

DESIGN AND SYNTHESIS OF PHOSPHORUS-BASED  
INHIBITORS FOR THE HIV-1 PROTEINASE

David Alan Perrey

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**DESIGN AND SYNTHESIS  
OF PHOSPHORUS-BASED INHIBITORS  
FOR THE HIV-1 PROTEINASE.**

a thesis presented by

David Alan Perrey

to the

UNIVERSITY OF ST. ANDREWS

in application for

THE DEGREE OF DOCTOR OF PHILOSOPHY

St Andrews

January 1995



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Signature of supervisor

Date 24/1/1995

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## Abstract

A number of peptide fragments including (2S)-Pro-(2S)-Ile-NHiBu (72) and (2S)-Phe-(2S)-Ile-NHiBu (73) and 1-(benzyloxycarbonyl)-aminophosphinic acid methyl esters (analogues of Phe (76), Cha (77) and Leu (78)) have been synthesised and coupled to give a series of phosphoramidate methyl ester-based peptide inhibitors of HIV-1 proteinase. These compounds were tested against the proteinase enzyme *in vitro* using a spectrophotometric assay and displayed activities in the 1-100  $\mu\text{M}$  range. Remarkably, comparison of these data with data obtained for activities against HIV-1 in cultured human lymphocytes showed an *in vitro:in vivo* ratio of approximately 1:1. These results are indicative of a highly efficient cell uptake mechanism and exceed the reported *in vitro:in vivo* ratios for HIV proteinase inhibitors by a factor of  $10^3$ . It is also apparent from the results that the compounds are not rapidly degraded in human lymphocytes. The activities of these compounds, however, was rather insensitive to small structural changes, undermining our attempts to optimise them. A phosphoramidate ethyl ester (Cbz-Phe-PO(OCH<sub>2</sub>CH<sub>3</sub>)NH-(2S)-Phe-(2S)-Ile-NHiBu (94)) was prepared and this possessed an IC<sub>50</sub> of 4.5  $\mu\text{M}$ , indicating that it might be possible to incorporate larger ligands onto the ester portion of the molecule.

Due to the problems associated with peptidic compounds as therapeutic agents, a number of non-peptidic targets were designed. 3,3'-Di-(benzyloxycarbonyl)-aminobenzoin (103) was synthesised in three steps from *m*-nitrobenzaldehyde and this displayed an IC<sub>50</sub> of 8  $\mu\text{M}$ .

Molecular modelling of a second target, a bicyclic phosphorodiamidate (106), showed that this compound should adopt a skewed position within the enzyme's active site, with the OH group between the catalytic aspartates (Asp 25 and Asp 25') and the P=O strongly hydrogen-bonded to one of the flap Ile's but relatively distant from the other. A synthesis of this molecule from *tris* (hydroxymethyl)amino-methane was undertaken, but problems with this led to the design of the less-substituted phosphorodiamidate (116). An alternative route from diethyl malonate was begun, although to date this has not been completed. This work is now under investigation by others in our group.

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Abbreviation	Meaning
AIDS	acquired immune deficiency syndrome
AZT	3'-azido-3'-deoxythymidine
Boc	tertiarybutoxy carbonyl
CA	capsid protein
Chex	cyclohexyl
Cbz	carbobenzyloxy
ddI	2', 3'-dideoxyinosine
diast.	diastereomer
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	D,L dithiothreitol
EDTA	ethylene diaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPNP	1, 2-epoxy-3-(4-nitrophenoxy)-propane
eq.	equivalents
FAB	fast atom bombardment
gp	glycoprotein
HIV	human immunodeficiency virus
HMPA	hexamethylphosphoramide
HPLC	high performance liquid chromatography
iBu	<i>iso</i> -butyl

IC <sub>50</sub>	concentration required to reduce enzyme activity by 50%
IN	integrase
K <sub>i</sub>	enzyme inhibition constant
K <sub>M</sub>	Michaelis-Menten constant
MA	matrix protein
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
N.D.	not determined
NMM	N-methylmorpholine
NMR	nuclear magnetic resonance
Noa	naphthyloxyacetyl
PR	proteinase
Qua	quinoline-2-carbonyl
RF	replicative form
RN	ribonuclease
RNA	ribonucleic acid
RT	reverse transcriptase
SIV	simian immunodeficiency virus
THF	tetrahydrofuran
Thfg	tetrahydrofuranylglycine
TLC	thin layer chromatography
UV	ultraviolet

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

## **CHAPTER ONE**

### **INTRODUCTION TO HIV-1**

## **1.0 Introduction.**

### **1.1 General Introduction - The Scale of the Problem**

At the end of 1988, around 7 million people world-wide were HIV positive. Four years later, this figure had almost trebled to 19.5 million, including almost 3 million cases of full-blown AIDS, most of whom are already dead.<sup>1</sup> Early predictions have proved inadequate and the number of people anticipated to be HIV positive by the end of the decade ranges from 40 to 110 million people;<sup>1</sup> this latter figure represents 2 % of the current population of the world. In 1990, the World Health Organisation predicted that the cumulative number of cases of AIDS would reach 6 million; less than a quarter of these are considered preventable.<sup>2</sup> Whilst it is true that other diseases, for example malaria, kill more people, the rapid spread of HIV, together with the lack of satisfactory treatment, means that for the foreseeable future, it will continue to be one of the most important areas of research world-wide.

### **1.2 The Life and Times of HIV.**

It is widely believed that the etiological agent responsible for AIDS is the human immunodeficiency virus (HIV), although some hold this point in contention. Originally referred to as HTLV-III or LAV, infection by the virus eventually produces profound defects in cell-mediated immunity.<sup>3</sup> Over time, there is a severe depletion of the number of CD4<sup>+</sup> T-lymphocytes which leads to opportunistic infections, neurologic and neoplastic disease and, ultimately, death.

Although AIDS first came to light in 1981, it was two years before the cause was identified.<sup>4</sup> The first class, known now as HIV-1, was found in the Western World and in Central Africa.<sup>4</sup> Soon afterwards, another major sub-type, HIV-2, was discovered in West Africa.<sup>5</sup> The genomes of the two classes are around 40 % identical, although interestingly HIV-2 is almost 80 % identical to the simian immunodeficiency virus (SIV). This infects certain African monkeys in the wild

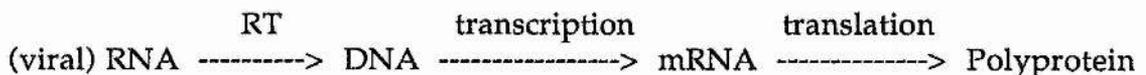
with no pathological consequence.<sup>6</sup> However, in Asian macaques held in captivity, and thus not exposed in their native habitat, an AIDS-like disease has been observed.<sup>7</sup> HIV-2 has infected a vast population in Western Africa and several cases have been documented in the West<sup>8</sup> but, as HIV-1 is the pathogen of greater consequence, it is with this strain that the following discussion will be mostly concerned.

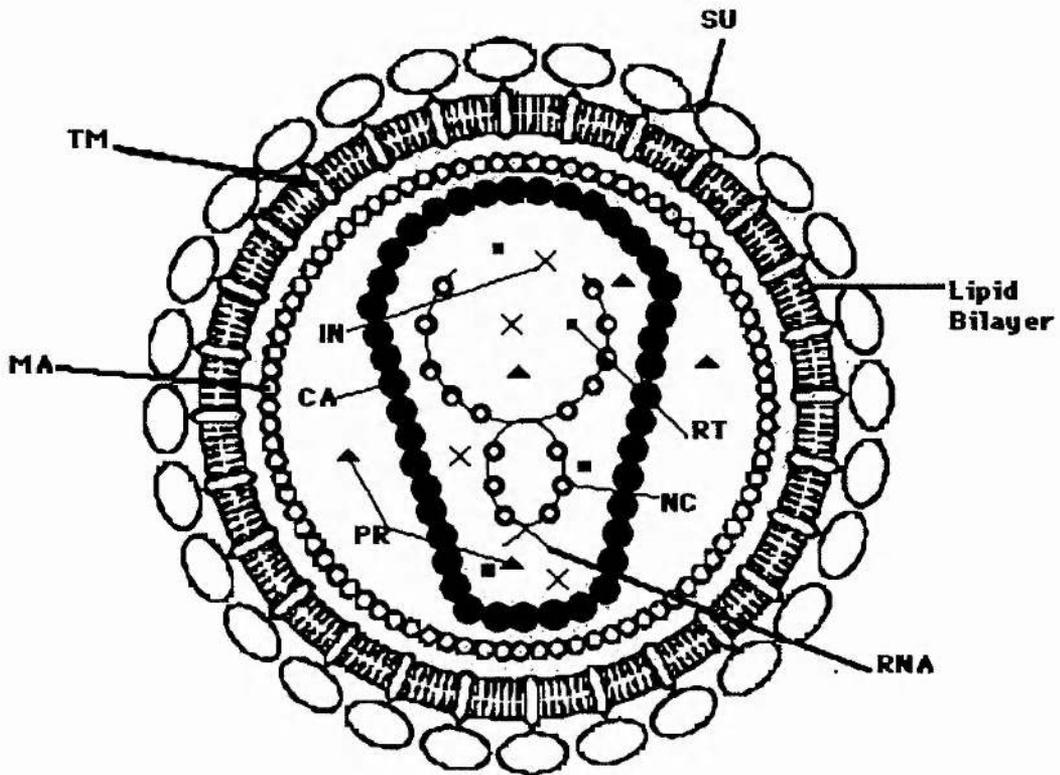
HIV was found to be a lentivirus, a member of the *Lentiviridae* family of retroviruses; members of this family include both HIV sub-types, SIV and also equine infectious anaemia virus (EIAV).

The virus consists of an envelope and a core (see Fig. 1.1). The envelope is made up of a lipid bilayer and three proteins, the surface glycoprotein gp120 on the outside, a transmembrane gp41 and a matrix protein p17 coating the inside. (The p notation used here refers simply to a protein, gp means a glycoprotein and the number following refers to its approximate weight in kilodaltons, as determined by electrophoresis).

The core is roughly bullet shaped. It has an outer skin of capsid protein p24, inside which are two strands of RNA, each tightly covered by the nucleocapsid protein p7. There are three additional enzymes present: the proteinase p11, reverse transcriptase p66 and integrase p32, and a further protein p6 of unidentified function.

The genome of the virus is encoded in RNA which it transcribes into DNA within the host cell for incorporation into the host genome. Subsequent expression is then performed by the host cell, under the supervision of the viral genes.





*Figure 1.1: Schematic Representation of the Human Immunodeficiency Virus. SU = surface protein gp120, TM = transmembrane gp41, MA = matrix protein p17, CA = capsid protein p24, NC = nucleocapsid protein p7, IN = integrase p32, RT = reverse transcriptase & PR = proteinase.*

Within each class, there exist many strains of HIV, each of which is distinct but clearly related to one or other of the classes. These are brought about by errors in conversion of viral RNA to DNA (reverse transcription). This process is highly prone to error,<sup>9</sup> as the viral reverse transcriptase has no proof-reading ability. Errors in base incorporation can occur with a frequency of up to one base per 1700,<sup>10</sup> and in a genome of about 9200 bases, this means an average of five mismatches per cycle of replication. Furthermore, the process has no DNA repair action so each error is retained. So within any one individual patient, several distinct variants will exist; in one case, 39 strains in two infected patients were reported,<sup>11</sup> and in another, a variation in the isolated viruses has been observed in the same patient at different times.<sup>12</sup> The pathological consequences of such variability go far to explain the seemingly random timing of the

development of full-blown AIDS in HIV-positive individuals. Obviously, many other factors may influence longevity, but evidence exists to suggest that long-term survivors are infected by less virulent strains, and also that virulence increases with time.<sup>13</sup>

With such heterogeneity and mutability inherent in the nature of the virus, it is clear that a thorough understanding of the viral life cycle is vital in order that therapeutic measures will be effective regardless of the strain or strains present.

### 1.3 The Life Cycle

The life cycle is still not fully understood and several aspects remain elusive. However, the major events in the life cycle are summarised below (see Fig. 1.2).

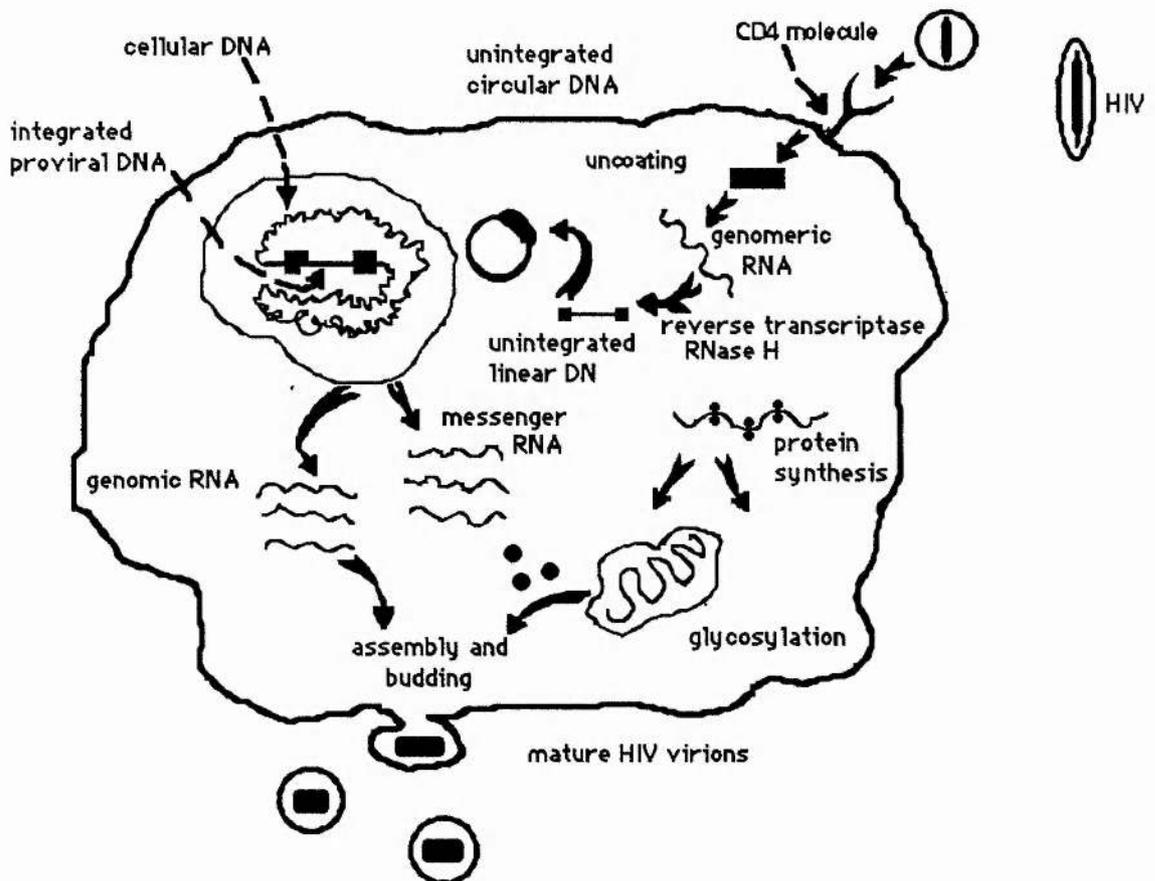


Figure 1.2: Diagram of the Viral Life Cycle.

The external portion of the viral envelope glycoprotein gp120 recognises and binds with high affinity to the receptor protein CD4. The T-helper (T4) lymphocytes, first to be identified as targets for HIV,<sup>5, 14</sup> have this surface marker, as do other targets such as macrophages and monocytes.<sup>15</sup> However, since these were discovered, many other cell types have been shown to be infected, including cells in the brain,<sup>16</sup> gastrointestinal tract,<sup>17</sup> kidney,<sup>18</sup> liver,<sup>19</sup> lung<sup>20</sup> and nervous system,<sup>21</sup> and some of these do not display CD4. Furthermore, some T-cells with high expressions of CD4 have been found to be HIV resistant.<sup>22</sup> This suggests that a second receptor is required for viral entry into the cell.

The transmembrane gp41 penetrates the cell membrane and initiates a process of membrane fusion. This causes the viral core to be expelled into the cell.

The nucleocapsid protein needs to be altered so that the RNA within is accessible to the reverse transcriptase. This could be carried out by cellular proteinases, but there exists *in vitro* evidence to suggest that the viral proteinase is responsible.<sup>23</sup>

The reverse transcriptase enzyme polymerises deoxynucleotides on the RNA template giving single strand DNA. A complementary strand is also synthesised and the now double-stranded DNA is transported to the cell nucleus. The integrase enzyme is also transported there, where it inserts the viral DNA into the host genome to form integrated viral DNA. This process is irreversible and thus the presence of the HIV genome in the cell is permanent. This integrated retroviral DNA piece is called the provirus.<sup>24</sup>

The cell may harbour the provirus for a long time and it is not understood what activates it. When it is activated, however, it begins to produce RNA molecules corresponding to the total length of the viral genome using cellular polymerases. Some of these will serve as genetic material for a new generation of viruses while the rest, the messenger RNA, migrates to the ribosomes in the

cytoplasm where synthesis of the polyproteins takes place using the mRNA as a template.

Open reading frames (ORF) are regions of the DNA where a substantial sequence of amino acids are coded between stop codons.<sup>24a</sup> When genes overlap in the genome, it means that they are encoded in different reading frames. Each amino acid is coded by three codons so the reading frames can be either one or two nucleotides out of phase.

When the proviral DNA is inspected, several ORFs are revealed. The proteins found in the intact virus are derived from three of these: *gag*, *pol* and *env* (see Fig. 1.3). Other ORFs encode proteins involved with controlling the expression of the provirus and are not packaged in the viral particle. The presence of these added genes make HIV one of the most complex of all known retroviruses.

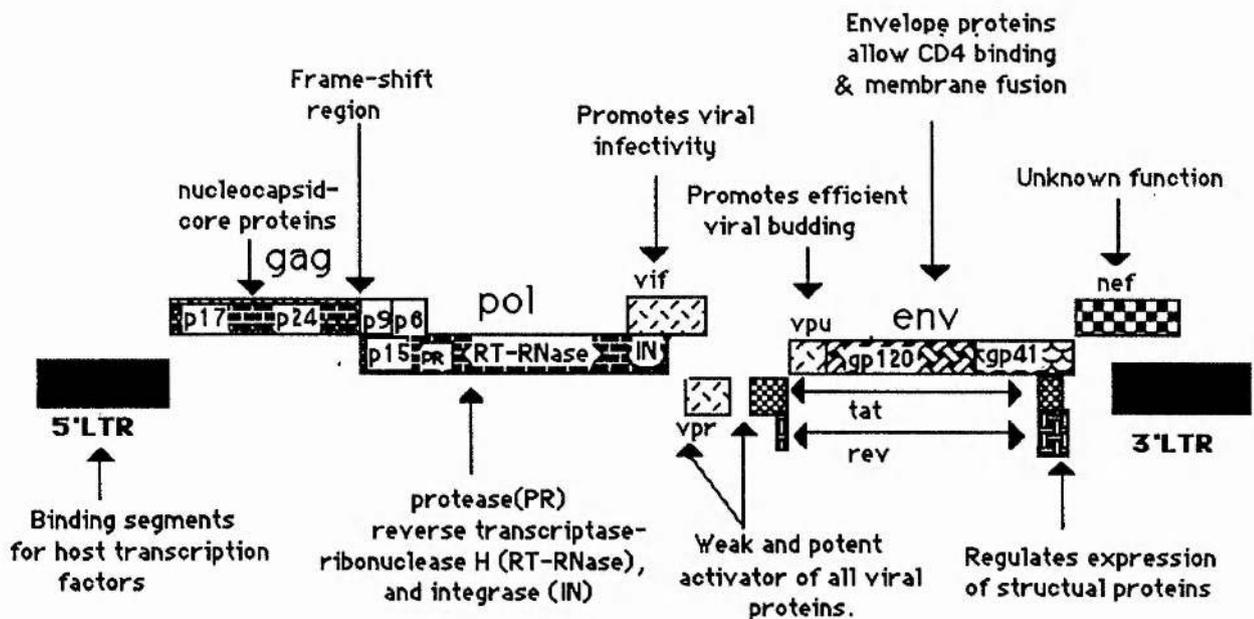


Figure 1.3: The HIV-1 genome.

The *gag* ORF (1536 nucleotides) is translated into a 55 kDa polyprotein including all the structural proteins.<sup>24</sup> The *pol* ORF (3045 nucleotides) is only translated as a *gag-pol* fusion protein.<sup>24</sup> It bypasses the *gag* stop codon due to a

frame-shift slightly before the stop codon. By moving one base backwards (towards the 5' end of the mRNA) the translation can continue smoothly until the *pol* stop codon is reached. This frame-shift is uncommon and results in a *gag* to *gag-pol* ratio of about 10:1. The *pol* region encodes the proteinase, reverse transcriptase and integrase, *i.e.* the viral enzymes. Since enzymes are catalytic, a stoichiometric amount is not required and using this frame-shift is a subtle and efficient way to generate them.

The *env* ORF is also translated, as a 160 kDa polyprotein.<sup>24</sup> This encompasses the surface protein gp120 and the transmembrane protein gp41.

Cellular enzymes carry out post-translational modification. The *env* polyprotein is glycosylated and cleaved into its two component proteins. Myristoylation of the N-terminus of the *gag* and *gag-pol* polyproteins also takes place. This is a non-polar fatty acid which is used to attach the polyproteins to the inside of the cell membrane.

Once the poly-proteins and some genomic RNA have associated near the cell membrane, the area 'buds' and a small portion of membrane pinches off to form a densely packed sphere about 0.1 microns in diameter.

This non-infectious immature viral particle then undergoes a series of processing steps catalysed by the viral proteinase. The proteinase catalyses its own release and, once free, catalyses a series of further hydrolytic cleavages. The matrix protein remains attached to the envelope while the rest of the released proteins assemble in the core, along with the two RNA molecules. It is possible to watch this maturation process through an electron microscope as the doughnut-like centre of the immature particle transforms to the bullet-shaped core of the mature virus.<sup>24a</sup>

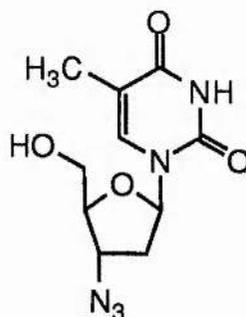
A single cell can produce several hundred new viruses, usually killing the cell in the process.<sup>24a</sup>

## 1.4 Therapeutic Targets

Examining the viral life cycle has revealed a number of possible stages for intervention.<sup>25</sup>

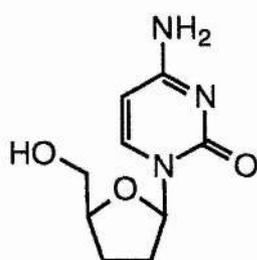
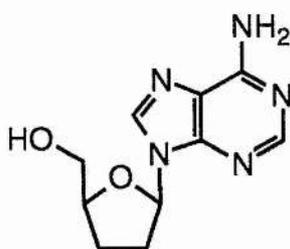
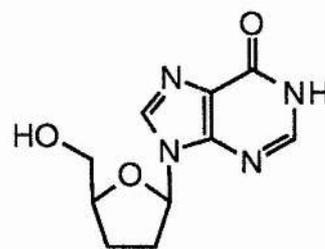
### 1.4.1 Reverse Transcriptase

Early work on anti-HIV agents was centred on the reverse transcriptase enzyme. The process of converting viral RNA to DNA is unique to retroviruses, so an inhibitor of this enzyme should not interfere in the body's other processes. This research resulted in the development of the first AIDS therapeutic agent, Zidovudine or 3'-azido-2',3'-dideoxythymidine (AZT) (1).



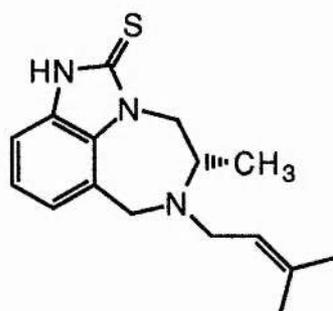
(1): Zidovudine (AZT).

AZT was originally discovered to be an anti-tumour agent<sup>26</sup> and was later found to be active against the Friend leukaemia virus.<sup>27</sup> In 1985, cytopathogenic effect on HIV was found *in vitro*<sup>28</sup> and it was subsequently given to AIDS patients.<sup>29</sup> Other nucleotides also went to clinical trials, such as 2',3'-dideoxyadenosine (ddA) (2), 2',3'-dideoxyinosine (ddI) (3) and 2',3'-dideoxycytidine (ddC) (4). These latter two have both been approved by the US Food and Drug Administration (FDA).

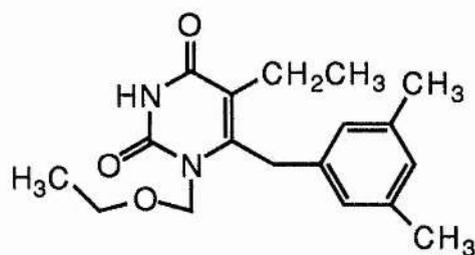
(2): *ddA*.(3): *ddI*.(4): *ddC*.

However, AZT has shown significant toxicity; indeed some patients cannot tolerate AZT therapy at all. With long term AZT treatment, its benefits begin to wane. Some mutant resistance has already been observed which does not bode well for either ddI or ddC.<sup>30</sup>

Some non-nucleotides have also shown activity against reverse transcriptase. In particular some benzodiazepines and dipyrodiazepinones have shown potent *in vitro* activity as well as low cytotoxicity and may be clinically useful.<sup>31</sup> The tetrahydroimidazo-[4, 5, 1-jk] [1, 4]-benzo-diazepin-2(1H)-one and thione (TIBO) derivatives were identified from an evaluation of over 600 classes of compounds.<sup>32</sup> R-82150 (5) is one such TIBO derivative and is as potent as AZT and has a therapeutic index that is five times higher.

(5): *R-82150*.

Amongst the 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) derivatives<sup>33</sup> was one derivative (6) which showed an *in vitro* therapeutic index of over 100,000.<sup>34</sup>



(6): HEPT derivative.

Nevirapine (7) has also been shown to target reverse transcriptase<sup>35</sup> and it has been shown to have activity against an AZT-resistant strain.<sup>36</sup> However, *in vitro* HIV-1 resistance to TIBO derivatives and nevirapine has been reported<sup>37</sup> and it remains to be seen whether this will also manifest itself *in vivo*. However, both TIBO and HEPT derivatives show no activity against HIV-2.<sup>32, 33a</sup> This may mean that the inhibitors cannot compensate for mutations in later generations of the virus and that it will be only a matter of time before resistant strains will develop.



(7): Nevirapine.

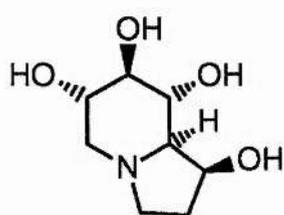
In addition, some findings point to their association with the development of resistant strains of HIV. All this has somewhat dampened enthusiasm for this series of compounds.

#### 1.4.2 Proteinase

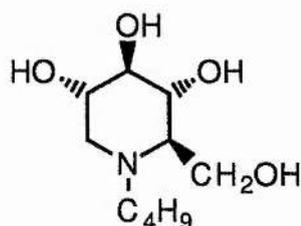
See sections 1.5 and 1.6 for a fuller discussion.

### 1.4.3 Glucosidase Inhibitors

A critical step in the life cycle is the N-glycosylation of the envelope protein followed by processing of the product by a number of enzymes. This latter part involves  $\alpha$ -glucosidases I and II which remove one or two glucose units from the oligosaccharide chain. Inhibitors of these enzymes such as castanospermine (8) and N-butyl deoxynojirimycin (N-butyl DNJ) (9) interfere with this process and N-butyl DNJ is undergoing clinical evaluation.<sup>38</sup>



(8): *Castanospermine*.



(9): *N-butyl DNJ*.

However, there are problems with these compounds: they require high doses and also they are non-specific in their recognition of viral and cellular glucosylation.

### 1.4.4 Inhibitors of Gene Expression

A major question in the fight against AIDS is whether it is actually possible to cure the disease. This is an issue because of the irreversible nature of the integration of the proviral DNA into the host cell DNA, guaranteeing life-long infection. A cure is therefore only possible if an agent can remove the viral genome from the host cells. This has not yet been achieved, however, there has been some success in blocking expression of viral genes. While this does not remove the infection, it may delay or even prevent the onset of full-blown AIDS.

In 1978, the first *in vitro* experiment was carried out that demonstrated the feasibility of anti-sense nucleotides.<sup>39</sup> These are nucleotide analogues which are designed to complement a specific segment of genome. This is then hybridised with the target sequence which effectively blocks expression. Problems with

rapid *in vivo* metabolism by cellular exonucleases were avoided by replacing the phosphate linkage with methyl phosphonates (10), phosphorothioates (11) or phosphoramidates (12) (see Fig. 1.4).<sup>40</sup>

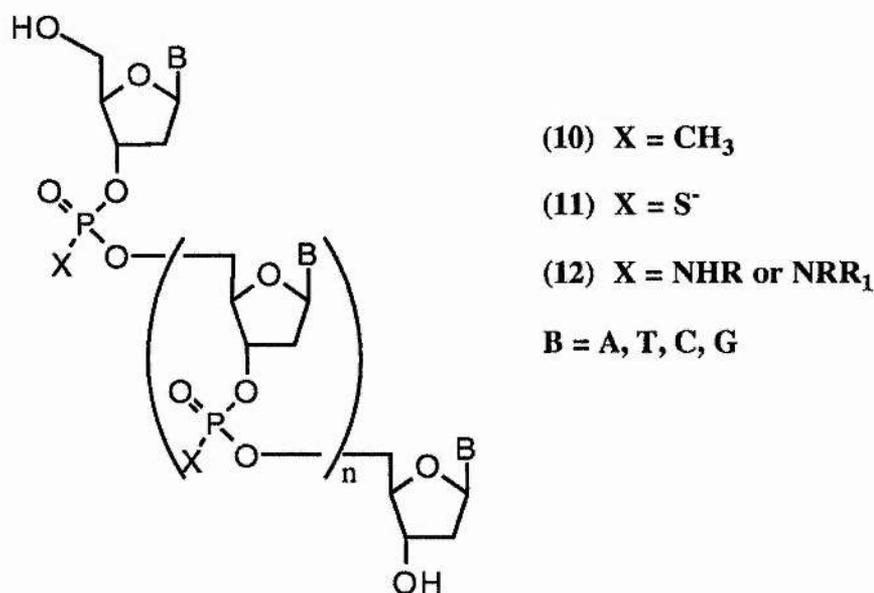


Figure 1.4: Anti-sense oligonucleotides.

To improve cell uptake properties, the oligonucleotides have been linked to poly-L-lysine and also to cholesterol. Agents targeting the *rev* gene have been prepared.

There are some other points to consider, such as the susceptibility of these compounds to endonucleases and also whether hybridisation will occur *in vivo*. It remains to be seen whether these problems can be overcome.

#### 1.4.5 Viral Binding Inhibition

The high affinity interaction between the surface glycoprotein gp120 and the CD4 receptor is vital for infection to occur. Soluble CD4 derivatives, which compete with the T-cells for the gp120, have been prepared and show anti-viral activity *in vitro*.<sup>41</sup>

While these results were encouraging, *in vivo* tests showed much poorer activity. A further problem with this approach is that HIV is unable to infect

some cells which display CD4 receptors and is able to infect some CD4 negative cells, so this approach cannot be widely applicable.

#### 1.4.6 *Tat* Regulatory Protein

Some of the benzodiazepines used as non-nucleotide reverse transcriptase inhibitors were found to block the viral protein synthesis which is enhanced by the *tat* protein.<sup>42</sup> The exact nature of the activity is unclear, but phase I clinical trials on Ro-24-7429 have been successfully completed.

#### 1.4.7 Other Targets

Other areas have the potential for anti-HIV action, although work in these areas is very much in its initial stages. The regulatory genes *tat* and *rev* are possible sites for drug intervention. The p15 ribonuclease H domain of reverse transcriptase is another area and with the availability of its X-ray crystal structure,<sup>43</sup> the impetus to design inhibitors should be provided.

Finally, there is the retroviral integrase enzyme, inhibition of which should block incorporation of viral DNA into the host cell DNA. Also, there may be other areas which can be exploited as greater understanding of the virus emerges.

### 1.5 HIV-1 Proteinase .

It has been known for over twenty years that viruses express their genomic products as large polyproteins.<sup>44</sup> It was also postulated that they might encode a proteolytic enzyme to regulate conversion of these polyproteins to the various structural and functional proteins. Such an enzyme would eliminate a reliance on cellular proteinases for this process and thus increase its ability to infect and replicate in numerous cell types.

Since that was proposed, it has been found that a wide range of viruses do indeed encode their own proteinase, including HIV. The first observation of a proteinase in a retrovirus was in Rous sarcoma virus, an avian retrovirus.<sup>45</sup>

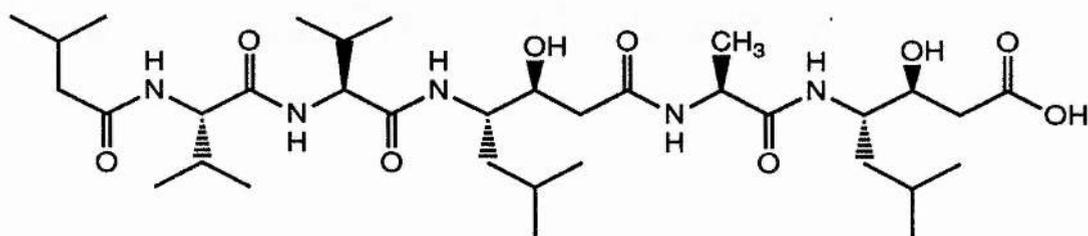
With the beginnings of the AIDS pandemic, researchers looking for potential therapies predicted the presence of a viral proteinase and that an inhibitor of this might provide anti-viral activity with minimal disruption to normal cellular processes.

### 1.5.1 Classification

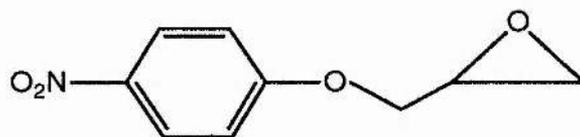
There are four broad classes of proteinase: aspartic, cysteine, serine and metallo proteinases. The HIV proteinase was found to be of the aspartic class, so named because of the presence of a pair of highly conserved aspartyl residues in the active site of the enzyme. Other, non-viral, aspartic proteinases, such as pepsin and renin, have been extensively studied with obvious benefit to the initial study of HIV proteinase. The enzyme was so classified on the basis of several pieces of evidence:

(1) Examination of the sequence homology of the HIV proteinase enabled the identification of a DTG triad observed in the catalytic site of other proteinases belonging to the aspartic family.<sup>46</sup>

(2) The enzyme was inhibited by the prototypical aspartic proteinase inhibitor, pepstatin (13)<sup>47</sup> and also irreversibly inactivated by 1,2-epoxy-3-(4-nitrophenoxy)-propane (EPNP) (14)<sup>48</sup> which had previously been shown to inhibit pepsin by esterification of one or both of the active site aspartate residues.<sup>49</sup>



(13): *Pepstatin A*.



(14): EPNP.

(3) Site-directed mutagenesis of Asp-25 to Asn completely abolished activity, indicating still further that the enzyme was an aspartic proteinase.<sup>50</sup>

(4) Conclusive evidence was provided when the X-ray crystal structure of the proteinase was solved.<sup>51</sup> From this, it was found that it was bilobal and was comprised of two identical 99 amino acid monomers (mass 11.5 kDa each) with each sub-unit contributing one conserved DTG triad to the active site, as had been predicted by Pearl and Taylor.<sup>52</sup> Though structurally similar to bilobal mammalian aspartic proteinases, the retroviruses have evolved a unique method for synthesising a protein not unlike the mammalian equivalent but carrying only half of the genetic information.<sup>53</sup>

### 1.5.2 Preparation and Purification

In order to study an enzyme, a ready supply of that enzyme must be available. HIV-1 proteinase has been purified by a number of methods:

(1) It has been isolated directly from the virus itself.<sup>54</sup> However, aside from the danger of using this method, it gave a very poor yield of the enzyme and that obtained showed very poor activity.

(2) More user-friendly methods utilise standard biochemical techniques of cloning and expressing the enzyme in bacteria and yeast. These have been reviewed.<sup>24b, 55</sup>

(3) The small size of the enzyme means it is also amenable to a chemical synthesis and this has been effected by a number of researchers using solid state resin techniques.<sup>47b, 56</sup>

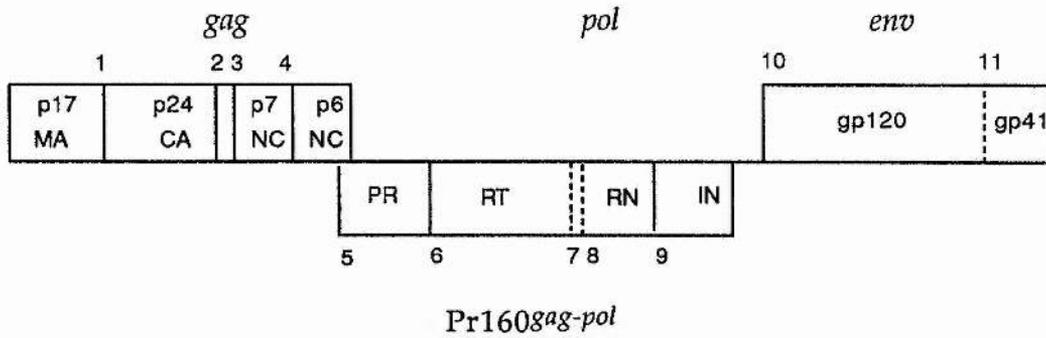
HIV-2 proteinase has received less attention than its counterpart, but this has also been expressed in bacteria and yeast and has been chemically synthesised.<sup>57</sup>

### 1.5.3 Substrate Specificity

Understanding the enzyme's preferences for particular residues in particular sub-sites is obviously desirable, as it gives a clearer picture of the binding sites available which can then be exploited in the design of inhibitors. However, such a task is not so straightforward, especially with an enzyme which recognises a variety of substrates. Inferences have been drawn from the results of enzymatic hydrolysis of peptides based on the natural substrates. The different cleavages have been classified into three general types based on the pattern of amino acid residues spanning  $P_4$  to  $P_4'$  (the  $P_n, P_n'$  terminology of Schechter and Berger<sup>58</sup> has been adopted and prescribes that amino acid residues are numbered  $P_1 P_2 \dots$  from the scissile bond to the amino terminus and  $P_1' P_2' \dots$  from the scissile bond towards the carboxy terminus) and attempts have also been made to explain them in terms of the distribution of hydrophilic and hydrophobic side chains.

The sequences of the natural substrates seem to suggest an enzyme that is quite specific; however that is not the case. The proteinase has been shown to cleave a variety of peptides and non-viral proteins.<sup>24b</sup> Although it has a pH optimum of around 5 or 6, it can, depending on the substrate, process peptides at up to pH 7 or at low pH.

A minimum of seven residues is required for cleavage to occur, spanning either  $P_3$  to  $P_4'$  or  $P_4$  to  $P_3'$ .<sup>59</sup> Although smaller peptides are not processed, a number of potent inhibitors have been made which contain fewer than seven residues (see section 1.6). This is in agreement with crystallographic studies which suggested the existence of multiple hydrogen bonds to the backbone of inhibitors spanning these sites (see Section 1.5.4). The natural substrates are shown in Figs. 1.5 and 1.6.



where MA = matrix protein

CA = capsid protein

p7 NC = nucleocapsid protein

p6 NC = nucleocapsid protein

PR= proteinase

RT = reverse transcriptase

IN = integrase

RN = ribonuclease

*Figure 1.5: The gag, pol and env polyproteins; 1-11 represent the sites of cleavage (see Fig. 1.6).*

The cleavage sites (\*) of the HIV-1 proteinase within the Pr160gag-pol are as follows:

	(P <sub>4</sub> )-(P <sub>1</sub> )	*	(P <sub>1</sub> ')-(P <sub>4</sub> ')
1.	Ser-Gln-Asn-Tyr	*	Pro-Ile-Val-Gln-
2.	Ala-Arg-Val-Leu	*	Ala-Glu-Ala-Met-
3.	Ala-Thr-Ile-Met	*	Met-Gln-Arg-Gly-
4.	Pro-Gly-Asn-Phe	*	Leu-Gln-Ser-Arg-
5.	Ser-Phe-Asn-Phe	*	Pro-Gln-Ile-Thr-
6.	Thr-Leu-Asn-Phe	*	Pro-Ile-Ser-Pro-
7.	Leu-Glu-Lys-Glu	*	Pro-Ile-Val-Gly-
8.	Ala-Glu-Thr-Phe	*	Tyr-Val-Asp-Gly-
9.	Arg-Lys-Ile-Leu	*	Phe-Leu-Asp-Gly-

*Figure 1.6: Cleavage Sites in HIV-1 Proteinase.*

The substrates are often divided into three classes. The first is in general Ser/Thr.Xaa.Yaa.Phe/Tyr \* Pro.Zaa (where \* represents the scissile bond and Xaa, Yaa and Zaa represent any amino acid). This sequence has been observed in a number of retroviruses but is unknown in other systems. Class two has Arg at P<sub>4</sub> and Phe-Leu in P<sub>1</sub>'-P<sub>2</sub>'. The final class has either Gln or Glu at P<sub>2</sub>'.

HIV-1 proteinase will cleave HIV-2 substrates (or *vice versa*), albeit at a reduced rate.<sup>60</sup> A more detailed series of studies looked at P<sub>3</sub>,<sup>61</sup> P<sub>2</sub>, P<sub>1</sub><sup>62</sup> and P<sub>2</sub>'.<sup>63</sup> From this work, it was concluded that a branched residue, such as Ile, is preferred at P<sub>2</sub>', although Ala and Leu are also tolerated. The presence of Phe and Gly at P<sub>2</sub>' gives poor substrates while Trp gave rise to a peptide that was not cleaved. Ile or Val at P<sub>1</sub>, on the other hand, resulted in no cleavage whereas Leu, norleucine, Met, Phe or Tyr all make excellent substrates. P<sub>3</sub> showed very little selectivity, only substitution by Pro gave an inactive peptide. P<sub>2</sub> also preferred branched residues, but also tolerated Leu, Phe, Ala, Asp or Asn. Gly and Pro were unfavourable. In another study, it was found that replacing the Pro at P<sub>1</sub>' with pipercolinic acid (the homoanalogue of Pro) gave an inhibitor, rather than a substrate.<sup>64</sup>

The subtle interactions of the substrate with the enzyme are still not well understood. Indeed, sub-site specificities for different cleavage sites are often not in parallel with each other.<sup>65</sup> Despite this, the information is useful to the medicinal chemist as it gives some clues about the sort of functionality to incorporate into inhibitors. This also shows that the enzyme is able to tolerate residues other than the natural ones thus allowing greater flexibility in inhibitor design. This is demonstrated in the specificity studies of the proteinase in recombinant proteins. Random mutagenesis of the P<sub>1</sub>-P<sub>1</sub>' positions produced a variable susceptibility to cleavage.<sup>66</sup> Although in some junctions (for example the PR-RT or RT-IN junctions), little or no change was tolerated, in the p6-PR junction a variety of changes were accepted. Replacing the natural Phe-Pro

cleavage sequence with residues such as Ala-Ile or even Gly-His resulted in substrates that were processed at an equivalent rate to the natural substrate.

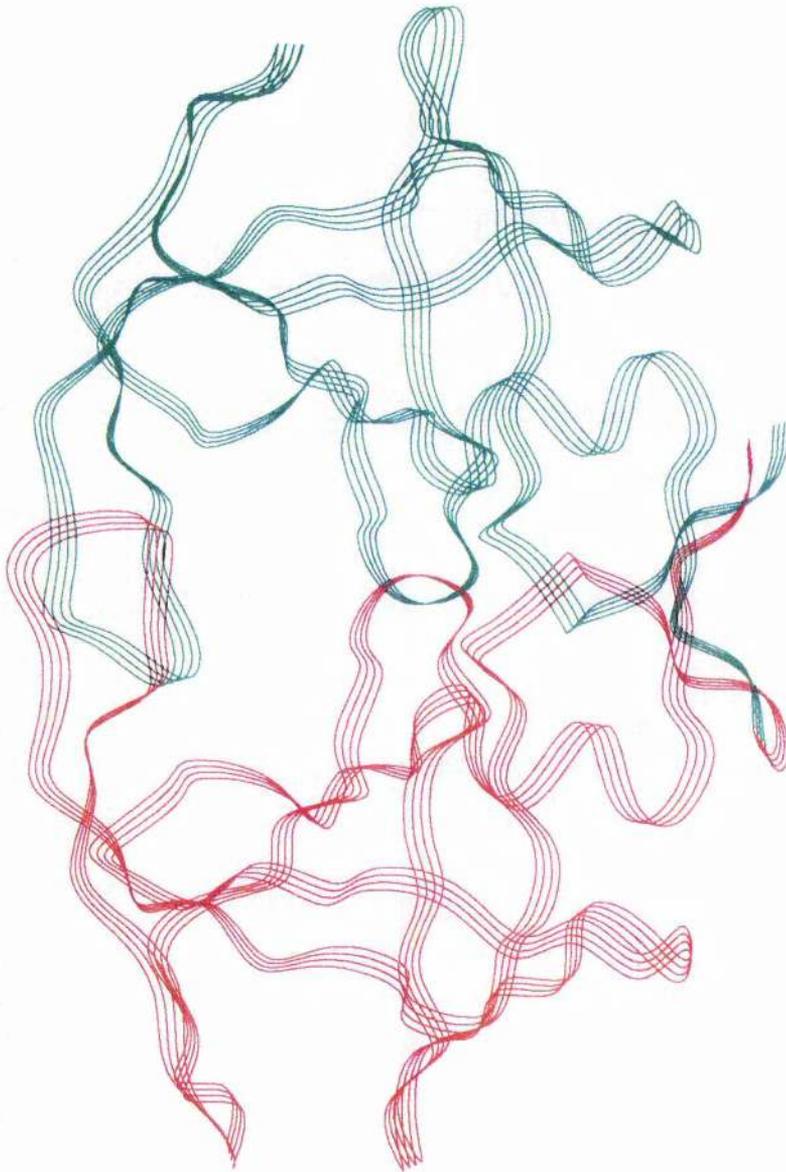
#### 1.5.4 Structural Properties of HIV-1 Proteinase

Non-viral proteinases are monomeric, contain more than 300 amino acids and are 'pseudosymmetric' in that they are bi-lobal.<sup>67</sup> One catalytic aspartate is contributed by each lobe and these are situated at the bottom of the cleft formed by these lobes.

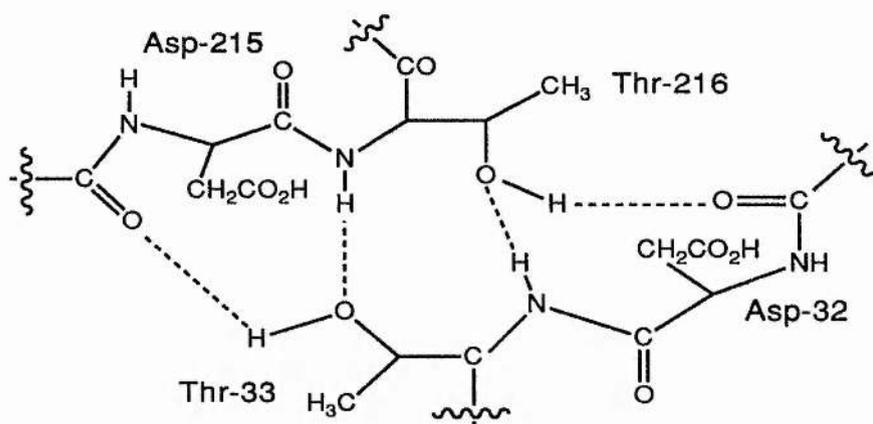
The retroviral proteinases on the other hand have fewer than 130 amino acids and have minimal sequence homology with their non-viral counterparts, with only two domains conserved. The first of these, Leu-Leu(Val)-Asp-Thr(Ser)-Gly-Ala contains the catalytic triad and is identical to the domain found in the non-viral proteinases. The second domain is Ile(Leu)-Leu-Gly-Arg-Asp. The structure and function of retroviral proteinases has been recently reviewed.<sup>68</sup>

Pearl and Taylor proposed that the retroviral proteinases achieve structures equivalent to their non-viral counterparts by dimerising.<sup>52</sup> This results in an overall similarity to the bilobal structure. The HIV-1 proteinase is roughly ellipsoid, measuring approximately 55 x 35 x 25 Å (see Fig. 1.7).

When unbound, it is C<sub>2</sub> symmetric, a symmetry which is perturbed upon binding of an unsymmetrical substrate.<sup>69</sup> It is very similar to the equivalent proteinase from the Rous sarcoma virus (RSV) as well as archetypal fungal proteinases.<sup>70</sup> This is particularly striking when the two active sites are superimposed; indeed the features of the HIV-1 proteinase were correctly predicted by modelling studies based upon the crystal structure of RSV proteinase.<sup>71</sup> The enzyme has a β-sheet structure, typical of aspartic proteinases, in which the two catalytic triads are in a loop which forms a number of hydrogen bonds to the opposite loop, giving the 'fireman's grip' that is a feature of the active site of aspartic proteinases (see Fig. 1.8).<sup>72</sup>



*Figure 1.7: The HIV-1 Protease.*



**Figure 1.8:** The 'Fireman's Grip', the Interaction of the Two Catalytic Triads, Shown in Pepsin.

The termini of each monomer interact strongly with each other and this interaction is thought to be important in maintaining the stability of the dimer. They are arranged in a network of anti-parallel  $\beta$ -sheets, forming four interdigitated strands. It is in this region of the viral poly-protein where the initial cleavage takes place, freeing the proteinase in an autoproteolytic event. However, this region of the enzyme is somewhat remote from the active site, so it is probable that the mechanism for cleavage is intermolecular. The stability conferred on the enzyme by this interaction also allows an alternative strategy to be envisaged that might deactivate the enzyme.<sup>51c, 73</sup> If the enzyme cannot form the dimer, it is unable to function. Evidence for the potential success of this strategy comes from an experiment which complexed a mixture of HIV-1 and HIV-2 proteinases.<sup>74</sup> The resulting dimer was found to be inactive. However, no further advances on this have appeared so far.

Another feature of the proteinase is the presence of two identical 'flap' regions, analogous to the single flap of the non-viral proteinases.

Loeb and co-workers made non-conservative replacements for each residue in the proteinase and found some regions to be critical.<sup>75</sup> Upon replacement of a residue with a residue of different polarity, the proteinase was found to be

inactive. The regions found were 22-33 (the catalytic domain), 47-52 (the flap region) and 77-87.

Enzyme-inhibitor complexes can give a medicinal chemist a good deal of information about how inhibitors interact with the enzyme and thus some clues as how to go about improving these interactions. A number of such complexes have been studied with a variety of structurally diverse inhibitors.<sup>69, 76</sup> These have revealed a number of common factors in enzyme-inhibitor complexes. First, despite the range of inhibitors that have been complexed, the overall proteinase structure is similar in all cases. Only a slight reorganisation of the core is observed, although the flaps can move by as much as 7 Å upon binding.<sup>69</sup> In this contracted conformation, the flaps form the top half of a protected hydrophobic tube extending roughly from P<sub>3</sub> to P<sub>3</sub>'. The effect of this is to shield about 80 % of an inhibitor from the surrounding solvent.

Secondly, structural studies have also revealed the presence of two active site water molecules in the inhibitor-enzyme complexes. One is hydrogen-bonded to the amide NHs of Ile-50 and Ile-50' (in the flap region) in a tetrahedral fashion which seems to act as a latch to keep the flaps closed. The other lies between P<sub>1</sub>-P<sub>2</sub> and P<sub>1</sub>'-P<sub>2</sub>' carbonyl oxygens in the inhibitor and seems to assist in ensuring a β-sheet-like conformation for the inhibitor.

All the inhibitors are bound in an extended conformation spanning P<sub>4</sub> to P<sub>3</sub>' with a network of hydrogen bonds mostly from the backbone of the 'floor' (which is the bottom of the cleft formed by the flaps closing) and from the flap region. The studies showed no apparent interactions between the active site aspartates and the amine function of either the reduced amide or hydroxyethylamine inhibitors (see section 1.6 for examples of these types of inhibitor), with the binding pockets for P<sub>2</sub> through to P<sub>2</sub>' almost entirely hydrophobic in nature.<sup>69, 76b</sup>

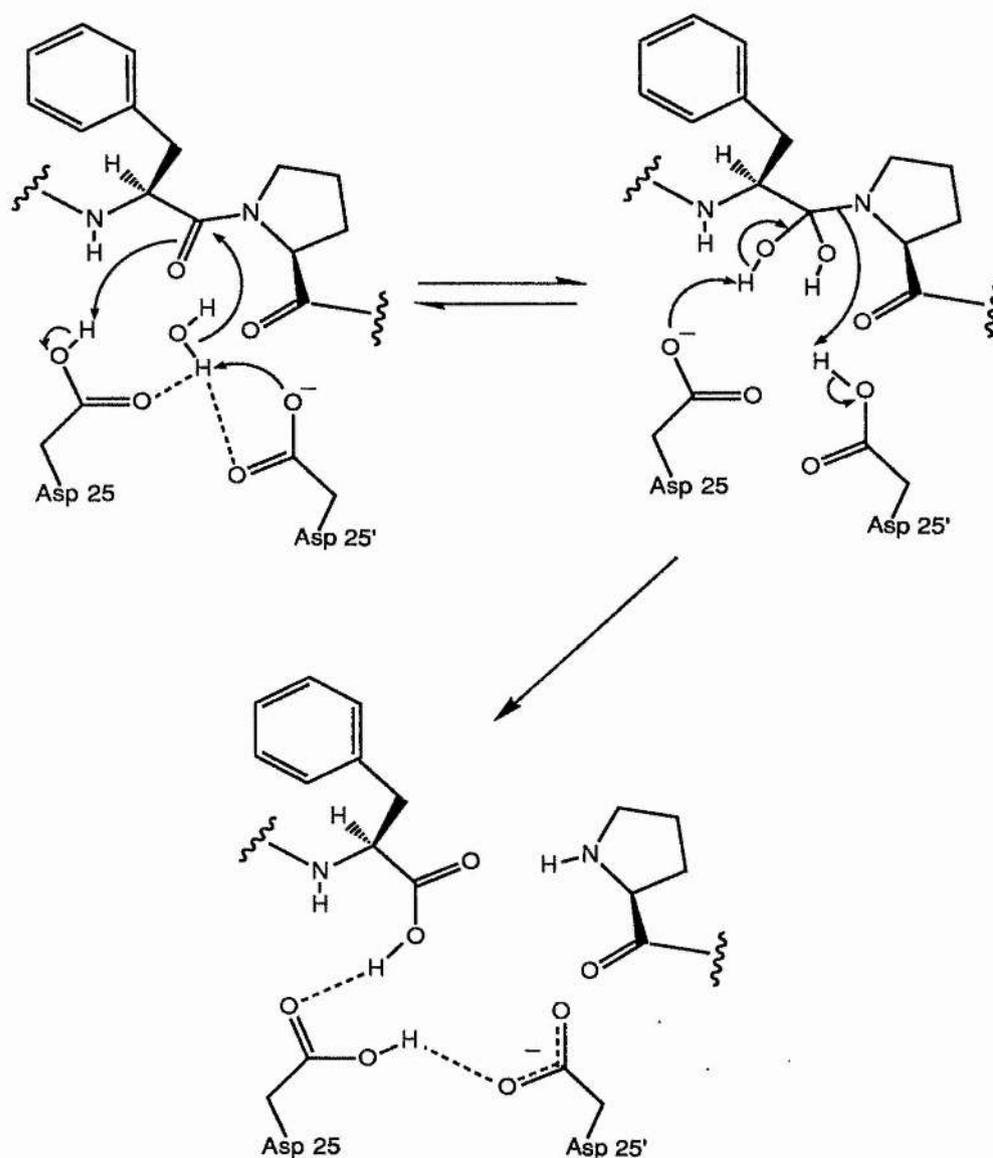
### 1.5.5 Catalytic Mechanism

An understanding of the mechanism by which an enzyme acts is crucial in order to design an inhibitor to counter it. For this reason, numerous kinetic studies have been undertaken in order to elucidate this mechanism.<sup>77</sup> Results include the inhibition of the enzyme by pepstatin ( $K_i < 2 \mu\text{M}$ ),<sup>78</sup> a pH optimum of 4.5 to 6.0<sup>77</sup> and a time dependent inactivation by EPNP.<sup>77b</sup> Furthermore, the pH dependence of the kinetic constant  $\log(k_{\text{cat}}/K_m)$  for several oligopeptide substrates constituted a bell-shaped curve of a pH range of 3.4 to 6.5. This indicated that there was an unprotonated group with a  $\text{p}K_a$  of 3.4 and a protonated group with a  $\text{p}K_a$  of about 6.5 that were required for catalysis.<sup>77f</sup> This, together with the identification of the proteinase as being of the aspartic family, indicated that Asp-25 and Asp-25' were involved and that one was protonated and the other was not. Substrate cleavage experiments done in  $\text{H}_2^{18}\text{O}$  resulted in the incorporation of  $^{18}\text{O}$  into the product and, by way of the reverse reaction, into reformed substrate.<sup>77e</sup> This indicated the presence of a nucleophilic water molecule, especially since there was no  $^{18}\text{O}$  incorporation into the enzyme.

These results showed the chemical mechanism to be of the general acid-general base type (see Fig. 1.9). This only shows the general features and there is some debate over the precise details, such as the protonation state of the tetrahedral intermediate, the timing of proton switches and the orientation of the scissile carbonyl with respect to the catalytic aspartates. The proposals of Davies,<sup>79</sup> Blundell,<sup>80</sup> Pearl,<sup>81</sup> Polgar<sup>82</sup> and James<sup>83</sup> give a more thorough treatment of the mechanistic problem.

However, the above mechanisms all contain a tetrahedral intermediate generated by a nucleophilic water molecule (catalysed by the aspartates) so in accordance with theories regarding the enzymatic stabilisation of the transition state, the amide hydrate intermediate would be expected to be very tightly bound by the enzyme.<sup>84</sup> Therefore, an inhibitor which mimics this transition state would be expected to be similarly tightly bound. The concept of transition state

mimicry has been used by the great majority of research groups synthesising and testing inhibitors for HIV-1 proteinase.



*Figure 1.9: The Mechanism of General Acid-General Base Catalysis.*

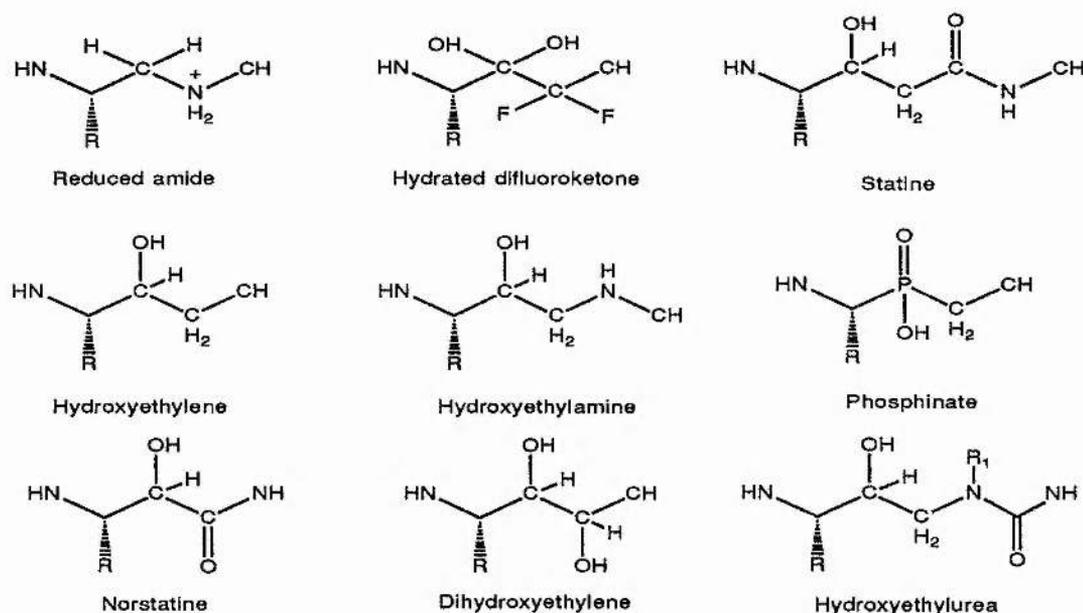
## 1.6 Proteinase Inhibitors

Since Kramer proposed that the viral proteinase was a viable target for anti-viral agents,<sup>85</sup> a great deal of effort has been directed towards the design and synthesis of proteinase inhibitors. The classical strategy makes use of the concept

of a transition state mimic, in which the transition state is replaced by a non-hydrolysable moiety. Although this could be any functionality, one which has similar structural and electronic features is obviously a more realistic mimic.

This strategy worked well for renin,<sup>86</sup> a mammalian aspartic proteinase, and that work has served as something of a blue-print for HIV proteinase inhibitors.

There are a wide range of isosteres utilised; some of the more common ones are shown below:



*Figure 1.10: Transition State Mimics.*

A study by Dreyer and co-workers<sup>87</sup> compared five of these isosteres by incorporating them into the same (or at least highly similar) peptide backbone (see Fig. 1.11). This study showed the hydroxyethylene moiety to be most potent, followed by difluoroketones, then statines and phosphinates and reduced amides as the least potent. While the peptide backbone was not precisely the same for each mimic, it does convey a good impression of the relative efficacy of each moiety and other comparisons have shown similar trends.<sup>88</sup>

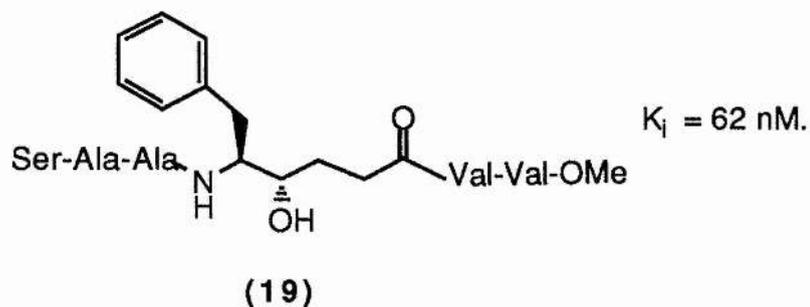
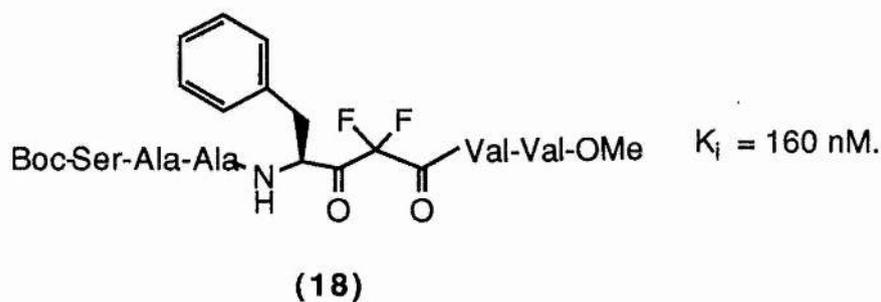
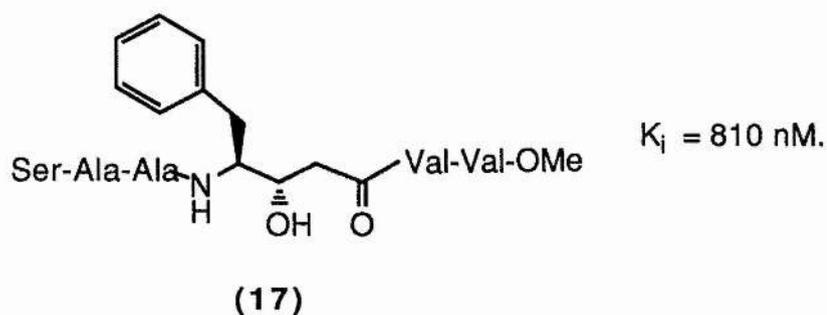
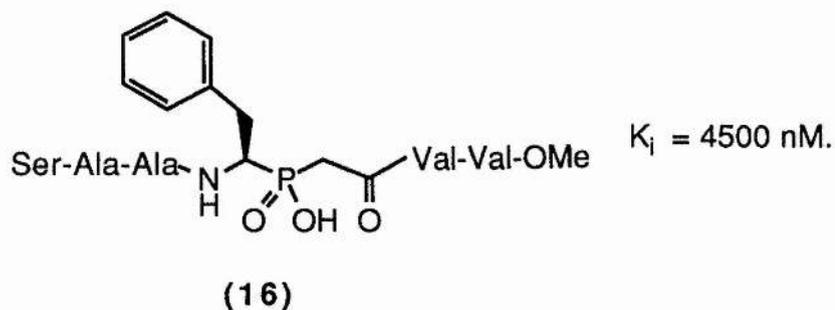
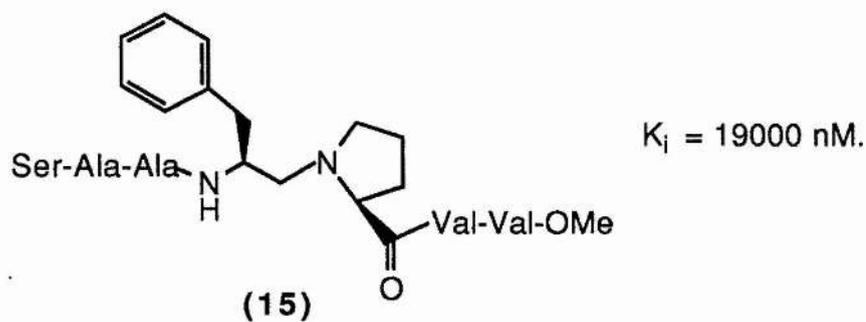
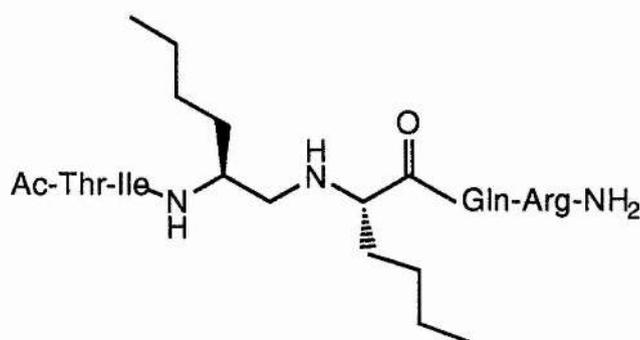


Figure 1.11: Comparison of Transition State Mimics.

### 1.6.1 Reduced Amides

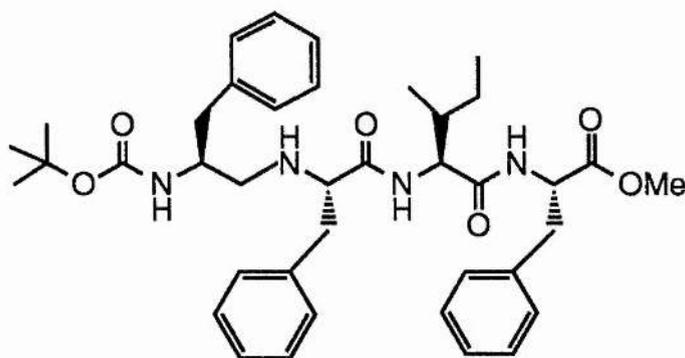
This class of inhibitors proposes a methylene group ( $\text{CH}_2$ ) as a transition state mimic of  $\text{C}(\text{OH})_2$ . There are obviously severe drawbacks with this proposal; it doesn't possess the hydrogen-bonding capability and is not very similar structurally. That said, it can be an effective inhibitor and its potency against renin was thought to be due to the amine being protonated to give an electrostatic interaction with the anionic aspartates. According to Hyland and co-workers,<sup>89</sup> there is a requirement that both aspartates be unprotonated, which is not the case for HIV proteinase and this may go some way to explaining the reduced amide's lower potency against this enzyme.

However, that is not to say that no effective inhibitors have been made. A synthetic substrate derived from the p1-p7 cleavage in Pr55<sup>gag</sup>, one of the best for HIV-1 proteinase, was converted into its reduced amide (20) and this became an inhibitor with a  $K_i$  of 780 nM.<sup>90</sup>

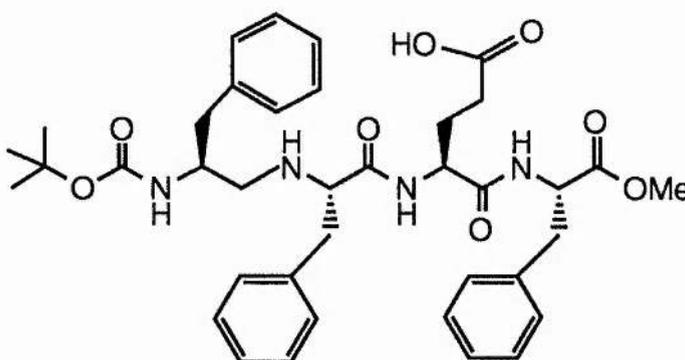


(20): *Norleucine-based inhibitor* ( $K_i = 780 \text{ nM}$ ).

Urban and co-workers improved this further;<sup>91</sup> compound (21) had a  $K_i$  of 23 nM while compound (22) was the most potent with a  $K_i$  of only 0.2 nM.



(21): A potent reduced amide inhibitor ( $K_i = 23 \text{ nM}$ ).

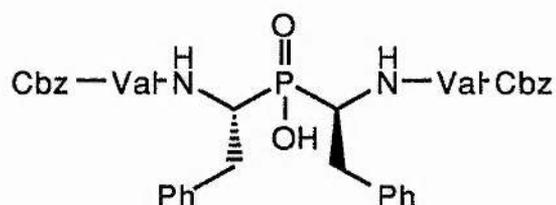


(22): A highly potent reduced amide ( $K_i = 0.2 \text{ nM}$ ).

### 1.6.2 Phosphinic Acids

The phosphinic acid class has been very successful in the past and phosphinic acid inhibitors of pepsin were amongst the most potent reported.<sup>92</sup> However, initial attempts to get similar results with HIV-1 proteinase were disappointing. This is possibly due to the anionic nature of the phosphinate at the assay pH of 6.0. The  $pK_a$  of a phosphinic acid is in the region of 3 to 3.5, so it would be predominantly ionised and there is precedent for the repulsion of negatively-charged species away from a negative aspartate.<sup>93</sup> With the lower pH optimum of pepsin, more protonated phosphinate was present which led to greater inhibition. This theory is backed up by an experiment with the HIV-1 proteinase; lowering the assay pH from 6.5 to 4.5 gave a 500-fold increase in potency.<sup>94</sup>

A potent phosphinic acid has been reported by Abdel-Meguid and co-workers.<sup>95</sup> SB 204144 (**23**) had a  $K_i$  of 2.8 nM at pH 6. Their pH analysis failed to unambiguously determine the question of whether the inhibitor would be much more potent at lower pH, however, crystallographic results indicated the presence of a protonated phosphinate.

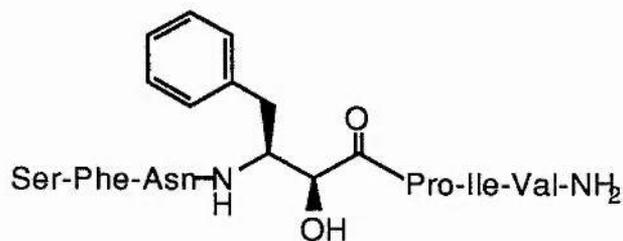


(**23**): SB 204144, a potent symmetrical phosphinic acid inhibitor ( $K_i = 2.8$  nM).

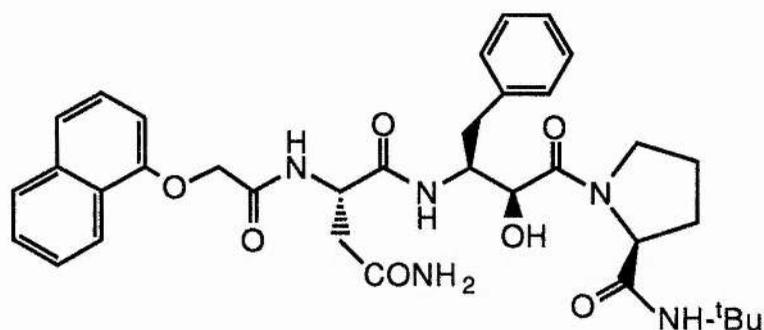
### 1.6.3 Statines

The archetypal aspartic proteinase inhibitor is Pepstatin A (**13**) and this was used as part of HIV-1 proteinase's characterisation as belonging to that class. Pepstatin A shows a  $K_i$  of approximately 1  $\mu$ M. A substrate analogue (**17**) (which pepstatin is not) only gave a  $K_i$  of 0.81  $\mu$ M.<sup>87</sup> More remarkably still, acetyl pepstatin gave a  $K_i$  of 20 nM,<sup>96</sup> despite only the addition of an extra acetyl group at  $P_4$ .

A related group are the norstatines; these have been shown to be effective inhibitors. Compound (**24**) showed a  $K_i$  of 5 nM<sup>97</sup> whereas compound (**25**) showed a  $K_i$  of 0.58 nM.<sup>98</sup> Both show a preference for the (S)-hydroxyl; when this is changed to (R), there is a 20-fold drop in potency.



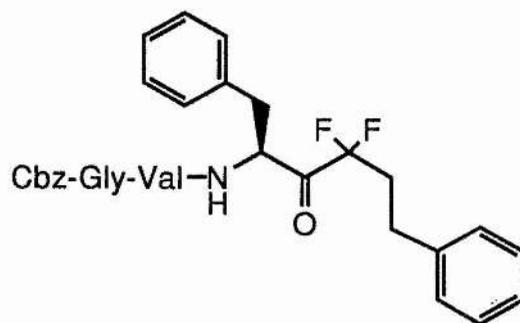
(**24**): Allophenylnorstatine based inhibitor ( $K_i = 5$  nM).



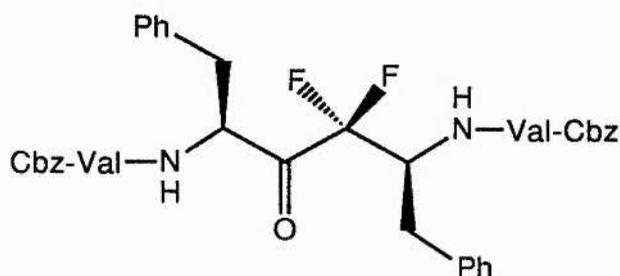
(25): Highly potent norstatine based inhibitor ( $K_i = 0.58$  nM).

#### 1.6.4 Difluoroketones

This is a highly potent class of inhibitors. Their potency stems from their ability to form a stable hydrate species, which is obviously a very close mimic of the substrate transition state. Two examples are shown below: compound (26) has an  $IC_{50}$  of 1.0 nM<sup>99</sup> and compound (27) has a  $K_i$  of 0.1 nM.<sup>100</sup>



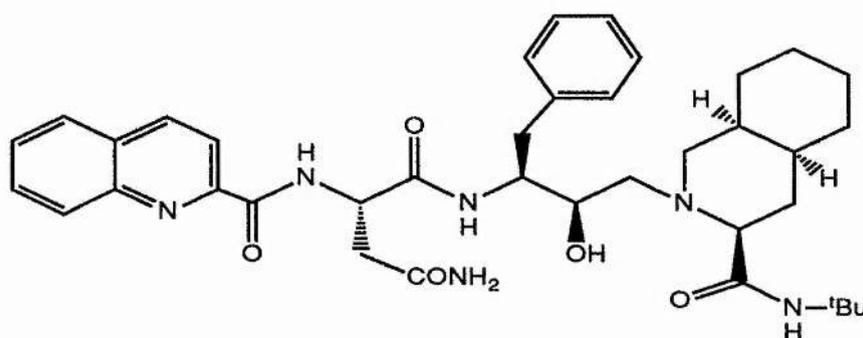
(26): Possessing an  $IC_{50}$  value of 1.0 nM.



(27): Symmetry-based difluoroketone ( $K_i = 0.1$  nM).

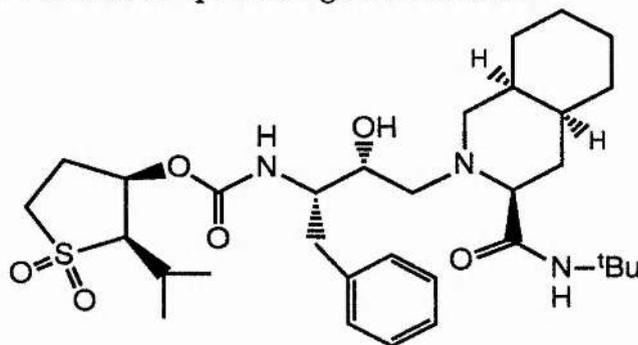
### 1.6.5 Hydroxyethylamines

Another highly potent class. Their efficacy is demonstrated by Ro-31-8959 (28)<sup>101</sup> which is currently being evaluated in a clinical setting and looks likely to be the first HIV proteinase drug on the market. It is highly potent *in vitro* ( $K_i = 0.12$  nM) but it also displays *in vivo* activity in the low nanomolar range against HIV-1, HIV-2, SIV and also some AZT resistant strains.<sup>102</sup>

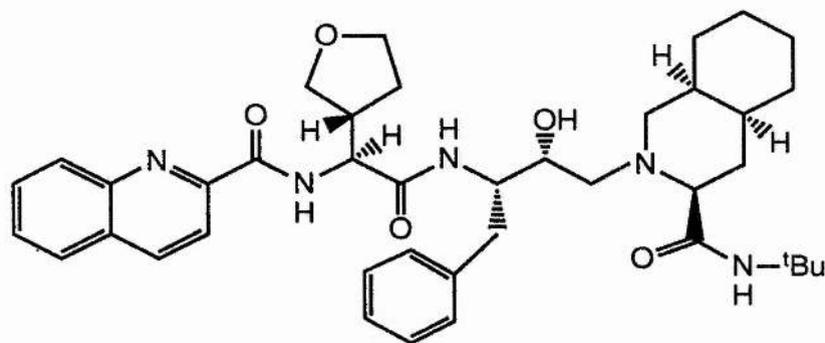


(28): Ro 31-8959, a highly potent inhibitor ( $K_i = 0.12$  nM).

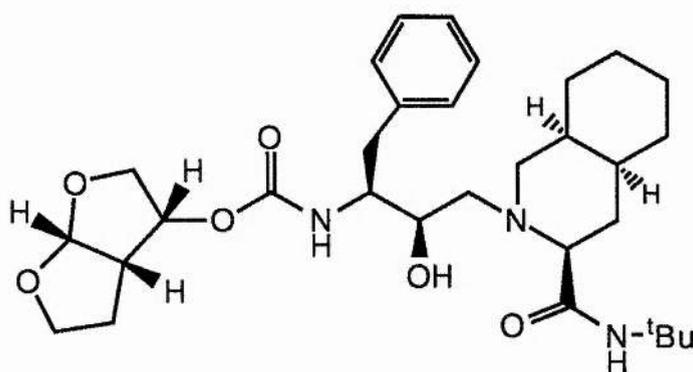
The success of Ro-31-8959 has prompted a good deal of derivation. Notable work comes from Merck's laboratories at West Point including the report of a sulfolane (29) ( $K_i = 3$  nM).<sup>103</sup> Also, incorporation of a tetrahydrofuranylglycine in place of the Asn in Ro-31-8959 led to the extremely potent inhibitor (30), which has an  $IC_{50}$  of 0.054 nM.<sup>104</sup> More recently they have entirely replaced the quinoline-2-carbonyl-Asn moiety with a fused *bis*-tetrahydrofuran ligand, giving a potency equivalent to (28), despite being a lot shorter.<sup>105</sup>



(29): Possessing a sulfolane ligand ( $K_i = 3$  nM).



(30): Incorporating the (3'R)-Thfg ligand ( $IC_{50} = 0.054 \text{ nM}$ ).

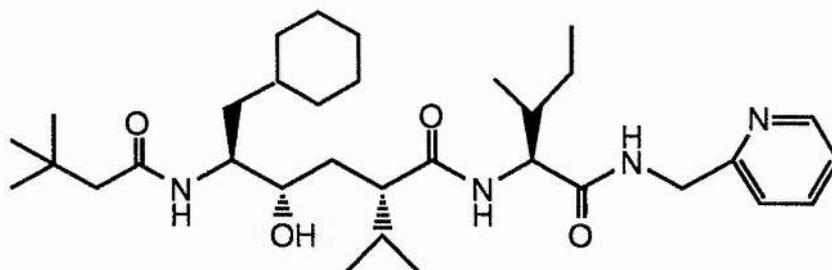


(31): Incorporating a fused bis-THF ligand ( $IC_{50} = 1.8 \text{ nM}$ ).

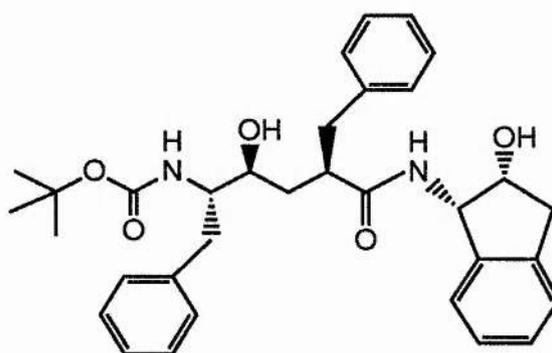
### 1.6.6 Hydroxyethylenes

As might be expected after Dreyer's study found it to be the most potent isostere, the hydroxyethylene is the most used of all the transition state mimics. Popular residues include benzyl in  $P_1$  and  $P_1'$  for a phenylalanine analogue, although cyclohexylmethyl is occasionally employed with equal effectiveness. At  $P_2'$ , the 1-amino-2-hydroxy-indan is a potent addition as is amino-benzimidazole. At  $P_3'$ , the amino-methyl-pyridine is a popular choice. These are illustrated below: compound (32) has a  $K_i$  of  $70 \text{ nM}$ ,<sup>106</sup> compound (33) has an  $IC_{50}$  of  $0.3 \text{ nM}$ <sup>107</sup> and compound (34) is still the most potent HIV-1 inhibitor to date with an  $IC_{50}$  of  $0.03 \text{ nM}$ .<sup>108</sup> This last inhibitor has the unusual styrene at  $P_1'$ , which shows that the pocket to be of large size. This last point is emphasised by

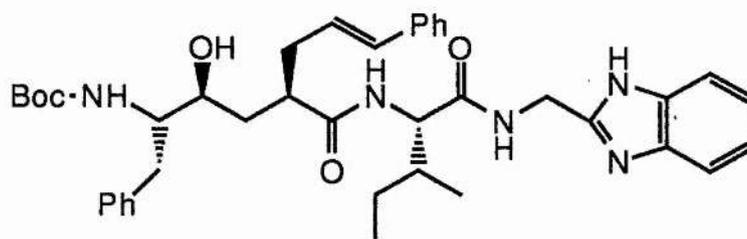
modelling studies that showed that C<sub>60</sub> derivatives will fit snugly into the active site.<sup>109</sup>



(32): *Cha-Val hydroxyethylene inhibitor* ( $K_i = 70 \text{ nM}$ ).

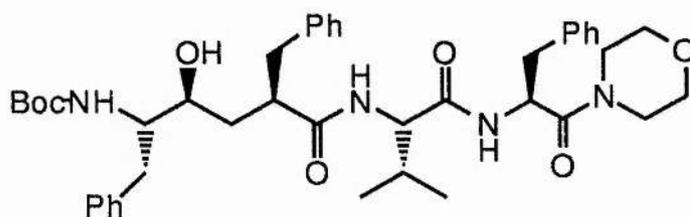


(33): *Highly potent pentapeptide inhibitor* ( $IC_{50} = 0.3 \text{ nM}$ ).



(34): *The most potent inhibitor of HIV-1 protease to date.*

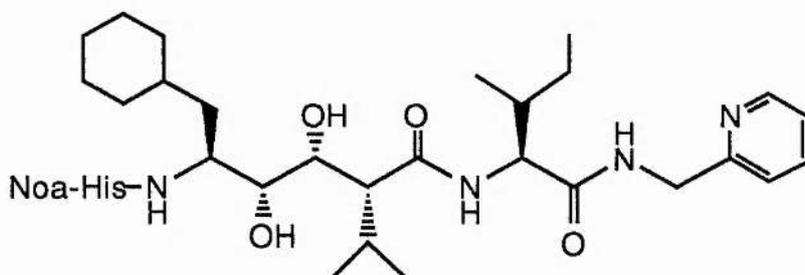
Another notable hydroxyethylene is CGP53437 (35) which is potent ( $K_i = 0.2 \text{ nM}$ ) but more interestingly, displays potent *in vivo* activity and oral bioavailability in mice.<sup>110</sup>



(35): CGP53437, orally bioavailable hydroxyethylene.

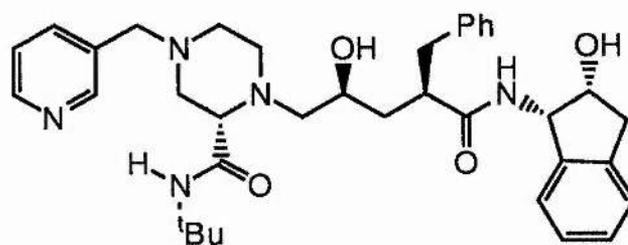
### 1.6.6.1 Modified Hydroxyethylenes

A number of subtle changes to the basic hydroxyethylene template are now being made in order to alter the properties of the compounds such that they will make good drugs. One example is the dihydroxy-ethylene (36) which has a  $K_i < 1$  nM.<sup>111</sup> Other compounds of note are the hydroxyethyl-urea of Getman and co-workers<sup>112</sup> and the substituted hydrazine of Sham and co-workers.<sup>113</sup>



(36): Dihydroxyethylene inhibitor ( $K_i$  value  $< 1$  nM).

Another orally active compound is the hydroxy-amino-pentane-amide of Vacca and co-workers, L-735,524 (37).<sup>114</sup> This had a  $K_i$  of 0.34 nM against HIV-1 proteinase and 3.3 nM against HIV-2 proteinase. It also showed effective anti-viral activity and displayed oral bioavailability of between 14.2 % in monkeys and 71.6 % in dogs and it is now being evaluated in phase I clinical studies.



(37): L-735,524, a potent orally available hydroxy-amino-pentane-amide.

### 1.6.7 C<sub>2</sub>-Symmetric Inhibitors

The HIV-1 proteinase is a homodimer; it is symmetric about a C<sub>2</sub> axis. Therefore, there could be a strategic advantage in designing inhibitors which are similarly symmetric. Kempf and co-workers first proposed this,<sup>115</sup> suggesting that there might be an increase in potency as well as an increase in specificity, since mammalian aspartic proteinases, though bilobal, are not symmetric. There is also a reduction in peptidic character.

They proposed two possible inhibitor cores, as shown in Fig. 1.12.

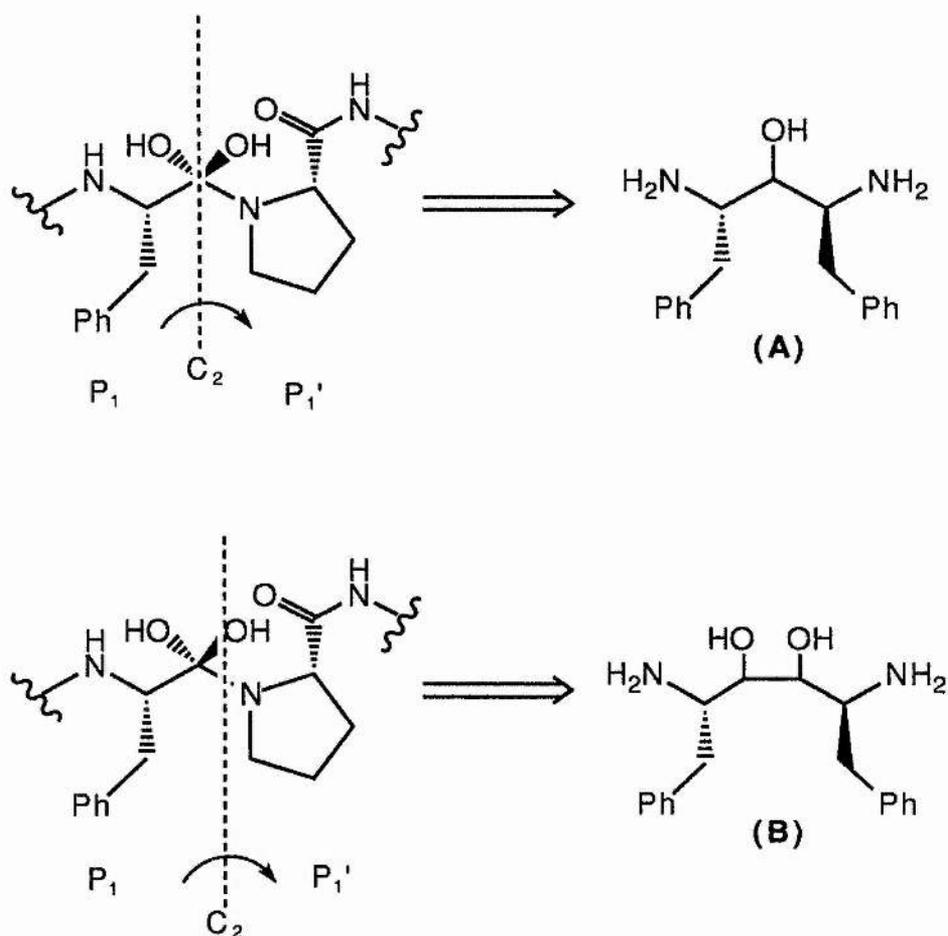
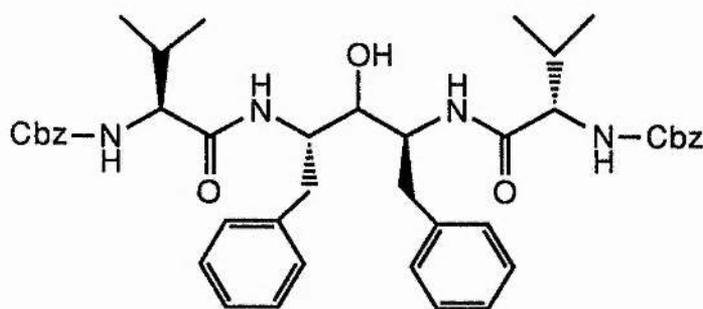


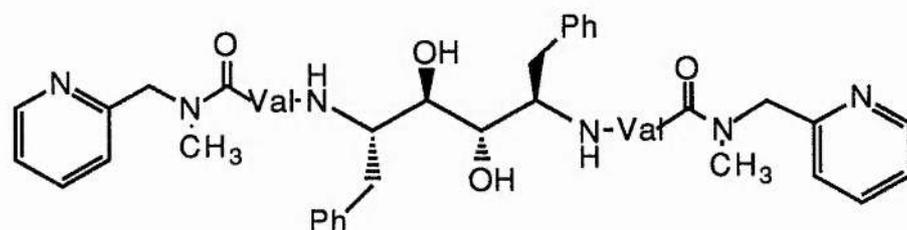
Figure 1.12: Design of  $C_2$ -symmetric inhibitors.

Strategy A gave A-74704 (**38**), a potent inhibitor of HIV-1 proteinase *in vitro* ( $IC_{50} = 3$  nM) and possessing sub-micromolar anti-viral activity.



(**38**): A-74704,  $C_2$ -Symmetric inhibitor ( $IC_{50} = 3$  nM).

Using the second strategy gave diols which were generally 10-50 times more potent than its equivalent alcohol. For example, the diol equivalent to (38) possessed an  $IC_{50}$  of 0.22 nM. Interestingly, both (3R, 4R) and (3R, 4S) diols were equi-potent. However, poor solubility meant they were unsuitable as anti-viral agents. Studies on these compounds showed that the terminal positions were exposed to the solvent. So solubility enhancing efforts were directed at those portions of the molecule. This did lead to improved solubility and the potency was retained. The most potent of this series was A-77003 (39) and this was put into phase I and phase II clinical trials.<sup>116</sup>

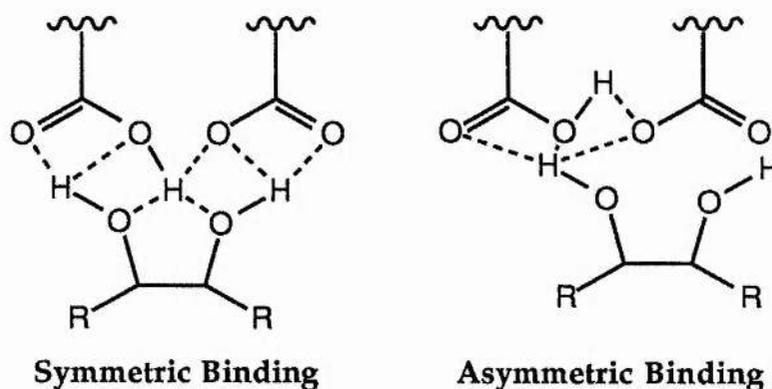


(39): A-77003,  $C_2$ -symmetric diol ( $IC_{50} < 1$  nM).

Although some AZT resistant strains of HIV have been observed, it was hoped that a similar situation would not occur for the anti-proteinase drugs; however, there have been reports of a proteinase strain with increased resistance to  $C_2$  symmetric inhibitors. One report found substantial decrease in inhibitory activity in A-77003 as well as several other diol-based inhibitors, although Ro-31-8959 was equally effective against all strains.<sup>117</sup> The resistant mutant was the result of a single amino acid substitution from Arg-8 to Gln or Lys, but it should be noted that Arg-8 is highly conserved. Another report isolated five strains that were 6 to 8-fold less susceptible to P9941, a compound not dissimilar to A-77003.<sup>118</sup> Again, Ro-31-8959 was unaffected.

Further, crystallographic studies have shown that the electronic environment around the active site aspartates is asymmetric which may promote asymmetric binding, even in inhibitors assuming an exact  $C_2$  symmetric

conformation.<sup>119</sup> Dreyer and co-workers confirmed this,<sup>120</sup> saying that one hydroxyl binds between the aspartates (see Fig 1.13) while the other one can only make a minor contribution and may contribute negatively.

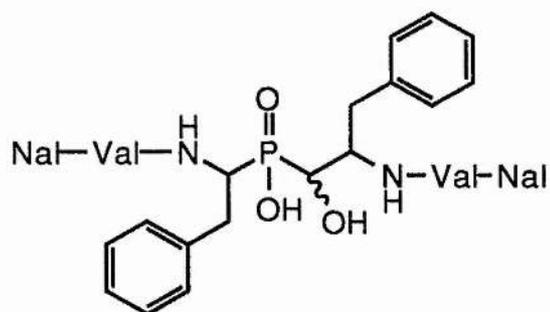


*Figure 1.13: Symmetric and Asymmetric Binding in Symmetric Diols.*

To test this theory, Kempf and co-workers synthesised A-78791, the deshydroxy analogue of A-77003 and found it to have greater potency than the diol.<sup>121</sup> This led to the discovery of A-80987, which is a shorter, bioavailable analogue of A-78791 and has now been entered into phase I clinical trials.<sup>122</sup>

#### 1.6.7.1 Other C<sub>2</sub> Symmetric Inhibitors

Other reported symmetrical inhibitors include the difluoroketones of Sham and co-workers (see section 1.6.4) and the phosphinic acid analogue of A-74704 (see section 1.6.2). Another notable inhibitor is the combined phosphinic acid/hydroxyethylene (41) of Stowasser and co-workers, which was designed to specifically examine asymmetric binding and gave an IC<sub>50</sub> of 0.5 nM.<sup>123</sup>



(41): Combined Phosphinic acid/ Hydroxyethylene ( $IC_{50} = 0.5 \text{ nM}$ ).

Following on from the work of Kempf and co-workers, Barrish and co-workers proposed a third axis of rotation, Axis C (see Fig. 1.14). This led them to synthesise the amino-diol (42) which showed an  $IC_{50}$  of 125 nM.<sup>124</sup>

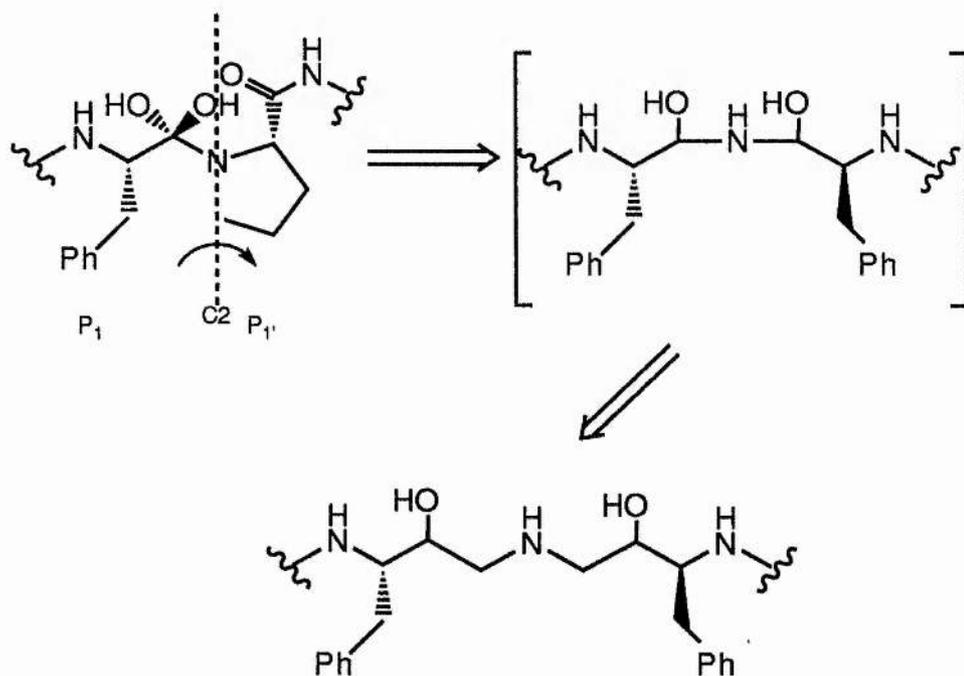
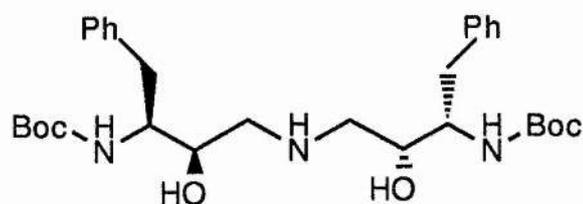


Figure 1.14: Axis C.



(42): Amino-diol inhibitor derived from Axis C ( $IC_{50} = 125 \text{ nM}$ ).

### 1.6.8 Non-Peptides

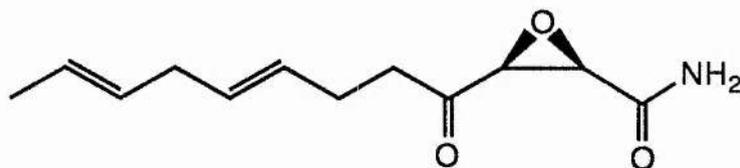
Though peptides can be extremely potent enzyme inhibitors, they rarely make good drugs. What is required is a combination of good biological activity and good bioavailability; it must not only be an effective agent, it must also be able to reach the target cell. Peptides tend not to meet these requirements because they are often broken down either by proteolytic enzymes or simply due to instability under the biological conditions. They also tend to be rapidly eliminated from plasma and have poor trans-membrane transport properties.<sup>125</sup>

There are ways round this; this has met with limited success with renin. A reduction in molecular weight, less amide bonds and greater water solubility all enhance the pharmacological properties of compounds. Likewise, blocking the terminal positions to avoid enzymatic degradation is similarly useful.<sup>126</sup>

However, it is often the case that peptides are used to pinpoint the binding requirements for an enzyme and what is learnt from these studies is applied to the design of non-peptide inhibitors which will hopefully have the correct biological properties to make good drugs.

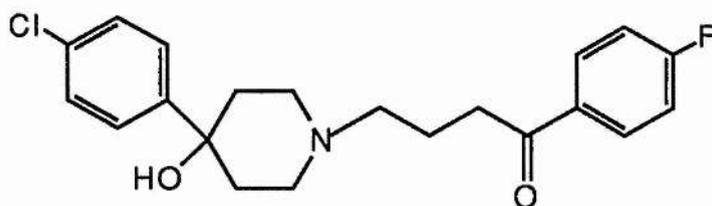
#### 1.6.8.1 Natural Products

One of the first non-peptide inhibitors of HIV-1 proteinase discovered was the anti-fungal antibiotic Cerulenin (43), a weak inhibitor with an  $IC_{50}$  of 2.5 mM.<sup>127</sup>



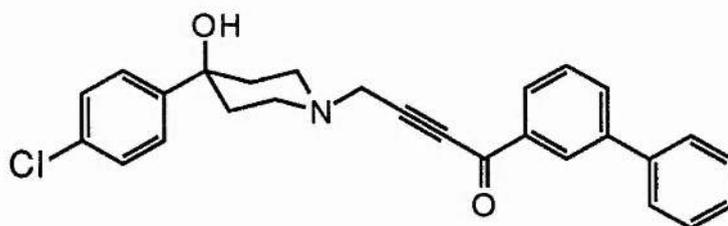
(43): Antifungal antibiotic Cerulenin ( $IC_{50} = 2.5$  mM).

Molecular modelling studies, in which molecules were docked into the enzyme active site and evaluated for complementarity, revealed Haloperidol (44), which had a  $K_i$  of 100  $\mu$ M.<sup>128</sup>



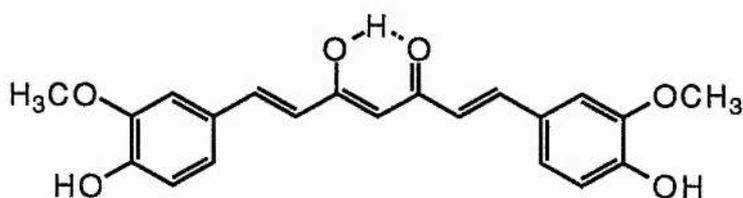
(44): *Haloperidol* ( $K_i = 100 \mu\text{M}$ ).

A number of haloperidol-related inhibitors have since been produced, the most potent being UCSF 142 (45) which was an irreversible inhibitor with a  $K_i$  of approximately  $10 \mu\text{M}$  against both HIV-1 and HIV-2 proteinases.<sup>129</sup>



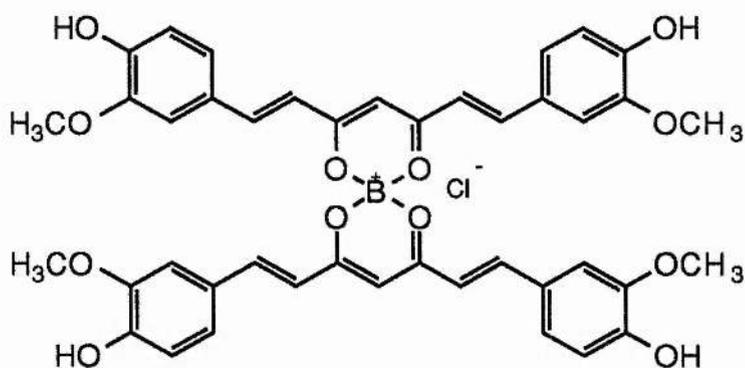
(45): *UCSF 142, Haloperidol-related inhibitor* ( $K_i = 10 \mu\text{M}$ ).

Curcumin (46) was also found to be a weak inhibitor of HIV-1 proteinase with a potency comparable to Haloperidol.<sup>130</sup>



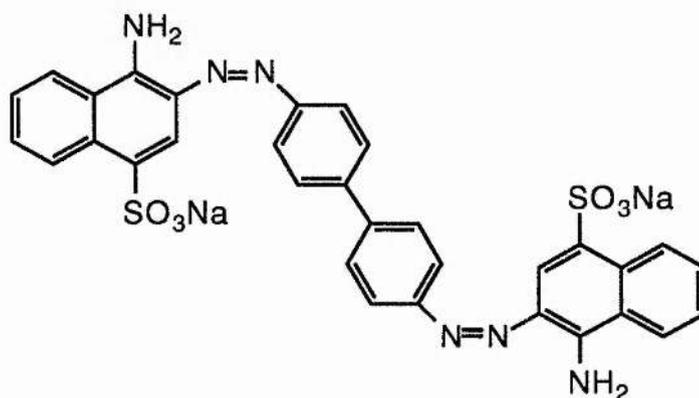
(46): *Curcumin* ( $IC_{50} = 100 \mu\text{M}$ ).

The authors also made a variety of boron complexes; the best one was (47) below, with an  $IC_{50}$  of  $6 \mu\text{M}$ .



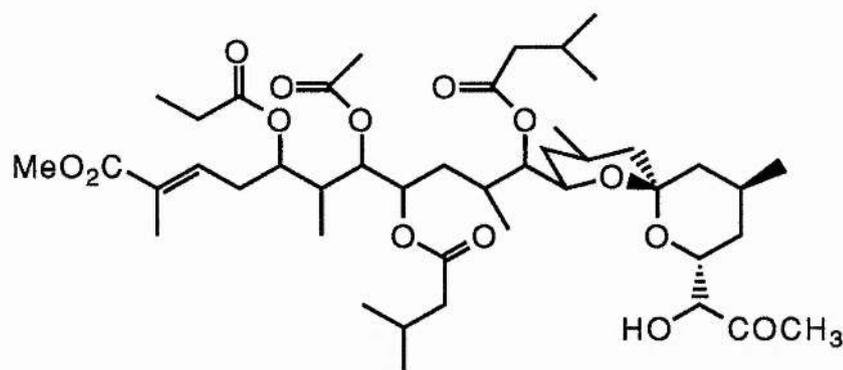
(47): *Curcumin Boron Complex* ( $IC_{50} = 6 \mu M$ ).

A remarkable result was that the dicarboxylic acid  $HO_2C-(CH_2)_{20}-CO_2H$  also inhibited the proteinase with an  $IC_{50}$  of  $12 \mu M$ .<sup>131</sup> Brinkworth and co-workers proposed this to be due to the dianion and thus synthesised a wide range of disulfonates.<sup>132</sup> This led to (48) which had an  $IC_{50}$  of  $0.78 \mu M$ .

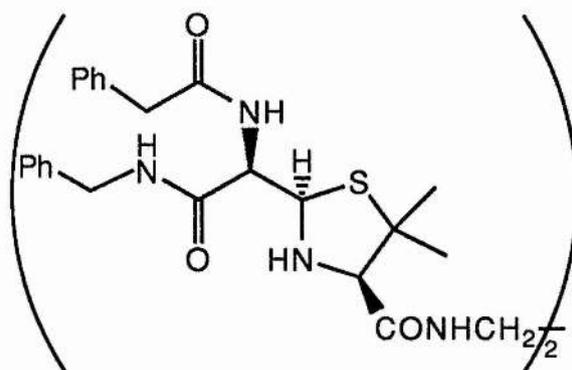


(48): *Disulfonate* ( $IC_{50} = 0.78 \mu M$ ).

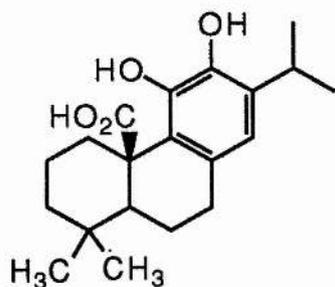
Potts and Faulkner isolated didemnaketals which showed activity against the enzyme.<sup>133</sup> Didemnaketal A (49) had an  $IC_{50}$  of  $2 \mu M$ .

(49): *Didemnaketal A*.

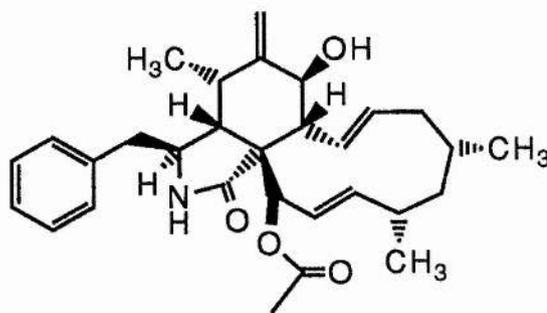
Humber and co-workers came upon a penicillin derivative. During a screening program, they found a crude penicillin dimer to be active, but on isolation found that the main component to be inactive. The active component was eventually found to be a diester derived from the opening of the  $\beta$ -lactam. A number of derivatives were prepared to increase the activity leading to (50) with an  $IC_{50}$  of 0.9 nM.<sup>134</sup>

(50): A Penicillin-derived symmetrical dimer ( $IC_{50} = 0.9$  nM).

Other natural products have been found to be inhibitors of HIV-1 proteinase: these include carnosolic acid (51),<sup>135</sup> cytochalasins such as L-696,474 (52)<sup>136</sup> and tetronic acid homologues (53) - (58) (see Fig. 1.15).<sup>137</sup>



(51): Carnosolic acid.



(52): L-696,474.

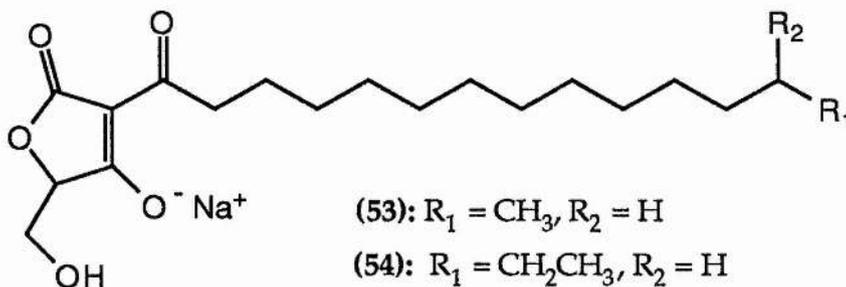
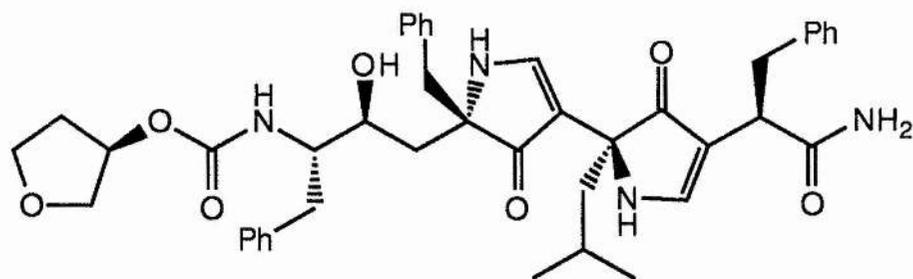
(53):  $R_1 = \text{CH}_3, R_2 = \text{H}$ (54):  $R_1 = \text{CH}_2\text{CH}_3, R_2 = \text{H}$ (55):  $R_1 = \text{CH}_3, R_2 = \text{CH}_3$ (56):  $R_1 = \text{CH}_2\text{CH}_2\text{CH}_3, R_2 = \text{H}$ (57):  $R_1 = \text{CH}(\text{CH}_3)_2, R_2 = \text{H}$ (58):  $R_1 = \text{CH}(\text{CH}_3)(\text{CH}_2\text{CH}_3), R_2 = \text{H}$ 

Figure 1.15: Tetronic Acid Homologues.

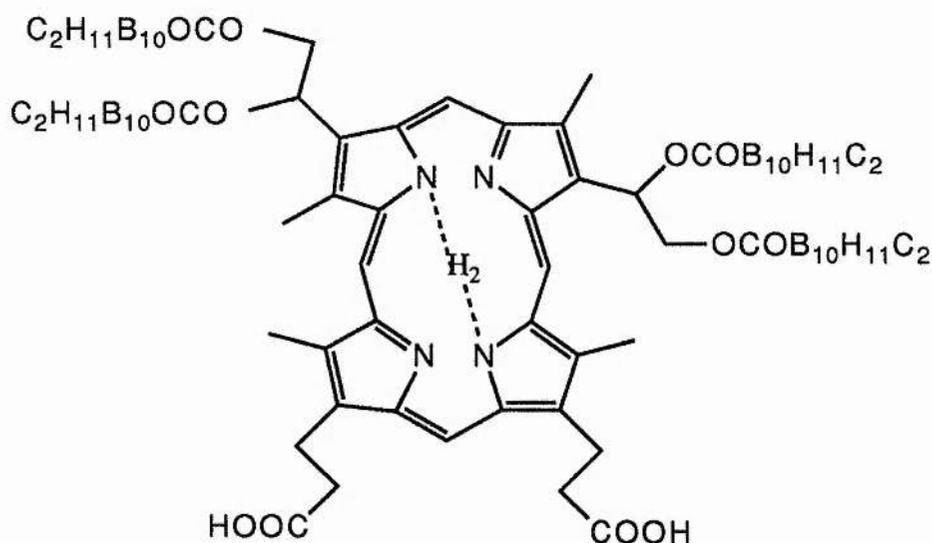
### 1.6.8.2 Heterocyclic Inhibitors

A number of heterocyclic compounds have been prepared as HIV-1 proteinase inhibitors. Amos B. Smith III and co-workers have been developing peptidomimetic molecules, in particular  $\beta$ -sheet mimics, and applied their work to HIV proteinase.<sup>138</sup> Their initial target was at least of comparable potency to the hydroxyethylene they had based their design on, but only a minor amount of optimisation led to (59) which was potent *in vitro* ( $\text{IC}_{50} = 1.3 \text{ nM}$ ) but also displayed good transport properties, having a  $\text{CIC}_{95}$  of 800 nM ( $\text{CIC}_{95}$  is the cellular inhibition concentration to reduce enzyme activity by 95 %).



(59):  $\beta$ -Sheet mimic ( $IC_{50} = 1.3$  nM).

A number of porphyrins have also been prepared, but it was the boronated porphyrin (60) that was found to be most potent of the series ( $IC_{50} = 185$  nM).<sup>139</sup>



(60): Boronated Porphyrin ( $IC_{50} = 185$  nM).

Lam and co-workers synthesised a series of cyclic ureas which were designed to displace the structural water molecule that binds the flap isoleucines and thus keeps the flaps closed.<sup>140</sup> The resulting molecules were highly potent and very selective, with no inhibition of renin, pepsin or cathepsin D at concentrations greater than 3000 times the  $K_i$  they showed against HIV-1 proteinase. Furthermore, X-ray crystallography showed that the structural water was indeed displaced, lending credence to this strategy. (61), containing a cyclopropane side-chain, showed a  $K_i$  of 2.14 nM and was 100 % bioavailable in rats, although this

figure was somewhat lower in dogs. DMP 323 (62) was of ten times greater in vitro potency ( $K_i = 0.27$  nM) and, although its bioavailability in rats was less, in dogs it was of equivalent bioavailability and this compound has been entered into phase I clinical trials.

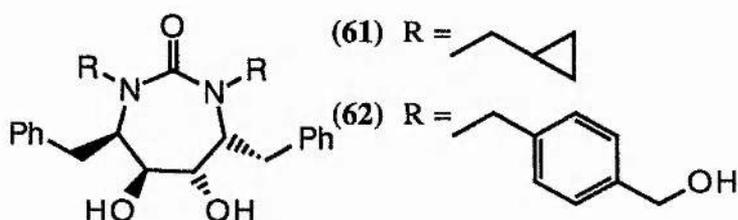
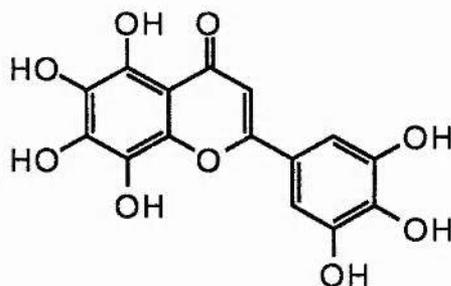
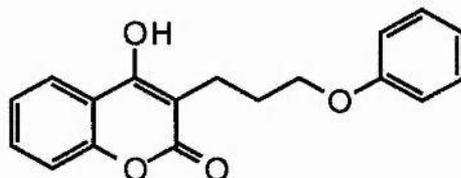


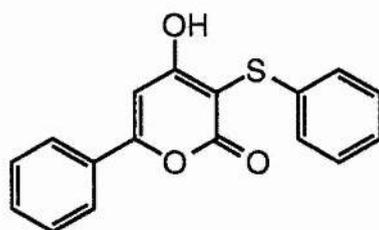
Figure 1.16: Cyclic Ureas.

Flavone and pyranone-type inhibitors have also been a popular target. A range of natural and modified flavones have been tested and the best of that series was the fully demethylated Gardenin A (63), with an  $IC_{50}$  of 11  $\mu$ M.<sup>141</sup>



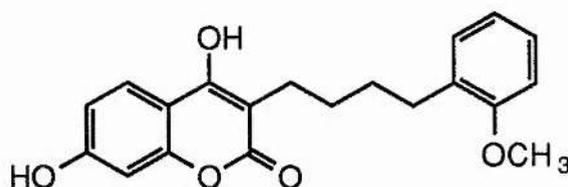
Many groups took up this lead. Tummino and co-workers have reported 4-hydroxy-pyranones such as (64) and (65) which display low micromolar inhibition but are completely achiral.<sup>142</sup>





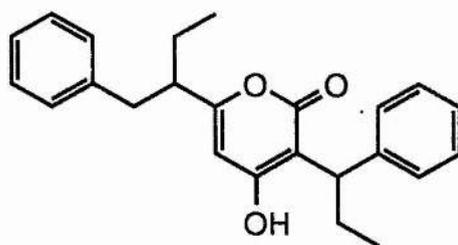
(65): *4-Hydroxy-6-Phenyl-pyran-2-one* ( $IC_{50} = 3.5 \mu M$ ).

Lunney and co-workers prepared a range of hydroxy-coumarins which were sub-micromolar, the best being (66) shown below which had an  $IC_{50}$  of  $0.52 \mu M$ .<sup>143</sup>



(66): *4-Hydroxy-Coumarin* ( $IC_{50} = 0.52 \mu M$ ).

The best of this series of compounds, however, is U-96988 (67) which, as a mixture of diastereomers possessed a  $K_i$  of 38 nM (32 nM against HIV-2 proteinase).<sup>144</sup> It was also selective; at  $10 \mu M$  it didn't inhibit either renin or gastricsin, and only inhibited pepsin and cathepsin D approximately 50%. Furthermore it was found to be orally bioavailable in rats and dogs and has now entered phase I clinical trials.



(67): *U-96988, potent Hydroxy-Pyranone* ( $K_i = 38 \text{ nM}$ ).

## **CHAPTER TWO**

### **RESULTS AND DISCUSSION**

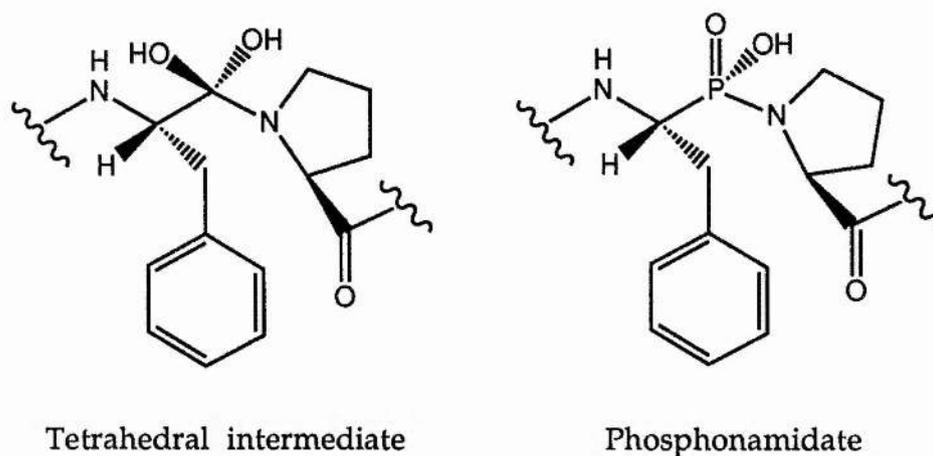
## 2.0 Results and Discussion

### 2.1 Design of HIV-1 Proteinase Inhibitors

The identification of a viral proteinase in HIV revealed a promising new target for therapeutic intervention. It has been shown that proteinase activity is vital for viral maturation<sup>50</sup> so inhibitors of the enzyme should slow or even stop the spread of infection and the onset of AIDS.

The strategy used by the majority of research groups was the same as was used so successfully with renin,<sup>86</sup> in which a good peptide substrate sequence is identified and the scissile bond replaced by a non-hydrolysable moiety.

We were interested in synthesising inhibitors for HIV-1 proteinase, in order to both study the enzyme and evaluate a novel series of inhibitors. The phosphoramidate isostere was selected for incorporation into a series of HIV-1 proteinase inhibitors due to close geometric and electronic resemblance to the transition state of the substrate (see Fig. 2.1). It has the correct tetrahedral



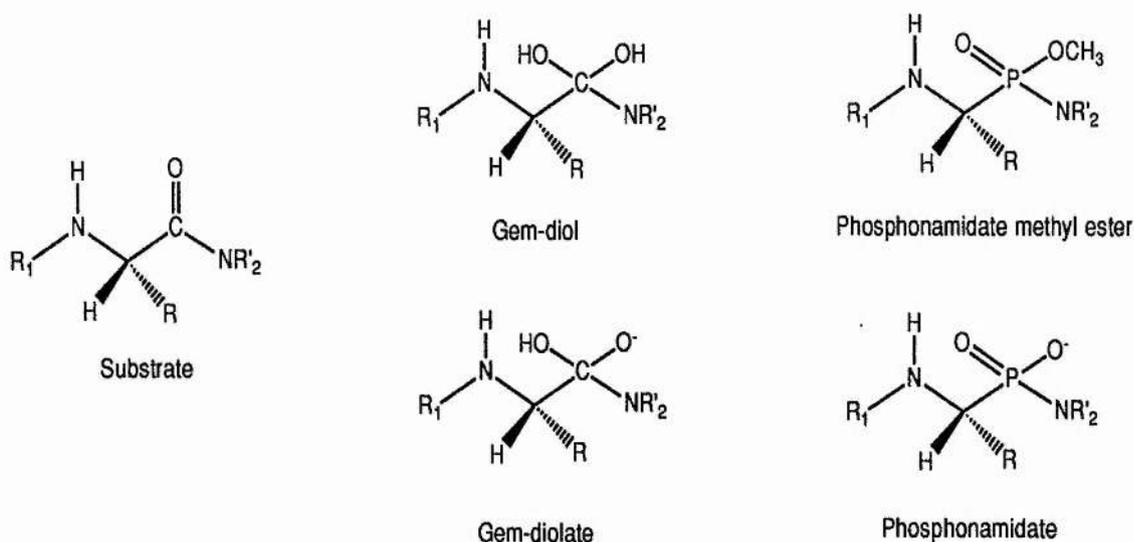
*Figure 2.1: Comparison of Tetrahedral Intermediate and Phosphoramidate Isostere.*

geometry as well as providing both oxygens present in the *gem*-diol. It is worth noting that phosphorus-oxygen bonds are approximately 10-15 % longer than their corresponding carbon-oxygen ones.<sup>145</sup> This means that the P-O double bond can conceivably mimic the lengthening carbonyl as it is attacked by the

nucleophilic water molecule and the P-O single bond can mimic the approaching water.

The phosphoramidate, unlike many other transition state mimics, also contains the nitrogen in P<sub>1</sub>' which is a potentially important source of hydrogen-bond interactions within the enzyme's active site.

A number of problems do exist; at the assay pH, the phosphoramidate will be negatively charged (the pK<sub>a</sub> of a phosphoramidate is approximately 3 to 3.5<sup>146</sup>) and this will interact unfavourably with a similarly negatively-charged aspartate. Also there is a question over the exact nature of the transition state or high energy intermediate, as to whether it is a *gem*-diol or a *gem*-diolate (see Fig. 2.2). A simple way to circumvent this problem is to investigate the methyl ester (which is an intermediate in the synthesis of the free phosphoramidate anyway) and compare their interactions.

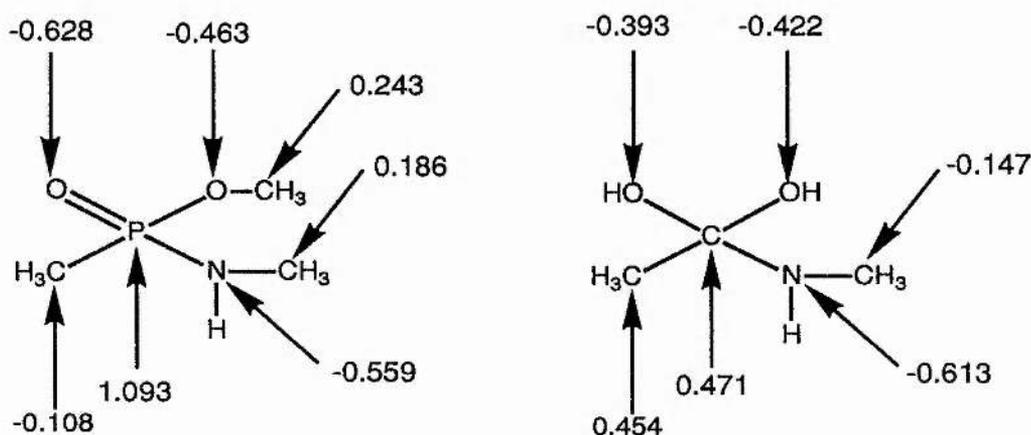


*Figure 2.2: Comparison of Gem-Diol and Gem-Diolate with Phosphoramidate Methyl Ester and Phosphoramidate Respectively.*

McLeod *et al.* found that the methyl esters are much more acid stable<sup>147</sup> which is immediately advantageous. The P-N bond in the phosphoramidate is acid-labile and possesses a short half life in acidic solution. Furthermore, although it

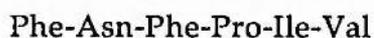
can accept hydrogen-bonds from the active site aspartate, the phosphoramidate has no hydrogen-bond donating ability unlike, for example, a hydroxyethylene. The fact that the hydroxyl-containing compounds tend to produce the most potent inhibitors suggests that both hydrogen-bond acceptor and donor ability is crucial for optimal interaction with the active site aspartates.

Despite this, in terms of charge density distribution, the phosphoramidate methyl ester has been shown to be a good mimic of a *gem*-diol intermediate. There is overall agreement between the relative charges on each atom as calculated by semi-empirical quantum mechanics<sup>148</sup> (see Fig. 2.3). Particularly notable are the oxygens and nitrogen, for which the phosphoramidate moiety was first chosen for investigation. *Ab initio* calculations have shown similar results to those shown above.<sup>149</sup>



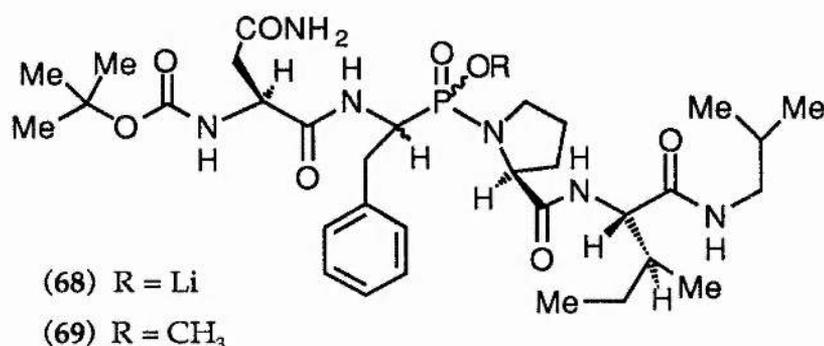
**Figure 2.3:** Comparison of the Relative Charges on a Phosphoramidate Methyl Ester and a *Gem*-Diol.

The design of an initial target was based on the sequence:



Each of these residues appears at least twice in their respective positions in the natural substrates (see Fig. 1.6). The Phe-Pro amide is the scissile bond in this sequence, which was replaced with a phosphoramidate. At the C-terminal

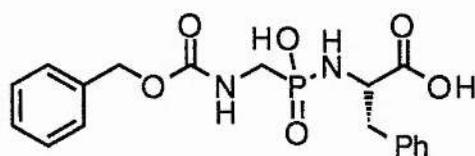
position was the Val surrogate, *iso*-butylamine and in P<sub>3</sub> was a benzyloxycarbonyl (Cbz) group. Camp<sup>150</sup> and Hawkins<sup>151</sup> had begun this synthesis and Camp had found that the Cbz group was unstable under the conditions of phosphoramidate methyl ester deprotection, therefore this was substituted with a *tert*-butyloxycarbonyl (Boc) group. The initial target thus became (68), with the intention to also examine the methyl ester (69).



This target had been reached by Camp *et al.*<sup>152</sup> and the synthesis of a series of phosphoramidate-based inhibitors was then undertaken. This included the synthesis of small peptide fragments for incorporation on the C-terminal side of the inhibitor and also synthesis of phosphoamino acids for incorporation at P<sub>1</sub>.

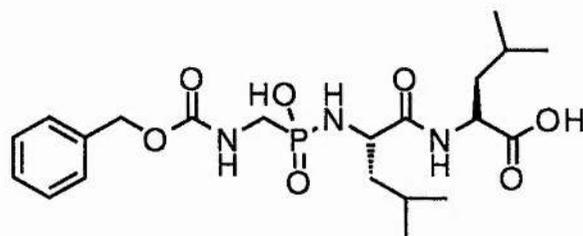
## 2.2. Synthesis and Testing of Phosphoramidate-Based Inhibitors for HIV-1 Proteinase

Phosphoramidates were first used as inhibitors of the zinc protease carboxypeptidase A. Although different in many respects, zinc and aspartic proteases do share similar mechanisms. The dipeptide (70) synthesised by Bartlett and Jacobsen incorporating the phosphoramidate gave a K<sub>i</sub> of 6 nM at pH 6.0,<sup>153</sup> which is also the pH for the HIV protease assay.



(70): *Dipeptide Phosphonamidate Inhibitor of Carboxypeptidase A.*

Bartlett contributed further to the area of phosphorus-containing inhibitors with the synthesis of (71), the most potent inhibitor yet for the zinc peptidase thermolysin, which had a  $K_i$  of 9.1 nM.<sup>154</sup>



(71): *Phosphonamidate Inhibitor of Thermolysin.*

More relevant to HIV-1, McLeod *et al.* synthesised racemic dipeptide phosphonamidates which showed  $K_i$  values around 50  $\mu\text{M}$ <sup>147</sup> (see Fig. 2.4). Compared with other inhibitors in the literature (see Section 1.6), this is not outstanding inhibition, but that such a small molecule bound at all was interesting. It was observed that the methyl esters of the phosphonamidates also inhibited, in fact the symmetric Phe-Phe analogue inhibited better as the methyl ester. Furthermore, the inhibitors tested were racemic, so contained only a fraction of the preferred diastereomer. This all proved very encouraging for the successful use of phosphonamidates as inhibitors of the HIV-1 proteinase.

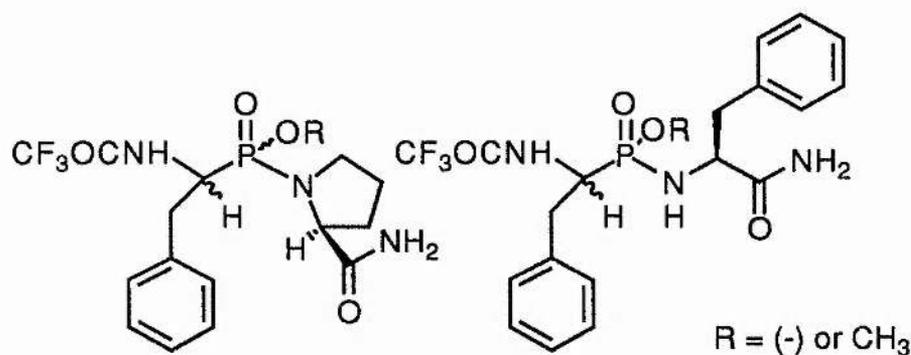
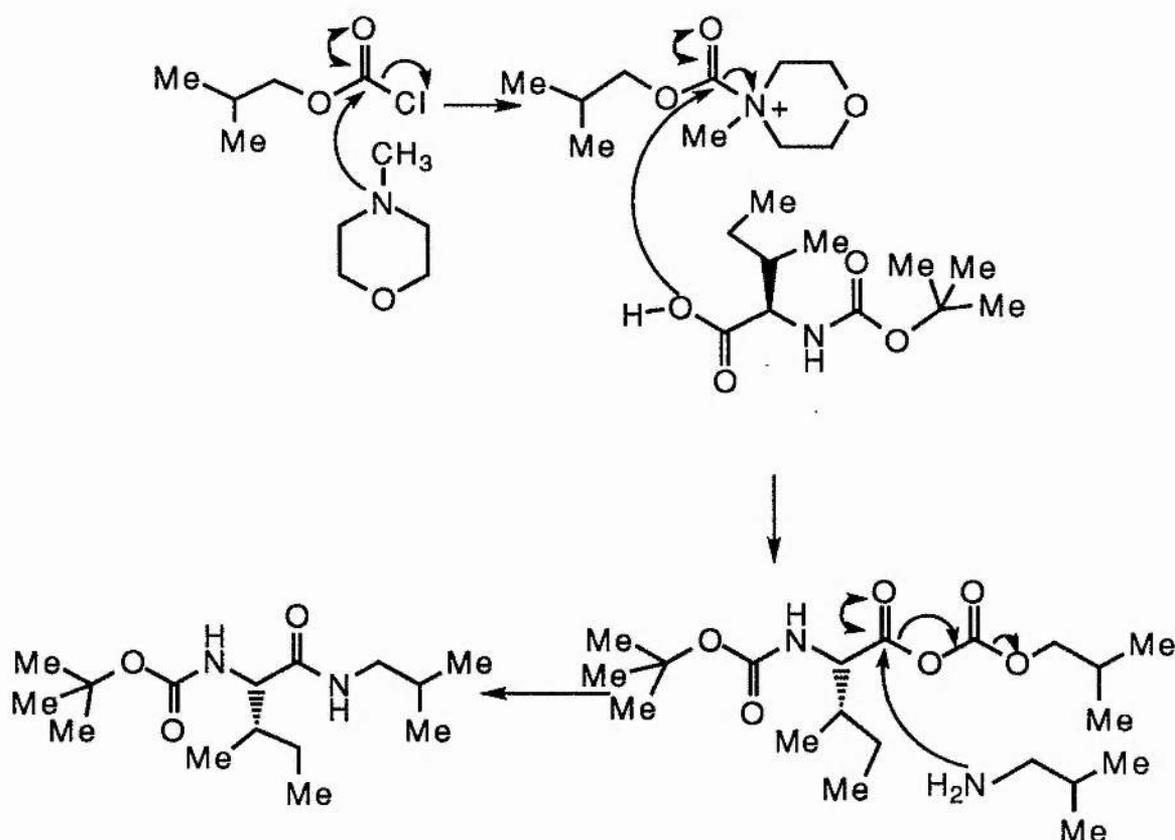


Figure 2.4: McLeod et al.'s Dipeptide Inhibitors of HIV-1 Proteinase.

### 2.2.1. Solution-Phase Peptide Synthesis.

A number of excellent methods exist for making peptides in the solution phase, including the use of acid chlorides or carbodi-imide coupling methods. For our work, the mixed anhydride procedure was used (see Scheme 2.1).<sup>150</sup>



Scheme 2.1: Mechanism of the Mixed Anhydride Coupling Between (2S)-Isoleucine and iso-Butylamine.

What is required in a coupling reaction is consistently high yields with no racemisation, which can be a problem where a base is employed. While methylamine gave a very rapid reaction, it also gave maximum racemisation. For this reason, *N*-methyl morpholine and *iso*-butyl chloroformate have been found to be the best combination.<sup>155</sup> For some combinations of amino acids, the mixed anhydride method has been less useful, particularly in the case when one or both starting materials are barely soluble in THF; however, for our work, no such problems were encountered and its good yields and ease of use made it the method of choice for this part of the synthesis. The newly formed Boc-protected peptide, easily recognisable by its distinctive *t*-butyl peak at approximately 1.5 ppm in its <sup>1</sup>H nmr spectrum, was then deprotected using hydrogen chloride gas in ethyl acetate.

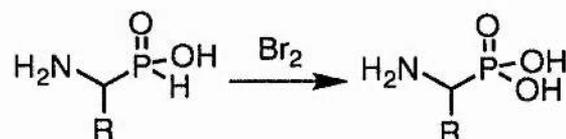
This sequence of reactions was used to synthesise the initial target of (2*S*)-Pro-(2*S*)-Ile-NHiBu (72) and also (2*S*)-Phe-(2*S*)-Ile-NHiBu (73) which incorporates a degree of symmetry into the final inhibitor. These peptides could then be used in the key step, that of formation of the phosphoramidate.

### 2.2.2. Synthesis of Phosphinic Acid Esters.

To synthesise a phosphoramidate, it is vital that a reliable method of obtaining its precursor is available. The classical route to the racemic 1-aminoalkyl phosphinic acids is by the addition of trialkyl phosphites to imines under rather harsh conditions.<sup>156</sup> A milder method utilised the addition of the trimethyl silyl esters of the phosphorous acids to imines.<sup>157</sup> The three component condensation of carbamate, aldehyde and phosphite has also proven useful.<sup>158</sup> This method provides an access to higher alkane phosphinic acids but it cannot be used in the synthesis of amino methane phosphinic acid. However, to complement this methodology, Oleksyszyn and Subotkowska developed a parallel synthesis, again using a carbamate and phosphite, plus

paraformaldehyde and acetic anhydride in glacial acetic acid.<sup>159</sup> Yuan *et al.* provided a direct route to the phosphinic half acid half methyl ester using phosphorous trichloride and acetyl chloride, instead of the phosphite used above, followed by methanolysis.<sup>160</sup> The role of the acetyl chloride is thought to be associated with the formation of a metaphosphate species which affords the monoesters in the subsequent alcoholysis.

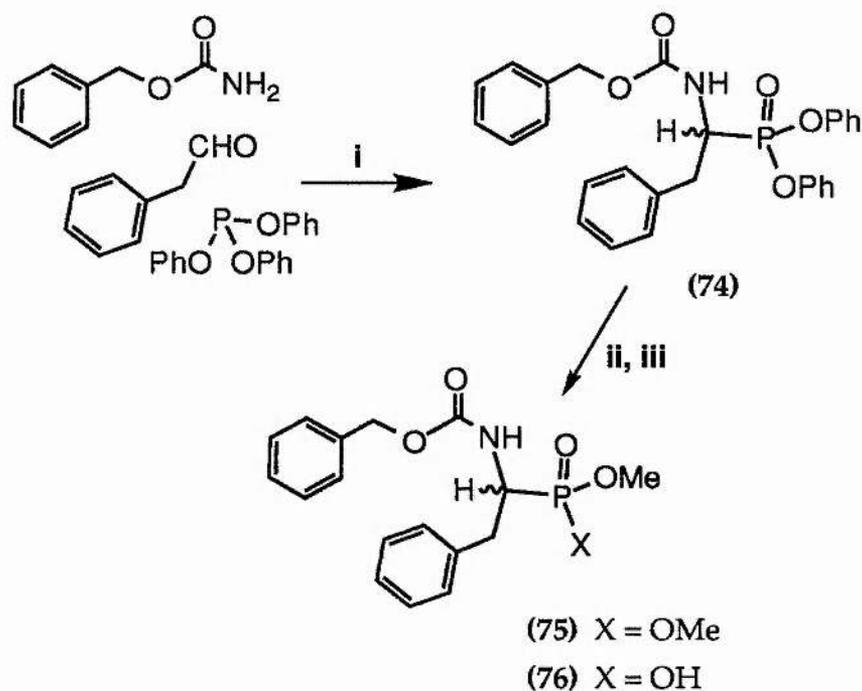
The aminoalkyl phosphinic acids are also accessible *via* the equivalent phosphonous acids by an extremely facile oxidation step using aqueous bromine (see Scheme 2.2).<sup>161</sup>



*Scheme 2.2: Oxidation of Amino-Phosphonous Acid to Amino-Phosphinic Acid.*

Virtually all the phosphonous amino acid analogues have been synthesised by addition of a suitable aldehyde to aminodiphenylmethane and 50 % aqueous hypophosphonous acid under reflux, followed by a deprotection step in aqueous hydrogen bromide.<sup>161</sup> Fmoc-protected phosphonous acids have been used in obtaining the mono-O-alkylated phosphinic acids.<sup>162</sup>

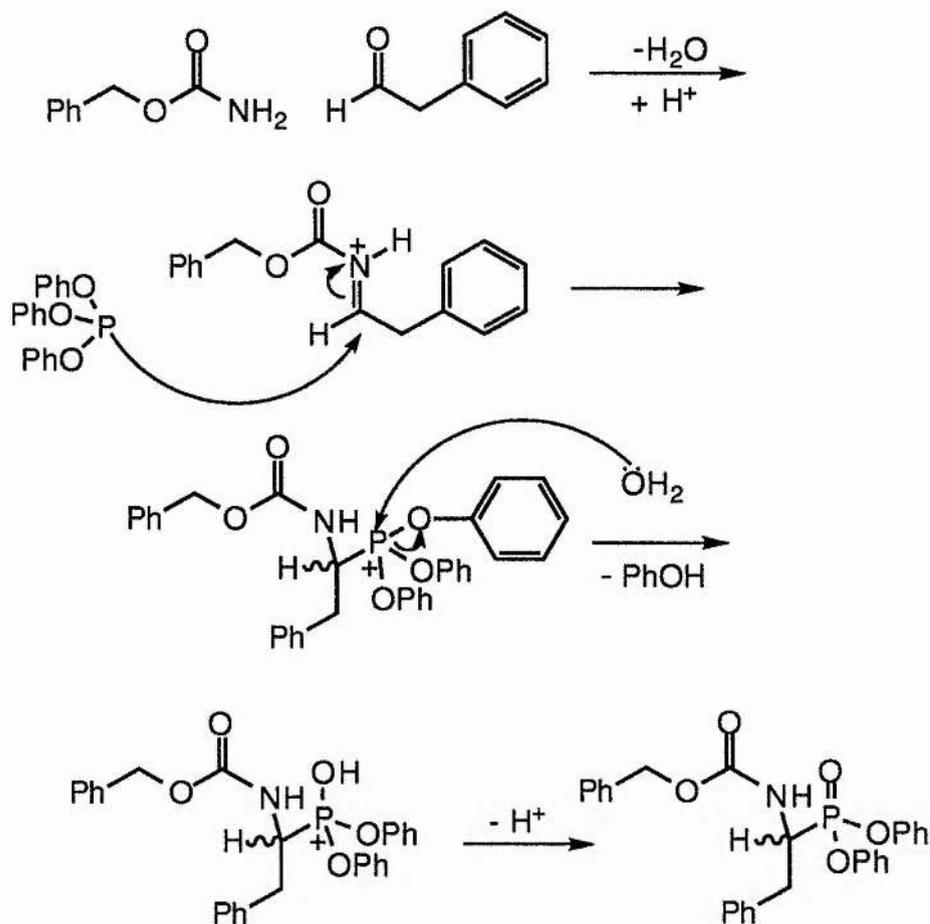
To obtain the required phosphonate, a three step synthesis was used (see Scheme 2.3).



Reagents : (i) AcOH, 80 °C; (ii) NaOMe, MeOH, r.t.;  
(iii) 2 M NaOH, MeOH, r.t.

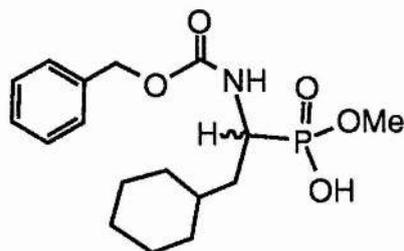
*Scheme 2.3: Synthesis of Phosphinic Acid Methyl Ester.*

Following the three-component coupling procedure of Oleksyszyn *et al.*<sup>158b</sup> using phenylacetaldehyde, triphenyl phosphite and benzyl carbamate in glacial acetic acid gave the diphenyl phosphonate (74) in 29 % yield (see Scheme 2.4).



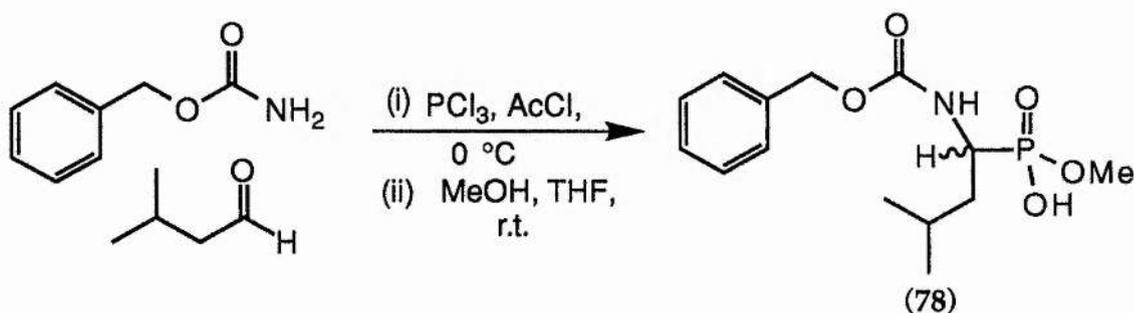
*Scheme 2.4: Mechanism of Formation of Diphenyl Phosphonate (74).*

*Trans*-esterification using sodium methoxide in methanol,<sup>163</sup> followed by removal of the phenol side-product by chromatography on silica, gave the dimethyl phosphonate (75) in 67 % yield. An alternative procedure using potassium fluoride and 18-crown-6 in methanol was also employed<sup>164</sup> but gave inconsistent yields. Finally, base hydrolysis gave the phosphinic acid methyl ester (76) in 77 % yield. The equivalent compound (77), derived from cyclohexylacetaldehyde, was also synthesised *via* this route.



(77): Cyclohexylalanine analogue.

The phosphinic acid methyl esters were also synthesised using the one-pot procedure of Yuan and co-workers (see Scheme 2.5).<sup>160</sup> The reaction did not proceed using phenylacetaldehyde, although it gave a 36 % yield when isovaleraldehyde was used, giving the Leu analogue (78).



Scheme 2.5: One-Pot Procedure For the Synthesis of Phosphinic Acid Methyl Esters.

### 2.2.3. *In Vitro* Testing.

Camp<sup>150</sup> took these two portions and coupled them using a modification of the method of Bartlett.<sup>153</sup> He also deprotected and extended the compounds with Boc-Asn. These compounds were taken and tested against HIV-1 proteinase *in vitro* using a spectrophotometric assay which involved measuring the decrease in absorbance at 300 nm of the decapeptide K-A-R-V-Nle-F(NO<sub>2</sub>)-D-A-Nle-G-NH<sub>2</sub> as it is cleaved at the Nle-F(NO<sub>2</sub>) peptide bond.<sup>165</sup>

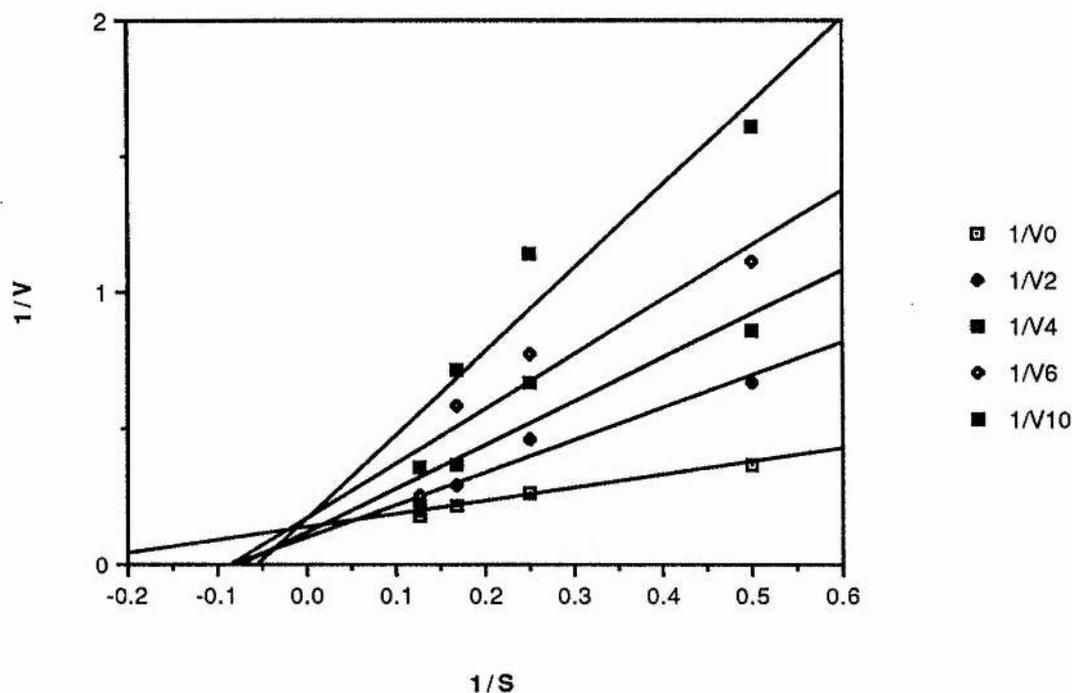
The data obtained were analysed according to the method of Dixon.<sup>166</sup> The assay was performed over a range of inhibitor concentrations and then 1/initial rate was plotted against inhibitor concentration to give an IC<sub>50</sub>.

Repeating this over a range of substrate concentrations gave a value for  $K_i$ ; alternatively, it can be calculated via the following equation:

$$K_i = \frac{IC_{50}}{(1+S/K_m)}$$

Initially, 1 % DMSO was used as a co-solvent; however, results obtained were poor and inconsistent. This was found to be due to an absorption by DMSO at 300 nm; there have also been reports of DMSO acting as an inhibitor for HIV-1 proteinase.<sup>167</sup>

The insolubility of these compounds in water proved to be a limiting step in determining their inhibition constants. Thus, an alternative co-solvent was sought to overcome this problem. Methanol was found to affect enzyme activity at 5 % concentration, although at concentrations of less than 2 % methanol, there was no appreciable loss in activity. Assaying using 1 % methanol proved satisfactory and a number of compounds were tested. These results are summarised in Table 1. Lineweaver-Burk double reciprocal plot of (79) showed the phosphoramidates to be competitive inhibitors of HIV-1 proteinase (see Fig. 2.8).

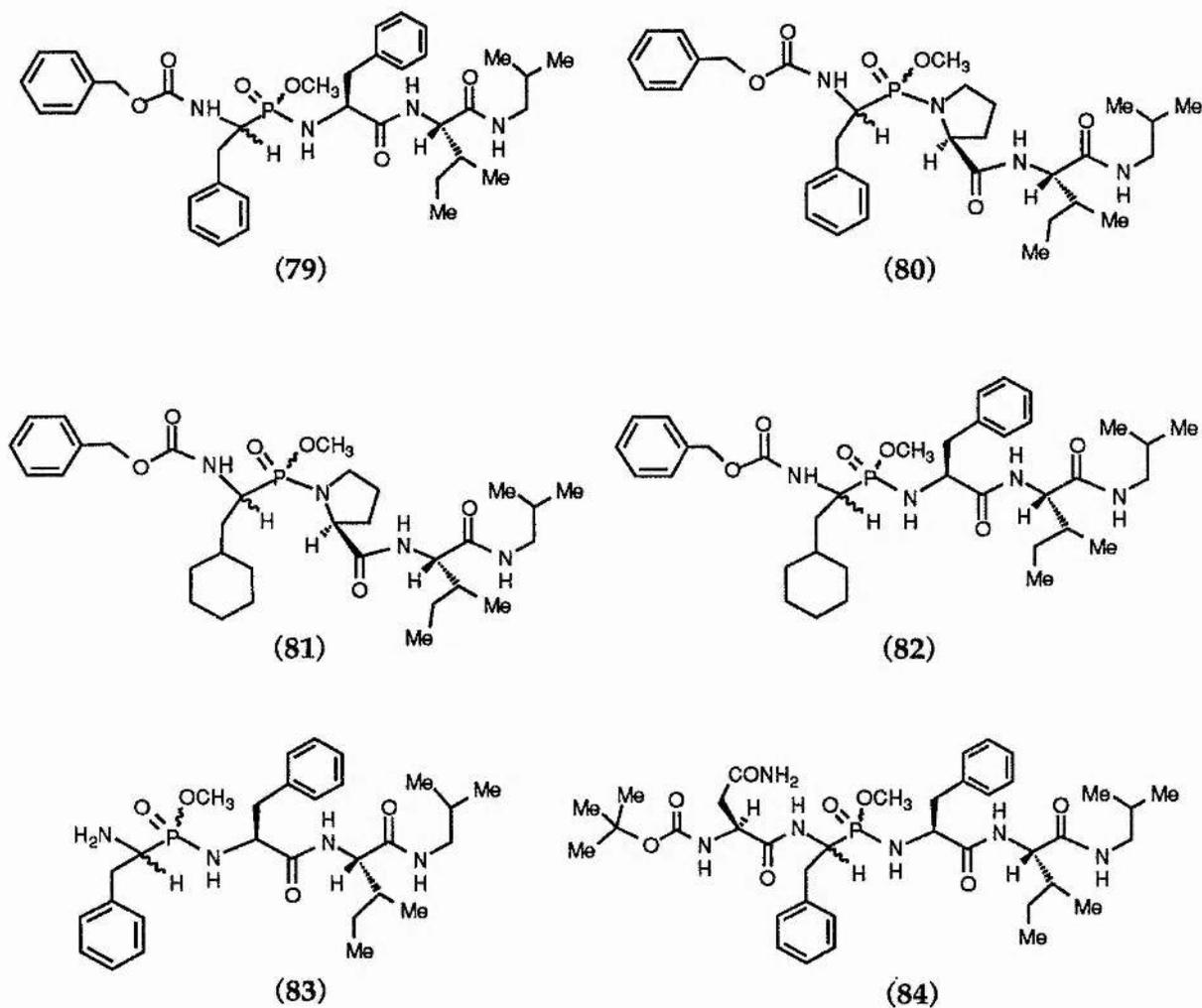


**Figure 2.5:** Lineweaver-Burk Double Reciprocal Plot For Phosphonamidate (79). (where  $1/V_2 = 0.2\%$  inhibitor,  $1/V_4 = 0.4\%$  inhibitor, etc.).

The free phosphonamidate (68) had a relatively high  $IC_{50}$ , most probably due to its anionic nature at the assay pH of 5.5. Therefore, our attention was turned to the methyl esters.

The target phosphonamidate methyl ester (69) had been separated into its individual diastereomers. It was expected that D-amino acid residues (equivalent to (S) configuration in a phospho-amino acid) would be poor inhibitors of HIV-1 proteinase. For example, Cushman *et al.* found that D-Phe at  $P_1'$  increases  $IC_{50}$  by 11-fold compared with the L-Phe epimer in a series of hydroxyethylenes.<sup>168</sup> We observed a slight preference for the (R,R) diastereomer, with an  $IC_{50}$  of  $30\ \mu\text{M}$  compared with  $80\ \mu\text{M}$  for the (R,S) diastereomer, but the selectivity between different diastereomers was not all that marked (see Table 1).

Why this contrast exists is not clear but it is likely to be due to a difference in the way the two different classes bind and/ or interact within the active site.



*Figure 2.6: Some Phosphoramidate Methyl Ester Inhibitors of HIV-1 Proteinase.*

**Table 1. Summary of Inhibition data.**

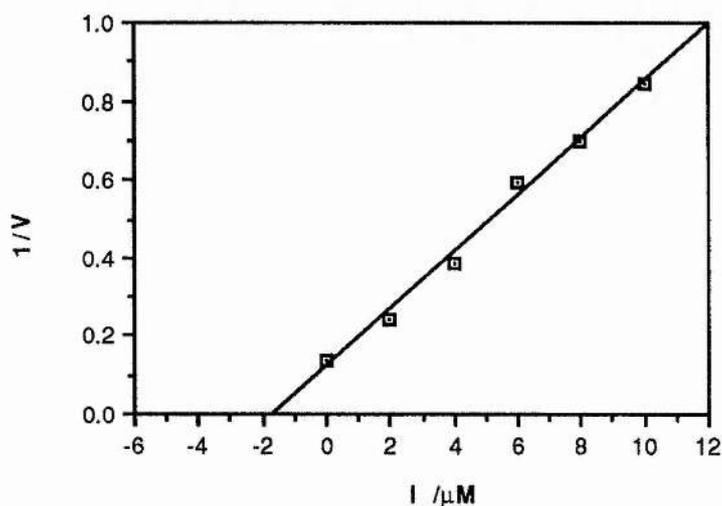
Entry Number	Compound	Configuration at (C <sup>α</sup> ,P)	IC <sub>50</sub> (μM) *	
			HIV Protease Inhibition	Antiviral activity
1	Boc-N-F-PO <sub>2</sub> -P-I-NH-iBu (68)	(epimeric at C <sup>α</sup> )	90	N.D.
2	Boc-N-F-[ψ]-P-I-NH-iBu (69)	(R, R)	30	45
3	(69)	(R, S)	80	>100
4	(69)	(S, S)	100	>100
5	(69)	(S, R)	>100	>100
6	Cbz-F-[ψ]-P-I-NH-iBu. (80)	(R, R)	5	3
7	(80)	(R, S)	25	25
8	(80)	(S, S)	N.D.	25
9	(80)	(S, R)	N.D.	>100
10	Cbz-Cha-[ψ]-P-I-NH-iBu. (81)	(mix. 2 diast.)	16	8
11	Cbz-F-[ψ]-(2S)-F-I-NH-iBu. (79)	(mix. 4 diast.)	2	2
12	Cbz-Cha-[ψ]-(2S)-F-I-NH-iBu. (82)	(mix. 4 diast.)	1	5
13	H <sub>2</sub> N-F-[ψ]-(2S)-F-I-NH-iBu. (83)	(mix. 4 diast.)	30	20
14	Boc-N-F-[ψ]-(2S)-F-I-NH-iBu. (84)	(mix. 4 diast.)	100	>100
15	Qua-N-F-[ψ]-(2S)-F-I-NH-iBu. (85)	(mix. 4 diast.)	45	50
16	Qua-N-Cha-[ψ]-P-I-NH-iBu. (86)	(1 diast. unknown)	20	15
17	Cbz-F-[ψ]-(2R)-F-I-NH-iBu. (87)	(mix. 4 diast.)	1	N.D.
18	Cbz-F-[ψ]-G-I-NH-iBu. (88)	(mix. 4 diast.)	>30	N.D.
19	Cbz-F-[ψ]-(2S)-F-NH-iBu. (89)	(mix. 4 diast.)	40	N.D.
20	Cbz-F-[ψ]-(2S)-F-(OMe). (90)	(mix. 4 diast.)	100	100

\* Values of IC<sub>50</sub> are estimated to be accurate to within ± 10%.

[ψ] = -PO(OCH<sub>3</sub>)-.



group interacts unfavourably when put in the P<sub>3</sub> position and that with this group deleted then the interactions are correspondingly more favourable. The various diastereomers tested also showed the lack of selectivity that the longer inhibitors did, with the (R,R) isomer possessing an IC<sub>50</sub> of 5 μM and the (R,S) isomer having an IC<sub>50</sub> of 25 μM (see Table 1). The (2S)-Phe-(2S)-Ile-NHiBu peptide (73) was incorporated into the inhibitors (79) and (84) and this showed a similar trend between the longer and shorter compounds. It was also more potent; as a mixture of diastereomers it had an IC<sub>50</sub> of 2 μM. The cyclohexylalanine analogue was also incorporated and was found to be a good replacement for Phe, with (79) and (82) displaying very similar IC<sub>50</sub> values.



*Figure 2.8: Plot of 1/Initial Rate Against Inhibitor Concentration For Phosphonamidate (79).*

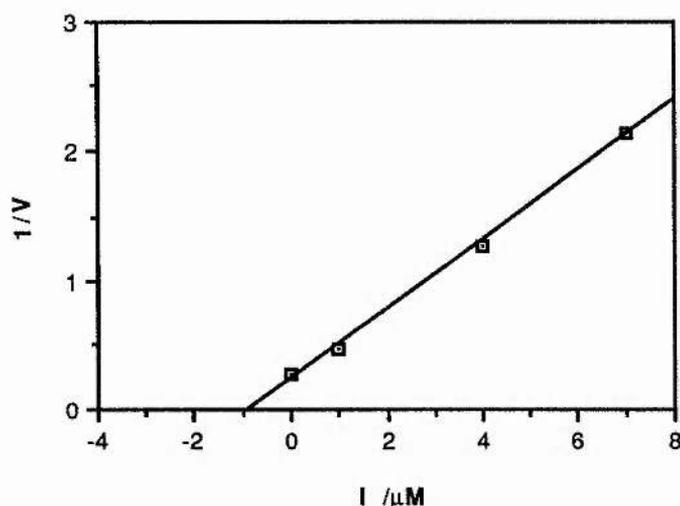


Figure 2.9: Plot of 1/Initial Rate Against Inhibitor Concentration For Phosphonamidate (82).

This series of compounds have also been evaluated in cells by D. Kinchington at St. Bartholomew's Hospital in London (see Table 1). Comparison with our *in vitro* data showed a remarkable correlation between the *in vitro* and *in vivo* values. The reason for this correlation is not clear and it is tempting to suggest that it is due to some property of the phosphonamidate methyl ester that allows for highly efficient cell-uptake.

The full details of this work have been published.<sup>169</sup>

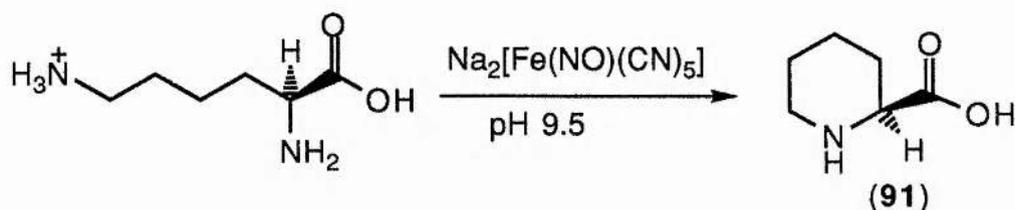
#### 2.2.4. Incorporation of Piperidine-2-Carboxylic Acid

As an alternative to Pro at P<sub>1</sub>', piperidine-2-carboxylic acid (91) was incorporated into the peptide. When it replaced Pro in a substrate, the resulting peptide was an inhibitor with a K<sub>i</sub> of ~ 1 μM<sup>64</sup> and the hydroxyethylamine Ro-31-8959 (28) contains the somewhat similar decahydroquinoline residue in the same position.

However, enantiomerically pure piperidine-2-carboxylic acid (which is known commercially as pipercolinic acid) is extremely expensive and so efforts were made to obtain the single enantiomer by other means.

The racemic pipercolinic acid is readily available and the peptide (2R/S)-Pip-(2S)-Ile-NHiBu was prepared via a similar protocol to the other peptides. It was hoped that separation of the diastereomers so formed would be possible; however, by t.l.c. in a variety of solvent systems, there was only very limited resolution at best.

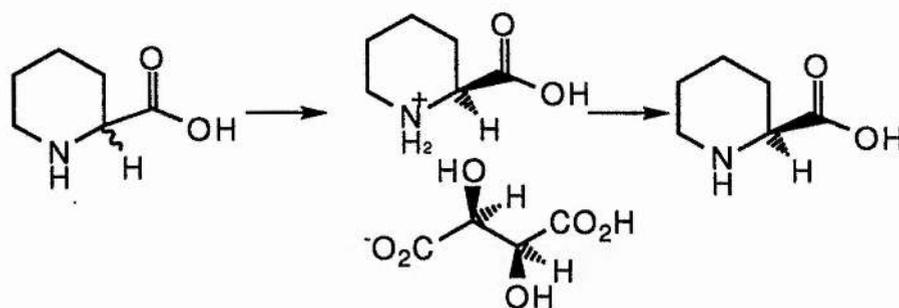
Kisfaludy *et al.* demonstrated the cyclisation of (2S)-lysine to give (2S)-pipercolinic acid using sodium nitroprusside under strict pH control (see Scheme 2.6).<sup>170</sup>



*Scheme 2.6: Preparation of (2S)-Pipercolinic Acid From (2S)-Lysine.*

When this procedure was followed, it was evident from the colour change in the reaction vessel that some kind of reaction had taken place. However, it proved impossible to separate the required product from the inorganic side-products, either by extraction into an organic solvent or by silica or fluorosil column.

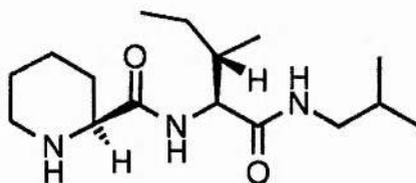
We turned instead to the resolution procedure of Hardtmann *et al.* (see Scheme 2.7).<sup>171</sup> The racemic amino acid and (2S,3S)-tartaric acid were dissolved in methanol, resulting in the formation of the tartrate salt. The (2S)-pipercolinic-(2S,3S)-tartrate precipitated out and could be collected by filtration. After drying, the purity of this salt was checked by melting point (195-196 °C, lit.<sup>171</sup> 195-196 °C) and optical rotation ( $\alpha_D = -20$  (c 2 in H<sub>2</sub>O), lit.<sup>171</sup> -21 (c 2 in H<sub>2</sub>O)).



*Scheme 2.7: Resolution of (2R/S)-Pipicolinic Acid Using (2S,3S)-Tartaric Acid.*

The free acid was obtained by passing the tartrate salt through an IR-120H Amberlite ion exchange column in its H<sup>+</sup> form, under which conditions the positively charged pipicolinic ion remains on the column and the tartrate anion is washed off. Elution with 10 % ammonia solution then yielded the pure (2S)-pipicolinic acid in 57 % yield (m.p. 268-270 °C, lit.<sup>171</sup> 270-272 °C,  $\alpha_D = -23$  (c 1.5 in H<sub>2</sub>O), lit.<sup>171</sup> -26 (c 1.5 in H<sub>2</sub>O)). Washing the column thoroughly with distilled water was found to be necessary to ensure that no tartrate remained on the column.

The amino acid so obtained was N-protected using a Boc group and coupled to (2S)-Ile-NHiBu.HCl (**92**) using the mixed anhydride method. Subsequent deprotection yielded the required dipeptide amide (**93**).



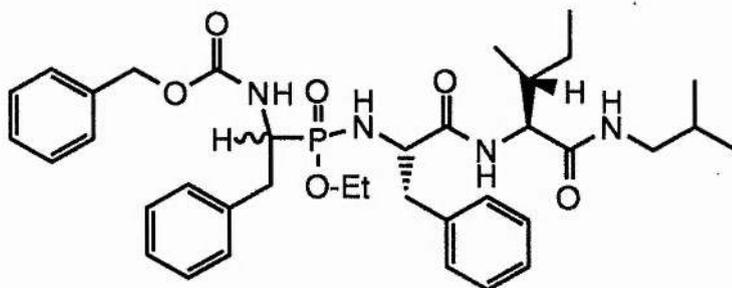
**(93): (2S)-Pip-(2S)-Ile-NHiBu.**

The crucial step of the synthesis was to couple the phospho-amino acid to the dipeptide amide (**93**), which was achieved *via* the phosphonic acid chloride. Earlier work had shown that the reaction does proceed very well, although steric

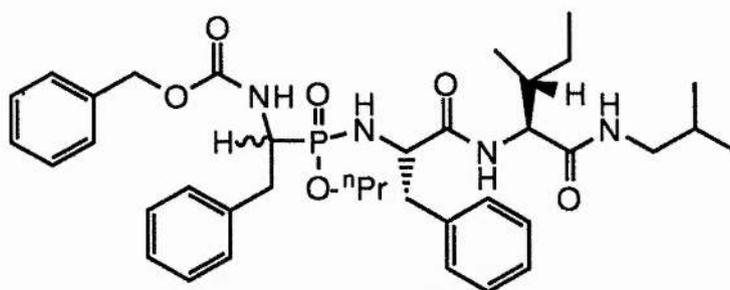
factors are very important to the overall yield.<sup>150</sup> It has been found that both the (2S)-Phe-(2S)-Ile-NHiBu (73) and the original peptide (2S)-Pro-(2S)-Ile-NHiBu (72) couple satisfactorily, in approximately 50 % yield. However, the coupling of the (2S)-Pip-(2S)-Ile-NHiBu (93) with the phosphophenylalanine analogue (76) proceeded very poorly, even at elevated temperature. From models of the reactants, we found that in the proline case, the strain of the ring pulls back the peptidic side chain, and keeps it out of the way so that the reaction could proceed. However, in the six-membered ring of the pipecolic acid, this strain is released, and the (2S)-Ile-NHiBu causes severe steric interaction with the phospho-amino acid, making the coupling a slow and difficult process. (2S)-pipecolic acid methyl ester was prepared in an attempt to reduce this unfavourable interaction, but even this was enough to cause very little product to be formed.

### 2.2.5. Extending the Chain.

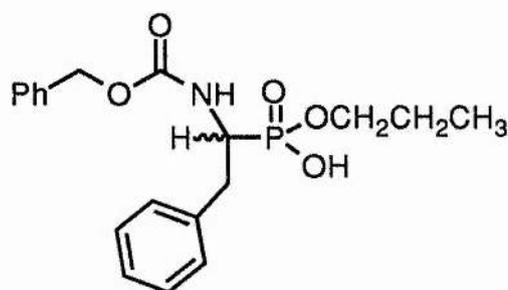
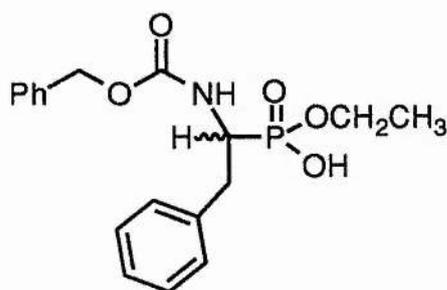
The methyl group on the phosphoramidate methyl ester should cause some unfavourable steric interactions in the active site. However, *in vitro* assays have shown that they have equal to or greater potency than the free phosphoramidate. While there may be something of an exchange of one bad interaction for another in this, it was decided to investigate the scope for extending this chain to ethyl or larger groups.



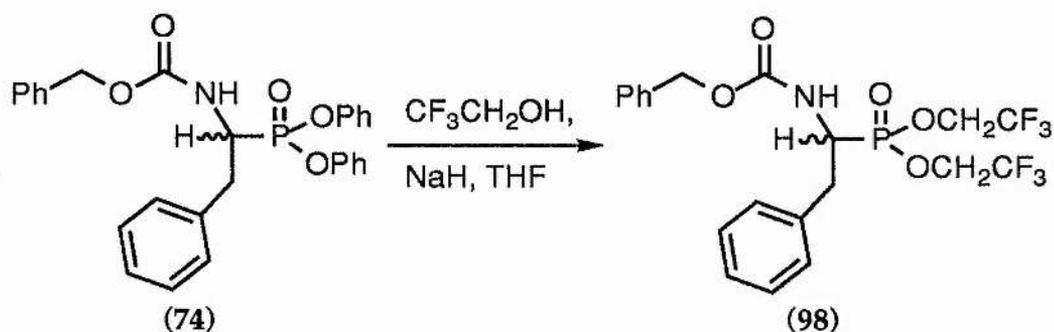
(94): Phosphoramidate Ethyl Ester.

(95): *Phosphonamidate n-Propyl Ester.*

The ethyl phosphinic acid ester (96) was prepared in an almost identical fashion to the methyl analogue, in a slightly lower overall yield of 36 %. The *n*-propyl analogue (97) was also available by this route, except that sodium hydride rather than sodium metal was used to generate sodium propoxide and the base hydrolysis to the phosphinic acid ester proceeded in very poor yield.

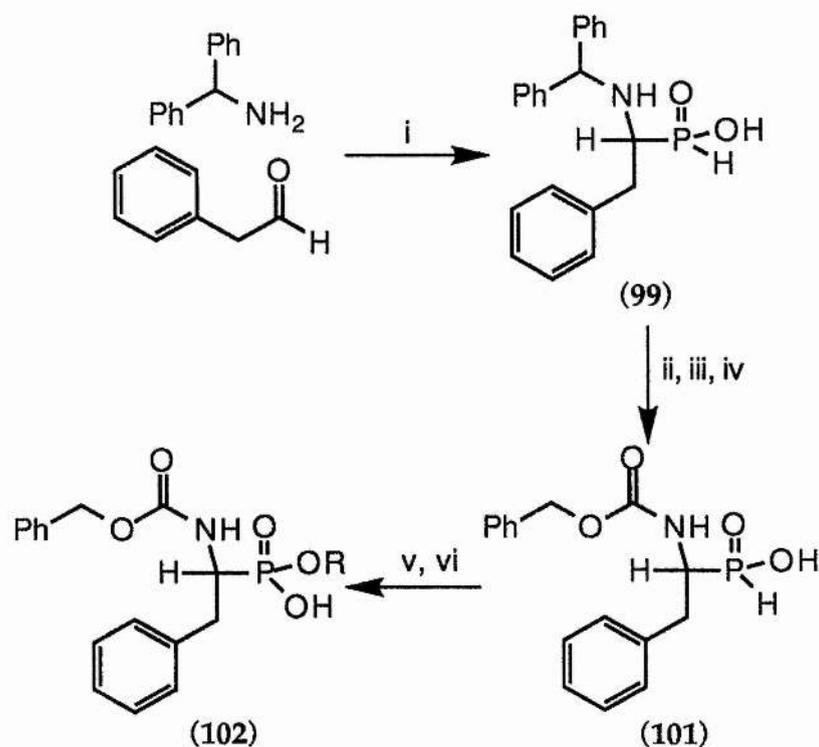
(96): *Ethyl Phosphinic Acid Ester.* (97): *n-Propyl Phosphinic Acid Ester.*

However, attempts to synthesise the trifluoroethoxy analogue (98) by reaction of the diphenyl phosphonate with trifluoroethoxide (produced from trifluoroethanol and sodium hydride) in THF were unsuccessful (see Scheme 2.8).



*Scheme 2.8: Attempted Synthesis of Trifluoroethoxy Analogue (98).*

An alternative route was investigated, using the methodologies of Dingwall<sup>161</sup> and Dumy<sup>162</sup> (see Scheme 2.9). The diphenylmethylphosphonous acid (99) was synthesised by adding phenylacetaldehyde to a refluxing mixture of aminodiphenylmethane and 50 % hypophosphonous acid. This is thought to proceed *via* an imine to which a hypophosphite anion adds. Addition of 1 M HCl was found to improve yields by suppressing a competing reduction of the imine by the hypophosphite. The diphenylmethyl group was removed by treatment with aqueous hydrogen bromide, followed by treatment with propylene oxide to give the free phosphonous acid (100) in 83 % yield.



Reagents: (i)  $\text{H}_3\text{PO}_2$ , 1 M HCl,  $\Delta$ ; (ii) 48 % aq. HBr; (iii) Propylene oxide, EtOH; (iv)  $\text{PhCH}_2\text{OCOCl}$ , 5 %  $\text{NaHCO}_3$ ; (v) DCC, ROH,  $\text{CH}_2\text{Cl}_2$ ; (vi) aq.  $\text{Br}_2$ .

*Scheme 2.9: Synthesis of Cbz-Amino-Phosphinic Acid Esters via Phosphonous Acid.*

The amino group was re-protected with a benzyloxycarbonyl group and then a DCC coupling of the phosphonous acid with trifluoroethanol was attempted. A product was isolated that possessed the expected  $\text{CH}_2$  singlet at 4.9 ppm in its  $^1\text{H}$  nmr spectrum but no  $\text{CF}_3$  signal could be observed by  $^{13}\text{C}$  nmr spectroscopy.

The phospho-amino acids so obtained were coupled to (2S)-Phe-(2S)-Ile-NHiBu (73) by forming the phosphoryl acid chloride using thionyl chloride in dichloromethane, followed by addition of the peptide at  $0^\circ\text{C}$ , along with 2.5 equivalents of triethylamine as an HCl scavenger.<sup>150</sup> During the acid chloride formation, it was found to be important to flush the flask with nitrogen to remove the acidic gases formed during the reaction, as this flushing procedure

led to much cleaner final products. The resulting inhibitors could be directly compared with the methyl ester (79).

The phosphoramidate ethyl ester (94) gave an  $IC_{50}$  of  $4.5 \mu\text{M}$  (see Fig. 2.10). Thus there is a slight drop in potency, not surprisingly, although the drop is not especially great. There is evidently some leeway for incorporation of functional groups onto the phosphoramidate and this is explored further in section 2.4.

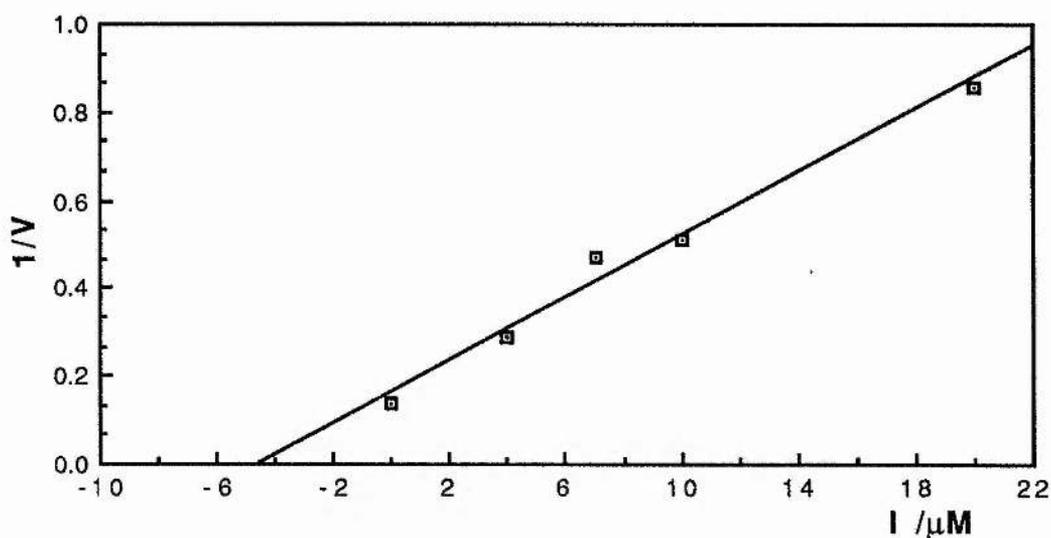
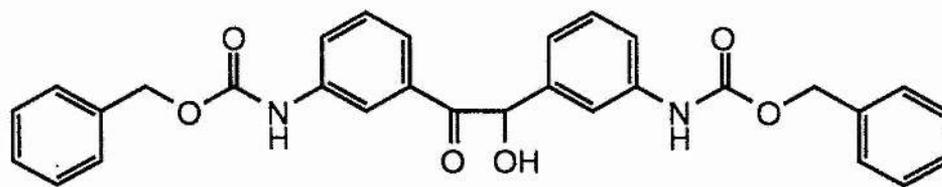


Figure 2.10: Plot of  $1/\text{Initial Rate}$  Against Inhibitor Concentration for Phosphoramidate Ethyl Ester (94).

### 2.3. Interlude - Benzoin Inhibitors of HIV-1 Proteinase.

As has already been noted, peptidic compounds rarely make good drugs. Ideally what is required is a simple and easily accessible non-peptide which displays potent anti-viral activity and low toxicity. While it is difficult to predict the behaviour of inhibitors *in vivo*, it should be possible to incorporate the features that make a potent inhibitor into a low molecular weight molecule which hopefully will lead to the required biological profile.

An interesting lead compound in this search is the benzoin (103). This has a high degree of  $C_2$  symmetry to reflect that in the enzyme, which is even greater if within the active site, the benzoin can enolise (see Fig. 2.11).

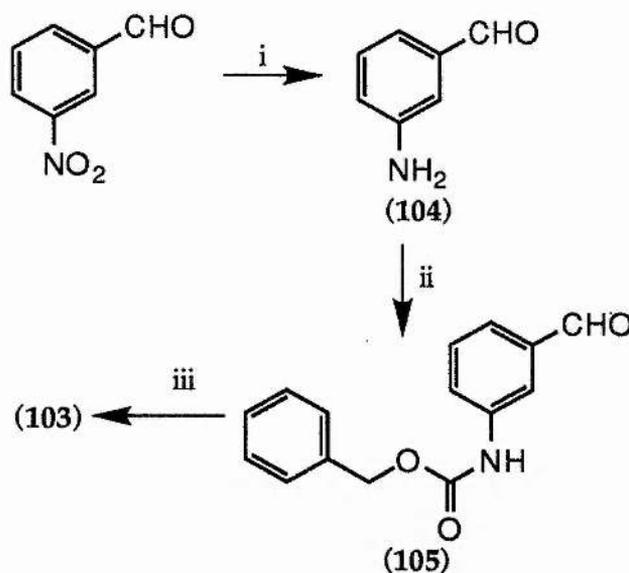


(103): Benzoin Inhibitor of HIV-1 Proteinase.



Figure 2.11: Enolisation of Benzoin.

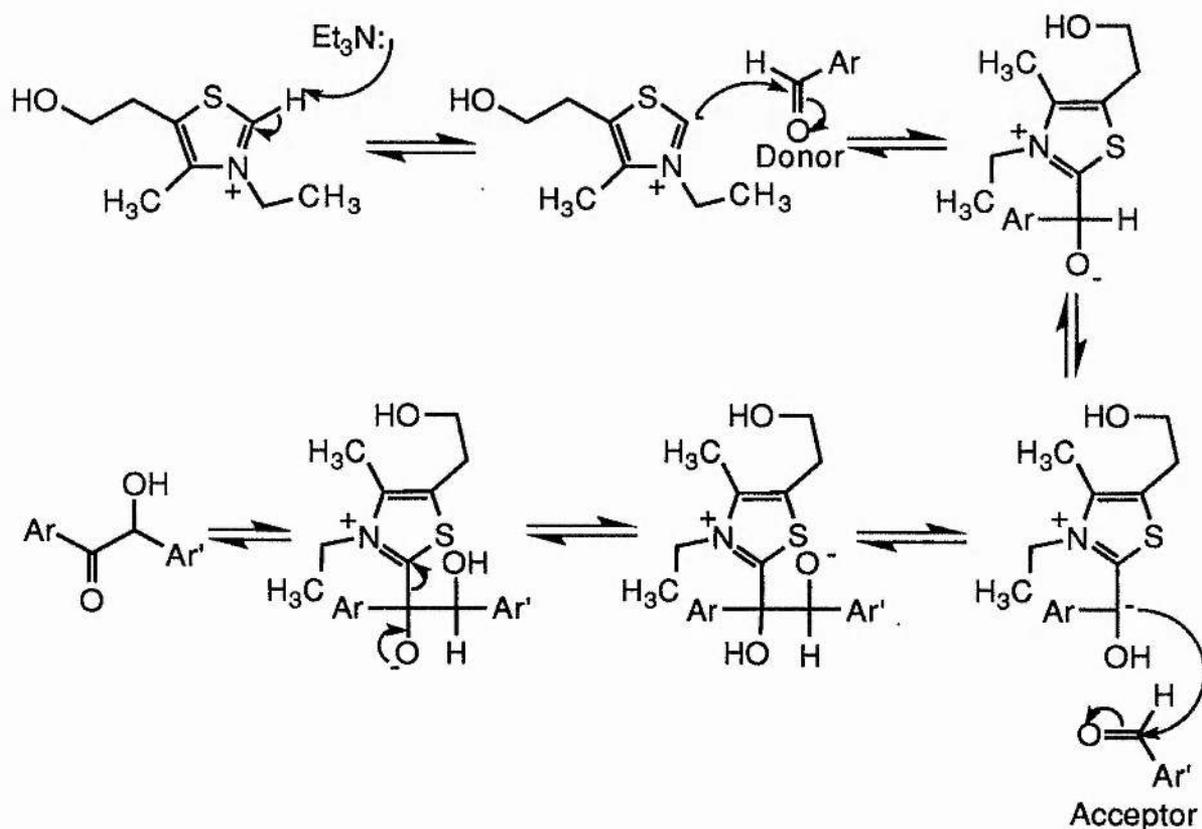
The compound is readily available in a three step synthesis from *m*-nitrobenzaldehyde.



Reagents: (i)  $\text{SnCl}_2$ , conc.  $\text{HCl}$ ; (ii)  $\text{PhCH}_2\text{OCOCl}$ , 2 M  $\text{NaOH}$ ; (iii) 3-Ethyl-5-(2-hydroxyethyl)-4-methyl-thiazolium bromide,  $\text{Et}_3\text{N}$ ,  $\text{EtOH}$ , reflux, 40 h.

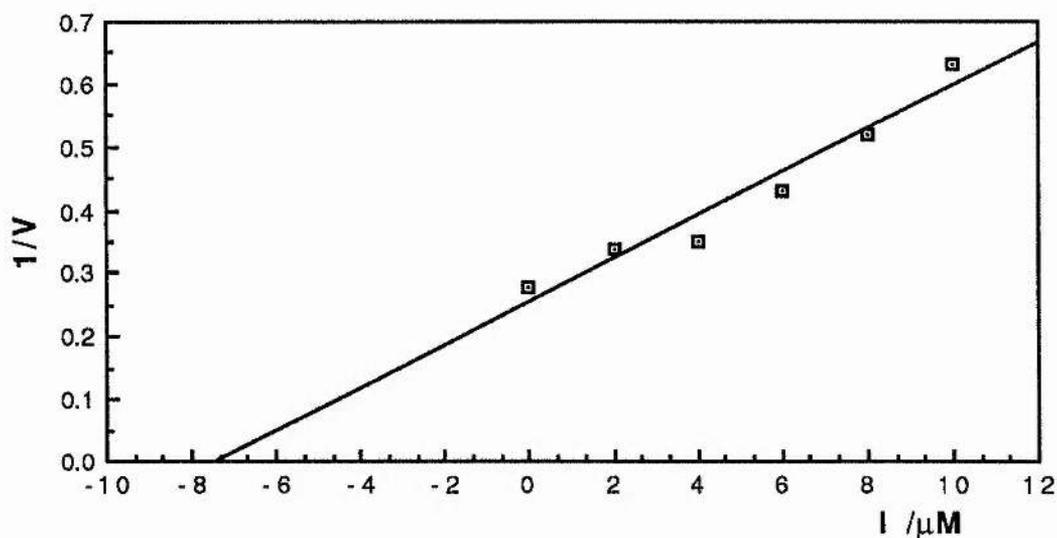
Scheme 2.10 Synthesis of 3, 3'-Di-[(Benzyloxycarbonyl)-amino]-benzoin.

Stannous chloride reduction in concentrated hydrochloric acid gives *m*-aminobenzaldehyde (104).<sup>172</sup> As this can polymerise if allowed to stand, this was immediately N-protected using benzylchloroformate in sodium hydroxide solution. This was achieved in 51 % overall yield after column chromatography. The final step is a benzoin condensation, in which two molecules of starting material condense with each other, catalysed by a thiazolium salt<sup>173</sup> (see Scheme 2.11). This reaction is very much substrate dependent since some aldehydes make good acceptors and some good donors, with benzaldehyde itself somewhere in the middle. After 40 hours reflux, followed by column chromatography, the reaction gave only a 25 % yield of final product.



Scheme 2.11: Mechanism of the Benzoin Condensation.

The benzoin was tested *in vitro* and was found to possess an  $IC_{50}$  of about  $7 \mu M$  (see Fig. 2.12).



*Figure 2.12: Plot of 1/Initial Rate Against Inhibitor Concentration for Benzoin (103).*

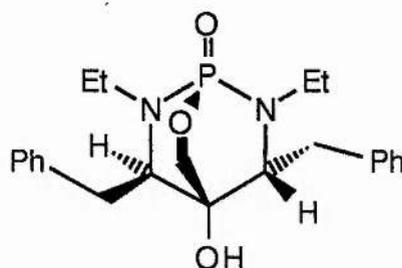
Although no further work has yet been done on this type of compound, it makes for an interesting lead compound and scope exists for considerable improvement in potency in a future study.

#### **2.4. Bicyclic Phosphorodiamidates.**

As part of the ongoing phosphoramidate program, a new design of inhibitor was proposed. This originated partly from wanting a non-peptidic target and also from molecular modelling that showed that the flap structural water molecule could be displaced.

The cyclic ureas (61) and (62) (see Section 1.6.8) have since shown that the hypothesis was a feasible one.<sup>140</sup> Other desirable design features included a degree of  $C_2$  symmetry and the introduction of a strained system as this would only be able to adopt a minimal number of conformations, which should enhance binding. Such a molecule could also find use as an active site probe. Having shown that it was possible to extend the ester chain in the

phosphoramidates, extrapolation of this led to the design of the phosphorodiamidate (106).



(106): *Bicyclic Phosphorodiamidate.*

Molecular dynamics calculations on (106) by J. Wilkie showed that it should take up a skewed conformation in the active site of the enzyme (see Figs. 2.13 and 2.14). The OH group positions itself between the catalytic Asp 25 and 25' whereas the P=O forms a strong hydrogen bond interaction with one of the flap Ile's (approx. 2-2.5 Å apart), but it is relatively distant (> 6 Å) from the other flap Ile (see Fig. 2.13). Despite this, the benzyl groups seem to make a good hydrophobic interaction in the P<sub>1</sub> and P<sub>1</sub>' pockets (see Fig. 2.14). This shows that the molecule won't take up a strictly C<sub>2</sub> symmetric conformation, but the results of Kempf and co-workers (see Section 1.6.7) have shown that it is virtually impossible to do this. It otherwise seems to be a good fit, but whether it will be able to hold both flaps closed, or whether holding one flap closed is enough, remains to be seen.

A proposed synthesis of this compound is shown in Scheme 2.12.

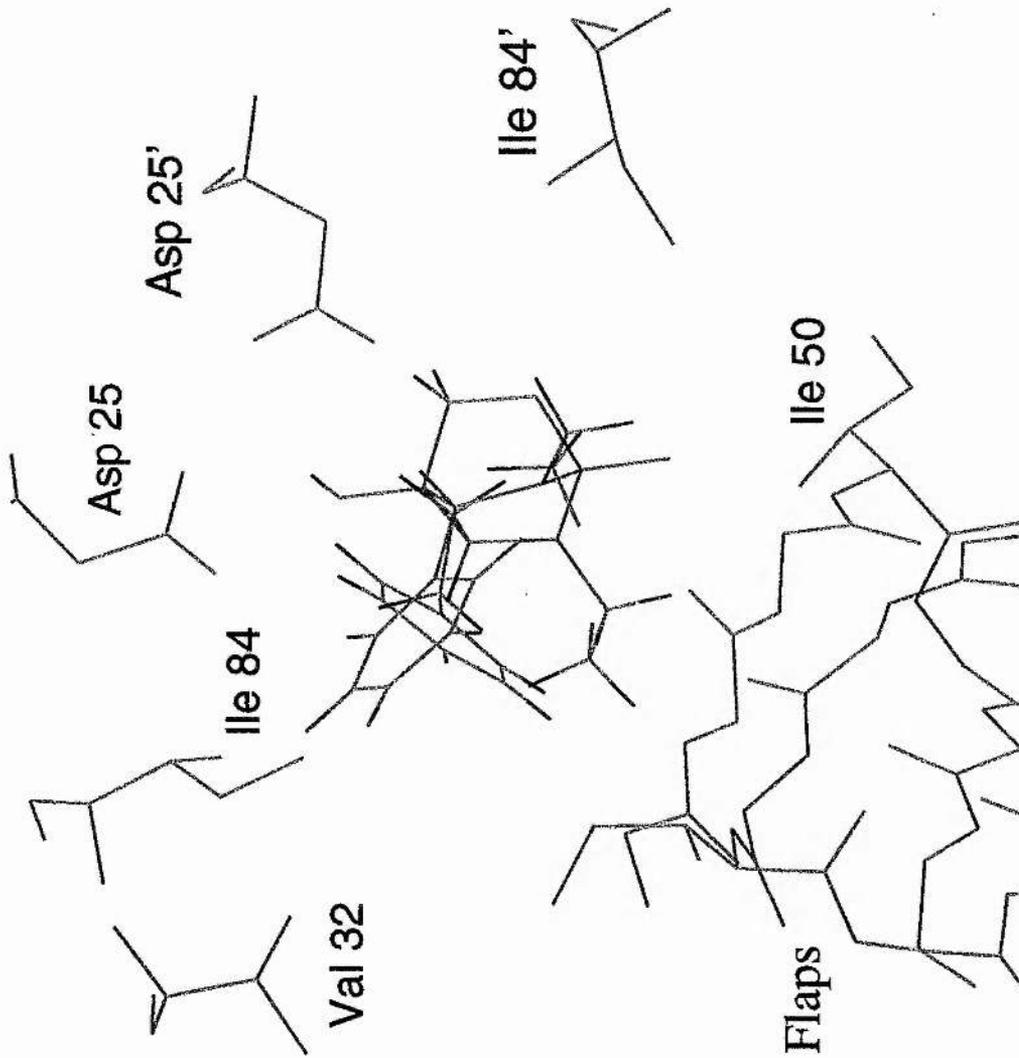


Figure 2.13: Phosphorodiamidate (106) Bound into the Active Site of HIV-1 Proteinase.

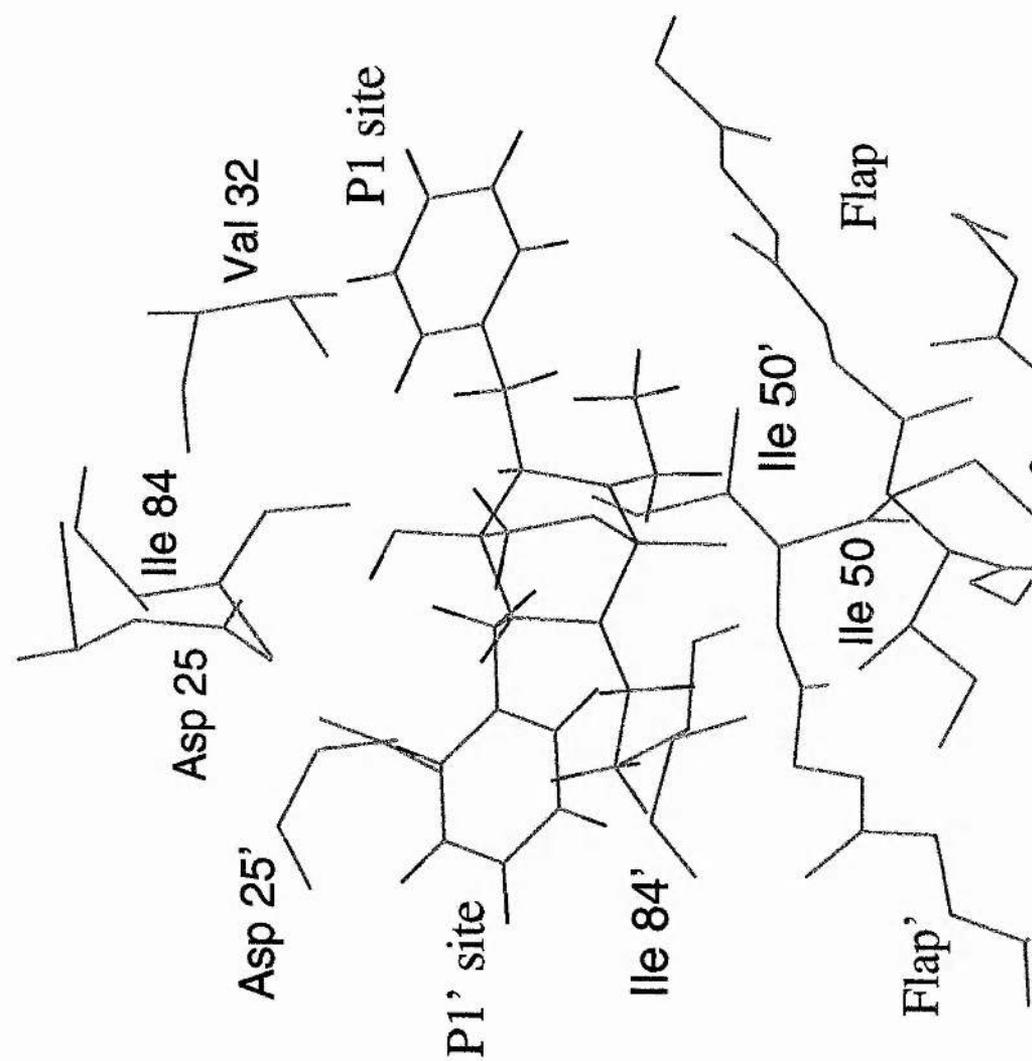
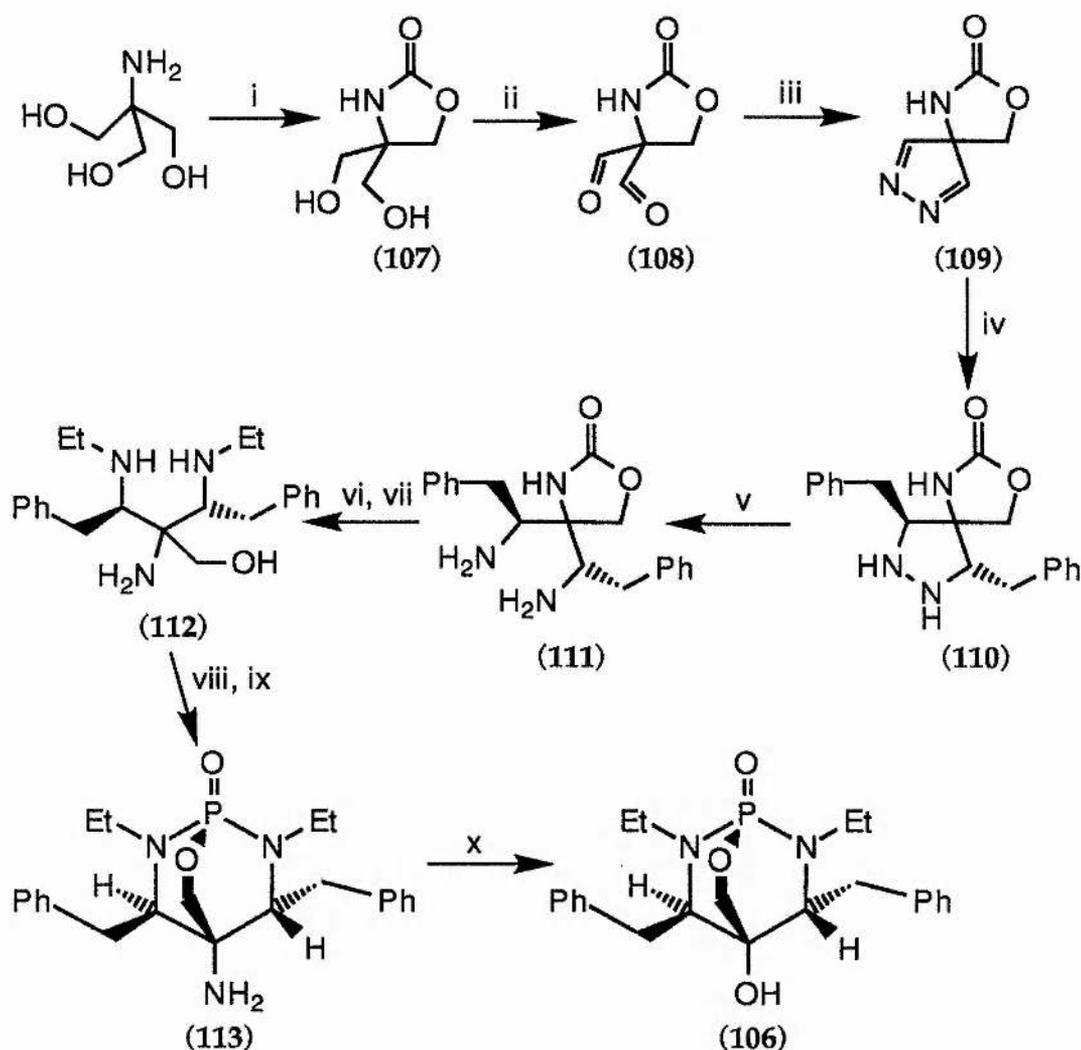


Figure 2.14: Phosphorodiamidate (106) Bound into the Active Site of HIV-1 Proteinase (Alternate View).



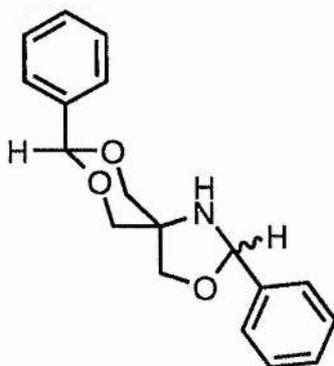
Reagents: (i)  $\text{CO}(\text{OEt})_2$ ,  $\text{K}_2\text{CO}_3$ ,  $130\text{ }^\circ\text{C}$ ; (ii)  $(\text{COCl})_2$ ,  $\text{DMSO}$ ,  $\text{Et}_3\text{N}$ ,  $\text{CH}_2\text{Cl}_2$ ; (iii)  $\text{H}_2\text{N}-\text{NH}_2 \cdot \text{H}_2\text{O}$ ,  $\text{CH}_2\text{Cl}_2$ ; (iv)  $\text{BnMgBr}$ ,  $\text{THF}$ ,  $-78\text{ }^\circ\text{C}$ ; (v)  $\text{H}_2$ ,  $\text{Pd/C}$ ,  $\text{MeOH}$ ; (vi)  $\text{CH}_3\text{CHO}$ ,  $p\text{-TsOH}$ ,  $\text{PhCH}_3$ ; (vii)  $\text{LiAlH}_4$ ,  $\text{THF}$ ; (viii)  $\text{PCl}_3$ ,  $\text{Et}_3\text{N}$ ,  $\text{CH}_2\text{Cl}_2$ ,  $-20\text{ }^\circ\text{C}$ ; (ix)  $m\text{-CPBA}$ ,  $\text{CH}_2\text{Cl}_2$ ; (x)  $\text{NaNO}_2$ ,  $\text{HCl}$ ,  $\text{H}_2\text{O}$ .

**Scheme 2.12:** Proposed Synthesis of Phosphorodiamidate (106).

The nitrogen and one oxygen of *tris*-hydroxymethylaminomethane were protected as the oxazolidinone (107) by the method of Evans *et al.* using diethyl carbonate and potassium carbonate in 76 % yield.<sup>174</sup> Efficient mechanical stirring of this reaction is vital since the mixture quickly becomes too thick for a magnetic stirrer to operate effectively.

Oxidation to the di-aldehyde (108) proved problematic. The starting material was highly insoluble in organic solvents and initial efforts used a variety of chromate oxidation procedures.<sup>175</sup> While the colour change in the reaction vessel indicated some kind of reaction had taken place, no product could be extracted from the reaction mixture. The reason for this could be that the di-aldehyde is sufficiently polar that it is very difficult to remove it from the metal surface or from an aqueous solution. <sup>1</sup>H nmr spectroscopy of an aqueous layer indicated a peak corresponding to the aldehyde CH at 8.4 ppm. However, all attempts to isolate or extract this compound, including saturation of the aqueous layer with sodium chloride, resulted in failure. Although highly polar, the diol was sparingly soluble in DMSO and so a Swern oxidation was attempted.<sup>176</sup> By t.l.c., a new spot that was positive to both permanganate and Brady's reagent was observed; however, all attempts to isolate the di-aldehyde resulted in failure.

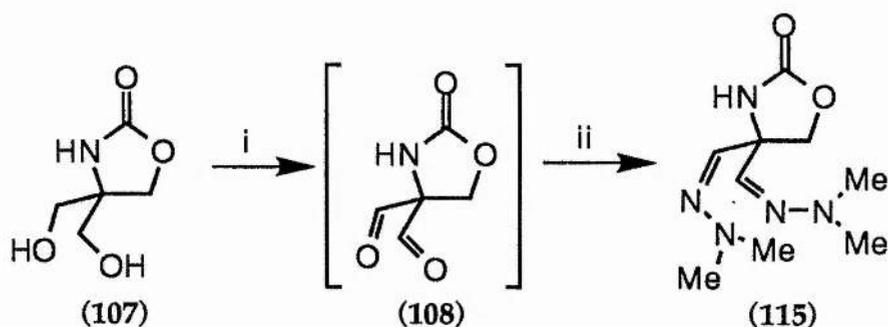
The main problem seemed to be the diol's insolubility, so attempts to increase its organic solubility were made by benzylating the nitrogen using benzaldehyde, which has been previously demonstrated on serine.<sup>177</sup> Mass spectrometry on the product of this reaction showed a peak at 298 a.m.u., which corresponds to  $[M + H]^+$  of the di-acetal (114). In an attempt to form the mono-N,O-acetal selectively, only one equivalent of benzaldehyde was used but this also led to the di-acetal.



(114): *Diacetal Derived From Tris Hydroxymethyl Amino Methane.*

Baker and Condon reported a series of hydroxyethylene inhibitors of renin and HIV-1 proteinase which were relevant to our own work.<sup>178</sup> They used a one-pot procedure, taking a diol, forming a di-aldehyde *in situ*, then reacting this with *N,N*-dimethyl-hydrazine to form a tetra-methyl-di-hydrazone. Grignard addition to the dihydrazone resulted in the formation of a single dihydrazone, thus displaying the extremely good diastereoselectivity in this reaction.

This protocol was adapted for use with the oxazolidinone-diol (107) (see Scheme 2.13). It was essentially a Swern oxidation followed by addition of *N,N*-dimethyl-hydrazine and anhydrous magnesium sulfate at room temperature. In order to solvate the starting material, it was necessary to perform the Swern oxidation in a 40:60 mixture of DMSO:dichloromethane at -50 °C; at any lower temperature than this, the solution froze. The mixture was allowed to warm to room temperature, the hydrazine and magnesium sulfate added and the mixture stirred overnight. Purification by column chromatography yielded the tetra-methyl-di-hydrazone (115) as an orange oil in 47 % yield.



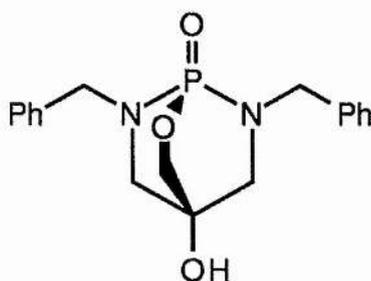
Reagents: (i)  $(\text{COCl})_2$ , DMSO,  $\text{Et}_3\text{N}$ ,  $\text{CH}_2\text{Cl}_2$ , -50 °C  $\rightarrow$  r.t.;  
(ii)  $\text{Me}_2\text{NNH}_2$ ,  $\text{MgSO}_4$ .

*Scheme 2.13: Preparation of Di-Hydrazone (115).*

Grignard addition was then attempted, but this led to a mix of products. It was suggested that the conditions were too harsh, thus the milder cuprate was tried in its place.<sup>179</sup> However, there was still no sign of the required product; it is possible that this system is too sterically demanding for the required *bis*-addition

to take place; in Baker and Condon's inhibitors, there were an extra two carbons between the two reacting centres.

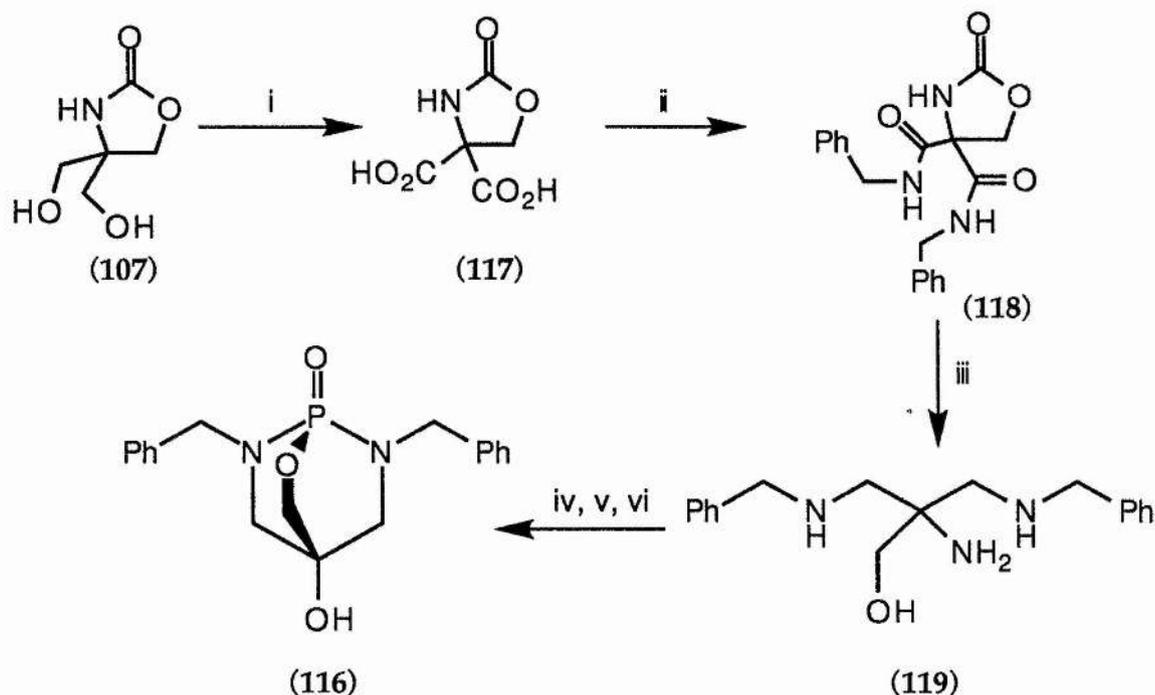
In order to get around this problem, a simpler analogue (116) was devised. This meant that the phosphorodiamidate moiety could still be studied as an inhibitor of HIV-1 proteinase.



(116): *Simplified Phosphorodiamidate.*

This could be accessible by oxidation of the diol to the di-acid, followed by formation of the amide, reduction with lithium aluminium hydride and, finally, cyclisation (Scheme 2.14).

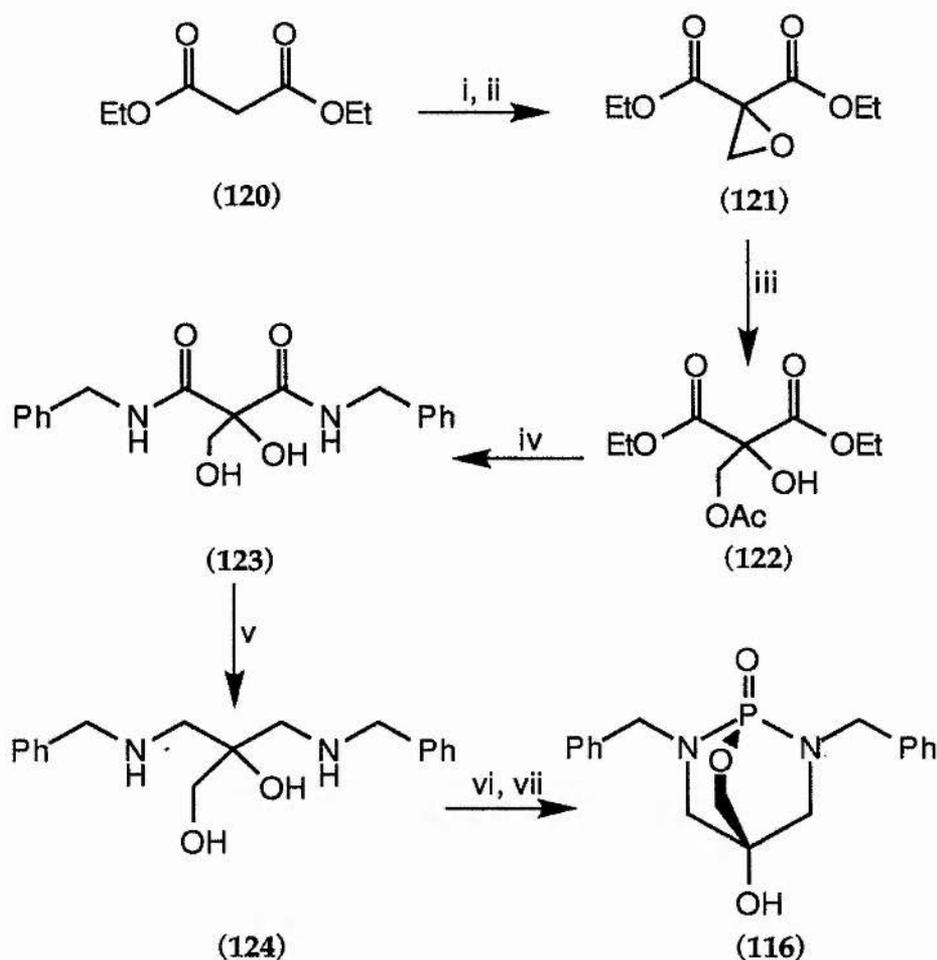
Oxidation of the diol (107) to the diacid (117) was thus attempted. Again there seemed to be a good deal of difficulty in extracting the product from the reaction mixture, either from chromate oxidative procedures<sup>180</sup> or using basic permanganate.<sup>181</sup> However, acidic permanganate<sup>182</sup> seemed to give the required product by <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy. However, mass spectrometry did not give the required molecular ion peak and attempted derivatisation with diazomethane to give the di-methyl di-ester further indicated that it was not the di-acid at all, although the identity of this product has eluded determination.



Reagents: (i)  $\text{KMnO}_4$ , aq.  $\text{NaOH}$ , acetone; (ii) NMM,  $\text{Me}_2\text{CHCH}_2\text{OCOC}$ ,  $\text{BnNH}_2$ , THF,  $-15^\circ\text{C}$ ; (iii)  $\text{LiAlH}_4$ , THF; (iv)  $\text{PCl}_3$ ,  $\text{Et}_3\text{N}$ ,  $\text{CH}_2\text{Cl}_2$ ,  $-20^\circ\text{C}$ ; (v) *m*-CPBA,  $\text{CH}_2\text{Cl}_2$ ; (vi)  $\text{NaNO}_2$ ,  $\text{HCl}$ ,  $\text{H}_2\text{O}$ .

*Scheme 2.14: Proposed Route to the Simpler Phosphorodiamidate (116).*

Therefore, an alternative synthesis from diethyl malonate (120) was proposed (Scheme 2.15). Knoevenagel condensation by the method of Bachman and Tanner gave diethyl methylene malonate.<sup>183</sup> This is a potent Michael acceptor so it was immediately epoxidised by the method of Foucaud<sup>184</sup> using 10 % sodium hypochlorite solution on an alumina surface to give (121) in 35 % overall yield. Opening the epoxide with sodium hydroxide was ineffective but with sodium acetate, the reaction proceeded smoothly,<sup>185</sup> giving rise to a new peak in the  $^1\text{H}$  nmr spectrum at 2.1 ppm, corresponding to the acetate, and a shift in the  $\text{CH}_2$  signal from 3.2 ppm in the epoxide to 4.6 ppm.



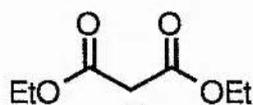
Reagents: (i) Paraformaldehyde,  $\text{Cu}(\text{OAc})_2$ ,  $\text{KOAc}$ ,  $\text{AcOH}$ ,  $90\text{ }^\circ\text{C}$ ; (ii) Neutral alumina,  $\text{CH}_3\text{CN}$ , 10%  $\text{NaOCl}$ , r.t.; (iii)  $\text{NaOAc}$ ,  $\text{AcOH}$ ,  $90\text{ }^\circ\text{C}$ ; (iv)  $\text{BnNH}_2$ ,  $\text{EtOH}$ ,  $\Delta$ ; (v)  $\text{LiAlH}_4$ ,  $\text{THF}$ , r.t.; (vi)  $\text{PCl}_3$ ,  $\text{Et}_3\text{N}$ ,  $\text{THF}$ ,  $-20\text{ }^\circ\text{C}$ ; (vii) *m*-CPBA,  $\text{CH}_2\text{Cl}_2$ ,  $0\text{ }^\circ\text{C} \rightarrow \text{r.t.}$

*Scheme 2.15: Proposed Synthesis of N,N'-Dibenzyl Phosphorodiamidate (116).*

To obtain the diamide, the malonate derivative (122) was refluxed in freshly distilled ethanol with an excess of benzylamine.<sup>186</sup> This had worked well when used with diethyl malonate, giving *N,N'*-dibenzyl malonamide as a white crystalline solid, so no problems were anticipated. Unfortunately, although the expected reaction did occur, so did a deformylation, as detected by the appearance of a CH signal at 4.5 ppm in the  $^1\text{H}$  nmr spectrum. This was confirmed by an

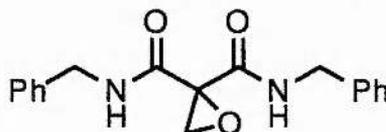
APT experiment in the  $^{13}\text{C}$  nmr spectrum which showed a down peak at 73 ppm. The method of Seebach, which is at room temperature, was also tried<sup>187</sup> but gave similar results.

It appears to be that the retro-aldol reaction is facile for the diester. Since the acetate is the most accessible of the three esters, it will be this that will react first. Due to the stability of the malonate anion (125), this molecule can then deformylate. If the opening of the epoxide and amide formation were switched, then the acetyl group will remain intact and thus there will be no opportunity for a deformylation to take place. Also, it could be that with a diamide instead of a diester, the retro-aldol will not be so facile and the deformylation will not take place.

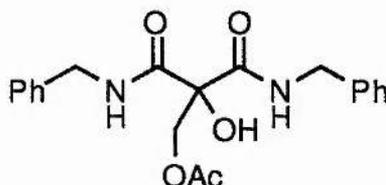


(125): *Diethyl Malonate Enolate.*

Therefore, the epoxide diester (121) will be refluxed in ethanol with an excess of benzylamine to give the epoxide diamide (126) and then this can be treated with sodium acetate in acetic acid at 90 °C to give the acetate diamide (127).



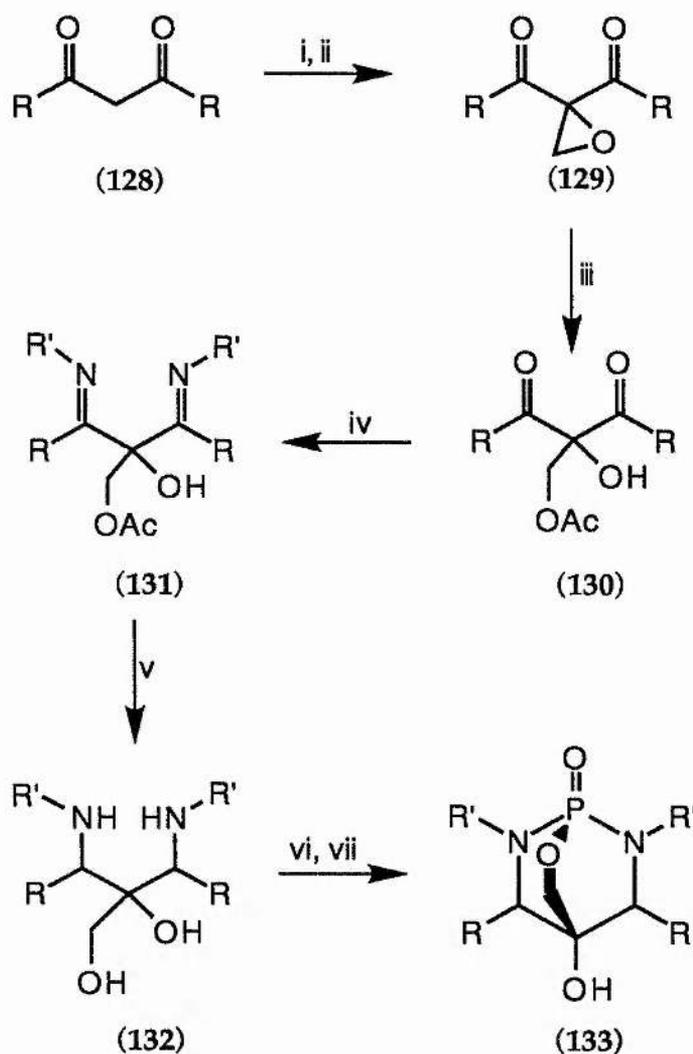
(126): *N, N'-Dibenzyl-Epoxymethylene Malonamide.*



(127): *N, N'-Dibenzyl-Acetoxyethyl-Hydroxy-Malonamide.*

Reduction of this compound should give the dihydroxy-diamine (124), which can then be cyclised using phosphorus trichloride followed by *m*-CPBA oxidation to give the phosphorodiamidate (116). This part of the synthesis is being carried out by others in our group.

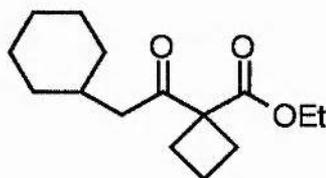
To obtain the original target, it was initially thought that a similar methodology could be used (see Scheme 2.16).



Reagents: (i) Paraformaldehyde,  $\text{Cu}(\text{OAc})_2$ ,  $\text{KOAc}$ ,  $\text{AcOH}$ ,  $90\text{ }^\circ\text{C}$ ; (ii) Neutral alumina,  $\text{CH}_3\text{CN}$ , 10%  $\text{NaOCl}$ , r.t.; (iii)  $\text{NaOAc}$ ,  $\text{AcOH}$ ,  $90\text{ }^\circ\text{C}$ ; (iv)  $\text{R}'\text{NH}_2$ ,  $\text{PhCH}_3$ , cat. *p*- $\text{TsOH}$ ,  $\Delta$ ; (vi)  $\text{LiAlH}_4$ ,  $\text{THF}$ , r.t.; (vii)  $\text{PCl}_3$ ,  $\text{Et}_3\text{N}$ ,  $\text{THF}$ ,  $-20\text{ }^\circ\text{C}$ ; (viii) *m*-CPBA,  $\text{CH}_2\text{Cl}_2$ ,  $0\text{ }^\circ\text{C} \rightarrow \text{r.t.}$ ;

Scheme 2.16: Proposed Route to the Generalised Phosphorodiamidate (133).

The 1,3-diketone is available by cuprate addition<sup>188</sup> onto malonyl dichloride.<sup>189</sup> However, some difficulty was encountered with the Knoevenagel condensation. Alternatively, the diester (122) could be used, as there are a number of procedures that add organometallics to esters to give ketones.<sup>187,190</sup> The simplest of these is that of Kikkawa *et al.*, in which a Grignard reagent is added to the ester in the presence of triethylamine.<sup>190a</sup> This was tried using diethyl cyclobutane-1,1-dicarboxylate as a model and gave a t.l.c. spot that was positive to Brady's reagent. However, upon isolation, <sup>1</sup>H nmr spectroscopy and mass spectrometry revealed it to be the keto-ester (134).



(134): Keto-ester Derived From Diester.

Adjustment of the conditions of the Grignard addition may allow for the required diketone to be obtained. Unfortunately, there was not sufficient time to complete this part of the study. This will also be continued by others in our group. This can then be applied to the synthesis of diketone (130), leading to the substituted phosphorodiamidate (133).

## **CHAPTER THREE**

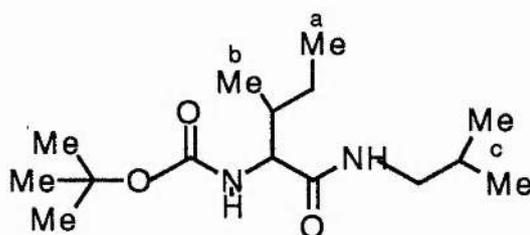
### **EXPERIMENTAL**

### 3.0. Experimental

NMR Spectra were recorded on a Bruker AM-300 F.T. spectrometer ( $^1\text{H}$ , 300 MHz;  $^{13}\text{C}$ , 75 MHz;  $^{31}\text{P}$ , 121.5 MHz) and a Varian Gemini F.T. spectrometer ( $^1\text{H}$ , 200 MHz;  $^{13}\text{C}$ , 50 MHz). High field NMR spectra were obtained on a service basis at the University of Edinburgh ( $^1\text{H}$ , 600 MHz;  $^{13}\text{C}$ , 150 MHz;  $^{31}\text{P}$ , 243 MHz) and the University of Warwick ( $^1\text{H}$ , 400 MHz;  $^{13}\text{C}$ , 100 MHz).  $^1\text{H}$  NMR spectra were referenced on chloroform, methanol or DMSO,  $^{13}\text{C}$  NMR spectra were referenced on chloroform, methanol or DMSO and  $^{31}\text{P}$  spectra on external  $\text{H}_3\text{PO}_4$ . NMR spectra are described in parts per million downfield shift from TMS and are reported consecutively as position ( $\delta_{\text{H}}$  or  $\delta_{\text{C}}$ ), relative integral, multiplicity (s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, d of d-doublet of doublets and br-broad), coupling constant ( $J_{\text{X,Y}}$  if applicable) and assignment. Infrared spectra were recorded using a Perkin Elmer 1420 ratio recording spectrometer and a Perkin Elmer 1710 f.t. i.r. spectrometer. The samples were prepared as Nujol mulls or thin films between sodium chloride discs. Absorption maxima are given in wavenumbers ( $\text{cm}^{-1}$ ) relative to a polystyrene standard. UV spectra were recorded on Pye-Unicam SP8-500 or SP8-100 spectro-photometers. Melting points were measured using electrothermal melting point apparatus and are uncorrected. Optical rotations were measured on an Optical Activity Ltd. AA-100 polarimeter using 10 cm path length cells at room temperature. Mass spectra were recorded on a Kratos MS50 and obtained on a S.E.R.C. service basis at the University of Swansea using a VG ZAB E. Major fragments are given as percentages of the base peak intensity. Where appropriate, all solvents and reagents were freshly distilled prior to use. THF and ether were distilled from sodium/ benzophenone under a nitrogen atmosphere; DMF and  $\text{CH}_2\text{Cl}_2$  were distilled from  $\text{CaH}_2$ . Flash chromatography was performed according to the procedure of Still<sup>191</sup> using Sorbisil C60 (40-60  $\mu\text{m}$ ) silica gel. Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (MN SIL G/UV<sub>254</sub>) and

compounds were visualised by UV fluorescence, iodine vapour, ethanolic phosphomolybdic acid, aqueous potassium permanganate, Brady's reagent or ninhydrin.

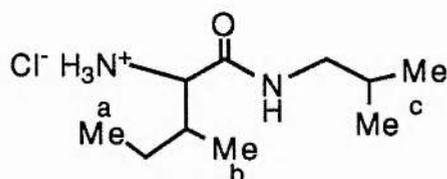
### Boc-(2S)-isoleucine-isobutylamide (135).<sup>150</sup>



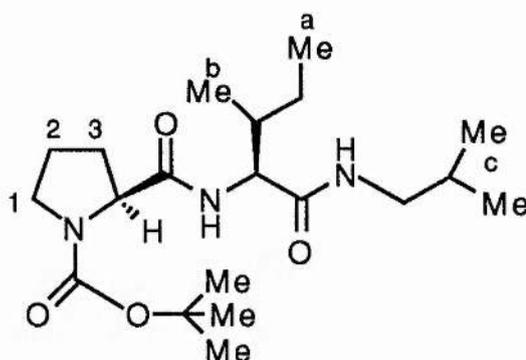
Boc-(2S)-isoleucine (4.62 g, 20 mmol) was dissolved in anhydrous THF (100 cm<sup>3</sup>), N-methylmorpholine (2.18 cm<sup>3</sup>, 20 mmol) was added and the solution cooled to -15 °C. Isobutylchloroformate (2.72 cm<sup>3</sup>, 20 mmol) was added with stirring. The solution was left for 5 min. Isobutylamine (1.99 cm<sup>3</sup>, 20 mmol) was added at -15 °C and the solution left for 5 min. The solution was allowed to warm to room temperature over a further 15 min. The hydrochloride salts were filtered off and the THF removed under reduced pressure to yield a white solid which was recrystallised from methanol/ water or ethyl acetate/ light petroleum (4.85 g, 85%). m.p. 158-160 °C (lit.<sup>150</sup> 158-160 °C). *m/z* (Found: [M + H]<sup>+</sup> 287.2335. C<sub>15</sub>H<sub>31</sub>N<sub>2</sub>O<sub>3</sub> requires 287.23347); [α]<sub>D</sub> -27.1 (c 1 in MeOH); ν<sub>max</sub> (Nujol) /cm<sup>-1</sup> 3335 (NH), 1682 (Urethane CO) & 1655 (Amide CO); δ<sub>H</sub> (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 0.9 (12H, m, CH<sub>3</sub>'s (a,b,c)), 1-1.25 (1H, m, CH (iBu)), 1.42 (9H, s, Boc), 1.7-1.9 (3H, m, CH<sub>2</sub> & CH (Ile)), 2.98-3.20 (2H, m, CH<sub>2</sub> (iBu)), 3.89 (1H, d of d, *J* 7, 9, α-CH), 5.19 (1H, d, *J* 8.6, NH (urethane)) & 6.10 (1H, t, *J* 5, NH (amide)). δ<sub>C</sub> (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) (12.0 (CH<sub>3</sub> (a)), 16.2 (CH<sub>3</sub> (b)), 21.0 (CH<sub>3</sub>'s (c)), 25.2 (CH<sub>2</sub> (Ile)), 28.9 (Boc CH<sub>3</sub>'s), 29.0 (CH<sub>3</sub> (c)CH), 37.9 (CH(CH<sub>3</sub> (b))), 47.4 (CH<sub>2</sub> (iBu)), 60.2 (α CH), 80.8 (C(CH<sub>3</sub>)<sub>3</sub>) & 156.6 (CO

urethane), 172.2 (CO amide);  $m/z$  (CI) 287 ( $[M + H]^+$ , 8%), 231 (48,  $[M + H - H_3CCCH_2]^+$ ), 213 (42,  $[M + H - H_3CCCH_2 - H_2O]^+$ ) & 187 (100,  $[M + H - Boc]^+$ ).

**(2S)-isoleucine-isobutylamide hydrochloride (92).<sup>150</sup>**



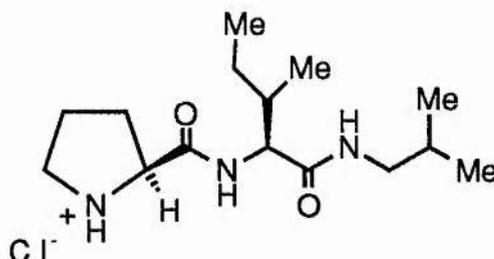
HCl gas was bubbled into ethyl acetate (150 cm<sup>3</sup>) at 0 °C for 1h. Boc-(2S)-isoleucine-isobutylamide (135) (4.94 g, 17 mmol) was added to the acidic solution and left for 1.5 h. at room temperature. The solvent was removed under reduced pressure to yield an off-white gummy solid (3.45g, 90%).  $m/z$  (Found:  $[M + H]^+$  187.1810. C<sub>10</sub>H<sub>23</sub>N<sub>2</sub>O requires 187.1810);  $\nu_{\max}$  (Nujol) /cm<sup>-1</sup> 3200 (NH), 1665 (CO) & 1558 (Amide II);  $\delta_H$  (200 MHz, *d*<sub>6</sub>-DMSO) 0.9 (12H, m, CH<sub>3</sub>'s), 1-1.25 (1H, m, CH (iBu)), 1.5-1.9 (3H, m, Ile CH<sub>2</sub> & CH), 2.84 & 3.07 (2H, m, CH<sub>2</sub> (iBu)), 3.65 (1H, t, *J* 4.8, Ile  $\alpha$ -CH), 8.31 (3H, s, NH<sub>3</sub><sup>+</sup>) & 8.64 (1H, t, *J* 5.4, iBu NH);  $\delta_C$  (50.3 MHz, *d*<sub>6</sub>-DMSO) 12.02 (CH<sub>3</sub> (a)), 15.17 (CH<sub>3</sub> (b)), 20.89 (CH<sub>3</sub> 's (c)), 25.42 (CH<sub>2</sub> (Ile)), 28.69 (iBu CH), 37.19 (CH(CH<sub>3</sub> (b))), 47.86 (CH<sub>2</sub> (iBu)), 58.48 ( $\alpha$  CH) & 168.91 (CO amide);  $m/z$  (EI) 187 ( $[M + H - HCl]^+$ , 100%), 86 (41,  $[M + H - HCl - C(O)NH\text{iBu}]^+$ ).

**Boc-(2S)-prolyl-(2S)-isoleucyl-isobutylamide (136).**<sup>150</sup>

Boc-(2S)-proline (430 mg, 2 mmol) was dissolved in anhydrous THF (20 cm<sup>3</sup>), N-methylmorpholine (218  $\mu$ l, 2 mmol) was added and the solution cooled to -15 °C. Isobutylchloroformate (272 cm<sup>3</sup>, 2 mmol) was added with stirring and the solution left for 5 min at -15 °C. (2S)-Ile-NH-iBu hydrochloride (92) (450 mg, 2 mmol) and N-methylmorpholine (218  $\mu$ l, 2 mmol) were dissolved in THF and added to the cold solution. The reaction was left at -15 °C for 5 min and a further 15 min at room temperature. The hydrochloride salts were filtered off and the THF removed under reduced pressure to yield a white solid which was recrystallised from ethyl acetate/ light petroleum (698 mg, 91%), m.p. 126-128 °C (lit.<sup>150</sup> 126-128 °C);  $m/z$  (Found:  $[M + H]^+$  384.286. C<sub>20</sub>H<sub>38</sub>N<sub>3</sub>O<sub>4</sub> requires 384.2862);  $[\alpha]_D$  -85.5° (c 1 in MeOH);  $\nu_{\max}$  (Nujol)/cm<sup>-1</sup> 3292 & 3089 (NH str.), 1706 (CO str. (urethane)), 1645 (CO str. (amide)) and 1553 (NH bend);  $\delta_H$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 0.85 (12H, m, CH<sub>3</sub>'s (a,b,c)), 1.05 (1H, m, 1  $\times$  CH<sub>2</sub> (Ile)), 1.40 (1H, m, 1  $\times$  CH<sub>2</sub> (Ile)), 1.45 (9H, s, Boc), 1.7-2.3 (6H, m, CH (iBu), CH (Ile) & CH<sub>2</sub>'s (2) & (3) (Pro)), 2.85-3.2 (2H, m, CH<sub>2</sub> (iBu)), 3.45 (2H, br, CH<sub>2</sub> (1) (Pro)), 4.25 (2H, br,  $\alpha$  CH's) and 6.67 & 6.94 (2H, br, NH's);  $\delta_C$  (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 12.0 (CH<sub>3</sub> (a)), 16.0 (CH<sub>3</sub> (b)), 20.8 (CH<sub>3</sub> 's (c)), 24.6 (CH<sub>2</sub> (2) Pro), 25.2 (CH<sub>2</sub> (Ile)), 28.6 (CH (iBu)), 28.7 (Boc CH<sub>3</sub>'s), 29.3 (CH<sub>2</sub> (3) Pro), 36.4 (CH (Ile)), 47.4 (CH<sub>2</sub> (iBu)), 47.7 (CH<sub>2</sub> (1) Pro), 58.6 ( $\alpha$  CH (Ile)), 61.0 ( $\alpha$  CH (Pro)), 81.3

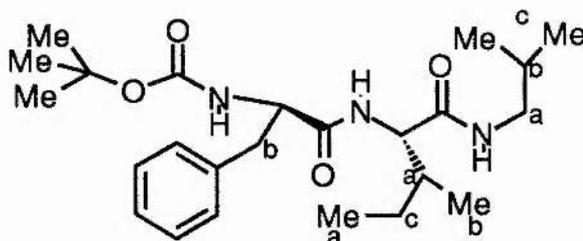
( $\underline{\text{C}}(\text{CH}_3)_3$ ), 156.2 ( $\underline{\text{C}}\text{O}$  urethane), 171.4 ( $\underline{\text{C}}\text{O}$  (Ile)) and 172.4 ( $\underline{\text{C}}\text{O}$  (Pro));  $m/z$  (CI) 384 ( $[\text{M} + \text{H}]^+$ , 60%), 328 (66,  $[\text{M} + \text{H} - (\text{H}_3\text{C})_2\text{CCH}_2]^+$ ) and 284 (53,  $[\text{M} + \text{H} - \text{Boc}]^+$ ).

**(2S)-Prolyl-(2S)-isoleucyl-isobutylamide hydrochloride (72).<sup>150</sup>**

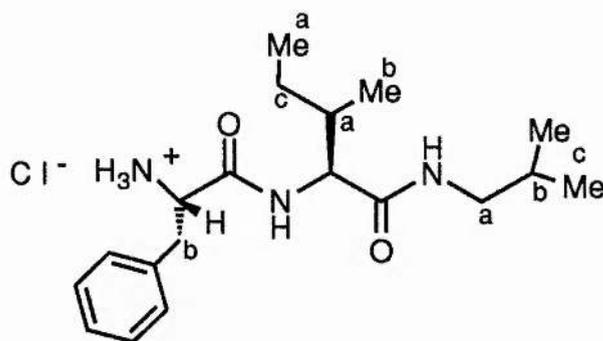


HCl gas was bubbled into ethyl acetate (50 cm<sup>3</sup>) at 0 °C for 1 h. Boc-(2S)-Pro-(2S)-Ile-NH-iBu (**92**) (1.0 g, 2.6 mmol) was added to the acidic solution and left for 1.5 h. at room temperature. The solvent was removed under reduced pressure to yield a white solid, which was recrystallised from methanol/ ethyl acetate (675 mg, 81%), m.p. 220 °C (decomp.) (lit.<sup>150</sup> 220 °C); (Found: C, 56.3; H, 9.25; N, 12.95. C<sub>15</sub>H<sub>30</sub>N<sub>3</sub>O<sub>2</sub>Cl requires C, 56.4; H, 9.45; N, 13.15%);  $m/z$  (Found:  $[\text{M} + \text{H} - \text{HCl}]^+$  284.2338. C<sub>15</sub>H<sub>30</sub>N<sub>3</sub>O<sub>2</sub> requires 284.2338);  $[\alpha]_{\text{D}}$  -66.1 (c 1 in MeOH);  $\nu_{\text{max}}$  (Nujol)/ cm<sup>-1</sup> 3280 (NH str.), 1655 & 1637 (CO str. (amides)) and 1538 (NH bend);  $\delta_{\text{H}}$  (600 MHz, *d*<sub>6</sub>-DMSO) 0.85 (12H, m, Ile & iBu CH<sub>3</sub>'s), 1.10 (1H, m, 1 × CH<sub>2</sub> (Ile)), 1.45 (1H, m, 1 × CH<sub>2</sub> (Ile)), 1.67 (1H, m, CH (iBu)), 1.74 (2H, m, 1 × CH<sub>2</sub> (Pro 3-H) & CH (Ile)), 1.84 (2H, m, CH<sub>2</sub> (Pro 4-H)), 2.29 (1H, m, 1 × CH<sub>2</sub> (Pro 3-H)), 2.80 (1H, m, 1 × CH<sub>2</sub> (iBu)), 2.95 (1H, m, 1 × CH<sub>2</sub> (iBu)) 3.15-3.3 (2H, m, CH<sub>2</sub> (Pro 5-H)), 4.18 (1H, d of d, *J* 7.80, 8.50, α CH (Ile)), 4.23 (1H, d of d, *J* 6.90, 8.10, α CH (Pro)), 8.15 (1H, t, *J* 5.85, NH (iBu) and 8.65 (1H, d, *J* 8.50, NH (Ile));  $\delta_{\text{C}}$  (50.3 MHz, *d*<sub>6</sub>-DMSO) 11.0 ( $\underline{\text{C}}\text{H}_3$  (Ile C-5), 15.3 ( $\underline{\text{C}}\text{H}_3$  (Ile C-3')), 20.0 ( $\underline{\text{C}}\text{H}_3$  (iBu)), 23.6 ( $\underline{\text{C}}\text{H}_2$  (Pro C-4)), 24.4 ( $\underline{\text{C}}\text{H}_2$  (Ile)), 28.0 ( $\underline{\text{C}}\text{H}$  (iBu)), 29.9 ( $\underline{\text{C}}\text{H}_2$  (Pro C-3)), 36.8 ( $\underline{\text{C}}\text{H}$  (Ile)), 45.4 ( $\underline{\text{C}}\text{H}_2$  (Pro C-5)), 45.9 ( $\underline{\text{C}}\text{H}_2$  (iBu)), 57.8 (α  $\underline{\text{C}}\text{H}$  (Ile)), 58.6 (α  $\underline{\text{C}}\text{H}$  (Pro)), 168.2 ( $\underline{\text{C}}\text{O}$  (Ile)) and 170.8 ( $\underline{\text{C}}\text{O}$  (Pro));  $m/z$  (CI) 284 ( $[\text{M} + \text{H} - \text{HCl}]^+$ , 100%).

## Boc-(2S)-phenylalanine-(2S)-isoleucine-isobutylamide (137).

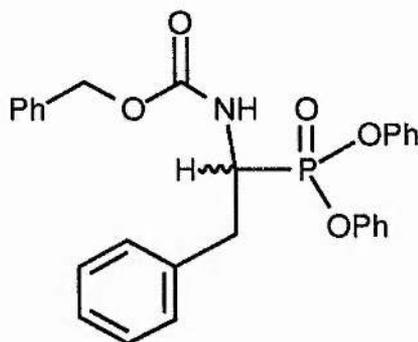


Boc-(2S)-phenylalanine (5.30 g, 20 mmol) was dissolved in THF (80 cm<sup>3</sup>), N-methylmorpholine (2.18 cm<sup>3</sup>, 20 mmol) was added and the solution cooled to -15 °C. Isobutylchloroformate (2.72 cm<sup>3</sup>, 20 mmol) was added with stirring and the solution left for 5 min at -15 °C. Isoleucine-isobutylamide.HCl (92) (4.50 g, 20 mmol) and N-methylmorpholine (2.18 cm<sup>3</sup>, 20 mmol) were dissolved in THF (~ 20 cm<sup>3</sup>) and added to the cold solution. The reaction was left at -15 °C for 5 min and a further 15 min at room temperature. The hydrochloride salts were filtered off and the THF removed under reduced pressure to yield a white solid (7.36 g, 85%). mp 167-169 °C; *m/z* (Found [M + H]<sup>+</sup> 434.3019. C<sub>24</sub>H<sub>40</sub>N<sub>3</sub>O<sub>4</sub> requires 434.3019).  $\nu_{\max}$  (Nujol) /cm<sup>-1</sup> 3323 (NH), 1689 (Urethane CO), 1645 (Amide COs), 1535 & 1510 (Amide II & Ar);  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 0.82-0.96 (12H, m, CH<sub>3</sub>'s), 1.40 (9H, s, BOC), 1.0-2.0 (4H, m, CH(a), CH(b), CH<sub>2</sub>(c)), 2.87-3.20 (4H, m, CH<sub>2</sub>(a), CH<sub>2</sub>(b)), 4.26 (1H, t, *J* 6, Phe  $\alpha$ -CH), 4.39 (1H, q, *J* 8, Ile  $\alpha$ -CH), 5.13 (1H, d, *J* 8, NH), 6.42 (1H, t, *J* 6, NH<sub>i</sub>Bu), 6.71 (1H, d, *J* 8, NH) & 7.12-7.35 (5H, m, Ar);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 11.27 (CH<sub>3</sub>(a)), 15.49 (CH<sub>3</sub>(b)), 20.04 (CH<sub>3</sub>(c)s), 24.48 (CH<sub>2</sub>(c)), 28.10 (BOC CH<sub>3</sub>s), 28.25 (CH(b)), 36.37 (CH(a)), 37.69 (CH<sub>2</sub>(b)), 46.78 (CH<sub>2</sub>(a)), 55.89 (Phe  $\alpha$ -CH), 58.12 (Ile  $\alpha$ -CH), 80.33 (BOC  $\underline{\text{C}}(\text{CH}_3)_3$ ), 126.87 (Ar p-CH), 128.57 (Ar m-CH), 129.15 (Ar o-CH), 136.23 (Ar  $\underline{\text{C}}-\text{CH}_2$ ), 155.48 (urethane CO) & 170.51 & 171.24 (amide COs); *m/z* (EI) 434 (M<sup>+</sup>, 23%), 378 (18, [M + 2H - tBu]<sup>+</sup>), 360 (86, [M + 2H - O-tBu]<sup>+</sup>), 334 (35, [M + 2H - BOC]<sup>+</sup>).

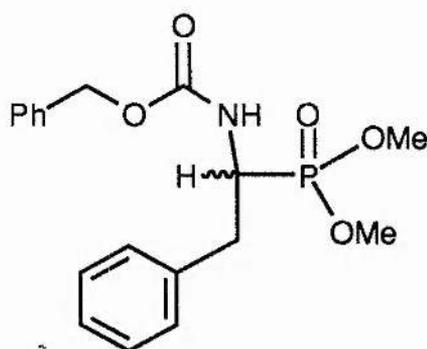
**(2S)-Phenylalanine-(2S)-isoleucine-isobutylamide hydrochloride (73).**

This deprotection was carried out in an identical manner to (92), using (137) (1.0 g, 2.3 mmol) in acidic ethyl acetate (50 cm<sup>3</sup>) to yield a white solid, which was recrystallised from methanol/ ethyl acetate (765 mg, 90 %). m.p. 142-143 °C (decomp.); (Found: C, 61.20; H, 8.95; N, 11.27. C<sub>19</sub>H<sub>32</sub>N<sub>3</sub>O<sub>2</sub>Cl requires C, 61.69; H, 8.72; N, 11.36%); *m/z* (Found: [M + H - HCl]<sup>+</sup> 334.2495. C<sub>19</sub>H<sub>32</sub>N<sub>3</sub>O<sub>2</sub> requires 334.2494); [α]<sub>D</sub> -9.4 (c 1 in MeOH); ν<sub>max</sub> (Nujol) /cm<sup>-1</sup> 3303 (NH), 1653 (COs), 1559 (Amide II) & 1502 (Ar); δ<sub>H</sub> (600 MHz, *d*<sub>6</sub>-DMSO) 0.81-0.84 (12H, m, CH<sub>3</sub>'s), 1.08 & 1.48 (2H, m, CH<sub>2</sub>(c)), 1.65-1.70 (2H, m, CH(a), CH(b)), 2.83 & 2.92 (2H, m, CH<sub>2</sub>(a)), 2.97 (1H, ABX splitting, *J*<sub>AX</sub> 6, *J*<sub>AB</sub> 13.8, 1 of CH<sub>2</sub>(b)), 3.08 (1H, ABX splitting, *J*<sub>AX</sub> 6, *J*<sub>AB</sub> 13.8, 1 of CH<sub>2</sub>(b)), 4.12 (1H, t, *J* 7.2 Hz, Phe α-CH), 4.16 (1H, app. t, *J* 8.4, Ile α-CH), 7.21-7.27 (5H, m, Ar), 8.08 (1H, t, *J* 5.4, NH*i*Bu), 8.21 (3H, s, NH<sub>3</sub><sup>+</sup>) & 8.6 (1H, d, *J* 9, NH-Ile); δ<sub>C</sub> (50.3 MHz, *d*<sub>6</sub>-DMSO) 11.36 (CH<sub>3</sub>(a)), 15.58 (CH<sub>3</sub>(b)), 20.49 (CH<sub>3</sub>(c)s), 24.71 (CH<sub>2</sub>(c)), 28.21 (CH(b)), 37.01 (CH<sub>2</sub>(c)), 37.15 (CH(a)), 46.31 (CH<sub>2</sub>(a)), 53.25 (Phe α-CH), 57.56 (Ile α-CH), 127.29 (Ar *p*-CH), 128.69 (Ar *m*-CH), 129.89 (Ar *o*-CH), 135.04 (Ar C-CH<sub>2</sub>) & 167.79 & 170.46 (COs). *m/z* (CI) 334 ([M + H - HCl]<sup>+</sup>, 100%), 316 (15, [M + 2H - HCl - NH<sub>3</sub>]<sup>+</sup>), 261 (6, [M + H - HCl - NH*i*Bu]<sup>+</sup>), 187 (6, [Ile-NH*i*Bu+H]<sup>+</sup>), 86 (35, [H<sub>2</sub>NCHCH(CH<sub>3</sub>)Et]<sup>+</sup>), 74 (10, [*i*Bu-NH<sub>3</sub>]<sup>+</sup>).

**Diphenyl [1-(N-benzyloxycarbonyl)-aminol]-2-phenylethylphosphonate**  
(74).<sup>158b</sup>



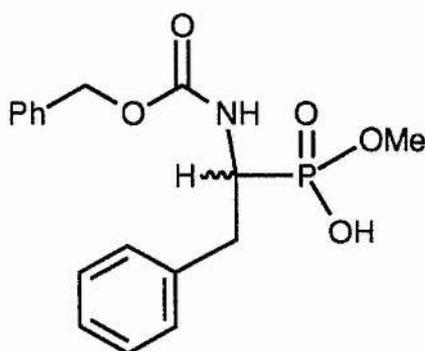
Triphenyl phosphite (31.0 g, 0.1 mol), phenylacetaldehyde (18 g, 0.15 mol) and benzyl carbamate (15.1 g, 0.1 mol) were dissolved in glacial acetic acid (15 cm<sup>3</sup>) and stirred at room temperature until the exothermic reaction had ceased (about 1 h). The mixture was then heated to 80 °C for a further 1 h and the volatile products were then removed under reduced pressure on a boiling water bath. The yellow residue was dissolved in methanol (150 cm<sup>3</sup>) and left to crystallise at -10 °C for 3 h. After this time the crystalline product was collected by filtration, dissolved in the minimum quantity of hot chloroform and a 4 fold excess of methanol added to give the product as a white solid after storage overnight at -10 °C (14.1g, 29%), m.p. 120-2 °C (lit.<sup>158b</sup> 119-120 °C); *m/z* (Found: [M + H]<sup>+</sup> 487.1549. C<sub>28</sub>H<sub>26</sub>NO<sub>5</sub>P requires 487.1549); *v*<sub>max</sub> (Nujol) /cm<sup>-1</sup> 3287 (NH), 1720 (urethane CO), 1590 (amide II), 1262 (P=O) and 1212 (P-OPh); δ<sub>H</sub> (300 MHz, C<sup>2</sup>HCl<sub>3</sub>) 3.1-3.5 (2H, m, CH<sub>2</sub>-CH), 4.82 (1H, m, CH), 5.12 (2H, s, CH<sub>2</sub>-O), 5.34 (1H, d, *J* 10.5, NH) & 7.02-7.40 (20 H, m, aromatic); δ<sub>C</sub> (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 36.5 (PhCH<sub>2</sub>CH), 49.8 (d, *J*<sub>P-H</sub> 158.1 PhCH<sub>2</sub>CH), 67.7 (PhCH<sub>2</sub>-O), 120.8-121.2 (aromatic C-4), 125.8-130.3 (aromatic C-2,3,5,6), 136.1 & 136.7 (quaternary aromatic) & 150.4 (urethane CO); δ<sub>P</sub> (121.5 MHz, C<sup>2</sup>HCl<sub>3</sub>) 17.17 (88%) & 16.74 (12%); *m/z* (EI) 487 (M<sup>+</sup>, 6%), 394 (32, [M - OPh]<sup>+</sup>), 379 (15, [M - PhCH<sub>2</sub>OH]<sup>+</sup>), 336 (100, [M - H<sub>2</sub>NCO-OCH<sub>2</sub>Ph]<sup>+</sup>), 234 (13, [PO(OPh)<sub>2</sub>]<sup>+</sup>), 145 (37, [PhCH<sub>2</sub>CH-NHCO]<sup>+</sup>), 94 (65, [PhOH]<sup>+</sup>) & 77 (15, Ph<sup>+</sup>).

Dimethyl [1-(N-benzyloxycarbonyl)-amino]-2-phenylethylphosphonate (75).<sup>151</sup>

The diphenyl compound (74) (15.4 g, 31 mmol) was dissolved with warming in dry methanol (200 cm<sup>3</sup>) and sodium (3.12 g, 64 mmol) in dry methanol (30 cm<sup>3</sup>) added dropwise with stirring. The solution was stirred at room temperature overnight, then the volume reduced to 20 cm<sup>3</sup> on a rotary evaporator and ethyl acetate (200 cm<sup>3</sup>) added. The solution was washed with 5 % sodium hydrogen carbonate solution (3 × 80 cm<sup>3</sup>), 0.1 M hydrochloric acid (1 × 80 cm<sup>3</sup>), water (1 × 80 cm<sup>3</sup>) and brine (1 × 80 cm<sup>3</sup>). The solution was dried (MgSO<sub>4</sub>) and the solvent removed under reduced pressure to give a yellow oil. This was purified by flash chromatography on silica, eluting with ethyl acetate, to give the product as a white solid after trituration with petroleum ether (7.56 g, 67%), m.p. 67-69 °C (lit.<sup>151</sup> 67-69 °C); *m/z* (Found [M + H]<sup>+</sup> 364.1314. C<sub>18</sub>H<sub>23</sub>NO<sub>5</sub>P requires 364.1315);  $\nu_{\max}$  (Nujol) /cm<sup>-1</sup> 3224 (NH), 1710 (urethane CO), 1586 (amide II), 1258 (P=O) & 1057 (P-OMe);  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 2.84-3.3 (2H, m, PhCH<sub>2</sub>CH), 3.62 & 3.68 (6H, d, *J*<sub>P-H</sub> 10.6, P(O)(OCH<sub>3</sub>)<sub>2</sub>), 4.40 (1H, dt, *J* 7, 8.6, PhCH<sub>2</sub>CH), 4.97 (2H, s, PhCH<sub>2</sub>O), 5.81 (1H, d, *J* 7.1, -NH) & 7.20 (10H, m, Ar);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 34.5 (PhCH<sub>2</sub>CH), 49.0 (d, PhCH<sub>2</sub>CH, *J*<sub>P-H</sub> 156), 52.8 (OMe), 65.5 (PhCH<sub>2</sub>O), 126.7-129.3 (aromatic), 137.4 & 137.7 (quaternary aromatic) & 156.1 (urethane CO);  $\delta_{\text{P}}$  (121.5 MHz, C<sup>2</sup>HCl<sub>3</sub>) 26.83 (91%) & 26.21 (9%); *m/z* (CI) 364 ([M + H]<sup>+</sup>, 100%), 259 (12, [M + 2H -

PhCH<sub>2</sub>O]<sup>+</sup>), 230 (3, [M + H - PhCH<sub>2</sub>OCO]<sup>+</sup>), 120 (17, [PhCH<sub>2</sub>CHNH<sub>2</sub>]<sup>+</sup>), 108 (11, [PhCH<sub>2</sub>OH]<sup>+</sup>) & 91 (13, [PhCH<sub>2</sub>]<sup>+</sup>).

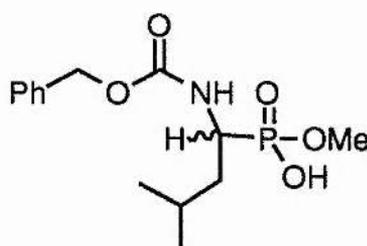
**Methyl hydrogen [1-(N-benzyloxycarbonyl)-amino]-2-phenyl-ethyl-phosphonate (76).<sup>151</sup>**



The dimethyl compound (75) (3.63 g, 10 mmol) was dissolved in methanol (30 cm<sup>3</sup>) and 2 M sodium hydroxide solution (15 cm<sup>3</sup>) added with stirring. The solution was stirred at room temperature for 12 h and then diluted with water (60 cm<sup>3</sup>) and washed with ethyl acetate (100 cm<sup>3</sup>). The aqueous layer was acidified to pH 2 with concentrated hydrochloric acid and extracted with ethyl acetate (3 x 70 cm<sup>3</sup>). The organic extracts were dried (MgSO<sub>4</sub>) and evaporated under reduced pressure to give the product as a white solid (2.69 g, 77%). m.p. 126-127 °C (lit.<sup>151</sup> 126.5-127.5 °C). *m/z* (Found: [M + H]<sup>+</sup> 350.1152. C<sub>17</sub>H<sub>21</sub>NO<sub>5</sub>P requires 350.1154);  $\nu_{\max}$  (Nujol) /cm<sup>-1</sup> 3289 (OH), 2665 & 2320 (P-OH), 1687 (urethane CO), 1546 (amide II), 1219 (P=O), 1041 (P-OH) and 982 (PO-C);  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 2.93 (2H, m, PhCH<sub>2</sub>CH), 3.62 (3H, d, *J*<sub>PH</sub> 10.6, P(O)OCH<sub>3</sub>), 4.40 (1H, dt, *J*<sub>NH</sub> 7.1, *J*<sub>CH</sub> 6.3, PhCH<sub>2</sub>CH), 4.97 (2H, s, PhCH<sub>2</sub>O), 5.64 (1H, br, OH), 5.81 (1H, d, *J* 7.1, NH) and 7.15 (10H, m, Ar);  $\delta_{\text{C}}$  (50.31 MHz, C<sup>2</sup>HCl<sub>3</sub>) 38.55 (PhCH<sub>2</sub>CH), 53.33 (d, *J*<sub>PC</sub> 103, PhCH<sub>2</sub>CH), 56.37 (d, *J*<sub>PC</sub> 21.1, OMe), 69.21 (PhCH<sub>2</sub>O), 126.71-129.36 (aromatic), 141.34 & 142.40 (quat. aromatic) and 156.17 (urethane CO);  $\delta_{\text{P}}$  (121.5 MHz, C<sup>2</sup>HCl<sub>3</sub>) 22.55 (9.5%) and 23.11 (90.5%); *m/z* (EI)

350 ( $[M + H]^+$ , 5.4%), 320 (100,  $[M - OMe + 2H]^+$ ), 242 (18,  $[M - OMe - PhCH_2O + 2H]^+$ ) and 91 (49,  $PhCH_2^+$ ).

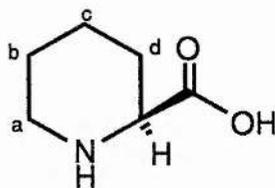
**Methyl Hydrogen [1-(N-Benzoyloxycarbonyl)-amino]-3-methyl-butylphosphonate (78).**



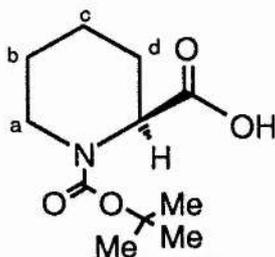
To a stirred solution of benzyl carbamate (0.76 g, 5 mmol) and  $PCl_3$  (0.68 g, 5 mmol) in acetyl chloride ( $10\text{ cm}^3$ ), was added dropwise isovaleraldehyde (0.52 g, 6 mmol, 1.2 eq) at  $0^\circ\text{C}$  over a period of 10 minutes. The solution was then stirred at room temperature for 1 h. After removal of the volatile compounds under reduced pressure, the resultant solid was suspended in dry THF ( $10\text{ cm}^3$ ) and an excess of dry methanol (10-20 mmol) was added slowly. The solution was then stirred at room temperature for 1.5 to 2 h. The solvent and volatile products were removed under reduced pressure to yield the product as a yellowy oil which was recrystallised from ether / light petroleum (0.57 g, 36 %). m.p.  $106\text{-}108^\circ\text{C}$ .  $m/z$  (Found:  $[M + H]^+$ , 316.1314.  $C_{14}H_{23}NO_5P$  requires 316.1314);  $\nu_{\text{max}}$  (Nujol) /  $\text{cm}^{-1}$  3281 (NH), 1689 (CO), 1543 (Amide II), 1270 (PO) & 1038 (P-OMe);  $\delta_{\text{H}}$  (200 MHz,  $C^2HCl_3$ ) 0.73-1.06 (6H, m,  $CH_3$ s), 1.15-1.83 (3H, m,  $CH_2$ , CH), 3.67 (3H, d,  $J$  16, O- $CH_3$ ), 4.0-4.25 (1H, m,  $\alpha$ -CH), 5.08 (2H, s,  $PhCH_2O$ ), 5.1 (1H, obscured, NH), 5.5 (1H, br, OH) & 7.28-7.45 (5H, m, Ar);  $\delta_{\text{C}}$  (50.3 MHz,  $C^2HCl_3$ ) 21.8 & 24.1 ( $CH_3$ ), 25.04 & 25.31 (CH), 36.59 ( $CH_2$ ), 44.91 & 48.16 (d,  $J_{\text{P-H}}$  164,  $\alpha$ -CH), 53.3 (OMe), 67.8 ( $PhCH_2O$ ), 128.62-129.22 (Aromatics), 137.12 (Quaternary aromatics) & 157.09 (Urethane CO).  $m/z$  (CI)

316 ( $[M + H]^+$ , 77%), 298 (3,  $[M + H - OH]^+$ ), 225 (15,  $[M + H - Bz]^+$ ), 208 (8,  $[M + H - OBz]^+$ ).

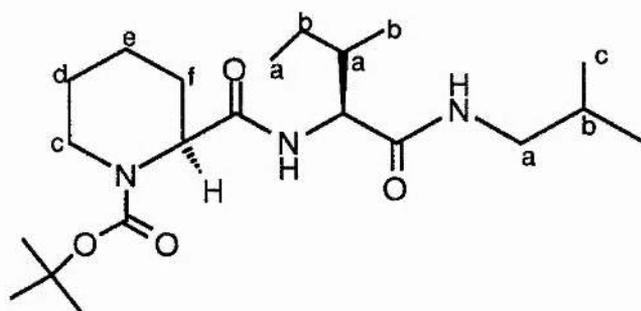
**(S)-Pipcolinic Acid (91).**<sup>171</sup>



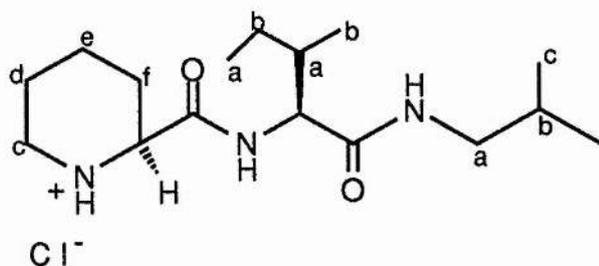
(R/S)-pipcolinic acid (2.0 g, 15.5 mmol) was suspended in hot methanol (7 cm<sup>3</sup>) and (2S,3S)-tartaric acid (2.33 g, 15.5 mmol) was added with stirring. The solution was then cooled and allowed to crystallise. The crystals were filtered off and recrystallised from water and acetone to give (S)-pipcolinic-(2S,3S)-tartrate (m.p. 195-196 °C (lit.<sup>171</sup> 195-196 °C);  $[\alpha]_D = -19.7$  (c 2 in H<sub>2</sub>O) (lit.<sup>171</sup> -21 (c 2 in H<sub>2</sub>O)).  $\nu_{\max}$  (Nujol)/ cm<sup>-1</sup> 3422 (NH) & 1713 (COs).  $\delta_H$  (200 MHz, <sup>2</sup>H<sub>2</sub>O) 1.51-1.84 & 2.15-2.21 (6H, m, CH<sub>2</sub>'s (b), (c) & (d)), 2.97, 3.61 & 3.66 (3H, m,  $\alpha$ -CH & CH<sub>2</sub>(a)) & 4.61 (2H, s, Tartrate CH's);  $\delta_C$  (50.3 MHz, <sup>2</sup>H<sub>2</sub>O) 24.31 & 24.57 (CH<sub>2</sub>s (b) & (c)), 29.07 (CH<sub>2</sub> (d)), 46.56 (CH<sub>2</sub> (a)), 61.06 ( $\alpha$ -CH), 74.96 (Tartrate CH's), 176.44 (Pip CO) & 177.86 (Tartrate COs). Pipcolinic tartrate was dissolved in water and then applied to a Amberlite IR-120 (H<sup>+</sup>) ion exchange resin (100 cm<sup>3</sup> of resin). The column was washed with distilled water to remove the tartrate and then eluted with 10% aqueous ammonia solution to give the (S)-pipcolinic acid (0.57 g, 57 % yield), m.p. 268-270 °C (lit.<sup>171</sup> 270-272 °C);  $[\alpha]_D = -23^\circ$  (c 1.5 in H<sub>2</sub>O) (lit.<sup>171</sup> -26 (c 2 in H<sub>2</sub>O)).  $\nu_{\max}$  (Nujol)/ cm<sup>-1</sup> 3060 (NH) & 1647 (CO);  $\delta_H$  (200 MHz, <sup>2</sup>H<sub>2</sub>O) 1.65 (5H, m) & 2.10 (1H, m) (CH<sub>2</sub>'s (b)-(d)), 2.85 (1H, m) & 3.37 (2H, m) (CH<sub>2</sub>(a) &  $\alpha$ -CH);  $\delta_C$  (50.3 MHz, <sup>2</sup>H<sub>2</sub>O) 24.39 & 24.67 (CH<sub>2</sub>s (b) & (c)), 29.37 (CH<sub>2</sub> (d)), 46.48 (CH<sub>2</sub> (a)), 61.84 ( $\alpha$ -CH) & 177.43 (Pip CO).

**Boc-(2S)-Pipicolinic acid (138).**

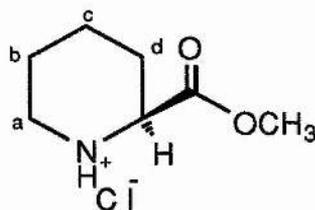
(S)-Pipicolinic acid (**91**) (2.58 g, 20 mmol) was dissolved in 1 M NaOH (20 cm<sup>3</sup>) and *t*-butyl alcohol (20 cm<sup>3</sup>) added with stirring. Di-*tert*-butyl-dicarbonate (5.8 g, 26 mmol, 1.3 eq) dissolved in *t*-butyl alcohol (10 cm<sup>3</sup>) was then added, with any resulting precipitate dissolved with 1 M NaOH. The mixture was stirred at r.t. for 3 h and then the alcoholic layer was removed under reduced pressure. The aqueous layer was washed with light petroleum (30 cm<sup>3</sup>) and then acidified to pH 3 with 1 M sulphuric acid in an ice bath. The turbid mixture was extracted with diethyl ether (3 x 50 cm<sup>3</sup>) and the combined organic layers washed with distilled water (2 x 50 cm<sup>3</sup>). The organic layer was dried (MgSO<sub>4</sub>) and evaporated under reduced pressure to give a clear oil, which was recrystallised from ether/ light petroleum to give a white crystalline solid (2.93 g, 64 %), m.p. 116-118 °C; (Found: C, 57.75; H, 8.15; N, 6.05. C<sub>11</sub>H<sub>19</sub>NO<sub>4</sub> requires C, 57.65; H, 8.3; N, 6.1 %); *m/z* (Found: [M + H]<sup>+</sup> 230.139. C<sub>11</sub>H<sub>20</sub>NO<sub>4</sub> requires 230.1392); [α]<sub>D</sub> -27.3° (c 1 in MeOH); ν<sub>max</sub> (Nujol)/ cm<sup>-1</sup> 3200-3000 (OH, br), 1747 (Acid CO), 1661 (Urethane CO), 1634, 1349, 1317, 1268, 1257, 1198, 1162, 1144 & 1037; δ<sub>H</sub> (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 1.25-1.80 (5H, m) & 2.21-2.27 (1H, m) (CH<sub>2</sub>'s (a), (b) & (c)), 1.46 (9h, s, Boc CH<sub>3</sub>'s), 2.96 (2H, t, *J* 7.5, CH<sub>2</sub> (d)) & 3.97 (1H, m, α-CH); δ<sub>C</sub> (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 21.24 (CH<sub>2</sub> (c)), 25.18 (CH<sub>2</sub> (b)), 27.09 (CH<sub>2</sub> (a)), 28.82 (Boc CH<sub>3</sub>s), 42.65 (CH<sub>2</sub> (d)), 54.15 (α-CH), 80.80 (Boc C(CH<sub>3</sub>)<sub>3</sub>), 156.07 (urethane CO) & 178.38 (acid CO); *m/z* (CI) 247 ([M + NH<sub>4</sub>]<sup>+</sup>, 3%), 230 (10, [M + H]<sup>+</sup>), 174 (27, [M - *t*Bu]<sup>+</sup>), 130 (100, [M + H - Boc]<sup>+</sup>).

**Boc-(S)-pipecolinic acid-(2S)-isoleucine-isobutylamide (139).**

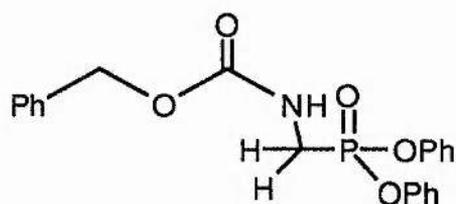
This compound was prepared in an identical manner to (135), except using (138) (4.12 g, 18 mmol) and (92) (3.34 g, 18 mmol) in dry THF (100 cm<sup>3</sup>); the crude white solid was recrystallised from ethyl acetate/ light petroleum (5.36 g, 75 %);  $\nu_{\max}$  (C<sup>2</sup>HCl<sub>3</sub>)/ cm<sup>-1</sup> 2968 (NH), 1678 (COs), 1368 & 1162;  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 0.90 (12H, m, CH<sub>3</sub>s), 1.12-2.23 (10H, m, CH's (a) & (b), CH<sub>2</sub>'s (b), (d), (e) & (f)), 1.47 (9H, s, Boc CH<sub>3</sub>'s), 2.96-3.12 (4H, m, CH<sub>2</sub>'s (a) & (c)), 4.25 (1H, app. t, Pip  $\alpha$ -CH), 4.71 (1H, m, Ile  $\alpha$ -CH), 6.29 (1H, br, *i*-Bu NH) & 6.72 (1H, br d, *J* 8.9, Ile NH);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 11.60 (CH<sub>3</sub> (a)), 16.18 (CH<sub>3</sub> (b)), 20.57 (CH<sub>3</sub> (c)), 20.95 (CH<sub>2</sub> (e)), 25.20 (CH<sub>2</sub> (d)), 25.31 (CH<sub>2</sub> (b)), 28.72 (CH<sub>2</sub> (f)), 28.90 (Boc CH<sub>3</sub>s), 29.22 (CH (b)), 36.98 (CH (a)), 44.34 (CH<sub>2</sub> (c)), 47.32 (CH<sub>2</sub> (a)), 51.40 & 54.20 ( $\alpha$ -CHs), 81.29 (C(CH<sub>3</sub>)<sub>3</sub>), 156.64 (urethane CO), 171.40 & 172.11 (amide COs).

**(S)-Pipecolinic acid-(2S)-isoleucine-isobutylamide hydrochloride (93).**

This compound was prepared in an identical manner to (92), using boc-(S)-pipicolinic acid-(2S)-isoleucine-isobutylamide (139) (1.98 g, 5 mmol); the crude off white solid was recrystallised from methanol/ acetone (1.5 g, 90 %), m.p. 140 °C (decomp.).  $m/z$  (Found:  $[M + H - HCl]^+$  298.2495.  $C_{16}H_{32}N_3O_2$  requires 298.2494).  $\nu_{max}$  (Nujol)/  $cm^{-1}$  3380, 3233 (NHs), 1670, 1632 (COs) & 1546 (Amide II);  $\delta_H$  (400 MHz,  $d_6$ -DMSO) 0.81-0.85 (12 H, m,  $CH_3$ s), 1.06-1.13 (1 H, m, CH (b)), 1.42-1.50 & 1.62-1.75 (9 Hs, m, CH (a),  $CH_2$ 's (b), (d), (e) & (f)), 2.77-2.98 (3 H, m) & 3.16-3.19 (1H, m) ( $CH_2$ 's (a) & (c)), 3.78-3.80 (1 H, m, Ile  $\alpha$ -CH), 4.17 (1H, t,  $J$  8.2, Pip  $\alpha$ -CH), 8.11 (1 H, t,  $J$  6, NH<sub>i</sub>Bu), 8.57 (2 H, d,  $J$  8.2,  $NH_2^+$ ) & 9.2-9.4 (1 H, br, Ile NH);  $\delta_C$  (400 MHz,  $d_6$ -DMSO) 11.11 ( $CH_3$ (a)), 15.46 ( $CH_3$ (b)), 20.17 ( $CH_3$ (c)), 21.29 & 21.68 ( $CH_2$ (e) & (f)), 24.45 ( $CH_2$ (b)), 27.26 (CH(b)), 28.03 ( $CH_2$ (d)), 36.74 (CH(a)), 43.35 ( $CH_2$ (b)), 46.05 ( $CH_2$ (a)), 56.89 & 57.37 ( $\alpha$ -CHs), 168.37 & 170.41 (COs);  $m/z$  (CI) 298 ( $[M + H - HCl]^+$ , 83 %), 197 (2,  $[M + H - HCl - CONHiBu]^+$ ), 84 (100, Piperidine ring).

**(S)-Pipicolinic acid methyl ester hydrochloride (140).**

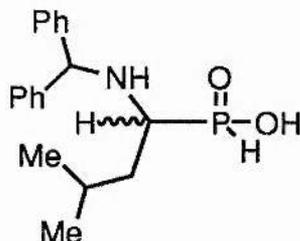
(S)-Pipicolinic acid (91) (2.58 g, 20 mmol) was dissolved in freshly distilled methanol (80 cm<sup>3</sup>). Distilled thionyl chloride (3.19 cm<sup>3</sup>, 44 mmol, 2.2 eq) was added dropwise with stirring at 0 °C, the solution allowed to warm to room temperature and then refluxed gently for 90 min. The solvent was removed under reduced pressure to yield a white solid which was recrystallised from methanol/ diethyl ether (2.19 g, 61 %);  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>H<sub>3</sub>O<sup>2</sup>H) 1.64-1.93 (5H, m) & 2.26-2.32 (1H, m) (CH<sub>2</sub> (b), (c) & (d)), 3.11 & 3.39 (2H, m, CH<sub>2</sub> (a)), 3.86 (3H, s, OCH<sub>3</sub>) & 4.08 (1H, ABX,  $J_{\text{AX}}$  3.64,  $J_{\text{AB}}$  11.25,  $\alpha$ -CH);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>H<sub>3</sub>O<sup>2</sup>H) 23.04 & 23.13 (CH<sub>2</sub> (b) & (c)), 27.42 (CH<sub>2</sub> (d)), 45.50 (CH<sub>2</sub>(a)), 54.04 (OCH<sub>3</sub>), 58.15 ( $\alpha$ -CH) & 170.64 (CO).

**Diphenyl 1-(N-benzyloxycarbonyl)-amino-methane phosphonate (141).<sup>159</sup>**

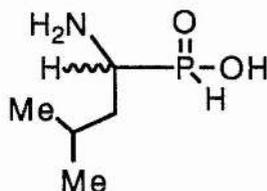
A mixture of benzyl carbamate (5.03 g, 33 mmol), paraformaldehyde (1 g, 33 mmol), acetic anhydride (8 cm<sup>3</sup>) and glacial acetic acid (5 cm<sup>3</sup>) was stirred for 3 h at 60-70 °C. Triphenyl phosphite (10.33 g, 33 mmol) was then added and the mixture stirred for a further 2 h at 110-120 °C. After removal of the acetic acid and acetic anhydride under reduced pressure, the mixture was

dissolved in methanol and allowed to stand at  $-10\text{ }^{\circ}\text{C}$  overnight. The precipitate formed was collected by filtration and washed with methanol. This was then recrystallised from chloroform/ methanol to give a white solid (2.70 g, 20 %), m.p.  $113\text{-}114\text{ }^{\circ}\text{C}$  (lit.<sup>159</sup>  $114\text{-}116\text{ }^{\circ}\text{C}$ ).  $m/z$  (Found:  $[M + H]^+$  398.1155.  $\text{C}_{21}\text{H}_{21}\text{NO}_5\text{P}$  requires 398.1152);  $\nu_{\text{max}}$  (Nujol)  $/\text{cm}^{-1}$  3273 (NH), 1721 (urethane CO), 1594 (aromatic), 1558 (amide II), 1226 (P=O), 953 (P-OPh) and 766 (aromatic);  $\delta_{\text{H}}$  (200 MHz,  $\text{C}^2\text{HCl}_3$ ) 3.98 (2H, d,  $J$  6.2,  $\text{CH}_2\text{PO}$ ), 5.13 (2H, s,  $\text{CH}_2\text{-Ar}$ ), 5.32 (1H, br s, NH) and 7.08-7.41 (15H, m, aromatic);  $\delta_{\text{C}}$  (50.3 MHz,  $\text{C}^2\text{HCl}_3$ ) 35.97 and 39.15 (d,  $J_{\text{PC}}$  160,  $\text{CH}_2\text{-PO}$ ), 67.91 ( $\text{CH}_2\text{-Ar}$ ), 120.9-130.3 (aromatic) and 163.1 (urethane CO);  $\delta_{\text{P}}$  (121.49 MHz,  $\text{C}^2\text{HCl}_3$ ) 15.82 (96.2 %) and 15.24 (3.8 %);  $m/z$  (CI) 415 ( $[M + \text{NH}_4]^+$ , 2 %), 290 (100,  $[\text{OCNCH}_2\text{PO}(\text{OPh})_2 + \text{H}]^+$ ), 264 (7,  $[M + \text{H} - \text{Cbz}]^+$ ), 94 (16, PhOH) & 91 (33,  $\text{PhCH}_2^+$ ).

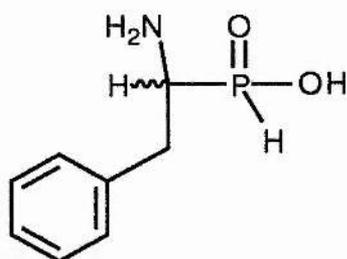
### 1-Diphenylmethyl-amino-3-methyl-butane phosphonous acid (142).<sup>161</sup>



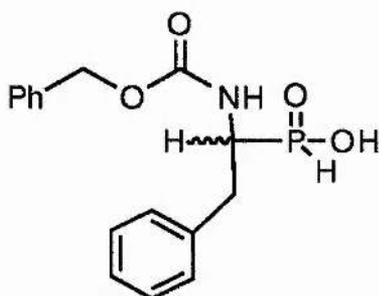
To a refluxing solution of aminodiphenylmethane (1.84 g, 10 mmol), aqueous hypophosphonous acid (50 %, 1.72 g, 10 mmol) and 1 M hydrochloric acid (10  $\text{cm}^3$ , 1 eq) in water (20  $\text{cm}^3$ ), was added dropwise with stirring. isovaleraldehyde (0.86 g, 10 mmol) in water (10  $\text{cm}^3$ ). Heating was continued for 2 h during which time a white solid precipitated out. The solution was cooled, then the solid collected by filtration and washed with acetone (2.50 g, 79 %), m.p.  $215\text{-}217\text{ }^{\circ}\text{C}$  (lit.<sup>161</sup>  $215\text{-}218\text{ }^{\circ}\text{C}$ ).  $\delta_{\text{H}}$  (200 MHz,  $^2\text{H}_2\text{O}$ ), 0.4-0.6 (6H, m,  $\text{CH}_3$ 's), 1.2-1.6 (3H, m, CH &  $\text{CH}_2$  on alkyl chain), 2.3 (1H, m,  $\alpha\text{-CH}$ ), 4.9 (1H, s,  $\text{Ph}_2\text{CHN}$ ), 6.7-7.1 (10H, m, aromatics), 5.5 & 8.0 (1H, d,  $J$  500, P(O)-H).

**1-Amino 3-methyl butane phosphonous acid (143).<sup>161</sup>**

The diphenylmethylaminomethyl butane phosphonous acid (142) (2.50 g, 8 mmol) was heated together with an excess of hydrobromic acid (48%, 6.75 g, 40 mmol, 5 eq) at 100 °C for 2-3 h until 2 distinct phases could be observed. The mixture was then evaporated to dryness under reduced pressure and the residue taken up in water. The aqueous solution was then washed with ether (3 x 30 cm<sup>3</sup>) to remove the diphenylmethyl bromide and then evaporated to dryness. The oily residue was dissolved in ethanol (25 cm<sup>3</sup>) and propylene oxide added until precipitation was observed. The mixture was then allowed to stand until complete precipitation had occurred. The solid was filtered off, washed in turn with ethanol and ether and then dried to give a white solid (0.43 g, 35 %), m.p. 222-224 °C (lit.<sup>161</sup> 222-223 °C).  $\delta_{\text{H}}$  (200 MHz, <sup>2</sup>H<sub>2</sub>O) 0.8-0.9 (6H, m, CH<sub>3</sub>'s), 1.5-1.7 (3H, m, CH<sub>2</sub> & CH), 3.12 (1H, m,  $\alpha$ -CH), 5.6 & 8.2 (1H, d, *J* 520, P(O)-H).  $\delta_{\text{C}}$  (50.31 MHz, <sup>2</sup>H<sub>2</sub>O) 23.3 & 24.7 (CH<sub>3</sub>'s), 26.6 (CH), 37.6 (CH<sub>2</sub>), & 50.4 & 52.2 ( $\alpha$ -CH, *J*<sub>PC</sub> 91).

**1-Amino-2-phenyl-ethyl phosphonous acid (100).**<sup>161</sup>

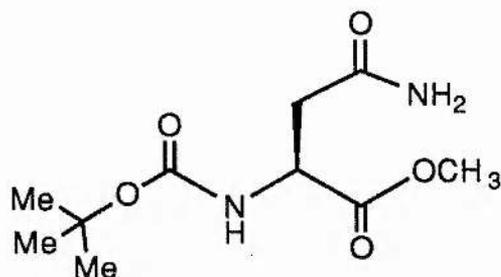
This was prepared as for (143), except using 1-(diphenylmethyl)-amino-2-phenyl-ethyl phosphonous acid (99) (4.14 g, 11.8 mmol), yielding the product as a white solid (1.81 g, 83 %), m.p. 226-228 °C (lit.<sup>161</sup> 227-228 °C);  $\delta_{\text{H}}$  (200 MHz,  $^2\text{H}_2\text{O}$ ) 2.82 (1H, m, 1 of benzyl  $\text{CH}_2$ ), 3.31 (2H, m, 1 of benzyl  $\text{CH}_2$  &  $\alpha$ -CH), 6.99 (1H, d,  $J$  536.7 Hz, PH) & 7.25-7.40 (5H, m, aromatic CHs);  $\delta_{\text{C}}$  (50.3 MHz,  $^2\text{H}_2\text{O}$ ) 34.72 (benzyl  $\text{CH}_2$ ), 54.77 (d,  $J$  88 Hz,  $\alpha$ -CH), 130.39-132.20 (aromatic CHs) & 137.87 (quaternary aromatic);  $\delta_{\text{P}}$  (121.5 Hz,  $^2\text{H}_2\text{O}$ ) 18.94 (100 %).

**1-Benzyloxycarbonyl-amino-2-phenyl-ethyl phosphonous acid (101).**<sup>161</sup>

1-Amino-2-phenyl-ethyl phosphonous acid (100) (555 mg, 3 mmol) was dissolved in water (10  $\text{cm}^3$ ) and the pH of the resultant solution adjusted to 9.5 with 5 M NaOH. Benzyl chloroformate (665 mg, 555  $\mu\text{l}$ , 3.9 mmol, 1.3 eq) was added slowly and the solution stirred at r.t. for 4 h. During this time, the pH was periodically checked and restored to 9-9.5 *via* the addition of 5 M NaOH solution. The aqueous solution was washed with diethyl ether

(10 cm<sup>3</sup>) and then slowly added to a 2:3 mixture of 6 M HCl and ice. The resulting precipitate was collected by filtration and recrystallised from ethyl acetate/ light petroleum (813 mg, 85 %);  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>H<sub>3</sub>O<sup>2</sup>H) 2.82 & 3.15 (2H, m, benzyl CH<sub>2</sub>), 3.99-4.12 (1H, m,  $\alpha$ -CH), 4.87-5.06 (2H, m, Cbz CH<sub>2</sub>), 6.99 (1H, d, *J* 553.5 Hz, PH) & 7.13-7.29 (10H, m, aromatic CH's);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>H<sub>3</sub>O<sup>2</sup>H) 34.03 (benzyl CH<sub>2</sub>), 53.83 (d, *J* 108,  $\alpha$ -CH), 67.92 (Cbz CH<sub>2</sub>), 128.06-130.55 (aromatic CH's), 138.41 & 138.61 (quaternary aromatics) & 157.25 (urethane CO).

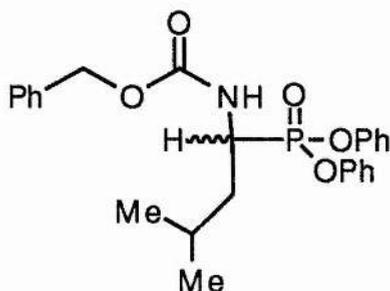
#### Boc-(2S)-Asparagine-methyl ester (144).



A solution of boc-(2S)-asparagine (0.42 g, 1.8 mmol) in dichloromethane (7 cm<sup>3</sup>) was cooled to 0 °C and diazomethane added dropwise with stirring until the yellow colour remains. The mixture was then allowed to stir at room temperature for 2 h. The excess diazomethane was flushed from the flask using nitrogen, and the solvent removed under reduced pressure to yield a yellowy oil. Careful recrystallisation from ethyl acetate/ light petroleum yielded a white solid (375 mg, 85 %), m.p. 85-87 °C. *m/z* (Found: [M + H]<sup>+</sup> 247.1294. C<sub>10</sub>H<sub>19</sub>N<sub>2</sub>O<sub>5</sub> requires 247.1294).  $\nu_{\text{max}}$  (Nujol) /cm<sup>-1</sup>: 3406, 3353 (NHs), 1742 (Ester CO), 1703 (Urethane CO), 1657 & 1626 (Amide I and II) & 1525 (Urethane Amide II);  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 1.4 (9H, s, Boc CH<sub>3</sub>'s), 2.7 (1H, ABX splitting, 1 of CH<sub>2</sub>), 2.9 (1H, ABX splitting, *J*<sub>AX</sub> 4, *J*<sub>AB</sub> 16.3, 1 of CH<sub>2</sub>), 3.7 (3H, s, OMe), 4.5 (1H, m,  $\alpha$ -CH), 5.75 (1H, d, NH, *J* 8.2) & 6.0 (2H, s, NH<sub>2</sub>).

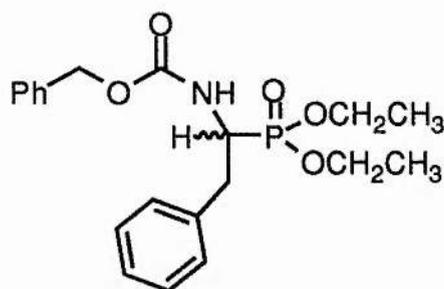
$\delta_C$  (50.3 MHz,  $C^2HCl_3$ ) 28.8 (Boc  $CH_3$ 's), 37.9 ( $CH_2$ ), 50.9 ( $\alpha$ -CH), 53.1 ( $OCH_3$ ), 80.6 (Boc  $C(CH_3)_3$ ) & 172.4, 172.6, 173.0 (3 COs).  $m/z$  (CI) 247 ( $[M + H]^+$ , 40%), 191 (58,  $[M + H - tBu]^+$ ), 176 (6,  $[M + H - O-tBu]^+$ ), 147 (100,  $[M + H - Boc]^+$ ).

**Diphenyl 1-[N-Benzyloxycarbonyl]-amino-3-methyl-butyl phosphonate (145).**<sup>158b</sup>



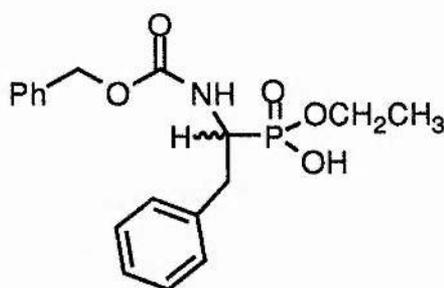
This compound was prepared in a manner identical with that for (74) except using iso-valeraldehyde (12.9 g, 100 mmol) in 55 % yield. m.p. 123-125 °C (lit.<sup>158b</sup> 122-123 °C).  $m/z$  (Found:  $[M + H]^+$  454.1783.  $C_{25}H_{29}NO_5P$  requires 454.1783);  $\nu_{max}$  (Nujol) / $cm^{-1}$  3270 (NH), 1713 (CO), 1591 (Ar C-C), 1550 (Amide II), 1270 (P=O), 1230 (P-OPh) & 946 (P<sup>v</sup>-OPh);  $\delta_H$  (200 MHz,  $C^2HCl_3$ ) 0.84-0.98 (6H, m,  $CH_3$ 's), 1.70-1.92 (3H, m, CH &  $CH_2$ ), 4.59 (1H, m,  $\alpha$ -CH), 5.12 (2H, s,  $CH_2O$ ), 5.34 (1H, d,  $J$  10.4, NH) & 7.01-7.33 (15H, m, aromatics).  $\delta_C$  (50.3 MHz,  $C^2HCl_3$ ) 21.62 & 23.81 ( $CH_3$ 's), 24.77 & 25.05 (CH), 39.03 ( $CH_2$ ), 45.81 & 48.95 (d,  $J_{P-H}$  158.3,  $\alpha$ -CH), 67.79 ( $PhCH_2O$ ), 120.85-130.29 (aromatics), 137.2 (Quaternary aromatic) & 158.42 (urethane CO).  $m/z$  (CI) 454 ( $[M + H]^+$ , 100%), 360 (8,  $[M + H - OPh]^+$ ), 346 (7,  $[M + H - OBz]^+$ ), 252 (2,  $[M + H - OPh - OBz]^+$ ).

## Diethyl [1-(benzyloxycarbonyl)-amino]-2-phenyl-ethyl phosphonate (146).

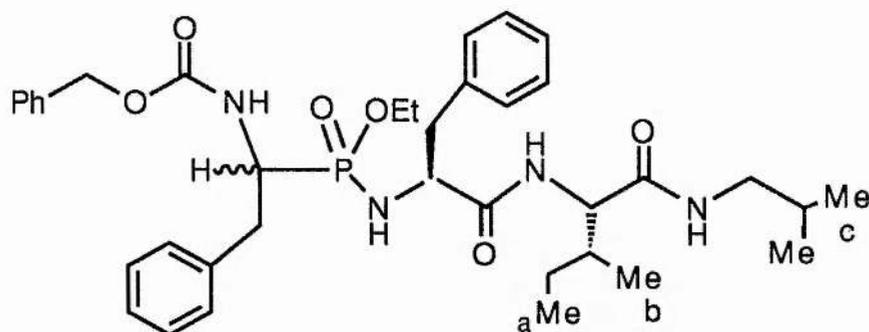


This compound was prepared in a manner identical to that used for (75), except using dry ethanol (150 cm<sup>3</sup>) and to it was added sodium (1.52 g, 66 mmol) in dry ethanol (40 cm<sup>3</sup>). After work-up and column chromatography (eluting with ethyl acetate), the product was isolated as a clear oil which could be further purified by recrystallisation using ether/ light petroleum (8.7 g, 74 %), m.p. 64-66 °C; *m/z* (Found: [M + H]<sup>+</sup> 392.1627. C<sub>20</sub>H<sub>27</sub>NO<sub>5</sub>P requires 392.16267);  $\nu_{\max}$  (Nujol) /cm<sup>-1</sup> 3233 (NH), 1722 (CO), 1548 (Amide II) & 1221 (PO);  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 1.26 (6H, t, *J* 7, OCH<sub>2</sub>CH<sub>3</sub>'s), 2.87 & 3.24 (2H, m, benzyl CH<sub>2</sub>), 4.08 (4H, q, *J* 7, OCH<sub>2</sub>CH<sub>3</sub>'s), 4.38 (1H, ddd, *J* 4.5, 10.5, 15.9,  $\alpha$ -CH), 5.00 (2H, s, Cbz CH<sub>2</sub>), 5.19 (1H, d, *J* 10.5, NH) & 7.10-7.32 (10H, m, aromatic CHs);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 16.92 (OCH<sub>2</sub>CH<sub>3</sub>'s), 36.45 (benzyl CH<sub>2</sub>), 49.09 (d, *J* 157,  $\alpha$ -CH), 63.08 (OCH<sub>2</sub>CH<sub>3</sub>'s), 67.40 (Cbz CH<sub>2</sub>), 127.27-129.73 (aromatic CHs), 137.20 (quaternary aromatic) & 156.15 (urethane CO);  $\delta_{\text{P}}$  (121.5 MHz, C<sup>2</sup>HCl<sub>3</sub>) 24.36 (90 %) & 23.77 (10 %); *m/z* (CI) 392 (100 %, [M + H]<sup>+</sup>), 301 (3, [M + H - PhCH<sub>2</sub>]<sup>+</sup>), 284 (31, [M + H - PhCH<sub>2</sub>OH]<sup>+</sup>).

**Ethyl hydrogen [1-(benzyloxycarbonyl)-amino]-2-phenyl-ethyl phosphonate (96).**



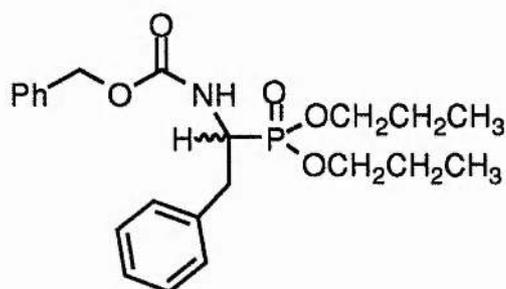
This compound was prepared in a manner identical to that used for (76), except using diethyl 1-(N-benzyloxycarbonyl)-amino-2-phenyl ethyl phosphonate (146) (3.91 g, 10 mmol) in ethanol (50 cm<sup>3</sup>). The product was recrystallised from dichloromethane/ light petroleum to yield a white solid (1.78 g, 49 %), m.p. 127-128 °C; *m/z* (Found: [M + H]<sup>+</sup> 364.1314. C<sub>18</sub>H<sub>23</sub>NO<sub>5</sub>P requires 364.13137);  $\nu_{\max}$  (Nujol) /cm<sup>-1</sup> 3285 (NH), 1688 (CO), 1539 (Amide II) & 1214 (PO);  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 1.27 (3H, t, *J* 7, OCH<sub>2</sub>CH<sub>3</sub>), 2.84 & 3.23 (2H, m, benzyl CH<sub>2</sub>), 4.13 (2H, q, *J* 7, OCH<sub>2</sub>CH<sub>3</sub>), 4.43 (1H, m,  $\alpha$ -CH), 4.91-5.06 (2H, m, Cbz CH<sub>2</sub>), 5.45 (1H, d, *J* 10, NH) & 7.17-7.31 (10H, m, aromatic CH's);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 16.83 (OCH<sub>2</sub>CH<sub>3</sub>), 36.19 (benzyl CH<sub>2</sub>), 49.34 (d, *J* 158.5  $\alpha$ -CH), 63.32 (OCH<sub>2</sub>CH<sub>3</sub>), 67.43 (Cbz CH<sub>2</sub>), 127.26-129.69 (aromatic CHs), 136.78 & 137.17 (quaternary aromatics) & 156.53 (urethane CO);  $\delta_{\text{P}}$  (121.5 MHz, C<sup>2</sup>HCl<sub>3</sub>) 25.93 (80 %), 25.23 (20 %); *m/z* (CI) 364 ([M + H]<sup>+</sup>, 18%), 273 (91, [M + H - PhCH<sub>2</sub>]<sup>+</sup>), 256 (49, [M + H - PhCH<sub>2</sub>OH]<sup>+</sup>), 108 (100, PhCH<sub>2</sub>OH).

**Cbz-(R/S)-Phe-[PO(OEt)-NH]-(2S)-Phe-(2S)-Ile-NH-iBu (94).**

To ethyl hydrogen 1-(N-benzyloxycarbonyl)-amino-2-phenyl-ethyl phosphonate (96) (545 mg, 1.5 mmol) in dry dichloromethane (15 cm<sup>3</sup>) was added thionyl chloride (357 mg, 219  $\mu$ l, 3 mmol, 2 eq) dropwise with stirring at 0 °C. The flask was flushed with nitrogen to remove the acidic gases formed during the reaction. The solution was then allowed to stir at r.t. for 4 h, with periodic flushing during that time. The solvent was removed under reduced pressure, the residue taken up in more dry dichloromethane (15 cm<sup>3</sup>) and flushed again. The solvent was again removed, more dichloromethane was added and the flask was flushed with nitrogen. The solution was cooled to 0 °C in an ice-bath and (2S)-Phe-(2S)-Ile-NHiBu.HCl (73) (554 mg, 1.5 mmol) and triethylamine (379 mg, 522  $\mu$ l, 3.75 mmol, 2.5 eq) in dry dichloromethane (10 cm<sup>3</sup>) was added dropwise with stirring. The solution was allowed to warm to r.t. and stirred overnight. The organic solution was washed with 1 M HCl (2 x 10 cm<sup>3</sup>), 5 % sodium bicarbonate solution (10 cm<sup>3</sup>) and saturated brine (10 cm<sup>3</sup>). The organic layer was dried (MgSO<sub>4</sub>), filtered and evaporated to yield a clear oil which resisted crystallisation (397 mg, 39 %); (Found: C, 65.3; H, 7.75; N, 8.05. C<sub>37</sub>H<sub>51</sub>N<sub>4</sub>O<sub>6</sub>P requires C, 65.45; H, 7.6; N, 8.25 %); *m/z* (Found: [M + H]<sup>+</sup> 679.3623. C<sub>37</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub>P requires 679.3624);  $\nu_{\max}$  (Nujol) /cm<sup>-1</sup> 3331 (NH), 1729 (urethane CO), 1699 & 1675 (amide CO's) & 1265 (PO);  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 0.82-0.93 (12H, m, Ile & iBu CH<sub>3</sub>s), 0.98-1.52 (3H, m, Ile CH

& CH<sub>2</sub>), 1.18-1.33 (3H, m, OCH<sub>2</sub>CH<sub>3</sub>), 1.68-1.87 (1H, m, *i*Bu CH), 2.70-3.32 (6H, m, benzyl CH<sub>2</sub>s & *i*Bu CH<sub>2</sub>), 3.63-3.77 (2H, m, Phe & Ile α-CHs), 4.11 (2H, q, J 7.6, OCH<sub>2</sub>CH<sub>3</sub>), 4.31-4.55 (1H, m, CH-P), 5.02 (2H, s, Cbz CH<sub>2</sub>), 7.09-7.42 (15H, m, aromatic CHs); δ<sub>C</sub> (75 MHz, C<sup>2</sup>HCl<sub>3</sub>) 10.89 (CH<sub>3</sub> (a)), 15.22 (CH<sub>3</sub> (b)), 16.83 (PO(OCH<sub>2</sub>CH<sub>3</sub>)), 18.92 (CH<sub>3</sub> (c)), 24.11 (Ile CH<sub>2</sub>), 27.56 (*i*-Bu CH), 36.64 & 36.79 (ArCH<sub>2</sub>CH\* & Ile CH), 39.28 (Phe CH<sub>2</sub>), 45.0 (*i*-Bu CH<sub>2</sub>), 48.9-51.7 (CH\* & PO(OCH<sub>2</sub>CH<sub>3</sub>)) 55.37 & 56.82 (α-CH's), 64.51 (Cbz CH<sub>2</sub>), 125.62-129.41 (aromatic CH's), 137.61-138.10 (quaternary aromatics), 154.96 (urethane CO) and 170.41 & 172.10 (amide CO's); δ<sub>P</sub> (121.5 MHz, C<sup>2</sup>HCl<sub>3</sub>) 29.52, 29.74, 29.89, 29.99; *m/z* (FAB) 701 ([M + Na]<sup>+</sup>, 22 %), 679 (72, [M + H]<sup>+</sup>)

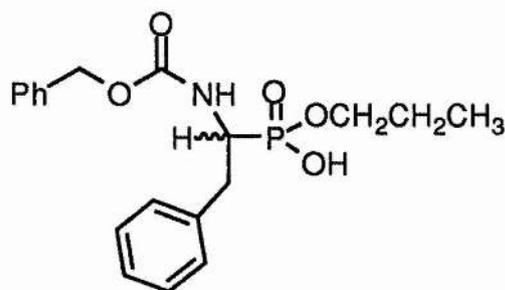
#### Dipropyl [1-(benzyloxycarbonyl)-amino]-2-phenyl-ethyl phosphonate (147).



To dry *n*-propanol (70 cm<sup>3</sup>), was added sodium hydride (40 % dispersion in oil, 1.32 g, 22 mmol, 2.2 eq). Once the solution was homogeneous, diphenyl 1-(*N*-benzyloxycarbonyl)-amino-2-phenyl-ethyl phosphonate (74) (4.87 g, 10 mmol) was added in one portion with stirring at r.t. The solution was allowed to stir overnight, then the volume reduced to 20 cm<sup>3</sup> on a rotary evaporator and ethyl acetate (70 cm<sup>3</sup>) added. The solution was washed with 5 % sodium hydrogen carbonate solution (3 x 30 cm<sup>3</sup>), 0.1 M hydrochloric acid (30 cm<sup>3</sup>), water (30 cm<sup>3</sup>) and brine (30 cm<sup>3</sup>). The solution was dried (MgSO<sub>4</sub>), filtered and the solvent removed under reduced pressure to give a yellow oil. This was purified by column chromatography (1:1 ethyl acetate:light

petroleum) to give a white solid which was recrystallised from ether/ light petroleum (2.30 g, 55 %), m.p. 55-57 °C;  $m/z$  (Found:  $[M + H]^+$  420.1940.  $C_{22}H_{31}NO_5P$  requires 420.19397);  $\nu_{\max}$  (Nujol) / $cm^{-1}$  3205 (NH), 1711 (CO), 1557 (Amide II), 1268 & 1215 (PO);  $\delta_H$  (200 MHz,  $C^2HCl_3$ ) 0.93 (6H, t,  $J$  7.7,  $OCH_2CH_2CH_3$ 's), 1.52-1.75 (4H, m,  $OCH_2CH_2CH_3$ 's), 2.78-2.95 & 3.17-3.31 (2H, m, benzyl  $CH_2$ ), 4.01 (4H, q,  $J$  6.6,  $OCH_2CH_2CH_3$ 's), 4.42 (1H, ddd,  $J$  4.4, 10.3, 26.7,  $\alpha$ -CH), 5.00 (2H, s, Cbz  $CH_2$ ), 5.13 (1H, d,  $J$  10.3, NH) & 7.11-7.29 (10H, m, aromatic CH's);  $\delta_C$  (50.3 MHz,  $C^2HCl_3$ ) 10.48 ( $OCH_2CH_2CH_3$ 's), 24.37 ( $OCH_2CH_2CH_3$ 's), 36.57 (benzyl  $CH_2$ ), 49.13 (d,  $J_{CP}$  156.8,  $\alpha$ -CH), 67.38 (Cbz  $CH_2$ ), 68.51 ( $OCH_2CH_2CH_3$ 's), 127.25-129.72 (aromatic CH's), 136.67 & 136.87 (quaternary aromatic) & 156.12 (urethane CO);  $\delta_P$  (121.5 MHz,  $C^2HCl_3$ ) 24.57 (11 %), 24.32 (81 %), 23.71 (8 %);  $m/z$  (EI) 419 ( $M^+$ , 1%), 312 (1,  $[M - OCH_2Ph]^+$ ), 91 (100,  $PhCH_2^+$ ), 43 (73,  $CH_2CH_2CH_3^+$ ).

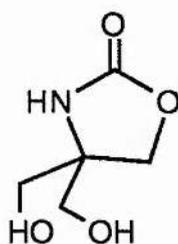
**Propyl hydrogen [1-(benzyloxycarbonyl)-amino]-2-phenyl-ethyl phosphonate (97).**



This compound was prepared in a manner identical to that used for (76), except using di-n-propyl 1-(benzyloxycarbonyl)-amino-2-phenyl-ethyl phosphonate (147) (2.10 g, 5 mmol), n-propanol (15  $cm^3$ ) and 2 M sodium hydroxide (10  $cm^3$ ). The resulting solid was recrystallised from dichloromethane/ light petroleum to yield the product as a white solid (338 mg, 18 %), m.p. 120-122 °C;  $m/z$  (Found:  $[M + H]^+$  378.1470.  $C_{19}H_{25}NO_5P$

requires 378.1470);  $\nu_{\max}$  (Nujol) / $\text{cm}^{-1}$  3290 (NH), 1719 (CO), 1540 (Amide II) & 1252 (PO);  $\delta_{\text{H}}$  (200 MHz,  $\text{C}^2\text{HCl}_3$ ) 0.90 (3H, t,  $J$  7.3,  $\text{OCH}_2\text{CH}_2\text{CH}_3$ ), 1.64 (2H, tq,  $J$  6.6, 7.3,  $\text{OCH}_2\text{CH}_2\text{CH}_3$ ), 2.83 & 3.25 (2H, m, benzyl  $\text{CH}_2$ ), 4.01 (2H, q,  $J$  6.6,  $\text{OCH}_2\text{CH}_2\text{CH}_3$ ), 4.32-4.45 (1H, m,  $\alpha$ -CH), 4.91-5.05 (2H, m, Cbz  $\text{CH}_2$ ), 5.37 (1H, d,  $J$  10.4, NH), 7.04-7.45 (10H, m, aromatic CH's);  $\delta_{\text{C}}$  (50.3 MHz,  $\text{C}^2\text{HCl}_3$ ) 10.46 ( $\text{OCH}_2\text{CH}_2\text{CH}_3$ ), 24.25 ( $\text{OCH}_2\text{CH}_2\text{CH}_3$ ), 36.24 (benzyl  $\text{CH}_2$ ), 49.41 (d,  $J_{\text{CP}}$  158,  $\alpha$ -CH), 67.42 (Cbz  $\text{CH}_2$ ), 68.42 ( $\text{OCH}_2\text{CH}_2\text{CH}_3$ ), 127.22-129.65 (aromatic CH's), 137.20 (quaternary aromatic), 156.45 (urethane CO);  $\delta_{\text{P}}$  (121.5 MHz,  $\text{C}^2\text{HCl}_3$ ) 25.86 (76 %), 25.06 (24 %);  $m/z$  (CI) 378 ( $[\text{M} + \text{H}]^+$ , 41%), 287 (52,  $[\text{M} + \text{H} - \text{PhCH}_2]^+$ ), 270 (26,  $[\text{M} + \text{H} - \text{PhCH}_2\text{OH}]^+$ ), 108 (100,  $\text{PhCH}_2\text{OH}^+$ ), 91 (83,  $\text{PhCH}_2^+$ ).

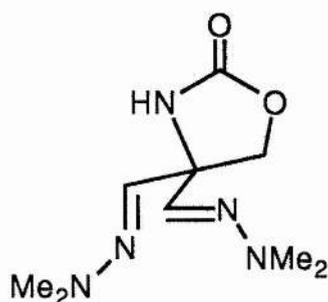
#### 4,4-Di-hydroxymethyl oxazolidinone (107).



Tris (hydroxymethyl) aminomethane (60.5 g, 0.5 mol), anhydrous potassium carbonate (7.0 g, 0.05 mol) and diethyl carbonate (64.9 g, 66.6  $\text{cm}^3$ , 0.55 mol) were heated together at 135  $^{\circ}\text{C}$  under nitrogen and stirred using a mechanical stirrer. This was stirred until all had dissolved and with the receiver flask cooled in ice, ethanol was collected over several hours until approx. 57  $\text{cm}^3$  had collected. At this point, the oil bath was removed and the thick glutinous mass dissolved as far as possible in methanol. The remaining solid (mostly potassium carbonate) was removed by filtration and the filtrate was evaporated under reduced pressure to yield 93 g of crude product, which was recrystallised from methanol (55.93 g, 76 %), m.p. 105-107  $^{\circ}\text{C}$ ; (Found: C, 41.1; H, 6.3; N, 9.5.  $\text{C}_5\text{H}_9\text{NO}_4$  requires C, 40.85; H, 6.2; N, 9.5 %);  $m/z$  (Found:  $[\text{M} +$

$\text{NH}_4]^+$  165.0875.  $\text{C}_5\text{H}_{13}\text{N}_2\text{O}_4$  requires 165.0875);  $\nu_{\text{max}}$  (Nujol)  $/\text{cm}^{-1}$  3600-3000 (br, OH), 3357, 3235 (NH), 1742, 1733 (CO), 1423, 1378, 1281, 1183, 1137 & 1055;  $\delta_{\text{H}}$  (200 MHz,  $d_6$ -DMSO) 3.37 (4H, s,  $\text{CH}_2\text{OHs}$ ) & 4.07 (2H, s, ring  $\text{CH}_2$ );  $\delta_{\text{C}}$  (50.3 MHz,  $d_6$ -DMSO) 62.49 (Quaternary carbon), 63.34 ( $\text{CH}_2\text{OHs}$ ), 68.30 (ring  $\text{CH}_2$ ) & 158.99 (Urethane CO);  $m/z$  (CI) 165 ( $[\text{M} + \text{NH}_4]^+$ , 83%), 148 (100,  $[\text{M} + \text{H}]^+$ ) & 122 (23,  $[\text{Tris} + \text{H}]^+$ ).

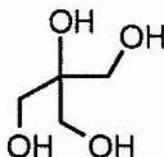
#### 4, 4-(N,N,N',N'-Tetramethyl)-di-hydrazone oxazolidinone (115).



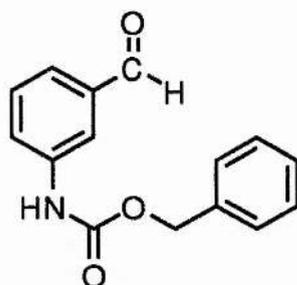
To a stirred solution of oxalyl chloride (5.59 g, 3.78  $\text{cm}^3$ , 44 mmol) in dichloromethane (40  $\text{cm}^3$ ) at  $-40$  to  $-50$   $^\circ\text{C}$  was slowly added dimethyl sulphoxide (20  $\text{cm}^3$ ) in dichloromethane (10  $\text{cm}^3$ ). After 3 min, the diol (107) (2.94 g, 20 mmol) was added in a mixture of dimethyl sulphoxide (20  $\text{cm}^3$ ) and dichloromethane (10  $\text{cm}^3$ ). After stirring the reaction mixture for about 15 min, triethylamine (8.08 g, 11.1  $\text{cm}^3$ , 80 mmol) was added and the reaction mixture stirred for an additional 15 min before being allowed to warm to r.t. 1,1-Dimethyl hydrazine (9.6 g, 12.2  $\text{cm}^3$ , 160 mmol) and magnesium sulphate (9.6 g, 80 mmol) were added with stirring and the reaction mixture allowed to stir at r.t. for 2-3 h. Water (40  $\text{cm}^3$ ) was added and the organic layer separated, washed with saturated brine, dried ( $\text{MgSO}_4$ ) and filtered. Removal of the solvent under reduced pressure yielded an orange-yellow oil which was purified by column chromatography on silica, eluting with ethyl acetate:light petroleum (7:3) (2.15 g, 47 %), b.p.  $120$ - $130$   $^\circ\text{C}/10$  mm Hg; (Found: C, 47.3; H,

7.95; N, 31.05.  $C_9H_{17}N_5O_2$  requires C, 47.55; H, 7.55; N, 30.85%);  $m/z$  (Found:  $[M + H]^+$ , 228.1461.  $C_9H_{18}N_5O_2$  requires 228.1460);  $\nu_{\max}$  (neat) /  $cm^{-1}$  3271 (NH), 1754 (C=N), ~1700 (obscured, C=O), 1589, 1268, 1189, 1138 & 1038;  $\delta_H$  (200 MHz,  $C^2HCl_3$ ) 2.80 (12H, s, N- $CH_3$ s), 4.56 (2H, s,  $CH_2$ ), 5.95 (1H, br, NH) & 6.49 (2H, s, hydrazone CHs);  $\delta_C$  (50.3 MHz,  $C^2HCl_3$ ) 43.17 (N- $CH_3$ s), 63.38 (Quaternary C), 72.56 ( $CH_2$ ), 132.52 (hydrazone C=N) & 159.73 (urethane C=O);  $m/z$  (CI) 228 ( $[M + H]^+$ , 17%), 200 (13,  $[M + H - CO]^+$ ) & 89 (100,  $[M + H - CO - NN(CH_3)_2 - N(CH_3)_2 + 5H]^+$ ).

### Tris-hydroxymethyl-hydroxymethane (148).



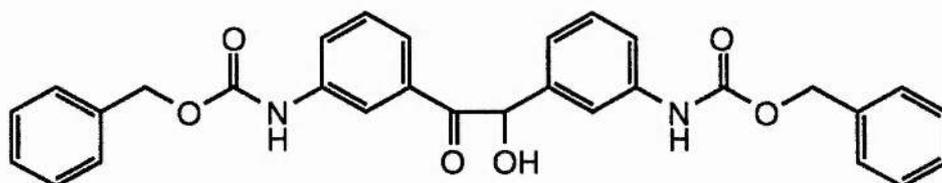
Tris-hydroxymethyl-aminomethane (0.61 g, 5 mmol) was dissolved in 1 M HCl (20  $cm^3$ ) and cooled to 0 °C. To this was added sodium nitrite (450 mg, 6.5 mmol, 1.3 eq) portionwise and with stirring. This was allowed to warm to r.t. and stirred until no further gas was evolved. The reaction was then quenched by the addition 2 M NaOH (20  $cm^3$ ). The solvent was removed under reduced pressure, acetone (20  $cm^3$ ) was added and the solution decanted. The solvent was removed under reduced pressure to yield a white solid (281 mg, 46 %);  $m/z$  (Found:  $[M + NH_4 - H_2O]^+$  122.082.  $C_4H_{12}NO_3$  requires 122.0817);  $\nu_{\max}$  (Nujol) /  $cm^{-1}$  2350-3300 (br, OH's), 1652, 1634, 1557, 1553 & 1297;  $\delta_H$  (200 MHz,  $^2H_2O$ ) 3.78 (6H, s,  $CH_2$ 's);  $\delta_C$  (50.3 MHz,  $^2H_2O$ ) 62.40 ( $CH_2$ 's) & 64.51 (quaternary C);  $m/z$  (CI) 122 ( $[M + NH_4 - H_2O]^+$ ), 100%, 104 (3,  $[M - H_2O]^+$ ), & 90 (7,  $CO(CH_2OH)_2^+$ ).

**3-[(Benzyloxycarbonyl)-amino]-benzaldehyde (105).**

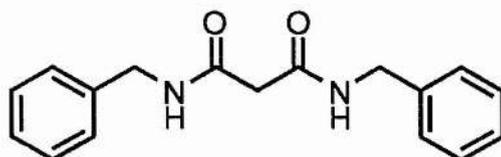
Stannous chloride dihydrate (6.77 g, 30 mmol) was dissolved in concentrated hydrochloric acid (9 cm<sup>3</sup>) and cooled in an ice bath. To this was added 3-nitrobenzaldehyde (1.51 g, 10 mmol) in one portion and the mixture was stirred vigorously until the solid had dissolved, leaving a clear red solution. Recooling the solution in ice to 2 °C resulted in the formation of an orange-red paste which was filtered through a sintered glass filter funnel and used immediately. The orange-red solid was dissolved in 2 M sodium hydroxide solution (10 cm<sup>3</sup>) and cooled in an ice bath. Benzyl chloroformate (2.22 g, 1.85 cm<sup>3</sup>, 13 mmol) was added in 6 portions over 25 min and the solution was then stirred at r.t. overnight. The pH was maintained above 7 by the periodic addition of sodium hydroxide solution. On completion of the reaction (by t.l.c.), the solution was acidified and extracted with dichloromethane (3 × 20 cm<sup>3</sup>). The combined organic extracts were dried (MgSO<sub>4</sub>), concentrated under reduced pressure and then purified by column chromatography on silica, eluting with ethyl acetate:light petroleum (3:2) to give a white solid (1.3 g, 51%), m.p. 102-104 °C; (Found: C, 70.7; H, 5.15; N, 5.35. C<sub>15</sub>H<sub>13</sub>NO<sub>3</sub> requires C, 70.6; H, 5.15; N, 5.5 %); *m/z* (Found: [M + H]<sup>+</sup> 256.0978. C<sub>15</sub>H<sub>14</sub>NO<sub>3</sub> requires 256.0974);  $\nu_{\max}$  (Nujol) /cm<sup>-1</sup> 3268 (NH), 1729 (Aldehyde), 1700 (Urethane), 1683, 1594, 1558 (Amide II and aromatics), 1326, 1294, 1271, 1227, 1169, 1154 & 1047;  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 5.21 (2H, s, benzyl CH<sub>2</sub>), 7.25-7.94 (9H, m, aromatic CH's) & 9.95 (1H, s, aldehyde CH);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 68.20 (benzyl CH<sub>2</sub>),

120.29, 125.35, 125.60, 129.27, 129.40, 129.58 & 130.69 (aromatic CHs), 136.69, 138.00 & 139.81 (quaternary aromatics), 154.29 (urethane CO) & 193.15 (aldehyde CO);  $m/z$  (CI) 256 ( $[M + H]^+$ , 57%) & 91 (100,  $\text{PhCH}_2^+$ ).

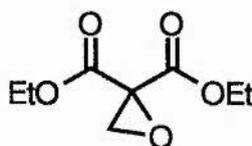
### 3, 3'-[(Benzyloxycarbonyl)-amino]-benzoin (103).



To 3-ethyl-5-(2-hydroxyethyl)-4-methyl-thiazolium bromide (26 mg, 0.1 mmol) in dry ethanol (10 cm<sup>3</sup>) under nitrogen was added triethylamine (60 mg, 84  $\mu\text{l}$ , 0.6 mmol). To this solution was added 3-(N-benzyloxycarbonyl)-aminobenzaldehyde (105) (510 mg, 2 mmol) and the mixture refluxed for 40 h. The mixture was allowed to cool and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica, eluting with ethyl acetate/ light petroleum (2:3) (130 mg, 25 %), m.p. 50 °C (decomp.); (Found: C, 70.2; H, 5.05; N, 5.4.  $\text{C}_{30}\text{H}_{26}\text{N}_2\text{O}_6$  requires C, 70.55; H, 5.15; N, 5.5 %);  $\nu_{\text{max}}$  (Nujol) /cm<sup>-1</sup> 3500-3200 (br, OH), 3311 (NH), 1730 (ketone CO), 1701 (urethane CO), 1595, 1546 & 1491 (Amide II and Aromatics), 1309, 1219 & 1065;  $\delta_{\text{H}}$  (200 MHz,  $\text{C}^2\text{HCl}_3$ ) 4.69 (1H, s, OH), 5.09-5.16 (4H, m, benzyl  $\text{CH}_2$ 's), 5.86 (1H, s, CH), 6.96-7.96 (18H, m, aromatic CH's);  $\delta_{\text{C}}$  (50.3 MHz,  $\text{C}^2\text{HCl}_3$ ) 67.64 & 67.70 (benzyl  $\text{CH}_2$ 's), 76.86 (CH(OH)), 112.80-130.25 (aromatic CH's), 134.07-140.06 (quaternary aromatics), 153.98 & 154.24 (urethane CO's) & 198.51 (ketone CO);  $m/z$  (CI) 511 ( $[M + H]^+$ , 11%).

**N, N'-Dibenzylmalonamide (149).**

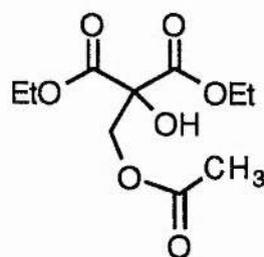
To diethyl malonate (800 mg, 5 mmol) in dry ethanol (10 cm<sup>3</sup>) was added benzylamine (1.07 g, 1.09 cm<sup>3</sup>, 10 mmol) and the mixture was refluxed for 2 h. The reaction was allowed to cool and the solvent removed under reduced pressure. A white crystalline solid formed on standing and was further purified by recrystallisation from ethanol/ diethyl ether (462 mg, 33 %), m.p. 135-137 °C; (Found: C, 72.25; H, 6.35; N, 9.9. C<sub>17</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> requires C, 72.3; H, 6.45; N, 9.9 %); *m/z* (Found: [M + H]<sup>+</sup>, 283.1446. C<sub>17</sub>H<sub>19</sub>N<sub>2</sub>O<sub>2</sub> requires 283.1447);  $\nu_{\max}$  (Nujol) /cm<sup>-1</sup> 3284 (NH), 1658 (amide CO), 1627 (amide II), 1548, 1245 & 1229;  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 3.22 (2H, s, malonic CH<sub>2</sub>), 3.39 & 4.36 (4H, 2 × s, benzyl CH<sub>2</sub>'s), 7.22-7.36 (10H, m, aromatic CH's) & 7.74 (2H, br, NH's);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 43.47 (malonic CH<sub>2</sub>), 44.05 (benzyl CH<sub>2</sub>'s), 127.96, 128.13, 129.17 (aromatic CH's), 138.28 (quaternary aromatics) & 167.89 (amide CO); *m/z* (CI) 283 ([M + H]<sup>+</sup>, 100%).

**Diethyl epoxyethylene malonate (121).**

A flask containing diethyl malonate (1.6 g, 10 mmol), paraformaldehyde (0.6 g, 20 mmol), cupric acetate monohydrate (100 mg, 0.5 mmol), potassium acetate (100 mg, 1 mmol) and glacial acetic acid (5 cm<sup>3</sup>) was heated to 90 - 100 °C in an oil bath until the mixture became clear, then for a further hour

(approx. 2-3 h in all). Acetic acid and other volatile materials were distilled out at 90 - 100 °C. The residue was distilled again using a bunsen, to yield a greenish-yellow distillate of diethyl methylene malonate. The distillate was taken up in acetonitrile (6 cm<sup>3</sup>) and neutral alumina (9.0 g) and 10% sodium hypochlorite solution (15 cm<sup>3</sup>, 20 mmol) was added with stirring. A further equivalent (7.5 cm<sup>3</sup>) of hypochlorite solution was added an hour later. The reaction mixture was then allowed to stir at r.t. for 1 h, before being extracted with ether (3 x 50 cm<sup>3</sup>). The combined organic layers were washed with 5% sodium hydrogen carbonate solution, dried (MgSO<sub>4</sub>), filtered and evaporated under reduced pressure to yield a clear liquid which was purified by column chromatography on silica, eluting with 1% EtOH in CH<sub>2</sub>Cl<sub>2</sub> (660 mg, 35%); *m/z* (Found: [M + H]<sup>+</sup> 189.0761. C<sub>8</sub>H<sub>13</sub>O<sub>5</sub> requires 189.0763);  $\nu_{\max}$  (neat)/ cm<sup>-1</sup> 2986, 2942 (CH), 1747 (CO), 1468, 1448, 1377, 1353, 1319, 1241 & 1104;  $\delta_{\text{H}}$  (200 MHz, C<sup>2</sup>HCl<sub>3</sub>) 1.30 (6H, t, *J* 7.1, CH<sub>3</sub>'s), 3.21 (2H, s, epoxide CH<sub>2</sub>) & 4.27 (4H, q, *J* 7.1, malonate CH<sub>2</sub>'s);  $\delta_{\text{C}}$  (50.3 MHz, C<sup>2</sup>HCl<sub>3</sub>) 14.39 (CH<sub>3</sub>'s), 51.46 (epoxide CH<sub>2</sub>), 55.98 (Quaternary C), 62.94 (malonate CH<sub>2</sub>'s) & 165.90 (CO's); *m/z* (CI) 189 ([M + H]<sup>+</sup>, 100%).

#### Diethyl acetoxymethylhydroxymalonate (122).



The epoxide (121) (940 mg, 5 mmol) was dissolved in glacial acetic acid (125 cm<sup>3</sup>) and to this was added sodium acetate (10.25 g, 125 mmol). This was heated to 90-100 °C for 4 h, then the solvent was removed under reduced pressure. The residue was dissolved in water (50 cm<sup>3</sup>) and the aqueous

solution extracted with diethyl ether ( $3 \times 50 \text{ cm}^3$ ). The combined organic layers were washed with 5 % sodium hydrogen carbonate solution ( $2 \times 100 \text{ cm}^3$ ), dried ( $\text{MgSO}_4$ ), filtered and the solvent removed to yield the product as a clear oil (781 mg, 63 %);  $m/z$  (Found:  $[\text{M} + \text{H}]^+$ , 249.0970.  $\text{C}_{10}\text{H}_{17}\text{O}_7$  requires 249.0974);  $\nu_{\text{max}}$  (neat)/  $\text{cm}^{-1}$  3488 (br, OH), 2986 (CH), 1749 (CO), 1448, 1371, 1232 & 1161;  $\delta_{\text{H}}$  (200 MHz,  $\text{C}^2\text{HCl}_3$ ) 1.31 (6H, t,  $J$  7.1, ester  $\text{CH}_3$ 's), 2.08 (3H, s, acetate  $\text{CH}_3$ ), 4.32 (4H, q,  $J$  7.1, ester  $\text{CH}_2$ 's) & 4.59 (2H, s,  $\text{CH}_2$ );  $\delta_{\text{C}}$  (50.3 MHz,  $\text{C}^2\text{HCl}_3$ ) 14.41 (ester  $\text{CH}_3$ 's), 21.09 (acetate  $\text{CH}_3$ ), 63.44 (ester  $\text{CH}_2$ 's), 63.94 (quaternary C), 65.75 ( $\text{CH}_2$ ), 168.56 & 170.83 (CO's);  $m/z$  (CI) 249 ( $[\text{M} + \text{H}]^+$ , 14%) & 231 (100,  $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$ ).

#### HIV-1 proteinase assays.

The assays are based upon the hydrolytic cleavage of the decapeptide Lys-Ala-Arg-Val-Nle-( $\text{NO}_2$ )Phe-Glu-Ala-Nle-Gly- $\text{NH}_2$  between the norleucine and nitrophenylalanine residues which leads to a reduction in absorbance at 300 nm. The peptide was stored as a  $10 \text{ mg ml}^{-1}$  solution in water and  $2 \mu\text{l}$  of this solution was added to  $1 \text{ cm}^3$  aliquot of assay buffer (100 mM NaOAc, 200 mM NaCl, 1mM DTT at pH 5.6). The inhibitors to be tested were made up as 10 mM stock solutions in methanol and were used at concentrations over the range 1-100  $\mu\text{M}$ . Methanol was added to a final concentration of 2% in the assay mixture and the solution was allowed to thermally equilibrate to  $37^\circ\text{C}$  before the reaction was initiated by the addition of  $15 \mu\text{L}$  of enzyme. The enzyme was stored as a  $0.993 \mu\text{M}$  solution in 10mM NaOAc containing 0.05% 2-mercaptoethanol, 1mM EDTA, 20% glycerol and 5% ethylene glycol.

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**Anti-viral assays: acute infection of cells (C8166 cells / HIV-1IIB)**

This work was performed by D. Kinchington at St. Bartholomew's Hospital in London and is included here for completeness.

High titre virus stocks of the human immunodeficiency virus HIV-1 (HTLV-III RF) were grown in H9 cells with RPMI 1640 (Flow laboratories) supplemented with 10% fetal calf serum. Cell debris was removed by low speed centrifugation, and the supernatant stored at  $-70^{\circ}\text{C}$  until required. In a typical assay, C8166 T-lymphoblastoid  $\text{CD4}^{+}$  cells were incubated with  $\text{TCID}_{50}$  HIV-1 at  $37^{\circ}\text{C}$  for 90 min and then washed three times with medium. Cell aliquots ( $2 \times 10^5$ ) were suspended in  $1.5 \text{ cm}^3$  growth medium in  $6 \text{ cm}^3$  tubes, and compounds in log dilutions [ $200 \mu\text{M}$  to  $0.2 \mu\text{M}$ ] were added immediately. The cells were then incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . After 72 h post-infection 200  $\mu\text{l}$  of supernatant was taken from each culture and assayed for HIV<sup>192</sup> using an antigen capture ELISA which recognises all the core proteins equally (Coulter Electronics, Luton, UK). The following controls were used: supernatants taken from uninfected and infected cells, infected cells treated with Ro-31-8959 (**28**) (Roche Products UK Ltd), AZT (**1**) (Roche Products UK, Ltd) and ddC (**4**) (Roche Products UK Ltd). The activities of 8959, AZT and ddC in infected cells each gave an  $\text{IC}_{50}$  of 3, 20 and 200 nM respectively. The ELISA plates were read with a spectrophotometer. Compounds were tested in duplicate at each concentration, and the data shown are the average of two assays.

To test for compound toxicity, aliquots of  $2 \times 10^5$  uninfected cells were cultured with the compounds in the same half log dilutions for 72 h. The cells were then washed with medium and resuspended in 200  $\mu\text{l}$  of growth medium containing  $^{14}\text{C}$  protein hydrolysate. After 12 h the cells were harvested and the  $^{14}\text{C}$  incorporation measured. Uninfected, untreated cells were used as controls.

## **CHAPTER FOUR**

## **REFERENCES**

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**4.0. References**

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## APPENDIX A

### Publications

1. Camp, N. P., Perrey, D.A., Kinchington, D., Paul C. D. Hawkins and D. Gani, *Bioorg. Med. Chem.*, in press.

## APPENDIX B

### *In Vitro* Assay Control Experiment

This was performed in an identical manner to the *in vitro* assay described in the experimental section, except the inhibitor used was Ro-31-8959 (**28**) (for which we thank Roche Ltd. for the supply of).

This gave an  $K_i$  value in this experiment of 7 nM (lit. value<sup>101</sup> of 0.12 nM).