4%, $[M + H]^+$), 398 (25, $[M + H/ 2]^+$), 368 (2, [phosphorylthreonyl-valyl-alanyl]$^+$) and 193 (100, [valyl-alanyl + 6H]$^+$).} (Scheme 2.32).

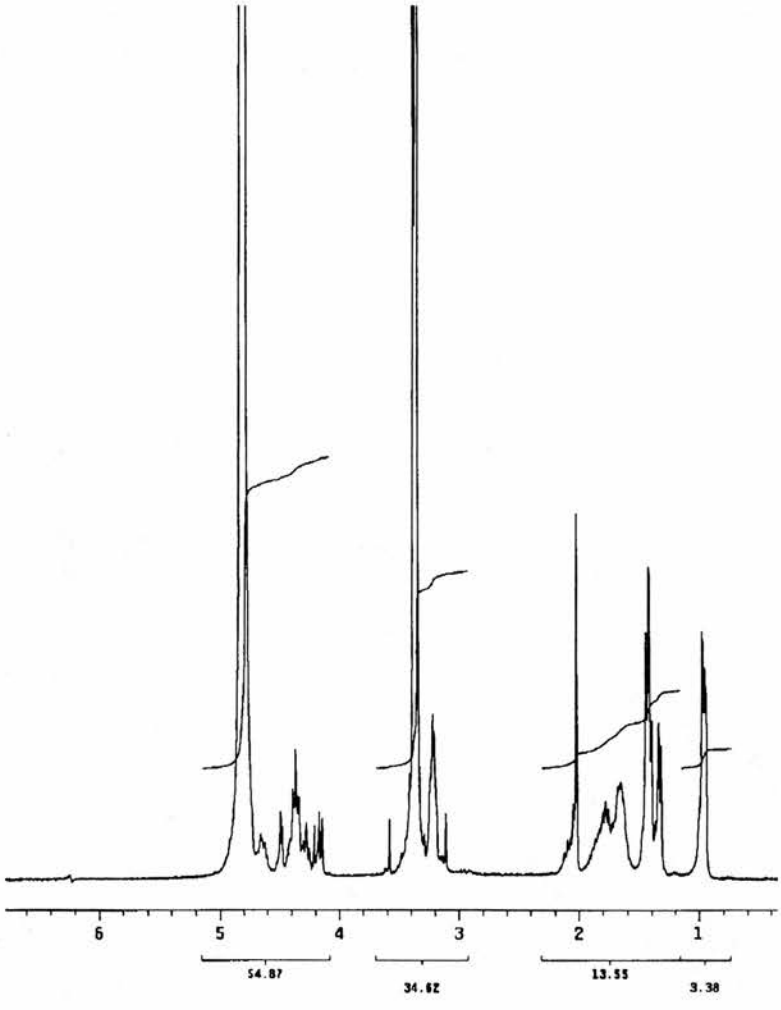
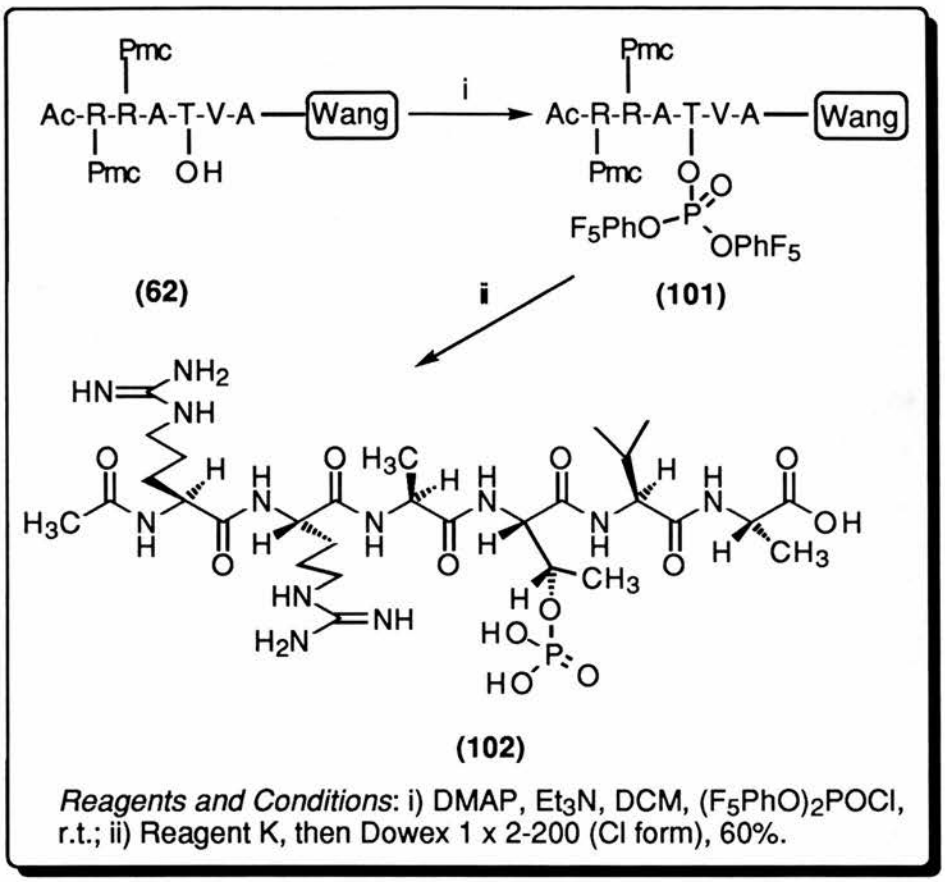


Figure 2.6: *^1H nmr spectrum of the phosphopeptide* ***(102)***.



Scheme 2.32: *Synthesis of the phosphopeptide substrate* ***(102)****.*

The applicability of dipentafluorophenylphosphorochloridate as a general phosphorylating agent was then investigated. The serine analogue of the substrate , *i.e.* Ac-RRASVA-OH **(105)** (Fig. 2.7) {m.p. 177-178 °C; *m/z* (E/S) 701 (13%, $[M + H]^+$) and 354 {100, [M - (alanyl-seryl-valyl-alanyl)]$^+$}}, was synthesised using Fmoc solid phase chemistry on Wang resin and cleavage was effected using TFA/ TES/ H_2O (90: 5: 5) as discussed previously. The resin-bound peptide, Ac-R(Pmc)R(Pmc)ASVA-Wang resin **(104)** was phosphorylated as for the threonine analogue to give the phosphopeptide **(107)** (Fig. 2.8) in an unoptimised yield of 16% yield, compared with the unphosphorylated compound, (over 14 solid phase steps) {m.p. 200 °C (decomp.); δ_P (121.41 MHz; ^2H_2O) 0.397 ppm; *m/z* (E/S) 781 (27%, $[M + H]^+$) and 354 {100, [M - (alanyl-phosphorylseryl-valyl-alanyl)]$^+$}} (Scheme 2.33).

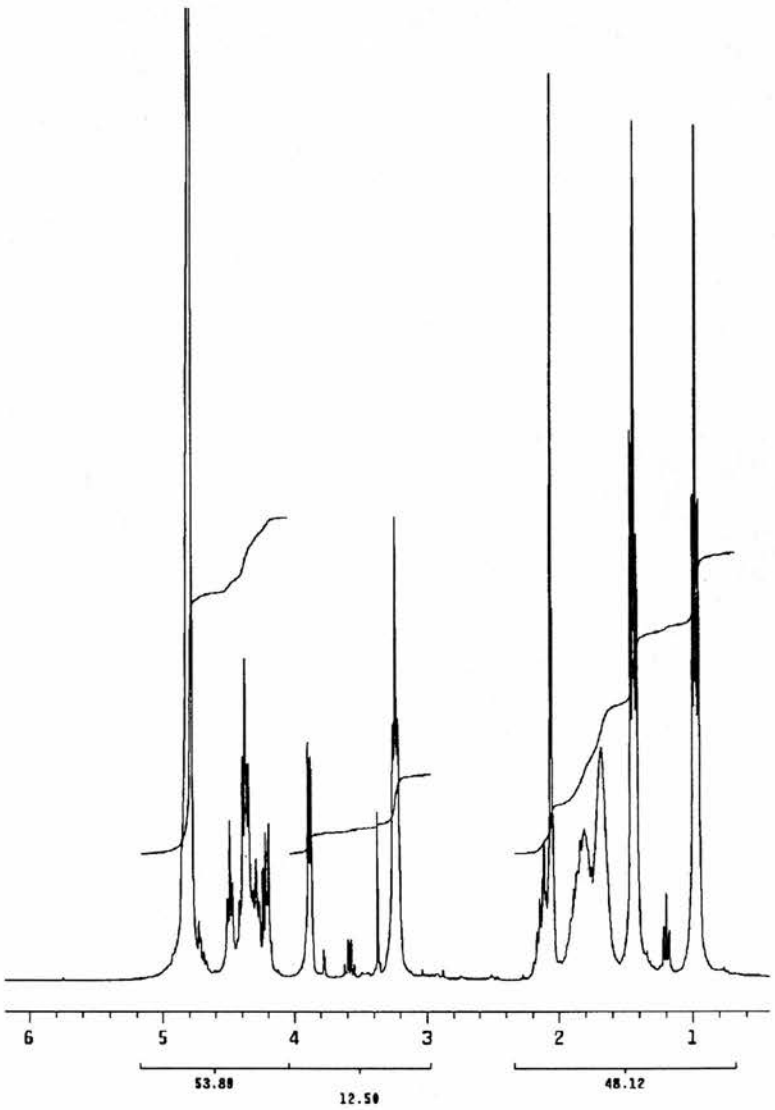
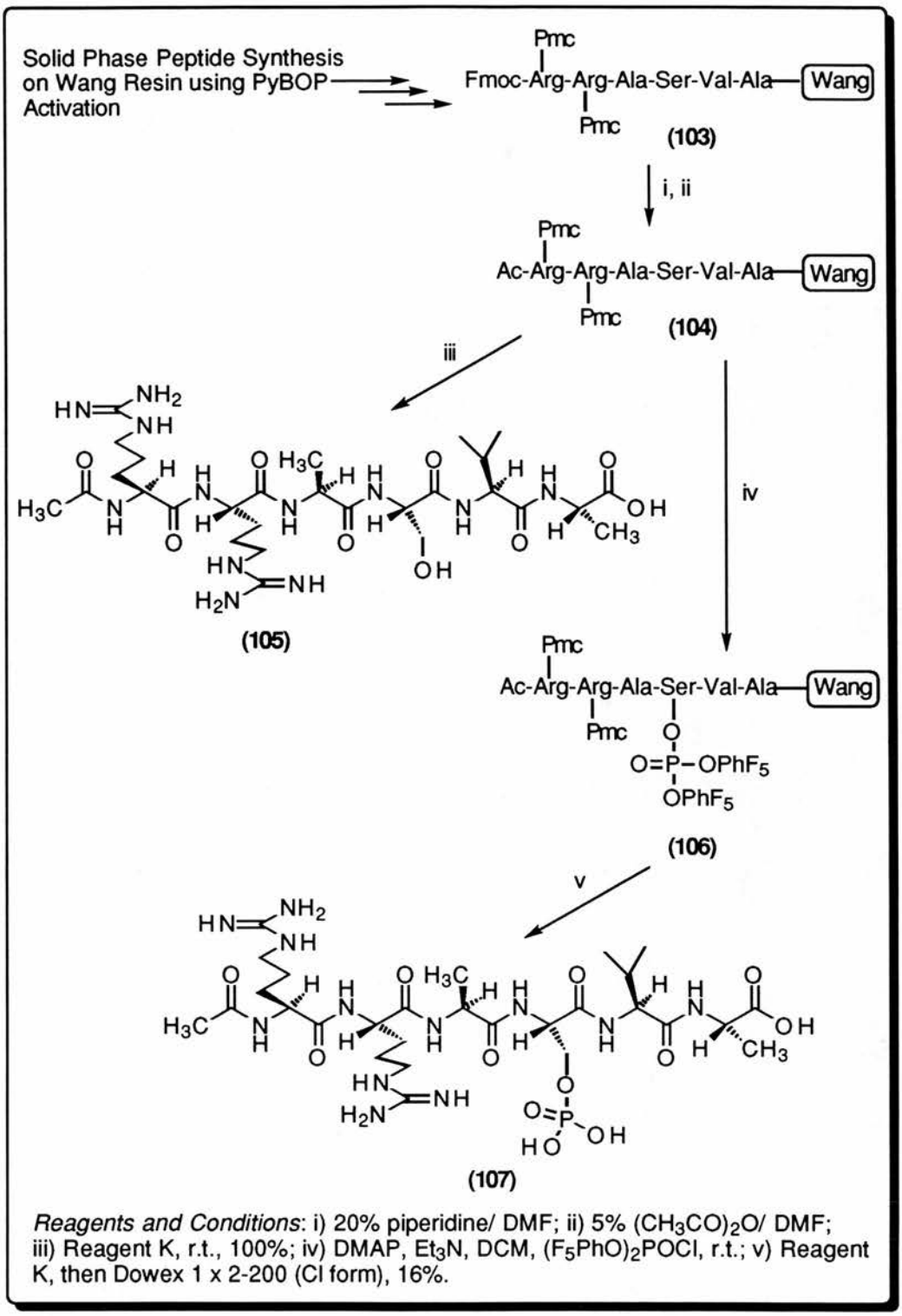


Figure 2.7: *^1H nmr spectrum of* ***(105)***.



Scheme 2.33: *Synthesis of the substrate serine analogue* ***(107)***.

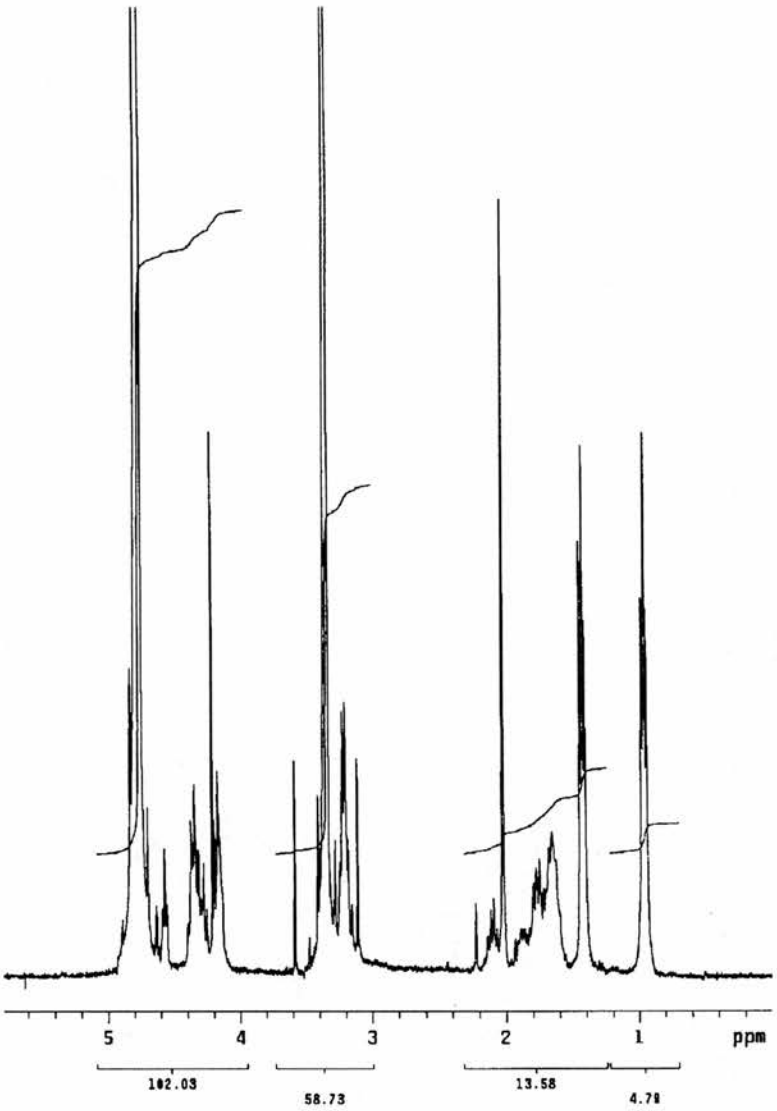


Figure 2.8: *^1H nmr spectrum of the phosphopeptide (**107**).*

2.10 Ac-RRAT(OPO_3H_2)VA-OH as a Substrate for PPλ

To assess whether the synthetic threonine phosphopeptide was, indeed, a substrate for the protein phosphatases, initial assays using the protein phosphatase PPλ were undertaken. PPλ is encoded by an open reading frame in bacteriophage λ. Amino acid sequence alignments of PPλ (Appendix 1) indicated that this enzyme is most closely related to PP1, PP2A and PP2B, although its mass is much lower than the eukaryotic enzymes. PPλ is in fact homologous in sequence to the N-terminal half of PP1 and has been used as a useful model for mechanistic studies.[308] Recombinant PPλ has been overexpressed and purified

from *E. coli* and requires Mn^{2+} or Ni^{2+} for activation of phosphatase activity. The recombinant enzyme is active towards Ser, Thr and Tyr phosphopeptide and phosphoproteins. The similarities between PPλ, PP1, 2A and 2B suggest that all these enzymes may utilize a common catalytic mechanism. Site-directed mutagenesis studies have defined the amino acid residues in PPλ that are required for metal ion binding, substrate binding and catalysis.[308] These studies identified amino acid residues in PPλ that are also invariant residues in all the phosphoprotein phosphatases, confirming similar substrate binding and catalytic behaviour for PPλ and PP1, 2A and 2B (Fig. 2.9). For this reason and due to the comparatively low cost of PPλ compared with the other protein phosphatases, PPλ was used in the initial enzyme nmr studies.

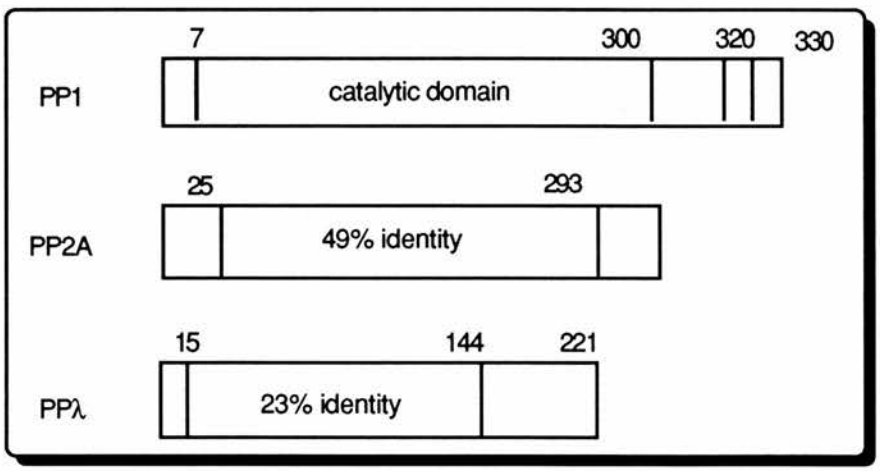


Figure 2.9: *Schematic diagram showing the domain structure of the Ser/ Thr protein phosphatases. The residue numbers given refer to PP1 from rabbit muscle, humna PP2A and the phosphatase encoded by bacteriophage λ .*[54]

A solution of the phosphopeptide Ac-RRAT(OPO_3H_2)VA-OH, adjusted to pH 7.8 (the optimal pH for the enzyme) with 1M NaOH was treated with 10 mm^3 of PPλ (equivalent to 200 units of enzyme). The progress of the reaction was monitored by nmr spectroscopy (Appendix 3). Initial studies using PPλ and our phosphopeptide indicated that the phosphorylated sequence is a substrate for the protein phosphatase and is turned over by

the enzyme to yield the dephosphorylated product as judged by ^1H nmr spectroscopy (500 MHz).

Similar experiments highlighted the absolute requirement of this enzyme for Mn^{2+} for activation of phosphatase activity (Appendix 3). Treatment of the substrate **(102)** with PPλ (200 units) in an assay buffer containing Mg^{2+} instead of Mn^{2+} and monitoring of the reaction by ^1H nmr spectroscopy for 24 h failed to show any change in the substrate. This indicated that the enzyme had failed to dephosphorylate the substrate and two possibilities could be forseen: either the enzyme in the reaction mixture was not in sufficient quantities for dephosphorylation to take place, or the optimal conditions for enzyme activity were not achieved within the reaction medium. To ensure adequate enzyme concentration in the reaction medium, more enzyme (equivalent to 200 units) was added and the reaction monitored for a further 4 h. Again no change was observed, at which point an aliquot of a 2 mM $MnCl_2.6H_2O$ solution was added to the mixture. Within 5 min of this addition a change was observed in the ^1H nmr of the substrate. Although Mn^{2+} ions are known to broaden nmr signals, the change observed was consistent with the disappearance of the phosphorylated Thr CH_3 signal and an emergence of the unphosphorylated Thr CH_3 signal, as compared with the ^1H nmr spectrum of the unphosphorylated sample (Appendix 4).

The nmr assay experiments were then repeated using the substrate **(102)** incubated with PP2A (3 units) (Appendix 5). The progress of the reaction was again monitored by ^1H nmr spectroscopy (500 MHz) and within 30 min of the start of the incubation, almost all of the substrate was shown to have been converted to the unphosphorylated compound (Fig. 2.10). The nmr spectrum of the phosphopeptide **(102)** (Fig. 2.10 a) is at a different pH (pH 2.9) to the nmr spectra in Fig. 2.10 b, which are at the optimal pH of the enzyme, *i.e.* pH 7.8. This difference in pH causes the shift of the Ala and Thr CH_3 signals as observed in Fig. 2.10 b. In a similar assay, microcystin-LR was added to the incubation mixture of substrate and PP2A, and monitoring of the reaction failed to show any change in the ^1H

nmr spectrum of the substrate. This confirms microcystin-LR as an inhibitor of the protein phosphatase 2A, thereby preventing the enzyme from dephosphorylating the phospho-peptide (Fig. 2.11).

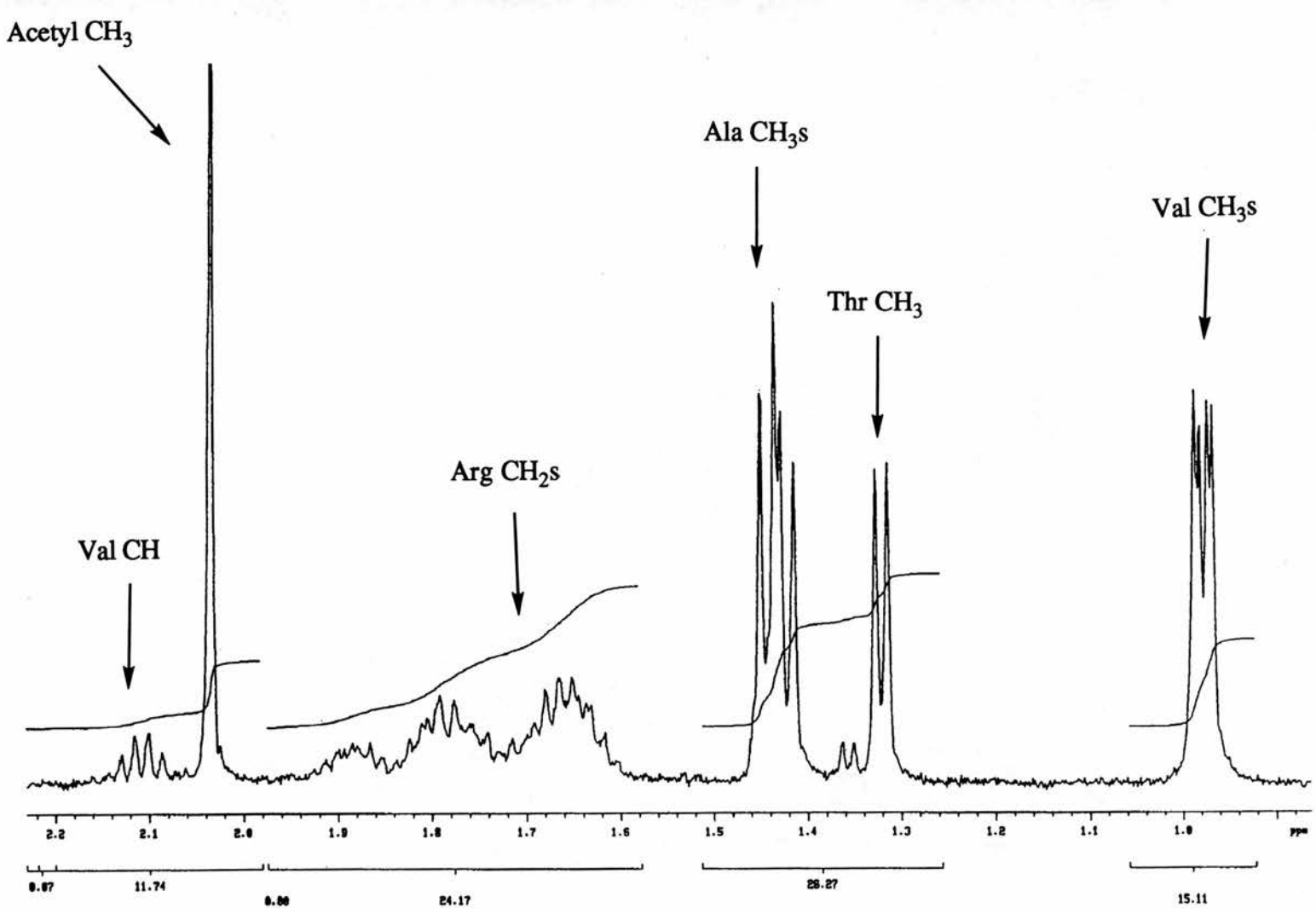


Figure 2.10 (a): *Expansion of a region of the ^1H nmr of the phosphopeptide* ***(102)*** *prior to the addition of PP2A and overleaf* **Figure 2.10 (b):** *after the addition of PP2A.*

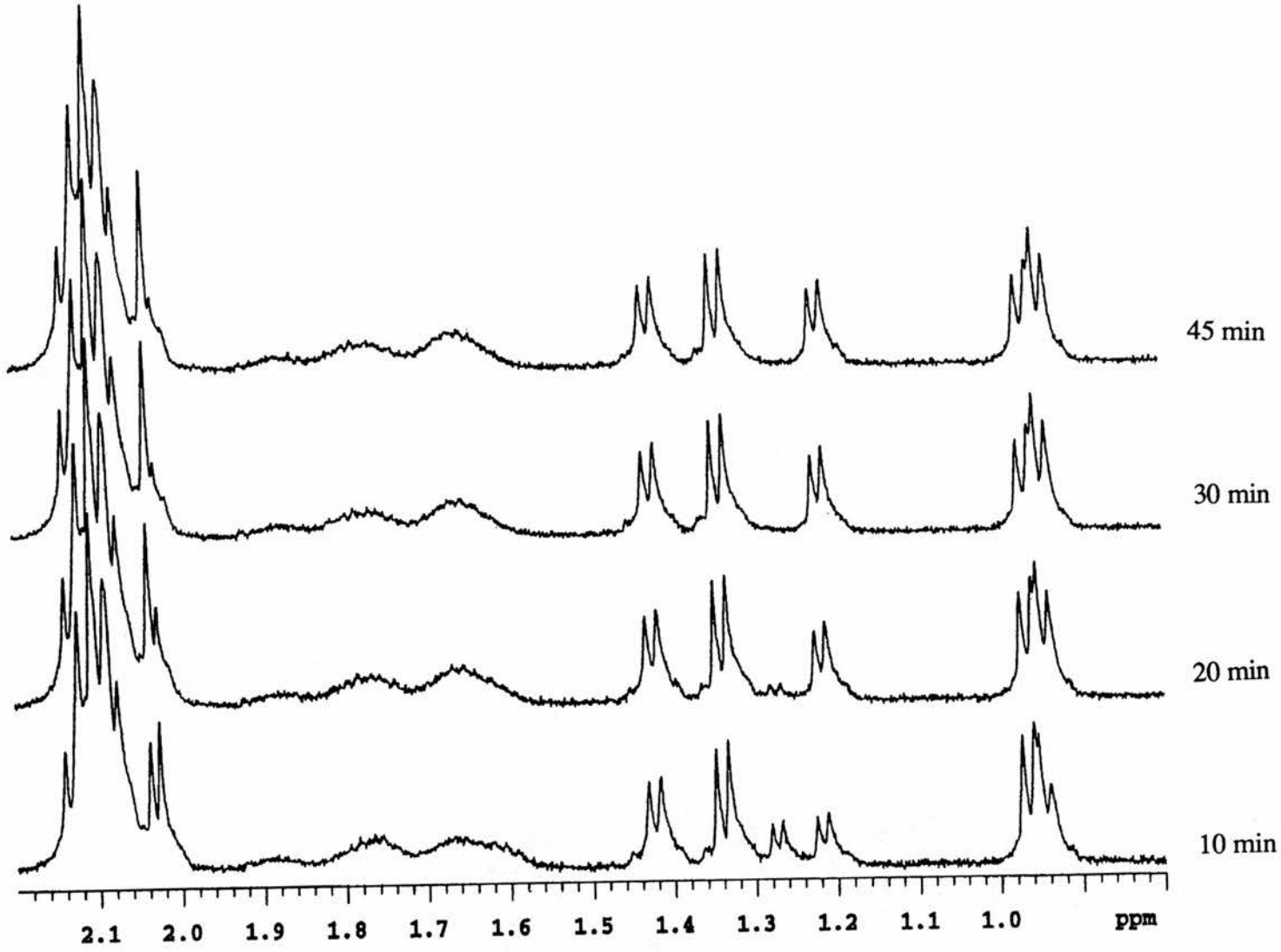


Figure 2.10 (b)

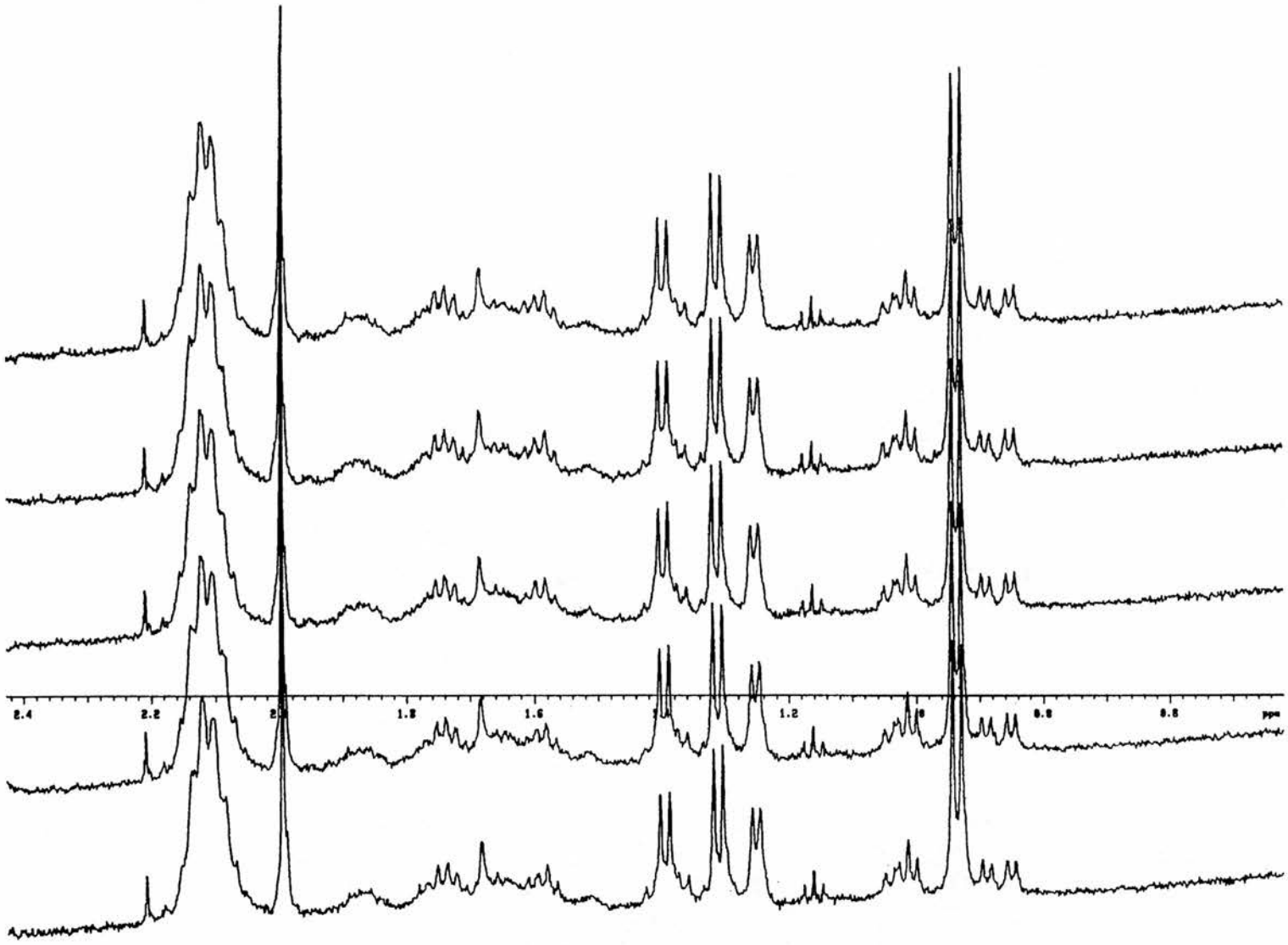


Figure 2.11: *Time-course ^{1}H nmr spectra of substrate, PP2A and microcystin-LR.*

CONCLUSIONS and FUTURE WORK

The main objective of the present work, *i.e.* the synthesis of phosphopeptides *via* a non-enzymic method was accomplished. This involved the solid phase synthesis of the peptide sequence using PyBOP activation on Wang resin. Phosphorylations on the solid phase using dipentafluorophenylphosphorochloridate followed by simultaneous deprotection and removal from the resin gave the required phosphopeptide product (**102**) in 60% overall yield (over 14 solid phase steps). The protocol employed was shown to be applicable to the synthesis of a serine-containing peptide and yielded the phosphoserine-containing peptide analogue in an overall unoptimised yield of 16%. The phosphothreonine peptide synthesised was shown to be a substrate for the protein phosphatases PPλ {using 200 units at 30 °C (pH 7.8)} and PP2A {using 3 units at 30 °C (pH 7.8)} in nmr spectroscopic assays.

Traditionally, studies on the dephosphorylation rate of the phosphatases involve the synthesis of ^{32}P-labelled peptides using protein kinases. ^{32}P has a short half life of 14.8 days and for kinetic data obtained from the use of these peptides to be accurate, repeated batch preparations must be made. A more stable isotope, *e.g.* ^{14}C, is therefore more desirable. This would allow a single batch preparation of a ^{14}C-containing phosphopeptide that can be used in assays and kinetic studies of the protein phosphatases. The synthesis of the ^{14}C-labelled substrate (**30**) is the immediate objective for the future work to be carried out.

Synthesising analogues of the substrate (**102**) would allow a structure-activity relationship study to be carried out. In view of the information available on the crystal structure of PP1,[54,55] an SAR study would enable a direct comparison of the different sequences and their interaction with the enzyme. This can shed light on the primary requirements for interaction and binding to the enzyme and the common concensus sequence required for such binding. The substrates synthesised could also be used in inhibition assays of the

microcystin and nodularin analogues being prepared in the group. This would provide information on inhibitor binding and mechanism of inhibition by these hepatotoxins. In view of the fact that these naturally occuring compounds are potent and selective inhibitors of the serine/ threonine protein phosphatases and that protein phosphorylation is known to be a regulatory element in carcinogenesis, infomation on the mode of inhibition by the microcystins and nodularins can offer important and novel therapeutic opportunities in the management of oncology as well as several other areas of medicine.

CHAPTER THREE

EXPERIMENTAL

3.0 EXPERIMENTAL

Elemental microanalyses were performed in the departmental microanalytical laboratory. Nmr spectra were recorded on Bruker AM-300 or Varian gemini 300 (300 MHz; f.t. ^{1}H-nmr, and 74.76 MHz; ^{13}C-nmr), or Varian gemini 200 (200 MHz; f.t. ^{1}H-nmr and 50.31 MHz; ^{13}C-nmr) spectrometers. ^{1}H-Nmr spectra are described in parts per million downfield shift from TMS and are reported consecutively as position (δ_H or δ_C), relative integral, multiplicity (s -singlet, d -doublet, t -triplet, q -quartet, dd -double of doublets, sep -septet, hep -heptet, m -multiplet, and br -broad), coupling constant (Hz) and assignment (numbering according to the IUPAC nomenclature for the compound). ^{1}H-Nmr were referenced internally on ^{2}HOH (4.68 ppm), $CHCl_3$ (7.27 ppm) or DMSO (2.47 ppm). ^{13}C-Nmr were referenced on CH_3OH (49.3 ppm), $C^{2}HCl_3$ (76.9 ppm), or DMSO (39.70 ppm).

I.R. spectra were recorded on a Perkin-Elmer 1710 f.t. IR spectrometer. The samples were prepared as Nujol mulls, solutions in chloroform or thin films between sodium chloride discs. The frequencies (υ) as absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard. Mass spectra and accurate mass measurements were recorded on VG 70-250 SE, Kratos MS-50, VG Platform E/S or by the EPSRC service at Swansea using VG AZB-E. Fast atom bombardment spectra were recorded using NOBA as a matrix. 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.*[309] using Sorbsil C 60 (40-60 μm mesh) silica gel. Analytical thin layer 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, 10% ammonium ceric nitrate in 10% sulfuric acid or ninhydrin.

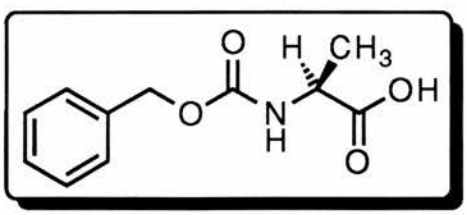
Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured at 23 °C on a Optical Activity AA-1000 polarimeter using 10 or 20 cm path length cells.

The solvents used were either distilled or of analar quality and light petrol ether 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. Isopropanol, isopentanol, DMF, toluene, CH_2Cl_2, acetonitrile, diisopropylamine, triethylamine, and pyridine were distilled over CaH_2. THF and diethylether were dried over sodium/ benzophenone and distilled under nitrogen. Thionyl chloride was distilled over sulphur, and the initial fractions were always discarded. BuLi was titrated according to the method of Lipton.[310]

Diazomethane preparation

N-Nitroso-N-methyl-4-toluene-sulphonamide (Diazald) (21 g, 98 mmol) in ether (150 cm^3) was added slowly to a solution of potassium hydroxide (4.8 g, 86 mmol) in water (8 cm^3) and isopropanol (12 cm^3). The solution was heated in a clear jointed distillation apparatus and the ethereal diazomethane distilled into ether (10 cm^3) at 0 °C. The yellow solution obtained was used in the synthesis of methyl ester derivatives.

N-α-Cbz-(2*S*)-alanine (108)



(2*S*)-Alanine (0.45 g, 5.0 mmol) was dissolved in aqueous sodium hydrogen carbonate (1.05 g, 12.5 mmol, 50 cm^3) and benzylchloroformate (0.8 cm^3, 5.5 mmol) was added in four portions over a period of 15 min with vigorous stirring. The reaction was allowed to stir for 3-4 h, and then washed with ether (2 x 25 cm^3). The aqueous phase was acidified

with 1 M HCl and then extracted with ether (3 x 50 cm^3). The ethereal extracts were washed with saturated brine, dried ($MgSO_4$) and the solvent removed under reduced pressure to give a colourless oil. Crystallisation from ether/ light petroleum afforded the protected alanine as a white solid (0.39 g, 35%), m.p 86-87 °C (lit.,[311] 87 °C); $[\alpha]_D$ -14.3 (*c* 2, acetic acid) {lit.,[311] -13.9 (*c* 2, acetic acid)}; υ_{max} (Nujol)/cm^{-1} 3333 (NH) and 1701 & 1696 (carbonyls); δ_H (200 MHz; C^2HCl_3) 1.46 (3H, d, *J* 7, CH_3), 4.36 (1H, qd, J_{NH} 7.2, J_{CH} 7.0, CH), 5.13 (2H, s, CH_2), 5.36 (1H, d, *J* 7.2, NH), 6.88 (1H, br. s, CO_2H) and 7.35 (5H, s, aromatic); δ_C (50.3 MHz; C^2HCl_3) 18.90 (CH_3), 49.99 (CH), 67.67 (CH_2O), 128.67, 128.79 & 129.07 (aromatic), 136.55 (quat. aromatic), 156.38 (CONH) and 178.35 (CO_2H); *m/z* (CI) 224 (100%, $[M + H]^+$), 180 (12, $[M - CO_2H + 2H]^+$) and 88 {12, $[NHCH(CH_3)CO_2H]^+$}.

N-α-Cbz-glycine (109)

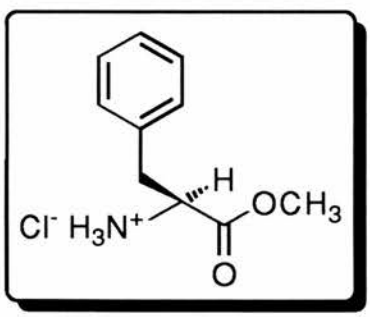
This compound was prepared in a manner identical to that for **(108)**, using glycine (0.38 g, 5.0 mmol) to give a white crystalline precipitate which was filtered and dried (0.61 g, 58%), m.p. 119-120 °C (lit.,[312] 120 °C); *m/z* (Found: $[M + H]^+$, 210.0766. Calc. for $C_{10}H_{12}NO_4$: *m/z*, 210.0766); υ_{max} (Nujol)/cm^{-1} 3328 (NH), 2620-2500 (aromatic), 1729 (CO_2H) and 1681 (urethane CO); δ_H (200 MHz; d_6-DMSO) 3.68 (2H, d, *J* 6.2, $C\underline{H}_2NH$), 5.06 (2H, s, CH_2O), 7.37 (5H, s, aromatic) and 7.60 (1H, t, *J* 6.2, NH); δ_C (50.3 MHz; d_6-DMSO) 42.41 (CH_2NH), 65.73 (CH_2O), 127.99, 128.08 & 128.62 (aromatic), 137.28 (quat. aromatic), 156.76 (urethane CO) and 171.85 (CO_2H); *m/z* (CI) 227 (97%, $[M + NH_4]^+$), 210 (74, $[M + H]^+$), 166 (78, $[M - CO_2H + 2H]^+$), 119 (15, $[M - PhCH_2 + H]^+$), 108 (100, $[PhCH_2 + NH_4]^+$), 91 (38, $PhCH_2^+$) and 76 (39, $CO_2HCH_2NH_3^+$).

N-α-Cbz-(2*S*)-glutamic acid (110)

(2*S*)-Glutamic acid (1.47 g, 10 mmol) was dissolved in water (30 cm^3), and MgO (1.21 g, 30 mmol) was added. The mixture was overlaid with ether (5 cm^3) and stirred vigorously.

Benzylchloroformate (2.85 cm^3, 20 mmol) was added in portions over 30 min with constant stirring and cooling in ice. The mixture was allowed to stir for 3 days at room temperature. The mixture was then washed with ether (30 cm^3), acidified with 1 M HCl and extracted with ethyl acetate (3 x 30 cm^3). The organic extract was washed with 1 M HCl (30 cm^3), brine (30 cm^3), dried ($MgSO_4$) and the solvent removed under reduced pressure. DCM (10 cm^3) was added and removed under reduced pressure to give a pale yellow oil, which was crystallised from ether/ light petroleum to give the product as a white solid (1.81 g, 64%), m.p. 118-119 °C (lit.,[312] 120-121 °C); *m/z* (Found: $[M + H]^+$, 282.0980. Calc. for $C_{13}H_{16}NO_6$: *m/z*, 282.0977); $[\alpha]_D$ -6.9 (*c* 8, acetic acid) {lit.,[312] -7.1 (*c* 8, acetic acid)}; υ_{max} (Nujol)/cm^{-1} 3305 (NH stretch), 2650 (OH) and 1549 (NH bend); δ_H (200 MHz; d_6-DMSO) 1.77 (2H, m, half of $C\underline{H}_2CH$), 1.93 (2H, m, half of $C\underline{H}_2CH$), 2.33 (2H, dd, *J* 5.9, 7.9, CH_2CO), 4.00 (1H, m, CH), 5.05 (2H, s, CH_2O), 7.38 (5H, s, aromatic) and 7.66 (1H, d, *J* 8.1, NH); δ_C (50.3 MHz; d_6-DMSO) 26.33 ($\underline{C}H_2CH$), 30.34 ($\underline{C}H_2CO$), 53.29 (CH), 65.70 (CH_2O), 127.99, 128.10 & 128.63 (aromatic), 137.23 (quat. aromatic), 156.45 (CONH) and 173.90 & 174.02 (CO_2H); *m/z* (CI) 299 (76%, $[M + NH_4]^+$), 282 (89, $[M + H]^+$), 238 (100, $[M - CO_2H + 2H]^+$), 108 (42, $[PhCH_2 + NH_4]^+$) and 91 (14, $PhCH_2^+$).

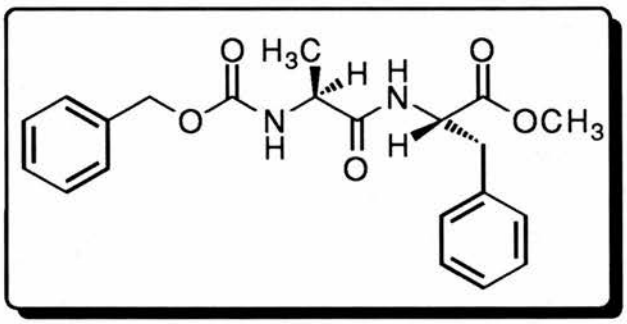
Methyl (2*S*)-phenylalaninate hydrochloride (111)



Thionyl chloride (6.5 g, 55 mmol) was added dropwise to a solution of (2*S*)-phenylalanine (4.1 g, 25 mmol) in dry methanol (30 cm^3) at 0 °C. The solution was refluxed for 2 h and then the solvent removed under reduced pressure. The pale yellow solid obtained was

recrystallised from methanol/ ether to give the product as a white solid (4.77 g, 88%), m.p. 158 °C (lit.,[313] 159-161 °C); $[\alpha]_D$ -4.5 (*c* 5, H_2O) {lit.,[313] -4.6 (*c* 5, H_2O)}; υ_{max} (Nujol)/cm^{-1} 2628-2430 (aromatic CH), 1747 (CO), 1585 (NH) and 760 (aromatic); δ_H (200 MHz; ^2H_2O) 3.07 (1H, dd, J_{AB} 14.7, J_{AX} 7.6, half of CH_2Ph), 3.22 (1H, dd, J_{AB} 14.7, J_{BX} 5.8, half of CH_2Ph), 3.70 (3H, s, CH_3O), 4.29 (1H, dd, J_{AX} 7.6, J_{BX} 5.8, CH) and 7.12-7.25 (5H, m, aromatic); δ_C (50.3 MHz; ^2H_2O) 38.40 (CH_3O), 56.40 & 56.93 (CH & CH_2Ph), 130.96, 132.13 & 132.22 (aromatic), 136.54 (quat. aromatic) and 172.89 (CO); *m/z* (CI) 180 (57%, $[M - Cl]^+$) and 91 (100, CH_2Ph^+).

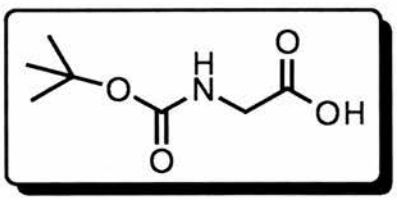
Methyl N-α-Cbz-(*S*)-alanyl-(*S*)-phenylalaninate (112)



To a stirred solution of Cbz-alanine (**108**) (0.56 g, 2.5 mmol) in THF (10 cm^3) was added NMM (275 mm^3, 2.5 mmol) and the solution cooled to -15 °C. IBCF (324 mm^3, 2.5 mmol) was then added and the solution stirred at -15 °C for 5 min. Phe.OMe.HCl (**111**) (0.54 g, 2.5 mmol) and NMM (275 mm^3) dissolved in THF was added to the reaction mixture in one portion. The mixture was stirred at -15 °C for 5 min and at room temperature for 20 min. The hydrochloride salts were then filtered off and the solvent removed under reduced pressure to give a colourless oil which was crystallised from ethanol/ H_2O to give the product as a white solid (0.58 g, 60%), m.p. 98-100 °C (lit.,[314] 97-98 °C, for the ethyl ester derivative); *m/z* (Found: $[M + H]^+$, 385.1761. $C_{21}H_{25}N_2O_5$ requires: *m/z*, 385.1763); $[\alpha]_D$ 18.9 (*c* 0.5, DCM); υ_{max} (Nujol)/cm^{-1} 3290 & 3320 (NH), 2728-2450 (aromatics), 1744 (ester CO), 1690 (amide CO) and 1661 (urethane CO); δ_H (200 MHz; C^2HCl_3) 1.33 (3H, d, *J* 7.0, $C\underline{H}_3CH$), 3.15 (1H, dd, *J* 5.7 & 13.9, half of

CH_2Ph), 3.06 (1H, dd, *J* 6.2 & 13.9, half of CH_2Ph), 3.72 (CH_3O), 4.24 (1H, m, $CH_3C\underline{H}$), 4.86 (1H, m, $C\underline{H}CH_2$), 5.10 (2H, s, CH_2O), 5.30 (1H, d, *J* 7.0, urethane NH), 6.51 (1H, d, *J* 6.2, amide NH) and 7.06-7.36 (10H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 19.00 ($\underline{C}H_3CH$), 38.30 ($CO_2\underline{C}H_3$), 50.93 ($\underline{C}HCH_2Ph$), 52.92 ($CH_3\underline{C}H$), 53.67 (CH_2Ph), 67.56 (CH_2O), 127.67-129.74 (aromatic), 136.10 (quat. aromatic), 160.83 (OCONH) and 172.16 & 172.26 (CO's); *m/z* (CI) 385 (100%, $[M + H]^+$), 277 (67, $[M - C_7H_7O]^+$) and 91 (15, $PhCH_2^+$).

N-α-Boc-glycine (113)



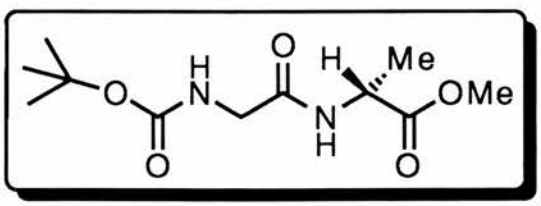
To a stirred solution of glycine (0.75 g, 10 mmol) in 5 M NaOH (5 cm^3) was added tertiary butyl alcohol (10 cm^3). Di-tertiary butyl dicarbonate (2.8 g, 13 mmol) was dissolved in tertiary butyl alcohol (4 cm^3) and added to the solution. The resulting precipitate was dissolved in 5 M NaOH (5-10 cm^3) and the reaction mixture was then stirred overnight at room temperature. The alcoholic layer was then removed under reduced pressure. The resulting aqueous layer was washed with light petroleum (20 cm^3), cooled to 0 °C and acidified to pH 3 with concentrated H_2SO_4. The mixture was then extracted with DCM (3 x 30 cm^3) and the organic layer washed with saturated brine and dried ($MgSO_4$). The solvent was removed under reduced pressure and the colourless oil obtained crystallised from ether/ light petroleum to give the product as a white crystalline solid (1.09 g, 62%), m.p. 87-88 °C (lit.,[315] 88.5-89 °C); *m/z* (Found: $[M + H]^+$ 176.0923. Calc. for $C_7H_{14}NO_4$: *m/z*, 176.0923); υ_{max} (Nujol)/cm^{-1} 3407 & 3401 (NH conformers), 3116 (OH), 1749 (CONH) and 1669 (CO_2H); δ_H (200 MHz; C^2HCl_3) 1.44 (9H, s, $C(C\underline{H}_3)_3$), 3.96 (2H, d, *J* 5.3, CH_2), 5.07 & 6.84 (1H, br. s, NH conformers) and 8.8 (1H, br. s, CO_2H); δ_C (50.3 MHz; C^2HCl_3) 28.76 ($C(\underline{C}H_3)_3$), 42.72 & 43.91

(CH_2 conformers), 80.95 & 82.28 ($\underline{C}(CH_3)_3$ conformers), 156.51 (CONH) and 174.44 & 175.40 (CO_2H conformers); *m/z* (CI) 193 (47%, $[M + NH_4]^+$), 176 (58, $[M + H]^+$), 120 (36, $[M - NHCH_2CO_2H + H + NH_4]^+$), 93 (46, $[M - BOC + H + NH_4]^+$) and 76 (100, $[M - BOC + 2H]^+$).

Methyl (2*R*)-alaninate hydrochloride (114)

This compound was prepared in a manner identical to that for Phe.OMe.HCl (**111**) using (2*R*)-alanine (0.89 g, 10 mmol) to give a pale yellow oil. Crystallisation from dry methanol/ dry ether gave the product as a white crystalline solid (0.84 g, 60%), m.p. 153 °C (lit.,[316] 154-155 °C); *m/z* (Found: $[M - Cl]^+$ 104.0712. Calc. for $C_4H_{10}NO_2$:*m/z*, 104.0711); $[\alpha]_D$ -5.9 (*c* 0.3, MeOH); υ_{max} (Nujol)/cm^{-1} 3393 (NH) and 1735 (CO); δ_H (200 MHz; ^2H_2O) 1.45 (3H, d, *J* 7.2, $C\underline{H}_3CH$), 3.74 (3H, s, CH_3O) and 4.10 (1H, q, *J* 7.2, $CH_3C\underline{H}$); δ_C (50.3 MHz; ^2H_2O) 17.85 (CH_3), 51.54 (CH), 56.33 (CH_3O) and 174.05 (CO); *m/z* (CI) 121 (7%, $[M - Cl + NH_3]^+$), 104 (100, $[M - Cl]^+$) and 45 (3, $[M - Cl - CO_2CH_3]^+$).

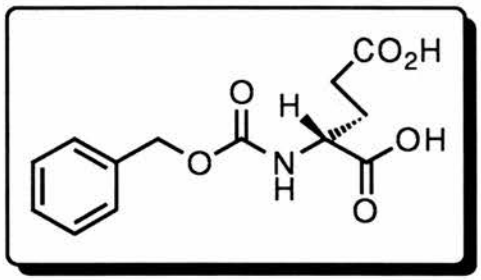
Methyl N-α-Boc-glycyl-(2*R*)-alaninate (115)



This compound was prepared in a manner identical to that for the fully protected dipeptide (**112**), using boc-glycine (**113**) (0.44 g, 2.5 mmol) and Ala.OMe.HCl (**114**) (0.35 g, 2.5 mmol) to give a yellow coloured oil which was refractory to crystallisation (0.57 g, 88%); *m/z* (Found: $[M + H]^+$, 261.1450. Calc. for $C_{11}H_{21}N_2O_5$: *m/z*, 261.1450); δ_H (200 MHz; C^2HCl_3) 1.43 (12H, m, CH_3 & $C(CH_3)_3$), 3.74 (3H, s, CH_3O), 3.82 (2H, s, CH_2), 4.60 (1H, m, CH), 5.18 (1H, t, *J* 4.9, $N\underline{H}CH_2$) and 6.69 (1H, d, *J* 5.9, $N\underline{H}CH$);

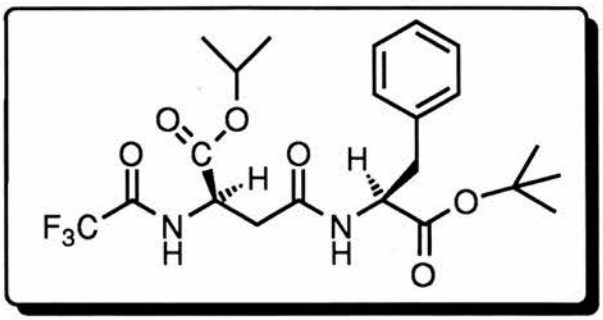
δ_C (50.3 MHz; C^2HCl_3) 18.92 (CH_3), 28.77 ($C(\underline{C}H_3)_3$), 44.78 (CH_2), 48.47 (CH_3O), 54.37 ($\underline{C}(CH_3)_3$), 65.7 (CH), 156.41 (ester CO), 169.48 (amide CO) and 173.72 (urethane CO); *m/z* (CI) 278 (11%, $[M + NH_4]^+$), 261 (100, $[M + H]^+$), 161 (99, [M - BOC + 2H]$^+$) and 102 (7, $[BOC + H]^+$).

N-α-Cbz-(2*R*)-glutamic acid (116)



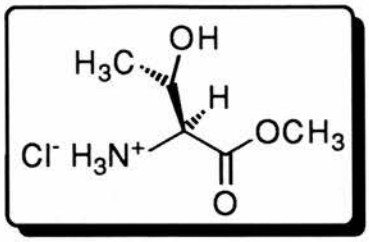
This compound was prepared in a manner identical to that for the (2*S*) isomer **(110)** using (2*R*)-glutamic acid (1.47 g, 10 mmol) to give a pale yellow oil, which was crystallised from ether/ light petroleum to give the product as a white solid (1.63 g, 58%), m.p. 116-117 °C (lit.,[317] 117.5-119 °C); $[\alpha]_D$ +6.7 (*c* 8.0, AcOH){lit.,[318] +7.2 (*c* 10.0, AcOH)}; υ_{max} (Nujol)/cm^{-1} 3304 (NH stretch), 1704 (CO) and 1550 (NH bend); δ_H (200 MHz; d_6-DMSO) 1.77 (2H, m, half of $C\underline{H}_2CH$), 1.93 (2H, m, half of $C\underline{H}_2CH$), 2.33 (2H, dd, *J* 5.9, 7.9, CH_2CO), 4.00 (1H, m, CH), 5.05 (2H, s, CH_2O), 7.38 (5H, s, aromatic) and 7.66 (1H, d, *J* 8.1, NH); δ_C (50.3 MHz; d_6-DMSO) 26.33 ($\underline{C}H_2CH$), 30.34 ($\underline{C}H_2CO$), 53.29 (CH), 65.70 (CH_2O), 127.99, 128.10 & 128.63 (aromatic), 137.23 (quat. aromatic), 156.45 (CONH) and 173.90 & 174.02 (CO_2H); *m/z* (CI) 282 (44%, $[M + H]^+$), 147 (29, $[C_5H_8NO_4 + H]^+$), 130 (100, $[M - C_8H_9NO_2]^+$), 91 (13, $PhCH_2^+$) and 73 (10, $[CH_2CH_2CO_2H]^+$).

^{t}Butyl N-trifluoroacetyl-(*R*)-(α-*iso*propyl)-β-aspartyl-(*S*)-phenylalaninate (117)



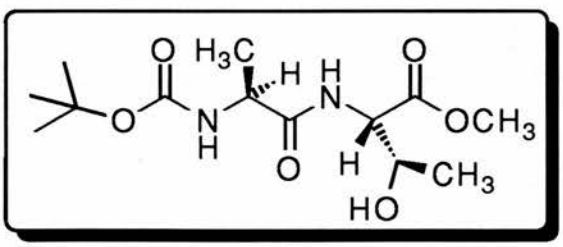
This compound was prepared in a manner identical to that for the protected dipeptide **(112)**, using α-*iso*propyl N-trifluoroacetyl-(2*R*)-aspartate (0.68 g, 2.5 mmol) and (2*S*)-phenylalanine *tert*-butyl ester hydrochloride (0.64 g, 2.5 mmol) to give a colourless oil which was crystallised from ethanol/ water to yield a white crystalline solid (0.42 g, 35%), m.p. 138-9 °C; *m/z* (Found: $[M + H]^+$, 475.2056. Calc. for $C_{22}H_{30}N_2O_6F_3$: *m/z*, 475.2056); $[\alpha]_D$ +16.27 (*c* 0.5, EtOH); υ_{max} (Nujol)/cm^{-1} 3329 & 3240 (NH), 2800-2600 (aromatic) and 1739-1717 (carbonyls); δ_H (300 MHz; C^2HCl_3) 1.16 (6H, 2 x d, *J* 6.3, $CH(C\underline{H}_3)_2$), 1.37 (9H, s, $C(C\underline{H}_3)_3$), 2.85 (1H, dd, J_{AB} 16.3, J_{AX} 4.3, half of $C\underline{H}_2CH$), 2.91 (1H, obscured dd, J_{AX} 4.1, half of $C\underline{H}_2CH$), 3.05 (2H, m, $C\underline{H}_2Ph$), 4.64 (2H, m, $C\underline{H}CH_2CO$ & $C\underline{H}CH_2Ph$), 5.05 (1H, hep, *J* 6.3, $OC\underline{H}(CH_3)_2$), 6.11 (1H, d, *J* 7.7, $CH_2CON\underline{H}$), 7.01-7.04 (5H, m, aromatic) and 7.74 (1H, d, *J* 7.9, $F_3CCON\underline{H}$); δ_C (75.5 MHz; C^2HCl_3) 21.43 ($C(\underline{C}H_3)_2$), 27.50 ($C(\underline{C}H_3)_3$), 36.04 ($\underline{C}H_2CH$), 37.78 ($\underline{C}H_2Ph$), 49.21 ($\underline{C}H(CH_3)_2$), 53.51 ($\underline{C}(CH_3)_3$), 70.17 ($\underline{C}HCH_2CO$), 82.63 ($\underline{C}HCH_2Ph$), 115.56 (q, CF_3, *J* 287.53), 127.00-129.22 (aromatic), 135.60 (quat. aromatic), 159.75 (q, $CF_3\underline{C}O$, *J* 37.7), 168.79 (CO's), 170.26 (amide CO); *m/z* (CI) 492 (4%, $[M + NH_4]^+$), 475 (76, $[M + H]^+$), 419 (100, $[M - C(CH_3)_3) + 2H]^+$), 401 (3, $[M - PhCH_2 + NH_4]^+$) and 91 (3, $PhCH_2^+$).

Methyl (2*S*)-threoninate hydrochloride (37)



This compound was prepared in a manner identical to that for **(111)** using (2*S*)-threonine (1.19 g, 10 mmol) to give a yellow coloured oil which was refractory to crystallisation (1.25 g, 74%); *m/z* (Found: $[M - Cl]^+$, 134.0817. Calc. for $C_5H_{12}NO_3$: *m/z*, 134.0817); $[\alpha]_D$ -9.07 (*c* 0.7, MeOH) {lit.,[319] +5.0 (*c* 3, MeOH) for the 2R isomer}; υ_{max} (Nujol)/cm^{-1} 3390 (NH) and 1739 (CO); δ_H (200 MHz; ^2H_2O) 1.27 (3H, d, *J* 6.6, $C\underline{H}_3CH$), 3.81 (3H, s, OCH_3), 4.04 (1H, d, *J* 3.7, $C\underline{H}NH$) and 4.36 (1H, dq, *J* 6.6, 3.7, $C\underline{H}CH_3$); δ_C (50.3 MHz; ^2H_2O) 21.61 ($CH\underline{C}H_3$), 56.53 (α-CH), 61.20 (OCH_3), 68.03 (β-CH) and 172.04 (CO_2); *m/z* (EI) 134 (10%, $[MH - HCl]^+$), 74 (100, $[M - Cl - CO_2CH_3]^+$) and 59 (71, $[CO_2CH_3]^+$).

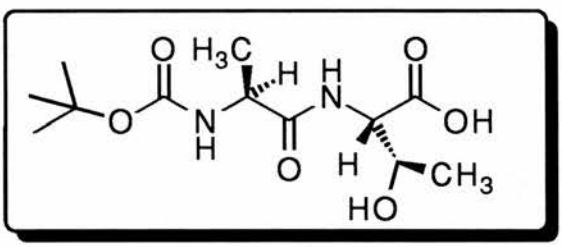
Methyl N-α-Boc-(*S*)-alanyl-(*S*)-threoninate (38)



This compound was prepared in a manner identical to that for the protected dipeptide **(112)** using N-α-Boc-(2*S*)-alanine (1.89 g, 10 mmol) and Thr.OMe.HCl **(37)** (1.695 g, 10 mmol), except that **(37)** was dissolved in DMF and the product was recrystallised from ethyl acetate/ petroleum ether to give a solid (3.04 g, 100%), m.p. 103-104 °C (lit.,[215] 104.5-105 °C); *m/z* (Found: $[M + H]^+$, 305.1709. Calc. for $C_{13}H_{25}N_2O_6$: *m/z*, 305.1712);

$[\alpha]_D$ -15.0 (*c* 2.61, $CHCl_3$) {lit.,[215] -15.3 (*c* 2.61, $CHCl_3$); υ_{max} ($CHCl_3$)/cm^{-1} 3430-3250 (secondary CONH), 2850-2800 (OCH_3), 1700-1667 (urethane) and 1368 ($C(CH_3)_3$); δ_H (200 MHz; C^2HCl_3) 1.21 (3H, d, *J* 6.4, thr CH_3), 1.36 (3H, d, *J* 5.6, ala CH_3), 1.41 (9H, s, $C(CH_3)_3$), 3.74 (3H, s, OCH_3), 4.21 & 4.32 (2H, m, C<u>H</u>CH_3 & C<u>H</u>OH), 4.60 (1H, 2 x d, *J* 2.56, thr α-CH), 5.2 (1H, d, *J* 7.32, urethane NH) and 6.98 (1 H, d, *J* 8.8, amide NH); δ_C (50.3 MHz; C^2HCl_3) 18.81 (ala CH_3), 20.31 (thr CH_3), 28.75 ($C(\underline{C}H_3)_3$), 50.68 (ala α-CH), 52.97 (thr α-CH), 57.90 (OCH_3), 68.63 (thr β-CH), 80.62 ($\underline{C}(CH_3)_3$), 150.06 (urethane CO), 171.82 (amide CO) and 173.93 (ester CO); *m/z* (CI) 305 (34%, $[M + H]^+$), 249 (100, $[M - C(CH_3)_3 + 2H]^+$), 205 (66, [M - Boc + $NH_3]^+$) and 187 (14, $[M - H - Boc]^+$).

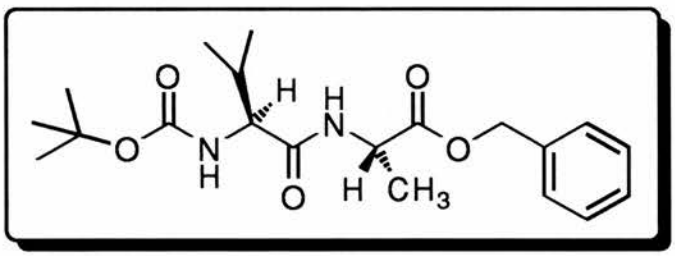
N-α-Boc-(*S*)-alanine-(*S*)-threonine (39)



To a stirred solution of the protected dipeptide (**38**) (1.00 g, 3.3 mmol) in ethanol (20 cm^3) was added aqueous potassium carbonate (0.5 g, 3.63 mmol) and the reaction left to stir for 4-5 h. The reaction mixture was washed with diethyl ether (20 cm^3) and the aqueous layer acidified with 1 M HCl and then extracted with DCM (2 x 20 cm^3), the organic layer washed with 1 M HCl (20 cm^3), water (20 cm^3), brine (20 cm^3) and dried ($MgSO_4$). The solvent was removed to give a white solid which was crystallised from ether/ light petroleum to give the product as a white crystalline solid (0.508 g, 53%), m.p. 77-80 °C; υ_{max} (Nujol)/cm^{-1} 3460-3360 (secondary CONH), 2480 (OH stretch), 1734 (urethane) and 1377 ($C(CH_3)_3$); δ_H (200 MHz; C^2HCl_3) 1.20 (3H, d, *J* 6.2, C<u>H</u>$_3$CHOH), 1.39 (3H, d, *J* 7.0, NHC<u>H</u>$_3$CH), 1.44 (9H, s, $C(C\underline{H}_3)_3$), 4.24 (2H, m, C<u>H</u>CH_3 & C<u>H</u>OH), 4.58 (1H, dd, *J* 2.2 & 1.6, C<u>H</u>CHOH), 5.16 (1H, d, *J* 7.4, urethane NH) and

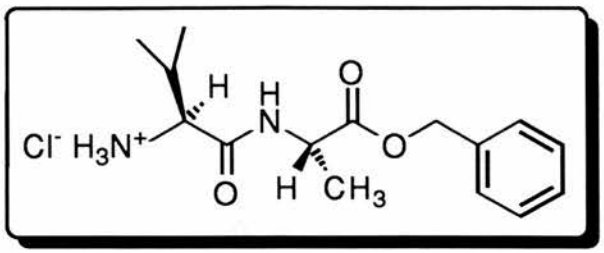
6.90 (1H, d, *J* 8.0, amide NH); δ_C (75.5 MHz; C^2HCl_3) 13.99 (ala CH_3), 19.81 (thr CH_3), 28.17 ($C(\underline{C}H_3)_3$), 57.20 (one of α-CH), 61.58 (one of α-CH), 68.25 (thr β-CH), 80.14 ($\underline{C}(CH_3)_3$), 155.43 (urethane CO), 170.66 (amide CO) and 173.03 (CO_2H); *m/z* (EI) 291 (16%, $[M + H]^+$), 290 (79, M^+), 218 {22, $[M - C(CH_3)_3O + H]^+$}, 174 (3, [M - BOC]$^+$), 116 (11, BOC^+), 57 (86, $C(CH_3)_3^+$) and 44 (100, CO_2).

Benzyl N-α-Boc-(*S*)-valyl-(*S*)-alaninate (41)



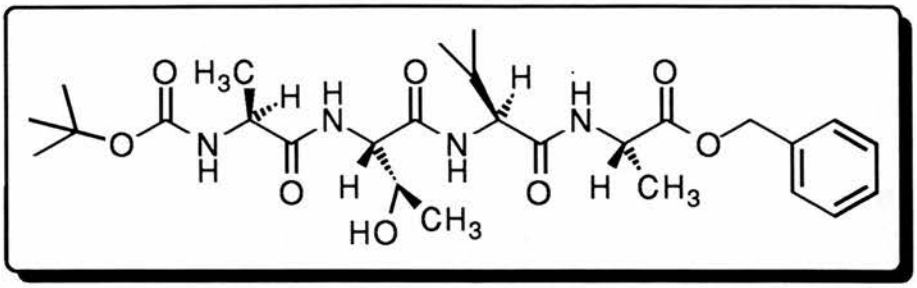
This compound was prepared in a manner identical to that for the protected dipeptide (**112**) using N-α–Boc-(2*S*)-valine (2.17 g, 10 mmol) and benzyl-(2*S*)-alaninate hydrochloride (2.16 g, 10 mmol), except that the latter was dissolved in DMF and the product was recrystallised from ethanol/ H_2O to give a white crystalline solid (3.56 g, 94%), m.p. 84 °C (lit.,[216] 82-83 °C); *m/z* (Found: $[M + H]^+$, 379.2233. Calc. for $C_{20}H_{31}N_2O_5$: *m/z*, 379.2233); $[\alpha]_D$ -110 (*c* 1, MeOH) {lit.,[216] -114 (*c* 1, MeOH)}; υ_{max} (Nujol)/cm^{-1} 3334 (NH), 3000-2800 (aromatic) and 1758-1731 (carbonyls); δ_H (200 MHz; C^2HCl_3) 0.93 (6H, 2 x d, *J* 6.9, $CH(C\underline{H}_3)_2$), 1.44 (12H, m, $C(C\underline{H}_3)_3$ & $C\underline{H}_3CH$), 2.11 (1H, m, $C\underline{H}(CH_3)_2$), 3.92 (1H, dd, *J* 5.9, 5.8, $C\underline{H}CONH$), 4.64 (1H, m, $C\underline{H}CH_3$), 5.18 (1H, d, *J* 5.0, $N\underline{H}CHCH_3$), 5.18 (2H, s, $C\underline{H}_2Ph$), 6.47 (1H, d, *J* 5.4, $OCON\underline{H}$) and 7.35 (5H, s, aromatic); δ_C (75.5 MHz; C^2HCl_3) 17.56 (one diastereomer of $CH(\underline{C}H_3)_2$), 18.19 (CH_3), 19.07 (one diastereomer of $CH(\underline{C}H_3)_2$), 28.18 ($C(\underline{C}H_3)_3$), 30.88 ($\underline{C}H(CH_3)_2$), 47.98 (one of α-CH), 59.67 (one of α-CH), 67.07 ($\underline{C}H_2Ph$), 79.78 ($\underline{C}(CH_3)_3$), 128.06-128.50 (aromatic), 135.13 (quat. aromatic), 155.70 (urethane CO), 171.02 (amide CO) and 172.40 (ester CO); *m/z* (CI) 379 (11%, $[M + H]^+$), 323 (47, $[M + H - CHCH(CH_3)_2]^+$), 279 (100, [M + H - BOC]$^+$) and 91 (7, CH_2Ph^+).

Benzyl-(*S*)-valyl-(*S*)-alaninate hydrochloride (42)



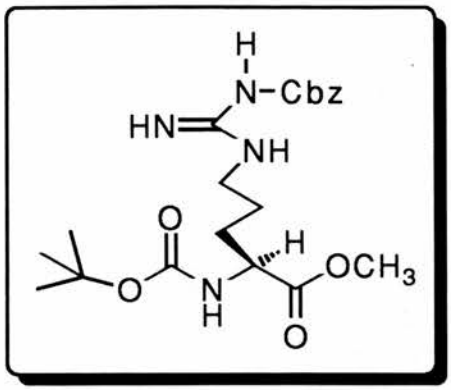
A solution of the boc-protected dipeptide (**41**) (100 mg, 0.265 mmol) in ethyl acetate (15-20 cm^3) was cooled to 0 °C. HCl gas was bubbled through the solution for 30 min, the reaction allowed to warm up to room temperature and the solution then left stirring for 1 h. Excess HCl gas was removed by a stream of nitrogen and the solvent then removed under reduced pressure to give a pale yellow oil which was refractory to crystallisation (80 mg, 96%); *m/z* (Found: $[M - Cl]^+$, 279.1709. $C_{15}H_{23}N_2O_3$ requires *m/z*, 279.1709); $[\alpha]_D$ -19.8 (*c* 0.25, H_2O); υ_{max} (Nujol)/cm^{-1} 3400 (NH stretch), 1750 (CO_2) and 1377 ($CH(CH_3)_2$); δ_H (200 MHz; ^2H_2O) 0.87 (6H, d, *J* 6.6, $CH(C\underline{H}_3)_2$), 1.37 (3H, d, *J* 6.8, $CHC\underline{H}_3$), 2.05 (1H, m, $C\underline{H}(CH_3)_2$), 3.71 (1H, d, *J* 4.6, $C\underline{H}CONH$), 4.44 (1H, q, *J* 6.2, $C\underline{H}CH_3$), 5.12 (2H, s, CH_2Ph) and 7.35 (5H, s, aromatic); δ_C (50.3 MHz; ^2H_2O) 16.86 (one diastereomer of $CH(\underline{C}H_3)_2$), 17.79 (CH_3), 18.44 (one diastereomer of $CH(\underline{C}H_3)_2$), 49.91 (ala α-CH), 59.32 (val α-CH), 68.88 (CH_2Ph), 129.49-129.83 (aromatic), 136.11 (quat. aromatic), 169.96 (amide CO) and 174.77 (ester CO); *m/z* (EI) 279 (11%, $[M - Cl]^+$), 144 (14, $[M - Cl - CO_2CH_2Ph]^+$), 108 (8, $[CH_2Ph + NH_3]^+$), 91 (50, CH_2Ph^+), 72 (100, $[C_3H_5NO + H]^+$) and 44 (63, CO_2).

Benzyl N-α-Boc-(*S*)-alanyl-(*S*)-threonyl-(*S*)-valyl-(*S*)-alaninate (43)



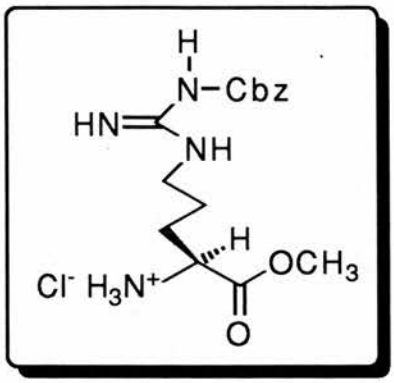
This compound was prepared in a manner identical to that for **(112)** using boc-protected dipeptide **(39)** (0.607 g, 2.1 mmol) and the dipeptide **(42)** (0.66 g, 2.1 mmol) to give a colourless oil which was recrystallised from acetone/ H_2O to yield a white crystalline solid (0.942 g, 82%), m.p. 166-168 °C; (Found: C, 58.8; H, 7.6; N, 10.05. $C_{27}H_{42}N_4O_8$ requires C, 58.9; H, 7.7; N, 10.15%); *m/z* (Found: $[M + Na]^+$, 573.2903. $C_{27}H_{42}N_4O_8Na$ requires: *m/z*, 573.3000); $[\alpha]_D$ -99.75 (*c* 0.2, $CHCl_3$); υ_{max} (Nujol)/cm^{-1} 1688 (CO_2Bn), 1623 (aromatic), 1377 ($C(CH_3)_2$) and 1267 (OH bending); δ_H (200 MHz; C^2HCl_3) 0.92 (6H, 2 x d, *J* 5.6, val $CH(C\underline{H}_3)_2$), 1.44 (18H, m, $C(CH_3)_3$ & 2 x ala CH_3 & thr CH_3), 2.15 (1H, m, $C\underline{H}(CH_3)_2$), 3.85 (1H, d, *J* 6.6, val α-CH), 4.16 (1H, obscured q, *J* 6.4, ala α-CH), 4.28 (1H, q, *J* 6.4, thr β-CH), 4.61 (1H, quintet, *J* 7.2, thr α-CH), 5.02 (1H, d, *J* 7.0, urethane NH), 5.17 (2H, d, *J* 3.6, CH_2Ph), 6.65 (1H, d, *J* 6.4, NH), 6.79 (1H, d, *J* 5.4, NH) and 7.35 (5H, s, aromatic); δ_C (50.3 MHz; C^2HCl_3) 18.12 (val $CH(\underline{C}H_3)_2$), 18.88 & 19.32 (2 x ala CH_3), 19.89 (thr CH_3), 29.09 ($C(\underline{C}H_3)_3$), 31.22 (val $\underline{C}H(CH_3)_2$), 48.58 & 50.40 (2 x ala α-CH), 59.40 (val α-CH), 64.48 (thr α-CH), 67.62 (CH_2Ph), 68.29 (thr β-CH), 80.34 ($\underline{C}(CH_3)_3$), 128.65-129.03 (aromatic), 135.7 (quat. aromatic), 156.09 (urethane CO), 170.99, 171.88 &172.97 (amide CO's) and 174.14 (ester CO); *m/z* (FAB) 573 (100%, $[M + Na]^+$), 551 (9, $[M + H]^+$) and 495 (9, $[M - BOC + 2H]^+$).

Methyl N-α-Boc-N-ω-Cbz-(2*S*)-argininate (45)



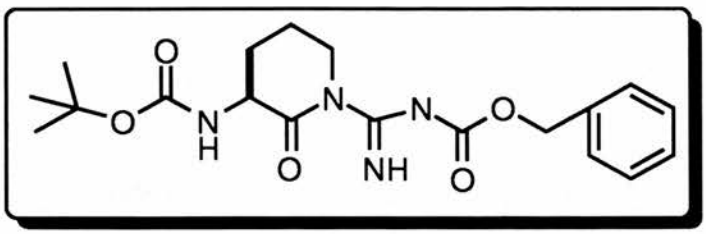
To a stirred solution of N-α-Boc-N-ω-Cbz-(2*S*)-arginine (1.02 g, 2.5 mmol) in ether (20 cm^3) and THF (20 cm^3), cooled to 0 °C was added an excess of ethereal diazomethane (50 cm^3). The reaction mixture was allowed to warm up to room temperature and then left to stir for 1 h. Excess diazomethane was removed by bubbling nitrogen gas through the solution. The solvent was then removed under reduced pressure and the resultant white solid recrystallised from DCM/ ether to give a white solid (0.75 g, 71%), m.p. 75-77 °C; (Found: C, 56.9; H, 7.05; N, 12.95. $C_{20}H_{30}N_4O_6$ requires C, 56.85; H, 7.15; N, 13.25%); *m/z* (Found: $[M + H]^+$, 423.2244. $C_{20}H_{31}N_4O_6$ requires *m/z*, 423.2247); $[\alpha]_D$ -9.0 (*c* 0.3, $CHCl_3$); υ_{max} (Nujol)/cm^{-1} 3305 (primary NH_2), 1720-1670 (urethane), 1645 (CN) and 1377 ($C(CH_3)_3$); δ_H (300 MHz; C^2HCl_3) 1.49 (9H, s, $C(CH_3)_3$), 1.62 (2H, m, CH_2), 3.07 & 3.28 (4H, m, 2 x CH_2), 3.74 (3H, s, OCH_3), 4.28 (1H, d, *J* 9, α-CH), 5.06 (2H, s, CH_2Ph) and 7.38-7.42 (5H, m, aromatic); δ_C (75.5 MHz; C^2HCl_3) 15.14 ($\underline{C}H_2CH_2$), 24.83 ($CH\underline{C}H_2$), 28.20 ($C(\underline{C}H_3)_3$), 30.64 (CH_2NH), 39.90 (α-CH), 52.36 (OCH_3), 66.00 (CH_2Ph), 80.31 ($\underline{C}(CH_3)_3$), 127.50-128.20 (aromatic), 137.43 (quat. aromatic), 155.85 (CN), 161.48 (urethane CO), 163.63 (CN$\underline{C}$O) and 172.75 (ester CO); *m/z* (CI) 423 (3%, $[M + H]^+$), 391 (7, $[M - OCH_3]^+$), 147 (100, $[M - C_{14}H_{17}N_3O_3]^+$) and 91 (38, CH_2Ph^+).

Methyl N-ω-Cbz-(2*S*)-argininate hydrochloride (46)



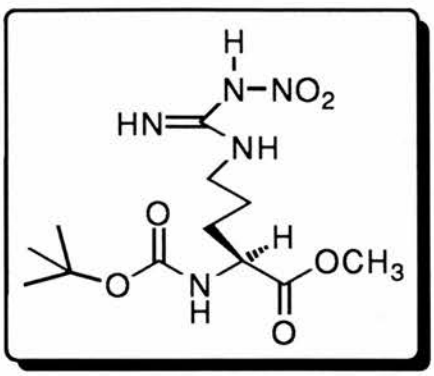
This compound was prepared in a manner identical to that for the dipeptide **(42)** using the boc-protected arginine derivative **(45)** (1.075 g, 2.55 mmol) to give a colourless oil which was refractory to crystallisation (0.843 g, 94%); $[\alpha]_D$ 10.9 (*c* 0.5, MeOH); υ_{max} (Nujol)/cm^{-1} 3400 (NH stretch), 2690-2430 (NH_2^+), 1675 (CN) and 1600 (NH_3^+); δ_H (200 MHz; ^2H_2O) 1.75 & 1.95 (4H, m, $C\underline{H}_2CH_2$ & CH_2NH), 3.33 (2H, m, $CHC\underline{H}_2$), 4.13 (1H, t, *J* 6.0, α-CH), 5.23 (2H, s, CH_2Ph) and 7.38 (5H, s, aromatic); δ_C (75.5 MHz; ^2H_2O) 13.46 ($\underline{C}H_2CH_2$), 21.96 ($CH\underline{C}H_2$), 24.92 (CH_2NH), 38.77 (α-CH), 51.78 (OCH_3), 66.97 (CH_2Ph), 126.43-127.11 (aromatic), 132.88 (quat. aromatic), 151.61 (CN) and 168.38 (CO); *m/z* (EI) 322 (7%, $[M - HCl]^+$), 108 (33, $[CH_2Ph + NH_3]^+$) and 91 (100, CH_2Ph^+).

1-Benzyloxycarbonylamidino-3(*S*)-butoxycarbonylamino-piperdin-2-one (47)



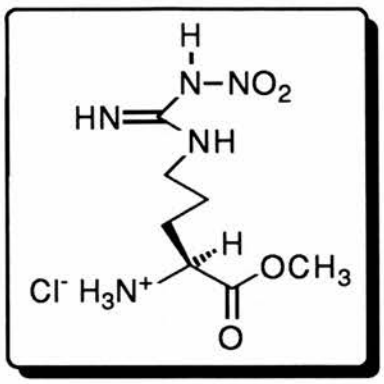
To a stirred solution of N-α-Boc-N-ω-Cbz-(2*S*)-arginine (0.05 g, 0.12 mmol) in dry THF (10 cm^3) was added N-methylmorpholine (13.2 cm^3, 0.12 mmol) and the solution cooled to -15 °C. Isobutylchloroformate (15.6 cm^3, 0.12 mmol) was added and the solution stirred at -15 °C for 5 min and then at room temperature overnight. The salts were filtered off and the solvent removed under reduced pressure to give a colourless oil (0.02 g, 43%); *m/z* (Found: $[M + H]^+$, 391.1975. $C_{19}H_{27}N_4O_5$ requires *m/z*, 391.1981); $[\alpha]_D$ 5.38 (*c* 0.4, $CHCl_3$); υ_{max} (Nujol)/cm^{-1} 2853 (CH stretch) and 1162-1263 (ester C-O stretch); δ_H (200 MHz; C^2HCl_3) 1.37 (9H, s, $C(CH_3)_3$), 1.68 (4H, br. s, $C\underline{H}_2CH_2$ & $C\underline{H}_2CH$), 5.17 (2H, s, CH_2Ph), 5.80 (1H, d, *J* 2.2, urethane NH) and 7.35-7.40 (5H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 24.09 ($\underline{C}H_2CH_2$), 28.16 ($C(\underline{C}H_3)_3$), 29.88 ($\underline{C}H_2CH$), 40.62 (CH_2NH), 53.87 (α-CH), 68.08 (CH_2Ph), 79.39 ($\underline{C}(CH_3)_3$), 128.06-128.46 (aromatic), 134.61 (quat. aromatic), 154.30 (CN), 154.44 (urethane CO), 155.82 (CN$\underline{C}$O) and 177.90 (ester CO); *m/z* (CI) 391 (14, $[M + H]^+$), 274 (18, $[C_{14}H_{16}N_3O_3]^+$), 178 (15, $[C_9H_9N_2O_2 + H]^+$), 115 (22, $[C_5H_7NO + NH_4]^+$) and 91 (100, $PhCH_2^+$).

Methyl N-α-Boc-N-ω-nitro-(2*S*)-argininate (49)



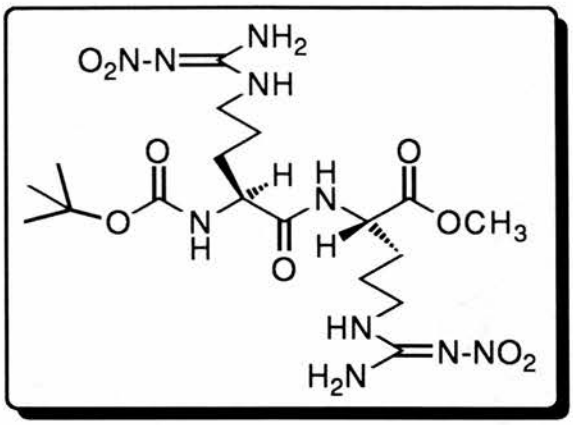
This compound was prepared in a manner identical to that for the methyl ester **(45)** using N-α-Boc-N-ω-nitro-(2*S*)-arginine (1.60 g, 5 mmol) to give an off-white solid which was recrystallised from DCM/ ether to give a white solid (1.43 g, 86%), m.p. 74-76 °C (lit.,[320] 98-100 °C, for the ethyl ester derivative); (Found: C, 43.15; H, 6.85; N, 20.85. $C_{12}H_{23}N_5O_6$ requires C, 43.25; H, 6.95; N, 21.0%); *m/z* (Found: $[M + H]^+$, 334.1733. $C_{12}H_{24}N_5O_6$ requires *m/z*, 334.1727); $[\alpha]_D$ -41.5 (*c* 0.5, $CHCl_3$); υ_{max} (Nujol)/cm^{-1} 3401 (NH stretch), 2953 (CH stretch), 1730 (CO_2CH_3) and 1560-1625 & 1268 ($N\text{-}NO_2$); δ_H (200 MHz; C^2HCl_3) 1.44 (9H, s, $C(CH_3)_3$), 1.84 (4H, m, $C\underline{H}_2CH_2$ & $C\underline{H}_2CH$), 3.50 (2H, m, $C\underline{H}_2NH$), 3.78 (3H, s, OCH_3), 4.34 (1H, t, *J* 8.4, α-CH), 5.38 (1H, d, *J* 8.2, urethane NH), 7.62 (1H, s, $CH_2N\underline{H}$) and 8.75 (1H, s, NH_2); δ_C (50.3 MHz; C^2HCl_3) 25.0 ($\underline{C}H_2CH_2$), 28.2 ($C(\underline{C}H_3)_3$), 31.9 ($\underline{C}H_2CH$), 40.77 (CH_2NH), 52.15 (α-CH), 53.19 (OCH_3), 81.26 ($\underline{C}(CH_3)_3$), 156.98 (CN), 159.86 (urethane CO) and 173.27 (ester CO); *m/z* (CI) 334 (7%, $[M + H]^+$), 234 (24, $[M - BOC + NH_3]^+$), 217 (10, $[M - BOC]^+$), 172 (100), 73 (51, $[C(CH_3)_3O]^+$) and 60 (9, $[CO_2CH_3 + H]^+$).

Methyl N-ω-nitro-(2*S*)-argininate hydrochloride (50)



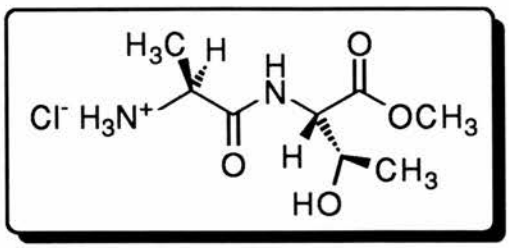
This compound was prepared in a manner identical to that for the dipeptide **(42)** using the methyl ester **(49)** (1.00 g, 3 mmol) to give an oil which was refractory to crystallisation (0.61 g, 75%); $[\alpha]_D$ 7.31 (*c* 1.3, MeOH); υ_{max} (Nujol)/cm^{-1} 3302-3376 (NH stretch), 2898-2927 (CH_2), 1751 (CO_2CH_3) and 1600 (N-NO_2); δ_H (200 MHz; ^2H_2O) 1.70 (2H, m, C$\underline{H}_2$CH_2), 1.98 (2H, m, C$\underline{H}_2$CH), 3.29 (2H, m, C$\underline{H}_2$NH), 3.78 (3H, s, OCH_3) and 4.14 (1H, t, *J* 6.2, α-CH); δ_C (75.5 MHz; ^2H_2O) 21.52 ($\underline{C}H_2CH_2$), 25.01 ($\underline{C}H_2CH$), 30.33 (CH_2NH), 50.60 (α-CH), 51.74 (OCH_3), 157.02 (CN) and 168.50 (ester CO); *m/z* (CI) 234 (7%, [M - Cl]$^+$), 186 (100, [M - $NH_3^+Cl^-$ - OCH_3]$^+$) and 60 (11, [CO_2Me + H]$^+$).

Methyl N-α-Boc-N-ω-nitro-(*S*)-arginyl-N-ω-nitro-(*S*)-argininate (51)



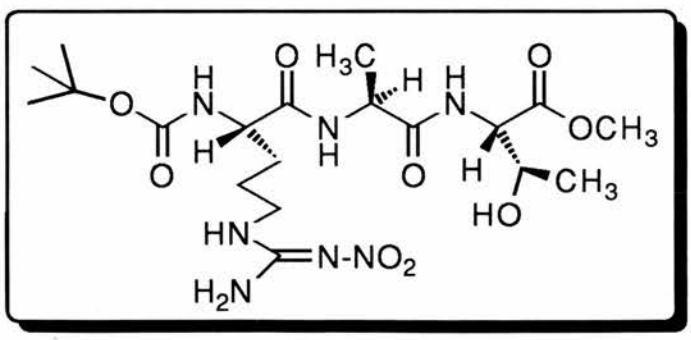
To a stirred solution of N-α-Boc-N-ω-nitro-(2*S*)-arginine (1.60 g, 5 mmol) in DMF was added PyBOP (2.60 g, 5 mmol), the methyl ester **(50)** (1.48 g, 5.5 mmol) and triethylamine (1.92 cm^3, 13.75 mmol). The solution was then left to stir overnight at room temperature. The salts were filtered off and the solvent removed under reduced pressure to give a viscous orangy-brown oil which was refractory to crystallisation and purification by silica chromatography (0.88 g, 33%); *m/z* (Found: $[M + Na]^{+\cdot}$ 557.2397. $C_{18}H_{34}N_{10}O_9Na$ requires *m/z*, 557.2500); $[\alpha]_D$ -4.25 (*c* 1, MeOH); υ_{max} (neat)/cm^{-1} 3391(NH stretch), 1739 (CO_2CH_3) and 1263 (N-NO_2); δ_H (200 MHz; $C^2H_3O^2H$) 1.43 (9H, s, $C(CH_3)_3$), 1.85 (8H, m, 4 x CH_2), 3.30 (4H, m, 2 x CH_2), 3.72 (3H, s, OCH_3) and 4.08 & 4.47 (2H, m, 2 x α-CH); δ_C (50.3 MHz; $C^2H_3O^2H$) 26.3 & 27.6 (2 x $\underline{C}H_2CH_2$), 27.76 & 29.95 (2 x $\underline{C}H_2CH$), 29.05 ($C(CH_3)_3$), 32.02 & 41.96 (2 x CH_2NH), 53.40 & 55.86 (2 x α-CH), 53.48 (OCH_3), 80.1 ($C(CH_3)_3$), 156.8 & 157.0 (2 x CN), 161.25 (urethane CO), 175.44 (amide CO) and 180.04 (ester CO); *m/z* (FAB) 557 (20%, $[M + Na]^+$), 535 (8, $[M + H]^+$) and 187 (100).

Methyl (*S*)-alanyl-(*S*)-threoninate hydrochloride (54)



This compound was prepared in a manner identical to that for the dipeptide **(42)** using the fully protected dipeptide **(38)** (0.76 g, 2.5 mmol) to give an oil which was refractory to crystallisation (0.597 g, 99%); $[\alpha]_D$ -26.0 (*c* 1.5, H_2O) {lit.,[215] -7 (*c* 2.97, MeOH) for the TFA salt or +17.2 (*c* 3, MeOH) for the (*R*)-threonyl isomer[321]}; υ_{max} (Nujol)/cm^{-1} 3339 (OH), 1746 (CO_2), 1377 (OH bending) and 1089 (CO stretch); δ_H (200 MHz; ^2H_2O) 1.22 (3H, d, *J* 6.8, thr CH_3), 1.55 (3H, d, *J* 7.0, ala CH_3), 3.76 (3H, s, OCH_3), 4.17 (1H, q, *J* 7.0, ala α-CH) and 4.4 (2H, m, thr α- & β-CH); δ_C (75.5 MHz; ^2H_2O) 16.98 (ala CH_3), 19.15 (thr CH_3), 49.42 (ala α-CH), 53.51 (thr α-CH), 58.75 (OCH_3), 67.29 (thr β-CH), 171.82 (amide CO) and 172.55 (ester CO); *m/z* (CI) 205 (85%, $[M - Cl]^+$), 74 (100, $[alanyl + 3H]^+$) and 60 (35, $[CO_2CH_3 + H]^+$).

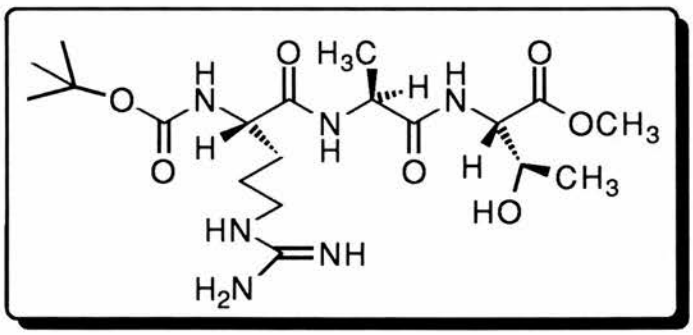
Methyl N-α-Boc-N-ω-nitro-(*S*)-arginyl-(*S*)-alanyl-(*S*)-threoninate (55)



This compound was prepared in a manner identical to that for the dipeptide **(112)** using N-α-Boc-N-ω-nitro-(2*S*)-arginine (0.87 g, 2.73 mmol) and the methyl ester **(54)** (0.66 g, 2.73 mmol), except that DMF was used to dissolve **(54)** and the compound was obtained

as an oil (0.99 g, 72%); υ_{max} (DCM)/cm^{-1} 3460 (secondary CONH), 3300-3400 (NH stretch), 1382 (CH_3 symmetrical deformation), 1330 (OH bending) and 1275 (N-NO_2); δ_H (200 MHz; C^2HCl_3) 0.93 (3H, d, *J* 2.2, thr CH_3), 1.27 (3H, obscured dd, *J* 7.2, ala CH_3), 1.43 (9H, s, $C(CH_3)_3$), 1.74 (4H, m, $C\underline{H}_2CH_2$ & $C\underline{H}_2CH$), 3.35 (2H, m, $C\underline{H}_2NH$), 3.75 (3H, s, OCH_3), 3.86 (1H, m, arg α-CH), 4.16 (1H, q, *J* 7.2, ala α-CH), 4.34 (1H, m, thr β-CH), 4.51 (1H, m, thr α-CH), 5.55 (1H, d, *J* 7.4, urethane NH), 5.65 (1H, d, *J* 7.4, amide NH), 5.85 (1H, d, *J* 7.8, amide NH), 7.7 (2H, br. s, NH_2) and 8.65 (1H, br. s, $CH_2N\underline{H}$); δ_C (50.3 MHz; C^2HCl_3) 17.81 (ala CH_3), 19.48 (thr CH_3), 24.80 (arg $\underline{C}H_2CH_2$), 28.71 ($C(\underline{C}H_3)_3$), 30.80 (arg $\underline{C}H_2CH$), 40.93 (CH_2NH), 48.67 (ala α-CH), 53.0 (arg α-CH), 53.99 (thr α-CH), 62.12 (OCH_3), 67.0 (thr β-CH), 80.47 ($\underline{C}(CH_3)_3$), 156.52 (CN), 159.75 (urethane CO), 163.39 & 172.69 (amide CO's) and 173.29 (ester CO); *m/z* (E/S) 529 (11.5%, $[M + Na]^+$), 506 (5.1, $[M + H]^+$), 475 (3.2, $[M - OMe + H]^+$) and 102 (100, $[Boc + H]^+$).

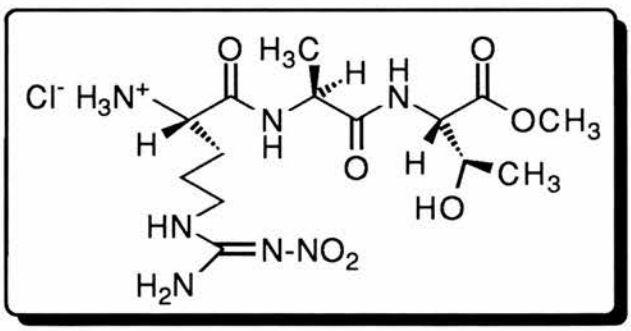
Methyl N-α-Boc-(*S*)-arginyl-(*S*)-alanyl-(*S*)-threoninate (118)



A solution of the protected peptide (**55**) (200 mg, 0.4 mmol) in 4.4% formic acid/ methanol (3 cm^3) was added to a 25 cm^3 flask containing 10% palladium on activated charcoal (200 mg) and 4.4% formic acid/ methanol (10 cm^3). The mixture was stirred under nitrogen for 5-6 h. The Pd/ C was then filtered off and washed with methanol (10 cm^3) and distilled water (10-20 cm^3). The combined filtrates were concentrated under reduced pressure to 5 cm^3 and the product extracted into ethyl acetate (2 x 10 cm^3). The organic layer was then washed with water (10 cm^3), saturated brine (10 cm^3) and dried

($MgSO_4$). The solvent was removed under reduced pressure to give an off-white foamy solid (143 mg, 78%); δ_H (200 MHz; C^2HCl_3) 0.91 (3H, d, *J* 6.6, thr CH_3), 1.24 (3H, dd, J_{AB} 6.2, J_{AX} 7.0, ala CH_3), 1.41 (9H, s, $C(CH_3)_3$), 1.72 (4H, br. s, arg $C\underline{H}_2CH_2$ & $CH_2\underline{C}H$), 3.33 (2H, br. s, arg $C\underline{H}_2NH$), 3.72 (3H, q, *J* 7.0, OCH_3), 3.86 (1H, d, *J* 6.6, arg α-CH), 4.14 (1H, obscured qd, *J* 7.2, ala α-CH), 4.32 (1H, br. s, thr β-CH), 4.49 (1H, m, thr α-CH), 5.64 (1H, d, *J* 6.8, urethane NH), 5.73 (1H, d, *J* 6.4, amide NH), 5.85 (1H, d, *J* 6.8, amide NH), 7.39 (1H, d, *J* 5.0, C=NH), 7.66 (2H, br. s, NH_2) and 8.57 (1H, br. s, $CH_2N\underline{H}$); δ_C (50.3 MHz; C^2HCl_3) 17.98 (ala CH_3), 19.46 (thr CH_3), 24.98 (arg $\underline{C}H_2CH_2$), 28.79 ($C(\underline{C}H_3)_3$), 30.95 (arg $\underline{C}H_2CH$), 40.99 (CH_2NH), 49.48 (ala α-CH), 53.0 (arg α-CH), 53.51 (thr α-CH), 62.15 (OCH_3), 68.5 (thr β-CH), 80.89 ($\underline{C}(CH_3)_3$), 156.71 (CN), 159.74 (urethane CO), 164.43 & 173.0 (amide CO's) and 173.42 (ester CO); *m/z* (CI) 461 (1%, $[M + H]^+$), 429 (3, $[M - OMe]^+$), 257 {100, [M - (alanyl-threonyl.OMe)]$^+$} and 58 (76, $[C(CH_3)_3 + H]^+$).

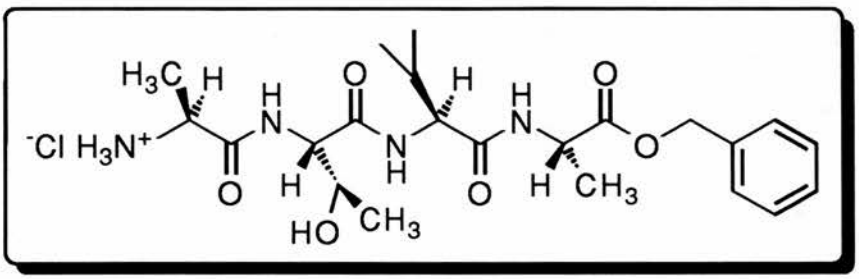
Methyl N-ω-nitro-(*S*)-arginyl-(*S*)-alanyl-(*S*)-threoninate hydrochloride (56)



This compound was prepared in a manner identical to that for the dipeptide (**42**) using the tripeptide (**55**) (0.993 g, 1.97 mmol) to give a colourless oil which was refractory to crystallisation (0.87 g, quantitative); δ_H (200 MHz; ^2H_2O) 0.96 (3H, d, *J* 6.2, thr CH_3), 1.35 (3H, d, *J* 6.6, ala CH_3), 1.76 (2H, br. s, arg $C\underline{H}_2CH_2$), 2.0 (2H, m, arg $C\underline{H}_2CH$), 3.35 (2H, m, $C\underline{H}_2NH$), 4.10 (2H, m, arg α-CH & ala α-CH) and 4.45 (2H, m, thr α & β-CH); δ_C (75.5 MHz; ^2H_2O) 16.22 (ala CH_3), 17.05 (thr CH_3), 20.99 (arg $\underline{C}H_2CH_2$), 26.24 (arg $\underline{C}H_2CH$), 38.44 (arg CH_2NH), 46.76 (arg α-CH), 50.75 (ala α-CH), 51.85

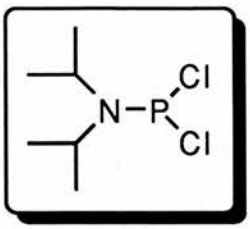
(thr α-CH), 56.53 (OCH_3), 67.56 (thr β-CH), 156.93 (CN), 163.73 & 170.25 (amide CO's) and 173.86 (ester CO); *m/z* (FAB) 465 (6%, $[MH + Na]^+$), 442 (23, $[M + H]^+$) and 102 (100).

Benzyl (*S*)-alanyl-(*S*)-threonyl-(*S*)-valyl-(*S*)-alaninate hydrochloride (119)



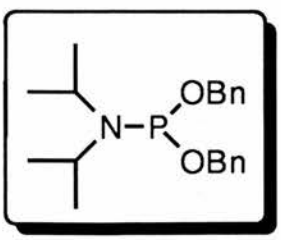
This compound was prepared in a manner identical to that for the dipeptide **(42)** using the fully protected tetrapeptide **(43)** (67 mg, 0.12 mmol) to give an oil (58 mg, quantitative); $[\alpha]_D$ -11.68 (*c* 2.5, MeOH); υ_{max} (DCM)/cm^{-1} 3064 (NH_3^+), 1713 (CO_2Ar), 1545 (NH bending) and 1378 ($C(CH_3)_2$); δ_H (200 MHz; $C^2H_3O^2H$) 0.93 (6H, 2 x d, *J* 3.6, $CH(C\underline{H}_3)_2$), 1.40 (9H, m, thr & two ala CH_3), 2.12 (1H, m, $C\underline{H}(CH_3)_2$), 3.73 (1H, d, *J* 3.8, val α-CH), 4.16 (2H, m, ala α-CH & thr β-CH), 4.45 (2H, m, ala α-CH & thr α-CH), 5.16 (2H, s, CH_2Ph) and 7.36 (5H, s, aromatic); δ_C (50.3 MHz; $C^2H_3O^2H$) 19.07 (val $CH(\underline{C}H_3)_2$), 19.76 (ala CH_3), 20.11 (thr CH_3), 32.50 (val $\underline{C}H(CH_3)_2$), 50.0 & 60.3 (2 x ala α-CH), 62.25 (val α-CH), 65.61 (thr α-CH), 68.26 (CH_2Ph), 129.71-129.94 (aromatic), 134.2 (quat. aromatic), 171.8 (amide CO) and 174.02 (ester CO); *m/z* (FAB) 469 (18%, $[M - Cl - NH_4]^+$), 451 (5, $[M - Cl]^+$), 279 (17, {M - Cl - (NH_2-alanyl-threonyl-CO)]$^+$}, 118 (49, [threonine]$^+$) and 91 (100, CH_2Ph^+).

N,N-Diisopropyl dichlorophosphoramidite (67)[239]



To a vigorously stirred solution of phosphorus trichloride (13.5 cm^3, 0.155 mol) in dry ether (75 cm^3) at -20 °C, under a nitrogen atmosphere, was added dropwise a solution of dry diisopropylamine (44.3 cm^3, 0.315 mol) in dry ether (75 cm^3). The mixture was stirred at -20 C for 2 h. It was then allowed to warm up to room temperature and the mixture stirred for a further hour. The salts were removed by filtration and the solvent removed under reduced pressure. The residue was fractionally distilled at reduced pressure to yield the product as a colourless oil (b.p. 80-81 °C/ 8 mmHg) (13.99 g, 45%) {lit,[249] 72-74 °C/ 7 mmHg}; *m/z* (Found: $[M + H]^+$, 201.0246. Calc. for $C_6H_{14}NPCl_2$: *m/z*, 201.0241); δ_H (200 MHz; C^2HCl_3) 1.28 (12H, d, *J* 6.9, ^{i}Pr-CH_3) and 3.93 (1H, sept., *J* 6.6, ^{i}Pr-CH); δ_C (50.3 MHz; C^2HCl_3) 23.94 (^{i}Pr-CH_3) and 48.66 (^{i}Pr-CH); δ_P (121.5 MHz; C^2HCl_3) 169.84 ppm; *m/z* (EI) 203 & 201 (2 & 3%, [M^+, chlorine isotopes]), 186 (18, $[Cl_2PN^iPr_2 - Me]^+$), 166 (11, $[ClNP^iPr_2]^+$), 144 (13, $[Cl_2P^iPr]^+$), 88 (7, $[PN^iPr]^+$), 43 (13, $[^iPr]^+$) and 36 (100, HCl^+).

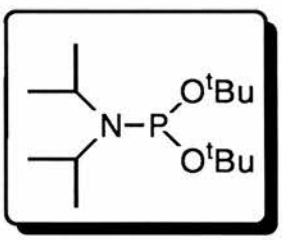
N,N-Diisopropyl dibenzylphosphoramidite (69)[239,322]



To a stirred solution of N,N-diisopropyldichlorophosphoramidite (5.05 g, 25 mmol) in dry DCM (20 cm^3) at -10 °C, under a nitrogen atmosphere, was added a solution of benzyl

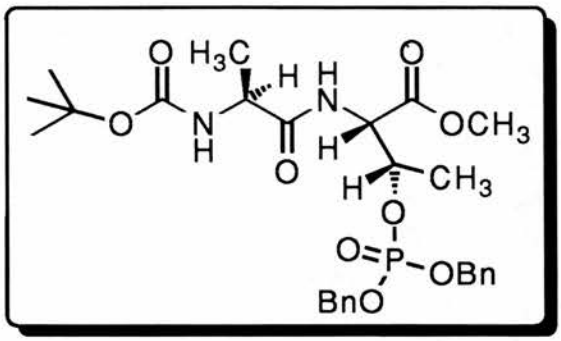
alcohol (6.8 cm^3, 65 mmol) and anhydrous triethylamine (9.01 cm^3, 65 mmol) in dry DCM (20 cm^3). The reaction mixture was stirred for 30 min at -10 °C, then allowed to warm up to room temperature and stirred for a further 5 h. The mixture was then diluted with DCM (50 cm^3), washed with 5% sodium bicarbonate solution (50 cm^3), brine (50 cm^3) and dried ($MgSO_4$). The solvent was removed under reduced pressure to give the product as a pale yellow oil in quantitative yield; *m/z* (Found: $[M + H]^+$, 346.1923. Calc. for $C_{20}H_{28}NPO_2$: *m/z*, 346.1935); υ_{max} (neat)/ cm^{-1} 2966 (CH), 1248 (P-O stretch) and 1201 (P-O aryl); δ_H (200 MHz; C^2HCl_3) 1.28 (12H, d, $J_{Me\text{-}H}$ 6.6, ^{i}Pr-CH_3), 4.78 (2H, m, CH_2OP) and 7.27-7.39 (10H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 25.25 (^{i}Pr-CH_3), 43.6 (d, ^{i}Pr-CH, J_{PC} 48.5), 65.77 (d, benzyl CH_2, J_{PC} 71.6), 127.57-129.05 (aromatic) and 140.0 (quat. aromatic); δ_P (121.5 MHz, C^2HCl_3) 148.1 ppm; *m/z* (CI) 346 (89%, $[M + H]^+$), 238 (100, $[M - BnO]^+$) and 91 (59, CH_2Ph^+).

N,N-diisopropyl di-^{t}butylphosphoramidite (120)



This compound was prepared in a manner identical to that for **(69)** to give the product as an oil (4.83 g, 87%); δ_H (300 MHz; C^2HCl_3) 1.12 (6H, d, *J* 6.9, ^{i}Pr CH_3), 1.29 (18H, s, $C(\underline{C}H_3)_3$) and 3.56 (2H, m, ^{i}Pr CH); δ_C (75.5 MHz; C^2HCl_3) 23.9 (d, ^{i}Pr CH_3, J_{PC} 7.6), 30.77 (d, $C(\underline{C}H_3)_3$, J_{PC} 7.6), 42.82 (d, ^{i}Pr CH, J_{PC} 11.93) and 74.22 (d, $\underline{C}(CH_3)_3$, J_{PC} 9.82); δ_P (121.41 MHz; C^2HCl_3) 129.84 ppm; *m/z* (EI) 277 (8%, M^+), 150 (100, [2 x ^{t}BuOH + 2H]$^+$), 148 (32, [2 x ^{t}BuOH + 2H]$^+$), 86 (26, [2 x ^{i}Pr]$^+$) and 43 (7, [^{i}Pr]$^+$).

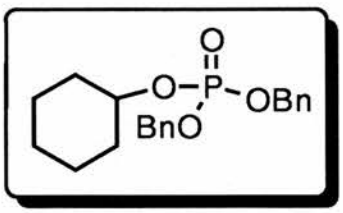
Methyl N-α-Boc-(*S*)-alanyl-(*S*)-threonyl dibenzyl phosphate (70)



The protected dipeptide **(38)** (0.304 g, 1 mmol) and tetrazole (0.14 g, 2 mmol) were dissolved in dry acetonitrile (10 cm^3) under a nitrogen atmosphere, and a solution of N,N-diisopropyldibenzyl phosphoramidite (0.45 g, 1.3 mmol) in dry acetonitrile (5 cm^3) was added. The reaction mixture was stirred at room temperature for 2 h and then cooled to -10 °C. A solution of *m*CPBA (0.26 g, 1.5 mmol) in DCM (10 cm^3) was added dropwise and the resulting solution stirred at 0 °C for 1 h. The reaction mixture was then diluted with DCM (60 cm^3), washed with 10% sodium sulfite solution (3 x 30 cm^3), 5% sodium bicarbonate solution (2 x 20 cm^3), brine (30 cm^3) and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a pale yellow oil (0.55 g, 98%); *m/z* (Found: $[M + H]^+$, 565.2311. $C_{27}H_{38}N_2O_9P$ requires *m/z*, 565.2314); $[\alpha]_D$ -1.41 (*c* 3.3, DCM); υ_{max} (neat)/cm^{-1} 2876-2935 (CH_3), 1383 (Boc), 1251 (P=O) and 1212 (P-OAryl); δ_H (200 MHz; C^2HCl_3) 1.17 (3H, dd, J_{AB} 3.3, J_{AX} 6.3, thr CH_3), 1.31 (3H, m, ala CH_3), 1.39 (9H, s, $C(CH_3)_3$), 3.66 (3H, s, OCH_3), 4.30 (1H, m, ala α-CH), 4.57 (1H, 2 x d, *J* 1.87, thr α-CH), 4.72 (1H, m, thr β-CH), 4.95 (4H, d, J_{PH} 8.2, 2 x benzyl CH_2), 5.50 (1H, d, *J* 7.6, urethane NH), 5.64 (1H, d, *J* 7.6, amide NH) and 7.22-7.29 (10H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 19.14 (ala CH_3), 20.43 (thr CH_3), 28.78 ($C(\underline{C}H_3)_3$), 50.64 (ala α-CH), 53.0 (d, thr β-CH, J_{PC} 11.8), 59.50 (OCH_3), 68.4 (thr α-CH), 69.9 (d, benzyl CH_2, J_{PC} 31.4), 80.17 ($\underline{C}(CH_3)_3$), 128.4-128.9 (aromatic), 136.07 (quat. C), 155.92 (urethane CO), 171.78 (amide CO) and 174.11 (ester CO); δ_P (121.5 MHz; C^2HCl_3) -0.75ppm; *m/z* (CI) 565 (100%, $[M + H]^+$), 509 (31, $[M - C(CH_3)_3 + 2H]^+$),

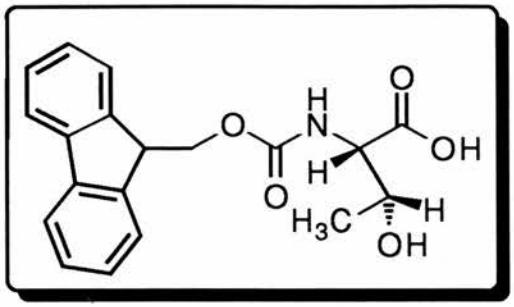
465 (64, [M - BOCNH + NH_3]$^+$), 411 (9, [M - $PO(OBn)_2$ + NH_3]$^+$), 205 (20, [187 + NH_4]$^+$), 187 (34, [M - $C_{19}H_{22}O_6P$]$^+$) and 91 (17, $PhCH_2^+$).

Cyclohexyl dibenzyl phosphate (73)



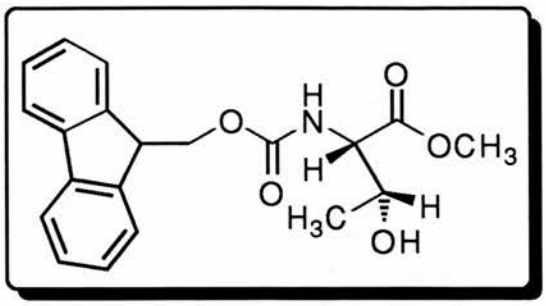
To a stirred solution of diisopropylethylamine (0.68 cm^3, 4 mmol), DMAP (48.87 mg, 0.4 mmol) and N,N-diisopropyldichloro phosphoramidite (0.263 g, 1.3 mol) in dry THF (10 cm^3) under an argon atmosphere was added a solution of cyclohexanol (0.1 g, 1 mmol) in dry THF and the mixture stirred for 3-4 h. Dry benzyl alcohol (310.5 mm^3, 3 mmol) was then added and the mixture stirred overnight. *m*CPBA (0.517 g, 3 mmol) in DCM (5-10 cm^3) was added dropwise at 0 °C and then stirred at room temperature for 1-2 h. The reaction mixture was diluted with DCM (30 cm^3), washed with 10% sodium sulfite (2 x 30 cm^3), 5% sodium bicarbonate (2 x 30 cm^3), brine (30 cm^3) and dried ($MgSO_4$). The solvent was removed under reduced pressure to give an oil which was purified by silica column chromatography using ethyl acetate/ petroleum ether (6/ 4) as the eluant to give the product as a pale oil (258 mg, 72%); *m/z* (Found: [M + H]$^+$, 361.1557. $C_{20}H_{26}PO_4$ requires *m/z*, 361.1568); υ_{max} (neat)/cm^{-1} 2861-2938 (CH stretch), 1585 (aromatic), 1238 (PO) and 1208 (P-OAryl); δ_H (200 MHz; C^2HCl_3) 1.52-1.93 (10H, m, CH_2), 4.35-4.40 (1H, m, CHOP), 5.04 (4H, m, CH_2Ph) and 7.30-7.37 (10H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 23.12 ($\underline{C}H_2CH_2$), 25.01 ($\underline{C}H_2CH_2$), 32.90 ($\underline{C}H_2CH$, d, J_{PC} 6.4), 64.45 (CH_2Ph), 69.03 (CHOP, d, J_{PC} 5.7), 126.58-128.24 (aromatic) and 140.94 (quat. aromatic); δ_P (121.41 MHz; C^2HCl_3) main peak at 8.80 ppm; *m/z* (CI) 361 (80%, [M + H]$^+$), 279 (35, [$P(O)(OBn)_2OH$ + H]$^+$), 102 (16, [cyclohexanol + 2H]$^+$) and 91 (68, CH_2Ph^+).

N-α-Fmoc-(2*S*)-threonine (121)



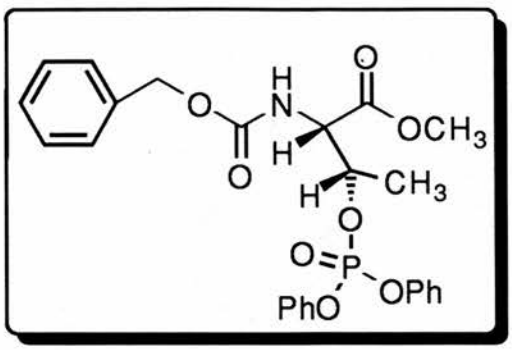
(2*S*)-Threonine (1.19 g, 10 mmol) dissolved in 10% Na_2CO_3 solution (25 cm^3) and dioxan (5 cm^3), was cooled to 0 °C. A solution of Fmoc-Cl (2.72 g, 10.5 mmol) in dioxan (10 cm^3) was added dropwise, the reaction allowed to warm to room temperature and then stirred overnight. The reaction mixture was diluted with water (100 cm^3), washed with diethyl ether (2 x 50 cm^3) and the aqueous layer acidified (pH 1). The aqueous phase was then extracted with ethyl acetate (3 x 50 cm^3) and the organic layer washed with distilled water (30 cm^3), brine (30 cm^3) and dried ($MgSO_4$). The solvent was then removed under reduced pressure to give a white solid (2.34 g, 69%), m.p. 102-105 °C (lit.,[212] 102-106 °C); *m/z* (Found: $[M + H]^+$, 342.1336. Calc. for $C_{19}H_{20}NO_5$: *m/z*, 342.1341); $[\alpha]_D$ -5.0 (*c* 1, MeOH) {lit.,[212] -4.8 (*c* 1, MeOH)}; υ_{max} (Nujol)/cm^{-1} 3377-3649 (OH), 2855-2927 (CO_2H, OH stretch) and 1597-1676 (aromatic); δ_H (200 MHz; C^2HCl_3) 1.24 (3H, d, *J* 6.2, CH_3), 4.22 (1H, t, *J* 6.8, Fmoc CH), 4.41-4.66 (4H, m, Fmoc CH_2, α- & β-CH), 5.93 (1H, d, *J* 8, NH) and 7.24-7.76 (8H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 19.9 (CH_3), 47.47 (Fmoc CH_2), 59.65 (β-CH), 67.98 (Fmoc CH), 68.38 (α-CH), 120.5, 125.65, 127.61 & 128.25 (aromatic), 141.74 & 144.03 (quat. aromatic), 157.82 (urethane CO) and 175.05 (carboxyl CO); *m/z* (CI) 342 (9%, $[M + H]^+$), 179 (100, $[9\text{-fluorene}]^+$), 164 (18, $[MH - 9\text{-fluorene} + H]^+$) and 120 (67, $[\text{threonine} + H]^+$).

Methyl N-α-Fmoc-(2*S*)-threoninate (71)



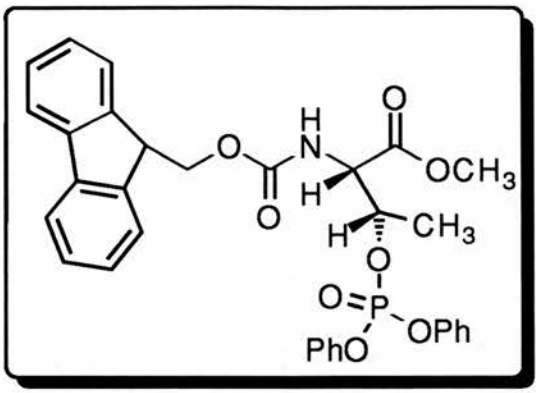
This compound was prepared in a manner identical to that for the methyl ester (**45**) using N-α-Fmoc-(2*S*)-threonine (0.2 g, 0.6 mmol) to give an off-white solid (0.19 g, 89%), m.p. 118-119 °C; *m/z* (Found: $[M + H]^+$, 356.1503. Calc. for $C_{20}H_{22}NO_5$: *m/z*, 356.1498); $[\alpha]_D$ -12.8 (*c* 1, $CHCl_3$); υ_{max} (Nujol)/cm^{-1} 3399 (thr OH), 2854 (OCH_3), 1752 (urethane), 1512 (aromatic) and 1377 (CH_3); δ_H (200 MHz; C^2HCl_3) 1.25 (3H, d, *J* 6.2, CH_3), 3.76 (3H, s, OCH_3), 4.21- 4.44 (3H, m, thr α- & β-CH & Fmoc CH), 5.84 (1H, d, *J* 9.2, urethane NH) and 7.27- 7.78 (8H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 20.37 (CH_3), 47.62 (CH_2), 53.14 (OCH_3), 59.77 (β-CH), 67.71- 68.36 (α-CH & Fmoc CH), 120.51-128.25 (aromatic), 141.79-144.35 (quat. aromatic), 157.40 (urethane CO) and 172.32 (ester CO); *m/z* (CI) 356 (21%, $[M + H]^+$), 196 (6, $[C_{14}H_{11}O + H]^+$), 179 (76, [9-fluorene]$^+$), 160 (65, $[M - C_{14}H_{11}O]^+$), 134 (100, $[C_9H_8 + NH_4]^+$), 116 (16, $[C_9H_8]^+$) and 45 (7, $[CHOHCH_3]^+$).

Methyl N-α-Cbz-(2*S*)-threonyl diphenyl phosphate (76)



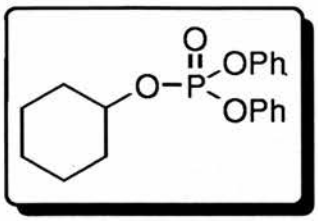
Methyl N-Cbz-(2*S*)-threoninate (80.6 mg, 0.3 mmol) and DMAP (3.66 mg, 0.030 mmol) were dissolved in dry pyridine (3-5 cm^3) under a nitrogen atmosphere. Diphenyl phosphorochloridate (115.0 mm^3, 0.555 mmol) was added and the reaction mixture stirred at room temperature for 3-4 h. The reaction mixture was then diluted with DCM (10 cm^3), washed with 0.1 M Hcl (10 cm^3), water (10 cm^3) and the organic phase removed under reduced pressure. The residue was taken up in EtOAc (20 cm^3) and washed with distilled water (20 cm^3). The aqueous phase was then extracted with EtOAc (3 x 30 cm^3) and the combined organic fractions washed with brine (30 cm^3) and dried ($MgSO_4$). The solvent was then removed under reduced pressure to give a pale yellow oil (100 mg, 67%); *m/z* (Found: $[M + H]^+$, 500.1478. $C_{25}H_{27}NO_8P$ requires *m/z*, 500.1474); υ_{max} (neat)/ cm^{-1} 3377 (OH), 1550 (aromatic), 1260 (P=O) and 1205 (P-OAryl); δ_H (200 MHz; C^2HCl_3) 1.47 (3H, d, *J* 6.6, CH_3), 3.71 (3H, s, OCH_3), 4.54 (1H, d, *J* 8.6, α-CH), 5.02 (1H, m, β-CH,), 5.09 (2H, s, CH_2Ph), 5.79 (1H, d, *J* 8.4, urethane NH) and 7.15-7.37 (15H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 18.94 (CH_3), 53.11 (OCH_3), 58.91 (d, β-CH, J_{PC} 6.5), 67.69 (α-CH), 76.99 (CH_2Ph), 120.54-121.03 (phenyl-aromatic), 126.01-130.31 (Cbz-aromatic), 136.53 (Cbz-quat. aromatic), 150.86 (d, phenyl-quat. aromatic, J_{PC} 6.19), 156.16 (urethane CO) and 169.28 (ester CO); δ_P (121.5 MHz; C^2HCl_3) -12.25 ppm; *m/z* (CI) 500 (71%, $[M + H]^+$), 392 (7, $[M - PhCH_2O]^+$), 250 (100, $[OP(OPh)_2OH]^+$) and 91 (30, CH_2Ph^+).

Methyl N-α-Fmoc-(2*S*)-threonyl diphenyl phosphate (77)



This compound was prepared in a manner identical to that for the methyl ester **(76)** using the Fmoc-protected **(71)** (0.2 g, 0.56 mmol) to give a yellow oil which was purified by silica column chromatography using ethyl acetate/ light petroleum (1:1) as the eluant to give the product (R_f 0.56) as a colourless oil (0.126 g, 38%); *m/z* (Found: $[M + H]^+$, 588.1778. $C_{32}H_{31}NO_8P$ requires *m/z*, 588.1787); υ_{max} (neat)/cm^{-1} 3399 (thr OH), 2854 (OCH_3), 1580 (aromatic) and 1377 (CH_3); δ_H (200 MHz; C^2HCl_3) 1.46 (3H, d, *J* 6.4, thr CH_3), 3.63 (3H, s, OCH_3), 4.25 (1H, apparent t, *J* 7.0, Fmoc CH), 4.45 (2H, d, *J* 7.0, Fmoc CH_2), 4.54 (1H, m, α-CH), 5.30 (1H, m, β-CH), 5.60 (1H, d, *J* 9.6, urethane NH) and 7.20-7.80 (19H, m, Fmoc & phenyl aromatics); δ_C (50.3 MHz; C^2HCl_3) 18.99 (CH_3), 47.64 (α-CH), 53.32 (OCH_3), 58.88 (d, β-CH, J_{PC} 7.2), 67.88 (Fmoc CH_2), 120.52 (phenyl aromatic), 125.6-130.34 (Fmoc aromatic), 160.5 (urethane CO) and 170.09 (CO_2H); δ_P (121.5 MHz; C^2HCl_3) -12.52 ppm; *m/z* (CI) 588 (51%, $[M + H]^+$), 251 (63, $[OP(OPh)_2 + NH_4]^+$), 179 (50, [9-fluorene]$^+$) and 160 (100, $[C_6H_{10}NO_4]^+$).

Cyclohexyl diphenyl phosphate (78)



Cyclohexanol (0.1 g, 1 mmol), DMAP (12.22 mg, 0.1 mmol) and dry Et_3N (503.16 mm^3, 3.61 mmol) were dissolved in dry DCM (10 cm^3) under an argon atmosphere. Diphenylphosphorochloridate (383.5 mm^3, 1.85 mmol) was added and the reaction mixture stirred at room temperature for 5-6 h. The reaction mixture was worked up as the methyl ester (**76**) to give an oil which was purified by silica chromatography using ethyl acetate/ petroleum ether (4:6) as the eluant (R_f 0.49).The crude diphenyl phosphate was recrystallised from ether/ light petroleum to give the product as a solid (228 mg, 69%), m.p. 33 °C (lit.,[323] 34-35 °C); *m/z* (Found: $[M + H]^+$, 333.1249. Calc. for $C_{18}H_{22}PO_4$: *m/z*, 333.1255); υ_{max} (DCM)/cm^{-1} 2861-2941 (CH stretch), 1591 (aromatic), 1266 (PO) and 1193-1221 (P-OAryl); δ_H (200 MHz; C^2HCl_3) 1.2-1.95 (10H, m, CH_2), 4.62 (1H, m, CH) and 7.12-7.36 (10H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 23.08 & 24.72 (CH_2), 32.85 (d, CH_2, J_{PC} 4.6), 79.15 (d, CH, J_{PC} 6.6), 119.85, 124.92 & 129.43 (aromatic, J_{PC} 5.18) and 150.41 (d, quat. aromatic, J_{PC} 7.14); δ_P (121.41 MHz; C^2HCl_3) -12.06 ppm; *m/z* (CI) 333 (10%, $[M + H]^+$) and 251 (100, $[OP(OPh)_2]^+$).

Solid phase peptide synthesis and peptide removal from Wang resin

Solid phase synthesis of the hexapeptides described below were carried out using the Rainin PS3 automated peptide synthesizer. The synthesis employed Fmoc chemistry and the C-terminal end amino acids were linked to Wang resin. Amino acids and the activating agent PyBOP were purchased from Novabiochem chemicals, solvents DMF, piperidine and NMM from Sigma-Aldrich. A four fold excess of the activated 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. Where coupling of an amino acid was known to be difficult or require a prolonged reaction time, a double coupling of that amino acid was performed, *i.e* for residues threonine, arginine and valine.

The peptides were cleaved from the resin using one of the following three cleavage mixtures:

A. The peptide-Wang resins were treated with the cleavage mixture TFA/ anisole/ DMS/ water (85:5:5:5) (10-15 cm^3) at room temperature for 1-2 h. The resin was then filtered off and the solvent concentrated under reduced pressure (4-5 cm^3). The peptides were then precipitated with excess dry diethyl ether, filtered and dried; or

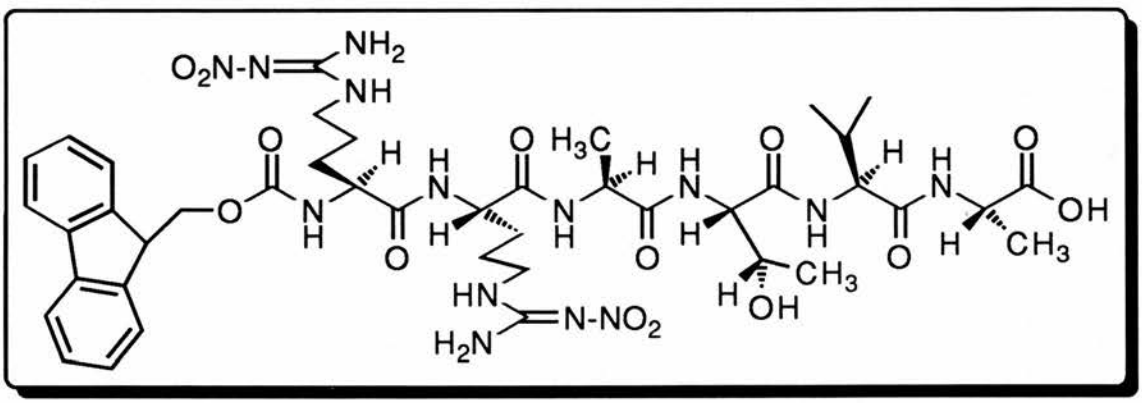
B. The peptide-Wang resins were cooled in an ice bath. The cleavage mixture, crystalline phenol/ 1,2-ethanedithiol/ thioanisole/ water/ TFA (0.75 g/ 0.25 cm^3/ 0.5 cm^3/ 0.5 cm^3/ 10 cm^3), was cooled in an ice bath and then added to the cooled peptide-resins to give a total volume of 10 cm^3 per 0.1-1.5 g of peptide-resin. The mixture was then allowed to warm up to room temperature and stirred for 1-2 h. The resin was then filtered off, washed with DCM (5-10 cm^3) and the combined filtrate concentrated under reduced pressure (4-5 cm^3). The peptide was then isolated by either:

(i) precipitation with excess dry diethyl ether, or

(ii) dilution of the residual solution with a tenfold excess of water followed by repeated extractions of the scavengers with an equivalent amount of cold diethyl ether. The aqueous layer is then lyophilised and the peptide recovered as a white solid; or

C. The peptide-Wang resins were treated in a manner identical to that for procedure B, but the cleavage mixture used was TFA/ H_2O/ TES (90: 5: 5).

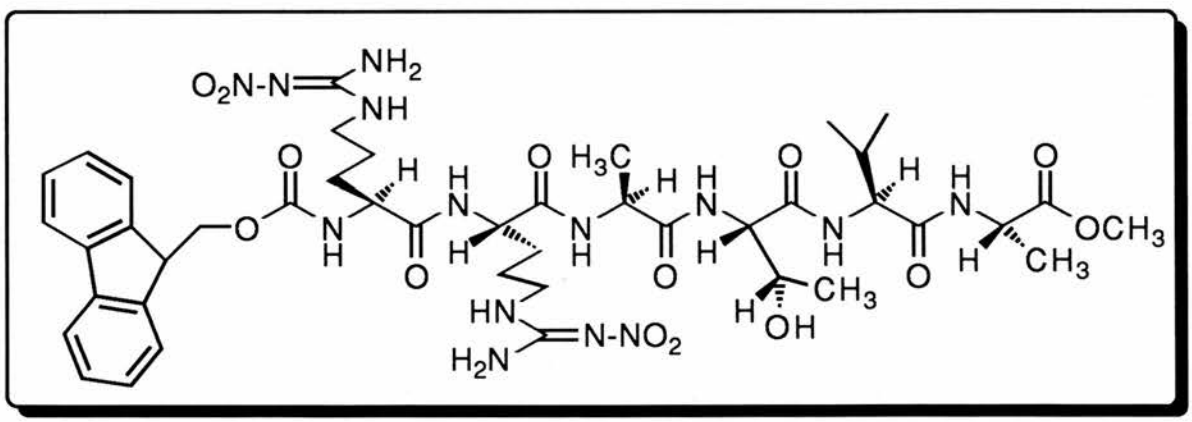
N-α-Fmoc-N-ω-nitro-(*S*)-arginyl-N-ω-nitro-(*S*)-arginyl-(*S*)-alanyl-(*S*)-threonyl-(*S*)-valyl-(*S*)-alaninate (122)



N-α-Fmoc-R(NO_2)R(NO_2)ATVA-Wang resin (200 mg, 30 mg petidyl content, 0.05 mmol) was treated with the cleavage mixture A (10-15 cm^3) at room temperature for 1 h. The resin was then filtered off and the solvent concentrated under reduced pressure (4-5 cm^3). The peptide was then precipitated with excess dry diethyl ether to give an off-white solid (30 mg, quantitative), m.p. 216 °C; *m/z* (Found: $[M + Na]^+$, 1007.4342. $C_{42}H_{60}N_{14}O_{14}Na$ requires *m/z*, 1007.4311); υ_{max} (Nujol)/cm^{-1} 3297 (NH stretch), 1634 (N-NO_2), 1602 (aromatic), 1377 ($C(CH_3)_2$) and 1268 (OH bending); δ_H (300 MHz; d_6-DMSO) 0.98 (6H, 2 x d, *J* 6.7, val $CH(C\underline{H}_3)_2$), 1.15 (3H, d, *J* 6.27, thr CH_3), 1.37 (6H, m, 2 x ala CH_3), 1.65 (4H, br s, 2 x arg $C\underline{H}_2CH_2$), 1.73 (4H, m, 2 x arg $C\underline{H}_2CH$), 2.10 (1H, m, val $C\underline{H}(CH_3)_2$), 3.26 (4H, br s, 2 x arg $C\underline{H}_2NH$), 3.51 (3H, q, *J* 7.0, ala α-CH), 3.72 (1H, m, val α-CH), 4.10-4.53 (5H, m, 2 x arg α-CH, thr α- & β-CH, ala α-CH), 7.04 (1H, d, *J* 8.7, urethane NH), 7.41-7.77 (9H, m, Fmoc) and 7.81-8.32 (11H,

m, amide and arg NH & NH_2); δ_C (75.5 MHz; d_6-DMSO) 16.96 & 18.09 (val $CH(\underline{C}H_3)_2$), 17.66 & 19.04 (2 x ala CH_3), 19.44 (thr CH_3), 24.62 & 25.0 (2 x arg $\underline{C}H_2CH_2$), 29.19 & 29.39 (2 x arg $\underline{C}H_2CH$), 30.89 (val $\underline{C}H(CH_3)_2$), 46.73 (thr α-CH), 47.49 & 48.22 (2 x ala α-CH), 54.32 & 56.99 (2 x arg α-CH), 57.87 (val α-CH), 65.68 (thr β-CH), 66.47 (Fmoc CH_2), 120.04-127.60 (Fmoc), 140.71-143.87 (Fmoc quat. aromatics), 155.92 (urethane CO), 158.46 & 159.31 (2 x arg CN), 169.63-172.27 (amide CO's) and 173.76 (CO_2H); *m/z* (FAB) 1029 (78%, $[M + 2Na]^+$), 1007 (100, $[M + Na]^+$) and 985 (19, $[M + H]^+$).

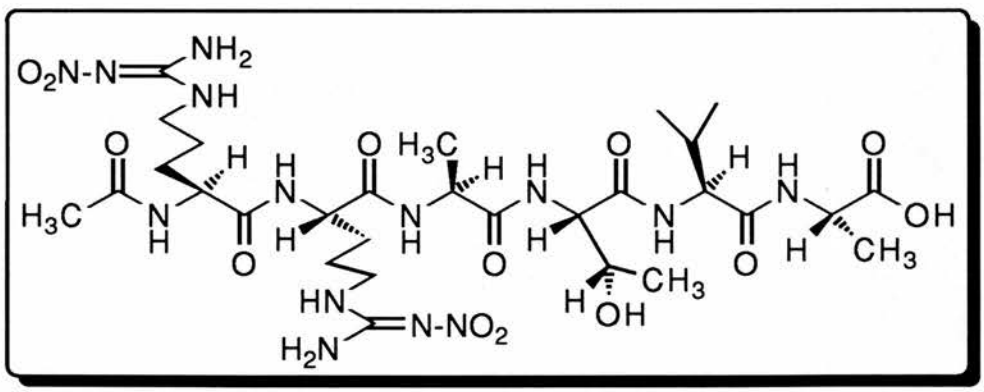
Methyl N-α-Fmoc-N-ω-nitro-(*S*)-arginyl-N-ω-nitro-(*S*)-arginyl-(*S*)-alanyl-(*S*)-threonyl-(*S*)-valyl-(*S*)-alaninate (123)



This compound was prepared in a manner identical to that for the methyl ester **(45)** using N-α-Fmoc-R(NO_2)R(NO_2)ATVA-OH (30 mg, 0.03 mmol) in diethyl ether and THF to give a white solid (29.94 mg, quantitative), m.p. 200-202 °C; *m/z* (Found: $[M + H]^+$, 999.4616. $C_{43}H_{63}N_{14}O_{14}$ requires *m/z*, 999.4648); υ_{max} (DMSO)/cm^{-1} 3331 (NH stretch), 2850 (OCH_3) and 1652 (N-NO_2); δ_H (300 MHz; d_6-DMSO) 0.95 (6H, 2 x d, *J* 6.8, val $CH(C\underline{H}_3)_2$), 1.11 (3H, d, *J* 6.27, thr CH_3), 1.31 (3H, d, *J* 7.1, ala CH_3), 1.36 (3H, d, *J* 7.4, ala CH_3), 1.61 (4H, br s, 2 x arg $C\underline{H}_2CH_2$), 1.75 (4H, m, 2 x arg $C\underline{H}_2CH$), 2.1 (1H, m, val $C\underline{H}(CH_3)_2$), 3.23 (4H, br s, 2 x arg $C\underline{H}_2NH$), 3.3 (3H, s, OCH_3), 4.10-4.50 (7H, m, 2 x arg α-CH, thr α- & β-CH, ala α-CH & Fmoc CH_2),

7.39-7.99 (9H, m, Fmoc) and 8.10-8.52 (11H, m, amide and arg NH & NH_2's); δ_C (75.5 MHz; d_6-DMSO) 15.59 & 16.94 (val CH($\underline{C}H_3)_2$), 16.52 & 17.83 (2 x ala CH_3), 18.27 (thr CH_3), 23.82 & 24.0 (2 x arg $\underline{C}H_2CH_2$), 28.06 & 28.25 (2 x arg $\underline{C}H_2CH$), 29.73 (val $\underline{C}H(CH_3)_2$), 45.58 (thr α-CH), 46.42 & 47.08 (2 x ala α-CH), 50.54 (OCH_3), 53.18 & 55.83 (2 x arg α-CH), 56.70 (val α-CH), 64.54 (thr β-CH), 65.32 (Fmoc CH_2), 118.90-126.46 (Fmoc), 139.57-142.73 (Fmoc quat. aromatics), 154.79 (urethane CO), 158.19 (2 x arg CN), 168.49-171.12 (amide CO's) and 171.58 (CO_2H); *m/z* (FAB) 1021 (70%, [M + Na]$^+$), 999 (61, [M + H]$^+$) and 154 (100, [2 x C_6H_5]$^+$).

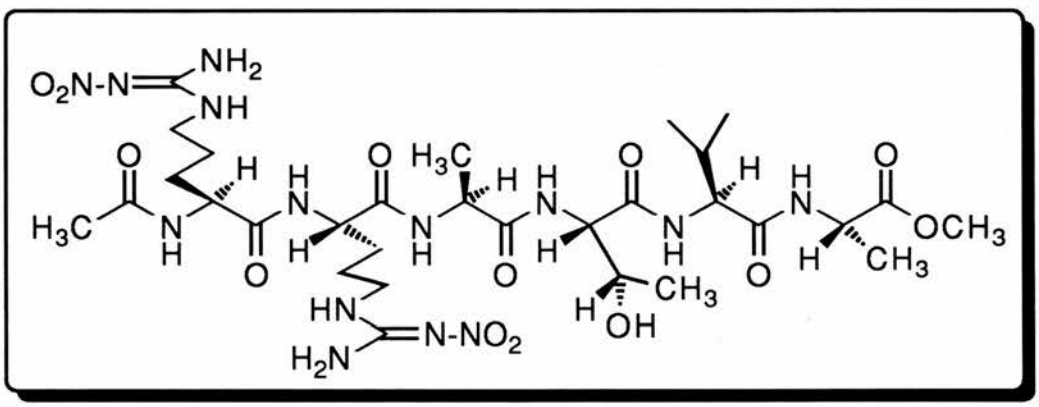
N-α-Acetyl-N-ω-nitro-(*S*)-arginyl-N-ω-nitro-(*S*)-arginyl-(*S*)-alanyl-(*S*)-threonyl-(*S*)-valyl-(*S*)-alaninate (124)



N-α-Fmoc-R(NO_2)R(NO_2)ATVA-Wang resin (0.44 g, 0.11 mmol) was treated with 20% piperidine/ DMF (10 cm^3) for 1/2 h, followed by 5% acetic anhydride/ DMF (10 cm^3) for 1/2 h with DMF washes of the resin after each treatment. This was then treated with cleavage mixture A (10 cm^3) to give a solid (149.1 mg, quantitative), m.p. 240 °C (decomp.); υ_{max} (Nujol)/cm^{-1} 3301 (NH stretch), 1645 (NH_2), 1377 ($C(CH_3)_2$) and 1273 (N-NO_2); δ_H (300 MHz; d_6-DMSO) 0.86 (6H, 2 x d, *J* 6.75, val CH($\underline{C}H_3)_2$), 1.03 (3H, d, *J* 6.1, thr CH_3), 1.24 (6H, m, 2 x ala CH_3), 1.51 (4H, br s, 2 x arg $\underline{C}H_2CH_2$), 1.70 (4H, m, 2 x arg $\underline{C}H_2CH$), 1.86 (3H, s, acetyl CH_3), 2.0 (1H, m, val $\underline{C}H(CH_3)_2$), 3.14 (4H, br s, 2 x arg $\underline{C}H_2NH$), 3.61 (2H, m, ala & val α-CH), 4.0-4.35 (5H, m, 2 x arg α-CH, thr α- & β-CH & ala α-CH) and 7.25-8.16 (12H, m, NH & NH_2's); δ_C (75.5 MHz;

d_6-DMSO) 17.25 & 18.27 (val CH($\underline{C}H_3)_2$), 17.98 & 19.34 (2 x ala CH_3), 19.76 (thr CH_3), 22.72 (acetyl CH_3), 26.60 & 26.68 (2 x arg $\underline{C}H_2CH_2$), 29.32 & 29.46 (2 x arg $\underline{C}H_2CH$), 31.12 (val $\underline{C}H(CH_3)_2$), 42.65 (arg CH_2NH), 44.0 (thr α-CH), 47.92 & 49.0 (2 x ala α-CH), 52.78 & 57.51 (2 x arg α-CH), 58.35 (val α-CH), 66.8 (thr β-CH), 159.62 (2 x arg CN), 167.0 ($CH_3\underline{C}O$), 170.11-172.77 (amide CO's) and 174.15 (CO_2H); *m/z* (E/S) 805 (7%, $[M + H]^+$) and 359 (6, [alanyl-threonyl-valyl-alanyl]$^+$).

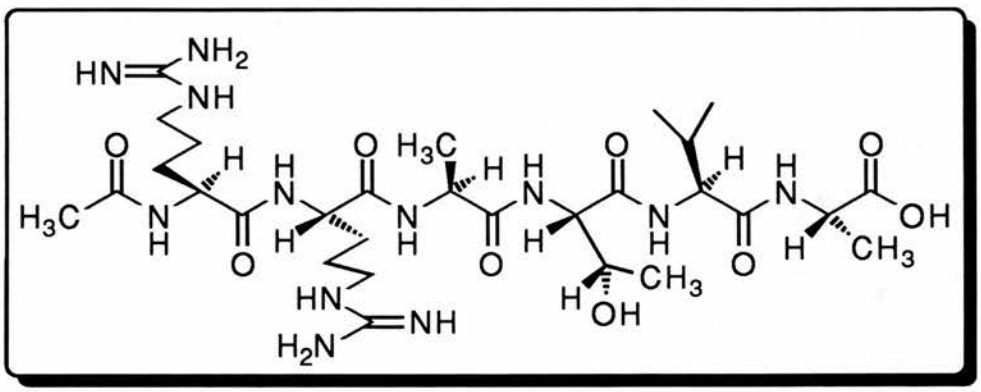
Methyl N-α-acetyl-N-ω-nitro-(*S*)-arginyl-N-ω-nitro-(*S*)-arginyl-(*S*)-alanyl-(*S*)-threonyl-(*S*)-valyl-(*S*)-alaninate (60)



This compound was prepared in a manner identical to that for the methyl ester (**45**) using the hexapeptide (**124**) (129 mg, 0.16 mmol) to give a solid (59.4 mg, 46%), m.p. 206 °C (decomp.); υ_{max} (DCM)/cm^{-1} 3342 (NH stretch), 1652 (N-NO_2), 1274 (OH bending) and 1381 ($C(CH_3)_2$); δ_H (300 MHz; d_6-DMSO) 0.91 (6H, 2 x d, *J* 6.3, val CH($C\underline{H}_3)_2$), 1.1 (3H, d, *J* 5.7, thr CH_3), 1.32 (6H, t, *J* 6.3, 2 x ala CH_3), 1.59 (8H, m, 2 x arg $C\underline{H}_2CH_2$ & 2 x arg $C\underline{H}_2CH$), 1.94 (3H, s, acetyl CH_3), 2.05 (1H, m, val CH), 3.22 (4H, m, 2 x arg $C\underline{H}_2NH$), 3.66 (3H, s, OCH_3), 4.1-4.4 (7H, m, α- & β-CH's), 7.69 (1H, d, *J* 5.7, NH), 7.75-8.17 (12H, m, NH & NH_2's) and 8.39 (1H, d, *J* 7.5, NH); δ_C (75.5 MHz; d_6-DMSO) 17.4 & 18.5 (val CH($\underline{C}H_3)_2$), 18.4 & 19.7 (2 x ala CH_3), 20.2 (thr CH_3), 22.9 (acetyl CH_3), 26.6 & 26.68 (2 x arg $\underline{C}H_2CH_2$), 28.3 & 29.6 (2 x arg $\underline{C}H_2CH$), 31.5 (val $\underline{C}H(CH_3)_2$), 48.77 & 49.6 (2 x ala α-CH), 52.9 (OCH_3), 53.24 & 53.6 (2 x arg α-CH), 58.5 (val α-CH), 59.11 (thr β-CH), 67.49 (thr α-CH), 160.04 (2 x arg CN), 171.07

($CH_3\underline{C}O$), 171.99-173.83 (amide CO's) and 173.99 (CO_2H); *m/z* (E/S) 819 (21%, [M + H]$^+$) and 200 (25, [threonyl-valyl]$^+$).

N-α-Acetyl-(*S*)-arginyl-(*S*)-arginyl-(*S*)-alanyl-(*S*)-threonyl-(*S*)-valyl-(*S*)-alaninate (63)

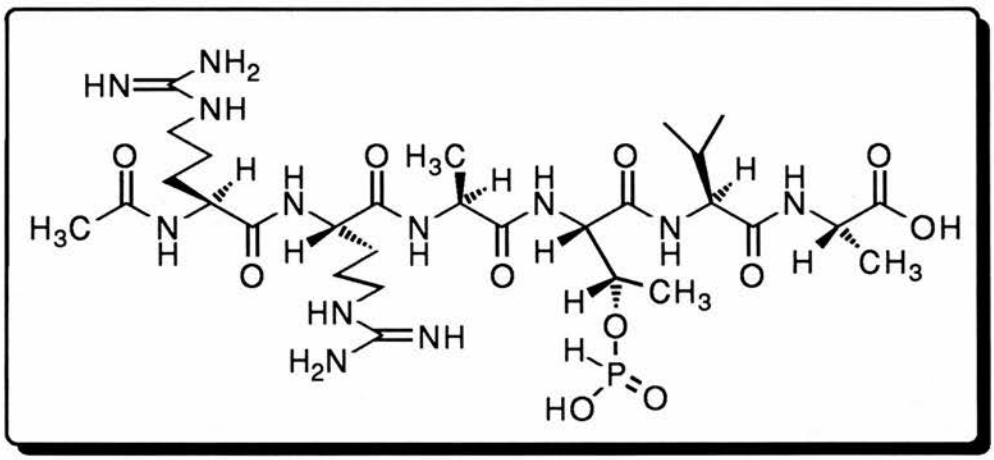


This compound was prepared in a manner identical to that for the hexapeptide **(124)** using N-α-Fmoc-R(Pmc)R(Pmc)ATVA-Wang resin (1.15 g peptide-resin, peptidyl content 97.18 mg, 0.162 mmol).

Ac-R(Pmc)R(Pmc)ATVA-Wang resin (0.35 g peptide-resin, peptidyl content 32.09 mg, 0.053 mmol) so prepared was treated with cleavage mixture B (10 cm^3) to give a white solid on lyophilization (32.09 mg, quantitative), m.p. 223 °C; υ_{max} (MeOH)/cm^{-1} 3418 (NH stretch), 1647 (NH_2) and 1558 (CO_2^- ion); δ_H (500 MHz; d_6-DMSO) 0.98 (6H, 2 x d, *J* 6.8, val CH($C\underline{H}_3$)$_2$), 1.16 (3H, d, *J* 6.7, thr CH_3), 1.38 (6H, 2 x d, *J* 7.4, 2 x ala CH_3), 1.62 (4H, m, 2 x arg $C\underline{H}_2CH_2$), 1.78-1.83 (4H, br s, 2 x arg $C\underline{H}_2CH$), 1.98 (3H, s, acetyl CH_3), 2.13 (1H, hep, *J* 6.8, val CH), 3.21 (4H, m, 2 x arg $C\underline{H}_2NH$), 4.14-4.52 (7H, m, 2 x arg α-CH, thr α- & β-CH, 2 x ala α-CH & val α-CH), 5.06 (1H, s, thr OH), 7.18 (1H, br s, CO_2H), 7.38-7.66 (9H, m, amide and arg NH's), 7.96 (1H, d, *J* 8, NH), 8.12 (3H, apparent d, *J* 8, NH's) and 8.30 (1H, d, *J* 6.8, NH); δ_C (75.5 MHz; d_6-DMSO) 16.22 & 17.55 (val CH($\underline{C}H_3$)$_2$), 16.53 & 18.24 (2 x ala CH_3), 18.80 (thr CH_3), 21.55 (acetyl CH_3), 24.33 (2 x arg $\underline{C}H_2CH_2$), 28.08 (2 x arg $\underline{C}H_2CH$), 30.32 (val CH), 48.89

& 48.99 (2 x ala α-CH), 53.07 & 53.51 (2 x arg α-CH), 59.05 (val α-CH), 59.19 (thr β-CH), 67.14 (thr α-CH), 156.90 (2 x arg CN), 171.72-175.14 (CO's) and 176.80 (CO_2H); *m/z* (FAB) 714 (13%, M^+), 656 (15, $[M - Ac]^+$) and 133 (100, $[C_4H_6NO_3 + NH_3]^+$).

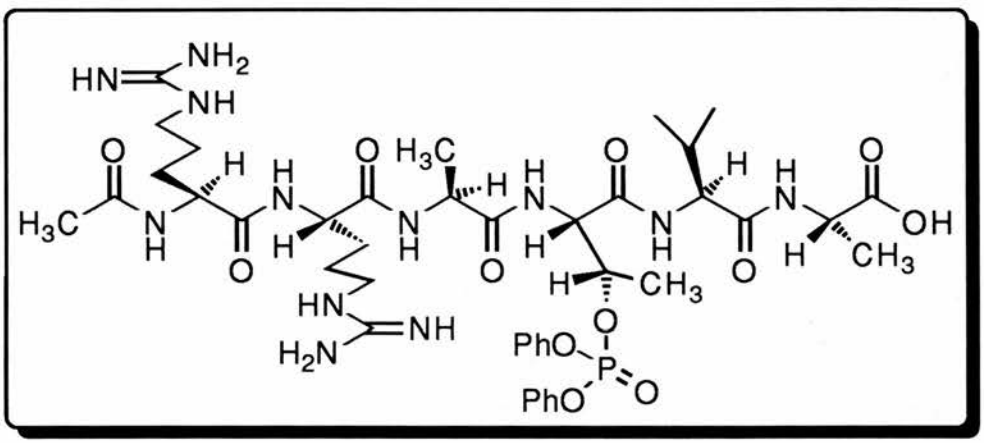
N-α-Acetyl-(*S*)-arginyl-(*S*)-arginyl-(*S*)-alanyl-(*S*)-(phosphonyl threonyl)-(*S*)-valyl-(*S*)-alaninate (79)



Ac-R(Pmc)R(Pmc)ATVA-Wang resin (0.53 g peptide-resin, 97.55 mg peptidyl content, 0.163 mmol) and 1H-tetrazole (0.571 g, 8.15 mmol) in dry acetonitrile (10 cm^3) was treated with a solution of N,N-diisopropyldi-^{t}butyl phosphoramidite (0.90 g, 3.26 mmol) in dry acetonitrile (10 cm^3) and left to stir overnight. The resin was then treated with ^{t}BuOOH (3 M in isooctane) (1.1 cm^3, 3.26 mmol) and stirred for 2 h. The oxidation procedure was repeated once more and the resin then washed and dried with DCM and MeOH. The resin obtained (0.32 g peptide-resin, 97.55 mg peptidyl content, 0.163 mmol) was deprotected using cleavage mixture B to give a solid on lyophilisation (88 mg, 90%), m.p. 196 °C; δ_H (300 MHz; ^2H_2O) 0.96 (6H, t, *J* 6.3, val CH(C<u>H</u>$_3$)$_2$), 1.2 (3H, d, *J* 6.3, thr CH_3), 1.30 (3H, t, *J* 4.2, ala CH_3), 1.43 (3H, d, *J* 7.2, ala CH_3), 1.66 (4H, br s, 2 x arg C<u>H</u>$_2$CH$_2$), 1.80 (4H, m, 2 x arg C<u>H</u>$_2$CH), 2.03 (3H, s, acetyl CH_3), 2.1 (1H, m, val CH), 3.21 (4H, m, 2 x arg C<u>H</u>$_2$NH), 4.19-4.65 (7H, m, α- & β-CH's) and 5.62 & 7.76 (1H, d, J_{PH} 641.4, P-H); δ_C (75.5 MHz; ^2H_2O) 16.46 & 18.11 (val CH(<u>C</u>H$_3$)$_2$), 16.93 &

18.68 (2 x ala CH_3), 19.24 (thr CH_3), 21.98 (acetyl CH_3), 24.80 (2 x arg $\underline{C}H_2CH_2$), 28.62 (2 x arg $\underline{C}H_2CH$), 30.90 (val CH), 49.08 & 49.33 (2 x ala α-CH), 53.54 & 53.82 (2 x arg α-CH), 58.63 (d, thr α-CH, J_{PC} 4.88), 59.50 (d, thr β-CH, J_{PC} 5.97), 67.53 (val α-CH), 157.35 (2 x arg CN), 171.27 ($CH_3\underline{C}O$), 173.08-176.71 (amide CO's) and 178.27 (CO_2H); δ_P (121.41 MHz; ^2H_2O) main peak at 5.21 ppm; *m/z* (E/S) 779 (11%, $[M + H]^+$), 778 (11, $[M]^+$), 715 (8, $[MH - PO_2H_2 + H]^+$) and 128 (10, [arginyl]$^+$).

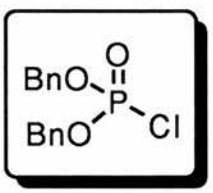
N-α-Acetyl-(*S*)-arginyl-(*S*)-arginyl-(*S*)-alanyl-(*S*)-diphenoxyphosphoryl threonyl-(*S*)-valyl -(*S*)-alaninate (80)



This compound was prepared in a manner identical to that for the phoshate triester **(78)** using Ac-R(Pmc)R(Pmc)ATVA-Wang resin (1.9 g peptide-resin, peptidyl content 195.08 mg, 0.33 mmol), DMAP (80.6 mg, 0.66 mmol), dry triethylamine (3.32 cm^3, 23.83 mmol) and diphenylphosphorochloridate (2.53 cm^3, 12.21 mmol) under an argon atmosphere at room temperature, stirring overnight. This solid phase phosphorylation was repeated 2-3 times to give Ac-RRAT(OP(OPh)$_2$)VA-Wang resin (3.65 g peptide-resin, peptidyl content 195.08 mg, 0.33 mmol) which was deprotected using cleavage mixture B (10 cm^3) to give a solid (156.2 mg, 80%), m.p. 128-130 °C; υ_{max} (DCM)/cm^{-1} 3421 (NH stretch), 3000-2500 (CO_2H, OH stretch), 1645 (NH_2), 1539-1505 (aromatic) and 1206 (P-OAryl); δ_H (300 MHz; ^2H_2O) 0.91 (6H, t, *J* 7.0, val CH($C\underline{H}_3$)$_2$), 1.20 (3H, d, *J* 7.0, thr CH_3), 1.40 (6H, m, 2 x ala CH_3), 1.51-1.80 (8H, m, 2 x arg $C\underline{H}_2CH_2$ & 2 x arg

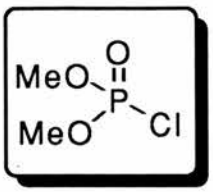
$C\underline{H}_2CH$), 2.02 (3H, s, acetyl CH_3), 2.1 (1H, m, val CH), 3.09 (4H, m, 2 x arg $C\underline{H}_2NH$), 3.9 (1H, br s, val α-CH), 4.1-4.61 (6H, m, α- & β-CH's), 6.87 (1H, d, *J* 7.6, NH) and 7.14-7.46 (10H, m, aromatic); δ_C (75.5 MHz; ^2H_2O) 17.50 & 18.4 (val $CH(\underline{C}H_3)_2$), 18.1 & 18.8 (2 x ala CH_3), 19.25 (thr CH_3), 22.0 (acetyl CH_3), 25.22 (br, 2 x arg $\underline{C}H_2CH_2$), 29.12 (br, 2 x arg $\underline{C}H_2CH$), 31.21 (val CH), 50.79 & 50.81 (2 x ala α-CH), 54.04 & 54.5 (2 x arg α-CH), 58.02 (val α-CH), 59.62 (d, thr β-CH, J_{PC} 18.85), 67.5 (thr α-CH), 120.97, 127.28 & 131.18 (aromatic), 150.0 (quat. aromatic), 163.52 (2 x arg CN), 171.93 ($CH_3\underline{C}O$), 173.08-276.0 (amide CO's) and 176.59 (CO_2H); δ_P (121.42 MHz; ^2H_2O) -11.44 ppm; *m/z* (FAB) 993 (7%, $[M + H + Na]^+$), 970 (5, $[M + H + Na]^+$), 947 (3, $[M + H]^+$), 251 (31, $[PO(OPh)_2(OH) + H]^+$), 199 (17, [arginyl-alanyl]$^+$) and 111 (100, $[alanyl + Na]^+$).

Dibenzylphosphorochloridate (82)



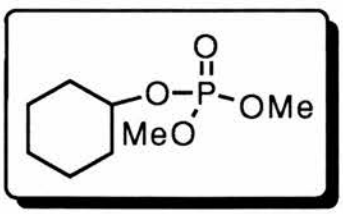
To N-chlorosuccinimide (2.70 g, 20.2 mmol) was added dibenzylphosphite (4.42 cm^3, 20 mmol) in dry toluene (10 cm^3) and the reaction stirred at room temperature for 2 h.[288] The succinimide salts were then filtered off and the filtrate used as a solution of phosphorylating agent in toluene; δ_H (200 MHz; C^2HCl_3) 5.09 (4H, d, J_{PH} 4.2, CH_2OP) and 7.34-7.50 (10H, m, aromatic); δ_C (50.3 MHz; C^2HCl_3) 69.0 (d, CH_2OP, J_{PC} 5.4), 128.05-129.47 (aromatic) and 136.39 (quat. aromatic); δ_P (121.41 MHz; $d_8$toluene) main peak at 4.997 ppm; *m/z* (CI) 190 (8%, $[M - OBn]^+$), 179 (100, $[M - H_2PO_3Cl]^+$), 100 (43, $[M - (OBn)_2 + NH_3]^+$) and 83 (18, $[M - (OBn)_2]^+$).

Dimethylphosphorochloridate (84)



Sulfuryl chloride (4.18 cm^3, 52 mmol) was added dropwise to dimethylphosphite (4.58 cm^3, 50 mmol) at 0-5 °C over 30 min.[289] The reaction mixture was stirred at 0-5 °C for 20 min and then allowed to warm to room temperature. The product was then distilled to give the product as a clear liquid (b.p. 75-80 °C/ 0.6 mmHg) {lit.,[324] b.p. 70 °C/ 10 mmHg} (1.26 g, 17%); *m/z* (Found: $[M - Cl]^+$, 109.0050. Calc. for $C_2H_6PO_3$: *m/z*, 109.0054); υ_{max} (neat)/cm^{-1} 2859 (OCH_3), 1299(P=O) and 1044 (P-OAryl); δ_H (200 MHz; C^2HCl_3) 3.86 (6H, d, J_{PH} 14.2, OCH_3); δ_C (50.3 MHz; C^2HCl_3) 55.37 (d, OCH_3, J_{PC} 6.94); δ_P (121.41 MHz; C^2HCl_3) main peak at 7.73 ppm; *m/z* (EI) 126 (44%, $[M - Cl + NH_3]^+$), 109 (55, $[M - Cl]^+$), 96 (100, $[PO(OMe)(OH) + H]^+$) and 65 (22, [2 x MeOH + $H]^+$).

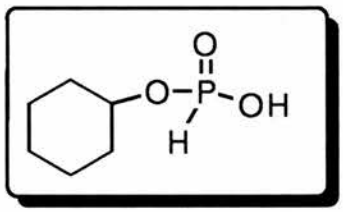
Cyclohexyl dimethyl phosphate (85)



To a solution of cyclohexanol (1.0 g, 10 mmol), DMAP (122.17 mg, 1 mmol) and dry triethylamine (5.03 cm^3, 36.1 mmol) in dry DCM (10 cm^3) was added a solution of dimethylphosphorochloridate (9.25 cm^3, 18.5 mmol) in dry DCM . The organic solvents were removed under reduced pressure to give the product as an oil (0.817 g, 39%) {lit.,[325] 106 °C/ 1.5 mmHg}; *m/z* (Found: $[M + H]^+$, 209.0947. Calc. for $C_8H_{18}PO_4$: *m/z*, 209.0943); υ_{max} (DCM)/cm^{-1} 2857 (OCH_3), 2857 (CH stretch), 1452 (CH defrmation),

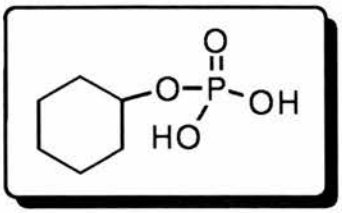
1266 (P=O) and 1063 (P-OAryl); δ_H (200 MHz; C^2HCl_3) 1.06-1.74 (10H, m, CH_2's), 3.29 (6H, d, J_{PH} 5.6, OCH_3) and 4.02 (1H, m, CHOP); δ_C (50.3 MHz; C^2HCl_3) 22.55 ($\underline{C}H_2CH_2$), 24.1 ($\underline{C}H_2CH_2$), 32.78 (d, CH_2CH, J_{PC} 3.8), 50.76 (d, OCH_3, J_{PC} 5.58) and 74.93 (d, CHOP, J_{PC} 6.19); δ_P (121.41 MHz; C^2HCl_3) main peak at 0.37 ppm; *m/z* (CI) 209 (15, $[M + H]^+$), 127 (76, $[P(O)(OCH_3)_2OH + H]^+$) and 83 (11, $[C_6H_{11}]^+$).

Cyclohexyl phosphonate (92)[326]



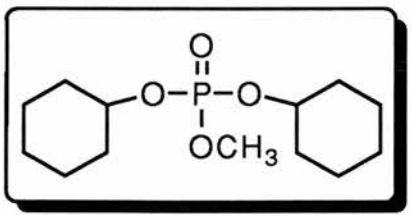
To a stirred solution of cyclohexanol (1.0 g, 10 mmol) and dry triethylamine (2.78 cm^3, 20 mmol) in dry 1,4-dioxane (10 cm^3), was added 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (salicylchlorophosphite) (2.43 g, 12 mmol) in dry 1,4-dioxane (10 cm^3) and the mixture stirred for 30 min. Water (xs) was added and the reaction mixture stirred overnight. The reaction mixture was then extracted with DCM (3 x 20 cm^3), washed with brine (20 cm^3) and dried ($MgSO_4$). The solvent was removed under reduced pressure to give an oil which was purified by silica chromatography using ethyl acetate/ DCM/ methanol (6:3:1) to give the product (R_f 0.72) as an oil (0.58 g, 35%); *m/z* (Found: $[M + H]^+$, 165.0683. Calc. for $C_6H_{14}PO_3$: *m/z*, 165.0681); υ_{max} (neat)/cm^{-1} 2936-2861 (CH stretch), 2437 (P-H) and 1222 (P=O stretch); δ_H (200 MHz; C^2HCl_3) 1.16-1.9 (10H, m, CH_2's), 4.38 (1H, m, CHOP) and 5.1 & 8.54 (1H, d, J_{PH} 688.4, PH); δ_C (50.3 MHz; C^2HCl_3) 22.76 ($\underline{C}H_2CH_2$), 24.37 ($\underline{C}H_2CH_2$), 32.78 & 32.97 (2 x d, $\underline{C}H_2CH$, J_{PC} 4.48 & 3.82) and 74.79 (d, CHOP, J_{PC} 5.89); δ_P (121.41 MHz; C^2HCl_3) 4.47 ppm; *m/z* (CI) 165 (18%, $[M + H]^+$) and 83 (100, $[C_6H_{11}]^+$).

Cyclohexyl phosphate (93)[327]



To a vigorously stirred solution of cyclohexanol (1.0 g, 10 mmol) and dry triethylamine (5.58 cm^3, 40 mmol) in dry DCM (10 cm^3) under an argon atmosphere at -10 °C, was added dry $POCl_3$ (1.03 cm^3, 11 mmol) over 5 min. The mixture was stirred at -10 °C for a further 30 min and then allowed to warm to room temperature and stirred for 3-4 h. Water (xs) was then added and the reaction stirred for a further 3 h. The salts were removed by filtration and the solvent removed under reduced pressure to give the crude product (79 mg, 4%); *m/z* (Found: $[M + H]^+$, 181.0634. Calc. for $C_6H_{14}PO_4$: *m/z*, 181.0630); υ_{max} (DCM)/cm^{-1} 3294 (H-bonded OH), 2888 (CH stretch) and 1274 (P=O); δ_H (200 MHz; C^2HCl_3) 1.24-1.92 (10H, m, CH_2's) and 4.29 (1H, m, CHOP); δ_C (50.3 MHz; C^2HCl_3) 23.37 ($\underline{C}H_2CH_2$), 25.21 (d, $\underline{C}H_2CH_2$, J_{PC} 4.88), 33.18 (d, $\underline{C}H_2CH$, J_{PC} 4.58) and 76.60 (d, CHOP, J_{PC} 6.29); δ_P (121.41 MHz; C^2HCl_3) -0.19 ppm; *m/z* (CI) 181 (7%, $[M + H]^+$), 102 (28, $[cyclohexanol + 2H]^+$) and 83 (7, $[C_6H_{11}]^+$).

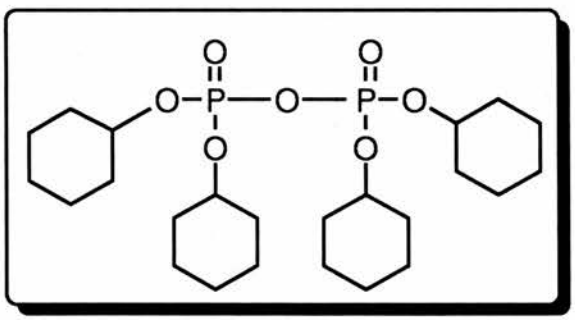
Dicyclohexyl methyl phosphate (96)



To a solution of cyclohexyl phosphonate (45 mg, 0.28 mmol) in pyridine (3 cm^3) was added 5% w/v I_2 in pyridine:water (98:2) (3 cm^3). The reaction mixture was stirred overnight and then poured into ice water (10 cm^3) and stirred for a further 2 h. The

precipitated solid was filtered off and the solvent removed under reduced pressure. The compound isolated was then dissolved in ether/ THF (2 cm^3) and treated with ethereal diazomethane (20 cm^3) at 0 °C for 2-3 h. Excess diazomethane was then removed by a stream of nitrogen and the solvent removed under reduced pressure to give the product as an oil (24 mg, 31%); *m/z* (Found: $[M + H]^+$, 277.1572. $C_{13}H_{26}PO_4$ requires *m/z*, 277.1569); υ_{max} (DCM)/cm^{-1} 2943-2862 (CH stretch), 1266 (P=O) and 736 (CH_2 rocking); δ_H (200 MHz; C^2HCl_3) 1.2-1.97 (22H, m, CH_2's), 3.72 (3H, d, J_{PH} 11.2, OCH_3) and 4.34 (2H, m, CH's); δ_C (50.3 MHz; C^2HCl_3) 24.02 & 25.60 (CH_2's), 33.79 (d, $\underline{C}H_2CH$, J_{PC} 4.6), 54.31 (d, OCH_3, J_{PC} 6.54) and 77.55 (d, CH, J_{PC} 2.21); δ_P (121.41 MHz; C^2HCl_3) main peak at -0.995 ppm; *m/z* (CI) 277 (18%, $[M + H]^+$), 111 (16, $[CH_3PO_4 + H]^+$), 97 (21, $[CH_3PO_2 + NH_4 + H]^+$) and 73 (100, $[C_5H_{10} + 3H]^+$).

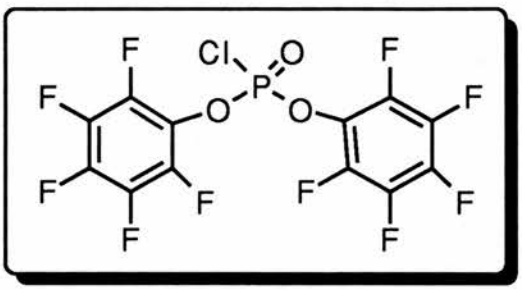
Tetracyclohexyl pyrophosphate (97)



To a solution of cyclohexyl phosphonate (45 mg, 0.28 mmol) in pyridine (3 cm^3) was added 5% w/v I_2 in pyridine:water (98:2) (3 cm^3) and the reaction mixture stirred overnight. The mixture was then washed with sodium thiosulfate (10 cm^3), water (10 cm^3) and the aqueous phase extracted with DCM (2 x 10 cm^3) and washed with brine (10 cm^3). The solvent was then removed under reduced pressure and the residue taken up in ether/ THF (2 cm^3) to give the product as a yellow-brown oil (54 mg, 38%); *m/z* (Found: $[M + H]^+$, 507.2651. $C_{24}H_{45}P_2O_7$ requires *m/z*, 507.2641); υ_{max} (DCM)/cm^{-1} 1422 (CH deformation), 1266 (P=O) and 739 (CH_2 rocking); δ_H (200 MHz; C^2HCl_3) 1.15-1.96 (44 H, m, CH_2's) and 4.51-4.54 (4 H, m, CH's); δ_C (50.3 MHz; C^2HCl_3)

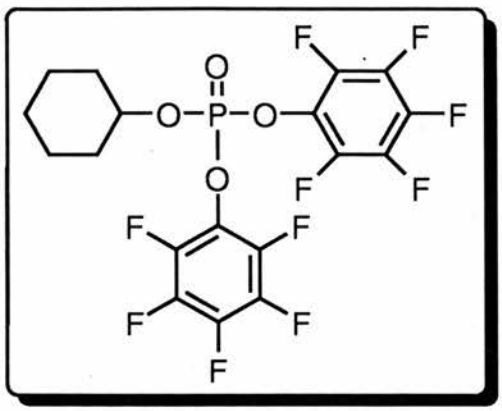
23.4 & 25.07 (CH_2's), 32.92 (d, $\underline{C}H_2CH$, J_{PC} 7.44) and 78.52 (d, CH, J_{PC} 3.07); δ_P (121.41 MHz; C^2HCl_3) main peak at -14.73 ppm; *m/z* (CI) 507 (2%, $[M + H]^+$), 166 (5, $[2 \times C_6H_{11}]^+$), 99 (28, $[C_6H_{11}O]^+$) and 57 (100, $[C_4H_8 + H]^+$).

Dipentafluorophenylphosphorochloridate (99)[303-307]



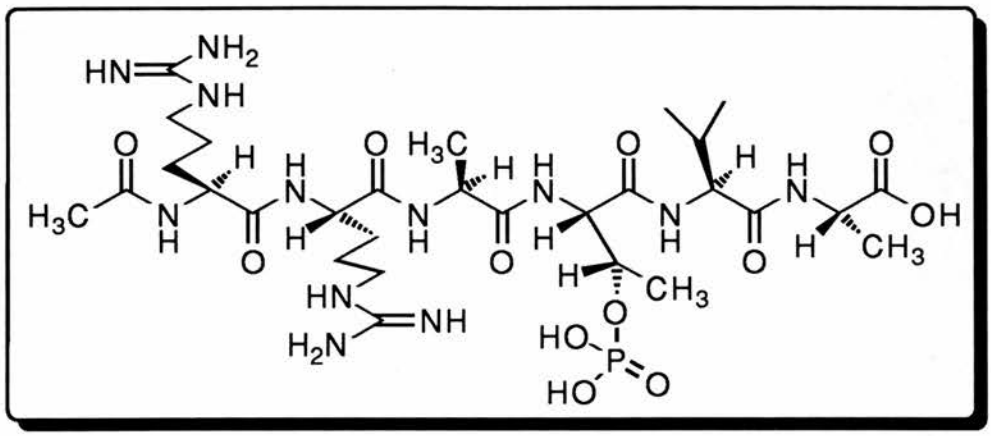
Using a modification of the procedure of Baer,[303] $POCl_3$ (10 cm^3, 0.107 mol) and pentafluorophenol (35.91 g, 0.195 mol) were refluxed overnight at 140 °C under a nitrogen atmosphere. The contaminants were then distilled off at the pump to leave the product as a brown oil that solidifies when kept in the freezer for long storage (23.24 g, 48%); *m/z* (Found: M^+, 447.9118. Calc. for $C_{12}PO_3F_{10}Cl$: *m/z*, 447.9114); υ_{max} (neat)/cm^{-1} 1240 (P-OAryl) and 1266 (P=O); δ_C (75.5 MHz; C^2HCl_3) 136.43-142.62 (aromatic); δ_P (121.41 MHz; C^2HCl_3) main peak at -15.05 ppm; δ_F (282.21 MHz; C^2HCl_3) 190.53-190.67, 194.94-195.11 & 198.05-198.14 (10F, all m, CF's); *m/z* (EI) 448 (43%, M^+), 413 (32, $[M - Cl]^+$), 265 (22, $[M - OC_6F_5]^+$) and 183 (100, $[OC_6F_5]^+$).

Cyclohexyl dipentafluorophenyl phosphate (100)



To a solution of cyclohexanol (0.12 g, 1.2 mmol) and dry triethylamine (167.3 μl, 1.2 mmol) in dry DCM (5-10 cm^3), under an argon atmosphere, was added a solution of dipentafluorophenylphosphorochloridate (0.54 g, 1.2 mmol) in dry DCM (10 cm^3) and the reaction left to stir for 4-5 h. The salts were then filtered off and the solvent removed under reduced pressure to give an oil which was purified by basified silica chromatography using ethyl actetate/ hexane (1:9) as the eluant to give the product (R_f 0.53) as an oil (110 mg, 18%); *m/z* (Found: M^+, 512.0228. $C_{18}H_{11}PO_4F_{10}$ requires *m/z*, 512.0235); υ_{max} (DCM)/cm^{-1} 2864 (CH stretch) and 1265 (P=O); δ_H (300 MHz; C^2HCl_3) 1,26-1.95 (10H, m, CH_2's) and 4.8 (1H, m, CHOP); δ_C (75.5 MHz; C^2HCl_3) 23.01 ($\underline{C}H_2CH_2$), 24.67 ($\underline{C}H_2CH_2$), 32.73 (d, $\underline{C}H_2CH$, J_{PC} 3.07), 82.65 (d, CHOP, J_{PC} 4.88) and 136.16-142.68 (m, aromatic); δ_P (121.41 MHz; C^2HCl_3) -11.225 ppm; δ_F (282.21 MHz; C^2HCl_3) 189.08-189.22, 192.51-192.67 & 197.39-197.46 (10F, m, CF's); *m/z* (EI) 512 (5%, M^+), 431 (100, $[P(O)(OPhF_5)_2OH + H]^+$), 184 (22, $[C_6F_5OH]^+$) and 83 (9, $[C_6H_{11}]^+$).

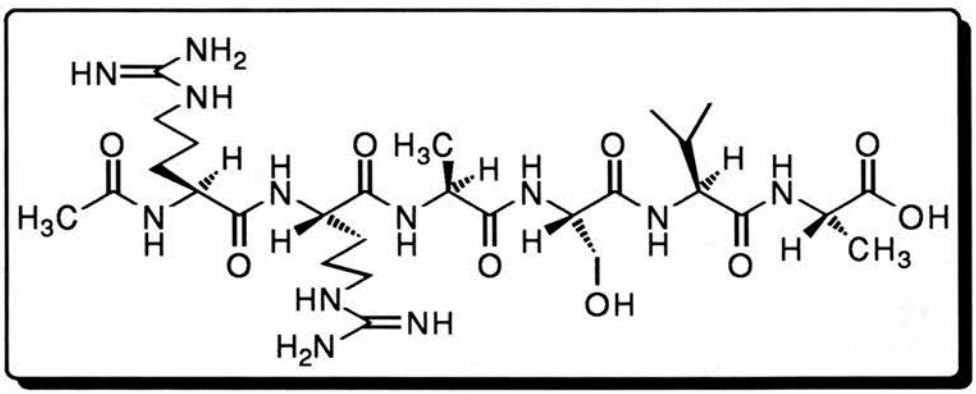
N-α-Acetyl-(*S*)-arginyl-(*S*)-arginyl-(*S*)-alanyl-(*S*)-dihydroxyphosphoryl threonyl-(*S*)-valyl-(*S*)-alaninate (102)



Ac-R(Pmc)R(Pmc)ATVA-Wang resin (0.95 g peptide-resin, 202.8 mg peptidyl content, 0.338 mmol), DMAP (41.3 mg, 0.338 mmol) and dry triethylamine (1.7 cm^3, 12.2 mmol) in dry DCM, under an argon atmosphere, was treated with a solution of dipentafluorophenyl phosphorochloridate (2.80 g, 6.253 mmol) in dry DCM and left to stir overnight. The resin was then washed with DCM and dried with MeOH. The dried resin (0.4 g peptide-resin, 95.4 mg peptidyl content, 0.159 mmol) was then treated with cleavage mixture C for 2 h and the aqueous layer lyophilised. The solid obtained was purified by ion exchange chromatography using the strong anion exchanger resin Dowex 1 x 2-200 (chloride form) to give the product as a white solid (57.24 mg, 60%), m.p. 205 °C (decomp.); υ_{max} (Nujol)/cm^{-1} 3386 (NH stretch), 2923-2855 (CH stretch), 1272 (P=O) and 1158-1049 (C-F); δ_H (300 MHz; ^2H_2O) 0.97 (6H, 2 x d, *J* 6.4, val $CH(C\underline{H}_3)_2$), 1.33 (3H, d, *J* 6.27, thr CH_3), 1.42 (6H, t, *J* 7.0, 2 x ala CH_3), 1.68 (4H, br s, 2 x arg $C\underline{H}_2CH_2$), 1.80 (4H, m, 2 x arg $C\underline{H}_2CH$), 2.02 (3H, s, acetyl CH_3), 2.1 (1H, m, val CH), 3.21 (4H, m, 2 x arg $C\underline{H}_2NH$) and 4.14-4.49 (7H, m, α- & β-CH's); δ_C (75.5 MHz; ^2H_2O) 16.42 & 18.08 (val $CH(\underline{C}H_3)_2$), 16.93 & 18.20 (2 x ala CH_3), 18.63 (thr CH_3), 21.99 (acetyl CH_3), 24.76 (br, 2 x arg $\underline{C}H_2CH_2$), 28.58 (2 x arg $\underline{C}H_2CH$), 30.81 (val CH), 49.10 & 50.16 (2 x ala α-CH), 53.44 & 53.82 (2 x arg α-CH), 58.66 (d, thr β-CH, J_{PC} 6.96), 59.64 (val α-CH), 72.84 (d, thr α-CH, J_{PC} 4.80), 157.14 (2 x arg

CN), 170.93 ($CH_3\underline{C}O$), 173.09-175.36 (amide CO's) and 176.59 ($\underline{C}O_2H$); δ_P (121.41 MHz; ^2H_2O) -0.616 ppm; *m/z* (E/S) 795 (4%, $[M + H]^+$), 398 (25, $[M + H/ 2]^+$), 368 (2, [phosphorylthreonyl-valyl-alanyl]$^+$) and 193 (100, [valyl-alanyl + 6H]$^+$).

N-α-Acetyl-(*S*)-arginyl-(*S*)-arginyl-(*S*)-alanyl-(*S*)-seryl-(*S*)-valyl-(*S*)-alaninate (105)

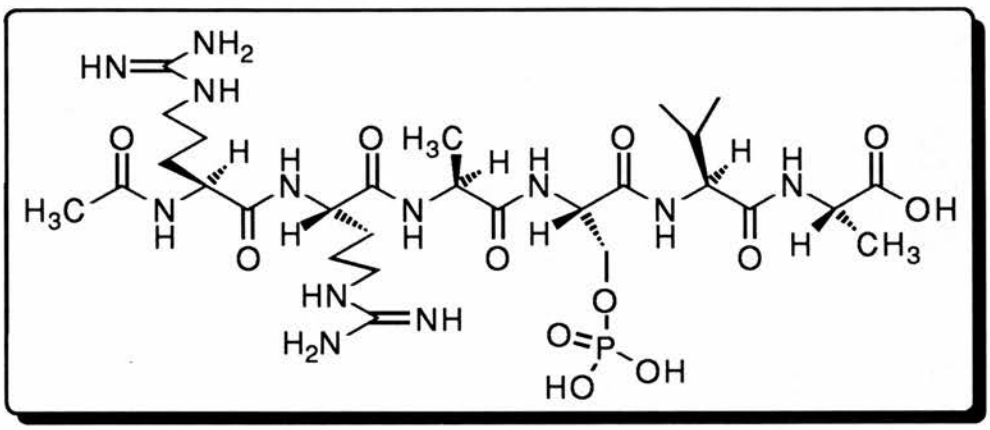


This compound was prepared in a manner identical to that for the hexapeptide **(124)**, using Fmoc-R(Pmc)R(Pmc)ASVA-Wang resin (0.77 g peptide-resin, 150 mg, peptidyl content, 0.25 mmol).

Ac-R(Pmc)R(Pmc)ASVA-Wang resin (0.33 g peptide-resin, 75 mg peptidyl content, 0.125 mmol) so obtained was treated with cleavage mixture C to give the product as a white solid on lyophilisation (75 mg, 100%), m.p. 177-178 °C; δ_H (300 MHz; ^2H_2O) 0.98 (6H, t, *J* 6.6, val $CH(C\underline{H}_3)_2$), 1.44 (6H, t, *J* 6.6, 2 x ala CH_3), 1.68 (4H, br s, 2 x arg $C\underline{H}_2CH_2$), 1.81 (4H, m, 2 x arg $C\underline{H}_2CH$), 2.06 (3H, s, acetyl CH_3), 2.12 (1H, m, val CH), 3.24 (4H, t, *J* 6.3, 2 x arg $C\underline{H}_2NH$), 3.89 (1H, d, *J* 5.7, val α-CH), 4.2-4.42 (4H, m, α-CH's), 4.49 (2H, t, *J* 5.6, ser CH_2) and 4.72 (1H, m, ser α-CH); δ_C (75.5 MHz; ^2H_2O) 16.03 & 17.42 (val $CH(\underline{C}H_3)_2$), 16.52 & 18.25 (2 x ala CH_3), 21.58 (acetyl CH_3), 24.35 (2 x arg $\underline{C}H_2CH_2$), 28.06 (2 x arg $\underline{C}H_2CH$), 30.27 (val CH), 48.66 & 49.72 (2 x ala α-CH), 53.17 & 53.63 (2 x arg α-CH), 55.46 (ser α-CH), 59.13 (ser CH_2), 61.03 (val α-CH), 156.92 (2 x arg CN), 171.74 ($CH_3\underline{C}O$), 173.04-176.36 (amide CO's) and 179.11

(CO_2H); *m/z* (E/S) 701 (13%, $[M + H]^+$), 375 (23, [Ac-arginyl-arginly-alanyl + 5H]$^+$), 354 {100, [M - (alanyl-seryl-valyl-alanyl)]$^+$} and 304 (8.92, [Ac-arginyl-arginyl + 5H]$^+$).

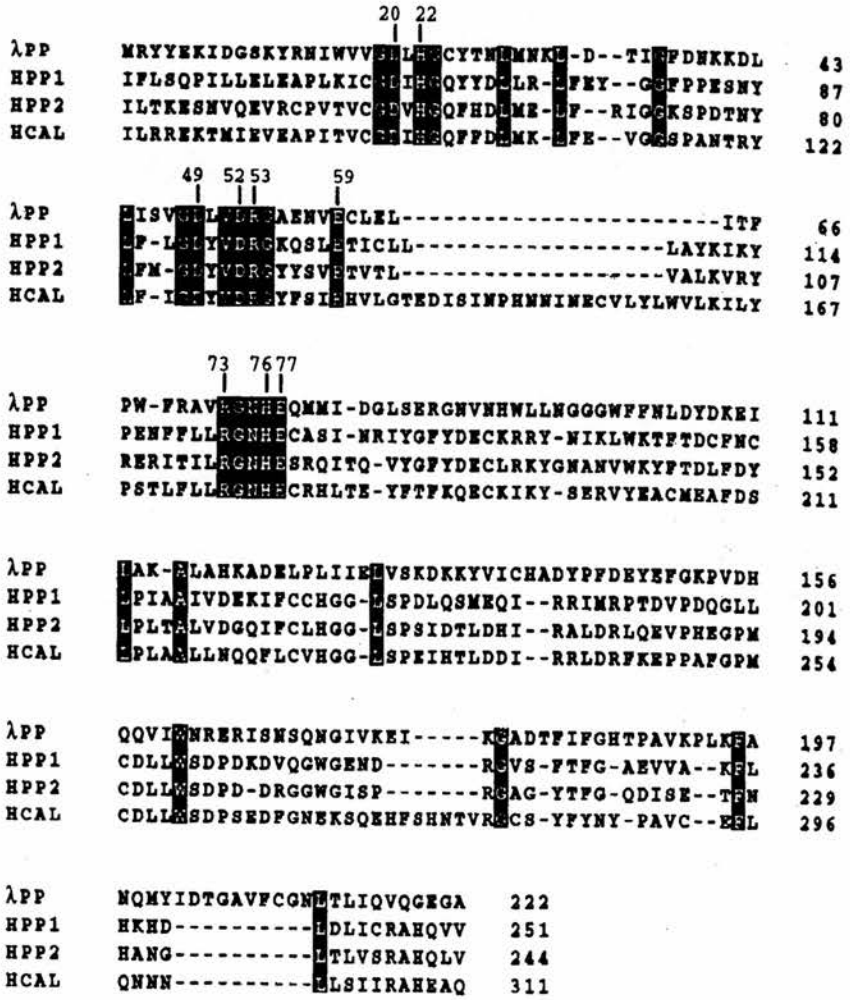
N-α-Acetyl-(*S*)-arginyl-(*S*)-arginyl-(*S*)-alanyl-(*S*)-dihydroxyphosphoryl seryl-(*S*)-valyl-(*S*)-alaninate (107)



This compound was prepared in a manner identical to that for the phosphorylated hexapeptide **(102)** using Ac-R(Pmc)R(Pmc)ASVA-Wang resin (0.33 g peptide-resin, 75 mg peptidyl content, 0.125 mmol), DMAP (30.54 mg, 0.25 mmol), dry triethylamine (1.26 cm^3, 9.025 mmol) and dipentafluorophenylphosphorochloridate (2.07 g, 4.625 mmol). The peptide-resin (0.31 g) obtained was treated with cleavage mixture C and purified by ion exchange chromatography using Dowex 1 x 2-200 (chloride form) to give the product as an off-white solid (12 mg, 16%), m.p. 200 °C (decomp.); δ_H (300 MHz; ^2H_2O) 0.96 (6H, t, *J* 6.0, val $CH(C\underline{H}_3)_2$), 1.43 (6H, 2 x d, *J* 6.6 & 7.2, 2 x ala CH_3), 1.72 (8H, m, 2 x arg $C\underline{H}_2CH_2$ & 2 x arg $C\underline{H}_2CH$), 2.02 (3H, s, acetyl CH_3), 2.11 (1H, m, val CH), 3.2 (4H, m, 2 x arg $C\underline{H}_2NH$), 4.16-4.40 (5H, m, α-CH's), 4.57 (2H, m, ser CH_2) and 4.70 (1H, m, ser α-CH); δ_C (75.5 MHz; ^2H_2O) 16.45 & 18.07 (val $CH(\underline{C}H_3)_2$), 16.82 & 18.70 (2 x ala CH_3), 21.97 (acetyl CH_3), 24.78 (2 x arg $\underline{C}H_2CH_2$), 28.63 (2 x arg $\underline{C}H_2CH$), 30.80 (val CH), 49.12 & 50.39 (2 x ala α-CH), 53.49 & 53.81 (2 x arg α-CH), 54.78 (d, ser α-CH, J_{PC} 7.34), 59.67 (d, ser CH_2, J_{PC} 3.84), 64.58 (val α-CH), 157.15 (2 x arg CN), 171.18 ($CH_3\underline{C}O$), 173.15-175.45 (amide CO's) and 176.65

(CO_2H); δ_P (121.41 MHz; ^2H_2O) 0.397 ppm; *m/z* (E/S) 781 (27%, $[M + H]^+$) and 354 {100, [M - (alanyl-phosphorylseryl-valyl-alanyl)]$^+$}.

APPENDIX 1



The aligned sequences are λPP, PPλ; HPP1, *Homo sapiens* protein phosphatase-1; HPP2, *Homo sapiens* lung protein phosphatase-2A catalytic subunit; HCAL, *Homo sapiens* calcineurin A.[308] Invariant amino acids are boxed. The numbers on the right indicate amino acid positions in the protein, whereas numbers above the sequences refer to the corresponding amino acid positions in PPλ.

APPENDIX 2

Enzymic Phosphorylations

Procedure 1

The phosphorylation of the synthetic peptide Ac-RRATVA-OH was carried out with cAMP-dependent protein kinase (from Sigma) or the catalytic subunit of protein kinase from bovine heart (from Boehringer Mannheim). The incubation mixture which was a final volume of 0.25 cm^3, contained 100 μM ATP, 100 mM Tris-HCl buffer (pH 7.5), 12 mM $MgCl_2$, 1 μM cAMP, 1 mM sodium vanadate, synthetic peptide (0.5 mg) and cAMP-dependent protein kinase (10 μg protein) or the catalytic subunit of protein kinase from bovine heart (10 μg protein). Incubations were carried out at 37 °C for 15-45 min, then terminated with 0.25 M HCl (50 mm^3) and the pH adjusted to 9 with 0.05 M NaOH (50 mm^3). The incubation mixture was then applied to an AG1 x 2 anion exchanger resin (acetate form), the phosphorylated peptide eluted with 30% acetic acid and the fractions subjected to LDMS.

Procedure 2

The synthetic peptide (2 mg/ cm^3) in a final volume of 0.25 cm^3 was phosphorylated with cAMP-dependent protein kinase (20 μg protein), 200 μM ATP, 70 mM Mes (pH 6.5), 15 mM $MgCl_2$, 2 μM cAMP and 250 μM sodium vanadate. The incubations were carried out at 30 °C for 2-3 h and then worked up as per procedure 1.

APPENDIX 3

PPλ NMR ASSAYS

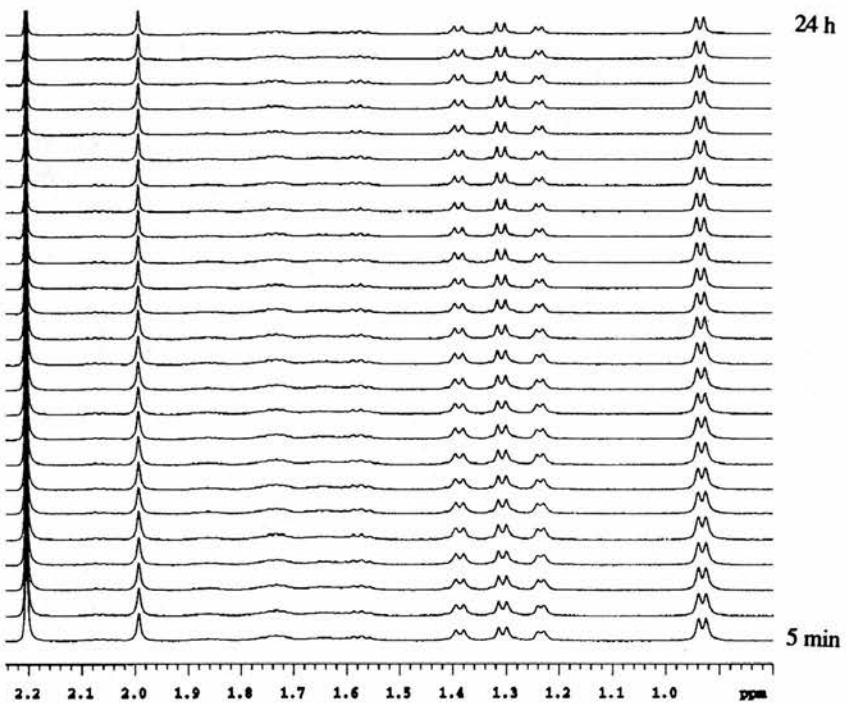
Ac-RRAT(OPO_3H_2)VA-OH as a Substrate

The substrate (1 mg) was dissolved in D_2O (0.6 cm^3) and the pH of the solution adjusted to 7.8. A solution of PPλ (10 mm^3, equivalent to 200 units) in buffer containing 50 mM Tris-HCl, 250 mM NaCl, 5mM DTT, 2 mM $MnCl_2$, 1 mM Na_2EDTA and 50% glycerol, was then added to the reaction medium and the progress of the reaction monitored by ^1H nmr spectroscopy (500 MHz) for several hours.

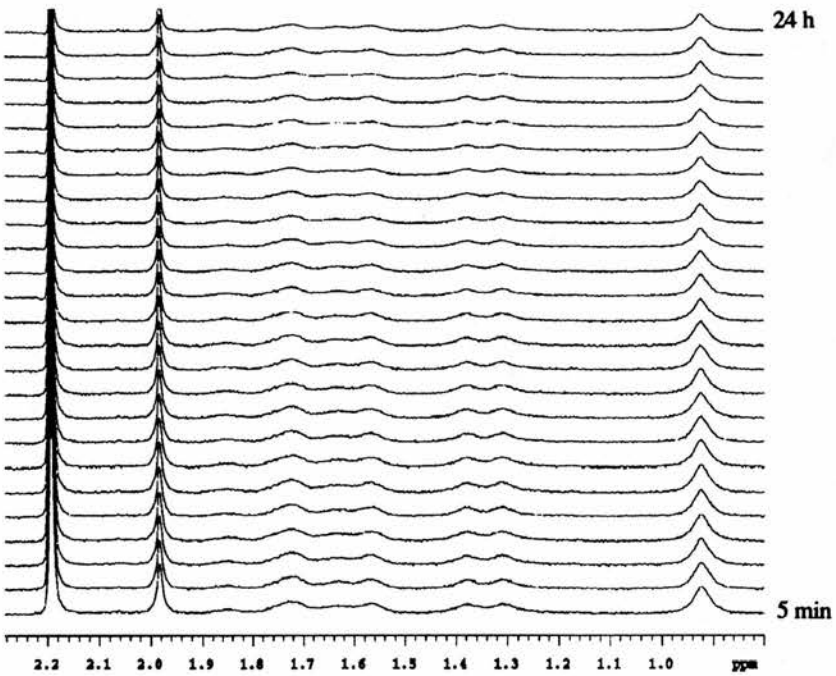
Absolute Requirement for Mn^{2+}

To a solution of the substrate (1 mg) dissolved in a buffer containing 50 mM deuterated Tris, pH 7.8, and 2 mM $MgCl_2$, was added PPλ (200 units). The reaction was monitored by ^1H nmr spectroscopy for 24 h, then more PPλ (200 units) was added to the medium. The progress of the reaction was recorded for a further 4 h. $MnCl_2$ (10 mm^3, 2 mM final concentration) was added to the reaction and then monitored for changes by ^1H nmr spectroscopy (500 MHz) (Appendix 4).

APPENDIX 4



Time-course experiment showing the effect of Mg^{2+} on PPλ. No change in the ^{1}H nmr spectra of the substrate was observed.



Time-course experiment showing the effect of Mn^{2+} on PPλ. The presence of Mn^{2+} in the incubation mixture resulted in the broadening of the signals. However, the shift of the thr CH_3 signal of the phosphopeptide can be observed.

APPENDIX 5

PP2A NMR ASSAYS

The substrate, Ac-RRAT(P)VA-OH (0.7 mg), was dissolved in D_2O (0.7 cm^3). PP2A (300 mm^3, 3 units) in a buffer containing 50% glycerol, 20 mM MOPS, pH 7.5, 60 mM 2-mercaptoethanol, 1 mM $MgCl_2$ and 0.1 mg/ cm^3 serum albumin, was added to the medium (pH 7.8). A time-course experiment was set up (30 °C) and ^1H nmr spectra (500 MHz) were recorded at 10, 20, 30, 45, 60, 75, 90, 150, 210 and 270 min after the initial addition of the enzyme (Fig. 2.10 b).

In a similar experiment, to the substrate (0.7 mg) dissolved in D_2O (0.7 cm^3) at 30 °C, pH 7.8, was added PP2A (300 mm^3, 3 units) and microcystin-LR (500 μg). The progress of the reaction was monitored using ^1H nmr (500 MHz) spectroscopy using the time-course experiment above (Fig. 2.11).

APPENDIX 6

^{18}O-Water/ Phosphate Ligand Exchange

An incubation solution containing homogeneous PPλ (200 units), potassium dihydrogen phosphate (10 mM), manganese chloride (2 mM), dithiothreitol (5 mM), bovine serum albumin (100 μg/ cm^3) and Tris-HCl (50 mM), pH 7.8, in a total volume of 500 mm^3 of 30% [^{18}O]water, was prepared. The control contained no enzyme. The reaction solutions were kept at 30 °C and at intervals over a period of 24 h, aliquots (100 mm^3) were quenched by cooling in liquid nitrogen. The frozen solutions were lyophilised and the residues redissolved in water (100 mm^3) and MeOH/ $HCl_{(aq)}$ (90:10; 100 mm^3). Excess ethereal diazomethane (3-5 cm^3) was added and the reaction left to proceed for 2-3 h. The unreacted diazomethane was removed in a stream of dinitrogen and the solvent removed under reduced pressure. The residues were dissolved in diethyl ether (300 mm^3) and the solutions subjected to analysis by GCMS under conditions previously optimised using authentic samples of trimethyl phosphate. The mass spectra for the trimethyl phosphate isolated from the control reactions and from the incubations, at t=2 h to t=24 h, showed the expected parent ion at 140 amu. However, the samples prepared did not show any ^{18}O-enrichment, as judged by the absence of ions at 142, 144 and 146 amu.

Similar ^{18}O-exchange experiments incorporating the unphosphorylated peptide, Ac-RRATVA-OH, in the incubation mixture were carried out. The control contained no enzyme. The aliquots removed (100 mm^3) were treated as before and the final solutions subjected to GCMS analysis. The mass spectra for the samples containing the free alcohol, Ac-RRATVA-OH, showed the expected parent ion at 140 amu as well as ^{18}O-enrichment judged by the presence of ions at 142, 144 and 146 amu. The presence of the peptide, Ac-RRATVA-OH, seems to have facilitated the incorporation of ^{18}O into the samples of trimethyl phosphate. The mode of action of the enzyme PPλ, therefore, could tentatively be considered to involve a ternary complex.

PUBLICATIONS

1. Pantea Hormozdiari and David Gani,
   Highly efficient solid-phase phosphopeptide synthesis using bis-(polyfluorophenyl) chlorophosphates: Preparation of serine/ threonine protein phosphatase substrates, *Tetrahedron Lett.*, 1996, **37**, 8227-8230.
2. The use of the phosphorylating agent dipentafluorophenylphosphorochloridate in "Solid Phase Phosphorylations" has been patented for such use:
   British Patent Application No. 9619768.6.

CHAPTER FOUR

REFERENCES

4.0 REFERENCES

1. M. J. Hubbard and P. Cohen, *Trends Biochem. Sci.*, 1993, **18**, 172-177.
2. P. Cohen, *Trends Biochem. Sci.*, 1992, **17**, 408-413.
3. P. Cohen, *Annu. Rev. Biochem.*, 1989, **58**, 453-508.
4. T. S. Ingebritsen and P. Cohen, *Science*, 1983, **221**, 331-338.
5. P. Cohen, S. Klumpp and D. L. Schelling, *FEBS Lett.*, 1989, **250**, 596-600.
6. S. Wera and B. A. Hemmings, *Biochem. J.*, 1995, **311**, 17-29.
7. P. T. W. Cohen, *FEBS Lett.*, 1988, **232**, 17.
8. M. Bollen and W. Stalmans, *Crit. Rev. Biochem. Mol. Biol.*, 1992, **27**, 227-281.
9. Z. Zhang, S. Zhao, G. Bai and E. Y. C. Lee, *J. Biol. Chem.*, 1994, **269**, 13766-13770.
10. V. Dombradi, M. Axton, D. M. Glover and P. T. W. Cohen, *Eur. J. Biochem.*, 1989, **183**, 603.
11. S. Tamura, K. R. Lynch, J. Larner, J. Fox, A. Yasui, K. Kikuchi, Y. Suzuki and S. Tsuiki, *Proc. Natl. Acad. Sci. U.S.A.*, 1989, **86**, 1796.
12. P. M. Tang, J. A. Bondor, K. M. Swiderek and A. A. DePaoli-Roach, *J. Biol. Chem.*, 1991, **266**, 15782-15789.
13. P. Stralfors, A. Hiraga and P. Cohen, *Eur. J. Biochem.*, 1985, **149**, 295-303.
14. M. J. Hubbard and P. Cohen, *Eur. J. Biochem.*, 1989, **186**, 711-716.
15. M. J. Hubbard, P. Dent, C. Smythe and P. Cohen, *Eur. J. Biochem.*, 1990, **189**, 243-249.
16. L. K. MacDougall, L. R. Jones and P. Cohen, *Eur. J. Biochem.*, 1991, **196**, 725-734.
17. D. Alessi, M. M. Sola, L. K. MacDougall, M. Ikebe and P. Cohen, *Eur. J. Biochem.*, 1992, **210**, 1023-1035.
18. P. Dent, L. K. MacDougall, C. MacKintosh, D. G. Campbell and P. Cohen, *Eur. J. Biochem.*, 1992, **210**, 1037-1044.

19. A. Fernandez, D. L. Brautigan and N. J. C. Lamb, *J. Cell Biol.*, 1992, **116**, 1421-1430.

20. M. Beullens, A. Van Eynde, M. Bollen and W. Stalmans, *J. Biol. Chem.*, 1993, **268**, 13172-13177.

21. J. Goris, E.Waelkens and W. Merlevede, *FEBS Lett.*, 1985, **188**, 262-266.

22. M. J. Hubbard and P. Cohen, *Eur. J. Biochem.*, 1989, **186**, 701-709.

23. S. Shenolikar and A. C. Nairn, Protein Phosphatases: Recent Progress, in *Advances in Second Messenger and Phosphoprotein Research*, ed. P. Greengard and G. A. Robison, Raven Press Ltd., New York, 1991, vol. 23, pp. 1-121.

24. J. H. Doonan and N. R. Morris, *Cell*, 1989, **57**, 987-996.

25. P. Hendrix, R. E. Mayer-Jaekel, P. Cron, J. Goris, J. Hofsteenge, W. Merlevede and B. A. Hemmings, *J. Biol. Chem.*, 1993, **268**, 15267-15276.

26. J. M. Axton, V. Dombradi, P. T. W. Cohen and D. M. Glover, *Cell*, 1990, **63**, 33-46.

27. A. D. Deana and L. A. Pinna, *Biochim. Biophys. Acta*, 1988, **968**, 179-185.

28. J. Chen, B. L. Martin and D. Brautigan, *Science*, 1992, **257**, 1261-1264.

29. H. Guo and Z. Damuni, *Proc. Natl. Acad. Sci. U.S.A.*, 1993, **90**, 2500-2504.

30. C. Jessus, J. Goris, S. Staquet, X. Cayla, R. Ozon and W. Merlevede, *Biochem. J.*, 1989, **260**, 45-51.

31. X. Cayla, J. Goris, J. Hermann, P. Hendrix, R. Ozon and W. Merlevede, *Biochemistry*, 1990, **29**, 658-667.

32. X. Cayla, C. Van Hoof and M. Bosch, *J. Biol. Chem.*, 1994, **269**, 15668-15675.

33. W. van Zyl, W. Huang and A. A. Sneddon, *Mol. Cell. Biol.*, 1992, **12**, 4946-4959.

34. D. L. Merat, Z. Y. Hu, T. E. Carter and W. Y. Cheung, *J. Biol. Chem.*, 1985, **260**, 11053-11059.

35. Y. Hashimoto, B. A. Perrino and T. R. Soderling, *J. Biol. Chem.*, 1990, **265**, 1924-1927.

36. W. Li and E. Handschumacher, *J. Biol. Chem.*, 1993, **268**, 14040-14044.

37. T. Nakamura, Y. Liu, D. Hirata, H. Namba, S. Harada,T. Hirokawa and T. Miyakawa, *EMBO J.*, 1993, **12**, 4063-4071.

38. D. B. Burnham, *Biochem. J.*, 1985, **231**, 335-341.

39. S. Shenolikar, *Annu. Rev. Cell Biol.*, 1994, **10**, 55-86.

40. R. E. Honkanen, J. Zwiller, S. L. Daily, B. S. Khatra, M. Dukelow and A. L. Boynton, *J. Biol. Chem.*, 1991, **266**, 6614-6619.

41. N. D. Brewis, A. J. Street, A. R. Prescott and P. T. W. Cohen, *EMBO J.*, 1993, **12**, 987-996.

42. P. T. W. Cohen, *Biochem. Soc. Trans.*, 1993, **21**, 884-888.

43. M. X. Chen, A. E. McPartlin, L. Brown, Y. H. Chen, H. M. Barker and P. T. W. Cohen, *EMBO J.*, 1994, **13**, 4278-4290.

44. M. S. Boguski, R. S. Sikorski, P. Hieter and M. Goebl, *Nature*, 1990, **346**, 114.

45. P. T. W.Cohen, J. F. Collins, A. F. W. Coulson, N. Berndt and O. B. da Cruz e Silva, *Gene*, 1988, **69**, 131-134.

46. P. T. W. Cohen and P. Cohen, *Biochem. J.*, 1989, **260**, 931-934.

47. E. F. Da Cruz e Silva, V. Hughes, P. McDonald, M. J. R. Stark and P. T. W. Cohen, *Biochem. Biophys. Acta*, 1991, **1089**, 269-272.

48. F. Posas, A. Casamayor, N. Morral and J. Arino, *J. Biol. Chem.*, 1992, **267**, 11734-11740.

49. F. Posas, A. Casamayor and J. Arino, *FEBS Lett.*, 1993, **318**, 282-286.

50. M. X. Chen, Y. H. Chen and P. T. W. Cohen, *Eur. J. Biochem.*, 1993, **218**, 689-699.

51. F. Posas, J. Clotet, M. T. Muns, J. Corominas, A. Casamayor and J. Arino, *J. Biol. Chem.*, 1993, **268**, 1349-1354.

52. J. B. Vincent and B. A. Averill, *FEBS Lett.*, 1990, **263**, 265-268.

53. D. Barford and J. C. Keller, *J. Mol. Biol.*, 1994, **235**, 763-766.

54. J. Goldberg, H. Huang, Y. Kwon, P. Greengard, A. C. Nairn and J. Kuriyan, *Nature*, 1995, **376**, 745-753.

55. M. P. Egloff, P. T. W. Cohen, P. Reinemer and D. Barford, *J. Mol. Biol.*, 1995, **254**, 942-959.

56. W. P. Taylor and T. S. Widlanski, *Chem. & Biol.*, 1995, **2**, 713-718.

57. C. R. Kissinger, H. E. Parge, D. R. Knighton, C. T. Lewis, L. A. Pelletier, A. Tempczyk, V. J. Kallsh, K. D. Tucker, R. E. Showalter, E. W. Moomaw, L. N. Gastinel, N. Habuka, X. Chen, F. Maldonado, J. E. Barker, R. Bacquet and J. E. Villafranca, *Nature*, 1995, **378**, 641-644.

58. N. Strater, T. Klabunde, P. Tucker, H. Witzel abd B. Krebs, *Science*, 1995, **268**, 1489-1492.

59. R. L. Stone and J. E. Dixon, *J. Biol. Chem.*, 1994, **269**, 31323-31326.

60. K. Guan, S. S. Broyles and J. E. Dixon, *Nature*, 1991, **350**, 359-362.

61. P. J. Rohan, P. Davis, C. A. Moskaluk, M. Kearns, H. Krutzsch, U. Siebenlist and K. Kelly, *Science*, 1993, **259**, 1763-1766.

62. K. Galaktionov and D. Beach, *Cell*, 1991, **67**, 1181-1194.

63. G. Francis, *Nature*, 1878, **18**, 11-12.

64. H. Konst, P.D. McKercher, P. R. Gorham, A. Robertson and J. Howell, *Can. J. Comp. Med. Vet. Sci.*, 1965, **29**, 221-228.

65. L. A. Lawton and G. A. Codd, *J. IWEM*, 1991, **5**, 460-465.

66. W. W. Carmichael, in *Natural Toxins: characterisation, Pharmacology and therapeutics*, ed. C. L. Ownby and G. V. Odell, Pergamon Press, Oxford, 1988, pp. 3-16.

67. W. W. Carmichael, *J. Appl. Bacteriol.*, 1992, **72**, 445-459.

68. K. Tacibana, P. J. Scheuer, H. Kikuchi, Y. Tsukitani, D. Van Engen, J. Clardy, Y. Gopichand and F. J. Schmitz, *J. Am. Chem. Soc.*, 1981, **103**, 2469-2471.

69. M. Murata, M. Shimatani, H. Sugitani, Y. Oshima and T. Yasumoto, *Bull. Jpn. Soc. Sci. Fish*, 1982, **48**, 549-552.

70. F. J. Schmits, R. S. Prasad, Y. Gopichand, M. B. Hossain, D. van der Helm and P. Schmidt, *J. Am. Chem. Soc.*, 1981, **103**, 2467-2469.

71. M. Isobe, Y. Ichikawa, D. Bai, H. Masaki and T. Goto, *Tetrahedron*, 1987, **43**, 4767-4776.

72. H. Fujiki and T. Sugimura, *Adv. Cancer Res.*, 1987, **49**, 223-264.

73. H. Fujiki, M. Suganuma, S. Nishiwaki, S. Yoshizawa, J. Yatsunami, R. Matsushima, H. Furuy, S. Okabe, S. Matsunaga and T. Sugimura, in *Relevance of animal studies to the evaluation of human cancer risk*, ed. R. D'Amato, T. J. Slaga, W. Farland and C. Henry, Wiley-Liss Inc., 1992, pp 337-350.

74. M. Suganuma, H. Fujiki, H. Suguri, S. Yoshizawa, M. Hirota, M. Nakayasu, M. Ojika, K. Wakamatsu, K. Yamada and T. Sugimura, *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 1768-1771.

75. M. Suganuma, M. Tatematsu, J. Yatsunami, S. Yoshizawa, S. Okabe, D. Uemura and H. Fujiki, *Carcinogenesis*, 1992, **13**, 1841-1845.

76. S. Nishiwaki, H. Fujiki, M. Suganuma, S. H. Furuya, R. Matsushima, Y. Iida, M. Ojika, K. Yamada, D. Uemura, T. Yasumoto, F. J. Schmitz and T. Sugimura, *Carcinogenesis*, 1990, **11**, 1837-1841.

77. H. Fujiki, M. Suganuma, H. Suguri, S. Yoshizawa, K. Takagi, T. Sassa, N. Uda, K. Wakamatsu, K. Yamada, T. Yasumoto, Y. Kato, N. Fusetani, K. Hashimoto and T. Sugimura, in *Mycotoxins and phycotoxins '88*, ed. S. Natori, K. Hashimoto and Y. Ueno, Elsevier Science Publishers B.V., Amsterdam, 1989, pp 453-460.

78. M. Suganuma, M. Suttajit, H. Suguri, M. Ojika, K. Yamada and H. Fujiki, *FEBS Lett.*, 1989, **250**, 615-618.

79. J. Hescheler, G. Mieskes, J. C. Rüegg, A. Takai and W. Trautwein, *Pflügers Arch*, 1988, **412**, 248-252.

80. R. E. Honkanen, H. Zwiller, R. E. Moore, B. S. Khatra, M. Dukelow and A. L. Boynton, *Mol. Pharmacol.*, 1991, **40**, 577-583.

81. R. E. Honkanen, J. Zwiller, R. E. Moore, S. L. Daily, B. S. Khatra, M. Dukelow and A. L. Boynton, *J. Biol. Chem.*, 1990, **265**, 19401-19404.

82. C. MacKintosh, K. A. Beattie, S. Klumpp, P. Cohen and G. A. Codd, *FEBS Lett.*, 1990, **264**, 187-192.

83. C. MacKintosh and S. Klumpp, *FEBS Lett.*, 1990, **277**, 137-140.

84. M. Suganuma, H. Fujiki, H. Furuya-Suguri, S. Yoshizawa, S. Yasumoto, Y. Kato, N. Fusetani and T. Sugimura, *Cancer Res.*, 1990, **50**, 3521-3525.

85. S. Yoshizawa, R. Matsushima, M. F. Watanabe, K. I. Harada, A. Ichihara, W. W. Carmichael and H. Fujiki, *J. Cancer Res. Clin. Oncol.*, 1990, **116**, 609-614.

86. J. Magae, H. Osada, H. Fujiki, T. C. Saido, K. Suzuki, K. Nagai, M. Yamasaki and K. Isono, *Proc. Jpn. Acad. B*, 1990, **66**, 209-212.

87. H. Fujiki, M. Suganuma, S. Nishiwaki, S. Yoshizawa, B. Winyar, F. J. Schmitz and T. Sugimura, in *Advances in second messenger and phosphoprotein research*, ed. Y. Nishizuka, M. Endo and C. Tanaka, Raven Press, New York, 1990, pp 340-344.

88. M. Suganuma, H. Fujiki, S. Okabe, S. Nishiwaki, D. Brautigan, T. S. Ingebritsen and M. R. Rosner, *Toxicon*, 1992, **30**, 873-878.

89. C. Bialojan and A. Takai, *Biochem. J.*, 1988, **256**, 283-290.

90. R. E. Honkanen, J. Zwiller, S. L. Daily, B. S. Khatra, M. Dukelow and A. L. Boynton, *J. Biol. Chem.*, 1991, **266**, 6614-6619.

91. Y. Kato, N. Fusetani, S. Matsunaga and K. Hashimoto, *J. Am. Chem. Soc.*, 1986, **108**, 2780-2781.

92. Y. Kato, N. Fusetani, S. Matsunaga, K. Hashimoto and K. Koseki, *J. Org. Chem.*, 1988, **53**, 3930-3932.

93. S. Matsunaga, H. Fujiki, D. Sakata and N. Fusetani, *Tetrahedron*, 1991, **47**, 2999-3006.

94. S. Matsunaga and N. Fusetani, *Tetrahedron Lett.*, 1991, **32**, 5605-5606.

95. B. M. Trost and J. A. Flygare, *Tetrahedron Lett.*, 1994, **35**, 4059-4062.

96. N. Tanimoto, S. W. Gerritz, A. Sawabe, T. Noda, S. A. Filla and S. Masamune, *Angew. Chem. Int. Ed. Engl.*, 1994, **33**, 673-675.

97. D. A. Evans, J. R. Gage and J. L. Leighton, *J. Am. Chem. Soc.*, 1992, **114**, 9434-9453.

98. H. Ishihara, B. L. Martin, D. L. Brautigan, H. Karaki, H. Ozaki, Y. Kato, N. Fusetani, S. Watabe, K. Hashimoto, D. Uemura and D. J. Hartshorne, *Biochem. Biophys Res. Commun.*, 1989, **159**, 871-877.

99. X.-C. Cheng, T. Kihara, H. Kusakabe, J. Magae, Y. Kobayashi, R. P. Fang, Z. F. Ni, Y. C. Shen, K. Ko, I. Yamaguchi and K. Isono, *J. Antibiot.*, 1987, **40**, 907-909.

100. M. Ubukata, X.-C. Cheng and K. Isono, *J. Chem. Soc., Chem. Commun.*, 1990, 244-246.

101. J. Magae, C. Watanabe, H. Osada, X.-C. Cheng and K. Isono, *J. Antibiot.*, 1988, **41**, 932-937.

102. M. Oikawa, H. Oikawa and A. Ichihara, *Tetrahedron Lett.*, 1993, **34**, 4797-4800.

103. Y. Ichikawa, K. Tsuboi, A. Naganawa and M. Isobe, *Synlett*, 1993, 907-908.

104. S. Nakamura and M. Shibasaki, *Tetrahedron Lett.*, 1994, **35**, 4145-4148.

105. M. Oikawa, H. Oikawa, T. Ueno and A. Ichihara, *J. Org. Chem.*, 1995, **60**, 5048-5068.

106. M. Ubukata, X.-C. Cheng, M. Isobe and K. Isono, *J. Chem. Soc., Perkin Trans. I*, 1993, 617-624.

107. M. Hori, J. Magae, Y.-G. Han, D. J. Hartshorne and H. Karaki, *FEBS Lett.*, 1991, **285**, 145-148.

108. Y. Sugiyama, I. I. Ohtani, M. Isobe, A. Takai, M. Ubukata and K. Isono, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 3-8.

109. K. I. Harada, K. Ogawa, Y. Kimura, H. Murata, M. Suzuki, P. M. Thorn, W. R. Evans and W. W. Carmichael, *Chem. Res. Toxicol.*, 1991, **4**, 534-540.

110. K. I. Harada, K. Ogawa, K. Matsuura, H. Nagai, H. Murata, M. Suzuki, Y. Itezono, N. Nakayama, M. Shirai and M. Nakano, *Toxicon*, 1991, **29**, 479-489.

111. J. Kiviranta, M. Namikoshi, K. Sivonen, W. R. Evans, W. W. Carmichael and K. L. Rinehart, *Toxicon*, 1992, **30**, 1093-1098.

112. T. Krishnamurthy, L. Szafraniec, D. F, Hunt, J. Shabanowitz, J. R. III Yates, C. R. Hauer, W. W. Carmichael, O. Skulberg, G. A. Codd and S. Missler, *Proc. nat. Acad. Sci. U.S.A.*, 1989, **86**, 770-774.

113. J. A. O. Meriluoto, A. Sandstrom, J. E. Eriksson, G. Remaud, A. Grey Craig and J. Chattopadhyaya, *Toxicon*, 1989, **27**, 1021-1034.

114. M. Namikoshi, K. L. Rinehart, R. Sakai, K. Sivonen and W. W. Carmichael, *J. Org. Chem.*, 1990, **55**, 6135-6139.

115. M. Namikoshi, K. L. Rinehart, R. Sakai, R. R. Stotts, V. R. Beasley, A. M. Dahlem, W. W. Carmichael and W. R. Evans, *J. Org. Chem.*, 1992, **57**, 866-872.

116. M. Namikoshi, K. Sivonen, W. R. Evans, W. W. Carmichael, L. Rouhiainen, R. Luukkainen and K. L. Rinehart, *Chem. Res. Toxicol.*, 1992, **5**, 661-666.

117. K. Sivonen, S. I. Niemela, R. M. Niemi, L. Lepisto, T. Luoma and L. A. Rasanen, *Hydrobiologia*, 1990, **190**, 267-275.

118. K. Sivonen, W. W. Carmichael, K. L. Rinehart, M. Namikoshi, A. M. Dahlem and S. I. Niemela, *Appl. Environ. Microbiol.*, 1990, **56**, 2650-2657.

119. K. Sivonen, M. Namikoshi, W. R. Evans, M. Fardig, W. W. Carmichael and K. L. Rinehart, *Chem. Res. Toxicol.*, 1992, **5**, 464-469.

120. K. Sivonen, M. Namikoshi, W. R. Evans, W. W. Carmichael, L. Rouhiainen, R. Luukkainen and K. L. Rinehart, *Appl. Environ. Microbiol.*, 1992, **58**, 2495-2500.

121. M. Namikoshi, K. Sivonen, W. R. Evans, W. W. Carmichael, F. Sun, L. Rouhiainen, R. Luukkainen and K. L. Rinehart, *Toxicon*, 1992, **30**, 1457-1464.

122. M. Namikoshi, K. Sivonen, W. R. Evans, W. W. Carmichael, F. Sun and K. L. Rinehart, *Toxicon*, 1992, **30**, 1473-1479.

123. K. Sivonen, M. Namikoshi, W. R. Evans, W. W. Carmichael, B. V. Gromov and K. L. Rinehart, *Toxicon*, 1992, **30**, 1481-1485.

124. K. Sivonen, O. M. Skulberg, M. Namikoshi, W. R. Evans, W. W. Carmichael and K. L. Rinehart, *Toxicon*, 1992, **30**, 1465-1471.

125. R. Luukkainen, M. Namikoshi, K. Sivonen, M. Fardig, K. L. Rinehart and S. I. Niemela, *Appl. Environ. Microbiol.*, 1993, **59**, 2204-2209.

126. M. Craig, T. L. McCready, H. A. Luu, M. A. Smillie, P. Dubord and C. F. B. Holmes, *Toxicon*, 1993, **31**, 1541-1549.

127. M. Namikoshi, F. Sun, B. W. Choi, W. R. Evans, W. W. Carmichael, K. L. Rinehart and V. R. Beasley, *J. Org. Chem.*, 1995, **60**, 3671-3679.

128. K. L. Rinehart, K. I. Harada, M. Namikoshi, C. Chen, C. A. Harvis, M. H. G. Munro, J. W. Blunt, P. E. Mulligan, V. R. Beasley, A. M. Dahlem and W. W. Carmichael, *J. Am. Chem. Soc.*, 1988, **110**, 8557-8558.

129. K. Sivonen, K. Kononen, W. W. Carmichael, A. M. Dahlem, K. L. Rinehart, J. Kiviranta and S. I. Niemea, *Appl. Environ. Microbiol.*, 1989, **55**, 1990-1995.

130. W. W. Carmichael, V. R. Beasley, D. L. Bunner, J. N. Eloff, I. Falconer, P. Gorham, K. I. Harada, T. Krishnamurthy, M-J. Yu, R. E. Moore, K. Rinehart, M. Runnegar, O. M. Skulberg and M. F. Watanabe, *Toxicon*, 1988, **26**, 971-973.

131. E. D. de Silva, D. E. Williams and R. J. Andersen, *Tetrahedron Lett.*, 1992, **33**, 1561-1564.

132. R. J. Valentekovich and S. L. Schreiber, *J. Am. Chem. Soc.*, 1995, **117**, 9069-9070.

133. M. Namikoshi, B. W. Choi, R. Sakai, F. Sun, K. L. Rinehart, W. W. Carmichael, W. R. Evans, P. Cruz, M. H. G. Munro and J. W. Blunt, *J. Org. Chem.*, 1994, **59**, 2349-2357.

134. D. P. Botes, A. A. Tuinman, P. L. Wessels, C. C. Viljoen, H. Kruger, D. H. Williams, S. Santikarn, R. J. Smith and S. J. Hammond, *J. Chem. Soc., Perkin Trans. I*, 1984, 2311-2318.

135. K. I. Harada, K. Matsuura, M. Suzuki, M. F. Watanabe, S. Oishi, A. M. Dahlem, V. R. Beasley and W. W. Carmichael, *Toxicon*, 1990, **28**, 55-64.

136. K. I. Harada, K. Matsuura, K. Ogawa, H. Murata, M. Suzuki, M. F. Watanabe, Y. Itezono and N. Nakayama, *Chem. Res. Toxicol.*, 1990, **3**, 473-481.

137. A. M. Dahlem, V. R. Beasley, K. I. Harada, K. Matsuura, M. Suzuki, C. A. Harvis, K. L. Rinehart and W. W. Carmichael, *Toxicologist*, 1989, **9**, 168.

138. A. M. Dahlem, V. R. Beasley, S. B. Hooser, K. Harada, K. Matsuura, M. Suzuki, C. A. Harvis, K. L. Rinehart and W. W. Carmichael, *Chem. Res. Toxicol.*, 1989.

139. T. K. Chakraborty and S. P. Joshi, *Tetrahedron Lett.*, 1990, **31**, 2043-2046.

140. T. Tsukuda, H. Kakisawa, P. Painuly and Y. Shimizu, *Tetrahedron Lett.*, 1989, **30**, 245-4248.

141. M. Namikoshi, K. L. Rinehart, A. M. Dahlem, V. R. Beasley and W. W. Carmichael, *Tetrahedron Lett.*, 1989, **30**, 4349-4352.

142. R. E. Moore, J. L. Chen, B. S. Moore and G. M. L. Patterson, *J. Am. Chem. Soc.*, 1991, **113**, 5083-5084.

143. M. F. Beatty, C. Jennings-White and M. A. Avery, *J. Chem. Soc., Perkin Trans. I*, 1992, 1637-1641.

144. H. Y. Kong and P. L. Toogood, *Tetrahedron Lett.*, 1996, **37**, 2349-2352.

145. V. R. Beasley, W. O. Cook, A. M. Dahlem, S. B. Hooser, R. A. Lovell and W. M. Valentine, *Vet. Clin. North AM [Food Anim. Pract.]*, 1989, **5**, 345-361.

146. S. B. Hooser, V. R. Beasley, R. A. Lovell, W. W. Carmichael and W. M. Haschek, *Vet. Pathol.*, 1989, **26**, 246-252.

147. R. Matsushima, S. Yoshizawa, M. F. Watanabe, K. I. Harada, M. Furusawa, W. W. Carmichael and H. Fujiki, *Biochem. Biophys. Res. Commun.*, 1990, **171**, 867-874.

148. R. Nishiwaki-Matsushima, T. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W. W. Carmichael and H. Fujiki, *J. Cancer Res. Clin. Oncol.*, 1992, **118**, 420-424.

149. B. W. Choi, M. Namikoshi, F. Sun, K. L. Rinehart, W. W. Carmichael, A. M. Kaup, W. R. Evans and V. R. Beasley, *Tetrahedron Lett.*, 1993, **34**, 7881-7884.

150. R. E. Moore, J. L. Chen, B. S. Moore and G. M. L. Patterson, *J. Am. Chem. Soc.*, 1991, **113**, 5083-5084.

151. G. Hartrampf and W. Buckel, *FEBS Lett.*, 1984, **171**, 73-78.

152. K. L. Rinehart, M. Namikoshi and B. W. Choi, *J. Appl. Phycology*, 1994, **6**, 159-176.

153. H. Shima, H. Tohda, S. Aonuma, M. Nakayasu, A. De Paoli-Roach, T. Sugimura and M. Nagao, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 9267-9271.

154. R. W. MacKintosh, K. N. Dalby, D. G. Campbell, P. T. W. Cohen, P. Cohen and C. MacKintosh, *FEBS Lett.*, 1995, **371**, 236-240.

155. M. Runnegar, N. Berndt, S. M. Kong, E. Y. C. Lee and L. Zhang, *Biochim. Biophys. Res. Commun.*, 1995, **216**, 162-169.

156. R. Nishiwaki-Matsushima, S. Nishiwaki, T. Ohta, S. Yoshizawa, M. Suganuma, K. I. Harada, M. F Watanabe and H. Fujiki, *Jpn. J. Cancer Res.*, 1991, **82**, 993-996.

157. R. J. Quinn, C. Taylor, M. Suganuma and H. Fujiki, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 1029-1034.

158. T. Lanaras, C. M. Cook, J. E. Eriksson, J. A. O. Meriluoto and M. Hotokka, *Toxicon*, 1991, **29**, 901-906.

159. R. J. Quinn, C. Taylor, R. McCulloch, R. Nishiwaki-Matsushima and H. Fujiki, *Bioorg. Med. Chem. Lett.*, 1992, **2**, 299-302.

160. R. Nishiwaki-Matsushima, H. Fujiki, K. I. Harada, R. J. Quinn and C. Taylor, *Bioorg. Med. Chem. Lett.*, 1992, **2**, 673-676.

161. S. Rudolph-Bohner, D. F. Mierke and L. Moroder, *FEBS Lett.*, 1994, **349**, 319-323.

162. D. F. Mierke, S. Rudolph-Bohner, G. Müller and L. Moroder, *Biopolymers*, 1995, **36**, 811-828.

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

164. M. P. Boland, M. F. J. R. Taylor and C. F. B. Holmes, *FEBS Lett.*, 1993, **334**, 13-17.

165. R. Nishwaki-Matsushima, T. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W. W. Carmichael and H. Fujiki, *J. Cancer Res. Clin. Oncol.*, 1992, **118**, 420-424.

166. H. Fujiki, *Mol. Carcinogen.*, 1992, **5**, 91-94.

167. S. Nishiwaki, H. Fujiki, M. Suganuma, M. Ojika, K. Yamada and T. Sugimura, *Biochem. Biophys. Res. Commun.*, 1990, **170**, 1359-1364.

168. R. Nishiwaki, T. Ohta, E. Sueoka, M. Suganuma, K. I. Harada, M. F. Watanabe and H. Fujiki, *Cancer Lett.*, 1994, **83**, 283-289.

169. J. E. Eriksson, D. Toivola, J. A. O. Meriluoto, H. Karaki, Y. G. Yan and D. Hartshorne, *Biochem. Biophys Res. Commun.*, 1990, **173**, 1347-1353.

170. I. R. Falconer and D. S. K. Yeung, *Chem. Biol. Interactions*, 1992, **81**, 181-196.

171. T. Ohta, R. Nishiwaki, J. Yatsunami, A. Komori, M. Suganuma and H. Fujiki, *Carcinogenesis*, 1992, **13**, 2443-2447.

172. S. Z. Yu, in *Primary liver cancer*, ed. Z. Y. Tang, M.C. Wu and S. S. Xia, China Academic Publishers, Springer, Berlin Heidelberg New York, 1989, pp 30-37.

173. J. M. Gregson, J. L. Chen, G. M. L. Patterson and R. E. Moore, *Tetrahedron*, 1992, **48**, 3727-3734.

174. M. Murakami, H. Matsuda, K. Makabe and K. Yamaguchi, *Tetrahedron Lett.*, 1991, **32**, 2391-2394.

175. M. Satake, M. Murata, T. Yasumoto, T. Fujita and H. Naoki, *J. Am. Chem. Soc.*, 1991, **113**, 9859-9861.

176. M. Murakami, K. Makabe, K. Yamaguchi, S. Konosu and M. R. Walchli, *Tetrahedron Lett.*, 1988, **29**, 1149-1152.

177. R. E. Moore, S. Banarjee, V. Bornemann, F. R. Caplan, J. L. Chen, D. G. Corley, L. K. Larsen, B. S. Moore, G. M. L. Patterson, V. J. Paul, J. B. Stewart and D. E. Williams, *Pure & Appl. Chem.*, 1989, **61**, 521-524.

178. R. E. Moore, V. Bornemann, W. P. Niemczura, J. M. Gregson, J.-L. Chen, T. R. Norton, G. M. L. Patterson and G. L. Helms, *J. Am. Chem. Soc.*, 1989, **111**, 6128-6132.

179. M. Murakami, H. Matsuda, K. Makabe and K. Yamaguchi, *Tetrahedron Lett.*, 1991, **32**, 2391-2394.

180. T. Okino, M. Murakami, R. Haraguchi, H. Munekata, H. Matsuda and K. Yamaguchi, *Tetrahedron Lett.*, 1993, **34**, 8131-8134.

181. S. Tsukamoto, P. Painuly, K. A. Young, X. Yang and Y. Shimizu, *J. Am. Chem. Soc.*, 1993, **115**, 11046-11047.

182. M. O. Ishitsuka, T. Kusumi, H. Kakisawa, K. Kaya and M. M. Watanabe, *J. Am. Chem. Soc.*, 1990, **112**, 8180-8182.

183. T. Okino, H. Matsuda, M. Murakami and K. Yamaguchi, *Tetrahedron Lett.*, 1993, **34**, 501-504.

184. M. E. Bunnage, A. J. Burke, S. G. Davies and C. J. Goodwin, *Tetrahedron: Asymmetry*, 1994, **5**, 203-206.

185. F. Matsuura, Y. Hamada and T. Shioiri, *Tetrahedron*, 1994, **50**, 11303-11314.

186. M. Murakami, Y. Okita, H. Matsuda, T. Okino and K. Yamaguchi, *Tetrahedron Lett.*, 1994, **35**, 3129-3132.

187. W. G. Dauben, C. R. Kessel and K. H. Takemura, *J. Am. Chem. Soc.*, 1980, **102**, 6893-6894.

188. W. G. Dauben, J. M. Gerdes and D. B. Smith, *J. Org. Chem.*, 1985, **50**, 2576-2578.

189. Y.-M. Li and J. E. Casida, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 11867-11870.

190. R. E. Honkanen, *FEBS Lett.*, 1993, **330**, 283-286.

191. Y.-M. Li, C. MacKintosh and J. E. Casida, *Biochem. Pharm.*, 1993, **46**, 1435-1443.

192. P. R. Hawkins, M. T. C. Runnegar, A. R. B. Jackson and I. R. Falconer, *Appl. Environ. Microbiol.*, 1985, **50**, 1292-1295.

193. I. Ohtani, R. E. Moore and M. T. C. Runnegar, *J. Am. Chem. Soc.*, 1992, **114**, 7941-7942.

194. R. E. Moore, I. Ohtani, B. S. Moore, C. B. de Koning, W. Y. Yoshida, M. T. C. Runnegar and W. W. Carmichael, *Gazzetta Chimica Italiana*, 1993, **123**, 329-336.

195. L. Murray, A. T. R. Sim, J. A. P. Rostas and R. J. Capon, *Aust. J. Chem.*, 1993, **46**, 1291-1294.

196. S. Miao and R. J. Andersen, *J. Org. Chem.*, 1991, **56**., 6275-6280.

197. R. J. Andersen, D. L. Burgoyne, D. E. Williams, F. Kong, E. D. de Silva, S. Miao, T. M. Allen, C. F. B. Holmes, D. Chen and M. Kent, *Gazzetta Chimica Italiana*, 1993, **123**, 293-299.

198. J. Kunz and M. N. Hall, *Trends Biochem. Sci.*, 1993, 334-338.

199. N. H. Sigal and F. J. Dumont, *Annu. Rev. Immunol.*, 1992, **10**, 519-560.

200. S. L. Schreiber, *Cell*, 1992, **70**, 365-368.

201. J. Liu, J. D. Farmer, W. S. Lane, J. Friedman, I. Weissman and S. L. Schreiber, *Cell*, 1991, **66**, 807-815.

202. M. W. Brooks and J. M. Clark, *Pestic. Biochem. Physiol.*, 1987, **28**, 127-139.

203. E. Enan and F. Matsumura, *Biochemical Pharmacology*, 1992, **43**, 1777-1784.

204. H. Fujiki and M. Suganuma, *J. Biochem.*, 1994, **115**, 1-5.

205. P. Agostinis, J. Goris, E. Waelkens, L. A. Pinna, F. Marchiori and W. Merlevede, *J. Biol. Chem.*, 1987, **262**, 1060-1064.

206. L. A. Pinna, P. Agostinis, A. Donella-deana and F. Marchiori, *Adv. Prot. Phosphatases*, 1989, **5**, 51-74.

207. A. Donella-Deana, C. H. MacGowan, P. Cohen, F. Marchiori, H. E. Meyer and L. A. Pinna, *Biochim. Biophys. Acta*, 1990, **1051**, 199-202.

208. P. Agostinis, J. Goris, L. A. Pinna, F. Marchiori, J. W. Perich, H. E. Meyer and W. Merlevede, *Eur. J. Biochem.*, 1990, **189**, 235-241.

209. S. J. McNall and E. H. Fischer, *J. Biol. Chem.*, 1988, **263**, 1893-1897.

210. A. Donella-Deana, M. H. Krinks, M. Ruzzene, C. Klee and L. A. Pinna, *Eur. J. Biochem.*, 1994, **219**, 109-117.

211. L. A. Pinna and A. Donella-Deana, *Biochim. Biophys. Acta*, 1994, **1222**, 415-431.

212. M. Bodanszky and A. Bodanszky, in *The Practice of Peptide Synthesis*, eds. K. Hafner, C. W. Rees, B. M. Trost, J. M. Lehn, P. von Rague Schleyer and R. Zahradnik, Springer-Verlag, Berlin, 1984, 101-104.

213. G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Am. Chem. Soc.*, 1967, **89**, 5012-5017.

214. F. M. F. Chen, Y. Lee, R. Steinauer and N. L. Benoiton, *Can. J. Chem.*, 1987, **65**, 613-618.

215. G. R. Pettit, S. K. Gupta and R. H. Ode, *J. Chem. Soc., Perkin Trans. I*, 1973, **9**, 950-954.

216. K. Suzuki, Y. Sasaki, N. Endo and Y. Mihara, *Chem. Pharm. Bull.*, 1981, **29**, 233-237.

217. M. Bodanszky, in *Principles of Peptide Synthesis*, Springer-Verlag, Berlin, 1984, p. 137-139.

218. L. Zervas, T. T. Otani, M. Winitz and J. P. Greenstein, *J. Am. Chem. Soc.*, 1959, **81**, 2878-2884.

219. D. T. Gish and F. H. Carpenter, *J. Am. Chem. Soc.*, 1953, **75**, 5872-5877.

220. L. Zervas, M. Winitz and J. P. Greenstein, *J. Org. Chem.*, 1957, **22**, 1515-1521.

221. J. Ramachandran and C. H. Li, *J. Org. Chem.*, 1962, **27**, 4006-4009.

222. R. E. Plapinger, M. M. Nachlas, M. L. Seligman and A. M. Seligman, *J. Org. Chem.*, 1965, **30**, 1781-1785.

223. I. Photaki and A. Yiotakis, *J. Chem. Soc., Perkin Trans. I*, 1976, 259-264.

224. J. Coste, D. Le-Nguyen and B. Castro, *Tetrahedron Lett.*, 1990, **31**, 205-208.

225. G. B. Fields and R. L. Noble, *Int. J. Peptide Protein Res.*, 1990, **35**, 161-214.

226. R. B. Merrifield, *J. Am. Chem. Soc.*, 1963, **85**, 2149-2154.

227. E. Atherton, H. Fox, D. Harkiss, C. J. Logan, R. C. Sheppard and B. J. Williams, *J. Chem. Soc., Chem. Commun.*, 1978, 537-539.

228. E. Atherton, H. Fox, D. Harkiss and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1978, 539-540.

229. D. S. King, C. G. Fields and G. B. Fields, *Int. J. Peptide Protein Res.*, 1990, **36**, 255-266.

230. R. Ramage and J. Green, *Tetrahedron Lett.*, 1987, **28**, 2287-2290.

231. J. Green, O. M. Ogunjobi, R. Ramage and A. S. J. Stewart, *Tetrahedron Lett.*, 1988, **29**, 4341-4344.

232. R. Ramage, J. Green and A. J. Blake, *Tetrahedron*, 1991, **47**, 6353-6370.

233. J. W. Perich and R. B. Johns, *Tetrahedron Lett.*, 1988, **29**, 2369-2372.

234. J. W. Perich and R. B. Johns, *J. Org. Chem.*, 1989, **54**, 1750-1752.

235. P. F. Alewood, J. W. Perich and R. B. Johns, *Tetrahedron Lett.*, 1984, **25**, 987-990.

236. J. W. Perich and R. B. Johns, *Aust. J. Chem.*, 1990, **43**, 1623-1632.

237. J. W. Perich and R. B. Johns, *Aust. J. Chem.*, 1990, **43**, 1633-1642.

238. L. Otvos, I. Elekens and V. M. Y. Lee, *Int. J. Peptide Protein Res.*, 1989, **34**, 129-133.

239. K. L. Yu and B. Fraser-Reid, *Tetrahedron Lett.*, 1988, **29**, 979-982.

240. W. Bannwarth and E. Küng, *Tetrahedron Lett.*, 1989, **30**, 4219-4222.

241. K. C. Ross, D. L. Rathbone, W. Thomson and S. Freeman, *J. Chem. Soc., Perkin Trans. I*, 1995, 421-426.

242. G. D. Prestwich, J. F. Marecek, R. J. Mourey, A. B. Theibert, C. D. Ferris, S. K. Danoff and S. H. Synder, *J. Am. Chem. Soc.*, 1991, **113**, 1822-1825.

243. P. Westerduin, G. H. Veeneman, J. E. Marugg, G. A. van der Marel and J. H. van Boom, *Tetrahedron Lett.*, 1986, **27**, 1211-1214.

244. M. W. Schwarz and W. Pfleiderer, *Tetrahedron Lett.*, 1984, **25**, 5513-5516.

245. T. Dorper and E. L. Winnacker, *Nucleic Acids Res.*, 1983, **11**, 2575-2584.

246. C. M. dreef-Tromp, A. W. M. Lefeber, G. A. van der Marel and J. H. van Boom, *Synthesis*, 1992, 1269-1272.

247. N. Usman, K. K. Ogilvie, M. Y. Jiang and R. J. Cedergren, *J. Am. Chem. Soc.*, 1987, **109**, 7845-7854.

248. S. A. Scaringe, C. Francklyn and N. Usman, *Nucleic Acids Res.*, 1990, **18**, 5433-5441.

249. G. R. Baker, "Mechanistic studies on *myo*-inositol monophosphatase from bovine brain", University of Southampton, PhD. thesis, 1991.

250. M. Mutter, V. N. R. Pillai, H. Azinger, E. Bayer and C. Toniolo, in *Peptides 1980*, ed. K. Brunfeldt, Scriptor, Copenhagen, 1981, 660-665.

251. F. Maser, B. Klein and M. Mutter, *Biopolymers*, 1983, **22**, 233-240.

252. M. H. Baron, C. DeLoze, C. Toniolo and G. D. Fasman, *Biopolymers*, 1978, **17**, 2225-2239.

253. S. B. H. Kent, in *Peptides: Structure and Function*, ed. V. J. Hruby, C. M. Deber and K. D. Kopple, Pierce Chemical Company, Rockford, IL, 1985, 407-414.

254. S. Abdel Rahman, H. Azinger and M. Mutter, *Biopolymers*, 1980, **19**, 173-187.

255. R. Anschütz and W. O. Emery, *Liebigs Ann. Chem.*, 1889, **253**, 105-121.

256. M. Rapp, *Liebigs Ann. Chem.*, 1884, **224**, 156-159.

257. G. Jacobsen, *Ber. Dtsch. Chem. Ges.*, 1875, **8**, 1519-1523.

258. P. Brigl and H. Müller, *Ber. Dtsch. Chem. Ges.*, 1939, **72**, 2121-2130.

259. *Reagents for organic synthesis*, ed. L. F. Fieser and M. Fieser, John Wiley & Sons Inc., New York London Sydney, 1967, p.345.

260. F. R. Atherton, H. T. Openshaw and A. R. Todd, *J. Am. Chem. Soc.*, 1945, 382-385.

261. W. Autenrieth and H. Hefner, Ber. Dtsch. Chem. Ges., 1925, **58**, 2151-2156.

262. F. Meggio, G. Chessa, G. Borin, L. A. Pinna and F. Marchiori, *Biochim. Biophys. Acta*, 1981, **662**, 94-101.

263. P. Agostinis, J. Goris, L. A. Pinna and W. Merlevede, *Biochem. J.*, 1987, **248**, 785-789.

264. T. Vorherr and W. Bannwarth, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 2661-2664.

265. W. Bannwarth and A. Trzeciak, *Helv. Chim. Acta*, 1987, **70**, 175-186.

266. E. A. Kitas, R. Knorr, A.Trzeciak and W. Bannwarth, *Helv. Chim. Acta*, 1991, **74**, 1314-1328.

267. D. M. Andrews, J. Kitchin and P. W. Seale, *Int. J. Peptide Protein Res.*, 1991, **38**, 469-475.

268. J. W. Perich, M. Ruzzene, L. A. Pinna and E. C. Reynolds, *Int. J. Peptide Protein Res.*, 1994, **43**, 39-46.

269. J. M. Lacombe, F. Adriamanampisoa and A. A. Pavia, *Int. J. Peptide Protein Res.*, 1990, **36**, 275-280.

270. Y. Ueno, F. Suda, Y. Taya, R. Noyori, Y. Hayakawa and T. Hata, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 823-826.

271. J. W. Perich, F. Meggio and L. A. Pinna, *Bioorg. Med. Chem.*, 1996, **4**, 143-150.

272. D. B. A. de Bont, W. J. Moree, J. H. van Boom and R. M. J. Liskamp, *J. Org. Chem.*, 1993, **58**, 1309-1317.

273. E. A. Ottinger, L. L. Shekels, D. A. Bernlohr and G. Barany, *Biochemistry*, 1993, **32**, 4354-4361.

274. R. M. Valerio, P. F. Alewood, R. B. Johns and B. E. Kemp, *Int. J. Peptide Protein Res.*, 1989, **33**, 428-438.

275. J. W. Perich, R. M. Valerio and R. B. Johns, *Tetrahedron Lett.*, 1986, **27**, 1377-1380.

276. J. H. van der Neut, J. H. Uhlenbroek and P. E. Verkade, *Recl. Trav. Chim. Pays-Bas*, 1953, **72**, 365-376.

277. J. H. Uhlenbroek and P. E. Verkade, *Recl. Trav. Chim. Pays-Bas*, 1953, **72**, 395-410.

278. J. W. Perich and R. B. Johns, *J. Chem. Soc., Chem. Commun.*, 1988, 664-666.

279. J. W. Perich and R. B. Johns, *J. Org. Chem.*, 1988, **53**, 4103-4105.

280. A. Paquet and M. Johns, *Int. J. Peptide Protein Res.*, 1990, **36**, 97-103.

281. M. Jones, K. K. Rana, J. G. Ward and R. C. Young, *Tetrahedron Lett.*, 1989, **30**, 5353-5356.

282. J. G. Ward and R. C. Young, *Tetrahedron Lett.*, 1988, **29**, 6013-6016.

283. S. J. Hecker, M. L. Minich and K. Lackey, *J. Org. Chem.*, 1990, **55**, 4909-4911.

284. N. Mora, J. M. Lacombe and A. A. Pavia, *Tetrahedron Lett.*, 1993, **34**, 2461-2464.

285. J. W. Perich, P. F. Alewood and R. B. Johns, *Tetrahedron Lett.*, 1986, **27**, 1373-1376.

286. T. B. Johnson and J. K. Coward, *J. Org. Chem.*, 1987, **52**, 1771-1779.

287. T. Baasov, S. Sheffer-Dee-Noor, A. Kohen, A. Jakob and V. Belakhov, *Eur. J. Biochem.*, 1993, **217**, 991-999.

288. F. R. Atherton, *Biochem. Prep.*, 1957, **5**, 1-4.

289. E. N. Walsh, *J. Am. Chem. Soc.*, 1959, **81**, 3023-3026.

290. G. W. Daub and E. E. van Tamelen, *J. Am. Chem. Soc.*, 1977, **99**, 3526-3528.

291. J. P. G. Hermans, E. de Vroom, C. J. J. Elie, G. A. van der Marel and J. H. van Boom, *Recl. Trav. Chim. Pays-Bas*, 1986, **105**, 510-511.

292. J. E. Marugg, A. Burik, M. Tromp, G. A. van der Marel and J. H. van Boom, *Tetrahedron Lett.*, 1986, **27**, 2271-2274.

293. P. Westerduin, G. H. Veeneman, G. A. van der Marel and J. H. van Boom, *Tetrahedron Lett.*, 1986, **27**, 6271-6274.

294. J. E. Marugg, M. Tromp, E. Kuyl-Yeheskiely, G. A. van der Marel and J. H. van Boom, *Tetrahedron Lett.*, 1986, **27**, 2661-2664.

295. K. K. Ogilvie and M. J. Nemer, *Tetrahedron Lett.*, 1980, **21**, 4145-4148.

296. A. V. Nikolaev, I. A. Ivanova, V. N. Shibaev and N. K. Kochetkov, *Carbohydrate Res.*, 1990, **204**, 65-78.

297. A. V. Nikolaev, T. J. Rutherford, M. A. J. Ferguson and J. S. Brimacombe, *J. Chem. Soc., Perkin Trans. I*, 1995, 1977-1987.

298. E. Larsson and B. Lüning, *Tetrahedron Lett.*, 1994, **35**, 2737-2738.

299. R. Anschütz and W. O. Emery, *Liebigs Ann. Chem.*, 1887, **239**, 301-303.

300. R. W. Young, *J. Am. Chem. Soc.*, 1952, **74**, 1672-1673.

301. P. J. Garegg, T. Regberg, J. Stawinski and R. Stromberg, *J. Chem. Soc., Perkin Trans. I*, 1987, 1269-1273.

302. J. Ludwig and F. Eckstein, *J. Org. Chem.*, 1989, 54, 631-635.

303. E. Baer, *Biochem. Prep.*, 1951, **1**, 50.

304. R. H. Boschan and J. P. Holder, *Chem. Abs.*, 1968, **68**, P77961a.

305. R. H. Boschan and J. P. Holder, *U.S. Patent Office 3,341,630*, 1967.

306. A. V. Podolskii and M. A. Bulatov, *Chem. Abs.*, 1976, **85**, 32557h.

307. M. I. Kabachnik, L. S. Zakharov, I. Yu. Kudryavtsev, A. P. Kharchenko, G. P. Tataurov and L. I. Korobeinikova, *Izv. Akad. Nauk. SSSR, Ser. Khim.*, 1979, **4**, 896-900.

308. S. Zhuo, J. C. Clemens, R. L. Stone and J. E. Dixon, *J. Biol. Chem.*, 1994, **269**, 26234-26238.

309. W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.

310. M. F. Lipton, C. M. Sorensen and A. C. Sadler, *J. Organomet. Chem.*, 1980, **186**, 155.

311. M. Hunt and V. du Vigneaud, *J. Biol. Chem.*, 1938, **124**, 699.

312. M. Bergmann and L. Zervas, *Ber. Dtsch. Chem. Ges.*, 1932, **65**, 1192.

313. H. Schwarz, F. M. Bumpus and I. H. Page, *J. Am. Chem. Soc.*, 1957, **79**, 5697.

314. a) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, 1942, **145**, 247; b) L. Heslinga and J. F. Arens, *Rec. Trav. Chim. Pays-Bas*, 1957, **76**, 982 (for the ethyl ester derivative).

315. G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, 1957, **79**, 6180.

316. A. L. Barker and G. S. Skinner, *J. Am. Chem. Soc.*, 1925, **46**, 403.

317. P. H. Bentley and A. V. Stachulski, *J. Chem. Soc., Perkin Trans. I*, 1983, 1187-1192.

318. L. Zervas, M. Winitz and J. P. Greenstein, *J. Org. Chem.*, 1957, **22**, 1515-1521.

319. K. Vogler and P. Lanz, *Helv. Chim. Acta*, 1960, **43**, 270.

320. K. Inouye, *Bl. Chem. Soc. Japan*, 1965, **38**, 1148.

321. T. Wieland and R. Sarges, *Ann. Chemie*, 1962, **658**, 181-193.

322. T. Tanaka, S. Tamaksukuri and M. Ikehara, *Tetrahedron Lett.*, 1986, **27**, 199.

323. a) L. Lecocq and A. R. Todd, *J. Chem. Soc. Trans.*, 1954, 2381-2384; b) A. B. Foster, W. G. Overend and M. Stacey, *J. Chem. Soc., Trans. II*, 1951, 980-987.

324. G. M. Steinberg, *J. Org. Chem.*, 1950, **15**, 637-647.

325. H. E. Baumgarten and R. A. Setterquist, *J. Am. Chem. Soc.*, 1957, **79**, 2605-2608.

326. J. O. Clayton and W. L. Jensen, *J. Am. Chem. Soc.*, 1948, **70**, 3880-3882.

327. a) J. F. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, 1957, **79**, 3741-3746; b) H. A. C. Montgomery and J. H. Turnbull, *Proc. Chem. Soc.*, 1957, 178.