SYNTHESIS AND APPLICATION OF FLUOROPHENYL BASED PHOSPHORYLATING AGENTS IN SOLUTION AND SOLID PHASE PHOSPHORYLATIONS

A thesis presented by

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to the

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in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY

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DECLARATION

I, Mark Hillier, hereby certify that this thesis which is approximately 50,000 words in length, has been written by me, and is a record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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I was admitted as a research student in October 1994 as a candidate for the degree of Ph. D. in August 1995; the higher study for which this is a record was carried out by the University of St. Andrews between 1994 and 1999.

Date..... Signature of Candidate.....

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

Date..... Signature of Supervisor.....

DEDICATED TO THE MEMORY OF REGINALD DAVIES 1912-1999

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ABSTRACT

Reversible phosphorylation of proteins on serine, threonine and tyrosine residues is now widely accepted as the principal mechanism for the control of intracellular events in eukaryotic and prokaryotic cells. The exact biochemical roles of many of the phosphatases and kinases remains unknown. Elucidation of the chemical mechanism of action of these enzymes is dependent on the availability on sufficient quantities of phosphopeptides and phosphoproteins.

Traditionally the chemical synthesis of phosphopeptides has been achieved by the 'global phosphorylation' approach, in which the desired peptide sequence is synthesised by solid phase peptide synthesis (SPPS) on a functionalised polymer support. Whilst still attached to the support, the peptide sequence is phosphorylated on the desired residue by reaction with an excess of phosphorylating reagent.

Many of the currently available phosphorylating reagents, save for a number developed by R. B. Johns, suffer from low reactivity, requiring multiple applications of the reagent to ensure complete phosphorylation of the substrate, or require harsh conditions to remove the phosphate protecting groups. This has limited their application to the synthesis of sterically hindered or sensitive sequences.

The reagent *bis*(pentafluorophenyl) chlorophosphate **90** was shown to be effective in the phosphorylation of sterically hindered sequences containing serine or threonine. However, the phosphorylation of tyrosine residues in SPPS had not been investigated, and solution phase phosphorylation produced only a low yield of phosphorylated substrate.

It was therefore decided to investigate the application of **90** in the solid phase phosphorylation of tyrosine residues. Phosphorylation was achieved using standard phosphorylation conditions. However, removal of both of the pentafluorophenyl groups was not achieved under a variety of conditions.

Phosphorylation of cyclohexanol was achieved in essentially quantitative yield. However, the order of addition of reagents is shown to be very important. Removal of the pentafluorophenyl groups was achieved through the use of refluxing sulphuric acid, or acidic hydrogenation, unsuitable for sensitive sequences.

The synthesis of a new phosphorylating agent, benzyl pentafluorophenyl chlorophosphate **118**, was developed. It was envisaged that this reagent would be applicable to the phosphorylation of tyrosine residues and would require milder deprotection conditions. Synthesis of benzyl pentafluorophenyl chlorophosphate **118** was achieved in four steps starting from dibenzyl phosphite. This reagent was shown to be effective in the SPPS of phosphoserine, -threonine and -tyrosine containing peptides and in solution.

A mechanism is also postulated to explain the acid lability of the pentafluorophenyl groups compared to phenyl groups, involving chelation of an acid proton by an *ortho*-fluorine and a phosphate oxygen. Synthesis of a series of resin bound *bis*(fluorophenyl) threonyl phosphate triesters are described.

Monitoring of the reaction between these triesters and a cleavage mixture containing TFA, by ^{31}P NMR spectroscopy, revealed the requirement for two *ortho*-fluorine substituents on the phenyl ring for partial cleavage to occur. However, there is no direct evidence for chelation of a proton, as postulated. Whilst there is a definite increase in acid lability as fluorine substitution increases, it is apparent that there may be other mechanisms operating during the experiments, and the results obtained to date are complex and far from unambiguous.

CONTENTS

ABBREVIATIONS

Ac	acetyl
Acm	acetamidomethyl
Adda	(2*S*, 3*S*, 8*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-2(*E*), 6(*E*)-dienoic acid.
ADP	Adenosine 5'-diphosphate
Alloc	allyloxycarbonyl
ATP	Adenosine 5'-triphosphate
Bn	benzyl
Boc	tertiary-butoxycarbonyl
BOP	benzotriazole-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophoshate
cAMP	adenosine 3', 5'-cyclic monophosphate
Cbz	benzyloxycarbonyl
DBU	1,8-diazobicyclo[5.4.0]undecene
DCC	1,3-dicyclohexylcarbodiimide
DCM	dichloromethane
DIPEA	*N*,*N*-diisopropylethylamine
DNA	deoxyribonucleic acid
DMAP	4-dimethylaminopyridine
DMBA	7,12-dimethylbenz[a]anthracene
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
EDCI	1-(3-dimethylaminopropyl)-3-ethyl carbodiimide methiodide
EDTA	ethylenediaminetetraacetic acid

Fmoc	9-fluorenylmethoxycarbonyl
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMP	hydroxymethylpolystyrene
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
I-1	inhibitor-1
I-2	inhibitor-2
IC_{50}	concentration required to reduce enzyme activity by 50%
Lev	levulinoyl
Maq	methylanthraquinoyl
Masp	(2*R*, 3*S*)-3-methylaspartic acid
Mbh	4,4'-dimethoxybenzhydryl
*m*CPBA	4-chloroperoxybenzoic acid
MeCN	acetonitrile
MNNG	*N*-methyl-N^1-nitro-*N*-nitrosoguanidine
Mtr	methoxytrityl
N-Mdha	*N*-methyldehydroalanine
N-Mdhb	*N*-methyldehydrobutyric acid
NMM	4-methylmorpholine
NMP	N-methylpyrrolidine
NMR	nuclear magnetic resonance
PFP	pentafluorophenyl
O/N or o/n	overnight
P_i	inorganic phosphate

PKA	protein kinase A
PKC	protein kinase C
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
PP1c	catalytic unit of PP1
PP1G	glycogen bound PP1 holoenzyme
PP1M	myosin bound PP1 holoenzyme
PP1S	cytosolic bound PP1 holoenzyme
PPX	protein phosphatase X
Ppase	protein phosphatase
PTKs	protein tyrosine kinase
PTPase	protein tyrosine phosphatase
PyBOP	benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate
PyBrOP	bromo-tris-pyrrolidinophosphonium hexafluorophosphate
RNA	ribonucleic acid
SPPS	solid phase peptide synthesis
TBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TES	triethylsilane
TFA	trifluoracetic acid
TFMSA	trifluoromethanesulphonic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
Trt	trityl (triphenylmethyl)

Wang	*p*-benzyloxybenzyl functionalised polystyrene

AMINO ACID ABBREVIATIONS

AMINO ACID	THREE LETTER ABBREVIATION	ONE LETTER SYMBOL
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Norleucine	Nle	-
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1.0 Introduction

1.1 General Introduction

Many biological processes are regulated by a simple chemical event – the cleavage or formation of a phosphate ester. Reversible phosphorylation is now known to play an important role in cellular transduction processes.[1, 2] This places the protein phosphatases, in conjunction with the protein kinases, in a position of central importance, due to their ability to act in opposition to each other, Fig. 1.1.

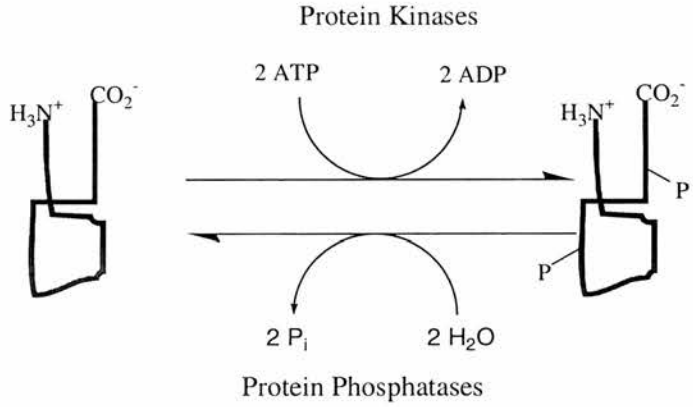


Figure 1.1: *Schematic representation of reversible protein phosphorylation*

1.2 The Phosphatases

The phosphatases can be classified according to two criteria – by substrate specificity or by mechanism of action.

Phosphatases have a wide range of substrates, varying from small phosphorylated metabolites (glucose-6-phosphate), to secondary messengers (phosphoinositol) upto very large substrates such as phosphoproteins. Using substrate specificity as a criterion, the phosphatases can be grouped into 3 main categories, Fig 1.2:

- non specific phosphatases, which catalyse the hydrolysis of almost any phosphate ester;
- phosphoprotein specific phosphatases, which utilise phosphopeptides and phosphoproteins as their preferred substrates; and
- small molecule specific phosphatases, which hydrolyse one substrate or groups of similar substrates

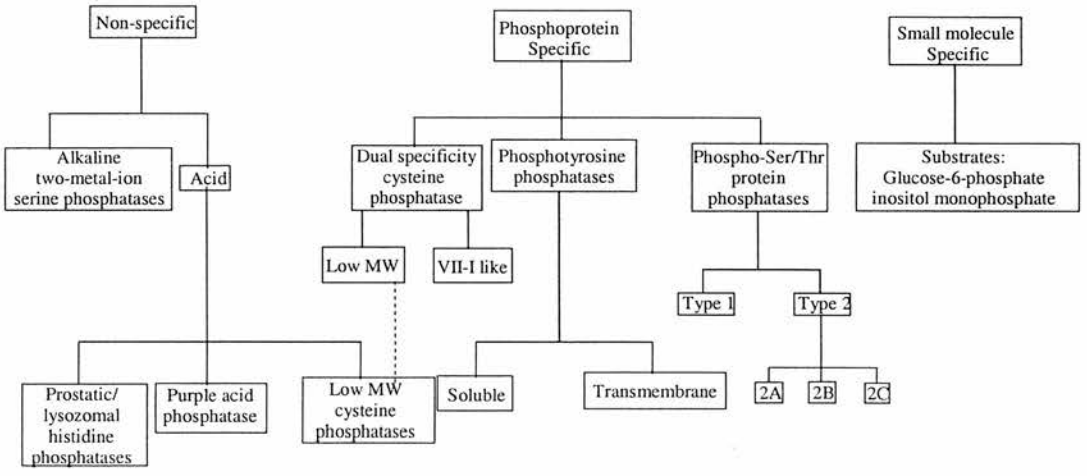


Figure 1.2: *The major phosphatases*[3]

A secondary classification, according to the mechanism of action of each phosphatase, is also employed.

Either an active site nucleophile is the initial phosphoryl group acceptor (see Fig. 1.3a) below), or alternatively a water molecule acts as the initial phosphoryl group acceptor, normally catalysed by the presence of metal ions in the active site (Fig. 1.3b).

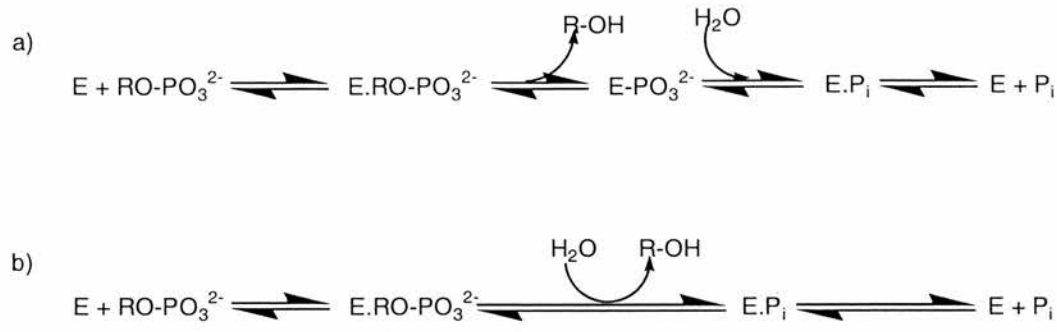


Figure 1.3: *The two mechanisms of phosphate hydrolysis: a) Hydrolysis via phosphoenzyme intermediate; b) Direct transfer to water*

Of those phosphatases that fall into category a) above, it is possible to further subdivide the phosphatases according to the nature of the phosphoryl group acceptor (Cys, His or Ser). The phosphatases operating by the second mechanism usually use a two metal ion diad to bind the ester, and catalyse subsequent hydrolysis. This metal ion motif is ubiquitous in phosphoryl group transfer biochemistry, and is used by phosphodiesterases (*i.e.* nucleases),[4] and phosphotriesterases.[5] Phosphatases utilising two metal ions in the active site are very heterogeneous, varying in metal ion type, protein sequence, structure, ligands, active site catalytic residues and mechanism.

Phosphoprotein phosphatases are subdivided further into the protein serine/ threonine phosphatases,[6] protein tyrosine phosphatases (PTPs),[7] and dual specificity phosphoprotein phosphatases.[8-10] The phosphotyrosine phosphatases and dual specificity phosphatases are mechanistically related, utilising an active site cysteine residue located in a phosphate binding loop as a phosphoryl group acceptor. However, the PTPs are specific for phosphotyrosine residues, whereas the dual specificity phosphatases hydrolyse phosphotyrosine and phosphoserine/ phosphothreonine residues.

There are approximately 100 protein phosphatases known,[6, 11] and about twice that number of kinases.[12-14] Two thirds of the kinases phosphorylate serine and threonine residues, yet less than a third of the protein phosphatases hydrolyse phosphoserine and phosphothreonine residues. Most of the known serine and threonine phosphatases contain one of just 4 main types of catalytic subunit, usually associated with regulatory subunits and other components.[15] These 4 main subgroups were originally classified by biochemical criteria as PP1, PP2A, PP2B (calcineurin) and PP2C. PP1 and PP2A are responsible for most of the protein dephosphorylation events occurring within the cell. It therefore appears that a few protein phosphatases are committed to reversing the effects of a huge number of Ser/ Thr protein kinases, whereas the number of Tyr specific protein kinases and phosphatases match one another.[7] This might reflect the substantial difference in mechanism controlling the reversible phosphorylation on Tyr and Ser/ Thr residues. Tyrosine kinases are generally not endowed with site specificity as stringent as most of the Ser/ Thr protein kinases. This is consistent with the notion that many PTKs recruit substrates through interaction of autophosphorylated domains with so called *src* homology 2 (SH2) domains (see Section 1.4, Protein Tyrosine Phosphatases).

1.3 Protein Serine / Threonine Phosphatases

1.3.1 Introduction

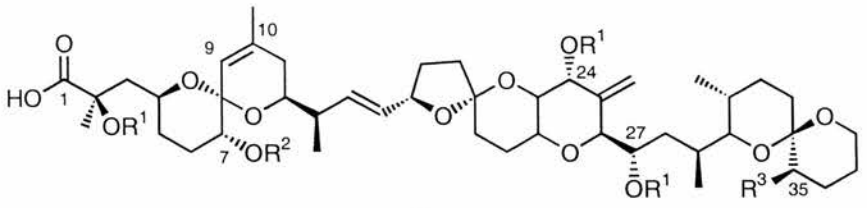
Serine and threonine phosphatases are present in all eukaryotic cell types and participate in a variety of cellular processes. These include cell proliferation, protein synthesis, regulation of transcription and neurotransmission.[6] However, it is only recently that tyrosine kinases have been identified and phosphorylation of tyrosine

residues has been recognised in connection with cellular processes.[14] The serine/ threonine protein phosphatases are unrelated in sequence to the tyrosine phosphatases and have been characterised into groups on the basis of biochemical properties.[16]

1.3.2 General Classification

Due to the broad and overlapping substrate specificity of the protein phosphatases *in vitro*, they cannot be classified by substrate alone. Therefore, the action of specific inhibitor is used as a criterion for classification. Based on biochemical parameters,[16] the Ser/ Thr protein phosphatases were divided into two classifications. Type-1 protein phosphatases, such as PP1, are inhibited by two heat- and acid- stable proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2) and preferentially dephosphorylate the β-subunit of phosphorylase kinase.[6,16] Type-2 phosphatases, such as members of the PP2 family, are insensitive to I-1 and I-2, and preferentially dephosphorylate the α-subunit of phosphorylase kinase.[16] Further classification of type-2 phosphatases is based upon their requirements for divalent cations. PP2A has no absolute requirement for divalent cations, though it is stimulated by Mn^{2+}, and by polycationic compounds under certain conditions (hence its name of PCS – polycation stimulated – protein phosphatase) whereas PP2B is dependent on Ca^{2+} and PP2C is dependent on Mg^{2+}.[16]

Discrimination between classes is further facilitated by the use of the natural tumour promoter okadaic acid **1**.[17]



1

PP1, 2A and 2B (calcineurin) have highly homologous catalytic domains. PP2C is unrelated to these enzymes. Included with PP1 and relatives is an enzyme encoded by bacteriophage λ (PP_λ), homologous in sequence to the N-terminal half of PP1, which has been a useful model for recombinant studies.[18] Recently a number of other protein phosphatases have been isolated. PP3 (see Section 1.3.7) was isolated from bovine brain and showed no homology to any known Ser/ Thr protein phosphatase.[19] PP4 (Section 1.3.8), a phosphatase found in the cytoplasm of human cells, shows a number of similarities of PP2A, but does not find the regulatory subunit of PP2A.[20]

1.3.3 Protein Phosphatase 1 (PP1)

PP1 is the most reluctant of the Ser/ Thr protein phosphatases to dephosphorylate phosphopeptide substrates, even if they are quite long. This suggests a higher order of structure in protein substrates is need to optimise PP1 catalytic activity. The inactivity towards peptides is due to autoinhibition by a domain of the catalytic subunit that can be removed proteolytically or displaced by high Mn^{2+} concentrations.

PP1 (similar to PP2A and PP2C) far prefers phosphothreonine substrates to phosphoserine substrates and perceives a prolyl residue immediately C-terminal to the phosphoamino acid as a strong negative determinant.[21] PP1 activity (unlike that

of PP2B and C) is not abolished by an acidic cluster C-terminal to the phosphoamino acid,[22] and seems to be increased greatly by multiple basic residues N-terminal to the phosphoamino acid.[23]

1.3.3.1 Structure of PP1

PP1 exists as a 1:1 complex between the catalytic subunit ($PP1_C$, molecular mass 27 kDa) and a number of regulatory subunits (also known as 'targeting subunits'). Two isoforms of $PP1_C$, α (330 resides) and β (311 residues) have been identified, differing in the N-terminal sequence.[24] From residue 34 in $PP1_C\alpha$, the two isoforms are coded for by an identical nucleotide sequence. This suggests that both $PP1_C\alpha$ and $PP1_C\beta$ are generated from the same gene by differential transcription and/ or splicing of mRNA.[24]

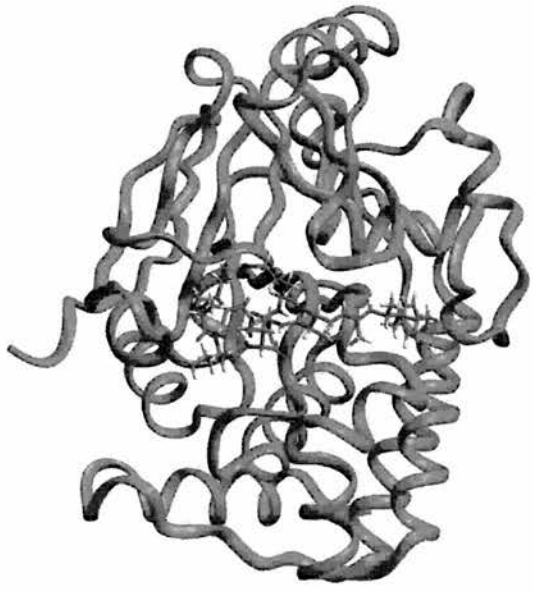
In mammalian cells, PP1 is associated with a number of subcellular compartments, mediated by a number of targeting proteins. The glycogen binding, or G-subunit (G_M), confers PP1 association with skeletal muscle glycogen.[25] The G-subunit also enhances the dephosphorylation of phosphoproteins found in glycogen particles. The 161kDa G_M subunit is only expressed in adult skeletal muscle,[26] PP1 association with liver glycogen being mediated through a different protein, the G_L subunit. Phosphorylation of PP1 by a cAMP dependent protein kinase (PKA) is promoted by hormones which cause an increase in intracellular cAMP levels, and leads to the displacement of PP1 from glycogen. Translation of PP1 to the cytosol decreases its activity towards the enzymes of glycogen metabolism, especially phosphorylase kinase, glycogen synthase and glycogen phosphorylase. PKA also activates and phosphorylates I-1. This should lead to rapid inactivation of cytosolic PP1. The G_M subunit is also involved with PP1 association with the sarcoplasmic

reticulum in skeletal and cardiac muscle.[27,28] Here PP1 regulates the phosphorylation state of phospholamban and calcium uptake.

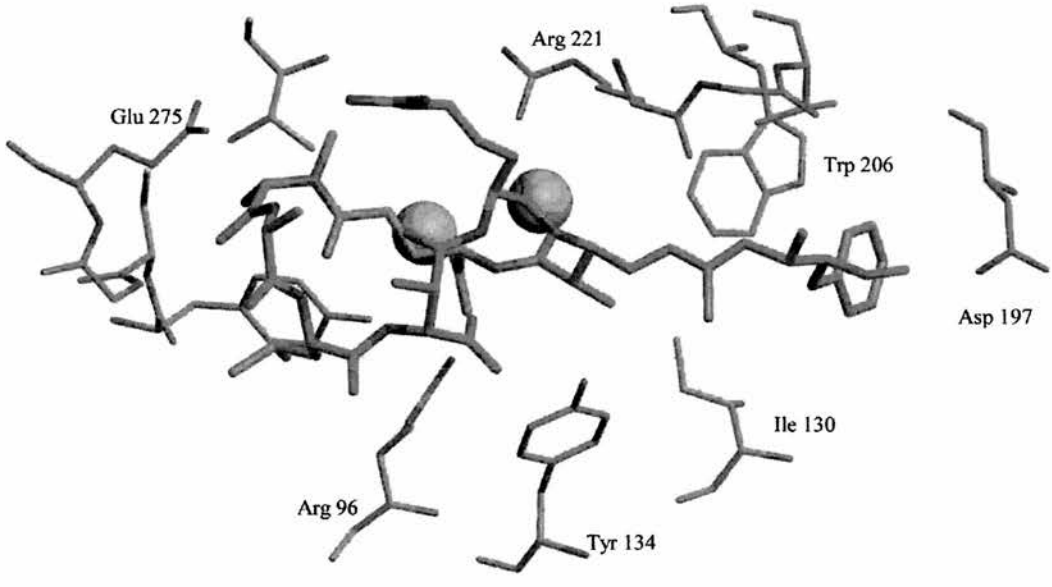
PP1 binding to smooth muscle myosin is mediated by two polypeptides, of 130 and 25 kDa, that comprise the M complex (M_{110}). This association enhances the dephosphorylation of smooth muscle myosin by PP1 but has no effect on the dephosphorylation of skeletal muscle myosin. I-2 acts as a chaperone to refold PP1 catalytic subunits to their native conformation.[29] Following reactivation, PP1 catalytic subunits can be displaced from the PP1/I-2 complex by the G-subunit or M complex. In this way, the PP1/I-2 complex may serve to deliver PP1 to different subcellular compartments. The structural differences in the two isoforms of PP1 do not contribute significantly to their activity or regulation. The physiological relevance of the two isoforms is thus far unclear.

Each form of $PP1_C$ isolated contains just one $PP1_C$-binding subunit, suggesting that the interaction of different targeting subunits with $PP1_C$ is mutually exclusive and that the binding sites for different subunits are identical or overlapping.[30,31]

The crystal structure of PP1 has been determined from data collected on PP1 complexed with microcystin-LR **2** at 2.8 Å and 2.1 Å, Fig 1.4.[32,33] These structures revealed that PP1 is a metalloenzyme unrelated in architecture to the tyrosine phosphatases, but sharing high sequence homology to PP2B, with very similar overall architectures and nearly identical active sites. Tyrosine phosphatases do not require a metal ion and use a cysteine residue as a nucleophile to effect catalysis (see Section 1.4.5).

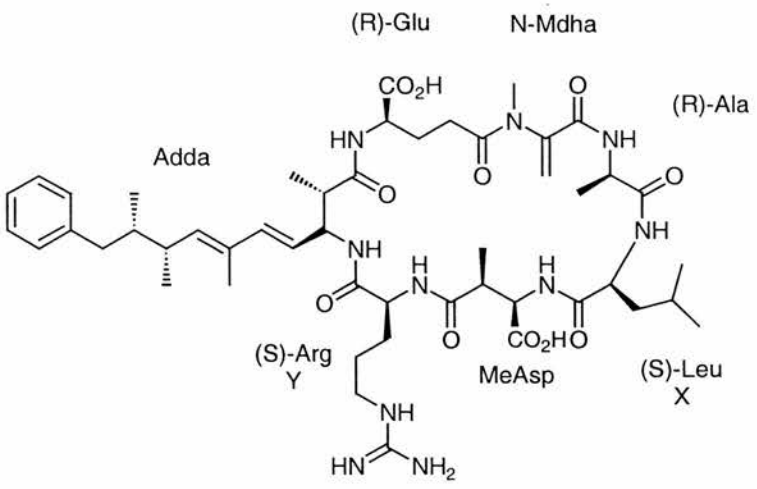


a)



b)

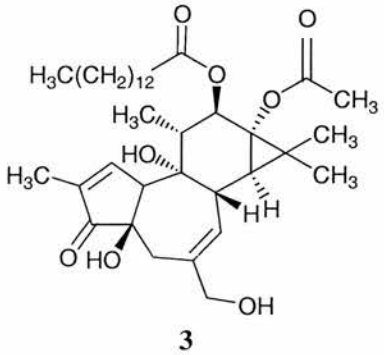
Figure 1.4:*Catalytic Subunit of PP1.* ***a)*** *Ribbon diagram of catlytic subunit of PP1, with stick model of microcystin-LR* ***2*** *bound in the active site;* ***b)*** *ball and stick view of active site (thin lines) with microcystim-LR* ***2*** *bound (thick lines) and two metal ions (purple spheres).*



Microcystin-LR **2**

1.3.3.2 Inhibitors of PP1

Tumour promoters can be divided into two classes; either TPA-type or non-TPA-type (TPA: 12-*O*-tetradecanoylphorbol-13-acetate, **3**). The TPA-type promoters are those that bind to the phorbol ester receptors in cell membranes which activate the Ca^{2+} activated protein kinase C (PKC) *in vitro*.[34]

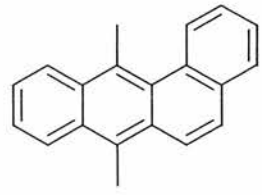


3

*(TPA, 12-O-tetradecanoylphorbol-13-acetate, **3**)*

Investigation of okadaic acid and its derivatives revealed a new mode of tumour promotion distinct from the TPA-type.[35] Okadaic acid was found to bind to

specific receptors in the particulate and cytosolic fractions of mouse skin and diverse other organs, and to inhibit activity within these fractions. Okadaic acid and dinophysistoxin-1 (35-(R)-methylokadaic acid) were reported to induce tumour promotion in two stage carcinogenesis experiments on mouse skin initiated with DMBA (7,12-dimethylbenz[a]anthracene) **4** and MNNG (*N*-methyl-N^1-nitro-*N*-nitrosoguanidine) **5**. The target receptors were later shown to be the protein phosphatases PP1 and PP2A (See Section 1.3.4).



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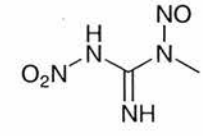

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Several other compounds were shown to inhibit PP1 and PP2A in a similar manner to okadaic acid. These compounds belonged to four structurally diverse groups of compounds, represented by okadaic acid **1**, microcystin **2**, calyculin **6** (Fig. 1.5), and tautomycin **7** (Fig. 1.5).

Okadaic acid, being the first tumour promoter of this class, has given its name to the whole group, the okadaic acid class of compounds. Their mechanism of inhibition, now recognised as the general mechanism of tumour promotion in a number of organs, is called the okadaic acid pathway of tumour promotion. Unlike okadaic acid, the microcystins, calyculins and tautomycins are equally effective against both PP1 and PP2A (Section 1.3.4)

Okadaic acid **1** is a C_{38} spiroketal polyether isolated from two sponges, *Halichondria okadai* and *H. melanodocia*.[36] Okadaic acid and its 35-methyl

derivative (dinophysistoxin-1, DTX-1) were first isolated as causative agents of diarrhetic shellfish poisoning (DSP) in Japan.[37]

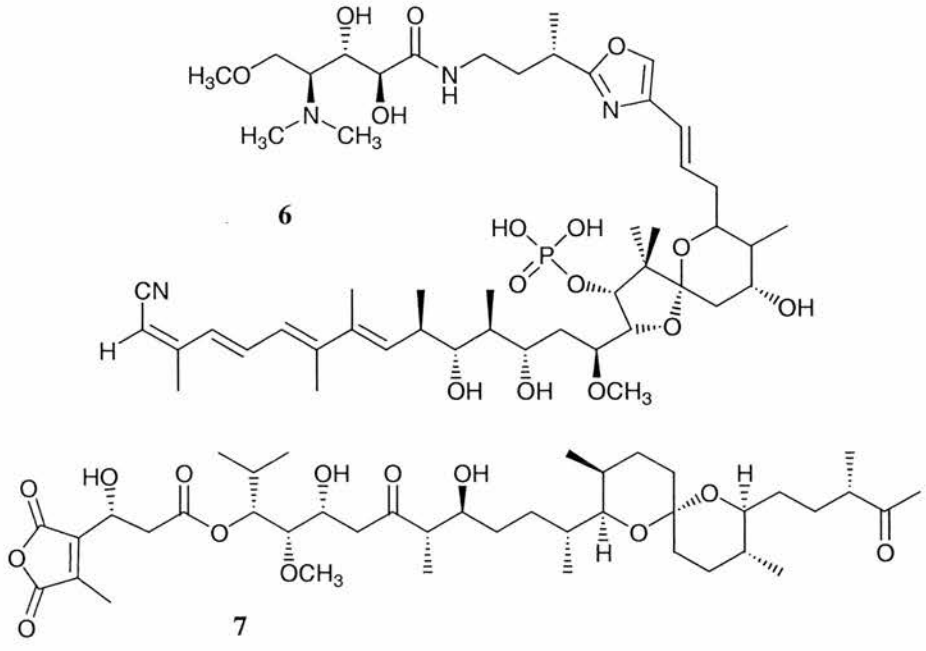


Figure 1.5: *calyculin A* **6** *and tautomycin* **7**

Calyculin A **6** was isolated from the marine sponge, *Discodermia calyx*, and found to be a strongly toxic compound against leukemia cells.[38] Its structure was found to contain an octamethyl-polyhydroxylated C_{28} fatty acid, linked to two γ-amino acids and esterified as the phosphate monoester. Calyculin A was also found to be a potent inhibitor of PP1 and PP2A, but with a profile different from that of okadaic acid, being equally effective against both PP1 and PP2A.[39, 40]

The microcystins and their structurally related counterparts, the nodularins, have been isolated from the cyanobacteria of genera *Nodularia*, *Nostoc*, *Oscillatoria* and the most widely studied, *Microcystis*. The toxins isolated belong to a family of closely related heptaisopeptides (the microcystins) and pentaisopeptides (the

nodularins). The most well known and studied of the toxins are microcystin-LR **2** and nodularin **8** (Fig 1.6).

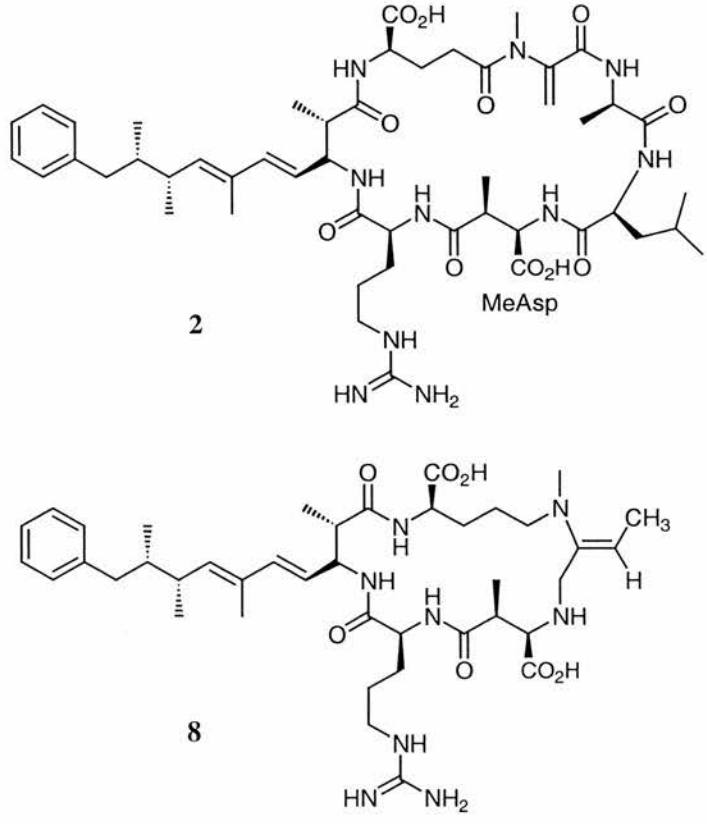


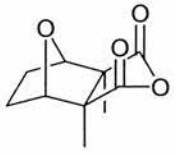
Figure 1.6: *Microcystin* **2** *and Nodularin* **8**

The general structure of microcystins is *cyclo*(-*R*-Ala[1]-*S*-X[2]-*R*-βMeAsp[3]-*S*-Y[4]-Adda[5]-*R*-*iso*Glu[6]-Mdha[7]), where X and Y are variable S amino acids, *R*-MeAsp is *R*-*erythro*-β-methylaspartic acid and Mdha is *N*-methyldehydroalanine. The two variable amino acids at positions 2 (X) and 4 (Y) form the basis for the nomenclature. Nodularin **8** lacks the variable S-amino acid X and the *R*-Ala residue, whilst the Mdha residue is replaced by a methylated analogue, *N*-methyldehydroaminobutyric acid (Mdhb).

Tautomycin **7**, is an antifungal antibiotic isolated from a culture of *Streptomyces spiroverticillatus*.[41] Besides its antifungal activity, tautomycin was

shown to induce a morphological change in human leukemia cells, which correlated with protein phosphorylation.[42] Tautomycin inhibits PP1 and PP2A with IC_{50} values of 20 nM and binds to the same site as okadaic acid.[43]

Cantharidin, (*exo*,*exo*-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride, **9**) is a naturally occurring toxin present in many species of the Chinese blister beetle (*Mylabris phalerata*) and has also been isolated from the Spanish fly (*Catharis vesicatoria*). It is a millimolar inhibitor of both PP1 and PP2A.[44]



9

Cantharidin **9**

Three structurally different compounds, okadaic acid, microcystin-LR and calyculin A all bind to the same modulatory site of the protein phosphatases, suggesting that they may possess a common pharmacophore. Computer generated minimum energy conformations of these three compounds indicated a pharmacophore consisting of a central core containing one conserved acidic group, two potential hydrogen bonding sites and a non polar side chain, Fig 1.7.[45]

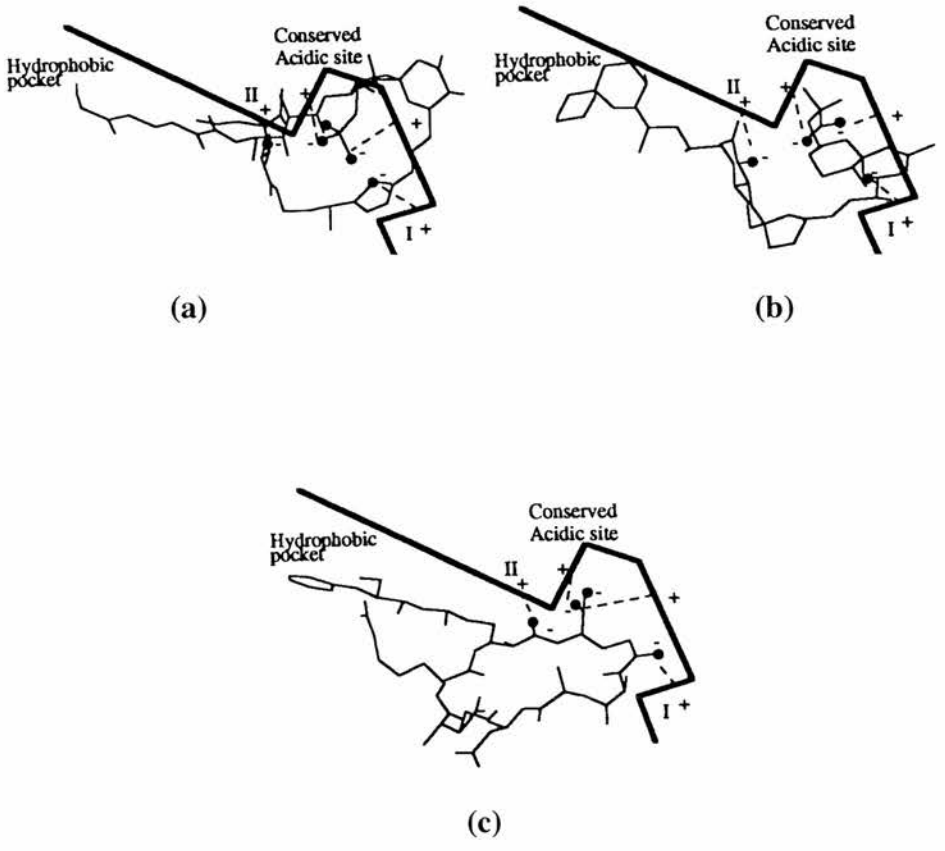


Fig. 1.7: *Superimposition of okadaic acid, calyculin A and microcystin-LR (molecular modeling above).The conserved acid binding domains of* ***(a)*** *calyculin A,* ***(b)*** *okadaic acid and* ***(c)*** *microcystin-LR, showing the potential hydrogen binding areas I and II, the conserved acids and the hydrophobic side chains. The protein is thought to lie above the three compounds.*

PP1 and PP2A are also regulated by a number of endogenous protein inhibitors, Fig. 1.8.

Inhibitor 1 (I-1) is an 18.7 kDa protein activated as an inhibitor of PP1 after phosphorylation of Thr35 by a cAMP-dependent protein kinase. Inhibitor 2 (I-2) is a 22.8 kDa protein that differs in sequence to I-1. The method of inhibition of $PP1_C$ by I-2 is different from that of I-1, since a greater concentration of I-2 is required to cause inhibition. DARPP-32 (dopamine and cAMP-regulated phosphoprotein) is a

22.6 kDa isoform of I-1 with equal inhibitory potency. The N-terminal regions of I-1 and DARPP-32, the most conserved regions, also contain the inhibitory domain. NIPP-1 (nuclear inhibitor of protein phosphatase 1) was found bound to the chromatin of the nucleus. Unlike DARPP-32 and I-1, NIPP-1 requires dephosphorylation to become an active inhibitor. I-1, I-2, DARPP-32 and NIPP-1 specifically inhibit PP1 but do not inhibit the other phosphatases.

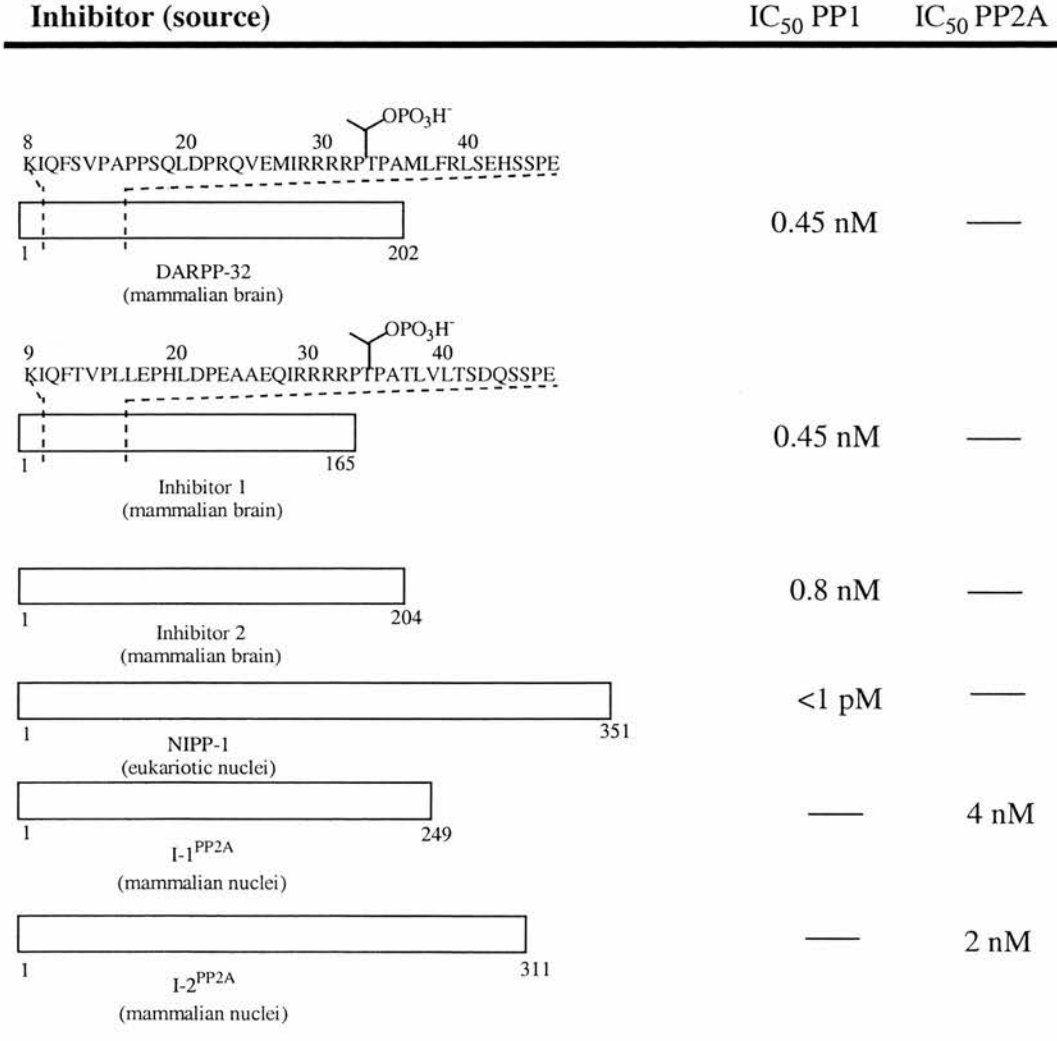


Figure 1.8: *The known endogenous inhibitors of PP1 and PP2A*[46]

Deinhibitor is a thermostable 9 kDa polypeptide shown to prevent inhibition of $PP1_C$ by I-1 or I-2.[47] Deinhibitor not only reduced inhibition of $PP1_C$ by I-1 but

also promoted dephosphorylation of I-1 by $PP1_C$ in the absence of Mn^{2+}.[48,49] Deinhibitor may therefore act to antagonise the actions of I-1. Deinhibitor may be reactivated by dephosphorylation only by the high molecular weight 'native' form of PP2A.[50]

Table 1.1: *Summary of Ser/ Thr specific phosphatases and inhibitors. NI, not inhibitory; ND, not determined; data are reported as IC_{50} values.*[46]

Inhibitor	**PP1**	**PP2A**	**PP2B**	**PP2C**	**PP4**	**PP5**
Microcystin-LR	0.1 nM	0.1 nM	8.7 nM	NI	8 pM	>1 nM
Nodularin-Y	1.8 nM	0.03 nM	8.7 mM	NI	ND	ND
Okadaic Acid	3 nM	0.2-1 nM	>10 mM	NI	0.2 nM	<3 nM
Calyculin A	0.3-0.7 nM	0.2-1 nM	>10 mM	NI	ND	ND
Tautomycin	0.7 nM	0.7 nM	~70 mM	NI	ND	ND
Cantharidin	0.5-2 mM	0.2 mM	>1 mM	NI	ND	ND
I-1/ DARPP	0.4-0.8 nM	NI	NI	NI	ND	ND
I-2	0.4-0.8 nM	NI	NI	NI	ND	ND
NIPP	<1 pM	NI	NI	NI	ND	ND

1.3.3.3 Phosphorylation of $PP1_C$ by Tyrosine Protein Kinase.

$PP1_C$ is the target for *in vitro* phosphorylation by a tyrosine protein kinase with the viral oncogene $pp60^{v\text{-}src}$. The tyrosine residue phosphorylated is 25 residues from the C-terminus. The inability of the PTK to phosphorylate the $PP1_C$/I-2 complex with $pp60^{v\text{-}src}$ suggests that association of I-2 primarily occurs at the domain of $PP1_C$ containing the phosphorylation site.[51] $pp60^{v\text{-}src}$ also phosphorylates free I-2 on a tyrosine residue near or at the domain interacting with $PP1_C$.[52]

1.3.3.4 Regulation of PP1

PP1 is subject to regulation by a number of hormones. Glucocorticoids and insulin are involved in the induction and/ or maintenance of hepatic $PP1_G$. Ca^{2+} also regulates $PP1_G$, as $PP1_G$ suffers inhibition by Ca^{2+} when glycogen synthase is used as a substrate.[53] Molecular mechanisms involved in insulin activation of hepatic PP1 remain to be elucidated. A decrease in hepatic protein phosphatase has been experimentally linked with *diabetes mellitus*. Activity has been restored to near normal by insulin replacement therapy.[54] Loss in enzyme activity is primarily a result of a reduction in PP1.[55] No change in PP2A is seen.

Selective changes in liver PP1 are associated with the onset of diabetes. Circulatory insulin levels correlate with hepatic $PP1_G$ content suggesting a role for insulin in the maintenance of $PP1_G$ activity.

1.3.4 Protein Phosphatase 2A (PP2A)

There are 2 main isoforms of the catalytic subunit of PP2A, α & β, which share about 97 – 8% identity, and are variably associated with non-catalytic subunits of type A or B (B, B' and B") to form dimeric or trimeric complexes.

1.3.4.1 Structure of PP2A

Several trimeric holoenzyme forms of PP2A exist and have been characterised.[6] The core consists of a 36 kDa catalytic subunit ($PP2A_C$) complexed with a regulatory subunit of 65 kDa (PR65 or A subunit).[56] This dimer associates with a number of different regulatory subunits of 55 kDa (PR55 or B subunit), 54 kDa (B' subunit), 72 kDa (PR72), or 74 kDa (B'' subunit),[57] Fig. 1.9. The exact function of the regulatory subunits is not yet fully known.

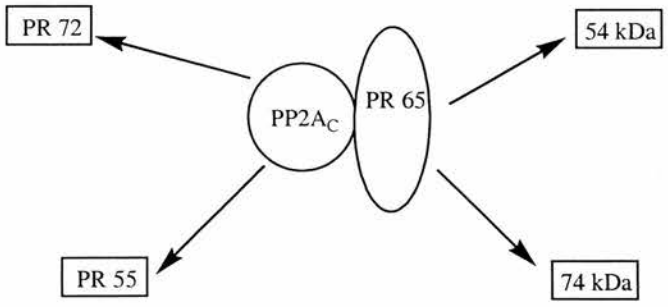


Figure 1.9: *Association of PP2A catalytic subunit with regulatory subunits*

PP2A is also termed polycation stimulated (PCS) protein phosphatase, based on its stimulation by cations.

Molecular cloning has revealed the existence of a number of isoforms of each subunit.[58] Two isoforms of the catalytic subunit (α, β),[59] two isoforms of PR65 (α, β), three isoforms of PR55 (α, β, γ) and two isoforms of PR72 exist. Most PP2A subunits seem to be ubiquitously expressed. The α and β isoforms of $PP2A_C$ show 97% homology but are distinct gene products. The variable regions are concentrated in the N-terminal 40 residues. The situation is much less complex in *Drosophila melanogaster*, since every subunit is encoded by only a single gene. The *Drosophila* PP2A catalytic subunit shares 94% homology with the mammalian enzyme, perhaps reflecting a remarkable degree of evolutionary conservation.

Three forms of PP2A, $PP2A_0$, $PP2A_1$ and $PP2A_2$ have been isolated from rabbit skeletal muscle.[60] All share common C (35 kDa) and A (60 kDa) subunits. $PP2A_0$ and $PP2A_1$ contain additional subunits B' (54 kDa) and B (55 kDa) respectively.

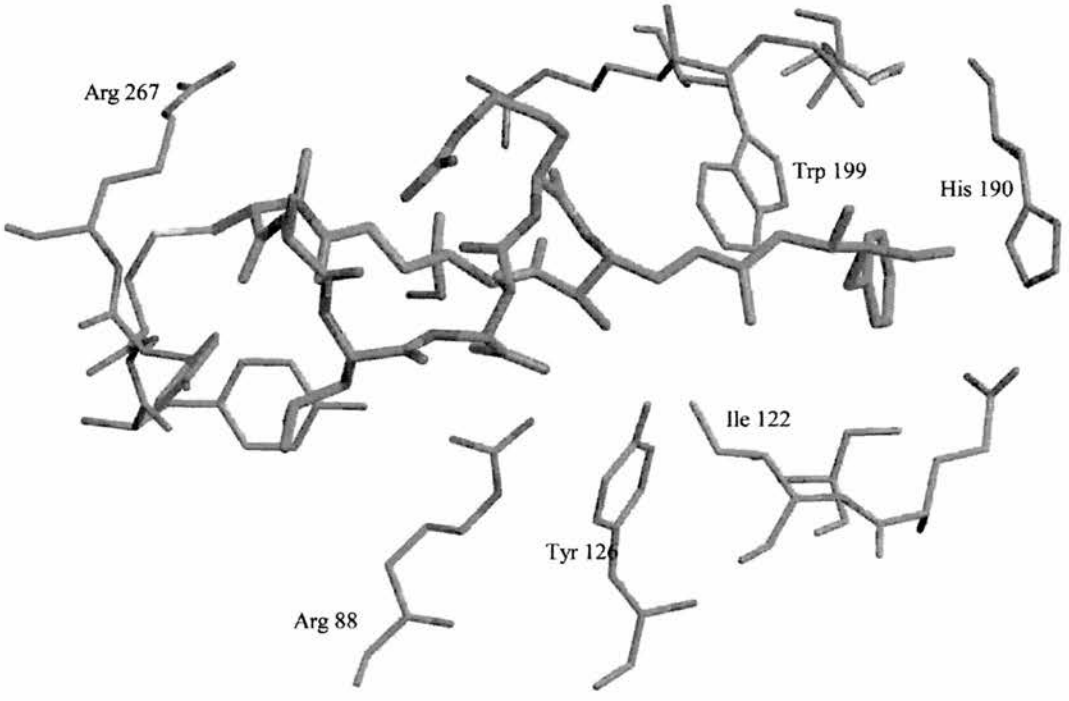


Figure 1.10: *Active site of catalytic subunit of PP-2A (thin lines) with microcystin-LR bound (thick lines)*

1.3.4.2 Regulation of PP2A

1.3.4.2.1 Regulation by polycations

PP2A is markedly inhibited by polycations such as protamine polylysine and histone H1, which have been used to characterise the enzyme.[61] The effect of polycations varied with the different forms of PP2A, with $PP2A_1$ more susceptible than $PP2A_2$, which was more susceptible than $PP2A_C$. The exact mechanism of inhibition is not known, though it is believed to occur primarily through interaction of these polycations with the catalytic subunit and does not involve dissociation of the enzyme complex. The substrate used also affects the efficacy of the polycations, suggesting that substrate interactions may also occur.

1.3.4.2.2 Regulation by ATP

$PP2A_2$ is more sensitive to ATP inhibition than $PP1_C$.[62] ATP kinetically distinguishes between the different enzyme forms as it exerts both substrate and enzyme directed effects on $PP2A_C$ and $PP2A_2$ but only substrate directed effects on $PP2A_1$. The physiological concentration of ATP may serve to influence the substrate specificity of the enzyme.

1.3.4.2.3 Regulation by Inhibition

PP2A is inhibited by a heat stable protein from rat liver (molecular weight 20 kDa).[63] This inhibitor is not phosphorylated by cAMP dependent protein kinase or GSK-3, distinguishing it from I-1 and I-2. A phosphoprotein from the cerebellum, termed the G-substrate, is specifically phosphorylated by a cGMP-dependant protein kinase.[64] The amino acid sequence surrounding the 2 phosphorylated threonine residues possesses a high degree of homology with that in I1 and DARRP-32.[65] The G-substrate, like I-1 and DARPP-32, is a good substrate for PP2B.[66] In contrast to I-1 and DARPP-32, the phosphorylated G-substrate inhibits $PP\text{-}2A_C$ and $PP\text{-}1_C$. The dephosphorylated protein is completely inactive.

1.3.4.3 Substrate Specificity

Higher order structure is important in defining the substrate specificity of the protein phosphatases. Studies of peptide sequences confirmed that PP1 was unable to dephosphorylate these substrates,[21] except in the presence of Mn^{2+}.[67] Limited information is available concerning the dephosphorylation of synthetic peptide substrates for PP2A and PP2B.

A threonine specific protein phosphatase has been isolated from rat liver using the synthetic peptide RRApTVA as substrate. The peptide RRPpTPA (the phosphorylation site in I-1 and DARPP-32) is not effectively dephosphorylated.

PP2A is able to phosphorylate a number of short phosphopeptides as effectively as protein substrates or even faster. Short phosphothreonine peptides are dephosphorylated much more effectively than the phosphoserine counterparts,[21] especially RRApTVA and RRREEEpTEEEAA.[68] The presence of multiple basic residues upstream from the phosphoamino acid greatly enhances the dephosphorylation rate.[22] A prolyl residue immediately C-terminal to the phosphoamino acid has a negative influence on dephosphorylation rate,[21, 68] which could be overcome by the addition of Mn^{2+}, and cationic side chains are most beneficial between the -3 and -6 positions.

1.3.4.4 Physiological Role of PP-2A

The discovery that PP2A (as well as PP1) is remarkably sensitive to inhibition by okadaic acid, a marine polyether, *in vitro* and *in vivo,* facilitated the analysis of the role of the enzyme in cell extracts and in intact cells.[69] Several regulatory enzymes involved in glycolysis and gluconeogenesis, fatty acid synthesis and amino acid breakdown - pyruvate kinase, 6-phosphofructo-1-kinase, acetyl CoA carboxylase, ATP citrate lyase, phenylalanine hydroxylase and hormone sensitive lipase - are excellent substrates for PP2A.[6] In intact cells, okadaic acid stimulates phosphorylation of several of these regulatory enzymes, resulting in stimulation of glucogenesis and lipolysis, and inhibition of fatty acid synthesis.[70] This suggests that PP2A may be the major protein phosphatase regulating these enzymes *in vivo*.[6] PP2A is the major phosphatase enzyme acting on the smooth muscle myosin.[71]

PP2A is found to dephosphorylate proteins that are themselves involved in signal transduction pathways. PP2A is the only phosphatase that significantly dephosphorylates the deinhibitor protein,[50] thus potentially regulating PP1 activity.

1.3.5 Protein Phosphatase 2B (PP2B)

PP-2B, also termed calcineurin, is a Ca^{2+}/ calmodulin regulated protein phosphatase, Fig 1.11. Calcineurin is a heterodimer composed of a 60 kDa catalytic subunit (calcineurin A), and a 19 kDa calcium binding subunit (calcineurin B). Calmodulin binds to the A subunit and contains the catalytic activity, and the B subunit is the Ca^{2+} binding, regulatory protein.[6] Sequencing of the B subunit from bovine brain identified it as a Ca^{2+} binding protein showing a high degree of identity in 4 putative Ca^{2+} binding loops with well characterised Ca^{2+} binding proteins such as calmodulin.[69]

1.3.5.1 Activation of PP2B

The affinity of purified PP2B has been shown to be activated by Mn^{2+} and Ni^{2+}. Whilst Mn^{2+} and Ni^{2+} are able to fully activate the enzyme, Ca^{2+} has little effect on its own. Activation was synergistic with Ca^{2+} suggesting a distinct metal binding site.

1.3.5.2 Role of PP2B

PP2B is found in highest concentrations in the nervous system, where it is localised to neurons. This suggests that the enzyme plays a role in neuronal functions. PP2B has been isolated from bovine cardiac tissue where it dephosphorylates substrates for a cAMP-dependent protein kinase. In mouse

exocrine pancreas, PP2B has been identified and could participate in the control of hormonal secretion.[72] T-cell activation, in response to the interaction of the T-cell receptor with antigen, is mediated by induction of interleukin-2 gene expression.[69]

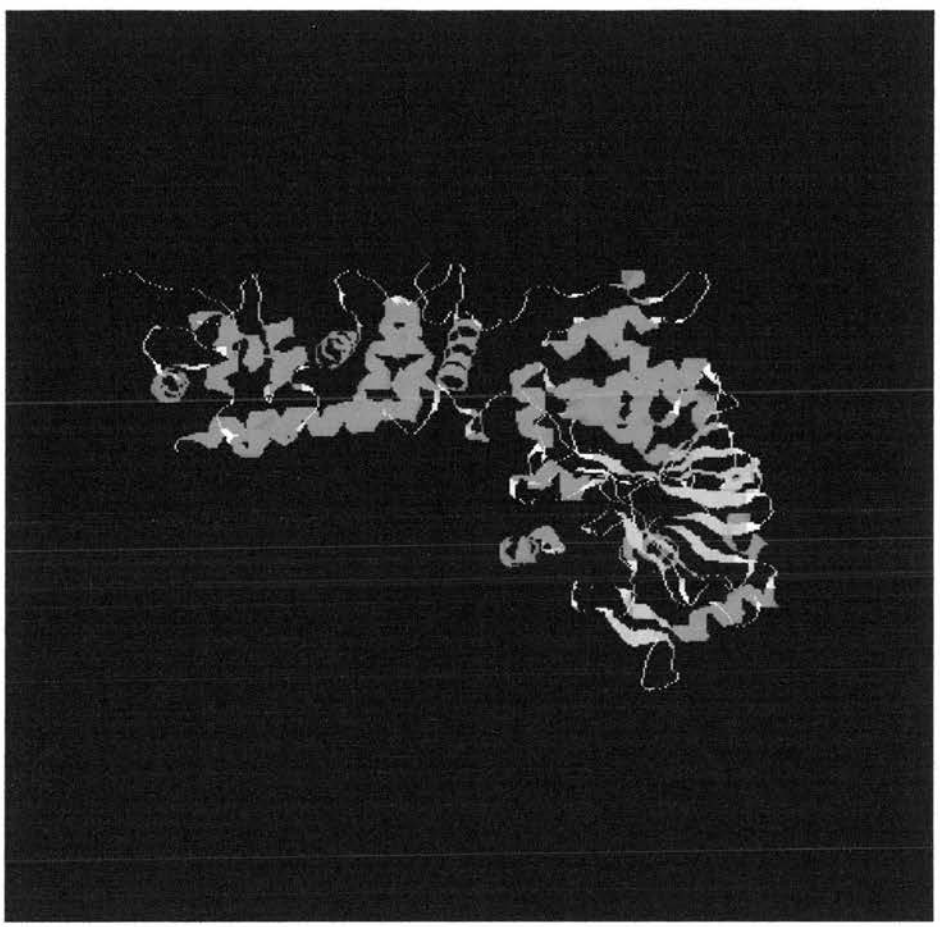


Figure 1.11: *Ribbon diagram of PP2B (calcineurin)*

1.3.5.3 Inhibitors of PP2B

Two drug-protein complexes inhibit PP-2B, cyclosporin A – cyclophilin and FK506 – FKBP.[73] These complexes bind to and inhibit the Ca^{2+} activated PP2B (calcineurin) with nanomolar affinities.[73] This prevents interleukin-2 expression and T-cell activation.

Cyclosporin A **10** (Fig. 1.12) is a lipid soluble cyclic undecapeptide produced by *Tolypocladium inflatum*, a fungus isolated from a Norwegian soil sample. Cyclosporin A has found widespread use in organ transplants as an immunosuppressant drug. FK506 **11** (Fig. 1.12) is produced by *Streptomyces tsukubaensis*, a soil bacterium from Northern Japan.[74, 75] FK506, although an immunosuppressant, is still under evaluation as a therapeutic agent.

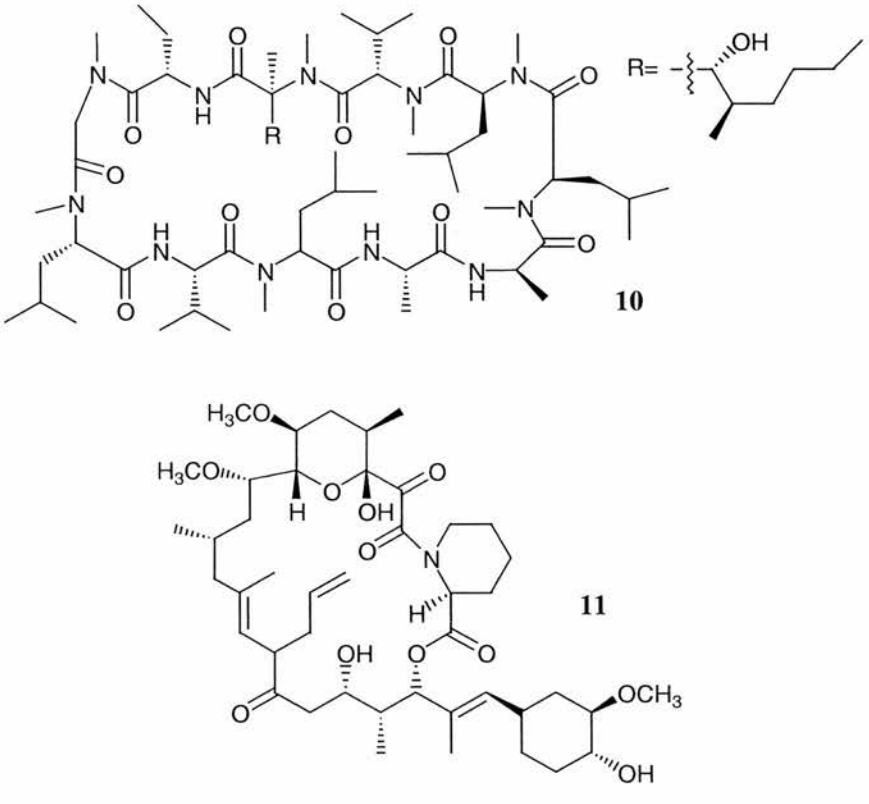


Figure 1.12: *Inhibitors of PP-2B-Cyclosporin* **10** *and FK506* **11**

Upon entering eukaryotic cells, cyclosporin A and FK506 bind to a distinct protein before binding to PP2B. Cyclosporin A binds to the protein cyclophilin, whereas FK506 binds to the protein FKBP (FK506-binding protein).[76,77]

1.3.6 Protein Phosphatase 2C (PP2C)

PP2C is a monomeric enzyme (Mr 43 kDa) requiring Mg^{2+} for activity, differing from the other Ser/ Thr protein phosphatases. It is a member of a multigene family and has a wide substrate specificity.[6]

1.3.7 Protein Phosphatase 3 (PP3)

A novel Ser/ Thr protein phosphatase, designated PP3, was isolated from bovine brain.[19] It was found to have Mr 36 kDa and to have no requirement for divalent cations to be active. It was found to preferentially dephosphorylate the β subunit of phosphorylase kinase and to be stimulated by I-2 rather than inhibited. It was inhibited by both okadaic acid and microcystin-LR with IC_{50} values between those for PP1 and PP2A, whilst for nodularin, it had a similar IC_{50} value to PP1, but lower than PP2A.[78] It was not inhibited by calyculin or tautomycin.[78] The substrate specificity, immunoblotting with wild-type antisera and the amino acid sequence of peptides derived from PP3 indicated that it was not an isoform of any known Ser/ Thr protein phosphatase.

1.3.8 Protein Phosphatase 4 (PP4)

Protein phosphatase [4], also termed PPX, is present in the cytoplasm of human cells, and more strongly in the nucleus, localised extensively into the centrosomes.[20] The centrosomes are one of the microtubule organising centres of the interphase cell and at mitosis it duplicates to form spindle-pole bodies. PP4 shares 65% amino acid identity to PP2Aα and PP2Aβ, and 45% identity to PP1 isoforms, and preferentially dephosphorylates the α subunit of phosphorylase kinase 2 fold faster than the β subunit. Comparison of the PP4 gene structure with that of

PP2A and PP1 suggests that PP4 is more closely related to PP2A than to PP1. Though PP4 shares 65% sequence activity with PP2A, it does not bind the 65kDa regulatory subunit of PP2A. PP4 is concentrated at the centromeres, PP2A is widely distributed throughout the cell. PP4 is therefore a more likely candidate as the phosphatase involved in microtubule organisation.

1.3.9. Protein Phosphatase 5 (PP5)

A human protein phosphatase (PP5) was identified from cDNA with a predicted molecular mass of 58kDa.[79] A structurally related phosphatase in *S. cerevisiae*, PPT1, was identified from its gene.[80] The polypeptide chain consists of a C terminal phosphatase catalytic domain and an N terminal domain which has 4 repeats of a 34 amino acid sequence, three of them arranged tandemly. The phosphatase domain possesses all the invariant motifs of the PP1/ PP2A/ PP2B family but less than 40% identity with any other known member. This is evidence that both PP5 and PPT1 are members of a new subfamily. The repeats in the N terminal domain are similar to tetratricopeptide repeat (TPR) motifs found in several proteins required for mitosis, transcription and RNA splicing.[79] PP5 is localised predominantly to the nucleus. Like other TPR containing proteins, PP5 may play a role in the regulation of RNA biogenesis and/ or mitosis.[79] TPR motifs have been shown to be absolutely essential for the biological function of the proteins in which they occur. Interestingly, FKBP59 (the target of the immunosuppressant FK506) possesses only 3 TPR motifs and is known to interact with PP2B.

1.3.10 Protein Phosphatase 6 (PP6)

PP6 is a 35 kDa protein with high homology with PP2A (57%), PP4 (59%), and rat PPV (98%), yet it is a distinct subgroup of the PP1/ PP2A/ PP2B family.[81] All the 42 amino acids invariant amongst the protein phosphatases and essential for catalytic activity are also present in PP6. The carboxy terminal sequence (YFL) is highly conserved amongst PP2A-like proteins and is shown to be subject to tyrosine phosphorylation in PP2A. This suggests that such a modification may be present in PP6. Three different mRNA's have been isolated, which may be additional isoforms or alternatively processed forms of PP6.

1.3.11 Protein Phosphatase λ (PPλ)

The N terminal half of the protein encoded by *orf* 221 in bacteriophage λ shows 35% identity to either PP1 or PP2A in this region.[82, 83] The phosphatase was stimulated 30-fold by Mn^{2+}, whereas Mg^{2+} and Ca^{2+} were much less effective. The phosphatase activity was unaffected by I-1, I-2, and okadaic acid.[82]

1.4 Protein Tyrosine Phosphatases

1.4.1 Introduction

Although tyrosine phosphorylation occurs to a much smaller extent than serine and threonine phosphorylation, it has become clear that Tyr phosphorylation is involved in the regulation of numerous cellular functions, such as cell cycle progression, proliferation and differentiation, metabolism, motility, cytoskeletal organisation, neuronal development, cell-cell interactions, gene transcription and the immune response.

It was originally thought that protein tyrosine kinases (PTKs) were the key enzymes controlling the *in vivo* phosphorylation of tyrosine, and that PTPases (protein tyrosine phosphatases) were small in number and functioned as 'house keeping' enzymes in opposing the action of the PTKs. However, since the isolation and characterisation of the first PTPase, PTP1B,[84, 85] cDNA cloning using the polymerase chain reaction and low stringency hybridisation have led to the discovery of more than 100 PTPases.

Amino acid sequence comparisons of the catalytic domains of the PTPases with the catalytic subunits of the protein Ser/Thr phosphatases have shown no sequence similarity. In contrast, the PTKs share sequence similarity with the serine/ threonine specific kinases. Therefore, although the two classes of phosphatases catalyse the same reaction, they have evolved completely different strategies to carry out the same task.

In addition to the differences in enzymatic mechanism, the protein Ser/ Thr phosphatases and the PTPases also differ in the structure of the holoenzyme. Whilst most Ser/ Thr phosphatases consist of multiple subunits, the PTPases are proteins consisting of a single polypeptide chain. The functional diversity within the PTPase family arises from the structural variation of regulatory and targeting sequences that are built into the same polypeptide chain as the catalytic domain, whereas in the Ser/ Thr phosphatases, the association of a limited number of catalytic subunits with a large number of regulatory and targeting subunits provides the functional diversity. Many PTPases are proteins of more than 400 amino acids, but their catalytic domains are usually contained in a span of 250 residues. This domain is the only structural element that is conserved amongst all PTPases from mammal to bacteria.

The unique feature that defines the PTPases is the active site sequence (H/V)**C**XXXXX**R**(S/T) in the catalytic domain, known as the signature motif.[86]

1.4.2 Classification

The PTPases can be primarily categorised into receptor-like and intracellular PTPases, Fig 1.13. The receptor-like PTPases, such as the leukocyte phosphatase CD45,[87] generally have an extracellular domain, a single transmembrane region and one or two cytoplasmic PTPase domains. The intracellular PTPases, such as PTP1B,[88] and the *Yersinia* PTPase,[89] contain a single catalytic domain and various amino or carboxyl terminal extensions flanking the catalytic domains, including SH2 domain (*src* homology 2), membrane associated domain, nuclear-localisation domain and cytoskeletal domains that may have targeting or regulatory functions. The PTPase signature motif can also be found in the structures of two additional groups of phosphatases that can hydrolyse phosphotyrosine residues, namely the VH1-like dual specificity phosphatases,[8] and the low-molecular-weight phosphatases.[90] The low-molecular-weight and dual specificity phosphatase share little amino acid sequence identity with the intracellular and transmembrane PTPases. The only similarities are the relative placements of the essential Cys and Arg residues in the active site that constitute the signature motif. The dual specificity phosphatases are able to utilise phosphothreonine and phosphoserine containing substrates as well as those containing phosphotyrosine. The low-molecular-weight phosphatases show substrate specificity to phosphotyrosine, though they also show weak activity towards phosphothreonine and phosphoserine.[90] The phosphotyrosine superfamily is summarised schematically in Fig 1.13 below.

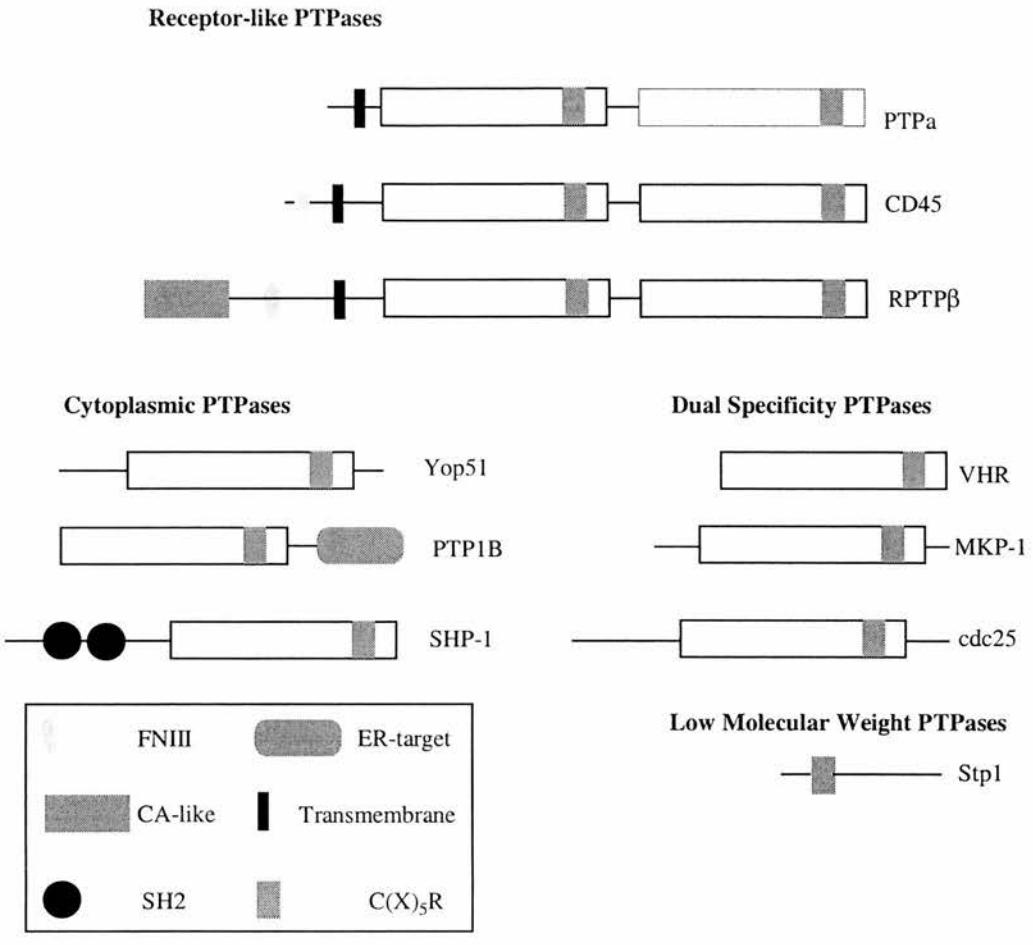


Figure 1.13: *The protein-tyrosine phosphatase superfamily.*

The crystal structures of the catalytic domains of three PTPases, PTP1B (Fig. 1.14),[91] the *Yersinia* PTPase,[92] and PTPα,[93] have been determined. Structural comparisons between these structures indicate that key structural features that are important for catalysis are also conserved amongst the three groups of phosphatases. Therefore, based on the similar biochemical and structural properties, the PTPases can be grouped into three sub-families: the tyrosine specific phosphatases (the classic PTPases), the dual specificity phosphatases and the low-molecular-weight phosphatases, Fig 1.13. The N-terminal domains of tensin and auxilin also show amino acid sequence similarity to the PTPases, though it is not clear whether they possess any phosphatase activity.[94] Therefore, it is important to characterise

enzymatic activity and test for substrate specificity, and not to make functional assignments of newly discovered gene products based purely on sequence homology analysis.

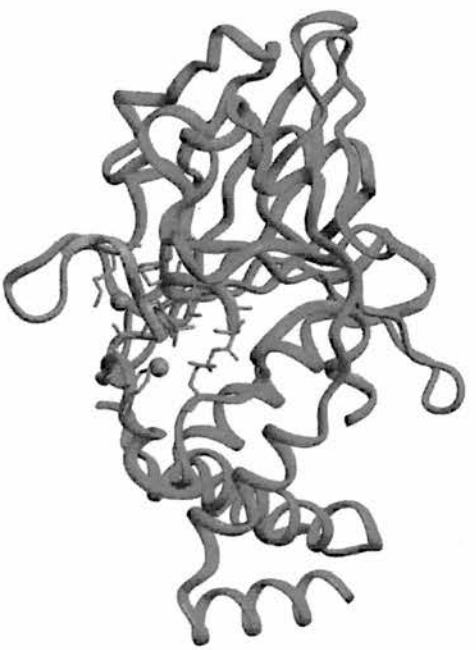


Figure 1.14: *Ribbon diagram of PTP1B (green ribbon) with substrate DADEpYL hexapeptide (purple ribbon) and structural water molecules (red spheres) bound.*

1.4.3 PTPases and Disease

1.4.3.1 PTPases and Cancers

The level of tyrosine phosphorylation and therefore the strength and duration of the signal transmitted, is balanced by the opposing action of PTKs and PTPases. Deregulated PTKs, such as *src*, *lck* and *neu* can function as dominant oncogenes,[95] and thus it has been expected that some PTPases function as products of tumour suppressor genes. The gene for the receptor like PTPγ has also been proposed to be a tumour suppressor gene, which is located on chromosome 3p21, a segment frequently altered in renal and lung carcinomas.[96] In addition, expression of PTP1B

has been shown to block transformation mediated by *neu*,[97] and partially revert transformation by *src*.[98]

There is mounting evidence that some PTPases can potentiate, rather than antagonise, the action of PTKs. This behavior enhances mitogenic signalling and can lead to cellular transformation.

1.4.3.2 PTPases and Diabetes

Tyrosine phosphorylation is a key reaction in the initiation and propagation of insulin action.[99] Insulin action is mediated by the insulin receptor, a transmembrane glycoprotein with intrinsic PTK activity. The insulin receptor is activated by insulin binding and undergoes autophosphorylation, leading to tyrosine phosphorylation of intracellular proteins. In most cell types, the predominant substrate for this phosphorylation is the protein known as insulin receptor substrate 1. The phosphorylation on Tyr residues on the insulin receptor and insulin receptor substrate 1 generate docking sites for other enzymes and effector molecules containing SH2 domains or phosphotyrosine domains to propagate the signal. The termination of insulin action, after insulin withdrawal, requires the dephosphorylation of both insulin receptor and insulin receptor substrate 1. Several PTPases, such as PTP1B,[100] and PTPα,[101] have been implicated as negative regulators of the insulin receptor signalling. Therefore the development of specific inhibitors of the PTPases acting on the insulin receptor or its substrates may enhance insulin action in diseases with insulin resistance such as NIDDM (non-insulin dependent *diabetes mellitus*).

1.4.3.3 PTPases and Infectious Diseases

A number of bacterial and viral pathogens have developed sophisticated strategies to subvert the host-call signaling pathways for their own benefit. The pathogenic bacterium *Yersinia* encodes a PTPase essential for its virulence. The genus *Yersinia* comprises three species of bacteria causing diseases ranging from gastrointestinal syndromes to the Bubonic Plague.[102] *Yersinia pestis* is the causative agent of the Black Death (the Bubonic Plague), so known because it was responsible for the death of some 25 million inhabitants of Europe in the 15th Century.[102] The *Yersinia* PTPase is obligatory for pathogenesis and the phosphatase activity of the PTPase is essential for virulence. As bacteria are believed to contain no tyrosine phosphorylated proteins, the targets for the *Yersinia* PTPase are likely to be host proteins. One of the substrates of the *Yersinia* PTPase in human epithelial cells was identified as p130^{cas}. This is a novel docking protein localised to focal adhesions and is phosphorylated on numerous Tyr residues in response to integrin engagement. This explains the ability of *Yersinia* PTPase to promote cell detachment from the extracellular matrix. The *vaccinia* virus encodes a dual specificity phosphatase, VH1, essential for viral transcription and infectivity. PTPase activity has also been detected in parasites such as *Leishmania donovani*, *Trypanosoma brucei*, and *Trypanosoma cruzi*.

1.4.4 Structure of the PTPases.

1.4.4.1 Overview

The tyrosine specific phosphatases, the VH1-like dual specificity phosphatases and the low-molecular-weight phosphatases share little amino acid sequence identity. The only similarity amongst the three classes of phosphatases are

the relative placement of the essential cysteine and argenine residues in the active sites that constitute the PTPase signature motif (H/V)**C**XXXXX**R**(S/T) (Figure 1.15).[103, 104]

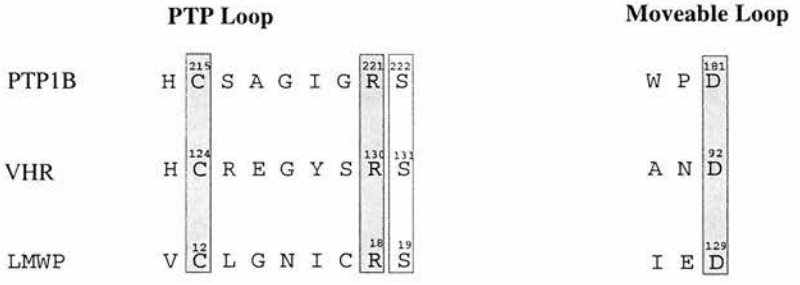


Figure 1.15: *Conserved structural elements for catalysis for the PTPases (PTP1B), dual specificity phosphatases (VHR) and the low-molecular-weight phosphatases (LMWP)*

The *Yersinia* PTPase shares only 5% sequence homology with the mammalian PTPases, PTP1 and VHR,[105] but 20% homology within the catalytic domain.[106] However, the structures share a common secondary structure, with close similarity in tertiary structure. The PTPases are $\alpha + \beta$ proteins with tertiary folds composed of a highly twisted mixed β-sheet flanked by α-helices on both sides.[92] The three-dimensional structure of the dual specificity phosphatase VHR reveals a general fold that occurs in *Yersinia* PTPase, PTP1B, and PTPα. As well as the lack of sequence homology between the low-molecular-weight phosphatases and the PTPases, the low-molecular-weight phosphatases are also smaller in size and contain the PTPase signature motif close to the amino terminus of the protein, whereas the signature motif occurs closer to the carboxy terminus of the PTPases and the dual specificity phosphatases. The residues of the signature motif form a loop structure that is defined as the PTP loop that binds the phosphoryl group of the substrate.

This PTP loop is located between the β-turn of the carboxy terminus of a β-strand and the first turn of the α-helix. The different phosphatase structures are striking examples of convergent evolution achieving highly similar active site clefts, and the similarity in active site motifs may suggest a common mechanism to bring about phosphate monoester hydrolysis in the disparate molecules.

1.4.4.2 The Active Site

The phosphatase active site is located within a crevice on the molecular surface, ~9 Å deep for the tyrosine specific phosphatases,[107] and ~6 Å deep for VHR.[105] The central feature is a strand-loop-helix element where the loop (PTP loop) contains the PTPase signature motif ((H/V)**C**XXXXX**R**(S/T)).[103, 104] The active site pocket is formed by the PTP loop at its base and surrounded by loops that provide an essential Asp general acid and residues that interact with the pTyr or pSer/ pThr in peptide substrates, Fig 1.16. The oxygen atoms of the oxyanion make two hydrogen bonds with the guanadinium group of the invariant Arg residue in the PTP loop. Kinetic studies have shown that the invariant Arg residue in the signature motif plays an important part in substrate binding and transition state stabilisation.[86] The oxygen atoms of the oxyanion are also hydrogen bonded to the NH amides of the peptide backbone making up the PTP loop.

The invariant Cys residue in the PTP loop has been shown to be essential for phosphatase activity and formation of the covalent cysteinyl phosphoenzyme intermediate.[108-111] The apparent thiol pK_a of the active site Cys was found to be 4.7 in the *Yersinia* PTPase,[103] 5.4 in PTP1,[112] and 5.6 in VHR,[113] indicating that the Cys exists as a thiolate ion at physiological pH. The thiolate is stabilised by a number of structural elements within the active site. The Cys Sγ is at the centre of

the PTP loop and within 3-4 Å from every amide nitrogen of the backbone of the PTP loop. The orientation of these microdipoles is essential for phosphate binding and thiolate stabilisation. Finally, the guanidinium group of the Arg residue and the hydroxyl group in the Ser/ Thr residue of the PTP loop may also help to lower the thiol pK_a.[91]

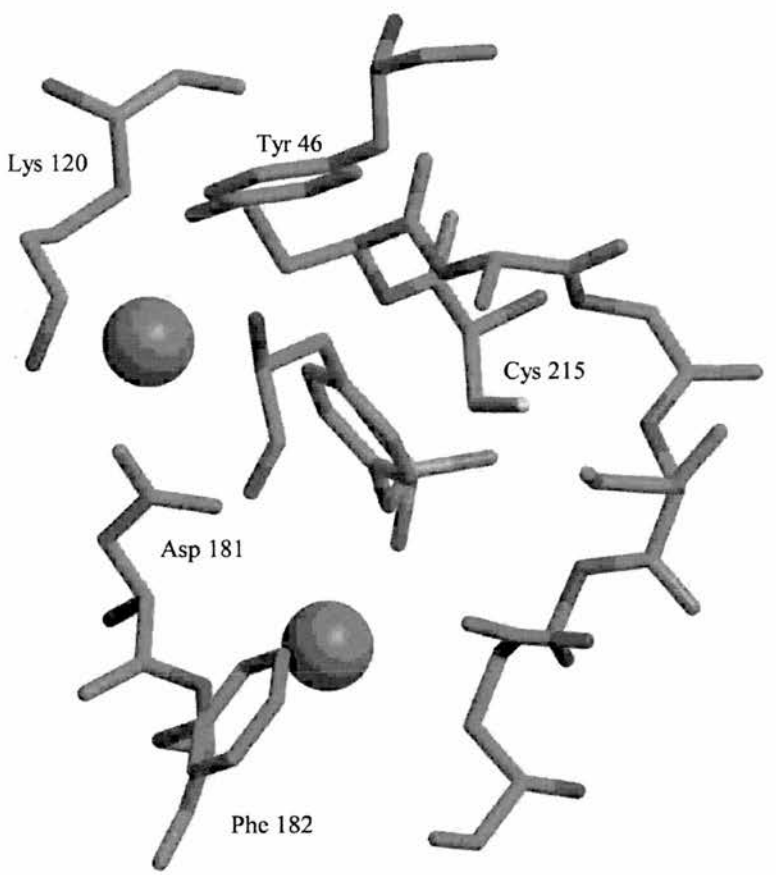


Figure 1.16: *Active site of PTP1B, showing catlytic residues, structural water molecules (red spheres) and phosphotyrosine substrate..*

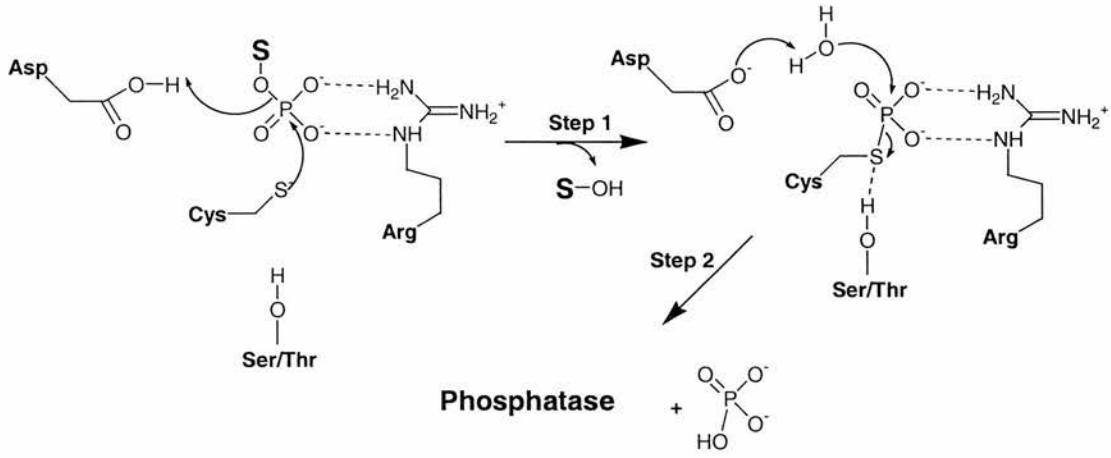
1.4.5 Mechanism of Catalysis

The mechanism of phosphate monoester hydrolysis has been the subject of intense investigation for over 40 years. Enzymological and structural studies have

led to the conclusion that the reactions catalysed by the PTPases, dual specificity phosphatases and low-molecular-weight phosphatases share a common mechanism.[114] Site directed mutagenesis show that the Cys residue is required for phosphatase activity.[111, 115] The Cys residue is phosphorylated through a thiophosphate linkage ($-S-PO_3^{2-}$) during catalysis, which suggests a double displacement mechanism, in which the phosphate group is first transferred to the thiol of the Cys residue, forming a phosphoenzyme intermediate, which is then hydrolysed by water in the second step, Scheme 1.1.

Mutation of the invariant Arg residue in the PTPase motif resulted in loss of activity for two receptor like PTPases, LAR and CD45,[116, 117] and bovine low-molecular-weight phosphatase. It was not clear however, whether the Arg was required for structure or catalysis or both. The high intrinsic activity of the *Yersinia* PTPase has made it possible to examine the effect of changing the invariant Arg residue upon the rate of catalysis. Upon mutation of Arg409 to alanine (R409A), an 8200-fold decrease in k_{cat} and a 26-fold increase in k_m was observed.[86] Similar results were obtained for the PTP1B.[118] This suggests that although the Arg residue in the PTP loop plays a role in substrate binding, it plays a much more important role in transition state stabilisation. In addition to the essential Cys and Arg residues, a conserved Ser or Thr can also be found in the PTPase signature motif immediately after the Arg residue. The role of this residue is to facilitate the breakdown of the phosphoenzyme intermediate through the conserved hydroxyl group, Scheme 1.1. This involves the Cys residue acting as a nucleophile to attack the phosphate ester, forming a thiophosphate intermediate and the arginine residue playing a role in substrate recognition and transition state stabilisation. Protonation of the leaving group oxygen by the conserved Asp residue, acting as a general acid,

facilitates the formation of the cysteinyl phosphate intermediate. After formation of the phosphoenzyme intermediate, the dephosphorylation occurs by attack of a water molecule that approaches from the site just vacated by the leaving group, resulting in the release of inorganic phosphate.



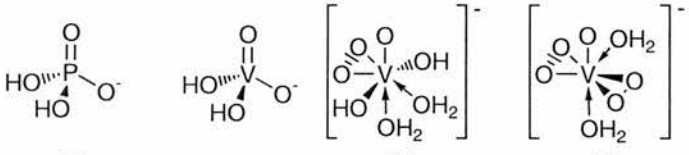
Scheme 1.1: *Common chemical mechanism for PTPase catalysed hydrolysis*

The same Asp residue also functions as a general base in the second hydrolysis step, by activating and/ or positioning the water molecule for hydrolysis of the phosphoenzyme intermediate. The conserved Ser/ Thr residue immediately following the invariant Arg residue in the PTP loop functions to facilitate the breakdown of the phosphoenzyme intermediate.

1.4.6 Inhibitors of PTPases

Specific inhibitors of protein tyrosine phosphatases are invaluable tools for elucidating the function of individual PTPases within cells and for studying the catalytic mechanism of the phosphatase enzymes. Inhibitors of PTPases fall into two broad categories; low-molecular-weight transition metal-containing complexes and low-molecular-weight non-metal containing complexes.

A number of groups report the efficacy of transition metal oxo-anions as inhibitors of protein tyrosine phosphatases.[119-121] Vanadate, molybdate and tungstate may inhibit tyrosine phosphatase by forming complexes which resemble the trigonal bipyramidal geometry of the $S_N2(P)$ transition state, Fig. 1.17. The crystal structure of low-molecular-weight phosphotyrosyl phosphatase complexed with vanadate and molybdate shows that vanadate forms a covalent linkage to Cys12 of the active site and displays the predicted trigonal bipyramidal geometry.[119] The weaker molybdate complex, however, exhibits pyramidal geometry. Therefore vanadate inhibits the PTPases by acting as a transition state analogue (K_i 0.38±0.02 μM).[120] In comparison, pervanadate, the complex formed by vanadate and hydrogen peroxide, inhibits PTPases by irreversibly oxidising the catalytic Cys residue in the active site.

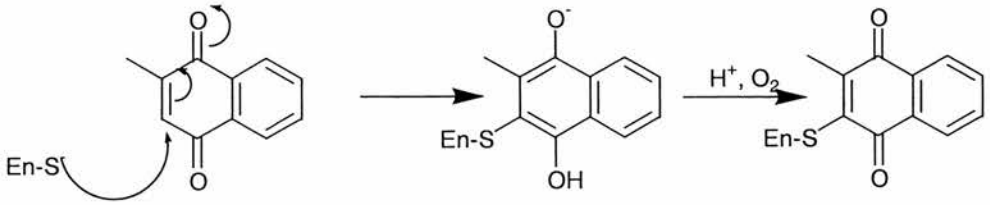


12 **13** **14a** **14b**

Figure 1.17:*Phosphate **12**, vanadate **13** and pervanadate **14**. Pervanadate exists in a number of complexes. Shown here are the monoperoxo (VL, **14a**) and diperoxo (VL_2, **14b**) forms. Exact co-ordination geometry, number and arrangement of water ligands is uncertain.*

Non-metal-containing inhibitors are predominantly based on phosphonates, non-hydrolysable phosphotyrosine mimetics, though there are two notable exceptions. Menadione (vitamin K_3, **15**) has been shown to exhibit a broad range of antitumour activity in human cells.[122] This is believed to be due to the irreversible

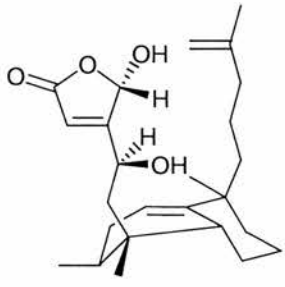
inhibition of cd25 by modification of the active site Cys thiolate of cd25 (Scheme 1.2, below).



15

Scheme 1.2: *Mechanism of inhibition of cd25 by menadione* ***15***.

Dysidiolide, **16**, is a sesteterpene isolated from the Caribbean sponge *Dysidea etheria.*[123] Dysidiolide has been shown to block the action of the protein phosphatase cd25a, responsible for signalling cells to undergo mitotic division. The method of inhibition is not known at present.



16

There have been a number of reports of phosphonate based inhibitors of the PTPases. Phosphonates are good substrate mimics for the phosphatases with a non-hydrolysable P-C bond in place of the scissile P-O bond present in the phosphate.

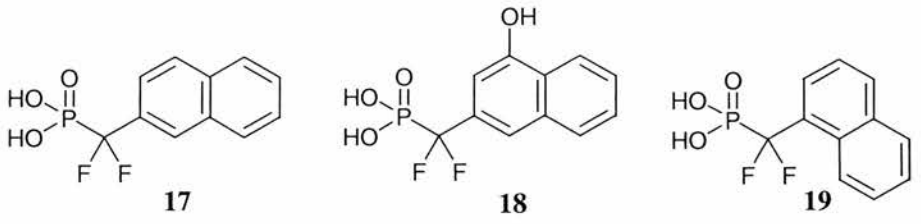
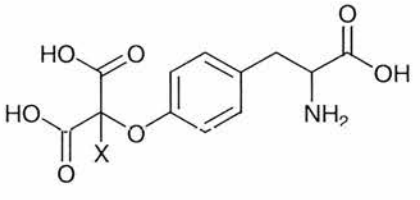


Figure 1.18: *Difluoromethylenenapthylphosphonate-based inhibitors of PTPases.*

The three difluoromethylene phosphonate inhibitors **17-19** (Fig. 1.18) all show micromolar inhibition of protein tyrosine phosphatases.[124, 125] The presence of the *m*-OH group in **18** allows it to displace a water molecule and hydrogen bond to Lys120 and Tyr46 in PTP1B, giving it a doubling in inhibitor strength over the non hydroxyl analogue **17**.[125] The use of fluoromethylene phosphonates reinstates hydrogen-bonding interactions lost on going from the phosphate to the phosphonate. The analogous dihydromethylene phosphonates typically inhibit in the millimolar range. The difluorophosphonate inhibitors have poor cellular penetration and a lack of suitable bioreversible protection. This has led to the development of the non-phosphonate containing phosphotyrosine mimetics *O*-malonyltyrosine (OMT, **20**) and *O*-fluoromalonyltyrosine (FOMT, **21**), Fig. 1.19.[126]



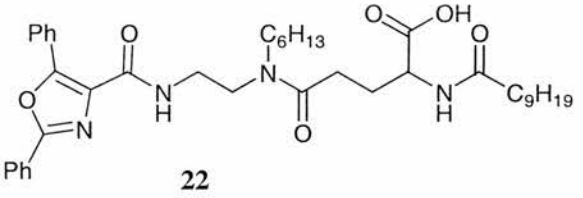
X=H, OMT **20** X=F, FOMT **21**

Figure 1.19: O-*malonyltyrosine **20** & O-fluoromalonyltyrosine **21***

The dicarboxylic acid motif offers prodrug protection strategies. When **20** and **21** were incorporated into a substrate sequence corresponding to one of the

autophosphorylation sites of EGF receptor ($EGFR_{988-993}$),[127] AcDADE-**Xaa**-L-amide, the resultant peptides were shown to be micromolar inhibitors of PTP1B (**Xaa** = **20**, IC_{50}=10 μM; **Xaa** = **21**, IC_{50}=1 μM). The hydrogen-bonding effect of the replacement of the proton by fluorine can be seen here in the 10-fold increase in potency of **21** over **20**.

Recently, the power of combinatorial chemistry has been harnessed in the search for new inhibitors. Using a pharmacophore modelled on natural product inhibitors of the phosphothreonine phosphatases, a refined library of phosphate free small molecule compounds were synthesised in a parallel, solid phase combinatorial-based approach.[128] Of the members of the library, AC-αα69 **22**, was shown to be a micromolar inhibitor of Cdc25a (K_i=10 μM) and a sub-micromolar inhibitor of PTP1B (K_i=0.85 μM)



22

1.5 Phosphorylating Reagents

1.5.1 Oligonucleotide Synthesis

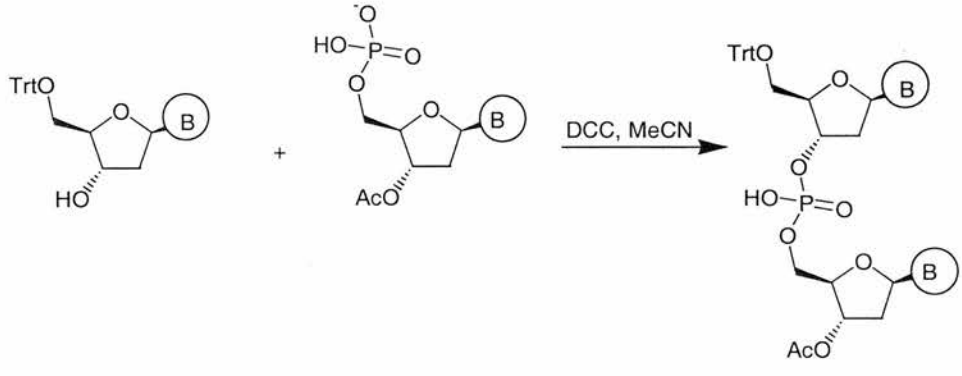
In the 1950s, after the 2° structure of DNA was discovered by Watson and Crick,[129] the chemical synthesis of DNA and RNA became a mission for chemists. The initial challenge was to achieve controlled polymer assembly through high yielding, rapid coupling of monomeric units. The monomeric units would be nucleotides, obtained either from natural sources or by chemical synthesis, which could then be 'zipped' together through the formation of internucleotidic phosphate

linkages in a rapid and effective manner. At first, this may appear to be deceptively simple, but given the high degree of functionality of the nucleotide, the cross reactivity of the various functionalities and the requirement for quick and near quantitative 'zipping' reactions, the synthesis of nucleic acids turned out to be far from facile.

Initial studies of phosphorylation reactions resulted in the introduction of diphenyl chlorophosphate and phenyl dichlorophosphate. Initial studies by Todd *et al.* on the synthesis of ADP and ATP revealed the complexity of the phosphate linkage.[130, 131] The reactivity of the phosphoryl group had to be precisely controlled to limit the formation of by-products and allow sequential addition of nucleosidic units. To overcome these limitations, Khorana *et al.* developed the phosphodiester approach to 'gene synthesis'.[132, 133]

1.5.1.1 The Phosphodiester Approach

The phosphodiester approach, as developed by Khorana,[132, 133] involves reacting a nucleoside having a 5'-hydroxyl group protected with a trityl group and a free 3'-hydroxyl with a nucleotide bearing a 5'-phosphate group and a 3'-acetyl protecting group, Scheme 1.3.



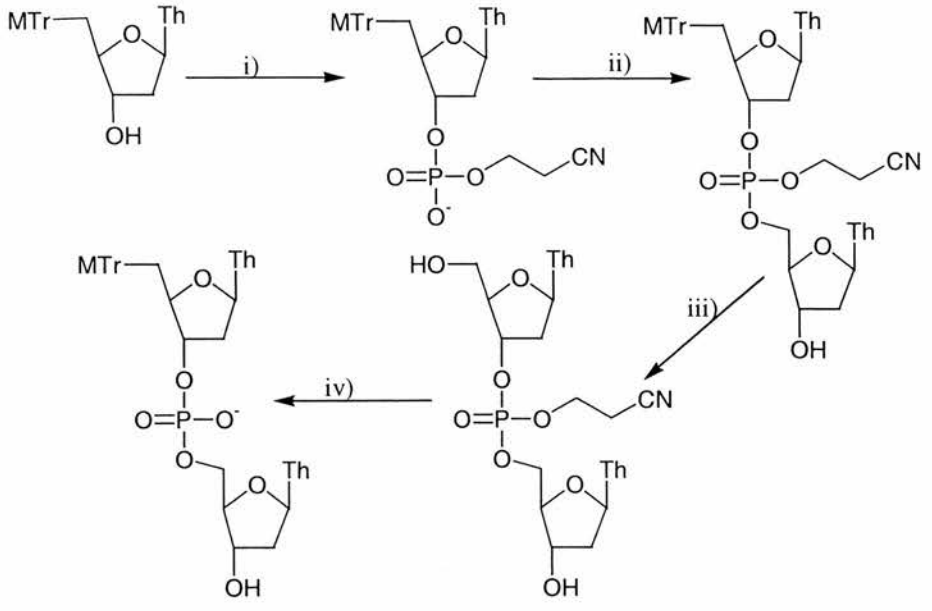
Scheme 1.3: *Phosphodiester approach to oligonucleotide synthesis*

Activation of the nucleoside-5'-phosphate in the presence of DCC followed by reaction with the nucleoside gave the nucleoside phosphodiester. Hydrolysis of the acetyl protecting group under mildly basic conditions, followed by addition of another nucleoside-5'-phosphate unit allows elongation of the polymer. This method became an established procedure and culminated in the landmark synthesis of genes encoding alanine tRNA from yeast and suppressor tRNA from *E. coli*.[133] However, the presence of unprotected phosphates and an internucleotidic phosphodiester group led to the formation of oligomers having pyrophosphate linkages and chain branched products and required time consuming chromatographic separation of the products. The limitations of the phosphodiester approach led to the development of a practical phosphotriester approach by Letsinger,[134] and later by Reese.[135]

1.5.1.2 The Phosphotriester Approach

In the phosphotriester approach, a nucleoside with the 3'-hydroxyl moiety derivatised as the β-cyanoethyl phosphodiester and the 5'-hydroxyl protected with the methoxytrityl group is coupled to a nucleoside having a free 5' hydroxyl group, Scheme 1.4. The resulting dinucleoside phosphotriester could be readily purified by silica gel chromatography and reacted on to form oligonucleotides.

Both the phospho-diester and –triester were developed for solution phase synthesis, imposing a necessary requirement for purification of the products after each step, a tedious and time consuming process in oligonucleotide synthesis.



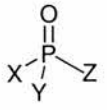
Scheme 1.4: *Phosphotriester approach to oligonucleotide synthesis*

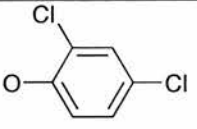
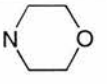
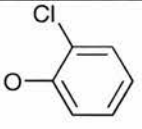
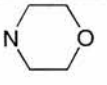
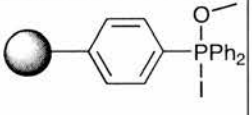
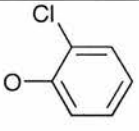
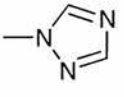
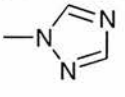
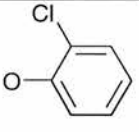
Following Merrifield's seminal work on solid phase peptide synthesis,[136] Letsinger and Mahadevan investigated the use of organic polymers as solid supports.[137, 138]

In addition to the production of various primers, probes and linkers, advances in solid phase oligomer synthesis has provided molecular biologists and biochemists with modified oligonucleotides to study protein DNA-interactions.[139]

A table of phosphorylating agents that have appeared in the literature is given below, Table 1.2.

Table 1.2: *Phosphorylating Agents for Oligonucleotide Synthesis.*

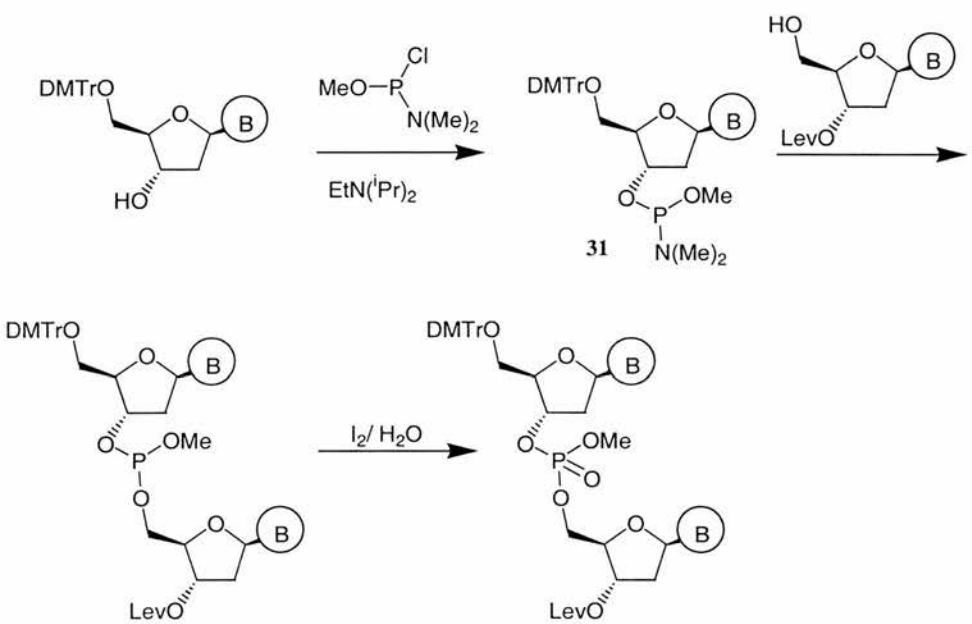


X	Y	Z	Ref.
Cl		SMe	**23**[140]
OBt	OBt		**24**[140]
$MTrNH(CH_2)_2S$	O^-		**25**[141]
Cl	OCH_2CCl_3		**26**[142]
	OPh	OPh	**27**[143]
MeC_6H_4S	OBt	OBt	**28**[140]
			**29**[144]
	OBt	OBt	**30**[145]

1.5.1.3 The Phosphoramidite (Phosphite Triester) Approach

The higher reactivity of P(III) species compared to P(V) is well known. This led Letsinger and Lunsford to investigate the application of phosphite triester

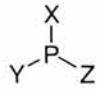
methodology in oligonucleotide synthesis.[146] Attempts by the Caruthers group to automate this methodology using silica gel as a solid support were unsuccessful, as the chlorophosphite intermediates used were highly reactive and difficult to manipulate.[147, 148] Work by Beaucage and Caruthers led to the development of nucleoside phosphoramidites of the general structure **31**, Scheme 1.5.[149] These phosphoramidites are readily prepared and stored as dry powders under an inert atmosphere.



Scheme 1.5: *Phosphoramidite approach to oligonucleotide synthesis*

Coupling of the phosphoramidite to the 3'-protected nucleoside is achieved by activation of the phosphoramidite by a weak acid such as 1-*H*-tetrazole, to give the phosphite triester. After oxidation to the phosphate triester, and deprotection of the 3'-terminus, further chain elongation is possible. The phosphite triester approach, as originally devised by Letsinger *et al.* for synthesis of

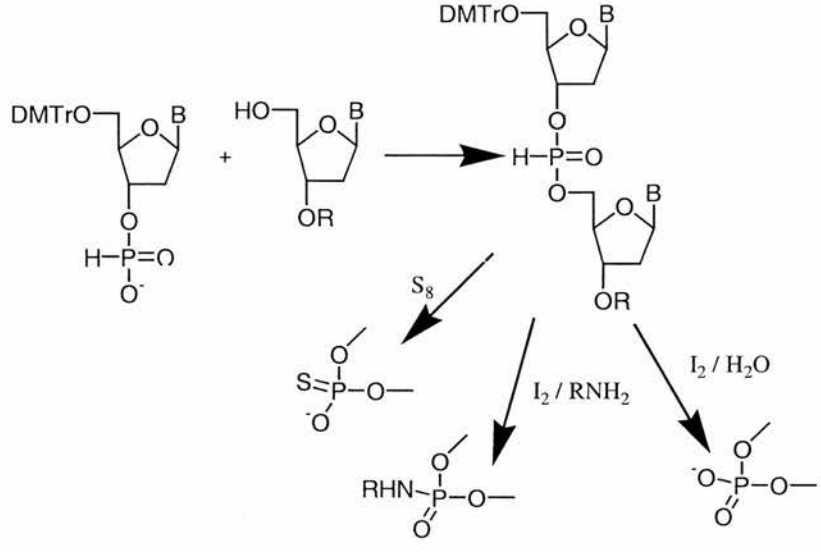
deoxyoligonucleotides on the solid support,[150] generally utilises phosphitylating reagents of the general structure:



A table of phosphitylating reagents that have appeared in the literature is given in Table 1.3, overleaf.

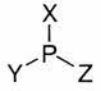
1.5.1.4. The H-phosphonate Approach.

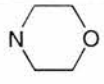
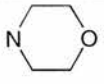
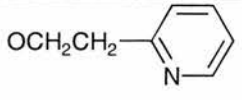
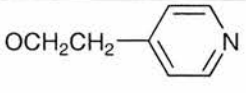
In the *H*-phosphonate approach, a deoxyribonucleoside 3'-*H*-phosphonate is condensed with a 3'-hydroxyl protected deoxyribonucleoside to produce a dinucleoside phosphotriester which after deprotection gives the dinucleotide. A distinctive feature of the *H*-phosphonate approach is that the oxidation of the internucleotidic *H*-phosphonate is performed only once, at the end of chain assembly. In addition, the *H*-phosphonate can be converted into a variety of functionalities by simply choosing the appropriate reagents, Scheme 1.6.[151]

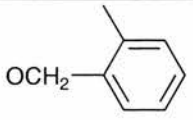
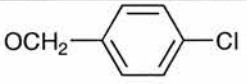
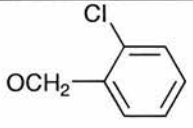
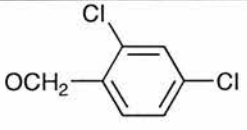
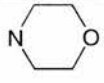
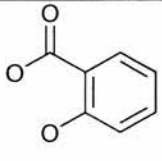


Scheme 1.6: *H-Phosphonate method of oligonucleotide synthesis*

Table 1.3: *Phosphitylating Reagents for Oligonucleotide Synthesis.*



X	Y	Z	Ref.
Cl	$N(^iPr)_2$	OMe	**32**[152]
Cl	$N(^iPr)_2$	OCH_2CH_2CN	**33**[153]
$N(^iPr)_2$	$N(^iPr)_2$	OMe	**34**[154]
Cl	$N(^iPr)_2$	$N(^iPr)_2$	**35**[155]
$N(^iPr)_2$	$N(^iPr)_2$	OCH_2CH_2CN	**36**[156]
Cl	Cl	$N(^iPr)_2$	**37**[157]
Cl	Cl	OCH_2CCl_3	**38**[158]
$H_2C=CHCH_2O$	$N(^iPr)_2$	$H_2C=CHCH_2O$	**39**[159]
OCH_2CH_2CN	$N(iPr)_2$	$OCH_2CH_2SiPh_3$	**40**[160]
NEt_2	NEt_2	$OCH_2CH_2SO_2Me$	**41**[155]
$N(^iPr)_2$	$N(^iPr)_2$	$OCH_2CH_2SO_2Me$	**42**[155]
		$OCH_2CH_2SO_2Me$	**43**[161]
$N(^iPr)_2$	$N(^iPr)_2$	OCH_2CCl_3	**44**[162]
$N(^iPr)_2$	$N(^iPr)_2$	$OCH_2CH_2C_6H_4NO_2$	**45**[162]
$N(^iPr)_2$	$N(^iPr)_2$		**46**[162, 163]
$N(^iPr)_2$	$N(^iPr)_2$		**47**[164]

$N(^iPr)_2$	$N(^iPr)_2$		**48**[161]
$N(^iPr)_2$	$N(^iPr)_2$		**49**[161]
$N(^iPr)_2$	$N(^iPr)_2$		**50**[161]
$N(^iPr)_2$	$N(^iPr)_2$		**51**[161]
NEt_2	NEt_2	$OCH_2CH{=}CH_2$	**52**[165]
NEt_2	NEt_2	OPh	**53**[166]
NEt_2	NEt_2	$OC_6H_4NO_2$	**54**[166]
NEt_2	NEt_2	OC_6F_5	**55**[166]
NEt_2	NEt_2	OC_6Cl_5	**56**[166]
NEt_2	NEt_2	$OCH(CF_3)_2$	**57**[167]
	Cl	OMe	**58**[168]
Cl			**59**[157, 169]

1.5.2 Modified Oligonucleotides

As the synthetic methods for oligonucleotide synthesis become more firmly established, there has been a greater demand for the synthesis of modified oligonucleotides. Modified oligonucleotides and synthetic DNA have a number of

applications in molecular biology, such as DNA sequencing primers, probes, adapters in gene synthesis.[170] Applications are also possible in diagnostics and forensic testing.[171, 172] There is therapeutic potential in the form of 'antisense oligonucleotides', ribosymes and triplex DNA.[171-173] Modification of the 5'-hydroxyl group into an aminoalkyl, mercapto, aldehyde or phosphoryl group has been reported.[174] An aliphatic amino- or mercapto- group at the 5' end, where the mercapto- or amino- group is attached to a fluorescent compound, is used as a primer in DNA sequence determination. When the amino group is linked to biotin or EDTA it is used as a hybridisation probe for certain DNA or in DNA strand cleavage studies. Where the 5' substituent is a phosphoryl group, it is used as a substrate for DNA ligase reactions and is used directly in the synthesis of protein genes.

1.5.3 Synthesis of RNA

The presence of the 2'-hydroxyl group in ribonucleosides introduces an added facet of complexity in the synthesis of RNA. The vicinal 2'-and 3'-hydroxyl groups are in a *cis*- orientation and consequently the 2'-hydroxyl can participate in neighbouring group interactions with many 3'-substituents. This can result in 3'- to 2'- migration or chain cleavage of the RNA under certain conditions.[175]

The chemical synthesis of RNA fragments is further complicated by the presence of the 2'-hydroxyl group which must be transiently protected during oligonucleotide synthesis. This is usually achieved by the use of silyl-based protecting groups, which can be selectively removed by such reagents as TBAF (tetra-*n*-butylammonium fluoride).

Both chloro-β-cyanoethyl-*N*,*N*-diisopropylamino-phosphoramidite **36** and salicylchlorophosphite **59** (Table 1.3) have been used in the synthesis of fragments of the cell wall of *Staph. lactis*.[176] This involves the introduction of an α(1-6) interglycosidic bond between two suitably protected *N*-acetyl-D-glucosamine units.

1.5.4 Phosphopeptide Synthesis

As has been mentioned previously (Section 1.1), protein phosphorylation is now widely recognised as one of the major processes regulated by protein kinases and phosphatases. Protein phosphorylation has been linked to carcinogenesis and there is also evidence for its role in certain diseases. The recognition that the protein phosphatases play a fundamental part in human metabolism has prompted immense interest in the physiological targets of these phosphatases. Elucidation of the chemical mechanism of action of these enzymes is dependent on the availability in sufficient quantities of phosphopeptides and phosphoproteins. Traditionally, the synthesis of phosphopeptides has been performed using either enzymatic methods, using ATP dependent protein kinases, or chemical methods, involving incorporation of phosphoamino acids in the growing peptide chain, or phosphorylation of the fully assembled peptide prior to purification.

Enzymatic methods, utilising protein kinases, are limited in two ways. Firstly, though enzymatic phosphorylation is attractive, it is limited by the substrate specificity of the available protein kinases. Secondly, the amount of phosphorylated substrate attainable is limited by the amount of kinase isolable at any one time. With the availability of recombinant protein kinases, the problem of obtaining sufficient quantity of phosphoprotein has been overcome, yet the restricted sequences available remains a major problem. Unlike protein phosphatases, protein

kinases display a marked preference for serine residues over threonine residues, therefore the amount of peptide containing a phosphorylated threonine residue will be quite low. Consequently, the synthesis of appreciable quantities of phosphopeptide requires multiple incubations of the substrate with the appropriate protein kinase.

The chemical synthesis of phosphopeptides currently employs two synthetic strategies, the building block approach, which uses preformed phosphoamino acids with suitably protected functional groups, and global phosphorylation, whereby phosphorylation occurs after complete assembly of the peptide sequence, Scheme 1.7.

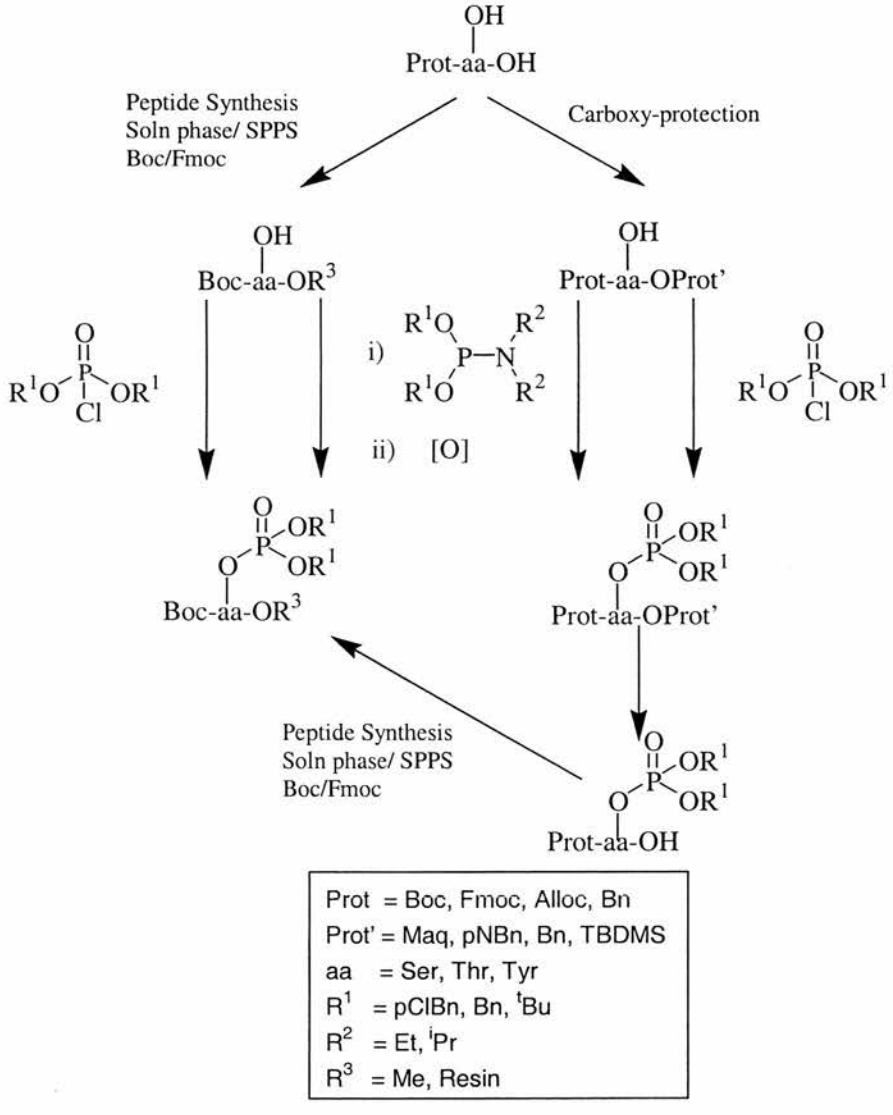
Each method has its advantages and drawbacks, and the choice of which method is most applicable will depend on the sequence under consideration.

1.5.4.1 Building Block Approach

This is, in principle, the more straightforward approach to preparing phosphopeptides, since it involves only incorporation of a suitably protected phosphoamino acid into the growing peptide chain. However, the synthesis of this phosphoamino acid may involve a number of synthetic steps and laborious purification steps.

A wide range of protecting groups have been evaluated for masking of the phosphate moiety, and these include phenyl,[177] trichloroethyl,[178] methyl,[153] benzyl,[179] and t-butyl.[180] The phenyl group has been widely used in the protection of phosphoserine in Boc based synthesis both in solution and on the solid phase.[177] Removal of the phenyl groups is achieved by hydrogenation over PtO_2.[181]

Cleavage of the resin is mediated by hydrogenolysis over a Pd catalyst,[181] since treatment with HF has been shown to cause substantial dephosphorylation of the phosphopeptide. The trichloroethyl protecting group is claimed to be superior to the phenyl protecting group as it does not require substantial amounts of expensive PtO_2, but can be removed under mild conditions using Zn dust in AcOH, or by hydrogenolysis over Pd in aqueous ethanol.[182]



Scheme 1.7: *Synthesis of Phosphopeptides by the global phosphorylation and phosphoamino acid strategies.*

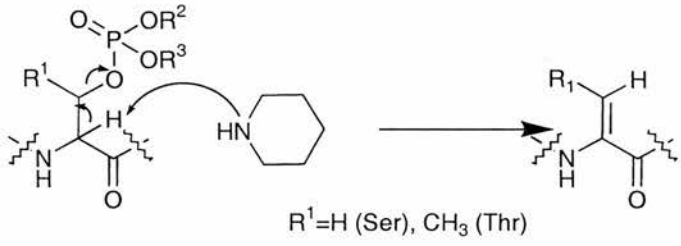
In the Boc based synthesis of phosphotyrosine containing peptides, ethyl, benzyl and methyl derivatives have been considered for the introduction of the phosphotyrosine into the peptide chain.[183] The methyl derivative, Boc-Tyr(OPO_3Me_2)-OH, has been found to be the most suitable with respect to stability during chain extension and ease of removal during acidolysis. Kitas *et al.* found a mixture of TFMSA/PhSMe/TFA or TMSBr/PhSMe/TFA to be best for deprotection.[184] A 2-step TMSOTf/TFA protocol has also been employed in the deprotection of Ser(OPO_3Me_2).

In the Fmoc based synthesis of phosphotyrosine containing peptides, methyl,[185] benzyl,[186] and t-butyl[180] derivatives are routinely used. However, the benzyl and methyl derivatives are subject to monodealkylation by piperidine during *N*-terminal deprotection. This may be overcome by the use of the non-nucleophilic base DBU.[186] Tyr(OPO_3Me_2) containing peptides require TFMSA or TMSBr to achieve demethylation. The TFA labile t-butyl group is stable to synthesis conditions, but degrades upon storage and therefore must be used immediately after it has been synthesised. The unprotected phosphotyrosine derivative Fmoc-Tyr(OPO_3H_2)-OH has been used in the synthesis of phosphotyrosine containing peptides, despite the lack of phosphate protection.[186] However, adjacent tyrosine residues may form a pyrophosphate linkage.

It was originally thought that the use of preformed phosphoserine and phosphothreonine building blocks was not compatible with the Fmoc based solid phase strategy. This was because the conditions required for removal of the Fmoc group, treatment with a 20% solution of piperidine in DMF, would cause β-elimination resulting in loss of the phosphate group and formation of a

dehydroamino acid, Scheme 1.8.[187] This has indeed been shown to be the case where the phosphoserine is protected as the phosphate triester.

However, Wakamiya *et al.*[188] have shown that if a mono protected derivative such as Fmoc-SerO(PO(OBn)OH)-OH **60** is used for incorporation of phosphoserine, then the formation of the dehydroalanine can be totally suppressed. White and Beythien have shown that both Fmoc-SerO(PO(OBn)OH)-OH **60** and Fmoc-ThrO(PO(OBn)OH)-OH **61**, Fig. 1.20, can be used to synthesise a range of peptides, some involving multiple phosphorylation sites.[189]



Scheme 1.8: *Phosphoamino acid undergoing β-elimination to the corresponding dehydroamino acid.*

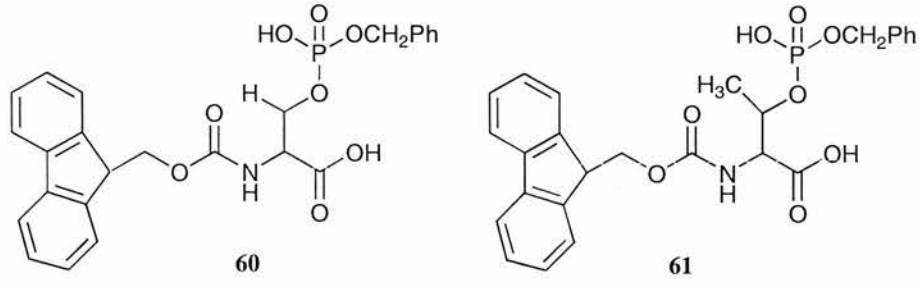


Figure 1.20: *Fmoc-SerO(PO(OBn)OH)-OH* ***60*** *and Fmoc-ThrO(PO(OBn)OH)-OH* ***61***

1.5.4.2 The Global Phosphorylation Approach

This strategy involves the post-synthetic phosphorylation of unprotected hydroxyl groups of the fully formed peptide on the solid support,[190, 191] or in solution.[192] Of the two strategies, this is the most widely applicable, as it is suited to the synthesis of peptides containing phosphorylated serine, threonine and tyrosine residues in both Fmoc and Boc based methodologies.

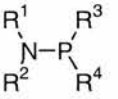
The residue to be phosphorylated is normally incorporated in its free hydroxyl form, without side chain protection, though a selectively protected derivative such as Fmoc-Ser(Trt)-OH can be used.[193] Phosphorylation of the free hydroxyl has been carried out using chlorophosphates,[191] *H*-phosphonates,[194] or phosphoramidites.[190] The first two methods afford the phosphate triester directly, whereas the third method requires oxidation of the phosphite triester to the phosphate triester, prior to deprotection.

The *H*-phosphonates and phosphoramidites are generally regarded as superior to the chlorophosphates as the latter are reported to be slow,[187, 191] and can give rise to side products.[178] The most useful reagents described in the literature are the dimethyl, di-^{t}butyl, di-*p*-chlorobenzyl and dibenzyl *N*,*N*-dialkyl phosphoramidites **62-70**, Table 1.4. The benzyl and t-butyl derivatives are compatible with the Fmoc strategy as the protecting groups are removed during the course of TFA acidolysis. Several research groups have investigated the utility of these two reagents in the global phosphorylation of serine, threonine and tyrosine residues.[195-198] Peptides of 5-15 residues have been routinely made on Kieselguhr,[195-197] and polystyrene supports,[198, 199] sometimes involving multiple phosphorylation sites.[199] The use of t-butyl phosphoramidite is limited, as it can give rise to *H*-phosphonate side products.[199] Diallyl *N*,*N*-

diisopropylphosphoramidite, **64**,[159] provides a further degree of synthetic flexibility as the allyl groups are orthogonal to both Boc and Fmoc. Removal of the allyl group is achieved by hydrostannolysis or tetrakis(triphenylphosphine) Pd(0)

The majority of the literature on the synthesis of phosphopeptides has involved the use of phosphoramidites or phosphites as phosphitylating agents in the 'phosphite-triester' approach. In this approach, the target alcohol is phosphitylated to afford the phosphite triester, which is then oxidised to the phosphate triester using an external oxidising agent, before removal of the phosphate protecting groups, either during peptide chain elongation, or at the final deprotection step. Phosphitylation of the target alcohol is usually achieved in the presence of 1*H*-tetrazole, which is sufficiently acidic to protonate the nitrogen of the phosphoramidite, allowing release of amine upon attack of the target alcohol. There are a number of different oxidising agents mentioned in the literature, with differing reports of efficacy. The three main reagents are *m*CPBA, ^{t}BuOOH and an aqueous solution of I_2, with different authors preferring different reagents.

Table 1.4: *Phosphoramidites used in phosphopeptide synthesis*



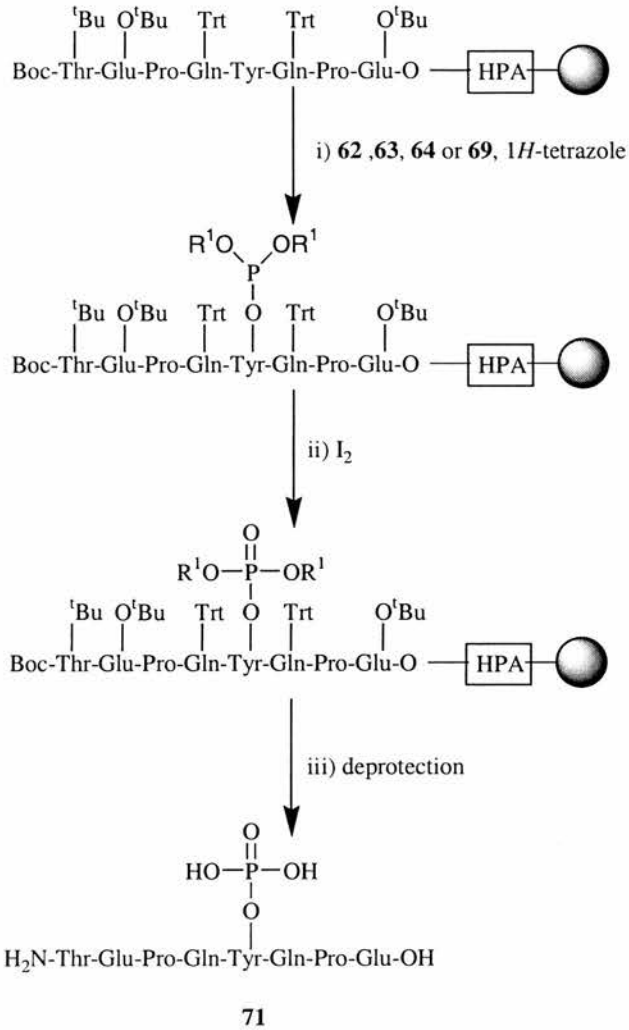
R^1	R^2	R^3	R^4	Ref	R^1	R^2	R^3	R^4	Ref
^{i}Pr		OBn		**62**[196, 200]	Et		O^{t}Bu		**67**[201-204]
^{i}Pr		O^{t}Bu		**63**[195]	Et		Oallyl		**68**
^{i}Pr		Oallyl		**64**[205]	Et		OMe		**69**
^{i}Pr		4-ClBn		**65**[197]	Et		OPh		**70**[206]
Et		OBn		**66**[201]					

Use of the phosphoramidites above, leads to the formation of the corresponding phosphite triester, which is subsequently oxidised to the phosphate triester, Scheme 1.9.

Oxidation can be carried out with a number of oxidising agents, the common reagents being aqueous iodine,[195] *m*CPBA,[198] and ^{t}BuOOH[196, 197, 199] though the use of iodobenzene diacetate,[207-209] and tetrabutylammonium periodate[209, 210] have also been reported. Oxidation with I_2 in THF/ 2,6-lutidine/ water has been reported to lead to side products in the oxidation of the phosphate triesters.[146] The side products were the *H*-phosphonate resulting from hydrolysis of excess phosphoramidite, and the phosphoramidate resulting from oxidation of excess phosphoramidite. Use of *m*CPBA, iodobenzene diacetate and tetrabutyl ammonium periodate, but not ^{t}BuOOH, were reported to have given good yield of the required phosphate triester.

The alternative approach is to incorporate the phosphorus in the required oxidation state directly, that is as the phosphate rather than the phosphite. This is achieved through the use of chlorophosphates or pyrophosphates.

The use of chlorophosphates in the synthesis of phosphopeptides has not been reported widely in the literature. The phosphorylation of alcohols with chlorophosphates has been reported to proceed only slowly, and with variable efficiency. However a separate oxidation step is not required and the reactions are generally a lot cleaner than the oxidation of the corresponding phosphite triester.

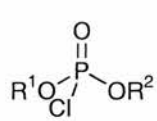


71

Deprotection: Reagent **62**, **63**: CF_3COOH/ thiophenol; Reagent **64**: CF_3COOH followed by $[Pd^0(PPh_3)_4]$; Reagent **69**: Me_3SiBr/ CF_3COOH/ thioanisole/ m-cresol

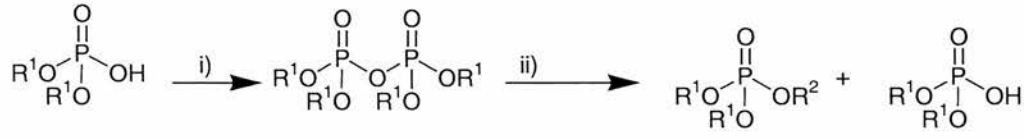
Scheme 1.9: *Solid phase peptide synthesis of* O^{527}*-phospho-p60*^{src}*-(523-531)-nonapeptide* ***71***[195]

Table 1.5: *Chlorophosphates used in phosphopeptide synthesis*



R^1	R^2	Ref.	R^1	R^2	Ref.
Me	Me	**72**	tBu	tBu	**75**
Ph	Ph	**73**[177, 206]	Allyl	allyl	**76**
Bn	Bn	**74**[191]	C_6F_5	C_6F_5	**77**[211]

The pyrophosphates, tetrabenzyl- and tetra(*p*-nitrophenyl)-pyrophosphate have both been utilised in the synthesis of phosphopeptides.[212, 213] These are synthesised from the corresponding phosphoric acid diester and a coupling reagent (DCC or di-*p*-tolyl carbodiimide). However, the use of pyrophosphates in the synthesis of phosphopeptides is not widely reported.



Reagents and Conditions: i) RN=C=NR, Et_2O; ii) R^2OH, base, DMAP

Scheme 1.10: *Synthesis of Pyrophosphates and reaction with alcohol substrate*

(R^1 = Bn or p-NO_2Ph)

1.6 Solid Phase Peptide Synthesis

1.6.1 Introduction

Solid phase peptide synthesis (SPPS) is based on the sequential addition of *N*-α and side chain protected amino acids to an insoluble polymeric support, Scheme 1.11. Acid labile *tert*-butoxycarbonyl (Boc-) or base labile 9-fluorenylmethoxycarbonyl (Fmoc-) groups are utilised for *N*-α protection, and the side chain protecting groups are chosen so as to be orthogonal to the *N*-α protecting group employed. After *N*-α deprotection, the resin is washed thoroughly and the next amino acid attached either by using a coupling reagent or as a preactivated derivative, such as a pentafluorophenyl ester, or preformed symmetrical anhydride (PSA), Section 1.6.2.

This cycle of deprotection and chain elongation is repeated until the desired sequence is obtained. The peptide is attached to the resin at the *C*-terminus, *via* a cleavable linker, and may be cleaved to yield the required peptide acid or amide, depending on the choice of linking agent used. Ideally, this cleavage also removes the protecting groups on the side chain functionalities of the amino acids in the peptide sequence. These protecting groups are chosen to be stable to the *N*-α deprotection and coupling conditions, thus preventing side chain acylation during chain elongation.

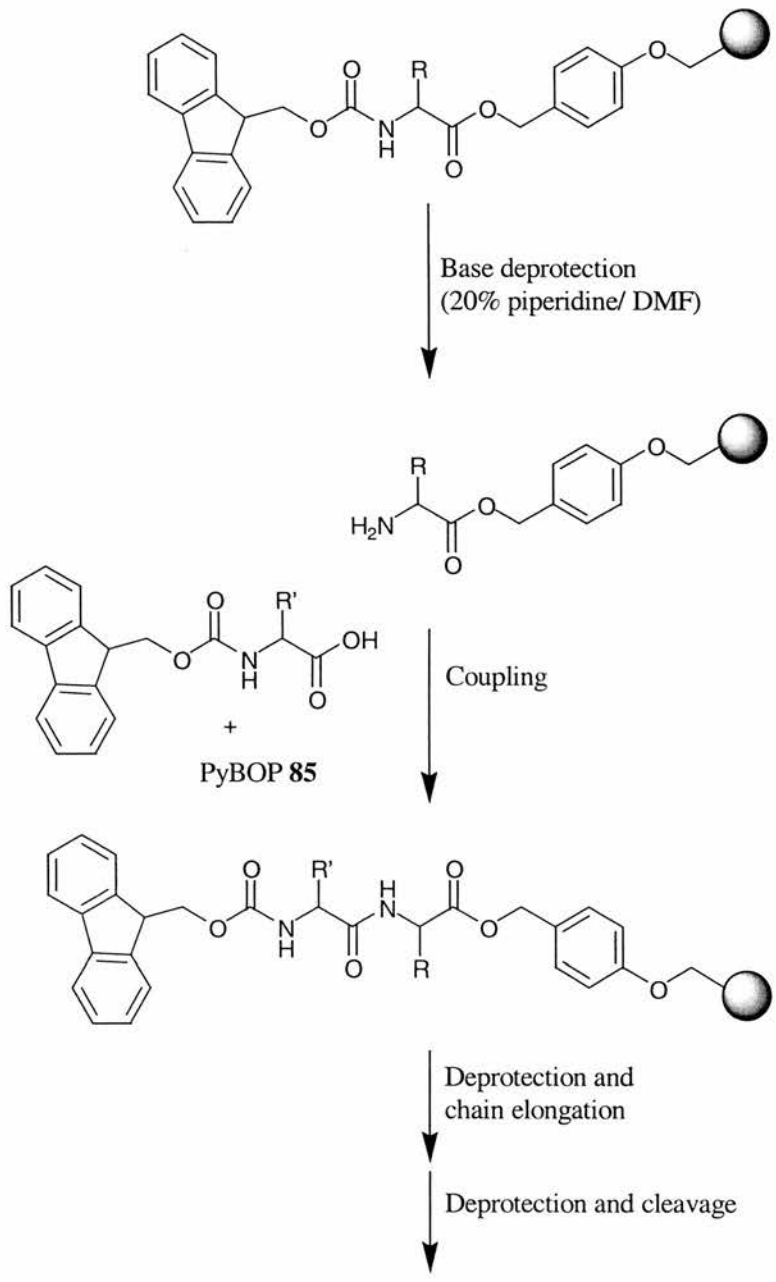
These processes may be carried out as:

- a batch process, where the resin is contained within a filter reaction vessel and reagents are added and removed under manual or computer control, and the support is filtered between each step,

or as

- a continuous flow process, where the resin is contained within a column and the resin is solvated during reagent exchange. Only Fmoc- based peptide synthesis is generally compatible with continuous flow processes.

In its infancy, the solid phase peptide synthesis method as developed by Merrifield utilised *N*-α ^{t}Boc protected amino acids.[136] Deblocking of the *N*-terminal residue was achieved through acidolysis by trifluoroacetic acid (TFA). This was a major cause for concern, in that repeated TFA acidolysis would result in side reactions and degradation in the quality of the final product. In addition, side chain deprotection and resin cleavage utilised HF, which is highly corrosive, expensive and requires specialised laboratory equipment which is not always available, or trifluoromethanesulfonic acid (TFMSA), which is also highly corrosive.



Scheme 1.11: *Generalised strategy of Fmoc solid phase peptide synthesis*

This prompted the development of protecting groups that could be removed under milder conditions. The most widely used of these is the 9-fluorenylmethoxycarbonyl (Fmoc) group,[214, 215] which is rapidly removed by primary amines and some secondary amines, but only slowly by tertiary amines.

The Fmoc group is usually removed in SPPS by treatment with a 20% solution of piperidine in DMF for 15 - 30 minutes, though other amines can be used.

The standard resin used in Fmoc based batch SPPS is Wang Resin (hydroxymethylpolystyrene [HMP] resin),[216] Fig. 1.21. This consists of polystyrene beads (100-200 mesh, 1% divinyl benzene, DVB) onto which the acid labile *p*-hydroxybenzyl alcohol linker is attached. The method used for the preparation of unsubstituted Wang resin is based on the improved synthetic method of Merrifield *et al.*[217] Attachment of the first amino acid is usually achieved by reaction of the resin with the symmetrical anhydride of the desired amino acid, in the presence of a catalytic amount of DMAP (4-dimethylaminopyridine). However, racemisation and dipeptide formation may occur.[218] A more effective but longer method involves the use of 2,6-dichlorobenzoyl chloride in DMF/ pyridine.[219] This eliminates racemisation and dipeptide formation.

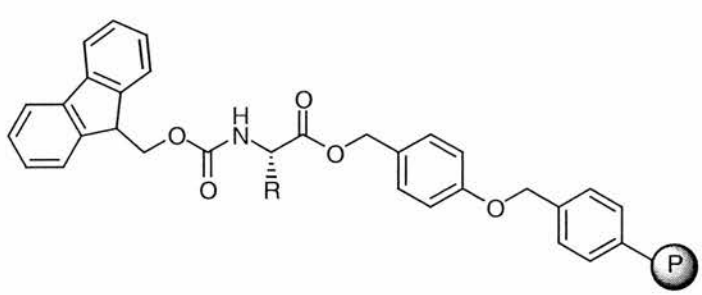
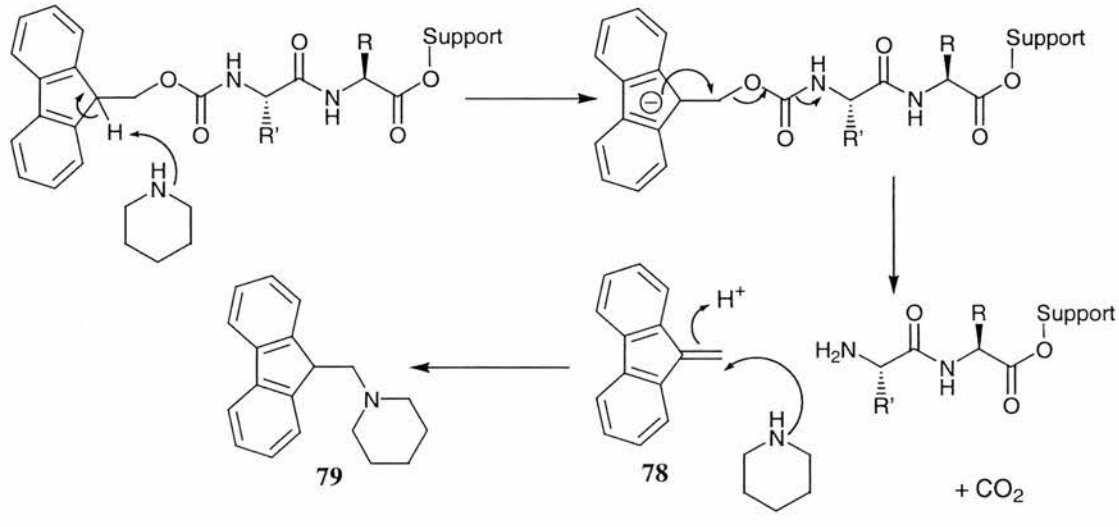


Figure 1.21: *Fmoc-amino acid pre-attached to Wang resin. The grey circle represents the polystyrene polymer matrix.*

In Boc- based SPPS, deprotection of the *N*-α terminus is achieved through the use of TFA in DCM, or neat TFA, and in Fmoc- based SPPS by the use of 20% piperidine in DMF, Scheme 1.12. The electron withdrawing effect of the fluorene ring system renders the hydrogens of the β-carbon susceptible to removal by weak

bases. β-Elimination and loss of carbon dioxide results in formation of the free amine and dibenzofulvene **78**. The dibenzofulvene can react with another equivalent of piperidine to form the fulvene-piperidine adduct **79**.

However, for long sequences, incomplete deprotection may occur even at high piperidine concentrations.[220] This problem is avoided by increasing the time for deprotection, or to use a stronger base such as DBU.[221] This excellent alternative causes rapid deprotection and less racemisation.[221]



Scheme 1.12: *Fmoc group removal and formation of adduct* ***79.***

Since no purification, other than washing of the resin with solvent, is performed during peptide assembly, the coupling reactions must be highly efficient to ensure complete coupling and high homogeneity of the final product. This is achieved by the use of extremely pure reagents and solvents and the use of an excess of both coupling reagents and amino acids.

Fmoc SPPS is designed for acidolysis of the peptide-linker. Non-acidic conditions are used for the removal of the Fmoc group and coupling of the next amino acid, therefore mild acids can be used for side chain deprotection and

cleavage from the resin. The majority of linkers and side chain protecting groups are therefore stable to alkali, but are rapidly removed upon treatment with weak acid (such as TFA).

The growing peptide chain may form secondary structures or aggregates with other peptide chains or with the polymer support. This lowers the rate of reaction and consequently results in lower yields. Incomplete solvation of the peptide-resin complex, sudden shrinkage of the gel matrix and reduced reagent penetration results in the failure of the deprotection and/ or alkylation steps. Introduction of dipolar aprotic solvents such as DMF, DMSO or NMP (*N*-methylpyrrolidine) can improve the situation. The degree of cross-linking of the resin should also be less than 1%, or proper swelling may be inhibited.[222] Aggregation of the growing peptide chain is also dependent on the nature of the peptide residues and the side chain protecting groups. A high proportion of Ala, Val, Ile, Asn and Gln residues will render the peptide chain prone to aggregation. Secondary amino acids, such as sarcosine (*N*-methylglycine) and proline, inhibit aggregation by imposing a turn on the peptide chain. Bulky hydrophobic groups (such as Trt) cause aggregation whereas small hydrophilic groups (such as acetyl or trifluoroacetyl) are a hindrance to aggregation.

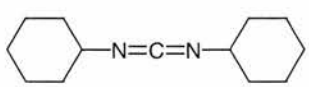
1.6.2 Methods for Peptide Coupling

Coupling may be carried out using one of four methods: carbodiimides, preformed symmetrical anhydrides, active esters, or *in-situ* coupling reagents.

1.6.2.1 Carbodiimides

DCC (dicyclohexylcarbodiimide) **80** was first described in the 1950's and still remains one of the most popular peptide coupling reagents.[223] It is particularly

appropriate for the apolar environment of the polystyrene resin, reactions generally being carried out at room temperature in DCM. A small amount DMF may be added to increase the solubility of reactive species. However, DMF slows down the reaction so minimal quantities should be used. The drawback to using DCC is the formation of dicyclohexylurea (DCU **81**) during activation and acylation. DCU is only soluble in some organic solvents. Both DIPCDI (di*iso*propylcarbodiimide),[224] and ^{t}butyl(methyl)carbodiimide are alternatives to DCC, as they form ureas which are more soluble in DCM than is DCU.



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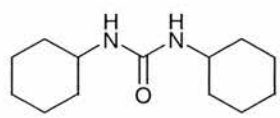
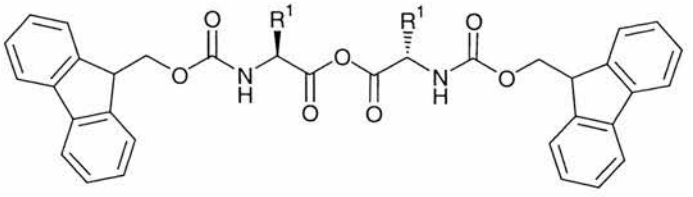


81

1.6.2.2 Preformed symmetrical anhydride (PSA)

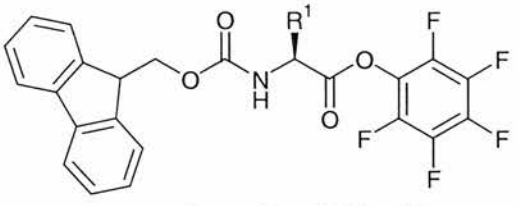
Preformed symmetrical anhydrides are formed from 2 equivalents of protected amino acid and 1 equivalent of DCC in DCM. After filtration to remove the DCU they are generally used immediately, due to their high reactivity, mainly in Boc SPPS. However, Fmoc based PSA's have also been studied.[225] Gly, Ala, Nle, Cys(Acm), Gln(Mbh), and Asn(Mbh) based PSAs are not generally available as they have limited solubility in DCM,[226] requiring significant amounts of DMF which slows down the reactions. The drawback to using PSAs is that the synthesis of them is both expensive and wasteful, as two equivalents of amino acid are consumed to provide one equivalent of PSA, and the PSA is generally used in large excess.



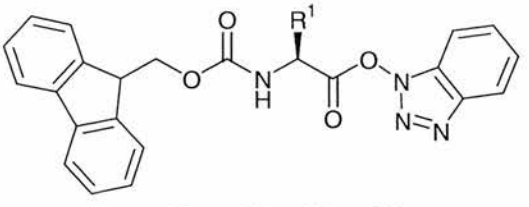
Preformed Symmetrical Anhydride

1.6.2.3 Active esters

The –OBt (benzotriazolyl) and –OPfp (pentafluorophenyl) esters of Fmoc amino acids are extremely useful and are now routinely used.[227] They are efficient acylating reagents with little chance of side reactions. However, not all amino acids are commercially available as –OPfp esters and they are slower than PSA's. The rate of reaction may, however, be significantly increased by the addition of HOBt.[228, 229]



Fmoc-Xaa-OPfp **82**



Fmoc-Xaa-OBt **83**

1.6.2.4. Coupling Reagents

The *in situ* activating agents **84-89**, Figure 1.22, are easy to use, promote fast reaction even between sterically hindered amino acids and are not prone to side reactions. The uronium reagents, HBTU **87** and TBTU **88**, must not be used in excess with respect to the amino acid as they will cap the *N*-terminus through guanidine formation. The phosphonium-based reagents are not prone to this problem, except PyBrOP® in DMF.[230]

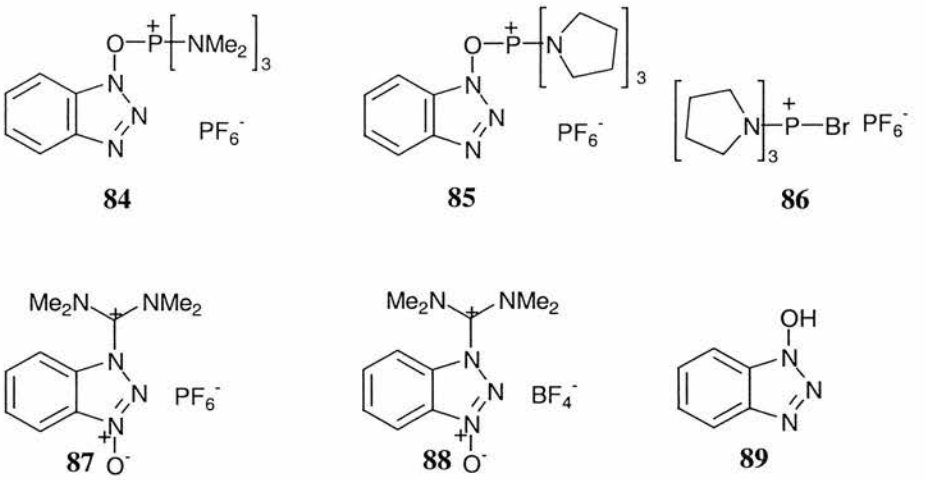


Figure 1.22: *Coupling Reagents: BOP* ***84****,*[231] *PyBOP® 85,*[232] *PyBrOP® 86,*[233] *HBTU 87,*[234] *TBTU 88,*[234] *and HOBt 89*[235]

The most commonly used coupling reagent is PyBOP **85**. This reagent has the advantage over its analogue BOP **84** (in which the three pyrrolidino groups are replaced by dimethylamino groups) in that it does not lead to the formation of carcinogenic by-products. PyBOP is especially suitable for solid phase peptide synthesis as it is soluble in a wide range of solvents such as DMF, THF, DCM and MeCN.

Coupling of *N*-methyl amino acids is a difficult and slow process. The use of PyBrOP® **86** allows fast and efficient racemisation free couplings.[236]

1.7 Phosphate Esters

1.7.1 Introduction

The main forms of phosphorus in biology are as orthophosphoric acid, H_3PO_4, its esters, anhydrides and occasionally amides. The oxidation state is P(V) and it has a coordination number of 4. The orthophosphates are tetrahedral at phosphorus, the single P-O bonds using sp^3 hybrid orbitals at phosphorus and are roughly 1.6 Å.[237] In triesters, the 'double' bond is shorter, about 1.46 Å, and

involves a phosphorus d-orbital in a p_π-d_π hybrid (Fig 1.23). Because phosphorus has five 3d orbitals, it can participate in such bonding simultaneously to more than one oxygen ligand.

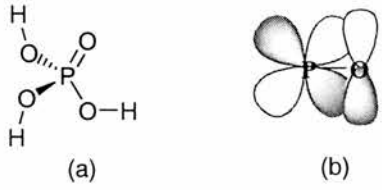


Figure 1.23: *(a) orthophosphoric acid; (b) P-O p_π-$d_\pi$bonding*

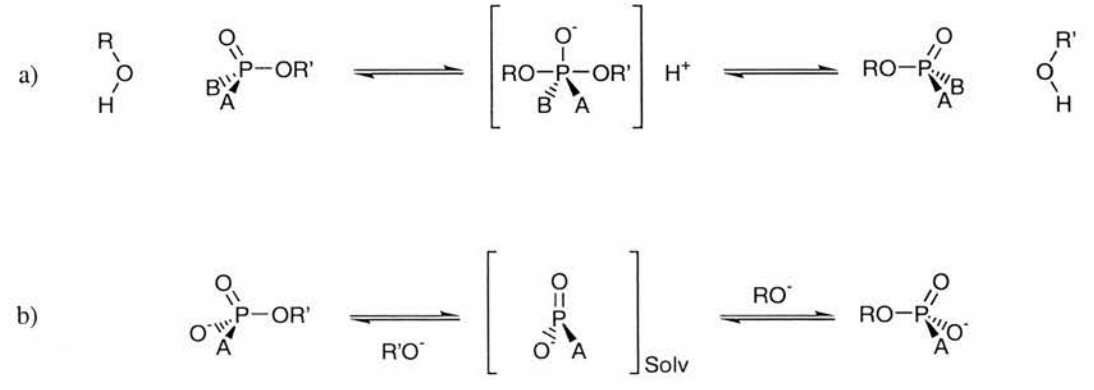
Phosphate triesters have all three hydrogens of phosphoric acid replaced by alkyl or aryl groups. They are non-ionic, soluble in many organic solvents, and sufficiently stable to be purified by chromatography. When all three ester groups are different, the phosphorus is a centre of asymmetry and optical isomers are possible.

Phosphate diesters have two alkyl groups and the remaining OH ligand is strongly acidic (pKa ~ 1.5).[238] Consequently, phosphate diesters exist as monoanions at pH.2, and are usually water-soluble.

Phosphate monoesters have a single aryl or alkyl group and two ionizable OH groups. These groups have pKa ~1.6 and pKa ~ 6.6, so there is an equilibrium in neutral solution between the monoanion and dianion.[238] The equivalent oxygens share the negative charge in both mono and dianions, and there is partial double bonding to each. In the monoanion, the hydrogen atom translocates rapidly between the three oxygens, making them equivalent in solution. The use of the three isotopes of oxygen, ^{16}O, ^{17}O and ^{18}O has been widely used in stereochemical synthesis and in analysis of substitution reactions of phosphates.[239]

1.7.2. Hydrolysis of Phosphate Esters

Both C-O and P-O cleavage reactions have been identified.[240] In general associative $S_N2(P)$ mechanisms are more common than dissociative $S_N1(P)$ ones, and they are usually linked to inversion of configuration at phosphorus. The associative process has a 5-coordinated species as intermediate or transition state in which ligand positional interchange – pseudorotation – is usually slower than breakdown of the trigonal bipyramidal species to form products, Scheme 1.13



Scheme 1.13: *Hydrolysis of phosphate esters* via *a) associative and b) dissociative pathways*

In dissociative reactions, a planar, 3-coordinate species, monomeric metaphosphate, can capture the incoming nucleophile on either face, resulting in the possibility of racemisation.

1.7.2.1. Hydrolysis of Phosphate Triesters

1.7.2.1.1 Alkyl Triesters

Trimethyl phosphate is slowly hydrolysed in alkaline solution in an $S_N2(P)$ process.[241] With $H_2{}^{18}O$ as solvent, no isotope exchange is observed showing that the P-O bond is cleaved exclusively.[242] Trimethyl phosphate is hydrolysed extremely slowly in neutral and acidic conditions with C-O cleavage. These reactions are

typical S_N2 processes. Alkyl phosphate triesters are also sensitive to β-elimination processes.[187] The cyanoethyl group has an acidic β-hydrogen and so is susceptible to cleavage under mildly basic conditions. Similarly, the 2,2,2-trichloroethyl ester group can be eliminated by reduction using either a zinc-copper couple or at a reducing anode using the appropriate potential.[162]

1.7.2.1.2 Aryl Triesters

Aryl phosphates are much more reactive than alkyl phosphates, and it is therefore possible to achieve selective, nucleophilic displacement of the phenolic residue in a dialkyl aryl phosphate triester due to its better leaving group ability.[240]

1.7.2.2 Hydrolysis of Phosphate Diesters

At pH > 2, phosphate diesters exist as their monoanions. Even in strong alkaline conditions, diesters hydrolyse more slowly than triesters with predominant C-O cleavage.[240] In acidic conditions, their hydrolysis is rather similar to that of trialkyl phosphates. The diaryl esters are rather more reactive under alkaline conditions, as would be expected for reactions involving a better leaving group.

The marked stability of the phosphate diester linkage to hydrolysis is an essential feature of the biological role of DNA. The position is dramatically changed for esters of 1,2-diols. Here, the vicinal hydroxy group enhances the rate of hydrolysis of di- and tri-esters some 10^7 times.[243, 244]

1.7.2.3 Hydrolysis of Phosphate Monoesters

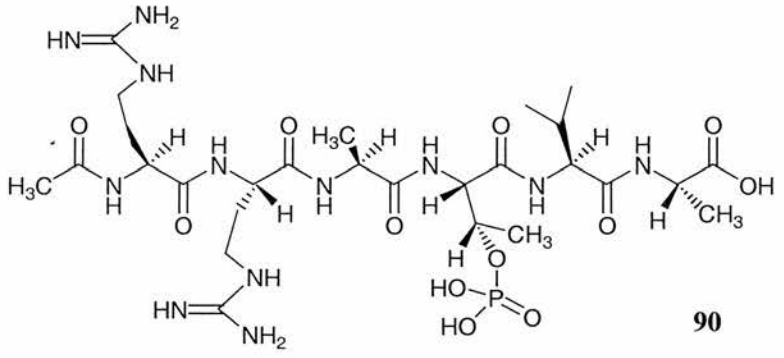
The hydrolysis of monoalkyl phosphates at low pH proceeds *via* the conjugate acid, $ROP(OH)_3^+$,[245] and is similar in mechanism to that of triesters.

These esters are very resistant to alkaline hydrolysis, as a result of anionic repulsion, but they show an unusually large reactivity for the neutral hydrolysis of the monoanion.

2.0 The Synthesis and Application of Fluorophenyl Based Chlorophosphate Reagents in Solid and Solution Phase Phosphorylations

2.1 Introduction

Previous work within the group has investigated the efficiency of a variety of phosphoramidites and chlorophosphates in the solid phase synthesis of the protein phosphatase substrate AcRRApTVA **90**.



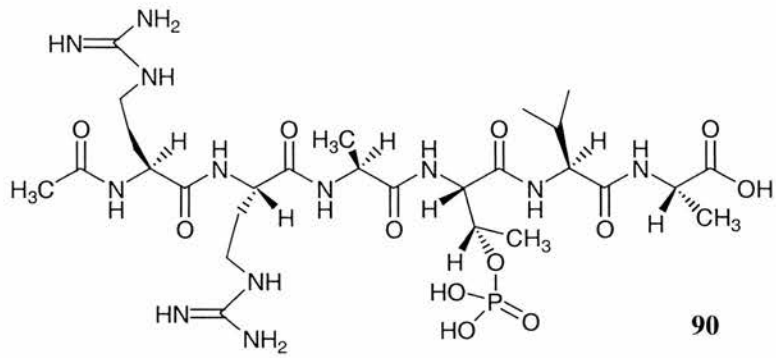
90

This work has lead to the discovery of a novel phosphorylating reagent, *bis*(pentafluorophenyl) chlorophosphate **90**, which has been shown to be highly effective in the synthesis of sterically hindered phosphoserine and phosphothreonine containing peptides.[211] It was therefore decided to investigate the application of this reagent in the solid phase synthesis of phosphotyrosine substrates and in solution phase synthesis.

2.2 *Bis*(pentafluorophenyl) chlorophosphate

2.2.1 Introduction

Work within our laboratory has been directed towards the elucidation of the role of the protein phosphatases PP1 and PP2A in cell signalling pathways. The approach undertaken has been to design a specific inhibitor for either PP1 or PP2A, to allow delineation of the roles of these two enzymes. In order to assess the potency of putative inhibitors in inhibition assays, a reliable source of substrate is required. Initial radiochemical assays have utilised ^{32}P labeled substrate. However, the short half-life ($t_{½}$) of ^{32}P means that the substrate must be prepared immediately prior to use, and cannot be stored for any length of time. However, a radiochemical assay has distinct advantages over an assay such as a colormetric assay, in that it is very sensitive and can be used at much lower concentrations of substrate. The radioactive isotope ^{14}C has a much greater $t_{½}$ and therefore, a substrate containing a ^{14}C label would have a substantially increased 'shelf life' over its ^{32}P containing counterpart. Incorporation of a ^{14}C label could be achieved by incorporating a ^{14}C *N*-acetyl cap to a peptide substrate. Therefore a reliable high yielding synthesis of phosphopeptides is required, amenable to the incorporation of a ^{14}C radiolabel. Accordingly, the synthesis of the protein phosphatase substrate AcRRApTVA **90**, was investigated by P. Hormozdiari.[246]



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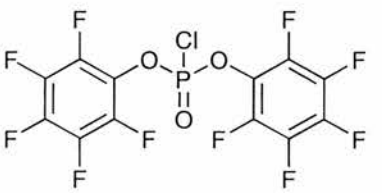
Attempted solution phase synthesis of the target sequence was unsuccessful, primarily due to the difficulty of coupling together the two arginine residues. Therefore a solid phase strategy was investigated, using Fmoc protection methodology, as described in Section 1.6.

In attempted solid phase phosphorylation of the resin bound peptide Ac-R(Pmc)R(Pmc)ATVA-OWang **93** (see Scheme 2.2, page 81), a range of phosphorylating reagents were investigated, including *N*,*N*-diisopropyl dibenzyl- and di^{t}butyl- phosphoramidite **62**, and **63**, and dimethyl, diphenyl and dibenzyl chlorophosphate **72**, **73** and **74**.[246]

Use of these reagents failed to achieve the desired outcome, due either to incomplete phosphorylation of the substrate (in the case of dimethyl chlorophosphate), side reactions upon workup (for phosphoramidites) or difficulties in removing the protecting groups after phosphorylation (diphenyl chlorophosphate). Therefore, a more reactive reagent with more labile protecting groups was required. Since the most encouraging results had been obtained with diphenyl chlorophosphate, a reagent based on diphenyl chlorophosphate was designed. The use of electron withdrawing substituents on the aromatic rings would increase the electrophilicity of the phosphorus centre, rendering it more reactive towards alcohol

substrates and hence increasing the reactivity of the reagent. Therefore, the reagent *bis*(pentafluorophenyl) chlorophosphate **77** was considered.



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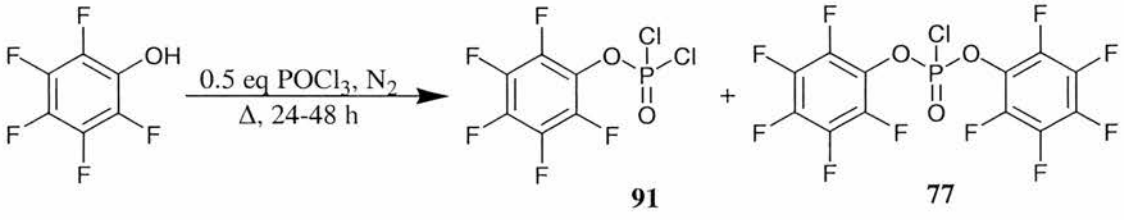
2.2.2 Synthesis

Boschan and Holder, in their 1967 patent, describe the use of pentafluorophenyl dichlorophosphate **91** and *bis*(pentafluorophenyl) chlorophosphate **77** as intermediates in the manufacture of pentafluorophenyl phosphate esters, and the use of these esters as the base stocks of hydraulic systems in aircraft.[247] The synthesis of **91** and **77** involved the heating of phosphoryl chloride and pentafluorophenol for approximately 90 hours under reflux, and isolating the two desired compounds by distillation. The amount of dichlorophosphate **91** and chlorophosphate **77** obtained was dependent on the ratio of the two starting materials. An equimolar amount of phosphoryl chloride and pentafluorophenol would afford mostly the dichlorophosphate **91**, whereas increasing the amount of pentafluorophenol to roughly two equivalents with respect to the phosphoryl chloride afforded mostly the chlorophosphate **77**.

It was felt that the rather excessive length of heating suggested by Boschan and Holder could be reduced substantially without appreciable loss in yield of the two products, as upon the mixture reaching reflux subsequent reaction between the pentafluorophenol and phosphoryl chloride would be rapid. Accordingly, pentafluorophenol and phosphoryl chloride were heated under reflux at 140 °C under

a nitrogen atmosphere for 24 to 48 hours, Scheme 2.1. After cooling, the remaining starting materials were removed by distillation. The remaining residue consisted of a mixture of pentafluorophenyl dichlorophosphate **91** and *bis*(pentafluorophenyl) chlorophosphate **77**. Distillation at low pressure (0.5–1.0 mmHg) afforded the pentafluorophenyl dichlorophosphate **91** as a colourless oil in 22% yield, bp 62 °C/ 0.7 mmHg {lit,[247] 112–125 °C/ 20 mm Hg}, purity 99.5 % by ^{31}P NMR spectroscopy, and *bis*(pentafluorophenyl) chlorophosphate **77** as a waxy white solid in 64% yield, bp 120 °C / 0.7 mm Hg {lit,[247] 181–3 °C/ 20 mm Hg}, purity 93.4 % by ^{31}P NMR spectroscopy. Both reagents could be stored under an inert atmosphere in the freezer for prolonged lengths of time with little decomposition.



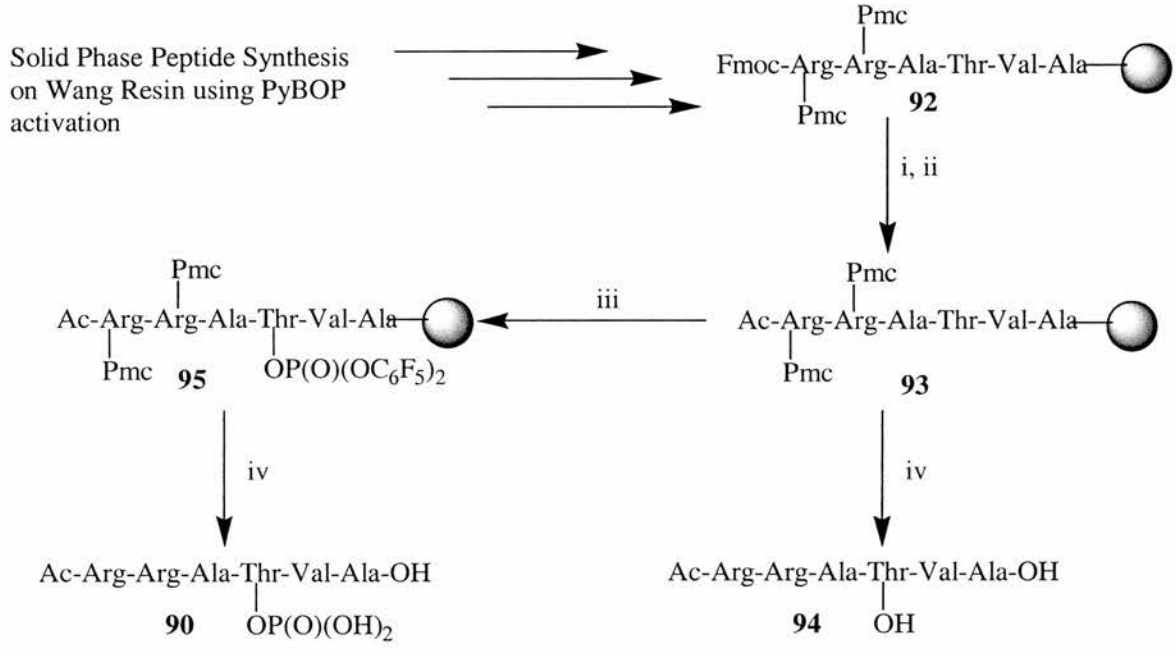
Scheme 2.1: *Synthesis of pentafluorophenyl dichlorophosphate **91** and* bis*(pentafluorophenyl) chlorophosphate **77**.*

2.2.3 Solid Phase Phosphorylations

2.2.3.1. Previous Work

Previous work within the group has concentrated on the synthesis of the substrate sequence Ac-RRApTVA **90**.[246] Phosphorylation of the fully protected resin bound peptide sequence Ac-R(Pmc)-R(Pmc)-ATVA-OWang **93**, Scheme 2.2, with the new reagent, *bis*(pentafluorophenyl) chlorophosphate **77**, afforded the resin bound phosphate triester **95**. Treatment of **95** with a cleavage mixture of 90% TFA,

5% Et_3SiH, 5% H_2O resulted in cleavage from the resin and removal of the Pmc and pentafluorophenyl groups. After purification, by ion exchange chromatography on Dowex-1 ion exchange resin, the desired phosphopeptide AcRRApTVA-OH **90** could be isolated in a 60% yield.[211] Prior to phosphorylation, a small amount of the resin **93** was treated with 20 % piperidine in DMF followed by 95% TFA/ 2.5% TES/ 2.5% H_2O and the resultant solid obtained after workup confirmed as AcRRATVA **94** confirming correct assembly of the desired sequence.



Reagents and Conditions: i. 20% piperidine/ DMF, RT, 15 min; ii. 5% Ac_2O/DMF, 0 °C, 15 min; iii. $(C_6F_5O)_2POCl$ **77**, Et_3N, DMAP, CH_2Cl_2, RT, 12 h; iv. CF_3CO_2H:Et_3SiH:H_2O (95:2.5:2.5), 60% overall

Scheme 2.2: *Synthesis of Ac-RRApTVA **90** using* bis*(pentafluorophenyl) chlorophosphate **77***

The serine analogue, AcRRApSVA-OH **97**, was also successfully prepared using an identical strategy, albeit in a lower un-optimised yield of 16% after purification. Therefore, it was decided to investigate the application of this novel reagent in the synthesis of phosphotyrosine containing peptides. If successful, this

would provide a powerful reagent for the synthesis of a variety of substrates, to probe the biological role of both the serine/threonine and tyrosine phosphatases, amongst the most important groups of enzymes. Access to a variety of phosphorylated substrates would allow substantial structure-activity analysis and investigation into substrate specificity.

2.2.3.2 Tyrosine Phosphorylation: Initial Attempted Synthesis of DADEpYL 98

The sequence chosen for assessment of the versatility of *bis*(pentafluorophenyl) chlorophosphate **77**, was the hexapeptide DADEpYL **98**. This corresponds to one of the autophosphorylation sites of Epidermal Growth Factor Receptor ($EGFR_{988\text{-}993}$),[127] a transmembrane tyrosine specific protein tyrosine kinase, which is the receptor for a small protein of 53 amino acids, EGF (Epidermal Growth Factor).[248] EGF binds to its receptor, EGFR, and stimulates the proliferation of epidermal cells and a variety of other cell types. There is evidence that EGFR also mediates the biological signals of at least two EGF-like growth factors – transforming growth factor α (TGFα),[249] and the *vaccinia* virus growth factor (VGF).[250] The EGFR is a single-pass transmembrane protein of 175 kDa, with a large glycosylated extracellular portion that binds EGF.[251] An intracellular tyrosine kinase domain is activated upon EGF binding, resulting in the transfer of a phosphate group, by the receptor, from ATP to selected tyrosine side chains, both on the receptor protein and on specific cellular proteins. The apparent activation of an intracellular domain by the binding of a factor to the extracellular domain is achieved by the dimerisation of the EGFR upon binding of EGF. The binding of EGF is thought to induce a conformational change in the extracellular domain of the receptor to induce receptor dimerisation.[252] This allows the two cytoplasmic

domains to cross-phosphorylate each other on multiple tyrosine residues, a phenomenon known as autophosphorylation. This creates specific binding sites for the SH2 domains of phospholipase C-γ (PLC-γ), specifically at 3 tyrosine autophosphorylation sites at the *C*-terminal tail of EGFR (Y992, Y1068, Y1173). The binding of the SH2 domains is crucial for efficient tyrosine phosphorylation of PLC-γ, by increasing its affinity for tyrosine phosphorylation by EGFR.

The $EGFR_{988\text{-}993}$ sequence is highly functionalised with a number of polar groups and presents a complex target for investigation of the application of *bis*(pentafluorophenyl) chlorophosphate **77** to phosphotyrosine synthesis.

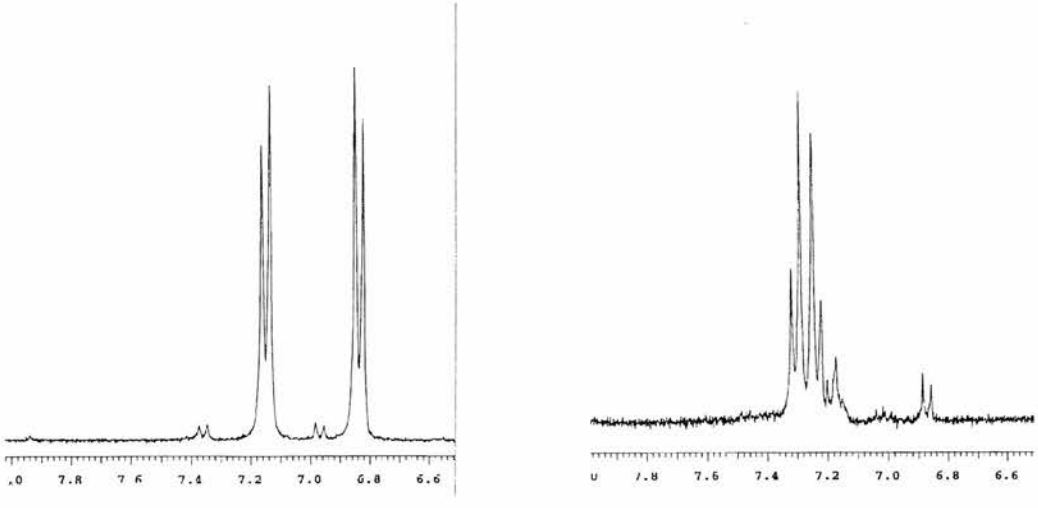
The synthesis of the peptide sequence was carried out utilising standard Fmoc- based solid phase peptide synthesis techniques, and peptide couplings were carried out *in situ*, using PyBOP **85**. The resin used was Wang resin, a polystyrene resin functionalised with the acid labile benzyloxybenzyl alcohol linker, Fig 1.21, page 65.

The Asp and Glu residues were incorporated with ^{t}Bu ester protection of the β-and γ–carboxyl side chains respectively. The Tyr residue was incorporate with no side chain protection, to facilitate phosphorylation and avoid tedious selective deprotection steps. The tyrosine hydroxyl group is not sufficiently reactive to react extensively with subsequent amino acid residues and therefore need not be protected. The stereochemistry of all amino acids is (*S*). *N*-α-Fmoc removal was achieved with a solution of 20% piperidine in DMF, and activation of the amino acids prior to coupling was achieved with 5% NMM in DMF and PyBOP **85**.

Fmoc-D(^{t}Bu)AD(^{t}Bu)E(^{t}Bu)YL-OWang **99** was synthesised as described above. A small amount of resin (20 mg or so) was treated with 20% piperidine in DMF followed by 95% TFA/2.5% TES/ 2.5% H_2O and the resultant solid obtained

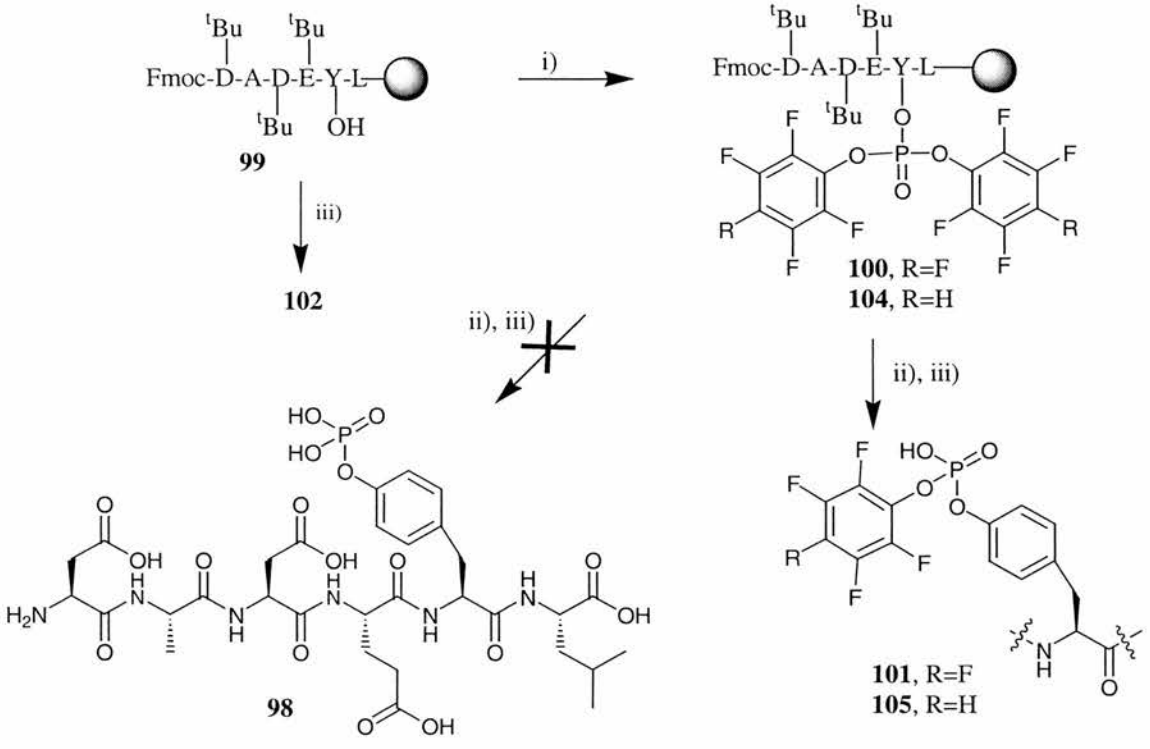
after standard workup was analysed by ^{1}H NMR spectroscopy to confirm that the sequence had been correctly assembled (i.e. the product of the cleavage was DADEYL **102**) .The resin **99** was phosphorylated with 10 equivalents of *bis*(pentafluorophenyl) chlorophosphate **77** to afford the resin bound phosphate triester **100**, Scheme 2.2. Incorporation of phoxphorus was confirmed by the presence of a signal in the gel phase ^{31}P NMR spectrum. Removal of the *N*-terminal Fmoc group by treatment with 20% piperidine in DMF and treatment with 95% TFA/ 2.5% H_2O/ 2.5% Et_3SiH for 2 hours followed by removal of the resin by filtration and lyopholisation of the filtrate afforded a white solid, {mp 120 °C, δ_P ($^{2}H_2O$) –8.75}.

Comparison of the ^{1}H NMR spectrum of the product **101** with that of the un-phosphorylated compound DADEYL **102** revealed a marked difference in the appearance of the aromatic regions of the two spectra, Fig. 2.1 below.



a) b)

Figure 2.1: *Aromatic region of ^{1}H NMR spectra for a) DADEYL **102**; and b) product (**101**) of phosphorylation of **99** with* bis*(pentafluorophenyl) chlorophosphate **77**. Horizontal scale is chemical shift, in parts per million (ppm).*

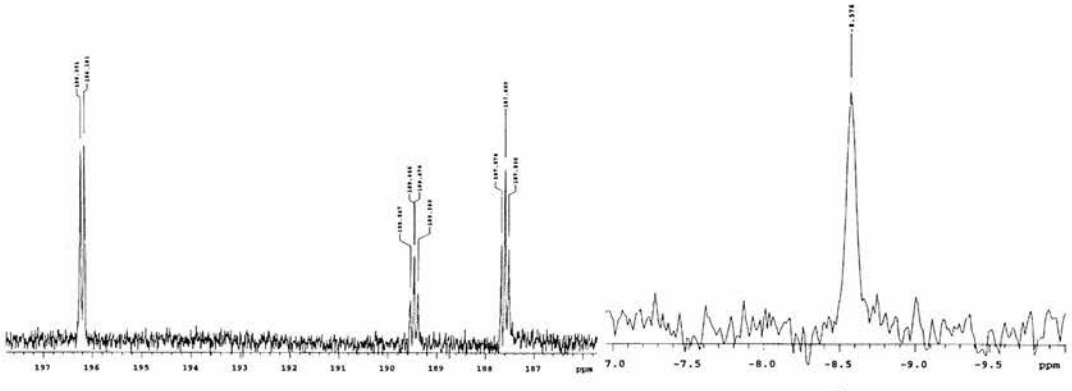


Reagents and Conditions: i) Et_3N, DMAP, CH_2Cl_2, $(C_6F_5O)_2P(O)Cl$ (**77**) or $(C_6HF_4O)_2P(O)Cl$ (**103**), rt., 4 h.; ii) 20% piperidine/ DMF, rt., 30 min.; iii) CF_3CO_2H:H_2O:Et_3SiH (95:2.5:2.5), 0 °C, 2h.

Scheme 2.3: *Tyrosine phosphorylation with* bis*(pentafluorophenyl) chlorophosphate 77 and* bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate* ***103*** *(Scheme 2.4)*

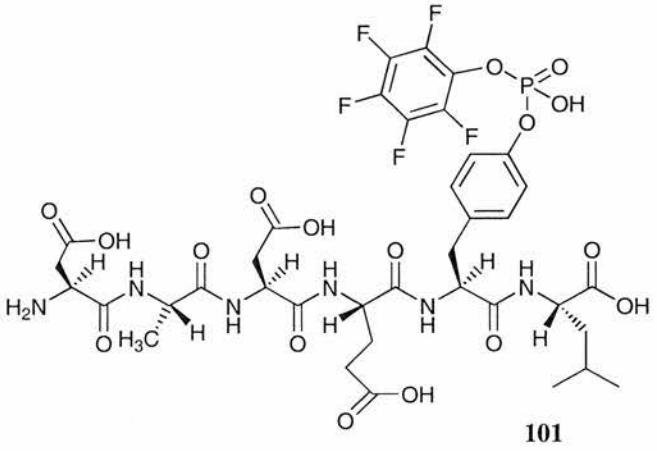
The change in coupling constants and difference in chemical shifts of the AB systems for b) (*J* 7.2 Hz; δ_H 7.24, 7.31 ppm [$\Delta\delta_H$ = 0.07 ppm]) compared to a) (*J* 8.4 Hz; δ_H 6.84, 7.15 ppm [$\Delta\delta_H$ = 0.31 ppm]) is indicative of a change in the chemical environment of the tyrosyl protons, attributed to phosphorylation of the hydroxyl moiety. However, it provides no information as to the number of pentafluorophenyl groups present in the product, as the pentafluorophenyl groups contain no integratable protons. Analysis of the ^{19}F and ^{31}P NMR spectra of the product **101**, Fig. 2.2 below, suggested the presence of one pentafluorophenyl group,

and therefore the product obtained was the tyrosyl pentafluorophenyl phosphate diester **101**. This was confirmed by negative ion electrospray mass spectrometry {*m/z* 969 $[M-2H]^-$}. In the ^{19}F NMR spectrum (Fig. 2.2a), a set of 3 signals at 187.60 - 196.21 ppm of relative integrals 2:1:2 signified the presence of a pentafluorophenyl group in the product. The peak at – 8.58 ppm in the ^{31}P NMR spectrum (Fig. 2.2b) is characteristic of the presence of a phosphotyrosine diester (diaryl phosphate diester).



(a) (b)

Figure 2.2: ***(a)*** *^{19}F and* ***(b)*** *^{31}P NMR spectra of* ***101****, the product of phosphorylation of* ***95*** *with* bis*(pentafluorophenyl) chlorophosphate* ***77****, and acidolytic workup. Horizontal scale is given in parts per million (ppm).*



101

Further analysis of the ^{13}C NMR spectrum revealed a series of peaks at 136-143 ppm, distinct from those identified as the tyrosyl resonances, signifying the presence of another aromatic group. Taken together, these three observations are consistent with the identity of the product as the pentafluorophenyl tyrosyl phosphate diester **101**. This suggested that treatment of the phosphate triester with the acidic cleavage mixture had resulted in the removal of only one of the pentafluorophenyl protecting groups. However, there was no way of verifying this with any degree of certainty, as the pentafluorophenyl groups lack any signals in the ^{1}H NMR spectrum, integratable with any signals in the ^{1}H NMR spectrum arising from the rest of the peptide. It was therefore decided to replace the *para*-fluorine of the pentafluorophenyl group with a hydrogen atom. This would provide a marker in the ^{1}H NMR spectrum for the presence of fluorophenyl protecting groups, simplifying the ^{1}H and ^{19}F resonances associated with the tetrafluorophenyl group, and should have only a negligible effect on the lability of the fluorophenyl group. We therefore envisaged the use of *bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate **103** in an analogous manner to that of *bis*(pentafluorophenyl) chlorophosphate **77**, Scheme 2.3. Comparison of the peak integrals within the aromatic region of the ^{1}H NMR spectrum would provide confirmation of the number of pentafluorophenyl groups still present in the product.

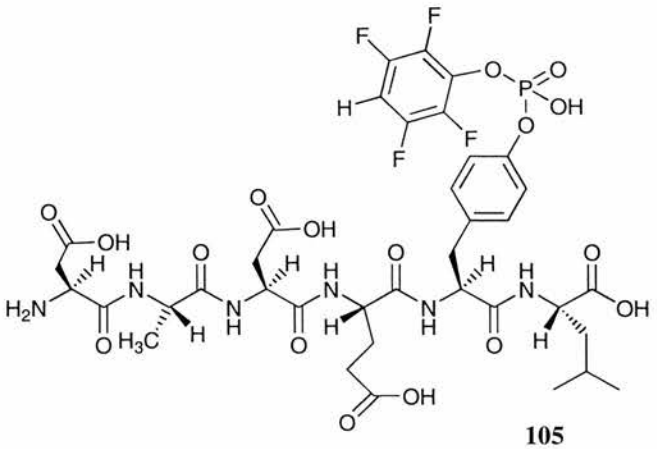
Accordingly, a synthetic approach analagous to that for the synthesis of *bis*(pentafluorophenyl) chlorophosphate **77** was utilised to synthesise *bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate **103**. Reaction of 2 equivalents of 2,3,5,6-tetrafluorophenol with $POCl_3$ under reflux in a nitrogen atmosphere gave the required *bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate **103** as the major product in

62 % yield after distillation (Scheme 2.4) {bp 160 °C/ 0.7 mm Hg, *m/z* (Found M^+ 411.9297, $C_{12}H_2O_3F_8P_1{}^{37}Cl_1$ requires 411.9302)}, purity 92.1 % by ^{31}P NMR spectroscopy.

103

Scheme 2.4: *Synthesis of* bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate **103***

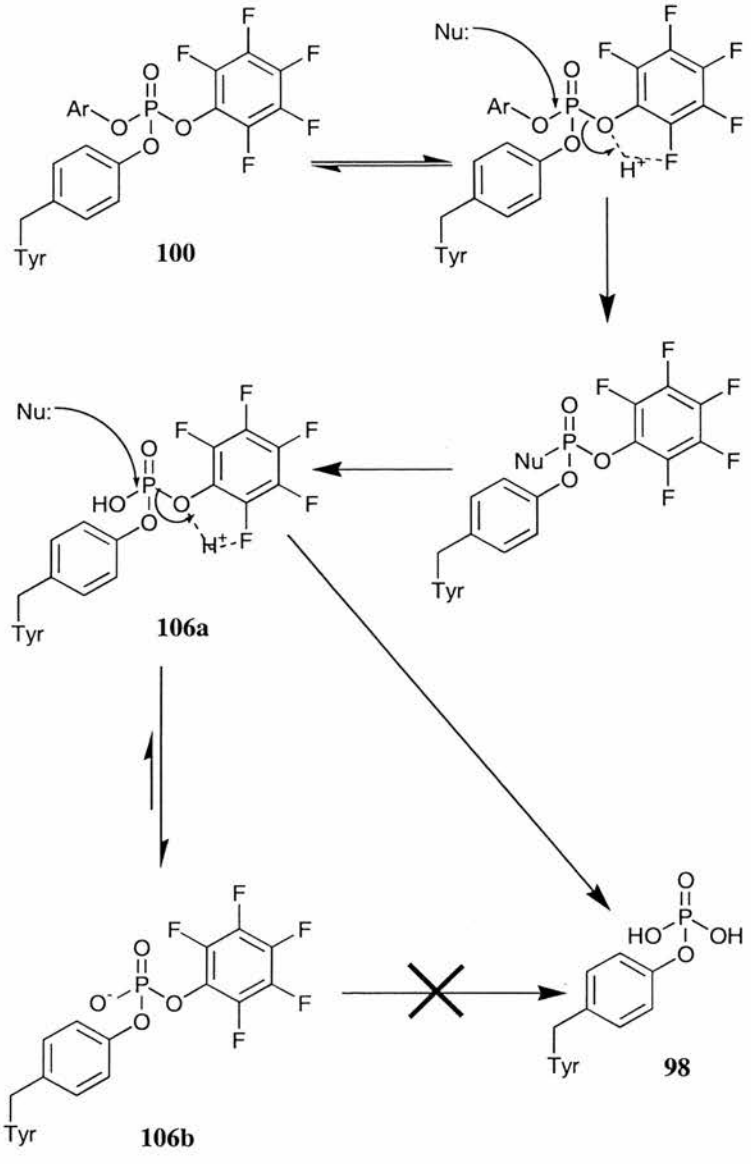
Phosphorylation of the fully protected resin bound peptide Fmoc-D(^{t}Bu)AD(^{t}Bu)E(^{t}Bu)YL-OWang resin **99** with *bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate **103** in a manner analagous with that for *bis*(pentafluorophenyl) chlorophosphate, Scheme 2.3, page 84, followed by filtration, washing and treatment with the standard cleavage mixture (95% TFA, 2.5% H_2O, 2.5% Et_3SiH) and lyophilisation of the filtrate, afforded a white hygroscopic solid, {mp. 130-134 °C, δ_P (^2H_2O) –9.54}. Analysis of the ^1H spectrum, showed a set of signals in the aromatic region corresponded to 5 protons, 4 from the tyrosine residue, and 1 from a single tetrafluorophenyl group. Thus, complete deprotection of the phosphate moiety had not occurred and the product of the reaction was the tyrosyl tetrafluorophenyl phosphate diester **105**. The identity of the product was further confirmed by ^{19}F and ^{31}P NMR spectroscopy and electrospray mass spectrometry {*m/z* 951 $[M - H]^-$}.



105

The use of TFMSA and TMSBr as strong acids has been employed in circumstances where prohibitively long cleavage times are required for certain residues such as Arg(Mtr) when TFA is used.[253] However, in both these reactions, the product was the tyrosyl tetrafluorophenyl phosphate **105**.

The incomplete deprotection of the phosphate group can be rationalised in terms of the enhanced stability of the fluorophenyl tyrosyl phosphate diester monoanion **106b**, Scheme 2.5. Protonation of a phosphate oxygen of the triester **100** is followed by nucleophilic attack (by H_2O or $CF_3CO_2^-$) to give the phosphate diester **106a** (after hydrolysis, if the attacking nucleophile is the trifluoroacetate anion). This species is in equilibrium with the monoanionic species **106b**. After removal of one of the pentafluorophenyl groups, the electron withdrawing nature of the aromatic ring of the tyrosyl phenyl group and the remaining pentafluorophenyl group lowers the pK_a of the phosphate diester **106a**.



Scheme 2.5: *Acid hydrolysis of tyrosyl* bis*(pentafluorophenyl) phosphate triester* ***100***

Therefore the diester will be predominantly present in the ionised form, *i.e.* the main species present will be the phosphate diester monoanion **106b**. This moves the equilibrium between the phosphate diester anion **106b** and the phosphate diester monoacid **106a** towards the anionic form, lowering the affinity of the phosphate diester anion **106b** to subsequent protonation. For acid mediated cleavage to occur, the phosphate monoanion must be protonated, followed by protonation of one of the

other phosphate oxygens. Attack of the nucleophile may then proceed, with loss of the second fluorophenyl group, Scheme 2.5. Due to the low pK_a of the fluorophenyl tyrosyl phosphate diester, this sequence of events is unlikely to occur, preventing attack of the incoming nucleophile and subsequent loss of the second pentafluorophenyl group.

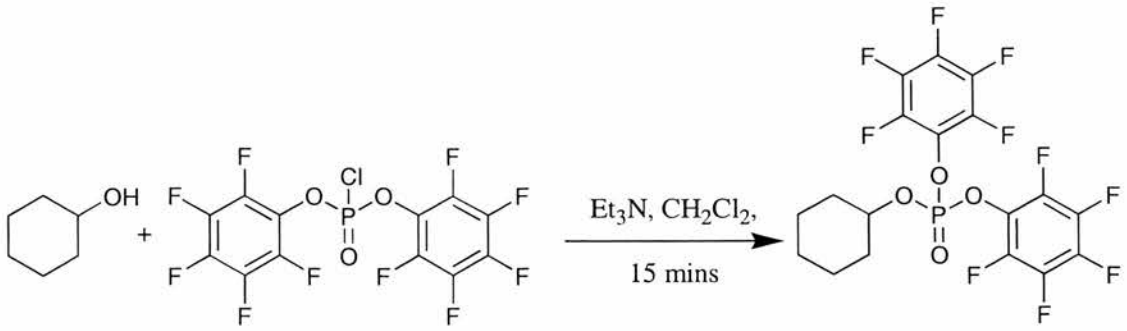
There is no literature evidence for the above-mentioned mechanism, it is merely postulated as a possible explanation.

2.3 Solution Phase Phosphorylations

2.3.1 Dependence of Reaction Yield on Order of Reagent Addition

P. Hormozdiari, in earlier work within our laboratories,[246] had used cyclohexanol as a threonine mimic to investigate the efficiency of *bis*(pentafluorophenyl) chlorophosphate **77** in solution phase phosphorylations. However, the product cyclohexyl *O,O'-bis*(pentafluorophenyl) phosphate **107** (Scheme 2.8), was only obtained in 15% yield, and an effective deprotection strategy was not found. We therefore turned our efforts towards optimising the phosphorylation conditions and investigating further the mechanism of deprotection.

Following the procedure of P. Hormozdiari,[246] a solution of *bis*(pentafluorophenyl) chlorophosphate **77** in DCM was added to a stirred solution of cyclohexanol and Et_3N and allowed to stir for 15 minutes (Scheme 2.6).



77 **107**

Scheme 2.6: *Synthesis of cyclohexyl* O,O'-bis*(pentafluorophenyl) phosphate **107***

After removal of the solvent by concentrating under reduced pressure, analysis of the reaction mixture by ^{1}H NMR spectroscopy showed the presence of two cyclohexyl species in the reaction mixture with α-H peaks at 4.8 ppm and 3.6 ppm, corresponding to phosphorylated and unphosphorylated cyclohexanol, respectively. Reaction of cyclohexanol with increasing amounts of *bis*(pentafluorophenyl) chlorophosphate **77** did not produce a concomitant increase in the amount of phosphorylated product **107** obtained.

When cyclohexanol and Et_3N were added to the phosphorylating reagent (a reversal in the order of addition) quantitative phosphorylation was seen to occur within 15 minutes, as judged by the appearance of the signal at 4.8 ppm in the ^{1}H NMR spectrum, and the disappearance of the signal at 3.6 ppm. Removal of the solvent and purification by column chromatography afforded the required phosphate **107** in 98% yield {mp. 28-32 °C, Found 512.0223, $C_{18}H_{11}O_4F_{10}P_1$ requires 512.0235}, Scheme 2.6. The difference in yield observed when the order of addition of reagents is altered suggests either a change in mechanism or the predomination of other side reactions.

Analysis of the reaction of cyclohexanol with diphenyl chlorophosphate under identical conditions showed the reaction to be only 40% complete after 9 hours as calculated from the integrals of the peaks in the ^{31}P NMR spectrum corresponding to the chlorophosphate and the phosphorylated cyclohexanol, Fig. 2.3 below. The tall peak occurs at –5.0 ppm and corresponds to the diphenyl chlorophosphate, and the peak that is seen to increase in intensity is attributed to diphenyl cyclohexyl phosphate and occurs at –12.0 ppm.

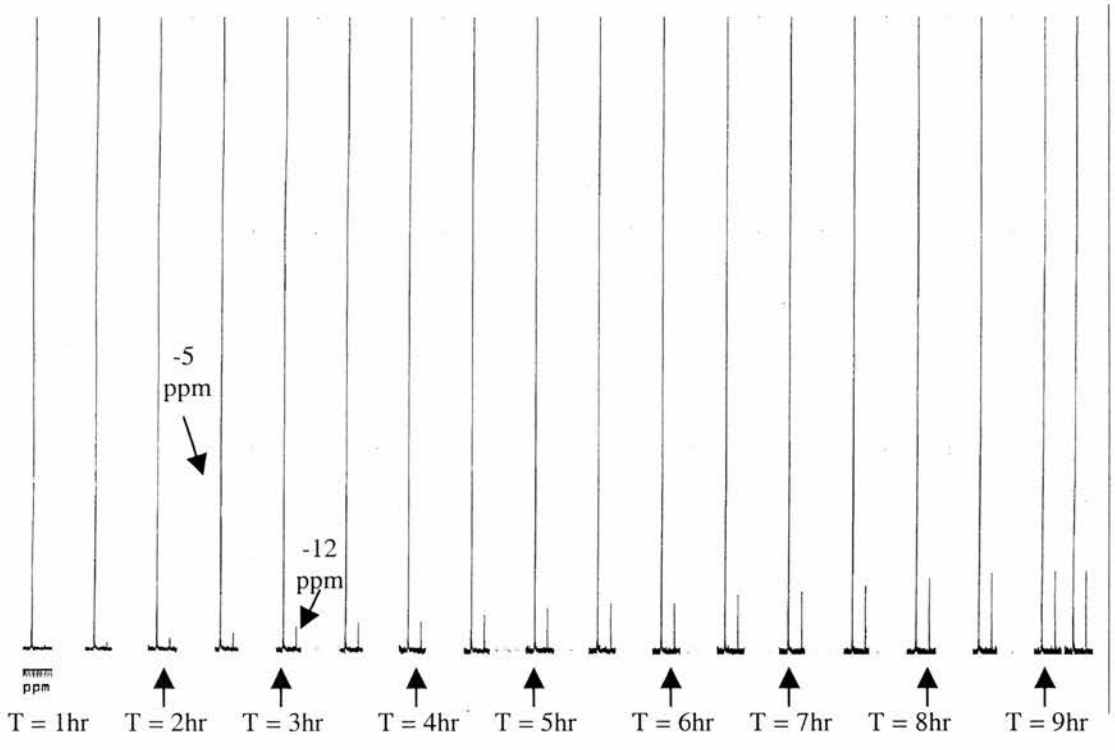


Figure 2.3: *Arrayed ^{31}P NMR spectra following the phosphorylation of cyclohexanol with diphenyl chlorophosphate.*

^{31}P NMR spectra were acquired every 15 minutes and are arrayed horizontally. For clarity, only alternate spectra are displayed. The reaction was performed with 2.5 equivalents of diphenyl chlorophosphate, hence the large intensity of the signal at –5.0 ppm. With *bis*(pentafluorophenyl) chlorophosphate,

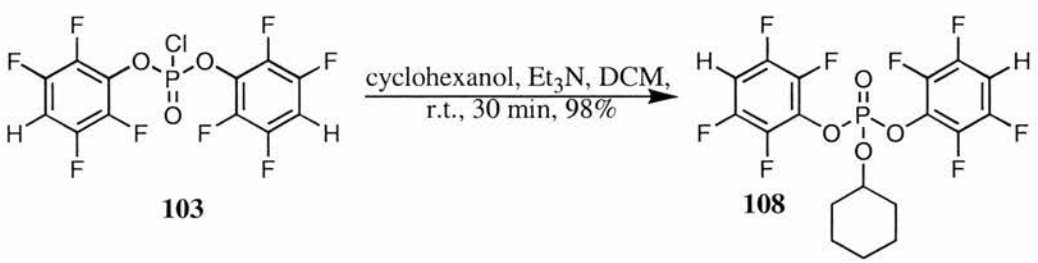
complete phosphorylation is achieved within 15 minutes; whereas with diphenyl chlorophosphate, only after 9 hours has the reaction reached completion (though not complete phosphorylation). The presence of the two pentafluorophenol groups has therefore caused an increase in reactivity of more than 30-fold. This can be attributed to the enhancement of the nucleophilicity of the phosphorus centre of the chlorophosphate **77** due to the electron withdrawing nature of the fluorine substituents on the two pentafluorophenyl rings.

When the phosphorylating agent is added to the substrate, it is being added to a vast excess of triethylamine and alcohol. Initial reaction between the *bis*(pentafluorophenyl) chlorophosphate **77** and triethylamine leads to expulsion of the chlorine group and the formation of a phospho-ammonium intermediate. This activated species may then either react with the alcohol substrate to form the desired phosphate triester product **107**, or may react either with more triethylamine or with the alcohol substrate, both of which are present in vast excess, to form an undesired product. The electron withdrawing nature of the fluorine atoms on the pentafluorophenyl group activates the phosphorus towards nucleophilic attack and the displacement of one of the pentafluorophenyl groups by a nucleophile such as triethylamine. The electron withdrawing nature of the fluorines also improve the leaving group ability of the pentafluorophenol. Therefore, under conditions of excess substrates and base, alternative side reactions may occur.

When the order of addition is reversed, the alcohol and triethylamine are added to an excess of chlorophosphate with respect to the amount that is added at any one time. As the addition proceeds, the chlorophosphate is always in excess, therefore, the chlorophosphate reacts with all the triethylamine present to form the mono-ammonium species. There is no more triethylamine present, therefore

reaction occurs between the phospho-ammonium adduct and the alcohol, to form the desired product, *bis*(pentafluorophenyl) cyclohexyl phosphate **107**.

In an analagous reaction, addition of cyclohexanol and triethylamine (1.2 equivalents) in DCM to a solution of *bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate **103** (1.4 equivalents) in DCM afforded the *bis*(2,3,5,6-tetrafluorophenyl) cyclohexyl phosphate triester **108** as a colourless oil in 98 % yield after purification by column chromatography, Scheme 2.9 {*m/z* (Found M^+, 476.0429. $C_{18}H_{13}O_4F_8P_1$ requires 476.0424)}.



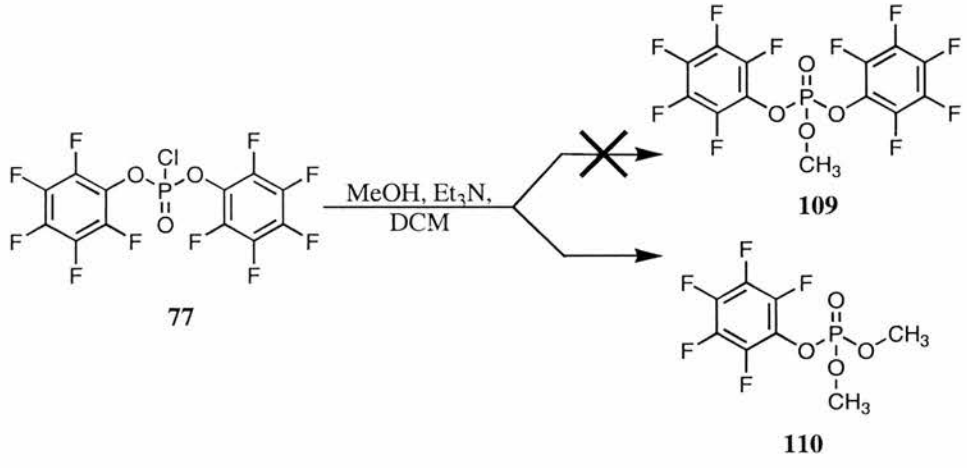
103 **108**

Scheme 2.7: *Synthesis of* bis*(2,3,5,6-tetrafluorophenyl) cyclohexyl phosphate **108**.*

When the substrate to be phosphorylated is a small primary alcohol such as methanol, an unexpected result is obtained. Reaction of *bis*(pentafluorophenyl) chlorophosphate **77** with methanol, did not give the expected *bis*(pentafluorophenyl) methyl phosphate **109** as the major product, but instead afforded the dimethyl pentafluorophenyl phosphate **110** as the isolated product as a colourless oil in 77 % yield, {*m/z* (Found M^+, 292.9928. $C_8H_6O_4F_5P_1$ required 291.9924)}, Scheme 2.8.

The lack of steric constraints when the nucleophile is small, such as is the case with methanol, allows a second molecule of methanol to attack the *bis*(pentafluorophenyl) methyl phosphate **109**, displacing one of the pentafluorophenyl groups, to form dimethyl pentafluorophenyl phosphate **110**.

Indeed, no *bis*(pentafluorophenyl) methyl phosphate **109** is observed in the product mixture, the reaction having proceeded through to the dimethyl pentafluorophenyl phosphate **110**.



Scheme 2.8: *Synthesis of dimethyl pentafluorophenyl phosphate **110***

2.3.2 Solution Phase Deprotection Studies

2.3.2.1 Deprotection under basic conditions

Preliminary investigations by P. Hormozdiari into the hydrolysis of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** under basic conditions had not yielded an effective deprotection protocol.[246] It was reasoned that the electron withdrawing nature of the fluorine atoms on the pentafluorophenyl ring would enhance the stability of the pentafluorophenolate anion $C_6F_5O^-$, thereby facilitating the removal of the pentafluorophenyl groups under basic conditions. Therefore, the deprotection of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** was investigated under a variety of basic conditions.

Initially a sample of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** was stirred with 2.2 equivalents of triethylamine in aqueous THF. In this system, the attacking nucleophile would be the hydroxide anion, not triethylamine, which would

abstract a proton from the water upon addition. Immediately upon addition of the cleavage mixture, a milky white suspension was observed, which later disappeared as the reaction proceeded. After stirring overnight, the reaction mixture was concentrated under reduced pressure and analysed by NMR spectroscopy. The ^{31}P NMR spectrum showed the presence of two peaks at –9.2 and –4.2 ppm, of relative integrals 23 and 77 respectively, corresponding to the starting material and pentafluorophenyl cyclohexyl phosphate monoanion. In an attempt to drive the reaction to completion, a variety of conditions were investigated, and these are summarised in Table 2.1 below.

Table 2.1: *Alkaline cleavage conditions evaluated.*

Base	Equiv.	Solvent	Temp	Time
Et_3N	2.2	THF/ H_2O	r.t.	O/N
NaOH	2.2, 5	THF/ H_2O	r.t.	O/N
KOH	5	THF/ H_2O	r.t.	O/N
$KHCO_3$	5, 10, 20	THF/ H_2O	r.t.	O/N

None of the conditions summarised above (Table 2.1) resulted in the formation of the desired cyclohexyl dihydrogen phosphate **111**. An increase in the amount of base used resulted in either incomplete reaction or the formation of a number of phosphorus containing species.

The pursuit of an alkaline hydrolysis protocol seemed to be ineffective so we therefore turned our efforts towards investigating the acid hydrolysis of *bis*(pentafluorophenyl) cyclohexyl phosphate **107**, as it has been

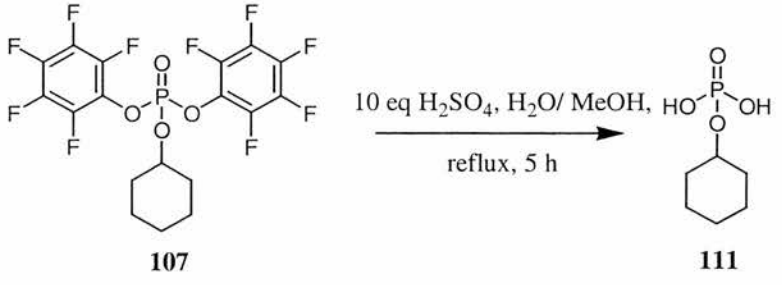
shown that, under solid phase synthesis conditions, the pentafluorophenyl groups are removed by treatment with aqueous acid.[211]

2.3.2.2 Deprotection under acidic conditions.

It has previously been shown that during solid phase synthesis of phosphothreonine and phosphoserine containing peptides employing *bis*(pentafluorophenyl) chlorophosphate **77**, deprotection of the phosphate moiety was achieved under acidolytic conditions.[211] Therefore it was envisaged that a similar deprotection strategy could be utilised in the solution phase deprotection of *bis*(pentafluorophenyl) phosphate triesters.

Accordingly, a range of acid conditions were evaluated and these are summarised in Table 2.2.

Treatment of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** with 10 equivalents of H_2SO_4 in refluxing aqueous methanol for 2 hours achieved the hydrolysis of the two pentafluorophenyl groups to afford cyclohexyl dihydrogen phosphate **111**, Scheme 2.9.



107 **111**

Scheme 2.9: *Cleavage of* bis*(pentafluorophenyl) cyclohexyl phosphate **107** with H_2SO_4 (aq.).*

Table 2.2: *Acid cleavage conditions evaluated.*

Acid	Equiv./ volume	Solvent	Temp (°C)	Time
H_2SO_4	10	H_2O/ MeOH	Reflux	2 h
TFA/H_2O/TES (90/5/5)	6 cm^3	N/A	r.t.	3 h
TFA/ H_2O (various ratios)	6 cm^3	N/A	r.t.	3 h
TFA/ HCl	10	THF	50	5 h
TFA/ H_2O (1/1)	6 cm^3	Toluene	r.t., reflux	3-5 h
TFA/ H_2O (9/1)	6 cm^3	Toluene	r.t., reflux	3-5 h
(0.5 M) TFMSA/ TFA	10	N/A	0	O/N
33% HBr/ AcOH[254]	5 cm^3	N/A	r.t.	O/N
(1 M) TMSOTf/ TFA/ PhSMe	3 cm^3	N/A	r.t.	O/N

However, these conditions were deemed too harsh to be generally applicable to the deprotection of more complex substrates and consequently, a milder deprotection method was sought.

In an analagous manner to the removal of pentafluorophenyl groups in solid phase peptide synthesis,[246] cyclohexyl phosphate triester **107** (0.4 mmol) was treated

with 6 cm^3 of a cleavage mixture of 90% TFA, 5% H_2O, 5% Et_3SiH for 3 hours. Analysis of the crude product by NMR spectroscopy revealed the presence of a new peak at 5.0 ppm, in the ^1H spectrum and a shift in the location of the pentafluorophenyl resonances in the ^{19}F NMR spectrum, speculatively assigned to either cyclohexyl pentafluorphenyl phosphate diester or *O*, *O*'-dicyclohexyl-*O*, *O*'-*bis*(pentafluorophenyl)-pyrophosphate.

Analysis of the cleavage products is hampered by the lack of an observable proton on the phosphate protecting groups. Use of *bis*(2,3,5,6-tetrafluorophenyl) cyclohexyl phosphate **108** as an analogue of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** provides a means of observing, *via* ^1H NMR spectroscopy, the extent of deprotection of the phosphate. Therefore, treatment of 0.25 mmol of *bis*(2,3,5,6-tetrafluorophenyl) cyclohexyl phosphate **108** with 20 equivalents of TFA : H_2O (1:1) for 5 hours, followed by removal of the solvent under reduced pressure resulted in the appearance of a new peak in the ^1H NMR spectrum at 4.8 ppm, but no new peak in the ^{31}P NMR spectrum. The main ^{31}P NMR peak occurred at –12.64 ppm, corresponding to the phosphate triester **108**. Reaction of triester **108** with 1:1 v/v TFA: H_2O and $MgCl_2$ resulted in the removal of one of the tetrafluorophenyl groups and formation of the cyclohexyl tetrafluorophenyl phosphate diester (δ_P –4.2).

A solution of cyclohexyl *bis*(pentafluorophenyl) phosphate triester **107** in dichloromethane was treated with 95% TFA / 5% H_2O (v/v) and allowed to stir for 5 hours. Analysis of the reaction mixture by ^1H and ^{31}P NMR spectroscopy, after removal of the solvent and excess reagent under reduced pressure, revealed peaks in the ^1H and ^{31}P spectra, at 4.5 ppm and –5.12 ppm respectively. The peaks corresponding to the starting material were also present. It was observed that very little of the starting material had reacted and this was attributed to the small amount

of unprotonated water present in the 95% TFA / 5% H_2O (v/v) mixture. Leaving the reaction overnight resulted in the disappearance of the signal at –11 ppm, and the appearance of multiple ^{31}P NMR signals.

Therefore, the effect of varying the relative proportions of water and TFA was investigated. Solutions of 50 mg of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** (0.1 mmol) in dichloromethane were treated individually with solutions of varying concentrations of trifluoroacetic acid in water, and the extent of reaction was judged by the relative integral of the new signal at 4.5 ppm in the ^1H NMR spectrum compared to the signal at 4.8 ppm corresponding to the starting material. The results are shown in the graph below (Fig. 2.4).

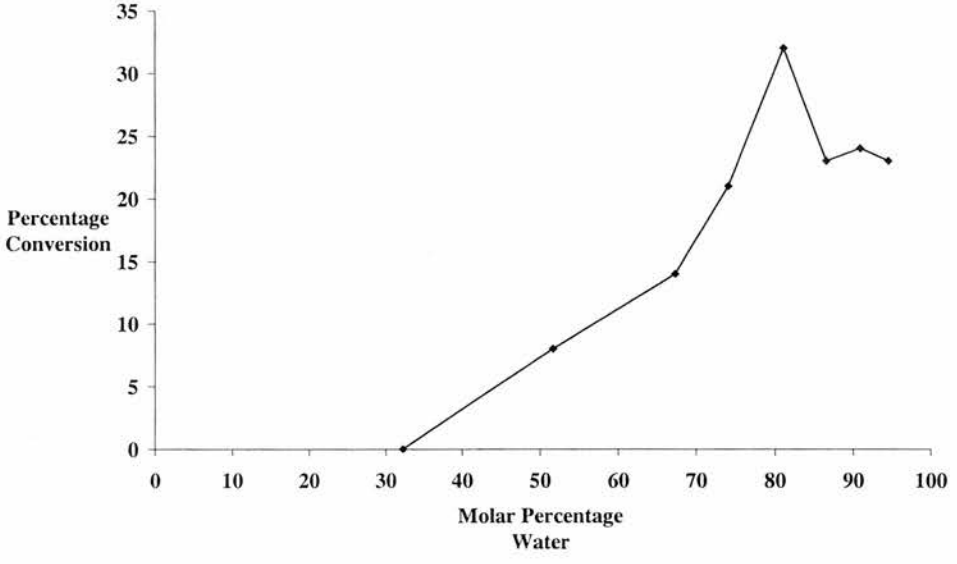


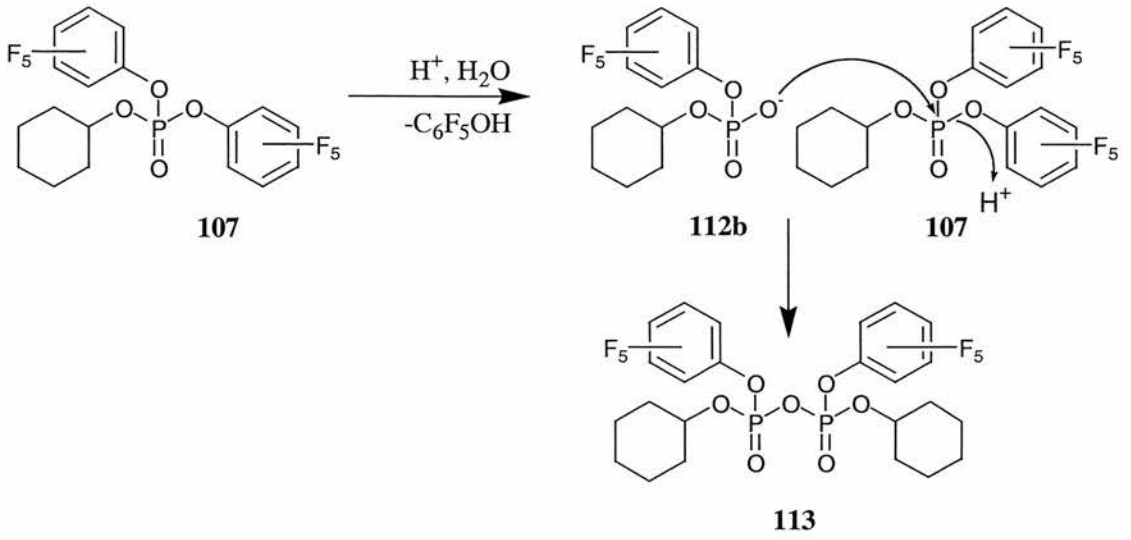
Figure 2.4: *Graph of percentage hydrolysis of phosphate **107** against molar percentage of H_2O in cleavage mixture*

It was initially assumed that this new peak was due to the presence of cyclohexyl pentafluorophenyl phosphate diester **112**, or cyclohexyl dihydrogen phosphate **111**. Comparison with a sample of cyclohexyl dihydrogen phosphate **111**

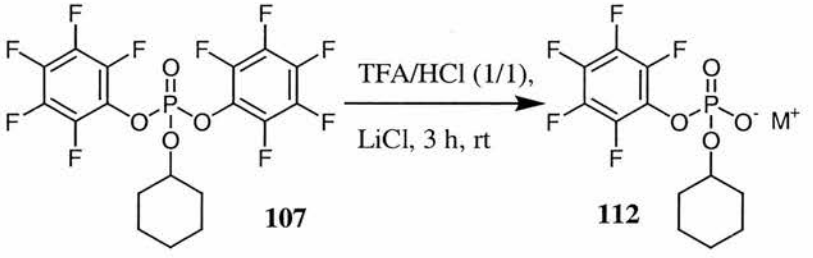
synthesised separately,[246] showed that this was not the product being observed. Analysis of a number of the reaction mixtures by mass spectrometry also failed to observe a peak corresponding to cyclohexyl dihydrogen phosphate **111** or the phosphate diester **112**. Therefore, it was rationalised that it may be the pyrophosphate **113** that was being formed, and not in fact the monoester **111** or diester **112a** being formed. Acidic hydrolysis of one pentafluorophenyl group from *bis*(pentafluorophenyl) cyclohexyl phosphate **107** leads to the formation of the pentafluorophenyl cyclohexyl phosphate diester **112a**. Due to the low pK_a of the phosphate diester **112a**, this almost immediately ionises to form the diester anion **112b**, which can then attack the phosphate triester **107** to displace a pentafluorophenyl group and form the pyrophosphate **113**, (Scheme 2.10).

Analysis of the mass spectrum, however, failed to show a peak corresponding to the pyrophosphate **113**.

It is a common motif in the Ser/ Thr protein phosphatases to utilise metal ions in the catalytic cycle.[255, 256] These metal ions often act either as Lewis acids, enhancing the electrophilicity of the phosphorus centre through increased polarisation of the P=O bond, or as a means of dissipating charge. Therefore, reactions were repeated in the presence of 1 or 2 equivalents of $MgCl_2$ or LiCl, Scheme 2.11. This led to the removal of only one of the pentafluorophenyl groups as judged by ^{31}P NMR spectroscopy, (δ_P -4 to -6), and indeed, reactions involving *bis*(2,3,5,6-tetrafluorophenyl) cyclohexyl phosphate **108** showed the presence of 1 tetrafluorophenyl group by ^{1}H and ^{19}F NMR spectroscopy.



Scheme 2.10: *Proposed mechanism of formation of* O,O'-bis*(pentafluorophenyl)*-O,O'-bis*(cyclohexyl) pyrophosphate* ***113*** *during acidic hydrolysis of bis(pentafluorophenyl) cyclohexyl phosphate* ***107***



Scheme 2.11: *Acidolysis of* bis*(pentafluorophenyl) cyclohexyl phosphate* ***107*** *in the presence of metal ions.*

The dissimilarity in reaction between the resin bound threonine and serine phosphate esters and *bis*(pentafluorophenyl) cyclohexyl phosphate may be due to the environment within the resin. Hodge has reported a number of examples of reactions carried out in homogeneous reaction systems and polymer support, which differed in reaction rates, reaction course or stereochemical result.[257] Wang resin is essentially a polystyrene matrix and any reaction taking place within it is doing so in an extremely aromatic and hydrophobic environment. In an attempt to simulate this

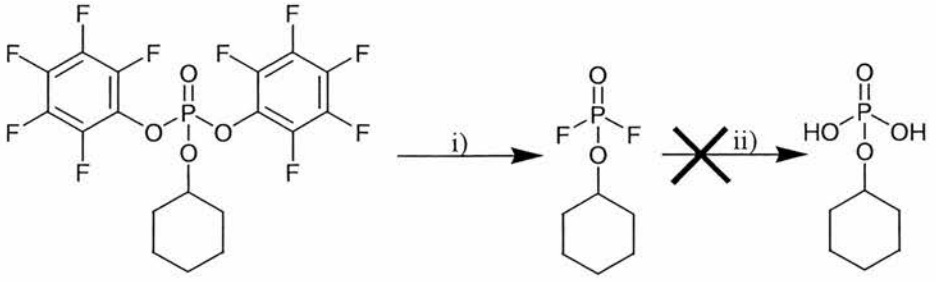
environment in solution, toluene was used as a solvent. However the desired cyclohexyl dihydrogen phosphate was not obtained. Therefore it seems that the conditions within the polymer matrix of the Wang resin are sufficiently fine tuned to allow acid cleavage of the pentafluorophenyl groups, but it is not possible to duplicate this environment in the solution phase.[257]

2.3.2.3 Deprotection via difluorophosphate intermediate

The synthesis of cyclohexyl dihydrogen phosphate **111** by Montgomery *et al* had utilised a dichlorophosphate intermediate. It has also been reported that fluorine has a great affinity for phosphorus. Therefore it was envisaged that treatment of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** with a source of fluoride such as TBAF would result in the formation of cyclohexyl difluorophosphate **115** which could then be hydrolysed to the dihydrogen phosphate **111** in an analogous manner to the dichlorophosphate, Scheme 2.12.

Accordingly, a solution of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** in THF was treated with 4 equivalents of a 1.0 mol dm^{-3} solution of TBAF in THF. Analysis of the reaction mixture by TLC after 1 hour showed the presence of two species. The less polar species co-eluted with pentafluorophenol, and the more polar species remained at the baseline. Further analysis by ^{1}H and ^{19}F spectroscopy showed the disappearance of the α-CH peak for the triester **107** at 4.8 ppm and the appearance of a new peak at 4.2 ppm. Analysis of the ^{31}P NMR spectrum showed 2 new peaks at –8.9 ppm and –1.3 ppm. This, however, is not evidence of formation of the fluorophosphates **115**. Stirring of the reaction mixture with water for 4 hours failed to achieve the hydrolysis of the putative difluorophosphate to the required

dihydrogen phosphate. As a number of fluorophoshates are known nerve agents, the failure of the reaction could be viewed as a blessing in disguise!



107 **115** **111**

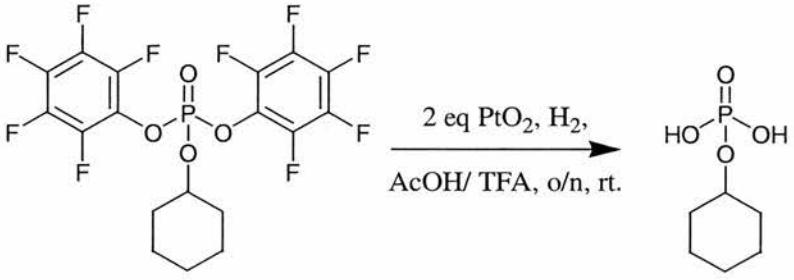
Reagents and Conditions: i) 4 eq. 1.0 mol dm^{-3} TBAF, THF, 1 h, rt.; ii) H_2O, 4h, rt.

Scheme 2.12: *Attempted synthesis of dihydrogen cyclohexyl phosphate **111*** via *difluorophosphate **115**.*

2.3.2.4 Hydrogenation of phosphate 107

Diphenyl phosphates may be deprotected by hydrogenation over Pd or Pt under a variety of conditions. Therefore, in the face of the lack of success with acidic or basic hydrolysis, it was decided to investigate whether the pentafluorophenyl groups could be removed by hydrogenolysis under acidic conditions. This would allow cleavage of the peptide from the resin (especially if a super-acid sensitive resin such as trityl resin is used). Hydrogenation of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** over 10% Pd/ C gave a complex mixture of products. Using Adams' catalyst (PtO_2) instead of Pd/C and using TFA : AcOH (8:7[258] or 1:1[259]), or $HCl_{(g)}$ in AcOH, resulted in quantitative conversion of the triester **107** to the desired dihydrogen phosphate **111**, Scheme 2.13. However, the use of a catalyst in the presence of resin may lead to problems in separating the catalyst from the resin, prior to treatment of the resin with a cleavage mixture.

Furthermore, it has been shown that resin bound diphenyl-protected phosphothreonine peptides are not deprotected under standard hydrogenation protocols.[246] It is therefore unlikely that this procedure can be applied to the solid phase. From this, it is apparent that *bis*(pentafluorophenyl) chlorophosphate **77** is not suitable for synthesis of phosphotyrosine containing peptides or for solution phase synthesis of phosphate containing molecules, and that an alternative reagent was required.

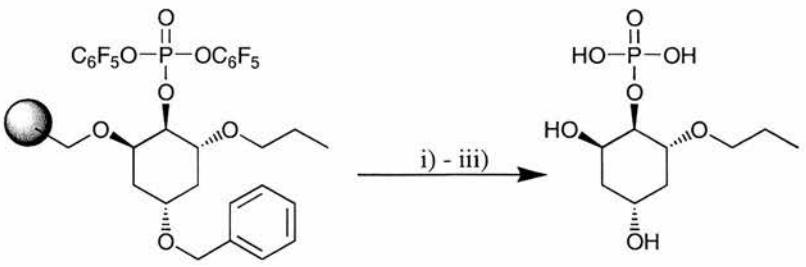


107 **111**

Scheme 2.13: *Acidic hydrogenation of triester **107**.*

2.3.3 Further applications of *bis*(pentafluorophenyl) chlorophosphate 77

As discussed in Section 2.1, *bis*(pentafluorophenyl) chlorophosphate **77** has been successfully utilised in the solid phase synthesis of phosphoserine and phosphothreonine containing peptides.[211] This reagent has also found use within our laboratories in the solid phase synthesis of proposed inositol monophosphatase inhibitors on Merrifield resin,[260] whereby removal of the pentafluorophenyl groups is achieved by the use of stannic chloride ($SnCl_4$) to afford the phosphate **117** from the resin bound phosphate triester **116**, Scheme 2.14.



116 **117**

*Reagents and Conditions:*i) 8 eq $SnCl_4$, DCM, 16 hours, rt;
ii) NH_4OH (aq); iii) cellulose phosphate cation exchange resin

Scheme 2.14: *Removal of pentafluorophenyl groups by stannic chloride ($SnCl_4$)*

2.4 Benzyl pentafluorophenyl chlorophosphate 118

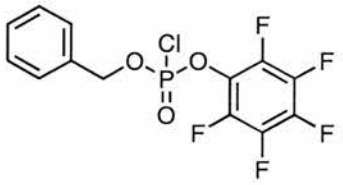
2.4.1. Design of the Reagent

Due to the enhanced reactivity of a reagent containing one or more pentafluorophenyl groups, it is advantageous to retain one pentafluorophenyl group in the new reagent. The pentafluorophenyl group will enhance the reactivity of the phosphorus centre and could also be easily removed by alkaline or acidic hydrolysis. The second protecting group should be able to be removed after the removal of the pentafluorophenyl group and would not rely on P-O bond fission for removal, as this would make it unsuitable for use in the preparation of phosphotyrosyl substrates.

From an examination of the literature, only five different chlorophosphates, $(RO)_2POCl$, have found general application in phosphopeptide synthesis, Table 1.5 p 61, **72-76** (R – Me, Ph, $PhCH_2$, ^tBu, allyl).

Removal of the methyl group requires the use of harsh acidic conditions, typically HF,[261] TMSBr/TFA[185] or TFMSA.[262] Removal of the allyl groups is achieved using tetra*kis*(triphenylphosphine) Pd(0).[205] However, for solid phase phosphorylations, an acid hydrolysis step is still required to remove other protecting groups and cleave the peptide from the resin. Some researchers have reported the

instability of di-^{t}butyl phosphotyrosyl containing peptides.[263] This means that further manipulation of the peptide after phosphorylation is likely to lead to degradation and unwanted side reactions. The removal of benzyl protecting groups from phosphates under acidic conditions has been well documented. From this it is evident that the benzyl group offers the greater scope for flexibility and ease of removal. The reagent so envisaged was benzyl pentafluorophenyl chlorophosphate **118**.



118

It was envisaged that the pentafluorophenyl group could be removed by treatment with aqueous alkali, and subsequent treatment with aqueous acid would result in either nucleophilic attack at the benzylic carbon, followed by subsequent C-O bond fission to give the phosphate anion and the benzylated nucleophile (S_N2 like mechanism) or S_N1 cleavage of the benzyl group to give the phosphate anion and the benzyl carbocation, which would be subsequently quenched by the 'nucleophile'.

2.4.2 Synthesis of Benzyl Pentafluorophenyl chlorophosphate 118

2.4.2.1 Direct reaction of chlorophosphate and alcohol

An initial analysis of the literature concerning the synthesis of chlorophosphates showed that the vast majority of the syntheses were of 'symmetrical' chlorophosphates (Fig 2.5 below, where $R^1 = R^2$). Syntheses of unsymmetrical chlorophosphates (Fig. 2.5 below, where $R^1 \neq R^2$) involved the

reaction of $POCl_3$ with the first alcohol (R^1OH), followed by isolation of the desired dichlorophosphate ($R^1OP(O)Cl_2$) and reaction of this with the second alcohol (R^2OH). From this reaction mixture the desired chlorophosphate ($(R^1O)(R^2O)P(O)Cl$) could be isolated.

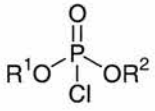
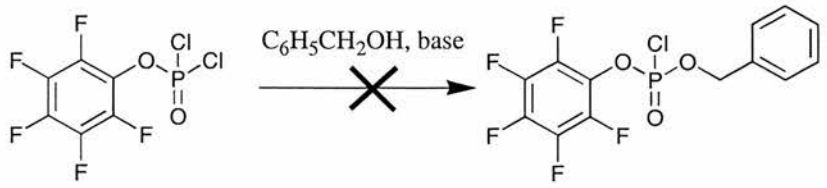


Figure 2.5: *Generalised chlorophosphate structure*

There are a number of problems with this approach. At both stages of the reaction, there may be considerable amounts of undesired byproducts - $(R^1O)_2P(O)Cl$ and $(R^1O)_3PO$ at the first stage, $(R^1O)(R^2O)_2PO$ and $(R^2O)_3PO$ at the second stage. This results in a poor yield of the desired product being obtained. An additional problem arises from the use of benzyl chlorophosphates. These have been shown to be unstable above 30 °C or so, and therefore distillation can not readily be used to purify them.

In the synthesis of *bis*(pentafluorophenyl) chlorophosphate **77**, the main byproduct was pentafluorophenyl dichlorophosphate **91**. It was therefore hoped that reaction of this byproduct with benzyl alcohol would yield the desired pentafluorophenyl benzyl chlorophosphate **118** directly (Scheme 2.15). This would allow a highly convergent synthesis of both *bis*(pentafluorophenyl) chlorophosphate **77** and pentafluorophenyl benzyl chlorophosphate **118**.



91 **118**

Scheme 2.15: *Attempted synthesis of benzyl pentafluorophenyl chlorophosphate* ***118*** *from pentafluorophenyl dichlorophosphate* ***91.***

Reaction of pentafluorophenyl dichlorophosphate **91** with benzyl alcohol under a number of conditions, (Scheme 2.15 and Table 2.3), yielded a variety of products, often in complex mixtures, the components of which were difficult to identify. When triethylamine and quinuclidine were used as bases, the isolated product was *bis*(pentafluorophenyl) benzyl phosphate **124**. In none of the reactions was the desired benzyl pentafluorophenyl chlorophosphate **118** isolated.

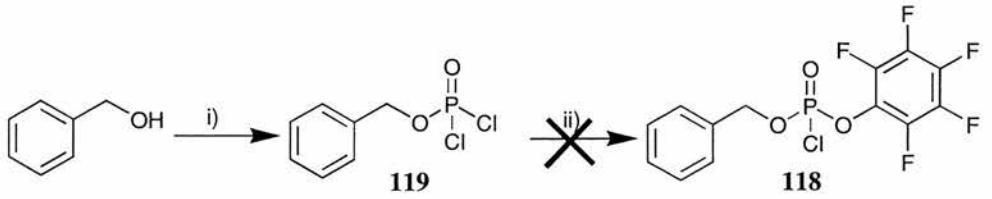
Table 2.3: *Base reagents and conditions evaluated.*

Base	**Solvent**	**Temp (°C)**	**Time**
2,6-lutidine	DCM	r.t	2 h
Et_3N	DCM	r.t	2 h
quinuclidine	DCM	r.t	2 h
pyridine	DCM	r.t	2 h
none	None	r.t	2 h
none	None	heating	2 h
none	Toluene	reflux	2 h

Therefore, the synthetic route from pentafluorophenyl dichlorophosphate **91** was abandoned and an alternative route to the target was investigated. It was decided to react all the constituents ($POCl_3$, pentafluorophenol and benzyl alcohol) together and then attempt to isolate the desired product as the final purification step. Kaz'menko *et al.* had reported the use of a catalytic amount of $AlCl_3$ to promote the reaction of $POCl_3$ with two alcohols.[264]

In order to investigate the requirement for $AlCl_3$ in the reaction, a control reaction was carried out in the absence of $AlCl_3$. Phosphoryl chloride, pentafluorophenol and benzyl alcohol were heated under reflux for 12 hours resulting in the formation of a black liquid. Distillation under reduced pressure afforded a colourless oil which later solidified. This was identified as *bis*(pentafluorophenyl) chlorophosphate **77** by NMR spectroscopy. Addition of benzyl alcohol to a mixture of pentafluorophenol, $POCl_3$ and $AlCl_3$ at low temperature followed by gentle heating caused the evolution of white fumes at 60 °C followed by a highly exothermic reaction which saw the temperature of the reaction rise to over 100 °C within a few minutes and the formation of an amorphous brown solid in the flask. The mixture was heated at 110 °C for 3 hours and then at 140 °C for a further 3 hours. The resultant black tar contained no identifiable product.

An alternative strategy was to form benzyl dichlorophosphate **119** first and add pentafluorophenol to it, in the presence of a suitable base if required, to form benzyl pentafluorophenyl chlorophosphate **118**, Scheme 2.16 and Table 2.4 below.



Reagents and Conditions: i) $POCl_3$, base; ii) C_6F_5OH, NaH, THF, 0°C

Scheme 2.16: *Attempted synthesis of benzyl pentafluorophenyl chlorophosphate* ***118*** *from benzyl alcohol.*

Table 2.4: *Base reagents and conditions evaluated.*

Base	**Solvent**	**Temp (°C)**
None	none	heating
Et_3N	THF	r.t.
quinuclidine	THF	r.t.
NaH	THF	r.t.
BuLi	THF	0
BuLi	THF	-78
KH	THF	r.t.
LiH	THF	r.t.
KO^tBu	THF/ ^{t}BuOH	r.t.

Quinuclidine and triethylamine were added to the $POCl_3$ and BzlOH mixture, before reaction with sodium pentafluorophenoxide (formed by the reaction of sodium hydride and pentafluorophenol) in THF, neither resulting in the formation of the desired product. Pre-formation of the benzyloxy anion, by reaction of benzyl

alcohol with a metal hydride or BuLi in THF, and addition to $POCl_3$ in THF, followed by reaction with sodium pentafluorophenoxide gave a mixture of products in all cases. Attempts to purify the reaction mixture by distillation resulted in the decomposition of the reaction products, due to the instability of the benzyl phosphate linkage above 30-40 °C.

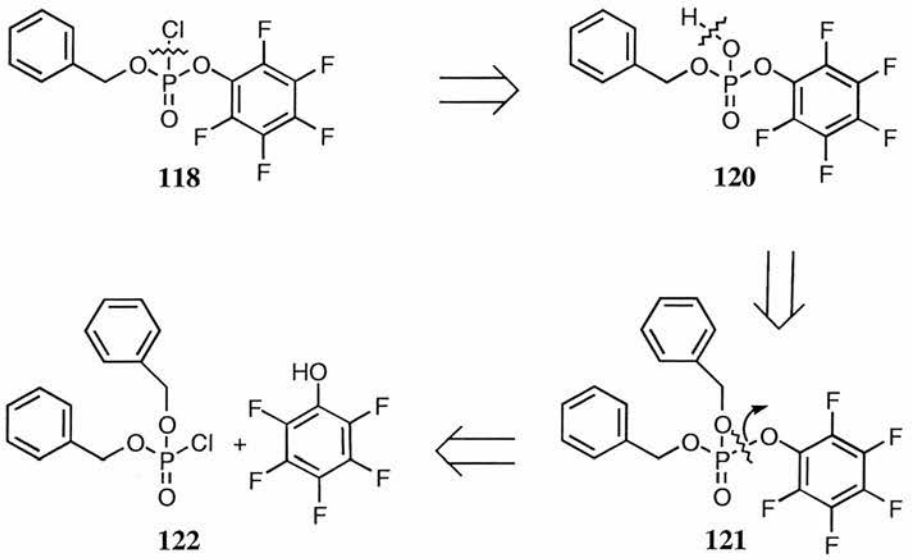
The three methods involving addition of an alcohol to $POCl_3$ or a dichlorophosphate all result in a variety of reaction products, from which it is not possible to isolate the desired product. Therefore, an alternative route is required, avoiding the formation of an intermediate dichlorophosphate.

2.4.2.2 Monodebenzylation of dibenzyl pentafluorophenyl phosphate triester 121

Both Lebeau *et al.*,[265] and Zervas *et al.*,[266] have reported the monodebenzylation of benzyl phosphate esters and the subsequent chlorination of the phosphate diester to form the chlorophosphate in essentially quantitative yield. It was therefore envisaged that benzyl pentafluorophenyl chlorophosphate **118** could be obtained from the chlorination of benzyl pentafluorophenyl hydrogen phosphate **120**. This, in turn, is the product of the monodebenzylation of dibenzyl pentafluorophenyl phosphate **121**, which can be synthesised from dibenzyl chlorophosphate **122** and pentafluorophenol, Scheme 2.17.

Dibenzyl chlorophosphate is unstable at more than 30 °C and is usually prepared *in situ* for phosphorylations.[267] Accordingly, dibenzyl phosphite **123** was treated with *N*-chlorosuccinimide in toluene, according to the method of Atherton,[267] to give dibenzyl chlorophosphate **122**, which was used for phosphorylations *in situ*, Scheme 2.18. Reaction of sodium hydride with pentafluorophenol in THF produced a clear solution of sodium

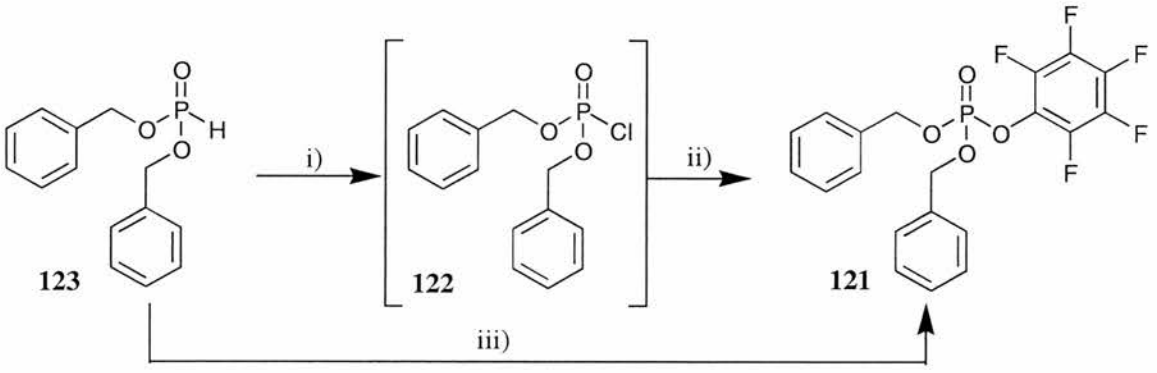
pentafluorophenolate. This was added to the dibenzyl chlorophosphate solution *via* cannula and allowed to stir for 30 minutes, Scheme 2.18. After removal of the THF and aqueous workup, dibenzyl pentafluorophenyl phosphate triester **121** was recrystallised from toulene / light petrol as a white solid in 78% yield (over 2 steps), {mp 60-62 °C, (Found C, 53.95; H, 3.05; $C_{20}H_{14}F_5O_4P_1$ requires: C, 54.05, H, 3.2 %)}



Scheme 2.17: *Retrosynthesis of benzyl pentafluorophenyl chlorophosphate **118***

The sodium pentafluorophenolate was added to the dibenzyl chlorophosphate, and not *vice versa*, to ensure that the pentafluorophenolate was only ever present in small amounts before it reacted with the dibenzyl chlorophosphate. If a substantial amount of pentafluorophenolate was present, there is a danger that displacement of a benzyl group from the triester **121** may occur, resulting in the formation of unwanted *bis*(pentafluorophenyl) benzyl phosphate **124**.

An alternative one-pot synthesis of dibenzyl pentafluorophenyl phosphate **121**, using CCl_4, Et_3N and DMAP, avoids the use of flammable metal hydrides, although it does involve the use of carcinogenic CCl_4,[268] Scheme 2.18. Reaction of dibenzyl phosphite with pentafluorophenol, CCl_4, Et_3N and DMAP in MeCN afforded a white solid after aqueous workup and recrystallisation, with identical spectral characteristics to the triester **121** prepared by the method above.



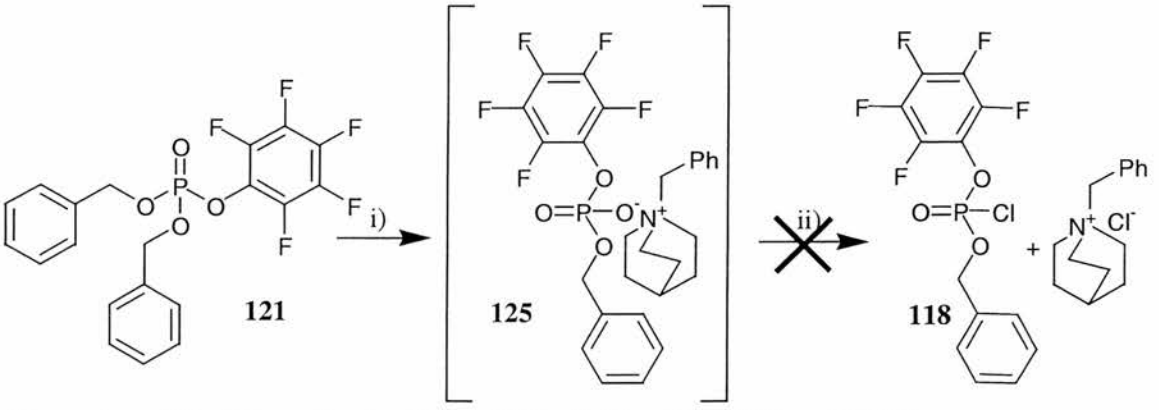
Reagents and Conditions: i) *N*-chlorosuccinimide, PhMe, r.t., 100%; ii) C_6F_5OH, NaH, THF, 80%; iii) C_6F_5OH, CCl_4, Et_3N, DMAP, MeCN, 85%

Scheme 2.18: *Synthesis of dibenzyl pentafluorophenyl phosphate triester **121***

The methods of Lebeau[265] and Zervas[266] both rely on the nucleophilic attack on the benzylic carbon rather than at the phosphorus centre. To ensure this they either use a hindered non-nucleophilic base (quinuclidine) or a soft nucleophile (I^-). Both of these prefer attack on the 'soft' benzylic carbon centre rather than the 'hard' phosphorus centre.

The one-pot debenzylation-chlorination method of Lebeau[265] was the initial method of choice, as it did not require the isolation of an intermediate. Treatment of the dibenzyl phosphate triester **121** with quinuclidine would form the benzyl quinuclidinium phosphate salt **125** and treatment of this with oxalyl chloride would

lead to the formation of benzyl pentafluorophenyl chlorophosphate **118** and benzylquinuclidinium chloride, Scheme 2.19 below. Accordingly, dibenzyl pentafluorophenyl phosphate **121** was treated with 1 equivalent of quinuclidine in refluxing toluene for 1 hour. After cooling to 0 °C, the solution was treated with oxalyl chloride and a catalytic amount of DMF for 30 minutes.

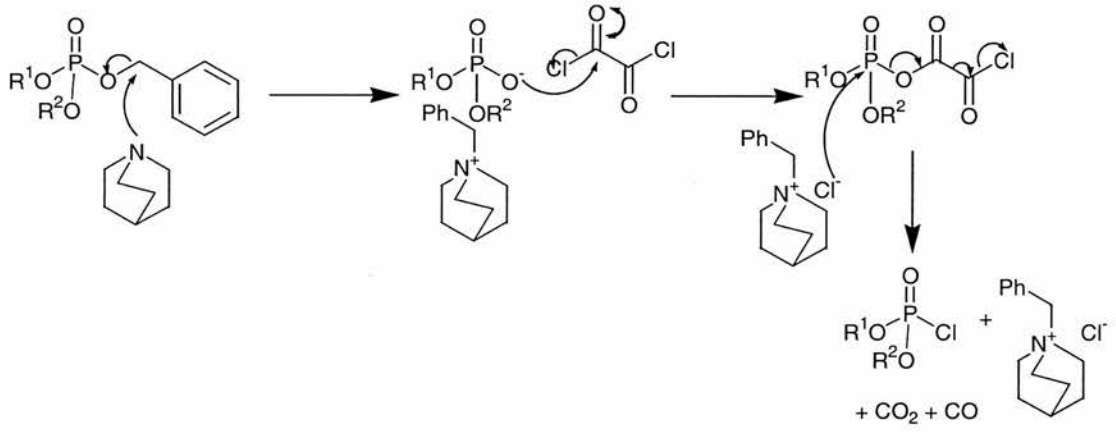


Reagents and Conditions: i) quinuclidine, PhMe, Δ, 1 h ; ii) $(COCl)_2$, cat. DMF.

Scheme 2.19: *Attempted synthesis of benzyl pentafluorophenyl chlorophosphate **118** by the method of Lebeau.*[265]

Removal of the chloride salts by filtration and concentration of the filtrate under reduced pressure afforded a colourless oil. Analysis of the reaction mixture after addition of the quinuclidine and heating for 1 hour showed that monodebenzylation had occurred and the quinuclidinium salt was present (δ_P –3.71, δ_H 5.20 [d, *J* 6.0]). However, subsequent chlorination with oxalyl chloride failed to give the desired chlorophosphate. The use of new quinuclidine and oxalyl chloride made no apparent change to the reaction. Analysis of the ^{31}P NMR spectrum revealed a set of peaks at –17.5 to –19.3 ppm, characteristic of the presence of a pyrophosphate. The formation of the pyrophosphate can be rationalised by an

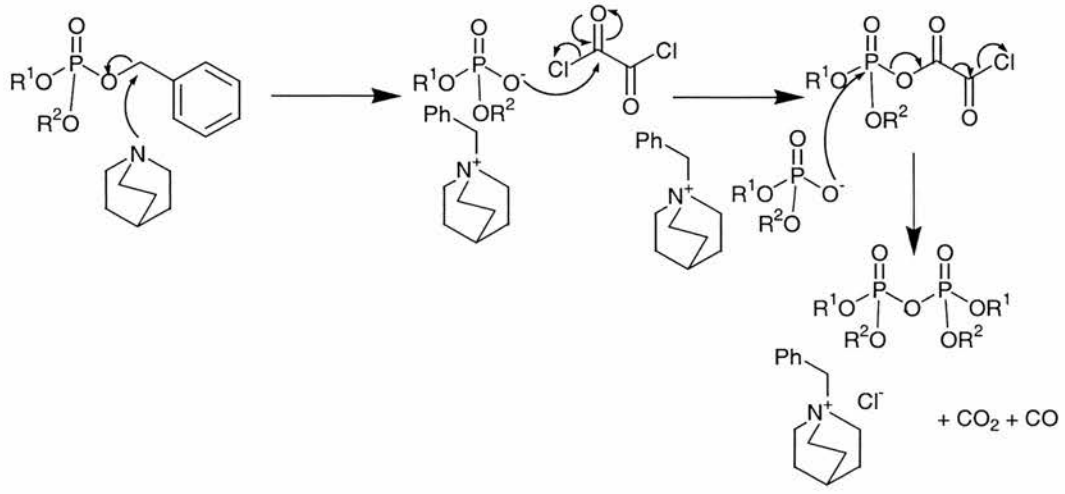
alternative mechanism to the one that normally operates. The standard mechanism is shown in Scheme 2.20. Under normal conditions, attack of the quinuclidine on the benzylic carbon leads to formation of the benzylquinuclidinium phosphate diester. This, in turn is acylated by the addition of oxalyl chloride, which liberates a chloride anion. Attack of this chloride anion on the phosphorus centre of the unstable acyl phosphate leads to decomposition, formation of the desired chlorophosphate and benzylquinuclidinium chloride with release of carbon dioxide and carbon monoxide, which are lost to the atmosphere.



Scheme 2.20: *Mechanism for synthesis of chlorophosphates by the method of Lebeau* et al. [265]

However, an alternative mechanism is possible, Scheme 2.21. Addition of a portion of oxalyl chloride results in the formation of a small amount of the unstable acyl phosphate, which is attacked by the remaining phosphate diester anion, and not by the chloride anion. This also leads to the formation of benzylquinuclidinium chloride, carbon dioxide and carbon monoxide, but does not form the desired

chlorophosphate. In this mechanism, the product is the pyrophosphate. Any excess oxalyl chloride, being extremely volatile, is lost to the atmosphere upon workup.



Scheme 2.21: *Alternative mechanism leading to formation of pyrophosphate.*

Whilst it was possible to obtain the chlorophosphate **118** using this method, the result could not be reproduced with any reliability. It was also noted that quinuclidine is both hygroscopic and expensive. Therefore, a cheaper and more robust alternative to quinuclidine was sought. The requirements were for a greater tolerance for atmospheric moisture, to aid in handling of the reagent, and a nucleophilicity similar to that of quinuclidine.

The nucleophilicity of a reagent may perhaps be expected to correlate with its basicity, in that both involve the availability of electron pairs and the ease with which they are donated. However, basicity involves lone pair donation to hydrogen, whereas nucleophilicity involves electron pair donation to another atom. Basicity involves an equilibrium (thermodynamic) situation whereas nucleophilicity usually involves a kinetic one. Basicity is likely to be little affected by steric influences whereas nucleophilicity may be markedly affected. However, provided like is being

compared with like (*i.e.* attacking atom is the same), then the two run reasonably in parallel and the stronger the base, the more powerful the nucleophile.

The reaction was repeated with NMM instead of quinuclidine. A solution of the phosphate triester **121** and 1 equivalent of NMM in toluene was refluxed for 4 hours, with a 1 cm^3 aliquot being removed every hour and analysed by ^1H and ^{31}P NMR spectroscopy, Table 2.5.

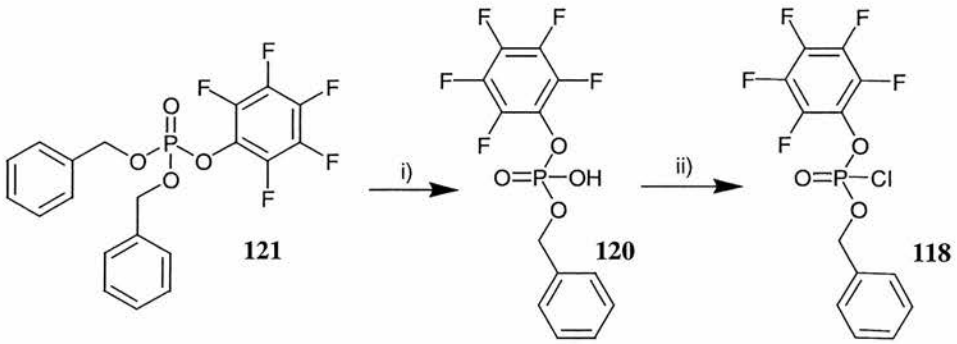
Table 2.5: *Ratio of **118** to debenzylated NMM salt as function of reaction time.*

Duration of heating	**Ratio of starting material 121 to NMM salt**
2 h	2.19:1
3 h	1.16:1
4 h	0.86:1

However, after 3 hours of heating, a number of other products were visible in the ^{31}P NMR spectrum. The substitution of *N*-phenylmorpholine for *N*-methylmorpholine resulted in the loss of any reaction. *N*-Phenylmorpholine has too much steric bulk compared to *N*-methylmorpholine and is not sufficiently nucleophilic even to attack the benzylic carbon of the benzyl group. Reaction of the phosphate triester **121** with pyridine in refluxing toluene for 1 hour led to 10% reaction as judged from ^1H and ^{31}P NMR spectroscopy. It was felt that the nucleophilicity of the pyridine could be enhanced by the presence of electron donating substituents on the pyridine ring. Therefore the reaction was repeated with DMAP instead of pyridine. Reaction of the phosphate triester **121** with 1 equivalent of DMAP in refluxing toluene for 2 hours led to the formation of a yellow solution

and a noticeable change in the appearance of the ^{13}C and ^{19}F NMR spectra. There was a slight shift in the position of the ^{31}P resonance, from –5.44 to –3.91 ppm, and a slight change in the ^{1}H NMR spectrum. This was reacted with oxalyl chloride to give a clear oil with a ^{31}P signal at –18 ppm, which corresponded to the dibenzyl-*bis*(pentafluorophenyl) pyrophosphate **126**. Formation of the phosphate anion was possible, but it seemed that it was more nucleophilic than chloride and reacted with the acyl intermediate instead of the chloride anion, leading to formation of the pyrophosphate. This method was therefore abandoned and we turned our attention to the method of Zervas *et al*,[266] which involved a two step strategy of debenzylation with anhydrous NaI, followed by chlorination with PCl_5.

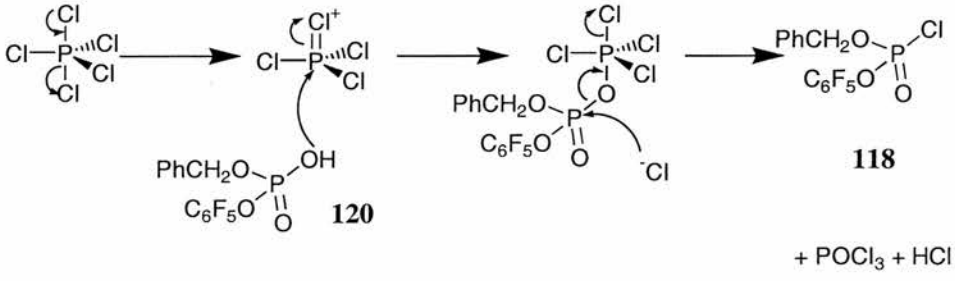
Accordingly, reaction of dibenzyl pentafluorophenyl phosphate **121** with 1 equivalent of NaI and acidification with 1 mol dm^{-3} HCl led to the isolation of benzyl pentafluorophenyl phosphoric acid diester **120** as a white solid in quantitative yield, {mp 112 °C, (Found C, 44.0; H, 2.15. $C_{13}H_8F_5O_4P_1$ requires C, 44.1, H 2.3%)}, Scheme 2.22. The yellow solution was concentrated under reduced pressure to afford a yellow oil identified as benzyl iodide.



Reagents and Conditions: i) NaI, acetone, Δ, 15 min, then $HCl_{(aq)}$, 100%; ii) PCl_5, CH_2Cl_2, 30 min, 70-90%

Scheme 2.22: *Successful synthesis of benzyl pentafluorophenyl chlorophosphate* ***118*** *by the method of Zervas.*[266]

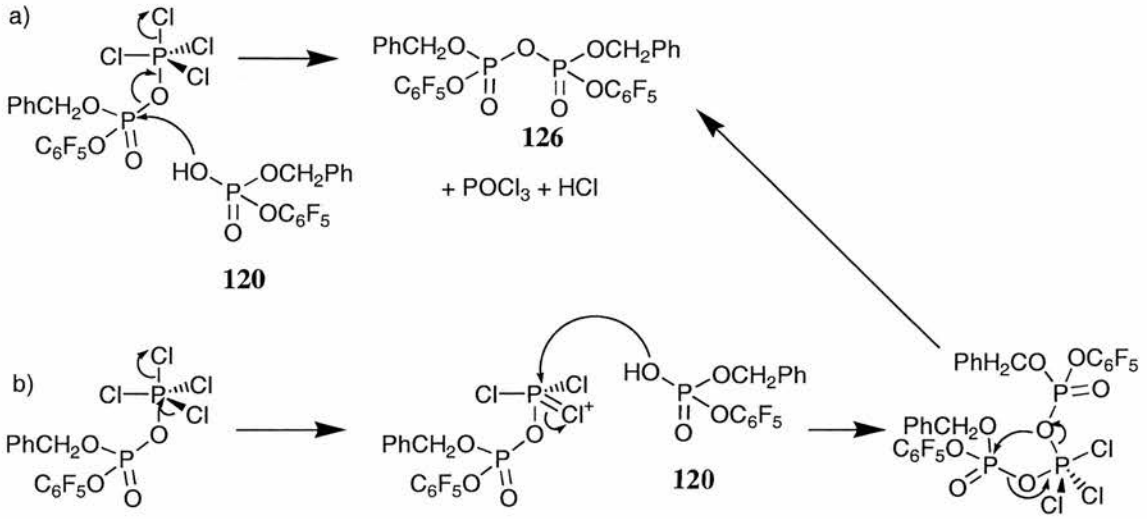
Reaction of a suspension of phosphoric acid diester **120** with 1 equivalent of PCl_5 in dichloromethane resulted in the reactants dissolving and the evolution of a colourless gas (HCl). The solvent was removed under reduced pressure (10 mmHg) to leave a colourless oil. This was expected to contain the product, benzyl pentafluorophenyl chlorophosphate **118** and any by-products of the reaction, most prominently $POCl_3$, Scheme 2.23.



Scheme 2.23: *Mechanism of synthesis of benzyl pentafluorophenyl chlorophosphate* ***118*** via *chlorination of phosphoric acid diester* ***120*** *with* PCl_5.[266]

Heating of the crude product to 45 °C at low pressure (0.5–1.0 mmHg) resulted in the removal of the $POCl_3$. Analysis of the remaining colourless oil by ^1H and ^{31}P NMR spectroscopy revealed the presence of two species within the mixture. The major product (75 – 80%) was identified as the desired product **118** {*m/z* (Found M^+, 371.9746. $C_{13}H_7{}^{35}Cl_1F_5O_3P_1$ requires 371.9742)}. The major contaminant was identified as *O,O'*-dibenzyl-*O,O'*-*bis*(pentafluorophenyl) pyrophosphate **126** by separate synthesis, Section 2.4.2.3.

A possible mechanism for the formation of the pyrophosphate is given below, Scheme 2.24.



Scheme 2.24: *Competing mechanism leading to formation of* O, O'*-dibenzyl*-O,O'-bis*(pentafluorophenyl)-pyrophosphate* ***126***

Chlorination was also attempted using oxalyl chloride in a manner analagous to the method of Lebeau *et al*, [265] described earlier, and also with thionyl chloride and Ph_3P/ CCl_4. Only the PCl_5 method was successful.

Increasing the amount of free phosphoric acid diester **120** relative to the activated intermediate at any one time can discourage formation of the pyrophosphate. If an excess of PCl_5 is used then all the available phosphoric acid diester **120** is consumed at the start of the reaction and the only available nucleophile is the chloride anion, attack of which leads to the desired product, the chlorophosphate **118**, rather than the pyrophosphate **126**. Therefore a suspension of the phosphoric acid diester **120** in CH_2Cl_2 was treated with varying amounts of PCl_5 and the resultant solution allowed to stir for 30 minutes. The CH_2Cl_2 and $POCl_3$ were removed by distillation at reduced pressure (10 mmHg and 0.5-1.0 mmHg respectively) and the proportions of the two products **118** and **126** estimated by comparison of the CH_2OP resonances in the ^1H NMR spectrum. It must be

remembered that each pyrophosphate molecule contributes 4 protons to the resonance and each diester molecule only 2.

Table 2.6: *Percentage purity of benzyl pentafluorophenyl chlorophosphate **118** with respect to equivalents of PCl_5 added*

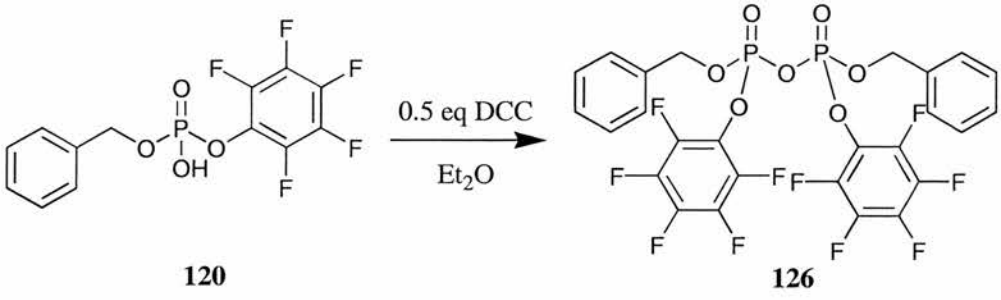
Equivalents of PCl_5	**% formation of 118**
1.5	11%
2.0	75%
3.0	74%
5.0	85%

It can be seen from Table 2.6 that the use of 5 equivalents of PCl_5 rather than 2 equivalents gives only a rather modest increase in the purity of the product. However, any excess PCl_5 must be removed by sublimation at reduced pressure at 40–45 °C. Heating the chlorophosphate **118** at this temperature for a prolonged length of time runs the risk of decomposition of the product. It is therefore advisable to heat the system for as little time as is necessary. Accordingly, it was decided that the modest increase in purity obtained using 5 equivalents over 2 equivalents was not worth the added risk of decomposition during purification. In all future phosphorylations, the reagent would be prepared *in situ* using 2 equivalents of PCl_5.

2.4.2.3. Synthesis of O,O'-dibenzyl-O,O'-bis(pentafluorophenyl)-pyrophosphate 126

In order to prove the identity of the side product of the PCl_5 chlorination of the phosphoric acid diester **120**, postulated as being the pyrophosphate **126**, as shown in Scheme 2.25, it was necessary to synthesise the pyrophosphate by a known route, to provide an authentic sample for comparison.

Pyrophosphate synthesis is normally achieved by the carbodiimide mediated coupling of 2 molecules of a phosphoric acid diester with the concomitant loss of water. The most commonly used carbodiimide is dicyclohexylcarbodiimide (DCC, **80**), as a solution in diethyl ether. This has the advantage that the dicyclohexylurea byproduct is insoluble in ether and can easily be removed by filtration. Literature accounts vary in the amount of DCC used, but it is generally accepted to be between 0.5 and 0.7 equivalents with respect to the phosphate diester.



120 **126**

Scheme 2.25: *Synthesis of* O, O'-*dibenzyl*-O,O'-bis*pentafluorophenyl-pyrophosphate* ***126***

In reactions between benzyl pentafluorophenyl phosphoric acid diester **120** and DCC the best yield of the pyrophosphate **126** was found to occur using 0.5 equivalents of DCC with respect to the phosphoric acid diester **120**, Scheme 2.25. The pyrophosphate **126** was found to be extremely unstable to hydrolysis and great

care had to be taken in the workup of the reaction. As a result of this, the pyrophosphate could not be isolated from starting material and remaining DCC. Accordingly, benzyl pentafluorophenyl phosphoric acid diester **120** was dissolved in Et_2O and treated with a solution of 0.5 equivalents of DCC in Et_2O. Dicyclohexyl urea immediately began to precipitate out of solution. When precipitation of the urea was complete, the precipitate was removed by filtration and the solvent removed at reduced pressure to afford the crude pyrophosphate as a colourless oil which could not be purified further, {*m/z* (CI^+) 708 (4%, $[M + NH_4]^+$)}

2.4.3 Phosphorylations with benzyl pentafluorophenyl chlorophosphate 118

2.4.3.1 Solution phase reaction with cyclohexanol as a serine and threonine mimic.

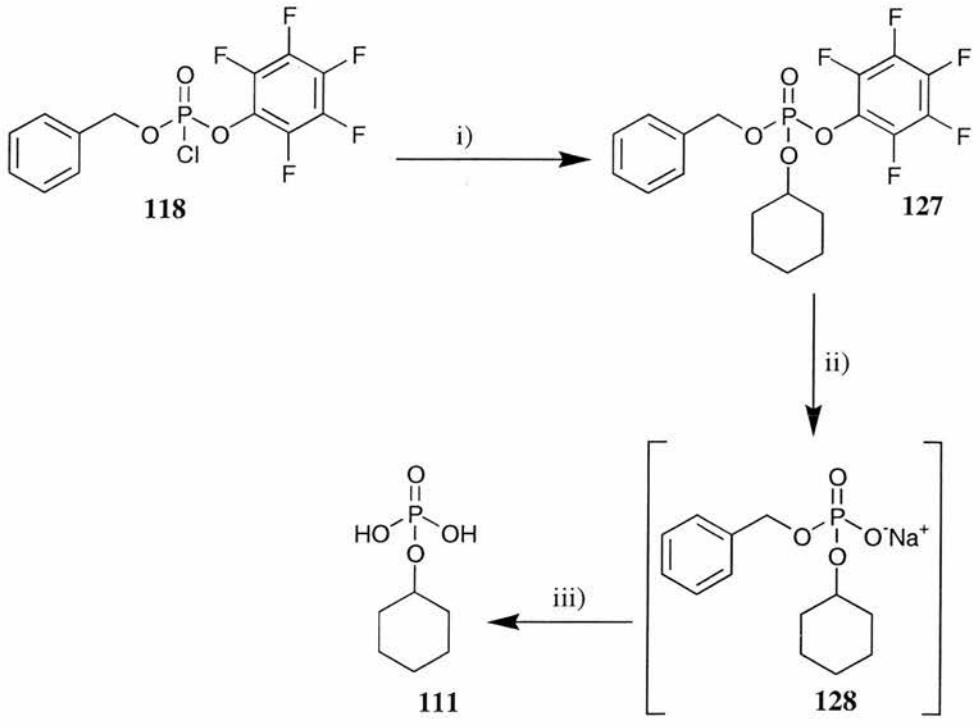
In order to model the phosphorylation of serine and threonine residues in solution phase reactions, cyclohexanol was used as a model for serine and threonine, as was the case for solution phase phosphorylations using *bis*(pentafluorophenyl) chlorophosphate **77**, Section 2.3.

A solution of cyclohexanol in DCM was quantitatively phosphorylated with 5 equivalents of **118** in the presence of Et_3N and DMAP to give the required triester **127**, Scheme 2.26. Care had to be taken when purifying the phosphate triester **127**, as it was prone to decomposition on the silica used for column chromatography

It should be noted that phosphorylations using dibenzyl chlorophosphate typically employ 10-20 equivalents relative to the target alcohol, and phosphorylation with *bis*(pentafluorophenyl) chlorophosphate **77** is observed to proceed quantitatively with 1.4 equivalents of the chlorophosphate relative to the target alcohol. Therefore, benzyl pentafluorophenyl chlorophosphate has a

reactivity intermediate between dibenzyl and *bis*(pentafluorophenyl) chlorophosphate, as would be expected.

Reaction of triester **127** with 10 equivalents of aqueous NaOH in DMSO gave the intermediate phosphate diester sodium salt **128**. The identity of the product was inferred from analysis of the ^{1}H, ^{13}C and ^{19}F NMR spectra. The product of the reaction was shown to have phosphorus coupled cyclohexyl resonances in both the ^{1}H and ^{13}C NMR spectra {δ_H 4.09 (m, C*H*OP); δ_C 75.60 (d, *J* 5.41, *C*HOP)}, and analysis of the ^{19}F NMR spectrum (change in position and order of peaks) confirmed there was no pentafluorophenyl groups connected to the phosphorus centre.

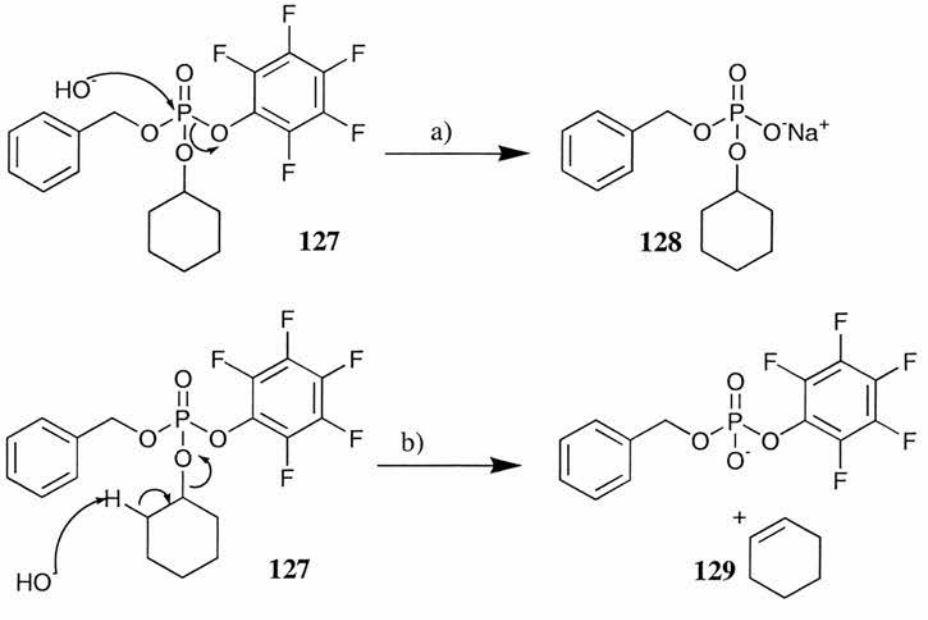


Reagents and Conditions: i) cyclohexanol, Et_3N, DMAP, DCM, 92 %;
ii) NaOH(aq), 12 h.; iii) TFA, DCM, 2 h, 85% (2 steps)

Scheme 2.26: *Synthesis of cyclohexyl dihydrogen phosphate **111***

It has been mentioned before that phosphate triester with β-CH_2 groups are prone to β-elimination. Were this to occur in the hydrolysis of triester **127**, the

products would be the benzyl pentafluorophenyl phosphate anion and cyclohexene **129**, Scheme 2.27, route b). Analysis of the reaction product by ^{1}H and ^{13}C NMR spectroscopy revealed the presence of phosphorus coupling in the α-CH and α-C resonances. The resonances have also shifted downfield compared to the triester **127**. This shows that the hydrolysis of the pentafluorophenyl group has occurred, rather than β-elimination. After removal of solvent under reduced pressure, the crude product was treated with 10 equivalents of 90% TFA in CH_2Cl_2 for 2 hours. Disappearance of the phosphorus coupled benzylic CH_2 resonances in the ^{1}H and ^{13}C NMR spectra showed that the benzyl group had been removed. After removal of volatiles under reduced pressure, purification of the crude product by ion exchange chromatography on Amberlite IR-120 afforded the product **111** in 85% yield (from the triester **127**), Scheme 2.26.



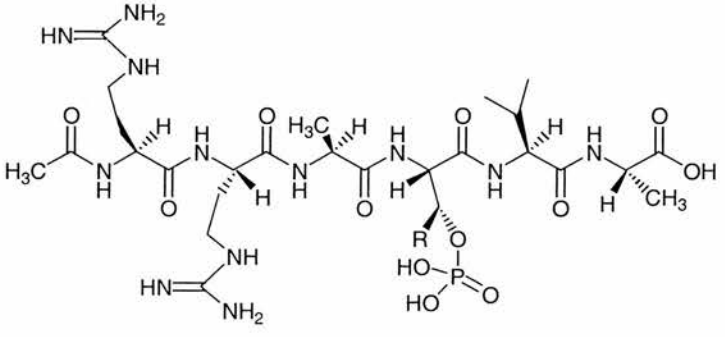
Scheme 2.27: *Alternative mechanisms of alkaline hydrolysis of benzyl pentafluorophenyl cyclohexyl phosphate* ***127****. a) hydrolysis of pentafluorophenyl group; b) β-elimination*

Comparison of the reaction product with a sample of cyclohexyl dihydrogen phosphate **111** prepared independently, Section 2.3.2.2 & 2.3.2.4, confirmed the identity of the product.

Reaction of 5 equivalents of benzyl pentafluorophenyl chlorophosphate **118** with methanol, in the presence of Et_3N and DMAP gave the expected product, benzyl pentafluorophenyl methyl phosphate **130** as a colourless oil in 76% yield, {*m/z* (Found M^+, 368.0232. $C_{14}H_{10}O_4F_5P_1$ requires 368.0237)}. This is in contrast to the reaction of *bis*(pentafluorophenyl) chlorophosphate **77**, which gave the dimethyl pentafluorophenyl phosphate triester **110** as the product, Scheme 2.8.

2.4.3.2 Solid phase phosphorylation of serine and threonine residues with benzyl pentafluorophenyl chlorophosphate 118

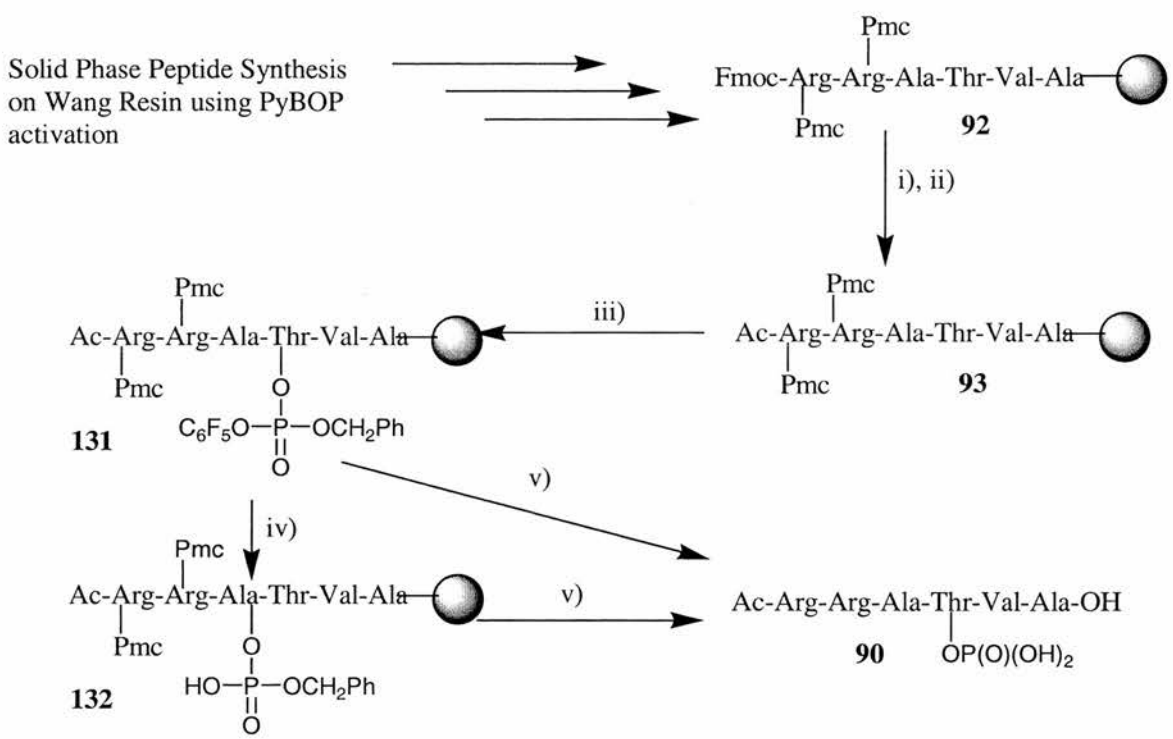
It has already been shown that *bis*(pentafluorophenyl) chlorophosphate **77** can be used in the synthesis of AcRRApTVA **90** and AcRRApSVA **97** by Fmoc based batch solid phase peptide synthesis. Therefore it was decided to investigate the scope of application of benzyl pentafluorophenyl chlorophosphate **118** in the synthesis of **90** and **97.**



90; R = CH_3
97; R = H

2.4.3.2.1 Synthesis of AcRRApTVA 90.

In a manner analagous to the synthesis of AcRRApTVA **90** using *bis*(pentafluorophenyl) chlorophosphate **77**, the fully protected peptide Ac-Arg(Pmc)-Arg(Pmc)-Ala-Thr-Val-Ala-OWang **93** was treated with 10-20 equivalents of benzyl pentafluorophenyl chlorophosphate **118**, Et_3N and DMAP to afford the resin bound phosphate triester **131**, Scheme 2.28. The presence of phosphorus was confirmed by gel phase ^{31}P NMR spectroscopy.



Reagents and Conditions: i. 20% piperidine/ DMF, RT, 15 min; ii. 5% Ac_2O/DMF, 0°C, 15 min; iii. $(C_6F_5O)(PhCH_2O)POCl$ **118**, Et_3N, DMAP, DCM, RT, 12 h; iv. NaOH, DMSO, 4h, RT; v. CF_3CO_2H:Et_3SiH:H_2O (95:2.5:2.5), 2h, 47%overall

Scheme 2.28: *Synthesis of AcRRApTVA **90** using benzyl pentafluorophenyl chlorophosphate **118***

There are two possible cleavage protocols available for deprotection of the phosphate moiety:

1. Acidic cleavage

   It has previously been shown that the pentafluorophenyl groups of phosphothreonine or phosphoserine containing peptides can be removed by treatment with 90%TFA/ 5% Et_3SiH/ 5% H_2O. Benzyl groups are also cleaved under acidic conditions. Therefore treatment of the resin bound phosphate triester **131** with the above mentioned cleavage mixture should result in the removal of both the benzyl and the pentafluorophenyl groups.

2. Alkaline – acidic cleavage

   The rationale behind the design of the chlorophosphate reagent **118** (section 2.5.1) was that the pentafluorophenyl group could be removed under alkaline conditions. However, phosphate triesters of both serine and threonine are prone to β-elimination. This reaction is competing with the hydrolysis of the pentafluorophenyl group from the phosphate triester. It was envisaged that the reactivity of the pentafluorophenyl group would be such that it would be removed from the phosphate faster than β-elimination occurred (as was observed in the deprotection of the cyclohexyl phosphate triester **127**). The resultant phosphate diester anion would not be susceptible to β-elimination. It has already been shown (Section 2.4.3.1) that treatment of benzyl pentafluorophenyl cyclohexyl phosphate **127** with aq. NaOH followed by TFA in DCM results in the formation of cyclohexyl dihydrogen phosphate **111** and not cyclohexene **129**, the product of β-elimination. This suggests that the removal of the pentafluorophenyl group is faster than β-elimination.

Treatment of the phosphate diester anion with 90%TFA/ 5% Et_3SiH/ 5% H_2O should then result in the removal of the benzyl and Pmc groups, cleavage from the resin and formation of the desired product.

Treatment of the resin bound phosphate triester **131**, according to protocol 1) above, with cleavage mixture 1) (See Chapter 4, page 170) followed by removal of the resin by filtration and lyophilisation of the filtrate produced a white hygroscopic solid. Purification of the crude peptide by ion-exchange chromatography afforded the desired sequence **90** in 47 % yield.

Treatment of the resin according to protocol 2) above, with 4 mol dm^{-3} aq. NaOH (100 equivalents) in DMSO for 4 hours was followed by filtration, washing to remove any vestiges of alkali, and drying. Treatment of the dried resin **129** with cleavage mixture 1) in an analogous manner to the acidic protocol, followed by removal of the resin by filtration and lyophilisation of the filtrate, afforded a white hygroscopic solid. Purification of the crude peptide by ion exchange chromatography afforded the target sequence **90** in 52% yield.

2.4.3.2.2 Synthesis of AcRRApSVA 94.

This was performed in a manner analogous to that for AcRRApTVA **90**, Scheme 2.26. Using protocol 1) above, the desired sequence **97** was obtained in 45% yield after purification, and under protocol 2), the target sequence **97** was obtained in 48% yield after purification.

2.4.3.3 Solid phase phosphorylation of tyrosine residues with benzyl pentafluorophenyl chlorophosphate 118.

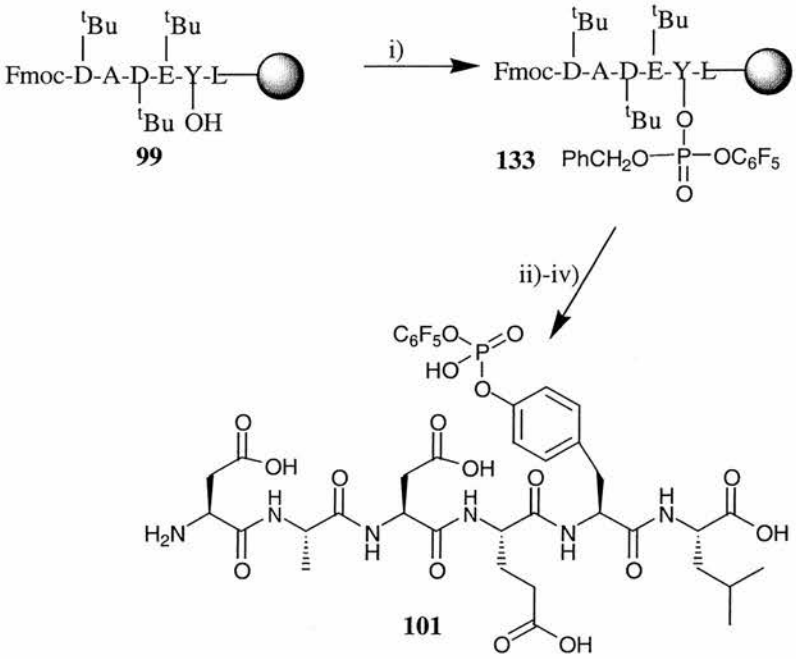
2.4.3.3.1 Initial attempted synthesis of DADEpYL 98.

As described previously (Section 2.2.3.2.1), the synthesis of DADEpYL **98**, a hexapeptide corresponding to one of the autophosphorylation sites of the Epidermal Growth Factor Receptor ($EGFR_{988-993}$), with *bis*(pentafluorophenyl) chlorophosphate **77** was unsuccessful.

In a manner analogous to the phosphorylation with *bis*(pentafluorophenyl) chlorophosphate **77**, the fully protected peptide Fmoc-Asp(^{t}Bu)-Ala-Asp(^{t}Bu)-Glu(^{t}Bu)-Tyr-Leu-OWang **99** was treated with a solution of 10-20 equivalents of benzyl pentafluorophenyl chlorophosphate **118** in DCM, prepared *in situ*, in the presence of Et_3N and DMAP at 0-5 °C for 1 hour, Scheme 2.29. At higher temperatures and longer reaction times, a yellow colour infused the solution, believed to be due to the decomposition of the phosphate triester, possibly by the debenzylation of the phosphate triester by excess triethylamine. Phosphorus incorporation was confirmed by gel phase ^{31}P NMR spectroscopy.

Treatment of the fully protected resin bound phosphate triester **131** with 20% piperidine in DMF resulted in removal of the *N*-α terminal Fmoc group. Subsequent treatment with 4 mol dm^{-3} aq. NaOH in DMSO followed by washing successively with CH_2Cl_2, MeOH, CH_2Cl_2 and treatment with 90% TFA/ 5% Et_3SiH/ 5% H_2O resulted in the isolation of a white solid. This compound was identified by NMR spectroscopy and found by to be the phosphoric acid diester **101**, and not the desired dihydrogen phosphate monoester **98**. This could be due to the unexpected removal of the benzyl group upon treatment of the phosphate triester **131** with 20% piperidine in DMF. Subsequent treatment of the resin with 90% TFA/ 5% Et_3SiH/

5% H_2O results in formation of the tyrosyl pentafluorophenyl phosphoric acid diester **101**, as described earlier, (Section 2.2.3.2.1).



Reagents and Conditions: i) Et_3N, DMAP, CH_2Cl_2, $(C_6F_5O)(BnO)P(O)Cl$ (**118**); ii) 20% piperidine/ DMF; iii) 4M NaOH (100 eq), 4 h; iv) CF_3CO_2H:H_2O:Et_3SiH (95:2.5:2.5)

Scheme 2.29: *Initial attempt at synthesis of DADEpYL **98** using benzyl pentafluorophenyl chlorophosphate **118**.*

In order to optimise the phosphorylation and cleavage protocols for the tyrosine containing substrates, it was decided to use a more simple substrate for optimisation and then to transfer the strategy back to the more complex sequence.

2.4.3.3.2 Synthesis of VpYL 138.

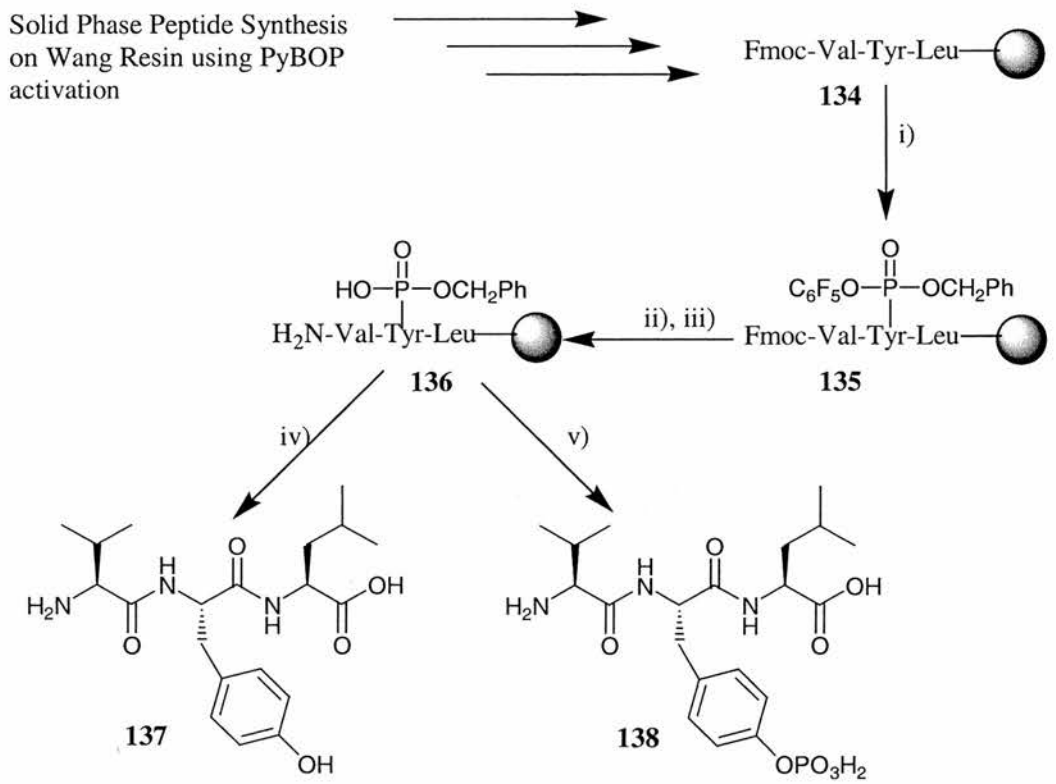
The sequence chosen for optimisation of deprotection conditions was VYL **137**. This sequence was chosen as the valinyl and leucinyl resonances are well defined and do not overlap with the tyrosyl resonances, making analysis of the tyrosine resonances straightforward.

The synthesis of Fmoc-VYL-OWang **134** was carried out on a Rainen PS3 automatic peptide synthesiser, Scheme 2.30. Fmoc protection was utilised and peptide coupling was carried out *in situ* using PyBOP **85**. Wang resin was used and the stereochemistry of all amino acids is (*S*). Correct assembly of the sequence was confirmed by analysis of the product of workup of the resin **134** which was shown to be VYL **137**.

Fmoc-VYL-OWang **134** was then phosphorylated by treatment with a solution of freshly prepared benzyl pentafluorophenyl chlorophosphate **118** (15-20 equivalents) in DCM and the suspension was allowed to stir for 4 hours, Scheme 2.32. Phosphorus incorporation was confirmed by gel phase ^{31}P NMR spectroscopy. After isolation and drying, the resin bound phosphate triester **135** was treated with 100 eq. 2 mol dm^{-3} NaOH in DMSO. Analysis of the resin by ^{31}P NMR spectroscopy confirmed that phosphorus was still present, and dephosphorylation had not occurred upon treatment with base. The *N*-α Fmoc group was removed by treatment with 2% DBU/ DMF, and the resin treated with 95% TFA/ 2.5% Et_3SiH/ 2.5% H_2O for 2 hours. Removal of the resin by filtration and lyophilisation of the filtrate gave an amorphous colourless solid.

Analysis by ^{1}H and ^{31}P NMR spectroscopy showed that dephosphorylation of the tyrosine residue had occurred to produce VYL **137**. There is some disagreement in the literature as to the susceptibility of benzyl tyrosyl phosphate diesters of the type **136** shown in Scheme 2.28 to dephosphorylation upon treatment with TFA/Et_3SiH/H_2O. Some researchers have reported a minor impurity resulting from dephosphorylation of the tyrosine residue,[269] whereas others report that formation of dihydroxyphosphotyrosine occurs.[270] In repeated acidolysis experiments, no VpYL **138** was isolated, dephosphorylation of the diester

intermediate **136** having occurred. Cleavage of the methyl groups from dimethyl phosphotyrosine containing peptides utilises a thiol nucleophile to attack the less electrophilic carbon centre of the methyl group, rather than the more electrophilic phosphorus centre, Scheme 2.31.



Reagents and Conditions: i) $(C_6F_5O)(PhCH_2O)POCl$ **118**, Et_3N, DMAP, CH_2Cl_2,4 h, RT; ii) 100 eq 2M NaOH, 4 h; iii) 2% DBU /DMF,RT, 30 min; iv) CF_3CO_2H:Et_3SiH:H_2O (95:2.5:2.5), 2 h, RT; v) 5% PhSMe/TFA, 30 min, 65%

Scheme 2.30: *Synthesis of VpYL 138.*

In an analogous manner, it is conceivable that the benzylic carbon of the benzyl group would be susceptible to an attack by a soft nucleophile, such as a thiol. Treatment of the phosphate diester **136** with 5% thioanisole in TFA gave VpYL **138**, in 65 % yield as an off white solid, with no dephosphorylation being observed (as judged from ^1H NMR spectroscopy), {*m/z* (Found $[M + H]^+$, 474.2010.

$C_{20}H_{22}N_3O_8P_1$ requires 474.2005)}. Treatment of the phosphate diester in an analogous manner with 5% anisole in TFA resulted in dephosphorylation of the diester **136**. Attack at the phosphorus centre by anisole, (a hard nucleophile), leads to dephosphorylation, whereas thioanisole, (a soft nucleophile), attacks the softer C centre of the benzyl group. However, treatment of the resin bound phosphate diester **136** with thioanisole for longer than 30 minutes led to dephosphorylation of the peptide sequence.

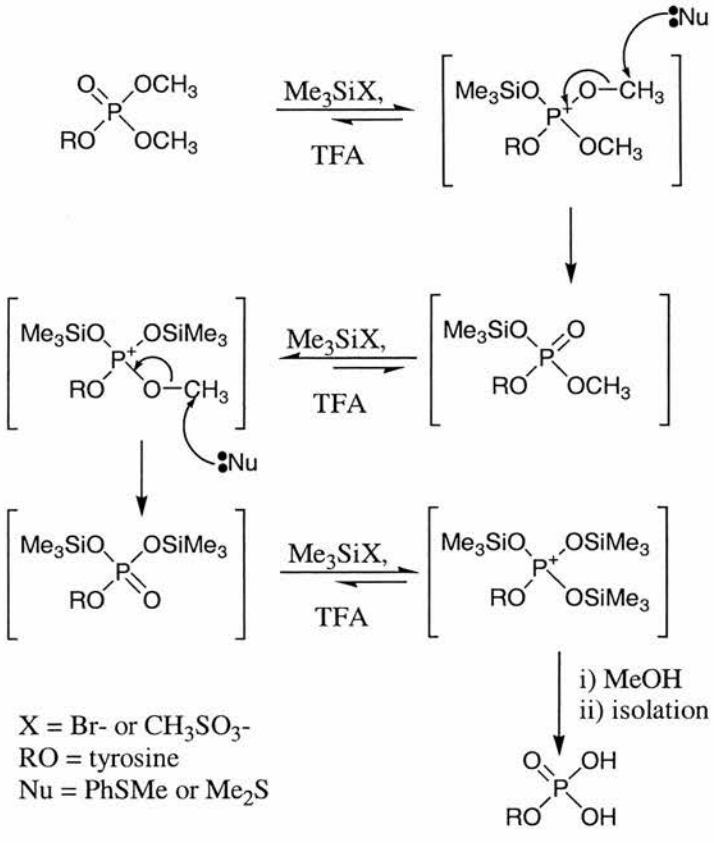
2.4.3.3.3 Synthesis of DADEpYL 98.

The above method was then applied to the synthesis of DADEpYL **98**. Accordingly, treatment of the resin bound phosphate triester **133** with 2 mol dm^{-3} aq. NaOH, then 2% DBU in DMF, then 5% thioanisole in TFA for 5 minutes, followed by removal of the resin by filtration and lypholisation of the filtrate led to the isolation of the desired sequence **98** in 52 % yield, {*m/z* (Found $[M + Na]^+$, 827.2444. $C_{31}H_{45}N_6O_{17}P_1Na_1$ requires 827.2477)}. After each step, the presence of phosphorus in the resin bound substrate was confirmed by ^{31}P NMR spectroscopy.

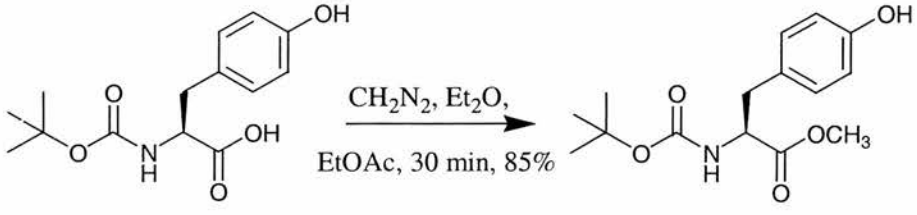
2.5.3.4 Solution phase reaction of Boc-Tyr-OMe 140 as a tyrosine mimic

Initial solution phase tyrosine phosphorylation reactions were carried out using phenol as a model for tyrosine residues. However, due to the highly hygroscopic nature of phenol, it was not possible to obtain the phenol sufficiently dry to achieve phosphorylation. Therefore, an alternative substrate was chosen, methyl-*N*-α-^{t}Boc-(2*S*)-tyrosinate **140**. This was prepared directly in a single step reaction by the esterification of commercially available *N*-α-^{t}Boc-(2*S*)-tyrosine **139** with ethereal diazomethane to afford methyl-*N*-α-^{t}Boc-(2*S*)-tyrosinate **140** as a

white solid in 85% yield after recrystallisation, mp. 114 °C (lit.[235] 114 °C), Scheme 2.32.



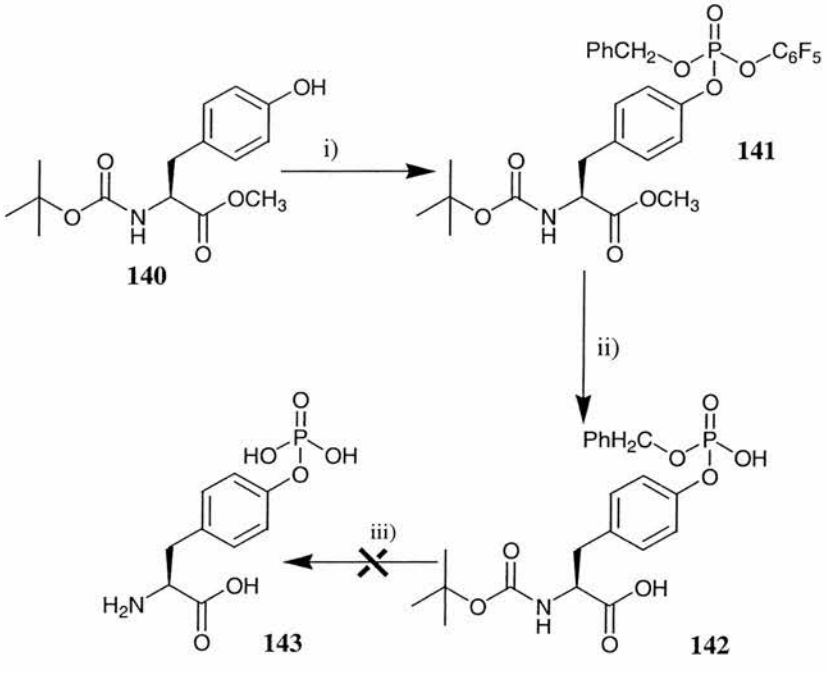
Scheme 2.31: *Deprotection of dimethyl phosphotyrosine.*[262]



139 **140**

Scheme 2.32: *Synthesis of methyl-*N-α-^{t}*Boc-(2*S*)-tyrosinate* ***140***

Phosphorylation of methyl-*N*-α-^{t}Boc-(2*S*)-tyrosinate **140** was achieved in a manner analagous to that for cyclohexanol (Section 2.4.3.1), with 5 equivalents of the chlorophosphate **118** in the presence of Et_3N and DMAP, to give the required triester **141** in 62 % yield after purification by column chromatography, Scheme 2.33. Reaction with 2 mol dm^{-3} aq. NaOH gave the intermediate phosphate diester sodium salt **142** (as determined from ^{31}P and ^{19}F NMR spectroscopy). Upon treatment with 5% thioanisole in TFA, in a manner analogous to the solid phase deprotection, the phosphate group was hydrolysed, and the desired product, dihydroxyphosphotyrosine **143** was not obtained. This is a further example of reactions in the solid phase resulting in a different outcome to the solution phase equivalent.



*Reagents and Conditions:*i) **118**, Et_3N, DMAP, CH_2Cl_2, 62 %; ii) 100 eq 2 M NaOH, 4 h, quant.; iii) PhSMe:TFA (1:19), 30 min.

Scheme 2.33: *Attempted synthesis of methyl dihydroxyphosphotyrosinate **140***

2.5 Conclusions

We have shown that the reagent *bis*(pentafluorophenyl) chlorophosphate **77**, whilst suitable for the solid phase synthesis of phosphoserine and –threonine containing peptides, is not suitable for the solid phase synthesis of phosphotyrosine containing peptides. In contrast, the pentafluorophenyl groups cannot be removed in solution upon treatment with acid, but removal requires prolonged hydrogenation under acidic conditions. It is also apparent that the conditions under which the phosphorylations are carried out are critically important in determining the extent of phosphorylation that occurs. The order of addition of reagents has been shown to have a profound effect on the amount of phosphorylation occurring.

The 'second generation' reagent, benzyl pentafluorophenyl chlorophosphate **118** has been shown to be of similar efficacy in the solid phase synthesis of phosphoserine and –threonine peptides as *bis*(pentafluorophenyl) chlorophosphate **77**, and to be applicable in the solid phase synthesis of phosphotyrosine containing peptides. It has also been shown to be effective in solution phase phosphorylations on serine/ threonine models and on a protected tyrosine residue.

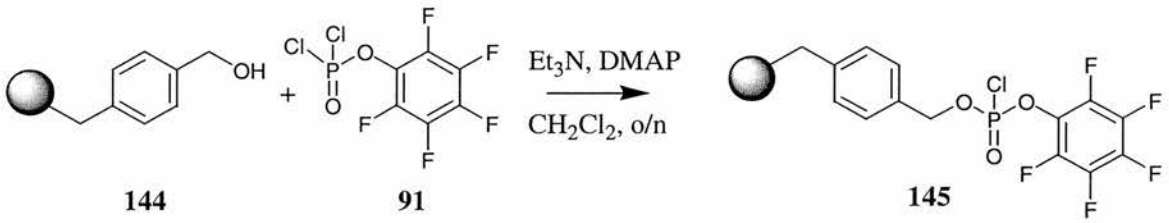
It is interesting to note that the phosphate triester, benzyl pentafluorophenyl cyclohexyl phosphate **111** does not undergo β-elimination when treated with base, unlike a number of reported phosphate triesters. This is believed to be due to the rapidity with which the pentafluorophenyl group is removed, forming the phosphate diester anion which is not prone to β-elimination.

2.6 Further work

There has been much interest lately in the use of supported reagents in organic synthesis. The use of reagents on heterogeneous supports leads to cleaner

reactions and allows the removal of the reagent from the reaction by simple filtration, obviating the need for protracted purification procedures.

As described in Section 2.4, the reagent benzyl pentafluorophenyl chlorophosphate **118** may be used in both solid and solution phase phosphorylations. It is, therefore, conceivable that the benzyl functionalised resin could act as the benzyl group in **118**, leading to the reagent **145** shown below, Scheme 2.34.



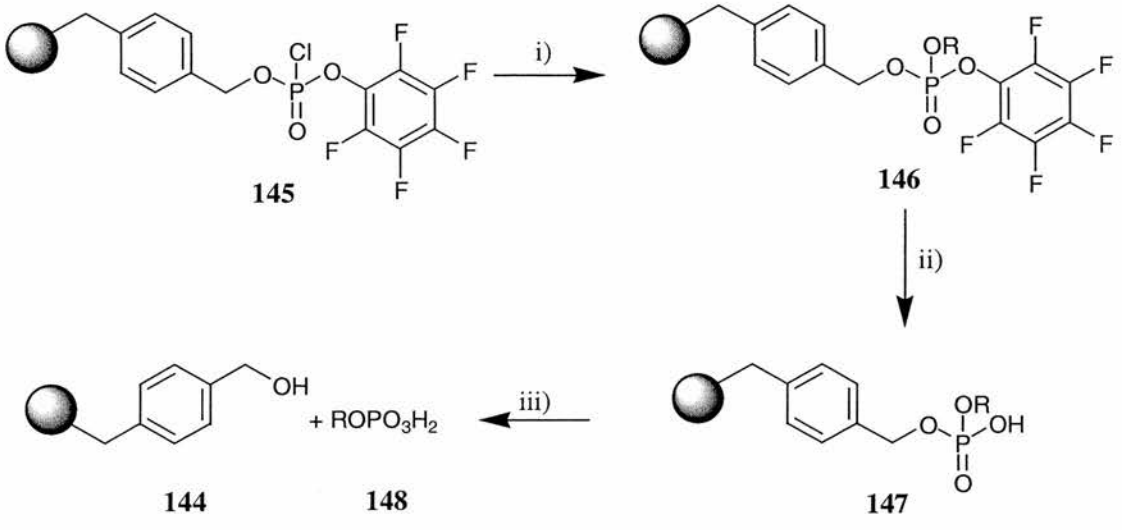
144 **91** **145**

Scheme 2.34: *Proposed synthesis of resin bound phosphorylating reagent **145***

Treatment of hydroxybenzyl polystyrene resin **144** with pentafluorophenyl dichlorophosphate **91** would lead to the formation of the resin bound pentafluorophenyl chlorophosphate **145**.

This phosphorylating reagent **145** can be reacted with a target alcohol, ROH, in the presence of Et_3N and DMAP, Scheme 2.35. Unreacted alcohol and byproduct are then simply removed by washing rather than having to resort to recrystallisation or chromatography. The resin bound phosphate triester **146** can then be treated with alkali to remove the pentafluorophenyl group to form the diester **147**, before treatment with TFA to cleave the phosphorylated alcohol **148** from the resin, with regeneration of the hydroxybenzyl resin **144**. This can then be recycled to form more chlorophosphate reagent **145**, Scheme 2.32 above.

The synthesis of the resin bound phosphorylating reagent **145** has been achieved within our laboratories, and is currently under investigation by Dr. A. P. Mehrotra in the solid phase synthesis of oligonucleotides.



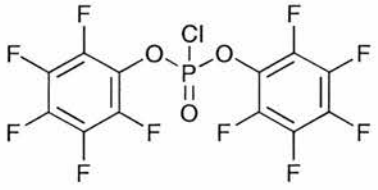
Reagents and Conditions: i) ROH, Et_3N, DMAP, DCM; ii) base; iii) TFA/ DCM

Scheme 2.35: *Use of resin bound phosphorylating agent **145** in phosphorylation of target alcohols, ROH*

3.0 Investigations into the Mechanism of Solid Phase Deprotection of *O*',*O*'-*Bis*(pentafluorophenyl) Phosphothreonine containing Peptides

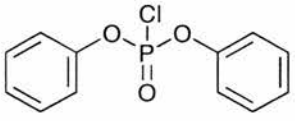
3.1 Introduction

The phosphorylating reagent *bis*(pentafluorophenyl) chlorophosphate **77**, has been shown to be more than 30 times more reactive in phosphorylation of cyclohexanol than its analogue diphenyl chlorophosphate **137**, see Section 2.4.1, page 84.



Bis(pentafluorophenyl) chlorophosphate,

77



diphenyl chlorophosphate,

137

In addition to the increased reactivity of *bis*(pentafluorophenyl) chlorophosphate **77**, phosphoserine and phosphothreonine containing peptides synthesised by Fmoc based solid phase peptide synthesis using *bis*(pentafluorophenyl) chlorophosphate **77** could be deprotected and cleaved from the resin with a single acidolytic treatment. Treatment of the phosphorylated resin bound peptide with 90% TFA/ 5% Et_3SiH/ 5% H_2O[211] resulted in removal of amino acid side chain protecting groups and the pentafluorophenyl groups from the phosphate in one reaction step (section 2.2.3.1, page 79). Conversely, repeated applications of diphenyl phosphate **137** are required to achieve complete

phosphorylation of hindered sequences, and prolonged treatment of the diphenyl phosphate analogues in a similar fashion did not result in removal of the phenyl groups.[246]

Any factors which influence the electron density in particular bonds, or at particular atoms, in a compound might be expected to affect considerably its reactivity towards a particular reagent. A number of factors have been recognised:

- *Inductive and field effects*: In a covalent single bond between unlike atoms, the electron pair forming the σ bond is never shared equally between the two atoms. It tends to be attracted a little more towards the more electronegative atom of the two. Thus in an alkyl fluoride the electron density tends to be nearer the fluorine than the carbon, as the former is the more electronegative. If the carbon attached to the fluorine is itself attached to another carbon, the effect can be transmitted further, down the carbon chain, Figure 3.1.

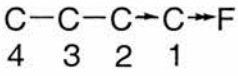


Figure 3.1: *Inductive effect of fluorine atom on carbon chain*

The effect of the fluorine atoms partial appropriation of the electron density of the C-F bond is to leave C_1 slightly electron deficient. This situation can be rectified by appropriation of some of the electron density in the C_1-C_2 bond, and so on down the chain. The effect of C_1 on C_2 is less than the effect of F on C_1 and in a saturated system the effect quickly dies away, though it may be more pronounced in an unsaturated system. These influences on the electron distribution in σ bonds are known as inductive effects. Functional groups can be classified as electron withdrawing (*-I*) or electron donating (*+I*) groups relative

to hydrogen. This means that F, a *–I* group, will draw electrons towards it more than a hydrogen atom would if it occupied the same position in the molecule. Though this inductive effect is quantitatively quite small, it is responsible for the increase in basicity that results from the replacement of one of the hydrogens of ammonia with an alkyl group.

- *Mesomeric (conjugative) effects*: These are essentially electronic redistribution that can take place in unsaturated, and especially conjugated, systems *via* their π orbitals. The stabilisation that can result by delocalisation of a positive or negative charge, *via* its π orbitals, can be a potent feature in making the formation of the ion possible in the first place. It is the stabilisation of the phenoxide anion, by delocalisation of its charge *via* the delocalised π orbitals of the nucleus, that is largely responsible for the acidity of phenol

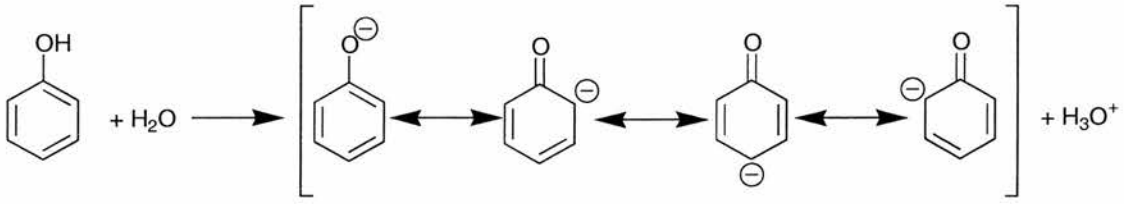


Figure 3.2: *Delocalisation of charge on phenoxide anion*

The essential difference between the inductive and mesomeric effect is that inductive effects can operate in both saturated and unsaturated compounds, whereas the mesomeric effect can only operate in unsaturated systems. The former involves electrons in σ bonds, the latter those in π bonds. Whilst inductive effects are transmitted over quite short distances in saturated systems before dying away,

mesomeric effects may be transmitted from one end to the other in quite large molecules, provided that the conjugation is present, through which they can proceed.

From this it would be expected that pentafluorophenol would be a stronger acid than both phenol and pentachlorophenol. However, pentafluorophenol is intermediate in acidity between phenol and pentachlorophenol (C_6F_5OH, pK_a: 5.32; C_6H_5OH, pK_a: 9.89; C_6Cl_5OH, pK_a: 5.26).[271] This is due to the greater power of electron release through back co-ordination in fluorine than in chlorine, Fig 3.3, which offsets the inductive effect of the aryl fluorines. Similar behavour is shown by *p*-fluorobenzoic acid and *p*-fluorophenol. Both are weaker acids than the corresponding chloro- compounds. Electron release from fluorine by this mechanism is such as to make *p*-fluorophenol a weaker acid than phenol itself. Aliphatic fluoro-alcohols are more acidic than the corresponding chloro-alcohols, (CF_3CH_2OH, pK_a: 11.32; CCl_3CH_2OH, pK_a: 11.80).[272]

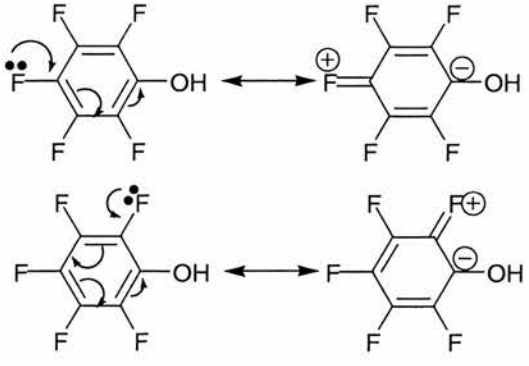


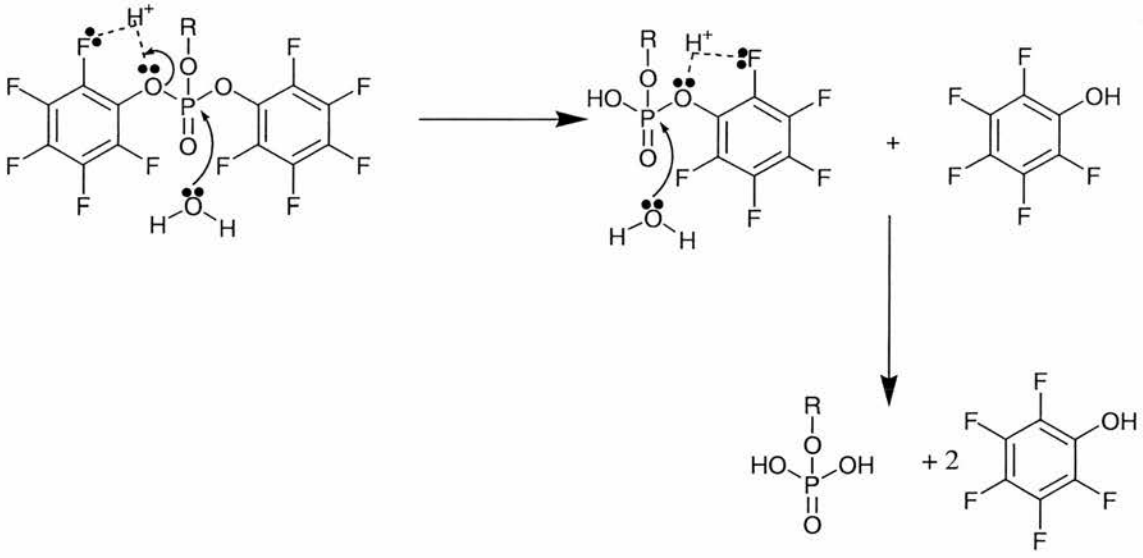
Figure 3.3: *Back co-ordination from fluorine in pentafluorophenol.*

The greater acidity of pentafluorophenol than phenol would suggest that the pentafluorophenyl groups would be less susceptible to acidic cleavage, as the pentafluorophenyl groups would be a worse leaving group than phenyl under acidic conditions.

Therefore a different mechanism must be proposed to account for the apparent difference in reactivity.

3.2 Proposed mechanism of pentafluorophenyl group cleavage in acidic media

The mechanism proposed involves chelation of a proton by one of the phosphate oxygens and one of the *ortho* fluorine atoms on the phenyl ring, (Scheme 3.1). This helps to stabilise the developing negative charge on the phosphate oxygen as the nucleophile (here water or trifluoroacetate anion) approaches and increases the electrophilicity of the phosphorus atom.



Scheme 3.1: *Acid catalysed deprotection of* bis*(pentafluorophenyl) phosphate esters*

There is evidence from infra-red spectroscopic studies on pentafluorophenol in liquid film and solution that there is co-ordination between the phenolic oxygen and an *ortho*-fluorine, Fig. 3.4.[273] The liquid film spectrum shows a sharp band at 3571 cm^{-1} and a broad band centred on 3425 cm^{-1}. The former is characteristic of

the 'free' hydroxyl stretching vibration band and the latter the typical hydrogen bonded hydroxyl group band. The spectrum of pentafluorophenol vapour shows a strong band at 3623 cm^{-1} and no band in the 3400 cm^{-1} region. The spectrum of a 1% solution in CCl_4 shows a sharp band at 3571 cm^{-1} with a weak shoulder at 3676 cm^{-1} and a weak broad bonded hydroxyl band at 3257 cm^{-1}. The bands at 3623 cm^{-1} and 3676 cm^{-1} are attributed to 'free' hydroxyl groups, and the sharp band at 3571 cm^{-1} is attributed to the hydrogen bond of the type shown below, Fig. 3.4.

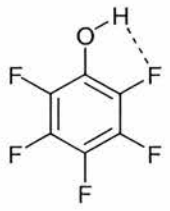


Figure 3.4: *Chelation of phenolic proton by ortho-fluorine.*

This intra-molecular hydrogen bonding leads to a decrease in inter-molecular hydrogen bonding, and has been cited as the reason for the lower boiling point of pentafluorophenol (142 °C) compared to phenol (182 °C).[274] It is also cited as the cause of the marked difference in boiling points between *o*-fluorophenol (152 °C) and *p*-fluorophenol (189 °C).[272, 274]

For the proposed mechanism to be correct it must be shown that:

- only fluorophenyl groups possessing *ortho*-fluorine atoms are subject to cleavage; and
- the cleavage is due to the presence of an *ortho*-fluorine atom and not to other steric and/or electronic effects.

To investigate the proposed mechanism, a number of resin bound phosphate triesters of the general formula shown below (Fig. 3.5) were synthesised, differing only in the number of fluorine atoms present in the ring, and their positions in the

ring. Reaction of these triesters with trifluoroacetic acid could be monitored by gel phase NMR spectroscopy to monitor the extent of reaction.

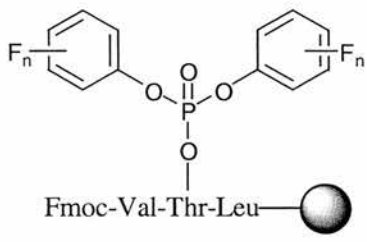


Figure 3.5: *Resin bound fluorophenyl phosphate triesters. The grey circle represents the Wang resin support*

3.3 Timecourse ^{19}F NMR spectroscopic analysis of reaction of fluorophenyl phosphate triesters with TFA.

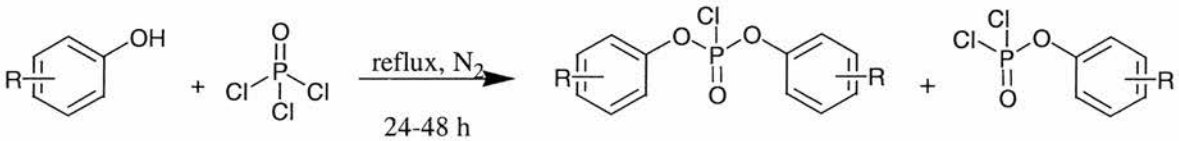
Initially it was decided to investigate the monofluorophenyl derivatives (2-, 3-, and 4-fluorophenyl phosphates). Were the mechanism to solely involve chelation of a proton by the *ortho*-fluorine, then cleavage of the *ortho*-fluorophenyl groups would occur, but cleavage of the *meta*- and *para*-fluorophenyl groups would not, as there is no scope for chelation of a proton at the correct position. It was expected that, upon treatment of the resin with the standard cleavage mixture of 95% TFA/ 2.5% H_2O/ 2.5% Et_3SiH, the 3- and 4- fluorophenyl derivatives would show no removal of the fluorophenyl groups, whereas the 2-fluorophenyl derivative should undergo removal of the fluorophenyl groups as well as removal from the resin. It was envisaged that the phosphate triesters could be prepared from a resin

bound threonine containing peptide and a suitable chlorophosphate using standard phosphorylation methodology developed earlier (section 2.3.3, page 74)

3.3.1. Acid lability of 2-, 3- & 4-fluorophenyl groups

3.3.1.1 Synthesis of the chlorophosphates 153 - 155

Accordingly, the *bis*(2-fluorophenyl), *bis*(3-fluorophenyl) and *bis*(4-fluorophenyl) chlorophosphates **153-155** were prepared according to the method of Boschan and Holder,[247] by heating a mixture of the appropriate fluorophenol **150-152** and $POCl_3$ under reflux, Scheme 3.2. After removal of starting materials, the crude mixtures obtained were purified by distillation at 0.5-0.7 mmHg to obtain the *bis*(fluorophenyl) chlorophosphates **153, 154** and **155** in 20% yield, 23% yield and 23% yield respectively. Analysis of purity by ^{31}P NMR spectroscopy gave purities of 92.6 % (**153**), 95.5 % (**154**) and 94.4 % (**155**).



150, R=2-F;
151, R=3-F;
152, R=4-F;

153, R=2-F;
154, R=3-F;
155, R=4-F;

156, R=2-F;
157, R=3-F;
158, R=4-F;

Scheme 3.2: *Synthesis of* bis*(fluorophenyl) chlorophosphates **153-155** and fluorophenyl dichlorophosphates **156-158***

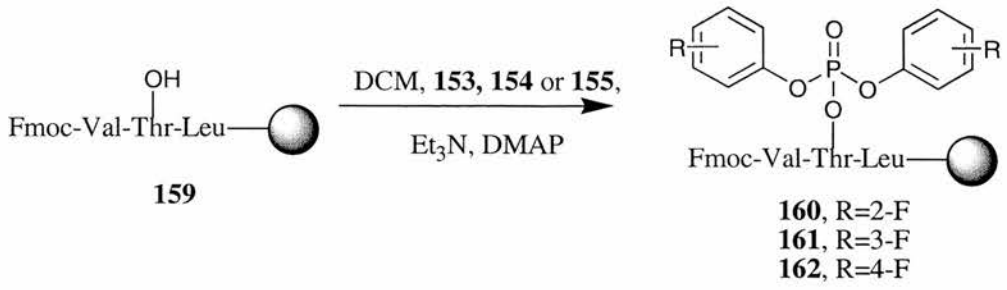
3.3.1.2 Formation of phosphate triesters.

The peptide sequence chosen for the study was Fmoc-VTL-OWang **159**. This sequence was chosen as it is a simple sequence containing no residues that may cause aggregation and side reactions. The synthesis of the sequence was carried out

on a Rainen PS3 automatic peptide synthesiser. Fmoc protection was utilised and peptide couplings were carried out *in situ*. The resin used was Wang resin (Fig 1.21, page 65) and the stereochemistry of all amino acids is (*S*). *N*-α-Fmoc group removal was achieved using a solution of 20% piperidine in DMF, and activation of the amino acids prior to coupling was achieved with 5% NMM in DMF and PyBOP **85**. A small amount of resin (20 mg) was treated with 95 % TFA/ 2.5 % TES/ 2.5 % H_2O and the product analysed by ^1H NMR spectroscopy to comfirm correct assembly of the tripeptide.

Fmoc-VTL-OWang **159** was phosphorylated with a solution of *bis*(2-fluorophenyl) chlorophosphate **153** (10 eq) in DCM to afford the *bis*(2-fluorophenyl) phosphate triester **160**. This was characterised by gel-phase ^{31}P and ^{19}F NMR spectroscopy, and single bead FT-IR {$\delta_P(C_6{}^2H_6)$: -15.05}

The 3-fluorophenyl and 4-fluorophenyl derivatives **161** and **162** were synthesised in an identical manner, using the appropriate chlorophosphate **154** or **155** respectively, Scheme 3.3. Both gave the expected spectral data {**161**, δ_P ($C_6{}^2H_6$): -12.58 ; **162**: δ_P ($C_6{}^2H_6$): -13.8}. The difference in phosphorus resonance is attributed to a concentration dependence in $C_6{}^2H_6$ and π stacking effects. In the synthesis of resin bound phosphate trimesters **160** – **162,** and the subsequent syntheses of phosphate trimesters **170, 171, 174, 175** and **176** phosphorylations were repeated until consistent results were obtained (typically twice) and the same batch of resin was used for analysis and cleavage experiments.



Scheme 3.3: *Synthesis of resin bound fluorophenyl phosphate triesters* ***160-162***

3.3.1.3 Timecourse NMR studies

In order to investigate the requirement for an *ortho*-fluorine on the phenyl ring, these phosphate triesters were assessed for the lability of the fluorophenyl groups under acidic conditions. The phosphate triesters **160-162** and the dihydrogen phosphate **163** have widely separated signals in their ^{31}P NMR spectrum. Therefore, it was envisaged that the reaction could be followed *in situ* by ^{31}P NMR spectroscopy. It was decided to utilise ^{31}P NMR rather than ^{19}F NMR, as the only source of phosphorus is the phosphates themselves, whereas analysis by ^{19}F NMR could be complicated, and indeed the signal from the substrates would be swamped by the signal from the trifluoroacetic acid present in the cleavage mixture.

Accordingly, 0.05 mmol (75 mg, 0.67 $mmolg^{-1}$) of the resin bound *ortho*-fluorophenyl substituted phosphate triester **160** was placed in an NMR tube containing an external standard, in this case a sealed capillary tube containing $^{2}H_2O$ to allow correct shimming and tuning of the coils, and 1 cm^3 of a CF_3CO_2H: H_2O: Et_3SiH (95: 2.5: 2.5) mixture was added. ^{31}P NMR spectra were collected every 15 minutes over the course of the next 10 hours, and a final spectrum being acquired 15-20 hours after the start of the reaction. By displaying the arrayed spectra in a stacked array, it was therefore possible to observe any time dependent change in the ^{31}P signals. Gel phase analysis of the cleavage reactions was hampered by a number

of factors. A limited amount of resin can be used, determined by the amount that can be put in the NMR tube and lie within the field of the acquisition coils. When the cleavage mixture is added and the tube shaken to ensure dispersion of the resin in the tube, some of the resin adheres to the side of the tube, out of the field of the acquisition coils. Attempts to push the resin down into the tube were met with only limited success. This reduces the amount of phosphorylated resin present within the field of the acquisition coils, reducing signal intensity. Additionally, over the course of the experiment, the resin may settle to the bottom of the tube or adhere to the walls of the tube, out of the field of the acquisition coils, again reducing signal intensity and increasing the signal-to-noise ratio. This high signal-to-noise ratio makes it difficult to accurately assess the relative heights of the peaks in the NMR spectra and thus the extent of reaction.

Upon analysis of the ^{31}P arrayed spectrum for the cleavage of the *ortho*-substituted triester **160**, it was evident that there was no cleavage of the fluorophenyl groups, as there was no change in the ^{31}P signal over the course of the reaction. Indeed, analysis of the supernatant of the reaction showed the cleavage product to be the phosphate triester $\{\delta_P -12.56\}$. The change in triester ^{31}P signal is due to a change in the solvent (d_6-benzene to aqueous).

An identical procedure was followed for the *meta*- and *para*- substituted fluorophenyl phosphate triesters **161** and **162**, and in both cases, no cleavage of the fluorophenyl groups were observed.

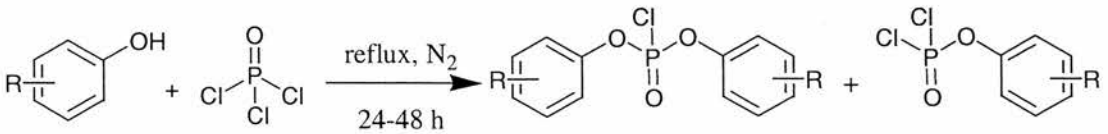
From this, it is possible to conclude that the presence of only one *ortho*-fluorine is insufficient to induce cleavage of the fluorophenyl groups. This means that either there is no requirement for an *ortho*-fluorine on the phenyl group, and the effect is purely electronic, or there is insufficient inducement for chelation to occur.

It was therefore decided to investigate the effect of two fluorine substituents on the phenyl ring. By using the 2,6- and 3,5-difluorophenyl analogues, it was hoped to answer both of the above questions. If neither the 2,6- nor the 3,5-difluorophenyl groups were removed by the acidic cleavage mixture, it would suggest that electronic effects were the cause of the difference and insufficient electron withdrawing ability was being provided by 1 or 2 fluorine atoms on the phenyl rings. However, if as was envisaged, the 2,6-difluorophenyl groups were removed, and the 3,5-difluorophenyl groups were not, then the lability of the pentafluorophenyl groups is due, at least in part, to the presence of the two *ortho*-fluorine substituents.

3.3.2. Acid lability of 2,6- & 3,5-difluorophenyl groups

3.3.2.1 Synthesis of the chlorophosphates 166, 167

The synthesis of *bis*(2,6-difluorophenyl) chlorophosphate **166** and *bis*(3,5-difluorophenyl) chlorophosphate **167** was carried out in an identical manner to that for the *bis*(fluorophenyl) chlorophosphates **153-155** (Scheme 3.2 above, page 148), using the procedure of Boschan and Holder.[247] A mixture of 2 equivalents of the alcohol **164** or **165** was heated with $POCl_3$ under reflux for 1 to 2 days. After cooling, starting materials were removed at reduced pressure (0.7 mm Hg) and the dichlorophosphates **168** and **169** were isolated by distillation, followed by the desired chlorophosphates **166** and **167** in 54% (92.3 % purity by ^{31}P NMR spectroscopy) and 47% yield (92.1 % purity by ^{31}P NMR spectroscopy) respectively, Scheme 3.4.



164, R=2,6-diF
165, R=3,5-diF

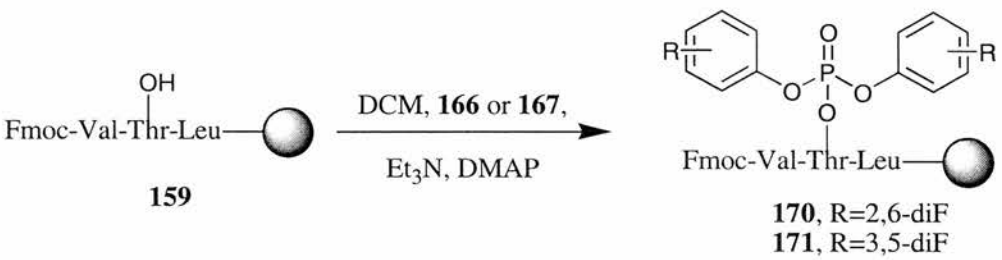
166, R=2,6-diF
167, R=3,5-diF

168, R=2,6-diF
169, R=3,5-diF

Scheme 3.4: *Synthesis of 2,6- and 3,5-* bis*(difluorophenyl) chlorophosphates **163** & **164** and difluorophenyl dichlorophosphates **165** & **166***

3.3.2.2 Formation of phosphate triesters.

To obtain the resin bound phosphate triesters **170** and **171**, a suspension of Fmoc-VTL-OWang resin **159** was phosphorylated by a procedure identical to that for the formation of the triesters **160-162**, Scheme 3.5. The resins were characterised by single bead FT-IR and gel phase ^{31}P and ^{19}F NMR spectroscopy {**170**: ($\delta_P(C_6{}^2H_6)$ -10.57; **171**: ($\delta_P(C_6{}^2H_6)$ -12.7)}.



159

170, R=2,6-diF
171, R=3,5-diF

Scheme 3.5: *Synthesis of resin bound difluorophenyl phosphate triesters **170** and **171***

3.3.2.3 Timecourse NMR studies

Reaction of the difluorophenyl phosphate triesters **170** and **171** under identical conditions to the fluorophenyl phosphate triesters **160-162** as described earlier (section 3.3.1.3) gave the arrayed ^{31}P NMR spectra, Figs. 3.6 and 3.7 respectively.

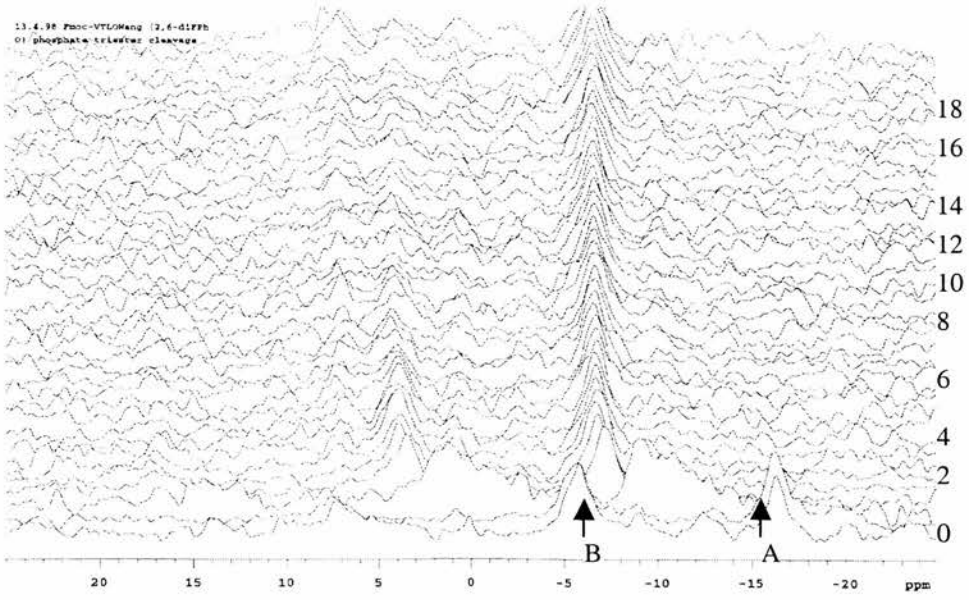


Figure 3.6: *Timecourse of acidic treatment of 2,6-difluorophenyl phosphate triester* ***167*** *with TFA/H_2O/Et_3SiH. Spectra are displayed every 30 minutes, and are stacked vertically.*

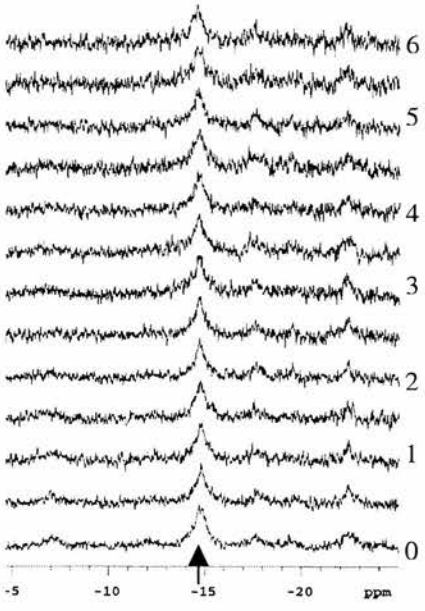


Figure 3.7*: Timecourse of acidic treatment of 3,5-difluorophenyl phosphate triester* ***168*** *with TFA/H_2O/Et_3SiH. Spectra are displayed every 30 minutes, and are stacked vertically.*

From Fig. 3.7 it can be seen that the 3,5-difluorophenyl phosphate triester **171** is not deprotected under acidic conditions, whereas the 2,6-difluorophenyl phosphate triester **170**, (peak A, Fig. 3.6), is seen to react, albeit slowly. The first difluorophenyl group is removed within an hour, to form the diester (peak B, Fig. 3.6), but the second group is not removed during the course of the experiment. However, peak B does not seem to increase in intensity as the intensity of peak A increases. Therefore, the presence of two *ortho*-fluorine atoms on the phenyl ring does seem to induce some acid lability, and this must be due to the chelation of a proton between the *ortho*-fluorine and the phenolic oxygen, see Fig. 3.4, page 146. In Fig. 3.7, the peak at –23 ppm may be due to pyrophosphate formation.

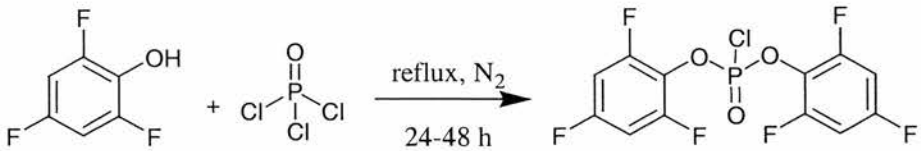
3.3.3 Acid lability of 2,4,6-trifluorophenyl groups

3.3.3.1 Synthesis of the chlorophosphate 173

To further investigate the requirement for two ortho-fluorine atoms on the phenyl ring, the 2,4,6-trifluorophenyl, 2,3,5,6-tetrafluorophenyl and 2,3,4,5,6-pentafluorophenyl phosphate triesters **174**, **175** and **176** were synthesised by an identical procedure to that for the other phosphate triesters, using Fmoc-VTL-OWang **159** and the appropriate *bis*(fluorophenyl) chlorophosphate **173**, **91** or **77**, see Sections 3.3.3.2 and 3.3.4.2, pages 140 and 143.

Bis(2,4,6-trifluorophenyl) chlorophosphate **173** was synthesised in an identical manner to that for the fluorophenyl chlorophosphates **153-155**, (Scheme 3.2, page 140). A mixture of 2 equivalents of 2,4,6-trifluorophenol **172** and $POCl_3$ was heated under reflux for 1 to 2 days, Scheme 3.6. The starting materials were removed under reduced pressure (0.5 mmHg) and the remaining mixture purified by

distillation to afford only the *bis*(2,4,6-trifluorophenyl) chlorophosphate **173** in 38% yield, 93.6 % purity by ^{31}P NMR spectroscopy. Interestingly, none of the (2,4,6-trifluorophenyl) dichlorophosphate was obtained in contrast to the synthesis of the *bis*(fluorophenyl) chlorophosphates **153-155**, in which variable amounts of the dichlorophosphates **156-158** were formed.

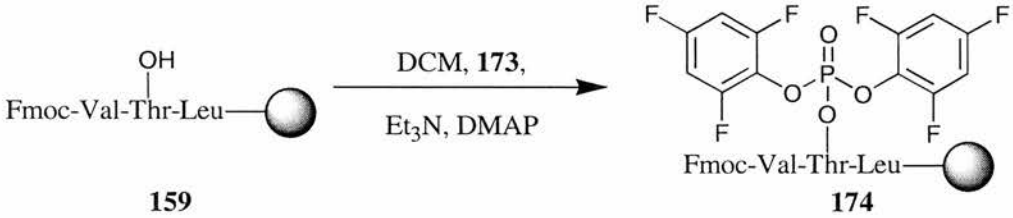


172 **173**

Scheme 3.6: *Synthesis of* bis*(2,4,6-trifluorophenyl) chlorophosphate **173***

3.3.3.2 Formation of phosphate triester.

Reaction of *bis*(2,4,6-trifluorophenyl) chlorophosphate **173** in CH_2Cl_2 with a suspension of Fmoc-VTL-OWang **159**, under standard conditions (see section 3.3.1.2) afforded the resin bound phosphate triester **174** after isolation by filtration and washing, Scheme 3.7.



159 **174**

Scheme 3.7: *Synthesis of resin bound* bis*(2,4,6-trifluorophenyl) threonyl phosphate triester **174***

3.3.3.3. Timecourse NMR studies

Reaction of the resin bound phosphate triester **174** under identical conditions as for the phosphate triesters **160-162** gave the arrayed spectrum shown, Figure. 3.8.

It can be seen from Fig. 3.8 that there is a noticeable change in the ^{31}P signal during the first 2 hours of the experiment. Initially one phenyl group is removed to form the phosphate diester {δ_P –6}, and the second fluorophenyl group is cleaved after about 60-90 minutes to form the phosphate monoester {δ_P –3}. This increase in rate is due to the presence of two *ortho*-fluorine atoms on the ring, as well as the electron withdrawing nature of the *para*-fluorine. These two factors both contribute to the enhanced electrophilicity of the phosphorus centre, resulting in rapid cleavage of the phenyl group.

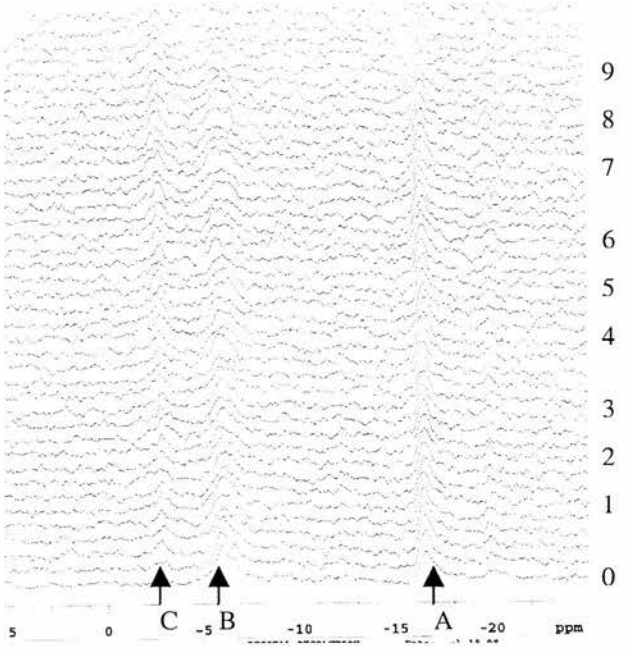


Figure 3.8: *Timecourse of acidic treatment of 2,4,6-trifluorophenyl phosphate triester* ***174*** *with TFA/H_2O/Et_3SiH. Spectra are displayed every 15 minutes, and are stacked vertically.*

It is again unclear as to whether the intensity of peak a decreases as the intensity of peaks B and C increase. However, as the intensity of peak C increases the intensity of peak B is seen to decrease, indicative of conversion of the diester (peak B) to the monoester (peak C).

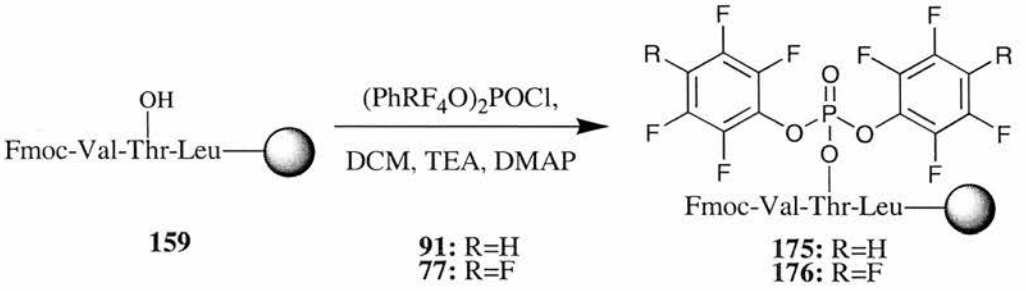
3.3.4 Acid lability of tetra- and penta-fluorophenyl groups

3.3.4.1 Synthesis of the chlorophosphates 91, 77

The synthesis of *bis*(tetrafluorophenyl) chlorophosphate **91** and *bis*(pentafluorophenyl) chlorophosphate **77** have already been described (Section 2.3.2, page 72 & section 2.3.3.2, page 81)

3.3.4.2 Formation of phosphate triesters 175 & 176

The 2,3,5,6-tetrafluorophenyl and pentafluorophenyl phosphate triesters **175** and **176** were synthesised in an identical manner to the phosphate triesters **148-150**, Scheme 3.8.



159

91: R=H
77: R=F

175: R=H
176: R=F

Scheme 3.8: *Synthesis of resin bound* bis*(2,3,5,6-tetrafluorphenyl) and* bis*(pentafluorophenyl) threonyl phosphate triesters **175** and **176***

3.3.4.3 Timecourse NMR studies

When the phosphate triesters **175** and **176** were treated with 95% CF_3CO_2H/ 2.5% H_2O/ 2.5% Et_3SiH, and upon investigation by ^{31}P NMR spectroscopy, a signal at approximately –12 ppm, corresponding to the triesters, was not observed, (Figs. 3.9 and 3.10 respectively). In Fig. 3.9, only the peak at –9 ppm is observed, corresponding to the fluorophenyl threonyl phosphate diester **177**, Scheme 3.9 (page 160). The high signal-to-noise ratio complicates accurate interpretation of the spectrum. The removal of the first tetrafluorophenyl group is so rapid, it is complete

before the end of the first ^{31}P acquisition. Similarly, in Fig. 3.10, the triester **176** is not observed, removal of the first pentafluorophenyl group being complete before the end of the first ^{31}P acquisition. Therefore, only the peaks at –9 ppm, corresponding to the phosphate diester **178**, and at –3 ppm , corresponding to the phosphate monoester **179**, are observed. Again, it is unclear as to whether the intensity of the triester peak (peak A) decreases as the intensities of the diester (B) and monoester (C) increase. The peak at –17 ppm may be due to pyrophosphate formation.

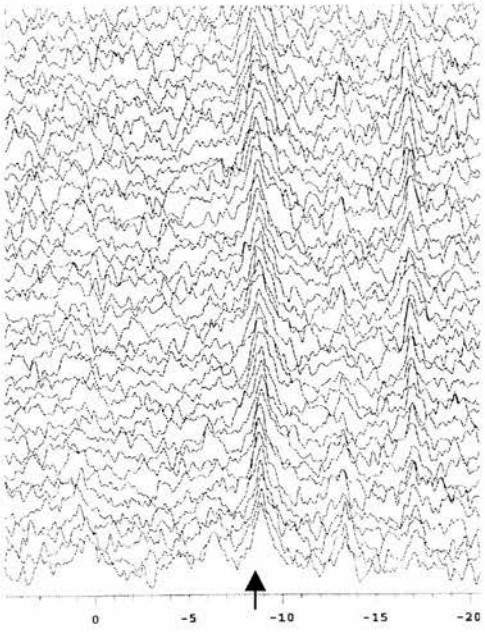


Figure 3.9: *Timecourse of acidic treatment of tetrafluorophenyl phosphate triester* ***175*** *with TFA/H_2O/Et_3SiH. Spectra are displayed every 15 minutes, and are stacked vertically.*

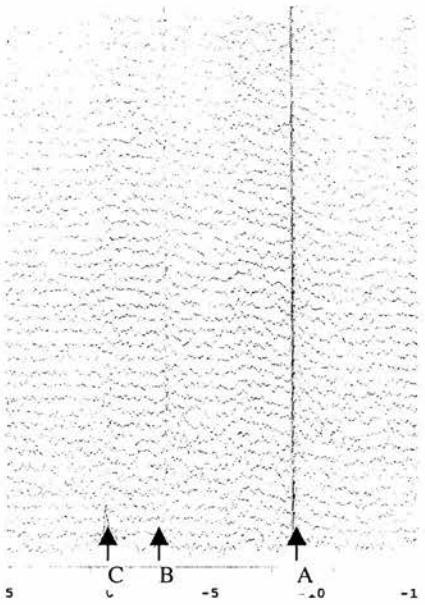
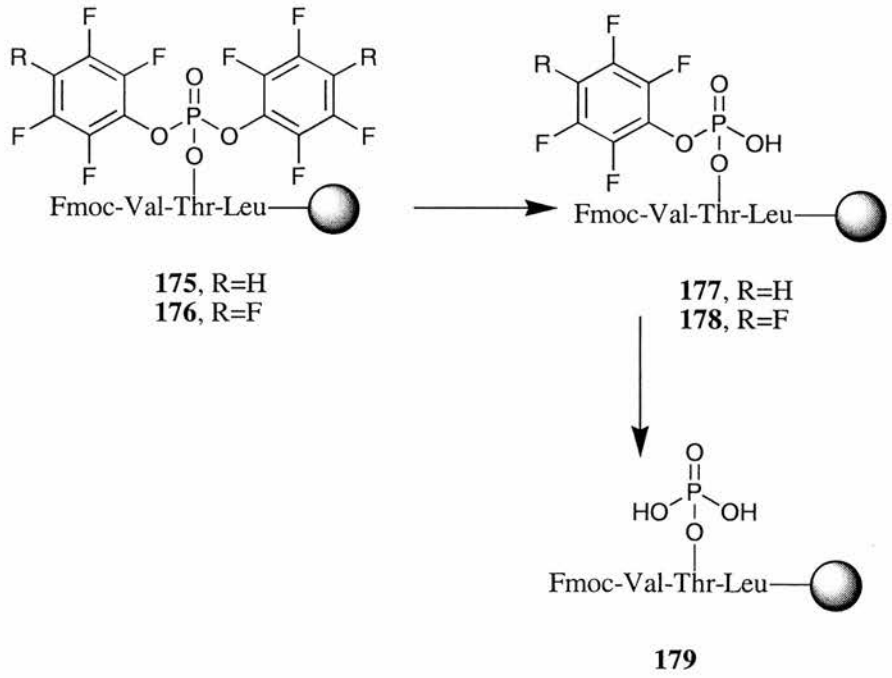


Figure 3.10: *Timecourse of acidic treatment of pentafluorophenyl phosphate triester* ***176*** *with TFA/H_2O/Et_3SiH. Spectra are displayed every 15 minutes, and are stacked vertically.*



175, R=H
176, R=F

177, R=H
178, R=F

179

Scheme 3.9: *Cleavage of tetra- and pentafluorophenyl groups from phosphate triesters.*

Cleavage of the first pentafluorophenyl or tetrafluorophenyl group is therefore extremely fast, and is complete during the time required to acquire the first of the ^{31}P NMR spectrum (within 15 minutes). The observed reaction is therefore the cleavage of the second pentafluorophenyl group from the phosphate diester {δ_P −9 ppm} to form the threonyl phosphate monoester {δ_P −3 ppm}. By reducing the amount of acid in the reaction mixture it was hoped to retard the rate of removal of the first pentafluorophenyl group so as to observe it during spectrum acquisition.

Accordingly, the experiment was repeated with 20% v/v CF_3CO_2H/ H_2O, instead of the 95% CF_3CO_2H mixture used previously, and spectra acquired every 15 minutes. It was then possible to observe the disappearance of the signal at −12 ppm (triester, peak A) and the appearance of a signal at −6 ppm (diester, peak B) followed by the appearance of a signal at −1 ppm (monoester, peak C), Fig. 3.11. There is an apparent change in signal position for the diester when the TFA concentration is decreased from 95% TFA to 20% TFA. This may be due to the extremely acidic environment of the 95% TFA reaction, which is less acidic in the 20% TFA reaction.

As a comparison, the same reaction was carried out, in solution phase, on a sample of cyclohexyl *bis*(pentafluorophenyl) phosphate **107**, Fig. 3.12 below. In this case, the product is the pyrophosphate {δ_P −21}, and not the phosphate diester or monoester as is the case in the solid phase reactions.

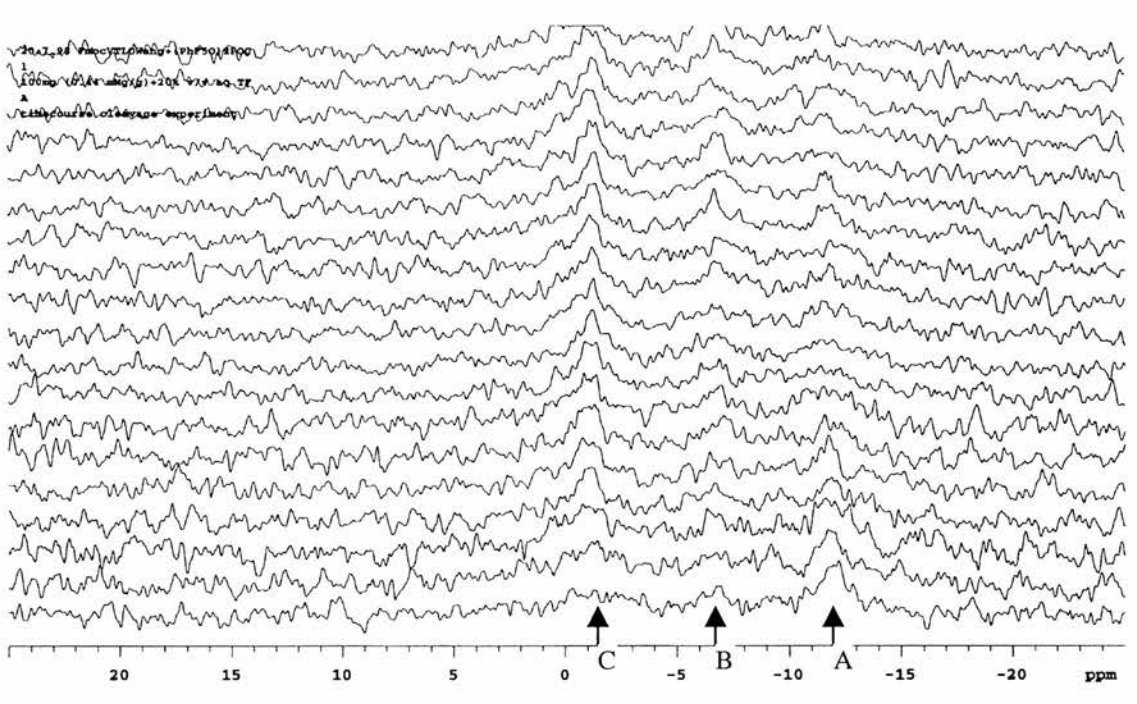


Figure 3.11: *Timecourse of acidic treatment of pentafluorophenyl phosphate triester* ***176*** *with 20 % v/v TFA/H_2O. Spectra are displayed every 15 minutes, and are stacked vertically*

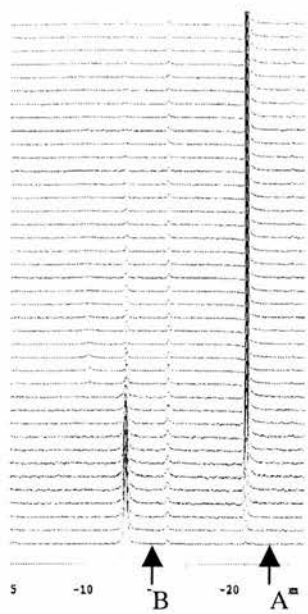


Figure 3.12: *Timecourse of acidic treatment of* bis*(pentafluorophenyl) cyclohexyl phosphate* ***107*** *with TFA/H_2O/Et_3SiH. Spectra are displayed every 15 minutes and are stacked vertically.*

This is a further example of solid and solution phase reactions following different reaction pathways.[257]

3.4 Evidence for proton chelation by variable temperature high field ^{1}H and ^{19}F NMR spectroscopy.

The experiments above suggest that the presence of two *ortho*-fluorine atoms on the phenyl ring induces acid lability. However, there is still no evidence for a formal interaction between the fluorine atoms and the acidic proton, Figure 3.13, as proposed in the mechanism.

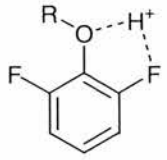


Figure 3.13: *Chelation of proton by 2,6-difluorophenyl group in acidic media.*

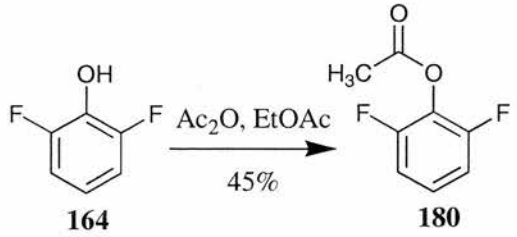
Chelation of a proton by either of the fluorine atoms may have one of two effects:

- Splitting of the ^{1}H NMR signal corresponding to the chelated acid proton.
- Shifting of the ^{19}F signal corresponding to the chelating fluorine and shifting of the ^{1}H signal of the chelated proton.

It was envisaged that analysis of a sample of protonated 2,6-difluorophenol by ^{1}H NMR spectroscopy would reveal the presence of the phenolic proton and the chelated proton, which would have different chemical shifts due to the slightly different chemical environments. However, at room temperature, rotation of the C-O bond is extremely rapid with respect to the NMR timescale, and therefore the two

protons will appear as equivalent. This rotation can be slowed down by cooling the sample. It was hoped that the rotation could be slowed sufficiently for the two individual resonances to be observed.

A sample of 0.2 mmol of 2,6-difluorophenol **164** and 0.2 mmol of TFMSA in $C^2H_2Cl_2$ (0.7 cm^3) was placed in a Varian Gemini 500 NMR spectrometer and the ^1H NMR spectrum acquired at room temperature (273 K). Two resonances were observed, a resonance, integral 3, corresponding to the hydrogens attached to the aromatic ring, and a resonance, integral 2, corresponding to the phenolic and acid protons. The sample was cooled down to 193 K in 10 K steps and the ^1H NMR spectrum acquired after each cooling step. Though the resonance of the phenolic and acidic protons began to broaden, it did not resolve into two separate peaks. From this, it is apparent that the barrier to rotation of the C-O bond is simply too low, and rotation cannot be slowed down even at extremely low temperatures. The barrier to rotation can be increased by adding steric bulk to the oxygen of the difluorophenol, for example by the addition of an ester group. Accordingly, treatment of 2,6-difluorophenol **164** with acetic anhydride afforded *O*-acetyl-2,6-difluorophenol **180** in 45 % yield as a colourless oil, Scheme 3.10.



164 **180**

Scheme 3.10: *Synthesis of O-acetyl-2,6-difluorophenol **180***

The variable temperature NMR experiment was repeated with 0.2 mmol of *O*-acetyl-2,6-difluorophenol **180** and 0.2 mmol TFMSA in $C^2H_2Cl_2$, and ^{19}F NMR

spectra were acquired at sample temperatures of 273 K, 253 K, 233 K, 213 K and 193 K, Fig. 3.14.

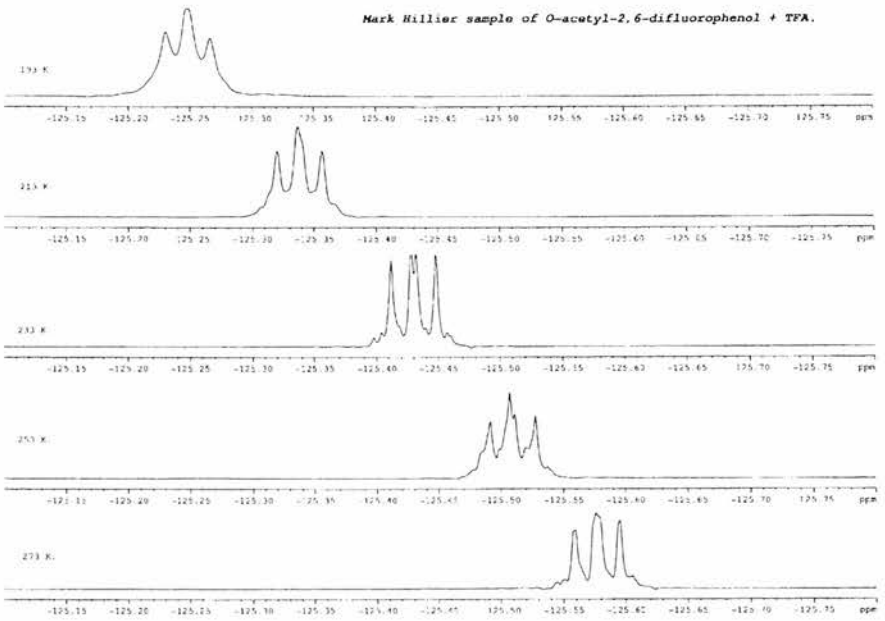


Figure 3.14: *Variable temperature ^{19}F NMR of 0.2 mmol* ***180*** *and 0.2 mmol TFA in $C^2H_2Cl_2$*

There is an observable shift in the ^{19}F frequency upon cooling the sample, and a splitting of the ^{19}F resonance is observable at 233 K. This may be due either to chelation of the TFMSA proton being observable at this temperature on the NMR timescale, with the result that the fluorines become non-equivalent, or may simply be due to a slowing of the rotation of the acetyl group about the phenolic C-O bond, resulting in the fluorine atoms being non-equivalent on the NMR timescale. The shift in ^{19}F signal on cooling, may be indicative of increased interaction between the *O*-acetyl-2,6-difluorophenol and the acidic proton of TFMSA.

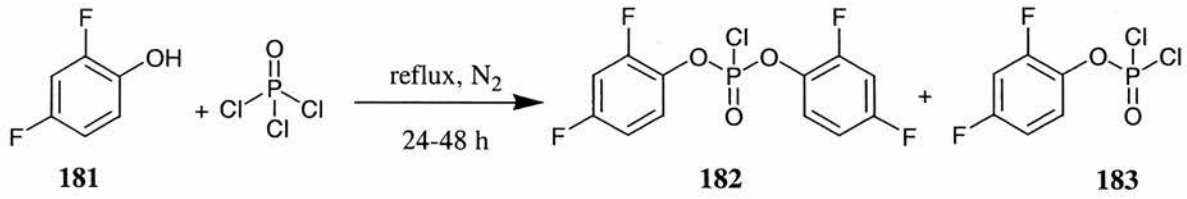
3.5 Conclusions

Whilst there is a definite increase in acid lability of the fluorophenyl groups with increasing fluorine substitution on the phenyl ring, there is no definite evidence that this is due to the chelation of a proton by the *ortho*-fluorine atoms and the phenolic oxygen or phosphate oxygen. However, given that the minimum requirement for acid cleavage is the presence of two *ortho*-fluorine atoms, it seems unlikely that their effect is solely an electronic one, and there is participation of the *ortho*-fluorine atoms in the mechanism of cleavage. However, the exact role is not known.

3.6 Further Work

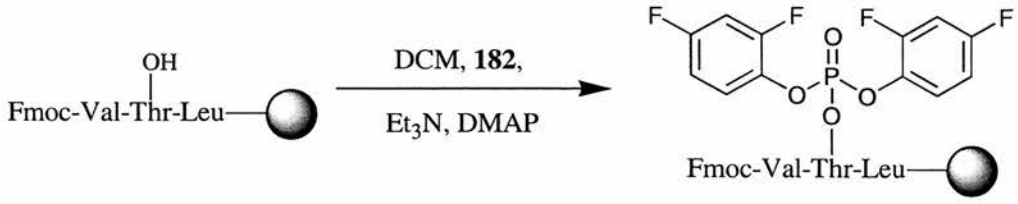
It has been shown that the *bis*(2,6-difluorophenyl) phosphate triester **170** and the *bis*(2,4,6-trifluorophenyl) phosphate triester **174** are deprotected under aqueous conditions whereas the *bis*(2-fluorophenyl) phosphate triester **160** is not, suggesting there is a minimum requirement for 2 *ortho*-fluorine atoms on the phenyl ring. Therefore it is necessary to examine the *bis*(2,4-difluorophenyl) phosphate triester **184** for lability in acid conditions, as this possesses only one *ortho*-fluorine on the phenyl ring, but has the *para*-fluorine present in the *bis*(2,4,6-trifluorophenyl) phosphate triester **174**.

Bis(2,4-difluorophenyl) chlorophosphate **182** can be obtained from the reaction of 2,4-difluorophenol **181** with $POCl_3$, Scheme 3.11, in a similar manner to the synthesis of *bis*(2,6-difluorophenyl) chlorophosphate **166** and *bis*(3,5-difluorophenyl) chlorophosphate **167**, Scheme 3.3. The *bis*(2,4-difluorophenyl) phosphate triester **184** can be obtained from the phosphorylation of Fmoc-VTL-Owang **159** with *bis*(2,4-difluorophenyl) chlorophosphate **182**, Scheme 3.12.



181 **182** **183**

Scheme 3.11: *Synthesis of* bis*(2,4-difluorophenyl) chlorophosphate* ***182*** *and (2,4-difluorophenyl) dichlorophosphate* ***183****.*



159 **184**

Scheme 3.12: *Synthesis of resin bound 2,4-difluorophenyl phosphate triester* ***184***

4.0 Experimental

Elemental microanalyses were performed in the departmental microanalytical laboratory. NMR spectra were recorded on a Varian Gemini 200 (^{1}H, 200 MHz; ^{13}C, 50.31 MHz), a Bruker AM-300 (^{1}H, 300 MHz; ^{13}C, 74.76 MHz), a Varian Gemini 300 or Bruker AL-300 (^{1}H, 300 MHz; ^{13}C, 74.76 MHz; ^{19}F, 282.3 MHz; ^{31}P, 121.4 MHz), a Bruker AMX-400 (^{1}H, 400 MHz; ^{13}C, 99.68 MHz; ^{19}F, 376.4 MHz; ^{31}P, 161.9 MHz) or a Varian Gemini 500 (^{1}H, 500 MHz; ^{13}C, 125.78 MHz) spectrometer. Chemical Shifts are described in parts per million downfield shift from $SiMe_4$ and are reported consecutively as position (δ_H, δ_C, δ_F, or δ_P), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = double of doublets, sep = septet, m = multiplet, and br = broad), coupling constant (*J*/Hz) and assignment (numbering according to the IUPAC nomenclature for the compound). ^{1}H NMR spectra were referenced internally on ^{2}HOH (δ 4.68), $CHCl_3$ (δ 7.27), CH_3O^2H (δ 3.35) or $(C^2H_3)_2SO$ (δ 2.47). ^{13}C NMR spectra were referenced on CH_3OH (δ 49.9), C^2HCl_3 (δ 77.5), or $(CH_3)_2SO$ (δ 39.70).

IR spectra were recorded on a Perkin-Elmer 1710 FT-IR or a Nicolet Avatar 360 FT-IR spectrometer. The samples were prepared as Nujol mulls, solutions in chloroform, thin films between sodium chloride discs, neat, or as single beads. 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 a VG 70-250 SE, a Kratos MS-50, a VG Platform E/S, VG Zabspec or by the EPSRC service at Swansea using a VG AZB-E. Fast atom bombardment spectra were recorded using *m*-nitrobenzyl alcohol (NOBA) as a matrix. Major fragments were given as percentages of the base peak intensity (100%).

Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Flash chromatography was performed according to the method of Still *et al.*,[275] using Sorbsil C60 (40 – 60 μm mesh) silica gel. Analytical thin layer chromatography (TLC) was carried out on 0.25mm precoated silica gel plates (Machery-Nagel SIL g/UV254) and compounds were visualised using UV fluorescence, iodine vapour, ethanolic phosphomolybdic acid, or ninhydrin.

The solvents used were either distilled or of analar quality and light petrol 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. DMF, DCM, acetonitrile, toluene, diisopropylethylamine, and triethylamine were distilled over CaH_2. THF and diethyl ether were dried over sodium / benzophenone and distilled under nitrogen. Thionyl chloride was distilled over sulphur and the initial fractions were always discarded. Phosphoryl chloride was distilled and the initial fractions always discarded.

Solid phase peptide synthesis was carried out on a Protein Technologies, Inc. Rainen P53 Automated Solid Phase Peptide Synthesiser. The NMM, DMF and piperidine used were all HPLC grade (99.9%).

All reactions were carried out at room temperature unless otherwise stated.

Solid phase peptide synthesis and peptide removal from Wang resin

Solid phase synthesis of the tripeptides and hexapaptides described below were carried out using the Rainin PS3 automated peptide synthesiser. The synthesis employed Fmoc chemistry and the *C*-terminal amino acids were linked to Wang resin. Amino acids and the activating agent PyBOP were purchased from NovaBiochem (Nottingham, UK), solvent 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 activation was achieved using a 5% NMM/ DMF solution. Where coupling of an amino acid was known to be difficult or require prolonged reaction times – *i.e.* for consecutive arginine residues – a double coupling of that amino acid was performed.

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

1) The peptide-resins were cooled in an ice bath. The cleavage mixture – 95% TFA/ 2.5% H_2O/ 2.5% triethylsilane – was cooled in an ice bath and then added to the cooled resin. The mixture was then allowed to warm to room temperature and stirred for 1–2 h. The resin was then removed by filtration and washed with DCM (5-10 cm^3) and the combined filtrates concentrated under reduced pressure (3-5 cm^3). The peptide was then isolated by either:

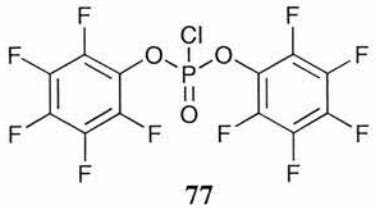
- precipitation with excess ice-cold dry ethyl ether, or
- dilution of the residual solution with a 5 – 10 fold excess of water followed by repeated extractions of the scavengers with an equivalent amount of dry ice cold diethyl ether. The aqueous layer is then concentrated to a small volume (5-10 cm^3) and lyophilised to afford the peptide; or

2) The peptide-resins were treated in a manner identical with that for procedure 1), but the cleavage mixture used was 5% thioanisole/ 95% TFA.

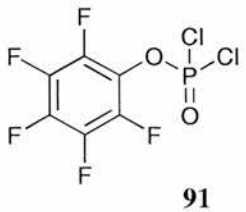
Diazomethane Preparation

N-Nitroso-*N*-methyl-4-toluenesulphonamide (Diazald) (21.0 g, 98.0 mmol) in ether (150 cm^3) was added slowly to a solution of potassium hydroxide (4.8 g, 80.0 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 and ether derivatives.

***Bis*(pentafluorophenyl) chlorophosphate 77 and pentafluorophenyl dichlorophosphate 91**[247]



77



91

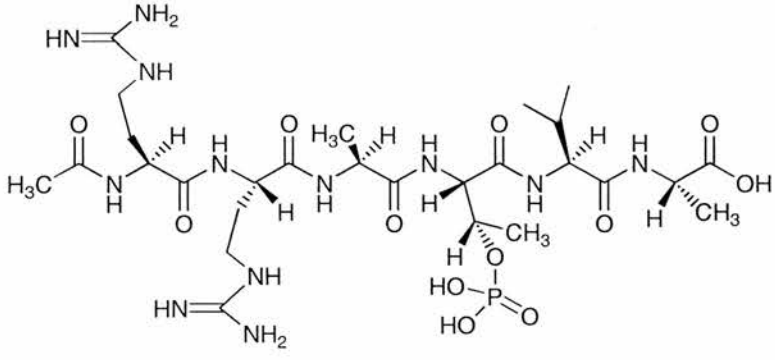
To freshly distilled $POCl_3$ (9.3 cm^3, 0.10 mol) was added pentafluorophenol (36.8 g, 0.20 mol) and the mixture refluxed for 24 h under a nitrogen atmosphere. Volatiles were removed under reduced pressure and the residue distilled to afford the dichlorophosphate **91** as the minor product (6.6 g, 22%) and chlorophosphate **77** as the major product (28.7 g, 64%).

Data for chlorophosphate **77**: bp 120 °C/ 0.7 mmHg (lit.,[247] 181–3 °C/ 20 mmHg) (Found: M^+, 447.9122. $C_{12}O_3F_{10}P_1Cl_1$ requires 447.9114), υ_{max}(thin film)/ cm^{-1} 1031 (P-O-C st), 1236 (ArC-F st), 1319 (P=O st) and 1522 (ArC-C); δ_C(75.4 MHz; C^2HCl_3) 136.51–142.77 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 190.5–190.7, 194.9–195.1 and 198.0–198.1 (10F, all m, Ar-C*F*); δ_P(121.41 MHz; C^2HCl_3) –15.1; *m/z* (CI^+) 448, 450 (9%, 3, M^+, [Cl isotopes]), 413 (49, $[M - Cl]^+$), 265 (6, [M

– $C_6F_5O]^+$), 246 (11, $[M - C_6F_5 - Cl]^+$), 230 (81, $[M - C_6F_5O - Cl]^+$) and 183 (100, $C_6F_5O^+$), purity 93.4 % by ^{31}P NMR spectroscopy.

Data for dichlorophosphate **91**: bp 62 °C/ 0.7 mmHg (lit.,[247] 112–125 °C/ 20 mmHg); (Found: M^+, 299.8936. $C_6O_2F_5P_1{}^{35}Cl_2$ requires 299.8933); υ_{max}(thin film)/ cm^{-1} 998 (P-O-C st), 1233 (ArC-F st), 1297 (P=O st) and 1522 (ArC-C). δ_C(75.4 MHz; C^2HCl_3) 138.69 –142.87 (Ar-*C*); δ_F(282.3 MHz; C^2HCl_3) 189.09, 192.62 and 198.84 (5F, m, Ar-C*F*); δ_P(121.41 MHz; C^2HCl_3) –11.23; *m/z* (EI^+) 300, 302, 304 (45%, 30, 5, M^+ [Cl isotopes]), 183 (32, $C_6F_5O^+$), 155 (52) and 117 (100, $POCl_2^+$), purity 99.5 % by ^{31}P NMR spectroscopy.

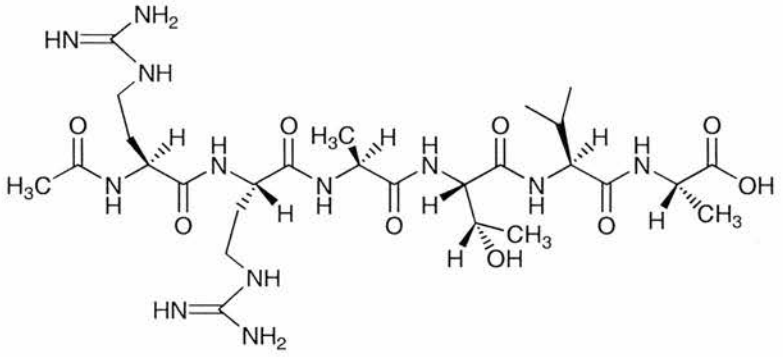
_N_-α-Acetyl-(*S*)-arginyl-(*S*)-arginyl-(*S*)-alanyl-(*S*)-dihydroxyphosphoryl threonyl-(*S*)-valyl-(*S*)-alaninate 90



To a stirred suspension of Ac-R(Pmc)R(Pmc)ATVA-Wang resin **93** (400 mg, 0.21 mmol), DMAP (40 mg, 0.4 mmol) and Et_3N (500 mm^3, 3.6 mmol) in CH_2Cl_2 (10 cm^3) was added a solution of benzyl pentafluorophenyl chlorophosphate **118** (2.25 mmol) in CH_2Cl_2 (10 cm^3) and the resulting mixture allowed to stir overnight. The resin was then washed with CH_2Cl_2 and dried with MeOH. The dried resin was treated with cleavage mixture 1 for 2 hours and the aqueous layer lyophilised. The solid obtained was purified by anion exchange chromatography using Dowex 1 x 2-200 (chloride form) to give the product **78** as a white solid (75

mg, 47.3%), mp 203-205 °C [lit,[246] 205 °C (decomp.)]; υ_{max}(Nujol)/ cm^{-1} 1256 (P=O), 1660 (C=O st) and 3292 (NH st); δ_H(300 MHz; ^2H_2O) 0.77 (6H, m, Val $CH(CH_3)_2$), 1.01 (3H, d, *J* 6.0, Thr CH_3), 1.23 (6H, t, *J* 7.5, 2 x Ala CH_3), 1.47-1.61 (8H, m, 2 x Arg CH_2CH_2 and 2 x Arg CH_2CH), 1.83 (3H, s, Acetyl CH_3), 1.90 (1H, m, Val CH), 3.02 (4H, m, 2 x Arg CH_2CN) and 3.95-4.21 (7H, m, α– and β–CH's); δ_C(75.5 MHz; ^2H_2O) 18.64 & 20.24 [Val $CH(CH_3)_2$], 19.16 & 20.86 [Leu $CH(CH_3)_2$], 21.45 (Thr CH_3), 29.68 (2 x Arg CH_2CH_2), 30.71 & 30.78 (Arg CH_2CN), 32.96 [Val $CH(CH_3)_2$], 51.28 & 52.31 (Ala α-CH), 55.69 & 56.17 (Arg α-CH), 56.37 (Val α-CH), 61.74 (d, *J* 5.59, Thr β-CH), 69.72 (Thr α-CH), 159.33 (2 x Arg CN), 174.18 (acetyl CO), 175.42, 175.82, 176.65, 176.96, 177.63 (CONH) and 178.82 (CO_2H); δ_P(121.4 MHz; ^2H_2O) –0.214; *m/z* (ES^+) 795 (10%, $[M + 2H]^+$), 715 (100, $[M + H - PO_3H_2]^+$) and 358 (30, $[\{M + 2H - PO_3H_2\}/2]^+$).

N-α-Acetyl-(S)-arginyl-(S)-arginyl-(S)-alanyl-(S)-threonyl-(S)-valyl-(S)-alaninate 94

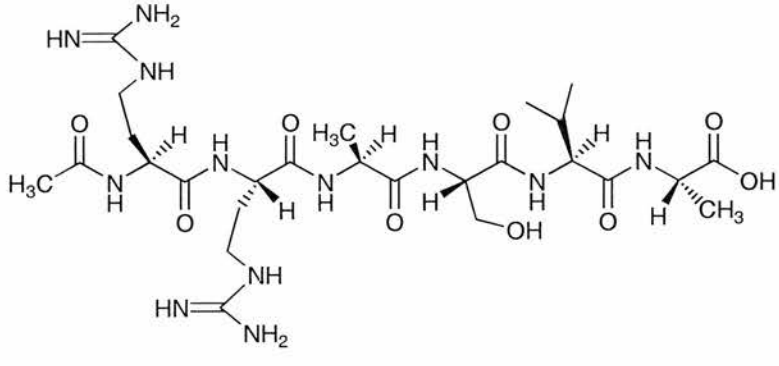


N-α-Fmoc-R(Pmc)R(Pmc)ATVA-Wang resin **92** (400 mg, 0.2 mmol) was treated with 20% DMF/ piperidine (10 cm^3) for 30 min., followed by 5% acetic anhydride/ DMF (10 cm^3) for 30 min., with DMF washes of the resin after each treatment. This was then treated with cleavage mixture 1 (10 cm^3) to give a white

solid (140 mg, 98.0%), mp 224-226 °C (decomp.) (lit,[246] 223 °C); υ_{max}(Nujol)/ cm^{-1} 1548 (CO_2^-), 1656 (NH_2), 1710 (amide NH) and 3313 (NH stretch); δ_H(300 MHz; ^2H_2O) 0.94-0.98 (6H, t, *J* 5.7 Hz, Val $(CH_3)_2$), 1.20 (3H, d, *J* 6.0, Thr CH_3), 1.39-1.44 (6H, dd, *J* 6.6, 7.2, 2 x Ala CH_3), 1.65-1.86 (8H, m, 2 x Arg CH_2CH_2 and 2 x Arg CH_2CH), 2.03-2.10 (4H, m, acetyl CH_3 and Val C*H*), 3.21 (4H, m, 2 x Arg CH_2NH) and 4.16-4.39 (7H, m, 2 x Arg α-C*H*, Thr α-C*H*, Thr β-C*H*, 2 x Ala α-C*H* and Val α-C*H*); δ_C (75.4 MHz; ^2H_2O) 16.15 (Val CH_3), 16.65 (Ala CH_3), 17.68 (Val CH_3), 18.34 (Ala CH_3), 18.92 (Thr CH_3), 21.67 (acetyl CH_3), 24.48 (2 x Arg CH_2CH_2), 28.21 & 28.30 (2 x Arg CH_2CH), 30.48 (Val *C*H), 48.79 (Ala α-*C*H), 49.83 (Ala α-*C*H), 53.23 & 53.70 (2 x Arg α-*C*H), 59.22 (Val α-*C*H), 59.29 (Thr β-*C*H), 67.27 (Thr α-*C*H), 157.07 (2 x Arg *C*N), 171.87-175.31 (*C*O's) and 176.52 (CO_2H); *m/z* (ES^+) 715 (55%, $[M + H]^+$) and 358 (100, $[\{M + 2H\}/2]^+$).

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

96

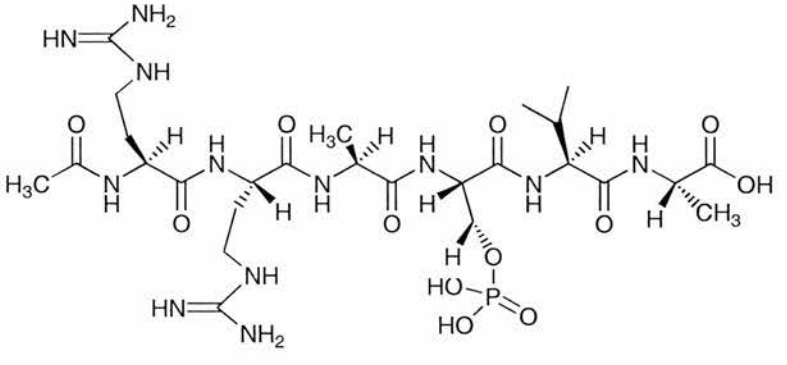


This compound was prepared in a manner identical to that for hexapeptide **94** using *N*-α-Fmoc-R(Pmc)R(Pmc)ASVA-Wang resin (385 mg, 0.2 mmol).

Ac-R(Pmc)R(Pmc)ASVA-Wang resin so prepared was treated with cleavage mixture 1 (10 cm^3) to give a white solid on lyophilisation (135 mg, 96.3%), mp 180

°C (lit.,[246] 177-178 °C), υ_{max} (Nujol)/ cm^{-1} 1541 (CO_2^-), 1657 (NH_2) and 3275 (NH stretch); δ_H(300 MHz; ^2H_2O) 0.95 (6H, t, *J* 6.87 Hz, Val (CH_3)$_2$), 1.41 (6H, t, *J* 7.01 Hz, 2 x Ala CH_3), 1.58-1.90 (8H, m, 2 x Arg CH_2CH_2 and 2 x Arg CH_2CH), 2.03-2.14 (4H, m, acetyl CH_3 and Val *CH*), 3.21 (4H, t, *J* 6.46, 2 x Arg CH_2NH), 3.86 (1H, d, *J* 5.49, Val α-*CH*), 4.17-4.37 (4H, m, 2 x Arg α-*CH* and 2 x Ala α-*CH*), 4.47 (2H, t, *J* 5.50, Ser CH_2) and 4.74 (1H, s, Ser α-*CH*); δ_C(75.4 MHz; ^2H_2O) 16.14 (Val CH_3), 16.64 (Ala CH_3), 17.55 (Val CH_3), 18.37 (Ala CH_3), 21.69 (acetyl CH_3), 24.46 (2 x Arg CH_2CH_2), 28.23 (2 x Arg CH_2CH), 30.41 (Val *CH*), 48.79 and 49.85 (2 x Ala α-*CH*), 53.27 & 53.72 (2 x Arg α-*CH*), 55.57 (Ser α-*CH*), 59.26 (Ser β-CH_2), 61.17 (Val α-*CH*), 157.09 (2 x Arg *CN*), 171.88 (CH_3CO), 173.20-175.23 (amide *CO*'s) and 176.53 (CO_2H); *m/z* (ES^+) 702 (12% , $[M + H]^+$) and 351 (100, $[\{M + 2H\}/2]^+$).

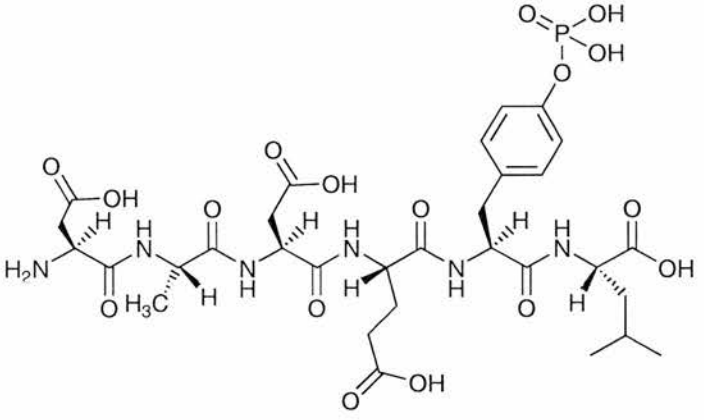
_N_-α-Acetyl-(*S*)-arginyl-(*S*)-arginyl-(*S*)-alanyl-(*S*)-dihydroxyphosphorylseryl-(*S*)-valyl-(*S*)-alaninate 97



This compound was prepared in a manner identical to that for the phosphothreonyl hexapeptide **90** using Ac-R(Pmc)R(Pmc)ASVA-Wang resin (385 mg, 0.2 mmol), DMAP (40 mg, 0.4 mmol), dry Et_3N (500 mm^3, 3.6 mmol) and benzyl pentafluorophenyl chlorophosphate **118** (2.37 mmol). The peptide resin so obtained was treated with cleavage mixture 1 (10 cm^3) and purified by ion exchange

chromatography using Dowex 1 x 2-200 (chloride form) to give the product **97** as an off-white solid (70 mg, 44.9%), mp 200 °C (decomp.) [lit.,[246] 200 °C (decomp.)], υ_{max}(Nujol)/ cm^{-1} 1260 (P=O st), 1654 (C=O st) and 3335 (NH st); δ_H(300 MHz; ^2H_2O) 0.76 [6H, t, *J* 6.0, Val CH(CH_3)$_2$], 1.22 (6H, 2 x d, *J* 3.9, 4.2, 2 x Ala CH_3), 1.45-1.68 (8H, m, 2 x Arg CH_2CH_2 and 2 x Arg CH_2CH), 1.82 (3H, s, acetyl CH_3), 1.88 (1H, m, Val C*H*), 3.01 (4H, m, 2 x Arg CH_2CN), 3.96-4.16 (7H, m, α-C*H*'s and Ser CH_2) and 4.24 (1H, m, Ser α-C*H*); δ_C(75.5 MHz; ^2H_2O) 18.69 & 20.01 [Val CH(CH_3)$_2$], 19.08 & 20.88 (2 x Ala CH_3), 24.12 (acetyl CH_3), 26.93 (2 x Arg CH_2CH_2), 30.65 & 30.70 (Arg CH_2CH), 32.79 (Val *C*H), 51.17 & 52.33 (2 x Ala α-*C*H), 55.70 & 56.15 (Arg α-*C*H), 56.39 (d, *J* 1.13, Ser CH_2), 58.03 (Ser α-*C*H), 61.90 (Val α-*C*H), 159.28 (Arg *C*N), 174.25 (acetyl *C*O), 175.49, 175.82, 176.62, 176.92, 177.53 (*C*ONH's) and 178.73 (CO_2H); δ_P(121.4 MHz, ^2H_2O) 0.33.

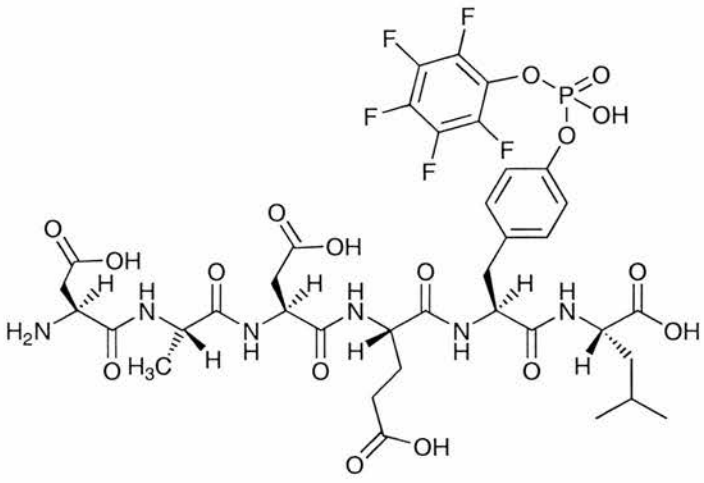
(*S*)-aspartyl-(*S*)-alanyl-(*S*)-aspartyl-(*S*)-glutamyl-(*S*)-dihydroxyphosphoryl tyrosyl-(*S*)-leucine 98



To a suspension of *N*-α-Fmoc-Asp(^{t}Bu)-Ala-Asp(^{t}Bu)-Glu(^{t}Bu)-Tyr-Leu-Wang resin **100** (180 mg peptide-resin, 0.1 mmol), Et_3N (200 mm^3, 1.4 mmol) and DMAP (11 mg, 0.1 mmol) in CH_2Cl_2 (10 cm^3) was added a solution of freshly prepared benzyl pentafluorophenyl chlorophosphate **118** (560 mg, 1.5 mmol) in

CH_2Cl_2 (5 cm^3), and the mixture allowed to stir for 4 –5 hours. The resin was filtered and washed with successive volumes of CH_2Cl_2 (10 cm^3), MeOH (10 cm^3) and CH_2Cl_2 (10 cm^3). The dried resin was treated with 2 mol dm^{-3} NaOH (5 cm^3) in THF: MeOH (4:1; 5 cm^3) for 4 hours. After filtering and washing (DCM, MeOH and DCM), the resin was dried and treated with 2% DBU in DMF (10 cm^3) for 15 minutes. After washing of the resin (DCM, MeOH, DCM), and drying, the resin was treated with cleavage mixture 2 for 30 min. After removal of volatiles under reduced pressure, and trituration with diethyl ether, the crude product was lyophilised to afford the product **98** as a white solid (42 mg, 52 %) mp 173-176 °C; (Found: $[M + Na]^+$, 827.2444. $C_{31}H_{45}N_6O_{17}P_1Na_1$ requires 827.2477), υ_{max}(Nujol)/ cm^{-1} 1272 (P=O st), 1504, 1648 (amide C=O), 1714 (amide NH) and 3310 (br., H-bonded CO_2H); δ_H(300 MHz; $C^2H_3O^2H$) 0.84 [6H, dd, *J* 5.1, 16.2, Leu CH(CH_3)$_2$], 1.36 (3H, d, *J* 7.8, Ala CH_3), 1.96 (2H, m, Glu β-CH_2), 2.6 (2H, m, Glu γ-CH_2), 2.81-3.06 (6H, m, 2 x Asp CH_2 & Tyr CH_2), 4.25-4.58 (6H, m, α-C*H*'s) and 7.12 (4H, dd, *J* 8.4, 33.3, Tyr Ar-*H*); δ_C(75.4 MHz; $C^2H_3O^2H$) 18.8 (Ala CH_3), 23.09 & 24.81 [Leu CH(CH_3)$_2$], 26.90 [Leu CH(CH_3)$_2$], 31.06 (Glu β-CH_2), 32.28 (Glu γ-CH_2), 37.96 (2 x Asp CH_2), 38.6 (Tyr CH_2), 42.01 (Leu CH_2), 52.01 (Leu α-CH), 52.79 (Asp α-CH), 53.89 (Ala α-CH) 55.67 (Glu α-CH), 57.45 (Thr α-CH), 122.92 (3-Ar-CH), 123.17 (2-Ar-CH), 129.97 (4-Ar-*C* Tyr), 133.0 (d, *J* 8.38, 1-Ar-*C*) and 168.53-178.47 (C=O amide & carboxyl); δ_P(121.4 MHz; $C^2H_3O^2H$) –3.64; *m/z* (ES^+) 827 (100%, $[M + Na]^+$), 805 (12, $[M + H]^+$) and 747 (13, $[M + H + Na - PO_3H_2]^+$).

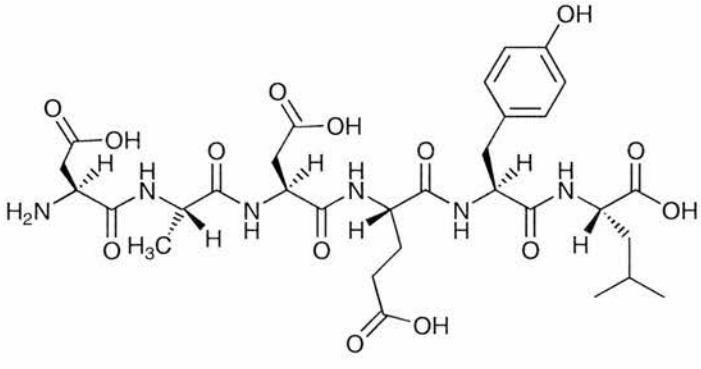
(*S*)-Aspartyl-(*S*)-alanyl-(*S*)-aspartyl-(*S*)-glutamyl-(*S*)-pentafluorophenylhydroxyphosphoryl tyrosyl-(*S*)-leucine 101



To a suspension of *N*-α-Fmoc-Asp(^{t}Bu)-Ala-Asp(^{t}Bu)-Glu(^{t}Bu)-Tyr-Leu-Wang resin **99** (500 mg peptide-resin, 0.3 mmol), Et_3N (503 mm^3, 3.2 mmol) and DMAP (37 mg, 0.3 mmol) in CH_2Cl_2 (10 cm^3) was added a solution of *bis*(pentafluorophenyl) chlorophosphate **77** (1.89 g, 4.2 mmol) in CH_2Cl_2 (10 cm^3) and the mixture allowed to stir for 4 –5 hours. The resin was filtered and washed with successive volumes of CH_2Cl_2 (10 cm^3), MeOH (10 cm^3), and CH_2Cl_2 (10 cm^3). The dried resin was treated with 20% piperidine in DMF for 15 minutes then filtered and washed (CH_2Cl_2, MeOH then CH_2Cl_2). After drying the resin was treated with cleavage mixture 1 for 2 hours and the resin removed by filtration. The resin was washed with CH_2Cl_2 (10 cm^3) and the filtrate concentrated under reduced pressure. Water (10 cm^3) was added and the aqueous layer washed with ice-cold ether (10 cm^3). The aqueous layer was concentrated under reduced pressure and lyophilised to give an extremely hygrosopic white solid **101** (226 mg, 77.6%), mp >120 °C (decomp.); υ_{max}(neat)/ cm^{-1} 1082 (Ar-CF stretch), 1269 (P=O stretch), 1666 (NH) and 3305 (H-bonded OH). δ_H(300 MHz; ^2H_2O) 0.93 (6H, dd, *J* 6.0, 15.6, 2 x Leu CH_3), 1.45 (3H, d, *J* 7.2, Ala CH_3), 1.67-1.74 (3H, m, Leu CH_2C*H*), 1.86-2.02

(2H, m, Glu β-CH_2), 2.34-2.40 (2H, m, Glu γ-CH_2), 2.82-3.16 (6H, m, 2 x Asp CH_2 and Tyr CH_2), 4.32-4.74 (6H, m, α-C*H*'s), 7.24 (2H, d, *J* 9.0, Tyr Ar-*H*) and 7.30 (2H, d, *J* 9.0, Tyr Ar-*H*); δ_C(75.4 MHz; ^2H_2O) 16.25 (Ala CH_3), 20.61 & 21.60 [Leu CH(CH_3)$_2$], 24.46 [Leu $CH(CH_3)_2$], 26.18 (Glu β-CH_2), 29.84 (Glu γ-CH_2), 34.73 (Asp CH_2), 35.00 (Asp CH_2), 36.19 (Tyr CH_2), 39.69 (Leu CH_2), 49.55 (Leu α-*C*H), 50.46 and 50.54 (Asp α-CH_2), 51.35 (Ala α-CH_2), 53.36 (Glu α-*C*H), 54.78 (Tyr α-*C*H), 120.49 (2-Ar-*C*H), 130.59 (4-Ar-*C*), 132.79 (3-Ar-*C*H),136.73, 139.96, 143.43 (Ar-*C*F), 151.05 (d, *J* 6.27, 1-Ar-*C*) and 168.83-176.97 (CO_2H's and *C*ONH's); δ_F(282.2 MHz; ^2H_2O) 187.60 (2F, dd, *J* 19.8, 21.5, 3-Ar-C*F*), 189.47 (1F, t, *J* 21.5, 4-Ar-C*F*) and 196.21 (2F, d, *J* 19.8, 2-Ar-C*F*); δ_P(121.4 MHz; ^2H_2O): -8.57; *m/z* (ES^-): 969 (70%, $[M - H]^-$) and 484 (100, $[\{M - 2H\}/2]^-$)

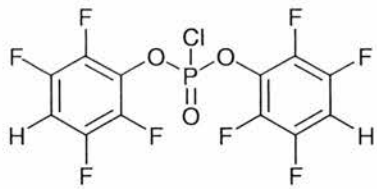
(*S*)-Aspartyl-(*S*)-alanyl-(*S*)-aspartyl-(*S*)-glutamyl-(*S*)-tyrosyl-(*S*)-leucine 102



N-α-Fmoc-Asp(^{t}Bu)-Ala-Asp(^{t}Bu)-Glu(^{t}Bu)-Tyr-Leu-Wang resin **99** (250 mg peptide-resin, 0.44 mmol g^{-1}, 0.1 mmol) was treated with 20% piperidine in DMF for 20 minutes, then filtered, washed with DCM, MeOH and DCM, and then dried. The resin so obtained was treated with cleavage mixture 1 to give a white solid on lyophilisation (70 mg, 97%), mp 150-154 °C, υ_{max}(nujol)/cm^{-1} 1660 (amide C=O), 1715 (amide NH) and 3305 (br., h-bonded CO_2H); δ_H(300 MHz; $^2H_20$) 0.89

(6H, dd, *J* 6.3, 15.3, 2 x Leu C*H*$_3$), 1.42 (3H, d, *J* 6.9, Ala C*H*$_3$), 1.51-1.67 (3H, m, Leu C*H*$_2$C*H*), 1.85-2.02 (2H, m, Glu β-C*H*$_2$), 2.19-2.36 (2H, m, Glu γ-C*H*$_2$), 2.79-2.97 (3H, m, Asp C*H*$_2$ and 1 of Tyr C*H*$_2$), 3.02-3.19 (3H, m, Asp C*H*$_2$ and 1 of Tyr C*H*$_2$), 4.19-4.67 (6H, m, α-C*H*'s), 6.84 (2H, d, *J* 8.4, Tyr Ar-*H*) and 7.16 (2H, d, *J* 8.4, Tyr Ar-*H*); δ_C(75.4 MHz; ^2H_2O): 16.32 (Ala *C*H$_3$), 20.58 & 22.29 [Leu CH(*C*H$_3$)$_2$], 24.37 [Leu *C*H(CH$_3$)$_2$], 26.12 (Glu β-*C*H$_2$), 29.72 (Glu γ-*C*H$_2$), 34.76 (Asp *C*H$_2$), 35.07 (Asp *C*H$_2$), 35.92 (Tyr *C*H$_2$), 39.49 (Leu *C*H$_2$), 49.52 (Leu α-*C*H), 50.26 & 50.32 (Asp α-*C*H$_2$), 51.41 (Ala α-*C*H$_2$), 53.36 (Glu α-*C*H), 54.94 (Tyr α-*C*H), 115.70 (2-Ar-*C*H), 128.40 (4-Ar-*C*), 130.82 (3-Ar-*C*H), 154.76 (1-Ar-*C*) and 168.83-177.21 (*C*O$_2$H's and *C*ONH's); *m/z* (ES$^-$): 723 (8%, [M – H]$^-$).

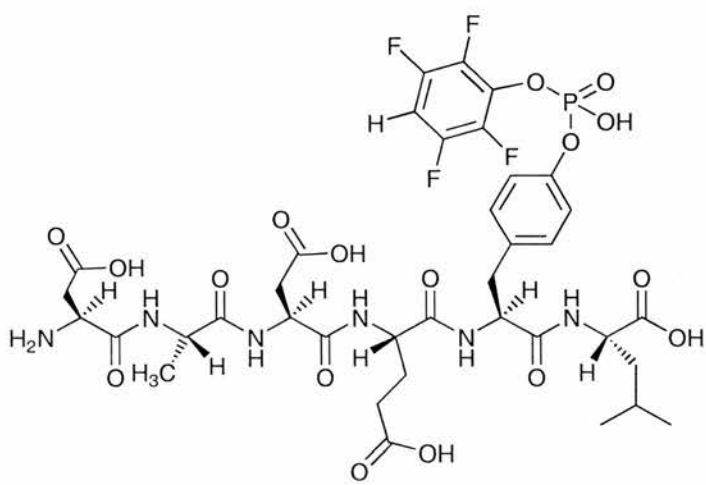
***Bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate 103**



Bis(2,3,5,6-tetrafluorophenyl) chlorophosphate **103** was prepared in a manner identical with that for *bis*(pentafluorophenyl) chlorophosphate **77**, using $POCl_3$ (2.9 cm^3, 31.0 mmol) and 2,3,5,6-tetrafluorophenol (9.11 g, 55.0 mmol) instead of pentafluorophenol. Purification by distillation under reduced pressure afforded the chlorophosphate **103** as the major product (7.08 g, 62.4%), bp 160 °C/ 0.7 mmHg (Found: M$^+$, 411.9297. $C_{12}H_2O_3F_8P_1{}^{37}Cl_1$ requires 411.9302); υ_{max}(thin film)/ cm^{-1} 1182 (Ar C-F), 1263 (P-O-C$_{ar}$ st) and 1325 (P=O st); δ_H(300 MHz; C^2HCl_3) 7.01–7.13 (2H, m, 2 x 3-Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 103.93 (t, $J_{C\text{-}F}$ 23.25, 4-Ar-*C*), and 138.45–148.12 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3)

198.10–198.15 and 214.12–214.19 (8F, m, Ar-C*F*); δ_P(121.41 MHz; C^2HCl_3) −16.72; *m/z* (EI^+) 412, 414 (42%, 14, M^+ [Cl isotopes]), 394 (11, $[M - Cl + OH]^+$), 245, 247, (23, 8, $[M - C_6F_4HO]^+$ {Cl isotopes}), 212 (25, $[M - C_6F_4HO - Cl]^+$) and 166 (100, $C_6F_4HOH^+$), purity 92.1 % by ^{31}P NMR spectroscopy.

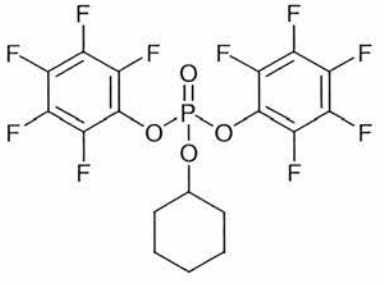
(*S*)-Aspartyl-(*S*)-alanyl-(*S*)-aspartyl-(*S*)-glutamyl-(*S*)-2′,3′,5′,6′-tetrafluorophenylhydroxyphosphoryl tyrosyl-(*S*)-leucine 105



This was prepared in a manner identical to (*S*)-aspartyl-(*S*)-alanyl-(*S*)-aspartyl-(*S*)-glutamyl-(*S*)-pentafluorophenylhydroxyphosphoryl tyrosyl-(*S*)-leucine **101** using *N*-α-Fmoc-Asp(^{t}Bu)-Ala-Asp(^{t}Bu)-Glu(^{t}Bu)-Tyr-Leu-Wang resin **99** (350 mg, 0.2 mmol), DMAP (37 mg, 0.3 mmol), Et_3N (334 mm^3, 3.3 mmol), and *bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate **103** (1.15 g, 2.8 mmol) to give the product **105** as a very hygroscopic white solid (105 mg, 96.1%), mp 130-134 °C; υ_{max}(nujol)/cm^{-1} 1076 (Ar-CF st), 1273 (P=O st), 1713 (NH) and 3358 (H-bonded OH); δ_H(300 MHz, ^2H_2O) 0.87 (6H, dd, *J* 3.5, 14.0, 2 x Leu CH_3), 1.40 (3H, d, *J* 7.2, Ala CH_3), 1.51-2.06 (2H, m, Glu β-CH_2), 2.24-2.32 (2H, m, Glu γ-CH_2), 2.84-3.22 (6H, m, 2 x Asp CH_2 & Tyr CH_2), 4.28-4.63 (6H, m, α-C*H*'s) and 7.08-7.25 (5H, m, Tyr Ar-*H* & POC_6F_4H); δ_C(75.4 MHz; ^2H_2O) 16.20 (Ala *C*H_3), 20.55 & 22.21 [Leu

$CH(CH_3)_2$], 24.37 [Leu $CH(CH_3)_2$], 26.11 (Glu β-CH_2), 29.78 (Glu γ-CH_2), 34.64 & 34.96 (Asp CH_2), 36.13 (Tyr CH_2), 39.58 (Leu CH_2), 49.52 (Leu α-CH), 50.46 (Asp α-CH_2), 51.37 (Ala α-CH_2), 53.38 (Glu α-CH), 54.82 (Tyr α-CH), 101.87 (t, *J* 23.22, 4-Ar-*C* PhF_4), 120.52 (2-Ar-CH Tyr), 130.62 (3-Ar-CH Tyr), 132.06 (4-Ar-*C* Tyr), 139.42 (d, *J* 14.61, Ar-CF), 142.70 (d, *J* 18.66, Ar-CF), 144.78 (d, *J* 12.77, Ar-CF), 148.01 (t, *J* 13.26, Ar-CF), 150.88 (d, *J* 7.58, 1-Ar-*C* Tyr) and 168.80-177.05 (CO_2H's and CONH's); δ_F(282.3 MHz; ^2H_2O) 195.781 (2F, 2 x d, *J* 9.03, 12.14, 3-Ar-C*F*) and 210.83 (2F, m, 2-Ar-C*F*); δ_P(121.4 MHz; ^2H_2O) -9.54; *m/z* (ES^-) 951 (19%, $[M - H]^-$), 475 (97, $[\{M - 2H\}/2]^-$) and 231 (22, $[\{M - 4H\}/4]^-$)

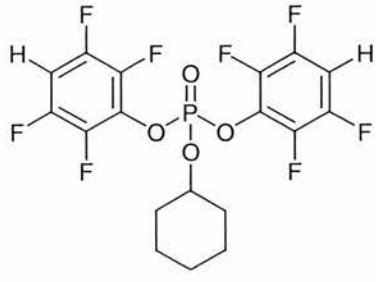
***Bis*(pentafluorophenyl) cyclohexyl phosphate 107**[246]



To a solution of cyclohexanol (104 mm^3, 1.0 mmol) and triethylamine (167 mm^3, 1.2 mmol) in DCM (10 cm^3) was added a solution of *bis*(pentafluorophenyl) chlorophosphate **77** (0.63 g, 1.4 mmol) in DCM (10 cm^3) and the solution allowed to stir for 15 minutes. The hydrochloride salts were filtered off and the filtrate concentrated to give the crude product as a yellow oil. Purification by flash column chromatography (ethyl acetate-hexane, 1:9) afforded the triester **107** as a colourless oil (435 mg, 98%) which later solidified, mp 28–32 °C (Found: M^+, 512.0223. $C_{18}H_{11}O_4F_{10}P_1$ requires 512.0235); υ_{max}(thin film)/ cm^{-1} 1025 (P-O-C_{al} st), 1101 (Ar C-F st), 1257 (P-O-C_{ar} st), 1268 (P=O st) and 1506 & 1520 (CH_2 asymm. st); δ_H(300

MHz; C^2HCl_3) 1.26–2.03 (10H, m, cyclohexyl C*H*$_2$'s) and 4.83 (1H, m, C*H*OP); δ_C(75.4 MHz; C^2HCl_3) 23.08 (γ–*C*H$_2$), 24.73 (δ–*C*H$_2$), 32.79 (d, J_{PC} 4.33, β–*C*H$_2$), 82.76 (d, J_{PC} 7.57, *C*H$_2$OP) and 133.62 – 143.62 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 189.7–189.6, 193.2–193.4 and 197.9–198.1 (10F, m, Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) −11.21; *m/z* (EI$^+$) 512 (4%, M$^+$), 431 (100, [M + 2H − C_6H_{11}]$^+$), 184 (39, $C_6F_5OH^+$) and 83 (38, $C_6H_{11}^+$).

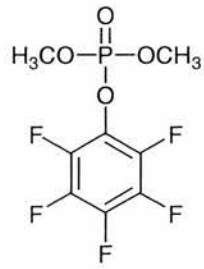
Bis(2,3,5,6-tetrafluorophenyl) cyclohexyl phosphate 108



This compound was prepared in a manner identical with that for *bis*(pentafluorophenyl) cyclohexyl phosphate **107**, using a solution of *bis*(2,3,5,6-tetrafluorophenyl) chlorophosphate **103** (825 mg, 2.0 mmol) in DCM (10 cm^3), and a solution of cyclohexanol (104 mm^3, 1.0 mmol) and Et_3N (167 mm^3, 1.2 mmol) in DCM (10 cm^3). The resultant oil was purified by flash column chromatography (ethyl acetate : hexane, 1:4) to afford the product **108** as a colourless oil (405 mg, 85 %), (Found: M$^+$, 476.0429. $C_{18}H_{13}O_4F_8P_1$ requires 476.0424); υ_{max}(thin film)/ cm^{-1} 1029 (P-O-C_{al} st), 1075 (Ar C-F st), 1278 (P-O-C_{ar} st), 1305 (P=O st), 1495 & 1525 (CH_2 asymm. st) and 2865 & 2945 (CH_2 st); δ_H(300 MHz; C^2HCl_3) 1.35–2.04 (10H, m, cyclohexyl C*H*$_2$'s), 4.82 (1H, m, C*H*OP) and 6.97–7.03 (2H, m, 2 x Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 23.04 (γ-*C*H$_2$), 24.74 (δ-*C*H$_2$), 32.75 (d, *J* 3.85, β-*C*H$_2$), 82.53 (d, *J* 7.62, α-*C*H) , 102.94 (t, *J* 23.22, Ar-*C*H), 140.67 (dd, *J* 15.68, 253.34,

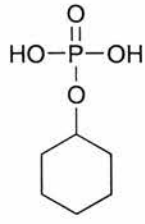
Ar-*C*-O-P), 144.61, & 147.86 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 197.80 (2F, m, *o*-Ar-C*F*) and 213.20 (2F, m, *m*-Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) −12.19; *m/z* (EI^+) 476 (5%, M^+), 395 (100, $[M + 2H - C_6H_{11}]^+$), 374 (25, $[M - F - C_6H_{11}]^+$), 228 (13, $[M - C_6H_{11} - C_6HF_4O]^+$, 166 (65, $C_6F_4HOH^+$) and 83(41, $C_6H_{11}^+$).

Dimethyl pentafluorophenyl phosphate 110



To a stirred solution of *bis*(pentafluorophenyl) chlorophosphate **77** (450 mg, 1.0 mmol) in DCM (5 cm^3) was added a solution of dry methanol (35 mm^3, 0.8 mmol), Et_3N (140 mm^3, 1.0 mmol) and DMAP (12 mg, 0.1 mmol) in DCM (5 cm^3) *via* cannula and the mixture stirred for 1 hour. The solvent was removed under reduced pressure and the resultant colourless oil was purified by column chromatography (ethyl acetate: hexane, 1:1) to afford the product **110** as a colourless oil (90 mg, 77.1 %), (Found: M^+, 291.9932. $C_8H_6O_4F_5P_1$ requires 291.9924.), υ_{max}(thin film)/ cm^{-1} 1024 (P-O-C_{al} st), 1294 (P=O st), 1482 (C-H bend), 1252 (CH_3 asymm. st), 2864 (C-H st) and 2968 (O-CH_3 st); δ_H(300 MHz; C^2HCl_3) 3.97 (6H, d, *J* 12.0,2 × POC*H*$_3$); δ_C(75.4 MHz; C^2HCl_3) 55.62 (d, *J* 5.43, PO*C*H$_3$) and 136.42 – 143.11 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 189.25 (2F, *m*-Ar-C*F*), 192.06 (1F, *p*-Ar-C*F*) and 196.63 (2F, *o*-Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) −3.49; *m/z* (EI^+) 292 (12%, M^+), 183 (12, $C_6F_5O^+$), 109 (100, $[M–C_6F_5O]^+$) and 79 (50, $[M + H - C_6F_5O - CH_3O]^+$).

Cyclohexyl dihydrogen phosphate 111



Method 1:

A suspension of platinum (IV) dioxide (PtO_2) (28 mg, 0.2 mmol) in glacial acetic acid (10 cm^3) was stirred under a hydrogen atmosphere for 20 min. (or until black). Gaseous HCl was bubbled through a stirred solution of *bis*(pentafluorophenyl) cyclohexyl phosphate **107** (50 mg, 0.1 mmol) in glacial acetic acid (10 cm^3) for 30 min., and then added to the suspension of PtO_2 *via* cannula. The reaction was then stirred under a hydrogen atmosphere overnight. The catalyst was removed by filtration through a celite pad and the volatiles removed under reduced pressure to afford the crude dihydrogen phosphate **111** as a colourless oil, (18 mg, quantitative), υ_{max}(thin film)/ cm^{-1} 1263 (P=O st); δ_H(300 MHz; $C^2H_3O^2H$) 1.33-1.96 (10H, m, cyclohexyl CH_2's) and 4.23 (1H, m, α–C*H*); δ_C(75.4 MHz; $C^2H_3O^2H$) 24.50 (γ–CH_2), 26.27 (δ–CH_2), 34.39 (β–CH_2) and 77.28 (d, *J* 5.46, α–CH); δ_P(121.4 MHz; $C^2H_3O^2H$) -0.21; *m/z* (EI^+) 149 (27 %, $[M + H - 2OH]^+$), 84 (82, $C_6H_{12}^+$) and 66 (100, $[P(OH)_2 + H]^+$).

Method 2:

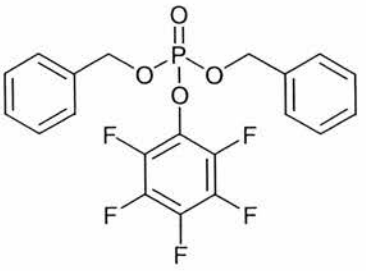
To a stirred solution of benzyl pentafluorophenyl cyclohexyl phosphate **127** (60 mg, 0.14 mmol) in THF (5 cm^3) was added 5 cm^3 of a mixture of 1.0 mol dm^{-3} NaOH:MeOH (4:1) and the solution allowed to stir overnight. The volatiles were removed under reduced pressure and the resulting solid was dissolved in 90% TFA/

H_2O (10 cm^3) and allowed to stir for 4 hours. The volatiles were removed under reduced pressure and the resultant oil redissolved in DCM (10 cm^3), washed with water (10 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure to afford the dihydrogenphosphate **111** as a white solid (20 mg, 79.4%). This method is cleaner than method 1, as no filtration to remove catalyst is required.

Dibenzyl chlorophosphate 122

To a cooled suspension of *N*-chlorosuccinimide (4.00 g, 29.5 mmol) in toluene (20 cm^3) was added dibenzyl phosphite **123** (5.0 cm^3, 29.5 mmol). The *N*-chlorosuccinimide gradually dissolved to form a clear solution, which soon began to deposit succinimide. After 2 hours the succinimide was filtered off and the solution of the chlorophosphate **122** used directly for phosphorylation without further purification. δ_H(300 MHz; C^2HCl_3) 5.22 (4H, dd, J_{PCH} 9.0, 6.6, CH_2OP) and 7.26–7.38 (10H, m, Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 70.93 (d, J_{PC} 6.56, CH_2OP), 128.27–129.09 (Ar-*C*H) and 134.25 (d, *J* 7.62, Ar-*C* quaternary); δ_P(121.4 MHz; C^2HCl_3) –5.23.

Dibenzyl pentafluorophenyl phosphate 121



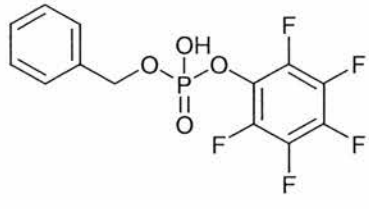
Method 1:

To a cooled suspension of NaH (60% suspension in mineral oil) (1.18 g, 29.5 mmol) in THF (20 cm^3) was added pentafluorophenol (5.43 g, 29.5 mmol) in THF (10 cm^3) over 30 minutes *via* cannula (CARE: evolution of H_2), to afford a clear solution. This solution was then added to a solution of freshly prepared dibenzyl chlorophosphate **122** (29.5 mmol) in toluene (20 cm^3) and allowed to stir under an argon atmosphere for 1 hour. The THF was removed under reduced pressure and the toluene layer washed with sat. $NaHCO_3$ (30 cm^3) and dried ($MgSO_4$). The organic layer was concentrated at reduced pressure to afford a yellow oil. Recrystallisation of the crude product from ethyl acetate/ hexane afforded the triester **121** as a white solid (10.22 g, 78.0%), mp 60–62 °C (Found: C, 53.95; H, 3.05; $C_{20}H_{14}F_5O_4P_1$ requires: C, 54.0; H, 3.2%), υ_{max}(Nujol)/ cm^{-1} 1022 (P-O-C_{al} st) and 1293 (P=O); δ_H(300 MHz; C^2HCl_3) 5.21 (4H, d, J_{PH} 8.7, CH_2OP) and 7.25–7.40 (10H, m, Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 70.88 (d, J_{PC} 6.5, CH_2OP), 128.24–129.04 (Ar-*C*H), 134.78 (Ar-*C* quaternary) and 136.00–139.50 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 189.3–189.4, 191.9–192.1 and 197.5–197.6 (5F, m, Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) –5.44; *m/z* (EI^+) 353 (95%, $[M - CH_2Ph]^+$), 107 (27, $PhCH_2O^+$) and 91 (100, $PhCH_2^+$).

Method 2:

To a solution of pentafluorophenol (2.87 g, 15.6 mmol) in anhydrous acetonitrile (100 cm^3), cooled to −10 °C, was added CCl_4 (7.5 cm^3, 78.04 mmol) with stirring. DMAP (191 mg, 1.56 mmol) and Et_3N (4.6 cm^3, 32.77 mmol) were added, and then after 1 minute, dibenzyl phosphite (5.0 cm^3, 22.6 mmol) was added dropwise, keeping the temperature below −10 °C by extra cooling or by slowing the rate of addition. After 1 hour, 0.5 mol dm^{-3} aqueous KH_2PO_4 (15 cm^3) was added and the reaction allowed to warm to room temperature. The mixture was extracted with ethyl acetate (3 x 50 cm^3) and the organic phase washed successively with water (50 cm^3) and saturated brine (50 cm^3), dried ($MgSO_4$), and then concentrated under reduced pressure. Recrystallisation from toluene-light petrol afforded the phosphate triester **121** as a white solid (3.54 g, 85%), which showed identical spectral characteristics to a sample prepared by Method 1 above.

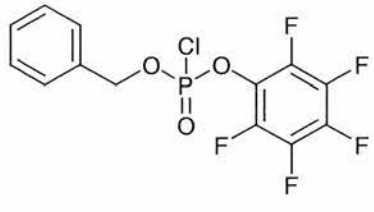
Benzyl pentafluorophenyl phosphoric acid 120



To a stirred solution of dibenzyl pentafluorophenyl phosphate **121** (5.0 g, 11.26 mmol) in dry acetone (40 cm^3) was added anhydrous sodium iodide (1.69 g, 11.26 mmol) and the solution refluxed for 15 minutes. After allowing to cool, the resultant suspension was placed in the fridge for several hours. The solid was isolated by filtration, washed with acetone and then dissolved in water. Hydrochloric acid (1 mol dm^{-3}) was added to the aqueous solution until precipitation was complete. After filtration and drying under vacuum at 40 °C, the product **120**

was obtained as a white solid in quantitative yield, mp 112 °C (Found: C, 44.0; H, 2.15. $C_{18}H_8F_5O_4P_1$ requires: C, 44.1; H, 2.3%); υ_{max}(Nujol)/ cm^{-1} 1273 (P=O) and 2731 (H-bonded OH); δ_H(300 MHz; C^2HCl_3) 5.20 (2H, d, J_{PH} 8.9, CH_2OP) and 7.25–7.40 (5H, m, Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 70.92 (d, J_{PC} 5.4, CH_2OP), 128.11–129.03 (Ar-*C*H), 134.67 (Ar-*C* quaternary) and 134.71–139.54 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 189.2–189.4, 192.3–192.4 and 197.6–197.7 (5F, m, Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) –4.66; *m/z* (EI^+) 354 (19%, M^+), 353(80, $[M - H]^+$) and 91 (100, $PhCH_2^+$).

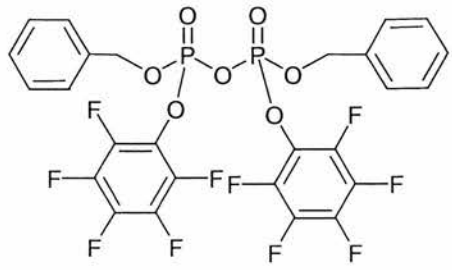
Benzyl pentafluorophenyl chlorophosphate 118



To a suspension of the phosphoric acid diester **116** (820 mg, 2.32 mmol) in dry CH_2Cl_2 (10 cm^3) was added PCl_5 (966 mg, 4.64 mmol). The suspension was allowed to stir until all the reactants had dissolved, and evolution of HCl had ceased. The solvent was removed by distillation under a nitrogen atmosphere at 10-20 mm Hg. Excess PCl_5 and the phosphoryl chloride byproduct were removed under reduced pressure (0.5 mm Hg) to afford the crude chlorophosphate **118** as an oil, in 70 – 85% purity, (Found: M^+, 371.9746. $C_{13}H_7{}^{35}Cl_1F_5O_3P_1$ requires 371.9742); υ_{max}(thin film)/ cm^{-1} 998 (P-O-C st), 1157 (Ar C-F), 1312 (P=O st) and 1522 (Ar C-C); δ_H(300 MHz; C^2HCl_3) 5.38 (2H, d, J_{PH} 8.9, CH_2OP) and 7.30–7.50 (5H, m, Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 72.91 (d, J_{PC} 7.54, CH_2OP), 128.23–129.74 (Ar-*C*H),

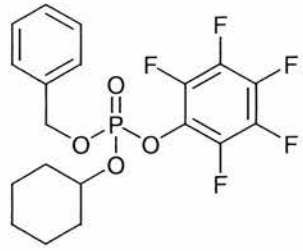
133.47 (Ar-*C* quaternary) and 136.36–142.80 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 190.2–190.4, 194.2–194.3 and 199.3–199.4 (5F, m, Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) 2.39; *m/z* (EI^+) 374, 372 (10%, 30, M^+ [Cl isotopes], 184 (30, $C_6F_5OH^+$) and 107 (100, OCH_2Ph^+).

***O,O'-bis*(Pentafluorophenyl)-*O,O'*-dibenzylpyrophosphate 126**



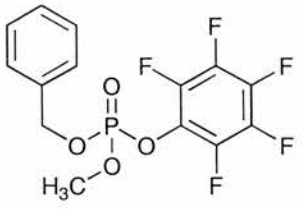
To a stirred solution of benzyl pentafluorophenyl phosphoric acid **120** (354 mg, 1.0 mmol) in dry ether (50 cm^3) was added a solution of dicyclohexylcarbodiimide **81** (104 mg, 0.5 mmol) in dry ether (2 cm^3) with stirring. The solution was allowed to stir for 30 minutes during which time the dicyclohexylurea precipitated out. The precipitate was removed by filtration and the filtrate concentrated at reduced pressure to give the crude pyrophosphate **126** as a colourless oil (700 mg yield) which could not be purified further, υ_{max}(thin film)/ cm^{-1} 909 (P-O-P st), 1025 (P-O-C_{al} st), 1262 (P-O-C_{ar} st) and 1287 (P=O st); δ_H(300 MHz; C^2HCl_3) 5.32 (4H, m, CH_2Ph) and 7.35 (10H, m, Ar-C*H*); δ_C(75.4 MHz; C^2HCl_3) 72.12 (*C*H_2Ph), 127.54–129.31, 133.60 (Ar-*C*H) and 136.15, 139.36 & 142.73 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 189.68 (2F, m, Ar-C*F*), 193.39 (1F, m, Ar-C*F*) and 198.04 (2F, m, Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) −17.54, −18.28, −18.66, & −19.86 (P-O-P); *m/z* (CI^+) 708 (4%, $[M + NH_4]^+$), 669 (7, $[M - 2F + H + NH_4]^+$) and 372 (24, $[(PhCH_2O)(C_6F_5O)PO + NH_4]^+$).

Cyclohexyl benzyl pentafluorophenyl phosphate 127



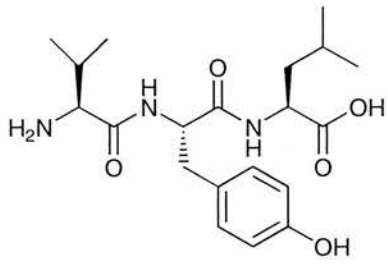
To a solution of cyclohexanol (47 mm^3, 0.44 mmol), DMAP (5.0 mg, 0.04 mmol) and Et_3N (140 mm^3, 1.0 mmol) in dry CH_2Cl_2 (10 cm^3), was added a solution of freshly prepared benzyl pentafluorophenyl chlorophosphate **118** (830 mg, 2.23 mmol) in dry CH_2Cl_2 (10 cm^3) *via* cannula. The solution was allowed to stir overnight, filtered, and volatiles removed under reduced pressure to afford a yellow oil. Purification by column chromatography eluting with dichloromethane: methanol (9:1) afforded the desired product **127** as a white amorphous solid (140 mg, 72.3%), $\upsilon_{max}(CH_2Cl_2)$/ cm^{-1} 1022 (P-O-C_{al}), 1265 (P=O st), and 1521 (CH_2); δ_H(300 MHz; C^2HCl_3) 1.26–1.94 (10H, m, CH_2's), 4.57 (1H, m, *CH*OP), 5.22 (2H, dd, *J* 2.8, 8.2, PhCH_2OP) and 7.27–7.42 (Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 23.20 (γ–CH_2), 24.85 (δ–CH_2), 32.96 (m, β–CH_2), 70.54 (d, *J* 5.41, CH_2OP), 80.06 (d, *J* 7.58, *C*HOP), 126.32–129.39 (Ar-*C*H) and 135.22–143.15 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 189.05 (2F, dd, *J* 18.35, 21.45, *m*-Ar-C*F*), 191.58 (1F, t, *J* 18.35, *p*-Ar-C*F*) and 197.66 (2F, d, *J* 21.45, *o*-Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) −6.26; *m/z* (EI^+) 353 (7%, $[M - C_6H_{11}]^+$), 265 (18, $[C_6F_5OPO_3H_2 + H]^+$), 184 (82, $C_6F_5OH^+$), 91 (100, $PhCH_2^+$) and 82 (43, $C_6H_{10}^+$).

Methyl benzyl pentafluorophenyl phosphate 130



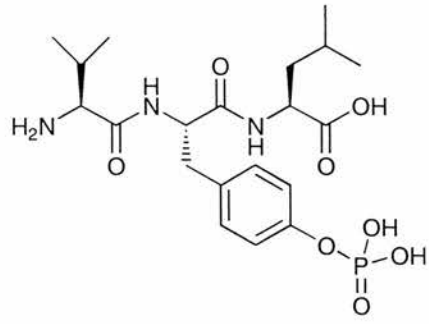
To a stirred solution of methanol (25 mm^3, 0.57 mmol), Et_3N (105 mm^3, 0.63 mmol) and DMAP (12 mg, 0.1 mmol) in DCM (5 cm^3) was added a solution of freshly prepared benzyl pentafluorophenyl chlorophosphate **118** (373 mg, 1.0 mmol) in DCM (5 cm^3) *via* cannula and the mixture allowed to stir overnight. The solvent was removed under reduced pressure and the resultant oil was redissolved in ethyl acetate (5 cm^3). The triethylammonium salts were removed by filtration and the solvent removed under reduced pressure. The resultant oil was purified by flash chromatography (ethyl acetate: hexane, 3:17) to give the required phosphate triester **130** as a colourless oil (158 mg, 75.6%), (Found: M^+, 368.0232. $C_{14}H_{10}O_4F_5P_1$ requires 368.0237), υ_{max}(thin film)/ cm^{-1} 1023 (P-O-C_{al} st) and 1293 (P=O st); δ_H(300 MHz; C^2HCl_3) 3.94 (3H, d, *J* 12.0, POC*H*$_3$), 5.26 (2H, dd, *J* 2.4, 8.4, POC*H*$_2$Ph) and 7.40 (5H, s, Ar-*H*); δ_C(74.76 MHz; C^2HCl_3) 55.55 (d, *J* 5.43, PO*C*H$_3$), 70.90 (d, *J* 5.43, PO*C*H$_2$Ph), 128.21, 128.61 & 129.07 (Ar-*C*H), 134.82 (d, *J* 6.57, 1-Ar-*C*H) and 136.30 – 139.61 (Ar-*C*F); δ_F(282.3 MHz; C^2HCl_3) 189.29 (2F, dd, *J* 18.35, 21.45, 3-Ar-C*F*), 192.00 (1F, t, *J* 18.35, 4-Ar-C*F*) and 197.15 (2F, d , *J* 21.17, 2-Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) -4.40; *m/z* (EI^+) 368 (9%, M^+), 107 (8, $PhCH_2O^+$) and 91 (100, $PhCH_2^+$).

(*S*)-Valyl-(*S*)-tyrosyl-(*S*)-leucine 137



N-α-Fmoc-Val-Tyr-Leu-Wang resin **134** (230 mg, 0.1 mmol, 0.44 mmol g^{-1}) was treated with 20% piperidine/ DMF (10 cm^3) then washed with CH_2Cl_2 and MeOH and dried. The dried peptide resin was treated with cleavage mixture 1) to give a white solid on lyophilisation (35 mg, 90%), mp >210 °C (decomp.), υ_{max}(Nujol)/ cm^{-1} 842 (aromatics), 1514 (aromatics), 1548 (CO_2^-), 1661 (NH_2), 1715 (amide NH) and 3294 (NH stretch); δ_H(300 MHz; ^2H_2O) 0.81-0.89 [6H, dd, *J* 6.3, 17.1, Leu CH(CH_3)$_2$], 0.98-1.01 [6H, dd, *J* 1.8, 6.9, Val CH(CH_3)$_2$], 1.43-1.66 (3H, m, Leu CH_2CH), 2.14-2.25 [1H, m, Val $CH(CH_3)_2$], 2.98-3.02 (2H, dd, *J* 5.4, 7.5, Tyr β-CH_2), 3.79 (1H, d, *J* 5.7, Val α-*CH*), 4.28-4.33 (1H, dd, *J* 5.55, 9.3, Tyr α-*CH*), 4.66 (1H, t, *J* 7.8, Leu α-*CH*), 6.84 (2H, d, *J* 8.55, Tyr Ar-*H*) and 7.16 (2H, d, *J* 8.55, Tyr Ar-*H*); δ_C(75.4 MHz; ^2H_2O) 16.67 & 17.35 [Val CH(CH_3)$_2$], 20.36 & 22.07 [Leu CH(CH_3)$_2$], 24.03 [Leu $CH(CH_3)_2$], 29.97 (Val β-*CH*), 36.03 (Tyr β-CH_2), 39.27 (Leu β-CH_2), 51.17 (Leu α-*CH*), 55.03 (Tyr α-*CH*), 58.22 (Val α-*CH*), 115.52 (3-Ar-*CH*), 127.87 (1-Ar-*CH*), 130.60 (2-Ar-*CH*), 154.60 (4-Ar-*CH*), 168.83 (CO_2H) and 172.24 & 175.74 (*C*ONH); *m/z* (ES^+) 394 (20%, $[M + H]^+$), 349 (30, $[M + H - CO_2H]^+$) and 196 (55, $[M/2]^+$).

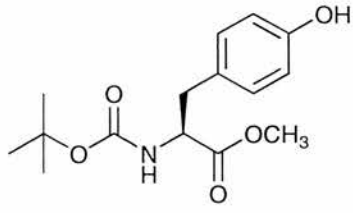
(*S*)-Valyl-(*S*)-dihydroxyphosphotyrosyl-(*S*)-leucine 136



N-α-Fmoc-VYL-Wang resin **134** (250 mg peptide-resin, 0.44 mmol g^{-1}, 0.1 mmol), DMAP (11 mg, 0.1 mmol) and Et_3N (200 mm^3, 1.44 mmol) in CH_2Cl_2 (10 cm^3) was treated with a solution of benzyl pentafluorophenyl chlorophosphate **118** (560 mg, 1.5 mmol) in CH_2Cl_2 (10 cm^3) and the mixture allowed to stir overnight. The resin was washed with CH_2Cl_2 and MeOH and then dried. The dried resin was treated with 2 mol dm-^{3} NaOH (0.5 cm^3, 1.0 mmol) in THF/ MeOH (4:1; 4 cm^3) for 4 hours, washed with CH_2Cl_2 and MeOH and then dried. The peptide resin so obtained was treated with cleavage mixture 2 to give the product **138** as an off white solid (30 mg, 65 %), mp 190 °C (decomp.), (Found: $[M + H]^+$, 474.2010. $C_{20}H_{33}N_3O_8P_1$ requires 474.2005), υ_{max}(Nujol)/ cm^{-1} 1272 (P=O st), 1714 (C=O st), and 3384 (H-bonded OH); δ_H(300 MHz; $C^2H_3O^2H$) 0.93-0.99 [6H, dd, *J* 6.0, 13.8, Leu CH(CH_3)$_2$], 1.02-1.09 [6H, dd, *J* 6.9, 13.5, Val CH(CH_3)$_2$], 1.64-1.84 (3H, m, Leu CH_2CH), 2.24 (1H, m, Val C*H*), 3.18 (2H, m, Tyr β-CH_2), 3.68 (1H, m, Val α-CH), 4.47 (1H, t, *J* 7.42, Leu α-CH), 4.87 (1H, m, Tyr α-CH) and 7.16-7.35 (4H, m, Tyr Ar-*H*); δ_C(75.4 MHz; $C^2H_3O^2H$) 17.57 & 18.84 [Val CH(CH_3)$_2$], 21.70 & 23.38 [Leu CH(CH_3)$_2$], 25.82 [Leu CH(CH_3)$_2$], 31.60 [Val CH(CH_3)$_2$], 38.04 (Tyr β-CH_2), 41.62 (Leu β-CH_2), 52.02 (Leu α-CH), 55.98 (Tyr α-CH), 59.38 (Val α-CH), 121.41 (3-Ar-CH), 131.39 (1-Ar-C), 131.72 (2-Ar-CH) and 151.93 (4-Ar-C);

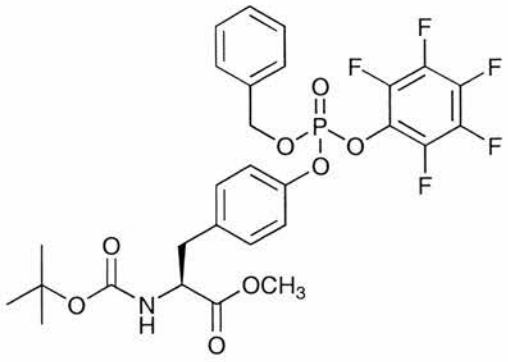
δ_P(121.4 MHz; $C^2H_3O^2H$) –4.60; *m/z* (FAB^+) 496 (67%, $[M + Na]^+$) and 474 (100, $[M + H]^+$).

Methyl *N*-^{t}Boc-(2*S*)-tyrosinate 140



To a cooled solution of *N*-^{t}Boc-(2*S*)-tyrosine **139** (400 mg, 1.42 mmol) in ethyl acetate (10 cm^3), was added excess ethereal diazomethane, until a persistent yellow colour was obtained. The solution was allowed to stir at 0 °C for 30 mins., then nitrogen gas was bubbled through the solution to remove unreacted diazomethane and the ethyl acetate was removed under reduced pressure to leave a yellow oil. Recrystallisation from ethyl acetate – light petrol afforded the methyl ester **140** as white crystals (366 mg, 87.1 %), mp 102-104 °C (lit.[235] 102-104 °C), υ_{max}(Nujol)/ cm^{-1} 1520 (amide CO), 1597 (aromatic CH), 1617 (aromatic CH), 1690 (urethane CO), 1714 (ester CO) and 3386 (phenol OH); δ_H(300 MHz; C^2HCl_3) 1.43 (9H, s, $C(CH_3)_3$), 2.98 (2H, m, β-CH_2), 3.72 (3H, s, OCH_3), 4.54 (1H, s, O*H*), 5.04 (1H, s, α-C*H*), 6.14 (1H, s, N*H*), 6.73 (2H, d, *J* 7.97, Ar-*H ortho*) and 6.97 (2H, d, *J* 8.24, Ar-*H meta*); δ_C(75.4 MHz; C^2HCl_3) 28.17 [$C(CH_3)_3$], 37.48 (β–CH_2), 52.20 (OCH_3), 54.57 (α-*C*H), 80.23 [$C(CH_3)_3$], 115.56 (Ar-*C*H *meta*), 127.51 (Ar-*C* quaternary *ortho*), 130.41 (Ar-*C*H *para*), 155.26 (Ar-*C* quaternary *ipso*), 155.44 [$(CH_3)_3CO_2C$] and 172.79 (CO_2CH_3); *m/z* (EI^+) 295 (5%, M^+), 239 (23, $[M + H - {}^tBu]^+$), 222 (46, $[M - O^tBu]^+$), 179 (58, $[M - CH_3 - {}^tBuO_2C]^+$), 136 (59, $[M + H - CO_2CH_3 - {}^tBuO_2C]^+$) and 57 (100, $^tBu^+$).

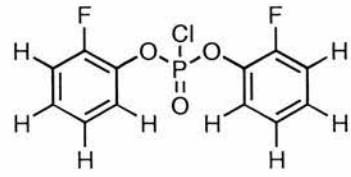
N-^{t}Boc-O'-benzyl-O'-pentafluorophenyl-(2*S*)-tyrosine-O-phosphate methyl ester 141



To a solution of methyl N-^{t}Boc-(2*S*)-tyrosinate **140** (106 mg, 0.36 mmol), DMAP (12 mg, 0.1 mmol) and Et_3N (100 mm^3, 0.72 mmol) in dry CH_2Cl_2 (10 cm^3) was added a solution of benzyl pentafluorophenyl chlorophosphate **118** (670 mg, 1.8 mmol) in dry CH_2Cl_2 (10 cm^3) *via* cannula. The solution was allowed to stir for 4 hours, filtered, and the volatiles removed under reduced pressure to leave a yellow oil. The crude product was purified by column chromatography eluting with dichloromethane: methanol (98:2) followed by ethyl acetate: hexane (1:4) to afford the desired phosphate triester **141** as a colourless oil (100 mg, 45 %), δ_H(300 MHz; C^2HCl_3) 1.42 [9H, s, C(CH_3)$_3$], 3.06 (2H, m, β-CH_2), 3.70 (3H, s, OCH_3), 4.56 (1H, m, α-C*H*), 4.97 (1H, m, N*H*), 5.32 (2H, dd, *J* 1.20, 8.70, PhCH_2), 7.10 (2H, d, *J* 8.55, Tyr Ar-*H*), 7.15 (2H, d, *J* 8.55, Tyr Ar-*H*) and 7.38 (5H, m, benzyl Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 28.17 [C(CH_3)$_3$], 37.57 (β–CH_2), 52.21 (OCH_3), 54.28 (α-*C*H), 71.59 (d, *J* 7.00, PhCH_2), 80.07 [*C*(CH_3)$_3$], 120.00 (d, *J* 4.49, Tyr 4-Ar-*C*H), 124.22, 128.32, 128.81, 129.19, 129.76, 130.78, & 133.92 (Ar-*C*H), 155.13 [(CH_3)$_3CO_2C$] and 172.18 (CO_2CH_3); δ_F(282.3 MHz; C^2HCl_3) 189.54 (2F, 2 x d, *J* 20.04, 21.17, *meta* Ar-C*F*), 192.49 (1F, 2 x d, *J* 21.17, 21.45, *para* Ar-C*F*) and

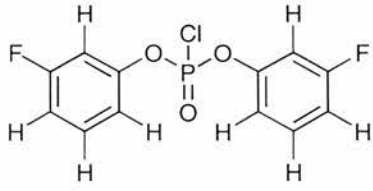
198.04 (2F, d, *J* 19.76, *ortho* Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) -10.91; *m/z* (EI) 482 {11%, $[M - H - C(CH_3)_3]^+$} and 441 {20, $[M + 2H - PhCH_2 - CO_2C(CH_3)_3]^+$}

Bis(2-fluorophenyl) chlorophosphate 156



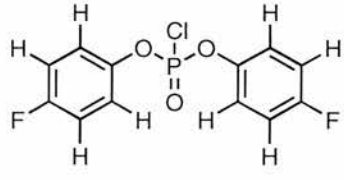
Bis(2-fluorophenyl) chlorophosphate **156** was prepared in a manner similar to that for *bis*(pentafluorophenyl) chlorophosphate **77**, using $POCl_3$ (9.4 cm^3, 0.1 mol) and 2-fluorophenol (22.42g, 0.2 mol) to afford 2-fluorophenyl dichlorophosphate (6.57g, 30 %) and the title chlorophosphate **156** (5.99g, 20 %), bp 170 °C/ 0.5 mmHg; (Found: M^+, 303.9866. $C_{12}H_8O_3F_2P_1{}^{35}Cl_1$ requires 303.9868); υ_{max}(thin film)/ cm^{-1} 972 (P-O-C st), 1174 (C-F st) and 1314 (P=O st). δ_H(300 MHz; C^2HCl_3) 7.09–7.47 (8H, m, Ar-C*H*); δ_C(75.4 MHz; C^2HCl_3) 117.19 (d, *J* 8.4), 122.30 (d, *J* 3.24), 124.71 & 126.84 (d, *J* 7.5) (Ar-CH's), 138.12 (m, Ar-*C*F) and 153.46 (d, J_{CP} 256.35, quaternary Ar-C); δ_F(282.3 MHz; C^2HCl_3) 221.78 (m, Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) -16.67; *m/z* (CI^+) 306, 304 (33, 100%, M^+, [Cl isotopes]), 284 (57, $[M + O - HCl]^+$), 158 (25, $[M - Cl - OC_6H_4F]^+$), 111 (23, $OC_6H_4F^+$) and 95 (24, $C_6H_4F^+$), purity 92.6 % by ^{31}P NMR spectroscopy.

***Bis*(3-fluorophenyl) chlorophosphate 157**



Bis(3-fluorophenyl) chlorophosphate **157** was prepared in a manner similar to that for *bis*(pentafluorophenyl) chlorophosphate **77**, using $POCl_3$ (2.85 cm^3, 45 mmol) and 3-fluorophenol (10.21 g, 90 mmol) to afford 3-fluorophenyl dichlorophosphate and the chlorophosphate **157** (3.22g, 22.8%), bp 140 °C/ 0.5 mmHg (lit.,[276] 119.5 – 125 °C/ 0.4 mmHg) (Found: M^+, 303.9860. $C_{12}H_8O_3F_2P_1{}^{35}Cl_1$ requires 303.9868); υ_{max}(thin film)/ cm^{-1} 984 (P-O-C st), 1159 (C-F st) and 1300 (P=O st). δ_H(300 MHz; C^2HCl_3) 6.92-7.18 (6H, m, 2 x 4-, 5-, 6-Ar-C*H*) and 7.3-7.42 (2H, m, 2 x 2-Ar-C*H*); δ_C(75.4 MHz; C^2HCl_3) 108.27 (dd, *J* 5.35, 26.01), 113.10 (d, *J* 20.58), 115.78 (t, *J* 4.30) & 136.85 (Ar-*C*H), 150.73 (Ar-*C*F) and 163.07 (d, J_{CP} 249.58, quaternary Ar-C); δ_F(282.3 MHz; C^2HCl_3) 241.95 (m, Ar-C*F*) ; δ_P(121.4 MHz; C^2HCl_3) -18.22; *m/z* (CI^+) 307, 305 (22, 66%, $[M+H]^+$ {Cl isotopes}), 268 (16, $[M + 2H - 2F]^+$), 112 (19, $HOC_6H_4F^+$), 95 (15, $C_6H_4F^+$) and 83 (11, $[M + H - C_6H_4FO - C_6H_4F]^+$), purity 95.5 % by ^{31}P NMR spectroscopy.

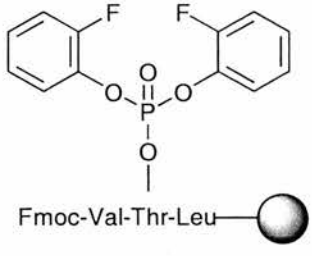
***Bis*(4-fluorophenyl) chlorophosphate 158**



Bis(4-fluorophenyl) chlorophosphate **158** was prepared in a manner similar to that for *bis*(pentafluorophenyl) chlorophosphate **77**, using $POCl_3$ (9.4 cm^3, 0.1 mol) and 4-fluorophenol (22.4 g, 0.2 mol) to afford 4-fluorophenyl

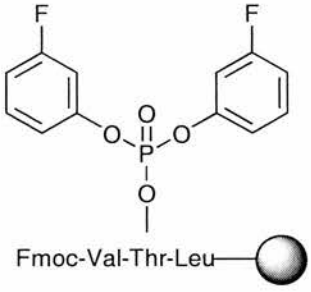
dichlorophosphate (10.05 g, 44 %) and the chlorophosphate **158** (6.94 g, 23%), bp 160-180 °C/ 0.5 mmHg (Found: M^+, 303.9859. $C_{12}H_8O_3F_2P_1{}^{35}Cl_1$ requires 303.9868), υ_{max}(thin film)/ cm^{-1} 960 (P-O-C st), 1130 (C-F st) and 1319 (P=O st); δ_H(300 MHz; C^2HCl_3) 7.07–7.27 (8H, m, Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 116.69 (d, *J* 23.83) & 121.65 (dd, *J* 5.66, 8.07) (Ar-*C*H's), 146.27 (dd, *J* 2.33, 7.54, Ar-*C*F) and 160.22 (d, J_{CP} 245.80, quaternary Ar-*C*); δ_F(282.3 MHz; C^2HCl_3) 234.67 (m, Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) -16.61; *m/z* (CI^+) 306, 304 (17, 52%, M^+ {Cl isotopes}), 268 (6, $[M + 2H - 2F]^+$), 111 (18, $OC_6H_4F^+$), 95 (7, $C_6H_4F^+$) and 83 (17, $[M - C_6H_4FO - C_6H_4F]^+$), purity 94.4 % by ^{31}P NMR spectroscopy.

Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(2-fluorophenyl)phosphate)-(2*S*)-Leu-Wang resin 160



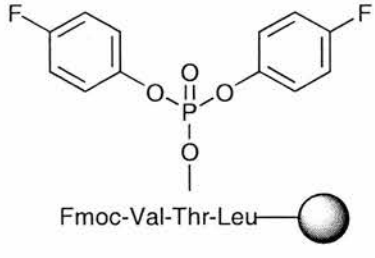
This was prepared in a manner identical with that for Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(pentafluorophenyl)phosphate)-(2*S*)-Leu-Wang resin **176** using *bis*(2-flourophenyl) chlorophosphate **156** (365 mg, 1.2 mmol) and Fmoc-Val-Thr-Leu Wang resin **159** (230 mg, 0.1 mmol). υ_{max}(single bead)/ cm^{-1} 1070 (Ar-CF) and 1263 (P=O); δ_P (121.4 MHz; $C_6{}^2H_6$) –15.05; δ_F(282.3 MHz; $C_6{}^2H_6$) 239.6.

Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(3-fluorophenyl)phosphate)-(2*S*)-Leu-Wang resin 158



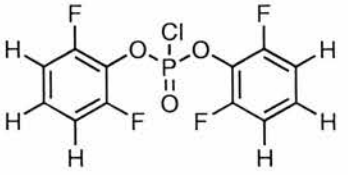
This was prepared in a manner identical with that for Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(pentafluorophenyl)phosphate)-(2*S*)-Leu-Wang resin **176** using *bis*(3-flourophenyl) chlorophosphate **157** (365 mg, 1.2 mmol) and Fmoc-Val-Thr-Leu Wang resin **159** (230 mg, 0.1 mmol). υ_{max} (single bead)/ cm^{-1} 1071 (Ar-CF) and 1243 (P=O st); δ_P(121.4 MHz; $C_6{}^2H_6$) –12.58; δ_F(282.3 MHz; $C_6{}^2H_6$) 241.46.

Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(4-fluorophenyl)phosphate)-(2*S*)-Leu-Wang resin 162



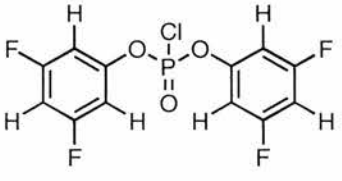
This was prepared in a manner identical with that for Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(pentafluorophenyl)phosphate)-(2*S*)-Leu-Wang resin **176** using *bis*(4-flourophenyl) chlorophosphate **157** (365 mg, 1.2 mmol) and Fmoc-Val-Thr-Leu Wang resin **159** (230 mg, 0.1 mmol). υ_{max}(single bead)/ cm^{-1} 1027 (Ar-CF) and 1178 (P=O st); δ_P (121.4 MHz; $C_6{}^2H_6$) –13.76; δ_F(282.3 MHz; $C_6{}^2H_6$) 240.0.

Bis(2,6-difluorophenyl) chlorophosphate 166



Bis(2,6-difluorophenyl) chlorophosphate **166** was prepared in a manner similar to that for *bis*(pentafluorophenyl) chlorophosphate **77**, using $POCl_3$ (9.4 cm^3, 0.1 mol) and 2,6-difluorophenol (26.0 g, 0.2 mol) to afford the 2,6-difluorophenyl dichlorophosphate and the chlorophosphate **166** (18.3g, 53.9%), bp 140 °C/ 0.5 mmHg; (Found: M^+, 339.9683. $C_{12}H_6O_3F_4P_1{}^{35}Cl_1$ requires 339.9679); υ_{max}(thin film)/ cm^{-1} 959 (P-O-C st), 1196 (C-F st) and 1303 (P=O st); δ_H(300 MHz; C^2HCl_3) 6.97-7.05 (4H, m, 2 x 3-Ar-C*H* & 2 x 5-Ar-C*H*) and 7.17-7.24 (2H, m, 2 x 4-Ar-C*H*); δ_C(75.4 MHz; C^2HCl_3) 112.54 (Ar-*C*H), 126.93 (td, *J* 8.76, 2.57, 2-Ar-C*F* + 6-Ar-C*F*) and 152.83 (d, *J* 4.53) & 156.20 (d, *J* 3.39, quaternary Ar-*C*); δ_F(282.3 MHz; C^2HCl_3) 226.04 (4F, m, Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) –15.69; *m/z* (CI^+) 342, 340 (22, 66%, M^+, [Cl isotopes]), 211 (25, $[M - C_6F_2H_3O]^+$), 129 (38, $C_6F_2H_3O^+$) and 101 (100), purity 92.3 % by ^{31}P NMR spectroscopy.

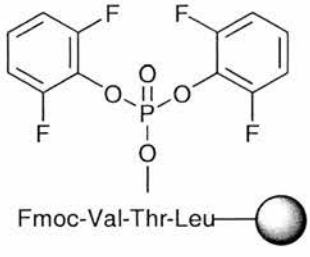
Bis(3,5-difluorophenyl) chlorophosphate 167



Bis(3,5-difluorophenyl) chlorophosphate **167** was prepared in a manner similar to that for *bis*(pentafluorophenyl) chlorophosphate **77**, using $POCl_3$ (3.8 cm^3, 0.04 mmol) and 3,5-difluorophenol (10.5 g, 0.08 mmol) to afford 3,5-difluorophenyl dichlorophosphate and the title chlorophosphate **167** (6.32g, 46.5%), bp 128-38 °C/

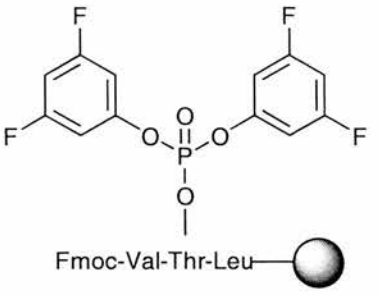
0.5 mmHg (Found: M^+, 339.9668. $C_{12}H_6O_3F_4P_1{}^{35}Cl_1$ requires 339.9679), υ_{max}(thin film)/cm^{-1} 1002 (P-O-C stretch), 1124 (C-F stretch) and 1313 (P=O stretch); δ_H(300 MHz; C^2HCl_3) 6.75-6.95 (6H, m, 2 x Ar-C*H*); δ_C(75.4 MHz; C^2HCl_3) 102.26 (2 x dd, *J* 24.88, 25.94, 4-Ar-*C*H), 104.23 (d, *J* 4.30) & 104.62 (d, *J* 5.43, 2-Ar-*C*H & 6-Ar-*C*H), 150.94 (Ar-*C*F) and 161.69 (d, *J* 15.16) & 165.02 (d, *J* 14.02, quaternary Ar-*C*); δ_F(282.3 MHz; C^2HCl_3) 244.90 (4F, dd, J_{CF} 6.21, 9.03, Ar-C*F*); δ_P(121.4 MHz; C^2HCl_3) -19.18; *m/z* (CI^+) 342, 340 (33, 100%, M^+ [Cl isotopes]), 113 (36, $C_6F_2H_3^+$) and 101 (28), purity 92.1 % by ^{31}P NMR spectroscopy.

Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(2,6-difluorophenyl)phosphate)-(2*S*)-Leu-Wang resin 170



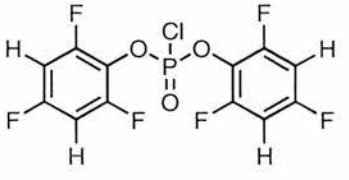
This was prepared in a manner identical with that for Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(pentafluorophenyl)phosphate)-(2*S*)-Leu-Wang resin **176** using *bis*(2,6-diflourophenyl) chlorophosphate **168** (420 mg, 1.2 mmol) and Fmoc-Val-Thr-Leu Wang resin **159** (230 mg, 0.1 mmol). υ_{max}(single bead)/ cm^{-1} 1246 (P=O st); δ_P(121.4 MHz; $C_6{}^2H_6$) –10.57; δ_F(282.3 MHz; $C_6{}^2H_6$) 226.1.

Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(3,5-difluorophenyl)phosphate)-(2*S*)-Leu-Wang resin 171



This was prepared in a manner identical with that for Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(pentafluorophenyl)phosphate)-(2*S*)-Leu-Wang resin **176** using *bis*(3,5-diflourophenyl) chlorophosphate **169** (420 mg, 1.2 mmol) and Fmoc-Val-Thr-Leu Wang resin **159** (230 mg, 0.1 mmol). υ_{max}(single bead)/ cm^{-1} 1018 (Ar-CF) and 1225 (P=O st); δ_P(121.4 MHz; $C_6{}^2H_6$) –12.71; δ_F(282.3 MHz; $C_6{}^2H_6$) 224.6.

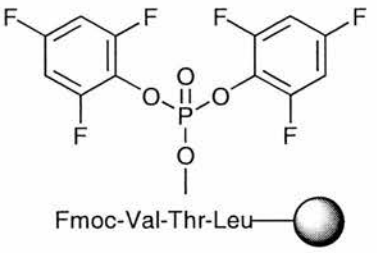
***Bis*(2,4,6-trifluorophenyl) chlorophosphate 173**



Bis(2,4,6-trifluorophenyl) chlorophosphate **173** was prepared in a manner identical with that for *bis*(pentafluorophenyl) chlorophosphate **77**, using $POCl_3$ (3.2 cm^3, 33.7 mmol) and 2,4,6-trifluorophenol (10.0g, 67.5 mmol) to afford the title chlorophosphate **173** after distillation at reduced pressure (4.82g, 38.0%), bp 160-164 °C/ 0.5-0.7 mm Hg (Found: M^+, 375.9488. $C_{12}H_4O_3F_6P_1{}^{37}Cl_1$ requires 375.9491); υ_{max}(Nujol)/ cm^{-1} 957 (P-O-C st), 1128 (C-F st) and 1312 (P=O st); δ_H(300 MHz; C^2HCl_3) 6.7-7.0 (4H, m, Ar-C*H*); δ_C(75.4 MHz; C^2HCl_3) 101.25 (t, *J* 26.01, 3-Ar-*C*H & 5-Ar-*C*H), 115.59 (quaternary Ar-*C*) and 153.14 (d, *J* 13.5) & 156.32 (d, *J* 16.6, Ar-CF); δ_F(282.3 MHz; C^2HCl_3) 228.25 (2F, m, 2, 6-Ar-C*F*) and

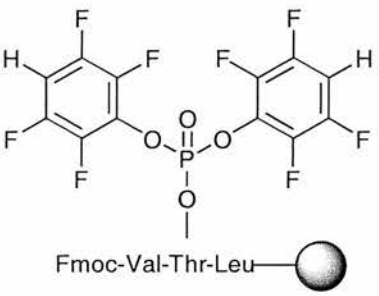
241.67 (1F, m, 4-Ar-C*F*); δ_P(121.4 MHz, C^2HCl_3) -14.74; *m/z* (CI^+) 378, 376 (33, 100%, M^+, [Cl isotopes]), 229 (30, [M – $C_6F_3H_2O]^+$), 194 (12, [M – Cl – $C_6F_3H_2O]^+$), 147 (48, $C_6H_2F_3O^+$) and 119 (29, H_2POCl^+), purity 93.6 % by ^{31}P NMR spectroscopy.

Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(2,4,6-trifluorophenyl)phosphate)-(2*S*)-Leu-Wang resin 174



This was prepared in a manner identical with that for Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(pentafluorophenyl)phosphate)-(2*S*)-Leu-Wang resin **176** using *bis*(2,4,6-triflourophenyl) chlorophosphate **173** (450 mg, 1.2 mmol) and Fmoc-Val-Thr-Leu Wang resin **159** (230 mg, 0.1 mmol). δ_P(121.4 MHz; $C_6{}^2H_6$) –16.5; δ_F(282.3 MHz; $C_6{}^2H_6$) 225.3 (2-Ar-CF & 6-Ar-CF), and 235.1 (4-Ar-CF).

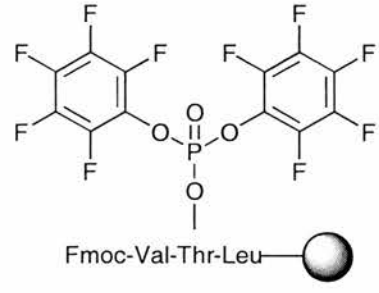
Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(2,3,4,6-tetrafluorophenyl)phosphate)-(2*S*)-Leu-Wang resin 175



This was prepared in a manner identical with that for Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(pentafluorophenyl)phosphate)-(2*S*)-Leu-Wang resin **176** using

bis(2,3,5,6-tetraflourophenyl) chlorophosphate **103** (500 mg, 1.2 mmol) and Fmoc-Val-Thr-Leu Wang resin **159** (230 mg, 0.1 mmol). υ_{max}(single bead)/ cm^{-1} 1099 (Ar-CF) and 1239 (P=O st); δ_P(121.4 MHz; $C_6{}^2H_6$) –8.89; δ_F(282.3 MHz; $C_6{}^2H_6$) 187-214.

Fmoc-(2*S*)-Val-(2*S*)-Thr-(*O*',*O*'-*bis*(pentafluorophenyl)phosphate)-(2*S*)-Leu-Wang resin 176



To a stirred suspension of Fmoc-Val-Thr-Leu Wang resin **159** (230 mg, 0.1 mmol) and DMAP (12 mg, 0.01 mmol) in CH_2Cl_2 (5 cm^3) was added dry Et_3N (195 mm^3, 1.4 mmol) followed by a solution of *bis*(pentafluorophenyl) chlorophosphate **77** (540 mg, 1.2 mmol) in CH_2Cl_2 (5 cm^3). The suspension was allowed to stir for 4 hours, then the resin isolated by filtration. The resin was thoroughly washed successively with CH_2Cl_2 (10 cm^3), MeOH (10 cm^3), then with CH_2Cl_2 (10 cm^3). The resin was dried under reduced pressure to give the title compound **176** in essentially quantitative yield. υ_{max}(single bead)/ cm^{-1} 1024 (Ar-CF) and 1225 (P=O); δ_P(121.4 MHz; $C_6{}^2H_6$) –10.39; δ_F(282.3 MHz; $C_6{}^2H_6$) 182-193.

Typical timecourse ^{31}P NMR experiment.

A sample of the resin bound phosphate triester (0.1 mmol) was placed in an NMR tube containing an external ^2H_2O standard in a sealed capillary tube and the NMR tube placed in the Varian Gemini 300 spectrometer. After locking and shimming the

^2H_2O standard, the NMR tube was removed and 1.0 cm^3 of a cleavage mixture containing 95% CF_3CO_2H, 2.5% Et_3SiH and 2.5% H_2O was added. The tube was replaced in the spectrometer without shaking, the lock signal readjusted, and ^{31}P NMR spectra acquired every 15 minutes.

_O_-Acetyl-2,6-difluorophenol 180[277]

To a stirred solution of 2,6-difluorophenol **164** (1.3 g, 10.0 mmol) and DMAP (1.5 g, 12.0 mmol) in CH_2Cl_2 (20 cm^3) was added acetic anhydride (1.22 cm^3, 13.0 mmol). After 2 hours, the solvent was removed under reduced pressure to leave a white solid residue. This was suspended in diethylether (50 cm^3), washed with 1 $moldm^{-3}$ HCl (50 cm^3) and aq. $NaHCO_3$ (50 cm^3), dried and concentrated under reduced pressure to afford the ester **180** as a clear oil (710 mg, 41.0%), (Found: M^+, 172.0339. $C_8H_6O_2F_2$ requires 172.0336); υ_{max}(thin film)/ cm^{-1} 739 (Ar-C-C), 777 (Ar-C-C), 1187 (Ar-C-F st), 1247 (C-O st) and 1778 (C=O st); δ_H(300 MHz; C^2HCl_3) 2.34 (3H, s, OCH_3), 6.94 (2H, m, 3-Ar-*H* & 5-Ar-*H*) and 7.12 (1H, m, 4-Ar-*H*); δ_C(75.4 MHz; C^2HCl_3) 19.80 ($OCCH_3$), 112.01 (2 x d, *J* 22.7, 6.5, 3-Ar-*C* & 5-Ar-*C*), 126.38 (t, *J* 9.2, 4-Ar-*C*), 155.36 (dd, *J* 4.30, 250.9, 2-Ar-*C*F & 6-Ar-*C*F) and 167.50 ($OCCH_3$); δ_F(282.2 MHz; C^2HCl_3) 225.17 (2F, t, *J* 6.5, 2-Ar-C*F* & 6-Ar-C*F*); *m/z* (EI^+) 172 (29%, M^+), 130 (76, $[M + H - OCCH_3]^+$) and 43 (100, $OCCH_3^+$).

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