INVESTIGATIONS AND SYNTHESIS
OF SOME NOVEL PHOSPHATE DERIVATIVES
AS POTENTIAL ANTI-HIV AGENTS

BY

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Abstract

The synthesis of a number of phosphoramidate derivatives of the anti-AIDS drug 3'-azido-3'-deoxythymidine (AZT) bearing an alkyl chain and a single N-linked, carboxyl-protected amino acid has been described. The derivatives were found to possess potent activity against the established etiologic agent for the disease, the Human Immunodeficiency Virus (HIV).

A high-yielding method for the preparation of the synthetic precursors to these phosphoramidate derivatives - the alkyl amino acyl phosphorochloridates - has been developed.

The mechanism of action of the phosphoramidate derivatives was probed by the synthesis of a series of compounds in which the distance between the amino acid carboxyl moiety and the phosphoramidate bond was varied. Activity was found to decrease on increasing the length of the methylene spacer. This is consistent with a mechanism of action involving cleavage of the phosphoramidate bond.

Further investigations revealed that an L-amino acid is not required for activity. Incubation of one of the phosphoramidate compounds with HIV-1 protease revealed very little decomposition. These findings seem to preclude HIV-specific activation of the compounds. However, investigation of the stability of the derivatives in aqueous media showed them to be resilient to chemical hydrolysis over a period of about five days, suggesting that other mechanisms may play a role in the activation of these compounds.

Various unsuccessful attempts were made to conjugate a dipeptide to a nucleoside via a phosphoramidate linkage.

Variations in the nucleoside moiety of the phosphoramidate compounds were also explored. It appears that a bioactive nucleoside is not a prerequisite for activity, thus illustrating the potential utility of the phosphoramidate approach in conferring activity on nucleoside analogues hitherto considered ineffective against HIV.

A Table of the phosphoramidate derivatives prepared and the results of their biological evaluation against HIV-1 in vitro can be found on page 226.
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Introduction

The AIDS pandemic

Acquired Immunodeficiency Syndrome (AIDS) was first recognised as a distinct clinical entity in 1981\textsuperscript{1-3}. Over 484,000 cases have been reported to the World Health Organisation\textsuperscript{4} and the incidence of this fatal disease in the world population continues to increase. There is at present no known cure.

The nature of the disease

AIDS is characterised by a depletion of T4 helper/inducer cells\textsuperscript{1,5} in patients suffering from the disease. These cells are a subset of thymus-derived (T) lymphocytes and they play a central role in immune response. When they are stimulated by contact with an antigen, they respond by cell division and the production of lymphokines, such as interleukin-2 and interferons. These lymphokines act as local hormones controlling the growth and maturation of other lymphocyte types, in particular the cytotoxic-suppressor (CD8) T cells and antibody producing B lymphocytes. T4 cells also influence the activity of the mobile scavengers known as monocytes and macrophages, which engulf infected cells and foreign particles. T4 cells are thus essential to the proper regulation of the human immune system.

The depletion of T4 cells leaves AIDS sufferers in an immunocompromised state, with the body no longer able to defend itself from invasion by infectious agents. Thus, the term "opportunistic infections" has been adopted for the diseases which take advantage of the body's immune dysfunction. These life-threatening infections include pneumocystis carinii pneumonia (PCP), toxoplasmosis (which often infects the brain and can lead to seizures and coma) and cryptosporidiosis (which typically attacks the intestinal tract, causing chronic diarrhoea)\textsuperscript{5,6}. Common fungal infections are cryptococcosis (which frequently causes meningitis but may also damage the liver, bone, skin and other tissues) and histoplasmosis (which causes a disseminated infection of the liver and bone-marrow in AIDS patients, and is a frequent cause of chronic fevers). A common viral infection is cytomegalovirus, which may result in pneumonia, encephalitis, blindness and inflammation of the gastrointestinal tract. Herpes simplex and Epstein-Barr viruses may also occur. Mycobacterial diseases usually infect patients in the later stages of AIDS, although tuberculosis may present
earlier.

Patients with AIDS are at increased risk of developing certain malignant tumours in addition to their risk of opportunistic infection. A typical example is Kaposi's sarcoma, which produces tumours in the skin and in the linings of internal organs, and is an unusual form of cancer occurring in non-AIDS patients receiving immunosuppressive therapy. Malignant lymphoma (cancers of the lymphoid tissue) may also develop.

In the terminal stages of AIDS many people suffer from AIDS dementia complex\(^7\), a syndrome characterised by a gradual loss of precision in both thought and motion. Eventually, some patients are unable to walk or communicate effectively.

In principle, therapies for AIDS could be based on at least three strategies. First, treatment of malignancies and/or opportunistic infections\(^6\). Most of these infections are due to reactivation of latent organisms in the host or, in some cases, due to ubiquitous organisms to which we are continuously exposed. In general the treatment of these infections suppresses rather than eradicates the organisms, so relapse is common when treatment is stopped.

Second, reconstitution of the cellular immune status, or enhancement of the immune functions by some other means\(^8\). Attempts at immune reconstitution in AIDS patients have been made using interleukin-2, interferons, thymic factors or bone marrow transplantation. However, none of these have been notably successful.

The third and probably most attractive means of therapy for AIDS must be to attack the causative agent for the disease.

The Human Immunodeficiency Virus: the putative cause of AIDS

In 1983 a new virus was identified and isolated from a man suffering from lymphadenopathy syndrome, a condition that often precedes full-blown AIDS\(^9\). This report was followed by several others\(^10,11\) confirming the presence of a virus in AIDS patients, and in 1986 the International Committee on the Taxonomy of Viruses agreed to call the virus HIV, the Human Immunodeficiency Virus\(^12\). HIV has also been referred to as lymphadenopathy-associated virus (LAV)\(^9\), AIDS-associated retrovirus (ARV)\(^13\) or human T-lymphotropic virus type III (HTLV-III)\(^10\). Two distinct subsets of HIV have emerged, termed HIV-1 and HIV-2. Several different strains of these two forms of the virus also exist\(^14\). HIV-1 is the virus responsible for the main AIDS
epidemic in the West. Evidence of HIV is found in almost every individual with AIDS or pre-AIDS. It is correlative data such as this which has led the majority of researchers to believe that HIV is the cause of AIDS, although this view has been challenged recently on the grounds that there is no direct evidence for HIV’s role as the sole cause of the disease\textsuperscript{15,16}.

The natural history of HIV infection illustrates the variety of clinical conditions which may be associated with AIDS\textsuperscript{17,18}. The virus can be spread by intimate sexual contact, by transmission of blood, blood products and organ donation, or by the maternal-foetal route. For an unknown time-span after infection the patient will not produce antibodies to the virus and may remain perfectly healthy. Some patients may then seroconvert, and become antibody positive. Although most people have no symptoms when HIV is first detected, some patients develop fatigue, fever and swollen glands, which may be accompanied by a rash. These symptoms usually disappear within a few weeks. Not all of those who have seroconverted progress to chronic infection. Patients may develop subclinical signs of immune deficiency, showing mild symptoms such as fever, sweats, fatigue, unexplained loss of weight, skin ailments, sickness and diarrhoea\textsuperscript{18}. The term AIDS-related complex (ARC) has been coined for AIDS-related illnesses of lesser clinical severity.

Those patients who remain symptomless probably go into a latent stage of infection, which may last for several years. Yet although the amount of virus in the circulation and cerebrospinal fluid may drop dramatically, the virus is still present. It can be found not only in the T4 lymphocytes but also in cells of the nervous system and intestine, and probably in some bone-marrow cells.

When replication of the virus flares again, chronic infection and progression to full-blown AIDS in almost all cases eventually leads to the death of the patient. The two main clinical manifestations of AIDS are tumours and the series of opportunistic infections previously described.

**HIV is a retrovirus with a complex structure and genetic organisation**

Viruses consist of packets of nucleic acid surrounded by a protective coat. They differ from ordinary cells in that they contain either DNA or RNA, but not both. The completed extracellular product of virus multiplication is called a virion (or virus particle).
Viruses are intracellular parasites. They hijack the enzymes of a host cell for their own replicative purposes. In the HIV virion, the core viral nucleic acid is RNA. The flow of genetic information in normal cells is from DNA to RNA and then to protein. Uninfected host cells do not possess enzymes to synthesise RNA (and hence protein) according to instructions given by an RNA template. Consequently, HIV must synthesise a DNA-provirus from its core RNA in order to replicate. HIV therefore encodes a unique type of enzyme, an RNA-directed DNA polymerase, or reverse transcriptase. This enzyme enables the virus to make the proviral DNA which is inserted into the host cell’s chromosomes, and then remains latent until it is activated to make new virus particles. Thus, genetic material flows from RNA to DNA ("retro" to the normal route for cells and many other viruses) and then to RNA and protein. Viruses which transcribe RNA to DNA using a reverse transcriptase enzyme are called retroviruses\textsuperscript{19,20}. HIV is the most complicated retrovirus that has been described to date\textsuperscript{21-23}. The structure of the HIV virion is shown in cross-section in Figure 1.

![Figure 1. Schematic diagram of the human immunodeficiency virus (HIV). The protein subunits p7 and p6 are associated with the RNA nucleocapsid and are omitted here.](image)

The membrane envelope consists of a lipid bilayer. A glycoprotein (gp) called gp41 spans the membrane and anchors on to knobs of another glycoprotein called gp120. Inside the membrane envelope are four types of structural protein (p) subunit, p17, p24, p7 and p6. The latter three proteins form the virion core, while the p17 is attached to the inside of the membrane. The core contains the viral enzymes (reverse transcriptase, integrase and protease) and two copies of the RNA genome, which carry the complement of the virus’s genetic information.
The information contained in the DNA derived from the RNA genome is arranged in genes. The genetic organisation of HIV-1 is shown in Figure 2. The genes seem to have one of two basic functions. They either contain information for the proteins that make up the structural components of the virus, or they encode for various regulatory proteins which control the virus's ability to infect a cell, incorporate its genetic material into the cell's genome, or reproduce new viruses. The genes are flanked at their ends by nucleic acid sequences called long terminal repeats (LTRs), which do not code for any protein but serve to initiate the expression of the viral genes. These regions may be likened to "on-off" switches; proteins from both the virus and the cell can trigger these switches by binding to the LTR sequences.

![Figure 2: Genetic organisation of HIV-1](image)

The three major structural genes of HIV are called gag, pol, and env. The gag (group-specific antigen) gene encodes a polyprotein of approximately 55 kd. This protein is subsequently cleaved (by a pol-gene encoded protease) to the core proteins (GAG proteins) which encapsidate the RNA genome: p17; p24 (the major structural protein of the mature virions); p7 and p6. The major viral enzymes are encoded by the pol (polymerase) gene; they are the reverse transcriptase that copies the RNA genome into DNA, the protease that processes the GAG and POL proteins to form mature infectious virions, and the integrase that is essential for the integration of the DNA-provirus into the host cell's chromosomes. The env (envelope) gene encodes for a protein (ENV protein) of about 90 kd. This protein is heavily glycosylated to a glycoprotein of about 160 kd (designated gp160). It gives rise to the gp120 and gp41 glycoproteins.

In addition to the gag, pol and env structural genes, there are a number of regulatory genes which encode for proteins which determine the levels of infectious...
virus\textsuperscript{25,26}. The roles of the tat, rev and nef genes will be discussed in more detail later. The specific functions of the other genes are as yet incompletely understood. The \textit{vpu} gene has recently been suggested to have a role in assembly or maturation of progeny viruses and thus to facilitate virus release from infected cells\textsuperscript{27-29}. However, it is still unclear whether this gene, encoding a 16 kd protein, is essential for replication of HIV. The \textit{vif} gene has been linked to the ability of HIV to replicate by a pathway of cell-free virion infection\textsuperscript{30,31}. This gene appears to play a crucial role in the efficient generation of infectious virus particles; however, the exact mechanism of the \textit{vif} function is still to be determined. Mutations within the \textit{vpr} gene\textsuperscript{32} do not appear to affect viral replication kinetics and cytopathogenicity, suggesting that the \textit{vpr} gene is not essential to viral multiplication\textsuperscript{33}. The significance of the presence of the above genes and the biochemistry and function of the ancillary proteins encoded by them will become clearer with further study.

The life-cycle of HIV
To develop effective therapies against any pathogen it is necessary to find vulnerable points of attack to selectively inhibit its replication, without harming the host cells. The life-cycle of HIV is in many respects different from the host cell replicative cycle, and it is these differences which have attracted most attention in attempts to combat AIDS. The life-cycle of HIV consists of a number of steps which are outlined below.

Binding
The initial event in the infection of human T-lymphocytes, macrophages and other cells by HIV is the attachment of the HIV-1 envelope glycoprotein gp120 to a receptor on the host cell's membrane. In the case of HIV, this receptor is the molecule known as the CD4 antigen\textsuperscript{34-37}. The ability of HIV-1 to selectively infect, replicate in and destroy CD4 T cells in part explains the specific loss of the T4 helper/inducer function characteristic of AIDS\textsuperscript{35}. The interaction of CD4 with gp120 also plays a critical role in the formation of multinucleated giant cells by cell fusion, which is a major cytopathic effect induced by HIV-1\textsuperscript{38,39}. These syncytia, clusters of many nuclei within a single cell membrane, are indicative of HIV infection in cell cultures. They form when infected cells, which make gp120 and carry it on their surface, fuse with healthy cells bearing the CD4 molecule.
The evidence that the CD4 molecule is the major cellular receptor for HIV comes from the ability of antibodies to CD4 to block HIV-1 infection and syncytia induction\textsuperscript{34,37,40}, and has been confirmed by the detection of CD4 binding to gp120\textsuperscript{41}. Furthermore, cells producing HIV-1 env polypeptides in the absence of other viral gene products efficiently induce the fusion of uninfected CD4 cells\textsuperscript{42,43}.

**Entry and uncoating**

Subsequent to the attachment step, all enveloped viruses require fusion between viral and cellular membranes for insertion of their genome into host cells. The simplest and most likely mechanism is that the viral envelope fuses directly with the cell plasma membrane, injecting the core of the virus (including its genetic material) into the cell\textsuperscript{44,45}. After the core of the virion enters the cell, the virus loses the envelope protein coat (uncoating) and releases its functional RNA genome into the cytoplasm. The mechanisms which bring about this release of RNA are unknown.

**Reverse transcription**

Once uncoating occurs, viral RNA is used as a template for the proviral DNA synthesis catalysed by reverse transcriptase. This process cannot occur naturally in the host cell because the reverse transcriptase enzyme is encoded only by the virus. This process has therefore proven to be one of the most attractive and successful targets for the development of anti-HIV agents, about which more will be discussed later.

DNA synthesis is normally initiated using an RNA primer. In the case of the synthesis of the first DNA strand - the minus (or antisense) strand - by reverse transcriptase, the primer used is a specific transfer RNA (tRNA) molecule: lysine transfer RNA\textsuperscript{22}. It has recently been shown that reverse transcriptase will bind directly to lysine transfer RNA even in the absence of the genomic RNA\textsuperscript{46}. A tertiary complex may thus be formed by the interaction of the reverse transcriptase, the tRNA and the viral genomic RNA. However, even in the presence of added deoxynucleotide triphosphates, this complex is not sufficient for initiation of DNA synthesis. The missing component appears to be a small protein product of the gag gene (p7)\textsuperscript{46}, but the interactions of this protein with the other components of the initiation complex have yet to be characterised.

The addition of deoxynucleotide triphosphates to the growing complementary
DNA strand thus involves at least the formation of a quaternary complex of enzyme, template, primer and nucleotides (Figure 3).

![Diagram](image_url)

**Figure 3.** The interaction between components necessary for initiation of minus strand DNA synthesis. Reverse transcriptase (RT) interacts with the genomic RNA template and there is also a direct interaction between the lysine transfer RNA (tRNA) and the protein. The small gag product p7 is necessary for initiation but its role is unclear. Deoxynucleotide triphosphates (dNTP) are presumed to interact with the template strand through base pairing and also directly with the enzyme.

The product of synthesis is initially a DNA/RNA heteroduplex, but associated with the reverse transcriptase is also a ribonuclease H activity, which degrades the RNA component of the heteroduplex to afford single-stranded DNA molecules. The ribonuclease H enzyme is also responsible for generating the primer for initiation of the plus (sense) DNA strand on the newly-synthesised minus strand. The final task of the enzyme is to remove the primer sequences once the DNA duplex has been completed.

**Integration of proviral DNA**

The viral genetic information, now in the form of double-stranded DNA, migrates to the host cell nucleus by an unknown mechanism. One of the critical steps in the lifecycle of a retrovirus is integration of the double-stranded DNA copy of their RNA genome into the host cell chromosome. Integration is essential for efficient production of progeny virus particles for most, if not all, retroviruses.

The integration reaction, whereby the HIV proviral DNA is spliced into the host cell's DNA, is dependent on another virus-specific enzyme encoded by the pol
gene: an integrase (or endonuclease). The integrase cleaves the terminal bases from each 3' end on the proviral DNA. The resulting viral 3' ends are then joined to the target cellular host DNA to form the recombination product.

Once integrated into the host's chromosomes, the viral DNA (provirus) will be duplicated together with the cell's own genes every time the cell divides. This provirus will also provide the template for the next generation of viral RNA.

Transcription and translation
At some time (in certain cases after a delay during which the virus may be considered latent) the proviral DNA is transcribed to messenger RNA (mRNA) and genomic RNA and ultimately translated to form viral proteins and infectious virions (Figure 4). These steps utilise host RNA polymerases and host ribosomes.

The latent stage of infection may vary markedly between individuals. This suggests that the start of HIV production within a cell is stimulated by an external or internal trigger, not associated directly with the virus. Antigens or regulatory interleukins have been suggested to activate infected cells in this way.

Figure 4. Representation of the steps involved in viral protein synthesis, i.e. transcription from proviral DNA to RNA by host RNA polymerases, and translation from RNA to protein by the host ribosomes.

Sequences in the LTR define the RNA initiation site: the starting point for the copy of the proviral DNA into mRNA, which carries the genetic information of the DNA from the nucleus into the cytoplasm. The sequences resemble those found at the initiation sites of host cellular genes. The interaction of cellular proteins with the LTR increases HIV expression in synergy with another gene product (TAT). For example, a host protein that recognises the HIV initiation sequences is NF-κB. This protein is activated
when lymphocytes are stimulated by an antigen and begin to multiply. The stimulation of infected T cells increases the binding of NF-κB to the viral LTR, thus precipitating viral growth. The sequence on the viral LTR to which NF-κB can bind is 11 base pairs long, and is called kB. This site functions as an enhancer element for HIV transcription, and can act synergistically with the product of the *tat* gene to increase viral replication. This is one possible mechanism by which T cell activation may lead to induction of latent virus.

When transcription has been initiated, the viral RNA is translated to form viral proteins using the biochemical apparatus of the host cell. The production of viral proteins is controlled by the regulatory genes mentioned earlier. Each regulatory gene encodes a protein that interacts specifically with a "responsive" element, which is a short sequence of nucleotides elsewhere in the genome.

The essential regulatory gene *tat* encodes for a diffusible protein (TAT) that, through binding to the LTR sequences of HIV, markedly enhances the expression of other viral genes and amplifies the production of new infectious virions. The *tat* gene mediates trans-activation of transcription - its mechanism of action affects the transcription of genes not in its immediate proximity. The TAT protein can boost expression of viral genes enormously. This stimulatory effect extends to all the viral proteins, both the structural components and the regulatory proteins, including the TAT protein itself. As a result of this positive feedback, the virus replicates very quickly when *tat* is activated.

To exert its effects, the TAT protein interacts with a short nucleotide sequence designated TAR (trans-acting responsive sequence), which is found at the start of the viral genome (in the 5'-LTR). It is not known exactly how TAT and the TAR sequence interact; the protein may directly bind to and activate elongation of TAR RNA. Alternatively, cellular RNA binding proteins may play a major role in mediating the *tat*-dependent LTR activation.

The *rev* gene encodes a small protein (116 amino acids) which is thought to function as a second trans-acting factor in viral replication. The REV protein is essential for efficient virion production; in the absence of this regulatory factor, *gag* and *env*-encoded protein synthesis is severely diminished.

The REV protein binds to the *rev*-responsive element, or RRE, which is located within the *env* region of the HIV-1 genome. In the presence of REV, RRE is
responsible for the increased levels of unspliced (GAG-producing) and singly-spliced (ENV-producing) mRNA. RRE sequences are built into the mRNAs that specify these structural virion proteins. The rev function operates by increasing the half-life of the unspliced viral mRNA. The rev gene also appears to promote the transport of unspliced viral mRNA containing RRE from the nucleus to the cytoplasm.

The multiply-spliced, short mRNAs for regulatory proteins such as TAT and REV lack the RRE sequence. The REV protein does not affect the half-life of these mRNA species, which are stable and do not require REV. So, in the absence of the REV protein, the truncated mRNAs that specify regulatory proteins build up and are translated to TAT and REV, for example.

The selective effects of REV on the levels of the viral mRNA suggest a model for feedback regulation by REV leading to a steady state of viral expression. A model summarising the function of the REV protein is shown in Figure 5.

![Figure 5. Model for the function of the rev protein. REV, or a cellular factor induced by REV, interacts with the cis-acting element (RRE) in the env region of HIV-1. This channels the unspliced mRNA towards transport and away from splicing. In addition, this interaction stabilises the unspliced mRNA.](image)

The interaction between the rev and tat mechanisms may thus hold viral growth in check. The two pathways can counteract each other: the tat product increases its own production and the production of the REV protein, whereas the REV protein slows its own synthesis and that of TAT because it favours the accumulation of unspliced mRNAs that form the structural proteins. This establishment of a steady state for the balanced expression of all viral proteins results in controlled virus growth, and enables...
the virus to reproduce itself for years without killing off its host.

In addition to an activator (tat) and a selective regulator (rev), HIV has an additional regulatory gene, designated nef (negative regulatory factor), which has been reported to function as a transcriptional silencer and down-regulate the expression of the viral genome. This gene may therefore be responsible for HIV’s ability to turn off its own growth and lie dormant in the genome of the host cell.

The three regulatory genes described above form a complex network of regulatory interactions. This picture will be complicated even further when the functions of other regulatory genes such as vif, vpu and vpr are clarified.

Once the proviral DNA has been transcribed to RNA by the host RNA polymerases, viral polyproteins can be translated using the host cell ribosomes. The proteins which ultimately comprise the virion core and the enzymes essential to viral replication are the products of the gag and pol genes. The pol reading frame is translated as a fusion polyprotein encoded by both the viral pol gene and the gag gene. These two genes lie in different translational reading frames, with the 3’ end of gag overlapping the 5’ end of pol by 241 nucleotides (Figure 6).

![Figure 6. The gag and pol open reading frames of HIV-1 and their polyprotein translation products.](image)

Thus, production of the gag-pol fusion protein requires either mRNA processing or translational frameshifting. The latter process involves the shift of ribosomes from one reading frame to another (as they move along mRNA), thus avoiding premature termination of polypeptide synthesis. This mechanism has been shown to operate in the synthesis of the gag-pol proteins of two other retroviruses, and has now been characterised in HIV-1 gag-pol expression. The translated gag-pol fusion product is a 160 kd polyprotein, designated Pr160gag-pol, containing in immature form the gag
proteins, the protease, reverse transcriptase-ribonuclease and integrase (endonuclease). This ribosomal frameshifting occurs with an efficiency of 11% resulting in a ratio of Pr55gag (the product of direct translation of the gag gene) to Pr160gag-pol (the product of the frameshift translation) of 8:1. Thus, by occasionally "frustrating" the termination codon of the gag gene through ribosomal frameshifting, the expression of two distinct genes regulated by a single set of genetic control elements is appropriately controlled to produce considerably more of the structural proteins of the virion than its retroviral enzymes.

**Transport, assembly, maturation and budding**

After the viral RNA has been translated on the host ribosomes, the resultant polyproteins must undergo post-translational modification to generate the functional forms of the constituent viral proteins. These secondary processes may involve host myristylating enzymes, host glycosylating enzymes and viral proteases.

The assembly of virion particles is apparently initiated by the migration of the retroviral polyproteins to the cellular membrane, an event which is preceded by enzyme-catalysed acylation of the proteins by a myristoyl (tetradecanoyl) group. Myristylation of the polyproteins is essential for their proper assembly into virion particles, presumably because this lipid substituent directs the gag and gag-pol polyproteins to the cell membrane, and anchors them into the lipid bilayer (Figure 7).

![Figure 7. Initiation of virion assembly at the cellular membrane of a T-lymphocyte by accumulation of the gag and gag-pol polyproteins and insertion of their N-terminal myristoyl groups into the membrane. Following cleavage from gp160, the envelope glycoprotein gp120 is situated on the outside of the membrane. The genomic RNA is attached to the nucleocapsid protein domain of Pr55gag.](image)

Upon concentration, the lipid-embedded polyproteins collect into a crescent shape and begin to bud from the cell. Eventually, a complete, spherical virion particle is formed which contains an annular core composed of the polyproteins.

Retroviral particles of this type are of immature morphology and are unable
to infect cells. Reverse transcriptase activity in immature HIV-1 particles is either diminished or almost completely absent, indicating that the precursor form of the enzyme within the \textit{gag-pol} polyprotein is in a less active form.

The proteolytic processing of the myristylated HIV polyproteins does not occur until a complete immature virion has become detached from the cell membrane. The maturation of the viral polyproteins is mediated by a viral protease. HIV-1 protease processes the \textit{gag-pol} polyprotein Pr160, and also carries out specific cleavages within the \textit{gag} precursor polyprotein Pr55 to yield the mature proteins p17, p24, p7 and p6. The HIV protease is thus vital to the successful replication of the virus.

HIV protease has been shown to be a \textit{pol} gene encoded product; the enzyme is therefore an intrinsic component of the \textit{gag-pol} polyprotein, implying that HIV-1 protease is released autocatalytically from Pr160.

It has been shown that HIV protease belongs to the mechanistic class of aspartic proteases. The highly conserved triad, Asp-Thr-Gly, found in retroviral protease sequences, was recognised to be homologous to the catalytic site of proteases belonging to the aspartic acid family. Also, HIV-1 protease was found to be inhibited \textit{in vitro} by pepstatin, a general inhibitor of aspartic proteases. Furthermore, single amino acid substitutions of the HIV-1 protease sequence in which the highly conserved Asp 25 was converted to Asn, Thr or Ala, resulted in elimination of protease activity.

Whereas fungal and mammalian enzymes of this class are generally composed of more than 200 amino acids, and consist of two homologous domains, retroviral proteases contain approximately half that number of residues. Furthermore, the catalytic triad which occurs twice in aspartic proteases such as pepsin and renin appears only once in the retroviral proteases.

It was proposed (and subsequently confirmed by elucidation of the crystal structure) that the catalytically competent form of retroviral proteases exists as a homodimer, with each monomer contributing one of the two aspartates to the active site. The structure of this active site was found to closely resemble the active site of pepsin-like aspartic proteases. This self-assembly of two identical monomers into a symmetric structure represents an efficient method for generating an active enzyme while encoding the minimum amount of genetic material.
The viral maturation process is manifested in the conversion of the annular virion to one containing a condensed, cone-shaped core, composed of p24 which constitutes the mature virion capsid. This core houses the retroviral enzymes, the nucleocapsid proteins p7 and p6 and the RNA genome.

The envelope proteins gp41 and gp120 are cleaved from the HIV env gene product gp160 and transported to the cell surface independently of the core proteins. These glycoproteins are synthesised from a lipid-linked oligosaccharide precursor, the carbohydrate of which is transferred to nascent polypeptide chains. Host glycosidase and transferase activities are responsible for the subsequent processing of the carbohydrate moiety. Indeed, viruses produced in the presence of inhibitors of trimming glycosidase, an enzyme involved in the normal processing of glycoproteins, are defective in their syncytia formation and have reduced infectivity.

Once the maturation process is complete and the structural proteins and enzymes have been released and activated, the virions are infectious and replication-competent.

The life-cycle of the virus is summarised in Figure 8.

Figure 8. The life-cycle of HIV.
Therapeutic agents effective against HIV

Each step in the viral replicative cycle presents a potential target for anti-HIV agents. A number of compounds have been shown to inhibit HIV replication in the laboratory, and several of these (alone or in combination) are now in clinical trials. In the following discussion of compounds known to be effective against HIV, it should be noted that anti-HIV activities should not strictly be compared unless tested under identical conditions. This is because the activity of a drug may be dependent on the cell type, culture medium, cell growth rates and the cell growth phase employed\textsuperscript{102,103}. Any anti-HIV concentrations given in this review should therefore be regarded as approximate.

Agents inhibiting HIV binding and other early steps in viral replication

HIV attachment to cells appears to require the specific interaction of the viral glycoprotein gp120 and the host cell CD4 receptor. Any molecules that inhibit this interaction may be expected to block HIV infection at the very first step in the viral replicative cycle.

Antibodies to HIV envelope glycoprotein or to CD4 can block viral binding and thus prevent infection\textsuperscript{104,105}. There are, however, inherent difficulties in creating an effective neutralising antibody to gp120. Not all antibodies to gp120 will block the CD4-binding site. Also, patients who produce antibodies (usually only in low concentrations, for reasons which are unclear) as a natural response to HIV infection may still develop AIDS. This may be due to the high rate of mutation of HIV; some variants of the virus may have an altered envelope glycoprotein that cannot be neutralised in this way.

Another approach involves the construction of a soluble form of CD4 that can bind to HIV, thereby monopolising its CD4 binding sites and preventing it from binding to the CD4 on a host cell. Recombinant soluble CD4 (rsCD4) derivatives are produced by genetic engineering; they consist of a CD4 molecule shortened to those peptide fragments that are involved in the binding of gp120\textsuperscript{106,107}. rsCD4 has been found to inhibit the infection of T cells by HIV at concentrations of 1 to 5 \(\mu\)g/ml\textsuperscript{108-112}. A potential advantage of this approach is that it will probably be difficult for the virus to mutate in such a way that it loses its affinity for the CD4 molecule while retaining its ability to infect T cells.
A drawback of rsCD4 is its plasma half-life of only 35 to 40 minutes in humans. To overcome this problem, hybrid molecules have been created by genetically combining the CD4 protein and the constant heavy chain domains of immunoglobulin (antibody) molecules to afford CD4-immunoadhesins\(^{113,114}\). Certain immunoglobulins have a long half-life in the bloodstream and also parts of the heavy chain domains of the immunoglobulins may be able to activate other parts of the immune system into destroying the virus. These hybrid (or chimeric) CD4-immunoglobulin (rsCD4-Ig) molecules retain antiviral activity against HIV-1 comparable to soluble CD4 in T cells and monocytes/macrophages\(^{113}\), and also gain other desirable properties such as a longer plasma half-life (7 to 48 hours)\(^{113}\).

Soluble CD4 can also be combined with toxins such as ricin\(^{115}\) or *pseudomonas* endotoxin\(^{116}\). Such CD4-toxin conjugates (CD4-immunotoxins) may bind selectively to those cells that express gp120 and because of the toxins then kill the cells. Thus, the main usefulness of these hybrid proteins is in destroying cells already infected with the virus. Certain cell types (such as macrophages) that are resistant to the cytopathic effects of HIV can produce virions for a long time, and destruction of such chronically infected cells may prove to be clinically useful.

One problem that may be associated with rsCD4 and related derivatives is that they may interfere with the natural immune responses involving the CD4 ligand, and hence exert immunosuppressive effects. Also, it now seems that CD4 is not the only target molecule for HIV infection: HIV can infect CD4 negative cells including neural cells, muscle cells or fibroblastoid cells *in vitro*\(^{117}\). Although the mechanism and significance of the CD4-independent transmission *in vivo* have yet to be determined, such a pathway may reduce the effectiveness of this therapeutic approach.

Phase I/II trials have been undertaken with rsCD4. Preliminary results suggest that the drug is without significant toxicity\(^{118}\). However, at the end of the 28-day period, only a slight decrease in the p24 antigen was noted.

Another class of compounds that specifically interfere with the virus adsorption process are the sulphated polysaccharides. Low molecular weight (7000 to 8000 daltons) dextran sulphate (Figure 9) may be regarded as the prototype of this class, and has been shown to block the binding of HIV virions to CD4 target cells, inhibit virally-induced syncytia formation and exert a potent anti-HIV effect *in vitro*\(^{119-122}\). Dextran sulphate consists of several repeating monosaccharide units with sulphate
groups. The sulphur content of dextran sulphate is approximately 19%, indicating that an average of two of the three hydroxyl groups per glucose moiety are substituted by sulphate groups (Figure 9). Compounds with similar anti-HIV activities in vitro include heparin and pentosan.

![Figure 9. Dextran sulphate.](image)

The sole clinical study that has been conducted with dextran sulphate showed that the compound had little toxicity but also little clinical effect. Dextran sulphate is very poorly absorbed when given orally, being almost totally degraded in the gastrointestinal tract of rats. Additional studies are proposed involving the intravenous administration of dextran sulphate. Dextran sulphates have been administered for some time as plasma expanders, anticoagulants and cholesterol-lowering drugs. The question therefore arises whether the usefulness of the sulphated polysaccharides as anti-HIV agents may be hampered by the possibility of coagulation abnormalities.

After binding to the receptor, the next step in the life-cycle of HIV is fusion of the virus with the target cell. The viral contents then enter the cell and the RNA is uncoated. Both of these stages may be targets for therapeutic intervention. The anti-influenza drug amantadine is believed to act by blocking viral uncoating, and it is possible that a similar approach will be effective against HIV.

**HIV reverse transcriptase inhibitors other than nucleoside analogues**

The process whereby the enzyme reverse transcriptase converts the information in the RNA genome of HIV into DNA is an essential step in the viral replicative cycle. This enzyme is a prime target for antiretroviral agents because it should be possible to find
inhibitors that will discriminate between it and the DNA polymerases of the host cell. Many of the drugs currently being studied act at this step. In particular, the nucleoside analogues have received a great deal of attention. The anti-HIV activity of the nucleoside analogues will be discussed later.

The compound suramin (Figure 10), used for over fifty years for the treatment of protozoal infections, inhibits reverse transcriptase and also inhibits HIV in vitro at concentrations far below those toxic for host cells\(^1\)\(^2\)\(^6\). However, in clinical trials major toxic effects were observed, including fever, rash, malaise, nausea, neurologic impairments and alterations in liver function\(^1\)\(^2\)\(^7\).

Another anionic compound is phosphonoformate (foscarnet) (Figure 11), which is a pyrophosphate analogue, and thus acts as a noncompetitive inhibitor of reverse transcriptase. Although phosphonoformate causes a 50% inhibition of HIV reverse transcriptase at 2 \(\mu\)M\(^1\)\(^2\)\(^8\)\(^9\), its effect on the replication of HIV in cell culture is less marked, where 50% inhibition of replication only occurs at concentrations greater than 100 \(\mu\)M\(^1\)\(^3\)\(^0\). Phosphonoformate is also known to concentrate in bone and to be eliminated very slowly over one year, thus limiting the long-term use of the drug as an anti-HIV agent\(^1\)\(^3\)\(^1\).

Antimoniotungstate (heteropolyanion-23, or HPA-23), full name ammonium-21-tungsto-9-antimoniate, is a cryptate mineral that in vitro inhibits HIV replication by acting as a competitive inhibitor of reverse transcriptase\(^1\)\(^3\)\(^1\). A clinical study of 69 AIDS patients showed a dose-dependent reduction of reverse transcriptase activity without any significant change in clinical symptoms. Side effects associated with HPA-23
include transient thrombocytopenia and renal toxicity\textsuperscript{32}.

A novel series of tetrahydro-imidazo[4,5,1-\textit{jk}][1,4]-benzodiazepin-2(1H)-one and -thione derivatives (TIBO derivatives) (Figure 12) were recently reported to be highly specific and potent inhibitors of HIV-1 replication\textsuperscript{33}.

![Figure 12. Structure of tetrahydro-imidazo[4,5,1-\textit{jk}][1,4]-benzodiazepin-2(1H)-one and -thione (TIBO) derivatives and compound R82150.](image)

These compounds, which are not structurally related to any other antiviral agents, were discovered by random screening. After the discovery of a lead compound, an extensive series of congeners was synthesised to maximise anti-HIV potency. One of the most active compounds, designated R82150, is shown in Figure 12. R82150 inhibited HIV-1 cytopathicity by 50\% at a concentration of 28 nM. Very surprisingly, none of the compounds in the series exhibited any activity against HIV-2.

HIV-1 reverse transcriptase was examined directly for its susceptibility to R82150\textsuperscript{34}. The compound inhibited HIV-1 reverse transcriptase activity by 50\% at a concentration of 3.1 \textmu M in one cell line, whereas no inhibitory effects of R82150 at concentrations of up to 350 \textmu M were observed on reverse transcriptase derived from HIV-2. The activity of the TIBO derivatives therefore seems to stem from a unique interaction with HIV-1 reverse transcriptase.

Studies of the pharmacokinetics of R82150 in dogs and man revealed no obvious adverse pharmacological effects, and the compound was well tolerated at the intravenous and oral dose levels used. Plasma drug levels were achieved in humans that were well above the virus-inhibiting concentration\textsuperscript{34}. Further studies on this series of compounds are underway to identify the analogue of choice for clinical trials in HIV-1 infected patients.

**Agents inhibiting the later stages of HIV replication**

Before the discovery of HIV, the replication of retroviruses was known to be inhibited by oligonucleotides\textsuperscript{35,36}, and the so-called antisense method has now been tested as
a potential therapy for AIDS. The strategy involves the synthesis of antisense (negative strand) mRNA; this then forms a duplex with the target (or sense) mRNA in the cell nucleus, and thus prevents its expression - a process termed translation arrest (or ribosomal-hybridisation arrest).

The problem with unmodified oligonucleotides is that they are unstable in vitro, due to the presence of cellular nucleases that cause hydrolytic degradation\textsuperscript{37}. Nuclease-resistant structures are therefore required for the antisense approach to work effectively, but sometimes chemical modification of oligomers leads to poor solubility and can mean that very high concentrations are required to achieve a biological effect. Oligonucleotides with a phosphorothioate modification - whereby an oxygen is substituted for sulphur (see Figure 13) - possess the negative charge necessary for aqueous solubility and also have the property of nuclease resistance\textsuperscript{38}.

\begin{center}
\includegraphics[width=0.5\textwidth]{phosphorothioate.png}
\end{center}

\textbf{Figure 13.} The structure of a phosphorothioate oligodeoxynucleotide (B = base).

A phosphorothioate analogue of 28-mer homo-oligodeoxycytidine (S-dC\textsubscript{28}) has been found to be potent against HIV-1 \textit{in vitro}\textsuperscript{39}. The mechanism of this sequence non-specific inhibition of HIV may arise from relatively selective inhibition of HIV reverse transcriptase, but interference with CD\textsubscript{4} recognition could also be involved\textsuperscript{37}. This mode of action is not the antisense mechanism desired, but could be useful as an anti-AIDS strategy.

A phosphorothioate hetero-oligomer has now been identified which has a
significant and sequence-specific inhibitory effect on the production of virally-encoded proteins in chronically HIV-1 infected T cells\textsuperscript{137}. The sequence adopted was complementary to the initiation sequence of HIV-1 rev. The 28-mer anti-rev sequence showed dose-dependent inhibition of the expression of the p24 gag protein. In contrast, the same antisense sequence with unmodified phosphodiester linkages; phosphorothioate oligomers containing the sense sequence, random or homopolymeric sequences; or an antisense sequence with N-methyl thymidine residues (thus precluding good hydrogen bonding) did not have an inhibitory effect on the viral expression. The antisense phosphorothioate oligomer induced an altered HIV-1 mRNA profile, suggesting that the mechanism for the inhibition of the observed viral expression is due to an interference with the regulatory rev gene and involves hybridisation to the relevant mRNA\textsuperscript{137}.

As regards in vivo activity, there are very little data available as to the toxicology and pharmacokinetics of the oligonucleotide analogues. The question of cell entry must also be addressed, since the compounds are relatively large and multiply charged. However, the development of antisense constructs to HIV genes as a means of inhibiting the virus is an attractive strategy which is being actively pursued.

Glycoprotein synthesis is important in the life-cycle of HIV and provides a potential target for therapeutic intervention. The enzymes which carry out elaboration of the polysaccharide component of the viral glycoproteins are host cell enzymes and the problem of achieving an antiviral effect without damaging the host cells arises. There are, however, several differences in viral and host glycoprotein structure which may be exploited. The surface glycoproteins of HIV-1 are heavily glycosylated with up to 50\% of their molecular mass comprising carbohydrate\textsuperscript{44,140,141}. The range of carbohydrate structures identified on gp120 appears to be very complex\textsuperscript{142,143}, and other features of viral glycoprotein synthesis such as intracellular transport\textsuperscript{144} may enable a satisfactory therapeutic index to be obtained.

Several trimming glycosidase inhibitors, including castanospermine (Figure 14) and deoxynojirimycin have been shown to block HIV-induced syncytia formation and interfere with HIV infectivity in vitro\textsuperscript{100,101}. N-butyldeoxynojirimycin (Figure 14), an analogue of deoxynojirimycin, has been reported to be more potent in inhibiting HIV replication by greater than five orders of magnitude at non-cytotoxic concentrations\textsuperscript{145}. N-butyldeoxynojirimycin is in Phase I clinical trials in the United States.
Figure 14. (a) Castanospermine (b) N-butyldeoxynojirimycin.

The role of proteolytic processing in HIV replication was discussed earlier. Inhibition of the virally encoded protease has become a major target in the search for effective anti-HIV agents.

Cleavage by HIV protease takes place at seven sites. These have little in common apart from the presence of Aromatic-Pro (Tyr-Pro or Phe-Pro) at three junctions, so the protease does not appear to have a stringent specificity146. The remaining cleavage sites show a variety of amino acids constituting the scissile bond: Leu-Ala; Leu-Phe; Met-Met and Phe-Leu dipeptides are all found in HIV-1 cleavage sites147. Further characterisation of the enzyme has been facilitated by the demonstration that synthetic oligopeptides that span each of the cleavage sites can be used as convenient substrates. Seven residues spanning the P4 to P3′ (notation of Schecter and Berger148) positions appear to be necessary to ensure specific and efficient cleavage of the scissile bond between the P1 and P1′ residues149-151. Thus, multiple hydrogen bonding contacts along the length of the substrate backbone are likely to be essential in promoting successful interaction with the active site of the enzyme. Also, not only the relative size/nature of the residues contributing the scissile peptide bond but also the identity of those in flanking positions has a considerable influence on the efficiency of cleavage147.

A classical strategy for designing enzyme inhibitors involves the incorporation of a transition-state mimic into substrate analogues. Non-hydrolysable dipeptide isosteres are substituted for the scissile amide bond. This approach has produced several inhibitors of HIV-1 and HIV-2 proteases.

Pepstatin A is a naturally occurring inhibitor of all aspartic proteases. It contains the dipeptide analogue statine (Sta, 4-amino-3-OH-6-methyl-heptanoic acid) (Figure 15(a)).
Pepstatin A is a relatively poor inhibitor of the proteases from HIV-1 and HIV-2$^{90-93}$. In contrast, acetyl pepstatin is an effective inhibitor ($K_i = 20 \text{nM}$)$^{91}$ of HIV-1 protease, and has been shown to be even more potent against HIV-2 protease$^{152}$ ($K_i = 5 \text{nM}$).

A further strategy involves replacement of the scissile -CONH- peptide bond with the reduced $\psi[-\text{CHOH-CH}_2]-($hydroxyethylene) moiety. Compound H261 (Figure 16) is a non-specific aspartic acid protease inhibitor. Inhibition of HIV-1 ($K_i = 5 \text{nM}$) and HIV-2 ($K_i = 35 \text{nM}$) protease was observed in vitro with this compound$^{152}$.

Figure 16. H261.

H261 and acetyl pepstatin are good inhibitors of most aspartic proteases and are thus not selective against HIV protease. Neither of these compounds has a sequence resembling those in HIV polyprotein cleavage junctions, suggesting that the active site of HIV protease does not appear to have a stringent requirement for particular primary structures in inhibitors$^{147}$. Thus it does not appear to be necessary to base potential inhibitors upon mimics of polyprotein cleavage sites in order to obtain favourable interactions within the active site of HIV protease.

The development of HIV protease inhibitors which do not interact with human aspartic proteases is an area in which there is much current research.

Nucleoside analogues as anti-HIV agents
The building blocks of both RNA and DNA are nucleotides, which consist of a purine
or pyrimidine base, a sugar and one or more phosphate groups. A nucleoside consists of a purine or pyrimidine base linked to a pentose sugar, either D-ribose in RNA or 2-deoxy-D-ribose in DNA. The major 2'-deoxynucleosides found in DNA are illustrated in Figure 17.

Figure 17. The structures of the major 2'-deoxynucleosides found in DNA: (a) thymidine; (b) 2'-deoxycytidine; (c) 2'-deoxyadenosine; (d) 2'-deoxyguanosine.

The 5'-O-triphosphate derivatives of the 2'-deoxynucleotides are the natural substrates for both cellular DNA polymerase α and viral reverse transcriptase. Both of these enzymes catalyse the formation of a phosphodiester bond between the 3'-hydroxyl group of the growing DNA chain and the 5'-O-phosphate group of the incoming nucleotide triphosphate (Figure 18).

Removal of the 3'-hydroxyl group in 2'-deoxynucleosides, or substitution by a different 3' moiety, generates 2',3'-dideoxynucleosides. Some but not all of these compounds can be metabolised by mammalian kinases to the 5'-triphosphate forms. They can then compete with the cellular 2'-deoxynucleoside 5'-triphosphates for incorporation into a growing DNA strand. When incorporated, the 2',3'-dideoxynucleosides may bring about chain termination, because a normal 5' to 3' phosphodiester linkage cannot be completed\textsuperscript{155-156}. Several of the 2',3'-
dideoxynucleoside triphosphates have higher affinities for HIV reverse transcriptase than for cellular DNA polymerase α, and this is probably one reason for the selective antiviral activity of these compounds.

Figure 18. Mode of action of DNA polymerase α and viral reverse transcriptase.

The nucleoside analogue 3'-azido-3'-deoxythymidine (AZT, also known as zidovudine or Retrovir) is currently one of only two drugs licensed for clinical use in the treatment of AIDS. AZT is an analogue of thymidine, in which the 3'-hydroxyl group is replaced by an azido group, Figure 19. AZT was found to be a potent inhibitor of HIV in vitro\textsuperscript{156}. In a phase I clinical trial to evaluate the safety, tolerance to and pharmacokinetics of AZT, the drug was well absorbed after an oral dose, increased the number of circulating CD4 lymphocytes and appeared to be associated
with clinical improvements in some patients\textsuperscript{157}. The phase II clinical data with AZT confirmed the observations made in the phase I trial, demonstrating a reduced mortality in patients receiving AZT\textsuperscript{158}. Patients showed at least a temporary increase in CD4 lymphocyte counts, fewer opportunistic infections and an average of 0.5 kg weight gain. In addition, some patients with HIV dementia had improved cognitive function. However, despite the significant clinical benefit of treatment with AZT, several serious adverse reactions are observed.

The most frequent toxicity is bone marrow suppression, leading to anaemia\textsuperscript{157,159}. Other toxic effects include nausea, vomiting, headaches and liver function abnormalities\textsuperscript{157,159-161}. Recent data suggest that lower doses of AZT benefit certain patients with a significant reduction of side-effects\textsuperscript{25}. However, even with the dose regimens recently employed in the clinic, virus can be isolated during therapy, which indicated that AZT may not completely suppress virus production \textit{in vivo}\textsuperscript{162}.

Pharmacokinetic studies with AZT indicated that the plasma half-life of the drug is approximately 1 h\textsuperscript{163}, thus necessitating frequent administration in order to maintain therapeutic drug levels. Although AZT does penetrate into the cerebrospinal fluid (CSF)\textsuperscript{163}, it does not penetrate into brain tissue\textsuperscript{164} and therefore may not suppress viral replication in the brain.

Another problem has emerged with the long-term administration of AZT. HIV strains have been isolated which are resistant to AZT \textit{in vitro}, usually from patients who had been receiving AZT therapy for more than six months\textsuperscript{165}. The mechanism responsible for the development of resistance appears to be related to cumulative mutation at at least three specific sites in the reverse transcriptase\textsuperscript{166}.

\textit{2',3'-Dideoxycytidine (ddCyd)} (Figure 20) was the second dideoxynucleoside
to be administered to patients with HIV infection\textsuperscript{167}. ddCyd can exert complete suppression of HIV replication at a half to one fifth of the effective concentration of AZT. The activity of ddCyd also appears to be more durable than that of AZT in vitro\textsuperscript{153,167}. Phase I clinical trials with ddCyd showed that the drug can suppress replication of HIV in vivo and improve clinical conditions in patients with advanced AIDS or ARC\textsuperscript{168,169}. Toxic effects of ddCyd, particularly at high doses, included skin eruptions, oral ulcerations, fever and malaise. Such symptoms usually resolved in one or two weeks, even with continuous therapy with ddCyd. However, the dose-limiting toxic effect was a painful peripheral neuropathy, which developed after 8-14 weeks of therapy with ddCyd.

![Chemical structures](image)

**Figure 20.** (a) 2'-Deoxycytidine (b) 2',3'-Dideoxycytidine.

Currently, studies with ddCyd involve periodical alternation of the drug with AZT, in an effort to reduce the hematopoietic suppressive effects of the latter\textsuperscript{168}. Other schedules involving administration of lower doses of ddCyd as a single agent are underway in phase II clinical trials.

2',3'-Dideoxyinosine (ddIno) is an analogue of the naturally occurring purine nucleoside inosine (Figure 21), and is the second drug to be licensed for AIDS therapy in the clinic. ddIno is closely related to 2',3'-dideoxyadenosine (ddAdo) (Figure 21), and indeed ddAdo is rapidly converted to ddIno by the ubiquitous enzyme adenosine deaminase\textsuperscript{170}, so these drugs can for many purposes be considered alternate forms. ddIno and ddAdo have been identified as equally potent inhibitors of HIV replication in vitro\textsuperscript{153}. ddIno has a relatively high therapeutic index in vitro as compared to the other dideoxynucleosides\textsuperscript{153,154}, and it has relatively little in vitro toxicity for human marrow progenitor cells\textsuperscript{171}. In human cells, ddIno is metabolised to its active form, 2',3'-dideoxyadenosine-5'-triphosphate\textsuperscript{172,173}, which has an intracellular half-life of more
than 12 h, and perhaps as long as 24 h, which compares favourably with the plasma half-life of AZT. Thus, even though the plasma half-life of ddIno is relatively short (about 35 min), ddIno may perhaps be administered with less frequent dosing schedules than AZT.

![Chemical structures](image)

**Figure 21.** (a) Inosine (b) 2',3'-Dideoxyinosine (c) 2',3'-Dideoxyadenosine.

In a phase I study, ddIno was administered to patients with AIDS or severe AIDS-related complex. The drug was orally bioavailable and penetrated into the cerebrospinal fluid. Although comparatively little evidence of an effect against HIV was seen at the lowest four doses, patients in the highest dose groups had increases in their CD4 T cells and an 80% decrease in serum HIV p24 antigen. The patients also had evidence of improved immunologic function, reporting increased energy and appetite and gaining an average 1.5 kg during the first six weeks of therapy. The most notable adverse effects directly attributable to ddIno administration included painful peripheral neuropathy, sporadic pancreatitis, mild headaches and insomnia. Further studies to define the safety and efficacy of ddIno are underway in the United States.

2',3'-Dideoxy-2',3'-didehydrothymidine (d4T) (Figure 22) has been shown to have an anti-HIV effect similar to that of AZT.

![Chemical structure](image)

**Figure 22.** 2',3'-Dideoxy-2',3'-didehydrothymidine (d4T).
In a test modelling bone marrow toxicity d4T was much less toxic to human haematopoietic progenitor cells than AZT\textsuperscript{79}. There are biochemical differences in the cellular metabolism of AZT and d4T and this may be the cause of the different toxicity profiles of the two drugs. Clinical trials with d4T have been initiated.

Many other nucleoside analogues have been found to have anti-HIV activity \textit{in vitro}. Of these, a selection of the most active compounds is shown in Figure 23.

![Chemical structures](image)

**Figure 23.** (a) 3'-Fluoro-3'-deoxymthymidine (b) 2',3'-Didehydro-2',3'-dideoxycytidine (c) 3'-Azido-2',3'-dideoxyuridine (d) 2,6-Diamino-2',3'-dideoxypurine (e) Carbocyclic 2',3'-didehydro-2',3'-dideoxyguanosine (f) Carbocyclic oxetanocin-A (g) Carbocyclic oxetanocin-G (h) 2',3'-Dideoxyguanosine (i) Phosphonylmethoxy ethyl adenine.
The reader is directed to more comprehensive reviews for in vitro data and detailed structure-activity analysis.\textsuperscript{25,180,181}

**Mechanism of action of the nucleoside analogues**
Most of the nucleoside analogues share a common mode of action. They must first undergo anabolic phosphorylation in target cells to their 5'-triphosphate forms, and after phosphorylation they may be incorporated into the viral DNA. This leads to premature termination of the elongating DNA chain and inhibition of DNA replication, as discussed earlier.

![Phosphorylation of AZT by host kinases.](image)

**Figure 24.** Phosphorylation of AZT by host kinases.

The phosphorylation of the nucleoside analogues is entirely dependent on the action
of mammalian enzymes: the host kinases. Experiments have shown that a virus-encoded enzyme plays no role in the anabolic activation of AZT, for example. AZT is phosphorylated to AZT triphosphate (AZTTP) by the same enzymes that phosphorylate thymidine\(^{182}\) (Figure 24). Thus, thymidine kinase catalyses the phosphorylation of AZT to its 5'-monophosphate (AZTMP)\(^{180,183}\). The second phosphorylation step is catalysed much less efficiently by cellular thymidylate kinase, and another kinase (probably thymidine diphosphate kinase\(^{183}\)) phosphorylates the AZT diphosphate (AZTDP) to AZTTP, the active form of the drug (Figure 24). AZTTP competes with thymidine 5'-triphosphate for incorporation into the lengthening viral DNA chain. This is evidenced by the reversal of the antiviral effect provided by AZT on addition of thymidine\(^{156}\). AZTTP is about 100-fold more inhibitory to HIV-1 reverse transcriptase than cellular DNA polymerase \(\alpha\)\(^{182}\), which explains its high selectivity. Although AZTTP is a reasonable substrate of DNA polymerases \(\beta\) and \(\gamma\), these polymerases are much less susceptible to inhibition by AZTTP than is reverse transcriptase\(^{184}\).

All of the nucleoside analogues currently under investigation as anti-HIV agents depend on host kinases for their anabolism to triphosphates.

**Limitations of the nucleoside analogues as anti-HIV agents**

5'-Phosphorylation is a prerequisite to the anti-HIV activity of all the nucleoside analogues. This puts severe constraints on the structure of any potential drug. Since HIV does not encode for nucleoside kinases it is necessary for these compounds to be phosphorylated by host cell enzymes. Cells vary as to their ability to accept nucleoside analogues as substrates for their kinases and these differences are reflected in the degree to which the drugs may be phosphorylated and hence exert their antiviral effect.

The 5'-triphosphates of 2',3'-dideoxyguanosine (ddGuo), 2',3'-dideoxythymidine (ddThyd), ddAdo and ddCyd all strongly inhibit HIV-1 reverse transcriptase. In terms of their inhibitory effects on the enzyme, the 5'-triphosphates can be ranked as follows: ddGuo = ddThyd > ddAdo = ddCyd. Contrast, however, the inhibitory effect of the parent nucleosides against the cytopathogenicity of HIV-1: ddCyd > ddAdo = ddGuo > ddThyd. A reason for this discrepancy was sought by Hao *et al*\(^{103}\). It was found that there was a direct correlation between the antiviral
activity of the compounds tested and their ability to generate their 5'-triphosphates. For example, ddCyd was found to generate the greatest amounts of its 5'-triphosphate intracellularly and this correlates with ddCyd displaying the greatest antiviral activity. On the other hand, it was found that the ability of ddThyd to generate its 5'-triphosphate was relatively poor and this correlates with the relatively low anti-HIV activity observed. It seems therefore that the potency displayed by the 2',3'-dideoxynucleosides and related compounds against HIV is strongly dependent on the generation of intracellular levels of their 5'-triphosphates. Two more examples serve to emphasise this point. The 5'-triphosphate of 3'-amino-3'-deoxythymidine (Figure 25) has a potent inhibitory effect on reverse transcriptase\textsuperscript{185}, and yet the nucleoside itself is totally inactive against HIV\textsuperscript{186}, which may be attributed to its inefficient phosphorylation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure25.png}
\caption{(a) 3'-Amino-3'-deoxythymidine (b) 3'-Amino-3'-deoxythymidine 5'-triphosphate.}
\end{figure}

3'-O-methylthymidine 5'-triphosphate (Figure 26) is another specific inhibitor of HIV reverse transcriptase\textsuperscript{187}, yet the parent nucleoside is inactive against HIV\textsuperscript{188}. Again, this is probably due to poor cellular phosphorylation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure26.png}
\caption{(a) 3'-O-methylthymidine (b) 3'-O-methylthymidine 5'-triphosphate.}
\end{figure}
**The Phosphate Triester Prodrug Approach**

Since most, if not all, nucleoside analogues require conversion into their 5'-phosphates as a vital step in their mode of action, the application of preformed 5'-phosphates as potential chemotherapeutic agents has been explored. However, little, if any, clinical benefit arises from the use of a preformed nucleotide in comparison to a nucleoside. Nucleotides are charged at physiological pH, making them poorly membrane permeable, and furthermore they are subject to rapid extracellular dephosphorylation by plasma enzymes.

The problem of drug delivery to a desired site of action is not a new one, and different strategies have been employed in order to improve drug bioavailability. One method involves chemical transformation of the active drug substance into *per se* inactive derivatives (prodrugs) that convert to the active compound by virtue of enzymic or chemical lability within the body system.

As discussed earlier, AZT therapy to HIV infection carries with it a number of problems in relation to the drug's toxicity, short plasma half-life and its poor permeability through the blood-brain barrier. Several groups have employed the prodrug approach in relation to improving the bioavailability of AZT. The compound 5'-((1,4-dihydro-1-methyl-3-pyridinylcarbonyl)-3'-azido-3'-deoxythymidin, Figure 27(a), for example, can be oxidised *in vivo* to yield the charged species shown in Figure 27(b), which itself is then slowly hydrolysed to yield AZT (the half-life for the prodrug in human serum is 7.7 h). The lipophilic dihydropyridine system readily penetrates the blood-brain barrier, which is particularly useful given the neurological disorders induced by HIV.

**Figure 27.** (a) 5'-((1,4-Dihydro-1-methyl-3-pyridinylcarbonyl) AZT (b) Quaternary pyridinium salt of (a).
When tested against HIV in vitro, the prodrug was found to be slightly more active than AZT\(^{199}\).

The prodrug approach may offer a solution to the problem of the poor bioavailability of charged nucleotides. Phosphate triesters may serve as potential nucleotide prodrugs, with careful design of the phosphate masking groups. Such compounds would be uncharged, thus facilitating membrane penetration. Lipophilic masking groups may allow the derivatives to cross the blood-brain barrier. Appropriately constructed compounds could also in theory penetrate cells using an active transport mechanism.

Following intracellular hydrolysis it should be possible to obtain the monophosphate of the nucleoside, which is of course of special interest with antitumoural and antiviral nucleosides such as AZT. As mentioned earlier, nucleoside analogues must serve as effective kinase substrates in order to be phosphorylated to the active entity, and normally kinases have such stringent specificity that any substantial deviation from the structure of the normal substrate results in loss of activity. 5'-Phosphorylation would in theory obviate this kinase dependence.

5'-Phosphate triester derivatives may show additional advantages over their parent nucleosides. For example, one of the major limitations of the drug 5-fluoro-2'-deoxyuridine in cancer chemotherapy is its rapid degradation caused by cleavage of the glycosidic bond. Phosphate triesters of this drug are entirely resistant to this form of deactivation\(^{201}\). Similarly, amino nucleosides such as ddCyd can be subject to metabolic deamination, a conversion which often brings about a diminution in biological activity\(^{202,203}\). Again, phosphate triesters have been shown to be entirely resistant to such enzymic deactivation\(^{204,205}\).

Having discussed the aims and potential advantages of the phosphate triester prodrug approach, the following section reviews and evaluates its application to antiviral and anticancer chemotherapy.

**Phosphate triesters and related derivatives as potential antiviral and anticancer agents**

Chawla et al. tested the ability of phosphate triesters to permeate mammalian cells and liberate 5'-mononucleotides intracellularly by loss of their phosphate masking groups\(^{206}\). Thymidine monophosphate derivatives (Figure 28), radioactively labelled
in the thymine base, were synthesised with masked phosphate moieties.

![Chemical structures](image)

**Figure 28.** (a) Thymidine 5'-phosphate (b) Thymidine 5'-bis(m-nitrophenyl) phosphate (c) Thymidine 5'-bis(p-nitrophenyl)phosphate.

The two compounds were evaluated in a bioassay of incorporation of radiolabelled thymidine into DNA in cultured cells. In the first study, a strain of cells was employed which lacked the cytoplasmic form of thymidine kinase, the enzyme responsible for generating thymidine 5'-monophosphate from thymidine. Under these conditions, labelling of DNA would be expected only as a result of the liberation of intracellular thymidine monophosphate by loss of the phosphate masking groups from the test compounds. The rate of incorporation of $^{3}H$ into the DNA from the compound shown in Figure 28(b) was very low, and it was therefore concluded that this phosphate triester was ineffective as a precursor for thymidine monophosphate in this study.
In the second section of this work, cells were cultured in the presence of an inhibitor of cytoplasmic thymidine kinase. The derivatives 28(b) and 28(c) were less effective than thymidine in labelling the DNA of the cells, and it was noted that the very small amount of labelling that did occur could have been due to the degradation of the test compounds to \[^{3}H\]thymidine prior to incorporation into the DNA.

Chawla et al. concluded that there was no evidence that 28(b) and 28(c) acted as sources of intracellular thymidine monophosphate by cell permeation followed by loss of the phosphate blocking groups. Although no lipophilicity data were given, it seems likely that compounds 28(b) and 28(c) would be quite polar in nature. No attempt was made to adapt these compounds to make them more lipophilic and therefore more compatible with the structural features of cell membranes. Since the penetration of a drug into a cell is the first necessary step for it to exert any biological activity, any potential phosphate triester prodrug must show hydrophobic character to enable it to pass through cell membranes.

Neumann and coworkers postulated\(^\text{207}\) that if a phosphate compound could be esterified with a drug, a sugar moiety and a fatty chain, then the composite structure might possess the following characteristics: (i) hydrophobic character due to the fatty hydrocarbon chain and the absence of charge, which is likely to allow the absorption and transport across membranes; (ii) hydrophilic solubility given by the carbohydrate (with the additional possibility of tailoring the carbohydrate to meet the specificity requirements of the receptors of properly targeted cells); (iii) active transport across a cell membrane by the glucose-phosphate or diglucosyl-phosphate transport proteins; (iv) among the different possibilities of in situ hydrolysis, the monophosphate of a nucleosidic drug might be obtained intracellularly.

A phosphate triester of thymidine was therefore synthesised\(^\text{208}\), Figure 29.

![Figure 29. 6-D-glucopyranosyl hexadecyl 5′-thymidinyl phosphate.](image)
Studies showed that the compound was more soluble than thymidine in water and more lipophilic than thymidine (as measured by partition coefficients in chloroform/water and octanol/water).

The interaction between this phosphotriester molecule and model membranes (large unilamellar vesicles) was studied by phosphorus, carbon and proton nmr\(^{\text{207}}\). The results demonstrated that the molecule was transported through the model membranes and into the internal milieu of the vesicles. Furthermore, no alteration of the membrane structure due to the presence of the compound was detected. The work of Neumann et al. therefore seems to provide good evidence for the ability of properly-designed phosphate triesters to cross cell membranes.

Engels and Schlaeger synthesised a series of triesters of adenosine cyclic 3',5'-monophosphate (cAMP)\(^{209}\). A representative compound is shown in Figure 30(b). cAMP plays an important role in regulating a variety of cellular processes, but in order to test cellular response to internal cAMP, one has to overcome the low cell membrane penetrating ability of exogenously added cAMP. Thus benzyl triesters of cAMP were synthesised as a transport medium to penetrate the cell membrane. Once inside the cell, it was postulated that the triester derivatives would be converted to cAMP by hydrolysis or enzymic degradation.

![Figure 30](image)

Figure 30. (a) Adenosine cyclic 3',5'-monophosphate (b) Benzyl ester of (a).
high cellular levels of cAMP. The triesters showed a complete lack of activity towards
the enzymes tested (cAMP-dependent protein kinase and cAMP phosphodiesterase),
while all the benzyl triesters were readily susceptible to aqueous hydrolysis. This
seems to support the conclusion that the benzyl triesters penetrated into the cells and
were then chemically hydrolysed to cAMP, which then produced the morphological
change. The benzyl triesters therefore act as a transport form of cAMP.

This approach was employed in related studies in which alkyl and benzyl
triesters of guanosine cyclic 3′,5′-monophosphate (cGMP) and o-nitrobenzyl triesters
of cAMP and cGMP were synthesised as membrane-transportable forms of the cyclic
nucleotides.\(^{210,211}\)

Hunston \textit{et al.} applied the phosphate drug delivery approach to the anticancer
drug 5-fluorouracil, shown in Figure 31(a).

![Figure 31. (a) 5-Fluorouracil (b) 5-Fluoro-2′-deoxyuridine 5′-phosphate.](image)

A good therapeutic response to this drug correlates with the persistence of 5-fluoro-2′-
deoxyuridine 5′-monophosphate (Figure 31(b)), a metabolite of the parent drug, in
tumour cells. However, it is not possible to administer this species directly because of
its charged nature at physiological pH and its susceptibility to rapid
dephosphorylation in plasma and in other tissues. A cyclic phosphate triester of 5-
fluoro-2′-deoxyuridine was prepared (Figure 32) in the hope that the monophosphate
would be generated directly from this compound within cells, thus obviating the
requirement for intracellular anabolism of 5-fluorouracil.

Hunston and coworkers envisaged bio-oxidation of the cyclic 5′-phosphate
triester in a similar manner to the known anticancer agent cyclophosphamide, which
is transformed \textit{in vivo} predominantly by cytochrome mixed function oxidase.
Thus bio-oxidation of an appropriate cyclic 5'-phosphoramidate of 5-fluoro-2'-deoxyuridine would give the hydroxy analogue as shown in Figure 33. Ring opening, elimination of acrolein and chemical or enzymic hydrolysis would then lead to the generation of 5-fluoro-2'-deoxyuridine 5'-monophosphate intracellularly (Figure 33).

The compound was tested for activity against S-180 Crocker sarcoma and L-1210 mouse leukaemia. In the latter the phosphate triester derivative showed no activity.
while in the former it showed only marginal activity at concentrations at which 5-fluorouracil was active. The reasons for the lack of activity were unclear. The absence of appropriate enzymes needed to oxidise the oxazaphosphacyclohexane ring in the test systems employed was mooted as a possible reason for the low activity.

Farquhar et al. independently synthesised the compound prepared earlier by Hunston et al. and the derivative shown in Figure 34\textsuperscript{213}.

![Phosphate triester derivative of 5-fluoro-2'-deoxyuridine](figure)

Figure 34. Phosphate triester derivative of 5-fluoro-2'-deoxyuridine synthesised by Farquhar et al.

Both compounds were screened against leukaemia P-388 implanted in mice. The compound shown in Figure 32 was only partially effective at prolonging the life spans of mice bearing the sensitive tumour, high dosages being required for optimal activity. The compound shown in Figure 34 was only marginally effective even at the highest doses tested. Significantly, both compounds were shown to be poorly susceptible to oxidative biotransformation in an enzyme study. Cyclophosphamide used as a control was extensively metabolised under the same conditions. Thus it appeared that the key primary step in the activation of these latent nucleotide precursors was blocked, since they did not appear to serve as adequate substrates for the oxidising enzyme.

In 1985 Farquhar and Smith applied the same strategy to a purine nucleoside analogue 9-[(\textbeta-D-arabinofuranosyl]adenine (araA), in order to determine whether the poor substrate affinities of the compounds shown in Figures 32 and 34 for mixed function oxidases were general or specific for 5-fluoro-2'-deoxyuridine\textsuperscript{214}.

AraA (Figure 35) is effective against human viral encephalitis. It has also been evaluated clinically as an antitumour agent and has shown modest activity. In common with most nucleoside analogues, araA is sequentially phosphorylated by cellular kinases to its 5'-triphosphate. It was hoped that the cyclic nucleotides synthesised would act as latent precursors of araA-monophosphate and thus preclude

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the requirement for the initial phosphorylation step in the activation sequence.

Figure 35. AraA.

Two compounds were synthesised and tested against murine leukaemia P-388 (Figure 36).

Figure 36. Phosphate triester derivatives of araA synthesised by Farquhar et al.

Compound 36(a) was marginally effective at prolonging the life span of mice bearing the tumour. AraA was similarly only marginally active. Compound 36(b) was completely ineffective even at the higher doses studied. Once again it was concluded that the cyclic compounds were not sufficiently biotransformed in vivo as anticipated. This interpretation was supported by the fact that the compounds shown in Figure 36 were only minimally transformed when incubated in vitro with mouse hepatic mixed function oxidase; under the same conditions cyclophosphamide was extensively degraded.

Further to their earlier work, Hunston et al. rationalised the preparation of a similar series of compounds on different grounds. Cyclic phosphotriester derivatives of 5-fluoro-2'-deoxyuridine were synthesised. The derivatives were designed to
hydrolyse slowly \textit{in vivo} to afford the nucleoside 5'-phosphate (thereby avoiding reliance on putative enzyme activation). The compound shown in Figure 34 was found to be too resistant to hydrolysis for this purpose, but it was thought that the introduction of suitable substituents into the phosphorus-containing ring would give compounds that would be hydrolysed more readily. To this end, the compounds shown in Figure 37 were prepared.

![Cyclic phosphate triesters derived from 5-fluoro-2'-deoxyuridine synthesised by Hunston et al.](image)

Studies of the inhibitory effects of the derivatives on the proliferation of murine leukaemia L1210 cells showed that the compound shown in Figure 37(b) was nearly as potent as the parent nucleoside, whereas those shown in Figures 34 and 37(a) were much less effective.

The cyclic phosphate triester derivatives were further examined in a mutant L1210 cell line deficient in thymidine kinase, which is the enzyme responsible for the conversion of 5-fluoro-2'-deoxyuridine to its active metabolite, the nucleoside 5'-monophosphate. The activity of the parent nucleoside was, as expected, much lower in this cell line. Compound 37(b) showed a similar drastic diminution in activity in this cell line, which seemed to suggest that it must be phosphorylated by cellular thymidine kinase in order to exert its activity. This would appear to negate any advantages of administering a preformed nucleoside phosphate, since it would appear that the phosphate moiety must be lost at some point before the active agent is formed. However, it is possible that the thymidine kinase deficient cell line has other properties which it does not share with the original cell line and it could be that the compounds were indeed acting in the manner envisaged.
Jones and his coworkers undertook to synthesise similar nucleotide derivatives containing five-membered phosphoramidate rings\textsuperscript{216}. It was hoped that the two compounds - shown in Figure 38 - would be slowly hydrolysed under physiological conditions to give a phosphodiester or an acyclic phosphoramidate.

\textbf{Figure 38.} Cyclic phosphoramidate derivatives of 5-fluoro-2'-deoxyuridine synthesised by Jones \textit{et al.}

Compound 38(b), when tested for antiproliferative activity against leukaemia L1210 cells, was active, showing about 3\% of the activity of 5-fluoro-2'-deoxyuridine. This compound had low activity in the mutant L1210 cell line deficient in thymidine kinase. One possible interpretation was that 38(b) was being degraded to 5-fluoro-2'-deoxyuridine which was then phosphorylated to 5-fluoro-2'-deoxyuridine-5'-phosphate, as opposed to the transformation of compound 38(b) directly into the 5'-phosphate.

Bères \textit{et al.} synthesised a number of 3',5'-cyclic monophosphates and neutral cyclic phosphate triesters of 5-alkyl-2'-deoxyuridines\textsuperscript{217}. Although the 5-alkyl-2'-deoxyuridines (such as the 5-ethyl and the 5-propyl compounds) are known to possess selective antiviral activity \textit{in vitro} and \textit{in vivo} (for example against herpes simplex viruses types 1 and 2), the antitumour properties of these thymidine analogues are poor. 5-Ethyl-2'-deoxyuridine also suffers from several limitations as a drug: it has low lipophilicity, is rapidly degraded to 5-ethyluracil and 5-(1-hydroxyethyl)uracil and suffers from the problem common to many nucleoside analogues of drug resistance arising from changes in enzyme activity. Also, the 5-alkyl-2'-deoxyuridines share with most nucleoside analogues a deleterious dependence on cellular kinases, since they must be phosphorylated at the 5'-position prior to incorporation into DNA.
Four cyclic triesters were synthesised as potential prodrugs of the relevant nucleoside 5'-phosphates (Figure 39).

![Cyclic phosphate triesters of 5-alkyl-2'-deoxyuridines synthesised by Bères et al.](image)

**Figure 39.** Cyclic phosphate triesters of 5-alkyl-2'-deoxyuridines synthesised by Bères et al. (a) $R_1=\text{Et}$, $R_2=\text{Me}$ (b) $R_1=\text{iPr}$, $R_2=\text{Me}$ (c) $R_1=\text{iPr}$, $R_2=\text{CH}_2\text{Ph}$ (d) $R_1=\text{nBu}$, $R_2=\text{Me}$.

All four compounds failed to demonstrate significant anticancer activity and all four were inactive against a number of herpes virus strains. Bères et al. do not discuss the reason for the lack of activity of these particular derivatives, but it would seem likely that they are relatively stable to intracellular hydrolysis, thus precluding formation of the free phosphate.

In a continuation of this work, several 5-halo and 5-(trifluoromethyl)-2'-deoxyuridine 3',5'-cyclic monophosphates and neutral triesters were synthesised, Figure 40.

![Cyclic phosphate triesters of 5-halo-2'-deoxyuridines synthesised by Bères et al. and their corresponding 3',5'-cyclic monophosphates](image)

**Figure 40.** Cyclic phosphate triesters of 5-halo-2'-deoxyuridines synthesised by Bères et al. and their corresponding 3',5'-cyclic monophosphates. (a) $X=\text{Br}$, $R=\text{CH}_3$ (b) $X=\text{I}$, $R=\text{CH}_3$ (c) $X=\text{I}$, $R=\text{CH}_2\text{Ph}$ (d) $X=\text{Br}$, $R=\text{H}$ (e) $X=\text{I}$, $R=\text{H}$.

The neutral cyclic triesters showed generally reduced antitumour activities compared
to the free (i.e. charged) cyclic monophosphates, shown in Figure 40 (d) and (e). The exception was the hydrolysable benzyl ester, Figure 40(c), which, in a particular cell line (human lymphoblast Raji cells), showed a cytostatic activity comparable to that of the diester. The antiviral activities of the neutral methyl esters 40(a) and 40(b) were similarly poor against a range of herpes viruses, although again the benzyl ester 40(c) was considerably more active than the methyl ester 40(b) and was in fact as potent as the free cyclic monophosphate 40(e) against HSV-1.

It appears from the work of Bères et al. that the nature of the phosphate masking group is crucial in retaining the activity of the parent nucleoside.

McGuigan and coworkers synthesised two series of uncharged phosphate triester derivatives of araA and the anticancer drug 9-\([\beta\text{-D-arabinofuranosyl}]\)cytosine (araC) as potential membrane soluble prodrugs of the nucleotides\(^2\text{04,205}\) (Figure 41). Both araA and araC share a dependence on kinase mediated activation to the 5' phosphate forms.

![Fig 41](image)

**Figure 41.** Phosphate triester derivatives of araA and araC synthesised by McGuigan et al. R=Et, nPr, nBu, n-pentyl, n-hexyl (araC derivative only).

Octanol/water partition coefficients were measured for each derivative and in all cases the derivatives showed enhanced lipophilicity over the parent nucleoside, which, it was postulated, would facilitate passive diffusion of the nucleotide triesters through cell membranes. The biological effects of the compounds were tested on a mammalian epithelial cell line employing a thymidine incorporation assay. The degree of inhibition of thymidine incorporation was found to be profoundly dependent on the structure of the phosphate moiety. In particular, there was a clear correlation between degree of inhibition and lipophilicity, activity rising with increasing lipophilicity in both series of compounds. McGuigan et al. suggested that this was strong evidence for the role of membrane penetration as a crucial step in the biological action of the triesters. The data obtained did not, however, distinguish between intracellular release of the
nucleotide or nucleoside, or direct action by the triesters themselves. It was hoped that
the triesters were acting as intracellular sources of araA-monophosphate and araC-
monophosphate via P-O-alkyl cleavage, thereby obviating the dependence on kinase-
mediated activation. However, equally possible was cleavage of the P-O-nucleoside
linkage.

In order to probe the mechanism of action of their compounds, McGuigan et
al. synthesised and evaluated a series of phosphinate derivatives of araA analogous
to the phosphate triesters prepared earlier, but bearing methylene bridges from the
phosphorus to the alkyl chains. The two phosphinate derivatives are shown below,
together with their phosphate analogues (Figure 42).

![Figure 42](image)

Figure 42. (a) Phosphinate and (b) phosphate derivatives of araA synthesised by
McGuigan et al.

The Van der Waals radii for the methylene and oxygen moieties are approximately the
same, and so similar biological activities might be expected for both phosphinates and
phosphates if the compounds were acting merely as depot forms of araA. However,
if the mode of action of the compounds were release of araA-monophosphate, the
phosphinate derivatives would be expected to show much less activity, since the P-C
bond has a high chemical stability and is not susceptible to enzymic cleavage.

Using the same biological testing procedures as before, it was found that the phosphinates retained an inhibitory effect on DNA synthesis, suggesting that the phosphate compounds do not act solely by release of araA-monophosphate via cleavage of the alkyl-phosphate bonds. However, in both cases the activities of the phosphinates were significantly lower than those of the analogous phosphates. It was concluded that a dual mechanism of action for the phosphate triesters was the most likely, with P-O-alkyl cleavage and intracellular free nucleotide release enhancing their activity relative to the phosphinates.

Farquhar et al. investigated the ability of acyloxymethyl groups to serve as biologically reversible phosphate-protecting groups. It was postulated that neutral acyloxymethyl phosphotriesters might penetrate cell membranes and revert, intracellularly, to the parent ionic phosphate after cleavage of the acyloxy moiety by carboxylate esterase and elimination of formaldehyde (Figure 43).

![Scheme proposed by Farquhar et al. for the liberation of ionic phosphates from phosphate triester precursors.](image)

Figure 43. Scheme proposed by Farquhar et al. for the liberation of ionic phosphates from phosphate triester precursors.
Following studies with phenyl phosphate as a model organophosphate monoester, the compound shown in Figure 44 was synthesised.

![Chemical structure](image)

**Figure 44.** Bis(pivaloyloxymethyl) nucleoside phosphate synthesised by Farquhar et al.

This derivative prevented the growth of Chinese hamster ovary cells in culture at a concentration of 5 μM (5-fluoro-2'-deoxyuridine control 1 μM).

Farquhar and colleagues then prepared the two pivaloyloxymethyl esters of 5-fluoro-2'-deoxyuridine monophosphate shown in Figure 45.

![Chemical structure](image)

**Figure 45.** Pivaloyloxymethyl esters of 5-fluoro-2'-deoxyuridine monophosphate.
These compounds were converted by carboxylate esterases under physiological conditions to unstable intermediates that rapidly yielded free 5-fluoro-2'-deoxyuridine monophosphate by elimination of formaldehyde and acrolein. Both compounds inhibited the growth of cell cultures resistant to 5-fluorouracil in vitro and were active in vivo against transplantable mouse tumours resistant to 5-fluorouracil and 5-fluoro-2'-deoxyuridine\textsuperscript{222,223}. In order to determine whether the compounds shown in Figure 45 formed 5-fluoro-2'-deoxyuridine monophosphate via degradation to 5-fluorouracil or 5-fluoro-2'-deoxyuridine, or whether they diffused into cells and then liberated the free nucleotide by loss of their esterifying groups (as hoped), the effects of the compounds on cells genetically devoid of thymidine kinase were studied\textsuperscript{224}. Such cells were incapable of converting the parent nucleoside to the nucleotide and thus any 5-fluoro-2'-deoxyuridine that arose from extracellular or intracellular degradation of the test compounds could not be directly phosphorylated in the cytoplasm. Similarly, the cells were incapable of converting thymidine to thymidine monophosphate. Thymidine monophosphate is essential for the synthesis of nuclear DNA. The sole alternative source of thymidine monophosphate derives from the action of thymidine monophosphate synthetase. Because 5-fluoro-2'-deoxyuridine inhibits thymidine monophosphate synthetase, the effects of the compounds on the rate of DNA synthesis were studied. Any inhibition observed could therefore be assigned to liberation of 5-fluoro-2'-deoxyuridine monophosphate from the test compounds by loss of their phosphate esterifying groups.

Both compounds inhibited DNA synthesis more rapidly and nearly ten times more effectively than the parent nucleoside. Compound 45(a) reduced the rate of DNA synthesis more rapidly and at a lower concentration than compound 45(b). This may reflect a difference in the rates at which the compounds penetrate the cell membranes; alternatively, it may relate to the observation that compound 45(a) was hydrolytically more stable than compound 45(b) and that it was more readily attacked by a hog liver esterase\textsuperscript{222}.

In the second section of this study the compound shown in Figure 46 was synthesised\textsuperscript{224}. The ability of the compound to induce DNA synthesis was assessed in cells devoid of thymidine kinase activity. Thymidine monophosphate synthesis from uridine monophosphate was blocked by the inhibition of thymidine monophosphate synthetase activity by aminopterin. The compound shown in Figure 46 induced DNA
synthesis at a rate appreciably faster than that produced by a much higher level of thymidine monophosphate.

![Figure 46. Pivaloyloxymethyl ester of thymidine monophosphate synthesised by Farquhar et al.](image)

It was concluded that this derivative appeared to furnish biologically active intracellular free thymidine monophosphate by the same mechanism as demonstrated by the compounds shown in Figure 45 in liberating 5-fluoro-2'-deoxyuridine monophosphate.

Farrow and coworkers devised an ingenious strategy for the intracellular liberation of nucleoside monophosphates from phosphate triesters. The synthesis and biological evaluation of some aryl bis(nucleosid-5'-yl) phosphates were described.

![Figure 47. Intracellular liberation of nucleotides from phosphate triesters as envisaged by Farrow et al.](image)
It was proposed that if an aryl group could be chosen so as to hydrolyse preferentially from such a compound under physiological conditions, then the ensuing enzymic phosphatase attack would always yield one equivalent each of nucleoside and nucleotide, Figure 47. A series of model compounds was prepared, and following hydrolysis experiments the best group R (see Figure 47) appeared to be the 4-(methylsulphonyl)phenyl moiety. Accordingly, the compounds shown in Figure 48 were prepared.

Figure 48. Phosphate triesters synthesised by Farrow et al. from (a) BVDU and (b) ACV.
The two parent nucleosides, (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) (Figure 48(a)) and 9-[(2-hydroxyethoxy)methyl]guanine (ACV) (Figure 48(b)) are herpes simplex virus (HSV) inhibitors which depend upon viral thymidine kinase for activation. Drug resistant strains have been isolated which owe this property to an inability to phosphorylate the nucleosides; thus, infection by these resistant virus strains is not affected by BVDU or ACV and if the monophosphate of either drug could be liberated in such an infected cell, in theory the infection should be susceptible.

Compounds 48(c) and 48(d) were evaluated for their antiherpes effect, both in vitro and in vivo. Although both compounds were markedly active against strains of HSV-1, neither compound showed appreciable activity against the strains lacking viral thymidine kinase. Furthermore, the antiviral activity spectrum of 48(c) was remarkably similar to that of BVDU and that of 48(d) was similar to ACV. Farrow et al. suggested that the 5'-5' dimers of BVDU and ACV were being degraded to their parent compounds and were therefore acting merely as prodrugs for the parent nucleosides. One problem with evaluating compounds in such a system is that a virus strain which is negative for thymidine kinase may also be negative for thymidylate kinase, in which case even if BVDU-monophosphate was liberated, its progress to BVDU-diphosphate would be blocked. Also, the biochemical transformation of a nucleotide depends upon its concentration and the $K_m$ for the relevant enzymes. Thus several factors may operate to render the conclusions drawn from the thymidine kinase negative strains invalid. Despite the inconclusive nature of the results obtained, this strategy was utilised in a recent drug patent, the content of which will be discussed later.

Having reviewed the status of phosphate triesters as potential antiviral and antitumour agents, their application specifically to anti-HIV therapy will now be considered.

McGuigan et al. synthesised a series of phosphate triesters as potential anti-HIV agents$^{226}$ (Figure 49). On evaluation in vitro all of the compounds were devoid of any anti-HIV activity. McGuigan et al. concluded that the stability of these simple trialkyl phosphates precluded metabolic conversion into the free 5'-phosphates. In an effort to overcome this problem, more labile phosphate derivatives were sought.
Thus, the bis(2,2,2-trihaloethyl) phosphate triesters of both AZT and ddCyd were synthesised\(^{27}\) (Figure 50).

All these 5'-phosphate derivatives of AZT and ddCyd displayed anti-HIV activity \textit{in vitro}. McGuigan \textit{et al.} conceded that the mechanism by which the compounds displayed their anti-HIV effect was uncertain. One possible mechanism would consist of membrane penetration, followed by intracellular cleavage of the (labile) substituted alkyl to phosphorus bonds. The resulting nucleotides might then be subject to further phosphorylation to the 5'-triphosphates, which would inhibit HIV reverse transcriptase. McGuigan and coworkers suggested that cleavage of the P-O-alkyl bonds and consequential nucleotide release was more likely than P-O-nucleoside bond cleavage and nucleoside release, basing this proposition on the greater labilising effect.
of halogen substitution on the P-O-alkyl bonds rather than on the more remote P-O-nucleoside bond.

Further to the work of Farrow et al. summarised earlier, Jones and Walker described the synthesis and anti-HIV activity of a number of bis(nucleosid-5'-yl) phosphates in a recent European patent. Examples of compounds claimed in their patent are shown in Figure 51.

![Figure 51. Bis(nucleosid-5'-yl) phosphates claimed by Jones and Walker in a recent drug patent.](image)

No detailed biological test results were given, but the inventors stated that the dosage of the compounds required for drug regimes will be lower than the corresponding amount for the parent nucleosides.

Hahn et al. synthesised a similar series of hetero and homodimers of
nucleosides\textsuperscript{229}, such as the example shown in Figure 52.

\[ \text{Figure 52. Example of a heterodimeric nucleoside phosphate triester synthesised by Hahn et al.} \]

On an equimolar basis, the phosphate triester dimers showed greater anti-HIV activity than the corresponding monomers. Furthermore, plasma and CSF levels of the dimers \textit{in vivo} were similar to those achieved by the monomers.

In an extension of their earlier work on masked phosphates, Farquhar and coworkers synthesised the bis(pivaloyloxymethyl) ester of 2',3'-dideoxyuridine monophosphate (Figure 53)\textsuperscript{230}.

\[ \text{Figure 53. Bis(pivaloyloxymethyl) ester of 2',3'-dideoxyuridine monophosphate synthesised by Farquhar et al.} \]

2',3'-Dideoxyuridine triphosphate is a powerful and selective inhibitor of HIV reverse transcriptase, yet the parent nucleoside is ineffective at blocking HIV infection of cells in culture\textsuperscript{231}. Studies have shown that 2',3'-dideoxyuridine (ddUrd) is not anabolised to the nucleotide, apparently because it is a poor substrate for cellular nucleoside kinases\textsuperscript{231}. Farquhar and colleagues hoped that the preparation of the appropriate phosphotriester of ddUrd would promote entry of the nucleotide into cells. The phosphotriester might then revert to the nucleoside monophosphate after cleavage of 61
the phosphate masking groups by carboxylate esters. Once liberated, the monophosphate could then be anabolised to the corresponding di- and triphosphates by cellular kinases.

The metabolism and anti-HIV activity of the phosphotriester were investigated in two human T cell lines, one with wild-type thymidine kinase activity and the other deficient in thymidine kinase activity. The mono-, di- and triphosphates of ddUrd were formed in both cell lines after exposure to the test compound. In contrast, phosphorylated metabolites were not observed in cells treated with ddUrd or ddUrd monophosphate alone. Furthermore, the test compound reduced the cytopathic effects of HIV-1 in T cells (ED_{50} = 4.75 μM), with no observable toxicity. The compound was similarly active in cells deficient in thymidine kinase activity. This work therefore provides good evidence for the viability of the phosphate triester prodrug approach in facilitating the development of anti-HIV nucleoside analogues hitherto considered inactive because of inherently poor substrate affinity for nucleoside kinases.

Phospholipid-nucleoside conjugates represent one of the latest developments in the application of innovative strategies towards anti-HIV therapy. Although the compounds reviewed here are not phosphate triesters, but rather diesters, their synthesis was prompted by similar hypotheses. Example compounds are shown in Figure 54.

The advantages of these liponucleotide prodrugs were suggested by Piantadosi et al. and Hostetler et al. Where both phospholipid component and nucleosidic component have anti-HIV activity then enhanced potency may be achieved over the parent compounds, since the agents exert their anti-HIV effects by different mechanisms. It was suggested that the conjugates would allow a larger concentration of drug to enter cells because of the lipophilic nature of the phospholipid moiety. Finally, the compounds potentially have the ability to by-pass the initial anabolic phosphorylation of the nucleoside analogue, possibly reducing viral resistance.

The conjugates described by the two groups were tested against HIV in vitro, with some showing activities greater than that of AZT. Significantly, the compounds seemed to show a reduction in toxicity when compared to the parent nucleoside. Phospholipid-nucleoside conjugates are therefore promising candidates for development as anti-HIV agents.
Figure 54. Two examples of phospholipid-nucleoside conjugates which have been synthesised and evaluated against HIV-1.

Finally, McGuigan et al. synthesised a series of phosphoramidate derivatives of AZT, designed as membrane-soluble prodrugs of the nucleotide (AZT-monophosphate). This work formed the immediate background to the studies described in this thesis. As mentioned earlier, simple dialkyl phosphate triesters of AZT were found to be inactive as anti-HIV agents. A phosphate protecting group was therefore sought which might be cleaved more rapidly in the HIV-infected cell. McGuigan and coworkers postulated that if an oligopeptide could be linked to a nucleoside via such a phosphate masking group, specific HIV protease-induced cleavage of the peptide from the conjugate compound might result in enhanced antiviral selectivity. A second rationale for the synthesis of a nucleotide-oligopeptide conjugate was the possibility of employing an HIV protease-specific inhibitor as the peptide moiety. In addition to all the advantages mentioned earlier in adopting the phosphate triester prodrug approach, a nucleotide-peptide conjugate might offer several other useful features in terms of its antiviral effect. First, peptides aimed at HIV protease target a late step, whereas the nucleoside analogues target an early step in HIV replication, enabling a two-pronged attack on the virus to be made from the
same drug. Second, the development of viral resistance to two active drugs would be likely to occur at a slower rate than to either compound alone, since the nucleoside and the peptide would act by different mechanisms. Third, the combination of compounds may reduce the toxicity associated with nucleoside analogues such as AZT. Fourth, the peptide prodrug moiety may facilitate exploitation of amino acid transport systems to deliver AZT (for example) to target cells at higher concentrations than can be achieved by AZT alone.

As an initial study, the synthesis of simple derivatives of AZT-monophosphate bearing an alkyl chain and a single N-linked, carboxyl-protected amino acid was pursued. The compounds which were prepared are shown in Figure 55.

![Figure 55. Structures of phosphoramidate derivatives of AZT and the results of their biological evaluation against HIV. AZT had an ED₅₀ of 0.03 μM in this test.](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R'</th>
<th>ED₅₀ (μM)</th>
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<tbody>
<tr>
<td>(a)</td>
<td>Me</td>
<td>Et</td>
<td>3</td>
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<td>(b)</td>
<td>iPr</td>
<td>Et</td>
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<td>(c)</td>
<td>H</td>
<td>Et</td>
<td>10</td>
</tr>
<tr>
<td>(d)</td>
<td>CH₂·iPr</td>
<td>Et</td>
<td>30</td>
</tr>
<tr>
<td>(e)</td>
<td>CH(Me)Et</td>
<td>Et</td>
<td>50</td>
</tr>
<tr>
<td>(f)</td>
<td>Bzl</td>
<td>Et</td>
<td>10</td>
</tr>
<tr>
<td>(g)</td>
<td>iPr</td>
<td>Me</td>
<td>3</td>
</tr>
<tr>
<td>(h)</td>
<td>iPr</td>
<td>iPr</td>
<td>10</td>
</tr>
<tr>
<td>(i)</td>
<td>iPr</td>
<td>nBu</td>
<td>3</td>
</tr>
<tr>
<td>(j)</td>
<td>iPr</td>
<td>nHex</td>
<td>3,30</td>
</tr>
</tbody>
</table>

The phosphoramidates shown in Figure 55 displayed potent anti-HIV activity. By comparison to the simple dialkyl phosphate derivatives of AZT, it can be seen that the introduction of a P-N linkage is clearly important for activity. Significantly, relatively minor modifications in the amino acid moiety led to marked changes in activity. Substituting alanine (55(a)) by valine (55(b)), glycine (55(c)) or phenylalanine (55(f)) led to little change in activity (ED₅₀ ca. 3-10 μM). However, substitution by leucine (55(d))
and especially isoleucine (55(e)) led to a substantial reduction in activity. There appeared to be diminishing activity with increasing steric bulk of the amino acid side chain. This suggested the possibility of enzymic cleavage of the amino acid moiety as an important step in the metabolic activation of these compounds. In contrast, the alkyl group could be varied considerably without greatly affecting activity. Thus lengthening the alkyl chain from methyl (55(g)) to ethyl (55(b)), propyl (55(h)), butyl (55(i)) and hexyl (55(j)) appeared to have no significant effect on activity.

The compounds were isolated in every case as a mixture of diastereoisomers, arising from the combination of multiple chiral centres. However, compound 55(j) was separated into two fractions by hplc, representing its two diastereoisomeric forms. The two separated isomers showed activities of 3 and 30 µM, thus providing further evidence for the importance of the precise stereochemical environment at phosphorus.

The precise mechanism by which these derivatives exert their anti-HIV activity is unknown. However, direct inhibition of HIV reverse transcriptase has been excluded. One of the purposes of the work described in this thesis was to investigate the mechanism of action of these compounds. The other main aim was to synthesize novel derivatives with a view to improving on the activities already achieved. The lead structure suggested a host of potential target compounds, some of which are indicated below (Figure 56).

![Figure 56.](image)

The synthesis, characterisation and biological evaluation of several of these target compounds will be described in the next section.
Results and Discussion

The method originally employed by McGuigan et al. in their synthesis of phosphoramidate derivatives of AZT bearing an alkyl chain and a single amino acid involved two steps. First, the synthesis of an alkyl amino acyl phosphorochloridate, as indicated in Figure 57.

![Figure 57. Structure of alkyl amino acyl phosphorochloridates (R = amino acid side chain, R' = alkyl chain).]

Second, the reaction of these phosphorochloridates with the nucleoside in THF containing N-methyl imidazole, Figure 58.

![Figure 58. Condensation of alkyl amino acyl phosphorochloridate and AZT to yield monophosphate triester derivatives bearing an alkyl chain and a single amino acid.]

The phosphorochloridates were prepared in crude form by an adaptation of a standard
procedure. Two equivalents of the free base of the appropriate carboxyl-protected amino acid were reacted with an alkyl phosphorodichloridate, the second equivalent being introduced to remove HCl by precipitation of the amino acid hydrochloride salt, Figure 59.

\[
\begin{align*}
2 \text{CH}_3\text{O} - &- \text{CH} - \text{NH}_2 + \text{ROP(O)Cl}_2 \to \text{CH}_3\text{O} - &- \text{CH} - \text{NH} - \text{P} - \text{Cl} + \text{CH}_3\text{O} - &- \text{CH} - \text{NH}_3\text{Cl} \\
- &- &- 40^\circ\text{C} & & &
\end{align*}
\]

**Figure 59.** Original method employed by McGuigan et al. in the synthesis of alkyl amino acyl phosphorochloridates.

The phosphorochloridates obtained by this method were frequently impure because of the difficulty in maintaining scrupulously dry conditions. Contamination by hydrolysed phosphate and pyrophosphate materials was commonly observed. A satisfactory method for the purification of these compounds has not been found: previous attempts at distillation had resulted in decomposition and Kosolapoff has noted the thermal instability of the P-N bond in related derivatives.

Chromatographic purification would appear to be incompatible with the hydrolytic sensitivity of the P-Cl bond.

The first aim of this work was therefore to find a new route to the alkyl amino acyl phosphorochloridates which would not require recourse to purification techniques.

An adaptation of the method of Brenner and Huber was used to protect the carboxyl termini of all amino acids. A typical procedure is given in experiment 1, which describes the preparation of L-phenylalanine methyl ester hydrochloride. All amino acid salts prepared in this way were isolated in high yields and characterised by proton nmr.

Ethyl phosphorodichloridate was prepared by the method given in experiment 2. One equivalent of ethanol and one equivalent of triethylamine were carefully added over a long period of time to phosphoryl chloride at low temperature, employing a large quantity of diethyl ether as solvent, Figure 60.
Figure 60. Reaction scheme for the synthesis of ethyl phosphorodichloridate (experiment 2).

Filtration, concentration and distillation afforded the product in good yield, with a boiling point of 74 °C/10 mm Hg as compared to the literature boiling point of 58 °C/13 mm Hg.

The first attempts at finding a novel synthesis for the alkyl amino acyl phosphorochloridates focused on the amino acid glycine. Glycine had caused particular problems when the free-base method was applied because glycine methyl ester rapidly undergoes self-polymerisation.

A comprehensive survey of Chemical Abstracts (1947 to the present) revealed that the alkyl amino acyl phosphorochloridates were novel species and so no alternative strategies for their synthesis were available from the literature. However, work describing the reaction of dibenzyl phosphorochloridate with protected amino acids provided a convenient starting point for a possible synthetic route. Li et al. had added the phosphorylating reagent to the amino acid hydrochloride in the presence of triethylamine in chloroform at 0 °C, Figure 61.

Thus, in experiment 3, ethyl phosphorodichloridate and two equivalents of triethylamine were added to one equivalent of glycine methyl ester hydrochloride (as a suspension in dichloromethane) at 0 °C, Figure 62. The addition of diethyl ether at the end of the reaction caused the triethylamine hydrochloride to precipitate out, and filtration and removal of the solvents afforded the product as an oil.

This new method had several potential advantages: the risk of formation of
hydrolysis products was reduced by avoiding the need to isolate the free base of the amino acid ester, the reaction was performed in 'one pot' and the instantaneous reaction of the amino acid methyl ester was expected to prevent polymerisation reactions from taking place.

\[
\begin{align*}
\text{CH}_2\text{O} & \quad \text{C} \quad \text{CH}_2\text{NH}_3\text{Cl} \quad + \quad \text{EtOP(O)Cl}_2 \quad + \quad 2 \text{Et}_3\text{N} \quad \xrightarrow{0 \, ^\circ \text{C}} \\
\text{CH}_2\text{O} & \quad \text{C} \quad \text{CH}_2\text{NH} \quad \text{P} \quad \text{Cl} \quad + \quad 2 \text{Et}_3\text{NHCl}
\end{align*}
\]

**Figure 62.** Reaction scheme for the synthesis of ethyl methoxyglycinyl phosphorochloridate (experiment 3).

The product of experiment 3 showed one major signal in its phosphorus nmr spectrum. The chemical shift of this major product was 11.68 ppm, which was in the region expected for the target molecule, ethyl methoxyglycinyl phosphorochloridate (the compound OP[N(CH_2CH_3)_2](OCH_2CH_3)Cl, for example, has a reported chemical shift of 14.7 ppm\(^{245}\)). The minor signals observed were probably due to unreacted ethyl phosphorodichloridate (at 4.15 ppm) and hydrolysed phosphate material (at -5.90 ppm), and another signal at 17.97 ppm indicated the formation of the fully substituted compound: OP[NHCH_2C(O)OCH_3]_2OCH_2CH_3 (OP[N(CH_3)_2]_2OCH_2CH_3 has reported chemical shifts of 18 and 16.3 ppm\(^{246-248}\)). The formation of this latter material suggested that the reaction was producing more substitutions at the P-Cl bonds than were required. Therefore in experiment 4 the reaction temperature was reduced to -60 °C, otherwise the conditions were the same as for experiment 3. However, the fully substituted compound persisted in the final product, albeit in a lower proportion.

A change in methodology was therefore adopted for the third attempt at the synthesis of pure ethyl methoxyglycinyl phosphorochloridate (experiment 5). Instead of adding the ethyl phosphorodichloridate to the amino acid salt, it was decided to stir the two together in dichloromethane and add triethylamine dropwise to this mixture at -78 °C. The ethyl phosphorodichloridate would thus be kept in excess and it was hoped that this would keep formation of the fully substituted material to a minimum. Indeed, the product of experiment 5 showed no trace of this compound, the only product contaminant being hydrolysed phosphate material.

It was found that by employing a very slow rate of addition of the
triethylamine (for example, by the use of a syringe pump on a timer), by scrupulous
drying of the amino acid salts (see Apparatus and Reagents section) and by removing
the solvent at the end of the reaction and extracting with carbon tetrachloride instead
of diethyl ether in the work-up, pure ethyl methoxyglycinyl phosphorochloridate
could be obtained, and a complete successful synthesis of this compound is given in
experiment 6.

The product was obtained in good yield (84%) and showed one signal in its
phosphorus nmr spectrum at 12.48 ppm. The carbon nmr spectrum was assigned by
analogy to a spectrum of N-glycyl-N-methyl glycine methyl ester from the literature and
by comparison to the carbon nmr spectrum of ethyl phosphorodichloridate
obtained earlier in experiment 2 (see Figure 63).

Three-bond phosphorus-carbon coupling was observed to the carbonyl carbon and to
the methyl carbon in the ethoxy chain. Two-bond phosphorus-carbon coupling was
observed to the methylene carbon in the ethoxy chain. There was no coupling
observed to the methylene carbon on the glycine moiety, a trend which would be
repeated consistently in later syntheses.

The proton nmr spectrum provided further confirmation that the target
compound had been successfully synthesised, with the signals due to the methoxy
protons, the methylene protons on the glycine moiety and the NH proton all
integrating correctly by comparison to the signals derived from the protons in the
ethoxy chain.

In the electron impact mass spectrum a molecular ion was seen and the measured accurate mass found to be within acceptable limits of the calculated mass of the target compound. Fragments corresponding to the loss of the ethoxy chain, the loss of the CH₃OC(O) moiety and the loss of the entire amino acid moiety were observed, the latter two fragments showing a chlorine isotope effect. The bulk purity of the compound was confirmed by chlorine analysis.

Experiment 7 reports the application of this procedure to the amino acid alanine. The method used was analogous to that used for the glycine compound: triethylamine was added slowly to a stirred mixture of the amino acid methyl ester hydrochloride salt and ethyl phosphorodichloridate in dichloromethane at low temperature (Figure 64). Removal of the solvent and extraction afforded the product - ethyl methoxy-L-alaninyl phosphorochloridate - in good yield.

![Figure 64. Reaction scheme for the synthesis of ethyl methoxy-L-alaninyl phosphorochloridate (experiment 7).](image)

The product appeared to be pure on observing the phosphorus nmr spectrum, there being one signal at 10.61 ppm. That there was only one signal present was unexpected, in view of the fact that the product was isolated as a mixture of two diastereoisomers arising from the linking of chiral centres in the amino acid and at the phosphorus (Figure 65).

![Figure 65. Ethyl methoxy-L-alaninyl phosphorochloridate with chiral centres indicated.](image)

However, in the carbon nmr spectrum all the signals except two (the methoxy carbon
and the methyl carbon in the ethoxy chain) showed a different shift for each
diastereoisomer, i.e. there were two resonances for each carbon. In addition,
phosphorus coupling complicated the spectrum still further, splitting the
diastereoisomeric signals of the carbonyl carbon, the methylene carbon in the ethoxy
chain and the alaninyl methyl side chain carbon. There was no two-bond phosphorus-
carbon coupling to the chiral amino acid carbon.

The proton nmr spectrum was similarly complicated by the presence of two
diastereoisomers and phosphorus-proton coupling. The alaninyl NH gave a broad, flat
signal as expected for an exchangeable proton; all the other moieties were clearly
discernible from the spectrum.

In the electron impact mass spectrum a protonated molecular ion was seen and
an accurate mass measurement obtained which corresponded well with the calculated
mass of the protonated target molecule. The expected 3:1 chlorine isotope effect was
observed for the protonated molecular ion and also for fragments corresponding to the
loss of the CH₃OC(O) moiety and the loss of the CH₃OC(O)CH₂CH₃ moiety. Other
fragmentations such as loss of chlorine, loss of the ethoxy chain (as ethanol), loss of
methanol and ethene and combinations of the above were also observed. The bulk
purity of the compound was confirmed by chlorine analysis.

In experiment 8 the same procedure was applied to the amino acid valine
(Figure 66).

![Figure 66. Reaction scheme for the synthesis of ethyl methoxy-L-valinyl phosphorochloridate (experiment 8).](image)

The target compound was obtained in good yield. Two signals corresponding to the
two diastereoisomeric products were observed in the phosphorus nmr spectrum in a
ratio of 4:3. Diastereoisomeric effects were also prominent in the carbon nmr spectrum,
with the 4:3 ratio shown in the phosphorus nmr being reproduced for most signals,
although only one of the isopropyl methyl groups on the valine moiety showed
different carbon nmr shifts for each diastereoisomer. Phosphorus coupling was
observed to the carbonyl carbon, the isopropyl carbon on the valine moiety and in the
ethoxy chain for both carbons. There was no two-bond phosphorus-carbon coupling
to the asymmetric carbon on the valine moiety.

In the proton nmr spectrum the NH proton was not seen but all the other
signals were appropriately assigned. The two isopropyl methyl groups in particular
gave two distinct doublets at high field (ca. 1 ppm).

The electron impact mass spectrum showed a fragmentation pattern diagnostic
of the amino acid valine. A protonated molecular ion was observed and a good
accurate mass reading obtained. The usual fragments corresponding to loss of chlorine,
loss of the CH\textsubscript{3}OC(O) moiety and loss of methanol were seen, but also prominent were
the loss of propane and propene fragments from the molecular ion, arising from the
breakdown of the valine isopropyl moiety. 3:1 Chlorine isotope effects were observed
in the fragmentation pattern.

Microanalysis showed that the product contained a percentage of chlorine
within the acceptable limit of that calculated for the target compound.

Having developed a good route to the amino acid-based phosphorochloridates,
the next section of work to be described deals with the application of this method to
the synthesis of several phosphoramidate derivatives of AZT and the results of the
biological evaluation of these compounds against HIV \textit{in vitro}.

As noted earlier, a range of compounds had been synthesised previously in
which both the amino acid and the alkoxy chain had been varied\textsuperscript{234}. In particular, it
was found that minor modifications in the amino acid side chain led to marked
changes in anti-HIV activity. To probe further the sensitivity of the amino acid side
chain to structural modification, it was decided to undertake a study of several
compounds in which the only variation was the increase in methylene spacer length
between the phosphoramidate moiety and the carboxyl group. Thus the five target
compounds were as shown in Figure 67.
Figure 67. (a) AZT (ethyl methoxyglycyl) phosphoramidate, (b) AZT (ethyl methoxy-β-alaninyl) phosphoramidate, (c) AZT (ethyl methoxy-4-aminobutyryl) phosphoramidate, (d) AZT (ethyl methoxy-5-aminovaleryl) phosphoramidate, (e) AZT (ethyl methoxy-6-aminocaproyl) phosphoramidate.
Before the synthesis of these target molecules could be attempted, it was first necessary to prepare the parent nucleoside, AZT. This was made by the method of Glinski et al.\textsuperscript{250} from 5'-O-trityl-2,3'-anhydrothymidine (experiment 9) (Figure 68).

![Figure 68. Preparation of AZT in two steps from 5'-O-trityl-2,3'-anhydrothymidine.](image)

AZT was obtained in 24% yield (which compares to a literature yield of 30%) with a melting point of 122 °C. The literature melting point of AZT is 113-115 °C, although Horwitz et al. report a melting point of 119-121 °C after thorough drying of the nucleoside\textsuperscript{251}.

The first target compound of the series, AZT (ethyl methoxyglycinyl) phosphoramidate (Figure 67(a)) was initially synthesised by Devine from AZT and the phosphorylating reagent prepared by the author in experiment 3\textsuperscript{34}. It was noted earlier that this latter material contained several minor impurities and this gave problems in the subsequent purification of the phosphoramidate compound\textsuperscript{238}. Preparative hplc was used in order to obtain material of sufficient purity for biological evaluation, but the compound was not fully characterised and it was therefore decided to repeat the synthesis using the ethyl methoxyglycinyl phosphorochloridate obtained in experiment 6, which was of a much higher standard of purity than the product of experiment 3.
Thus experiment 10 reports the synthesis of AZT (ethyl methoxyglycyl) phosphoramidate. An excess of ethyl methoxyglycyl phosphorochloridate was reacted with AZT in THF containing N-methyl imidazole (Figure 69).

![Reaction scheme for the synthesis of AZT (ethyl methoxyglycyl) phosphoramidate (experiment 10).](image)

The excess of phosphorylating reagent used in all the nucleoside condensation reactions was found to be necessary to drive the reactions to completion. When the reactions had finished, the solvent was removed and the reaction products dissolved in chloroform and subjected to several aqueous washes, the aim being to hydrolyse any remaining phosphorylating reagent and then to discard this unwanted material in the aqueous layer. However, hydrolysed phosphorus-containing material consistently co-eluted with the desired products during chromatography and the derivatives could frequently only be isolated after two successive silica gel column chromatographic purifications, using first a methanol/chloroform eluent and then a methanol/ethyl acetate eluent. This was indeed to prove necessary for the glycine analogue currently under consideration.

AZT (ethyl methoxyglycyl) phosphoramidate was eventually isolated in 70% yield and fully characterised.
The phosphorus nmr spectrum showed one signal at 6.71 ppm, the chemical shifts of the two diastereoisomeric forms of the product being apparently coincident. This was in the region appropriate for such species: the compound \( \text{OP(OCH}_2\text{CH}_3)_2\text{N(CH}_2\text{CH}_3)_2 \) has a reported chemical shift of 9 ppm\(^{252}\). In the carbon nmr spectrum there was clear evidence of the presence of two diastereoisomers in a ratio of 1:1, many signals on the base, sugar, alkoxy and amino acid moieties appearing as two peaks. Splitting of these signals by phosphorus coupling was noted for some of the resonances within three bonds of the phosphorus, for example, the carbonyl carbon on the amino acid, the \( \text{C}4' \) and \( \text{C}5' \) carbons on the nucleoside and the methylene carbon in the ethoxy chain.

Further confirmation of a 1:1 diastereoisomeric ratio was found in the proton nmr spectrum. The signal due to \( \text{H}6 \) on the nucleoside base appeared as two singlets in a ratio of 1:1 and the signal due to \( \text{H}1' \) on the nucleoside sugar appeared as two triplets in a similar 1:1 pattern. The resonances for the \( \text{H}5' \) protons and the ethoxy methylene protons were observed as complex multiplets arising from the presence of two diastereoisomers and phosphorus-proton coupling.

The electron impact mass spectrum provided further evidence for the correct identification of the product as the desired target compound. A molecular ion was observed and the measured mass found to be within the limits acceptable for the calculated mass of the target compound. Fragments corresponding to the loss of the thymine moiety and loss of the entire phosphoramidate moiety from the molecular ion were observed and the subsequent fragmentation of the latter species could also be detected, with the loss of the ethoxy moiety and the loss of the amino acid moiety. Fragments corresponding to the nucleoside base and sugar were also noted.

In the fast atom bombardment mass spectrum a protonated molecular ion and a fragmentation pattern similar to that observed for the electron impact mass spectrum were observed.

The purity of the compound was confirmed by microanalysis (acceptable results for C, H and P) and hplc, with the two diastereoisomers showing slightly different retention times. The levels of AZT were below detectable limits, which is important given the high activity of AZT in the biological assay used to test anti-HIV-1 potency.

Full details of the biological evaluation procedure are contained in the Appendix. Briefly, T-lymphoblastoid cells were incubated with HIV-1 for 90 minutes.
at 37 °C. The cells were then washed three times with saline solution and cell aliquots resuspended in growth medium with the compounds under study at two concentrations. The cells were incubated at 37 °C for 72 hours and then 200 µl of supernatant taken from each culture and assayed for HIV antigen. Supernatants taken from uninfected cells, untreated infected cells and infected cells treated with AZT and ddCyd were used as controls. Compounds were tested in duplicate at each concentration and each compound was tested on at least two separate occasions. AZT has an activity of 0.005 µM in this test. Toxicity tests were run in parallel to check that any activity observed was antiviral and not a toxic effect.

AZT (ethyl methoxyglycyl) phosphoramidate was tested as a crude mixture of its diastereoisomers and showed an antiviral activity of 10 µM (the concentration required to reduce HIV-1 antigen production by 50%). The compound was non-toxic at the concentrations tested (up to 100 µM). The results of this biological evaluation will be discussed in more detail later when the descriptions of the syntheses of the other compounds in the series have been completed.

The second target molecule - AZT (ethyl methoxy-β-alaninyl) phosphoramidate - was prepared in a manner analogous to the glycine derivative.

The required phosphorylating reagent, ethyl methoxy-β-alaninyl phosphorochloridate, was synthesised by the usual method in experiment 11 (Figure 70).

\[
\begin{align*}
\text{CH}_3\text{O} - \text{C} - \text{CH}_2 - \text{NH}_2\text{Cl} & \quad + \quad \text{EtOP(O)Cl}_2 & \quad + \quad 2 \text{Et}_3\text{N} \\
\text{CH}_3\text{O} - \text{C} - \text{CH}_2 - \text{NH} - P - Cl & \quad + \quad 2 \text{Et}_3\text{NHCl}
\end{align*}
\]

Figure 70. Reaction scheme for the synthesis of ethyl methoxy-β-alaninyl phosphorochloridate (experiment 11).

The phosphorus nmr spectrum of the crude material showed one major signal at 12.78 ppm. There was also a signal, smaller in intensity, which appeared to indicate the presence of unreacted ethyl phosphorodichloridate. This was removed by adding a small quantity of dry hexane (in which the alkyl amino acyl phosphorochloridates are insoluble), and shaking. The hexane containing the dissolved ethyl phosphorodichloridate was then decanted off and the resultant product found to be
pure by phosphorus nmr. However, the yield of the desired compound was considerably lower than the usual 80-90% after this additional procedure.

In the carbon nmr spectrum a three-bond phosphorus-carbon coupling to the methylene carbon adjacent to the carbonyl moiety (the α carbon) was observed, but there was no two-bond coupling to the β carbon (i.e. the carbon adjacent to the phosphoramidate moiety). This is consistent with the observations mentioned earlier that three-bond $P-N-C-C$ couplings and never two-bond $P-N-C$ couplings are seen for $P-N$ linked amino acid derived phosphorochloridates and nucleoside phosphoramidates.

In the proton nmr spectrum fine splitting due to phosphorus-proton coupling was observed for the resonances of the methylene protons in the ethoxy chain. Both the α protons and the β protons on the amino acid moiety appeared as multiplets, the latter group producing the more downfield of the two signals.

Satisfactory mass spectral and microanalytical data were not obtained for this compound.

The reaction of ethyl methoxy-β-alaninyl phosphorochloridate with AZT is reported as experiment 12. An excess of the phosphorylating reagent was reacted with the nucleoside in THF in the presence of N-methyl imidazole (Figure 71).

![Figure 71. Reaction scheme for the synthesis of AZT (ethyl methoxy-β-alaninyl) phosphoramidate (experiment 12).](image-url)
Two successive chromatographic purifications afforded the product in reasonable yield. The compound was fully characterised spectroscopically.

The phosphorus nmr spectrum showed one signal at 7.48 ppm, the chemical shifts of the two diastereoisomers being coincident as for the glycine analogue. In the carbon nmr spectrum there was evidence of a diastereoisomeric ratio of 1:1 and phosphorus-carbon coupling was observed to the α carbon on the amino acid, to both carbons in the ethoxy chain and to C5' on the nucleoside sugar, but not, unusually, to C4'. There was no phosphorus-carbon coupling observed to the carbonyl carbon, this atom now being too distant from the phosphorus for signal splitting to occur.

The proton nmr spectrum showed the presence of the two diastereoisomeric forms of the product in a ratio of 1:1; for example, two singlet resonances were observed for the methoxy protons, separated by 0.01 ppm. The signals due to the methylene protons in the ethoxy chain, the two H5' protons and the H4' proton all overlapped to give a complicated multiplet. Two separate resonances were observed for each proton at the 2' position.

The electron impact mass spectrum showed a pattern of fragmentation similar in nature to that observed for the glycine analogue. A molecular ion was seen and an accurate mass reading obtained which confirmed the identity of the product as the target compound. Loss of the thymine and azide moieties were evident and the loss of the entire phosphoramidate moiety and its subsequent fragmentation was clearly discernible, with loss of water, loss of methanol and loss of ethene all noted.

Microanalysis (acceptable data obtained for C, H and P) and analytical hplc confirmed the purity of the compound.

AZT (ethyl methoxy-β-alaninyl) phosphoramidate was tested as this diastereoisomeric mixture and showed an antiviral activity of 20 μM against HIV-1 in vitro (see later for full discussion).

The third compound in this series to be prepared was AZT (ethyl methoxy-4-aminobutyryl) phosphoramidate. The precursor molecule ethyl methoxy-4-aminobutyryl phosphorochloridate was synthesised by the usual method as described in experiment 13. The reaction scheme is indicated in Figure 72.

The product of the reaction was isolated in good yield and in pure form as evidenced by phosphorus nmr (one peak) and chlorine analysis. In the carbon nmr spectrum, three-bond phosphorus-carbon coupling to the β carbon resonance on the
amino acid was observed, with no coupling to the γ carbon closest to the phosphoramidate bond. The β carbon, being relatively shielded by the adjacent α and γ carbons, resonated upfield of both of the latter. The signals due to the carbons in the ethoxy chain both showed phosphorus-carbon coupling as usual.

\[
\begin{align*}
\text{CH}_3\text{O} & \text{C} \quad \text{CH}_2\text{C} \quad \text{CH}_2\text{NH_3} \quad + \quad \text{EtOP(O)Cl}_2 \quad + \quad 2 \text{Et}_3\text{N} \\
\rightarrow & \quad \text{dichloromethane} \\
\text{CH}_3\text{O} & \text{C} \quad \text{CH}_2\text{C} \quad \text{CH}_2\text{NH-P-Cl} \quad + \quad 2 \text{Et}_3\text{NHCl}
\end{align*}
\]

*Figure 72. Reaction scheme for the synthesis of ethyl methoxy-4-aminobutyryl phosphorochloridate (experiment 13).*

The proton nmr spectrum provided further confirmatory evidence for the proposed structure of this compound. All the protons in the molecule gave assignable resonances; the signal from the NH clearly integrated for one proton as expected, even though it was observed as a very broad (almost flat) singlet. The methylene protons in the ethoxy chain, the γ protons (closest to the P-N bond) and the β protons all gave multiplet signals. Both the α protons (closest to the carboxyl moiety) and the methyl protons in the ethoxy chain appeared as triplets. The methoxy protons as usual gave a sharp singlet resonance at ca. 3.6-3.7 ppm.

In the electron impact mass spectrum a protonated molecular ion was observed which showed a 3:1 chlorine isotope effect. The molecular ion was also seen and an accurate mass reading obtained in good agreement with the calculated accurate mass of the desired compound. Fragments corresponding to loss of methanol, ethanol and chlorine from the protonated molecular ion were observed and a pattern showing the fragmentation of various sections of the amino acid moiety from the protonated molecular ion was also clearly discernible (i.e. loss of CH$_3$OC(O)CH$_3$, loss of CH$_3$OC(O)CH$_2$CH$_3$ and loss of CH$_3$OC(O)CH$_2$CH$_2$CH$_3$).

The reaction of this phosphorylating reagent with AZT is reported as experiment 14. The usual THF/N-methyl imidazole conditions were employed; the reaction scheme is indicated in Figure 73.

The product was isolated in reasonable yield after the usual chromatographic purification procedures and spectroscopic data collected which appeared to be in agreement with the proposed structure.
The phosphorus nmr spectrum showed one signal at 7.75 ppm (coincident diastereoisomers). The carbon nmr spectrum suggested a 1:1 diastereoisomeric ratio, the only signals not illustrating this being C6 (on the nucleoside base), the methoxy carbon, the α carbon (adjacent to the carbonyl carbon on the amino acid) and the 5-CH3 carbon. Phosphorus couplings to C4' and C5', to the methylene and methyl carbons in the ethoxy chain and to the β carbon were observed.

The signals in the proton nmr spectrum corresponding to the H4' and H5' protons on the nucleoside and the methylene protons in the ethoxy chain overlapped to give a complex multiplet, as in the β-alanine analogue. As compared to the phosphorochloridate precursor, the chemical shift of the α protons (adjacent to the carbonyl moiety on the amino acid) had not altered, the β protons had moved very slightly upfield by ca. 0.06 ppm, while the γ protons (adjacent to the phosphoramidate bond) had moved more discernibly upfield by ca. 0.1 ppm.

In the electron impact mass spectrum a molecular ion was observed and the accurately measured mass found to be close in value to that calculated for the target compound. Loss of the phosphoramidate moiety from the molecular ion and its
subsequent fragmentation was the most prominent feature of the spectrum, but fragments corresponding to the nucleoside base and sugar were also observed. As in the precursor phosphorochloridate, a fragmentation pattern corresponding to various sections of the amino acid moiety was seen.

Microanalysis (C, H, N and P) and hplc confirmed the bulk purity of the compound. Both diastereoisomers had the same hplc retention time.

Biological evaluation of AZT (ethyl methoxy-4-aminobutyryl) phosphoramidate as a mixture of its diastereoisomers revealed an anti-HIV activity of 100 μM.

The fourth congener to be investigated was AZT (ethyl methoxy-5-aminovaleryl) phosphoramidate. The relevant phosphorochloridate, ethyl methoxy-5-aminovaleryl phosphorochloridate, was prepared by the method as described in experiment 15, with the reaction scheme as indicated in Figure 74.

![Figure 74. Reaction scheme for the synthesis of ethyl methoxy-5-aminovaleryl phosphorochloridate (experiment 15).](image)

The product was isolated in very good yield and gave one signal in its phosphorus nmr spectrum in the expected region. In the carbon nmr spectrum the carbonyl carbon resonance was as usual at ca. 170 ppm. The only carbon signal to display phosphorus-carbon coupling besides the carbons in the ethoxy chain was the γ carbon, which showed a three-bond coupling of 7.5 Hz. This is comparable in magnitude to the value of 8.0 Hz observed for the three-bond phosphorus-carbon coupling to the methyl carbon on the ethoxy chain. The β carbon resonated upfield at 21.70 ppm, while the δ carbon (adjacent to the phosphoramidate moiety) gave the most downfield signal of those in the amino acid chain at 41.28 ppm.

The β and γ protons overlapped in the proton nmr spectrum to give an upfield multiplet, although the most upfield signal was as usual derived from the methyl protons in the ethoxy chain, which resonated as a triplet (the signal splitting arising from three-bond coupling to the adjacent methylene protons). All the other moieties (including the NH) gave discernible and assignable signals.
A molecular ion and a protonated molecular ion were both observed in the electron impact mass spectrum and the accurate mass measurement obtained for the latter found to be in excellent agreement with that calculated for the proposed structure. Loss of methanol, HCl, ethanol and ethene from the protonated molecular ion were all observed and combinations thereof. Amino acid fragmentation was also in evidence from the electron impact mass spectrum, with the loss of several fragments differing by 14 mass units (corresponding to a methylene moiety). Chlorine isotope effects were also commonly observed. Chlorine analysis confirmed the bulk purity of this phosphorylating reagent.

The reaction of ethyl methoxy-5-aminovaleryl phosphorochloridate with AZT is reported as experiment 16 and the reaction scheme adopted is illustrated in Figure 75.

The target compound was isolated in good yield and showed one signal in its phosphorus nmr spectrum at 7.84 ppm. The presence of diastereoisomers in a 1:1 ratio was confirmed by both the carbon and the proton nmr spectra. Carbon resonances on the sugar (C1', C4', C5'), the base (C4, C5, 5-CH3), the amino acid (carbonyl, γ CH2, ...
δ CH₂) and ethoxy chain (methylene and methyl carbons) all consisted of two signals, one for each isomer. The carbon nmr spectrum was assigned by reference to two literature spectra. The four methylene moieties on the amino acid had chemical shifts of 40.89, 33.28, 31.02 and 21.72 ppm. The signal at 31.02 ppm was split into a doublet by phosphorus coupling, and so, based on the three-bond P-N-C-C coupling observed in earlier analogues, this signal was assigned to the γ methylene carbon. O,O-diethyl N-ethylphosphoramidate (Figure 76) has a reported chemical shift of 36.2 ppm for carbon X, and so by analogy the signal at 40.89 ppm in the spectrum of the product of experiment 16 was assigned to the δ methylene moiety.

![Figure 76. O,O-diethyl N-ethylphosphoramidate.](image)

This left methylene carbons α and β unassigned. The methyl ester of hexanoic acid (Figure 77) is reported to give signals at 34.1 and 24.8 ppm for carbons Y and Z respectively.

![Figure 77. Methyl hexanoate.](image)

The two unassigned signals on the 5-aminovaleryl moiety of the AZT phosphoramidate compound had chemical shifts of 33.28 and 21.72 ppm; these were therefore assigned as methylene carbons α and β respectively. The δ carbon (adjacent to the phosphoramidate bond) had moved upfield by ca. 0.4 ppm while the γ carbon had moved more prominently upfield by ca. 1 ppm, as compared to the phosphorochloridate precursor. The α and β carbons gave signals which were virtually identical in terms of their chemical shifts to those observed for the phosphorylating reagent. Phosphorus-carbon coupling was observed to C4' (three-bond coupling) and to the methylene carbon in the ethoxy chain and C5' (two-bond coupling).

In the proton nmr spectrum the presence of two diastereoisomers was confirmed by the signals from H6 (which appeared as two singlets) and H1' (which appeared as two triplets). The δ methylene protons (adjacent to the P-N bond) were
assigned to the resonance at 2.85 ppm by analogy with the 4-aminobutyryl phosphoramidate analogue, in which the $\gamma$ methylene protons (adjacent to the P-N bond) resonated slightly more downfield at 2.94 ppm. The signal from the $\alpha$ methylene protons overlapped with the signal from one of the protons at the 2' position on the nucleoside sugar at 2.26 ppm (as compared to 2.35 ppm in the 4-aminobutyryl analogue). The proton nmr signals from the two central methylene groups on the amino acid were unresolved at 200 MHz. At 400 MHz they were resolved into two multiplets at 1.61 and 1.48 ppm. Initially it was impossible to decide which of these resonances was due to the $\beta$ methylene protons and which was due to the $\gamma$ methylene protons. Homonuclear decoupling experiments were used to confirm the proposed assignments. Irradiation at ca. 2.26 ppm ($\alpha$ methylene protons) affected the resonance at 1.61 ppm, confirming the latter as being due to the $\beta$ methylene protons. Irradiation at ca. 2.85 ppm ($\delta$ methylene protons) affected the resonance at 1.48 ppm, confirming the latter as being due to the $\gamma$ methylene protons. This second irradiation also caused the signal from the amino acid NH to collapse to a simple triplet.

In the electron impact mass spectrum a molecular ion was observed and the accurate mass measurement obtained found to be in good agreement with the calculated value for the target compound. A fragment corresponding to the detached phosphoramidate moiety was observed, with the fragmentation of this species by the loss of small molecules such as methanol and ethene being evident. Loss of sections of the amino acid moiety from the phosphoramidate fragment (again the diagnostic difference of 14 between fragments corresponding to a methylene group) and fragments corresponding to the nucleoside base, sugar and azide group helped to confirm the structure as that of the target molecule.

The bulk purity of the compound was confirmed by hplc, although microanalysis gave satisfactory values for H and P only. Hplc also showed the presence of two diastereoisomers.

Biological evaluation of AZT (ethyl methoxy-5-aminovaleryl) phosphoramidate as a mixture of its diastereoisomers gave an anti-HIV-1 activity of 100 $\mu$M.

The final analogue in this series to be synthesised was AZT (ethyl methoxy-6-aminocaproyl) phosphoramidate. Ethyl methoxy-6-aminocaproyl phosphorochloridate was prepared by the method shown in Figure 78 (see experiment 17).
Figure 78. Reaction scheme for the synthesis of ethyl methoxy-6-aminocaproyl phosphorochloridate (experiment 17).

The product of this reaction was isolated in near-quantitative yield. One signal was observed in the phosphorus nmr spectrum. In the carbon nmr spectrum, three-bond phosphorus-carbon couplings were observed to the δ carbon (7.4 Hz) and to the methyl carbon in the ethoxy chain (8.0 Hz). A two-bond phosphorus-carbon coupling was as usual observed to the methylene carbon in the ethoxy chain. The two signals at 25.79 and 24.20 ppm were assigned to the β and γ carbons, but which resonance was derived from which moiety was impossible to discern.

In the proton nmr spectrum the signal due to the NH was the most downfield and appeared as a broad singlet. The methylene protons on the ethoxy moiety appeared as a multiplet, as did the ε methylene protons. The α methylene protons appeared as a triplet (coupling to the two β protons), while the δ and β methylene protons overlapped to give a multiplet resonance at 1.30 ppm. The γ methylene protons (the most shielded in the amino acid moiety) and the methyl protons in the ethoxy chain gave an upfield multiplet at 1.10 ppm.

The electron impact mass spectrum confirmed the identity of the product as the target phosphorochloridate, with an accurate mass reading for the protonated molecular ion in good agreement with that calculated for the proposed structure. Extensive fragmentation from the protonated molecular ion was observed: loss of the methoxy radical; loss of the ethoxy radical; loss of methanol and loss of ethene were all noted, together with combinations of the above and 3:1 chlorine isotope effects. A pattern arising from fragmentation of sections of the amino acid moiety was also observed, in common with the 4-aminobutyryl and 5-aminovaleryl analogues.

The reaction of this phosphorylating reagent with AZT is reported as experiment 18. The THF/N-methyl imidazole conditions were utilised as usual, as shown in Figure 79.

The product was isolated in a yield slightly lower than that normally obtained for these nucleoside phosphoramidates; however, the spectroscopic and analytical data confirmed the identification and bulk purity of the compound.
Figure 79. Reaction scheme for the synthesis of AZT (ethyl methoxy-6-aminocaproyl) phosphoramidate.

There was one resonance in the phosphorus nmr spectrum at 7.83 ppm. In the carbon nmr spectrum a 3:2 ratio of diastereoisomers was apparent and this was later confirmed in the proton nmr. Three-bond phosphorus-carbon couplings were observed to C4', to the δ methylene carbon on the amino acid and to the methyl carbon in the ethoxy chain. Two-bond coupling was observed to the methylene carbon in the ethoxy chain and to C5', but, as usual, there was no two-bond coupling to the carbon adjacent to the phosphoramidate moiety (the ε carbon). As in the precursor phosphorochloridate, two signals were assigned to the β and γ methylene carbons but which resonance was derived from which of these moieties could not be decided.

In the proton nmr spectrum the signals due to the NH on the nucleoside base, H6 and H1' all suggested a diastereoisomeric ratio of 3:2. There was a complex multiplet at ca. 4.1 ppm arising from the overlapping resonances of the methylene protons in the ethoxy chain and the H4' and H5' nucleoside sugar protons. The ε protons were assigned to the signal at 2.91 ppm and the signal from the α protons once again overlapped with one of the H2' resonances at 2.32 ppm. The γ methylene protons, being the most shielded of those on the amino acid moiety, were assigned to the upfield signal at 1.35 ppm. The remaining methylene moieties on the amino acid
were assigned by comparison to the 5-aminovaleryl analogue: the \( \beta \) methylene protons were assigned to the signal at 1.63 ppm (as compared to 1.61 ppm in the previous analogue); the \( \delta \) methylene protons were assigned to the signal at 1.53 ppm (as compared to 1.48 ppm for the methylene moiety in the equivalent position in the 5-aminovaleryl phosphoramidate compound).

The electron impact mass spectrum provided further evidence for the identification of the product as the target compound. A molecular ion was observed and the measured accurate mass found to be sufficiently close in value to the calculated mass of the target compound. Fragments corresponding to loss of thymine, loss of the azide moiety and loss of the phosphoramidate moiety from the molecular ion were all observed, and the subsequent fragmentation of the latter species by the loss of small molecules such as methanol, ethanol and ethene was also evident.

The bulk purity of the compound was confirmed by microanalysis and hplc. In contrast to the spectroscopic data, hplc seemed to suggest a diastereoisomeric ratio of 1:1.

Biological evaluation of AZT (ethyl methoxy-6-aminocaproyl) phosphoramidate as a mixture of its diastereoisomers gave an anti-HIV-1 activity of 100 \( \mu \)M.

Thus, AZT (ethyl methoxyglycyciny1) phosphoramidate was the most active compound of the series, causing 50% inhibition of viral replication at a concentration of 10 \( \mu \)M. AZT (ethyl methoxy-\( \beta \)-alaninyl) phosphoramidate was less active at 20 \( \mu \)M. The three compounds AZT (ethyl methoxy-4-aminobutyryl) phosphoramidate, AZT (ethyl methoxy-5-aminovaleryl) phosphoramidate and AZT (ethyl methoxy-6-aminocaproyl) phosphoramidate were equi-active only at the highest concentration studied (100 \( \mu \)M). It is clear from these results that the anti-HIV activity of the phosphoramidate derivatives declines with increasing separation of the phosphoramidate and carboxyl ester moieties. This is consistent with a mechanism of action involving intracellular cleavage of the P-N bond. Juodka and Smrt have shown that a phosphoramidate bond is labilised by an amino acid carboxyl moiety in a dialkyl alkoxyglycyciny1 phosphoramidate derivative\(^{25a}\). Increased separation of the carboxyl ester from the phosphoramidate bond would therefore be expected to stabilise the latter and thus reduce activity. Cleavage of the P-N bond is a crucial step in the proposed activation of these compounds. Such cleavage would release AZT ethyl phosphate, which might either act as such or undergo further hydrolysis to AZT.
or AZT monophosphate. Both of the latter species would give rise to AZT triphosphate if released in an intracellular environment, although the phosphate prodrug strategy depends on the release of the nucleotide rather than the nucleoside.

The proximity of the carboxyl group to the phosphoramidate bond therefore appears to be important to the activity of these compounds, activity decreasing rapidly when there are more than two methylene groups separating these moieties. However, given the poor activity of the latter three members of the series, it was considered possible that the presence of the distant ester group was no longer making any contribution to activity. To test this hypothesis, it was decided to synthesise an unsubstituted compound, AZT (ethyl n-propylamino) phosphoramidate (Figure 80).

![Figure 80. AZT (ethyl n-propylamino) phosphoramidate.](image)

To this end, ethyl n-propylamino phosphorochloridate was synthesised (experiment 19). Ethyl phosphorodichloridate was reacted with two equivalents of n-propylamine in diethyl ether at low temperature, Figure 81.

![Figure 81. Reaction scheme for the synthesis of ethyl n-propylamino phosphorochloridate.](image)

After work-up an oil was isolated in very good yield. The phosphorus nmr spectrum of this material contained one peak at 14.07 ppm, which was in the expected region
for the target molecule. Carbon and proton nmr confirmed the structure of the product as that of the desired phosphorochloridate. There was no phosphorus-carbon coupling observed to the carbon adjacent to the phosphoramidate bond, which had a chemical shift of 43.69 ppm (similar in value to the shifts seen for the carbons in analogous positions in the 4-aminobutyryl, 5-aminovaleryl and 6-aminocaproyl phosphorochloridate congeners considered earlier). However, three-bond phosphorus-carbon couplings of 7.8 Hz and 8.0 Hz were observed to the middle carbon in the n-propyl chain and the methyl carbon in the ethoxy chain respectively. A two-bond phosphorus-carbon coupling was observed to the methylene carbon in the ethoxy chain, which had a chemical shift of ca. 64 ppm as usual.

The proton nmr spectrum showed all the resonances for the appropriate moieties as expected: for example, the NH proton gave a broad singlet at 4.9 ppm and the methyl protons in the ethoxy chain gave a triplet of doublets, the fine splitting arising from four-bond phosphorus-proton coupling (0.9 Hz in magnitude).

In the electron impact mass spectrum a molecular ion was observed and an accurate mass reading obtained which was in good agreement with the calculated mass of the target phosphorochloridate. Fragmentation by loss of ethene, ethane, HCl and ethanol occurred and chlorine isotope effects were noted. The bulk purity of the compound was confirmed by chlorine analysis.

The synthesis of AZT (ethyl n-propylamino) phosphoramidate had been previously carried out by Devine\textsuperscript{2,3,8}, although his material was incompletely characterised and did not give consistent results when evaluated against HIV-1 \textit{in vitro} (possibly due to AZT contamination). It therefore proved necessary to prepare this compound again and this was accomplished by the author (reported as experiment 20). The reaction conditions adopted are illustrated in Figure 82.

The compound was isolated in substantially better yield than obtained by Devine (87% as compared to 68%) and was fully characterised spectroscopically and analytically. There was one signal in the phosphorus nmr spectrum at 9.77 ppm, which is a value slightly higher than that observed previously for the amino acid-based derivatives, showing that the environment at the phosphorus was different in this compound. The carbon and proton nmr spectra showed a 1:1 ratio of diastereoisomers, the data being similar to that obtained by Devine (see Appendix).
Figure 82. Reaction scheme for the synthesis of AZT (ethyl n-propylamino) phosphoramidate (experiment 20).

In the electron impact mass spectrum a molecular ion was observed with an accurately measured mass which was found to be sufficiently close in value to the calculated mass for AZT (ethyl n-propylamino) phosphoramidate. Loss of thymine and the azide moiety from the molecular ion confirmed the AZT part of the structure, and fragments corresponding to the phosphoramidate moiety and the loss of small molecules (methane, water, ethene, ethane and ethanol) therefrom were observed. Loss of the entire amine moiety from the phosphoramidate fragment was also evident, as were sugar fragments from the nucleoside.

The purity of the compound was confirmed by hplc (levels of AZT below detectable limits) and microanalysis.

AZT (ethyl n-propylamino) phosphoramidate, evaluated against HIV-1 as a mixture of its diastereoisomers, showed an activity of 50 µM.

This compound therefore displayed anti-HIV activity intermediate in magnitude between that of AZT (ethyl methoxy-β-alaninyl) phosphoramidate and the later members of the series (the 4-aminobutyryl, 5-aminovaleryl and 6-aminocaproyl...
analogues). That AZT (ethyl n-propylamino) phosphoramidate had an activity greater than that of the later compounds suggested that the remote ester group was no longer contributing to their activity. It was considered possible, however, that the different environment at the phosphorus meant that AZT (ethyl n-propylamino) phosphoramidate acts by a different mechanism to the amino acid-containing compounds, and therefore any role which the ester moiety plays in conferring anti-HIV activity on the latter cannot be ruled out. Furthermore, although a simple alkoxy group (as in the nucleoside dialkyl phosphates as synthesised by McGuigan et al. and discussed in the Introduction226) does not confer anti-HIV activity, a simple alkylamino group attached to the phosphorus does. The reasons for this are unclear but may relate to the greater susceptibility of the P-N bond to chemical hydrolysis.

The synthesis and anti-HIV evaluation of the six phosphoramidate compounds so far discussed were reported in the journal Antiviral Research; a copy of this paper may be found in the Appendix.

The activity displayed by AZT (ethyl methoxy-β-alaninyl) phosphoramidate suggested that an alpha-amino acid moiety was not required for the anti-HIV effectiveness of the nucleoside phosphoramidates. In the Introduction it was suggested that one of the rationales for preparing these derivatives was the possibility of HIV-specific enzymic activation of the compounds (for example by HIV protease). β-Alanine is known to occur biologically in free or combined form but never in proteins257. The activity of the β-alanine analogue therefore suggested that HIV-specific enzymic cleavage of the amino acid from the phosphoramidate was unlikely to be the sole mode of activation of these prodrugs. To test this proposition further it was decided to synthesise a nucleoside phosphoramidate derivative bearing an amino acid with D stereochemistry. D-amino acids do not occur in proteins and protease enzymes are generally specific only for peptides having L-α-amino acids in their amide linkages258. For example, when D-amino acids are the C-terminal component of dipeptides, the enzyme α-chymotrypsin can hydrolyse the peptide bond only slowly, if at all259,260. The reason for this was suggested to be the substrate specificity of α-chymotrypsin: the active site can only accept amino acids with a specific configuration261.

In the original series of monophosphate amino acid-containing derivatives synthesised by McGuigan et al., structure-activity correlations had been made by
keeping the amino acid constant (as L-valine) and varying the alkoxy group. For consistency, it was therefore decided to attempt the synthesis of AZT (ethyl methoxy-D-valinyl) phosphoramidate. The activity of this compound could then be compared with its L-valine congener.

The required phosphorochloridate was synthesised in experiment 21 according to the reaction scheme shown in Figure 83.

\[
\begin{align*}
\text{CH}_3\text{O} &\quad \text{CH} &\quad \text{NH}_2\text{Cl} &+ &\text{EtOP(O)Cl}_2 &+ &2 \text{Et}_3\text{N} \\
& & & &\text{dichloromethane} &\rightarrow &-78^\circ \text{C} \\
\text{CH}_3\text{O} &\quad \text{C} &\quad \text{CH} &\quad \text{NH} &\quad \text{P} &\quad \text{Cl} &+ &2 \text{Et}_3\text{NHCl} \\
& & & & & &\text{OEt} &\text{OEt}
\end{align*}
\]

**Figure 83.** Reaction scheme for the synthesis of ethyl methoxy-D-valinyl phosphorochloridate (experiment 21).

Ethyl methoxy-D-valinyl phosphorochloridate was obtained in good yield and showed two signals in a ratio of 1:1 in its phosphorus nmr spectrum (due to the presence of two diastereoisomers). The chemical shifts of the two isomers were 11.82 and 12.41 ppm, which compare closely to the shifts of 11.82 and 12.29 ppm observed for ethyl methoxy-L-valinyl phosphorochloridate synthesised in experiment 8. The 1:1 ratio of diastereoisomers was reflected in the carbon nmr spectrum, the carbonyl carbon, for example, gave two doublet resonances (three-bond phosphorus-carbon coupling causing the splitting of each signal) at ca. 172 ppm. The asymmetric carbon resonated as two signals at ca. 59 ppm and the isopropyl carbon gave two doublet resonances at ca. 31 ppm (again, three-bond phosphorus-carbon coupling causing the signal splitting). The diagnostic (of valine) isopropyl methyl moieties gave two sets of two singlets at ca. 18 and ca. 17 ppm, both sets showing the 1:1 ratio of diastereoisomers.

In the proton nmr spectrum the NH proton was seen as a multiplet at 4.46 ppm and the methylene protons in the ethoxy chain also appeared as a multiplet with a three-bond proton-phosphorus coupling of 2.46 Hz. The proton on the chiral carbon gave a multiplet resonance at 3.75 ppm, close to the singlet signal from the methoxy protons at 3.70 ppm. The isopropyl proton appeared as a multiplet at 2.09 ppm and the methyl protons in the ethoxy chain resonated as a triplet of doublets, the three-
bond proton-proton coupling being 7.19 Hz, the four-bond phosphorus-proton coupling being 0.81 Hz. The isopropyl methyl moieties unsurprisingly gave the most upfield resonances, close to 0.90 ppm.

In the electron impact mass spectrum a protonated molecular ion was observed and its mass found to be acceptably close in value to the accurately calculated mass of the target molecule. Fragments corresponding to loss of HCl, ethane, propane, ethene and propene from the protonated molecular ion were all observed, many such fragments showing chlorine isotope effects.

The synthesis of AZT (ethyl methoxy-D-valinyl) phosphoramidate from its phosphorochloridate precursor is described in experiment 22 (see the reaction scheme illustrated in Figure 84).

![Reaction scheme for the synthesis of AZT (ethyl methoxy-D-valinyl) phosphoramidate (experiment 22).](image)

The usual THF/N-methyl imidazole conditions were adopted and the product was isolated in reasonable yield. The phosphorus nmr chemical shifts of the two diastereoisomers were 6.93 and 6.66 ppm, which compare closely to the shifts observed for the L-valine congener: 6.87 and 6.73 ppm. The intensities of the resonances showed a 3:2 ratio.
In the carbon nmr spectrum, three-bond phosphorus-carbon coupling split both diastereoisomeric signals arising from the carbonyl carbon, the magnitude of the coupling being 2.9 Hz (as compared to ca. 3.6 Hz in the phosphorochloridate). Both the resonances at C4' and C5' showed phosphorus-carbon coupling, as did both carbons in the ethoxy chain. The carbon nmr chemical shifts displayed were virtually identical to the shifts observed previously by McGuigan et al. for AZT (ethyl methoxy-L-valinyl) phosphoramidate.

The proton nmr spectrum was also very similar to that observed previously by others for the L-valine analogue, except that the 3-NH on the nucleoside base resonated 1.1 ppm further downfield in the D-valine compound. Also, the multiplet signal at ca. 4.30 ppm was assigned by the author to the H3' proton (on the strength of homonuclear decoupling experiments), whereas McGuigan and coworkers had previously assigned this signal to H4'. The presence of two diastereoisomers was evident from the proton nmr spectrum, the signals from H6, H1', the methoxy protons and the 5-CH3 all showing more than one resonance, although the ratios between signals were not consistent. Both the isopropyl methyl groups appeared as doublets at high field, with three-bond proton-proton coupling constants of ca. 7 Hz.

In the electron impact mass spectrum the observed molecular ion provided further confirmatory evidence that the target compound had been successfully synthesised, the calculated mass corresponding well with the accurate mass observed. Fragmentation from the molecular ion by loss of the thymine base, loss of methanol, ethene and propene, loss of the azide moiety and loss of the phosphoramidate moiety were all detected, and the subsequent fragmentation of the latter was also noted. In particular, loss of ethanol (arising from the ethoxy group), loss of methanol (derived from the amino acid carboxyl protecting group) and loss of propene (caused by the fragmentation of the valine isopropyl moiety) were all observed.

Acceptable analytical data were only obtained after further chromatographic purification. Microanalysis and hplc (coincident diastereoisomeric retention times noted) then confirmed the bulk purity of the compound.

AZT (ethyl methoxy-D-valinyl) phosphoramidate was evaluated against HIV-1 in vitro as a mixture of its diastereoisomers and showed an activity of 10 µM. The activity of AZT (ethyl methoxy-L-valinyl) phosphoramidate in the same test was 10 µM.
It appears that a change in amino acid stereochemistry from L to D does not alter the activity of these phosphoramidate derivatives. This suggests that enzymic activation of the compounds by cleavage of the amino acid from the phosphoramidate moiety does not play a major role in enabling the prodrugs to exert their anti-HIV effects, since such enzymes would be unlikely to accept D-amino acids as substrates, as discussed earlier. However, this finding does not preclude the possibility of enzymes acting at other sites in the molecule, or enzymic activation following chemical hydrolysis of the phosphoramidate bond.

The susceptibility of the amino acid-based nucleoside phosphoramidates to direct cleavage by HIV-1 protease was examined by reverse phase hplc (experiment 23). To the compound AZT (ethyl methoxy-L-alaninyl) phosphoramidate (as synthesised by McGuigan et al.) was added an aliquot of the enzyme in a citrate/phosphate buffer with glycerol together with a small quantity of Tween 20. The molar ratio of enzyme/substrate was approximately 1:100. The mixture was stored at 37 °C and hplc traces recorded at various time points. For a reasonable substrate, HIV-1 protease turns over about 20 moles of substrate per mole of enzyme per second at high substrate concentrations (10 mM), as in this test. Although the incubation time required was essentially unknown, after over one hour hplc showed very little decomposition and at five hours there still appeared to be little change as monitored by hplc. Very small amounts (less than 0.1% of the mixture) of uv active products were produced with retention times higher than those of the parent diastereoisomers, and no decomposition to AZT was observed. This test therefore appears to confirm the apparent low susceptibility of these phosphoramidate compounds for the enzyme HIV-1 protease.

Given the importance of cleavage of the P-N bond as the first step in the proposed activation of these derivatives, and having established that this cleavage appears to take place non-enzymically, it was decided to investigate the stability of a typical derivative towards chemical hydrolysis in aqueous media at physiological temperature and a range of pH.

The compound employed for these studies was AZT (n-propyl methoxy-L-valinyl) phosphoramidate (Figure 85), as synthesised by McGuigan et al. This compound had been evaluated against HIV-1 in vitro and showed activity at 10 µM.
The study is reported as experiment 24. Aqueous, non-phosphate buffers were employed with pHs of 6, 7 and 8. The phosphoramidate derivative (as its diastereoisomeric mixture) was mixed with each buffer solution and the samples stored at 37 °C. Decomposition was monitored by phosphorus nmr spectroscopy.

The chemical shifts of the two diastereoisomers were coincident at ca. 10.7 ppm in the pH 6 and pH 8 buffer solutions at the start of the study. The pH 7 sample displayed two signals corresponding to the two diastereoisomers at 10.78 and 10.68 ppm. No change was observed in the phosphorus nmr spectrum of any of the samples after 1 hour and then 1 day, illustrating that the derivatives are reasonably stable to aqueous hydrolysis. However, after 5 days the phosphorus nmr spectra of the samples at pH 6 and pH 8 showed the presence of minor species giving rise to signals at 10.2, 10.1 and 3.3 ppm for the former and 10.2 and 7.8 ppm for the latter. The chemical shifts of less than 10 ppm are consistent with hydrolysis of the phosphoramidate compound. After 20 days the signals observed earlier at 5 days were slightly more pronounced, although still minor (i.e. less than 1% of the mixture) when compared to the resonances from the parent compound. At pH 6, resonances were recorded at 1.0, 3.4, 4.9 and 7.9 ppm, with several other signals appearing close to those observed for the parent compound at ca. 10 ppm. At pH 8, the signal at 7.8 ppm was still present but now there were also two more resonances at ca. 11 ppm, as well as several additional signals at ca. 10 ppm. The first signs of decomposition at pH 7 were evident after 20 days with additional resonances at 4.9, 7.8, 10.2 and 10.3 ppm.

After 103 days the samples were examined by hplc. For the sample at pH 6 there were three main decomposition products present in a proportion greater than 0.5% of the total mixture. AZT, with a retention time of 8.61 minutes, constituted
about 0.53% of the mixture. Two species with retention times of 17.96 and 18.15 minutes constituted 1.43 and 1.85% of the mixture respectively, with the starting material forming about 95% of the rest of the sample. For the pH 7 sample, AZT was present in a greater amount than at pH 6, constituting 1.1% of the mixture. Besides AZT, there was another species with a retention time of 17.52 minutes forming 0.45% of the mixture. The starting compound constituted greater than 98% of the sample. The presence of AZT in the sample was even more significant at pH 8, with the nucleoside constituting 3.06% of the mixture. One other species (besides the starting material - 96%) was present in a proportion of 0.74%, with a retention time of 17.59 minutes.

In order to correlate the hplc results with those obtained from the phosphorus nmr spectra, the samples were re-analysed by the latter technique at 110 days. For the pH 6 sample, decomposition products with chemical shifts of 1.3, 3.4, 7.8, 10.2, 10.3 and 10.5 ppm were noted. For the pH 7 sample, in addition to the decomposition product discovered earlier (at 7.8 ppm) there was also a further product observed with a chemical shift of 3.5 ppm. More significant change was observed for the pH 8 sample. Besides the signals at 7.8, 11 and ca. 10 ppm observed previously, there was a new resonance at ca. 9 ppm and another at 8.0 ppm.

Figure 86. Mode of aqueous hydrolysis proposed for phosphoramidate derivatives at pH 8 and pH 7 (and to a lesser extent at pH 6).
Considering the two sets of results (phosphorus nmr and hplc) together, the signal observed at 7.8 ppm may be tentatively assigned to the species shown in Figure 86, representing cleavage of the P-O-nucleoside bond. This hydrolysis appeared to take place at all three levels of pH, occurring to the greatest extent at pH 8 and to the least extent at pH 6. Such cleavage would of course liberate AZT, which was found in greatest proportion in the pH 8 sample.

Given the acid sensitivity of the P-N linkage, cleavage of this bond was more likely to occur in the pH 6 sample. Such cleavage would give rise to two decomposition products, a nucleoside phosphorus-containing species, which may be assigned to the resonance noted at ca. 3 ppm and the hplc retention time of 18.15 minutes, and the amino acid methyl ester (Figure 87). Indeed, this mode of decomposition appeared to predominate at pH 6, occurring to a much lesser extent at pH 7.

![Figure 87. Mode of aqueous hydrolysis at pH 6 (and to a lesser extent at pH 7) proposed for the phosphoramidate derivatives.](image)

There were several signals in the phosphorus nmr spectra and several hplc traces which could not be assigned to specific decomposition species. Other hydrolysis products which might be expected to occur would include materials formed by
hydrolysis of the n-propyl P-O-ester functionality and products formed by cleavage of the amino acid methoxy moiety.

By way of conclusion to these investigations, it appears that the favoured mode of aqueous hydrolysis of the phosphoramidate compounds is cleavage of the P-O-nucleoside bond, which occurred at all the levels of pH studied. The cleavage of the P-N bond appeared to be preferred in conditions of lower pH. Perhaps the most important observation was the discovery that these compounds are stable in aqueous media at neutral pH over a period of about 5 days. Given the timescale of the biological evaluation procedure (72 hours from addition of compounds to the time of HIV assay), it appears that activation of the prodrugs does not depend solely on chemical hydrolysis, but that other mechanisms may play a role in realising the anti-HIV activity of these derivatives.

To summarise the structure-activity data accumulated so far, it appears that an L-α-amino acid is not required for the activity of these prodrug compounds. Further to this conclusion, the question arises as to how much the structure of the amino acid (whatever its stereochemistry) can be altered without substantial loss of anti-HIV potency. It has been established that the closer the ester moiety is to the phosphoramidate bond, the greater is the activity. However, it seems that an ester moiety is not a prerequisite to activity, as exemplified by the compound AZT (ethyl n-propylamino) phosphoramidate, which was active at 50 μM. This still represents a much lower level of potency than that achieved by the compounds containing amino acids, so the P-N bond is not sufficient in itself for good anti-HIV activity.

The next two compounds to be synthesised probed the effect on activity of altering the ester moiety on the amino acid; the amino acid carboxyl group was reduced to a methylene group, while the methoxy linkage was retained. Thus the two target compounds shown in Figure 88 represent 'reduced' forms of the active species synthesised earlier, namely AZT (ethyl methoxyglycinyll) phosphoramidate (Figure 88(a)) and AZT (ethyl methoxy-L-alaninyl) phosphoramidate (synthesised by McGuigan et al., Figure 88(c)).
Figure 88. (a) AZT (ethyl methoxyglycyl) phosphoramidate and the target compound (b), which is analogous to (a) except that a methylene moiety replaces the amino acid carboxyl moiety; (c) AZT (ethyl methoxy-L-alaninyl) phosphoramidate and the target compound (d), which is analogous to (c) except that a methylene moiety replaces the amino acid carboxyl moiety.

The synthesis of the target compound shown in Figure 88(b) was the first to be attempted. The procedure adopted was as used previously: condensation of the appropriate phosphorochloridate with AZT in the presence of N-methyl imidazole, using THF as solvent. The synthesis of the phosphorylating reagent required for this compound is reported as experiment 25 (see Figure 89).
Figure 89. Reaction scheme for the synthesis of ethyl 2-methoxyethylamino phosphorochloridate (experiment 25).

Two molar equivalents of 2-methoxyethylamine were reacted with ethyl phosphorodichloridate in diethyl ether at -78 °C, the second equivalent being used to remove the HCl produced in the reaction by precipitation as the amine salt. Filtration of the salt and removal of the solvent afforded the product in good yield. Phosphorus and carbon nmr data were collected for this compound and confirmed the proposed structure. There was one signal in the phosphorus nmr spectrum confirming the purity of the phosphorochloridate. The chemical shift of this resonance was 13.43 ppm, consistent with shifts observed earlier for similar species. The carbon nmr spectrum showed phosphorus coupling to the PNHCH₂CH₃ carbon (7.9 Hz), to the POCH₂CH₃ carbon (8.0 Hz) and to the POCH₂ carbon (6.2 Hz), as expected for asymmetric phosphorylating reagents of this type. The chemical shift of the PNHCH₂CH₃ carbon was 71.61 ppm, showing the deshielding effect exerted by the adjacent methoxy group. The methoxy carbon itself resonated at 58.77 ppm which was more downfield than in the amino acid-based derivatives, showing perhaps a stronger deshielding arising from the greater proximity of the phosphorochloridate moiety.

The synthesis of the target phosphoramidate, AZT (ethyl 2-methoxyethylamino) phosphoramidate, is reported as experiment 26 (see the reaction scheme indicated in Figure 90).

Using THF/N-methyl imidazole condensation conditions the target compound was isolated in moderate yield. The compound was fully characterised by phosphorus, carbon and proton nmr, by electron impact mass spectrometry and by microanalysis.

The phosphorus nmr chemical shifts of the compound were 7.89 and 7.86 ppm, due to the presence of two diastereoisomers. These shifts were in the region observed previously for the amino acid-based phosphoramidates. The carbon nmr spectrum showed all the expected shifts and splittings; a diastereoisomeric ratio of 1:1 (as seen in the phosphorus nmr spectrum) was evident for the carbon signals of C4, C5, C1', C4', PNHCH₂CH₃, C5', POCH₂, C3', PNCH₂, POCH₂CH₃ and 5-CH₃. Splitting by
phosphorus-carbon coupling was observed for those carbons within three bonds of phosphorus - C4', C5', PNHCH₂CH₂, POCH₂ and POCH₂CH₃. The PNHCH₂CH₂ resonances had moved slightly (ca. 1 ppm) downfield as compared to the equivalent signal in the phosphorochloridate, while the methoxy carbon had virtually the same chemical shift and the PNHCH₂ carbon had moved slightly (0.3 ppm) upfield.

Figure 90. Reaction scheme for the synthesis of AZT (ethyl 2-methoxyethylamino) phosphoramidate (experiment 26).

In the proton nmr spectrum the 1:1 ratio of diastereoisomers was evident for the signals derived from H6, H1' and the methoxy protons. The methoxy protons resonated at 3.36 ppm, ca. 0.3 ppm more upfield than in the amino acid-containing nucleoside phosphoramidate compounds. The PNHCH₂CH₂ protons gave a triplet signal, with a three-bond proton-proton coupling constant of 5.05 Hz, while the PNHCH₃ protons gave a multiplet signal (arising from the presence of two diastereoisomers and phosphorus-proton coupling).

In the electron impact mass spectrum a molecular ion was observed; the accurately measured mass corresponded well with the calculated value for the target compound. Fragmentation from the molecular ion consisted of loss of 2-methoxyethyamine, loss of thymine and loss of the entire phosphoramidate moiety.
The subsequent fragmentation of the latter by loss of water, ethene, methanol and ethanol was noted. Nucleosidic sugar and base fragments were also observed.

Microanalysis (within acceptable limits for C, H and P) and hplc confirmed the bulk purity of the compound, no starting material (AZT) being detected in the sample by the latter technique.

AZT (ethyl 2-methoxyethylamino) phosphoramidate was evaluated against HIV-1 in vitro as a mixture of its diastereoisomers and showed an activity of 7 μM. This result together with the biological evaluation of the next target compound will be discussed once the synthesis and characterisation of the latter have been described.

The compound AZT (ethyl 2-methoxy-1-methylethylamino) phosphoramidate was synthesised by reaction of 2-methoxy-1-methylethylamino phosphorochloridate with the nucleoside in THF containing N-methyl imidazole. The phosphorylating reagent was synthesised according to the method described in experiment 27 - see Figure 91 for the appropriate reaction scheme.

\[
\begin{array}{c}
\text{2 CH}_3\text{O}-\text{CH}_2-\text{CH}-\text{NH}_2 + \text{EtOP(0)Cl} \\
\text{CH}_3 \\
\text{CH}_3\text{O}-\text{CH}_2-\text{CH}-\text{NH}-\text{P}-\text{Cl} + \text{CH}_3\text{O}-\text{CH}_2-\text{CH}-\text{NH}_3\text{Cl} \\
\text{CH}_3 \text{OEt} \text{H} \text{H} \text{H} \\
\end{array}
\]

Figure 91. Reaction scheme for the synthesis of ethyl 2-methoxy-1-methylethylamino phosphorochloridate (experiment 27).

The phosphorochloridate was isolated in good yield and the phosphorus and carbon nmr data collected were in agreement with the proposed structure. Because the starting material 2-amino-1-methoxypropane was used as its racemic mixture, the phosphorylating reagent was obtained as a mixture of its diastereoisomers and this was clearly evident from the spectroscopic data. Besides the two signals in the phosphorus nmr spectrum (at 12.40 and 12.14 ppm, with a 5:4 intensity ratio), the carbon nmr spectrum also showed the presence of the two diastereoisomers in a ratio of 5:4, specifically at the resonances arising from the methylene and methyl carbons adjacent to the asymmetric centre. As with ethyl 2-methoxyethylamino phosphorochloridate (experiment 25), the methylene carbon adjacent to the methoxy group was the most deshielded, with two chemical shifts of ca. 76 ppm. The PNH'CH carbon resonance had a chemical shift of 47.95 ppm, while the PNH'CHCH₃ carbon
resonated upfield at ca. 19 ppm as two doublets (ratio 5:4), the phosphorus-carbon couplings for each diastereoisomer being ca. 5 Hz.

The reaction of ethyl 2-methoxy-1-methylethylamino phosphorochloridate with AZT is reported as experiment 28, Figure 92.

The product of this reaction was isolated in somewhat low yield after the usual purification procedures, although the reasons for this were unclear. The spectroscopic data clearly showed the presence of several isomers arising from the combination of multiple chiral centres. The phosphorus nmr spectrum showed two signals in the expected region at 7.16 and 7.12 ppm, in a ratio of 6:5. In the carbon nmr spectrum there were four signals for the carbon at C5 on the nucleoside base - the maximum number expected since four possible diastereoisomers could be formed in the reaction. Some of the other resonances in the carbon nmr spectrum were not as clearly resolved due to phosphorus-carbon coupling complicating the signals still further (for example C5' and C4'). The methylene carbon adjacent to the methoxy group had moved slightly downfield as compared to the same moiety in the phosphorochloridate, by ca. 0.7 ppm. The asymmetric carbon had moved ca. 0.5 ppm upfield.

Figure 92. Reaction scheme for the synthesis of AZT (ethyl 2-methoxy-1-methylethylamino) phosphoramidate (experiment 28).
In the proton nmr spectrum the presence of several diastereoisomers was evident from the signals derived from H6 (two singlets) and especially from the signals due to the PNH proton, the methoxy protons, the PNHCH proton and the methylene protons adjacent to the methoxy group, which all overlapped to give a multiplet integrating for seven protons centred at 3.49 ppm. The methyl protons on the amine moiety resonated at typically high field - 1.19 ppm - as a multiplet.

The electron impact mass spectrum confirmed the identity of the product as the target phosphoramidate, with an accurate mass reading for the molecular ion in good agreement with that calculated for the target compound. Loss of thymine, the azide moiety, 2-amino-1-methoxy-propane and small molecules such as ethene, ethanol and methoxy methane from the molecular ion were observed. A fragment corresponding to the complete phosphoramidate moiety was noted, and extensive fragmentation of this species was also evident (especially by the loss of molecules such as water, methanol, ethene and ethanol). The base peak was assigned to a fragment of the nucleoside sugar.

Microanalysis results were acceptable for C, H and P, while hplc confirmed the bulk purity of the compound.

AZT (ethyl 2-methoxy-1-methylethylamino) phosphoramidate was tested as a mixture of its diastereoisomers against HIV-1 and showed an activity of 35 μM.

Comparing the anti-HIV activities of the two 'reduced' analogues with their amino acid-containing congeners, AZT (ethyl methoxyglycinyl) phosphoramidate had an activity of 10 μM, while its 'reduced' form, AZT (ethyl 2-methoxyethylamino) phosphoramidate had a similar activity of 7 μM. AZT (ethyl methoxy-L-alaninyl) phosphoramidate had an activity of 3 μM while its 'reduced' form, AZT (ethyl 2-methoxy-1-methylethylamino) phosphoramidate had an activity an order of magnitude less at 35 μM. The disparate activities shown by these 'reduced' compounds in relation to their amino acid-containing analogues are difficult to reconcile. One of the conclusions that may be drawn from these results is that it appears that anti-HIV potency is retained despite modification to the amino acid carbonyl group. The second of the two comparisons noted above is perhaps the most consistent with the structure-activity correlations drawn so far, in that an ester moiety clearly is not required for anti-HIV activity, but its presence does appear to confer substantially greater anti-HIV activity. This is supported by the poor activity noted earlier for the derivative AZT.
(ethyl \textit{n}-propylamino) phosphoramidate.

The wide variation in activity shown by these compounds modified in the amino acid moiety is consistent with cleavage of the P-N bond as an important step in their activation, since such variation would alter the strength of, and environment around, this bond. This cleavage would release AZT ethyl phosphate, which might either act as such or undergo further hydrolysis to AZT or AZT monophosphate. The latter species is preferable according to the prodrug strategy, since it could then be further metabolised to the reverse transcriptase inhibitor AZT triphosphate. In order to probe which of these two species is released preferentially following P-N cleavage it was decided to synthesise a derivative containing a phosphorus-carbon bond instead of a phosphorus-oxygen bond with respect to the alkoxy moiety in the nucleoside phosphoramidate compounds. The phosphorus-carbon bond has a high chemical and biological stability\textsuperscript{263,264}. Thus, if cleavage of the ethoxy P-O bond plays an essential role in the activation of these compounds, substitution by a P-C bond should lead to a diminution in biological activity, since activation of the derivatives would be impaired.

Thus the target compound is shown in Figure 93(a). The compound shown in Figure 93(b), AZT (methyl methoxy-L-valinyl) phosphoramidate (synthesised previously by McGuigan \textit{et al.}\textsuperscript{234}) represents the isosteric analogue of the compound shown in Figure 93(a), in that a CH\textsubscript{2} moiety replaces the methoxy oxygen. The biological activity of these two compounds will be compared when drawing mechanistic inferences. Isosterism strictly refers to compounds of identical size and shape; although compound 93(a) as compared to 93(b) does not meet these conditions rigorously, the bond angles and lengths involved are similar enough that the term may be reasonably applied\textsuperscript{263}. 

![Figure 93(a) and 93(b)](image-url)
The first step in the synthesis of the compound shown in Figure 93(a) was the preparation of the appropriate phosphonylating reagent, ethyl methoxy-L-valinyl phosphonochloridate (experiment 29). This was synthesised from ethyl phosphonic dichloride by the method used previously for the preparation of the amino acid-derived phosphorochloridates (Figure 94).

The product was isolated in very good yield and characterised by phosphorus nmr, carbon nmr and phosphorus analysis. The chemical shifts of the resonances in the phosphorus nmr spectrum were 48.43 and 47.50 ppm (a 1:2 diastereoisomeric mixture), which is in the correct region for compounds of this type. The carbon nmr spectrum revealed the presence of the two diastereoisomers in a 1:2 ratio very clearly, all the carbons except one of the isopropyl methyl groups appearing as two signals. Phosphorus coupling was observed to the carbonyl carbon, the asymmetric carbon, the isopropyl carbon and the methyl carbon in the ethyl chain. The magnitude of the one-bond phosphorus-carbon coupling to the methylene carbon in the ethyl chain was the most striking feature of the carbon nmr spectrum, values of 113.1 and 114.3 Hz being...
recorded for the two diastereoisomers. This magnitude of coupling has literature precedent: the compound indicated in Figure 95(a) shows a value of 104 Hz for one-bond phosphorus-carbon coupling\(^{266}\); the compound shown in Figure 95(b) shows a value of 142.2 Hz\(^{267}\).

(a) CH\(_3\)P(O)Cl\(_2\) (b) CH\(_3\)P(O)(OMe)\(_2\)

**Figure 95.** Compounds illustrating the large magnitude of one-bond phosphorus-carbon couplings in their carbon nmr spectra.

The methylene and methyl carbons in the ethyl chain resonated at significantly higher field than in the ethoxy analogues: for the methylene carbon, *ca.* 29 ppm as compared to 65 ppm; for the methyl carbon *ca.* 5.5 ppm as compared to *ca.* 16 ppm. The purity of the compound was confirmed by phosphorus analysis.

The reaction of ethyl methoxy-L-valinyl phosphonochloridate with AZT was first monitored in a tlc scale reaction (not reported). The THF/N-methyl imidazole conditions were employed, with initially 3 molar equivalents of phosphorylating reagent. After 5 hours tlc showed that little reaction had taken place and there was little further change after 32 hours. A further 6 equivalents of phosphorylating reagent were added, but this produced little further change on tlc. It appeared that the phosphonochloridate had decomposed on storage and this was confirmed when a phosphorus nmr spectrum of the material was recorded, showing a large number of signals below 30 ppm which indicated the presence of hydrolysis products. This observation suggested that the reaction with the nucleoside had to be carried out using freshly prepared ethyl methoxy-L-valinyl phosphonochloridate. The reagent was therefore prepared again using exactly the same method as in experiment 29 and was used freshly in experiment 30.

Thus experiment 30 records the full-scale reaction of the phosphonochloridate with AZT, as shown in Figure 96.

The THF/N-methyl imidazole method was again utilised, but this time the reaction appeared to proceed to completion. Purification of the compound proved extremely problematic. Three chromatographic purifications failed to afford pure material as observed by phosphorus nmr (not reported). An aqueous wash was to prove necessary in order to obtain the compound in spectroscopic purity. The phosphorus nmr spectrum displayed two (diastereoismeric) resonances in a ratio of 1:2, with chemical shifts of 36.22 and 35.79 ppm respectively.

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The carbon nmr spectrum also suggested a diastereoisomeric ratio of 1:2, with all the signals except that derived from the methoxy carbon showing the presence of the two isomers. Phosphorus-carbon coupling was observed to the carbonyl carbon and the isopropyl carbon on the valine moiety, and to the C4' and C5' on the sugar of the nucleoside. Large single-bond phosphorus-carbon couplings (133.6 and 130.05 Hz) were observed to the methylene carbon in the ethyl chain, as in the phosphorochloridate precursor. The methylene carbon in the ethyl chain showed a chemical shift of ca. 22 ppm; a shift of ca. 7 ppm upfield as compared to the phosphorochloridate. The shift of the methyl group in the ethyl chain was the most upfield signal at ca. 6 ppm (similar to the shift in the phosphorylating reagent). These chemical shifts compare closely to those observed for the resonances of the ethyl carbons in the compound shown in Figure 97; shifts of 19.0 and 6.6 ppm respectively were reported for the methylene and methyl carbons in the ethyl chain\textsuperscript{255}.

\[ (\text{CH}_3\text{CH}_2\text{O})_2\text{P(O)}\text{CH}_2\text{CH}_3 \]

Figure 97. Compound used for comparison of $^{13}$C nmr chemical shifts.
The effect on chemical shift of the removal of the electronegative oxygen from the ethyl chain was also prominent in the proton nmr spectrum, where the methylene protons in the ethyl chain resonated at 1.84 ppm, as compared to ca. 4.3 ppm for the methylene protons in the ethoxy side chain of one of the phosphoramidate compounds. The presence of two diastereoisomers in a 1:2 ratio was also evident from the proton nmr spectrum, with the signal for H6 resonating as two singlets and the signal for H1' resonating as two triplets.

The electron impact mass spectrum provided further evidence for the identification of the product as the target compound. A molecular ion was observed and the measured accurate mass found to be sufficiently close in value to the calculated mass of the target compound. A protonated molecular ion was also observed, fragmenting by loss of thymine and the azide moiety. Loss of the phosphoramidate moiety from the molecular ion was noted and the subsequent fragmentation of this section of the molecule by loss of water, propane, methanol and methoxy valine were observed. Other fragments corresponding to thymine, protonated thymine and the nucleoside sugar also featured prominently in the mass spectrum.

Despite the apparent purity of the compound as evidenced by spectroscopic techniques, hplc analysis revealed the presence of an impurity representing 15% of the material. The product was therefore purified by preparative hplc.

That the presence of a 15% impurity had not been detected by spectroscopy was indeed surprising. The analytical hplc had been performed using a reverse phase acetonitrile/water based system, which was the method of choice for detecting very small amounts of AZT in the final product. However, on re-analysing the product purified by preparative hplc in this reverse phase system it was discovered that these analytical conditions caused the material to decompose. This would explain why the analytical results for this compound previously obtained on spectroscopically pure samples indicated the presence of large amounts of impurity. When the purified sample was analysed under the same conditions as used for the preparative separation (normal phase), the bulk purity was confirmed (and the product was also found to be AZT-free).

In purifying the product by preparative hplc two separate fractions were collected corresponding to the two isomers in the mixture. Thus the two diastereoisomers of AZT (ethyl methoxy-L-valiny) phosphoramidate could be
evaluated separately for their anti-HIV activity.

Recalling the 2:1 ratio of diastereoisomers noted earlier for this compound, one isomer was labelled the major and one the minor according to their distribution in the original mixture. Separate phosphorus and proton NMR data were obtained for each isomer and these are included in the report of experiment 30.

The phosphorus NMR chemical shift of the major isomer was 33.21 ppm. Although this is different from the shift of the major isomer in the mixture, a different NMR spectrometer was used to record each spectrum and discrepancies such as this are not uncommon when different machines are used. Similarly the phosphorus NMR chemical shift of the minor isomer was 33.51 ppm, which again differs from its shift in the mixture.

The proton NMR data indicated several differences between the diastereoisomers. In the spectrum of the major isomer, H3', the two H5' protons and H4' gave four separate multiplet signals at 4.40, 4.27, 4.14 and 4.02 ppm respectively. In the spectrum of the minor isomer, the resonances of H3' and one of the H5' protons were coincident, giving a multiplet at 4.30 ppm, whereas the other H5' proton gave a signal which overlapped with that of H4' at 4.06 ppm.

The major diastereoisomer of AZT (ethyl methoxy-L-valinyl) phosphonamidate synthesised in experiment 30 was evaluated against HIV-1 in vitro and displayed an activity of 1 pM. The minor isomer was similarly evaluated and displayed an activity of 4 pM. The P-O bond-containing isoster of these compounds - AZT (methyl methoxy-L-valinyl) phosphoramidate - displayed an anti-HIV activity of 3 pM in the same test.

All three compounds therefore displayed a similar level of anti-HIV potency. It thus appears that cleavage of the P-O ethoxy bond in the phosphoramidate derivatives is not a strict criterion for activity. That the phosphonamidate isomers both show activity suggests a mechanism of action involving cleavage of the entire phosphonamidate moiety to liberate the parent nucleoside AZT, although the possibility of the phosphonamidate acting itself as a specific or non-specific inhibitor of one or more enzymic processes as distinct from the phosphoramidates cannot be ruled out.

How far the results obtained for the phosphonamidate can be extrapolated to the phosphoramidates is therefore unclear. It is possible, for example, that the activity
of the amino acid-containing phosphoramidates does indeed arise mainly from P-O bond cleavage in the ethoxy moiety of these compounds, with P-O-nucleoside cleavage playing only a minor role. However, the phosphonamidate derivative has to be cleaved at the P-O-nucleoside bond if P-O bond cleavage is to take place at all; the loss of the binding function of the esterified oxygen in the phosphonamidate analogue might mean that this compound can only act by this (possibly) less favourable (as compared to the phosphoramidates) mechanism. It has been noted previously that the presence of a carbon-phosphorus linkage does not preclude enzymic cleavage of phosphorus-ester linkages also present\textsuperscript{268}.

Summarising, and taking account of the previous work on phosphorus-carbon bond-containing derivatives discussed in the Introduction\textsuperscript{219}, the most likely mode of action of the nucleoside amino acid-containing phosphoramidates would appear to be a dual mechanism involving cleavage of the P-N bond followed by cleavage of either of the phosphorus-ester linkages. Thus, at least to some extent, the phosphoramidates may be acting as intracellular sources of AZT monophosphate.

It was noted earlier that HIV protease is unlikely to be involved in the activation of the phosphoramidate derivatives containing only one amino acid. This is unsurprising given the observation that a minimum of seven residues spanning the protease cleavage sites appear to be necessary to ensure specific and efficient cleavage of the scissile bond\textsuperscript{149-151}. It would thus appear that in order to promote enzyme-specific activation of the compounds it is necessary to extend the peptide moiety of the phosphoramidate prodrugs. Even if enzyme-specific cleavage cannot be achieved in this manner, such a nucleotide-oligopeptide conjugate might still possess several useful characteristics, as mentioned earlier in the Introduction. The attempts to increase the number of amino acid residues in the peptide moiety of the phosphoramidate compounds will now be described.

Devine had experimented with the synthesis of alkyl peptidyl phosphorochloridates, with the aim of then condensing these materials with nucleosides using THF/N-methyl imidazole conditions\textsuperscript{238}. These experiments were unsuccessful, the amide linkage apparently being incompatible with the P-Cl bond, and an alternative method was therefore sought.

Galardy \textit{et al.} have demonstrated that a t-butyl protecting group on the carboxyl moiety of an amino acid can be cleaved using trifluoroacetic acid in the
presence of the P-N bond. If this protecting group could be cleaved from the amino acid moiety of one of the nucleoside phosphoramidate derivatives, without perturbation of the rest of the bonds in the molecule, then the resultant intermediate compound could be coupled to another amino acid via standard procedures to afford a dipeptide-containing derivative. The next target compound was therefore as indicated in Figure 98.

![Figure 98. Nucleoside phosphoramidate derivative incorporating protection of the amino acid carboxyl terminus with a t-butyl moiety.](image)

The required phosphorylating reagent, ethyl t-butyl-L-valinyl phosphorochloridate, was prepared in experiment 31 according to the reaction scheme in Figure 99.

![Figure 99. Reaction scheme for the synthesis of ethyl t-butyl-L-valinyl phosphorochloridate (experiment 31).](image)

The standard method was adopted and the product isolated in excellent yield. The identity and purity of this material were confirmed by spectroscopy (phosphorus, carbon and proton nmr) and microanalysis, the observed value for chlorine being within 0.13% of the calculated value. There were two signals in the phosphorus nmr spectrum (at 12.67 and 12.17 ppm) in a ratio of 1:1, indicating the presence of two
diastereoisomeric forms of the product. The presence of two diastereoisomers in a ratio of 1:1 was also evident from various signals in the carbon nmr spectrum. The carbonyl carbon resonated as two doublets (one from each isomer), each signal being split by phosphorus-carbon coupling of approximately 4 Hz. Similarly, the methylene carbon in the ethoxy chain resonated as a pair of doublets, the phosphorus-carbon coupling constant for each isomer being approximately 3 Hz. The t-butyl moiety was clearly identifiable, with the tertiary carbon resonating at 82.32 and 82.21 ppm and the t-butyl methyl groups resonating upfield at 28.04 ppm. A slight inconsistency in the diastereoisomeric ratios observed was noted for both isopropyl methyl groups, which appeared as two signals each, but showing ratios of 2:3 rather than 1:1.

In the proton nmr spectrum the resonance of the exchangeable valine NH proton was not observed, but all the other protons in the product gave clearly assignable signals. The t-butyl methyl groups, for example, resonated as a singlet integrating for 9 protons at 1.48 ppm, while the valine isopropyl methyl groups gave the most upfield signals (both doublets, coupling to the isopropyl CH proton) at 1.00 and 0.92 ppm.

In the electron impact mass spectrum a protonated molecular ion was observed and fragments corresponding to the loss therefrom of chlorine, ethanol and 2-methylpropene (arising from fragmentation of the t-butyl moiety) were noted. The base peak was assigned to the (relatively stable) tertiary carbonium ion \( \text{C(CH}_3\text{)}_3^+ \).

The reaction of ethyl t-butyl-L-valinyl phosphorochloridate with AZT was performed using THF/N-methyl imidazole condensation conditions according to the reaction scheme in Figure 100 (experiment 32).

The desired product, AZT (ethyl t-butyl-L-valinyl) phosphoramidate, was isolated in good yield after chromatography. The phosphorus nmr spectrum displayed two signals in a ratio of 4:3, with chemical shifts in the expected region for the target molecule (ca. 7 ppm). The 4:3 ratio was confirmed in the carbon nmr spectrum, with the resonances \( \text{C}_5, \text{C}_1', \text{C}_2', \text{C}_3', \text{C}_4', \text{C}_5' \), the t-butyl tertiary carbon, the asymmetric carbon, the isopropyl carbon and the isopropyl methyl carbons all showing this distribution of isomers. The t-butyl carbon resonated at 82.07 and 82.03 ppm, which is about 0.2-0.3 ppm upfield of its position in the phosphorochloridate. The chemical shift of the signal arising from the t-butyl methyl groups was virtually unchanged from the phosphorochloridate precursor, at 28.00 ppm. Three-bond phosphorus-carbon
couplings of ca. 3 Hz (to the carbonyl carbon on the amino acid), ca. 6 Hz (to the isopropyl carbon), ca. 7 Hz (to the methyl carbon in the ethoxy chain) and ca. 8 Hz (to C4') were observed, as well as two-bond couplings of ca. 5 Hz (to C5' and the methylene carbon in the ethoxy chain).

![Chemical structure](image)

**Figure 100.** Reaction scheme for the synthesis of AZT (ethyl t-butyl-L-valinyl) phosphoramidate (experiment 32).

In the proton nmr spectrum the valine NH proton was noted as a multiplet at 3.60 ppm. The H6 proton, the H1' proton, the methyl group at position 5 on the nucleoside base and one of the isopropyl methyl groups all resonated as pairs of signals showing the 4:3 diastereoisomeric ratio observed in the carbon and phosphorus nmr spectra.

In the electron impact mass spectrum the observed molecular ion provided further confirmatory evidence that the target compound had been successfully synthesised, the calculated mass corresponding closely to the measured accurate mass. Fragments corresponding to loss of thymine, loss of the azide moiety and loss of the phosphoramidate moiety from the molecular ion were noted. The fragmentation of the phosphoramidate part of the molecule by the loss of t-butyl alcohol, ethene and propane was observed, and fragments corresponding to the nucleoside part of the
product were also evident, the base peak being assigned to a nucleoside sugar fragment.

Despite further purification by flash column chromatography, satisfactory microanalysis results were obtained only for the elements H and P. However, hplc confirmed the bulk purity of the compound, the two isomers showing retention times of 27.0 and 27.2 minutes.

Although the purpose of synthesising this analogue was to utilise it in further reactions, it was nevertheless tested against HIV-1 in vitro and showed an activity of 10 μM. The corresponding derivative, AZT (ethyl methoxy-L-valinyl) phosphoramidate, differing from the above compound only by the amino acid protecting group (methoxy instead of t-butyl), showed an activity of 10 μM.

It would appear from this comparison that alteration in the nature of the amino acid protecting group has little effect on activity. Synthesis of further derivatives modified in this manner should confirm this proposition.

Experiment 33 reports the reaction of AZT (ethyl t-butyl-L-valinyl) phosphoramidate with trifluoroacetic acid, as shown in Figure 101.

![Reaction scheme for the synthesis of AZT (ethyl L-valinyl) phosphoramidate.](image)

Figure 101. Reaction scheme for the synthesis of AZT (ethyl L-valinyl) phosphoramidate.
The phosphoramidate derivative was stirred in anhydrous trifluoroacetic acid for 20 minutes and the reaction monitored by tlc. There were at least two products formed and the presence of AZT in the mixture was also noted. An attempt to precipitate out the products by pouring the mixture into hexane was unsuccessful. It was decided not to pursue this trifluoroacetic acid cleavage methodology as it was not deemed to be sufficiently clean. Almost certainly cleavage of the P-N bond had taken place in addition to other side reactions. In the work carried out by Galardy et al., p-nitro-substituted aromatic moieties attached to the phosphorus would have made the amino acid nitrogen much less basic and hence less susceptible to protonation by the acid, thus making the phosphoramidate bond less easy to cleave.

A more convenient strategy for extending the peptide moiety of the nucleoside phosphoramidate derivatives was therefore sought.

Kosolapoff has reported the reaction of secondary phosphites with primary or secondary amines in the presence of an aliphatic polyhalide to afford phosphoramidates in excellent yield, Figure 102.

\[
\begin{align*}
(\text{RO})_2\text{POH} + \text{CCl}_4 + 2 \text{R'}\text{NH}_2 & \rightarrow (\text{RO})_2\text{P(O)HNHR}' + \text{CHCl}_3 + \text{R NH}_3\text{Cl}
\end{align*}
\]

**Figure 102.** Example of a typical reaction between a secondary phosphite and an amine in the presence of a polyhalo compound.

Ji et al. utilised basically the same methodology recently in their synthesis of N-(diisopropoxyphosphoryl) amino acids, Figure 103.

\[
\begin{align*}
(\text{PrO})_2\text{P(O)H} + \text{R} & \rightarrow 1. \text{Et}_3\text{N} / \text{CCl}_4 / \text{H}_2\text{O} / \text{EtOH} \\
& \rightarrow 0 - 20 ^\circ \text{C}, 4 - 16 \text{ h} \\
& \rightarrow 2. \text{H}_3\text{O}^+ 
\end{align*}
\]

**Figure 103.** Reaction scheme for the synthesis of N-(diisopropoxyphosphoryl) amino acids by the method of Ji et al.

It will be noted from the above that an amino acid with a free carboxyl moiety was linked successfully to a phosphate ester. Ji et al. then utilised their N-(diisopropoxyphosphoryl) amino acids as the building unit for the synthesis of N-(diisopropoxyphosphoryl) dipeptides, Figure 104.
It was reasoned that if a nucleoside 5'-alkyl hydrogen phosphonate (as shown in Figure 105(a)) could be synthesised, then this could be coupled to an unprotected amino acid by the method of Ji et al. (Figure 105(b)), leaving the carboxyl moiety free for building on an appropriate peptide.

Furthermore, nucleoside 5'-alkyl hydrogen phosphonates would themselves be suitable candidates for biological evaluation against HIV-1.

As a preliminary to the attempts at synthesising nucleoside 5'-alkyl hydrogen phosphonates...
phosphonates, it was decided to experiment with the methodology of Ji et al. in order to become familiar with the chemistry and to reveal any potential synthetic difficulties using inexpensive starting materials.

Thus experiment 34 reports the synthesis of N-(diethylphosphoryl) glycine according to the method shown in Figure 106.

![Figure 106. Reaction scheme for the synthesis of N-(diethylphosphoryl) glycine (experiment 34).](image)

The product was obtained in good yield (59%) although this represents a somewhat lower yield than those obtained by Ji et al. for their N-(diisopropoxycarbonyl) amino acids (71-95%). This might be due to faster hydrolysis of the diethyl phosphorochloridate intermediate formed in situ in the basic aqueous solution.

The high purity of the material formed in this reaction was confirmed by the phosphorus nmr spectrum, which showed only one resonance. The phosphorus nmr chemical shift of the product was 6.27 ppm, which was in the correct region for derivatives of this type. Ji et al. report phosphorus nmr chemical shifts of 5.6±0.5 ppm for N-(diisopropoxycarbonyl) amino acids.

In the carbon nmr spectrum the carbonyl carbon resonated downfield at 172.52 ppm, the signal being split into a doublet by three-bond phosphorus-carbon coupling. The methylene and methyl carbons in the ethoxy chains resonated at 62.49 and 15.79 ppm respectively, chemical shifts similar in value to those observed for the nucleoside phosphoramidates. Both signals were split into doublets by phosphorus-carbon coupling. The glycine methylene moiety gave a singlet signal at 42.18 ppm, as compared to, for example, 42.72 ppm in the compound AZT (ethyl methoxyglycylino) phosphoramidate.

In the proton nmr spectrum the exchangeable amino acid carboxylic proton was clearly seen as a broad singlet downfield at 12.43 ppm. All the other observed signals were assigned to moieties in the product.

Experiment 35 describes the coupling of N-(diethylphosphoryl) glycine prepared in experiment 34 with L-phenylalanine methyl ester, to afford the dipeptide.
compound \( N\)-(diethylphosphoryl) glycine-L-phenylalanine methyl ester (Figure 107).

\[
\text{(EtO)}_2\text{P(O)}\text{NHCH}_2\text{COOH} + \text{H}_2\text{NCH(CH}_3\text{Ph})\text{CO}_2\text{CH}_3 \to \text{(EtO)}_2\text{P(O)}\text{Cl}(1) / \text{Et}_3\text{N}(2)
\]

dichloromethane

\[
\text{(EtO)}_2\text{P(O)}\text{NHCH}_2\text{COOCH(CH}_3\text{Ph})\text{CO}_2\text{CH}_3
\]

**Figure 107.** Reaction scheme for the synthesis of \( N\)-(diethylphosphoryl) glycine-L-phenylalanine methyl ester (experiment 35).

Use of the coupling reagent ethyl phosphorochloridate/triethylamine followed by chromatographic purification afforded the product in 41% yield.

The compound isolated was fully characterised by spectroscopic and analytical techniques. The phosphorus nmr spectrum showed one signal at 6.20 ppm, indicating the bulk purity of the product. This was confirmed by hplc and microanalysis.

In the carbon nmr spectrum all the amino acid moieties gave clearly assignable resonances. The phenylalanine carboxylic carbon gave a singlet signal at 171.80 ppm, while the glycine carboxylic carbon resonated as a doublet at 170.01 ppm, the magnitude of the phosphorus-carbon coupling being 6.0 Hz. The aromatic carbons on the phenylalanine moiety resonated at typically low field, between 127.10 and 135.93 ppm. The methylene carbons in the ethoxy chains gave a multiplet resonance at 62.79 ppm, the signal being split due to phosphorus-carbon coupling. The methyl carbons in the ethoxy chains resonated as a doublet signal; the magnitude of the three-bond phosphorus-carbon coupling in this case was 5.8 Hz.

In the proton nmr spectrum the aromatic protons on the phenylalanine moiety and the amide proton resonated most downfield at ca. 7 ppm. The asymmetric CH proton resonated at 4.86 ppm as a multiplet, while the glycine CH\(_2\) protons resonated at 3.59 ppm, also as a multiplet, the deshielding effect of the ester moiety on the former accounting for the difference in chemical shift values. The methoxy protons gave a singlet signal at 3.70 ppm, while the methylene protons on the phenylalanine moiety resonated as a multiplet at 3.12 ppm. The signal due to the exchangeable glycine NH proton was noted at 3.84 ppm, appearing as a multiplet. Resonances for the methylene and methyl protons in the ethoxy chains gave multiplet signals at 4.00 and 1.27 ppm respectively.

In the electron impact mass spectrum a molecular ion was observed and the accurate mass measurement obtained found to be in good agreement with the
calculated value for the expected product. Fragmentation of the peptide part of the molecule predominated, with the most abundant ions retaining the phosphate part intact.

Although strictly a model compound for future synthetic work, N-(diethylphosphoryl) glycine-L-phenylalanine methyl ester was tested against HIV-1 \textit{in vitro} and showed an antiviral activity of 50 μM; however, this was noted to be a purely toxic effect. This suggests that toxicity may be a potential problem to be overcome for the nucleoside peptide-containing phosphoramidates.

Having concluded appropriate model studies it was decided to proceed to the synthesis of nucleoside 5′-alkyl hydrogen phosphonates and hopefully subsequent coupling reactions with amino acids and peptides.

The target compound decided upon was AZT ethyl hydrogen phosphonate (Figure 108). A Chemical Abstracts search revealed that this compound had not been synthesised before. It was therefore proposed to adopt the methodology of Garegg \textit{et al.}\textsuperscript{271}, who had synthesised 5′-protected 3′-alkyl hydrogen phosphonates.

![Figure 108. AZT ethyl hydrogen phosphonate.](image)

The proposed reaction scheme is indicated in Figure 109.

The strategy envisaged reaction of AZT with ethyl morpholinyl phosphorochloridite in the presence of diisopropylethylamine to afford a phosphoramidite intermediate. This would then be reacted with water and tetrazole in acetonitrile to give the desired product.
Figure 109. Proposed reaction scheme for the synthesis of AZT ethyl hydrogen phosphonate.

The preparation of the reactant ethyl morpholinyl phosphorochloridite necessitated first the synthesis of ethyl phosphorodichloridite, which is reported as experiment 36. Ethanol was added to a 10-fold excess of phosphorus trichloride at low temperature, employing diethyl ether as solvent, Figure 110.

\[
\text{EtOH} + \text{PCl}_3 \xrightarrow{\text{diethyl ether}} -78 \, ^\circ \text{C} \xrightarrow{} \text{EtOPCl}_2 + \text{HCl}
\]

Figure 110. Reaction scheme for the synthesis of ethyl phosphorodichloridite (experiment 36).

Distillation of the reaction mixture afforded the pure product, boiling at 116-120 °C (literature boiling point 116-118 °C\textsuperscript{272}).

The synthesis of ethyl morpholinyl phosphorochloridite by the method of Dörper and Winnacker\textsuperscript{273} is reported as experiment 37. The reaction scheme is indicated in Figure 111.
Two molar equivalents of morpholine were reacted with ethyl phosphorodichloridite in diethyl ether, the second equivalent being introduced to remove the HCl produced in the reaction by precipitation of morpholine hydrochloride. Filtration and distillation of the filtrate afforded the product as a colourless oil (boiling point 77-79 °C/0.05 mm Hg, as compared to the literature boiling point of 51-54 °C/0.03 mm Hg) in reasonable yield (37%, as compared to the literature yield of between 40 and 50%). The single resonance in the phosphorus nmr spectrum confirmed the purity of the product. The phosphorus nmr chemical shift was 168.60 ppm, which compares closely to the literature value of 172.1 ppm.

The carbon and proton nmr spectra confirmed the structure of the material; in the carbon nmr spectrum the morpholine moiety was clearly indicated, the O-methylene carbons resonating at 66.87 ppm as a doublet, the three-bond phosphorus-carbon coupling being 7.4 Hz. The N-methylene carbons resonated at 43.87 ppm also as a doublet, the two-bond phosphorus-carbon coupling being 9.6 Hz. The O-methylene protons gave a triplet signal at 3.66 ppm in the proton nmr spectrum, coupling to the N-methylene protons with a coupling constant of 5.07 Hz. The N-methylene protons resonated as a multiplet at 3.14 ppm, coupling to both the O-methylene protons and phosphorus.

The identity of the product was confirmed by the electron impact mass spectrum, which showed a molecular ion fragment the measured mass of which was in close agreement with the calculated mass for ethyl morpholinyl phosphorochloridite. Chlorine isotope effects were observed for several fragments, including the molecular ion itself. The presence of the morpholinyl moiety was also evident.

The first attempt at synthesising AZT ethyl hydrogen phosphonate via the intermediate AZT (ethyl morpholinyl) phosphoramidite is reported as experiment 38. AZT was reacted with ethyl morpholinyl phosphorochloridite in chloroform in the
presence of diisopropylethylamine, to afford the phosphoramidite intermediate (as shown in Figure 112), which was reacted in the next step without further purification.

Figure 112. Reaction scheme for the synthesis of the intermediate AZT (ethyl morpholinyl) phosphoramidite (experiment 38).

In the second stage of experiment 38, the phosphoramidite intermediate was activated with tetrazole and reacted with water in acetonitrile, Figure 113.

Figure 113. Reaction scheme for the proposed synthesis of AZT ethyl hydrogen phosphonate from the intermediate AZT (ethyl morpholinyl) phosphoramidite.

Concentration of the reaction mixture followed by chromatographic purification
afforded two main fractions, both in very low yield. The first fraction was found to be impure by phosphorus nmr spectroscopy, there being several signals in the range 4.21-11.90 ppm. The resonances at ca. 6 ppm were in the expected region for the desired hydrogen phosphonate: Garegg et al. report a phosphorus nmr chemical shift of 8.3 ppm for 5'-O-dimethoxytritylthymidine 3'-methyl hydrogen phosphonate\(^{271}\). However, the yield was so low (about 10 mg) that further purification was deemed impractical. The second fraction contained material contaminated with AZT; a range of signals was observed in the phosphorus nmr spectrum (-4.20 to 6.39 ppm), but again the poor yield precluded further purification.

The low yields and appearance of AZT in the later fractions during chromatography suggested that the product might be unstable, or that unwanted side reactions had occurred. Dörper and Winnacker acknowledge that the reaction of nucleoside with ethyl morpholinyl phosphorochloridite does not proceed cleanly, stating that phosphate impurities may amount to up to 15% of the material produced at the intermediate stage\(^{273}\). It is possible that impurities akin to those observed by Dörper were interfering in the subsequent reaction of the phosphoramidite with tetrazole and water in experiment 38. It was therefore decided to repeat the reaction, but this time the purity of the phosphoramidite intermediate would be checked by phosphorus nmr spectroscopy. Thus experiment 39 reports the reaction of AZT with ethyl morpholinyl phosphorochloridite in the presence of diisopropylethylamine using the same conditions as in the first stage of experiment 38. After work-up, the crude intermediate product was isolated. The phosphorus nmr spectrum of this material showed a series of signals at ca. 141 ppm, which was in the expected region for the desired phosphoramidite (Dörper and Winnacker report a phosphorus nmr chemical shift of 144.8 ppm for 5'-O-dimethoxytritylthymidine (methyl morpholinyl) phosphoramidite\(^{273}\)). There were several other signals between 6.04 and 30.33 ppm, corresponding to P(V) (phosphate) materials. It appeared that the desired intermediate species had been successfully formed, albeit of lower purity than achieved by others in the synthesis of trityl-protected nucleoside phosphoramidites\(^{273}\).

Reaction of the above product with tetrazole and water in acetonitrile, followed by work-up and chromatographic purification, gave two main fractions. The first fraction, again isolated in very poor yield, was highly impure when examined by phosphorus nmr spectroscopy, there being many signals in the range -3.10 to 11.54
ppm. The second fraction was similarly isolated in low yield and of low purity, chemical shift values between 0.82 and 19.70 ppm being observed for the resonances in the phosphorus nmr spectrum.

Decomposition of the product during chromatographic purification was considered a possible reason for the failure of this reaction to furnish materials of sufficient purity. Flushing the chromatographic column through with methanol and removal of the latter afforded a fraction of product showing signals at 0.82, 15.15 and 19.70 ppm in its phosphorus nmr spectrum, there being no trace of material with a chemical shift in the region 6-8 ppm corresponding to the desired product.

The experiment was repeated using a more polar eluent in the chromatographic purification to reduce the length of time that the product spent on the column, but this again afforded material in low yield and of low purity (this is not reported in the Experimental section).

The occurrence of side reactions during the formation of the phosphoramidite intermediate was considered to be the main reason for the failure to obtain the target compound using the above methodology. The large number of signals observed at ca. 141 ppm for the proposed intermediate AZT (ethyl morpholinyl) phosphoramidite and the occurrence of a high proportion of phosphate material at this early stage in the synthesis suggested that a competing reaction was taking place. Reaction of the nucleoside azide moiety with the desired P(III) phosphoramidite intermediate via the initial formation of a phosphite imine by analogy with the tertiary phosphines\textsuperscript{275} was considered the most likely possibility, since oxidation of such a species in the tetrazole/water stage might afford a phosphoramidate with a similar chemical shift to those observed for the final products of experiments 38 and 39, i.e. ca. 6 ppm, Figure 114.
Figure 114. Proposed side reaction occurring in the synthesis of AZT (ethyl morpholinyl) phosphoramidite; subsequent oxidation of the material produced would give a species with a chemical shift of ca. 6-8 ppm.

An alternative route to the nucleoside 5'-ethyl hydrogen phosphonates was therefore sought. Kluba and Zwierzak devised a general procedure for the synthesis of mixed dialkyl phosphites which afforded the products in reasonable yields and a high standard of purity.²⁷⁶ (Figure 115).
The readily prepared sodium alkyl hydrogen phosphites and tetra-\(n\)-butylammonium hydrogen sulphate were reacted together to afford tetra-\(n\)-butylammonium alkyl hydrogen phosphites. Good yields of the mixed dialkyl phosphites were then obtained by heating stoichiometric amounts of the tetra-\(n\)-butylammonium alkyl hydrogen phosphites and alkyl iodides together in acetonitrile at 50 °C for 5 hours. Kluba and Zwierzak state the latter reaction to be clean, straightforward and unaccompanied by undesirable side-processes, making it ideal for application to nucleoside chemistry. Thus the proposed reaction scheme for the synthesis of AZT ethyl hydrogen phosphonate by the above methodology is shown in Figure 116.

Sodium ethyl hydrogen phosphite was prepared by the method of Nylen in experiment 40. The product was isolated in good yield and in a pure form as evidenced by phosphorus nmr (one signal with a chemical shift of 7.12 ppm) and
melting point (182 °C; literature melting point 182-183 °C\textsuperscript{276}).

Tetra-n-butylammonium ethyl hydrogen phosphite was synthesised in experiment 41 according to the published procedure\textsuperscript{276}. The crude product was isolated as a yellow syrup in good yield (85% as compared to a literature yield of 79.5%) and an analytical state of purity. One resonance was observed in the phosphorus nmr spectrum at 1.62 ppm. The proton nmr spectrum was in close agreement with that reported in the literature for the target compound\textsuperscript{276}. In particular, the P(O)H proton resonated at 6.90 ppm as a doublet, with a one-bond phosphorus-proton coupling constant of 586 Hz (as compared to 6.88 ppm, 579 Hz\textsuperscript{279}). The methylene protons on the n-butyl moieties were unresolved at 80 MHz according to the literature; at 200 MHz they were resolved into three distinct multiplet signals.

It was decided to employ the 5'-deoxy-5'-iodo derivative of the relatively inexpensive 3'-O-acetylthymidine rather than AZT in the trial reactions with tetra-n-butylammonium ethyl hydrogen phosphite. 3'-O-acetyl-5'-deoxy-5'-iodothymidine was synthesised in experiment 42 by the procedure of Verheyden and Moffat\textsuperscript{279}. 3'-O-acetylthymidine (synthesis reported in experiment 50 - see later for discussion) was reacted with methyltriphenoxyphosphonium iodide in DMF according to the reaction scheme as indicated in Figure 117.

![Figure 117. Reaction scheme for the synthesis of 3'-O-acetyl-5'-deoxy-5'-iodothymidine (experiment 42).](image)

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Following chromatographic purification the product was isolated in good yield and was characterised by proton nmr spectroscopy and its melting point, which was in agreement with the value quoted in the literature\textsuperscript{280}.

The reaction of 3'-O-acetyl-5'-deoxy-5'-iodothymidine with tetra-n-butylammonium ethyl hydrogen phosphite was carried out in acetonitrile at 50 °C (Figure 118) (not reported in the Experimental section), using a 1:1 molar ratio of reactants. After 45 hours there was no reaction observed by tlc.

\[
\begin{align*}
\text{EtO}_2P(O)O^{-(nC_4H_{9})_4N^+} + I^- & \quad \xrightarrow{\text{CH}_3CN, 50 ^\circ \text{C}, 45 h} \quad \text{OEt} \quad \text{O} \\
& \quad \text{OC(O)CH}_3 \\
& \quad \text{OC(O)CH}_3
\end{align*}
\]

\textbf{Figure 118.} Proposed reaction scheme for the synthesis of 3'-O-acethylthymidine ethyl hydrogen phosphonate in acetonitrile.

The reaction was repeated using DMF as solvent and an excess of the phosphitylating reagent, but even after heating the mixture for several hours at 120 °C no reaction was observed by tlc.

Unfortunately, this strategy for the synthesis of nucleoside 5'-ethyl hydrogen phosphonates could not be pursued further. However, the above methodology may yet prove useful in future studies, not only in respect of the synthesis and evaluation of the hydrogen phosphonates, but also in the synthesis of alkyl peptidyl phosphoramidates mentioned earlier.

Having examined the effect of modification of the phosphoramidate moiety on the anti-HIV activity of the nucleoside monophosphate derivatives, the final section of work explored the structure-activity relationships arising from the variation of the nucleoside moiety. Osei-Kissi had shown\textsuperscript{281} that removal of AZT and replacement by simple methyl and ethyl chains (Figure 119) led to a complete abolition of activity.

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McGuigan et al. synthesised thymidine 5'-(butyl methoxy-L-valinyl) phosphoramidate\textsuperscript{234}, as shown in Figure 120, but unsurprisingly this derivative was inactive against HIV \textit{in vitro}. The inactivity of this compound suggested that the phosphoramidates do not possess anti-HIV activity in their own right, but rather that they require activation to either the nucleoside, the nucleotide or a closely related derivative.

The introduction of a bioactive nucleoside moiety other than AZT had not been attempted. The nucleoside analogue 3'-azido-2',3'-dideoxyuridine (AzddUrd) (Figure 121) has been evaluated as an anti-HIV agent and, although less potent than AZT \textit{in vitro}\textsuperscript{282-285}, it shows significantly lower toxicity towards bone marrow cells\textsuperscript{285}. AzddUrd is less effective than AZT against HIV in peripheral blood mononuclear cells; this appears to be related to the lower affinity of AzddUrd for the enzyme responsible for its initial phosphorylation\textsuperscript{286}.
AzddUrd was therefore deemed a suitable candidate for the synthesis of an appropriate phosphoramidate derivative, and the next target compound is illustrated in Figure 122(a). The analogous compound AZT (ethyl methoxy-L-valinyl) phosphoramidate (synthesised previously\textsuperscript{234}) is shown in Figure 122(b).

AzddUrd was synthesised by the method of Lin \textit{et al.}\textsuperscript{287}, according to the reaction scheme indicated in Figure 123.
1-(2-Deoxy-5-O-trityl-β-D-threo-pentofuranosyl)uracil (donated by Pfizer) was converted to the methanesulphonyl derivative in pyridine using methanesulphonyl chloride (experiment 43). Following chromatographic purification the intermediate product, 1-(2-deoxy-3-O-methanesulphonyl-5-O-trityl-β-D-threopentofuranosyl)uracil, was isolated in good yield. Confirmation of the structure and purity of the product was by proton nmr and melting point (157 °C; literature melting point 152-155 °C). Reaction of this compound with sodium azide in DMF (experiment 44) afforded 3'-azido-5'-O-trityl-2',3'-dideoxyuridine after recrystallisation, which was detritylated in aqueous acetic acid to give AzddUrd in reasonable yield. The melting point of 167-170 °C was in agreement with the value of Krenitsky et al., 166.5-168.5 °C. Hplc confirmed the bulk purity of the compound, which was also characterised by proton nmr spectroscopy.

![Reaction scheme for the synthesis of AzddUrd (experiments 43 and 44).](image)

Condensation of this nucleoside with ethyl methoxy-L-valinyl phosphorochloridate was performed in experiment 45, using the THF/N-methyl imidazole conditions, as shown in Figure 124.
The product was isolated in very good yield and spectroscopic data collected which were in agreement with the structure proposed for the target compound. The spectra were analogous to those obtained earlier for the AZT derivative prepared by McGuigan et al., except that in the carbon nmr spectrum the signal for C5 on the nucleoside base was upfield by ca. 8 ppm as compared to the C5 resonance on a thymine base. Also, in the proton nmr spectrum the appearance of a resonance due to the proton at position 5 on the uracil base was noted, appearing as a doublet (coupling to the proton at position 6, which also resonated as a doublet). The phosphorus nmr spectrum showed two signals (one for each diastereoisomer present in the mixture) in a ratio of 2:1 at 6.96 and 6.84 ppm. This ratio of diastereoisomers was confirmed in the carbon nmr spectrum (for example by the signal at C1) and the proton nmr spectrum (for example, by the signal from the proton at position 6 on the nucleoside base).

In the electron impact mass spectrum a molecular ion was observed; the accurately measured mass of this ion was in good agreement with the calculated
molecular mass of the target compound. Extensive fragmentation was noted. Loss of methanol, ethene, uracil, the azide moiety, water, propane and the entire phosphoramidate moiety from the molecular ion was observed. The subsequent fragmentation of the latter species was strongly evident as usual, with the loss of small molecules such as ethanol, methanol, water, propane and propene (the latter two indicative of the valine amino acid moiety). Loss of the entire valine amino acid ester from the phosphoramidate fragment was observed, and nucleosidic sugar and base fragments were also noted. Hplc data were obtained after further chromatography, whereupon the bulk purity and AzddUrd-free nature of the product were both confirmed.

AzddUrd (ethyl methoxy-L-valinyl) phosphoramidate was evaluated against HIV-1 in vitro as a mixture of its diastereoisomers and showed an activity of 110 μM.

Thus it appears that substitution of the thymine base in the compound AZT (ethyl methoxy-L-valinyl) phosphoramidate (active at 10 μM) with uracil leads to a substantial diminution in activity. This is perhaps surprising, in view of the fact that AzddUrd, while not usually as effective as AZT against HIV in vitro, nevertheless often retains at least a moderate potency. However, it should be noted that the biological evaluation procedure employed is particularly sensitive to AZT. Biological evaluation in other in vitro tests may therefore be necessary to assess the effectiveness of this compound.

In the next target analogue the nucleoside moiety was 2',3'-dideoxycytidine (ddCyd, Figure 125(a)). The in vitro and in vivo activity of this nucleoside analogue were discussed in the Introduction.

![Figure 125. (a) ddCyd (b) Target compound ddCyd (ethyl methoxy-L-valinyl) phosphoramidate.](image-url)
Reactions with ddCyd pose two problems. First, the nucleoside is poorly soluble in all the common solvents. Second, unlike AZT, ddCyd has two possible sites of reaction: the 5'-hydroxyl group and the 4-amino moiety on the nucleoside base.

The initial attempt at condensation of ddCyd with ethyl methoxy-L-valinyl phosphorochloridate is reported as experiment 46. Pyridine was adopted as the reaction solvent after observing that the dissolution of the nucleoside may be effected by heating a suspension of ddCyd in pyridine and then cooling the resultant solution to room temperature, whereupon the nucleoside stayed in solution. The reaction scheme is shown in Figure 126.

Two products were observed by tlc. Aqueous work-up and chromatographic purification afforded material which was impure according to its phosphorus nmr spectrum. The desired product seemed to be indicated by the signal at 8.19 ppm, but an impurity which gave a resonance at ca. 10 ppm could not be removed, despite several attempted chromatographic purifications. It was thought that this impurity was the N-phosphorylated nucleoside. Because this latter derivative would have a similar structure to the desired material, it was suspected that co-elution during chromatography was precluding good separation of the compounds.

In order to avoid the problem of base phosphorylation, it was decided to
protect the -NH₂ functionality of ddCyd before attempting condensation with a phosphorochloridate. An appropriate protecting group was therefore sought which could be removed from the final compound under mild (if possible, neutral) conditions, since the P-O bond is base sensitive and the P-N bond acid sensitive. Such a protecting group - the N-dimethylaminomethylene group - was developed by Zemlicka and Holy²⁸⁹,²⁹⁰, who reported its application to the protection of both ribo- and deoxynucleosides²⁹¹. Its removal can be performed simply by refluxing the base-protected compound in ethanol. Thus, the protection of the amino functionality of ddCyd is reported as experiment 47. Seven molar equivalents of dimethylformamide dimethyl acetal were reacted with ddCyd in DMF (Figure 127).

![Figure 127. Reaction scheme for the synthesis of N-dimethylaminomethylene-2',3'-dideoxycytidine (experiment 47).](image)

After work-up, N-dimethylaminomethylene-2',3'-dideoxycytidine was isolated in very good yield. This novel compound was characterised by proton and carbon nmr spectroscopy, fast atom bombardment mass spectrometry and microanalysis. In the proton nmr spectrum, all the base-protecting group protons gave assignable resonances: the N=CH proton resonated at 8.61 ppm and the two N-CH₃ groups resonated at 3.16 and 3.03 ppm. In the carbon nmr spectrum the resonances at 157.62 and 155.01 ppm could not be unequivocally assigned to either the C2 or the N=CH moieties. The two N-CH₃ groups resonated at 40.70 and 34.63 ppm.

In the fast atom bombardment mass spectrum a protonated molecular ion was observed and fragments corresponding to the nucleoside sugar and base were noted. Microanalysis figures were acceptable for the elements C, H and N.

The reaction of N-dimethylaminomethylene-2',3'-dideoxycytidine with ethyl methoxy-L-valinyl phosphorochloridate is reported as experiment 48, as shown in the reaction scheme indicated in Figure 128.

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In order to check whether the protecting group would be stable to the N-methyl imidazole condensation conditions, the protected nucleoside was stirred with the base in THF for one hour. No decomposition was observed by tlc and so ethyl methoxy-L-valinyl phosphorochloridate was added. After five hours the reaction had gone to completion, there being two products. It was decided to incorporate a citric acid wash into the usual work-up procedure, in the hope that removal of the protecting group could be achieved before purification. This was unsuccessful, and so the crude product was refluxed with ethanol overnight as described to afford the deprotected phosphoramidate. After chromatographic purification a product was isolated in low yield. Although the phosphorus nmr spectrum indicated that the product had been isolated in a pure form (two signals - two diastereoisomers), the carbon nmr spectrum showed the presence of an impurity; for example, there were four signals noted for C6 (there should have been a maximum of two) and there were five signals observed
for the valine isopropyl methyl groups (there should have been a maximum of four).

It appeared that N-dimethylaminomethylene protection of ddCyd followed by phosphorochloridate condensation did not give rise to the facile production of the phosphoramidate derivative that was hoped for. The product was therefore purified by preparative hplc.

The phosphorus nmr spectrum of the purified material showed one signal at 5.56 ppm. The structure of the compound was confirmed by proton nmr spectroscopy and fast atom bombardment mass spectrometry. In the proton nmr spectrum H2' and H3' resonated at low field as expected for 2',3'-dideoxynucleoside derivatives. The presence of the valinyl phosphoramidate moiety was confirmed by the observation of resonances corresponding to the methoxy, the isopropyl and the isopropyl methyl protons and the valine NH proton.

In the fast atom bombardment mass spectrum a protonated molecular ion and fragments corresponding to the loss therefrom of the nucleoside base and sugar moieties were noted. Extensive fragmentation of the phosphoramidate moiety indicative of the amino acid valine was also observed, with the loss of molecules such as ethanol, ethene, propene and propane from the main phosphoramidate fragment.

The biological evaluation of ddCyd (ethyl methoxy-L-valinyl) phosphoramidate against HIV-1 in vitro is awaited.

It was decided to continue the synthesis of analogues in this series with nucleosides which showed no activity against HIV. As mentioned in the Introduction, one of the aims of the phosphate prodrug approach is to obviate the dependence of nucleoside analogues on nucleoside kinases for their activation. If nucleoside monophosphates can be liberated intracellularly, any stringent nucleoside kinase specificity in theory becomes irrelevant. This is because, once so liberated, the nucleoside monophosphates can be anabolised to the corresponding di- and triphosphates by other cellular kinases.

Three non-bioactive nucleosides were therefore selected for utilisation in the synthesis of appropriate phosphoramidate derivatives.

The first nucleoside so chosen was 3'-O-acetylthymidine. The target molecule is indicated in Figure 129. For consistency with the bioactive nucleoside series of compounds, the ethyl methoxy-L-valinyl phosphoramidate moiety was retained.
3'-O-acetylthymidine was synthesised from thymidine in three steps by standard procedures\textsuperscript{280,292} (experiments 49 and 50, Figure 130).

The resultant material was characterised by proton nmr spectroscopy and its identity verified by comparison to the literature melting point (177 °C as compared to 176 °C reported by Michelson and Todd\textsuperscript{280}).
The synthesis of 3'-O-acetylthymidine (ethyl methoxy-L-valinyl) phosphoramidate using THF/N-methyl imidazole conditions is reported as experiment 51 (Figure 131).

Figure 131. Reaction scheme for the synthesis of 3'-O-acetylthymidine (ethyl methoxy-L-valinyl) phosphoramidate (experiment 51).

The product was isolated in somewhat low yield, but the reason for this was unclear. The compound was fully characterised by phosphorus, carbon and proton nmr spectroscopy, electron impact mass spectrometry, microanalysis and hplc. The phosphorus nmr spectrum showed one signal at 6.63 ppm, the two diastereoisomers giving coincident resonances. The presence of two isomers was, however, clearly evident from the carbon nmr spectrum. For instance, for C4' there were two signals in a 5:3 ratio and for C5' there was a pair of doublets (due to phosphorus-carbon coupling) in a 3:5 ratio. The C3' resonance occurred at 74.51 ppm (as compared to a chemical shift of ca. 60 ppm in the 3'-azido nucleosides), and the two carbons on the acetyl moiety were assigned to the resonances at 170.38 and 20.84 ppm.

In the proton nmr spectrum the acetyl methyl group was assigned to the singlet at 2.11 ppm. H3' resonated as two multiplets in a ratio of 3:2 at ca. 5.3 ppm (H3' in the analogous azide-containing compounds usually resonated at ca. 4.4 ppm, indicating the greater deshielding effect exerted by the acetyl moiety at this position).
An accurate mass measurement corresponding to the molecular ion of the target compound was observed in the electron impact mass spectrum and the usual phosphoramidate and nucleosidic fragments were noted. Fragmentation of the valine part of the molecule was evident from the loss of propene and propane from the molecular ion and the ion corresponding to the phosphoramidate moiety.

Microanalysis data were acceptable for C, H, N and P and hplc too confirmed the bulk purity of the compound, the two diastereoisomers showing retention times of 21.2 and 20.9 minutes.

3'-O-acetylthymidine (ethyl methoxy-L-valinyl) phosphoramidate was evaluated against HIV-1 in vitro and showed an activity of 40 μM.

While this level of activity was not especially high, that there was any activity at all shown by this derivative was particularly significant. Given the inactivity of the parent nucleoside towards HIV-1, the anti-HIV effectiveness of this phosphoramidate derivative lends support to the kinase by-pass theory mentioned earlier. This finding further suggests that dideoxynucleosides hitherto considered inactive because of inherently poor affinity for nucleoside kinases could now be developed as anti-HIV agents by employing the phosphoramidate prodrug strategy. The application of this approach to two further non-bioactive nucleosides will be discussed shortly.

It will be recalled from the Introduction that bis(2,2,2-trihaloethyl) phosphate triesters of both AZT and ddCyd displayed anti-HIV activity in vitro. In order to investigate whether a bis(2,2,2-trihaloethyl) phosphate group could impart activity to a non-bioactive nucleoside such as 3'-O-acetylthymidine in a manner similar to the phosphoramidate moiety discussed above, it was decided to synthesise the compound 3'-O-acetylthymidine bis(2,2,2-trifluoroethyl) phosphate, as shown in Figure 132.

![Figure 132. 3'-O-acetylthymidine bis(2,2,2-trifluoroethyl) phosphate.](image-url)
The method of Tollerfield was employed. The nucleoside was dissolved in pyridine and an excess of bis(2,2,2-trifluoroethyl) phosphorochloridate added (experiment 52) (Figure 133).

![Reaction scheme for the synthesis of 3'-O-acetylthymidine bis(2,2,2-trifluoroethyl) phosphate (experiment 52).](image)

Figure 133. Reaction scheme for the synthesis of 3'-O-acetylthymidine bis(2,2,2-trifluoroethyl) phosphate (experiment 52).

Following aqueous work-up and chromatographic purification the product was isolated in good yield. The phosphorus nmr chemical shift of the product was -3.53 ppm which compares to a shift of -2.52 ppm for AZT bis(2,2,2-trifluoroethyl) phosphate. The signal from the CF₃ carbons was split into a quartet of doublets in the carbon nmr spectrum, with coupling constants of 277.6 Hz for the one-bond carbon-fluorine coupling and 9.1 Hz for the three-bond carbon-phosphorus coupling. The signal from the methylene carbons in the trifluoroethyl chains was similarly split into a quartet of doublets, the two-bond carbon-fluorine coupling being 38.2 Hz and the two-bond carbon-phosphorus coupling being 4.0 Hz.

The proton nmr spectrum and the electron impact mass spectrum confirmed the identity of the product as the target molecule, the latter providing an accurate mass measurement which was in excellent agreement with the proposed molecular mass. Fragments corresponding to the phosphate moiety, the thymine base and the
sugar moiety were all noted in the electron impact mass spectrum and fragmentation by the loss of trifluoromethane and trifluoroethanol supported the structural characterisation.

Despite microanalysis results which were discrepant for carbon, the bulk purity of the compound was confirmed by hplc.

3'-O-acetylthymidine bis(2,2,2-trifluoroethyl) phosphate was found be inactive against HIV-1 \textit{in vitro}. The inactivity of this derivative suggests that the bis(2,2,2-trihaloethyl) phosphate moiety does not function in the same manner as a phosphoramidate moiety in conferring anti-HIV activity on nucleosides which are themselves ineffective against the virus. This difference may possibly be attributable to a different mechanism of action: liberation of a nucleoside rather than a nucleotide from a bis(2,2,2-trihaloethyl) phosphate ester would mean that such a compound would be dependent on substrate-specific nucleoside kinases for its activation, whereas liberation of the nucleotide from a phosphoramidate would involve no such dependence.

Returning now to the phosphoramidate series modified in the nucleoside moiety, the next target compound to be synthesised is indicated in Figure 134.

![Figure 134. Second phosphoramidate derivative to incorporate a non-bioactive nucleoside moiety, 3'-O-methanesulphonylthymidine.](image)

The nucleoside analogue 3'-O-methanesulphonylthymidine is ineffective against HIV \textit{in vitro}. The synthesis of this nucleoside in good yield from 5'-O-tritylthymidine using a literature procedure is described in experiment 53 according to the reaction scheme shown in Figure 135.

Melting point determination gave a value which was identical to that quoted in the literature, confirming both the identity and purity of the product.
The condensation of the nucleoside with ethyl methoxy-L-valinyl phosphorochloridate using THF/N-methyl imidazole conditions is described as experiment 54. The reaction scheme that was used is indicated in Figure 136.

Figure 135. Reaction scheme for the synthesis of 3'-O-methanesulphonylthymidine (experiment 53).

Figure 136. Reaction scheme for the synthesis of 3'-O-methanesulphonylthymidine (ethyl methoxy-L-valinyl) phosphoramidate (experiment 54).
The phosphoramidate product was isolated in rather poor (20%) yield after chromatography. The phosphorus nmr spectrum showed one resonance at 6.67 ppm. In the carbon nmr spectrum the C3' resonated downfield (as compared to the 3'-O-acetyl and 3'-azido derivatives) at 79.30 ppm, due to the strong deshielding effect exerted by the methanesulphonyl moiety. The methanesulphonyl methyl carbon resonated at 37.89 ppm. Three-bond phosphorus-carbon couplings to C4' (7.4 Hz), the carbonyl carbon on the valine moiety (2.8 Hz), the isopropyl carbon on the valine moiety (6.8 Hz) and the methyl carbon in the ethoxy chain (7.2 Hz) were noted, as well as two-bond phosphorus-carbon couplings to C5' (5.4 Hz) and the methylene carbon in the ethoxy chain (5.4 Hz). None of the resonances in the carbon nmr spectrum appeared as more than one signal to indicate the presence of the two diastereoisomers, and similar observations were made for the signals in the proton nmr spectrum. The H3' proton resonated at 5.42 ppm (downfield of the chemical shift of the analogous proton in the 3'-O-acetylthymidine derivative by 0.14 ppm). The methanesulphonyl methyl protons resonated as a singlet at 3.13 ppm.

In the fast atom bombardment mass spectrum no molecular ion was observed, but several diagnostic fragments indicated that the target compound had been formed. The highest molecular weight fragment corresponded to loss of the methanesulphonyl moiety from the molecular ion. The distinctive phosphoramidate valine-containing fragment was evident with its characteristic fragmentation pattern consisting of the loss of small molecules such as water, ethanol, propane, ethene, methanol and propene. The presence of the nucleosidic part of the target structure was confirmed by the presence of fragments corresponding to the thymine base and the nucleoside sugar.

Hplc confirmed the bulk purity of the compound and also indicated that two diastereoisomeric forms were present, with retention times differing by 12 seconds.

3'-O-methanesulphonylthymidine (ethyl methoxy-L-valinyl) phosphoramidate was evaluated against HIV-1 in vitro and showed activity at the highest concentration studied, 200 µM.

As noted earlier for the 3'-O-acetylthymidine phosphoramidate derivative, although the parent nucleoside shows no activity towards HIV in vitro, the phosphoramidate compound displayed (in this case weak) activity. That the derivative displayed any activity at all is significant, showing again the potential utility of the
phosphate prodrug approach in conferring activity on previously inactive compounds. However, the anti-HIV effectiveness of the methanesulphonyl compound was appreciably less than that of the acetyl compound.

Hydrolysis of the P-N bond as envisaged, followed by conversion of the nucleoside ethyl phosphate to the nucleotide by (for example) phosphodiesterase, would still leave the compounds dependent on cellular kinases for further activation to the triphosphate, which presumably would be the active entity. Thus any major structural deviation from the natural nucleotide, such as substitution of the 3'-hydroxyl moiety by the bulky and electronically dissimilar methanesulphonyl moiety, may prevent the compound from being accepted as a good substrate by these kinases, and thus preclude proper activation. The size and nature of the acetyl moiety may make the relevant nucleotide derivative more acceptable as a substrate to the putative kinase enzymes.

In order to probe further the effect on anti-HIV activity of variation in the nucleoside moiety, the phosphoramidate derivative of the highly modified nucleoside analogue 1-(2-deoxy-3-O-methanesulphonyl-β-D-threo-pentofuranosyl)thymine was synthesised. Thus in experiment 55, this nucleoside analogue (donated by Pfizer) was reacted with ethyl methoxy-L-valinyl phosphorochloridate using the THF/N-methyl imidazole condensation conditions, as shown in the reaction scheme indicated in Figure 137.

![Figure 137. Reaction scheme for the synthesis of 1-(2-deoxy-3-O-methanesulphonyl-β-D-threo-pentofuranosyl)thymine (ethyl methoxy-L-valinyl) phosphoramidate (experiment 55).](image-url)
The product was isolated in good yield following chromatography. The phosphorus nmr chemical shifts of the two diastereoisomeric forms of the product were 6.74 and 6.55 ppm (4:3 ratio), which were in the appropriate region for phosphoramidate derivatives. In the carbon nmr spectrum all the usual phosphorus-carbon couplings were observed. Some signals within three bonds of phosphorus (for example the carbonyl carbon on the amino acid and the C4' carbon) showed not only two distinct resonances (one for each isomer) but also signal splitting arising from phosphorus-carbon coupling. The C3' resonated at 77.85 ppm, as compared to 77.30 ppm in the α-methanesulphonyl derivative discussed earlier. The methanesulphonyl methyl carbon resonated at 38.45 ppm, downfield of the comparable signal in the α-methanesulphonyl compound by ca. 0.6 ppm, possibly because of the proximity of the aromatic nucleoside base in the β-methanesulphonyl derivative. Both of the isopropylmethyl groups on the valine moiety resonated upfield as two signals; both showed the 4:3 ratio of diastereoisomeric products.

In the proton nmr spectrum the methanesulphonyl methyl protons gave two distinct singlet resonances at 3.18 and 3.17 ppm, the signals appearing in a 4:3 ratio corresponding to the ratio of diastereoisomers isolated. H3' resonated at 5.33 ppm, upfield by ca. 0.1 ppm as compared to H3' in the α-methanesulphonyl derivative, again probably reflecting the proximity of the thymine base to the H3' proton in the latter compound. The exchangeable 3-NH proton on the thymine base was not observed; the signal due to the valine NH proton was probably coincident with that of the methylene protons in the ethoxy chain, giving a multiplet integrating for three protons at 4.06 ppm.

The electron impact mass spectrum confirmed the identity of the product as the target phosphoramidate, with an accurate mass reading for the molecular ion in good agreement with that calculated for the target derivative. Fragmentation by the loss of the methanesulphonyl moiety from the molecular ion provided further confirmatory evidence for the identity of the product as that of the target compound. Extensive fragmentation of the phosphoramidate moiety (as usual) was strongly evident and the 100% abundance of a nucleoside sugar fragment was observed. Microanalysis and hplc confirmed the bulk purity of the compound.

1-(2-Deoxy-3-0-methanesulphonyl-β-D-threo-pentofuranosyl)thymine (ethyl methoxy-L-valinyl) phosphoramidate was tested as a mixture of its diastereoisomers
and was inactive against HIV-1 \textit{in vitro}.

Even if hydrolysis of this derivative were to afford the nucleoside monophosphate, the structure of the nucleotide may be too remote from that of a natural substrate to be accepted by the kinases necessary for further phosphorylation. The bulky methanesulphonyl substituent at the 3'-\(\beta\)-position may, for instance, affect the conformation of the nucleoside sugar. Correlations between the preferred sugar ring conformation and the activity of the nucleoside analogues against HIV have been made\textsuperscript{296,297}. It is possible that an unfavourable sugar conformation in the present derivative may impair its ability to perform as a substrate for the relevant kinases. Alternatively, the inactivity of this derivative may stem from the proximity of the \(\beta\)-methanesulphonyl moiety to the nucleoside base, which might impede the free rotation of the latter about the glycosidic bond, thus preventing the nucleotide from serving as an appropriate substrate in the subsequent kinase-mediated phosphorylation.

Having prepared three derivatives containing a non-bioactive nucleoside and two derivatives containing a nucleoside known to be active against HIV, to complete the series it was decided to synthesise the compound shown in Figure 138, derived from the known anti-HIV agent 2',3'-dideoxy-2',3'-didehydrothymidine (d4T) (discussed in the Introduction).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure138.png}
\caption{Phosphoramidate derivative incorporating the bioactive nucleoside moiety d4T.}
\end{figure}

Herdewijn \textit{et al.} had reported the reaction of 1-(2-deoxy-3-O-methanesulphonyl-5-O-trityl-\(\beta\)-D-threo-pentofuranosyl)thymine with tetra-\(n\)-butylammonium fluoride (TBAF) in THF to afford 5'-O-trityl-2',3'-dideoxy-2',3'-didehydrothymidine\textsuperscript{188}, as shown in Figure 139.
Figure 139. Synthesis of 5'-O-trityl-2',3'-dideoxy-2',3'-didehydrothymidine by the method of Herdewijn et al.

Similar methodology was employed in experiment 56: 1-(2-deoxy-3-O-methanesulphonyl-β-D-threopentofuranosyl)thymine (ethyl methoxy-L-valinyl) phosphoramidate was reacted with TBAF in THF, Figure 140, with the phosphoramidate moiety used as a pseudo 5' protecting group.

Figure 140. Proposed reaction scheme for the synthesis of d4T (ethyl methoxy-L-valinyl) phosphoramidate (experiment 56).

Work-up and chromatography failed to produce the desired compound, no uv active materials being retrieved from the column. The most likely reason for the failure of this reaction to furnish the target compound was attack by fluoride ion on the phosphorus causing decomposition.

Another method for producing the nucleoside d4T from 1-(2-deoxy-3-O-
methanesulphonyl-5-O-trityl-β-D-threo-pentofuranosyl)thymine involves reaction of
the latter with two molar equivalents of potassium t-butoxide in DMSO, followed by
detritylation (Figure 141), the major problem in this synthesis being removal of the
DMSO.

Figure 141. Synthesis of d4T from 1-(2-deoxy-3-O-methanesulphonyl-5-O-trityl-β-
D-threo-pentofuranosyl)thymine.

In experiment 57 two molar equivalents of potassium t-butoxide were reacted with 1-(2-deoxy-3-O-methanesulphonyl-β-D-threo-pentofuranosyl)thymine (ethyl methoxy-L-valinyl) phosphoramidate in DMSO (Figure 142).

Figure 142. Reaction scheme for the synthesis of d4T (ethyl methoxy-L-valinyl)
phosphoramidate (experiment 57).

The reaction appeared to proceed to about 80% completion on tlc. The product was
then precipitated out by pouring the reaction mixture into diethyl ether. Chromatographic purification afforded a product with two signals in its phosphorus nmr spectrum, which were tentatively assigned to the two diastereoisomeric forms of the target compound. However, the proton nmr spectrum of the product indicated that a nucleosidic impurity was present, there being extra resonances with chemical shifts close in value to the signals assigned to the product's H1', H4' and H5' protons. The product was therefore purified by preparative hplc.

The phosphorus nmr spectrum of the purified material gave one resonance at 5.96 ppm. The proton nmr spectrum was used to confirm the identity of the product as the target compound. In the following comparison, the chemical shifts in parentheses are those recorded by Herdewijn et al. for the equivalent protons in 5'-O-trityl-2',3'-dideoxy-2',3'-didehydrothymidine (in deuterochloroform)\textsuperscript{188}. The broad singlet downfield at 8.30 ppm was assigned to the exchangeable thymine base 3-NH proton. The singlet at 7.38 ppm was assigned to the proton at position 6 on the thymine base. The multiplets at 7.04, 6.36, 5.88 and 4.99 ppm were assigned to H1', H3', H2' and H4' respectively (7.05, 6.36, 5.88 and 4.96 ppm respectively). The methylene protons in the ethoxy chain and the protons at the 5' position on the nucleoside sugar resonated as a multiplet at 4.14 ppm (3.39 ppm for the H5' protons). All the chemical shifts noted for the protons on the amino acid moiety were consistent with those observed in previous compounds. The methyl protons at the 5 position on the thymine base resonated as a singlet at 1.92 ppm (1.28 ppm). The methyl protons in the ethoxy chain gave a triplet of doublets, the three-bond proton-proton coupling being 7.05 Hz, the four-bond phosphorus-proton coupling being 0.7 Hz.

The excellent correlation with literature chemical shifts, particularly for the olefinic protons H2' and H3', confirmed the identity of the product of experiment 57 as d4T (ethyl methoxy-L-valinyl) phosphoramidate. That the H5' protons were shifted downfield in the phosphoramidate compound as compared to the trityl derivative was unsurprising, given the strong deshielding effect exerted by the former as noted previously.

No extra signals arising from the presence of more than one diastereoisomer were noted in either the phosphorus or the proton nmr spectrum, and so presumably only one isomer was isolated in the hplc purification.

In the fast atom bombardment mass spectrum a protonated molecular ion was
observed. Loss of the thymine base, the double bond-containing sugar moiety and the phosphoramidate moiety from the protonated molecular ion provided further evidence for the identity of the product as that of the target compound.

Biological evaluation of d4T (ethyl methoxy-L-valinyl) phosphoramidate against HIV-1 *in vitro* is awaited.

In summary, six compounds were synthesised in which only the nucleoside moiety was varied. It appears that a bioactive nucleoside is not a prerequisite for activity in the amino acid-containing phosphoramidate series of compounds. However, anti-HIV effectiveness does appear to depend strongly on the structure of the nucleoside analogue employed; major deviation from the structure of a natural nucleoside results in poor activity.

The temptation to attribute the modest activity shown by the two compounds 3′-O-acetylthymidine (ethyl methoxy-L-valinyl) phosphoramidate and 3′-O-methanesulphonylthymidine (ethyl methoxy-L-valinyl) phosphoramidate to the success of the phosphoramidate approach in achieving prodrug activation without reliance on nucleoside kinases is strong, but further mechanistic investigations need to be carried out before such a hypothesis can be supported. Nevertheless, the ability of the phosphoramidate moiety to impart activity to non-bioactive nucleosides by whatever mechanism remains a significant discovery which provides a basis for future research in this area.
Summary and Conclusions

A procedure for the synthesis of alkyl amino acyl phosphorochloridates in high yield and analytically pure form has been developed.

A series of amino acid-containing derivatives of AZT has been synthesised in which the distance between the amino acid carboxyl moiety and the phosphoramidate bond was varied. The anti-HIV effectiveness of these compounds was found to decrease on increasing the length of the methylene spacer. This observation is consistent with a mode of action involving cleavage of the phosphoramidate bond.

The synthesis and biological evaluation of several other derivatives has been accomplished in order to clarify whether the phosphoramidate bond is cleaved enzymically or chemically (or by a combination of these two mechanisms). Changes in the amino acid structure and stereochemistry were tolerated without substantial loss of anti-HIV activity, suggesting chemical hydrolysis. This was supported by the results of studies with HIV-1 protease, which had little effect on a typical derivative when incubated with the latter for five hours. However, investigation of the stability of the phosphoramidate compounds in aqueous media showed them to be resilient to chemical hydrolysis over a period of about five days, suggesting that other mechanisms may play a role in the activation of these compounds.

Attempts to conjugate a dipeptide to a nucleoside via a phosphoramidate linkage were unsuccessful. A method has been suggested for the synthesis of nucleoside 5'-alkyl hydrogen phosphonates. Besides their suitability for evaluation against HIV, these compounds may be of value in future peptide coupling reactions.

A series of phosphoramidate derivatives in which the nucleoside moiety was varied has been synthesised. Three bioactive and three non-bioactive nucleosides were employed for these studies. It appears that a bioactive nucleoside is not essential for activity. This indicates the potential utility of the phosphoramidate prodrug approach in conferring activity on nucleoside analogues hitherto considered ineffective against HIV.
Experimental

Apparatus and reagents
Merck Kieselgel 60 F₂₅₄ pre-coated silica plates were used for tlc and the components visualised by uv light. Flash column chromatography was carried out using Woelm silica as the stationary phase. When ethyl acetate was used as a component of the mobile phase, analytical grade reagent was distilled prior to use. Melting points were determined on a Riechert hot stage melting point apparatus and are uncorrected. EIMS and FABMS were carried out by Dr M. Mruzek on a VG 7070H mass spectrometer fitted with a Finnigan Incos II data system. Hplc was carried out on either a Gilson binary gradient system or on an ACS quaternary system. The author acknowledges the assistance and expertise of Mr S. Corker in conducting the hplc. ³¹P nmr spectra were either recorded by the author on a Varian XL-200 spectrometer operating at 82 MHz or by Mrs G. Maxwell on a Varian VXR-400 spectrometer operating at 164 MHz. The ³¹P nmr spectra obtained during the stability studies of nucleoside phosphoramidates were recorded by Dr B.C.N.M. Jones on a Varian VXR-400 spectrometer operating at 164 MHz and this assistance is gratefully acknowledged by the author. ³¹P nmr spectra are reported in units of δ relative to 85% phosphoric acid as external standard; positive shifts are downfield of the reference. ¹³C nmr spectra were either recorded by the author on a Varian XL-200 spectrometer operating at 50 MHz or by Mrs G. Maxwell or Mr D. Shipp on a Varian VXR-400 spectrometer operating at 100 MHz and are reported in units of δ relative to tetramethylsilane (TMS) as internal standard. Unless otherwise stated, both ³¹P and ¹³C nmr spectra were proton noise decoupled and all signals were singlets. ¹H nmr spectra were either recorded by the author on a Varian XL-200 spectrometer operating at 200 MHz or by Mrs G. Maxwell or Mr D. Shipp on a Varian VXR-400 spectrometer operating at 400 MHz and are reported in units of δ relative to TMS as internal standard. The abbreviations used in the assignment of nmr signals may be found in the Abbreviations Section. The observed microanalysis results for the diastereoisomeric mixtures of the nucleoside phosphoramidate products were sometimes found to be outside the limits normally considered acceptable. The difficulty in obtaining such derivatives free from solvents and water of hydrolysis is a frequently encountered problem in this
area of chemistry. The purity of the compounds was however always confirmed by analytical hplc.

All experiments involving water-sensitive reagents were carried out under scrupulously dry conditions. Molecular sieves used in the drying of solvents were activated by heating to ca. 160 °C in vacuo for a minimum of 12 hours. Anhydrous solvents and reagents were obtained in the following ways. Benzene, dichloromethane, diethyl ether, pyridine and hexane were heated under reflux over calcium hydride for several hours, distilled and stored over activated molecular sieves. Tetrahydrofuran was heated under reflux over lithium aluminium hydride for several hours, distilled and stored over activated molecular sieves. Triethylamine, morpholine and diisopropylethylamine were heated under reflux over calcium hydride for several hours and distilled immediately prior to use. Analytical grade carbon tetrachloride was stored over activated molecular sieves. N-methyl imidazole was distilled in vacuo and stored over activated molecular sieves. Chloroform was washed with water, dried with anhydrous calcium chloride, refluxed with phosphorus pentoxide for several hours, distilled and stored over activated molecular sieves in darkness. DMF was stored over activated molecular sieves overnight and distilled in vacuo on to activated molecular sieves. DMSO was distilled in vacuo on to activated molecular sieves, discarding the first 20% of distillate. After ca. 72 h the DMSO was poured on to fresh activated molecular sieves, and this was repeated after a further 72 h. Acetonitrile was distilled from phosphorus pentoxide twice, followed by storage over activated 3 Å molecular sieves. Trifluoroacetic acid was refluxed over phosphorus pentoxide and distilled. Anhydrous methanol and ethanol were obtained by heating with magnesium activated with iodine followed by distillation and storage over activated molecular sieves. Acetic anhydride, phosphoryl chloride, phosphorus trichloride, methanesulphonyl chloride, n-propylamine, 2-methoxyethylamine and 2-amino-1-methoxy-propane were distilled prior to use. The buffer solutions used in experiment 24 were prepared according to literature procedures. Anhydrous solids were obtained by heating (where appropriate) in vacuo in a vacuum pistol. Methyltriphenoxyphosphonium iodide was washed repeatedly with ethyl acetate until the crystalline material was yellow in colour. This was then dried briefly in vacuo.
1. L-phenylalanine methyl ester hydrochloride

Thionyl chloride (5 ml, 8.2 g, 0.07 mol, 2 equivs) was added dropwise to methanol (20 ml, 15.8 g, 0.49 mol, 16 equivs) at a temperature of -10 °C. L-phenylalanine (5.0 g, 0.03 mol) was added portionwise over 15 min at -10 °C. The mixture was then heated at 40 °C overnight. The solvent was removed in vacuo to afford a white solid, which was filtered, washed with diethyl ether (100 ml), and dried.
Yield: 6.5 g (99%).

^1H nmr δ(DMSO-d6): 8.71 (bs, 3H, NH₃), 7.31 (m, 5H, ArH), 4.19 (t, 1H, 'CH), 3.61 (s, 3H, -OCH₃), 3.11 (m, 2H, -Cl^-Ph).

2. Ethyl phosphorodichloridate

A solution of ethanol (12.6 ml, 9.9 g, 0.21 mol, 1 equiv) and triethylamine (30 ml, 21.8 g, 0.21 mol, 1 equiv) in diethyl ether (200 ml) was added to a vigorously stirred solution of phosphoryl chloride (20 ml, 32.9 g, 0.21 mol) in diethyl ether (200 ml) at -78 °C over a period of 7 h. The mixture was then allowed to warm to room temperature and stirred under nitrogen overnight. The white precipitate was filtered and the solvent removed from the filtrate in vacuo to afford an oil, which was distilled under reduced pressure. A colourless oil was obtained, b.p. 74 °C/10 mm Hg.
Yield: 27.7 g (79%).

^31P nmr δ(CDC1₃): 4.70.

^13C nmr δ(CDC1₃): 68.82 (d, POCH₂CH₃, 2JCP = 9.3 Hz), 15.65 (d, POCH₂CH₃, 3JCP = 8.8 Hz).

^1H nmr δ(CDC1₃): 4.44 (m, 2H, POCH₂), 1.50 (m, 3H, POCH₂CH₃).

3. Ethyl methoxyglycinyl phosphorochloridate (first attempt)

Ethyl phosphorodichloridate (0.73 ml, 1.0 g, 6.1 mmol) and triethylamine (1.75 ml, 1.27 g, 12.6 mmol, 2.05 equivs) were dissolved in dichloromethane (20 ml). This solution was then added dropwise, with stirring, over 0.5 h, to a suspension of glycine methyl ester hydrochloride (0.81g, 6.4 mmol, 1.05 equivs) in dichloromethane (20 ml) at 0 °C. The reaction was stirred at 0 °C for 1.5 h and was then allowed to warm to room temperature. The mixture was then stirred for 0.5 h and diethyl ether (150 ml) added. The resulting white precipitate was filtered
and the solvents removed from the filtrate in vacuo to afford a yellow oil. Yield: 1.19 g (90%).

$^31$P nmr $\delta$(CDCl$_3$): 17.97, 11.68, 4.15, -5.90.

4. Ethyl methoxyglycinyl phosphorochloridate (second attempt)
Ethyl phosphorodichloridate (0.73 ml, 1.0 g, 6.1 mmol) and triethylamine (1.71 ml, 1.24 g, 12.3 mmol, 2 equivs) were dissolved in dichloromethane (30 ml). This solution was then added dropwise, with stirring, over 0.5 h, to a suspension of glycine methyl ester hydrochloride (0.77 g, 6.1 mmol, 1 equiv) in dichloromethane (20 ml) at -60 °C. The reaction was stirred at -60 °C for 2 h and was then allowed to warm to room temperature. Diethyl ether (150 ml) was then added. The resulting white precipitate was filtered and the solvents removed from the filtrate in vacuo to afford a yellow oil. Yield: 1.23 g (93%).

$^31$P nmr $\delta$(CDCl$_3$) (not referenced): 20.10, 13.84, 6.50, -3.85.

5. Ethyl methoxyglycinyl phosphorochloridate (third attempt)
Triethylamine (1.88 ml, 1.36 g, 13.5 mmol, 2 equivs) was dissolved in dichloromethane (30 ml). This solution was added dropwise over a period of 4 h to a mixture of glycine methyl ester hydrochloride (0.84 g, 6.7 mmol, 1 equiv) and ethyl phosphorodichloridate (0.81 ml, 1.11 g, 6.80 mmol) in dichloromethane (30 ml) at -78 °C. The reaction was allowed to warm to room temperature over a period of 2 h. Diethyl ether (40 ml) was added and the white precipitate filtered. The solvents were then removed in vacuo. Some solid still remained and so carbon tetrachloride (10 ml) was added, the solid filtered and the carbon tetrachloride removed in vacuo to afford a yellow oil. Yield: 0.54 g (37%).

$^31$P nmr $\delta$(CDCl$_3$): 12.13, -5.02.

6. Ethyl methoxyglycinyl phosphorochloridate (fourth attempt)
Ethyl phosphorodichloridate (1.24 g, 7.6 mmol) was dissolved in dichloromethane (40 ml) and glycine methyl ester hydrochloride (0.96 g, 7.6 mmol, 1 equiv) added. This mixture was cooled to -78 °C and triethylamine (2.12 ml, 1.54 g, 15.2 mmol,
2 equivs) was added over a period of 3 h by means of a syringe pump. The reaction was then stirred at room temperature for 2 h. The solvent was removed in vacuo and carbon tetrachloride (20 ml) added to the resultant solid. The mixture was shaken and the solid filtered. The solvent was removed from the filtrate in vacuo to afford a yellow oil.

Yield: 1.38 g (84%).

$^{31}$P nmr $\delta$(CDCl$_3$): 12.48.

$^{13}$C nmr $\delta$(CDCl$_3$): 170.37 (d, CH$_3$OC(O), $^3$J$_{CP}$ = 10.4 Hz), 64.75 (d, POCH$_2$, $^3$J$_{CP}$ = 6.0 Hz), 52.61 (-OCH$_3$), 42.84 (PNHCH$_3$), 15.92 (d, POCH$_2$CH$_3$, $^3$J$_{CP}$ = 8.0 Hz).

$^1$H nmr $\delta$(CDCl$_3$): 4.23 (m, 2H, POCH$_2$), 3.84 (s, 1H, NH), 3.79 (s, 3H, -OCH$_3$), 3.71 (s, 2H, CH$_2$NH), 1.40 (t, 3H, POCH$_3$H).

EIMS m/e: 215.0126 (M$^+$, C$_6$H$_{11}$ClNO$_4$P requires 215.0114, 0.08%), 170 (M$^+$ - OCH$_2$CH$_3$, 2), 158 (M$^+$ - CH$_3$OC(O), $^{37}$Cl, 32), 156 (M$^+$ - CH$_3$OC(O), 100), 130 (M$^+$ - CH$_3$OC(O)CH$_2$N, $^{37}$Cl, 31), 128 (M$^+$ - CH$_3$OC(O)CH$_2$N, 98), 92 (C$_2$H$_5$O$_2$P$^+$, 7).

Analysis: Cl 16.29%. C$_6$H$_{11}$ClNO$_4$P requires Cl 16.45.

7. Ethyl methoxy-L-alaninyl phosphorochloridate

Triethylamine (1.85 ml, 1.34 g, 13.3 mmol, 2 equivs) was dissolved in dichloromethane (30 ml). This solution was added dropwise over a period of 2 h to a mixture of L-alanine methyl ester hydrochloride (0.93 g, 6.6 mmol, 1 equiv) and ethyl phosphorodichloridate (0.79 ml, 1.08 g, 6.6 mmol) in dichloromethane (30 ml) at -78 °C. The mixture was allowed to warm to room temperature over a period of 2.5 h. The dichloromethane was removed in vacuo and diethyl ether added (30 ml). The solid was filtered and the diethyl ether removed in vacuo to afford a yellow oil.

Yield: 0.95 g (62%).

$^{31}$P nmr: $\delta$(CDCl$_3$): 10.61.

$^{13}$C nmr: $\delta$(CDCl$_3$): 173.39, 173.26 (2d, 1:1, CH$_3$OC(O), $^3$J$_{CP}$ = 2.7 Hz and 4.1 Hz), 64.65, 64.60 (2d, 1:1, POCH$_2$, $^3$J$_{CP}$ = 2.9 Hz and 3.3 Hz), 52.51 (-OCH$_3$), 50.54, 50.34 (C, 1:1), 20.26 (m, CH-CH$_3$), 15.91 (d, POCH$_2$CH$_3$, $^3$J$_{CP}$ = 7.6 Hz).

$^1$H nmr $\delta$(CDCl$_3$): 4.60 (bs, 1H, NH), 4.12 (m, 2H, POCH$_2$), 3.88 (m, 1H, 'CH), 3.58 (s, 3H, -OCH$_3$), 1.28 (m, 6H, POCH$_2$CH$_3$ and 'CH-CH$_3$).

EIMS m/e: 232 (MH$^+$, $^{37}$Cl, 10%), 230.0321 (MH$^+$, C$_6$H$_{14}$ClNO$_4$P requires 230.0349,
28), 194 (M* - Cl, 1), 184 (MH* - CH₃CH₂OH, 2), 173 (MH* - CH₂OC(O), ³⁷Cl, 2), 172 (MH* - CH₂OH - CH₂=CH₂, ³⁷Cl, 49), 171 (MH* - CH₂OC(O), 5), 170 (MH* - CH₂OH - CH₂=CH₂, 100), 166 (MH* - Cl - CH₂CH₂, 20), 158 (MH* - CH₃OC(O) - CH₃, ³⁷Cl, 4), 156 (MH* - CH₂OC(O) - CH₃, 13), 144 (MH* - CH₃OC(O)CH₂CH₃, ³⁷Cl, 25), 142 (MH* - CH₃OC(O)CH₂CH₃, 70).

Analysis: Cl 15.17%. C₆H₁₃ClNO₄P requires Cl 15.44.

8. Ethyl methoxy-L-valinyl phosphorochloridate

Triethylamine (3.09 ml, 2.24 g, 0.022 mol, 2 equivs) was dissolved in dichloromethane (40 ml). This solution was added dropwise over a period of 2 h to a mixture of L-valine methyl ester hydrochloride (1.86 g, 0.011 mol, 1 equiv) and ethyl phosphorodichloridate (1.32 ml, 1.81 g, 0.011 mol) in dichloromethane (30 ml) at -78 °C. The mixture was allowed to warm to room temperature and stirring continued for another 2.5 h. The dichloromethane was removed in vacuo and carbon tetrachloride (20 ml) added. The solid was filtered and the carbon tetrachloride removed in vacuo, leaving a slightly yellow oil containing some traces of solid. More carbon tetrachloride (10 ml) was added, the solid filtered and the carbon tetrachloride removed in vacuo, to afford a yellow oil.

Yield: 2.51 g (88%).

³¹P nmr δ(CDCl₃): 12.29, 11.82 (4:3).

¹³C nmr δ(CDCl₃): 172.69, 172.40 (2d, 4:3, CH₃OC(O), ³JC,P = 3.4 Hz and 2.4 Hz), 64.71, 64.45 (2d, 4:3, POCH₂, ³JC,P = 2.4 Hz and 2.2 Hz), 60.33, 59.68 (C, 4:3), 52.33 (-OCH₃), 32.18, 32.04 (2d, 4:3, iPrCH, ³JC,P = 3.2 Hz), 19.00, 18.92 (4:3, iPrMe(1)), 17.41 (iPrMe(2)), 15.91 (d, POCH₂CH₃, ³JC,P = 7.5 Hz).

¹H nmr δ(CDCl₃): 4.34 (m, 2H, POCH₂), 4.04 (m, 1H, \( ^{-} \)CH), 3.78 (s, 3H, -OCH₃), 2.20 (m, 1H, iPrCH), 1.43 (t, 3H, POCH₂CH₃), 1.01 (d, 3H, iPrMe(1)), 0.93 (d, 3H, iPrMe(2)).

EI-MS m/e: 260 (MH⁺, ³⁷Cl, 11%), 258.0657 (MH⁺, C₈H₁₈ClNO₄P requires 258.0662, 32), 223 (MH⁺ - Cl, 1), 222 (M⁺ - Cl, 15), 216 (MH⁺ - C₃H₈, ³⁷Cl, 3), 214 (MH⁺ - C₃H₈, 13), 200 (MH⁺ - CH₃OH - CH₂=CH₂, ³⁷Cl, 33), 198 (MH⁺ - CH₃OH - CH₂=CH₂, 100), 194 (MH⁺ - HCl - CH₂=CH₂, 10), 188 (MH⁺ - CH₂=CH₂ - C₃H₈, ³⁷Cl, 4), 186 (MH⁺ - CH₂=CH₂ - C₃H₈, 12), 172 (MH⁺ - CH₂=CH₂ - CH₃OC(O)H, ³⁷Cl, 17), 170 (MH⁺ - CH₂=CH₂ - CH₃OC(O)H, 52), 129 (MH⁺ - C₂H₆ - CH₃OC(O) - CH₂=CH-CH₃, ³⁷Cl, 162)
Analysis: Cl 13.53%. C₇H₈ClNO₃P requires Cl 13.76.

9. 3'-Azido-3'-deoxythymidine

5'-O-Trityl-2,3'-anhydrothymidine (3.48 g, 7.5 mmol) and sodium azide (2.42 g, 37.3 mmol, 5 equivs) were heated to 120 °C in DMF (25 ml) for 4 h. The reaction mixture was then poured into ice/water (600 ml) and stirred for 1 h. The yellow precipitate was filtered and dried. The crude 5'-O-trityl-3'-azido-3'-deoxythymidine (5.34 g) was suspended in 80% acetic acid (50 ml) and heated to 85 °C for 0.75 h. The reaction mixture was then allowed to cool to room temperature. A little ice was added to assist in the precipitation of the trityl alcohol. The solid was filtered off and the solvents removed from the filtrate in vacuo. The product was then purified by flash column chromatography, using silica (60 g) and an eluent of 2% methanol/chloroform. Removal of solvents from the relevant fractions in vacuo afforded a white foam. The product was recrystallised from isopropyl alcohol.

Yield: 0.47 g (24% based on 5'-O-trityl-2,3'-anhydrothymidine).

Melting point: 122 °C.

1H nmr δ(CDCI₃): 8.54 (bs, 1H, NH), 7.35 (s, 1H, H₆), 6.04 (t, 1H, H₁', J₃H₋H₂ = 6.6 Hz), 4.39 (m, 1H, H₃'), 3.85 (m, 3H, H₄', H₅'), 2.55 (m, 1H, H₂'), 2.38 (m, 2H, H₂', 5'-OH), 1.90 (d, 3H, 5-CH₃, J₅-H₁ = 1.2 Hz).

10. 3'-Azido-3'-deoxythymidine (ethyl methoxyglycinyl) phosphoramidate

3'-Azido-3'-deoxythymidine (0.22 g, 0.8 mmol) was dissolved in THF (3 ml) and N-methyl imidazole (0.58 g, 7.0 mmol, 8.4 equivs) added. Ethyl methoxyglycinyl phosphorochloridate (0.76 g, 3.5 mmol, 4.2 equivs) was added and the reaction mixture stirred overnight. The reaction was concentrated in vacuo, dissolved in chloroform (20 ml) and washed with saturated sodium bicarbonate solution (15 ml), then water (3 x 15 ml). The organic layer was separated and dried (MgSO₄), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified twice by flash column chromatography, using first silica (70 g) and an eluent of 3% methanol/chloroform, then silica (60 g) and
an eluent of 2% methanol/ethyl acetate. Removal of the solvents in vacuo afforded
a colourless gum.
Yield: 0.23 g

$^{31}$P nmr δ(CDC$_3$): 6.71.

$^{13}$C nmr δ(CDC$_3$): 171.60, 171.54 (2d, 1:1, CH$_3$C(O), $^3$J$_{CP}$ = 2.9 Hz and 2.5 Hz),
164.31, 164.28 (1:1, C2), 150.68, 150.63 (1:1, C4), 135.52, 135.49 (1:1, C6), 111.41,
111.24 (1:1, C5), 85.01, 84.59 (1:1, C1'), 82.49, 82.42 (2d, 1:1, C4', $^3$J$_{CP}$ = 7.8 Hz and
7.4 Hz), 65.37, 65.06 (2d, 1:1, C5', $^3$J$_{CP}$ = 3.9 Hz and 4.1 Hz), 63.18, 63.11 (2d, 1:1,
POCH$_2$, $^3$J$_{CP}$ = 5.0 Hz and 4.9 Hz), 60.52, 60.40 (1:1, C3'), 52.40 (-OCH$_3$), 42.72, 42.65
(1:1, NCH$_2$), 37.37 (C2'), 16.16 (d, POCH$_2$CH$_3$, $^3$J$_{CP}$ = 5.6 Hz), 12.43 (5-CH$_3$).

$^1$H nmr δ(CDC$_3$): 7.46, 7.43 (2s, 1:1, 1H, H6), 6.27, 6.21 (2t, 1:1, 1H, H1'), 4.42 (m,
1H, H3'), 4.30 (m, 2H, H5'), 4.18 (m, 3H, POCH$_2$, valine NH), 4.05 (m, 1H, H4'),
3.74 (m, 5H, OCH$_3$, PNCH$_2$), 2.39 (m, 2H, H2'), 1.92 (s, 3H, 5-CH$_3$), 1.34 (t, 3H,
POCH$_2$CH$_3$, $^3$J$_{HH}$ = 7.04 Hz).

EI-MS m/e: 446.1342 (M*, C$_{15}$H$_{25}$N$_6$O$_8$P requires 446.1315, 1%), 321 (MH$^+$ - thymine,
1), 279 (MH$^+$ - thymine - N$_3$, 2), 278 (M$^+$ - thymine - N$_3$, 19), 263 (M$^+$ - thymine
- N$_3$ - CH$_2$=CH$_2$, 1), 250 (M$^+$ - thymine - N$_3$ - CH$_2$=CH$_2$, 1), 225 (M$^+$ - thymine
- N$_3$ - CH$_2$=CH$_2$, 1), 218 (M$^+$ - thymine - N$_3$ - CH$_2$=CH$_2$, 1), 198 (C$_7$H$_{13}$NO$_5$P$,^+$, 6),
180 (C$_7$H$_{13}$NO$_5$P$^-$ - H$_2$O, 5), 152 (C$_7$H$_{13}$NO$_5$P$^-$ - H$_2$O - CH$_2$=CH$_2$, 16),
135 (C$_7$H$_{13}$NO$_5$P$^-$ - H$_2$O - OCH$_2$CH$_3$, 3), 127 (thymineH$^+$, 14), 126 (thymine$^+$, 63),
110 (C$_2$H$_5$O$_3$P$,^+$, 57), 107 (C$_7$H$_{13}$NO$_5$P$^-$ - H$_2$O - CH$_3$OC(O)CH$_2$, 2), 81 (C$_5$H$_9$O$^-$, 100).

FAB-MS m/e: 447 (MH$^+$, 10), 404 (M$^+$ - N$_3$, 1), 278 (M$^+$ - thymine - N$_3$, 7), 250 (M$^+$
- thymine - N$_3$ - CH$_2$=CH$_2$, 3), 198 (C$_7$H$_{13}$NO$_5$P$,^+$, 6), 180 (C$_7$H$_{13}$NO$_5$P$^-$ - H$_2$O, 3),
165 (C$_7$H$_{13}$NO$_5$P$^-$ - CH$_3$, 1), 152 (C$_7$H$_{13}$NO$_5$P$^-$ - H$_2$O - CH$_2$=CH$_2$, 8), 135 (C$_7$H$_{13}$NO$_5$P$^-$
- H$_2$O - OCH$_2$CH$_3$, 2), 127 (thymineH$^+$, 5), 110 (C$_2$H$_5$O$_3$P$,^+$, 12), 82 (C$_5$H$_9$O$^-$, 100).

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 µm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions: A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention
time: 14.5, 14.8 min.

Analysis: C 39.42%, H 4.75, N 17.17, P 7.04. C$_{15}$H$_{25}$N$_6$O$_8$P[0.5H$_2$O] requires C 39.72,
H 5.31, N 18.46, P 6.80.
11. Ethyl methoxy-β-alaninyl phosphorochloridate
Triethylamine (1.36 ml, 0.98 g, 9.7 mmol, 2 equivs) was dissolved in dichloromethane (30 ml). This solution was added dropwise over 5.5 h to a mixture of β-alanine methyl ester hydrochloride (0.68 g, 4.9 mmol, 1 equiv) and ethyl phosphorodichloridate (0.58 ml, 0.79 g, 4.9 mmol) in dichloromethane (30 ml) at -78 °C. The mixture was then allowed to warm to room temperature and stirring continued for 1.25 h. The dichloromethane was removed in vacuo and carbon tetrachloride (20 ml) added. The solid was filtered and the carbon tetrachloride removed from the filtrate in vacuo to afford a yellow oil. 

$^{31}P$ nmr δ(CDCl₃): 12.83, 4.84, -5.96.

Hexane (20 ml) was added and the mixture vigorously shaken. The hexane was decanted off leaving an oil contaminated with some solid. Carbon tetrachloride (10 ml) was added, the solid filtered and the carbon tetrachloride removed from the filtrate in vacuo to afford a yellow oil.

Yield: 0.49 g (44%)

$^{31}P$ nmr δ(CDCl₃): 12.78.

$^{13}C$ nmr δ(CDCl₃): 172.30 (CH₂OC(O)), 64.34 (d, POCH₂, $^3J_{CP} = 6.2$ Hz), 51.81 (-OCH₃), 37.31 (βCH₂), 34.84 (d, αCH₂, $^3J_{CP} = 6.6$ Hz), 15.84 (d, POCH₂CH₃, $^3J_{CP} = 8.0$ Hz).

$^1$H nmr δ(CDCl₃): 4.16 (m, 2H, POCH₂), 3.65 (s, 3H, -OCH₃), 3.64 (s, 1H, NH), 3.23 (m, 2H, βCH₂), 2.55 (m, 2H, αCH₂), 1.37 (m, 3H, POCH₂CH₃).

12. 3'-Azido-3'-deoxythymidine (ethyl methoxy-β-alaninyl) phosphoramidate
3'-Azido-3'-deoxythymidine (0.10 g, 0.4 mmol) was dissolved in THF (3 ml) and N-methyl imidazole (0.30 ml, 0.31 g, 3.8 mmol, 10 equivs) added. To this solution was added ethyl methoxy-β-alaninyl phosphorochloridate (0.44 g, 1.9 mmol, 5 equivs) in THF (2 ml). After stirring for 24 h the reaction had proceeded to 95% completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (20 ml), then water (3 x 15 ml). The organic layer was separated and dried (MgSO₄), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off
and the gummy residue purified twice by flash column chromatography, using first silica (70 g) and an eluent of 2% methanol/chloroform, then silica (40 g) and an eluent of 5% methanol/ethyl acetate. Removal of the solvents in vacuo afforded a yellow gum.

Yield: 0.07 g (42%).

\(^{31}\text{P \text{nmr}}\) \(\delta(\text{CDCl}_3): 7.48.\)

\(^{13}\text{C \text{nmr}}\) \(\delta(\text{CDCl}_3): 172.59, 172.54 (1:1, \text{CH}_3\text{OC(O)}), 163.78, 163.76 (1:1, C2), 150.35, 150.30 (1:1, C4), 135.40 (C6), 111.52, 111.42 (1:1, C5), 85.22, 84.91 (1:1, C1'), 82.63, 82.55 (1:1, C4'), 65.28, 65.02 (2d, 1:1, C5', \(J_{\text{CP}} = 5.0\) Hz and 5.1 Hz), 63.10, 63.04 (2d, 1:1, POCH₂, \(J_{\text{CP}} = 5.4\) Hz and 5.6 Hz), 60.47, 60.42 (1:1, C3'), 51.99 (-OCH₃), 37.58 (C2'), 37.15, 37.13 (1:1, βCH₂), 35.80, 35.75 (2d, 1:1, αCH₂, \(J_{\text{CP}} = 2.0\) Hz and 2.1 Hz), 16.36 (d, POCH₂CH₂, \(J_{\text{H-H}} = 1.5\) Hz), 12.56 (5-CH₃).

\(^{1}\text{H \text{nmr}}\) \(\delta(\text{CDCl}_3): 9.3\) (bs, 1H, 3-NH), 7.45, 7.44 (2s, 1:1, 1H, H6), 6.25, 6.20 (2t, 1:1, 1H, H1'), 4.41 (m, 1H, H3'), 4.03 - 4.27 (m, 5H, POCH₂, H5', H4'), 3.71, 3.70 (2s, 1:1, 3H, -OCH₃), 3.22 (m, 2H, βCH₂), 2.55 (m, 2H, αCH₂), 2.45 (m, 1H, H2'), 2.33 (m, 1H, H2'), 1.94 (s, 3H, 5-CH₃), 1.37 (t, 3H, POCH₂CH₂, \(J_{\text{H-H}} = 7.02\) Hz).

EIMS m/e: 460.1515 (M⁺, \(\text{C}_{16}\text{H}_{25}\text{N}_6\text{O}_8\text{P}\) requires 460.1472, 1%), 335 (MH⁺ - thymine, 1), 293 (MH⁺ - thymine - N₃, 1), 292 (M⁺ - thymine - N₅, 10), 250 (M⁺ - \(\text{C}_6\text{H}_{13}\text{NO}_5\text{P}\), 2), 212 (\(\text{C}_6\text{H}_{13}\text{NO}_5\text{P}⁺, 2\)), 210 (\(\text{C}_6\text{H}_{13}\text{NO}_5\text{P}⁺, 1\)), 194 (\(\text{C}_6\text{H}_{13}\text{NO}_5\text{P}⁺\) - H₂O, 1), 180 (\(\text{C}_6\text{H}_{15}\text{NO}_5\text{P}⁺\) - CH₂OH, 3), 166 (\(\text{C}_6\text{H}_{15}\text{NO}_5\text{P}⁺\) - H₂O - CH₂=CH₂, 1), 152 (\(\text{C}_6\text{H}_{15}\text{NO}_5\text{P}⁺\) - CH₂OH - CH₂=CH₂, 5), 138 (\(\text{C}_6\text{H}_{15}\text{NO}_5\text{P}⁺\) - CH₃OC(O)CH₂, 6), 127 (thymineH⁺, 2), 126 (thymine⁺, 11), 110 (\(\text{C}_2\text{H}_4\text{O}⁺\), 9), 82 (\(\text{C}_2\text{H}_4\text{O}⁺\), 9), 81 (\(\text{C}_2\text{H}_4\text{O}⁺\), 100).

Analytical hplc: Column: 50 x 250 x 4.6 mm Spherisorb ODS2 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 23.1, 23.3 mins (1:1).

Analysis: C 42.07%, H 5.73, N 16.63, P 6.33. \(\text{C}_{16}\text{H}_{25}\text{N}_6\text{O}_8\text{P}\) requires C 41.74, H 5.47, N 18.25, P 6.73.

13. Ethyl methoxy-4-aminobutyryl phosphorochloridate

Triethylamine (2.13 ml, 1.54 g, 15.2 mmol, 2 equivs) was dissolved in dichloromethane (40 ml). This solution was added dropwise over 5.5 h to a
mixture of 4-aminobutyric acid methyl ester hydrochloride (1.71 g, 7.6 mmol, 1 equiv) and ethyl phosphorodichloridate (0.90 ml, 1.24 g, 7.6 mmol) in dichloromethane (30 ml) at -78 °C. The mixture was then allowed to warm to room temperature over a period of 2.5 h. The dichloromethane was removed in vacuo and carbon tetrachloride (20 ml) added. The solid was filtered and the carbon tetrachloride removed from the filtrate in vacuo to afford a yellow oil.

Yield: 1.52 g (82%).

$^{31}$P nmr $\delta$(CDCl$_3$): 13.82.

$^{13}$C nmr $\delta$(CDCl$_3$): 173.48 (CH$_3$O), 64.04 (d, POCH$_2$, $^3$J$_{CP}$ = 6.1 Hz), 51.44 (-OCH$_3$), 40.89 (CH$_3$), 30.75 (CH$_2$), 25.69 (d, $\beta$CH$_2$, $^3$J$_{CP}$ = 7.9 Hz), 15.71 (d, POCH$_2$CH$_3$, $^3$J$_{CP}$ = 8.0 Hz).

$^1$H nmr $\delta$(CDCl$_3$): 5.10 (bs, 1H, NH), 4.20 (m, 2H, POCH$_2$), 3.68 (s, 3H, -OCH$_3$), 3.04 (m, 2H, CH$_2$), 2.44 (t, 2H, $\alpha$CH$_2$, $^3$J$_{HH}$ = 7.3 Hz), 1.88 (m, 2H, $\beta$CH$_2$), 1.39 (t, 3H, POCH$_2$CH$_3$, $^3$J$_{HH}$ = 7.05 Hz).

EIMS m/e: 246 (MH$^+$, $^{35}$Cl, 21%), 245 (M$^+$, $^{37}$Cl, 5), 244 (MH$^+$, 64), 243.0549 (M$^+$, C$_7$H$_{15}$ClNO$_4$P requires 243.0427, 1), 214 (MH$^+$ - CH$_3$OH, $^{35}$Cl, 27), 212 (MH$^+$ - CH$_3$OH, 73), 208 (M$^+$ - Cl, 23), 198 (MH$^+$ - CH$_3$CH$_2$OH, 4), 186 (MH$^+$ - CH$_3$OH - CH$_2$=CH$_2$, $^{37}$Cl, 7), 184 (MH$^+$ - CH$_3$OH - CH$_2$=CH$_2$, 21), 172 (MH$^+$ - CH$_3$OC(O)CH$_3$, $^{37}$Cl, 17), 170 (MH$^+$ - CH$_3$OC(O)CH$_3$, 50), 158 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, $^{37}$Cl, 8), 156 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, 27), 144 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, $^{37}$Cl, 7), 142 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, 20).

Analysis: Cl 14.58%. C$_7$H$_{15}$ClNO$_4$P requires Cl 14.55.

14.3'-Azido-3'-deoxythymidine (ethyl methoxy-4-aminobutyryl) phosphoramidate

3'-Azido-3'-deoxythymidine (0.11 g, 0.4 mmol) was dissolved in THF (3 ml) and N-methyl imidazole (0.26 ml, 0.27 g, 3.3 mmol, 8 equivs) added. To this solution was added ethyl methoxy-4-aminobutyryl phosphorochloridate (0.54 g, 2.2 mmol, 5.5 equivs). An additional portion of N-methyl imidazole (0.10 ml, 0.10 g, 1.2 mmol) was added. After stirring for 21 h the reaction had proceeded to completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (3 x 20 ml), then water (2 x 15 ml). The organic layer was separated and dried (MgSO$_4$), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and
poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified twice by flash column chromatography, first using silica (70 g) and an eluent of 2% methanol/chloroform, then silica (70 g) and an eluent of 3% methanol/ethyl acetate. Removal of the solvents in vacuo afforded a colourless gum.

Yield: 0.13 g (66%).

$^{31}$P nmr $\delta$(CDCl$_3$): 7.75.

$^{13}$C nmr $\delta$(CDCl$_3$): 173.51, 173.47 (1:1, CH$_3$OC(O)) 164.03, 164.01 (1:1, C2), 150.51, 150.44 (1:1, C4), 135.37 (C6), 111.39, 111.24 (1:1, C5), 85.20, 84.80 (1:1, C1), 82.60, 82.53 (2d, 1:1, C4', $^3$J$_{CP}$ = 3.9 Hz and 3.7 Hz), 65.15, 64.94 (2d, 1:1, C5', $^3$J$_{CP}$ = 4.9 Hz and 5.1 Hz), 62.91, 62.84 (2d, 1:1, POCH$_2$, $^3$J$_{CP}$ = 5.4 Hz and 5.9 Hz), 60.47, 60.45 (1:1, C3'), 51.74 (-OCH$_3$), 40.74, 40.71 (1:1, $\gamma$CH$_2$), 37.50 (C2'), 30.95 ($\alpha$CH$_2$), 26.78, 26.72 (2d, 1:1, $\beta$CH$_2$, $^3$J$_{CP}$ = 3.7 Hz and 3.6 Hz), 16.27, 16.20 (1:1, POCH$_2$CH$_3$), 12.47 (5-CH$_3$).

$^1$H nmr $\delta$(CDCl$_3$): 9.90 (bs, 1H, 3-NH), 7.43, 7.38 (2s, 1:1, 1H, H6), 6.23, 6.18 (2t, 1:1, 1H, H1'), 4.40 (m, 1H, H3'), 4.00 - 4.25 (m, 5H, POCH$_2$, H5', H4'), 3.65 (s, 3H, -OCH$_3$), 3.53 (m, 1H, NH), 2.94 (m, 2H, $\gamma$CH$_2$), 2.28 - 2.42 (m, 4H, $\alpha$CH$_2$, H2') 1.90, 1.895 (2d, 1:1, 3H, 5-CH$_3$, $^4$J$_{HH}$ = 0.98 Hz and 1.04 Hz), 1.82 (m, 2H, $\beta$CH$_2$), 1.34 (t, 3H, POCH$_2$CH$_3$, $^3$J$_{HH}$ = 7.06 Hz).

EIMS m/e: 474.1566 (M*, C$_{17}$H$_{27}$N$_6$O$_8$P requires 474.1628, 1%), 250 (M' - C$_7$H$_{12}$NO$_3$P, 1), 226 (C$_7$H$_{17}$NO$_3$P', 1), 194 (C$_7$H$_{12}$NO$_3$P' - CH$_3$OH, 1), 180 (C$_7$H$_{12}$NO$_3$P' - CH$_3$CH$_2$OH, 1), 166 (C$_7$H$_{17}$NO$_3$P' - CH$_3$OH - CH$_2$=CH$_2$, 2), 152 (C$_7$H$_{12}$NO$_3$P' - CH$_3$OC(O)CH$_2$CH$_3$, 6), 138 (C$_7$H$_{17}$NO$_3$P' - CH$_3$OC(O)CH$_2$CH$_3$, 4), 127 (thymineH*, 2), 126 (thymine*, 18), 124 (C$_7$H$_{17}$NO$_3$P' - CH$_3$OC(O)CH$_2$CH$_2$CH$_3$, 5), 110 (C$_2$H$_7$O$_3$P*, 8), 109 (C$_7$H$_{17}$NO$_5$P' - CH$_3$OC(O)CH$_2$CH$_2$CH$_2$NH$_2$, 1), 82 (C$_3$H$_4$O*, 9), 81 (C$_3$H$_5$O*, 100).

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 21.4 min.

Analysis: C 41.66%, H 5.62, N 17.19, P 6.37. C$_{17}$H$_{27}$N$_6$O$_8$P requires C 41.47, H 5.94, N 17.07, P 6.29.
15. Ethyl methoxy-5-aminovaleryl phosphorochloridate

Triethylamine (1.42 ml, 1.30 g, 10.2 mmol, 2 equivs) was dissolved in dichloromethane (40 ml). This solution was added dropwise over 2.5 h to a mixture of 5-aminovaleric acid methyl ester hydrochloride (0.85 g, 5.1 mmol, 1 equiv) and ethyl phosphorodichloridate (0.60 ml, 0.82 g, 5.1 mmol) in dichloromethane (30 ml) at -78 °C. The mixture was then allowed to warm to room temperature over a period of 4 h. The dichloromethane was removed in vacuo and carbon tetrachloride (20 ml) added. The solid was filtered and the carbon tetrachloride removed from the filtrate in vacuo to afford a yellow oil.

Yield: 1.20 g (92%).

$^{31}$P nmr $\delta$(CDCl$_3$): 13.79.

$^{13}$C nmr $\delta$(CDCl$_3$): 173.73 (CH$_3$OC(O)), 64.03 (d, POCH$_2$, $^2$J$_{CP}$ = 6.1 Hz), 51.43 (-OCH$_3$), 41.28 ($\delta$CH$_2$), 33.32 (CH$_2$), 30.03 (d, $\gamma$CH$_2$, $^3$J$_{CP}$ = 7.5 Hz), 21.70 ($\delta$CH$_2$), 15.80 (d, POCH$_2$CH$_2$, $^3$J$_{CP}$ = 8.0 Hz).

$^1$H nmr $\delta$(CDCl$_3$): 4.5 (bs, 1H, NH), 4.16 (m, 2H, POCH$_2$), 3.60 (s, 3H, -OCH$_3$), 2.92 (m, 2H, $\delta$CH$_2$), 2.29 (m, 2H, $\alpha$CH$_2$), 1.49 - 1.63 (m, 4H, $\gamma$CH$_2$, $\gamma$CH$_2$), 1.36 (t, 3H, POCH$_2$CH$_2$, $^3$J$_{HH}$ = 6.98 Hz).

EIMS m/e: 260 (MH$^+$, $^{37}$Cl, 45), 259 (M$^+$, $^{37}$Cl, 14), 258.0662 (MH$^+$, C$_8$H$_{18}$ClNO$_4$P requires 258.0662, 70), 257 (M$^+$, 1), 229 (MH$^+$ - CH$_3$O, $^{37}$Cl, 2), 228 (MH$^+$ - CH$_3$OH, $^{37}$Cl, 28), 227 (MH$^+$ - CH$_3$O, 8), 226 (MH$^+$ - CH$_3$OH, 67), 222 (MH$^+$ - HCl, 6), 212 (MH$^+$ - CH$_3$CH$_2$OH, 2), 200 (MH$^+$ - CH$_3$OH - CH$_2$=CH$_2$, $^{37}$Cl, 3), 198 (MH$^+$ - CH$_3$OH - CH$_2$=CH$_2$, 8), 172 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, $^{37}$Cl, 2), 170 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, 5), 158 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, $^{37}$Cl, 12), 156 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, 41), 142 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, 3), 130 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, 100).

Analysis: Cl 13.50%. C$_8$H$_{17}$ClNO$_4$P requires Cl 13.76.

16. 3'-Azido-3'-deoxythymidine (ethyl methoxy-5-aminovaleryl) phosphoramidate

3'-Azido-3'-deoxythymidine (0.10 g, 0.4 mmol) was dissolved in THF (3 ml) and N-methyl imidazole (0.29 ml, 0.30 g, 3.6 mmol, 10 equivs) added. To this solution was added ethyl methoxy-5-aminovaleryl phosphorochloridate (0.48 g, 1.8 mmol, 5 equivs), the last portion being added in THF (2 ml). After stirring for 19 h the reaction had proceeded to completion on tlc. The reaction was concentrated in
vacuo, dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (20 ml), then water (3 x 15 ml). The organic layer was separated and dried (MgSO₄), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified twice by flash column chromatography, first using silica (70 g) and an eluent of 2% methanol/chloroform, then silica (70 g) and an eluent of 2% methanol/ethyl acetate. Removal of the solvents in vacuo afforded a colourless gum.

Yield: 0.13 g (70%).

³¹P nmr δ(CDCｌ₃): 7.84.

¹³C nmr δ(CDCｌ₃): 173.68, 173.66 (1:1, CH₂OC(O)) 163.99 (C2), 150.45, 150.37 (1:1, C4), 135.25 (C6), 111.27, 111.11 (1:1, C5), 85.04, 84.60 (1:1, C1'), 82.50, 82.42 (2d, 1:1, C4', 3J_C_P = 3.5 Hz and 3.3 Hz), 65.00, 64.78 (2d, 1:1, C5', 3J_C_P = 4.9 Hz and 4.7 Hz), 62.74, 62.66 (2d, POCH₂), 3J_C_P = 5.1 Hz and 4.8 Hz), 60.35 (C3'), 51.54 (-OCH₃), 40.89, 40.85 (1:1, δCH₃), 37.41 (C2'), 33.28 (αCH₃), 31.02, 30.96 (2d, 1:1, γCH₂, 3J_C_P = 5.9 Hz and 5.7 Hz), 21.72 (βCH₂), 16.18, 16.11 (1:1, POCH₂CH₃), 12.43, 12.40 (1:1, 5-CH₃).

¹H nmr δ(CDCｌ₃): 10.0 (bs, 1H, 3-NH), 7.42, 7.34 (2s, 1:1, 1H, H6), 6.21, 6.14 (2t, 1:1, 1H, H1', 3J_H1'-H2' = 6.74 Hz), 4.36 (m, 1H, H3'), 3.99 - 4.20 (m, 5H, POCH₂, H5', H4'), 3.61 (s, 3H, -OCH₃), 3.42 (m, 1H, NH), 2.85 (m, 2H, δCH₂), 2.37 (m, 1H, H2'), 2.23 - 2.29 (m, 3H, αCH₂, H2') 1.86 (s, 3H, 5-CH₃) 1.61 (m, 2H, βCH₂), 1.48 (m, 2H, γCH₂), 1.31 (t, 3H, POCH₂CH₃, 3J_H_H = 7.02 Hz).

EIMS m/e: 488.1733 (M⁺, C₁₈H₂₈N₆O₈P requires 488.1784, 1%), 363 (MH⁺ - thymine, 1), 321 (MH⁺ - thymine - N₃, 1), 320 (M⁺ - thymine - N₃, 7), 240 (C₈H₁₉NO₅P⁺, 1), 238 (C₈H₁₇NO₅P⁺, 1), 208 (C₈H₁₉NO₅P⁺ - CH₂OH, 1), 180 (C₈H₁₉NO₅P⁺ - CH₃OH - CH₂=CH₂, 1), 152 (C₈H₁₉NO₅P⁺ - CH₃OC(O)CH₂CH₃, 2), 138 (C₈H₁₉NO₅P⁺ - CH₃OC(O)CH₂CH₂CH₃, 4), 127 (thymineH⁺, 3), 126 (thymine⁺, 11), 124 (C₈H₁₉NO₅P⁺ - CH₃OC(O)(CH₂)₂CH₃, 1), 110 (C₂H₄O₃P⁺, 9), 109 (C₈H₁₉NO₅P⁺ - CH₃C(O)(CH₂)₄NH₂, 1), 82 (C₅H₆O⁺, 11), 81 (C₅H₅O⁺, 100).

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention
time: 22.8, 23.0 min (3:2).
Analysis: C 45.31%, H 6.38, N 15.02, P 6.16. C18H29N6O8P requires C 44.26, H 5.98, N 17.21, P 6.34.

17. Ethyl methoxy-6-aminocaproyl phosphorochloridate
Ethyl phosphorodichloridate (1.05 g, 6.4 mmol) was dissolved in dichloromethane (40 ml) and 6-aminocaproic acid methyl ester hydrochloride (1.17 g, 6.4 mmol, 1 equiv) added. This mixture was cooled to -78 °C and triethylamine (1.8 ml, 1.31 g, 12.9 mmol, 2 equivs) added over 3 h by means of a syringe pump. The reaction was then allowed to warm to room temperature over 1.5 h, and then stirred for 1 h. The solvent was removed in vacuo and carbon tetrachloride (20 ml) added. The solid was filtered and the solvent removed from the filtrate in vacuo to afford a yellow oil.
Yield: 1.74 g (99%).

$^{31}$P nmr $\delta$(CDCl$_3$): 13.85.

$^{13}$C nmr $\delta$(CDCl$_3$): 173.39 (CH$_3$OC(O)), 63.83 (d, POCH$_2$, $^3$J$_{CP}$ = 6.1 Hz), 51.22 (-OCH$_3$), 41.37 (PNHCH$_2$), 33.61 (CH$_3$OC(O)CH$_2$), 30.06 (d, PNHCH$_2$CH$_2$, $^3$J$_{CP}$ = 7.4 Hz), 25.79, 24.20 (PNHCH$_2$CH$_2$CH$_2$ and PNHCH$_2$CH$_2$CH$_2$CH$_2$), 15.60 (d, POCH$_2$CH$_3$, $^3$J$_{CP}$ = 8.0 Hz).

$^1$H nmr $\delta$(CDCl$_3$): 4.80 (bs, 1H, NH), 3.97 (m, 2H, POCH$_2$), 3.40 (s, 3H, -OCH$_3$), 2.71 (m, 2H, PNHCH$_2$), 2.06 (t, 2H, CH$_3$C(O)CH$_2$), 1.30 (m, 4H, PNHCH$_2$CH$_2$, PNHCH$_2$CH$_2$CH$_2$CH$_2$), 1.10 (m, 5H, POCH$_2$CH$_3$, PNHCH$_2$CH$_2$CH$_2$).

EI-MS m/e: 272.0779 (MH$^+$, C$_8$H$_{20}$ClNO$_4$P requires 272.0818, 1), 243 (MH$^+$ - CH$_3$O, $^{37}$Cl, 4), 241 (MH$^+$ - CH$_3$O, 16), 240 (MH$^+$ - CH$_3$OH, 2), 227 (MH$^+$ - CH$_3$CH$_2$O, 1), 215 (MH$^+$ - CH$_3$OC(O)O, $^{37}$Cl, 1), 214 (MH$^+$ - CH$_3$OH - CH$_2$=CH$_2$, $^3$J$_{CP}$, 3), 213 (MH$^+$ - CH$_3$OC(O), 3), 212 (MH$^+$ - CH$_3$OH - CH$_2$=CH$_2$, 10), 201 (MH$^+$ - CH$_3$OC(O)CH$_2$, $^{37}$Cl, 2), 199 (MH$^+$ - CH$_3$OC(O)CH$_2$, 12), 198 (MH$^+$ - CH$_3$OC(O)CH$_3$, 2), 187 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_2$, $^{37}$Cl, 1), 186 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, $^{37}$Cl, 5), 185 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_2$, 6), 184 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, 1), 171 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_2$, 6), 159 (MH$^+$ - CH$_3$OC(O)CH$_2$CH$_3$, 4), $^{37}$Cl, 42), 158 (MH$^+$ - CH$_3$OC(O)(CH$_2$)$_3$CH$_3$, 6), 157 (MH$^+$ - CH$_3$OC(O)(CH$_2$)$_4$, 76), 156 (MH$^+$ - CH$_3$OC(O)(CH$_2$)$_5$, 100).
18.3'-Azido-3'-deoxythymidine (ethyl methoxy-6-aminocaproyl) phosphoramidate
3'-Azido-3'-deoxythymidine (0.09 g, 0.3 mmol) was dissolved in THF (3 ml) and
N-methyl imidazole (0.23 ml, 0.24 g, 2.9 mmol, 8 equivs) added. To this solution
was added ethyl methoxy-6-aminocaproyl phosphorochloridate (0.53 g, 1.9 mmol,
5.5 equivs), the last portion being added in THF (2 ml). An additional portion of
N-methyl imidazole (0.08 ml, 0.08 g, 1.0 mmol, 3 equivs) was added. After stirring
for 21 h the reaction had proceeded to 95% completion on tlc. The reaction was
concentrated in vacuo, dissolved in chloroform (30 ml) and washed with saturated
sodium bicarbonate solution (3 x 20 ml), then water (2 x 15 ml). The organic layer
was separated and dried (MgSO₄), filtered and then concentrated in vacuo to a
gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum
spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator
overnight. The petroleum spirit was decanted off and the gummy residue purified
twice by flash column chromatography, first using silica (70 g) and an eluent of 2%
methanol/chloroform, then silica (70 g) and an eluent of 2% methanol/ethyl
acetate. Removal of the solvents in vacuo afforded a colourless gum.
Yield: 0.07 g (38%).

³¹P nmr δ(CDCI₃): 7.83.

¹³C nmr δ(CDCI₃): 173.97, 173.95 (2:3, CH₃OC(0)) 163.89, 163.86 (2:3, C2), 150.40,
150.32 (3:2, C4), 135.34 (C6), 111.39, 111.23 (3:2, C5), 85.17, 84.74 (2:3, C1'), 82.62,
82.60 (2d, 1:1, C4', ³JCP = 2.3 Hz), 65.09, 64.86 (2d, 2:3, C5', ²JCp = 5.0 Hz and 4.6
Hz), 62.84, 62.76 (2d, 2:3, POCH₂, ²JCp = 5.2 Hz and 5.0 Hz), 60.45 (C3'), 51.59 (-
OCH₃), 41.18, 41.14 (2:3, CH₂), 37.56 (C2'), 33.79 (αCH₂), 31.41, 31.35 (2d, 8CH₂,
³JCp = 4.4 Hz), 26.05 and 24.39 (γCH₂ and βCH₂), 16.30, (d, POCH₂CH₃, ³JCp = 6.9
Hz), 12.54, 12.50 (2:3, 5-CH₃).

¹H nmr δ(CDCI₃): 9.63, 9.58 (2s, 2:3, 1H, 3-NH), 7.48, 7.41 (2s, 3:2, 1H, H6), 6.27,
6.20 (2t, 3:2, 1H, H1', ³JH1'-H2' = 6.59 Hz), 4.39 - 4.44 (m, 1H, H3'), 4.04 - 4.26 (m, 5H,
POCH₂, H5', H4'), 3.67 (s, 3H, OCH₃), 3.17 (m, 1H, NH), 2.91 (m, 2H, CH₂), 2.43
(m, 1H, H2'), 2.28 - 2.35 (m, 3H, αCH₂, H2') 1.94, 1.93 (2d, 3H, 5-CH₃, ⁴JHH = 1.12
Hz and 1.96 Hz), 1.63 (m, 2H, CH₂), 1.53 (m, 2H, 5-CH₃, ¹JHH = 1.12 Hz
Hz), 1.31 - 1.39 (m, 5H,
POCH₂CH₃, γCH₂).

EIMS m/e: 503 (MH⁺, 0.23%), 502.1893 (M⁺, C₁₉H₂₈N₆O₄P requires 502.1941), 1), 377
(MH⁺ - thymine, 0.48), 334 (M⁺ - thymine - N₃, 11), 254 (C₆H₁₂NO₃P⁺, 3), 252
(C\textsubscript{9}H\textsubscript{19}NO\textsubscript{3}P\textsuperscript{+}, 2), 250 (M\textsuperscript{+} - C\textsubscript{9}H\textsubscript{19}NO\textsubscript{3}P, 1), 222 (C\textsubscript{9}H\textsubscript{21}NO\textsubscript{3}P\textsuperscript{+} - CH\textsubscript{3}OH, 4), 208 (C\textsubscript{9}H\textsubscript{21}NO\textsubscript{3}P\textsuperscript{+} - CH\textsubscript{3}CH\textsubscript{2}OH, 1), 194 (C\textsubscript{9}H\textsubscript{21}NO\textsubscript{3}P\textsuperscript{+} - CH\textsubscript{3}OH - CH\textsubscript{2}=CH\textsubscript{2}, 1), 180 (C\textsubscript{9}H\textsubscript{21}NO\textsubscript{3}P\textsuperscript{+} - CH\textsubscript{3}OC(O)CH\textsubscript{3}, 4), 166 (C\textsubscript{9}H\textsubscript{21}NO\textsubscript{3}P\textsuperscript{+} - CH\textsubscript{3}OC(O)CH\textsubscript{2}CH\textsubscript{3}, 3), 152 (C\textsubscript{9}H\textsubscript{21}NO\textsubscript{3}P\textsuperscript{+} - CH\textsubscript{3}OC(O)CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}, 6), 138 (C\textsubscript{9}H\textsubscript{21}NO\textsubscript{3}P\textsuperscript{+} - CH\textsubscript{3}OC(O)CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}, 36), 127 (thymineH\textsuperscript{+}, 6), 126 (thymine\textsuperscript{+}, 33), 124 (C\textsubscript{9}H\textsubscript{21}NO\textsubscript{3}P\textsuperscript{+} - CH\textsubscript{3}OC(O)(CH\textsubscript{2})\textsubscript{2}CH\textsubscript{3}, 5), 110 (C\textsubscript{2}H\textsubscript{7}O\textsubscript{3}P\textsuperscript{+}, 44), 109 (C\textsubscript{9}H\textsubscript{21}NO\textsubscript{3}P\textsuperscript{+} - CH\textsubscript{3}OC(O)(CH\textsubscript{2})\textsubscript{2}NH\textsubscript{2}, 4), 82 (C\textsubscript{5}H\textsubscript{4}O\textsuperscript{+}, 19), 81 (C\textsubscript{5}H\textsubscript{3}O\textsuperscript{+}, 1).

Analytical data were obtained after a further purification by flash column chromatography, using silica (30 g) and an eluent of 3.5% ethanol/chloroform.

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 23.2, 23.3 min (1:1).

Analysis: C 45.40%, H 6.37, N 16.25, P 6.49. C\textsubscript{19}H\textsubscript{31}N\textsubscript{8}O\textsubscript{8}P requires C 45.42, H 6.22, N 16.73, P 6.16.

19. Ethyl \textit{n}-propylamino phosphorochloridate

Ethyl phosphorodichloridate (1.79 g, 0.011 mol) was dissolved in diethyl ether (40 ml) and \textit{n}-propylamine (1.30 g, 0.022 mol, 2 equivs) in diethyl ether (40 ml) added dropwise over a period of 3 h at -78 °C. The reaction mixture was allowed to warm to room temperature and stirring continued for 1 h. The white solid was filtered and the diethyl ether removed from the filtrate \textit{in vacuo} to afford a colourless oil.

Yield: 1.94 g (95%).

\textsuperscript{31}P nmr δ(CDC\textsubscript{3}): 14.07.

\textsuperscript{13}C nmr δ(CDC\textsubscript{3}): 64.05 (d, POCH\textsubscript{2}, \textsuperscript{2}J\textsubscript{C-P} = 6.0 Hz), 43.69 (PNHCH\textsubscript{2}), 24.02 (d, PNHCH\textsubscript{2}CH\textsubscript{2}, \textsuperscript{3}J\textsubscript{C-P} = 7.8 Hz), 15.86 (d, POCH\textsubscript{2}CH\textsubscript{3}, \textsuperscript{3}J\textsubscript{C-P} = 8.0 Hz), 11.19 (nPr CH\textsubscript{2}).

\textsuperscript{1}H nmr δ(CDC\textsubscript{3}): 4.9 (bs, 1H, NH), 4.14 (m, 2H, POCH\textsubscript{2}), 2.86 (m, 2H, NHCH\textsubscript{2}), 1.51 (m, 2H, NHCH\textsubscript{2}CH\textsubscript{3}), 1.31 (td, 3H, POCH\textsubscript{2}CH\textsubscript{3}, \textsuperscript{3}J\textsubscript{H-H} = 7.12 Hz, \textsuperscript{4}J\textsubscript{H-P} = 0.9 Hz), 0.86 (t, 3H, NHCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}, \textsuperscript{3}J\textsubscript{H-H} = 7.33 Hz).

EIMS m/e: 187 (M\textsuperscript{+}, \textsuperscript{37}Cl, 1%), 186 (MH\textsuperscript{+}, 1), 185.0383 (M\textsuperscript{+}, C\textsubscript{9}H\textsubscript{13}ClNO\textsubscript{3}P requires 185.0372, 4), 159 (M\textsuperscript{+} - CH\textsubscript{3}=CH\textsubscript{2}, \textsuperscript{37}Cl, 1), 158 (MH\textsuperscript{+} - C\textsubscript{2}H\textsubscript{5}, \textsuperscript{37}Cl, 32), 157 (M\textsuperscript{+} - CH\textsubscript{3}=CH\textsubscript{2}, 4), 156 (MH\textsuperscript{+} - C\textsubscript{2}H\textsubscript{5}, 82) 150 (MH\textsuperscript{+} - HCl, 2), 142 (MH\textsuperscript{+} - CH\textsubscript{2}CH\textsubscript{2}OH, 173
$^{37}$Cl, 2), 140 (MH$^+$ - CH$_3$CH$_2$OH, 4), 130 (M$^+$ - CH$_3$CH$_2$CH$_2$N, $^{37}$Cl, 53), 128 (M$^+$ - CH$_3$CH$_2$CH$_2$N, 100).

Analysis: Cl 18.93%. C$_{13}$H$_{13}$ClNO$_2$P requires Cl 19.10.

20. 3'-Azido-3'-deoxythymidine (ethyl n-propylamino) phosphoramidate

3'-Azido-3'-deoxythymidine (0.12 g, 0.4 mmol) was dissolved in THF (3 ml) and N-methyl imidazole (0.28 ml, 0.29 g, 3.6 mmol, 8.2 equivs.) added. To this solution was added ethyl n-propylamino phosphorochloridate (0.33 g, 1.8 mmol, 4.1 equivs.). After stirring for 8 h the reaction had proceeded to completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (20 ml) and washed with saturated sodium bicarbonate solution (15 ml), then water (3 x 15 ml). The organic layer was separated and dried (MgSO$_4$), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified by flash column chromatography, using an eluent of 3% methanol/chloroform. Removal of the solvents in vacuo afforded a colourless gum.

Yield: 0.16 g (87%).

$^{31}$P nmr $\delta$(CDCl$_3$): 9.77.

$^{13}$C nmr $\delta$(CDCl$_3$): 164.37, 164.34 (1:1, C2), 150.76, 150.68 (1:1, C4), 135.30 (C6), 111.41, 111.23 (1:1, C5), 85.03, 84.62 (1:1, C1'), 82.63, 82.60 (2d, 1:1, C4', $^3$J$_{CP}$ = 5.2 Hz), 65.12, 64.87 (2d, 1:1, C5', $^3$J$_{CP}$ = 4.4 Hz and 3.6 Hz), 62.71, 62.65 (2d, 1:1, POCH$_2$, $^3$J$_{CP}$ = 7.6 Hz and 4.2 Hz), 60.61 (C3'), 43.19, 43.13 (1:1, PNCH$_2$), 37.56 (C2'), 24.94, 24.87 (2d, 1:1, PNCH$_2$CH$_2$, $^3$J$_{CP}$ = 7.3 Hz and 6.4 Hz), 16.29, 16.24 (2d, 1:1, POCH$_2$CH$_2$, $^3$J$_{CP}$ = 2.3 Hz and 6.8 Hz), 12.55, 12.52 (1:1, 5-CH$_3$), 11.20 (PNCH$_2$CH$_2$CH$_3$).

$^1$H nmr $\delta$(CDCl$_3$): 10.54 (bs, 1H, 3-NH), 7.51, 7.43 (2s, 1:1, 1H, H6), 6.29, 6.21 (2t, 1:1, 1H, H1'), 4.43 (m, 1H, H3'), 4.24 (m, 2H, H5'), 4.11 (m, 3H, POCH$_2$ and H4'), 3.70 (m, 1H, PNH), 2.85 (m, 2H, PNHCH$_2$), 2.43 (m, 1H, H2'), 2.32 (m, 1H, H2'), 1.93 (s, 3H, 5-CH$_3$), 1.52 (m, 2H, NHCH$_2$CH$_2$), 1.35 (m, 3H, POCH$_2$CH$_2$), 0.92 (t, 3H, NHCH$_2$CH$_2$CH$_3$), $^3$J$_{HH}$ = 7.36 Hz).

EIMS m/e: 416.1521 (M$^+$, C$_{15}$H$_{25}$N$_5$O$_6$P requires 416.1573, 1%), 291 (MH$^+$ - thymine, 1), 249 (MH$^+$ - thymine - N$_3$, 2), 248 (M$^+$ - thymine - N$_3$, 12), 168 (C$_{5}$H$_{13}$NO$_3$P$^+$, 11),

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152 (C₅H₁₂NO₄P⁺ - CH₄, 4), 150 (C₅H₁₂NO₄P⁺ - H₂O, 2), 140 (C₅H₁₅NO₃P⁺ - CH₂=CH₂, 9), 138 (C₅H₁₅NO₃P⁺ - CH₂, 38), 127 (thymineH⁺, 7), 126 (thymine⁺, 26), 122 (C₅H₁₅NO₃P⁺ - CH₃CH₂OH, 13), 110 (C₅H₁₂O₂P⁺, 51), 109 (C₅H₁₅NO₃P⁺ - CH₃CH₂CH₂NH₂, 9), 82 (C₅H₆O⁺, 28), 81 (C₅H₆O⁺, 100).

Analytical hplc: Column: 250 x 4.6 mm Techsphere ODS 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 80% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 19.7, 20.2 min (1:1).

Analysis: C 42.64%, H 6.06, N 18.27, P 7.30. C₁₃H₂₃N₆O₆P[0.5H₂O] requires C 42.35, H 6.16, N 19.76, P 7.28.

21. Ethyl methoxy-D-valinyl phosphorochloridate

Triethylamine (2.10 ml, 1.53 g, 15.1 mmol, 2 equivs) was dissolved in dichloromethane (30 ml). This solution was added dropwise over 1.5 h to a mixture of D-valine methyl ester hydrochloride (1.27 g, 7.5 mmol, 1 equiv) and ethyl phosphorodichloridate (0.90 ml, 1.23 g, 7.5 mmol) in dichloromethane (30 ml) at -78 °C. The mixture was then allowed to warm slowly to room temperature and stirring continued for 3 h. The dichloromethane was removed in vacuo and carbon tetrachloride (20 ml) added. The solid was filtered and the carbon tetrachloride removed from the filtrate in vacuo to afford a brown oil.

Yield: 1.63 g (84%).

³¹P nmr δ(CDCl₃): 12.41, 11.82 (1:1).

¹³C nmr δ(CDCl₃): 172.12, 171.88 (2d, 1:1, CH₂OC(O), ³JC₁P = 3.5 Hz and 3.8 Hz), 64.01, 63.88 (2d, 1:1, POCH₂, ³JC₁P = 6.6 Hz), 59.83, 59.35 (1:1, C), 51.63, 51.57 (1:1, -OCH₃), 31.46, 31.32 (2d, 1:1, iPrCH, ³JC₁P = 5.4 Hz and 5.8 Hz), 18.44, 18.37 (1:1, iPrMe(1)), 17.02, 16.92 (1:1, iPrMe(2)), 15.30 (d, POCH₂CH₂, ³JC₁P = 7.9 Hz).

¹H nmr δ(CDCl₃): 4.46 (m, 1H, NH), 4.28 (m, 2H, POCH₂, ³JH-P = 2.46 Hz), 3.75 (m, 1H, "CH), 3.70 (s, 3H, -OCH₃), 2.09 (m, 1H, iPrCH), 1.36 (td, 3H, POCH₂CH₂, ⁴JH-P = 0.81 Hz, ³JHH = 7.19 Hz), 0.94 (d, 3H, iPrMe(1)), 0.88, 0.84 (2d, 1:1, 3H, iPrMe(2)).

EIMS m/e: 258.0630 (MH⁺, C₈H₁₈ClNO₄P requires 258.0662, 0.34%), 222 (MH⁺ - HCl, 1), 216 (MH⁺ - C₃H₆, 37Cl, 4), 214 (MH⁺ - C₃H₆, 13), 201 (MH⁺ - CH₃OC(O), 37Cl, 2), 200 (MH⁺ - CH₃OH - CH₂=CH₂, 37Cl, 30), 199 (MH⁺ - CH₃OC(O), 7), 198 (MH⁺ - CH₃OH - CH₂=CH₂, 100), 194 (MH⁺ - HCl - CH₂=CH₂, 2), 188 (MH⁺ -
22. 3'-Azido-3'-deoxythymidine (ethyl methoxy-D-valinyl) phosphoramidate

3'-Azido-3'-deoxythymidine (0.12 g, 0.4 mmol) was dissolved in THF (4 ml) and N-methyl imidazole (0.44 ml, 0.45 g, 5.5 mmol, 12 equivs) added. To this solution was added ethyl methoxy-D-valinyl phosphorochloridate (0.72 g, 2.8 mmol, 6 equivs) in THF (1 ml). After stirring overnight the reaction had proceeded to completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (20 ml), then water (3 x 15 ml). The organic layer was separated and dried (MgSO₄), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified by flash column chromatography, using silica (70 g) and an eluent of 3% methanol/chloroform. Removal of the solvents in vacuo afforded a colourless gum.

Yield: 0.13 g (61%).

$^{31}P$ nmr δ(CDCl₃): 6.93, 6.66 (3:4).

$^{13}C$ nmr δ(CDCl₃): 173.78, 173.63 (2d, 3:2, CH₃OC(O)), $^3J_{C,P} = 2.9$ Hz), 164.03, 164.01 (3:2, C2), 150.55, 150.53 (3:2, C4), 135.41, 135.31 (3:2, C6), 111.52, 111.46 (3:2, C5), 84.76, 84.50 (1:1, C1'), 82.44, 82.36 (2d, 3:2, C4', $^3J_{C,P} = 2.2$ Hz), 65.40, 65.05 (2d, 3:2, C5', $^2J_{C,P} = 5.2$ Hz and 4.9 Hz), 63.34, 63.15 (2d, 2:3, POCH₂, $^3J_{C,P} = 5.4$ Hz and 5.0 Hz), 60.45, 60.30 (3:2, C3'), 59.82, 59.73 (3:2, ′C), 52.22 (-OCH₃), 37.39, 37.29 (1:1, C2'), 32.03, 31.96 (3:2, iPrCH), 19.12, 19.10 (3:2, iPrMe(1)), 17.37, 17.29 (3:2, iPrMe(2)), 16.26, 16.19 (2d, 3:2, POCH₂CH₃, $^3J_{C,P} = 3.9$ Hz and 3.8 Hz), 12.44 (5-CH₃).

$^1$H nmr δ(CDCl₃): 9.60 (bs, 1H, 3-NH), 7.38, 7.36 (2s, 1:1, 1H, H6), 6.21, 6.18 (2t, 1:1, 1H, H1', $^3J_{H1',Hz} = 6.45$ Hz), 4.30 (m, 1H, H3'), 4.04-4.20 (m, 4H, POCH₂, H5'), 3.96 (m, 1H, H4'), 3.65 (2m, 1H, 'CH), 3.67, 3.66 (2s, 1:1, 3H, -OCH₃), 3.43 (m, 1H, valine
NH), 2.37 (m, 1H, H2'), 2.25 (m, 1H, H3'), 2.02 (m, 1H, iPrCH), 1.90, 1.897 (2d, 3:4, 3H, 5-CH3, JH-H = 7.09 Hz, JH-H = 3.36 Hz), 0.94 (d, 3H, iPrMe(1), JH-H = 6.77 Hz), 0.87, 0.84 (2d, 3H, iPrMe(2), JH-H = 6.99 Hz).

EIMS m/e: 489 (MH+, 1%), 488.1735 (M+, C18H29N6O8P requires 488.1785, 2), 429 (MH+ - CH3OH - CH2=CH2, 4), 363 (MH+ - thymine, 1), 321 (MH+ - thymine - CH2=CH-CH3, 2), 320 (M+ - thymine - CH2=CH-CH3, 15), 315 (M+ - thymine - HN3, 2), 260 (M+ - thymine - CH2=CH-CH3 - N3 - H2O, 5), 250 (M+ - C8H17NO5P, 3), 240 (C8H19NO5P+, 2), 222 (C8H19NO5P+ - H2O, 4), 196 (C8H19NO5P+ - C3H8, 5), 194 (C8H19NO5P+ - CH3CHOH, 4), 180 (C8H19NO5P+ - CH3OH - CH2=CH2, 22), 178 (C8H19NO5P+ - C3H8 - H2O, 2), 168 (C8H19NO5P+ - C3H8 - CH2=CH2, 3), 166 (C8H19NO5P+ - CH3CHOH - CH2=CH2, 3), 164 (C8H19NO5P+ - C3H8 - CH3OH, 1), 152 (C8H19NO5P+ - CH3CHOH - CH2=CH-CH3, 17), 150 (C8H19NO5P+ - CH3CHOH - C3H8, 2), 136 (C8H19NO5P+ - C3H8 - CH2=CH2 - CH3OH, 3), 127 (thymineH+, 4), 126 (thymine+, 14), 124 (C8H19NO5P+ - CH3OC(O)CH2CH(CH3)2, 1), 110 (C2H7O3P+, 4), 109 (C8H19NO5P+ - CH3OC(O)CHCH(CH3)2N3H2, 4), 82 (C3H6O+, 18), 81 (C3H6O+, 100).

Analytical data were obtained after further purification by flash column chromatography, using silica (40 g) and an eluent of 1.5% ethanol/ethyl acetate. Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 24.0 min.

Analysis: C 44.07%, H 6.07, N 16.31, P 6.34. C18H29N6O8P[0.5H2O] requires C 43.46, H 6.08, N 16.89, P 6.23.

23. Investigation of the susceptibility of 3'-azido-3'-deoxythymidine (ethyl methoxy-L-alaninyl) phosphoramidate towards decomposition by HIV-1 protease

The enzyme preparation consisted of 50 μg of HIV-1 protease in 450 μl of citrate/phosphate buffer and glycerol. To 0.32 mg of 3'-azido-3'-deoxythymidine (ethyl methoxy-L-alaninyl) phosphoramidate were added 45 μl of the enzyme preparation together with 4 μl of Tween 20. The mixture was stored at 37 °C thereafter and hplc traces recorded at various time points.
Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 \mu m. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min.

Time 0: retention times (min): 20.69 (49.14 area %), 21.22 (46.35), 21.67 (3.15), 22.33 (1.01), 22.77 (0.17), 23.21 (0.11), 25.97 (0.03), 26.18 (0.04).

Time 1.25 h: 19.82 (0.03), 20.57 (48.98), 21.13 (46.12), 21.59 (3.29), 22.31 (1.03), 22.78 (0.15), 23.23 (0.09), 25.02 (0.01), 25.16 (0.02), 25.63 (0.07), 26.01 (0.08), 26.23 (0.12), 29.60 (0.01).

Time 2.5 h: 5.61 (0.48), 5.88 (0.07), 6.27 (0.35), 6.63 (0.02), 20.61 (0.03), 21.07 (42.58), 21.50 (46.09), 21.84 (8.67), 22.51 (0.99), 22.87 (0.28), 23.24 (0.15), 25.60 (0.05), 25.99 (0.07), 26.18 (0.17).

Time 3.75 h: 18.65 (0.02), 19.54 (49.56), 20.12 (45.99), 20.59 (2.97), 21.28 (1.07), 21.82 (0.14), 22.30 (0.09), 24.16 (0.01), 24.80 (0.03), 25.19 (0.04), 25.42 (0.06), 26.79 (0.01).

Time 5 h: 19.61 (49.47), 20.20 (46.14), 20.67 (2.97), 21.35 (1.05), 21.77 (0.05), 21.88 (0.08), 22.34 (0.09), 23.10 (0.01), 24.82 (0.03), 25.25 (0.04), 25.45 (0.06).

24. Stability studies on 3'-azido-3'-deoxythymidine (n-propyl methoxy-L-valinyl) phosphoramidate in aqueous media

(a) Preparation of buffer solutions.

(i) pH 6. A buffer solution of pH 5.93 was prepared by mixing 0.2 M sodium hydrogen maleate (25 ml), 0.1 M sodium hydroxide (26.9 ml) and methanol (10 ml), and diluting to a total volume of 100 ml with water.

(ii) pH 7. A buffer solution of pH 6.90 was prepared by mixing 0.2 M hydrochloric acid (17.5 ml), 0.2 M 2,4,6-collidine (29.25 ml) and methanol (10 ml), and diluting to a total volume of 100 ml with water.

(iii) pH 8. A buffer solution of pH 7.94 was prepared by mixing 0.2 M hydrochloric acid (5 ml), 0.2 M 2,4,6-collidine (48 ml) and methanol (10 ml), and diluting to a total volume of 100 ml with water.

(b) Preparation of samples.

(i) pH 6. To 3'-azido-3'-deoxythymidine (n-propyl methoxy-L-valinyl) phosphoramidate (0.014 g) was added pH 6 buffer (1.5 ml). The resulting mixture was transferred to a 10 mm nmr tube and D2O (1.5 ml) added. The tube was stoppered, sealed and stored at 37 °C.
(ii) pH 7. To 3′-azido-3′-deoxythymidine (n-propyl methoxy-L-valinyl) phosphoramide (0.019 g) was added pH 7 buffer (1.5 ml). The resulting mixture was transferred to a 10 mm nmr tube and D$_2$O (1.5 ml) added. The tube was stoppered, sealed and stored at 37 °C.

(iii) pH 8. To 3′-azido-3′-deoxythymidine (n-propyl methoxy-L-valinyl) phosphoramide (0.029 g) was added pH 8 buffer (1.5 ml). The resulting mixture was transferred to a 10 mm nmr tube and D$_2$O (1.5 ml) added. The tube was stoppered, sealed and stored at 37 °C.

(c) Recording of spectra.

Spectra were recorded on a Varian VXR-400 nmr machine operating at 162 MHz at 37 °C at times 0, 1 h, 1 day, 5 days, 20 days and 110 days.

(i) pH 6.

Time 0: δ$^{31}$P nmr: 10.71.

Time 1 h: δ$^{31}$P nmr: 10.70.

Time 1 day: δ$^{31}$P nmr: 10.57, 10.47.

Time 5 days: δ$^{31}$P nmr: 10.53, 10.43, 10.19, 10.10, 3.32.

Time 20 days: δ$^{31}$P nmr: 10.57, 10.47, 10.44, 10.31, 10.21, 10.17, 10.08, 7.90, 4.90, 3.37, 1.01.

Time 110 days: δ$^{31}$P nmr: 10.59, 10.52, 10.49, 10.28, 10.25, 7.88, 3.42, 1.18.

(ii) pH 7.

Time 0: δ$^{31}$P nmr: 10.78, 10.68.

Time 1 h: δ$^{31}$P nmr: 10.90, 10.82.

Time 1 day: δ$^{31}$P nmr: 10.59, 10.49.

Time 5 days: δ$^{31}$P nmr: 10.52, 10.41, 10.20.

Time 20 days: δ$^{31}$P nmr: 10.55, 10.45, 10.30, 10.19, 7.78, 4.94.

Time 110 days: δ$^{31}$P nmr: 10.61, 10.51, 7.78, 3.50.

(iii) pH 8.

Time 0: δ$^{31}$P nmr: 10.69.

Time 1 h: δ$^{31}$P nmr: 10.65.

Time 1 day: δ$^{31}$P nmr: 10.49, 10.39.

Time 5 days: δ$^{31}$P nmr: 10.52, 10.36, 10.16, 7.76.

Time 20 days: δ$^{31}$P nmr: 11.23, 11.09, 10.51, 10.41, 10.24, 10.14, 7.76.

Time 110 days: δ$^{31}$P nmr: 11.29, 11.16, 10.59, 10.49, 10.35, 10.25, 8.76, 8.05, 7.78.
(d) Analytical hplc.
The samples were analysed by hplc at 103 days.
Column: 50+250 x 4.6 mm Spherisorb ODS2 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 20% B; 10 min 20% B; 30 min 80% B. Detector: uv 254 nm. Flow rate: 1 ml/min.
(i) pH 6.
Retention times (min): 8.61 (0.53 area %), 17.96 (1.43), 18.15 (1.85), 26.28 (56.97), 26.56 (38.00).
(ii) pH 7.
Retention times (min): 8.60 (1.10), 17.52 (0.45), 26.09 (59.14), 26.37 (39.12).
(iii) pH 8.
Retention times (min): 8.55 (3.06), 17.59 (0.74), 26.18 (58.48), 26.45 (37.49).

25. Ethyl 2-methoxyethylamino phosphorochloridate
To a solution of ethyl phosphorodichloridate (0.60 ml, 0.82 g, 5.1 mmol) in diethyl ether (30 ml) was added a solution of 2-methoxyethylamine (0.90 ml, 0.76 g, 10.1 mmol, 2 equivs) in diethyl ether (30 ml) at -78 °C over a period of 1.5 h. The reaction was then stirred at -78 °C for a further 3 h and was then allowed to warm to room temperature. The solid was filtered and the solvent removed from the filtrate in vacuo, to afford a colourless oil.
Yield: 0.68 g (66%).
$^{31}$P nmr δ(CDCl$_3$): 13.43.
$^{13}$C nmr δ(CDCl$_3$): 71.61 (d, PNCH$_2$CH$_2$, $^3$J$_{CP}$ = 7.9 Hz), 64.30 (d, POCH$_2$, $^3$J$_{CP}$ = 6.2 Hz), 58.77 (-OCH$_3$), 41.40 (PNCH$_2$), 15.88 (d, POCH$_2$CH$_2$, $^3$J$_{CP}$ = 8.0 Hz).

26. 3'-Azido-3'-deoxythymidine (ethyl 2-methoxyethylamino) phosphoramidate
3'-Azido-3'-deoxythymidine (0.10 g, 0.4 mmol) was dissolved in THF (5 ml) and N-methyl imidazole (0.26 ml, 0.26 g, 3.2 mmol, 8 equivs) added. To this solution was added ethyl 2-methoxyethylamino phosphorochloridate (0.33 g, 1.6 mmol, 4 equivs). After stirring for 24 h the reaction had proceeded to completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (20 ml) and washed with saturated sodium bicarbonate solution (3 x 20 ml), then water (2 x 15 ml). The organic layer was separated and dried (MgSO$_4$), filtered and then concentrated in
vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified twice by flash column chromatography, first using silica (70 g) and an eluent of 4% methanol/chloroform then using silica (30 g) and an eluent of 5% methanol/ethyl acetate. Removal of the solvents in vacuo afforded a colourless gum.

Yield: 0.07 g (43%).

$^3$P nmr $\delta$(CDCl$_3$): 7.89, 7.86 (1:1).

$^{13}$C nmr $\delta$(CDCl$_3$): 164.00 (C2), 150.50, 150.44 (1:1, C4), 135.28 (C6), 111.42, 111.30 (1:1, C5), 84.94, 84.65 (1:1, C1'), 82.61, 82.53 (2d, 1:1, C4', $^3$J$_{CP}$ = 7.8 Hz), 72.50, 72.45 (2d, 1:1, PNHC$_2$CH$_2$, $^3$J$_{CP}$ = 3.3 Hz), 65.14, 64.91 (2d, 1:1, C5', $^2$J$_{CP}$ = 5.0 Hz and 4.6 Hz), 62.87, 62.81 (2d, 1:1, POCH$_2$, $^2$J$_{CP}$ = 7.5 Hz and 5.0 Hz), 60.52, 60.47 (1:1, C3'), 58.76 (-OCH$_3$), 41.15, 41.10 (1:1, PNHC$_2$), 37.56 (C2'), 16.25, 12.49, 12.46 (1:1, 5-CH$_3$).

$^1$H nmr $\delta$(CDCl$_3$): 9.81 (bs, 1H, 3-NH), 7.49, 7.43 (2s, 1:1, 1H, H6), 6.27, 6.22 (2t, 1:1, 1H, H1'), 4.41 (m, 1H, H3'), 4.04 - 4.26 (m, 5H, H5', POCH$_2$, H4'), 3.54 (bs, 1H, PNH), 3.45 (t, 2H, PNHC$_2$CH$_2$, $^3$J$_{HH}$ = 5.05 Hz), 3.37, 3.36 (2s, 3H, 1:1, -OCH$_3$), 3.11 (m, 2H, PNHC$_2$), 2.43 (m, 1H, H2'), 2.32 (m, 1H, H2'), 1.93 (s, 3H, 5-CH$_3$), 1.35 (t, 3H, POCH$_2$CH$_2$, $^3$J$_{HH}$ = 7.05 Hz).

EI MS m/e: 432.1509 (M$^+$, C$_{15}$H$_{25}$N$_6$O$_7$P requires 432.1522, 1%), 357 (M$^+$ - CH$_3$OCH$_2$CH$_2$NH$_2$, 1), 307 (MH$^+$ - thymine, 1), 264 (M$^+$ - C$_3$H$_{15}$NO$_3$P, 15), 184 (C$_3$H$_{15}$NO$_4$P$^+$, 5), 166 (C$_5$H$_{13}$NO$_4$P$^+$ - H$_2$O, 4), 156 (C$_5$H$_{15}$NO$_4$P$^+$ - CH$_2$=CH$_2$, 1), 152 (C$_5$H$_{15}$NO$_4$P$^+$ - CH$_3$OH, 4), 138 (C$_3$H$_{15}$NO$_4$P$^+$ - CH$_3$CH$_2$OH, 31), 127 (thymineH$^+$, 6), 126 (thymine$^+$, 25), 110 (C$_2$H$_5$O$_3$P$^+$, 37), 82 (C$_4$H$_6$O$^+$, 21), 81 (C$_5$H$_4$O$^+$, 100).

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 µm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 80% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 10.7, 11.3 min.

Analysis: C 41.72%, H 5.97, N 17.92, P 7.13. C$_{15}$H$_{25}$N$_6$O$_7$P requires C 41.67, H 5.83, N 19.44, P 7.16.
27. Ethyl 2-methoxy-1-methylethylamino phosphorochloridate
To a solution of ethyl phosphorodichloridate (0.80 ml, 1.10 g, 6.7 mmol) in diethyl ether (30 ml) was added a solution of racemic 2-amino-1-methoxy-propane (1.42 ml, 1.20 g, 13.5 mmol, 2 equivs) in diethyl ether (30 ml) at -78 °C over 1 h. The reaction was then stirred at -78 °C for 3 h and was then allowed to warm to room temperature. The solid was filtered and the solvent removed from the filtrate in vacuo to afford a yellow oil.
Yield: 1.27 g (88%).

\[^{31}\text{P nmr } \delta(\text{CDCl}_3): 12.40, 12.14 (5:4).\]

\[^{13}\text{C nmr } \delta(\text{CDCl}_3): 76.68, 76.34 (2d, 5:4, \text{CH}_2\text{C}_2, ^3J_{\text{CP}} = 7.3 \text{ Hz and } 7.7 \text{ Hz}), 64.20 (d, \text{POCH}_2, ^3J_{\text{CP}} = 6.6 \text{ Hz}), 58.91 (-\text{OCH}_3), 47.95 (\text{C}), 19.28, 19.07 (2d, 5:4, ^{1}\text{CHCH}_3, ^3J_{\text{CP}} = 4.6 \text{ Hz and } 5.1 \text{ Hz}), 15.93 (d, \text{POCH}_2\text{CH}_3, ^3J_{\text{CP}} = 7.9 \text{ Hz}).\]

28. 3'-Azido-3'-deoxythymidine (ethyl 2-methoxy-1-methylethylamino) phosphoramidate
3'-Azido-3'-deoxythymidine (0.11 g, 0.4 mmol) was dissolved in THF (5 ml) and N-methyl imidazole (0.26 ml, 0.27 g, 3.3 mmol, 8 equivs) added. To this solution was added ethyl 2-methoxy-1-methylethylamino phosphorochloridate (0.35 g, 1.6 mmol, 4 equivs). After stirring for 26 h the reaction had proceeded to completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (20 ml) and washed with saturated sodium bicarbonate solution (3 x 20 ml), then water (2 x 15 ml). The organic layer was separated and dried (MgSO4), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified twice by flash column chromatography, first using silica (50 g) and an eluent of 4% methanol/chloroform then using silica (70 g) and an eluent of 3% methanol/ethyl acetate. Removal of the solvents in vacuo afforded a colourless gum.
Yield: 0.05 g (27%).

\[^{31}\text{P nmr } \delta(\text{CDCl}_3): 7.16, 7.12 (6:5).\]

\[^{13}\text{C nmr } \delta(\text{CDCl}_3): 164.13 (\text{C2}), 150.59, 150.53 (\text{C4}), 135.27 (\text{C6}), 111.45, 111.42, 111.32, 111.28 (4 \text{ diast., C5}), 84.89, 84.87, 84.57 (\text{C1'}, 82.56 (\text{m, C4'}), 77.20 (\text{m,}}\]
\[ ^\text{CHCH}_2 \text{OCH}_3, 65.06 (\text{m, C}^5), 62.79 (\text{m, POCH}_2), 60.70, 60.62, 60.58, 60.53 (4 \text{ diast., C}^3), 58.92, 58.90 (-\text{OCH}_3), 47.50 (\text{m, PNH'C}), 37.51, 37.48 (5:2, C^2), 19.90 (\text{m, 'CHCH}_2\text{OCH}_3), 16.24 (\text{m, POCH}_2\text{CH}_3), 12.48, 12.46 (5-\text{CH}_3). \]

\[ ^1\text{H} \text{nmr } \delta(\text{CDCl}_3): 10.07 (\text{bs, 1H, 3-NH}), 7.50, 7.43 (2s, 1H, H_6), 6.25 (\text{m, 1H, H}_1'), 4.43 (\text{m, 1H, H}_3', 4.17 (\text{m, 5H, H}_5', \text{POCH}_2, \text{H}_4'), 3.49 (\text{m, 7H, PNH', -OCH}_3, \text{PNHCH}, \text{CH}_2\text{OCH}_3), 2.43 (\text{m, 1H, H}_2'), 2.31 (\text{m, 1H, H}_2'), 1.93 (\text{s, 3H, 5-CH}_3), 1.35 (\text{t, 3H, POCH}_2\text{CH}_3), J_\text{HH} = 7.06 \text{ Hz}, 1.19 (\text{m, 3H, 'CHCH}_2). \]

EIMS m/e: 446.1691 (M*, \text{C}_4\text{H}_7\text{N}_6\text{O}_2\text{P} \text{ requires } 446.1679, 1%), 401 (M' - \text{CH}_3\text{-OCH}_2, 1), 400 (M' - \text{CH}_3\text{OCH}_3), 357 (M' - \text{CH}_3\text{OCH}_2\text{CH}(\text{CH}_3)\text{NH}_2, 1), 320 (M' - \text{thymine, 1}), 314 (M' - \text{CH}_3\text{OCH}_2\text{CH}(\text{CH}_3)\text{NH}_2 - \text{HN}_3, 3), 278 (M' - \text{thymine - N}_3, 1), 277 (M' - \text{thymine - HN}_3, 11), 250 (M' - \text{thymine - N}_3 - \text{CH}_2=\text{CH}_2, 3), 249 (M' - \text{thymine - HN}_3 - \text{CH}_2=\text{CH}_2, 1), 232 (M' - \text{thymine - N}_3 - \text{CH}_3\text{CH}_2\text{OH, 9}), 231 (M' - \text{thymine - HN}_3 - \text{CH}_3\text{CH}_2\text{OH, 3}), 204 (M' - \text{thymine - N}_3 - \text{CH}_2=\text{CH}_2 - \text{CH}_3\text{OCH}_3, 1), 198 (\text{C}_6\text{H}_{17}\text{NO}_4\text{P}^+, 4), 180 (\text{C}_6\text{H}_{17}\text{NO}_4\text{P}^+ - \text{H}_2\text{O, 4}), 166 (\text{C}_6\text{H}_{17}\text{NO}_4\text{P}^+ - \text{CH}_3\text{OH, 2}), 152 (\text{C}_6\text{H}_{17}\text{NO}_4\text{P}^+ - \text{CH}_3\text{CH}_2\text{OH, 36}), 138 (\text{C}_6\text{H}_{17}\text{NO}_4\text{P}^+ - \text{CH}_3\text{OH - CH}_2=\text{CH}_2, 6), 127 (\text{thymineH}^+, 6), 126 (\text{thymine}^+, 26), 124 (\text{C}_6\text{H}_{17}\text{NO}_4\text{P}^+ - \text{CH}_3\text{OCH}_2\text{CH}_3\text{CH}_3, 43), 110 (\text{C}_2\text{H}_5\text{O}_3\text{P}^+, 4), 109 (\text{C}_6\text{H}_{12}\text{NO}_4\text{P}^+ - \text{CH}_3\text{OCH}_2\text{CH}(\text{CH}_3)\text{NH}_2, 5), 82 (\text{C}_6\text{H}_4\text{O}^+, 22), 81 (\text{C}_6\text{H}_5\text{O}^+, 100). \]

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 \text{ \mu m}. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 80% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 18.4, 18.8 min.

Analysis: C 41.46%, H 6.33, N 15.93, P 6.63. \text{C}_4\text{H}_7\text{N}_6\text{O}_2\text{P}[\text{H}_2\text{O}] \text{ requires } C 41.38, H 6.29, N 18.10, P 6.67.

29. Ethyl methoxy-L-valinyl phosphonochloridate

Triethylamine (0.82 ml, 0.60 g, 5.9 mmol, 2 equivs) was dissolved in dichloromethane (30 ml). This solution was then added dropwise over a period of 3 h to a mixture of L-valine methyl ester hydrochloride (0.49 g, 2.9 mmol, 1 equiv) in dichloromethane (30 ml) and ethylphosphonic dichloride (0.31 ml, 0.43 g, 2.9 mmol) at -78 °C. The reaction was then allowed to warm to room temperature over a period of 2 h. The dichloromethane was removed \textit{in vacuo} and diethyl ether (20 ml) added. The solid was filtered and the diethyl ether removed from the
filtrate in vacuo to afford a yellow oil which still showed some traces of solid on being allowed to stand. More diethyl ether (40 ml) was added, the solid filtered and the diethyl ether removed from the filtrate in vacuo. After being allowed to stand in the refrigerator overnight there were more traces of solid present. More diethyl ether (5 ml) was added, the solid filtered and the diethyl ether removed from the filtrate in vacuo to afford an oil.

Yield: 0.59 g (83%).

$^{31}$P nmr: $\delta$(CDCl$_3$): 48.43, 47.50 (1:2).

$^{13}$C nmr: $\delta$(CDCl$_3$): 172.22, 171.82 (2d, 3:2, CH$_3$O$\bigcirc$), $^3$$\nu$CP = 2.1 Hz), 58.44, 56.95 (2d, $^1$$\nu$C, $^3$$\nu$CP = 3.4 Hz), 51.32, 51.24 (3:2, -OCH$_3$), 31.06, 30.92 (2d, iPrCH, $^5$$\nu$CP = 6.1 Hz), 29.18, 28.99 (2d, PCH$_2$, $^5$$\nu$CP = 113.1 and 114.3 Hz), 18.02 (iPrMe(1)), 16.67, 16.42 (iPrMe(2)), 5.60, 5.48 (2d, PCH$_2$CH$_3$, $^5$$\nu$CP = 5.0 and 4.8 Hz).

Analysis: P 13.17%. C$_8$H$_{17}$ClNO$_3$P requires P 12.82.

3. Azido-3'-deoxythymidine (ethyl methoxy-L-valinyl) phosphonamidate

3'-Azido-3'-deoxythymidine (0.16 g, 0.6 mmol) was dissolved in THF (10 ml) and N-methyl imidazole (0.38 ml, 0.39 g, 4.8 mmol, 8 equivs) added. Ethyl methoxy-L-valinyl phosphonochloridate (0.60 g, 2.5 mmol, 4 equivs) was added, the last traces being added in THF (5 ml). The reaction was stirred overnight after which time it had proceeded to completion on tlc. The solvent was removed in vacuo and the residual gum dissolved in chloroform (30 ml), washed with saturated sodium bicarbonate solution (20 ml) and then water (3 x 15 ml). The organic layer was separated and dried (MgSO$_4$), filtered, and the solvent removed in vacuo to afford a gum. This was dissolved in chloroform (10 ml) and the product precipitated in petroleum spirit (bp 30-40 °C) (200 ml) overnight. The product was purified three times by flash column chromatography, first employing silica (40 g) and an eluent of 5% methanol/chloroform, then silica (80 g) and an eluent of 5% methanol/ethyl acetate, then silica (20 g) and an eluent of 10% methanol/chloroform (the last column was pre-eluted with chloroform (250 ml)). After removal of the solvents in vacuo, a colourless gum was obtained. This was dissolved in chloroform (50 ml) and washed with water (200 ml). The organic layer was separated, dried (MgSO$_4$), filtered, and the chloroform removed in vacuo to afford a colourless gum.

Yield: 0.10 g (37%)
$^3$P nmr $\delta$(CDCl$_3$): 36.22, 35.79 (1:2).

$^{13}$C nmr $\delta$(CDCl$_3$): 174.13, 174.0 (2d, CH$_3$O(O), $^3$J$_{CP} = 1.4$ Hz), 163.54, 163.51 (1:2, C2), 150.14 (C4), 135.33, 135.16 (1:2, C6), 111.45, 111.35 (1:2, C5), 85.39, 85.14 (1:2, C1'), 82.70, 82.57 (2d, 2:1, C4'), $^3$J$_{CP} = 6.4$ Hz and 7.2 Hz), 62.83, 62.72 (2d, 1:2, C5'), $^2$J$_{op} = 6.7$ Hz and 6.5 Hz), 60.84, 60.52 (1:2, C6), 150.14 (C4), 135.33, 135.16 (1:2, C6), 111.45, 111.35 (1:2, C5), 85.39, 85.14 (1:2, C1'), 82.70, 82.57 (2d, 2:1, C4'), $^3$J$_{CP} = 6.4$ Hz and 7.2 Hz), 62.83, 62.72 (2d, 1:2, C5'), $^2$J$_{op} = 6.7$ Hz and 6.5 Hz), 60.84, 60.52 (1:2, C6), 150.14 (C4), 135.33, 135.16 (1:2, C6), 111.45, 111.35 (1:2, C5), 85.39, 85.14 (1:2, C1'), 82.70, 82.57 (2d, 2:1, C4'), $^3$J$_{CP} = 6.4$ Hz and 7.2 Hz), 62.83, 62.72 (2d, 1:2, C5'), $^2$J$_{op} = 6.7$ Hz and 6.5 Hz), 60.84, 60.52 (1:2, C6), 150.14 (C4), 135.33, 135.16 (1:2, C6), 111.45, 111.35 (1:2, C5),

$^1$H nmr $\delta$(CDCl$_3$): 9.35 (bs, 1H, 3-NH), 7.38, 7.31 (2s, 1:2, 1H, H$_6$), 6.21, 6.15 (2t, 2:1, 1H, H$'$), 4.0 - 4.43 (m, 4H, H$'$), 3.73 (s, 3H, -OCH$_3$), 3.25 (m, 1H, valine NH), 2.48 (m, 1H, H$_{20}$, 2.32 (m, 1H, H$_{2'}$), 1.94, 1.93 (2d, 1:2, 3H, 5-CH$_3$), 1.84 (m, 2H, PCH$_2$), 1.25 (m, 3H, PCH$_2$), 1.20 (m, 3H, PCH$_2$), 0.99 (m, 3H, iPrMe(1)), 0.90 (m, 3H, iPrMe(2)).

EIMS m/e: 473 (MH*, 0.17%), 472.1777 (M*, Cl$_2$H$_2$N$_2$O$_7$P requires 472.1835), 413 (MH* - CH$_3$O(O)H, 3), 305 (MH* - thymine - N$_3$, 1), 304 (MH* - thymine - HN$_3$, 11), 250 (M$^*$ - C$_8$H$_{17}$NO$_4$P, 2), 244 (M$^*$ - thymine - N$_3$ - CH$_3$O(O)H, 3), 224 (C$_8$H$_{19}$NO$_4$P$^*$, 8), 204 (M$^*$ - C$_8$H$_{17}$NO$_4$P - HN$_3$, 3), 206 (C$_8$H$_{19}$NO$_4$P$^*$ - H$_2$O, 4), 188 (C$_8$H$_{17}$NO$_3$P$^*$ - H$_2$O, 2), 180 (C$_8$H$_{19}$NO$_4$P$^*$ - C$_3$H$_8$, 14), 164 (C$_8$H$_{19}$NO$_4$P$^*$ - CH$_3$O(O)H, 57), 148 (C$_8$H$_{19}$NO$_4$P$^*$ - C$_3$H$_8$ - CH$_3$OH, 2), 127 (thymineH$^*$, 4), 126 (thymine$^*$, 28), 124 (M$^*$ - C$_8$H$_{17}$NO$_4$P - thymine, 1), 120 (C$_8$H$_{19}$NO$_4$P$^*$ - C$_3$H$_8$ - CH$_3$O(O)H, 6), 110 (C$_2$H$_7$O$_3$P$^*$, 7), 108 (C$_8$H$_{19}$NO$_4$P$^*$ - CH$_3$O(O)CH$_2$CH(CH$_3$)$_2$, 1), 93 (C$_8$H$_{19}$NO$_4$P$^*$ - CH$_3$O(O)CHCH(CH$_3$)$_2$NH$_2$, 8), 82 (C$_3$H$_4$O$^*$, 14), 81 (C$_3$H$_5$O$^*$, 100).

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 23.5, 23.8 min (2:1). Impurities indicated at 24.8, 25.0 min.

The product was purified by preparative hplc.

Preparative hplc: Column: 2 x 250 x 10 mm Lichrosorb 5 μm silica. Mobile phase: 2.5% methanol/97.5% dichloromethane. Detector: uv 254 nm. Flow rate: 5.0 ml/min.

$^3$P nmr $\delta$(CDCl$_3$) (major isomer): 33.21.

$^1$H nmr $\delta$(CDCl$_3$) (major isomer): 8.87 (bs, 1H, 3-NH), 7.31 (s, 1H, H$_6$), 6.19 (t, 1H, 185
H1', 3JH1-H2 = 6.45 Hz), 4.40 (m, 1H, H3'), 4.27 (m, 1H, H5'), 4.14 (m, 1H, H5'), 4.02 (m, 1H, H4'), 3.88 (m, 1H, 'CH), 3.74 (s, 3H, -OCH3), 3.13 (t, 1H, valine NH), 2.43 (m, 1H, H2'), 2.28 (m, 1H, H2'), 2.11 (m, 1H, iPrCH), 1.93 (s, 3H, 5-CH3), 1.77 (m, 3H, PCH2CH3), 1.19 (m, 2H, PCH2), 0.98 (m, 3H, iPrMe(1)), 0.87 (m, 3H, iPrMe(2)).

31P nmr δ(CDC13) (minor isomer): 33.51.
1H nmr δ(CDC13) (minor isomer): 8.44 (bs, 1H, 3-NH), 7.37 (s, 1H, H6), 6.14 (t, 1H, H1', 3JH1-H2 = 6.37 Hz), 4.30 (m, 2H, H3', H5'), 4.06 (m, 2H, H5', H4'), 3.80 (m, 1H, 'CH), 3.75 (s, 3H, -OCH3), 3.09 (m, 1H, valine NH), 2.45 (m, 1H, H2'), 2.26 (m, 1H, H2'), 2.11 (m, 1H, iPrCH), 1.95 (s, 3H, 5-CH3), 1.77 (m, 3H, PCH2CH3), 1.17 (m, 2H, PCH2), 0.99 (m, 3H, iPrMe(1)), 0.88 (m, 3H, iPrMe(2)).

Analysis: C 45.05%, H 6.34, N 16.05, P 5.74. C18H29N6O7P[0.5H2O] requires C 44.91, H 6.28, N 17.46, P 6.43.

31. Ethyl t-butyl-L-valinyl phosphorochloridate
Ethyl phosphorodichloridate (0.37 g, 2.3 mmol) was dissolved in dichloromethane (25 ml) and L-valine t-butyl ester hydrochloride (0.48 g, 2.3 mmol, 1 equiv) added. This suspension was cooled to -78 °C and triethylamine (0.64 ml, 0.47 g, 4.6 mmol, 2 equivs) was added by means of a syringe pump. The reaction mixture was allowed to warm to room temperature over a period of 2 h and was then stirred for 2 h. The solvent was removed in vacuo, and carbon tetrachloride (20 ml) added. The solid was filtered and the solvent removed from the filtrate in vacuo to afford a colourless oil.

Yield: 0.71 g (92%).

31P nmr δ(CDC13): 12.67, 12.17 (1:1).
13C nmr δ(CDC13): 171.28, 170.93 (2d, 1:1, C(O), 3JC.P = 3.6 Hz and 3.8 Hz), 82.32, 82.21 (1:1 'BuC), 64.56, 64.44 (2d, 1:1, POCH3, 3JC.P = 3.4 Hz and 3.2 Hz), 60.74, 60.04 (1:1, 'C), 32.13, 31.99 (2d, iPrCH, 3JC.P = 2.5 Hz and 3.1 Hz), 28.04 ('Bu CH3), 19.03, 18.95 (2:3, iPrMe(1)), 17.40, 17.35 (2:3, iPrMe(2)), 15.96, 15.80 (2d, 1:1, POCH2CH3, 3JC.P = 2.8 Hz and 3.1 Hz).
1H nmr δ(CDC13): 4.25 (m, 2H, POCH3), 3.66 (m, 1H, 'CH), 2.09 (m, 1H, iPrCH), 1.48 (s, 9H, 'Bu CH3), 1.39 (m, 3H, POCH2CH3), 1.00 (d, 3H, iPrMe(1)), 0.92 (d, 3H, iPrMe(2)).

EIMS m/e: 300 (MH+, 1), 264 (M+ - Cl, 2), 219 (MH+ - Cl - CH3CH2OH, 2), 208
32. 3'-Azido-3'-deoxythymidine (ethyl t-butyl-L-valinyl) phosphoramidate

3'-Azido-3'-deoxythymidine (0.11 g, 0.4 mmol) was dissolved in THF (2 ml) and N-methylimidazole (0.27 ml, 0.27 g, 3.3 mmol, 8 equivs) added. To this solution was added ethyl t-butyl-L-valinyl phosphorochloridate (0.56 g, 1.9 mmol, 4.4 equivs) dissolved in THF (1.6 ml). After stirring for 64 h the reaction had proceeded to ca. 90% completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (20 ml) and washed with saturated sodium bicarbonate solution (15 ml), then water (3 x 15 ml). The organic layer was separated and dried (MgSO₄), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified by flash column chromatography, using silica (70 g) and an eluent of 3% methanol/chloroform. Removal of the solvents in vacuo afforded a colourless gum.

Yield: 0.18 g (82%).

$^{31}$P nmr $\delta$(CDCl₃): 7.20, 7.02 (4:3).

$^{13}$C nmr $\delta$(CDCl₃): 172.22 (d, CH₃OC(O)), $^{3}J_{CP} = 3.1$ Hz), 163.99 (C2), 150.50, 150.46 (2:1, C4), 135.20 (C6), 111.53, 111.43 (4:3, C5), 84.77, 84.55 (3:4, C1'), 82.50, 82.38 (2d, 4:3, C4'), $^{3}J_{CP} = 7.6$ Hz and 8.2 Hz), 82.06, 82.03 (3:4, iBuC), 65.29, 65.14 (2d, 3:4, C5', $^{2}J_{CP} = 5.1$ Hz and 4.7 Hz), 63.06 (d, POCH₂, $^{3}J_{CP} = 4.7$ Hz), 60.52, 60.49 (3:4, C3'), 60.28, 60.13 (3:4, 'C), 37.52, 37.44 (4:3, C2'), 32.13, 32.01 (2d, 3:4, iPrCH, $^{3}J_{CP} = 6.1$ Hz and 6.6 Hz), 28.00 (BuCH₂), 19.18, 19.03 (4:3, iPrMe(1)), 17.20, 17.10 (3:4, iPrMe(2)), 16.22 (d, 3:5, POCH₂CH₃, $^{3}J_{CP} = 7.1$ Hz), 12.52 (5-CH₃).

$^{1}$H nmr $\delta$(CDCl₃): 9.14 (bs, 1H, 3-NH), 7.50, 7.41 (2s, 4:3, 1H, H6), 6.26 (2t, 4:3, 1H, H1'), 4.39 (m, 1H, H3'), 4.15 (m, 6H, POCH₂, H5', H4', 'CH), 3.60 (m, 1H, valine NH), 2.42 (m, 1H, H2''), 2.28 (m, 1H, H2''), 2.10 (m, 1H, iPrCH), 1.96, 1.95 (2s, 4:3, 3H, 5-CH₃), 1.47 (s, 9H, iBuCH₃), 1.33 (m, 3H, POCH₂CH₃), 1.00, 0.98 (2d, 4:3, 3H, iPrMe(1)), 0.89 (m, 3H, iPrMe(2)).

EIMS m/e: 530.2200 (M⁺, C₂₁H₃₅N₄O₈P requires 530.2254, 1%), 429 (M⁺ - (CH₃)₃COC(O), 1), 260 (M⁺ - (CH₃)₃COC(O)H - thymine - N₃, 1), 250 (M⁺ -...
Analytical data were obtained after further purification by flash column chromatography, using silica (50 g) and an eluent of ethyl acetate.

Analytical hplc: Column: 250 x 4.6 mm Kromasil C18 5 µm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 20% B; 10 min 20% B; 30 min 80% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 27.0, 27.2 min.

Analysis: C 48.38%, H 6.79, N 14.52, P 5.45. (ethyl-L-valinyl) phosphoramidate requires C 47.54, H 6.65, N 15.84, P 5.84.

33. 3'-Azido-3'-deoxythymidine (ethyl L-valinyl) phosphoramidate (first attempt)

3'-Azido-3'-deoxythymidine (ethyl t-butyl-L-valinyl) phosphoramidate (0.05 g, 8.9 x 10^{-5} mol) was stirred with trifluoroacetic acid (2 ml) for 20 min, after which time all of the starting material had been consumed. The mixture was analysed by tlc in two solvent systems: A, chloroform/methanol/acetic acid 90:9:1; B, chloroform/acetonitrile/2-propanol/acetic acid 3:1:0.5:0.2. Tlc showed that at least two products had been formed and 3'-azido-3'-deoxythymidine was also present in the reaction mixture. In an attempt to precipitate out the product the reaction mixture was poured into hexane (100 ml) and stored in the refrigerator overnight. This was unsuccessful and the reaction was terminated.

34. N-(diethylphosphoryl)glycine

A suspension of glycine (1.50 g, 20 mmol) in triethylamine (10 ml), water (6 ml) and ethanol (4 ml) was cooled to 0 °C. A mixture of diethyl phosphite (2.76 g, 20 mmol) and carbon tetrachloride (8 ml) was added dropwise and the mixture stirred at 20 °C for 16.5 h. The reaction was quenched by acidifying the mixture to pH 2 with 2M dilute hydrochloric acid (ca. 32 ml). The mixture was then extracted with ethyl acetate (3 x 40 ml) and the extract dried (MgSO₄). The mixture was filtered and the solvent removed from the filtrate in vacuo to afford a
colourless oil.

Yield: 2.49 g (59%).

$^{31}$P nmr $\delta$(CDCl$_3$): 6.27.

$^{13}$C nmr $\delta$(CDCl$_3$): 172.52 (d, CH$_3$OOC(O), $^3$J$_{CP}$ = 11.0 Hz), 62.49 (d, POCH$_2$, $^3$J$_{CP}$ = 5.1 Hz), 42.18 (PNHCH$_2$), 15.79 (d, POCH$_3$CH$_3$, $^3$J$_{CP}$ = 6.9 Hz).

$^1$H nmr $\delta$(CDCl$_3$): 12.43 (bs, 1H, C(O)OH), 4.84 (bs, 1H, NH), 4.08 (m, 4H, POCH$_2$), 3.65 (d, 2H, PNHCH$_2$), 1.32 (t, 6H, POCH$_3$), $^3$J$_{HH}$ = 6.98 Hz).

35. N-(diethylphosphoryl)glycine-L-phenylalanine methyl ester

L-phenylalanine methyl ester hydrochloride (0.45 g, 2.1 mmol, 1.1 equivs) was stirred with triethylamine (0.63 g, 6.3 mmol, 3.3 equivs) in dichloromethane (2 ml) for 0.5 h. The triethylamine hydrochloride was filtered, washed with a little dichloromethane and the excess triethylamine and solvent removed from the filtrate in vacuo. Solid was evident in the product and so hexane (ca. 5 ml) was added and the solid filtered. The solvent was removed from the filtrate in vacuo to afford an oil. Separately, N-(diethylphosphoryl)glycine (0.4 g, 1.9 mmol) and triethylamine (0.19 g, 1.9 mmol, 1 equiv) were stirred together in dichloromethane (10 ml) at -10 °C. To this solution was added a solution of diethyl phosphorochloridate (0.33 g, 1.9 mmol, 1 equiv) and triethylamine (0.38 g, 3.8 mmol, 2 equivs) in dichloromethane (5 ml) over 30 min. The L-phenylalanine methyl ester (as a solution in a little dichloromethane) was then added and the mixture stirred overnight. The mixture was washed with dilute citric acid (2 x 50 ml), saturated sodium bicarbonate solution (50 ml) and water (50 ml). The organic layer was separated, dried (MgSO$_4$) and evaporated. The gummy residue was purified by flash column chromatography on silica (20 g), using ethyl acetate as eluent. The solvent was removed from the appropriate fractions in vacuo to afford a colourless gum.

Yield: 0.29 g (41%).

$^{31}$P nmr $\delta$(CDCl$_3$): 6.20.

$^{13}$C nmr $\delta$(CDCl$_3$): 171.80 (Phe C(O)), 170.01 (d, Gly C(O), $^3$J$_{CP}$ = 6.0 Hz), 127.10 - 135.93 (ArC), 62.79 (m, POCH$_2$), 53.29 (Phe 'CH), 52.28 (OCH$_3$), 44.66 (Gly CH$_2$), 37.84 (Phe CH$_2$), 16.15 (d, POCH$_3$CH$_3$, $^3$J$_{CP}$ = 5.8 Hz).

$^1$H nmr $\delta$(CDCl$_3$): 7.14 - 7.31 (m, 6H, ArH, Phe NH), 4.86 (m, 1H, Phe 'CH), 4.00
(m, 4H, POCH₂), 3.84 (m, 1H, Gly NH), 3.70 (s, 3H, -OCH₃), 3.59 (m, 2H, NCH₂), 3.12 (m, 2H, CH₂Ph), 1.27 (m, 6H, POCH₂CH₃).

EIMS m/e: 372.1454 (M⁺, C₁₆H₂₅N₂O₄P requires 372.1450, 5%), 341 (M⁺ - OCH₃, 1), 340 (M⁺ - CH₃OH, 3), 313 (M⁺ - C(O)OCH₃, 8), 312 (M⁺ - CH₃OC(O)H, 1), 282 (MH⁺ - CH₂Ph, 1), 281 (M⁺ - CH₂Ph, 14), 194 ([(CH₃CH₂O)₂P(O)NHCH₂C(O)]⁺, 44), 167 ([(CH₃CH₂O)₂P(O)N]⁺, 52), 166 ([(CH₃CH₂O)₂P(O)NHCH₂]⁺, 100), 138 ([(CH₃CH₂O)₂P(O)H]⁺, 58), 110 (C₂H₅O₃P⁺, 77), 91 (CH₂Ph⁺, 65).


36. Ethyl phosphorodichloridite
Phosphorus trichloride (105 g, 0.77 mol) was dissolved in diethyl ether (200 ml). A solution of ethanol (32 g, 0.70 mol) in diethyl ether (100 ml) was added over 0.5 h at -78 °C. The reaction was then allowed to warm to room temperature and stirring continued overnight under nitrogen. Distillation of the reaction mixture afforded the product as a colourless oil.

Yield: 62 g (93%).

³¹P nmr δ(CDCl₃): 177.15.

¹³C nmr δ(CDCl₃): 64.79 (d, POCH₂, ²J_C-P = 10.0 Hz), 15.78 (d, POCH₂CH₃, ³J_C-P = 3.2 Hz).

¹H nmr δ(CDCl₃): 4.39 (m, 2H, POCH₂), 1.45 (t, 3H, POCH₂CH₃, ³J_H-H = 6.7 Hz).

37. Ethyl morpholinyl phosphorochloridite
Ethyl phosphorodichloridite (39.5 g, 0.27 mol) was dissolved in diethyl ether (400 ml). Morpholine (47 ml, 46.8 g, 0.54 mol, 2 equivs) was added dropwise over a period of 0.75 h. Some external cooling was necessary to prevent the reaction from heating up. After stirring for 2.75 h, the white precipitate was filtered off and the residue washed with diethyl ether (100 ml). The diethyl ether was removed by distillation at atmospheric pressure. Hexane (ca. 100 ml) was added and the mixture placed in the refrigerator in order to precipitate out more solid. The mixture was allowed to warm
to room temperature and the solid was then filtered and the hexane removed from the filtrate in vacuo. The resultant viscous oil was distilled in vacuo and the fraction boiling at 77-79 °C/0.05 mm Hg collected.

Yield: 19.6 g (37%).

$^{31}$P nmr $\delta$(CDCl$_3$): 168.60.

$^{13}$C nmr $\delta$(CDCl$_3$): 66.87 (d, OCH$_2$ (morpholine), $^3$JC$_{CP}$ = 7.4 Hz), 62.38 (d, POCH$_2$, $^2$JC$_{CP}$ = 16.4 Hz), 43.87 (d, NCH$_2$, $^2$JC$_{CP}$ = 9.6 Hz), 16.27 (d, POCH$_2$CH$_3$, $^3$JC$_{CP}$ = 6.9 Hz).

$^1$H nmr $\delta$(CDCl$_3$): 3.94 (m, 2H, POCH$_2$), 3.66 (t, 4H, OCH$_2$ (morpholine), $^3$J$_{HH}$ = 5.07 Hz), 3.14 (m, 4H, NCH$_2$), 1.30 (t, 3H, POCH$_2$CH$_3$, $^3$J$_{HH}$ = 7.00 Hz).

EI-MS m/e: 199 (M+, $^{37}$Cl, 17%), 198 (MH+, 4), 197.0377 (M+, C$_6$H$_{13}$ClNO$_2$P requires 197.0372, 58), 162 (MH+ - HCl, 81), 154 (MH+ - CH$_3$CH$_2$OH, $^{37}$Cl, 5), 152 (MH+ - CH$_3$CH$_2$OH, 10), 134 (C$_4$H$_8$NO$_2$P+, 55), 113 ([CH$_3$CH$_2$OP$^{37}$Cl]+, 18), 111 ([CH$_3$CH$_2$OP$^{37}$Cl]+, 67), 86 (C$_4$H$_8$NO+, 96).

38. 3'-Azido-3'-deoxythymidine ethyl hydrogen phosphonate (first attempt)

3'-Azido-3'-deoxythymidine (0.15 g, 0.6 mmol) was added to chloroform (1 ml) and the resultant suspension gently heated to effect dissolution of the nucleoside. Diisopropylethylamine (0.39 ml, 0.29 g, 23 mmol, 4 equivs) was added. Ethyl morpholinyl phosphorochloridite (0.17 g, 0.9 mmol, 1.5 equivs) was then added over 1 min and the mixture stirred for 15 min. The resultant solution was transferred with ethyl acetate (30 ml) to a separating funnel and extracted five times with brine (30 ml). The organic phase was dried (Na$_2$SO$_4$), filtered and concentrated in vacuo to an oil. This was dissolved in acetonitrile (3 ml) and water (0.08 g, 4.4 mmol, 8 equivs) and tetrazole (0.06 g, 0.8 mmol, 1.4 equivs) added. The mixture was stirred for 0.5 h. The solvent was then removed in vacuo to afford the crude product.

$^{31}$P nmr $\delta$(CDCl$_3$): many signals in the range -3.80 to 11.90.

The product was purified by flash column chromatography, using silica (20 g) and an eluent of 1% methanol/chloroform. Two fractions were obtained. The first contained material with an R$_f$ of 0.26 (in ethyl acetate). The second contained material with an R$_f$ of 0.16 and 3'-azido-3'-deoxythymidine (R$_f$ 0.58) (in ethyl acetate).

Yield (first fraction): 0.011 g (5% based on expected product).

$^{31}$P nmr $\delta$(CDCl$_3$) (first fraction): 11.90, 6.40, 6.06, 5.76, 4.15, 3.68, -3.74, -4.21.

Yield (second fraction): 0.015 g (7%).
$^{31}$P nmr $\delta$(CDCl$_3$) (second fraction): 6.39, 6.13, 5.80, 3.52, -3.70, -4.20.

39. 3'-Azido-3'-deoxythymidine ethyl hydrogen phosphonate (second attempt)

3'-Azido-3'-deoxythymidine (0.22 g, 0.8 mmol) was added to chloroform (3 ml) and the resultant suspension gently heated to effect dissolution of the nucleoside. Diisopropylethylamine (0.56 ml, 0.42 g, 3.2 mmol, 4 equivs) was added. Ethyl morpholinyl phosphorochloridite (0.27 g, 1.4 mmol, 1.7 equivs) was then added over a period of 1 min and the mixture stirred for 15 min. The reaction was diluted with chloroform (30 ml) and washed with water (2 x 40 ml). The organic layer was separated and dried (MgSO$_4$), filtered and the solvent removed in vacuo. The last traces of solvent were removed on a high vacuum pump to afford a yellow foam.

$^{31}$P nmr $\delta$(CDCl$_3$): 141.70, 141.43, 141.21, 141.03, 140.60, 140.20, 30.33, 27.70, 10.43, 9.60, 6.29, 6.04.

This crude product was dissolved in acetonitrile (5 ml) and water (0.13 g, 7.2 mmol, 9 equivs) and tetrazole (0.08 g, 1.1 mmol, 1.4 equivs) added. The mixture was stirred for 1 h and the solvent then removed in vacuo. The gummy residue was applied to a flash column consisting of silica gel (40 g) and an eluent of 1% methanol/chloroform. The same elution pattern as in experiment 38 was observed. Two fractions were obtained.

Yield (first fraction): 0.036 g (12%).

$^{31}$P nmr $\delta$(CDCl$_3$) (first fraction): many signals in the range -3.10 to 11.54.

Yield (second fraction): 0.039 g (13%).

$^{31}$P nmr $\delta$(CDCl$_3$) (second fraction): many signals in the range 5.58 to 11.50.

The flash column was flushed through with methanol and a third fraction collected containing all the remaining uv active material.

$^{31}$P nmr $\delta$(CDCl$_3$) (third fraction): 19.70, 15.15, 0.82.

40. Sodium ethyl hydrogen phosphite

To diethyl phosphite (10 g, 0.07 mol) was added ethanol (10 ml) and water (10 ml). Sodium hydroxide (3 g, 0.07 mol, 1 equiv) was dissolved in water (10 ml) and added dropwise to the diethyl phosphite solution. The reaction mixture was then heated to 50 °C overnight. The solvents were then removed in vacuo to afford a colourless solid.

Yield: 8.3 g (88%).
$^{31}$P nmr δ(D$_2$O): 7.12.
Melting point: 182 °C.

41. Tetra-$n$-butylammonium ethyl hydrogen phosphite
A 20% aqueous solution of sodium hydroxide (2.5 ml) was added dropwise with
stirring and external cooling (ice bath) to a solution of tetra-$n$-butylammonium
hydrogensulphate (3.40 g, 0.01 mol, 1 equiv) in water (2 ml). A solution of sodium
ethyl hydrogen phosphite (1.32 g, 0.01 mol) in water (1.5 ml) was then added
dropwise. Stirring was continued for 15 min. The mixture was filtered and the filtrate
extracted with dichloromethane (4 x 10 ml), the extracts combined, dried (MgSO$_4$) and
evaporated in vacuo to afford a yellow syrup.
Yield: 2.97 g (85%).

$^{31}$P nmr δ(CDCl$_3$): 1.62.
$^1$H nmr δ(CDCl$_3$): 6.90 (d, 1H, P(O)H, J$_{HH}$ = 586 Hz), 3.88 (m, 2H, POCH$_2$), 3.32 (m, 8H,
'NCH$_2$), 1.65 (m, 8H, 'NCH$_2$CH$_2$), 1.45 (m, 8H, 'NCH$_2$CH$_2$CH$_2$), 1.23 (t, 3H,
POCH$_2$CH$_3$), $^3$J$_{HH}$ = 6.98 Hz), 1.01 (m, 12H, 'NCH$_2$CH$_2$CH$_2$CH$_3$).
Analysis: C 56.82%, H 11.99, N 3.58, P 8.06. C$_{18}$H$_{42}$N$_3$O$_3$P[1.5H$_2$O] requires C 57.11, H 11.98, N 3.70, P 8.18.

42. 3'-O-acetyl-5'-deoxy-5'-iodothymidine
3'-O-acetyltymidine (0.42 g, 1.5 mmol) was dissolved in DMF (5 ml) and
methyltriphenoxyposphonium iodide (1.0 g, 2.2 mmol, 1.5 equivs) added. The
reaction was kept at 25 °C for 1 h, methanol (0.5 ml) added and then the mixture was
evaporated to dryness, the last traces of DMF being removed by co-evaporation with
toluene. The residue was dissolved in chloroform (30 ml), washed with saturated
sodium thiosulphate solution (20 ml) and water (3 x 20 ml), the organic layer
separated and dried (MgSO$_4$) and the chloroform removed in vacuo to afford a
colourless gum. The product was purified by flash column chromatography using
silica (70 g) and an eluent of chloroform. The solvent was removed from the relevant
fractions in vacuo to afford a colourless gum. This became a foam when placed under
high vacuum.
Yield: 0.40 g (69%).
Melting point: 129 °C.
43. 1-(2-Deoxy-3-O-methanesulphonyl-5-O-trityl-D-threopentofuranosyl)uracil

1-(2-Deoxy-5-O-trityl-D-threopentofuranosyl)uracil (3.6 g, 7.7 mmol) was dissolved in pyridine (20 ml) and the solution cooled to -10 °C. Methanesulphonyl chloride (2.1 ml, 3.1 g, 27 mmol, 3.5 equivs) was added dropwise over 5 min. The reaction was allowed to warm to room temperature and stirring continued overnight. The reaction mixture was then poured into rapidly-stirred iced water (600 ml) and stirring continued for 1 h. The brown solid obtained was filtered and dried. The product was purified by flash column chromatography, using silica (70 g) and an eluent of chloroform/ethanol 8:1.

Yield: 3.0 g (72%).
Melting point: 157 °C.

1H nmr δ(CDC13): 8.39 (bs, 1H, 3-NH), 7.62 (s, 1H, H6), 6.34 (m, 1H, H1'), 5.07 (m, 1H, H3'), 3.90 (m, 1H, H4'), 3.58 (m, 2H, H5'), 2.33 (m, 2H, H2'), 2.12 (s, 3H, acetyl CH3), 1.97 (s, 3H, 5-CH3).

44. 3'-Azido-2',3'-dideoxyuridine

A mixture containing 1-(2-deoxy-3-O-methanesulphonyl-5-O-trityl-D-threopentofuranosyl)uracil (2.96 g, 5.4 mmol) and sodium azide (1.77 g, 27.2 mmol, 5 equivs) in DMF (30 ml) was heated at 85 °C for 4 h. The mixture was allowed to cool to room temperature and was then poured into rapidly-stirred iced water (800 ml) and stirring continued for 0.75 h. The solid was filtered off, washed with water (200 ml) and dried. The product was recrystallised from ethanol to afford 1.00 g (37%) of a yellow solid.

1H nmr: δ(CDC13): 9.10 (bs, 1H, 3-NH, abs. on D2O exchange), 7.35 (m, 16H, trityl H, H6), 6.25 (m, 1H, H1'), 5.68 (d, 1H, H5), 5.29 (m, 1H, H3'), 4.27 (m, 1H, H4'), 3.68 (m, 2H, H5'), 2.78 (s, 3H, methanesulphonyl H), 2.55 (m, 2H, H2').

A suspension of this 3'-azido-5'-O-trityl-2',3'-dideoxyuridine (0.97 g) in 80% acetic acid (40 ml) was refluxed at 110 °C for 0.5 h. The solution was allowed to cool to room temperature, and some solid fell out of solution. Water (10 ml) was added to assist in
the precipitation, and the mixture filtered. Activated charcoal was added and the mixture stirred for 0.3 h to clarify the filtrate. After two filtrations the filtrate was concentrated in vacuo to yield colourless crystals, which were dried in vacuo. The crystals were then washed with diethyl ether and ethanol and redried.

Yield: 0.16 g (32%).

Melting point: 167-170 °C

$^1$H nmr: $\delta$(CDCl$_3$): 8.0 (bs, 1H, 3-NH), 7.67 (d, 1H, H6), 6.12 (t, 1H, H1'), 5.76 (d, 1H, H5), 4.41 (m, 1H, 5'-OH), 4.0 (m, 1H, H3'), 3.87 (m, 1H, H4'), 2.52 (m, 2H, H5'), 2.17 (m, 2H, H2').

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 6.2 min.

45. 3'-Azido-2',3'-dideoxyuridine (ethyl methoxy-L-valinyl) phosphoramidate

3'-Azido-2',3'-dideoxyuridine (0.14 g, 0.5 mmol) was dissolved in THF (10 ml) and N-methyl imidazole (0.44 ml, 0.45 g, 5.5 mmol, 10 equivs) added. Ethyl methoxy-L-valinyl phosphorochloridate (0.71 g, 2.8 mmol, 5 equivs) was added. After stirring for 26 h the reaction had proceeded to 70% completion on tlc. Ethyl methoxy-L-valinyl phosphorochloridate (0.26 g, 1.0 mmol, 2 equivs) and N-methyl imidazole (0.16 ml, 0.17 g, 2.0 mmol, 4 equivs) were added and stirring continued for another 40 h, after which time the reaction had proceeded to completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (20 ml), then water (3 x 15 ml). The organic layer was separated and dried (MgSO$_4$), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified by flash column chromatography, using silica (70 g) and an eluent of 5% methanol/chloroform. Removal of the solvents in vacuo afforded a colourless gum.

Yield: 0.20 g (78%).

$^{31}$P nmr $\delta$(CDCl$_3$): 6.96, 6.84 (2:1).

$^{13}$C nmr $\delta$(CDCl$_3$): 173.60, 173.46 (2d, 2:1, CH$_3$OC(0), $^3$J$_{CP}$ = 3.1 Hz), 163.26 (C2), 150.26, 150.23 (2:1, C4), 139.61 (C6), 102.78, 102.68 (2:1, C5), 85.24, 85.09 (1:2, C1'), 82.68 (d,
C4', $J_{CP} = 7.6$ Hz, 65.21, 65.03 (2d, 1:2, C5', $J_{CP} = 5.2$ Hz and 5.0 Hz), 63.25 (m, POCH$_2$), 60.17 (C3'), 59.87 (C'), 52.17, 52.15 (1:1, -OCH$_3$), 37.75 (C2'), 32.02, 31.97 (2d, 1:2, iPrCH, $J_{CP} = 6.6$ Hz), 19.04, 19.00 (iPrMe(1)), 17.32 (iPrMe(2)), 16.19, 16.12 (2d, 3:2, POCH$_2$CH$_2$), $J_{CP} = 7.0$ Hz and 7.1 Hz).

$^1$H nmr (CDCl$_3$): 7.63, 7.58 (2d, 2:1, 1H, H6, $J_{H5-H6} = 8.22$ Hz and 8.14 Hz), 6.18, 6.14 (2t, 2:1, 1H, H1', $J_{H1'-H2'} = 6.42$ Hz), 5.75, 5.74 (2d, 2:1, 1H, H5, $J_{H5-H6} = 8.14$ Hz), 4.31 (m, 1H, H3'), 3.70, 3.69 (2s, 3:2, -OCH$_3$), 3.66 (m, 2H, 'CH, valine NH), 2.43 (m, 1H, H2'), 2.27 (m, 1H, H2'), 2.05 (m, 1H, iPrCH), 1.27 (m, 3H, POCH$_2$), 0.95 (m, 3H, POCH$_2$CH$_3$), 0.95 (m, 3H, iPrMe(1)), 0.87 (m, 3H, iPrMe(2)).

EI-MS m/e: 474.1576 (M\(^+\), C$_{17}$H$_{27}$N$_6$O$_8$P requires 474.1628, 0.3%), 415 (MH\(^+\) - CH$_3$OH - CH$_2$=CH$_2$, 2), 414 (M\(^+\) - CH$_3$OH - CH$_2$=CH$_2$, 14), 363 (MH\(^+\) - uracil, 1), 321 (MH\(^+\) - uracil - N$_3$, 1), 320 (M\(^+\) - uracil - N$_3$, 13), 301 (M\(^+\) - uracil - HN$_3$ - H$_2$O, 2), 276 (M\(^+\) - uracil - N$_3$ - C$_3$H$_6$, 2), 260 (M\(^+\) - uracil - N$_3$ - CH$_3$OH - CH$_2$=CH$_2$, 11), 240 (C$_8$H$_{19}$NO$_3$P$, 8), 238 (C$_8$H$_{17}$NO$_5$P$, 1), 236 (M\(^+\) - C$_8$H$_{17}$NO$_5$P, 4), 222 (C$_8$H$_{19}$NO$_5$P, - H$_2$O, 6), 208 (C$_8$H$_{19}$NO$_5$P, - CH$_2$OH, 2), 206 (C$_8$H$_{17}$NO$_5$P, - CH$_2$OH, 1), 196 (C$_8$H$_{19}$NO$_5$P, - C$_3$H$_6$, 12), 194 (C$_8$H$_{19}$NO$_5$P, - CH$_3$CH$_2$OH, 6), 180 (C$_8$H$_{19}$NO$_5$P, - CH$_3$OH - CH$_2$=CH$_2$, 52), 178 (C$_8$H$_{19}$NO$_5$P, - C$_3$H$_8$ - H$_2$O, 3), 168 (C$_8$H$_{19}$NO$_5$P, - C$_3$H$_8$ - CH$_2$=CH$_2$, 7), 166 (C$_8$H$_{19}$NO$_5$P, - CH$_3$CH$_2$OH - CH$_2$=CH$_2$, 8), 164 (C$_8$H$_{19}$NO$_5$P, - C$_3$H$_8$ - CH$_3$OH, 2), 152 (C$_8$H$_{19}$NO$_5$P, - CH$_3$CH$_2$OH - CH$_2$=CH$_2$, 33), 150 (C$_8$H$_{19}$NO$_5$P, - CH$_3$CH$_2$OH - C$_3$H$_6$, 4), 136 (C$_8$H$_{19}$NO$_5$P, - C$_3$H$_8$ - CH$_2$=CH$_2$ - CH$_3$OH, 7), 124 (C$_8$H$_{19}$NO$_3$P, - CH$_3$OC(O)CH$_2$CH(CH$_3$)$_2$, 2), 113 (uracilH\(^+\), 22), 112 (uracil\(^+\), 7), 110 (C$_2$H$_4$O$_3$P, 7), 109 (C$_8$H$_{19}$NO$_3$P, - CH$_3$OC(O)CHCH(CH$_3$)$_2$NH$_2$, 3), 82 (C$_3$H$_5$O, 20), 81 (C$_3$H$_6$O, 100).

Analytical data were obtained after further purification by flash column chromatography, using silica (40 g) and an eluent of 2.5% ethanol/ethyl acetate.

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 \(\mu\)m. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 22.9, 23.0 min (3:2).

46. 2',3'-Dideoxycytidine (ethyl methoxy-L-valinyl) phosphoramidate (first attempt)

2',3'-Dideoxycytidine (0.22 g, 1.0 mmol) was added to pyridine (20 ml) and the suspension heated to effect dissolution of the nucleoside. The resulting solution was cooled to -20 °C and ethyl methoxy-L-valinyl phosphorochloridate (1.15 g, 4.5 mmol,
4.4 equivs) added. The mixture was stirred at -20 °C for 5 min and was then allowed to warm to room temperature. TLC after 2 h showed the reaction to have proceeded to about 50% completion. After stirring overnight the reaction had proceeded to one major product (Rf 0.16) and one minor product (Rf 0.3) (5% methanol/chloroform). The reaction was quenched with deionised water (5 ml) and left to stir for 30 min. The pyridine and water were removed in vacuo, the last traces of pyridine being removed by co-evaporation with toluene (3 x 5 ml). The residual gum was purified by flash column chromatography using silica (40 g) and an eluent of 10% methanol/ethyl acetate.

\[ ^{31}P \text{ nmr } \delta(\text{CDCl}_3): 10.0 \text{ (broad signal), 8.19.} \]

47. N-dimethylaminomethylene-2',3'-dideoxycytidine

2',3'-Dideoxycytidine (0.49 g, 2.3 mmol) was suspended in DMF (5 ml) and N,N-dimethylformamide dimethyl acetal (2.20 ml, 1.97 g, 16.6 mmol, 7 equivs) added. The mixture was stirred for 17 h, after which time the resultant clear solution was evaporated in vacuo. The residual syrup was diluted with ethanol (12 ml) and diethyl ether (120 ml) added to precipitate the product but no solid was observed. Petroleum spirit (bp 60-80 °C) (50 ml) was added but still no precipitate was produced. The solvents were removed in vacuo to afford a white solid. This was washed with a little petroleum spirit (bp 60-80 °C), filtered and dried.

Yield: 0.53 g (86%).

\[ ^1H \text{ nmr } \delta(\text{DMSO-}d^6): 8.61 (s, 1H, N=CH), 8.15 (d, 1H, H6, J_{H5-H6} = 7.21 \text{ Hz}), 5.95 (m, 2H, H5, H1'), 5.05 (bs, 1H, 5'-OH, abs. on D$_2$O exchange), 4.06 (m, 1H, H4'), 3.72 (m, 1H, H5'), 3.57 (m, 1H, H5'), 3.16 (s, 3H, NCH$_3$(1)), 3.03 (s, 3H, NCH$_3$(2)), 2.33 (m, 1H) and 1.85 (m, 3H) (H2' and H3'). \]

\[ ^13C \text{ nmr } \delta(\text{DMSO-}d^6): 170.98 (C2), 157.62, 155.01 (C4 and C8), 142.10 (C6), 100.75 (C5), 86.20 (C1'), 81.91 (C4'), 61.72 (C5'), 40.70 (NCH$_3$(1)), 34.63 (NCH$_3$(2)), 32.72 (C2'), 24.28 (C3'). \]

FABMS m/e: 289 ([M+Na]$^+$, 21), 267 (MH$^+$, 27), 212 ([ddCyd]$^+$, 6), 167 (MH$^+$ - C$_5$H$_8$O$_2$, 100), 152 (MH$^+$ - C$_5$H$_8$O$_2$ - CH$_3$, 3), 137 (MH$^+$ - C$_5$H$_8$O$_2$ - 2 x CH$_3$, 15), 136 (M$^+$ - C$_5$H$_8$O$_2$ - 2 x CH$_3$, 25), 112 (cytosine$^+$, 38).

Analysis: C 53.92%, H 6.89, N 20.67. C$_{12}$H$_{19}$N$_4$O$_3$ requires C 54.12, H 6.81, N 21.04.

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48. 2',3'-Dideoxycytidine (ethyl methoxy-L-valinyl) phosphoramidate (second attempt)

N-dimethylaminomethylene-2',3'-dideoxycytidine (0.21 g, 0.8 mmol) was suspended in THF (5 ml). N-methyl imidazole (0.50 ml, 0.52 g, 6.3 mmol, 8 equivs) was added, causing some of the nucleoside to dissolve. The mixture was stirred for 1 h. An extra portion of THF was added (1 ml) but this did not effect complete dissolution of the nucleoside. There was no decomposition observed on tlc. Ethyl methoxy-L-valinyl phosphorochloridate (0.75 g, 2.9 mmol, 3.6 equivs) was added and after 5 h the reaction had proceeded to completion on tlc, there being two products running faster than the starting material. The solvent was removed in vacuo and the gummy residue dissolved in chloroform (20 ml). The chloroform solution was washed with aqueous citric acid (3 x 20 ml) and the organic layer separated. Examination by tlc showed the presence of two products running faster than the starting material. The chloroform layer was dried (MgSO₄), filtered and the solvent removed in vacuo to afford a gum. This was dissolved in ethanol (5 ml) and stored overnight, after which time tlc showed no change. The ethanol solution was refluxed for 2 h, after which time tlc showed the presence of a new product, running faster than unprotected 2',3'-dideoxycytidine but slower than N-dimethylaminomethylene-2',3'-dideoxycytidine. After 6 h at reflux the reaction had proceeded to ca. 80% completion on tlc, and after refluxing overnight there was little further change. The ethanol was removed in vacuo to afford a gum. The product was purified by flash column chromatography, using silica (20 g) and an eluent of 10% methanol/chloroform. The solvents were removed in vacuo to afford a yellow gum.

Yield: 0.05 g (14%).

³¹P nmr δ(CDCl₃): 6.64, 6.18.

¹³C nmr δ(CDCl₃): impurity indicated (see Results and Discussion section).

The product was purified by preparative hplc.


³¹P nmr δ(CDCl₃): 5.56.

¹H nmr δ(CDCl₃): 7.87 (m, 1H, H6), 6.07 (m, 1H, H5), 5.78 (d, 1H, H1'), 4.14 (m, 5H, POCH₂, H5', H4'), 3.71 (s, 3H, OCH₃), 3.65 (m, 1H, 'CH), 3.26 (m, 1H, valine NH), 2.44
(m, 1H, H2'), 2.05 (m, 1H, iPrCH), 1.91 (m, 3H, H2', H3'), 1.28 (t, 3H, POCH2CH3, 3JH-H = 7.23 Hz), 0.95 (d, 3H, iPrMe(1), 3JH-H = 6.66 Hz), 0.87 (d, 3H, iPrMe(2), 3JH-H = 6.66 Hz).

FABMS m/e: 455 ([M+Na]+, 0.2%), 433 (MH+, 1), 322 (MH+ - cytosine, 36), 262 (MH+ - cytosine - CH3OH - CH2=CH2, 1), 219 (MH+ - cytosine - C8H12O2, 2), 194 (C8H16NO3P+ - CH3OH, 1), 180 (C8H16NO3P+ - CH3OH - CH2=CH2, 6), 167 (C8H16NO3P+ - CH2=CH2, 1), 166 (C8H16NO3P+ - CH2=CH2, 1), 163 (C8H16NO3P+ - C3H8 - CH3OH, 1), 152 (C8H16NO3P+ - CH3OH - CH2=CH2, 6), 150 (C8H16NO3P+ - CH3CH2OH - C3H8, 2), 149 (C8H16NO3P+ - CH3CH2OH - C3H8, 4), 136 (C8H16NO3P+ - C3H8 - CH2=CH2 - CH3OH, 15), 135 (C8H16NO3P+ - C3H8 - CH2=CH2 - CH3OH, 1), 123 (C8H16NO3P+ - CH3OC(O)CH2CH(CH3)2, 2), 112 (cytosineH+, 23), 111 (cytosine*, 3), 109 (C8H16NO3P+ - CH3OC(O)CHCH(CH3)2NH2, 5), 85 (C3H6O+, 14), 83 (C5H7O+, 48), 81 (C3H5O+, 22).

49. 5'-O-tritylthymidine

Thymidine (5.2 g, 0.02 mol) and trityl chloride (6.7 g, 0.02 mol, 1.1 equivs) were dissolved in pyridine (50 ml) and the resultant solution heated to reflux for 2 h. The reaction mixture was cooled to room temperature and was then poured on to rapidly-stirred ice/water (600 ml) and blended for 0.5 h. The gelatinous precipitate was isolated by decanting off the water/pyridine and was then dissolved in chloroform (200 ml). The chloroform was washed with water (6 x 100 ml). The organic layer was separated, dried (MgSO4), filtered, and the solvent removed from the filtrate in vacuo to afford a yellow foam. This was recrystallised from benzene to afford a colourless solid.

Yield: 8.9 g (85%).

1H nmr δ(CDCl3): 8.54 (s, 1H, 3-NH), 7.57 (s, 1H, H6), 7.35 (m, 15H, trityl H), 6.42 (t, 1H, H1', 3JH1-H2 = 7.3 Hz), 4.58 (m, 1H, H3'), 4.05 (d, 1H, H4', 3JH4-H5' = 3.1 Hz), 3.43 (m, 2H, H5'), 2.35 (m, 2H, H2'), 2.22 (d, 1H, 3'-OH, 3JH-H = 4.13 Hz), 1.48 (s, 3H, 5-CH3).

50. 3'-O-acetylthymidine

A solution of 5'-O-tritylthymidine (8.4 g, 0.017 mol) in pyridine (60 ml) and acetic anhydride (6.5 ml, 7.0 g, 0.07 mol, 4 equivs) was kept at room temperature for 20 h.
The reaction mixture was then poured on to iced water (800 ml) and stirred for 0.5 h. The white precipitate was filtered and dried. This crude 5'-O-trityl-3'-O-acetylthymidine was heated in 80% acetic acid (30 ml) at 80 °C for 0.5 h. The reaction mixture was allowed to cool to room temperature and ice added to assist in the precipitation of the trityl alcohol. After the ice had melted the mixture was filtered and the filtrate evaporated in vacuo. The residue was purified by recrystallisation from acetone to afford a colourless solid.

Yield: 2.6 g (54%).
Melting point: 177 °C.

$^1$H nmr $\delta$(CDCl$_3$): 8.60 (bs, 1H, 3-NH), 7.51 (s, 1H, H6), 6.27 (t, 1H, H1'), 5.37 (m, 1H, H3'), 4.10 (m, 1H, H4'), 3.94 (m, 2H, H5'), 2.43 (m, 3H, H2', 5'-OH), 2.11 (s, 3H, acetyl CH$_3$), 1.93 (s, 3H, 5-CH$_3$).

51. 3'-O-acetylthymidine (ethyl methoxy-L-valinyl) phosphoramidate

3'-O-acetylthymidine (0.15 g, 0.5 mmol) was dissolved in THF (5 ml) and N-methyl imidazole (0.28 ml, 0.29 g, 3.6 mmol, 7 equivs) added. To this solution was added ethyl methoxy-L-valinyl phosphorochloridate (0.46 g, 1.8 mmol, 3.4 equivs) and after stirring for 23 h the reaction had proceeded to ca. 90% completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (3 x 20 ml), then water (2 x 15 ml). The organic layer was separated and dried (MgSO$_4$), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified by flash column chromatography, using silica (50 g) and an eluent of ethyl acetate. Removal of the solvent in vacuo afforded a colourless gum.

Yield: 0.07 g (28%).

$^{31}$P nmr $\delta$(CDCl$_3$): 6.63.

$^{13}$C nmr $\delta$(CDCl$_3$): 173.55, 173.44 (2d, 5:3, CH$_3$OOC(O), $^3J_{CP}$ = 2.7 Hz and 3.0 Hz), 170.38, 170.34 (5:3, acetyl C(O)), 163.90 (C2), 150.67, 150.62 (5:3, C4), 135.03, 134.93 (5:3, C6), 111.66, 111.43 (5:3, C5), 84.60, 84.42 (3:5, C1'), 82.85, 82.79 (2d, 5:3, C4', $^3J_{CP}$ = 7.6 Hz), 74.51, 74.42 (5:3, C3'), 65.78, 65.72 (2d, 3:5, C5', $^3J_{CP}$ = 5.3 Hz), 63.03, 62.97 (2d, 5:3, POCH$_2$, $^3J_{CP}$ = 5.4 Hz), 59.77, 59.67 (3:5, 'C), 52.15, 52.09 (3:5, -OCH$_3$), 37.34, 37.18 (3:5,
C2'), 31.95, 31.82 (2d, 3:5, iPrCH, JCP = 6.4 Hz and 6.8 Hz), 20.84 (acetyl CH3), 19.04, 18.95 (5:3, iPrMe(1)), 17.27, 17.17 (3:5, iPrMe(2)), 16.12, 16.05 (2d, 3:5, POCH2CH3, JCP = 5.3 Hz), 12.36, 12.25 (3:5, 5-CH3).

\[ {^1}H \text{ nmr } \delta(\text{CDCl}_3): 9.97, 9.94 (2bs, 2:3, 1H, 3-NH), 7.59, 7.52 (2s, 3:2, 1H, H6), 6.37 (m, 1H, H1'), 5.33, 5.27 (2d, 3:2, 1H, H3'), 4.17 (m, 5H, POCH2, H5', H4'), 3.74, 3.73 (2s, 2:3, 3H, -OCH3), 3.68 (m, 2H, valine NH and 'CH), 2.41 (m, 1H, H2'), 2.12 (m, 2H, H2' and iPrCH), 2.11 (s, 3H, acetyl CH3), 1.96 (s, 3H, 5-CH3), 1.33 (m, 3H, POCH2CH3), 0.98 (m, 3H, iPrMe(1)), 0.89 (m, 3H, iPrMe(2)).

\[ \text{EIMS } m/e: 505.1884 (\text{tvP, Q j H ^ A o P  requires 505.1825, 0.07%}), 446 (M^* - CH}_3\text{OC(O), 1), 320 (M^* - thymine - CH}_3\text{OC(O), 6), 267 (M^* - C}_8\text{H}_9\text{NO}_3\text{P, 1), 260 (M^* - thymine - CH}_2=\text{CH-CH}_3 - \text{CH}_3\text{OC(O) - H}_2\text{O, 3), 240 (C}_8\text{H}_9\text{NO}_3\text{P}^* - \text{H}_2\text{O, 1), 196 (C}_8\text{H}_9\text{NO}_3\text{P}^* - C}_3\text{H}_8, 1), 194 (C}_8\text{H}_9\text{NO}_3\text{P}^* - \text{CH}_3\text{CH}_2\text{OH, 1), 180 (C}_8\text{H}_9\text{NO}_3\text{P}^* - \text{CH}_3\text{OC(O)H, 4), 168 (C}_8\text{H}_9\text{NO}_3\text{P}^* - C}_3\text{H}_6 - \text{CH}_2=\text{CH}_2, 1), 166 (C}_8\text{H}_9\text{NO}_3\text{P}^* - \text{CH}_3\text{CH}_2\text{OH - CH}_2=\text{CH}_2, 1), 152 (C}_8\text{H}_9\text{NO}_3\text{P}^* - \text{CH}_3\text{CH}_2\text{OH - CH}_2=\text{CH-CH}_3, 4), 150 (C}_8\text{H}_9\text{NO}_3\text{P}^* - \text{CH}_3\text{CH}_2\text{OH - C}_3\text{H}_6, 1), 136 (C}_8\text{H}_9\text{NO}_3\text{P}^* - C}_3\text{H}_8 - \text{CH}_2=\text{CH}_2 - \text{CH}_3\text{OH, 1), 127 (thymineH^+, 1), 126 (thymine^+, 2), 110 (C}_2\text{H}_5\text{O}_3\text{P}^*, 2), 82 (C}_3\text{H}_6\text{O}^+, 9), 81 (C}_2\text{H}_5\text{O}^+, 100).\]

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 \( \mu \)m. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 80% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 20.9, 21.2 min. Analysis: C 45.04%, H 6.38, N 7.54, P 5.39. C20H32N3O10P[1.7H2O] requires C 44.81, H 6.60, N 7.84, P 5.78.

52. 3'-O-acetylthymidine bis(2,2,2-trifluoroethyl) phosphate

3'-O-acetylthymidine (0.11 g, 0.4 mmol) was dissolved in pyridine (10 ml). Bis(2,2,2-trifluoroethyl)phosphorochloridate (0.27 g, 1.0 mmol, 2.6 equivs) was added and stirring continued for 4 h. The reaction was quenched with water (0.04 ml, 1.9 mmol, 5.2 equivs) and the pyridine removed in vacuo, the last traces being co-evaporated with toluene (3 x 3 ml). The residue was purified twice by flash column chromatography, first using silica (70 g) and an eluent of 5% methanol/chloroform, then using silica (70 g) and an eluent of 1% methanol/chloroform. The relevant fractions containing the product were pooled and the solvents removed in vacuo to afford a colourless gum. Yield: 0.14 g (72%).

\[ {^{31}}P \text{ nmr } \delta(\text{CDCl}_3): -3.53.\]
$^{13}$C nmr δ(CDCl$_3$): 170.66 (acetyl C(O)), 163.88 (C2), 150.73 (C4), 134.77 (C6), 126.45, 126.35 (qd, CF$_3$, $J_{CP} = 277.6$ Hz, $J_{CP} = 9.1$ Hz), 112.10 (C5), 84.74 (C1), 82.47 (d, C4', $J_{CP} = 7.9$ Hz), 73.85 (C3'), 68.41 (d, C5', $J_{CP} = 5.9$ Hz), 64.89 (2 x qd, POCH$_2$, $J_{CF} = 38.2$ Hz, $J_{CP} = 4.0$ Hz), 36.87 (C2'), 20.85 (acetyl CH$_3$), 12.30 (5-CH$_3$).

$^1$H nmr δ(CDCl$_3$): 9.79 (bs, 1H, 3-NH), 7.34 (s, 1H, H6), 6.39 (m, 1H, H1'), 5.28 (m, 1H, H3'), 4.44 (m, 6H, POCH$_2$, H5'), 4.19 (m, 1H, H4'), 2.47 (m, 1H, H2'), 2.22 (m, 1H, H2'), 2.12 (s, 3H, 5-CH$_3$), 1.93 (s, 3H, acetyl CH$_3$).

EIMS m/e: 528.0669 (M$^+$, CuH$_{19}$F$_6$O$_9$P requires 528.0733, 0.4%), 263 (C$_4$H$_7$F$_6$O$_4$P$^+$, 6), 193 (C$_4$H$_7$F$_6$O$_4$P$^-$ - CHF$_3$, 3), 163 (C$_4$H$_7$F$_6$O$_4$P$^+$ - CF$_3$CH$_2$OH, 4), 127 (thymineH$^+$, 7), 126 (thymine$^+$, 8), 82 (C$_5$H$_9$O$^+$, 25), 81 (C$_5$H$_9$O$^+$, 100).

Analytical hplc: Column: 50+250 x 4.6 mm Spherisorb ODS2 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 82% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 25.5 min.

Analysis: C 37.43%, H 4.00, N 4.94, P 5.85. C$_{16}$H$_{19}$F$_6$O$_9$P requires C 36.38, H 3.63, N 5.30, P 5.86.

53. 3'-O-methanesulphonylthymidine

5'-O-tritylthymidine (1.02 g, 2.1 mmol) and triethylamine (0.88 ml, 0.64 g, 6.3 mmol, 3 equivs.) were dissolved in dichloromethane (100 ml). Methanesulphonyl chloride (0.18 ml, 0.26 g, 2.3 mmol, 1.1 equivs) in dichloromethane (20 ml) was added dropwise at -5 ºC over 10 min. The reaction was then allowed to warm to room temperature, and tlc showed that the reaction had proceeded to 95% completion. Methanesulphonyl chloride (0.03 ml, 0.05 g, 0.4 mmol, 0.2 equiv) was added and stirring continued for 10 min. Tlc showed that the reaction had gone to completion. The reaction mixture was washed with saturated sodium bicarbonate solution (100 ml) and brine (100 ml), the organic layer was separated and dried (MgSO$_4$) and the dichloromethane removed in vacuo. The white crystals obtained were dried thoroughly in vacuo and then suspended in 80% acetic acid (30 ml). This suspension was heated to ca. 80 ºC for 1 h, and was then allowed to cool to room temperature. Ice was added to precipitate the trityl alcohol. The solid was filtered and the solvents removed from the filtrate in vacuo to afford a gum. The product was recrystallised from ethanol.

Yield: 0.43 g (64%).

Melting point: 116 ºC.

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54. 3'-O-methanesulphonylthymidine (ethyl methoxy-L-valinyl) phosphoramidate
3'-O-methanesulphonylthymidine (0.12 g, 0.4 mmol) was suspended in THF (5 ml) and
N-methyl imidazole (0.26 ml, 0.27 g, 3.3 mmol, 9 equivs) added. To this solution was
added ethyl methoxy-L-valinyl phosphorochloridate (0.42 g, 1.6 mmol, 4.5 equivs) and
after stirring for 20 h the reaction had proceeded to completion on tlc. The reaction
was concentrated in vacuo, dissolved in chloroform (20 ml) and washed with saturated
sodium bicarbonate solution (15 ml), then water (3 x 15 ml). The organic layer was
separated and dried (MgSO₄), filtered and then concentrated in vacuo to a gum. This
was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40
°C) (450 ml). The product was precipitated in the refrigerator overnight. The
petroleum spirit was decanted off and the gummy residue purified by flash column
chromatography, using silica (50 g) and an eluent of ethyl acetate. Removal of the
solvent in vacuo afforded a colourless gum.

Yield: 0.04 g (20%).

$^{31}$P nmr $\delta$(CDCl₃): 6.67.

$^{13}$C nmr $\delta$(CDCl₃): 173.74, 173.71 (d, CH₃OC(O), $^3$J_C-P = 2.8 Hz), 163.50 (C2), 150.26 (C4),
135.08 (C6), 111.81 (C5), 84.60 (C1'), 82.88 (d, C4', $^3$J_C-P = 7.4 Hz), 79.30 (C3'), 65.16 (d,
C5', $^3$J_C-P = 5.4 Hz), 63.31 (d, POCH₂, $^3$J_C-P = 5.4 Hz), 59.83 (C), 52.29 (-OCH₃), 38.52
(C2'), 37.89 (methanesulphonyl-CH₃), 31.84 (d, iPrCH, $^3$J_C-P = 6.8 Hz), 19.18 (iPrMe(1)),
17.13 (iPrMe(2)), 16.19 (d, POCH₂CH₃, $^3$J_C-P = 7.2 Hz), 12.38 (5-CH₃).

$^1$H nmr $\delta$(CDCl₃): 8.79 (s, 1H, 3-NH), 7.51 (s, 1H, H6), 6.38 (m, 1H, H1'), 5.42 (d, 1H, H3'),
4.41 (s, 1H, H4'), 4.27 (m, 2H, H5'), 4.12 (m, 2H, POCH₂), 3.76 (m, 1H, 'CH), 3.73 (s, 3H, -OCH₃),
3.55 (m, 1H, valine NH), 3.13 (s, 3H, methanesulphonyl-CH₃), 2.33 (m, 1H, H2'), 2.12 (m, 1H, iPrCH),
1.96 (s, 3H, 5-CH₃), 1.33 (t, 3H, POCH₂CH₃), 1.00 (d, 3H, iPrMe(1)), 0.88 (d, 3H, iPrMe(2)).

FABMS m/e: 446 (M⁺ - SO₃CH₃, 1%), 401 (M⁺ - SO₃CH₃ - OCH₂CH₃, 1), 240
(C₈H₁₅NO₅P⁺, 1), 222 (C₈H₁₅NO₅P⁺ - H₂O, 1), 221 (C₈H₁₅NO₅P⁺ - H₂O, 2), 194
(C₈H₁₅NO₅P⁺ - CH₃CH₂OH, 1), 193 (C₈H₁₅NO₅P⁺ - CH₃CH₂OH, 3), 180 (C₈H₁₅NO₅P⁺ -
CH₃OC(O)H, 2), 179 (C₈H₁₅NO₅P⁺ - CH₃OC(O)H, 1), 178 (C₈H₁₅NO₅P⁺ - C₃H₈ - H₂O, 1),
177 (C₈H₁₅NO₅P⁺ - C₃H₈ - H₂O, 1), 168 (C₈H₁₅NO₅P⁺ - C₃H₈ - CH₂=CH₂, 1), 167
(C₈H₁₅NO₅P⁺ - C₃H₈ - CH₂=CH₂, 3), 166 (C₈H₁₅NO₅P⁺ - CH₃CH₂OH - CH₂=CH₂, 2), 165
(C₈H₁₅NO₅P⁺ - CH₃CH₂OH - CH₂=CH₂, 3), 164 (C₈H₁₅NO₅P⁺ - C₃H₈ - CH₃OH, 1), 163
(C₈H₁₅NO₅P⁺ - C₃H₈ - CH₃OH, 2), 152 (C₈H₁₅NO₅P⁺ - CH₃CH₂OH - CH₂=CH-CH₃, 5),
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151 (C₈H₁₈NO₃P⁺ - CH₂CH₂OH - CH₂=CH-CH₃, 2), 150 (C₈H₁₈NO₃P⁺ - CH₂CH₂OH - C₈H₁₈, 6), 149 (C₈H₁₈NO₃P⁺ - CH₃CH₂OH - C₈H₁₈, 16), 136 (C₄H₉NO₅P⁺ - C₃H₆ - CH₂=CH₂ - CH₃OH, 36), 135 (C₄H₉NO₅P⁺ - C₃H₆ - CH₂=CH₂ - CH₃OH, 6), 127 (thymineH⁺, 2), 110 (C₅H₆O₃P⁺, 2), 82 (C₅H₇O⁺, 68), 81 (C₅H₇O⁺, 4).

Analytical hplc: Column: 250 x 4.6 mm Techsphere ODS 5 µm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 18% B; 10 min 18% B; 30 min 80% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 21.1, 21.3 min.

55. 1-(2-Deoxy-3-O-methanesulphonyl-β-D-threo-pentofuranosyl)thymine (ethyl methoxy-L-valinyl) phosphoramidate

1-(2-Deoxy-3-O-methanesulphonyl-β-D-threo-pentofuranosyl)thymine (0.26 g, 0.8 mmol) was suspended in THF (5 ml) and N-methyl imidazole (0.54 ml, 0.56 g, 6.8 mmol, 8.5 equivs) added. To the resultant solution was added ethyl methoxy-L-valinyl phosphorochloridate (0.88 g, 3.4 mmol, 4.25 equivs) and after stirring for 32 h the reaction had proceeded to completion on tlc. The reaction was concentrated in vacuo, dissolved in chloroform (20 ml) and washed with saturated sodium bicarbonate solution (15 ml), then water (3 x 15 ml). The organic layer was separated and dried (MgSO₄), filtered and then concentrated in vacuo to a gum. This was dissolved in chloroform (10 ml) and poured on to light petroleum spirit (bp 30-40 °C) (450 ml). The product was precipitated in the refrigerator overnight. The petroleum spirit was decanted off and the gummy residue purified by flash column chromatography, using silica (50 g) and an eluent of 2% methanol/ethyl acetate. Removal of the solvents in vacuo afforded a colourless gum.

Yield: 0.35 g (82%).

³¹P nmr δ(CDCl₃): 6.74, 6.55 (4:3).

¹³C nmr δ(CDCl₃): 173.94, 173.62 (2d, 4:3, CH₃OC(O), ³JC_F = 3.1 Hz and 2.9 Hz), 164.22, 164.19 (4:3, C2), 150.88, 150.84 (3:4, C4), 135.30, 135.21 (3:4, C6), 111.36, 111.24 (1:2, C5), 83.81, 83.70 (2:1, C1'), 80.35, 80.08 (2d, 3:4, C4', ³JC_F = 8.2 Hz and 9.1 Hz), 77.85 (C3'), 63.12 (d, C5', ³JC_F = 5.4 Hz), 62.86, 62.44 (2d, POCH₂, ²JC_F = 4.5 Hz and 4.0 Hz), 59.91, 59.81 (4:3, C'), 52.15 (-OCH₃), 39.37 (C2'), 38.45 (methanesulphonyl CH₃), 31.90, 31.84 (2d, iPrCH, ³JC_F = 6.0 Hz and 6.0 Hz), 19.13, 19.06 (4:3, iPrMe(1)), 17.38, 17.29 (3:4, iPrMe(1)), 16.12 (d, POCH₂CH₃, ³JC_F = 7.2 Hz), 12.61, 12.59 (3:4, 5-CH₃).

¹H nmr δ(CDCl₃): 7.43 (s, 1H, H6), 6.32 (m, 1H, H1'), 5.33 (m, 1H, H3'), 4.33 (m, 3H,
H5', H4'), 4.06 (m, 3H, POCH2), 3.78, 3.65 (2s, 1H, 'CH), 3.74, 3.73 (2s, 4:3, 3H, methanesulphonyl CH3), 3.18, 3.17 (2s, 4:3, methanesulphonyl CH3), 2.88 (m, 1H, H2O, 2.47 (m, 1H, H2'), 2.09 (m, 1H, iPrCH), 1.94 (s, 3H, 5-CH3), 1.29 (m, 3H, POCHjCR2), 0.98 (m, 3H, iPrMe(1)), 0.90 (m, 3H, iPrMe(2)).

EIMS m/e: 541.1533 (M+, C19H32N3O11PS requires 541.1495, 0.26%), 483 (MH+ - CH3OC(O), 1), 320 (M+ - thymine - SO3CH3, 2), 303 (MH+ - thymine - SO3CH3 - H2O, 2), 260 (M+ - thymine - SO3CH3 - CH3OH - CH2=CH2, 2), 240 (C8H19NO3P+, 1), 222 (C8H19NO3P+ - H2O, 1), 194 (C8H19NO3P+ - CH3CH2OH, 2), 180 (C8H19NO3P+ - CH3OC(O)H, 4), 178 (C8H19NO3P+ - C8H8 - H2O, 2), 168 (C8H19NO3P+ - C8H8 - CH2=CH2, 1), 166 (C8H19NO3P+ - CH3CH2OH - CH2=CH2, 1), 152 (C8H19NO3P+ - CH3CH2OH - CH2=CH-CH3, 3), 136 (C8H19NO3P+ - C8H8 - CH2=CH2 - CH3OH, 1), 127 (thymineH+, 2), 126 (thymineH+, 6), 110 (C7H3O3P+, 2), 82 (C5H6O+, 9), 81 (C5H5O+, 100).

Analytical hplc: Column: 250 x 4.6 mm Kromasil C18 5 μm. Pumps: A=water, B=acetonitrile + 5% water. Gradient conditions, A/B: 0 min 20% B; 10 min 20% B; 30 min 80% B. Detector: uv 254 nm. Flow rate: 1 ml/min. Retention time: 21.1 min.


56. 2',3'-Dideoxy-2',3'-didehydrothymidine (ethyl methoxy-L-valinyl) phosphoramide (first attempt)

1-(2-Deoxy-3-0-methanesulphonyl-fl-D-threopentofuranosyl)thymine (ethyl methoxy-L-valinyl) phosphoramide (50 mg) was dissolved in THF containing 1.1 M tetra-n-butyl ammonium fluoride (5 ml). Tlc after 48 h showed the presence of one major product. The solvent was removed in vacuo to afford a brown gum. This was applied to a flash column consisting of silica (20 g) and an eluent of 3% methanol/chloroform. The fractions containing the major product were examined in a different tlc system (5% methanol/ethyl acetate) and showed the presence of two additional products. The solvents were removed in vacuo to afford a gum which was applied to a flash column consisting of silica (20 g) and eluent 2% methanol/ethyl acetate. No uv active material was obtained in any of the fractions collected.
57. 2',3'-Dideoxy-2',3'-didehydrothymidine (ethyl methoxy-L-valinyl) phosphoramidate (second attempt)

1-(2-Deoxy-3-O-methanesulphonyl-D-threo-pentofuranosyl)thymine (ethyl methoxy-L-valinyl) phosphoramidate (0.10 g, 0.2 mmol) was dissolved in DMSO (1 ml) and potassium t-butoxide (0.04 g, 0.4 mmol, 2 equivs) added, whereupon the reaction mixture turned brown. The mixture was stirred for 2 h, after which time the reaction had proceeded to ca. 80% completion on tlc, there being one product running faster than the starting material (solvent system 10% methanol/chloroform; Rf of product 0.51, Rf of starting material 0.44). There was no further change after 1 h. The reaction mixture was poured on to diethyl ether (450 ml) and the product precipitated in the refrigerator overnight. The diethyl ether was decanted off, and the residue purified by flash column chromatography, using silica (70 g) and an eluent of 3% methanol/chloroform. The solvents were removed in vacuo to afford a colourless gum.

Yield: 0.03 g (40%).

$^{31}P$ nmr $\delta$(CDCl$_3$): 6.95, 6.40 (2:1).

$^1$H nmr $\delta$(CDCl$_3$): impurity indicated (see Results and Discussion section).

The product was purified by preparative hplc.

Preparative hplc: Column: 250 x 20 mm Kromasil C18 10 µm. Pumps: A=H$_2$O, B=acetonitrile + 5% H$_2$O. Isocratic conditions: 75% A, 25% B. Detector: uv 263 nm. Flow rate: 15 ml/min (0-26 min), 25 ml/min (26-35 min). Retention time: 10.7 min.

$^{31}P$ nmr $\delta$(CDCl$_3$): 5.96.

$^1$H nmr $\delta$(CDCl$_3$): 8.30 (bs, 1H, 3-NH), 7.38 (s, 1H, H6), 7.04 (m, 1H, H1'), 6.36 (m, 1H, H3'), 5.88 (m, 1H, H2'), 4.99 (m, 1H, H4'), 4.14 (m, 4H, POCH$_2$, H5'), 3.73 (s, 3H, -OCH$_3$), 3.63 (m, 1H, 3CH$_3$), 3.23 (m, 1H, valine NH), 2.02 (m, 1H, iPrCH), 1.92 (s, 3H, 5-CH$_3$), 1.30 (td, 3H, POCH$_2$CH$_3$), $^3$J$_{H-H} = 7.05$ Hz, $^4$J$_{H-P} = 0.7$ Hz, 0.95 (d, 3H, iPrMe(1), $^3$J$_{H-H} = 6.81$ Hz), 0.86 (d, 3H, iPrMe(2), $^3$J$_{H-H} = 6.91$ Hz).

FABMS m/e: 446 (MH$^+$, 0.4%), 320 (MH$^+$ - thymine, 3), 240 (C$_8$H$_9$NO$_5$P$^+$, 1), 222 (C$_8$H$_9$NO$_5$P$^+$ - H$_2$O, 1), 219 (MH$^+$ - thymine - C$_8$H$_9$O$_2$, 7), 217 (MH$^+$ - thymine - C$_8$H$_9$O$_2$, 2), 205 (M$^+$ - C$_8$H$_9$NO$_5$P, 1), 194 (C$_8$H$_9$NO$_5$P$^+$ - CH$_3$CH$_2$OH, 1), 180 (C$_8$H$_9$NO$_5$P$^+$ - CH$_3$OH - CH$_2$=CH$_2$, 6), 167 (C$_8$H$_9$NO$_5$P$^+$ - C$_3$H$_8$ - CH$_2$=CH$_2$, 1), 166 (C$_8$H$_9$NO$_5$P$^+$ - CH$_3$CH$_2$OH - CH$_2$=CH$_2$, 1), 163 (C$_8$H$_9$NO$_5$P$^+$ - C$_3$H$_8$ - CH$_3$OH, 1), 152 (C$_8$H$_9$NO$_5$P$^+$ - CH$_3$CH$_2$OH - CH$_2$=CH-CH$_3$, 6), 150 (C$_8$H$_9$NO$_5$P$^+$ - CH$_3$CH$_2$OH - C$_3$H$_8$, 2), 85 (C$_5$H$_9$O$^+$, 10), 83 (C$_5$H$_7$O$^+$, 29), 81 (C$_5$H$_9$O$^+$, 73).
### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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pol polymerase gene
POL pol-encoded protein
ppm parts per million
pr polyprotein
Pro proline
q quartet
rev regulator of expression of virion proteins gene
REV rev-encoded protein
RNA ribonucleic acid
RRE rev-responsive element
rsCD4 recombinant soluble CD4
RT reverse transcriptase
s singlet
Ser serine
t triplet
TAR trans-acting responsive sequence
tat transactivator gene
TAT tat-encoded protein
TBAF tetra-n-butylammonium fluoride
THF tetrahydrofuran
Thr threonine
tlc thin layer chromatography
trityl triphenylmethyl
tRNA transfer ribonucleic acid
Trp tryptophan
Tyr tyrosine
uv ultraviolet
Val valine
vif virion infectivity factor
vpr viral protein R gene
vpu viral protein U gene
References


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236. F. Hamilton, personal communication.
238. K.G. Devine, unpublished observations.


262. N.A. Roberts, personal communication.


278. P. Nylen; *Chem. Zentr.* 1, 2736 (1936).
Table of derivatives prepared and the results of their biological evaluation *in vitro*.

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EC<sub>50</sub> values: the concentration required to reduce HIV-1 antigen production by 50%.
NT: not tested (results awaited); in: inactive.
Synthesis and anti-HIV evaluation of some phosphoramidate derivatives of AZT: studies on the effect of chain elongation on biological activity

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1 Department of Chemistry, University College London, London, U.K. and 2 Division of Virology, Department of Medical Microbiology, St. Mary's Hospital Medical School, London, U.K.

(Received 18 June 1990; revision accepted 24 August 1990)

Summary

A series of phosphoramidate derivatives of the anti-HIV drug AZT has been prepared as membrane soluble pro-drugs of the bio-active nucleotide forms and evaluated in vitro against HIV-1. Terminal substituted alkyl amines have a pronounced anti-HIV effect: this effect declines upon increasing the length of the methylene spacer. The results are consistent with a mechanism of action involving intracellular cleavage of the phosphoramidate bond, and release of the nucleotide, or a derivative thereof. Full spectroscopic data are included on the products and their phosphorochloridate precursors.

HIV; Phosphoramidate derivative; AZT

Introduction

Therapies for the acquired immunodeficiency syndrome (AIDS) have been dominated by strategies directed against the human immunodeficiency virus HIV-1. In particular, nucleoside analogues such as 3'-azido-3'-deoxythymidine (AZT) (1) are in clinical use (Yarchoan et al., 1986). Other nucleoside analogues, notably the
$2',3'$-dideoxy nucleosides, are also undergoing clinical evaluation (Merigan et al., 1989). However, all of these compounds share a number of problems: in particular, nucleoside analogues have a deleterious dependence on kinase mediated activation because the bio-active form is the nucleotide (Cooney et al., 1986). For AZT the triphosphate acts either by inhibition of the viral reverse transcriptase (RT), or by incorporation into the viral DNA chain (hence blocking further extension of the DNA) (Furman et al., 1986). It is well established that this dependence of chemotherapeutic nucleoside analogues on kinases may lead to poor activity, and the emergence of clinical resistance (Furman et al., 1979). Another common problem of nucleoside analogues is drug toxicity; for AZT this is manifested as neutropoenia and anaemia (Pinching et al., 1989).

As we have noted for the anti-herpes drug araA (McGuigan et al., 1989), and the anti-cancer drug araC (Jones et al., 1989), it is possible that the limiting kinase dependence of chemotherapeutic nucleoside analogues may be overcome by the synthesis of appropriate pro-drug forms; not of the nucleoside, but of the nucleotide. We have recently noted that simple dialkyl phosphate triester derivatives of AZT are inactive as anti-HIV agents (McGuigan et al., 1990a), whereas carboxy-protected amino-linked phosphoramidate derivatives (such as compound 2a) are potent anti-HIV agents (Devine et al., 1990). In particular, it was found that minor modifications in the amino acid side chain lead to marked changes in anti-HIV activity (McGuigan et al., 1990b). In this paper we report the synthesis and anti-HIV evaluation of several novel derivatives, which indicates a marked effect on anti-viral activity of increasing the methylene spacer length between the phosphoramidate moiety and the carboxyl ester group.

Materials and Methods

All reactions were carried out under scrupulously dry conditions unless otherwise indicated. THF was dried by distillation at atmospheric pressure from lithium aluminium hydride onto activated 4A molecular sieves. Dichloromethane was distilled from calcium hydride and stored over 4A molecular sieves. Analytical grade carbon tetrachloride was dried over 4A molecular sieves. $N$-Methylimidazole and triethylamine were distilled from calcium hydride. For tlc, Merck 60 F$_{254}$ precoated silica plates were employed. For flash column chromatography either Merck Kieselgel 60 or Woelm silica was used. AZT and ethyl phosphorodichloridate were supplied by Aldrich Chemical Company. For the nucleoside derivatives: proton nmr spectra were recorded on a Varian VXR400 spectrometer operating at 400 MHz, $^{13}$C spectra were obtained on this instrument, operating at 100 MHz, and $^{31}$P spectra on a Varian XL200 instrument operating at 82 MHz. For the phosphorochloridates, all nmr spectra were recorded on the latter instrument (200 MHz for $^1$H, 50 MHz for $^{13}$C and 82 MHz for $^{31}$P). Proton and carbon spectra were referenced to TMS, and phosphorus spectra to 85% phosphoric acid; positive shifts are downfield of the reference. All nmr spectra were run in CDCl$_3$. Mass spectra were recorded on a VG7070H spectrometer, courtesy of Dr M. Mruzek (EIMS). HPLC analyses were
conducted on all samples submitted for biological evaluation, using an ACS system with a 50+250 × 4.6 mm Spherisorb ODS2 5 μM column. Gradient elution was employed, using water (A), and acetonitrile (B) mixed as follows: 18% B for 0–10 min, then a linear gradient to 80% B at 30 min. Detection was by UV at 254 nm, with a flow rate of 1 ml/min. In every case the bulk purity exceeded 99%, with levels of AZT below detectable limits (<0.01%).

**Ethyl methoxyglycinyl phosphorochloridate**

Triethylamine (3.15 ml, 2.29 g, 22.6 mmol) in anhydrous dichloromethane (30 ml) was added dropwise with vigorous stirring over a period of 5.5 h to a mixture of glycine methyl ester hydrochloride (1.42 g, 11.3 mmol) and ethyl phosphorodichloridate (1.34 ml, 1.84 g, 11.30 mmol) in dichloromethane (30 ml) at −78°C. The mixture was allowed to warm to room temperature with stirring over 1.5 h. The solvent was then removed under reduced pressure, benzene (50 ml) added, and the mixture filtered. The filtrate was concentrated under reduced pressure, carbon tetrachloride (10 ml) added, and the mixture filtered once again. The filtrate was concentrated to dryness under reduced pressure, to yield the product as an oil (2.00 g, 82%). \( \delta_p +12.48; \delta_H 4.23(2H, m, CH_2OP), 3.84(1H, s, NH), 3.79(3H, s, OMe), 3.71(2H, s, CH_2N), 1.40(3H, m, CH_3CH_2); \delta_C 170.37(d, C=0, J=10.4 Hz), 66.74(d, POCH_2, J=6.0 Hz), 52.61(OMe), 42.84(CH_2N), 15.92(d, CH_3CH_3, J=8.0 Hz); EIMS m/e 215.0126(M+, C_5H_11ClNO_4P requires 215.0114, 0.1%), 170(M+ -OEt, 2), 158(M+ -MeOCO, 37Cl, 32), 156(M+ -MeOCO, base peak), 130(M+ -MeOCOCH_2N, 37Cl, 31), 128(M+ -MeOCOCH_2N, 98).

**3′-Azido-3′-deoxythymidine-5′-(ethyl methoxyglycinyl) phosphoramidate (2a)**

3′-Azido-3′-deoxythymidine (1) (0.20 g, 0.75 mmol) and ethyl methoxyglycinyl phosphorochloridate (0.81 g, 3.75 mmol) were stirred together in anhydrous tetrahydrofuran (THF) (5 ml) containing N-methylimidazole (0.60 ml, 7.48 mmol) for 16 h, at ambient temperature. The solvent was removed in vacuo, and the residue dissolved in chloroform (30 ml), and extracted with saturated sodium bicarbonate solution (15 ml), and water (2 × 15 ml). The organic phase was dried (MgSO_4) and evaporated in vacuo. The residue was re-dissolved in chloroform (10 ml), and precipitated with petroleum (bp 30–40°C) (500 ml). The precipitate was purified by chromatography on silica gel (30 g), using 4% methanol in chloroform as eluent. Pooling and evaporation of appropriate fractions gave the pure product (0.28 g, 84%), \( \delta_p +6.60; \delta_H \) (starred peaks are duplicated due to diastereoisomers) 8.45′(1H, s, N^3H), 7.35′(1H, s, H6), 6.15′(1H, t, H1′), 4.35′(1H, m, H3′), 4.20(2H, m, H5′), 4.10(2H, m, CH_2OP), 4.00(1H, m, H4′), 3.70(5H, m, glycine OMe, CH_2), 3.30(1H, m, glycine NH), 2.40(1H, m, H2′), 2.20(1H, m, H2′), 1.90(3H, s, 5-Me), 1.30(3H, m, CH_3CH_3); EIMS m/e 447.1348(MH^+, C_{15}H_{24}N_6O_8P requires 447.1393, 1%), 81(C_5H_3O, base peak); HPLC retention times 14.45, 14.79 min (1:1). Carbon-13 nmr data for this compound, and for the other nucleoside phosphoramidates, are in Table 1.
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Carbon-13 nmr data for compounds (2a–e) and (3), recorded in CDCl₃ at 100 MHz. Many peaks are split due to diastereomeric and coupling effects. Coupling constants to phosphorus, in Hz: a 4.5/5.2, b 8.2/7.9, c 5.4/6.2, d 4.8/5.1, e 3.1/4.2, f 7.5, g 5.0/5.1, h 5.4/5.6, i 1.5, j 2.0/2.1, k 3.9/3.7, l 1.4/9/5.1, m 5.4/5.9, n 7.0, o 3.7/3.6, p 3.5/3.3, q 4.9/4.7, r 5.1/4.8, s 7.1, t 5.9/5.7, u 7.5, v 5.0/4.6, w 5.2/5.0, x 6.9, y 4.4/4.4, z 7.9, a' 5.0/4.5, b' 5.0/4.8, c' 2.0/2.0, d' 6.7/7.2.
Ethyl methoxy-β-alaninyl phosphorochloridate

This was prepared by a method entirely analogous to the glycyl analogue above, except that the isolation procedure was modified slightly. Thus, the benzene treatment was omitted, and the volume of carbon tetrachloride was increased to 20 ml. Further trituration with hexane (20 ml) and carbon tetrachloride (10 ml) yielded the product as an oil. From 0.79 g of ethyl phosphorodichloridate was isolated 0.49 g (44%) of the title compound. δ_p +12.8; δ_H 4.16(2H, m, POCH_2), 3.65(3H, s, OMe), 3.64(1H, s, NH), 3.23(2H, m, NCH_2), 2.55(2H, m, NCH_2CH_2), 1.37(3H, m, CH_3CH_2); δ_C 172.30(C=O), 64.34(d, POCH_2, J=6.2 Hz), 51.81(OMe), 37.31(NCH_2), 34.84(d, NCH_2CH_2, J=6.6 Hz), 15.84(d, CH_3CH_2, J=8.0 Hz).

3'-Azido-3'-deoxythymidine-5'-(ethyl methoxy-β-alaninyl) phosphoramidate (2b)

This was prepared by an entirely analogous method to compound (2a) above, except that the reaction was stirred for 24 h, and the chromatographic purification was achieved on two successive columns, using eluents of 2% methanol in chloroform, followed by 5% methanol in ethyl acetate. Thus, from 0.1 g of (1) was isolated 0.06 g, (35%) of (2b). δ_p +7.48; δ_H (starred peaks are duplicated due to diastereoisomers) 9.30'(1H, s, N^3H), 7.45'(1H, s, H6), 6.23'(1H, t, H1'), 4.41(1H, m, H3'), 4.03-4.27(5H, m, POCH_2, H5', H4'), 3.71'(3H, s, OMe), 3.53(1H, bs, NH), 3.22(2H, m, NCH_2), 2.55(2H, m, NCH_2CH_2), 2.45(1H, m, H2'), 2.33(1H, m, H2'), 1.94(3H, s, 5-Me), 1.37(3H, t, CH_3CH_2, J=7.0 Hz); EIMS m/e 460.1515(M^+, C_{16}H_{25}N_6O_8P requires 460.1472, 1%), 81(C_5H_50 + , base peak); HPLC retention times 23.08, 23.27 min (1:1).

Ethyl methoxy-4-aminobutyryl phosphorochloridate

This was prepared by a method entirely analogous to the glycyl analogue above, except that the benzene treatment was omitted, and the volume of carbon tetrachloride was increased to 20 ml. Thus from 1.24 g of ethyl phosphorodichloridate was isolated 1.52 g (82%) of the title compound. δ_p +13.8; δ_C 173.48(C=O), 64.04(d, POCH_2, J=6.1 Hz), 51.44(OMe), 40.89(NCH_2), 30.75(CH_2CO), 25.69(d, NCH_2CH_2, J=7.9 Hz), 15.71(d, CH_3CH_2, J=8.0 Hz); EIMS m/e 246(MH^+, 37Cl, 21%), 244.0505(MH^+, C_7H_{16}ClNO_4P requires 244.0506, 64), 212(M^+-OMe, 73), 208(M^+-Cl, 23).

3'-Azido-3'-deoxythymidine-5'-(ethyl methoxy-4-aminobutyryl) phosphoramidate (2c)

This was prepared by an entirely analogous method to compound (2b) above, except that the reaction was stirred for 21 h, and the second chromatographic column was eluted with 3% methanol in ethyl acetate. Thus, from 0.11 g of (1) was isolated 0.13 g (66%) of (2c). δ_p +7.75; δ_H (starred peaks are duplicated due to
diastereoisomers) 9.90'(1H, s, N^3H), 7.41''(1H, s, H6), 6.21'(1H, t, H1'), 4.40(1H, m, H3'), 4.00-4.25(5H, m, POCH2, H5', H4'), 3.65(3H, s, OMe), 3.53(1H, bs, NH), 2.94(2H, m, NCH2), 2.28-2.42(4H, m, CH2CO, H2'), 1.90'(3H, d, 5-Me, J=1.0 Hz), 1.82(2H, m, NCH2CH2), 1.34(3H, t, CH3CH2, J=7.1 Hz); EIMS m/e 474.1666(M'^+, C17H27N6O8P requires 474.1628, 1%), 81(C5H5O, base peak); HPLC retention time 21.39 min.

*Ethyl methoxy-5-aminovaleryl phosphorochloridate*

This was prepared by a method entirely analogous to the 4-aminobutyryl analogue above. Thus from 0.82 g of ethyl phosphorodichloridate was isolated 1.20 g (92%) of the title compound. δ_p +13.79; δ_H 4.51(1H, bs, NH), 4.16(2H, m, POCH2), 3.60(3H, s, OMe), 2.92(2H, m, NCH2), 2.29(2H, m, CH2CO), 1.49-1.63(4H, m, CH2CH2CO, NCH2CH2), 1.36(3H, t, CH3CH2, J=7.0 Hz); δ_C 173.73(C=0), 64.03(d, POCH2, J=6.1 Hz), 51.43(OMe), 41.28(NCH2), 33.32(CH2CO), 30.03(d, NCH2CH2, J=7.5 Hz), 17.30(CH2CH2CO), 15.80(d, CH3CH2, J=8.0 Hz); EIMS m/e 260(MH'^+, 77Cl, 45%), 258.0662(MH'^+, C18H18ClN04P requires 258.0662, 70%), 226(M+ -OMe, 67), 222(M'^-OMe, 67), 222(M'^-Cl, 6).

3'-Azido-3'-deoxythymidine-5'-(ethyl methoxy-5-aminovaleryl) phosphoramidate (2d)

This was prepared by an entirely analogous method to compound (2a) above, except that the reaction was stirred for 19 h, and the chromatographic column was eluted with 2% methanol in chloroform. Thus, from 0.1 g of (1) was isolated 0.13 g (70%) of (2d). Analytical and biological data were obtained following further chromatographic purification on silica, using 2% methanol in ethyl acetate as eluent. δ_p +7.84; δ_H (starred peaks are duplicated due to diastereoisomers) 10.0(1H, bs, N^3H), 7.38'(1H, s, H6), 6.18'(1H, t, H1', J=6.7 Hz), 4.36(1H, m, H3'), 3.99-4.20(5H, m, POCH2, H5', H4'), 3.61(3H, s, OMe), 3.42(1H, m, NH), 2.85(2H, m, NCH2), 2.37(1H, m, H2'), 2.23-2.29(3H, m, CH2CO, H2'), 1.86(3H, s, 5-Me), 1.61(2H, m, CH2CH2CO), 1.48(2H, m, NCH2CH2), 1.31(3H, t, CH3CH2, J=7.0 Hz); EIMS m/e 488.1733(M'^+, C18H29N6O8P requires 488.1784, 1%), 81(C5H5O, base peak); PCLC retention times 22.83, 22.99 min (3:2).

*Ethyl methoxy-6-aminocaproyl phosphorochloridate*

This was prepared by a method entirely analogous to the 4-aminobutyryl analogue above. Thus from 1.05 g of ethyl phosphorodichloridate was isolated 1.74 g (99%) of the title compound. δ_p +13.85; δ_H 5.1(1H, bs, NH), 4.17(2H, m, POCH2), 3.60(3H, s, OMe), 2.91(2H, m, NCH2), 2.26(2H, m, CH2CO), 1.50(4H, m, CH2CH2CO, NCH2CH2), 1.30(5H, m, CH3CH2, PNHCH2CH2CH2); δ_C 173.79(C=0), 63.83(d, POCH2, J=6.1 Hz), 51.22(OMe), 41.37(NCH2), 33.61(CH2CO), 30.06(d, NCH2CH2, J=7.4 Hz), 25.79(NCH2CH2CH2), 24.20(CH2CH2CO), 15.60(d, CH3CH2, J=8.0 Hz); EIMS
m/e 272.0779(MH+, C₉H₂₀ClNO₄P requires 272.0818, 1), 243(M+-OMe, ³⁷Cl, 4),
241(M+-OMe, 16), 129(MeOCO-[CH₂]₅, base peak).

3'-Azido-3'-deoxythymidine-5'-{(ethyl methoxy-6-aminocaproyl) phosphoramidate (2e)

This was prepared by an entirely analogous method to compound (2c) above, except that the second chromatographic column was eluted with 2% methanol in ethyl acetate. Thus, from 0.094 g of (1) was isolated 0.067 g (38%) of (2e). Analytical and biological data were obtained following further chromatographic purification on silica, using 3.5% ethanol in chloroform as eluent. δₚ +7.83; δ_H (starred peaks are duplicated due to diastereoisomers) 9.61'(1H, s, N³H), 7.45'(1H, s, H6), 6.24'(1H, t, H1', J=6.6 Hz), 4.42(1H, m, H3'), 4.04-4.26(5H, m, POCH₂, H5', H4'), 3.67(3H, s, OMe), 3.17(1H, m, H3'), 2.91(2H, m, NCH₂), 2.43(1H, m, H2'), 2.28-2.35(3H, m, CH₂CO, H2'), 1.94'(3H, d, 5-Me, J=1.0 Hz), 1.63(2H, m, CH₂CH₂CO), 1.53(2H, m, NCH₂CH₂), 1.31-1.39(5H, m, CH₃CH₂,
CH₂CH₂CH₂NH); EIMS m/e 502.1893(M+, C₁₉H₂₅N₄O₈P requires 502.1941, 1%), 81(C₅H₅O+, base peak); HPLC retention times 23.21, 23.32 min (1:1).

Ethyl propylamino phosphorochloridate

n-Propylamine (1.30 g, 22 mmol) in anhydrous diethyl ether (40 ml) was added dropwise with vigorous stirring to a solution of ethyl phosphorodichloridate (1.79 g, 11 mmol) in diethyl ether (40 ml) over a period of 3 h at −78°C. The mixture was allowed to warm to room temperature with stirring over 1 h, and was then filtered. The filtrate was concentrated to dryness under reduced pressure, to yield the product as an oil (1.94 g, 95%). δₚ +14.07; δ_H 4.9(1H, bs, NH), 4.14(2H, m, CH₂OP), 2.86(2H, m, CH₂N), 1.51(2H, m, CH₂CH₂N), 1.31(3H, m, CH₃CH₂O), 0.86(3H, m, CH₃CH₂CH₂); δC 64.05(d, POCH₂, J=6.0 Hz), 43.69(CH₂N), 24.02(d, CH₂CH₂N, J=7.8 Hz), 11.19(CH₃CH₂CH₂); EIMS m/e 187(M+, ³⁷Cl, 1%), 185.0383(M+, C₃H₇ClNO₂P requires 185.0372, 4), 158(M+-Et, ³⁷Cl, 32), 156(M+-Cl, 82), 150(M+-Cl, 2), 130(MH+-PrNH, ³⁷Cl, 53), 128(MH+-PrNH, base peak).

3'-Azido-3'-deoxythymidine-5'-{(ethyl propylamino) phosphoramidate (3)

This was prepared by an entirely analogous method to compound (2a) above. Thus, from 0.20 g of (1) was isolated 0.21 g, (68%) of (3). δₚ +9.81; δ_H (starred peaks are duplicated due to diastereoisomers) 9.10'(1H, s, N³H), 7.35'(1H, s, H6), 6.15'(1H, t, H1'), 4.35(1H, m, H3'), 4.20(2H, m, H5'), 4.00-4.10(3H, m, POCH₂, H4'), 3.40(1H, m, NH), 2.80(2H, m, NCH₂), 2.40(1H, m, H2'), 2.30(1H, m, H2'), 1.90(3H, s, 5-Me), 1.50(2H, m, NCH₂CH₂), 1.40(3H, t, CH₃CH₂), 0.80(3H, t, NCH₂CH₂CH₂); EIMS m/e 416.1521(M+, C₁₃H₂₅N₆O₆P requires 416.1573, 1%), 81(C₅H₅O+, base peak); HPLC retention times 22.39, 22.59 min (3:2).
Biological evaluation

High titre virus stocks of the human immunodeficiency virus HIV-1 (RF strain) were grown in H9 cells with RPMI 1640 (Flow laboratories) supplemented with 10% fetal calf serum, penicillin (100 IU/l) and streptomycin (100 μg/ml). Cell debris was removed by low speed centrifugation, and the supernatant stored at −70°C until required. The target cell used in these assays was the C8166 CD4+ lymphoblastoid cell line. In a typical assay C8166 cells were incubated with 10 TCID50 HIV-1 at 37°C for 90 min and then washed three times with phosphate buffered saline (PBSA, Dulbecco A). Cell aliquots (2 × 10⁵) were resuspended in 1.5 ml growth medium in 6 ml tubes, and compounds in half log dilutions [100 μM to 0.1 μM; 200 μM to 0.1 μM in the case of compound (3)] were added immediately. The nucleoside phosphate triesters were sparingly soluble in aqueous solution, and 10 mM stock solutions of each compound were made up in DMSO. The final DMSO concentration in the tissue culture medium was 1%. The cells were then incubated at 37°C in a 95% air/5% CO₂ incubator. At 72 h post infection 200 μl of supernatant was taken from each culture and assayed for HIV (Kinchington et al., 1989) using an antigen capture ELISA (Coulter, Luton, U.K.). The following controls were used: supernatants taken from uninfected, and infected cells, infected cells treated with AZT (Roche Products U.K. Ltd.), and ddCyd (Roche). The activities of AZT and ddCyd on infected cells consistently gave an ED₅₀ of 0.005 and 0.2 μM respectively. The ELISA plates were read with a BioRad spectrophotometer. Compounds were tested in duplicate at each concentration, and each compound was tested on at least two different occasions. To test for compound toxicity, 2 × 10⁵ aliquots of uninfected cells were cultured with the compounds in the same half log dilutions for 72 h. The cells were then washed with PBSA and resuspended in 200 μl of growth medium containing ¹⁴C protein hydrolysate. After 12 h the cells were harvested and the ¹⁴C incorporation measured. Uninfected, untreated cells were used as controls. The compounds (2a–e, 3) showed a range of activities (Table 2), but none showed toxicity at 100 μM in this system.

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Shows the anti viral activity (EC₅₀ values; the concentration required to reduce HIV antigen production by 50%) for compounds (2a–e) and (3) in the assay system used. None of the compounds showed significant toxicity at 100 μM (¹⁴C protein hydrolysate uptake study) except (2c), which showed slight toxicity at 100 μM. The EC₅₀ values shown for the phosphoramidates are the average of three different assays; the values for AZT and ddCyd are the average of >10 assays.

*Number of methylene groups separating methyl ester from phosphoramidate in structure (2).
Results

A common, 2-stage synthetic procedure was adopted for each of the phosphoramidate compounds (2a–e). In the first step, ethyl phosphorodichloridate was reacted with the methyl ester of the appropriate ω-amino acid, in the presence of triethylamine. The resulting phosphorochloridates were fully characterised by spectroscopic methods (see experimental section). These materials reacted with AZT in anhydrous THF, containing an excess of N-methylimidazole (Van Boom et al., 1975). The products (2a–e) were isolated by precipitation from petrol and column chromatography. In each case, a number of successive column chromatographic purification steps were necessary to produce homogeneous material, using different eluants in each step. In every case, spectral data clearly indicate the presence of two diastereoisomers in the products (2a–e); resulting from mixed stereochemistry at the phosphorus centre. In this study, the crude mixtures of diastereoisomers were
tested for their anti-viral effect; it is quite possible that the separate isomers may differ markedly in their activities. In each case, the products were characterised by $^{31}$P nmr, chemical shift values of ca. δ 7.5–7.8 being noted. This compares closely to literature values for similar species (for example OP(NEt$_2$)(OEt)$_2$ δ$_P$ +9; Mark et al., 1969). In each case, the $^{31}$P nmr signals for the two diastereoisomers were coincident. However, in the $^{13}$C nmr spectra, many signals for the base, sugar, alkoxy, and amino moieties appeared as two peaks, whose ratios reflected the ratio of the diastereoisomers, which was usually close to 1:1. In some cases, for the resonances of carbon atoms within three bonds of the phosphorus, further splitting was noted, due to phosphorus coupling. Some resonances displayed both diastereomeric splitting, and phosphorus coupling. Carbon-13 nmr data for the products (2a–e) are listed in Table 1. Proton nmr spectra and mass spectra further support the structures of the products (2a–e), the former also confirming the presence, and ratio of, diastereoisomers. The purity of the products was confirmed by HPLC; in particular, AZT levels were below detectable limits. This is important given the high activity of AZT in the biological assay used.

**Discussion**

The anti-HIV-1 activity of the phosphoramidates (2a–e) was measured in vitro, by methods described previously (Kinchington et al., 1989), the results being summarised in Table 2. Thus, compound (2a) was the most active of the series, causing 50% inhibition of viral replication at a concentration of 10 μM. Compound (2b) was less active, and compounds (2c–e) were active only at high concentrations. The latter three compounds were in fact equi-active in the test system employed, each causing 50% inhibition only at the highest concentration studied (100 μM). It is clear that the anti-HIV activity of the phosphoramidate derivatives declines with increasing separation of the phosphoramidate and carboxyl ester moieties. This is consistent with a mechanism of action involving intracellular cleavage of the P-N bond: increased separation of the carboxyl ester might be expected to stabilise this bond, and thus reduce activity. This cleavage would release AZT ethyl phosphate, which might either act as such, or undergo further hydrolysis to AZT or AZT monophosphate. Both of the latter species at least would be potent anti-HIV agents if released in an intracellular environment.

Given the apparent plateau in the activity of the later members of the series (2c–e) it was considered possible that the presence of the distant ester group was no longer making a contribution to the activity of the phosphoramidates. To test this hypothesis, the unsubstituted propylamino compound (3) was prepared by analogous methodology, and evaluated in vitro. As noted in Table 2 this compound displays anti-HIV activity intermediate in magnitude between that of (2b) and (2c–e). The surprisingly high activity of (3) clearly suggests that the remote ester group in (2c–e) does not contribute to their activity. Indeed, the activity is higher in the absence of the ester group. The reasons for this are unclear; however, it is interesting to note that a simple alkylamino group attached to the phosphorus
(as in [3]) does confer anti-HIV activity, whereas a simple alkylx group in this position (as in a nucleoside dialkyl phosphate) does not (McGuigan et al., 1990a). Lastly, the activity of, particularly (2b), indicates that a natural alpha-amino acid moiety is not required for activity. This indicates that specific hydrolysis by HIV aspartate proteinase (Navia et al., 1989), the original rationale for preparing amino acid derivatives, is unlikely to be the sole mode of activation of these pro-drugs. Further studies are underway to probe the mechanism of action of these compounds.

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References


