



The Welsh School of Pharmacy
Cardiff University

**Design, Synthesis and Biological
Evaluation of Novel Bioactive Nucleosides
and Nucleotides**

A Thesis submitted to Cardiff University for the degree of
Philosophiae Doctor

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Abstract

At the present there are 36 approved antiviral drugs in the UK of which half are nucleoside analogues. However, the emergence of drug resistance and of new virus strains necessitates new drugs. In particular in this thesis, different nucleoside analogues were studied as potential antivirals.

One of the major issues related to nucleoside analogues is the emergence of resistance due to a lack of bioactivation to the monophosphate form. To overcome this issue, the phosphoramidate ProTide technology can be applied. This strategy allows the delivery of the monophosphate form directly inside the cell.

Bicyclic nucleoside analogues are a new class of anti-varicella zoster agents of which Cfl743 is the most potent anti-varicella zoster compounds reported to date. Its 5'-valyl derivative, FV100, is currently in phase II clinical trials. A series of derivatives to increase the activity and to investigate the mechanism of action of this new class of compound are reported. Moreover, attempts to improve the scale up synthesis of FV100 are described.

Ribavirin is a broad spectrum antiviral drug. The application of the ProTide approach to this compound was not successful. Enzymatic and molecular modelling studies have been performed in order to understand the lack of activity.

Acyclovir and its esters are currently the treatment of choice for herpes simplex and varicella-zoster infections. The application of the ProTide technology gave surprising results. In fact, these compounds have been found to be active against HIV, whilst ACV itself did not show any activity. Moreover, these compounds retained activity versus thymidine kinase deficient strains against which acyclovir lost activity. These striking results prompted us to investigate other different nucleoside analogues, through a virtual screening using reverse transcriptase, guanylate or adenylate kinase and human polymerase γ . The selected nucleoside analogues from this study include: ganciclovir, penciclovir and their derivatives. ProTides of these are thus pursued.

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Publication

A part of the work presented in this thesis has been published in the following journals:

1. McGuigan, C.; Derudas, M.; Bugert, J. J.; Andrei, G.; Snoeck, R.; Balzarini, J. Successful kinase bypass with new acyclovir phosphoramidate prodrugs. *Bioorg Med Chem. Lett.* **2008**, *18*, 4364-4367.
2. Lisco, A.; Vanpouille, C.; Tchesnokov, E. P.; Grivel, J. C.; Biancotto, A.; Brichacek, B.; Elliott, J.; Fromentin, E.; Shattock, R.; Anton, P.; Gorelick, R.; Balzarini, J.; McGuigan, C.; Derudas, M.; Götte, M.; Schinazi, R. F.; Margolis, L. Acyclovir is activated into a HIV-1 reverse transcriptase inhibitor in herpesvirus-infected human tissues. *Cell Host Microbe* **2008**, *4*, 260-270.
3. Tchesnokov, E. P.; Obikhod, A.; Massud, I.; Lisco, A.; Vanpouille, C.; Brichacek, B.; Balzarini, J.; McGuigan, C.; Derudas, M.; Margolis, L.; Schinazi, R. F.; Götte, M. Mechanisms associated with HIV-1 resistance to acyclovir by the V75I mutation in reverse transcriptase. *J. Biol. Chem.* **2009**, *284*, 21496-21504.
4. Derudas, M.; Carta, D.; Brancale, A.; Vanpouille, C.; Lisco, A.; Margolis, L.; Balzarini, J.; McGuigan, C. The application of phosphoramidate prodrug technology to acyclovir confers anti-HIV inhibition. *J. Med. Chem.* **2009**, *52*, 5520-5530.
5. McGuigan, C.; Derudas, M.; Quintiliani, M.; Andrei, G.; Snoeck, R.; Henson, G.; Balzarini, J. Fluorosugar analogues of the highly potent anti-VZV BCNAs. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6264-6267.

The full articles are referred in the Appendix I.

Abbreviations & Acronyms

ABC	abacavir
ACV	acyclovir
Ala	alanine
AZT	azidothymidine
BCNA	bicyclic nucleoside analogue
Bn	benzyl
BNF	British National Formulary
Boc	N-(tert-butoxycarbonyl)
BVdU	brivudin
BVU	(<i>E</i>)-5-(2-bromovinyl)-uracil
CAN	cerium (IV) ammonium nitrate
CLogP	calculated LogP
dATP	deoxyadenosine triphosphate
DBU	1,8-diazabicycloundec-7-ene
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
ddAMP	dideoxyadenosine monophosphate
ddI	2',3'-dideoxyinosine
dGTP	deoxyguanosine triphosphate
DIPEA	diisopropylethylamine
DMAP	dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DP	diphosphate
dTTP	deoxythymidine triphosphate
d4T	2',3'-didehydro-2',3'-dideoxythymidine
EPV	Epstein-Barr virus
Et	ethyl
Fmoc	N-9-fluorenylmethoxycarbonyl
FTC	(-)- β -L-3'-thia-2',3'-dideoxy-5-fluoro-cytidine

GCV	ganciclovir
GMP	guanosine monophosphate
HCMV	human cytomegalovirus
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HHV	human herpes virus
HPMPA	(S)-9-(2-phosphono-methoxypropyl)adenine
HSV	herpes simplex virus
ICTV	international committee on taxonomy of viruses
IN	integrase
IPA	isopropanol
ⁱ Pr	isopropyl
Me	methyl
MP	monophosphate
MRI	magnetic resonance imaging
mRNA	messenger RNA
NA	nucleoside analogue
Naph	1-naphthyl
NDK	nucleotide diphosphate kinase
NMI	N-methylimidazole
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NtRTI	nucleotide reverse transcriptase inhibitor
NtA	nucleotide analogue
OST	osteosarcoma
PCV	penciclovir
Ph	phenyl
Phe	phenylalanine
PK	protein kinase
PMEA	9-[2-(phosphonomethoxy)ethoxy]-adenine
PMEG	phosphonomethoxyethylguanine
POM	pivaloyloxymethyl

POC	isopropylloxycarbonyloxymethyl
PR	protease
ProTide	nucleotide prodrug
P-Tsa	para-toluenesulfonic acid
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT	reverse transcriptase
SAR	structure activity relationship
SATE	S-acyl-2-thioethyl
SDTE	S-dithioethyl
TBDMS	tetrabutyltrimethylsilyl
TBDMSCl	tetrabutyltrimethylsilyl chloride
^t Bu	tert-butyl
^t BuMgCl	tert-butylmagnesium chloride
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TI	therapeutic index
TK	thymidine kinase
TLC	thin layer chromatography
TNF	tenofovir
TP	triphosphate
VZV	varicella zoster virus
3TC	(-)-β-L-3'-thia-2',3'-dideoxycytidine

1. Introduction

1.1 Virus: classification, structure and replication

Viruses are considered to be the smallest organisms able to infect vertebrates, protists, plants, insects, yeasts, fungi, bacteria and other unicellular organisms. At present there are over 6000 viruses of which about 200 can infect human beings.^{1,2}

Viruses can be classified with two different systems: the international committee on taxonomy of viruses (ICTV) and the Baltimore classification.^{3,4}

1.1.1 Structure of the virus

Viruses are obligate parasites that need a cell host to reproduce their genome and they proliferate by invading the non-infected cells. They consist of a core of nucleic acid, DNA or RNA enclosed within a protein shell called a capsid. The capsid can also be coated by another glycoprotein layer called an envelope. The process of infection starts when viruses enter into contact with the surface of the cell. Firstly, viruses bind the receptors present in the cell membrane. These receptors can promote a series of functional changes in the membrane with the results of facilitating the permeation of the viruses into cells. Moreover, they give the selectivity to the virus to infect specific cells.⁵ A large number of viruses can interact with more than one receptor; for example, the capacity of herpes virus to infect different cells is due to its ability to interact with different receptors.⁶

Here, we report as an example the mechanism of replication of human immunodeficiency virus (HIV) with the potential targets for antiviral drugs. This replication cycle is different from the replication of DNA viruses. In fact, as HIV belongs to the retrovirus family and it is a RNA virus, it needs firstly a reverse transcription followed by an integration into the host genome whilst in the case of DNA viruses, the viral genome is replicated by a viral DNA polymerase.⁵

1.1.2 Mechanism of replication of HIV

As previously mentioned, viruses have to interact initially with the host cell by binding with the receptors situated in the cell membrane. **Fig 1.1** reports the mechanism of replication for HIV. The glycoprotein gp120 situated in the viral envelope interacts with the CD4 receptors and with a co-receptor, which is CXCR4

for T lymphocytes or CCR5 for macrophages, creating a bond necessary for the proceeding fusion step. The inhibition of this interaction might block the entry of the virus into the host cell. The interaction of gp120 with the membrane receptor is followed by the interaction of the subunit gp41 with the surface of the cell, which gives a series of changes of the external structure of the virus and the host cell membrane with the result of the virus-cell fusion and the entry of the capsid core inside the host cell. The blockade of this step might prevent the entry of the virus.

Once inside the cell, before the process of the replication begins, HIV has to lose the capsid in order to release the genetic material, constituted by two copies of the single-stranded RNA and the enzymes, reverse transcriptase (RT), integrase (IN) and protease (PR), necessary for replication.

In the case of DNA viruses, the replication of the genome is carried out by the viral DNA polymerase, which is expressed in the infected cells. The DNA polymerase is a well known target for antiviral therapy which will be discussed later in this chapter.

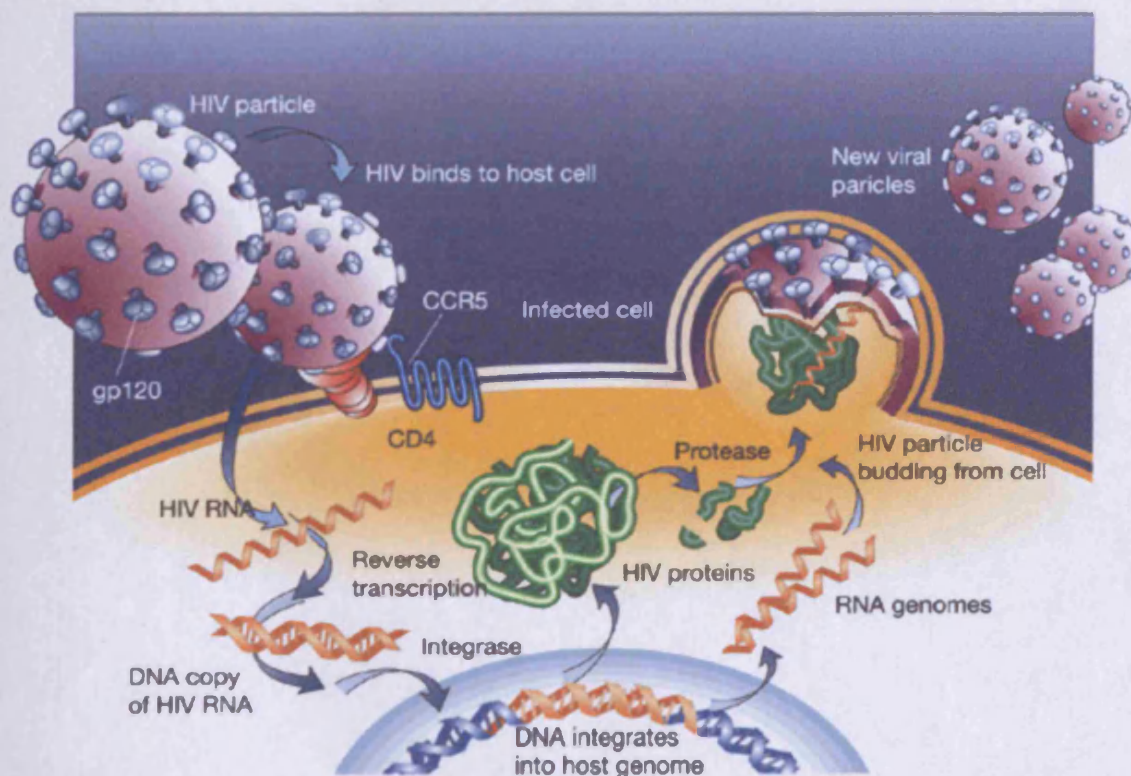


Fig. 1.1: mechanism of replication of HIV (from ref. 7).

In the case of HIV, its RNA genome needs to be converted to DNA by the RT. RT is a RNA-dependent DNA polymerase encoded by the virus. It consists of two catalytic sites: a polymerase active site and ribonuclease site. The transcription of a single

stranded RNA into a RNA-DNA hybrid is carried out by the polymerase site. The RNA-DNA hybrid is then hydrolysed to a single-stranded DNA by the ribonuclease H site. The single-stranded DNA is then converted to a double strand again by the RT in the polymerase site. This step is necessary for the proliferation of the virus in the host cell and it represents one well-known target for anti-HIV therapy, constituted by nucleoside reverse transcriptase inhibitors (NRTIs), as inhibitors of the catalytic binding site, and non-nucleoside reverse transcriptase inhibitors (NNRTIs), as inhibitors of the allosteric site. Being present only in the virus-infected cells, RT is a good target in terms of selectivity and therapeutic index. Once the double DNA strand is formed, it needs to be integrated into the host genome. This step requires the retroviral enzyme IN. IN works in two steps: in the first, it cleaves the 3'-end of the viral DNA. The second step consists of the integration, process called strand transfer, of the viral DNA in the cellular genome. IN is another valid target for anti-HIV therapy as it is present only in the infected cells.

The integrated viral DNA into the host genome is called proviral DNA and it might remain dormant in the cell host or it can be transcribed to viral messenger RNA (mRNA) or to viral genome RNA by the cellular enzymes. The viral messenger is now translated to polyproteins, structural proteins and enzymes. These polyproteins are cleaved by the PR enzyme into two different types of proteins: functional proteins, with enzymatic activity, or structural proteins, which will form part of the virion structure. The step mediated by the PR is crucial for the viral proliferation as it releases all the proteins necessary for the assembly of a new viral particle and it presents another target for antiviral therapy. It is not only a target for anti-HIV therapy but also has potential for hepatitis C virus (HCV) and herpesvirus for instance.

Two single RNA strands and the three enzymes necessary for the replication are now enclosed into the new capsid. The last step consist in the release of the capsid through the host cell membrane, which forms the envelope of the new viral particle, which once matured, can infect another cell. The inhibition of the assembly as well as the inhibition of the release of the newly formed viruses might block the progress of the infection.

This process happens billions of times per day and HIV is able to destroy the host immune system.^{5, 8-10}

1.2 Antiviral agents

Viral infections can be symptomatic or asymptomatic, and can cause mild to severe symptoms and even death. For most of them, the immune system of the host is able to react to the virus invasion producing the antibody necessary to fight the infection and protecting the organism from further infection by the same virus.

A possibility to prevent the viral infection is the administration of a vaccine, which gives the antibody necessary to recognize the virus by the immune system. There are several vaccines available such as those for hepatitis A and B, polio, tetanus, seasonal influenza, varicella zoster virus (VZV), etc.⁵

For some infections, for which the immune system is not able to react or vaccines are not available, the development of antiviral agents was and is currently necessary.

Also, for post-exposure treatment an antiviral agent may well be the only alternative.

As seen in **Fig. 1.1**, there are several potential targets to which a drug can interact and there is at least one drug from most of them. The potential targets are:

- viral attachment and entry;
- viral uncoating;
- viral genome replication;
- viral assembly and maturation;
- viral release.

Currently there are 36 approved antiviral drugs in the UK (British National Formulary (BNF) 56, October 2008) and several others are currently under clinical trial. In particular, acyclovir (ACV), ganciclovir (GCV), penciclovir (PCV), ribavirin and some of the RT inhibitors will be discussed in detail as they are part of the work presented in this thesis.

1.2.1 Inhibitors of viral attachment and entry

The inhibition of this early stage allowed the use of compounds that do not need to enter inside the cells, as they are preventing the first step of the viral infection.

The first approved drug able to inhibit HIV entry is enfuvirtide, which is a peptide containing 36 amino acids (**Fig. 1.2**).

Ac-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-Trp-Ala-Ser-Leu-Trp-Asn-Trp-Phe-NH₂

Fig. 1.2. Amino acids sequence of enfuvirtide.

motif) CCR5 coreceptor. This receptor is expressed on T cells and is used by the virus to bind to the cell surface and diffuse inside the cells.⁵

Fig. 1.3 reports the structure of maraviroc (**1**). It acts as CCR5 antagonist preventing the binding of the virus to the receptor.¹² Moreover, maraviroc is an oral bioavailable drug.

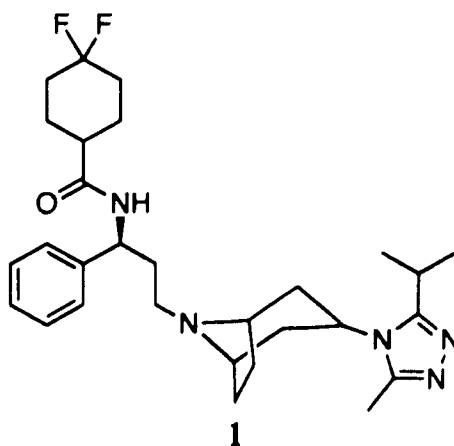


Fig. 1.3. Structure of maraviroc.

1.2.2 Inhibitors of viral uncoating

The viral uncoating is the process by which the virus releases the genetic material into the cytoplasm or into the nucleus, consenting the process of replication.

Currently there are two drugs approved (**Fig. 1.4**): amantadine (**2**), approved in 1966, and rimantadine (**3**) approved in 1994. Both drugs are active against influenza A virus.^{13, 14}

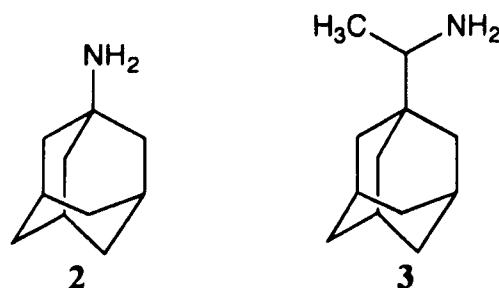


Fig. 4: amantadine and rimantadine structures.

These two adamantane derivatives act by inhibiting the ion channel in the cytoplasm leading to a decrease of the pH, which promotes the degeneration of the envelope on the virus with consequent release of the ribonucleoprotein into the cytoplasm. After its release, the ribonucleoprotein is transported into the nucleus for the transcription and replication.^{15,16}

Besides the antiviral activity, amantadine is also used in the treatment of Parkinson disease, however the mechanism of action is not fully understood and may involve the inhibition of the N-methyl-D-aspartate receptor.¹⁷

1.2.3 Inhibitors of the viral genome replication

As already discussed, once inside the cell, viruses start the process of replication of their genome. This process is different depending on the type of virus: DNA, RNA or retrovirus for instance.

The majority, 17 out of 36, of the current antiviral drugs are nucleoside analogues (NAs). These compounds act as analogues of the native nucleosides. **Fig. 1.5** reports the structures of the native nucleosides: adenosine or deoxyadenosine (**4**), guanosine or deoxyguanosine (**5**), cytidine or deoxycytidine (**6**), deoxythymidine (**7**) and uridine (**8**).

R = H or OH

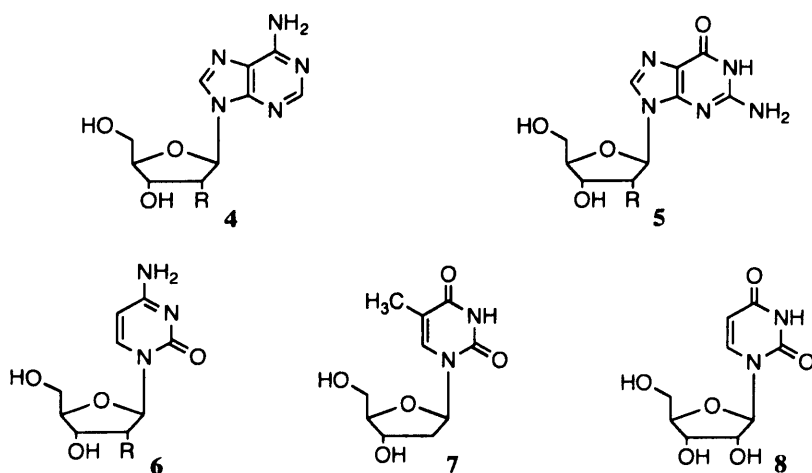


Fig. 1.5: the native nucleosides.

1.2.3.1 Viral DNA polymerase inhibitors

Viral DNA polymerase is the enzyme involved in the replication of the viral DNA. The compounds clinically approved (**Fig. 1.6**) in the UK as inhibitors of this polymerase are: ACV (**9**) and its prodrug valacyclovir (**12**), GCV (**10**) and its prodrug valganciclovir (**13**), PCV (**11**) and its prodrug famciclovir (**14**), the acyclic nucleoside phosphonate cidofovir (**15**) and the non-nucleoside foscarnet (**16**). Another compound, not licensed in the UK and in the USA but commercially available in several countries in Europe, is the deoxyuridine analogues brivudin (BVdU, **17**). All of these compounds inhibit the herpes polymerase.

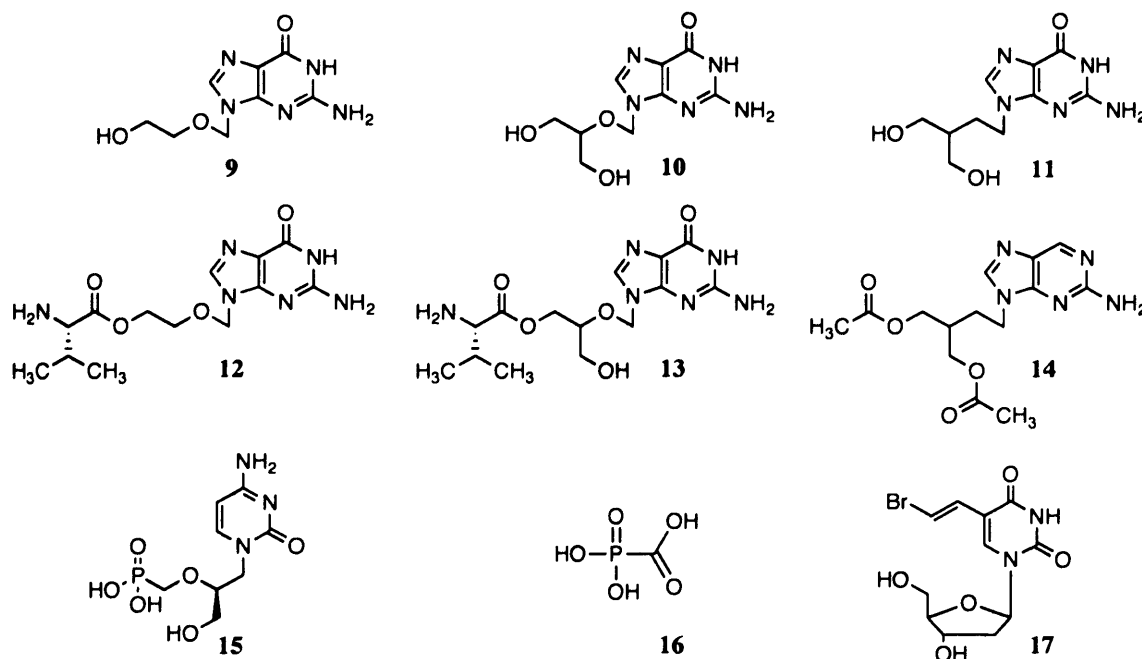


Fig. 1.6: Viral DNA-polymerase inhibitors.

1.2.3.1.1 Acyclovir: antiviral activity and mechanism of action

ACV (**9**) is a guanine analogue with activity against herpes simplex virus (HSV)-1 and 2 and VZV.¹⁸ Due to its poor bioavailability, a series of prodrugs have been synthesized. The L-valyl ester derivative, valacyclovir (**12**), showed the best profile. It is metabolised in the gut and in the liver by valacyclovir hydrolase to ACV increasing its bioavailability by *ca.* 4 fold.¹⁹

The mechanism of action of ACV (**Fig. 1.7**) involves activation to the monophosphate (MP) form (**18**) mediated specifically by HSV or VZV thymidine kinase (TK).¹⁸ TK-deficient strains show resistance to ACV due to the lack on bioactivation as the cellular kinases are unable to phosphorylate ACV.²⁰ Guanosine monophosphate

kinase converts ACV-MP to the diphosphate (DP) form (19),²¹ followed by conversion to the triphosphate (TP) form (20) mediated by nucleoside diphosphate kinase.²² In its triphosphate form, ACV is incorporated into the growing viral DNA competing with deoxyguanosine triphosphate (dGTP).^{18, 23} Once incorporated into the DNA the lack of the 3'-OH does not allow the addition of further deoxynucleoside which involves the termination of the DNA elongation.^{24, 25}

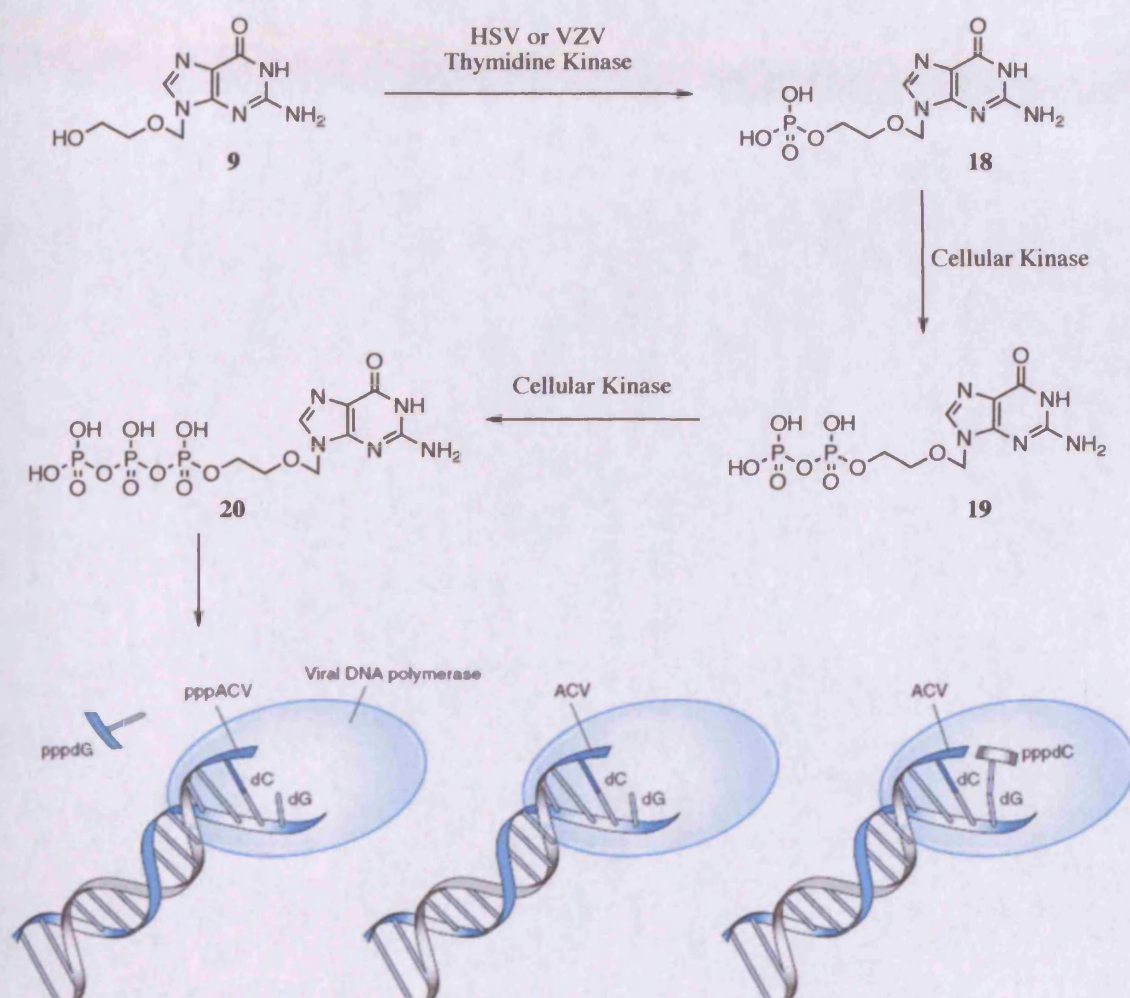


Fig. 1.7: mechanism of action of ACV (adapted from ref. 5).

The specific phosphorylation mediated by the viral TK, as well as the ability to inhibit the viral polymerase 10-30 times more specifically than cellular polymerase gives ACV a good therapeutic index (TI),¹⁸ and it remains still the treatment of choice for HSV and VZV infections, with sales of over \$1.6 billion for Valtrex and \$0.16 billion for Zovirax topical in 2008.²⁶ However, resistance to this drug has developed. The mechanisms of resistance to ACV can involve the TK and/or the viral polymerase.^{27, 28} With regard to TK, the resistance can be due to a total lack of the enzyme (TK

deficient strain), a decrease in the production of this enzyme or a decreased activity of this enzyme (TK low producer) or an alteration of the enzyme for which ACV is not a substrate (TK altered).²⁹ In the case of the viral polymerase, the resistance is due to a modification of the active site with consequent poor interaction with ACV-TP.²⁸

1.2.3.1.2 Ganciclovir: biological activity and mechanism of action

The discovery of ACV was quickly followed by the discovery of GCV (**10**). GCV showed improved activity against HSV-1 and 2 compared to ACV, and also showed potent activity against human cytomegalovirus (HCMV), another virus belonging to the herpesviridae family, against which ACV showed poor activity.³⁰ GCV was the first approved drug for the treatment of HCMV infections. GCV is an acyclic analogue of 2'-deoxyguanosine and it differs from ACV for the presence of the 3'-OH. As for ACV, the mechanism of action of GCV involves phosphorylation to the triphosphate form. In HSV-infection, the first step of phosphorylation is specifically mediated, as well as for ACV, by HSV-encoded TK.¹⁴ In the case of CMV, the conversion to the monophosphate is mediated by CMV-encoded protein kinase (PK). This protein is expressed by the gene UL97 and it has selectivity for GCV.³¹ In fact, the mutation of this gene gives resistance to GCV due to lack of phosphorylation.³¹ Further phosphorylation to the triphosphate are mediated by cellular kinases.³² The triphosphate is the active form of GCV and it acts as an inhibitor of the viral DNA polymerase. In contrast with ACV, which acts as chain terminator, GCV, due to the presence of the 3'-OH, showed incorporation of one additional nucleotide.³³ This specific viral polymerase is expressed by the UL54 gene and also in this case, mutation of this gene can give resistance to GCV.³²

GCV showed higher selectivity to the viral polymerase in comparison with the cellular polymerase.³⁴ However, this selectivity is not as great as the selectivity showed by ACV against HSV. In fact, GCV showed a greater toxicity compared to ACV, including bone marrow suppression (neutropenia, anemia and trombocytopenia).³²

One of the major issues for GCV, as well as for ACV, is its poor bioavailability, which is approximately 5%.³² As for ACV, the L-valinyl ester product of GCV, valganciclovir (**13**), has been synthesized showing a huge increase of bioavailability up to 60%.³²

GCV and valganciclovir are currently used for the treatment of HCMV infection particularly in immunocompromised patients.¹⁴

1.2.3.1.3 Penciclovir: antiviral activity and mechanism of action

Another acyclic nucleoside analogue discovered after ACV and strictly related to GCV, being its carbo analogue, is PCV (**11**). This compound showed antiviral activity slightly less active than ACV against HSV-1 and 2 and slightly more active than ACV against VZV.³⁵ PCV also showed activity against Epstein-Barr virus (EBV) and hepatitis B virus (HBV).³⁶ As for both ACV and GCV, the mechanism of action of PCV involves phosphorylation to the monophosphate form mediated by HSV or VZV-TK and it has been reported to be more specific for PCV than for ACV.³⁷ Further phosphorylation to PCV-TP gives the active form of PCV, which inhibits the viral DNA polymerase showing a similar mechanism of action of ACV.³⁸ In this case, ACT-TP is a better inhibitor of the polymerase than PCV-TP.³⁷ PCV is currently used topically for HSV infection. Its prodrug, famciclovir (**14**) is used for the treatment of VZV infections.³⁶ This prodrug consists in the diacetyl derivative of 6-deoxypenciclovir which has considerably improved oral bioavailability.³⁹ To release the parent, it needs a double activation: the hydrolysis of the two acetyl groups and the oxidation at the 6-position. The major bioactivation pathway firstly involves the hydrolysis of one acetyl group probably in the intestine, followed by the removal of the second acetyl in the liver.⁴⁰ The last step implicates the oxidation of the 6-position which is mediated by either the xanthine oxidase³⁶ or aldehyde oxidase.⁴¹

1.2.3.1.4 Cidofovir, foscarnet and brivudin: antiviral activity and mechanism of action

Cidofovir or (S)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (**15**) is a nucleotide analogue (NtA). In fact, cidofovir bears a phosphonate group, which is equivalent to the phosphate group but with the property of being stable to the hydrolysis mediated by phosphatase to which normal phosphate NAs are unstable.⁴² Contrary to the other NAs previously described in this chapter, cidofovir needs only two phosphorylations to be converted in the active form and in this form is able to inhibit viral DNA polymerase but also cellular polymerases.⁴² Cidofovir shows a very wide antiviral spectra including all the viruses belonging to the herpesviridae family,

poxviridae (for example cowpox, vaccinia) etc.⁴² It is currently approved mainly for the treatment of HCMV retinitis in patients with AIDS.³⁶

The only currently approved non-nucleoside inhibitor of viral polymerase is foscarnet or phosphonoformic acid (**16**).⁴³ The principal target of foscarnet is HCMV, but it has also activity against others herpesvirus, such as HSV-1 and 2, VZV, EBV, human herpes virus 6 (HHV). Foscarnet is also able to inhibit HIV.¹⁴ Foscarnet acts by blocking the pyrophosphate cleavage of the terminal nucleoside by binding the pyrophosphate binding site of the viral polymerase with consequent blockage of the DNA polymerization.¹⁴

BVdU or (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (**17**) is a deoxyuridine analogue that is currently approved in some territories mainly for the treatment of VZV infections but also against HSV-1 infections.^{44, 45} In common with the other NAs, BVdU needs to be bioactivated to the triphosphate form. In this form, BVdU acts either by competing with the natural nucleoside deoxythymidine triphosphate (dTTP) and inhibiting the viral polymerase or it can be incorporated into the growing DNA and it can affect the stability and the function of the viral DNA.⁴⁵

An important issue related to BVdU is the interaction with 5-fluorouracil. This interaction is related to the metabolism of BVdU to (*E*)-5-(2-bromovinyl)-uracil (BVU) and 2-deoxyribose-1-phosphate mediated by thymidine phosphorylase. BVU is a potent inhibitor of the enzyme responsible for the dihydropyrimidine dehydrogenase, a key enzyme in pyrimidine metabolism. This enzyme is involved in the metabolism of 5-fluorouracil, whose antitumor activity and toxicity is greatly enhanced by the inhibition of this enzyme by BVU.⁴⁵

1.2.3.2 Reverse transcriptase inhibitors

As already described previously in this chapter, RT is a fundamental enzyme for HIV replication; it transcribes a single-stranded RNA into a double-stranded DNA. After the discovery of HIV and the development of compounds able to inhibit the replication of the virus, RT became one of the most studied enzymes and currently there are 9 RT inhibitors approved in the UK of which 6 NRTIs, 1 nucleotide-RTIs (NtRTI) and 2 NNRTIs. Several others are currently approved in other countries or under clinical investigations.⁴⁶

1.2.3.2.1 Nucleoside reverse transcriptase inhibitors

The currently approved NRTIs are (**Fig. 1.8**): 3'-azido-2',3'-dideoxythymidine or azidothymidine (AZT) or zidovudine (**21**), 2',3'-dideoxyinosine or didanosine (ddI) (**22**), 2',3'-didehydro-2',3'-dideoxythymidine (d4T) or stavudine (**23**), (-)- β -L-3'-thia-2',3'-dideoxycytidine (3TC) or lamivudine (**24**), (1*S*, 4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol or abacavir (ABC) (**25**) and (-)- β -L-3'-thia-2',3'-dideoxy-5-fluoro-cytidine (FTC) or emtricitabine (**26**).

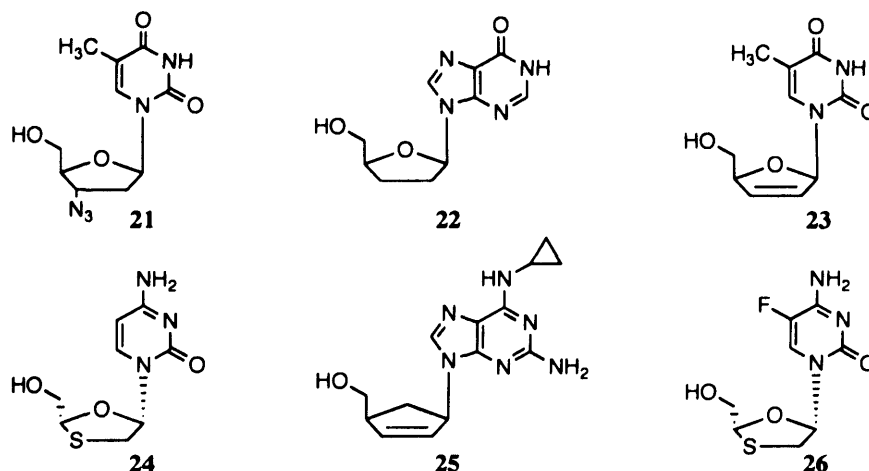


Fig. 1.8: currently approved NRTIs.

NRTIs, as already seen for the DNA polymerase inhibitors, need to be bioactivated to the triphosphate form to be able to inhibit the enzyme by acting as a chain terminator.

Fig. 1.9 illustrates the mechanism of action of the first approved anti-HIV compound: AZT (**21**), which inhibits HIV replication in the nM range (50-500 nM).⁴⁷ The first step of bioactivation is the conversion of AZT to its monophosphate form (**27**) mediated by cellular TK.⁴⁸ Contrary to the case of ACV, which is specifically phosphorylated by the HHV-TK and it gives the selectivity for ACV, AZT is as good a substrate as thymidine for cellular TK and moreover, there is no difference in phosphorylation between HIV-infected and non-infected cells.⁴⁸ The second phosphorylation, to obtain the AZT-DP (**28**), is mediated by cellular thymidylate kinase and this is the rate limiting step in the bioactivation of AZT. Nucleotide diphosphate kinase (NDK) converts AZT-DP to the triphosphate. AZT-TP (**29**) inhibits RT by acting as a chain terminator due to the absence of the 3'-OH⁴⁹ at a much lower concentration than that required to inhibit cellular polymerases showing a good TI.⁴⁸

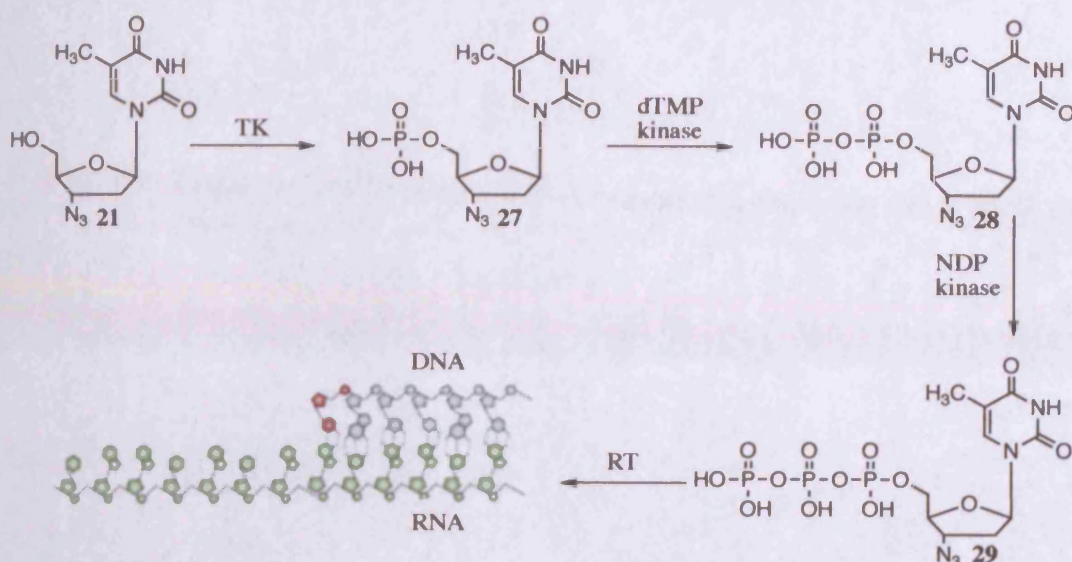


Fig. 1.9: mechanism of action of AZT.

In spite of its selectivity, AZT showed toxicity in patients including bone marrow suppression, which leads to anemia and neutropenia.⁵

ddI (22) was the second anti-HIV compound approved followed by d4T (23) in 1994.⁵⁰ Their mechanism of action is similar to AZT, however ddI needs first to be converted to the inosine monophosphate than to dideoxyadenosine monophosphate (ddAMP); in the triphosphate form, they compete with deoxyadenosine triphosphate (dATP) and dTTP respectively in the DNA synthesis acting as chain terminators due to the lack of the 3'-OH.¹⁴

Lamivudine (24) is a particular nucleoside as the 3'-carbon is substituted with sulphur and it is a L-stereoisomer, not the natural D-stereoisomer. The antiviral activity of lamivudine was first reported in 1991 and it was evaluated as a mixture of isomers.⁵¹ The separation of the two stereoisomer showed that both stereoisomers have anti-HIV activity but surprisingly the L-enantiomer showed less toxicity than the D-enantiomer. Moreover, although less active than AZT, the selectivity index of lamivudine is ~100 times greater than AZT.^{52, 53} The mechanism of action of lamivudine is similar to AZT and acts by inhibiting DNA synthesis.¹⁴

Lamivudine was found to be active against HBV⁵⁴ and it is currently approved also for the treatment of chronic HBV infections.⁴⁶

Abacavir (25) was approved in 1998 and it is the only guanosine analogue available for the treatment of HIV. ABC is a prodrug of the earlier reported carbovir, which is a carbocyclic guanosine analogue. First, carbovir was synthesized and biologically

evaluated as a mixture of two isomers, (+)-carbovir and (-)-carbovir,⁵⁵ but further studies showed that the (-)-isomer was the most potent anti-HIV inhibitor.⁵⁶

The poor bioavailability of carbovir prompts the design of a series of different prodrugs ending to the cyclopropyl amine derivative, ABC.⁵⁷ The mechanism of activation of ABC involves firstly the phosphorylation to the monophosphate by adenosine phosphotransferase followed by conversion to carbovir monophosphate mediated by a cytosolic enzyme.⁵⁸ Further phosphorylation to the di- and triphosphate led to the active form of ABC, which acts as competitive inhibitor of dGTP and as a chain terminator in the DNA synthesis.⁵⁹

Emtricitabine or FTC (**26**) is the latest NRTI approved. FTC is a cytidine derivative with the unusual oxathiolane ring. The (-)- β -isomer of FTC has been found to be more active than the other enantiomer by ~150 fold in the inhibition of HIV at non-toxic concentrations.⁶⁰ As for the others NRTI, the mechanism of action of FTC involves phosphorylation to the triphosphate form. FTC showed activity against HBV and in this case, as well, the (-)- β -isomer has been found to be more potent, ~40 fold, than the other enantiomer.⁶¹

1.2.3.2.2 Nucleotide reverse transcriptase inhibitors

Currently only one NtRTI has been approved for the treatment of HIV (**Fig. 1.10**): tenofovir disoproxil fumarate (**31**), which is a prodrug of tenofovir (TNF) (**30**).

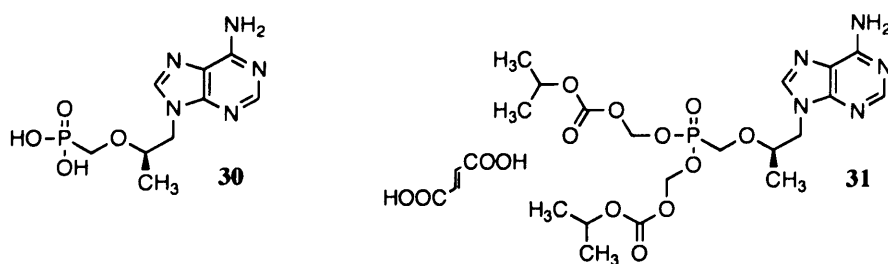


Fig. 1.10: tenofovir (**30**) and its disoproxil fumarate prodrug (**31**).

As already described for cidofovir, the particularity of this class of compounds is the by-pass of the first step of phosphorylation needing only two phosphorylation steps to the corresponding triphosphate analogue. TNF showed activity against HIV-1 and 2, and also against HBV.⁴² The chirality in TNF is extremely important as the (*R*)-enantiomer is more potent than the (*S*)-enantiomer.⁶² Due to the poor bioavailability

of TNF, for the presence of the negative charges at the phosphate moiety, a series of prodrugs has been designed. Amongst these, the bis disoproxil fumarate has been selected, showing increased bioavailability but also increased potency probably due to the increased uptake inside the cell.^{63, 64}

1.2.3.2.3 Anti-hepatitis nucleoside and nucleotide analogues

Three compounds belong to this class of compounds presently approved in the UK (**Fig. 1.11**): adefovir dipivoxil (**32**), entecavir (**33**) and telbivudine (**34**). These compounds have been included in this section as their target, HBV-RT, is very similar to HIV-RT.

Adefovir dipivoxil is an oral prodrug of adefovir. The prodrug, as noted for TNF, increased the bioavailability compared to the parent but also its potency against HIV.⁶⁵ Adefovir is a NtA bearing a phosphonate moiety as described above for cidofovir and TNF. After the phosphorylation to the corresponding triphosphate form, it acts by targeting the HBV-RT, but also HIV-RT, blocking the DNA synthesis. It also has activity against all the HHVs.^{42, 66}

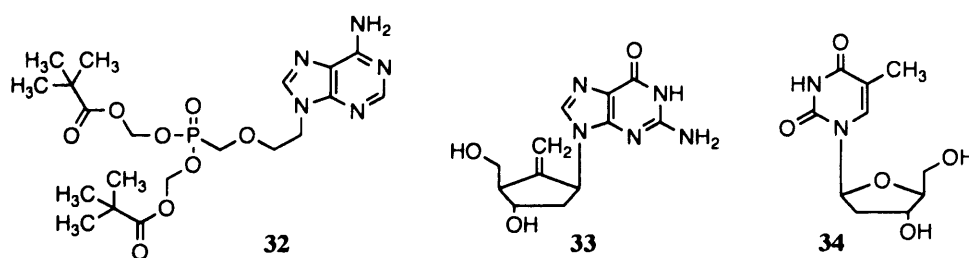


Fig. 1.11: anti-HBV drugs.

Entecavir (**33**) was firstly designed as an anti-HSV agent, but it was found to be active against HBV in the nM range.⁶⁷ The characteristic of this deoxyguanosine analogue is the unusual sugar moiety, which is a carbocycle bearing an exocyclic double bond. It is selectively active against HBV and it acts by inhibiting the RT.⁶⁷

Telbivudine (**34**) is the β -L-2'-deoxy-thymidine. It acts, after phosphorylation to the triphosphate form, as inhibitor of HBV-RT. It showed only activity against HBV and not against HIV.⁶⁸

1.2.3.2.4 Non-nucleoside reverse transcriptase inhibitors

At present, only two NNRTIs, nevirapine (**35**) and efavirenz (**36**), have been licensed in the UK (**Fig. 1.12**), with several others currently under clinical investigation.⁴⁶ Two more compounds have been licensed by the FDA: delavirdine (**37**), which is rarely used, and etravirine (**38**), which has been recently approved.⁴⁶

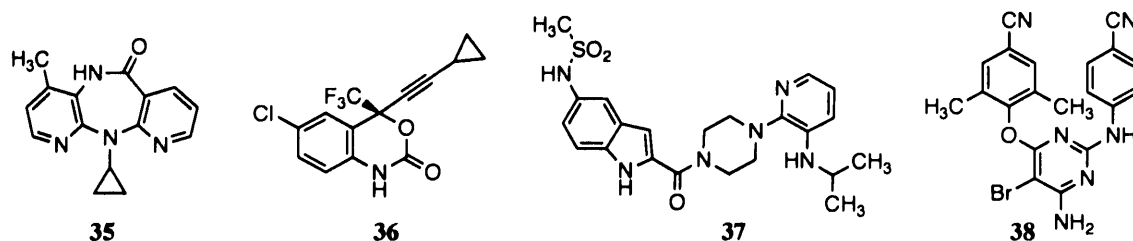


Fig. 1.12: non-nucleosides reverse transcriptase inhibitors.

NNRTIs interact with a binding site of RT, which is different from the active site of the enzyme. This interaction leads to a change of the conformation of the active site of the enzyme with the result to block the activity of RT. Resistance to the NNRTIs occurs very quickly due to the change of conformation of the binding site. NNRTIs inhibit only HIV-1 and they showed a very good cytotoxic profile as they do not interact with human polymerases.⁶⁹

The first NNRTI reported was nevirapine (**35**), which showed activity against HIV-1 without competing with dGTP.⁷⁰ Moreover, nevirapine has been found to be active against AZT-resistant strain and to be synergistic with AZT, confirming the difference in the mechanism of action of these two categories of anti-HIV drugs.⁷¹

The second NNRTI approved in the UK is efavirenz (**36**). Its mechanism of action and activity are similar to nevirapine. It showed also activity against several HIV-1 mutants strains; these results encourage the use of this compound in case of resistance.⁷²

1.2.4 HIV integrase inhibitors

The inhibition of the integrase blocks the integration of the viral DNA into the host genome. As this enzyme is only present in the HIV infected cells, it is considered an important target for the design of new anti-HIV agents.⁷³ Several integrase inhibitors have been synthesized and described,⁷⁴ but at present, only one has been licensed: raltegravir (**39**, **Fig. 1.13**).

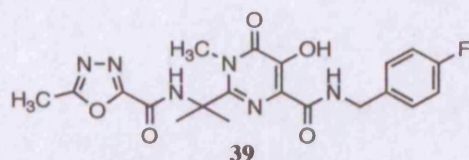


Fig. 1.13: currently approved integrase inhibitor raltegravir.

Raltegravir inhibits the strand transfer of integrase, it showed a good antiretroviral profile, also against multi-drug resistant strains.⁷⁵

1.2.5 Protease inhibitors

Another important target in the development of antiviral therapy, especially for HIV infections, is the enzyme PR. The catalytic site of PR has been identified and crystallized helping the design and synthesis of PR-inhibitors.¹⁰

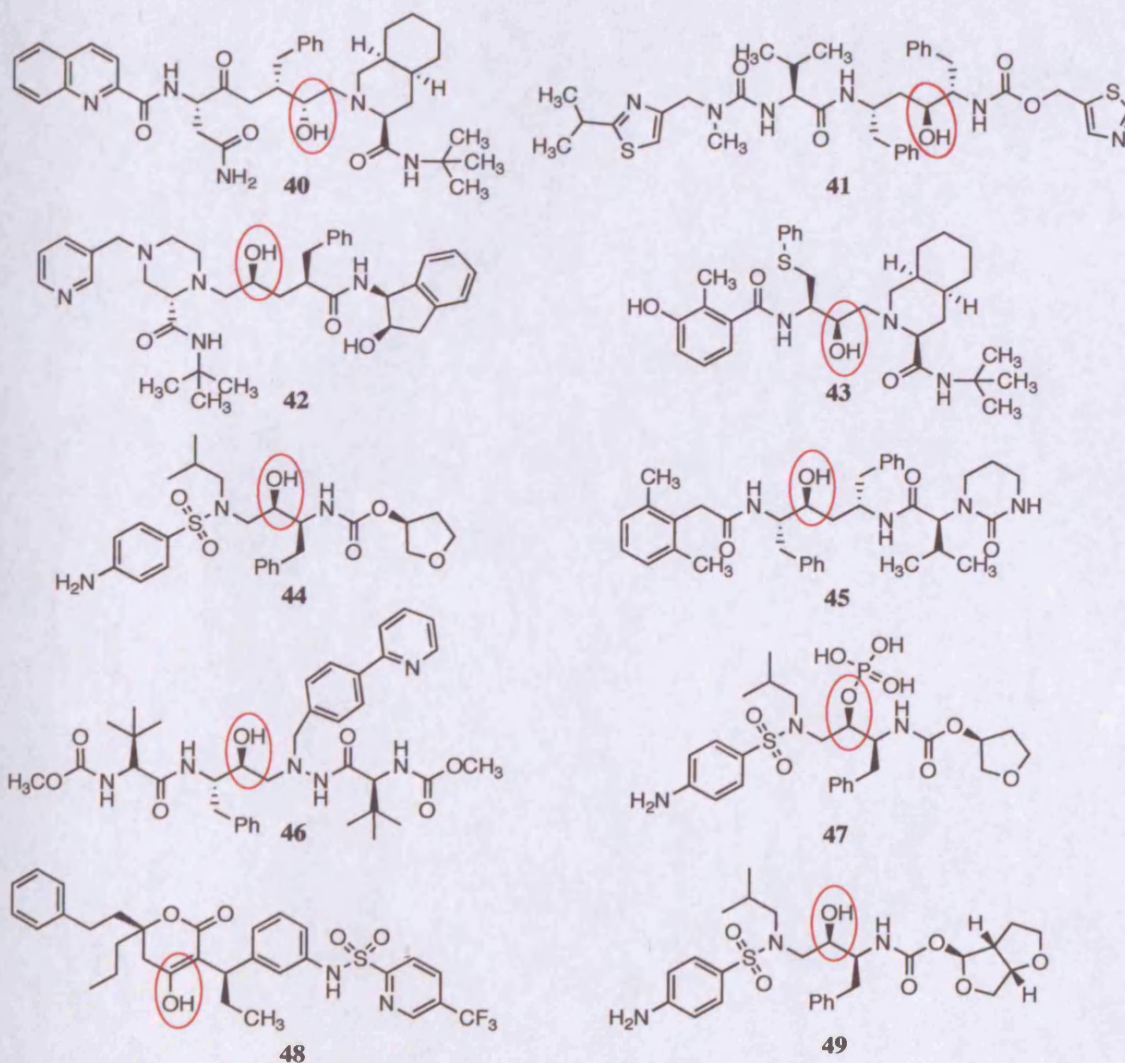


Fig. 1.14: anti-HIV protease inhibitors.

At this time ten PR inhibitors (**Fig. 1.14**) have been approved in the UK: saquinavir (**40**), ritonavir (**41**), indinavir (**42**), nelfinavir (**43**), amprenavir (**44**), lopinavir (**45**), atazanavir (**46**), fosamprenavir (**47**), tipranavir (**48**) and darunavir (**49**).^{14, 46} All the compounds currently approved share the same mechanism of action. These compounds bear a stable peptidomimetic moiety (red circle). The stability is due to the presence of a hydroxyethylene core that can not be cleaved by the enzyme, with the result of blocking its activity.¹⁰

1.2.6 Anti-HIV therapy

Having discussed all the classes of compounds used for the treatment of HIV infections, it is necessary to briefly report the different therapeutic regimens in use. Since the discovery of AZT and its approval for the treatment of HIV almost 20 years ago, several improvements have been made in this field. The emergence of resistance using only AZT mono therapy has been described.⁷⁶ However, the discovery of new RT inhibitors as well as the discovery of new classes of compounds, such as PR inhibitors, changed the way to treat HIV. The current treatment, called highly active antiretroviral therapy (HAART), includes the combination of NRTI, NNRTI and/or PR inhibitors. This combination has the aim not only to reduce the insurgence of resistance, but also acts in terms of synergy and reduction of toxicity.⁷⁷ There are several combination drugs on the market, such as: atipla (efavirenz, emtricitabine, TNF), combivir (lamivudine and AZT), trizivir (ABC, lamivudine and AZT), truvada (emtricitabine and TNF), etc.²⁶ One of the goals of this combination is also the compliance of patients being a longterm therapy.

1.2.7 Ribavirin: a broad antiviral with different potential targets

Ribavirin (**50**, **Fig. 1.15**) is a NA with antiviral activity against a number of DNA and RNA viruses in vitro and in vivo.⁷⁸

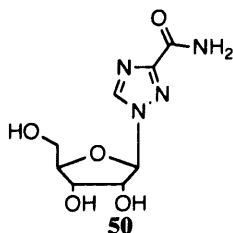


Fig. 1.15: the antiviral drug ribavirin,

As for the other NAs, ribavirin needs to be converted into its 5'-monophosphate form by intracellular phosphorylation mediated by adenosine kinase, followed by further phosphorylation to the di- and triphosphate.⁷⁹ Its mechanism of action is not completely understood and, depending on the virus examined, several mechanisms have been proposed:

- a) it has been found that in the monophosphate form, ribavirin inhibits inosine monophosphate dehydrogenase. The inhibition of this cellular enzyme leads to a reduction of GTP,^{80, 81} the decrease of GTP may promote the incorporation of ribavirin triphosphate into viral RNA;⁸²
- b) in the triphosphate form, ribavirin inhibits viral RNA synthesis by inhibition of the viral polymerase in influenza virus;⁸³
- c) in Dengue virus, ribavirin triphosphate has been found to inhibit viral 2'-O-methyltransferase while ribavirin monophosphate inhibits the viral guanylyl transferase;⁸⁴
- d) once incorporated in the viral RNA, ribavirin induces an accumulation of mutations, which, if lethal, impede viral replication (so called "error catastrophe");⁸⁵
- e) finally, in HBV and HCV affected patients, ribavirin modulates diverse immunomodulatory activities.^{86, 87}

Ribavirin is currently in used for the treatment of respiratory syncytial virus (RSV) and forms part of the only approved combination therapy for hepatitis C virus (HCV) infection in association with interferon- α .⁸⁸

1.2.8 Influenza virus neuraminidase inhibitors

Influenza virus neuraminidase is an important enzyme in the proliferation of the virus. The function of this enzyme, which is encoded by the virus, is to cleave the bond between the new hemagglutinin virion and the sialic acid of the host cells helping the release of the new virions and the consequent spread of infection. The blockade of this enzyme stops the proliferation of the virus.⁵

In the UK, two inhibitors of influenza virus neuraminidase have been approved (**Fig 1.16**): zanamivir (**51**) and oseltamivir (**52**), which is a prodrug of the free acid parent.



Fig. 16: neuraminidase inhibitors.

Zanamivir have been discovered through molecular modeling design from the crystal structure of the enzyme and it showed potent inhibitory activity against both influenza A and B virus.⁸⁹ Due to the poor bioavailability, zanamivir is administrated by inhalation.

Modification of the structure of zanamivir, to improve oral bioavailability, led to the parent of oseltamivir.⁹⁰ Oseltamivir is orally bioavailable and effective and safe for the treatment of influenza infections.⁹¹

1.2.9 Antiviral agents currently under clinical investigations

The need for new antiviral agents is always in continuous process, especially to treat HIV and HCV infections. Moreover, the emergence of drug resistance or new virus strains, such as swine influenza virus, or the need of new therapy with fewer adverse effects, pushes the pharmaceutical companies as well as the research groups to continue the fight against viruses.

In 2008, it has been reported by De Clercq and Field the current state of art of the current drugs in the market and those ones currently under clinical investigation.^{36, 46,}

⁸⁸ The major effort has been made for the treatment of HIV infection, for which there are 25 compounds currently approved (22 in the UK), other 3 are expected to be approved in the near future and another 15 (of which 7 NAs) are under clinical investigation. In the case of HCV infection, there are more than 10 compounds in clinical trials of which 5 are NAs.

It is clear from this data that NAs occupy a huge part of the antiviral treatment; to summarize, 18 out of 36 approved antiviral agents in the UK are NAs.

1.3 Phosphate Prodrugs

One of the major issues concerning the use of NAs is the innate or acquired resistance to the drug. In most of the cases, resistance can be due to:

- poor cell entry;
- transporter deficiency;
- poor bioactivation to the active form, usually in the first step of phosphorylation (TK or other cellular kinases);^{92, 93}
- poor affinity versus the target, ex. polymerase, or RT, etc.^{93, 94}

Mutations of these enzymes change the conformation of the active site and NAs are less recognized by the enzyme.

One of the major goals of medicinal chemists and biologists is to overcome this issue and in particular the new technologies to overcome the first step of phosphorylation will be described.

1.3.1 Phosphate prodrug technologies

The three steps of phosphorylation to the active form of NAs are mediated by kinases. NAs can be substrates for viral kinases, cellular kinases or both. The specificity for one or the other as well as the selectivity for the polymerase gives the selectivity of the drug. Moreover, some NAs, such as ACV, are selectively phosphorylated by HHV-encoded TK and this factor gives selectivity of ACV to these infections.²⁸

In addition, it has been described how the first step of activation is the key-limiting step in the process of bioactivation of NAs and the by-pass of this step could improve/extend the activity of NAs.⁹³

To overcome this issue several methodologies have been developed.

One of the easiest ways is the use of monophosphate NAs. However, monophosphates themselves can not be used to by-pass this step, because of their instability in biological media and poor passive diffusion through cellular membranes due to their extremely high hydrophilicity.

A second approach has been the use of phosphonates. These are particular analogues where the phosphorus-carbon bond is more resistant to degradation by cellular enzyme than the phosphorus-oxygen bond present in the phosphates.⁴² The issue related to the poor lipophilicity for these compounds has been solved by synthesising phosphanate prodrugs.

A third approach is the delivery of a monophosphate prodrug. Currently, there are different methodologies and they are used in both antiviral and anticancer fields (for reviews see Jones,⁹³ Meier,⁹⁵ Wagner,⁹⁶ and Hecker and Erion⁹⁷) and the following will be discussed:

- bis(pivaloyloxymethyl) (POM)- and bis(isopropylloxycarbonyloxymethyl) (POC) prodrug;
- bis(S-dithioethyl) (SDTE)- and bis(S-acyl-2-thioethyl) (SATE) prodrug;
- *cycloSal* prodrug;
- phosphoramidate diester;
- phospholipid conjugates;
- hepdirect prodrug;
- diamidate prodrug;
- arylphosphoramidate.

1.3.1.1 Bis(POM) and bis(POC) approach

This approach was firstly reported by Farquhar and coworkers and it consists of a POM phosphate masking group (Fig. 1.17). Once inside the cell, the POM-group is enzymatically cleaved by a carboxyesterase to release the monophosphate form of the NA.⁹⁸ This approach has been successfully applied to ddU (**53**),⁹⁸ AZT (**54**),⁹⁹ and 5-fluoro-2'-deoxyuridine (**55**).¹⁰⁰ One of the major successes is the application of this approach to TNF (bis-POC) (**31**) and to adefovir (bis-POM) (**32**), which are currently in the market.⁴⁶

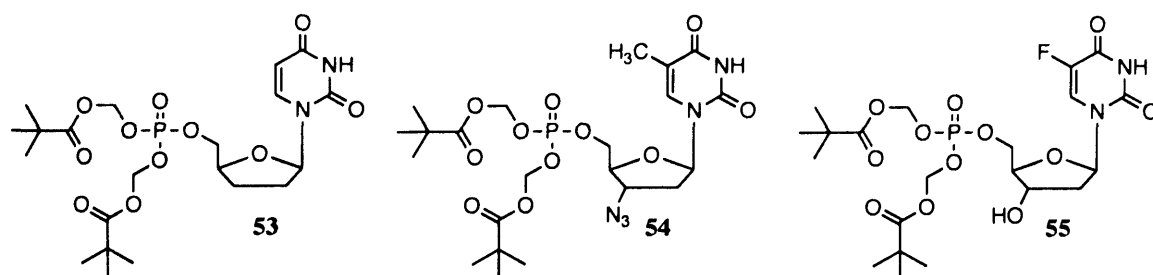


Fig. 1.17: Bis(POM) and Bis(POC) approach.

1.3.1.2 Bis(SDTE) and bis(SATE) approaches

Fig 1.18 reports the bis(SDTE) (**56**) and bis(SATE) (**57**) approaches, which have been described by Gosselin and Imbach. Both approaches need enzymatic bioactivation but while for bis(SDTE), it is a reductase that mediated the cleavage in the case of

bis(SATE), it is a carboxyesterase, showing similar bioactivation mechanism compared to bis(POM).¹⁰¹



Fig. 1.18: bis(SDTE) and bis(SATE) approach.

These approaches have been successfully applied to AZT, retaining good activity in the TK-deficient strain, to d4T, and to ACV, extending its activity to HBV.¹⁰¹

1.3.1.3 CycloSal approach

This approach has been developed by Meier and coworkers and it is completely different from the others as it requires chemical activation. The lability of the phenyl and the benzyl phosphate moieties controls the hydrolysis of the prodrug.⁹⁵ This method has been applied to several NAs such as d4T (**58**, **Fig. 1.19**),¹⁰² AZT,¹⁰³ etc. Following these results, a second and a third generations of cycloSal have been designed, with the introduction of esterase-cleavable functions (**Fig. 1.19**, here reported applied to d4T, **59** for the second generation, **60** and **61** for the third generation).¹⁰⁴⁻¹⁰⁶ Recently Meier and coworkers reported the application of this technology to mask NA diphosphates.¹⁰⁷

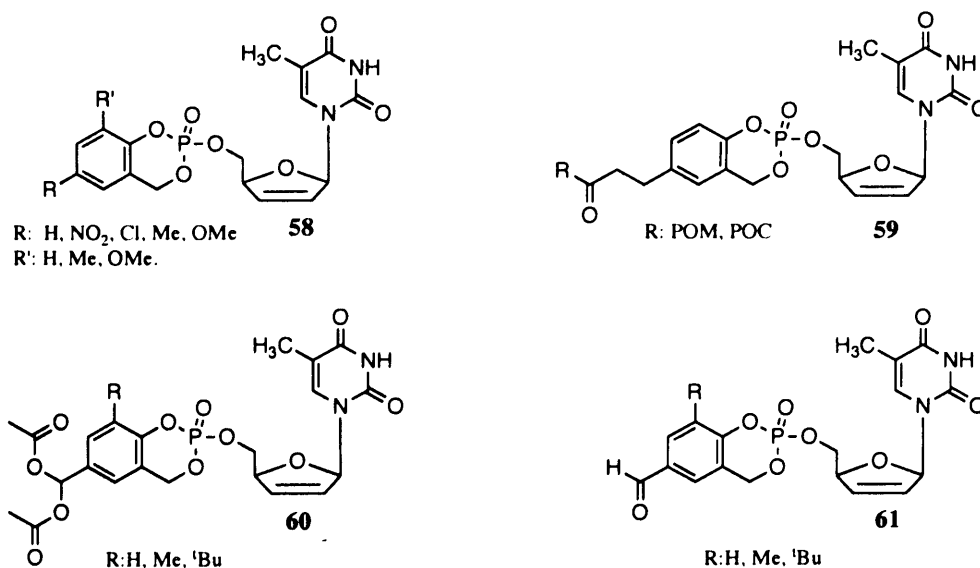


Fig. 1.19: cycloSal approach.

1.3.1.4 Phosphoramidate diester approach

This approach has been developed by Wagner *et al.*, and it is a variant of the technology developed by McGuigan *et al.* (arylphosphoramidate ProTide approach). In this strategy, only one negative charge in the phosphate moiety is masked by an amino acid, whilst the other is free (**Fig. 1.20**). The unmasked charge gives more water solubility and stability in human plasma.

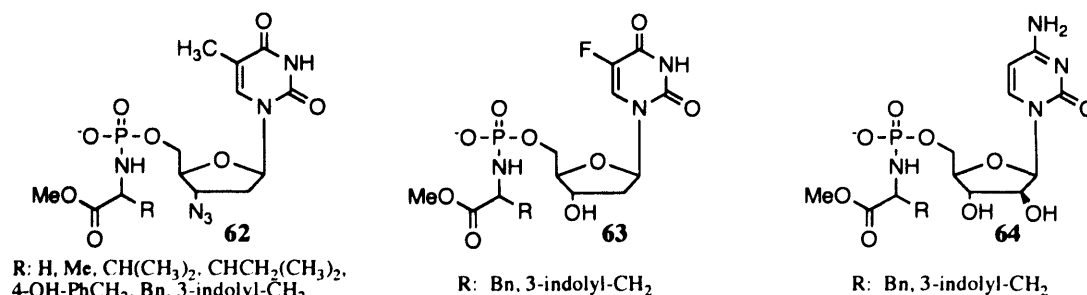


Fig. 1.20: phosphoramidate diester approach.

Notably, in this approach the chirality at the phosphorus is absent, and it differs from several other approaches in this regard. The bioactivation of this prodrug involved an hydrolysis of the P-N mediated by a phosphoramidase type enzyme. This approach has been applied for example to AZT (**62**),¹⁰⁸ 5-fluoro-2'-deoxyuridine (**63**) and 1-β-arabinofuranosylcytosine (**64**).¹⁰⁹

1.3.1.5 Phospholipid conjugates approach

This approach has been developed by Hostetler *et al.* and it has been applied specifically to acyclic nucleoside phosphonates (**Fig. 1.21**), such as cidofovir (**65** and **66**) and (S)-9-(2-phosphono-methoxypropyl)adenine (HPMPA) (**67** and **68**).¹¹⁰

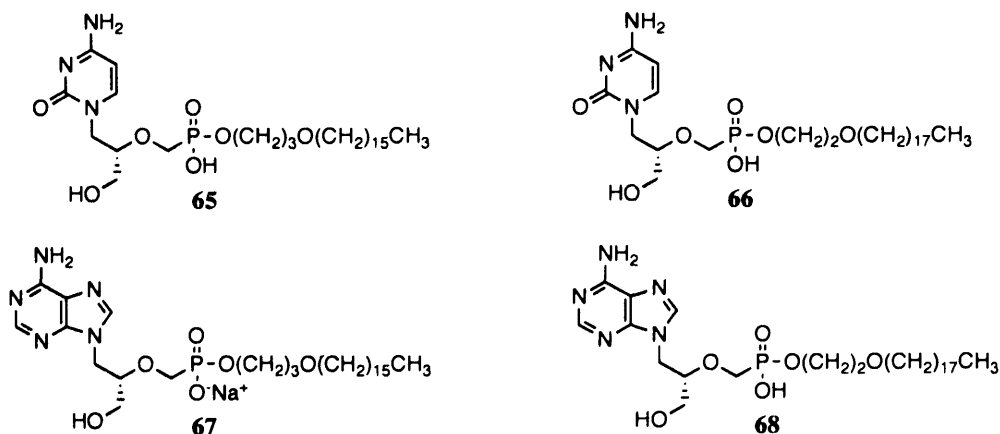


Fig. 1.21: phospholipid conjugates approach.

This strategy involves the masking of one negative charge with a phospholipid moiety. In particular, the hexadecyloxypropyl and octadecyloxyethyl esters gave the best results. The mechanism of bioactivation involves cleavage of the phospholipid part mediated by phospholipase C. The application of this approach to cidofovir gave a remarkable increase in the potency of this compound against several viruses, enhancing for example the activity against HCMV by 400 fold and against VZV by 1200 fold. In the case of HPMPA this approach enhanced the antiviral activity against HCMV by 270 fold and against HIV-1 by 11000 fold.¹¹⁰

1.3.1.6 HepDirect approach

Cyclic 1-aryl-1,3-propanyl ester (HepDirect) is a strategy developed by Metabasis. These prodrugs are specifically bioactivated by the cytochrome P₄₅₀ with the consequent delivery of the monophosphate form within the liver, decreasing the toxicity to other organs.⁹⁷ At present three HepDirect prodrugs are currently under clinical evaluation (**Fig. 1.22**): pradefovir (**69**), which is a prodrug of adefovir, for the treatment of HBV, MB07133 (**70**), prodrug of cytarabine monophosphate, for the treatment of primary liver cancer and the non-nucleoside MB07811 (**71**) for the treatment of hyperlipidemia.⁹⁷

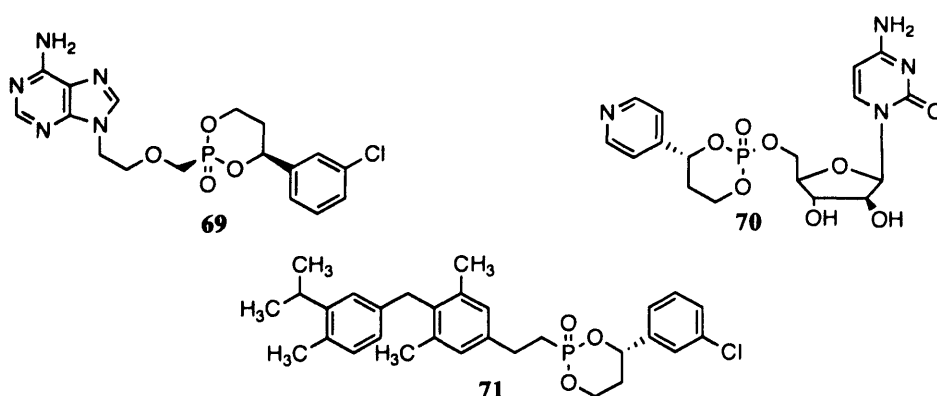


Fig. 1.22: hepdirect approach.

1.3.1.7 Diamidate prodrug

Firstly reported by McGuigan et al. applying this technology to AZT, it has not been greatly developed.⁹⁷ As already seen for the phosphoramidate diester approach, the phosphorus is no longer chiral.

Recently, this approach has been applied to a fructose-1,6-bisphosphatase inhibitor (**72**, **Fig. 1.23**) and advanced in clinical trial for the treatment of type 2 diabetes.

Moreover, Gilead has applied this strategy to 9-[2-(phosphonomethoxy)ethoxy]-adenine (PMEA) (**73**) and to phosphonomethoxyethylguanine (PMEG) (**74**). Further studies indicate the delivery of the parent into the cells.⁹⁷

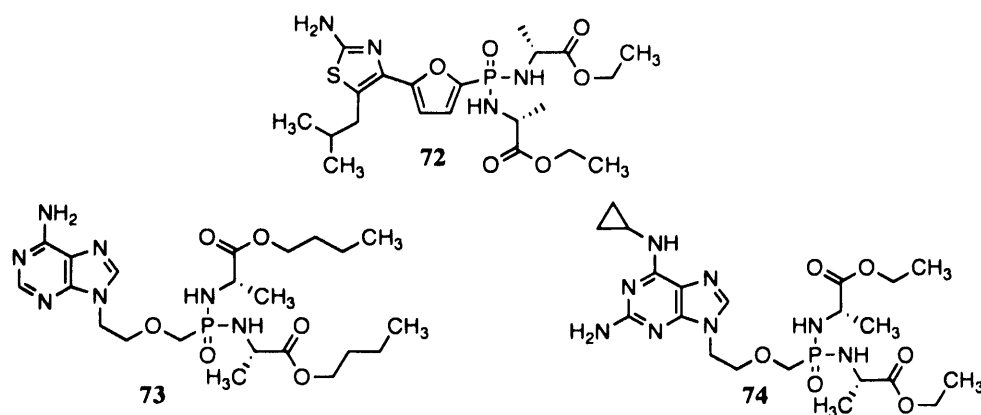


Fig. 1.23: phosphonic diamide approach.

1.3.1.8 Arylphosphoramidate ProTide approach

This approach has been developed by McGuigan and co-workers (for review see Cahard¹¹¹ and Mehellou¹¹²). Initially designed with the alkyl and haloalkyl phosphate triesters (**75**, **Fig. 1.24**) followed by the substitution of one of the chains with an amino acid giving the alkyloxyphosphoramidate (**76**) and haloalkyloxyphosphoramidate (**77**). Then the bis-aryloxy compounds (**78**) have been introduced and finally the arylphosphoramidate currently used (**79**).^{111,112}

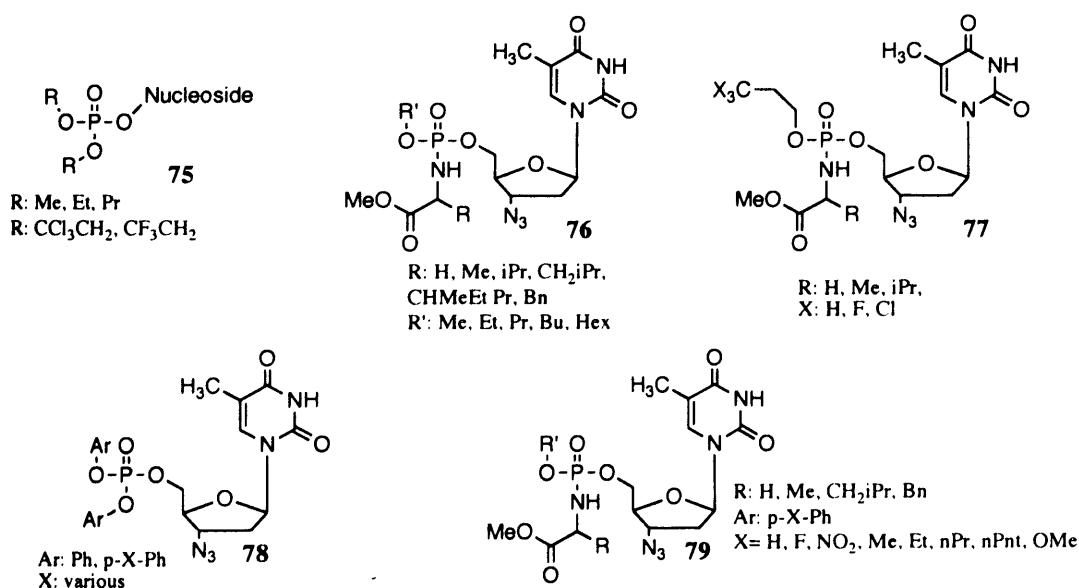


Fig. 1.24: the history of arylphosphoramidate ProTide approach.

This technology has been applied to several NAs showing in most of the cases improvement/extension of the activity and further activity against TK deficient strains, successfully by-passing the first step of phosphorylation. The literature reports many examples: d4T,^{113, 114} ddA,¹¹⁵ abacavir,¹¹⁶ 4'-azidouridine,¹¹⁷ *N*-acetylglucosamine,¹¹⁸ etc.

1.3.1.8.1 Mechanism of activation of arylphosphoramidate

Fig. 1.25 reports the putative bioactivation pathway for the arylphosphoramidate.

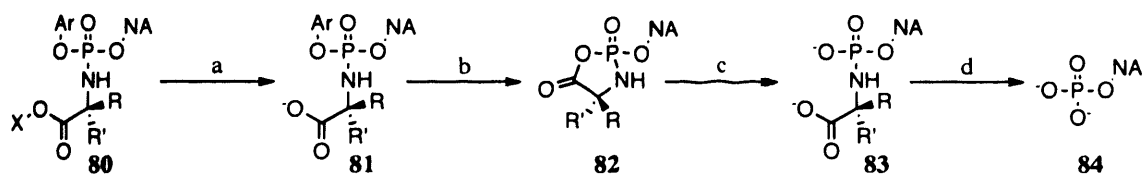


Fig. 1.25: mechanism of activation of arylphosphoramidate.

The first step (a) is the hydrolysis of the carboxylic ester moiety (81) hypothesised to be mediated by a carboxyesterase-type enzyme.^{119, 120} In particular, evidence indicated that this enzyme is cathepsin A, and its mechanism of action will be discussed later in this thesis.¹²¹

The second step (b) is a spontaneous cyclisation through an internal nucleophilic attack of the carboxylate on the phosphorus centre with displacement of the aryl moiety (82).

The third step (c) is the opening of the unstable putative cyclic mixed anhydride mediated by water (83).

The fourth step (d) is the cleavage of the P-N bond perhaps mediated by a phosphoramidase-type enzyme with the release of the monophosphate (84).¹¹⁹ More specifically, this final step is supposed to be mediated by human hint enzyme 1, which belongs to the HIT superfamily.¹²² Its mechanism will be discussed later in this thesis.

1.3.1.8.2 SAR of the arylphosphoramidate moiety

The arylphosphoramidate moiety consists of three variable components: the aryl, the amino acid and the ester. All these parts contribute to the activity of these prodrugs. In particular, the combination of these moieties plays a crucial role in terms of lipophilicity and pharmacokinetics.

1.3.1.8.2.1 Ester moiety

The first part to be considered is the ester, as it is involved in the first step of the bioactivation. Several esters have been considered: linear such as methyl, ethyl, n-propyl, pentyl, etc; branched such as 2-propyl, *tert*-butyl, etc; and the benzyl group. In particular, in most of the cases, phosphoramidate carrying the benzyl group proved to be highly potent, whilst in the case of the *tert*-butyl, the prodrugs are inactive, and this is probably due to a lack of bioactivation as the *tert*-butyl moiety is too bulky to be processed by the enzyme.^{111, 112}

1.3.1.8.2.2 Aryl moiety

The aryl component is the second part to be processed, more specifically released. Depending on the aryl group, it can be released faster or slower, being an equilibrium step. Several aryl moieties have been considered, especially the phenyl and 1-naphthol, and several substitutions to the phenyl ring have been considered in order to improve the activity.^{111, 112}

1.3.1.8.2.3 Amino acid moiety

The amino acid is probably the most important part of the arylphosphoramidate moiety. In this case as well, several amino acids have been considered and L-alanine gives, usually, the best results. The unnatural amino acid α,α -dimethylglycine is a good substituent of L-alanine in terms of activity. The other amino acids used often gave reduced activity compared to L-alanine. Another important aspect concerning the amino acid is its stereochemistry; in fact, the L-isomer is often more active than the D-isomer, and this is probably due to the interaction with the enzymes involved in the bioactivation.^{111, 112}

1.3.1.9 Summary of phosphate prodrug technologies

To summarize, **Table 1.1** reports the different phosphate prodrug approached discussed in this section with the enzyme involved in the bioactivation pathway and they are progressed to human trials.

Prodrug Class	Enzyme(s) involved	Taken to human trials
Bis(POM)/Bis(POC)	Esterase	Yes
Bis(SDTE)/Bis(SATE)	Esterase	No
CycloSal	Chemical	No
Phosphoramidate Diester	Esterase, amidase	No
Phospholipid Conjugate	Phosphodiesterase	Yes
HepDirect	Cytochrome P ₄₅₀	Yes
Phosphonic Diamide	Esterase, amidase	Yes
Arylphosphoramidate (ProTide)	Esterase, amidase	Yes

Table 1.1: summary of the phosphate prodrug approaches

1.4 Objectives

As previously mentioned, new antiviral agents are continuously required, especially for the treatment of HIV and HCV, but also against those viruses for which drug resistance emerges.

In the present work, the synthesis and the biological evaluation of novel antiviral drugs will be reported. In particular, the specific aims are:

- synthesis and biological evaluation of novel derivatives of bicyclic nucleoside analogues (BCNAs);
- attempts to improve the scale up synthesis of FV100, which is the most potent anti-VZV agent reported to date and currently in phase II clinical trials;
- the application of the phosphoramidate approach to ribavirin;
- the application of the ProTide approach to acyclovir;
- the identification of acyclovir derivatives through a virtual screening using RT, adenylate or guanylate kinase, and polymerase γ ;
- synthesis and biological evaluation of acyclovir derivatives and application of the phosphoramidate approach.

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2. Ten years of BCNAs: from the bench to clinical trials

2.1 BCNAs: a new class of potent and selective anti-VZV agents

In 1999 during the synthesis of novel 5-alkenyl-2'-deoxyuridine, a new class of potent and selective anti-VZV compound was discovered.¹ This new class of compounds bears an unusual fluorescent bicyclic furanopyrimidine base (bicyclic nucleoside analogues, BCNAs). It must be reported that BCNAs were previously obtained in several laboratories as a side product in the coupling of terminal alkynes to 5-iodo-2'-deoxyuridine, but were never identified as biologically active.^{2,3}

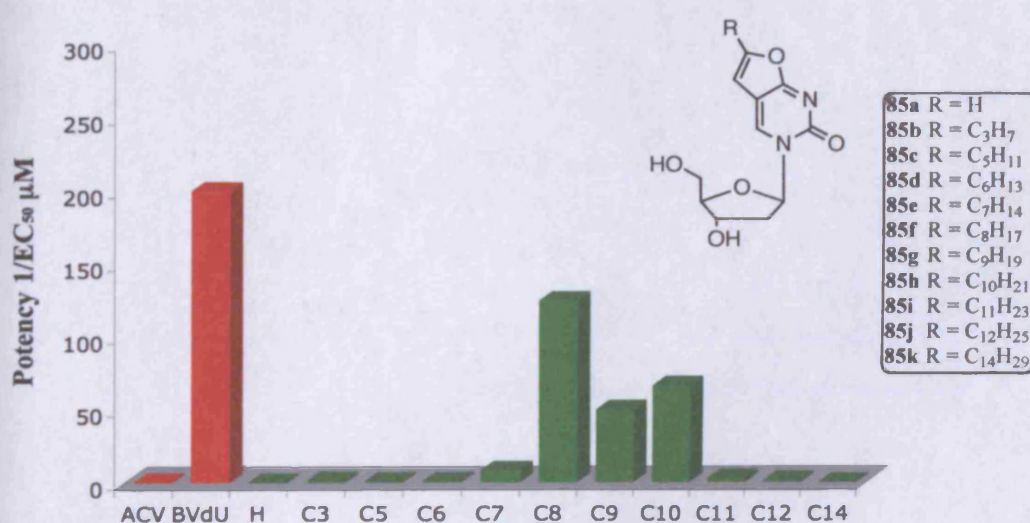


Fig. 2.1: SAR of the first series of BCNAs (data for VZV-OKA)

Fig 2.1 reports the first series of compounds made (**85a-k**). A clear structure activity relationship (SAR) of these compounds emerges (**Table 2.1**): with a short chain (< 6) little antiviral activity was found, similar to acyclovir (ACV); in the case of C7 (**85e**), C9 and C10 (**85g-h**) the activity was moderate. The optimal length of the chain was with C8 (**85f**) wherein the activity was roughly similar to BVdU and ca. 300 fold more than ACV.¹

		Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
		TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
		YS strain	OKA strain	07/1 strain	YS strain		
85a	H	33	28	>200	>200	>200	>200
85b	C3	0.8	0.8	>200	>200	>200	>200
85c	C5	1.8	1.8	>50	>50	>50	>50
85d	C6	1.3	1.3	>50	>50	200	>200
85e	C7	0.12	0.12	>50	>50	>50	>50
85f	C8	0.008	0.008	>50	>50	>50	>50
85g	C9	0.02	0.02	>200	>200	>200	>200
85h	C10	0.008	0.015	>50	>50	>50	>50
85i	C11	0.3	0.37	>50	>50	200	>200
85j	C12	1.2	0.8	>50	>50	50	>200
85k	C14	>50	>50	>50	>200	>200	>200
BVdU	-	0.005	0.005	>200	>200	>200	>200
ACV	-	1	2.9	74	125	>200	>200

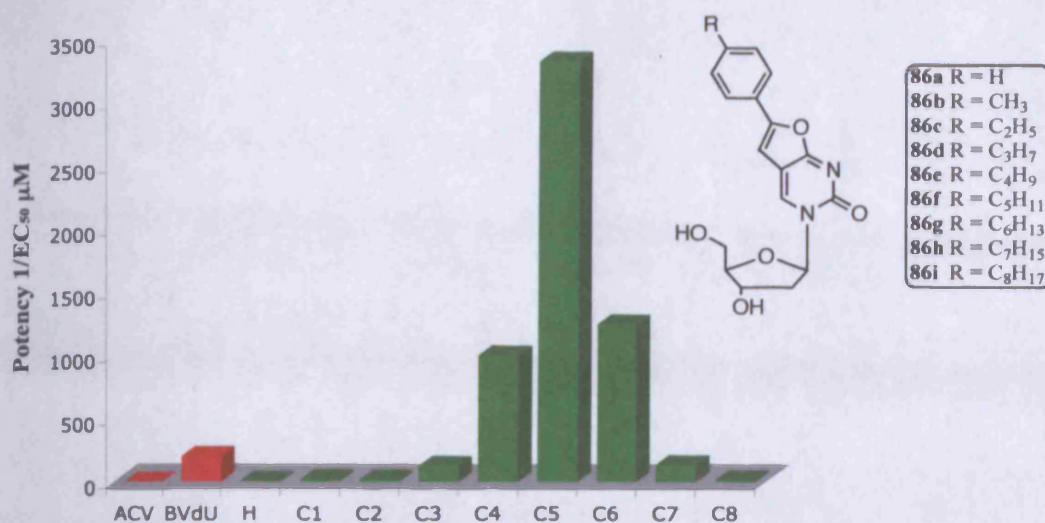
a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.1: antiviral activity against VZV for the compounds **85a-k** including BVdU and ACV as control.

The optimization of the lead compound resulted in a series of 6-*p*-alkylphenyl derivatives (**86a-i**, Fig. 2.2). From Table 2.2 it is possible to extrapolate a SAR of these compounds: as previously noted for the parent alkyl derivatives, there is a dependence between the length of the chain and the activity. In fact, in the case of chain length C4-C6 (**86e-g**) a subnanomolar activity against VZV has been found. The 6-pentylphenyl **86f**, called Cf1743, (EC₅₀ = 0.1 nM) is ca 10000 times more active than ACV and ca. 20 times more active than BVdU and it is the most active anti-VZV compound reported to date.⁴ All the compounds have been found to be inactive against the TK deficient strains; this result suggests the need of phosphorylation for these compounds to show antiviral activity.

Fig. 2.2: SAR of compounds **86a-i** (data for VZV-OKA).

		Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
		TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
		YS strain	OKA strain	07/1 strain	YS strain		
86a	H	0.28	0.16	>200	>162	>20	>200
86b	C1	0.06	0.06	103	>200	>200	>200
86c	C2	0.09	0.07	>50	>50	>20	123
86d	C3	0.01	0.008	>50	>20	>50	188
86e	C4	0.0008	0.001	>20	>20	>200	>200
86f	C5	0.0001	0.0003	>5	>5	>50	>200
86g	C6	0.0002	0.0008	>5	>5	>20	18
86h	C7	0.0057	0.0074	>5	>5	5	18
86i	C8	0.05	0.065	>20	>20	>20	>200
BVdU	-	0.005	0.005	>200	>200	>200	>200
ACV	-	1	2.9	74	125	>200	>200

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.2: antiviral activity against VZV for the compounds **86a-i** and BVdU and ACV as control.

The high selectivity and the lack of toxicity of Cfl743 promoted its development as a potential new anti-VZV drug (to be discussed later in this chapter).

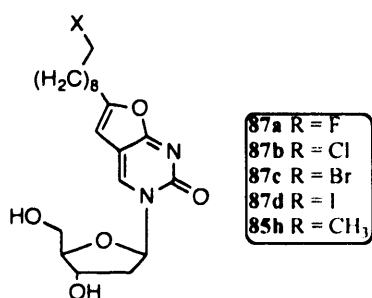
2.2 Modifications to the structure of BCNAs

After the discovery of the BCNAs, a significant SAR study, both to the alkyl derivatives and the phenyl-alkyl derivatives, has been made in order to improve the activity but also to investigate the mechanism of action.

2.2.1 Modifications of the side chain

Being fundamental for the activity of BCNAs, several modifications have been made in this regard.

The introduction of halogens (**87a-d**) at the end of the chain in the case of the nonyl derivatives gave compounds that did not significantly differ from the parent **85h** in potency (Table 2.3).⁵



		Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
		TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
		YS strain	OKA strain	07/1 strain	YS strain		
87a	F	0.014	0.022	>20	>20	>50	200
87b	Cl	0.012	0.007	15	13	>200	200
87c	Br	0.031	0.026	>50	50	>200	>50
87d	I	0.034	0.061	>50	>50	50	>200
85h	CH ₃	0.008	0.015	>50	>50	>50	>50

a) effective concentration required to reduce virus plaque formation by 50%.

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.3: antiviral activity against VZV of halogen terminal substituted BCNA.

Terminal insaturation, alkenyl (**88a-d**) or alkynyl (**89a-d**) in the chain led to a decrease of activity, with a loss of 4-40 fold for the alkenyl and 250-750 fold for the alkynyl compared to the parent (Table 2.4).⁶

88a n = 5
88b n = 6
88c n = 8
88d n = 10

89a n = 5
89b n = 6
89c n = 7
89d n = 8

		Antiviral Activity				Cytotoxicity	
		EC ₅₀ (μM) ^a				(μM)	
		TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
		YS strain	OKA strain	07/1 strain	YS strain		
88a	C5	>200	>200	>200	>200	>200	>200
88b	C6	13	14	>200	>200	>200	>200
88c	C8	0.06	0.27	>200	>50	≥200	>200
88d	C10	0.1	0.09	>200	>200	≥50	>200
89a	C5	10	8	>200	>200	>200	>200
89b	C6	33	25	>200	>200	>200	>200
89c	C7	37	79	>200	>200	>200	>200
89d	C8	4	5	>200	>200	>200	>200
85f	C8	0.024	0.008	>50	>50	>50	>50

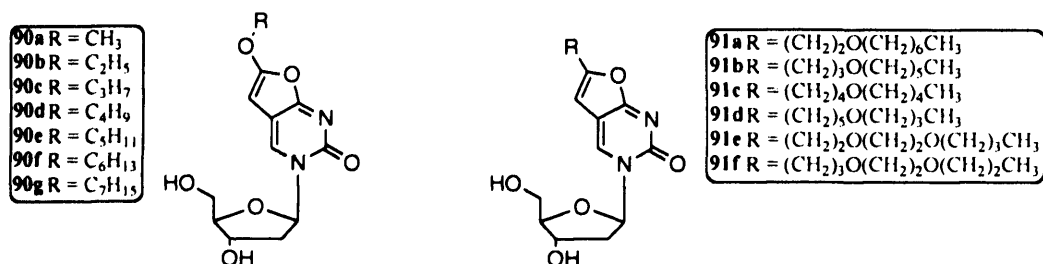
a) effective concentration required to reduce virus plaque formation by 50%.

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.4: antiviral activity against VZV of alkenyl and alkynyl terminal BCNA.

The introduction of an ether or a glycol chain either with or without the phenyl ring was investigated in order to increase the solubility of these compounds. In spite of the increase of water solubility, the ether derivatives (**90a-g**, **91a-d**) have been found to be slightly less active than the parent, whilst glycols (**91e, f**) have been found to be considerably less active (Table 2.5).⁷⁻⁹



	Antiviral Activity				Cytotoxicity	
	EC ₅₀ (μM) ^a				(μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
90a	0.045	0.05	>50	>50	200	>200
90b	0.012	0.009	45	>50	>50	>200
90c	0.002	0.002	11	>50	>200	>200
90d	0.0021	0.0015	-	-	>200	>200
90e	0.001	0.001	3.2	>20	>20	>200
90f	0.0014	0.0015	>5	>20	>20	>200
90g	0.006	0.006	>50	>20	>20	>200
91a	8.1	5.9	>50	>50	200	>200
91b	22	17	>50	>50	200	>200
91c	0.75	0.5	>50	>50	>200	>200
91d	9.3	7.9	>50	>50	>200	>200
91e	>200	>200	>200	>200	>200	>200
91f	97	120	>200	>200	>200	>200
85f	0.024	0.008	>50	>50	>50	>50

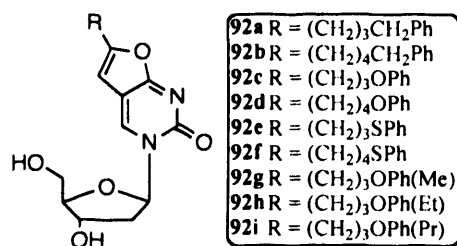
a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.5: antiviral activity against VZV of ether and glycol BCNA derivatives.

The movement of the phenyl ring at the end of the chain (92a,b), with different lengths, as well as the introduction of oxygen (92c,d,g-i) or sulphur (92e,f) led to a reduction of activity (Table 2.6).¹⁰



	Antiviral Activity				Cytotoxicity	
	EC ₅₀ (μM) ^a				(μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
92a	26	28	>200	>200	>200	>200
92b	9.2	9.4	>20	>20	50	84
92c	92	77	>200	>200	>200	>200
92d	13	25	>200	>200	≥200	>200
92e	0.67	0.90	>50	>50	200	>200
92f	-	-	-	-	-	-
92g	11	5	>50	>50	200	>200
92h	10.8	8.4	>50	>50	200	165
92i	2.8	3.2	>20	>20	≥50	>200
85f	0.024	0.008	>50	>50	>50	>50
86f	0.0001	0.0003	>5	>5	>50	>200

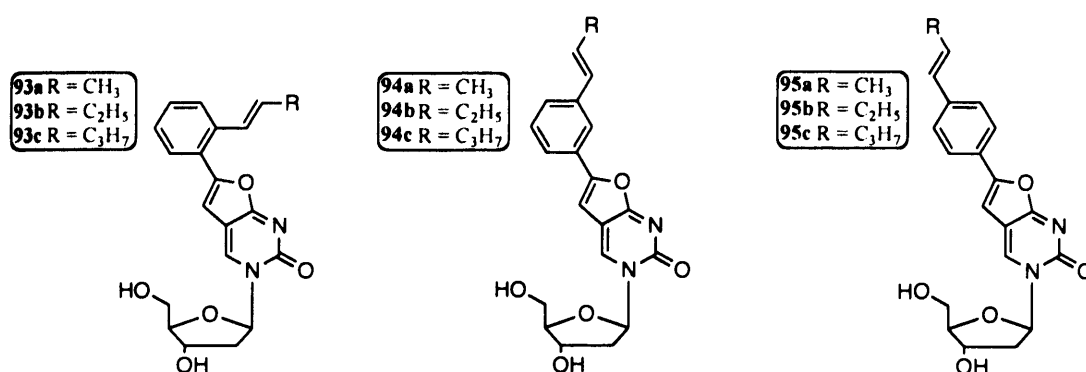
a) effective concentration required to reduce virus plaque formation by 50%.

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology.

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.6: antiviral activity against VZV of alkyl-aryl, -phenoxy and -thiophenyl BCNA derivatives.

Alkenyl chains, with a double bond at the α-position and *E*-configuration, led to a retention of activity in the case of *para* substitution (95a-c) with respect to the phenyl ring, and poor activity in the case of *ortho* (93a-c) and *meta* (94a-c) substitution, with the *meta* slightly more active than the *ortho* (Table 2.7).^{11, 12}



		Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
		TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
		YS strain	OKA strain	07/1 strain	YS strain		
93a	<i>ortho</i>	-	12	>50	>50	400	103
93b	<i>ortho</i>	-	33	>50	>50	400	102
93c	<i>ortho</i>	-	8.4	>50	>50	80	41
94a	<i>meta</i>	58	13	>50	>50	>200	>200
94b	<i>meta</i>	2.3	1.9	>50	>50	≥20	68
94c	<i>meta</i>	1.3	1.1	>50	>50	≥200	>200
95a	<i>para</i>	0.004	0.004	>50	>50	≥50	>200
95b	<i>para</i>	0.0006	0.0007	>50	>50	≥5	>200
95c	<i>para</i>	0.0003	0.0008	>50	>50	>5	>200
86f	<i>para</i>	0.0006	0.0005	>50	>50	≥50	>200

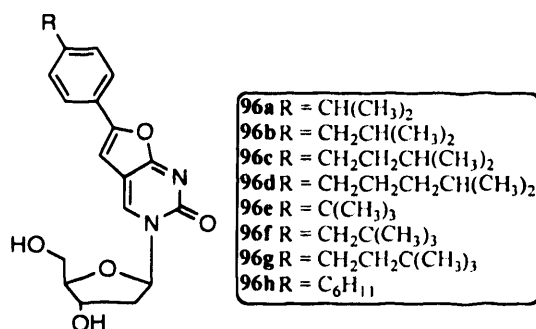
a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.7: antiviral activity against VZV of alkenyl BCNA derivatives.

The substitution of the linear chain with branched chains (**96a-g**) as well as the use of cyclohexyl (**96h**) showed lower activity (**Table 2.8**). This lack of activity can be due to steric hindrance of the bulky chain or to a different lipophilicity of these compounds compared to that of the linear parent.¹³



	Antiviral Activity				Cytotoxicity	
	EC ₅₀ (μM) ^a				(μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
96a	7.9	6.9	>20	>50	≥50	45
96b	0.051	0.048	>200	>2	>200	>200
96c	0.0011	0.0017	>50	>2	≥50	>200
96d	-	>80	>80	-	400	>200
96e	>20	>50	>50	>50	≥50	>200
96f	-	>80	>80	-	400	140
96g	0.31	0.33	≥80	-	≥80	>200
96h	3.1	1.4	>20	>20	>20	>200
86f	0.0001	0.0003	>5	>5	>50	>200

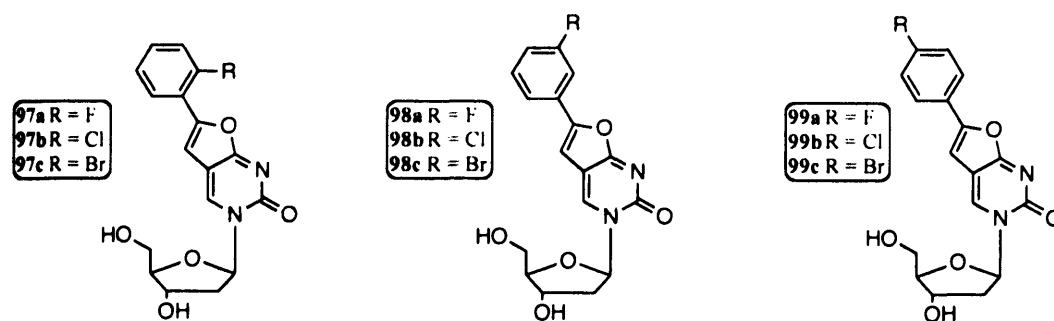
a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.8: antiviral activity against VZV of branched BCNA derivatives.

The substitution in the phenyl ring with fluorine (**97a**, **98a**, **99a**), chlorine (**97b**, **98b**, **99b**) and bromine (**97c**, **98c**, **99c**) gave different results compared to the unsubstituted ring (**Table 2.9**): the *para* substitution is tolerated for chlorine and bromine, while fluorine has been found to be inactive; the *meta* is tolerated for all three halogens used but they are less active than the parent compound; the *ortho* substitution led to an increase of activity for all the substitutions with a slightly better activity for the chlorine and bromine.¹⁴



		Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
		TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
		YS strain	OKA strain	07/1 strain	YS strain		
97a	<i>ortho</i>	0.031	0.04	>20	>20	>20	>200
97b	<i>ortho</i>	0.011	0.016	>20	>20	137	>200
97c	<i>ortho</i>	0.022	0.013	>5	>5	>5	>200
98a	<i>meta</i>	1.5	1.3	200	>200	>200	200
98b	<i>meta</i>	2.9	3.0	>20	>20	>20	>200
98c	<i>meta</i>	2.0	1.5	>5	>5	>5	36
99a	<i>para</i>	>50	>50	>50	>50	200	171
99b	<i>para</i>	0.08	0.09	>20	>20	≥20	>200
99c	<i>para</i>	0.2	0.29	>5	>5	>2	96
86a	-	0.16	0.28	>200	162	>200	>200
86f	<i>para</i>	0.0003	0.0001	>20	>5	≥20	>200

a) effective concentration required to reduce virus plaque formation by 50%.

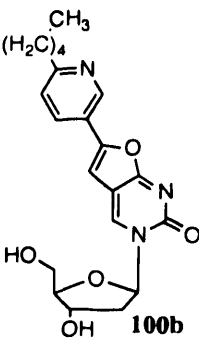
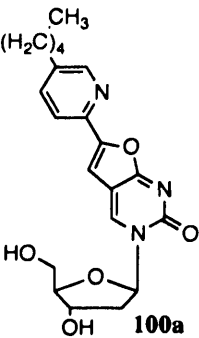
b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.8: antiviral activity against VZV of phenyl halogen BCNA derivatives.

2.2.2 Modification of the phenyl ring

The substitution of the phenyl ring with a pyridyl ring (**100a,b**) gave a loss of activity (**Table 2.9**).¹⁵



Antiviral Activity				Cytotoxicity	
EC ₅₀ (μM) ^a				(μM)	
TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
YS strain	OKA strain	07/1 strain	YS strain		
100a	-	>80	-	400	>200
100b	-	0.1	-	80	>200
86f	0.0001	0.0003	>5	>50	>200

a) effective concentration required to reduce virus plaque formation by 50%;
b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;
c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.9: antiviral activity against VZV of pyridyl BCNA derivatives.

The logical substitutions at the 7-position were the bioisosters of the furano ring: pyrrole and thiophene.

The substitution of the furano ring with pyrrole (**101a-d**) and N-alkylated pyrrole (**102a-d**) led to a loss of activity (**Table 2.10**).¹⁶

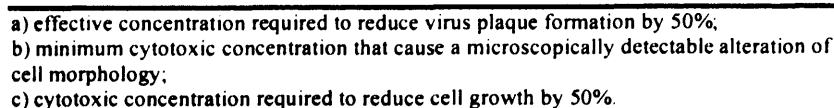
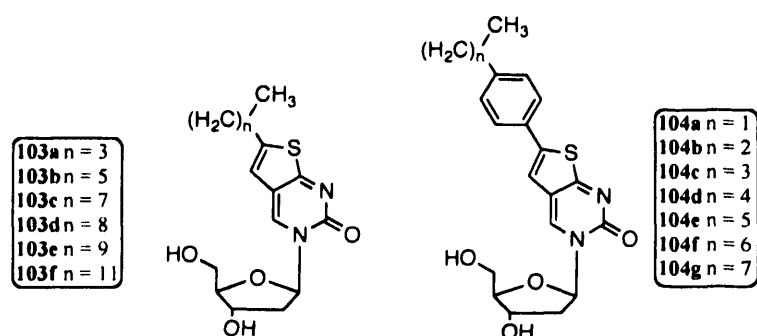


Table 2.10: antiviral activity against VZV of N-7 BCNA derivatives.

The replacement of the furano ring with a thiophene ring gave retention of activity in the case of the alkyl side chain (**103a-f**),¹⁷ while in the case of the alkylphenyl side chain (**104a-g**) it gave reduction of activity (**Table 2.11**).¹⁸



	Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
103a	0.15	0.16	≥200	157	≥100	>200
103b	0.14	0.14	>50	≥50	125	>200
103c	0.005	0.002	≥5	>5	20	53
103d	0.01	0.01	>20	>20	>20	>20
103e	0.06	0.03	>5	>5	20	54
103f	0.3	0.2	>5	>5	18	49
104a	0.15	0.2	20	-	>200	>200
104b	0.06	0.09	>16	-	80	>200
104c	0.02	0.028	>3.2	-	50	>200
104d	0.014	0.025	20	-	50	>200
104e	0.043	0.08	>50	-	200	>200
104f	0.18	0.27	>20	-	50	>200
104g	3.4	-	>20	-	≥20	>200
85f	0.0003	0.0001	>5	-	≥20	>200

a) effective concentration required to reduce virus plaque formation by 50%.

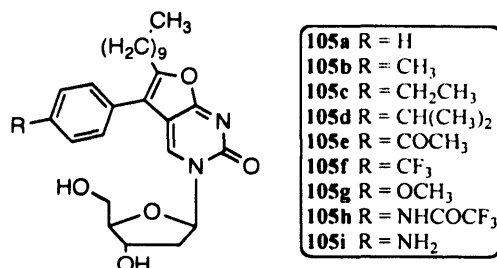
b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology.

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.11: antiviral activity against VZV of S-7 BCNA derivatives.

2.2.4 Modification at 5-position

The only substitution at the 5-position has been done with the BCNA bearing the alkyl chain (C₁₀H₂₁). This substitution included a phenyl ring or *para*-substituted phenyl ring (**105a-i**), which led to a considerably decreased in the anti-VZV activity (**Table 2.12**).¹⁹



	Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
105a	>5	>5	>5	>5	20	37
105b	9	9	>5	>5	≥20	89
105c	>20	>20	>20	>20	50	>200
105d	>5	>5	>5	>5	20	>200
105e	5	8	>50	>50	200	>200
105f	>20	>20	>20	20	50	>200
105g	4.4	6.7	-	>20	≥20	>200
105h	>200	>50	>50	>50	≥200	>200
105i	3.7	4.0	-	8.1	≥50	67
85h	0.008	0.015	>50	>50	>50	>50

a) effective concentration required to reduce virus plaque formation by 50%.

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

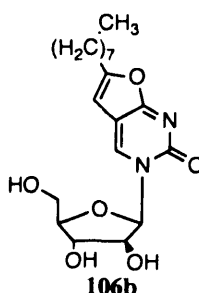
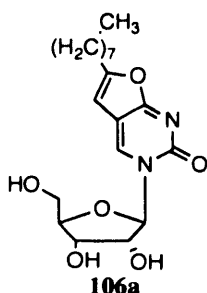
Table 2.12: antiviral activity against VZV of 5-substituted BCNA derivatives.

2.2.5 Sugar modifications

A major effort in the SAR studies of BCNA has been directed towards the sugar moiety. These modifications have been done to improve the activity but also to clarify the mechanism of action of this new class of compounds.

2.2.5.1 Modifications at the 2'-position

The introduction of hydroxyl group at the 2'-position, as ribo sugar (**106a**) or arabino sugar (**106b**), in the octyl derivative, led to a loss of activity (Table 2.13) showing that these modifications in this position are not tolerated.⁷



	Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
106a	21	23	>20	>20	50	>50
106b	3.3	3.6	-	-	-	>20
85f	0.024	0.008	>50	>50	>50	>50

a) effective concentration required to reduce virus plaque formation by 50%.

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

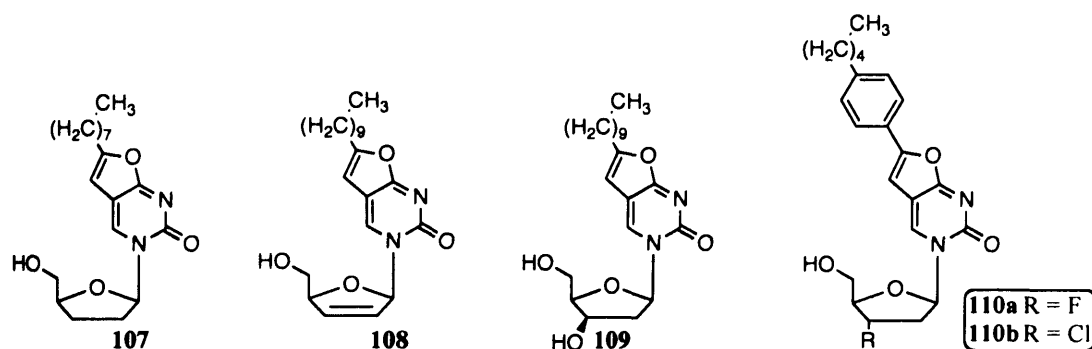
Table 2.13: antiviral activity against VZV of 2'-modified BCNA derivatives.

2.2.5.2 Modifications at the 3'-positions

The 3'-modifications made include (Table 2.14):

- the 3'-deoxy derivative (**107**) which led to a loss of the anti-VZV activity but surprisingly these compounds gained anti-HCMV activity;²⁰
- the d4 derivative (**108**) which was found to be completely inactive;
- the substitution of the alpha hydroxyl at the 3'-position with the beta hydroxyl (**109**) as well as the substitution with fluorine (**110a**) and chlorine (**110b**) led to a loss of activity.²¹⁻²³

These results indicate that the 3'-OH plays a crucial role in the anti-VZV activity.



	Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
107	20	40	>200	>50	>200	>200
108	>50	>50	>50	>50	200	16
109	>20	24	>50	>50	>50	>22
110a	>2	>2	>2	>2	-	5.2
110b	-	>20	>5	-	>200	>200
86f	0.0001	0.0003	>5	>5	≥20	>200

a) effective concentration required to reduce virus plaque formation by 50%;

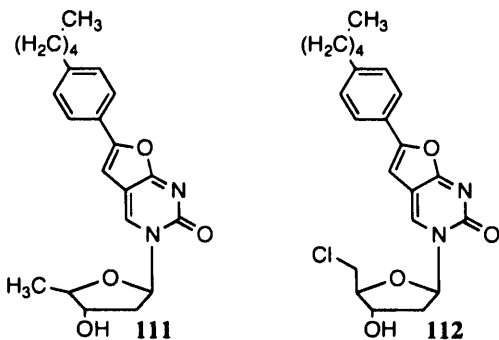
b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.14: antiviral activity against VZV of 3'-modified BCNA derivatives.

2.2.5.3 Modifications at the 5'-positions

A series of modifications has been done to the 5'-position (**Table 2.15**). The 5'-deoxy (**111**) as well as the 5'-Cl (**112**) led to a loss of activity indicating the fundamental role of this position in the antiviral activity of BCNA presumably due to a need for 5'-phosphorylation as confirmed by VZV-TK dependency.^{23, 24}



	Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
111	>5	>5	>5	>5	20	95
112	3.3	>5	>5	-	20	>50
86f	0.0001	0.0003	>5	>5	≥20	>200

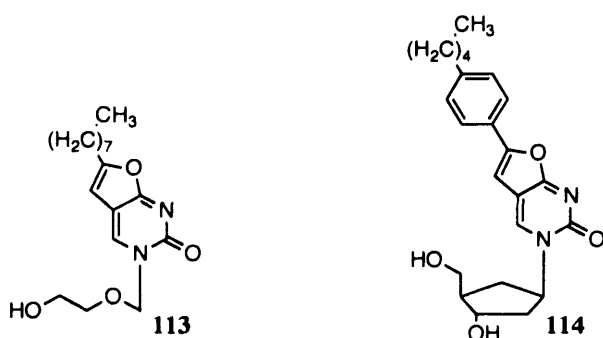
a) effective concentration required to reduce virus plaque formation by 50%;
b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;
c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.14: antiviral activity against VZV of 5'-modified BCNA derivatives.

2.2.5.4 Substitution of the sugar moiety

The substitution of the deoxyribose with an acyclic sugar (**113**) led to a loss of activity compared to the parent compound (**Table 2.15**).²⁵

The substitution of the sugar with a carbocycle (**114**), which is usually well tolerated, led to a loss of activity (**Table 2.15**).²⁶ From these results it is clear that substitution at the sugar moiety is detrimental to the anti-VZV activity.



	Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
113	-	11	>10	-	>80	>200
114	0.28	0.49	>50	-	>20	>50
86f	0.0001	0.0003	>5	>5	>20	>50

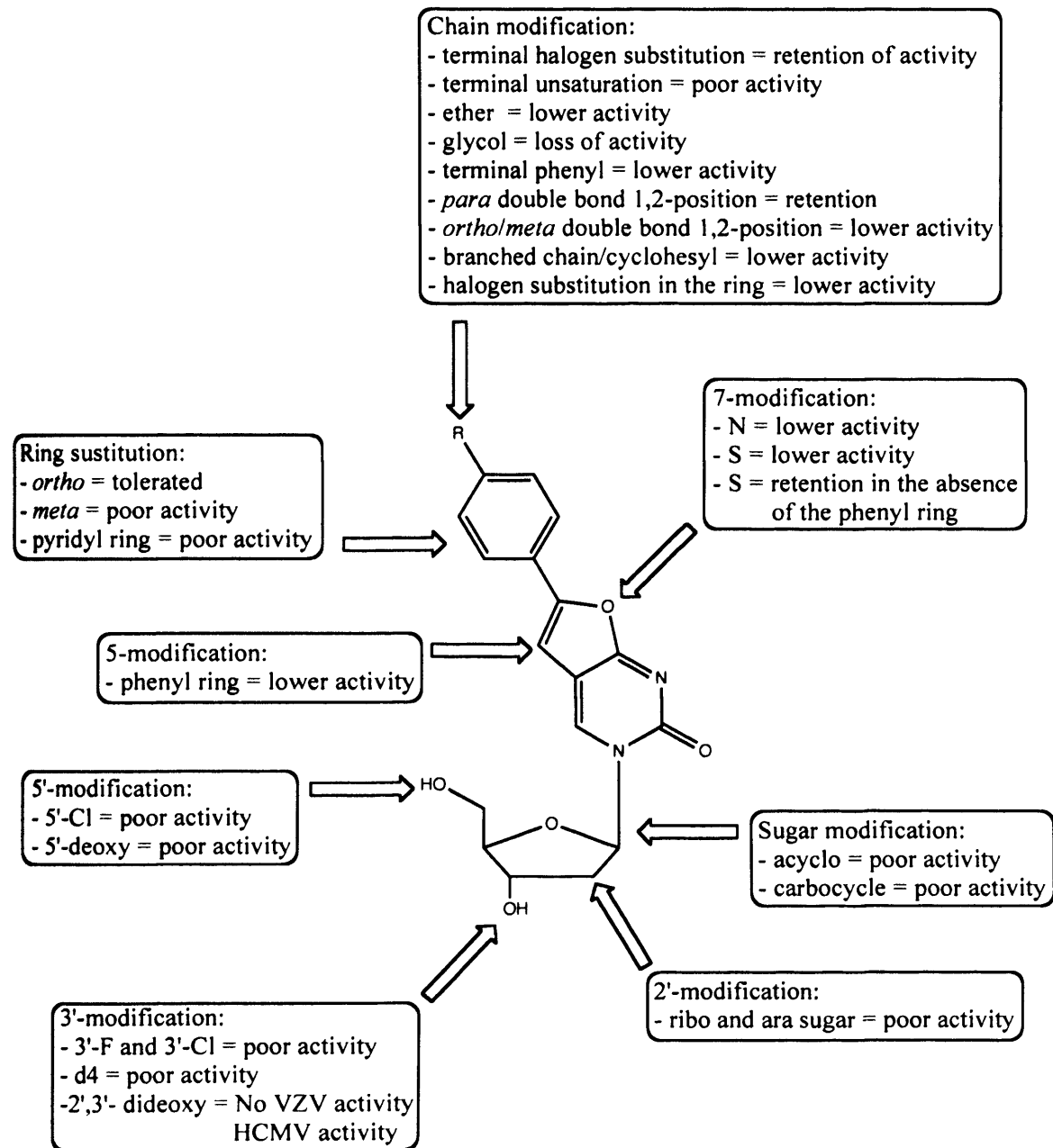
a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.14: antiviral activity against VZV of sugar-modified BCNA derivatives.

2.2.6 Summary of SAR for BCNAs



2.3 Mechanism of action of BCNAs

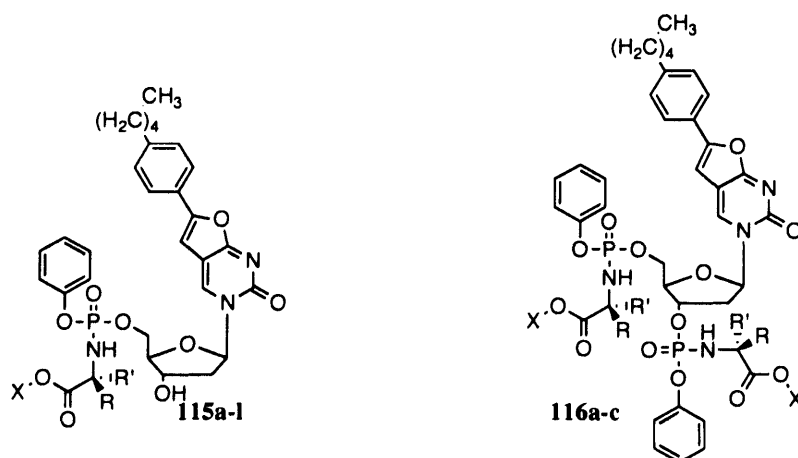
The mechanism of action of this new class of compounds is not completely understood. The total lack of activity in the TK deficient VZV-strain suggests an obligatory phosphorylation mediated by VZV-TK. This step is mediated specifically by the VZV-TK; in fact when incubated with either HSV-TK or cellular TK no phosphorylated BCNA metabolites, mono- or diphosphate, have been detected.²⁸ The lack in activity found for the 5'-deoxy and halogen derivatives indicates the need of the 5'-OH for the antiviral activity. Moreover, the 3'-OH is also fundamental for the activity as modifications in this position gave loss of activity. With regard to the alkyl chain, its length is critical for the activity and its variation led to a consistent variation of the anti-VZV activity. The possible mechanism of action so far hypothesized is probably the inhibition of the VZV-polymerase by the triphosphate form of BCNA.

2.4 ProTides of the BCNA lead compound

In order to investigate the mechanism of action of the BCNA lead compound **86f** a series of ProTide has been synthesized. These compounds have been made with the aim to understand if the second step of phosphorylation is mediated by VZV-TK or by cellular kinases as this technology is able to bypass the first step by delivering the monophosphate into the cell.

Initially a series of 5'-phosphoramidate (**Table 2.16**, **115a-l**) of **86f** have been made. However, during the synthesis 3',5'-diphosphoramidate (**116a-c**) were isolated and biologically evaluated.²⁷

Table 2.16 shows the biological activity for compounds **115a-l** and **116a-c**. Although, these compounds have been found to be less active than the parent **86f**, they still retain a good potency, which is higher than ACV. A possible explanation of these results could be that the phosphorylation mediated by VZV-TK is more efficient than the activation of the phosphoramidate. Moreover, the lack on activity in TK-deficient strain indicates that the second step of phosphorylation is probably still mediated by VZV-TK.



Cpds	R	R'	X	Antiviral Activity EC ₅₀ (μM) ^a			Cytotoxicity (μM)	
				TK ⁺ VZV		TK ⁻ VZV	MCC ^b	CC ₅₀ ^c
				OKA strain	YS strain	07 strain		
115a	Me	H	Me	0.031	0.024	> 16	80	41.5
115b	Me	H	Et	0.010	0.009	> 16	80	21
115c	Me	H	ⁿ Pr	0.0066	0.0046	> 3.2	16	9
115d	Me	H	ⁱ Pr	0.029	0.014	> 3.2	16	9.3
115e	Me	H	^t Bu	0.473	0.485	> 3.2	16	8.6
115f	Me	H	Bn	0.0067	0.0072	> 3.2	80	34
115g	Me	Me	Me	0.100	0.230	15	> 20	> 50
115h	Me	Me	Et	0.036	0.045	> 4	> 4	27
115i	Me	Me	Bn	0.010	0.024	> 4	> 4	26
115j	Bn	H	Me	0.015	0.032	> 4	20	24
115k	Bn	H	Et	0.016	0.036	> 4	20	12
115l	Bn	H	Bn	0.010	0.020	> 4	20	20
116a	Me	H	Me	0.43	-	> 100	> 100	> 100
116b	Me	H	ⁿ Pr	0.040	0.030	> 40	> 40	> 20
116c	Me	H	ⁱ Pr	0.180	0.118	> 40	> 40	> 50
ACV	-	-	-	2.9	1	74	> 200	> 200
86f	-	-	-	0.0003	0.0001	> 5	> 50	> 200

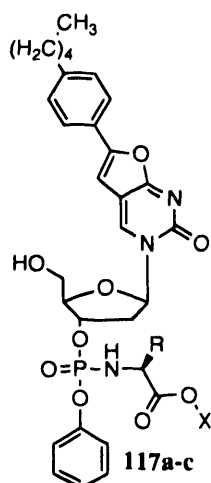
a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.16: biological results of BCNA ProTides 115a-l and 116a-c.

Interestingly, the diphosphoramidates **116a-c** still showed some antiviral activity and these results suggested to synthesize a series of 3'-phosphoramidate (**Table 2.17**, **117a-e**).²⁷



Cpds	R	X	Antiviral Activity EC ₅₀ (μM) ^a			Cytotoxicity (μM)	
			TK ⁺ VZV		TK ⁻ VZV	MCC ^b	CC ₅₀ ^c
			OKA	YS	07		
			strain	strain	strain		
117a	Me	Me	0.011	0.0094	>20	>5	>50
117b	Me	Et	0.015	-	>100	-	-
117c	Me	Bn	0.005	0.0059	>20	>20	>50
117d	Bn	Me	0.0041	0.005	>20	>20	>50
117e	Bn	Et	<0.009	<0.032	>20	>20	>50
ACV	-	-	2.9	1	74	>200	>200
86f	-	-	0.0003	0.0001	>5	>50	>200

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 2.17: biological results of 3'-phosphoramidates **117a-e** and **ACV** and **86f** as control.

The activity showed by the 3'-phosphoramidates **117a-e** is comparable with that of the 5'-phosphoramidates. This result is not easy to interpret; an explanation could be that inside the cell the 3'-phosphoramidate is metabolised to the monophosphate form and then converted to **86f** and at this point it is phosphorylated at the 5'-position by

VZV-TK or it is possible that the phosphate moiety migrates from the 3'-position to the 5'-position.²⁷

2.5 FV100: the valyl prodrug derivative of the lead compound Cf1743

As mentioned before, the high selectivity and no-toxicity of Cf1743 promote its development as potential new anti-VZV drug. However, the lipophilicity of this compound, although fundamental for the activity, implies very low water solubility. In order to improve the poor bioavailability of this compound, different prodrugs have been considered (Fig. 2.3), such as the tripeptide Val-Pro-Val (118), the valyl ester (119) and the monophosphate (120).²⁹

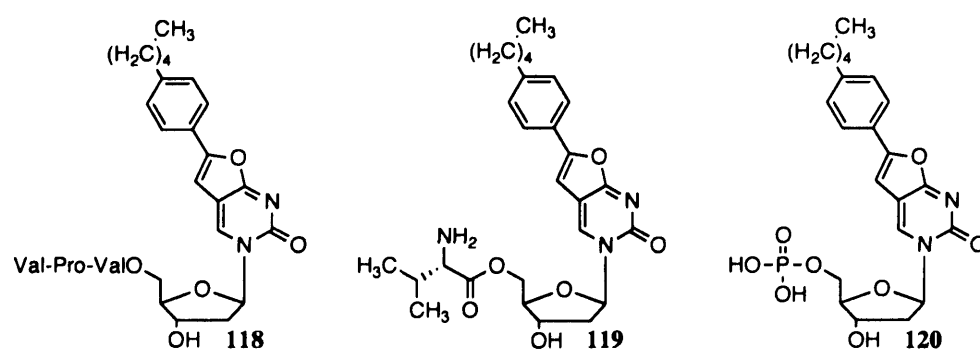


Fig. 2.3: prodrugs of BCNA lead compound Cf1743.

Pharmacokinetic studies showed the best results for the tripeptide Val-Pro-Val, for which the bioavailability was increased 20 fold more than the parent compound. For the valyl ester prodrug was increased 10 fold compare to the parent compound. The monophosphate prodrug instead did not show any significant improvement.

In spite of its higher bioavailability, the tripeptide prodrug has been discarded due to synthetic cost. The valyl ester has been chosen as prodrug of Cf1743 for its lower synthetic cost and for its efficacy showed with acyclovir (as valacyclovir)³⁰ and ganciclovir (as valganciclovir).³¹

In order to increase the water solubility of (119), the hydrochloride salt has been made, raising the solubility from 0.02 mg/mL to 0.49 mg/mL with an enhancement of 490 times. The antiviral activity for this compound, called FV-100, against VZV strain was 3 fold higher than the parent compound.

FV-100 has successfully concluded phase I of clinical trial and it is currently on phase II.

2.6 References

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3. Understanding the mechanism of action of BCNA

The mechanism of action of BCNA is not, as already mentioned in Chap. 2, fully understood. BCNAs probably need to be phosphorylated as previously described in the TK-deficient strains completely lose their activity. Moreover, the 3'- and 5'-positions play a crucial role in the activity of this compound. In fact, all the modifications done at these positions led to a decrease of activity. Interestingly, the application of the ProTide technology did not improve the activity of **86f**, but the loss of activity in the TK-deficient strains, may indicate that VZV-TK mediates also the second step of phosphorylation. Furthermore, the activity found for the 3'-ProTide is quite unusual and needs to be further investigated.

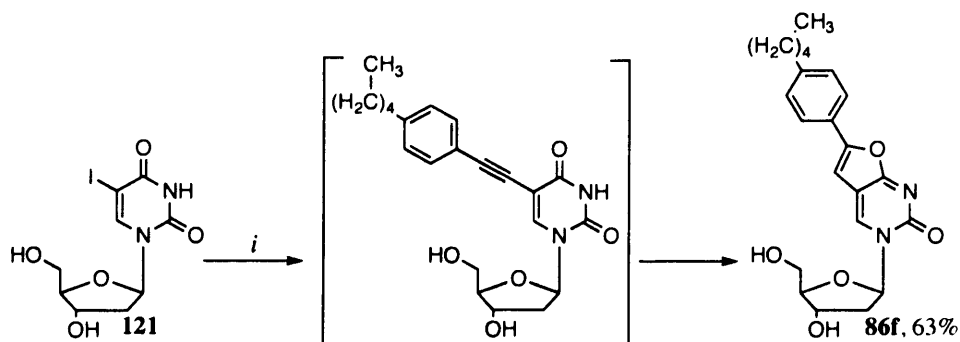
In this chapter the synthesis of a series of compounds in order to investigate the mechanism of action of BCNA will be described.

3.1 5'-modification of the BCNA lead compound

In order to understand the function of the 5'-OH and to obtain a suitable bioisostere for the synthesis of 5'-deoxy-3'-phosphoramidate of the lead compound **86f**, the 5'-iodo derivative was synthesised.

The general synthesis of BCNAs involves coupling of 5-iodo-2'-deoxyuridine with the appropriate alkyne using palladium as the coupling agent, copper (I) iodide and a base for the further cyclization.¹ The problem in this synthetic route is the purification by column chromatography which is followed by a trituration with an organic solvent. A new synthetic route, removing the column chromatography purification has been studied in order to obtain **86f**.²

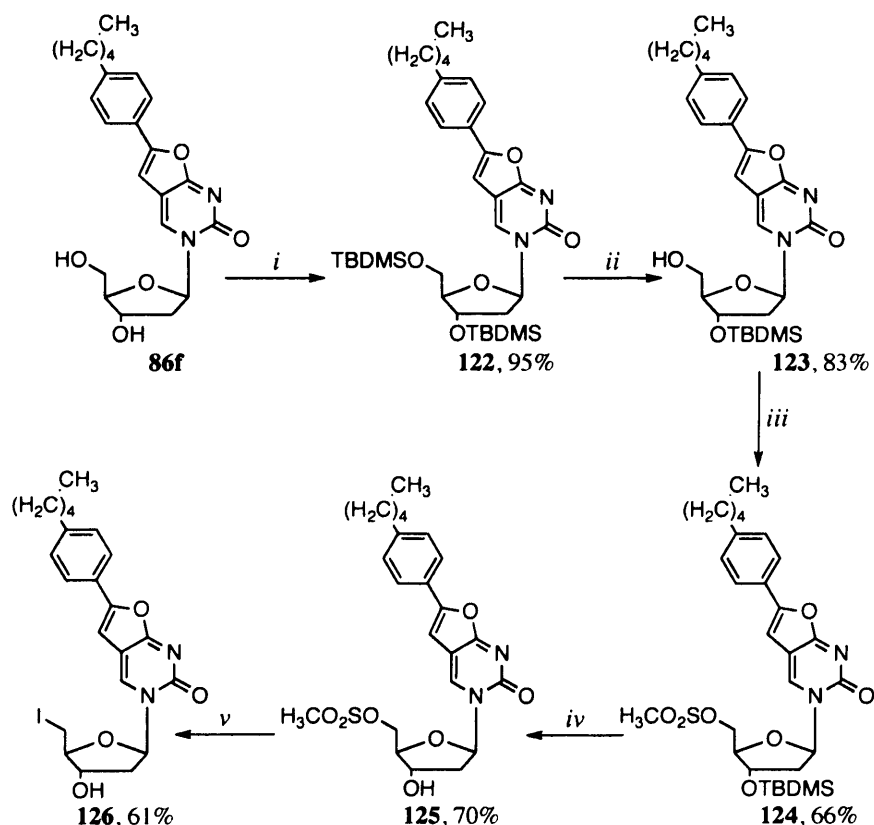
Scheme 3.1 reports the synthesis of **86f**. 5-iodo-2'-deoxyuridine **121** was dissolved in anhydrous dimethylformamide (DMF), followed by addition of: (4-pentylphenyl) acetylene, tetrakis(triphenylphosphine)-palladium(0) (tetrakis Pd(PPh₃)₄), copper (I) iodide (CuI) and anhydrous diisopropylethylamine (DIPEA). The reaction was stirred at room temperature overnight. Copper (I) iodide and anhydrous triethylamine (TEA) were added and the reaction was stirred at 85 °C for 7 h. After cooling, the solvent was removed and the residue was triturated with dichloromethane (DCM). The solid was filtered to give compound **86f** as pure product without further purification. Notably, this procedure is currently used in the scale up synthesis of this compound.



Reagents and Conditions: (i) (4-pentylphenyl)acetylene, Tetrakis Pd(PPh₃)₄, CuI, DIPEA, dry DMF, rt, overnight, then CuI, dry TEA, 85 °C, 7 h.

Scheme 3.1: synthesis of the lead compound **86f**.

For the synthesis of the desired 5'-iodo derivative different routes have been taken. Firstly, the direct iodination at the 5'-position under Mitsunobu condition using iodine and triphenylphosphine gave only a trace of the desired compound contaminated with triphenylphosphine. A different synthetic pathway was then performed (**Scheme 3.2**).



Reagents and Conditions: (i) TBDMSCl, imidazole, anhydrous pyridine, rt, 2 h; (ii) TFA/Water/THF (1/1/8), 0 °C, 2 h; (iii) methanesulfonyl chloride, anhydrous pyridine, rt, 20 h; (iv) TFA/THF/Water (1/2/4), rt, 2.5 h; (v) NaI, acetone, reflux.

Scheme 3.2: synthesis of 5'-iodo derivative of **86f**.

The 3' and 5'-positions were protected using tetrabutyltrimethylsilyl chloride (TBDMS-Cl) in the presence of imidazole to give **122**, followed by selective deprotection of the 5'-OH using trifluoroacetic acid (TFA) in water and tetrahydrofuran (THF), in a ratio of 1/1/8, at 0 °C to get **123** (this synthetic route will be described in Chap. 5). Compound **123** was then mesylated using methanesulfonyl chloride in anhydrous pyridine giving **124**, followed by 3'-deprotection of the TBDMS group to provide **125**, which has been tested as a prodrug of **86f**. Compound **125** was then treated with sodium iodide in acetone under reflux to give the final product **126**.

3.2 Biological evaluation of 5'-derivatives of BCNA lead compound

125 and **126** have been evaluated for their ability to inhibit VZV (Table 3.1).

Compounds	Antiviral Activity EC ₅₀ (μM) ^a		Cytotoxicity (μM)	
	TK ⁺ VZV TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	OKA strain	07/1 strain		
125	>20	-	>100	>100
126	0.97	6.8	>50	31.2
86f	0.0003	>20	>20	>200
ACV	3.5	74	1778	796

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 3.1: anti-VZV activity for compounds **125** and **126** including **86f** and ACV as reference.

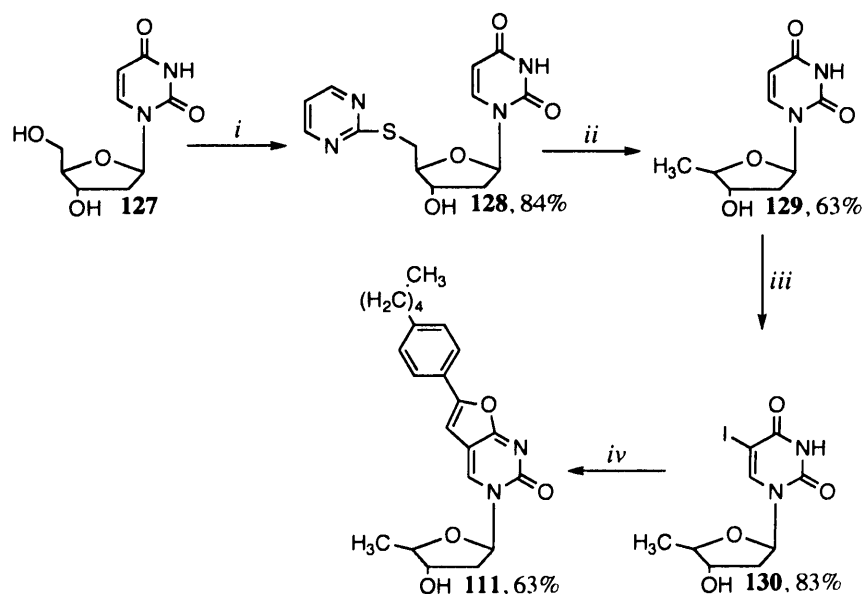
125 has been found to be inactive, as already seen for the 5'-chloro and 5'-deoxy derivatives (Chap. 2). Interestingly, **126** has been found to be active in the sub-μM range (0.97 μM VZV-OKA strain) and also it showed some activity against VZV-TK deficient strain (6.8 μM VZV-07/1 strain). However, its apparent antiviral activity may be simply cytotoxicity (CC₅₀ = 31 μM).

Due to synthetic problems, the 5'-deoxy derivative of **86f** was selected as an intermediate for the synthesis of the 3'-ProTides of the 5'-deoxy derivatives of **86f**.

3.3 Synthesis of the 5'-deoxy derivative of the BCNA lead compound

Initially, the procedure already described for the synthesis of **111** was used. The first step of the synthesis was the reaction of **86f** with 2-mercaptopyrimidine in the presence of N,N-dimethylformamide dineopentylacetal followed by reduction with Raney-Nickel providing **111**.³ However, due to a low overall yield (11%) for this route, a different strategy has been considered (**Scheme 3.3**).

2'-Deoxyuridine **127** was coupled with 2-mercaptopyrimidine in the presence of N,N-dimethylformamide dineopentylacetal, giving **128**, followed by reduction in ethanol using Raney-Nickel as catalyst providing **129**.⁴ Compound **129** was then selectively iodinated at the 5-position using iodine and cerium (IV) ammonium nitrate (CAN) in acetonitrile at reflux giving **130**.⁵ The last step involved the coupling of the base with (4-pentylphenyl)acetylene under standard conditions to give the desired compound **111**. The overall yield for this new synthetic route is 26% compared to 11% previously described.



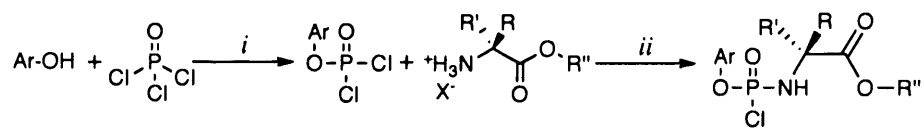
Reagents and Conditions: (i) 2-mecaptopyrimidine, N,N-dimethylformamide dineopentylacetal, acetonitrile, reflux, 4 h; (ii) Raney-Nickel, ethanol, reflux, 2 h; (iii) I₂, CAN, acetonitrile, reflux, 1.5 h; (iv) (4-pentylphenyl)acetylene, Tetrakis Pd(PPh₃)₄, CuI, DIPEA, dry DMF, rt, overnight, then CuI, dry TEA, 85 °C, 7 h.

Scheme 3.3: synthesis of 5'-deoxy derivative of our lead compound **86f**.

3.4 General synthesis of arylphosphorochloridates

The general synthesis of arylphosphorochloridate (**Scheme 3.4**) involves coupling of the appropriate aryldichlorophosphate with the appropriate amino acid ester using anhydrous TEA as a base. Phenylidichlorophosphate is commercially available, whilst

the naphthyl and *p*-fluorophenyl derivatives were obtained by coupling of 1-naphthol or *p*-fluorophenol with POCl₃ using anhydrous TEA as a base.



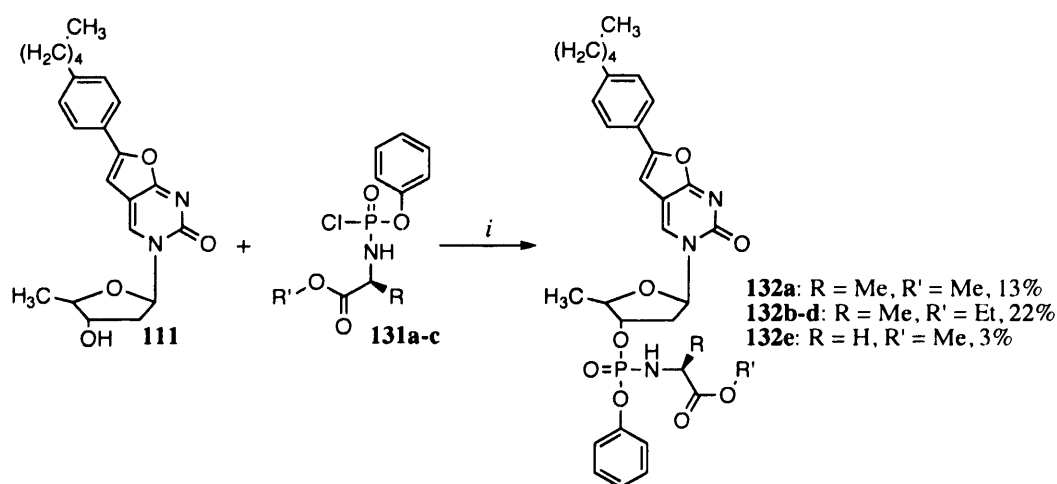
Reagents and Conditions: (i) anhydrous TEA, anhydrous Et₂O, -78 °C, 1 h, then rt, overnight; (ii) anhydrous TEA, anhydrous DCM, -78 °C, 1 h, then rt, 2h.

Scheme 3.4: general synthesis of phosphorodichloridates and phosphorochloridates.

The arylphosphorodichloridate were obtained as oil and used without further purification. The arylphosphorochloridates were usually purified by filtration through silica gel column chromatography or used as a crude in the next step and they were obtained as pale yellow oil. In the case of chiral amino acids, such as L-alanine, phosphorochloridates were obtained as a mixture of two diastereoisomers, which were detected in the ³¹P-NMR exhibiting the presence of two peaks.

3.5 Synthesis of the 5'-deoxy-3'-phosphoramidate of the BCNA lead compound

Coupling between compound **111** and the appropriate phosphorochloridate (**131a-c**) (**Scheme 3.5**) was performed using 1-methylimidazole (NMI) as the coupling reagent following the Van Boom procedure.⁶



Reagents and Conditions: (i) anhydrous NMI, anhydrous THF, rt, overnight.

Scheme 3.5: synthesis of phosphoramidates of compound **111**.

The desired compounds were obtained as a mixture of the two diastereoisomers confirmed by the presence of two signals in ³¹P NMR (in the range of 2.7 and 4.4

ppm). In the case of the ethyl ester derivative, the two diastereoisomers, after purification using preparative TLC, were isolated (**132b,c**) and one fraction was obtained as a mixture (**132d**). The structures of these compounds were confirmed by NMR spectroscopy (^1H , ^{13}C and ^{31}P NMR) and mass spectroscopy; whilst the purity was confirmed by elemental analysis.

3.6 Biological evaluation 5'-deoxy-3'-phosphoramidates of BCNA lead compound

The synthesised compounds **132a-e** were evaluated for their ability to inhibit the replication of VZV.

Compounds	Antiviral Activity EC ₅₀ (μM) ^a		Cytotoxicity (μM)	
	TK ⁺ VZV	TK ⁻ VZV	MCC ^b	CC ₅₀ ^c
	OKA strain	07/1 strain		
132a	>20	>20	100	52.8
132b	>20	>20	100	56.8
132c	>20	>20	100	>100
132d	>100	>20	≥100	>100
132e	>20	>20	100	70.3
86f	0.0003	>20	>20	>200

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 3.2: biological results for compounds **132a-e**.

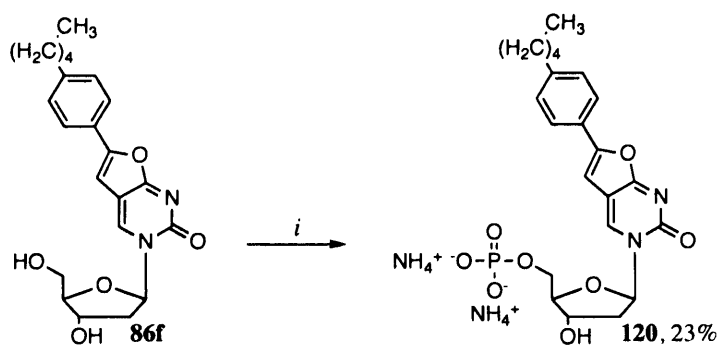
The compounds did not show any activity against VZV (**Table 3.2**). These results support the hypothesis that the activity of BCNA involves a 5'-phosphorylation and it does not involve a 3'-phosphorylation or could involve phosphorylation of both positions. The activity found for the 3'-ProTides compounds **117a-e** can be due to a migration of the phosphate moiety from the 3'-position to the 5'-position and this migration may help during the activation of the ProTide to release the monophosphate form. Another hypothesis is that these compounds act as a prodrug releasing the parent nucleoside **86f**, which will then be phosphorylated by VZV-TK.

3.7 Attempt to synthesise mono-, di-, tri- and bisphosphate of BCNA lead compound

The next step, in order to elucidate the mechanism of action of BCNA, was the synthesis of the possible metabolites of **86f** and this includes the synthesis of the mono-, di-, tri and bisphosphate. These compounds will then be used as a standard reference during the characterization of the metabolites obtained in *in vivo* experiments.

3.7.1 Synthesis of the monophosphate of BCNA lead compound

The monophosphate **120** was synthesised by coupling between **86f** and POCl₃ in triethylphosphate (**Scheme 3.6**) under Yoshikawa conditions following the procedure already described for this compound.²



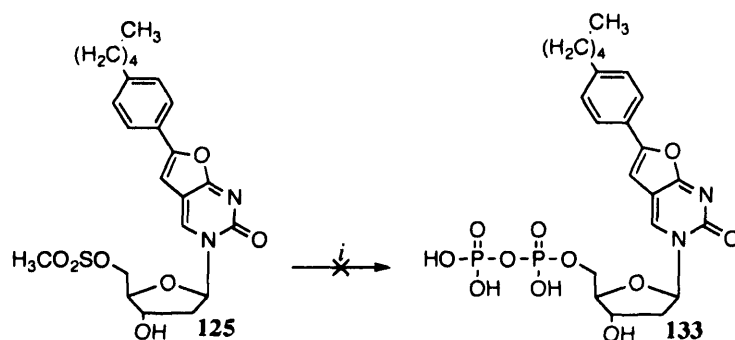
Reagents and Conditions: (i) POCl₃, triethylphosphate, 0 °C, 2.5 h, then 0.4 M aqueous solution of NH₄HCO₃.

Scheme 3.6: synthesis of the monophosphate.

The poor yield obtained for this compound is probably due to the relative instability of BCNA. In fact, the release of hydrochloric acid during the reaction could cleave the glycosidic bond of the compound, which is quite acid labile.

3.7.2 Attempt to synthesise the diphosphate of BCNA lead compound

Scheme 3.7 reports the synthesis of the diphosphate **133** which involves a nucleophilic displacement of the 5'-mesyl group of **125** by the tri(tetra-n-butylammonium) hydrogen pyrophosphate.⁷



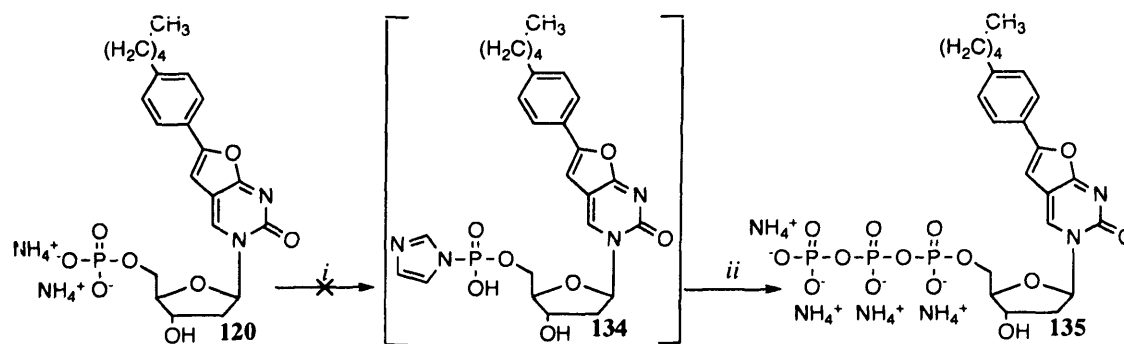
Reagents and Conditions: (i) anhydrous acetonitrile, tris-(tetra-*n*-butylammonium)hydrogen pyrophosphate, rt, 1 day.

Scheme 3.7: attempt to synthesize the diphosphate of **86f**.

No desired compound was isolated due to problem during the purification and probably to the poor stability of this compound.

3.7.3 Attempt to synthesise the triphosphate of BCNA lead compound

For the synthesis of the triphosphate **135**, two different routes have been considered. The first route considered (**Scheme 3.8**) was the activation of the monophosphate using 1,1'-carbonyldiimidazole to get compound **134** followed by attack of the pyrophosphate to give the desired triphosphate as the ammonium salt after quenching the reaction with an aqueous solution of ammonium bicarbonate.⁸



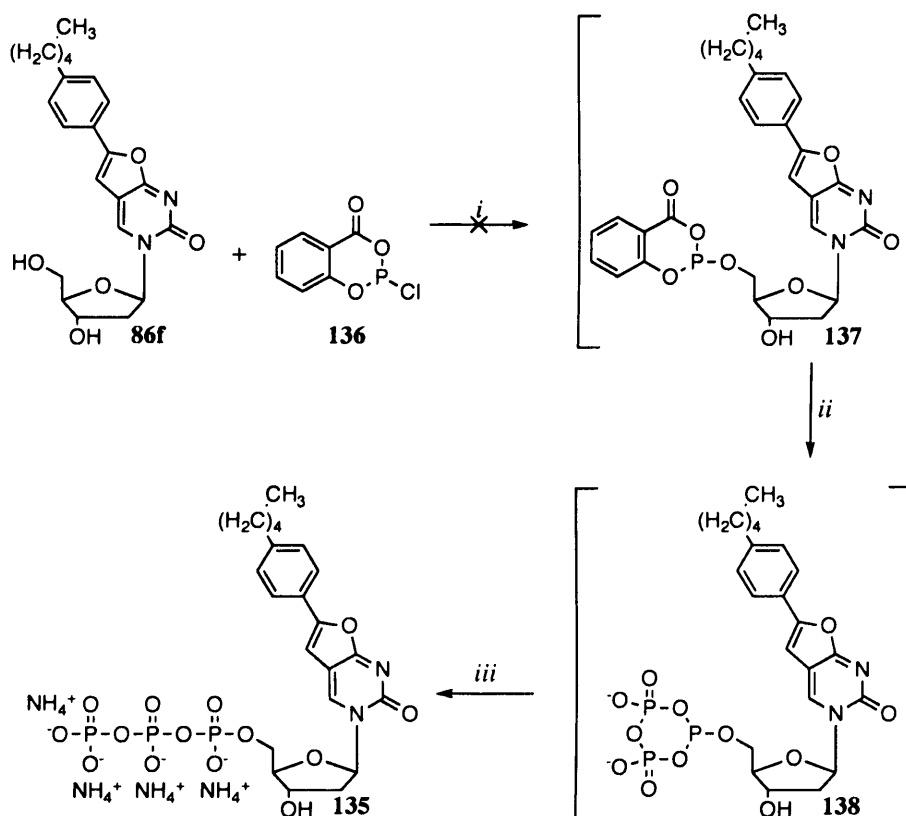
Reagents and Conditions: (i) 1,1'-carbonyldiimidazole, anhydrous DMF, rt, 4 h; (ii) MeOH, rt, 30 min; then tributylammonium pyrophosphate, rt, 24 h; then 0.4 M NH_4HCO_3 , rt, 10 min.

Scheme 3.8: first attempt to synthesise the triphosphate of **86f**.

The second route (**Scheme 3.9**) used was the “one-pot-three-step” reaction starting from the parent **86f**, which was converted to the intermediate **137** using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one **136** as phosphorylating agent. Compound **137** was then opened by the attack of the pyrophosphate with the displacement of the carbonyl group followed by cyclisation with the release of the phenolic group to obtain the

cyclic derivative **138**. This intermediate is then oxidized by addition of iodine to obtain the triphosphate, which is converted to the ammonium salt after the addition of concentrated ammonia.⁹

This procedure did not succeed probably due to a lack of reactivity in the first step as mainly starting material was observed on TLC. This could be due to a poor reactivity of BCNA due to the high lipophilicity of this compound compared to other NAs.



Reagents and Conditions: (i) anhydrous pyridine, anhydrous dioxane, anhydrous DMF, rt, 20 min; (ii) bis(tri-*n*-butylammonium)pyrophosphate, anhydrous DMF, anhydrous tributylamine, rt, 10 min; (iii) 1% solution of iodine in a mixture of pyridine/water (98/2), then 5% aqueous solution of thiosulphate, water and conc. ammonia.

Scheme 3.9: second attempt to synthesize the triphosphate of **86f**

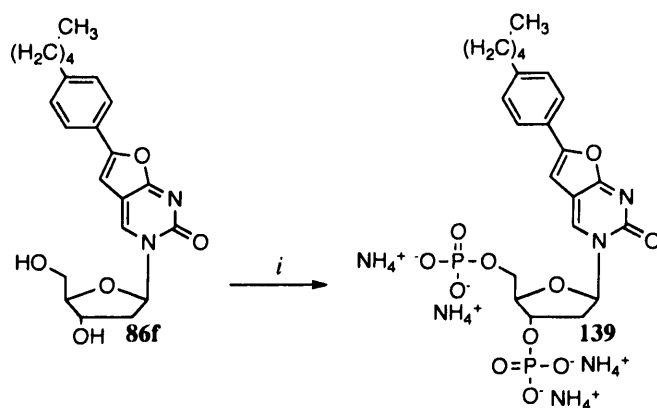
3.7.4 Attempt to synthesise bisphosphate of BCNA lead compound

Another potential metabolite of **86f** could be the bisphosphate (**139**, **Scheme 3.10**). In fact, from an analysis of the metabolism of a radiolabelled sample of **86f** incubated with cells that are TK deficient but VZV-TK efficient, performed by Prof Jan Balzarini at the Rega Institute, Leuven, Belgium an unknown peak on the HPLC was observed. From this experiment, the formation of the monophosphate form as well as the glycosidic bond cleavage was noted. However, a peak close to the triphosphate form was observed indicating a formation of a polar product, which, however, seems

not to have a phosphorus-oxygen-phosphorus linkage as it appeared to be insensitive to phosphodiesterase. A possible compound could be the 3',5'-bisphosphate.

Different strategies have been used for the synthesis of this compound.

The first route used (**Scheme 3.10**) was the same used for the synthesis of the monophosphate, starting from the parent **86f** using an excess of POCl_3 and using triethylphosphate as a solvent in one case or anhydrous pyridine. Initially, the compound was purified by silica gel column chromatography and the appropriate fractions were collected.



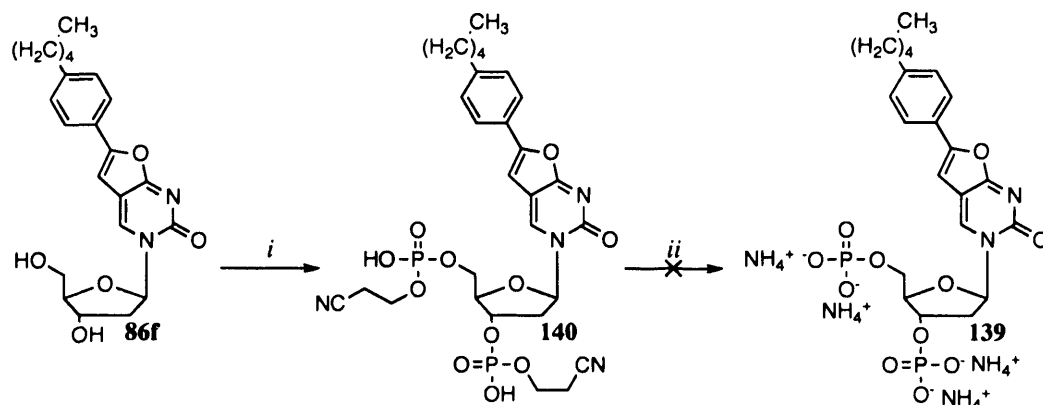
Reagents and Conditions: (i) POCl_3 , triethylphosphate or anhydrous pyridine, 0°C , 3-6 h, then 0.4 M aqueous solution of NH_4HCO_3 .

Scheme 3.10: first attempt to synthesise the 3',5'-bisphosphate of **86f**.

Mass spectrometry (EI) data showed two peaks: one corresponding to the free base, meaning that the BCNA is not stable under these conditions due to the release of HCl , and another peak corresponding to the sugar bisphosphate. This result indicates that this compound could be unstable.

The second route (**Scheme 3.11**) used to synthesised compound **139** was to use of 2-cyanoethyl phosphate as the phosphorylating reagent. The presence of an alkyl chain in the phosphate moiety could help in the purification step with the derivative being more lipophilic and more stable. For the synthesis, **86f** was coupled in the presence of dicyclohexylcarbodiimide (DCC) with 2-cyanoethylphosphate, which was obtained from the commercially available barium salt form which was converted to the free form by washing through a column of Dowex 50 $[\text{H}^+]$ resin.¹⁰ The deprotection step could be performed by refluxing the ester phosphate derivative in 0.1N NaOH ,¹⁰ but in this condition BCNAs have been found to be unstable. Another method is the use

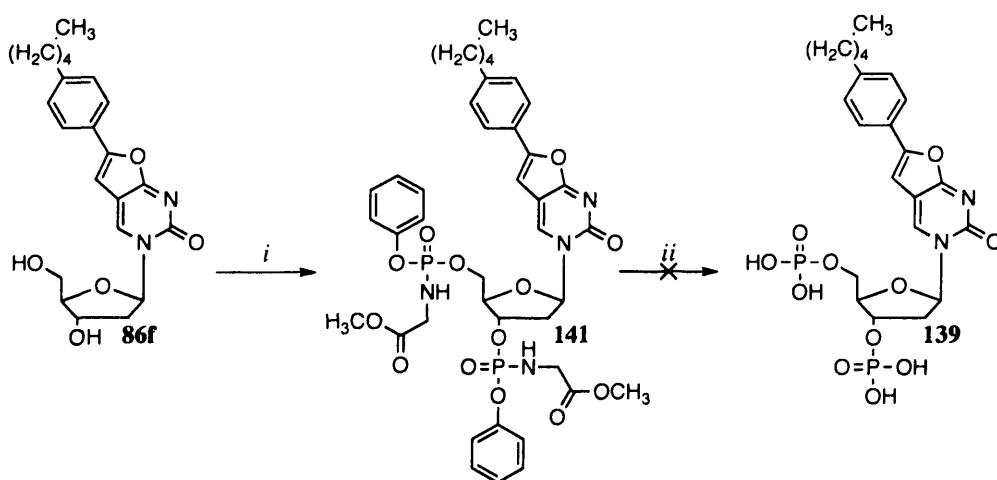
of 1,8-diazabicycloundec-7-ene (DBU). The starting material disappeared from the TLC but the compound isolated showed no phosphorus in ^{31}P -NMR.



Reagents and Conditions: (i) anhydrous pyridine, solution of 2-cyanoethylphosphate in anhydrous pyridine, DCC, rt, 44 h; (ii) DBU, anhydrous THF, 1.5 h, rt.

Scheme 3.11: second attempt to synthesise the 3',5'-bisphosphate of **86f**.

The third route used (**Scheme 3.12**) to synthesise the 3',5'-bisphosphate was the degradation of the 3',5'-diphosphoramidate of **86f**. Compound **141** was obtained by coupling between the parent **86f** and the phenyl-(methoxy-glycyl)-phosphorochloridate using tert-butyilmagnesium chloride ($t\text{BuMgCl}$) as a coupling reagent following Uchiyama condition.¹¹ The phosphoramidate obtained was degraded using basic and acid conditions and the reaction was followed by ^{31}P -NMR.¹²



Reagents and Conditions: (i) Phenyl-(methoxy-glycyl)-phosphorochloridate, anhydrous THF, anhydrous pyridine, $t\text{BuMgCl}$, rt, overnight; (ii) MeOD, 0.1 M NaOH, 4.5 h, 45 °C, then 0.1 M HCl, rt, 30 min.

Scheme 3.12: third attempt to synthesize the 3',5'-bisphosphate of **86f**

The starting material showed peaks ($\delta = 4$) at ^{31}P -NMR. The basic conditions hydrolysed the ester followed by displacement of the phenyl ring and in the ^{31}P -NMR a peak at ($\delta = 7$) was observed. In acidic condition, the cleavage of the phosphorus-nitrogen bond was observed with the release of the monophosphate, which was observed in the ^{31}P -NMR ($\delta = 0$). However, during purification by preparative HPLC the desired compound was not recovered but only the products of degradation. Although promising, this synthetic route was not further investigated due to time constraints.

3.8 Photoaffinity labeling

Another technique used to investigate the mechanism of action of BCNAs is photoaffinity labeling. This method allows the determination of the interaction between a ligand and an enzyme. To show their properties, these substrates need to be activated, through UV irradiation, to an active form, which is able to bind covalently with the target enzyme.¹³

Various photophores are currently in use,¹³ and in particular (**Fig. 3.1**) arylazide (**142**) and benzophenone (**143**) have been investigated for BCNA.

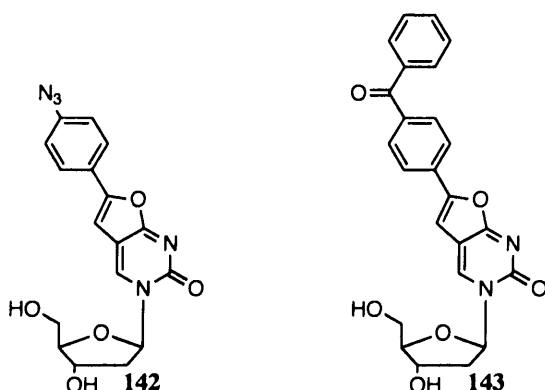
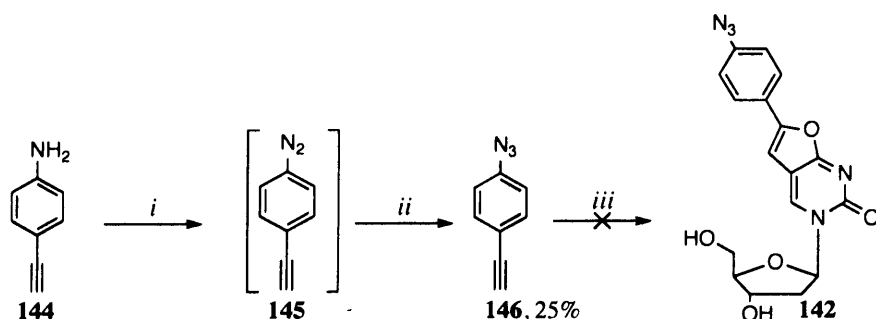


Fig. 3.1: proposed photoaffinity labeled BCNA derivatives.

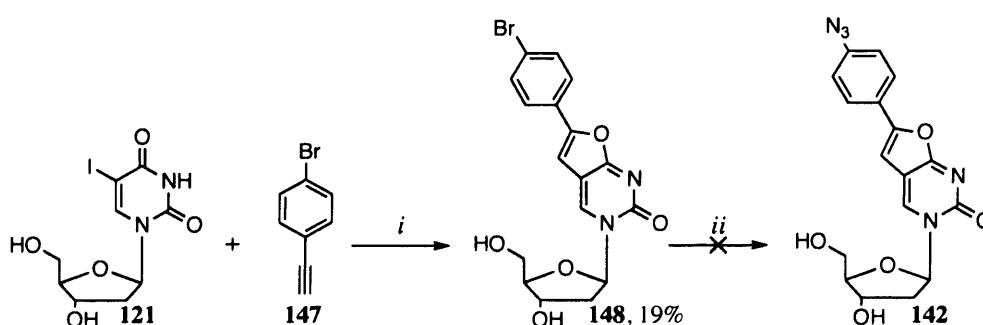
These two photophores have been chosen as they have been used in several substrates and on the basis of synthetic access. However, the synthesis of the arylazide derivative **142** (**Scheme 3.13**) has been found to be difficult due to the extreme reactivity of the azide. In fact, no coupling between the 5-iodo-2'-deoxyuridine and **146** has been observed. It may be that the azide reacts with the triple bond to form a triazole ring, which was not isolated.



Reagents and Conditions: (i) NaNO_2 , $\text{HCl}/\text{H}_2\text{O}$, 0°C 1h; (ii) NaN_3 , H_2O , 0°C , 1 h; (iii) 5-iodo-2'-deoxyU, Tetrakis $\text{Pd}(\text{PPh}_3)_4$, CuI , DIPEA , dry DMF , rt, overnight; then CuI , dry TEA , 85°C , 8 h.

Scheme 3.13: attempt to synthesise the arylazide derivative of **86f**.

Another attempt (**Scheme 3.14**) has been made starting from compound **148**, which was obtained by coupling between **121** and **147**. The substitution of bromine with azide has been previously performed following two different procedures already described for simple aromatic systems. In the first case, a mixture of copper iodide, L-proline sodium salt and sodium azide was used. However, the conditions required for the last step, involving the addition of an aqueous solution of NaOH, even if in small amount, are not suitable for BCNAs and the nucleoside degraded.¹⁴ In the second case, a direct substitution of the bromine using sodium azide in water and DMF at 100 °C was used. However, after three days no reaction occurred and the starting material was recovered.¹⁵

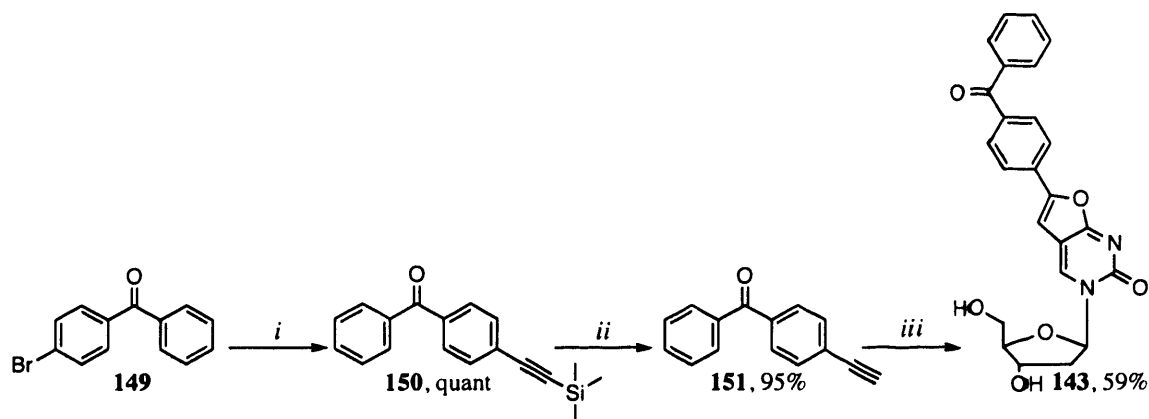


Reagents and Conditions: (i) Tetrakis Pd(PPh₃)₄, CuI, anhydrous DIPEA, anhydrous DMF, rt, overnight; then CuI, anhydrous TEA, 80 °C, 7 h. (ii) NaN₃, L-proline sodium salt, CuI, EtOH, H₂O, 90 °C, 1 day; then aqueous solution of NaOH or NaN₃, DMF, H₂O, 100 °C, 3 days.

Scheme 3.14: attempt to synthesise the arylazide derivative of **86f**.

As the use of azide resulted in a failure, the benzophenone was then considered.

For the synthesis (**Scheme 3.15**), the commercially available 4-bromo-benzophenone **149** was coupled with trimethylsilyl acetylene under Sonogashira conditions to provide compound **150**, in quantitative yield and it was used without further purification in the deprotection step using sodium methoxide to obtain compound **151**. Coupling with 5-iodo-2'-deoxyuridine and **151** was performed under standard conditions to obtain the desired compound **143** in good yield.



Reagents and Conditions: (i) ethynyltrimethylsilyl chloride, $\text{Pd(PPh}_3)_4$, CuI, anhydrous DIPEA, anhydrous DMF, rt, overnight; (ii) MeONa, anhydrous MeOH, rt, 1h; (iii) 5-iodo-2'-deoxyU, $\text{Pd(PPh}_3)_4$, CuI, anhydrous DIPEA, anhydrous DMF, rt, overnight; then CuI, anhydrous TEA, 85 °C, 8 h.

Scheme 3.15: synthesis of the compound **143**.

Biological evaluation and photoaffinity labeling experiments are currently underway.

3.9 References

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4. Fluoro derivatives of BCNA lead compound

4.1 2'-fluoro derivative of BCNA lead compound

As already mentioned in Chap. 2, all the modifications at the sugar moiety of the BCNAs are detrimental to anti-VZV activity of this class of compound. However, the introduction of fluorine at the 2'-position was not so far considered. Fluorine has the same size atomic radius of the hydrogen and could be therefore considered a potential favorable replacement for the hydrogen.

The literature reports several examples where the introduction of fluorine at the 2'-position of the sugar moiety gives enhancement of antiviral or anticancer activity, for example (**Fig. 4.1**): clevudine (**152**), currently in phase III clinical trial for the treatment of HBV infection;¹ RT7128 (**153**), prodrug of 2'-deoxy-2'-fluoro-2'-C-methylcytidine, at present in clinical trial as an anti-HCV agent;² gemcitabine (**154**), which is 2'-difluorocytidine and it is a potent anticancer compound licensed by Lilly.

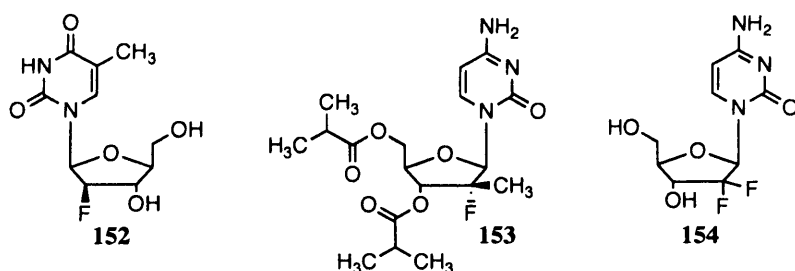


Fig. 4.1: examples of 2'-fluoro NAs.

In the case of compound **86f**, all the potential fluorine-substitutions at the 2'-position have been made (**Fig. 4.2**) obtaining compounds **155** (α -derivative), **156** (β -derivative) and **157** (gem-derivative).

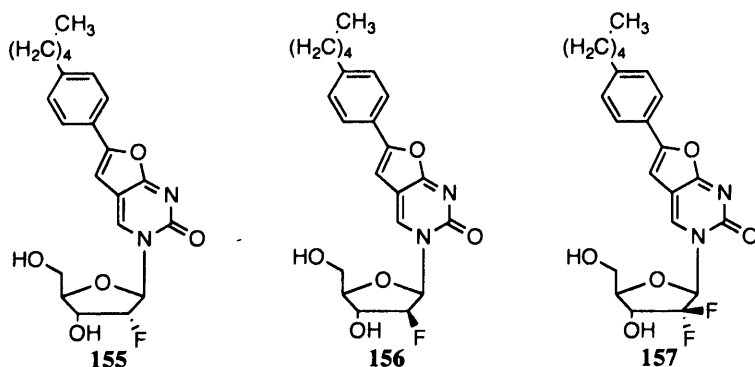
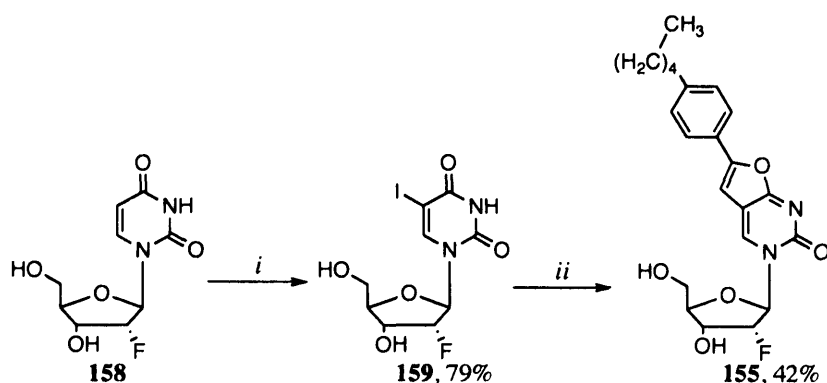


Fig. 4.2: 2'-fluoro derivatives of **86f**.

4.1.1 Synthesis of the 2'- α -fluoro derivative of the BCNA lead compound

Scheme 4.1 reports the synthesis of compound **155** starting from the commercially available 2'-fluoro-2'-deoxyuridine **158** which was selectively iodinated at the 5-position using iodine and CAN in acetonitrile at reflux giving **159** in good yield.⁴ **159** was then coupled with (4-pentylphenyl)acetylene under Sonogashira conditions to give the desired compound **155**.

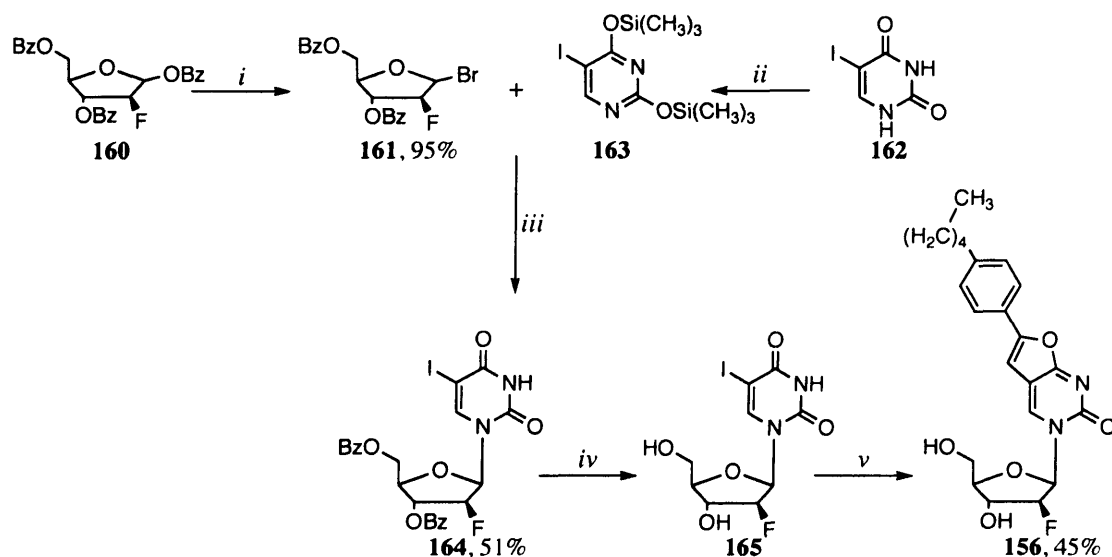


Reagents and Conditions: (i) I_2 , CAN, acetonitrile, reflux, 1 h; (ii) (4-pentylphenyl)acetylene, Tetrakis $Pd(PPh_3)_4$, CuI, anhydrous DIPEA, anhydrous DMF, rt, overnight, then CuI, anhydrous TEA, 85 °C, 7.5 h.

Scheme 4.1: synthesis of 2'- α -fluoro derivative **155**.

4.1.2 Synthesis of the 2'- β -fluoro derivative of the BCNA lead compound

Scheme 4.2 reports the synthesis of the 2'- β -fluoro derivative **156**.



Reagents and Conditions: (i) HBr in acetic acid, DCM, rt, 22 h; (ii) hexamethyldisilazane, ammonium sulphate, anhydrous acetonitrile, 70 °C, 5 h; (iii) NaI, anhydrous DCM, anhydrous acetonitrile, rt, 1 week; (iv) MeONa, anhydrous MeOH, rt, 1 h; (v) 4-pentylphenylacetylene, tetrakis $Pd(PPh_3)_4$, CuI, anhydrous DIPEA, anhydrous DMF, rt, overnight, then CuI, anhydrous TEA, 85 °C, 8 h.

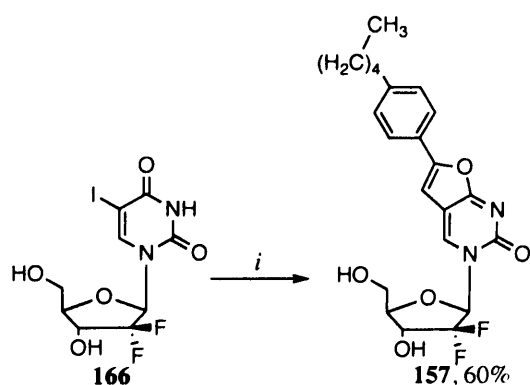
Scheme 4.2: synthesis of 2'- β -fluoro derivative **156**.

For this route the intermediate **165** needed to be prepared according to the procedure already described in the literature.⁵

The commercially available 2- β -fluoro-2-deoxy-1,3,5-tribenzoylribose **160** was converted to the 1-bromo derivative **161** using hydrobromic acid in acetic acid giving the pure compound without purification. **161** was then coupled in the presence of sodium iodide with the activated base **163**, which was obtained by silylation from 5-iodouracil **162** using hexamethyldisilazane in the presence of ammonium sulphate. The desired β -anomer **164** was obtained by precipitation during the reaction to give a white solid. However, from the filtrate another solid was obtained as a mixture of two anomers (α and β). The two anomers were characterised by NMR comparing the results with the data in the literature. The deprotection of the benzoyl group was performed using sodium methoxide in anhydrous methanol to give **165** which was used without further purification in the coupling step with (4-pentylphenyl)acetylene under standard conditions to give the desired compound **156**.

4.1.3 Synthesis of the 2'-gem-difluoro derivative of the BCNA lead compound

Scheme 4.3 reports the synthesis of the 2'-gem-difluoro derivative **157** starting from **166**, which was available in our laboratory. It was coupled with (4-pentylphenyl)acetylene under Sonogashira conditions to give the desired compound **157**.



Reagents and Conditions: (i) 4-pentylphenylacetylene, tetrakis Pd(PPh₃)₄, CuI, anhydrous DIPEA, anhydrous DMF, rt, overnight, then CuI, anhydrous TEA, 85 °C, 7.5 h.

Scheme 4.3: synthesis of 2'-gem-difluoro derivative **157**.

4.2 Enzymatic and biological evaluation of 2'-fluoro derivatives

The synthesised compounds and the parent **86f** were evaluated for their ability to inhibit VZV-TK. In addition, these compounds were evaluated for their ability to inhibit two different strains of TK-efficient VZV and one strain TK-deficient VZV (Table 4.1).

Cpds	F-position	Enzymatic Activity IC ₅₀ (μM)	Antiviral Activity EC ₅₀ (μM) ^a			Cytotoxicity (μM)	
		VZV-TK	TK ⁺ VZV		TK ⁻ VZV	MCC ^b	CC ₅₀ ^c
			YS strain	OKA strain	07/1 strain		
155	α-fluoro	2.7	-	0.75	>20	>50	>100
156	β-fluoro	38	0.011	0.007	>50	>50	-
157	Di-fluoro	~13	6.7	1.5	>50	≥50	-
86f	-	3.3	-	0.0097	>20	≥50	77

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 4.1: enzymatic and biological evaluation of 2'-fluoro derivatives of **86f**.

From Table 4.1, it is easy to note the differences for these compounds. In fact, in the case of the enzymatic test using VZV-TK, which as already seen plays a crucial role in BCNAs phosphorylation,⁶ the α-fluoro derivative **155** retains low inhibitorial activity, comparable to the parent **86f**. The β-fluoro derivative **156** showed a loss of potency (10 fold) whilst the gem-fluoro derivative **157** showed an inhibitory activity midway between the other two compounds being 4 fold less potent than the parent.

The antiviral activity showed interesting results. In fact, both α- and gem- derivatives showed a loss of activity, respectively of ~80 fold and ~150 fold, compare to the parent. By contrast, the β-derivative is highly active, being of anti-VZV activity comparable with the parent compound.

All the compounds showed no activity against VZV TK-deficient strain, supporting the fact that they need to be phosphorylated to show antiviral activity.

It is interesting to note that in spite of being a good substrate for the VZV-TK, compound **155** did not show the same potency in the antiviral assay. This aspect was already noticed with other BCNA derivatives.⁶

4.3 Molecular modeling studies on VZV-TK

It has been reported that the conformation of the sugar moiety (north or south) is fundamental for the interaction with the active site of target enzymes, such as TK or polymerase (viral or cellular), and usually one conformation is preferred to the other.⁷ Sugars are usually in dynamic equilibrium with both conformations as the energy barrier is quite low.⁸ To block the conformation either to the north or to the south conformation, locked NAs have been designed and in particular in this experiment the methanocarbothymidine has been used with the carbosugar moiety blocked in the north (**167**, **Fig. 4.3**) and the south conformation (**168**).⁸

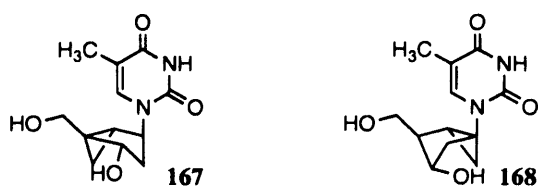


Fig. 4.3: locked NAs.

From these studies using HSV-TK, it appears that, for the first step of phosphorylation, the NA in the north conformation is a weak substrate for the HSV-TK whilst the south one does not bind. However, for the second step of phosphorylation mediated by HSV-TK, it has been found that the south conformer is a better substrate than the north. In the incorporation of both conformers into the DNA, the north was preferred to the south and this is also proved by the antiviral testing as the north conformer was found to be active whilst the south conformer was inactive.⁸ In order to understand the differences in the inhibitory activity versus VZV-TK for the BCNA fluoro-derivatives, the affinity with the enzyme using docking techniques has been performed.

Firstly, stochastic and systematic searches, with the aim to find the lowest energy conformation for these compounds, have been performed.

From these studies (**Fig. 4.4**) it has been found that the north conformation of the sugar is more stable for the α -fluoro derivative **155** and for the gem-fluoro derivative **157** compared to the south conformation by 1.8 and 1.0 kcal, respectively. On the contrary, for the β -fluoro derivative **156** the south conformation was more stable than the north by 1.2 kcal.

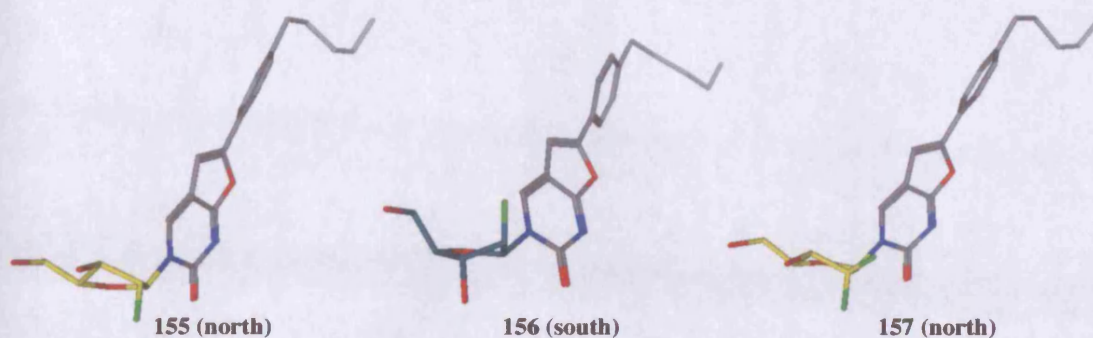


Fig. 4.4: lowest energy conformation for the BCNA fluoro-derivatives.

The lowest minimised structure of both north and south conformation for these compounds have been docked with VZV-TK (protein data bank (PDB) 1OSN).

In the case of compound **155** (**Fig. 4.5**) the north conformation displayed a better docking compared to the south, due to the formation of a hydrogen bond between the fluorine and the hydroxyl of the tyrosine 59 of the enzyme, which was of 2.78 Å in the case of the north conformation and 3.87 Å for the south conformation.

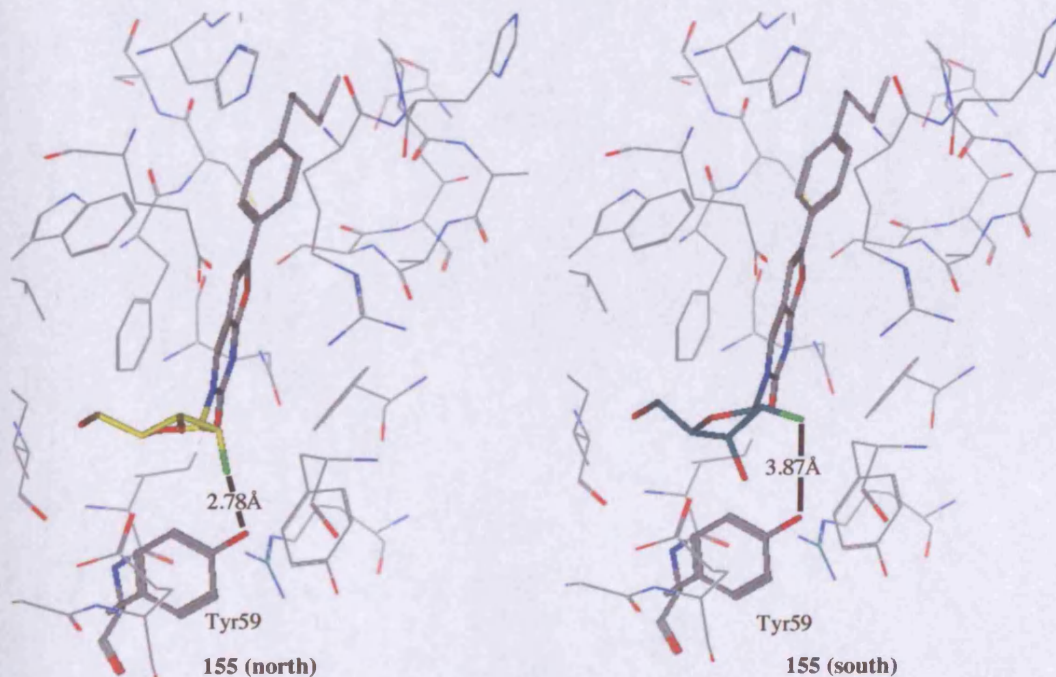


Fig. 4.5: VZV-TK docking results for the α -fluoro **155**.

In the case of the β -fluoro derivative **156**, the south conformation showed a better docking compared to the north (**Fig. 4.6**). However, the fluorine in the β -position is not able to interact with the tyrosine 59 of the enzyme.

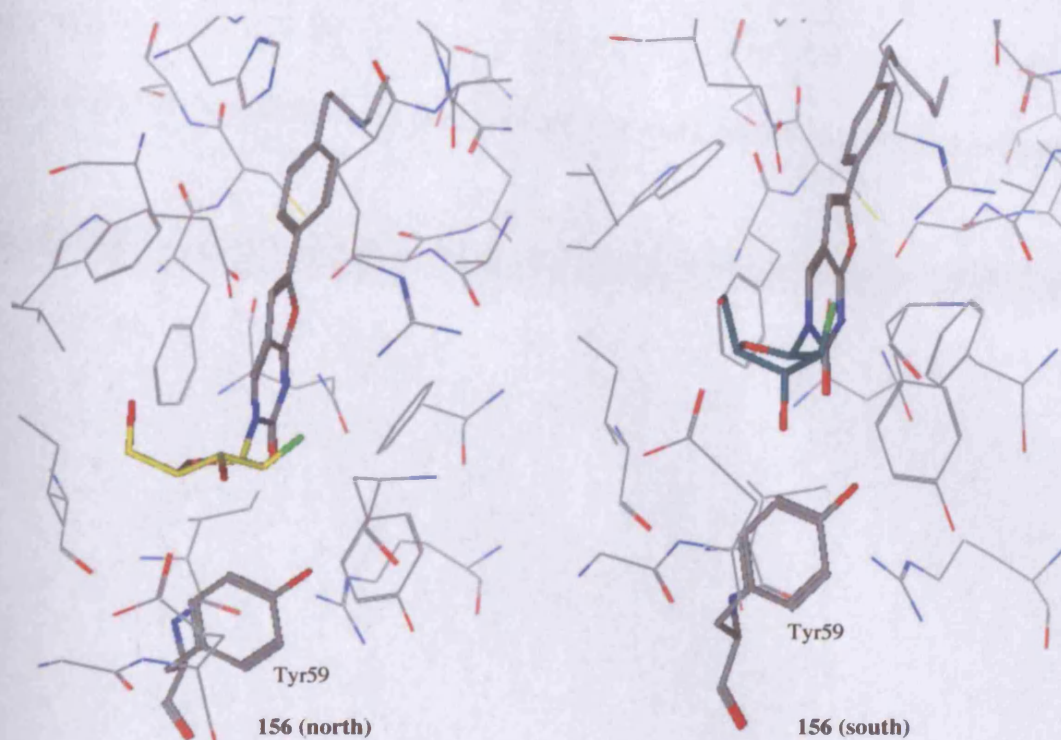


Fig. 4.6: VZV-TK docking results for the β -fluoro **156**.

In the case of the gem-fluoro **157**, both north and south conformations showed the same interaction with the active site of the enzyme (Fig. 4.7).

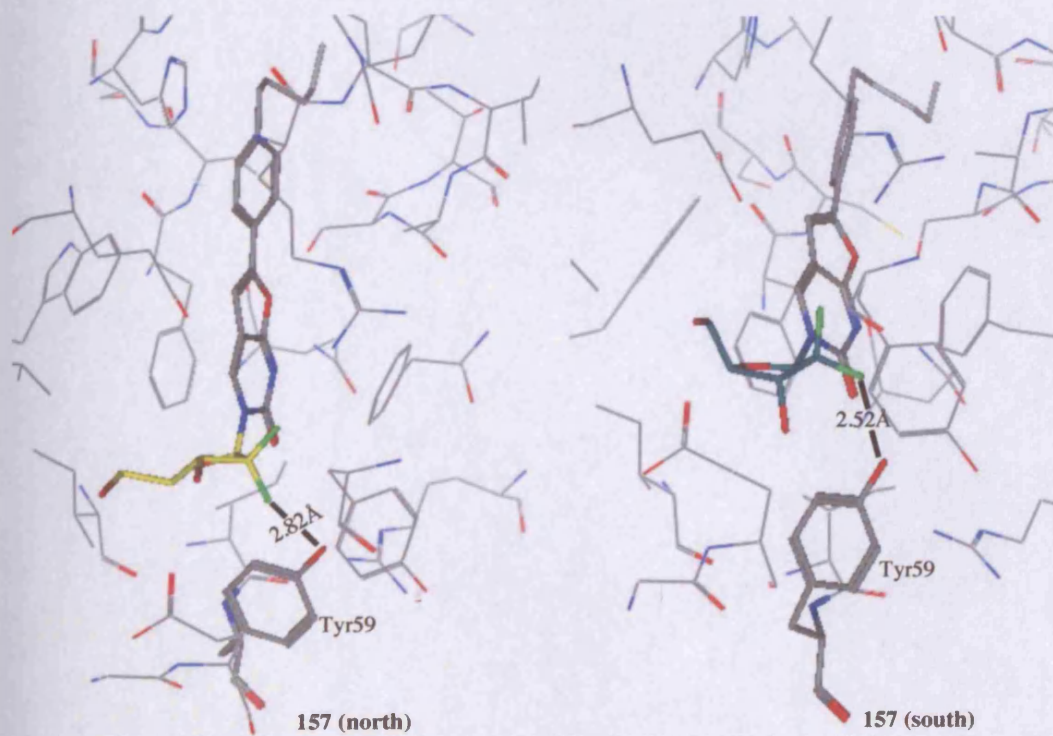


Fig. 4.7: VZV-TK docking results for the gem-fluoro **157**.



In fact, the formation of the hydrogen bond between the fluorine and the hydroxyl of the tyrosine 59, with a distance of 2.82 Å for the north conformation and 2.52 Å for the south was noticed.

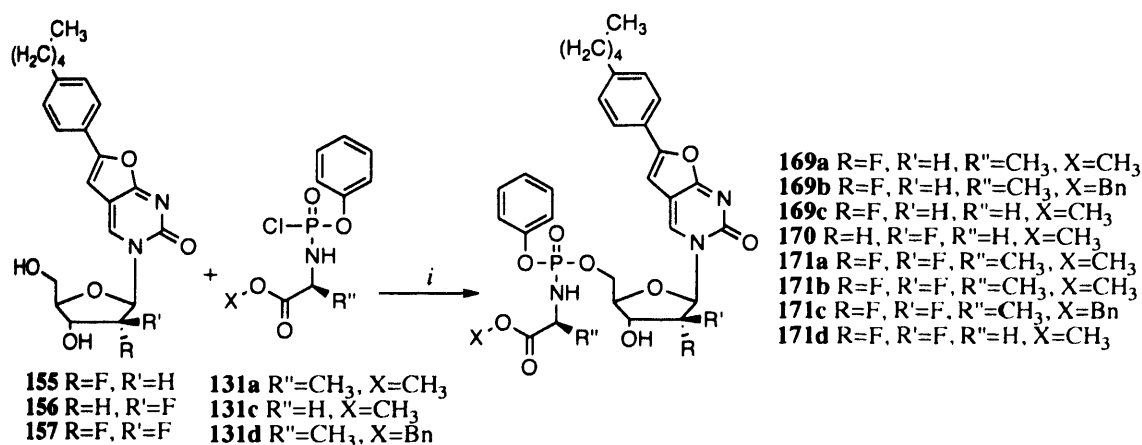
These studies support the results of the enzymatic experiment, which showed good affinity for **155**, poor affinity for compound **156**, whilst **157** showed an intermediate affinity for the enzyme.

These results are also in agreement with the result obtained using the locked NA, for which the north conformation is preferred to the south in the case of the first step of phosphorylation, whilst the south is preferred for the second phosphorylation. These results are in agreement with both enzymatic and biological results obtained.

4.4 Application of the phosphoramidate approach to the BCNA 2'-fluoro derivatives

In order to investigate the enzymatic activity and the antiviral activity found for these compounds, a series of phosphoramidates have been synthesised.

The synthesis of the phosphoramidates (**Scheme 4.4**) has been performed by coupling the appropriate nucleoside (**155-157**) with the appropriate phosphorochloridate (**131a, c, d**) using NMI as coupling reagent.



Reagents and Conditions: (i) anhydrous NMI, anhydrous THF, anhydrous pyridine, rt, 24-48 h.

Scheme 4.4: synthesis of the BCNA fluoro derivatives ProTides.

The coupling reaction proceeded well in the case of the glyciny derivatives, for which all the compounds have been isolated. In the case of the β -fluoro derivative only one compound was isolated, the glyciny derivative, whilst for the other two coupling reactions it was not possible to isolate the desired compounds as the reaction seemed not to work properly leaving starting material and only trace amount of final product on the TLC. A possible explanation for these results could be the conformation of the sugar of the β -fluoro derivative, which could be not very accessible for phosphorylation and this aspect is supported by the computational studies previously reported, showing the difference in the sugar conformation between the β -fluoro and the other two derivatives.

In the case of the gem-fluoro, the single diastereoisomer of the L-alanine methyl ester was isolated as well as the mixture of the two.

Table 4.2 reports the summary of the BCNA fluoro derivative phosphoramidates.

Cpds	F-position	Amino Acid	Ester	³¹ P NMR	¹⁹ F NMR	Yield
169a	α-fluoro	L-alanine	Me	3.90, 3.83	-203.40, -203.72	5%
169b	α-fluoro	L-alanine	Bn	3.94, 3.71	-200.55, -200.94	7%
169c	α-fluoro	Glycine	Me	5.24, 4.95	-203.47, -203.56	15%
170	β-fluoro	Glycine	Me	5.26, 5.15	-200.08	4%
171a	gem-fluoro	L-alanine	Me	3.98, 3.96	-118.46, -118.83	2%
171b	gem-fluoro	L-alanine	Me	3.89	-118.54	1%
171c	gem-fluoro	L-alanine	Bn	3.99, 3.79	-118.23, -118.44	4%
171d	gem-fluoro	Glycine	Me	5.42, 4.92	-118.13, -118.65	14%

Table 4.2: summary of BCNA fluoro derivatives phosphoramidates.

4.5 Biological evaluation of BCNA 2'-fluoro derivatives ProTides

The synthesised compounds were evaluated for their ability to inhibit VZV-TK (Table 4.3). In addition, these compounds were evaluated for their ability to inhibit two different strains of VZV TK-efficient and one strain VZV TK-deficient (Table 4.3). Compound **86f** and the parent fluoro derivatives **155**, **156** are included as a reference. The biological evaluation for the gem-fluoro ProTides is currently underway.

Compounds	Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
	TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
	YS strain	OKA strain	07/1 strain	YS strain		
169a	0.90	0.90	10.05	9.33	50	7.1
169b	-	2	10	-	50	11.3
169c	1.06	1.20	7.13	10.47	50	22.2
170	0.03	0.004	10.0	11.5	50	37.9
155	-	0.75	>20	-	>50	>100
156	0.011	0.007	>50	-	>50	-
86f	0.00031	0.00030	>2	>2	>2	>100

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 4.3: anti-VZV activity of BCNA fluoro derivatives ProTides.

From **Table 4.3** it is clear that the BCNA fluoro-derivatives ProTides retain the same activity of the corresponding parent compound. In the case of the TK-deficient strains, all the compounds showed some activity, losing, however, in potency compared to the TK-efficient strains. Interestingly, all the compounds showed cytotoxicity, and probably the activity found against the TK-deficient strains may be simply toxicity.

The antiviral evaluation for the gem-fluoro derivative ProTides is currently under way.

From these results it is clear that the phosphoramidate technology did not improve the activity for these compounds. However, these compounds have been found to be cytotoxic, in a range between 7-38 μM . Moreover, the activity found against the VZV-TK deficient strains may be toxicity.

4.6 Investigation of possible biodistribution study of a fluoro derivative of BCNA lead compound through MRI studies

Magnetic resonance imaging (MRI) is a common medical technique used in radiology. This technique allows the study of the structure and activity of the body; in fact using contrast agents is possible to enhance the appearance of specific area of the body such as blood vessels or tumors.

Using this technique it is possible to study the distribution of compounds in the body. In order to investigate the distribution of BCNA lead compound **86f** in mice a fluoro-derivative **172** has been designed and synthesised.

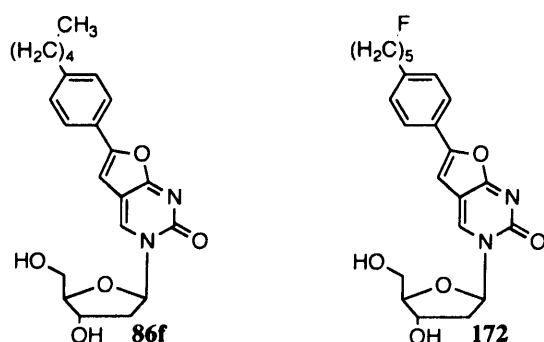


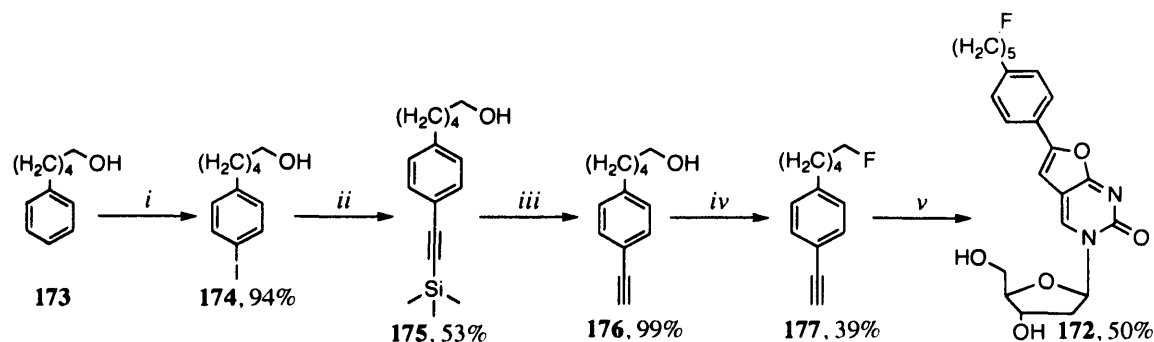
Fig. 4.8: the terminal fluoro derivative of BCNA lead compound **86f**.

Fluorine has been chosen for several reasons. Its magnetic properties can be detected by magnetic resonance and the substitution at the end of the chain with halogens gave retention of activity.

4.6.1 Synthesis of the terminal fluoro derivative of the BCNA lead compound

The synthesis of compound **172** (**Scheme 4.5**) involves a cross coupling reaction of 5-iodo-2'-deoxyuridine and 1-ethynyl-4-(5-fluoropentyl)benzene **177** under Sonogashira conditions. The alkyne derivative was synthesised in 5 steps starting from the commercially available 5-phenylpentanol-1-ol **173**. The iodination of **173** proceeded in excellent yield (94%) to give compound **174**, which was however, slightly contaminated with the *ortho*-iodo derivative. The cross-coupling of **174** with trimethylsilyl acetylene was performed under Sonogashira conditions to obtain compound **175**, which was deprotected to yield compound **176** using sodium

methoxide in methanol. The final synthone **177** was obtained by fluorination of **176** using diethylamino sulphur trifluoride (DAST).



Reagents and Conditions: (i) I_2 , sodium iodate, glacial acetic acid, H_2SO_4 , 105 °C, 1 h; (ii) trimethylsilylacetylene, tetrakis $Pd(PPh_3)_4$, CuI, anhydrous DIPEA, anhydrous DMF, rt, overnight; (iii) MeONa, anhydrous MeOH, rt, 1 h; (iv) DAST, anhydrous DCM, rt, 2 h; (v) 5-iodo-2'-deoxyuridine, tetrakis $Pd(PPh_3)_4$, CuI, anhydrous DIPEA, anhydrous DMF, rt, overnight; then CuI, anhydrous TEA, 85 °C, 7.5 h.

Scheme 4.5: synthesis of compound **172**.

4.6.2 Biological evaluation of the terminal fluoro derivative of the BCNA lead compound

The synthesised compound **172** was evaluated for its ability to inhibit the replication of VZV (Table 4.3). Data for VZV are given for the activity of these compounds versus two VZV-strains of TK-competent and also one strain of TK-deficient cells. The data includes **86f**, ACV and BVdU as a reference.

Compounds	Antiviral Activity			Cytotoxicity	
	EC ₅₀ (μM) ^a			(μM)	
	TK ⁺ VZV		TK ⁻ VZV		
	YS strain	OKA strain	07/1 strain	MCC ^b	CC ₅₀ ^c
172	0.004	0.0089	> 5	> 2	> 100
86f	0.0027	0.0014	> 0.2	> 0.2	-
ACV	4.4	16	61	>1778	1107
BVdU	0.013	0.017	4.2	>1201	1201

a) effective concentration required to reduce virus plaque formation by 50%.

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 4.3: antiviral activity against VZV for the compound **33**.

Compound **172** retains the anti-VZV activity. Unfortunately, preliminary MRI studies showed poor sensitivity for this compound probably due to the presence of just only one fluorine in the structure, which is not enough to be detected.

Further studies, including the synthesis of a trifluoroderivative and the synthesis of a radiolabeled ^{18}F derivative of **86f** have been considered.

4.7 References

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5. Improving the synthesis of FV100

FV100 is the valyl ester prodrug of Cf1743, the most active anti-VZV compound reported to date. As already mentioned, FV100 successfully completed phase I clinical trials and is currently on phase II.

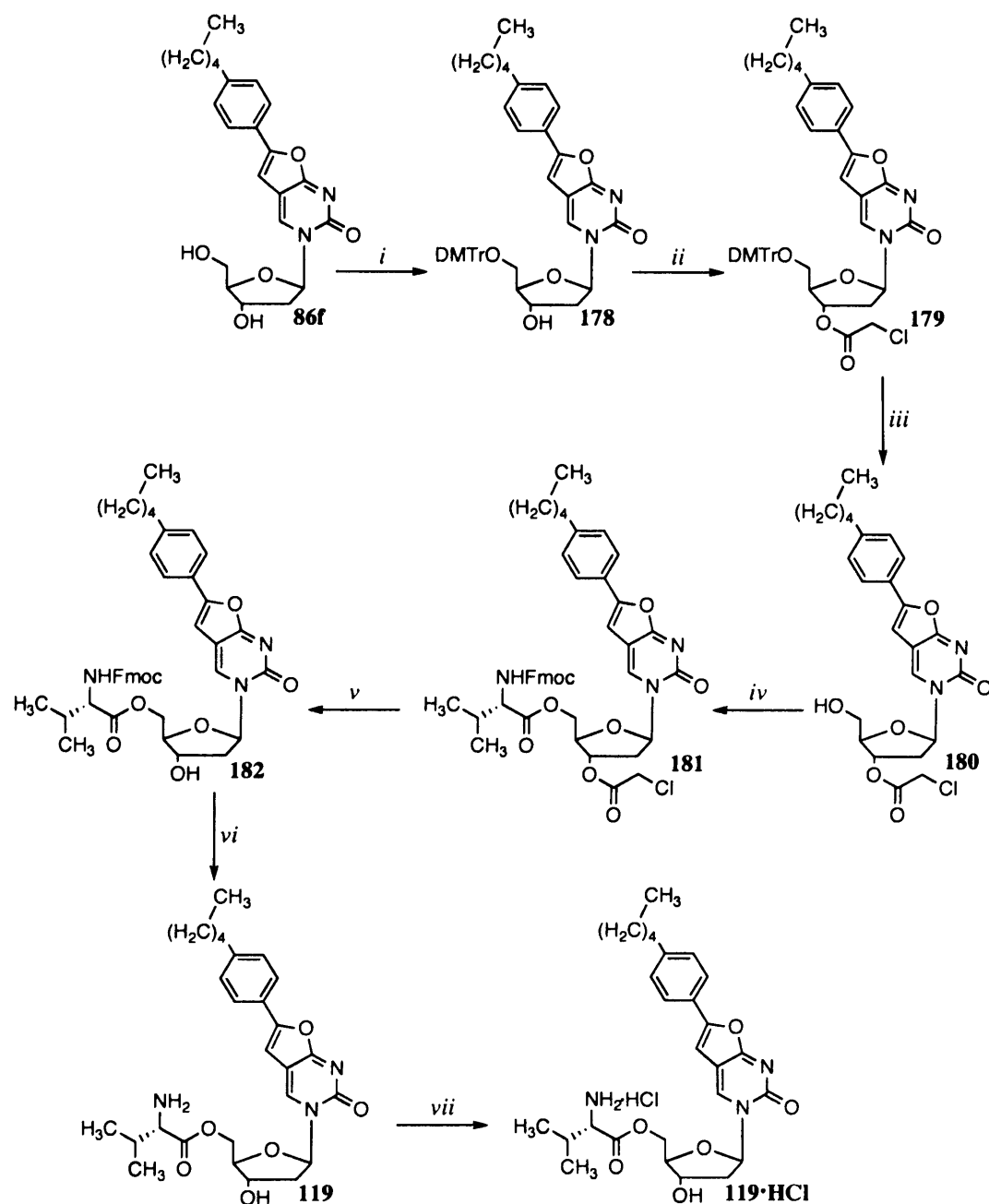
Its development as a new anti-VZV drug involves new synthetic strategies, which will cut the cost not only for trials but also for the market in the case this compound becomes clinically available.

5.1 Current scale up synthesis of FV100

The first step of the synthesis of FV-100 involves a Sonogashira cross-coupling reaction between the 5-iodo-2'-deoxyuridine and (4-pentylphenyl)acetylene in the same conditions described in Chap. 3, to provide the compound pure without recourse to chromatography.¹

The current synthetic scale up of **119** as a hydrochloride salt (**Scheme 5.1**) involves 7 steps starting from **86f**. The first step involves selective protection at the 5'-position with 4,4'-dimethoxytrityl chloride to obtain compound **178** followed by protection of the 3'-position with chloroacetic chloride to give compound **179** and selective 5'-deprotection in acidic conditions to obtain compound **180**. The overall yield for these three steps is 50%.

The coupling of compound **180** with N-9-fluorenylmethoxycarbonyl (Fmoc)-L-valine was performed using DCC and with dimethylaminopyridine (DMAP) as a catalyst to obtain compound **181**. This compound was treated with thiourea and sodium carbonate to deprotect the 3'-position giving **182** followed by deprotection of the Fmoc group using piperidine to yield compound **119**. The hydrochloride salt **119·HCl** was obtained by dissolving **119** in DCM and adding 20% HCl/isopropanol (IPA) with an overall yield from the coupling step of 74%. The compound was then further purified by crystallization to obtain the final compound. The yield for this last step was 80%. The overall yield for the synthetic route is 30%, providing 3-5 kg of **119·HCl**.



Reagents and Conditions: (i) DMTr-Cl, pyridine, DCM, rt, 30 min, then 0-5 °C, 30 min; (ii) chloroacetic anhydride, DMAP, DCM, rt; (iii) acetic acid/water, 25-45 °C, 6 h; (iv) Fmoc-L-valine, DCC, DMAP, THF, rt, 2.5 h; (v) thiourea, Na₂CO₃, DCM/EtOH, 40-45 °C, 2 h; (vi) piperidine, DCM, rt, 2.5 h; (vii) HCl/IPA, DCM, 5-10 °C, 1.5 h.

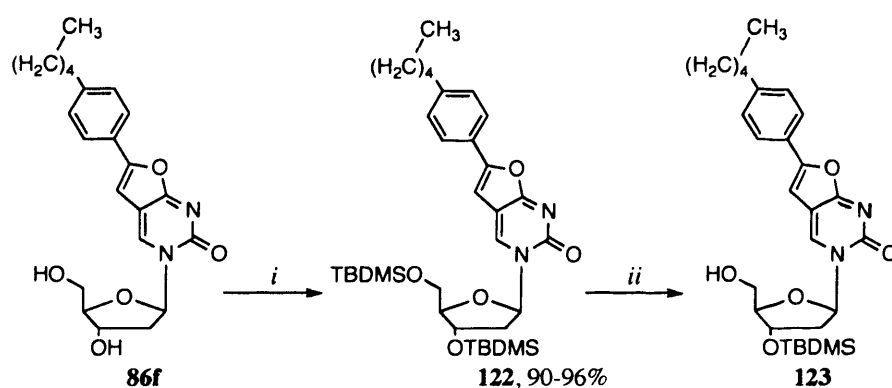
Scheme 5.1: current scale up synthesis of FV-100.

5.2 New potential synthetic routes for the synthesis of FV100

In order to improve the overall yield of this synthesis as well as to reduce the number of steps different synthetic routes have been considered.

5.2.1 Selective 5'-OTBDMS deprotection

Firstly, consideration is given to the synthesis of the first synthon, the 5'-OH-3'-protected derivative of **86f** and the synthetic route, already described in Chap. 3, was used (**Scheme 5.2**). This route involves a 3',5'-protection using TBDMS-Cl followed by selective deprotection at the 5'-position. In order to increase the overall yield, several conditions have been considered especially for the second step.



Reagents and Conditions: (i) TBDMS-Cl, imidazole, anhydrous pyridine or DMF, rt, overnight; (ii) different conditions see **Table 5.1**.

Scheme 5.2: synthetic route to obtain the first synthon **123**.

The first step involves a 3',5'-protection, to obtain compound **122**, using TBDMS-Cl in the presence of imidazole using DMF or pyridine.^{2,3} The reaction works with both solvent giving a yield of 90-96% in the case of pyridine and 89-94% in the case of DMF. However, the work-up using pyridine is easier than using DMF. Notably, the compound was obtained pure without need of purification.

For the second step several conditions have been used and they are summarized in **Table 5.1**. The best result has been obtained using a mixture a TFA/water in THF in different ratios. However, for this method, several aspects must be considered such as volume of solvent, addition of the reagents, temperature and time. The volume of solvent used is important in this step as the desired compound **123** precipitates during the reaction. It has been noted that in the presence of a large amount of solvent **123** is soluble and it can be fully deprotected. Concerning the addition of the reagents, optimum results have been obtained adding slowly cold TFA/H₂O at 0 °C and stirring

at this temperature followed by a slow increase to room temperature. As already mentioned, the desired compound precipitates during the reaction and it can be removed by filtration. The filtrate contains still starting material and the 3'-OH-5'-OTBDMS derivative as a side product can be reacted with TFA/H₂O to give a second portion of **123**, which again precipitates during the reaction. The combined fractions of **123** were washed with diethyl ether to remove the TFA, starting material and of 3'-OH-5'-OTBDMS side product. **123** was then dissolved in DCM leaving in the filter the parent compound **86f**. The compound was obtained pure without purification.

Reagents	Conditions	Yield
TFA/H ₂ O/THF = 1/1/8 ⁴	0 °C, 2 h then slowly rt, 4 h	75-83%
TFA/H ₂ O/THF = 1/1/5	0 °C, 7 h	51%
TFA/H ₂ O/THF = 1/1/6	rt, reagents add in three portions	55%
TFA/H ₂ O/THF = 3/3/10	Addition at -15 °C, then 0 °C, 4 h, then slowly rt, 3 h	80%
Acetic acid/H ₂ O/THF = 13/7/3 ⁵	rt, 5 days	65%
Acetic acid/H ₂ O/THF = 6/3/4	rt, 5 days	54%
90% TFA ⁶	0 °C, 20 min	Degradation
95% TFA/DCM = 1/1 ⁷	- 10 °C, 2 h	Degradation
TFA/DCM = 1/1 ⁸	rt, 20 min	Full deprotection
NH ₄ F, MeOH ⁹	rt, 3 days	48-58%
TBAF silica gel solid support, THF ¹⁰	rt, 6 h	Mixture of different compounds
Pyridinium- <i>p</i> -toluene sulfonate, EtOH ¹¹	50 °C, 3 h	Mixture of different compounds

Table 5.1: conditions used for the selective removal of 5'-OTBDMS.

Other methods have been used in order to increase the yield and to reduce the time. The use of more concentrated acid, which reduced the reaction time, gave degradation of the starting material, perhaps due to the acid labile glycosidic bond. In these conditions, the deprotection of the 3'-position was preferred to the 5'-position and moreover full deprotection was observed. Using mild conditions, such diluted acetic

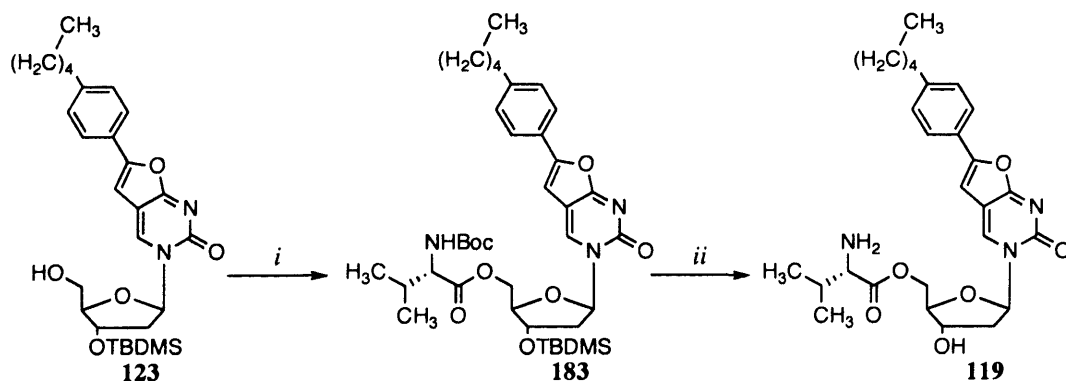
acid or ammonium fluoride, the desired compound was isolated, but the yield and the reaction time were not suitable for a scale up synthesis.

The average overall yield for these steps was 73%, with an increase of more than 20% compared to the current synthesis. Furthermore, the number of steps has been decreased from three to two.

5.2.2 Synthesis of FV100 from the Boc-L-valine derivative

N-(tert-butoxycarbonyl) (Boc) has been chosen as a protecting group in this particular route as it is an acid labile group so its deprotection as well as the deprotection of the 3'-OTBDMS may be achieved in one reaction (**Scheme 5.3**). Moreover, the use of hydrochloric acid could give directly FV100 as a salt reducing further the number of steps.

The coupling with **123** and Boc-L-valine was performed using the same conditions used in the current scale up synthesis using DCC and a catalytic amount of DMAP. The desired compound **183** was obtained and used without further purification in the deprotection step. In order to understand and to investigate the deprotection step, different conditions have been used (**Table 5.2**).



Scheme 5.3: synthesis of FV100 through Boc-L-valine derivative **183**.

The TBDMS group is quite labile and easy to remove under the majority of the conditions used (**Table 5.2**). By contrast, the Boc-moiety has been found to be particularly resistant to the conditions applied and in particular under conditions in which it is usually removed, such as 4N HCl in water, which is used in the synthesis of valaciclovir. Due to the poor stability of the glycosidic bond, it was not possible to increase either the reaction time or the concentration of the acid. In fact, in some cases

the cleavage of the glycosidic bond has been observed along with the formation of degradation products by TLC.

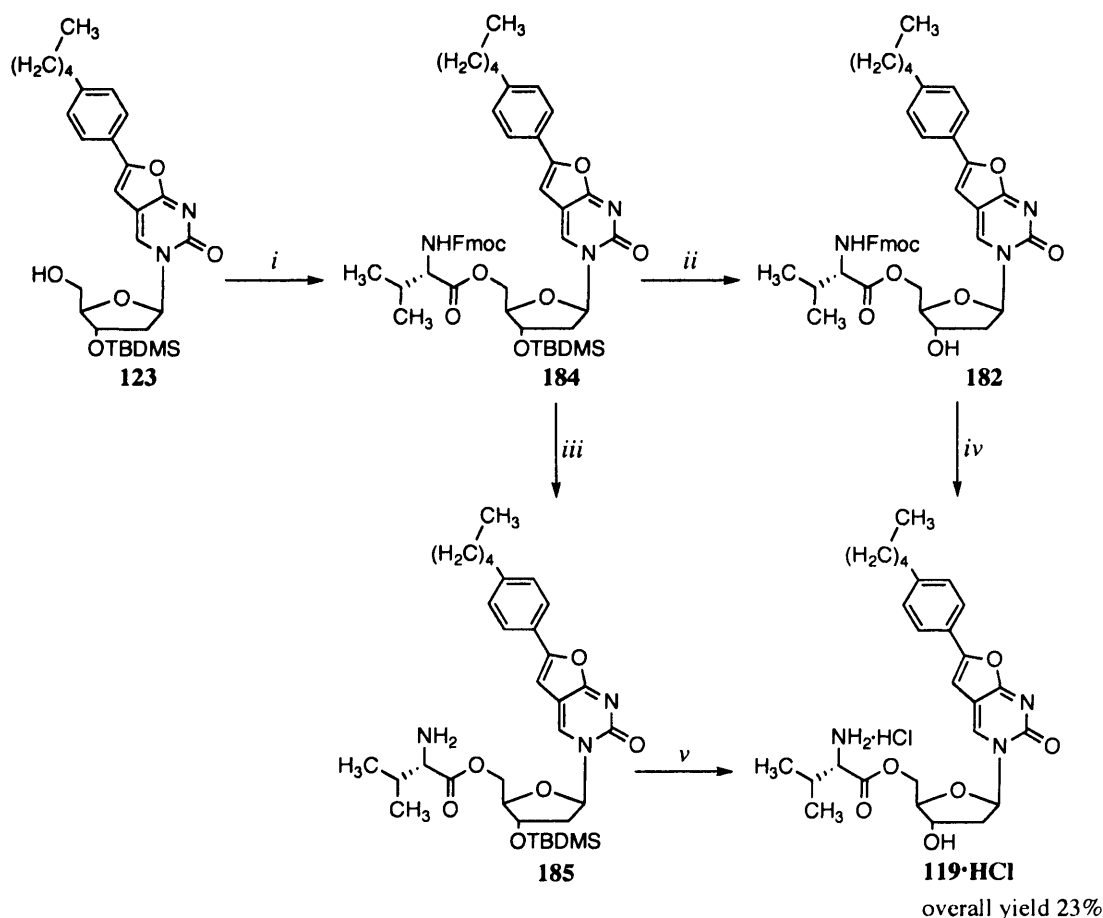
Reagents	Conditions	Results
TFA/H ₂ O/THF = 1/1/4 ⁴	rt, 24 h	TBDMS deprotection
TFA/DCM = 1/1 ¹²	rt, 1.5 h	Cleavage of glycosidic bond
TFA/MeOH = 1/1 ¹²	Sequential addition at 0 °C rt, 20 h	TBDMS deprotection and degradation
TFA/dioxane = 1/1 ¹²	Sequential addition at 0 °C rt, 20 h	TBDMS deprotection and degradation
1N HCl/THF = 2/1 ¹³	rt, 1 day	Degradation
4N HCl/ H ₂ O = 1/2 ¹⁴	rt, 1 day	Partial TBDMS deprotection
12N HCl/H ₂ O = 0.1/2 ¹⁴	rt, 1 day	No reaction
0.01 N HCl	rt, 1 day	Not soluble
20% HCl in IPA/IPA = 1/1 ¹⁵	Sequential addition, rt, 6 h	Partial TBDMS deprotection, cleavage of glycosidic bond
20% HCl in IPA/IPA = 5/3 ¹⁵	Sequential addition at 0 °C rt, 1 day	Partial TBDMS deprotection, cleavage of glycosidic bond
4N HCl/Dioxane = 1/2	Addition at 0 °C, rt, 36 h	Partial TBDMS deprotection, degradation
4N HCl/MeOH = 1/6	Addition at 0 °C, rt, 36 h	Partial TBDMS deprotection, degradation
10% H ₂ SO ₄ /Dioxane = 1/5 ¹²	rt, 5h	TBDMS deprotection
<i>p</i> -TsOH·H ₂ O, MeOH ¹²	rt, 22h	Degradation
<i>p</i> -TsOH·H ₂ O, EtOAc	50 °C, overnight	No reaction
TBDMSCl, MeOH ¹²	Reflux, 4.5 h	Cleavage of glycosidic bond
TBDMSCl, MeOH	rt, 22h	Degradation
TBAF solid support, THF ¹⁰	rt, 40 h	TBDMS deprotection
H ₂ O	Reflux, 4 days	No deprotection of Boc
CAN, acetonitrile ¹⁶	Reflux, 2 days	No reaction
1M BCl ₃ in DCM, DCM	Addition at 0 °C, rt 3 h	TBDMS deprotection, degradation

Table 5.2: conditions used for the deprotection of BOC and 3'-OTBDMS.

Although promising, in terms of number of steps, unfortunately this synthetic route failed.

5.2.3 Synthesis of FV100 from the Fmoc-L-valine derivative

The second route (**Scheme 5.4**) considered for the synthesis of FV100 used Fmoc as the protecting group of the L-valine. For the synthesis of **184**, the same procedure already described was used. The desired compound was obtained and used as crude, however, it was not completely pure and it was contaminated with Fmoc-L-valine.



Reagents and Conditions: (i) Fmoc-L-valine, DCC, DMAP, rt, overnight; (ii) TFA/H₂O/THF = 1/1/4, rt, overnight; (iii) piperidine, DCM, rt, 10 min; (iv) piperidine, DCM, rt, 10 min, then 20% HCl/IPA, 5-10 °C, 1.5 h; 20% HCl/IPA, rt, 2-4 h, then 0-5 °C, overnight.

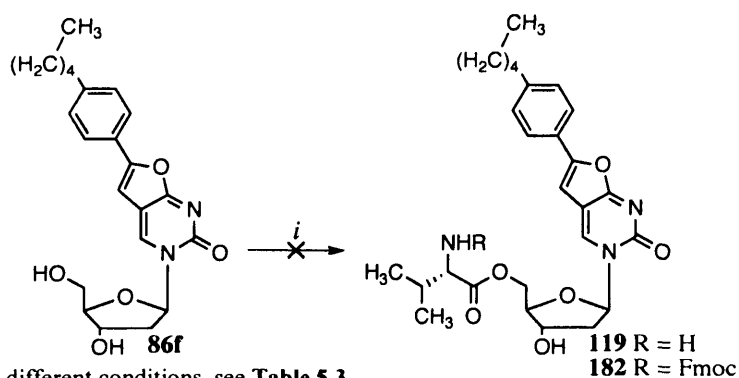
Scheme 5.4: synthesis of FV100 through Fmoc-L-valine derivative **184**.

To obtain compound **119**, two different strategies have been followed: the first one was the deprotection of the TBDMS group using TFA/H₂O/THF (1/1/8) followed by deprotection of the Fmoc group using piperidine and then the formation of the chloridate using 20% HCl/IPA. The second strategy was the removal of the Fmoc group followed by the deprotection of the TBDMS group and formation of the salt in

one reaction. This last reaction was successful and **119·HCl** was isolated pure without purification. The overall yield for this route was 23%. However, it must be said that compound **184** was used not completely pure and this could have influenced the deprotection step. Due to time constraints, this route has not been further investigated.

5.2.4 Direct esterification of the unprotected nucleoside using lipase

Another attempt to reduce the number of steps has been attempted using an enzymatic procedure. In this way, the specificity given by the enzyme in the esterification step may allow the use of unprotected nucleoside. Lipase has been used for its selectivity in the esterification process, for example, in the selective acylation of ribavirin with L-alanine¹⁹ or in the selective 5'-acetylation of 6-methoxy-ara-guanosine.²⁰ Different methods and conditions have been used for this route (**Scheme 5.5**) and **Table 5.3** summarises them.



Reagents and Conditions: (i) different conditions, see **Table 5.3**.

Scheme 5.5: synthesis of FV100 using unprotected **86f**.

Unfortunately, all the different conditions used in this new route were not successful for the synthesis of FV100. In fact, only starting material was recovered after the reaction.

However, there are several key factors to be considered in getting these lipase reactions to work in a synthetic direction. It has been reported that the solvent can widely affect the reaction in terms of selectivity and yield,²⁰ however being purely empirical, a number of different solvents need to be tried, especially considering the poor solubility of the parent compound **86f**.

The type of enzyme used is also critical. The lipase from *Candida antarctica* in solid support (Novozyme-435) is widely used and reported.¹⁸⁻²⁰ However, several other lipases are available, but not all lipases work well and several attempts using different

lipases need to be performed. It may be that the enzymes could be used on a more soluble and reactive precursor of **86f** and this is currently underway at our sponsor.

Reagents	Conditions	Results
L-valine, lipase from <i>Candida rugosa</i> type VIII, DCM/DMF, pH4 acetate buffer ¹⁷	Reflux, 3 days	Negative
Fmoc-L-valine, lipase from <i>Candida rugosa</i> type VIII, DCM/DMF, pH4 acetate buffer ¹⁷	Reflux, 3 days	Negative
L-valine, lipase acrylic resin from <i>Candida antarctica</i> , THF ¹⁸	Reflux, 3 days	Negative
L-valine ethyl ester, lipase acrylic resin from <i>Candida antarctica</i> , THF ¹⁸	Reflux, 3 days	Negative
Fmoc-L-valine, lipase acrylic resin from <i>Candida antarctica</i> , THF ¹⁸	Reflux, 3 days	Negative
Fmoc-L-valine-acetoxime, lipase acrylic resin from <i>Candida antarctica</i> , THF, DMF ^{19, 20}	65 °C, 1 week	Negative
Fmoc-L-valine-acetoxime, lipase acrylic resin from <i>Candida antarctica</i> , DMF	65 °C, 1 day, 85 °C 2 days	Negative

Table 5.3: conditions used for the synthesis of FV100 using unprotected **86f**.

5.3 Conclusion

The improvement of the synthesis of FV100, plays at this point of its development a crucial role in order to reduce the production cost. At present the current scale up synthesis involves seven steps: for the first three steps, to obtain the 5'-OH-3'-protected derivative of **86f**, the overall yield was 50%, for the following four steps, to obtain **119·HCl** was 74%, whilst the last crystallization step gave 80% yield. The corresponding overall yield for this synthetic route is 30%.

In the present work, a consistent improvement for the synthesis of the first synthon has been made improving by more than 20% the overall yield, from 50% to 73%, and reducing the number of steps from three to two.

For the second part of the synthetic route different strategies have been used.

The use of Boc protected amino acid was particularly fascinating in terms of further reduction of number of steps, being that Boc is an acid labile group. However, the deprotection step was not successful as the Boc group was extremely stable under the conditions in which the glycosidic bond was stable.

The use of Fmoc as the protecting group was more successful than the Boc route and the final compound was isolated with an overall yield of 23%, which is less than the overall yield obtained from the current scale up synthesis (74%).

The last attempt to reduce the number of steps and to increase the overall yield has been made using the direct esterification of the unprotected nucleoside using lipase. All the different conditions used resulted in failure. However, due to time constraints, this route has not been further investigated.

In conclusion, a new route for the synthesis of **119·HCl** has been preliminary developed. This route includes five steps with an overall yield of 17%, whilst the current synthesis involves seven steps and an overall yield of 30%.

In spite of having less synthetic yield, the new route needs to be further investigated especially in the second part of the synthesis in order to be used for a scale up synthesis.

5.4 References

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6. Ribavirin ProTides

6.1 Ribavirin background

As already seen in the introduction, ribavirin **50** is a nucleoside analogue with antiviral activity against a number of DNA and RNA viruses *in vitro* and *in vivo*.¹ Ribavirin is converted by intracellular phosphorylation (**Fig. 6.1**) mediated by adenosine kinase to the monophosphate **186**, followed by further phosphorylation mediated by cellular kinases to the diphosphate **187** and to the triphosphate **188**.²

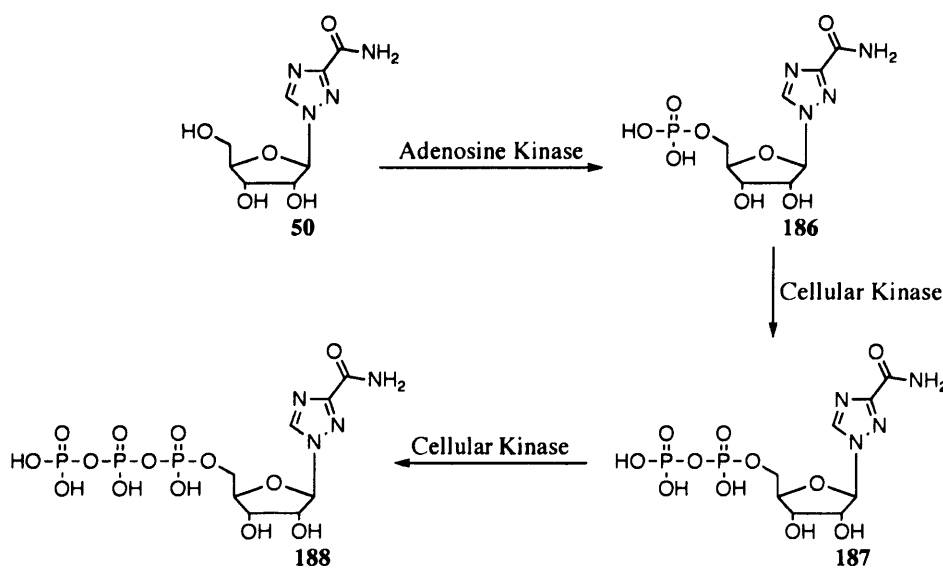


Fig. 6.1: ribavirin and its subsequent phosphorylation products.

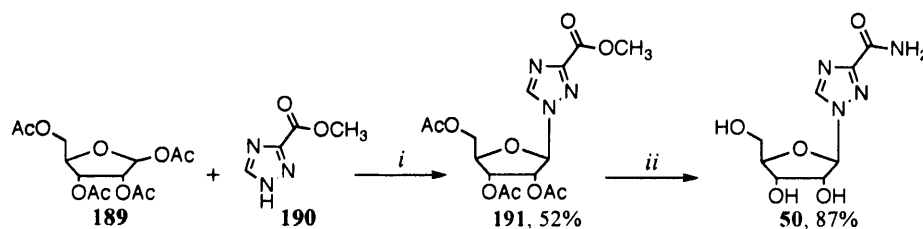
Phosphorylations to the phosphate forms of ribavirin are essential for its activity but the exact mechanism of action is unclear and may vary. The phosphorylation to monophosphate is the rate limiting step at least in some cases.³ The ProTide technology has been applied to ribavirin in order to improve and extend its activity against different viruses.

A small series of phosphoramidates with different lipophilicities (CLogP) has been synthesised with the aim to study the SAR of these compounds.

6.2 Synthesis of ribavirin

Ribavirin has been synthesized following the procedure reported by Ramasamy *et al.* (**Scheme 6.1**).⁴ Condensation of commercially available 1,2,3,5-tetra-O-acetyl- β -D-ribofuranose **189** and 1,2,4-triazole-3-carboxylate **190** in the presence of bis(*p*-nitrophenyl)phosphate provided compound **191** as pure β -anomer confirmed

by the chemical shift presented in the literature. Deprotection of **191** using methanolic ammonia at room temperature for 12 h gave ribavirin **50**.

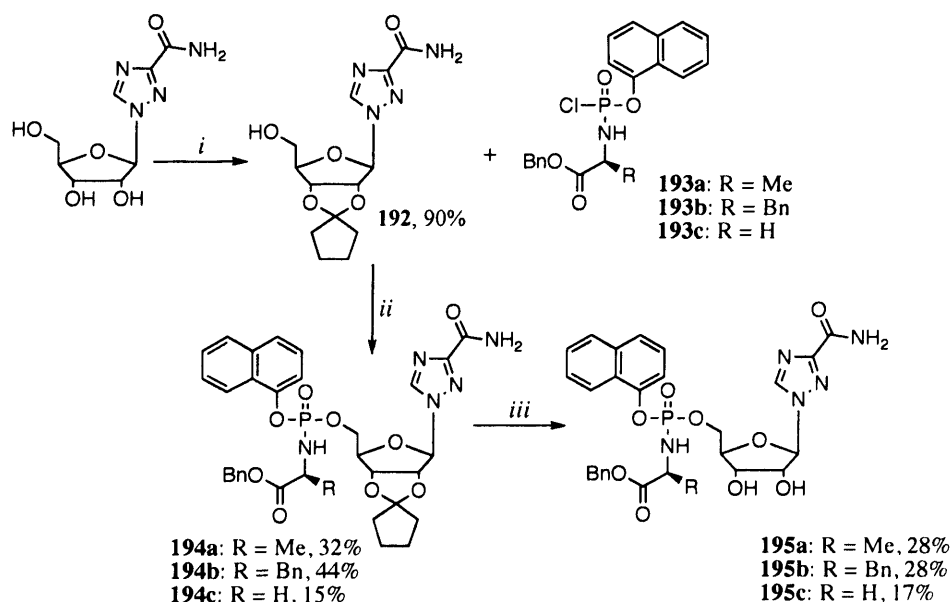


Scheme 6.1: synthesis of ribavirin.

6.3 Synthesis of ribavirin phosphoramidates

In order to improve the poor solubility of ribavirin and to prevent the formation of side products, the 2' and 3' positions of the sugar were protected with cyclopentanone using perchloric acid as catalyst to give the desired compound **192** (Scheme 6.2).

Compound **192** was coupled with the appropriate phosphorochloridates **193a-c** using ^tBuMgCl as the coupling reagent to give the desired compounds **194a-c** (Scheme 6.2).⁵



Scheme 6.2: Synthesis of ribavirin ProTides.

The protecting group was then removed using 60% formic acid at room temperature overnight giving the desired final compounds **195a-c**. All of these

compounds have been obtained as mixtures of two diastereoisomers, confirmed by the presence of two peaks on ^{31}P NMR. Mass spectroscopy, elemental analysis and HPLC confirmed the structures and the purity for these compounds.

6.4 Biological evaluation of ribavirin ProTides

The synthesised compounds **195a-c** were evaluated for their ability to inhibit the replication of different viruses (Table 6.1). All compounds display increased ClogP values compared to ribavirin, which, most likely, enhances their cellular permeability.

Cpds	CLogP	RSV	Antiviral Activity EC ₅₀ (μM) ^a			Cytotoxicity (μM)
			HCV Huh-5-2	Influenza A H3N2 subtype	Influenza B	
195a	0.96	45	68	18	14	>100
195b	2.38	>20	>70	>100	>100	>100
195c	0.84	58	-	>20	>20	>100
50	-2.85	19	87±22	9	9	>100

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

Table 6.1: biological evaluation of ribavirin ProTides.

Although active, the ProTides did not show any improvement of activity compared to the parent compound, their antiviral concentrations being, at best, in the 14-100 μM range. In the case of RSV, for which ribavirin is clinically approved, the L-alanine **195a** and glycine **195c** ProTides were about 3-fold less active than the parent nucleoside. Compound **195a** proved equipotent to ribavirin in inhibiting HCV subgenomic replicon replication. ProTide **195a** was active against influenza virus (its potency being ~2- fold lower than that of ribavirin). For all the viruses tested, the phenylalaninyl ProTide **195b** had no or borderline activity at the highest concentration tested (100 μM).

No relationship between lipophilicity and activity has been found for these compounds.

All three ProTides were non-toxic to uninfected cells at 100 μM, as was ribavirin.

No activity (>100 μM) has been found against parainfluenza-3-virus, reovirus-1,

sindbis virus, coxsackie virus B4, HIV-1, HIV-2, HSV-1, HSV-2, HSV-1 TK-deficient strain, CMV, feline corona virus, feline herpes virus (data not shown).

In order to understand the relatively poor activity of these ProTides, which is suggestive of ineffective activation, enzymatic and preliminary molecular modeling studies were performed.

6.5 Enzymatic studies using carboxypeptidase Y enzyme

The putative mechanism of activation of phosphoramidate prodrugs (**Fig. 6.2**), as mention in Chap. 1, involves an initial hydrolysis of the carboxylic ester by a carboxypeptidase type enzyme obtaining compound **196** with subsequent spontaneous cyclisation with displacement of the aryloxy group giving compound **197** followed by opening of the unstable ring mediated by water to get compound **198**. The last step in the bioactivation involves the hydrolysis of the P-N bond by a phosphoramidase-type enzyme to obtain the monophosphate form of ribavirin **186**.^{6, 7}

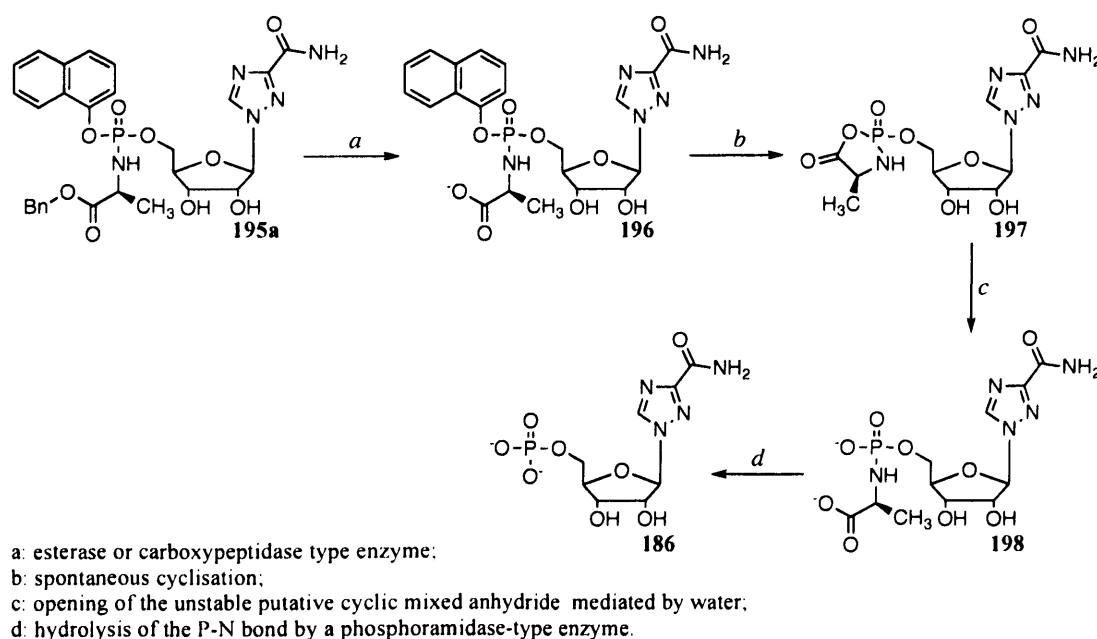


Fig. 6.2: putative mechanism of activation of ribavirin ProTide **195a**.

An enzymatic study using carboxypeptidase Y has been performed in order to investigate whether ribavirin ProTide can be metabolised, following the procedure already described by Birkus.⁸

195a was incubated with carboxypeptidase Y in *d*₆-acetone and Trizma buffer (pH = 7.6) and the enzyme reaction was followed by ³¹P NMR. The spectra (**Fig. 6.3**)

shows fast metabolism of the starting material ($\delta_P = 4$) to the putative intermediate **198** ($\delta_P = 7$), the reaction being completed within 1.5-2 h. The suggestion that the reaction product is structure **198** is based on two factors. Firstly, while **195a** contains a chiral phosphorus and displays two phosphorus signals in the NMR, the ($\delta_P = 7$) product elicits only one signal, suggestive of an achiral phosphorus, as in **198**. Secondly, other reports of amino aryl phosphates such as **198** indicate a chemical shift of this magnitude.⁹ Close examination of the early (3-30 min) spectra shows a double peak at $\delta_P < 5$, indicative of a chiral phosphorus and consistent with the proposed activation pathway. This species is considered to be the initial cleavage product **196** on the basis of similar analogues.⁹ This enzyme experiment indicates that the first activation step of Protide **195a** is sufficiently efficient and comparable in rate to cases where ProTides show good biological efficacy.¹⁰

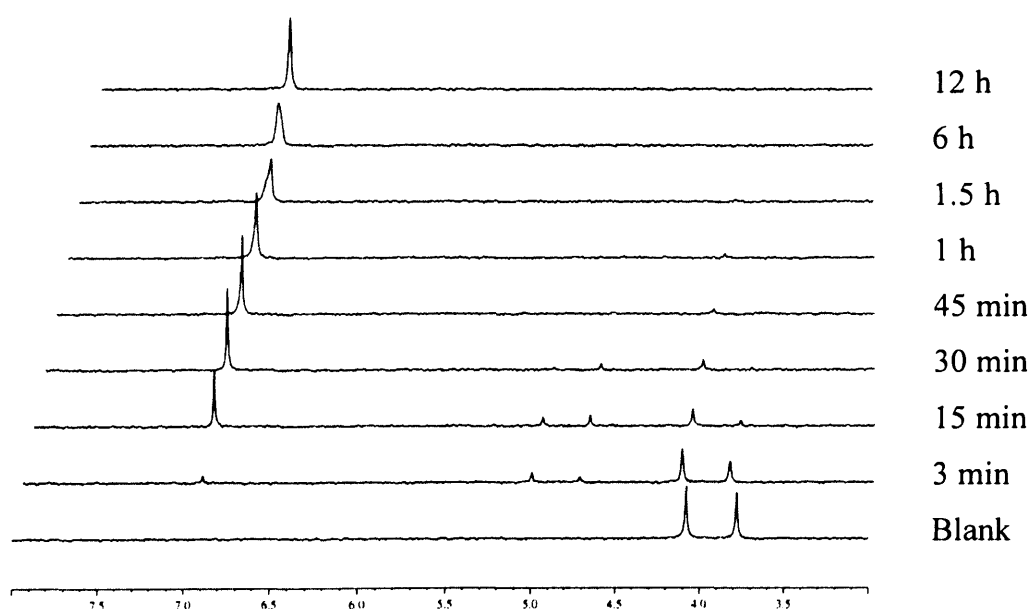


Fig 6.3: enzymatic experiment of compound **195a** with carboxypeptidase Y.

6.6 Molecular modelling studies using human hint enzyme

The last step in the bioactivation pathway involves hydrolysis of the P-N bond by a phosphoramidase-type enzyme.^{11, 12}

Preliminary molecular modelling studies using docking techniques were performed in order to understand if ribavirin L-alanine phosphate **198**, putative

intermediate obtained during the enzymatic experiment, could be a good substrate for the human hint enzyme.

This enzyme is considered to be responsible for the cleavage of the phosphorus-nitrogen bond in the final step of the phosphoramidate activation. It belongs to the HIT superfamily.¹³ The proposed mechanism of action of this enzyme (Fig. 6.4) involves an attack of the histidine 112 (red) to the phosphorous followed by the release of the amino moiety, which is favoured by the protonation mediated by the serine (green). Subsequently, a molecule of water attacks the phosphorous with the release of the histidine (red) and the protonation of the serine (green).¹³

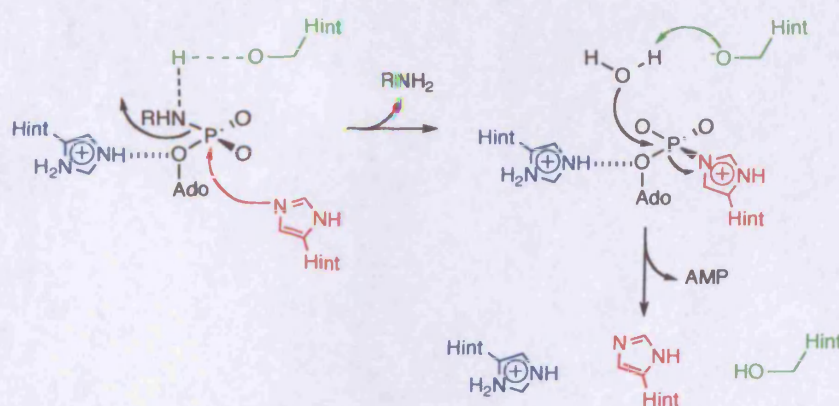


Fig. 6.4: proposed mechanism of action of Hint.

Docking studies were carried out using a co-crystallised structure of adenosine monophosphate (green structure on Fig. 6.5) with the enzyme in order to identify the catalytic site. From these studies (Fig. 6.5) it appears that ribavirin L-alanine phosphate is not able to interact with the catalytic site of the enzyme; in fact, the phosphate moiety is placed just outside the binding pocket. (see Chap.7 as a good example of a docking on Hint enzyme).

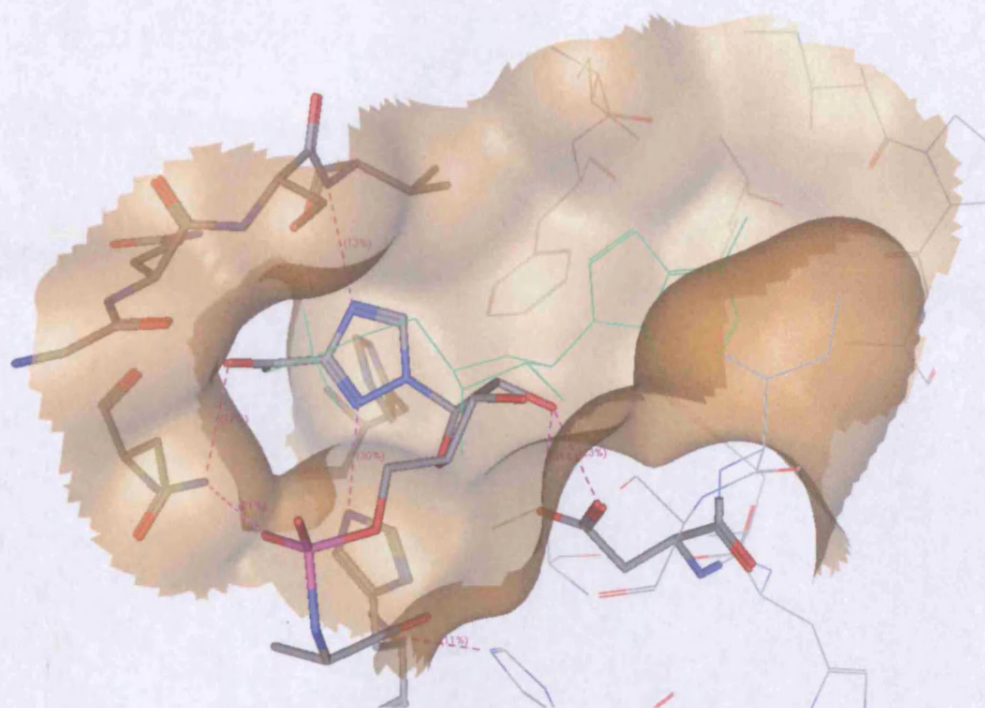


Fig 6.5: docking of compound 198 with Hint-enzyme.

These results support that the lack of activity found for this compound could be due to the poor deliver of the monophosphate form inside the cells.

6.7 Conclusion

To summarise, a series of novel ribavirin ProTides has been synthesised. No improvement/extension of activity has been found. A likely explanation for this aspect is the relatively poor activation of the ProTide to the free monophosphate. Enzyme assays indicate that the first step of activation is efficient and that the blockade may be at the final P-N cleavage step by HINT. This is consistent with our preliminary molecular modeling data with the HINT enzyme.

6.8 References

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7. Acyclovir ProTides

7.1 Acyclovir background

ACV (**Fig 7.1, 9**) (2-amino-9-[(2-hydroxyethoxy)methyl]-3,9-dihydro-6H-purin-6-one), as already seen in Chap. 1, is a guanine analogue antiviral drug. ACV and its prodrug are the current treatment of choice for HSV and VZV infections.¹

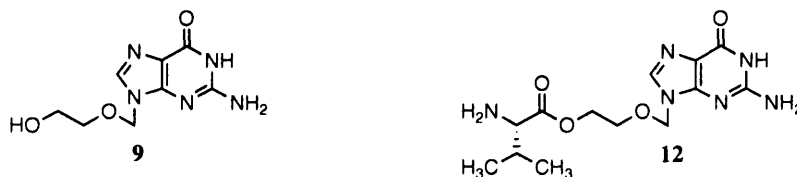


Fig. 7.1: ACV and its prodrug valacyclovir.

The mechanism of action involves phosphorylation to give the monophosphate, which is selective in the infected cells and is mediated by a herpes virus-specified TK.² The monophosphate is subsequently converted to the di- and triphosphate by cellular kinases. In the active triphosphate form, ACV inhibits the HSV polymerases 10-30 times more than cellular DNA polymerases.²

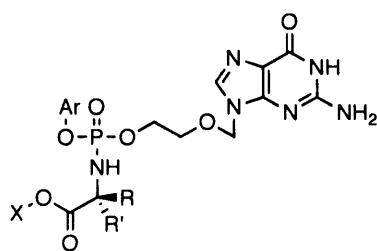
To overcome the major problem of ACV, its poor oral bioavailability, a series of prodrugs have been synthesised. The L-valyl ester of ACV (valacyclovir, **12**) is metabolised in the gut and in the liver by valacyclovir hydrolase to ACV,³ increasing its bioavailability by *ca.* 4 fold.⁴

HHV encoded TK specifically phosphorylates ACV to the monophosphate form. This step of phosphorylation gives the selectivity of this compound for certain viruses, especially HSV and VZV. However, due to the large use of ACV and valacyclovir, resistance to these drugs is increasing. The resistance is due to a lack of phosphorylation by a loss of viral TK activity or by an alteration of the viral polymerase for which ACV-TP is not a good substrate. The by-pass of the first step of phosphorylation may be achieved by applying the ProTide technology to ACV. The intracellular delivery of the monophosphate may lead to a reduced antiviral selectivity, but the activity against TK-deficient ACV resistant HSV can be enhanced. Moreover, activity may also be broadened to other viruses.

7.2 Design of acyclovir phosphoramidates

Previously, we reported the synthesis of a masked phosphate (**199a**, Fig. 7.2), using the phosphoramidate technology, with the purpose to obtain a nucleoside kinase-independent antiviral.⁵ The data on **199a** shows that the activity is similar to ACV against HCMV and VZV, whilst surprisingly the compound was totally inactive against HSV-2, while ACV is active.⁵

In order to improve the activity of **199a**, a new series of ACV phosphoramidates has been synthesised (**199b-m**, Fig. 7.2).



Compounds	Ar	R	R'	X	CLogP
199a	Ph	Me	H	Me	-0.82
199b	Ph	Me	H	Bn	0.89
199c	Ph	Me	H	ⁱ Pr	0.02
199d	Ph	H	Me	Bn	0.89
199e	<i>p</i> -F-Ph	Me	H	Bn	1.11
199f	Naph	Me	H	Me	0.35
199g	Naph	Me	H	ⁿ Pr	1.41
199h	Naph	Me	H	Bn	2.06
199i	Ph	Bn	H	Bn	2.31
199j	Naph	Me	Me	Bn	2.37
199k ⁶	Naph	Me	H	Et	0.88
199l ⁶	Naph	Me	H	ⁱ Pr	1.19
199m ⁶	Naph	Me	H	^t Bu	1.59

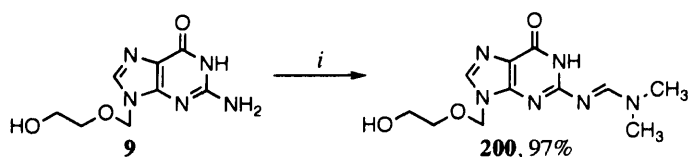
Fig. 7.2: ACV phosphoramidates.

As already seen in Chap. 1, the ProTide moiety has three different changeable parts: the aryl moiety, the amino acid and the ester. In the first part of this study L-alanine was chosen as the amino acid, as it has shown an optimal biological profile,⁷ whilst varying the other two components. For the aryl moiety were considered: phenol, 1-naphthol and *p*-fluoro-phenol and as ester moiety: methyl,

ethyl, *n*-propyl, *iso*-propyl, *tert*-butyl and benzyl and combinations thereof. All these combinations allowed us to extensively vary the CLogP for these compounds and to study how this variation can influence the antiviral activity. For example, 1-naphthol was chosen to improve the lipophilicity and for its leaving group ability, fundamental in the activation of phosphoramidates. Moreover, an enhancement of activity against a panel of cancer cell lines for naphthol phosphoramidates of other nucleosides has been reported.^{8, 9} In addition, L-phenylalanine and the non-natural amino acids D-alanine and dimethylglycine have been considered.

7.3 Synthesis of acyclovir ProTides

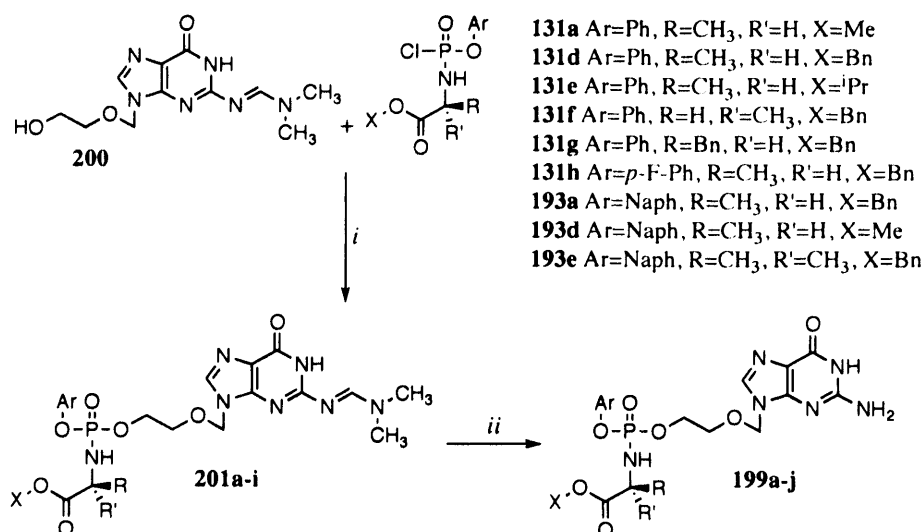
In order to improve the poor solubility of ACV in the optimal solvent (THF), usually used for the preparation of phosphoramidates, the guanine base was protected using *N,N*-dimethylformamide dimethyl acetal (**Scheme 7.1**).¹⁰



Reagents and Conditions: (*i*) *N,N*-dimethylformamide dimethyl acetal, DMF, rt, 1 day.

Scheme 7.1: protection of ACV with DMF.

The solubility was improved sufficiently and compound **200** was coupled (**Scheme 7.2**) with the appropriate phosphorochloridate (**131a**, **d-h**, **193a**, **d**, **e**) using ^tBuMgCl as a hydroxyl activator,¹¹ in anhydrous THF giving the desired compounds (**201a-j**). The deprotection step was initially carried out by refluxing the N₂-DMF-protected ProTides in *n*-propanol. However, due to a transesterification during the synthesis of **199h**, obtaining compound **199g**, the solvent was changed to 2-propanol obtaining the desired compounds (**199a-f**, **h**, **i**).



Reagents and Conditions: (i) ^tBuMgCl, anhydrous THF, rt, overnight; (ii) 1-propanol or 2-propanol, reflux, 18-96 h.

Scheme 7.2: synthesis of ACV ProTides.

All the compounds have been obtained as mixtures of two diastereoisomers confirmed by the presence of two peaks in the ³¹P-NMR, with the exception of the dimethylglycine derivative **199j**, due to the absence of a second chiral center.

Table 7.1 summarises the ACV ProTides made.

Compounds	Ar	R	R'	X	³¹ P NMR	Yield
199a	Ph	Me	H	Me	3.72, 3.55	13%
199b	Ph	Me	H	Bn	3.80, 3.50	14%
199c	Ph	Me	H	ⁱ Pr	3.80, 3.65	7%
199d	Ph	H	Me	Bn	3.80, 3.50	25%
199e	<i>p</i> -F-Ph	Me	H	Bn	4.02, 3.78	16%
199f	Naph	Me	H	Me	4.07, 4.05	7%
199g	Naph	Me	H	ⁿ Pr	4.09, 4.01	4%
199h	Naph	Me	H	Bn	4.13, 3.96	35%
199i	Ph	Bn	H	Bn	3.41, 3.31	14%
199j	Naph	Me	Me	Bn	2.52	23%

Table 7.1: summary of ACV ProTides

7.4 Biological evaluation of acyclovir ProTides

The synthesised compounds **199a-j** and the previously reported **199k-m**⁶ were evaluated for their ability to inhibit the replication of different viruses.

7.4.1 Anti-HSV activity of acyclovir ProTides

Table 7.2 reports the antiviral activity of ACV ProTides versus HSV-1 (TK efficient and TK deficient strains) and HSV-2.

Compounds	CLogP	Antiviral Activity EC ₅₀ (μM) ^a			Cytotoxicity (μM)	
		HSV-1	HSV-2	HSV-1 TK ⁻	MCC ^b	IC ₅₀ ^c
199a	-0.82	20±0	16±5.7	79±29	>100	-
199b	0.89	8±5.7	4±0	15±7.1	>50	91
199c	0.02	10±0	8.5±0.7	27±25	>50	>100
199d	0.89	2±0	1.4±0.8	23±16	>100	-
199e	1.11	0.9±0.1	0.5±0	1.5±0.7	>100	-
199f	0.35	16±5.7	10±2.1	79±29	>50	>100
199g	1.41	5.5±2.1	1.9±1.6	16±5.7	≥50	68
199h	2.06	2±0	1.4±0.8	10±2.1	≥20	20
199i	2.31	17±4.2	8±5.7	≥100	>50	87
199j	2.37	2.4±0	1.6±1.1	3.2±1.1	>50	>100
199k ⁶	0.88	32±25	9.5±0.7	32±18	>150	>100
199l ⁶	1.19	15±7.1	10±0	≥45	>50	>100
199m ⁶	1.59	>100	50	>100	>50	>100
ACV	- 2.42	0.4	0.2	50	>100	-

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) inhibitory concentration required to inhibit cell proliferation by 50%.

Table 7.2: antiviral activity against HSV-1 and 2 for ACV ProTides including ACV as a control.

The antiviral activity of ACV was in the sub-micromolar range versus HSV-TK efficient strain, whilst it lost activity versus the HSV-1 TK-deficient strain (50 μM). Interestingly, almost all the ACV ProTides showed activity against all the strains with some exceptions. The majority of the compounds showed activity in the range 1-30 μM and notably retaining a good activity against the TK-deficient

strain. Only one compound **199m**, bearing the ^tbutyl group as an ester did not show any activity. The lack on activity for this compound will be discussed later in this chapter. No relation between the lipophilicity and activity has been found for these compounds. The activity of several ACV ProTides versus the HSV-1 TK-deficient strain demonstrates a successful kinase bypass for these compounds. Moreover, in spite of being less selective due to the viral kinase bypass, these compounds did not show appreciable cytotoxicity.

7.4.2 Anti-VZV activity of ACV ProTides

Table 7.3 reports the antiviral activity of ACV ProTides versus VZV (two strains of TK efficient and two strains of TK deficient).

Cpds	CLogP	Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
		TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
		YS strain	OKA strain	07/1 strain	YS strain		
199a	-0.82	20	19	24	16	>100	162
199b	0.89	1.0	0.72	1.8	0.59	>50	>100
199f	0.35	10.9	7.6	22	6.1	>50	>100
199g	1.41	2.8	1.4	2.5	-	>50	-
199h	2.06	3.3	7.2	6.9	-	>50	20
199i	2.31	6.6	5.2	8.4	10.0	>50	86.9
199j	2.37	1.7	5.5	6.0	3.33	>100	>100
ACV	- 2.42	2.9	2.5	61	43	>500	1350

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 7.3: antiviral activity against VZV for ACV ProTides including ACV as a control.

Most of the compounds showed good potency in these assays, showing retention of activity compared to ACV. Notably, several of the agents retain activity versus TK deficient strains, whilst ACV showed a loss of activity. Interestingly, compound **199b** showed submicromolar activity and it retained activity versus TK deficient strains. Moreover, the compounds tested did not show cytotoxicity while compound **199h** did show some toxicity (20 μM).

In this case as well no clear relation between lipophilicity and activity has been found for these compounds. However, compound **199a**, which has the lowest CLogP, showed the lowest antiviral activity and it may be due to a poor intracellular uptake.

7.4.3 Anti-HCMV activity of ACV ProTides

Table 7.4 reports the antiviral activity of ACV ProTides versus HCMV.

Cpds	CLogP	Antiviral Activity EC ₅₀ (μM) ^a		Cytotoxicity (μM)	
		AD-169 strain	Davis strain	MCC ^b	CC ₅₀ ^c
199a	-0.82	>50	31.6	>100	162
199b	0.89	12.6	2.9	>50	>100
199f	0.35	39.8	10	>50	>100
199g	1.41	13.4	0.8	>100	67.5
199h	2.06	2.1	1.8	>100	59.4
199i	2.31	20	10	>50	86.9
199j	2.37	6.0	2.1	>100	>100
199k	20	2.5	>	>50	>100
199l	1.19	31.6	5.0	>50	>100
199m	1.59	>50	>50	>50	>100
GCV	-2.54	3.60	5.7	>200	857

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 7.4: antiviral activity against HCMV for ACV ProTides including GCV as a control.

The activity of ACV ProTides against HCMV again confirmed the success of the phosphoramidate technology when applied to ACV. In fact, some of the compounds showed inhibitory activity against HCMV comparable to GCV, which is currently the treatment of choice of this type of infection.

7.4.4 Biological activity versus osteosarcoma of ACV ProTides

Table 7.5 reports the activity against osteosarcoma (OST) cells; both a TK deficient strain and TK deficient/HSV-1 TK-competent strain.

Compounds	CLogP	Antiviral Activity	
		CC ₅₀ (μM)	
		OST TK ⁻	OST TK ⁻ /HSV-1 TK ⁺
199a	-0.82	6.6±4.1	2.7±0.5
199b	0.89	2.4±1.8	0.52±0.33
199f	0.35	0.85±0.30	0.40±0.24
199i	2.31	149±76	1.0±0.9
199l	2.37	0.65±0.30	0.14±0.04
199m	1.59	205±85	3.8±1.8
ACV	-2.42	102±64	0.14±0.06

Table 7.5. Antiviral activity against OST for ACV ProTides including ACV as a control.

The results from **Table 7.5** prove the successful by-pass of the first step of phosphorylation delivering the monophosphate form inside the cells. In fact, in the TK deficient strain, ACV is inactive, while most of its prodrugs are active. In the strain with HSV-1 TK, ACV becomes active and the ProTides retain activity. Interesting results are from two of these prodrugs: **199i** and **199m**. In fact, they did not show any activity against the TK deficient strain, this is due to the poor bioactivation of this prodrug to deliver the monophosphate. Compound **199i** bears the L-phenylalanine as amino acid, which is not the ideal amino acid and it showed loss of activity compared to the L-alanine derivatives for instance.¹² Compound **199m** bears the *tert*-butyl as amino acid ester, which is not processed by the carboxypeptidase esterase as the *tert*-butyl moiety is too bulky and is not able to interact with the active site of the enzyme.¹² The activity found for these compounds against TK competent strain can be due to the cleavage of the prodrug to deliver ACV followed by phosphorylation to the monophosphate form mediated by the HSV-1 TK.

No activity ($> 100 \mu\text{M}$) for these compounds has been found against: parainfluenza-3-virus, reovirus-1, sindbis virus, coxsackie virus B4, vesicular stomatitis, respiratory syncytial virus, punta toro virus, influenza A virus, influenza B virus, feline corona virus, feline herpes virus (data not shown).

7.5 Acyclovir ProTides as anti-HIV reverse transcriptase inhibitor

ACV is, as already described at the beginning of the chapter, the current treatment of choice for VZV and HSV infection. Its mechanism of action is well understood involving inhibition of the viral DNA polymerase and causing the termination of the nascent viral DNA chain. Due to the specificity of the phosphorylation mediated by specific HHV kinase, ACV shows activity only against HHVs.

However, clinical study indicates the possible inhibition of HIV-1 by ACV in patients coinfecting with HHV.¹³ The reduction of HIV-1 load in these patients has been recently reported.¹⁴ The mechanism of this suppression was not very clear. Further studies demonstrate that ACV is an anti-HIV agent only in the presence of HHV co-infections. In fact, in the presence of HHV kinase, ACV can be phosphorylated to the monophosphate form and then further is converted to the triphosphate form and inhibit the HIV-1 RT.

Since the first phosphorylation step of ACV is specifically carried out by HHV-TK, it was clear to test the anti-HIV activity of the ACV ProTides.

7.5.1 Biological evaluation versus HIV of acyclovir ProTides

The ACV ProTides were evaluated for their ability to inhibit the replication of HIV-1 and HIV-2 (CEM) and HIV-1 (MT-4) (**Table 7.6**).

ACV did not show any activity against HIV-1 and 2, whilst almost all of its ProTides showed activity. In particular, all the L-alanine derivatives with the exception of **199c**, **e**, **m**, showed inhibitory activity against HIV in a range of 6.2-17 μM against HIV-1 (CEM), in a range of 0.8-15 μM against HIV-1 (MT-4) and in a range of 8.9-42 μM against HIV-2 (CEM). No clear SAR could be observed for these compounds in terms of lipophilicity, and also regarding the aryl and ester moieties. The lack of activity obtained for compound **199m**, bearing the 'butyl group as an ester, is in agreement with the literature.¹² The lack of activity found for compound **199c** is quite surprising, as the naphthyl derivative **199i** was active. The substitution of L-alanine with L-phenylalanine **199i** led to a decrease of activity, whilst the substitution with the non-natural amino acid D-alanine **199d** and dimethylglycine **199j** led to a loss of activity. In the case of the dimethylglycine derivative, this lack of activity is quite surprising as from previous studies this type of substitution is usually well tolerated. The lack of

activity found for the D-alanine derivative is in agreement with the literature.¹² Thus L-alanine seems strongly preferred here.

Cpds	CLogP	Antiviral Activity EC ₅₀ (μM) ^a			Cytotoxicity (μM)		
		HIV-1 CEM	HIV-2 CEM	HIV-1 MT-4	IC ₅₀ ^b CEM	CC ₅₀ ^c MT-4	IC ₅₀ ^b MT-4
199a	-0.82	17±4.6	26±8.5	15	67±7.8	-	-
199b	0.89	16±14	11±4.9	5.7±1.6	42±11	>150	33.8±10.6
199c	0.02	>100	>100	>150	>100	>150	>150
199d	0.89	>250	>250	-	≥250	-	-
199e	1.11	>20	>20	-	76±13	-	-
199f	0.35	10±7.9	13±6.4	4.7±2.1	57	>150	18.7±3.2
199g	1.41	6.6±5.6	24±30	10	22	-	-
199h	2.06	15±14	8.9±6.3	0.8	17	-	>150
199i	2.31	26±11	34±24	16	42	-	-
199j	2.37	≥100	79±30	-	>100	-	-
199k⁶	0.88	12±9.8	42±13	1.7±0.8	32±7.8	>150	18.7±3.2
199l⁶	1.19	6.2±5.4	12±0.71	5.4	36±15	>150	72.5
199m⁶	1.59	>100	>100	>150	>100	>150	>150
ACV	- 2.42	>250	>250	>250	>250	>250	>250

a) effective concentration required to reduce virus plaque formation by 50%;

b) cytotoxic concentration required to decrease the viability of the cell cultures by 50%;

c) inhibitory concentration required to inhibit cell proliferation by 50%.

Table 7.6: anti-HIV activity for ACV ProTides.

One of the major issues for this new class of anti-HIV compounds is their cytotoxicity. In fact, all of these compounds showed an antiproliferative effect in the range 17-76 μM in the case of CEM and 12-34 μM in the case of MT-4. Thus, by-passing HHV-TK does have a potential toxicity penalty.

7.6 Acyclovir ProTides as anti-HCV inhibitors

After the discovery of the anti-HIV activity for the ACV ProTides, these compounds have been tested against HCV, with interesting results (Table 7.7).

Cpds	Aryl	Amino Acid	Ester	CLogP	Antiviral Activity EC ₅₀ (μM) ^a	Cytotoxicity (μM)
					HCV Huh-5-2	CC ₅₀ ^b
199b	Ph	L-Ala	Bn	0.89	12	>66
199c	Ph	L-Ala	ⁱ Pr	0.02	32	>33
199d	Ph	D-Ala	Bn	0.89	>33	>33
199e	<i>p</i> -F-Ph	L-Ala	Bn	1.11	22	>33
199f	Naph	L-Ala	Me	0.35	19	>33
199g	Naph	L-Ala	ⁿ Pr	1.41	10	>33
199h	Naph	L-Ala	Bn	2.06	8.7	>33
199i	Ph	L-Phe	Bn	2.31	25	>66
199j	Naph	DMG	Bn	2.37	11	>33
199k ⁶	Naph	L-Ala	Et	0.88	17	>66
199m ⁶	Naph	L-Ala	^t Bu	1.59	33	>33
ACV	-	-	-	-2.42	30	>33

a) effective concentration required to reduce virus plaque formation by 50%;

b) cytotoxic concentration required to decrease the viability of the cell cultures by 50%;

Table 7.7: anti-HCV activity for ACV ProTides.

ACV showed activity (30 μM) against HCV. All the compounds tested showed activity versus HCV in the range 8.7-33 μM, with the best result achieved with compound **199h**, which is 3 times more active than the parent. The D-alanine derivative **199d** did not show any activity. Also in this experiment, no clear-cut SAR could be observed with regard to the nature of the aryl moiety, the alaninyl ester moiety or lipophilicity. Notably, none of the compounds tested showed any cytotoxicity in this test.

7.7 Summary of ACV ProTides activity

The application of the phosphoramidate ProTide technology to ACV has been a success. In fact, in different tests this technology allowed us to extend and in some cases to improve the antiviral activity of ACV. In the case of the HSV, ACV ProTides showed a successful by-pass of the first step of phosphorylation, showing activity versus TK-deficient strains, for which ACV showed a loss of activity. These results have been confirmed also against VZV, with retention of activity against TK-deficient strains.

The extension of activity versus HCMV and HCV, but more importantly versus HIV, showed the success of this technology applied to ACV.

Analysing all the results, it is possible to summarise that the L-alanine is optimal for the antiviral activity, especially against HIV for which the substitution with other amino acids led to a decrease of activity. The anti-HSV and anti-VZV activity seems to tolerate some amino acid substitutions. This aspect may be due to a different bioactivation of the ACV ProTides in these two tests, due for example to different specificities and/or different intracellular levels of the necessary activating enzymes.

In order to investigate the differences in activity found for these compounds, enzymatic and molecular modelling studies have been performed.

7.8 Enzymatic studies using carboxypeptidase Y on ACV ProTides

The supposed mechanism of activation of ProTides has been already described in Chap. 1 and 6. To investigate the difference in activity found for some of the ACV ProTides enzymatic studies using carboxypeptidase Y, as already seen with the ribavirin ProTide, have been performed. For these experiments compounds **199b**, **c** and **m** were chosen because **199b** was active against all HSV, VZV, HIV, HCV; **199c** was against HSV, VZV and HCV but not versus HIV, whilst **199m** was inactive in all the biological assays.

The same procedure used in Chap. 6 was followed, dissolving the compound in acetone- d_6 and trizma buffer (pH = 7.6) and recording a blank before the addition of the enzyme.

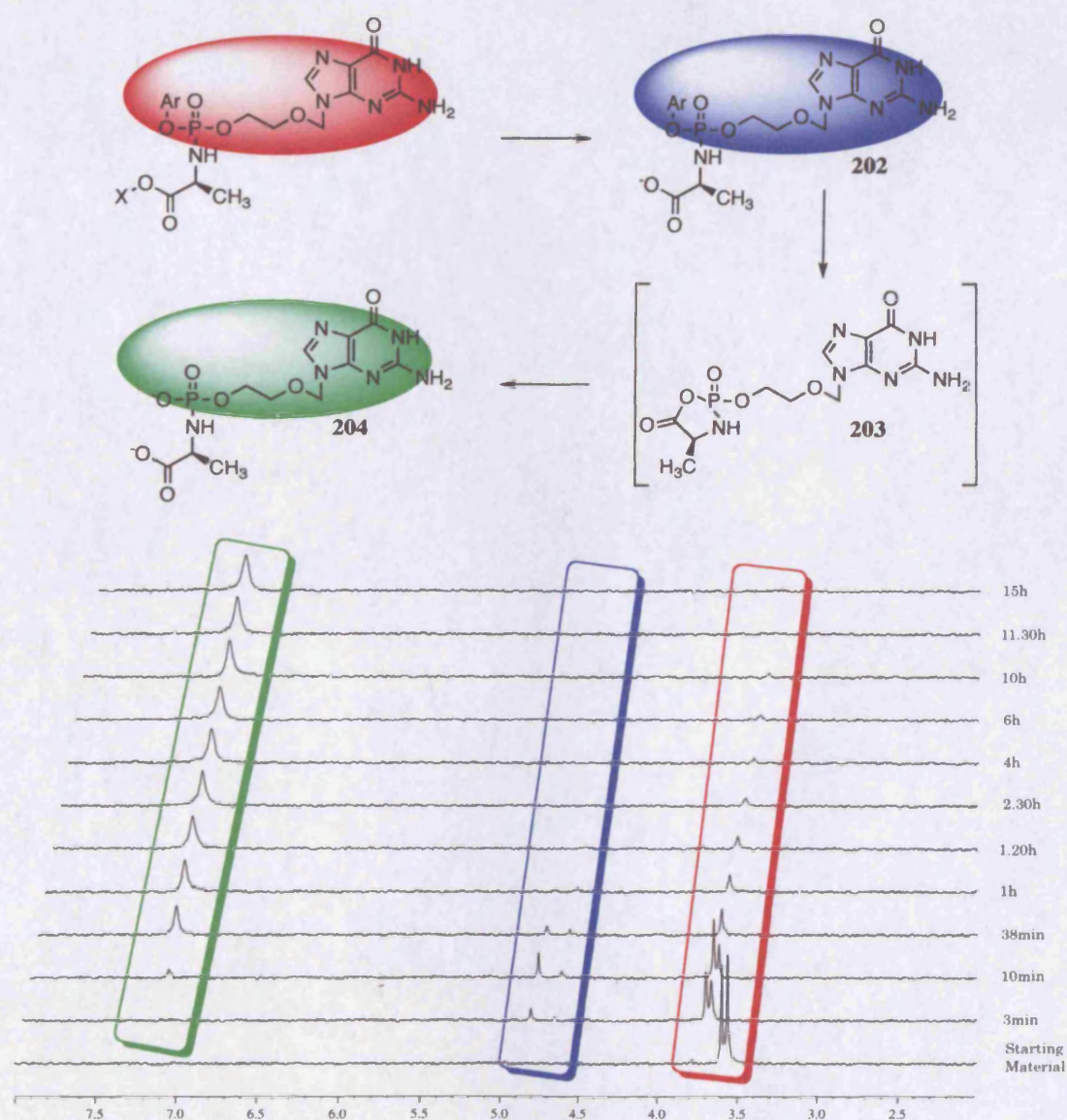


Fig. 7.3: carboxypeptidase-mediated cleavage of compound **199b**, monitored by ^{31}P NMR.

Compound **199b** (Fig. 7.3, $\delta_P = 3.65$ and 3.60) was fast hydrolysed to the intermediate **202** ($\delta_P = 4.85$ and 4.70), which is then further processed to the final compound **204** ($\delta_P = 7.10$). Notably, the intermediate **203** is detected by the ^{31}P NMR. The half-life of compound **199b** is 17 min.

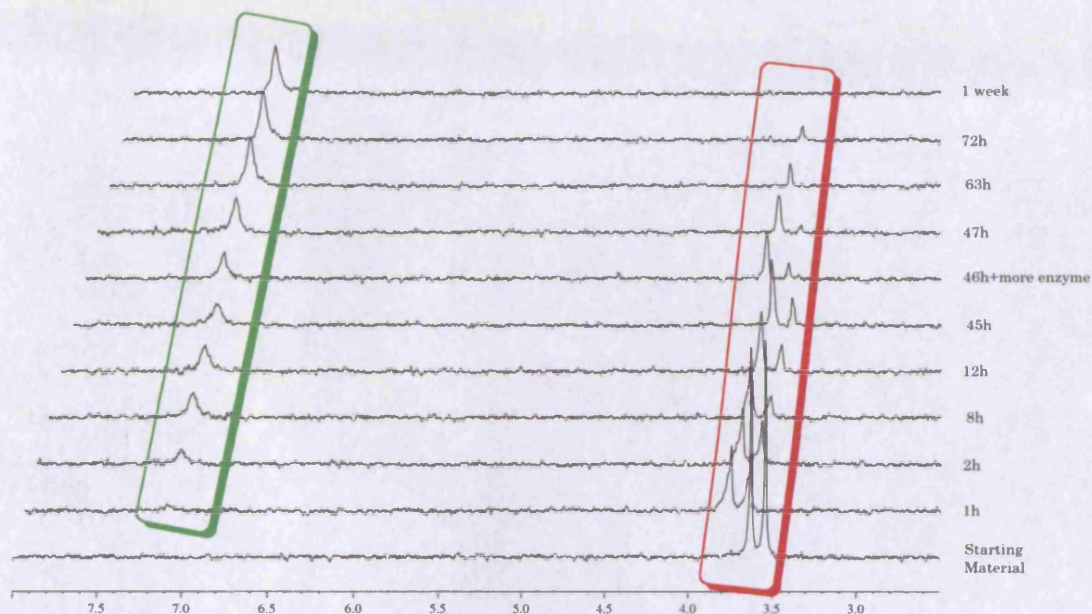


Fig. 7.4: carboxypeptidase-mediated cleavage of compound **199c**, monitored by ^{31}P NMR.

In the case of the isopropyl derivative **199c**, the experiment (Fig. 7.4) shows a slow conversion of the starting material to the desired compound **203** ($\delta_P = 7.10$) showing an half-life of 46 h and the need of more enzyme to complete the hydrolysis. Notably, it seems from the spectra that one of the two diastereoisomers is faster metabolised than the other.

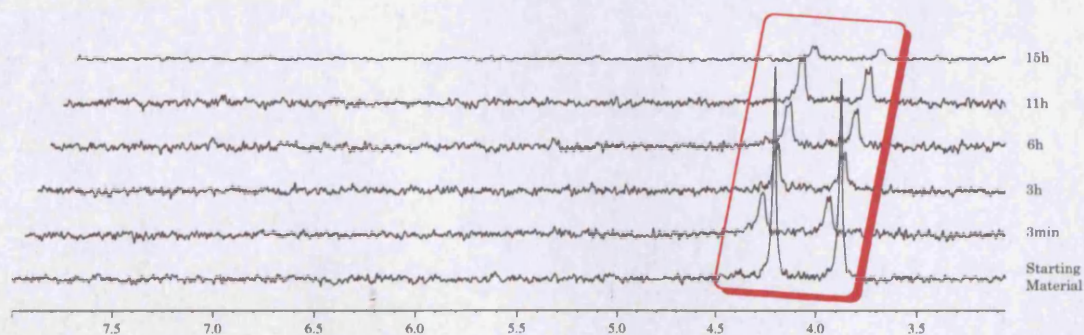


Fig. 7.5: carboxypeptidase-mediated cleavage of compound **199m**, monitored by ^{31}P NMR.

The experiment (**Fig. 7.5**) of the *tert*-butyl derivative **199m** showed no conversion at all, and this result is in accordance with the lack of antiviral activity for this compound and it is probably due to the presence of the *tert*-butyl moiety which is too bulky to be processed by the enzyme.

These experiments show that ACV ProTides need to be bioactivated in order to show the antiviral activity. The results found for these experiments are in agreement with the results found in the anti-HIV assay and the assay may be regarded as partially predictive of antiviral efficacy.

The different results obtained for these three compounds have been investigated using molecular modelling techniques.

7.9 Molecular modelling-1: carboxypeptidase Y enzyme

Carboxypeptidase Y is considered to be one putative enzyme responsible for the cleavage of the amino acid ester moiety. In order to understand the results obtained in the enzymatic experiments, molecular modelling studies using docking techniques have been performed on the crystal structure of the enzyme (PDB 1YSC).¹⁵ The mechanism of action of this enzyme involves firstly the attack of the Ser146 to the carbonyl of the ester, which is coordinated with the NH from Gly52 and Gly53.¹⁶ **Fig. 7.6** reports the catalytic site of the enzyme with the docking of compound **199b**.

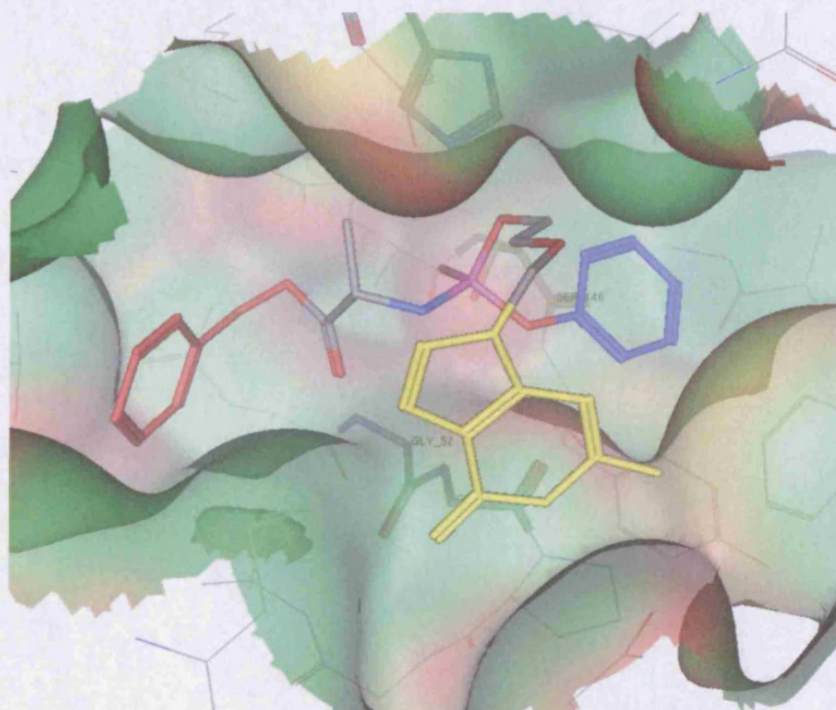


Fig. 7.6: catalytic site of carboxypeptidase Y.

The catalytic site consists of two different pockets. In the pocket on the left is placed the ester of the amino acid (red in the picture), whilst in the pocket on the right is placed the aryl moiety of the phosphoramidate (blue). The nucleoside base (yellow) is placed outside the pocket.

Compound **199b** showed a positive interaction, for both phosphate diastereoisomers, with the active site of the enzyme (**Fig. 7.7**, shown R_P diastereoisomer). In fact, the carbonyl group is in a favourable position for the coordination with the NH of both Gly52 and Gly53, and it is able to interact with the catalytic Ser146. This result supports the enzymatic result obtained for this compound.

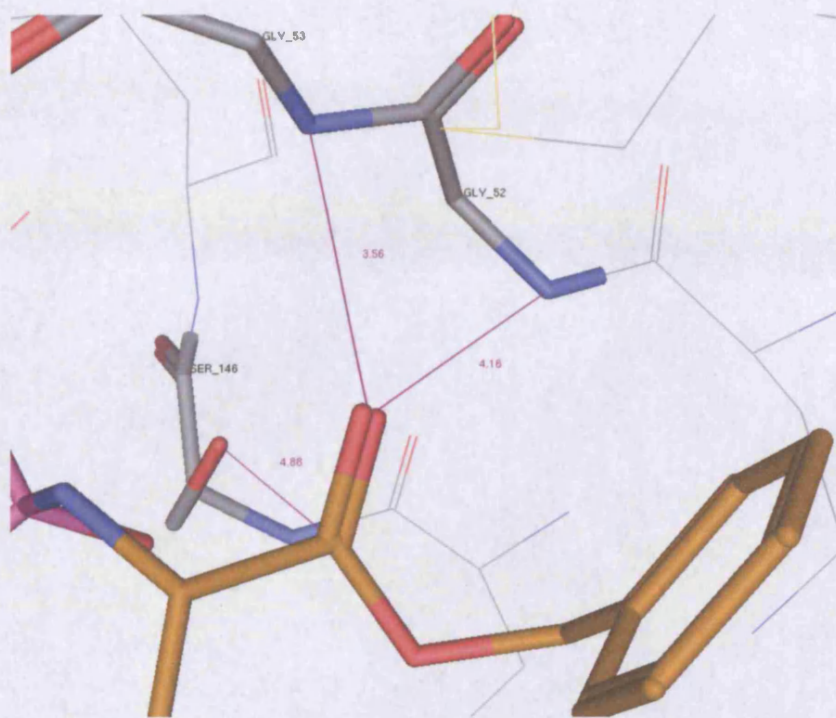


Fig. 7.7: docking of compounds **199b** (R_P) within the catalytic site of carboxypeptidase Y.

In the case of compound **199c** (Fig. 7.8, shown R_P diastereoisomer) different results have been obtained in the case of the R_P diastereoisomer and the S_P diastereoisomer.

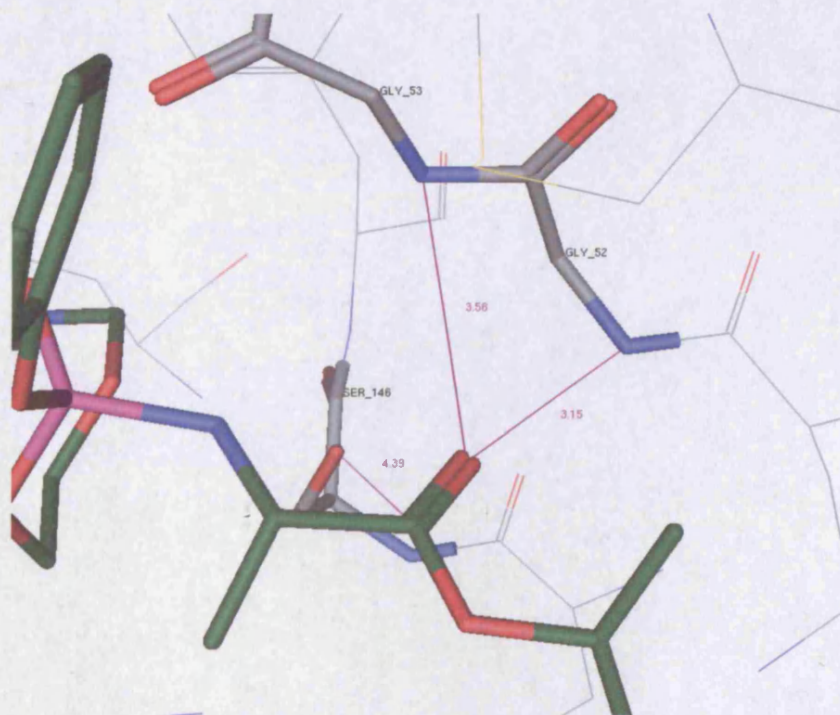


Fig. 7.8: docking of compounds **199c** (R_P) within the catalytic site of carboxypeptidase Y.

In fact, while for the R_P the carbonyl group is in a positive orientation for the attack of the Ser146, the carbonyl of the S_P diastereoisomer is situated in a different orientation and it is not able to coordinate properly with Gly52 and Gly53. This result support the difference in the metabolism found for this compound in the enzymatic assay, which showed a faster metabolism for one diastereoisomer compared to the other. It is striking that the docking predict an isomeric difference here, where observed in the assay, but not for compound **199b** earlier, where no difference was observed in rate.

The carbonyl of compound **199m** (Fig. 7.9, shown S_P diastereoisomer) is not in a favourable position due to the presence of the bulky *tert*-butyl ester. In fact, the carbonyl group is not able to interact with the two glycines with a consequent poor activation for this compound, in agreement with the enzymatic result.

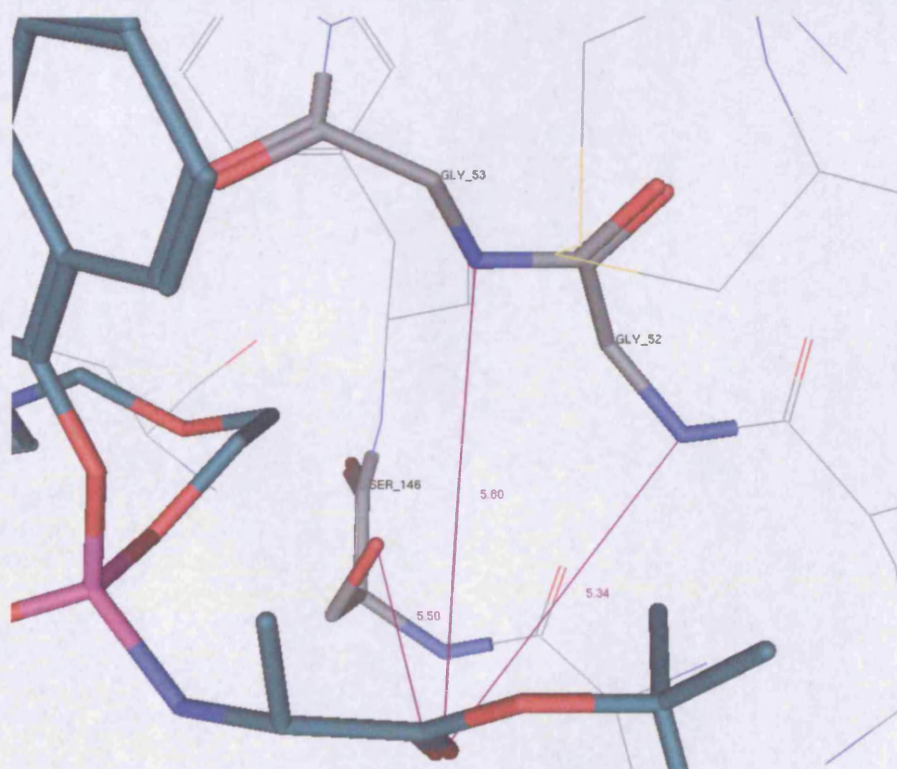


Fig. 7.9: docking of compounds **199m** (S_P) within the catalytic site of carboxypeptidase Y.

7.10 Molecular modelling-2: human Hint enzyme

As already mentioned in Chap.6, human Hint enzyme is considered to be responsible for the cleavage of the phosphorus-nitrogen bond with consequent release of the monophosphate form (**Fig. 7.10**).

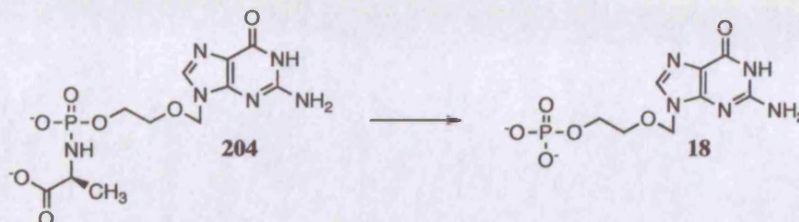


Fig. 7.10: cleavage of the phosphorus-nitrogen bond mediated by human Hint enzyme.

A molecular modelling study using docking techniques has been performed for compound **204**. (**Fig. 7.11** and **Fig. 7.12**).

Fig. 7.11 reports the interaction of compound **204** with the amino acid involved in the cleavage of the phosphorous-nitrogen bond.

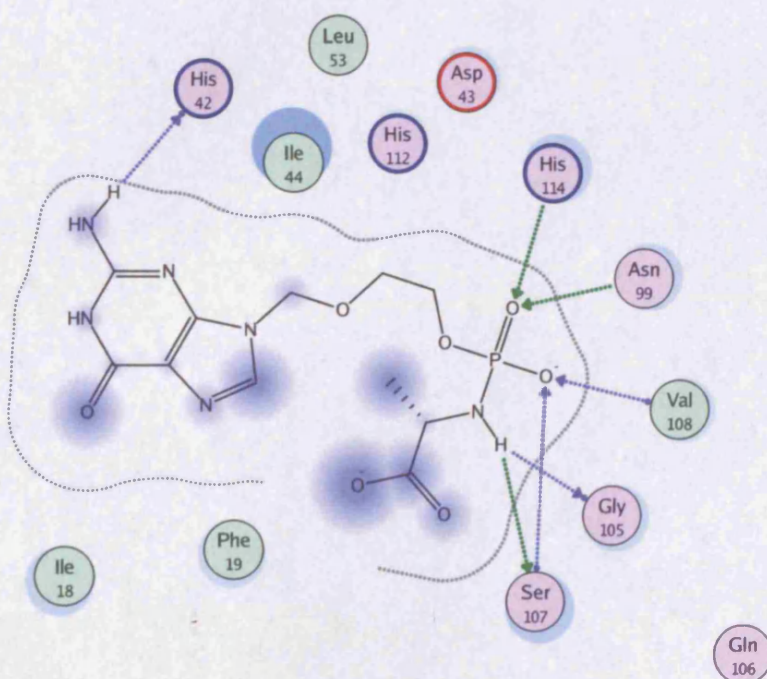


Fig. 7.11: interaction of compound **204** within the catalytic site of human HINT (I) enzyme

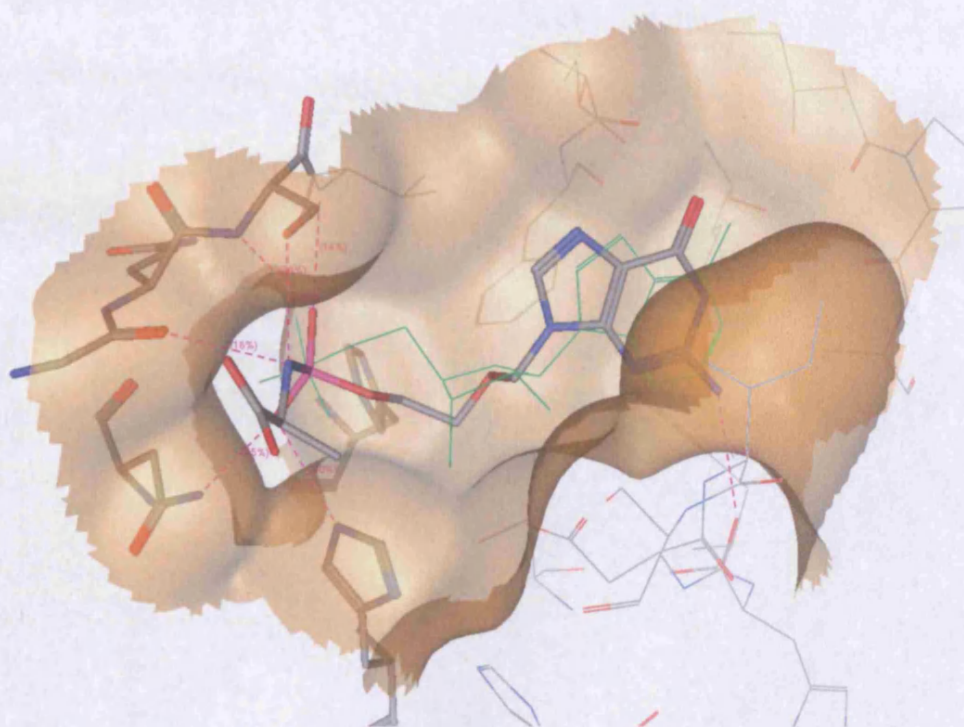


Fig. 7.12: docking of compound **204** within the catalytic site of human HINT (I) enzyme

The results from this experiment are quite clear. The compound binds in the active site of the enzyme. The phosphate moiety (pink) is in a suitable position for the cleavage of the P-N bond. The guanosine base positively interacts with the enzyme.

This experiment suggests that the last of the bioactivation of the ACV ProTides, in particular L-alanine derivatives, may proceed well, supporting the antiviral activity found for this compound.

7.11 References

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8. Virtual Screening on Acyclovir as an anti-HIV agent

The anti-HIV activity found for the ACV ProTides was extremely interesting and surprising as these compounds were never previously recognised as anti-HIV agents. However, these compounds have been found to be somewhat cytotoxic. In order to reduce the cytotoxicity and to boost the potency for these compounds, a virtual screening has been performed using docking techniques and using HIV-RT, adenylate or guanylate kinase and DNA polymerase γ . The goal is to increase the specificity for HIV-RT while retaining substrate action of adenylate or guanylate kinases.

8.1 Building the acyclovir derivatives database

A compound database has been built considering modifications at the nucleoside base and at the acyclic sugar moieties. **Fig. 8.1** reports the modifications considered and their possible combinations, including:

- simple and branched acyclic sugars;
- oxygen, methylene or sulphur at the 2'-position;
- substitution at the 8-position of the base including: hydrogen, methyl, fluorine, bromine or chlorine;
- substitution at the 7-position of the base including: nitrogen or carbon (hydrogen or methyl substituted);
- substitution at the 6-position of the base including: carbonyl, amine, chlorine, methoxide, ethoxide, phenoxy, methylamine, or cyclopropylamine;
- substitution at the 2-NH position of the base including: hydrogen, methyl, ethyl, phenyl or benzyl.

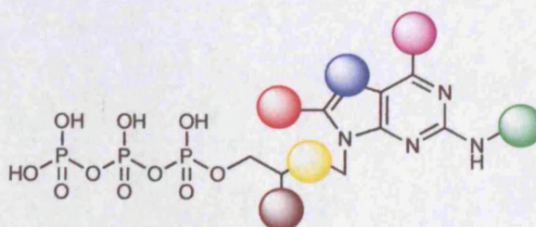
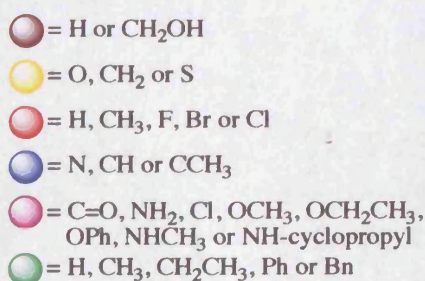


Fig. 8.1: ACV derivatives database.

The combination of all these substitutions gives a total of 3600 compounds. The database was divided in two different groups considering the modification at the 6-position of the base. The first group (3150 compounds) bears the substitution at the 6-position compared to ACV. The second group (450 compounds) bears the carbonyl moiety at the 6-position. The splitting in two different groups is due to a difference in the base pairing. In fact, while the 6-carbonyl compounds, being a guanosine analogues, have a base pairing with the cytidine, with the three hydrogen bonds, for the 6-modified derivatives, preliminary docking study, showed a better base pairing with the thymine (**Fig. 8.2**). In fact, in the case of the 6-O-modified derivatives two hydrogen bonds are observed, whilst in the 6-N-modified derivatives three hydrogen bonds between the bases are detected.

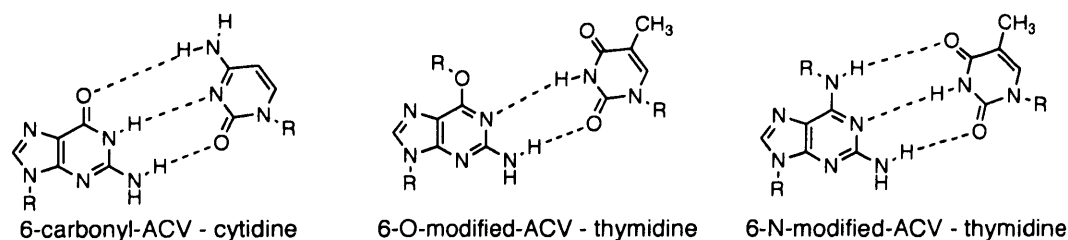


Fig. 8.2: base pairing of the 6-carbonyl and 6-modified ACV derivative.

Moreover, the stereochemistry of all the compounds bearing the substitution at the acyclic sugar moiety has been considered.

8.2 First screening: docking using HIV-RT enzyme

The first screening considered was using HIV-RT. For these experiments a crystal structure of HIV-RT co-crystallized with TNF diphosphate was used (PNB: 1T05).¹

The original structure was used for the docking of the 6-modified ACV derivatives, whilst for the 6-carbonyl derivatives the thymine of the enzyme was substituted with cytidine. All the compounds were docked in the triphosphate form, as they should interact with the catalytic site of RT and act as a chain terminator.

Fig. 8.3 reports the catalytic site of HIV-RT including TNF diphosphate.

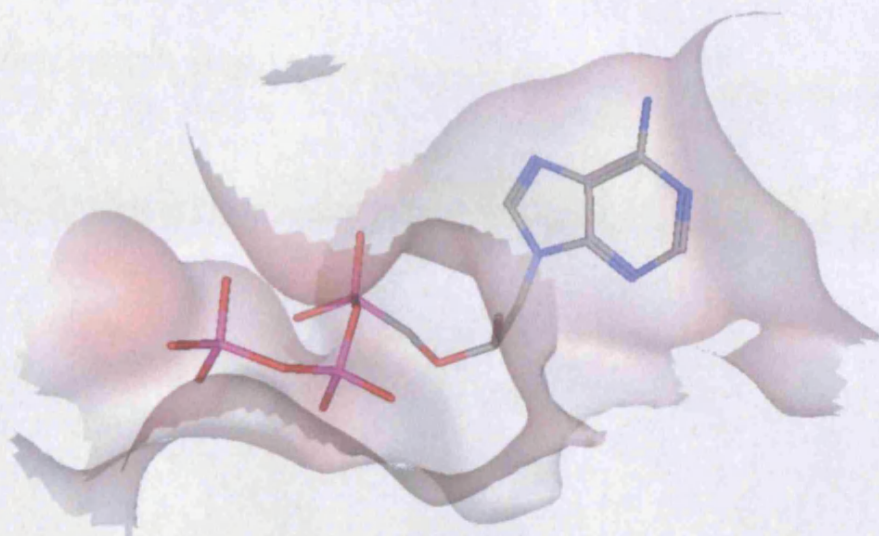


Fig. 8.3: the catalytic site of RT co-crystallized with TNF diphosphate.

For the first screening, a root mean square deviation (RMSD) $< 1 \text{ \AA}$, which results in a good base pairing, was considered. The RMSD measures the difference between the prediction and the compound crystallized, in this case respectively the compounds docked and TNF diphosphate. In particular, for this experiment the deviation from the base of the compounds docked and the base of TNF diphosphate has been considered. This allowed us to reduce the number of compounds to 782 for the 6-modified group and to 202 for the 6-carbonyl group, giving a total of 984 compounds.

Fig. 8.4 reports the best five results for the 6-modified derivatives.

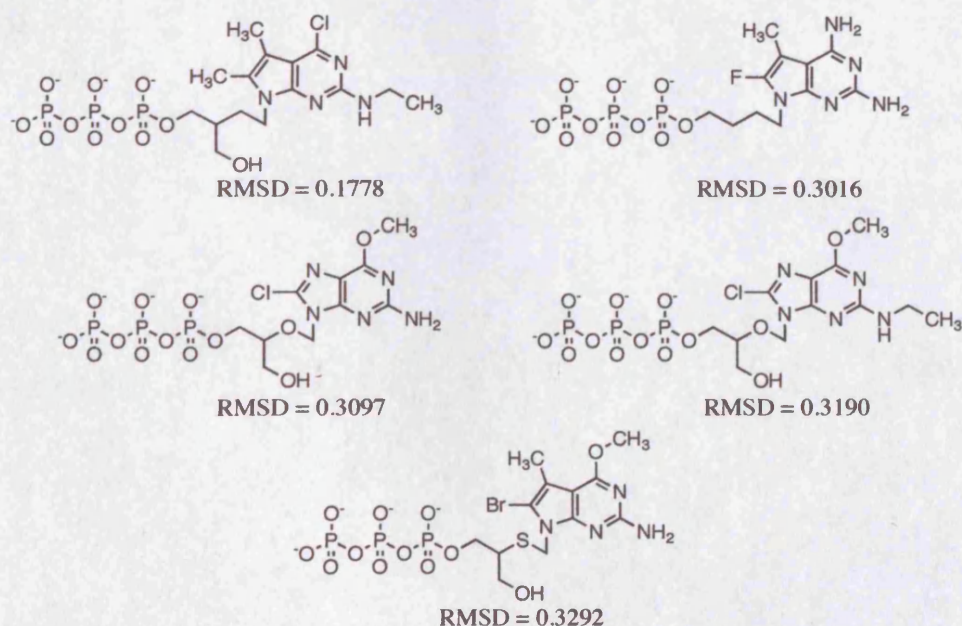


Fig. 8.4: best HIV-RT 6-modified docked compounds.

From this first screening, it has been observed that the branched sugar compounds are preferred. In fact, in the catalytic site of the enzyme the Tyr 115 and Phe 116 form a pocket (**Fig. 8.5**), which is able to accommodate a substitution in the sugar moiety. Moreover, another pocket can accommodate a potential substitution at the 2-amino position. In addition, the substitutions at the 8-position of the base are permitted, especially halogen substituents.

Fig. 8.5 reports the docking of the 6-modified derivative with the lowest RMSD. Notably, the position of the OH of the branched sugar and of the ethyl at the 2-amino position are observed.

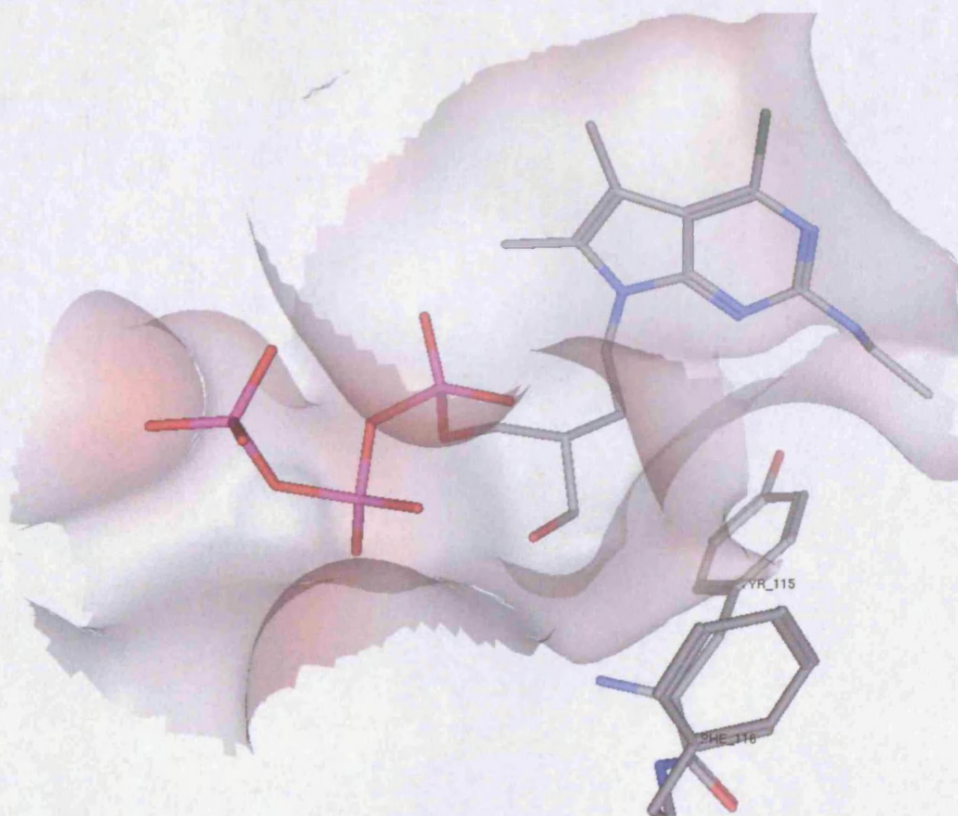


Fig. 8.5: docking of the 6-modified derivative with the lowest RMSD.

Fig. 8.6 reports the best five results for the 6-carbonyl derivatives. Also in this case, the substitution at the 8-position, the branched sugar and the substitution at the 2-amino position are preferred. Notably, the best result for this docking has been achieved with GCV.

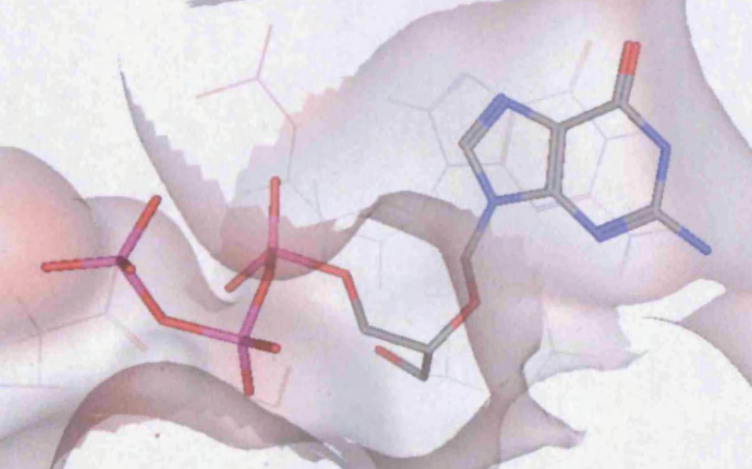


Fig 8.7: docking of the 6-carbonyl derivative with the lowest RMSD.

The branched sugar of (*R*)-GCV is accommodated in the pocket formed by the two residues Tyr115 and Phe116.

8.3 Second screening: docking using adenylate kinase or guanylate kinase

For the second docking adenylate kinase or guanylate kinase were considered. For these docking experiments the monophosphate form of the compounds were used as these two enzymes catalysed the second step of phosphorylation. The docking using kinases that catalysed the phosphorylation to the monophosphate were not considered as the phosphoramidate ProTides approach will be applied to the selected compounds.

8.3.1 Adenylate kinase docking

The first group of compounds, which bear the 6-modified base, was docked with adenylate kinase, as from preliminary docking they showed better results. The enzyme used was a crystal structure in complex with P1, P4-di(adenosine)tetraphosphate (PDB: 2C95).²

Fig. 8.8 reports the structure of the enzyme with adenosine diphosphate.

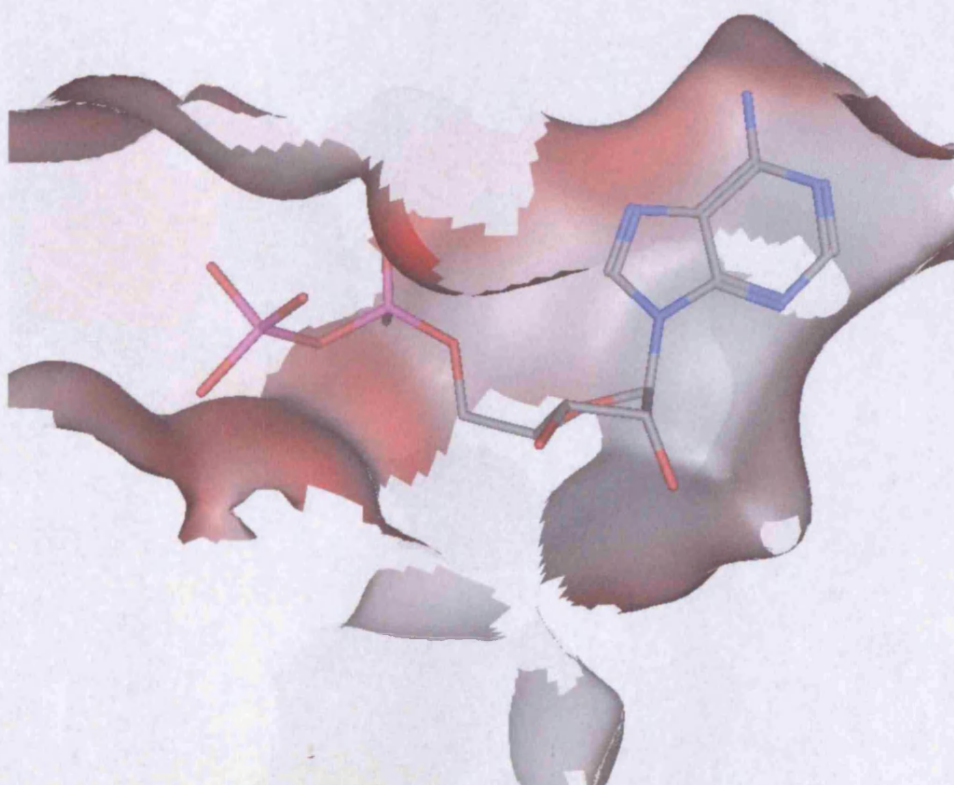


Fig. 8.8: crystal structure of adenylate kinase with adenosine diphosphate.

From **Fig. 8.8**, it is possible to see a pocket close to the sugar moiety and more specific to the 3'-position of the sugar. This pocket could allow substitution on the acyclic sugar moiety of the ACV derivatives. For these docking experiments, two

different RMSDs have been considered: the first regarding the position of the base (<1 Å) and the second considering the position of the phosphate moiety (<3 Å). These two values allowed a good position of the base and a reasonable position for the phosphate moiety, which, however, in some cases, was slightly moved compared to the crystallized compound.

Fig. 8.9 reports the best five results for the 6-modified derivatives considering the position of the base. In most of the cases, the branched sugar is still preferred. Notable from **Fig. 8.9** is the presence of the chlorine at the 6-position.

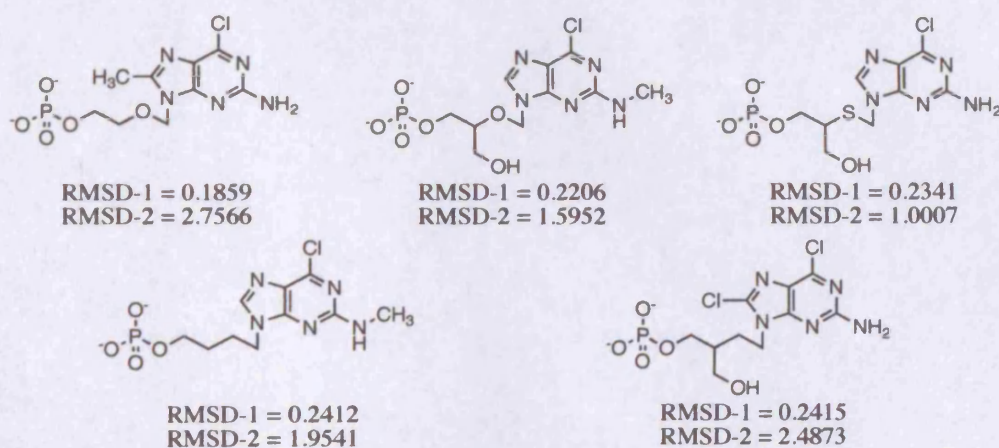


Fig. 8.9: best adenylylate kinase 6-modified docked compounds.

In **Fig. 8.10** is reported the compound within the catalytic site of the enzyme with lowest RMSD for the base.

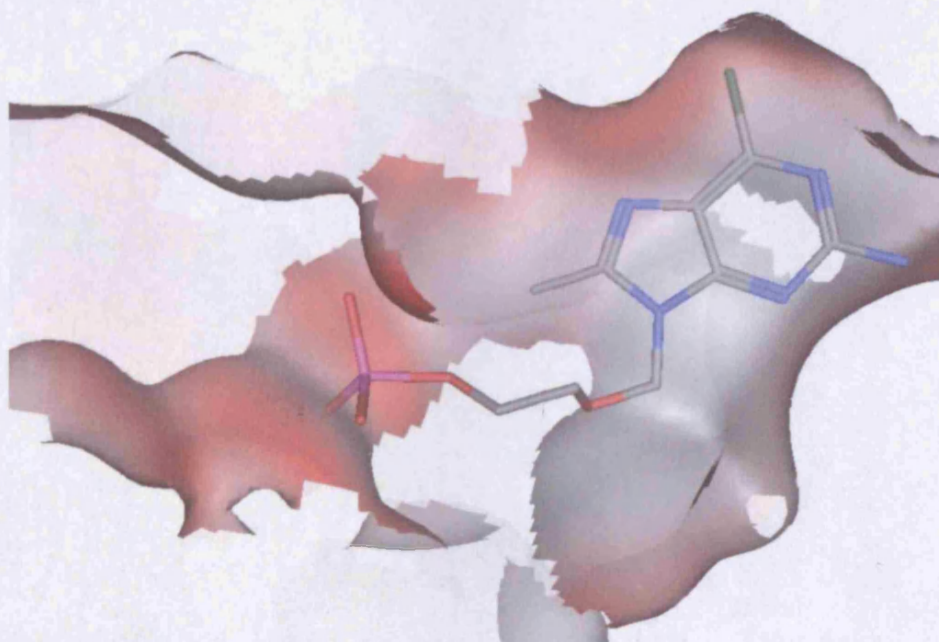


Fig. 8.10: adenylylate kinase docking of the 6-modified ACV derivative.

The compound showed a good position of the base, whilst the monophosphate moiety was found to be slightly in a different position compared to the crystallized compound.

8.3.2 Guanylate kinase docking

The second group of compounds, the 6-carbonyl ACV derivatives, has been docked using guanylate kinase. For this experiment the mouse enzyme co-crystallized with guanosine monophosphate GMP (PDB: 1LVG)³ was used showing a similarity with the human enzyme of 88%.

Fig. 8.11 reports the crystal structure of the catalytic site of the enzyme with GMP.

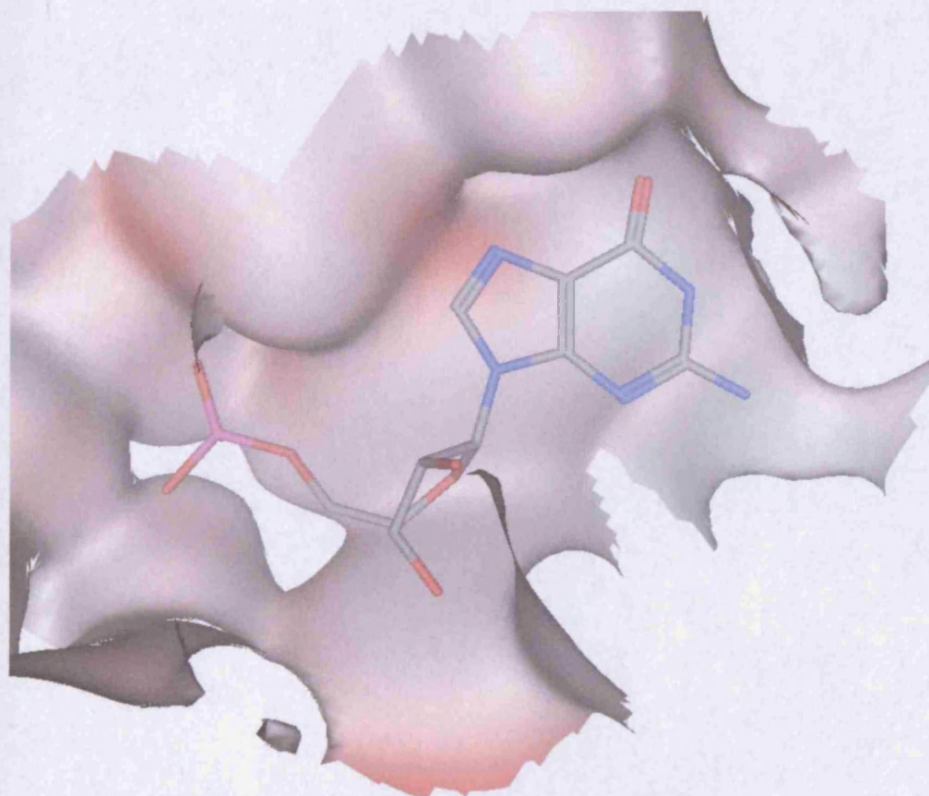


Fig. 8.11: catalytic site of guanylate kinase co-crystallized with GMP.

Notable is the presence of a pocket in the proximity of the 3'-position of the sugar.

Fig 8.12 reports the best results found for the 6-carbonyl derivatives considering the position of the base. Also in this case, $\text{RMSD} < 1$ and < 3 compared respectively to the position of the base and to the position of the phosphate of the crystallized compound were considered. From these results, the branched sugar is

still well tolerated and also the substitution at the 8-position with a preference to the halogens, whilst the substitutions at the 2-amino position are less tolerated.

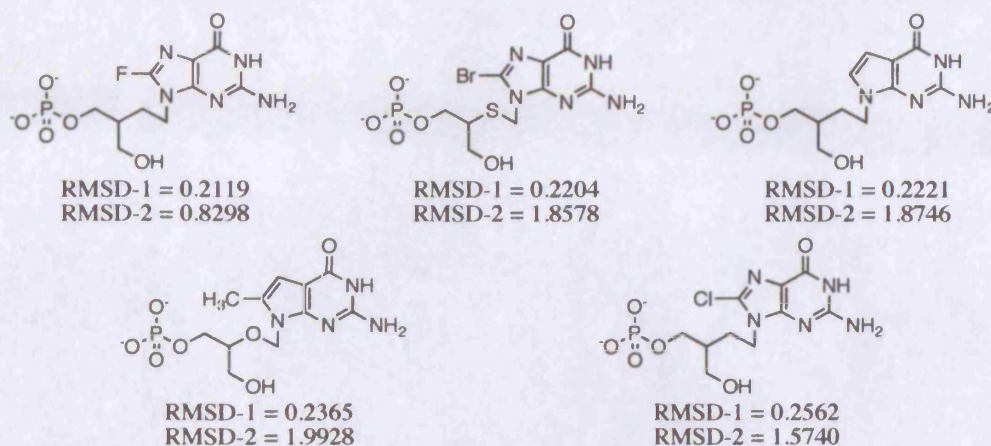


Fig. 8.12: best guanylate kinase 6-carbonyl docked compounds.

Fig 8.13 reports the docking of the compound with the lowest RMSD compared to the position of the base and also showing a good RMSD of the phosphate moiety position. In addition, the OH of the branched acyclic sugar is well positioned.

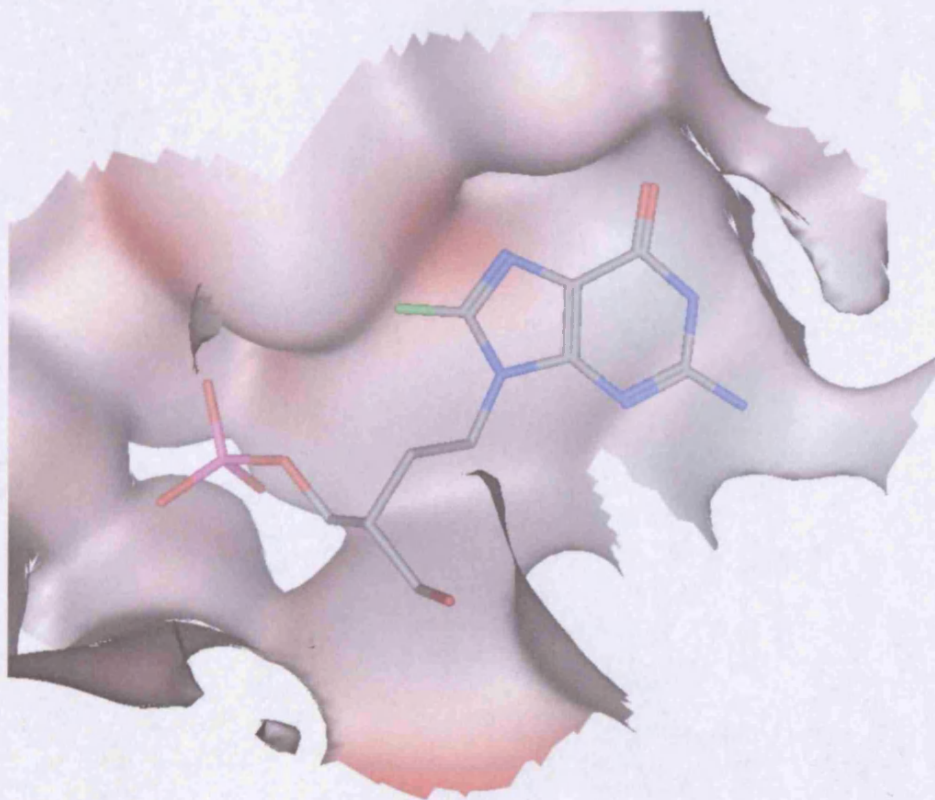


Fig. 8.13: guanylate kinase docking of the 6-carbonyl ACV derivative.

From the virtual screening using either the adenylate or the guanylate kinases the number of compounds drops to 218 compounds, of which 63 compounds belong to the 6-modified ACV derivative and 155 compounds belong to the 6-carbonyl derivatives.

From these first two results seems that the 6-carbonyl group is preferred to the modifications in this position. Moreover, good results have been obtained with the modification at the sugar moiety and also the substitution at the 8-position with halogens.

8.4 Third screening: docking using human polymerase γ

The last part of the virtual screening includes the use of the human polymerases. It is known that the toxicity of the NAs, especially RT inhibitors, is due to interactions with these enzymes.⁴⁻⁷ The subtypes of human polymerase mainly involved in the cytotoxicity are: α and γ . Unfortunately, the crystal structures for both human polymerases have not yet been identified. However, a model for the human polymerase γ is available.⁸ DNA polymerase γ is able to incorporate the antiviral NtAs, which act as a chain terminators and consequently show cytotoxicity.⁹⁻¹²

The two databases obtained after the first two screening were converted to the triphosphate form, as in this form NAs are able to interact with the polymerase. In this case, the results were analyzed visually, excluding all the compounds that showed good interactions with the catalytic site of the enzyme. From this last screening a total of 25 compounds were obtained. The last step of the screening was to choose the compounds with the best results with the other two screenings and finally those that were accessible from a synthetic point of view. The selected compounds are reported in **Fig. 8.14**.

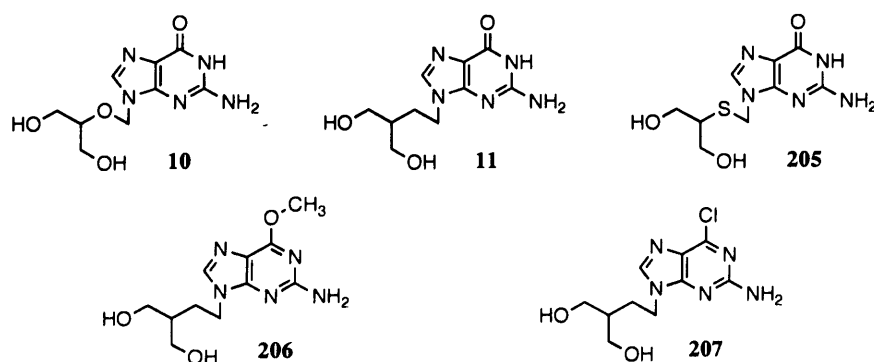


Fig. 8.14: selected compounds from the virtual screening.

Fig. 8.15 reports the virtual screening of ACV derivatives. The initial 3600 compounds were reduced to 984 after the screening using RT. The screening using either guanylate kinase or adenylate kinase reduced the number to 218 compounds. The final screening using human polymerase γ reduced the number to 25 compounds. Finally, the compounds were analysed for the best docking results with RT and guanylate or adenylate kinase and from a synthetic point of view.

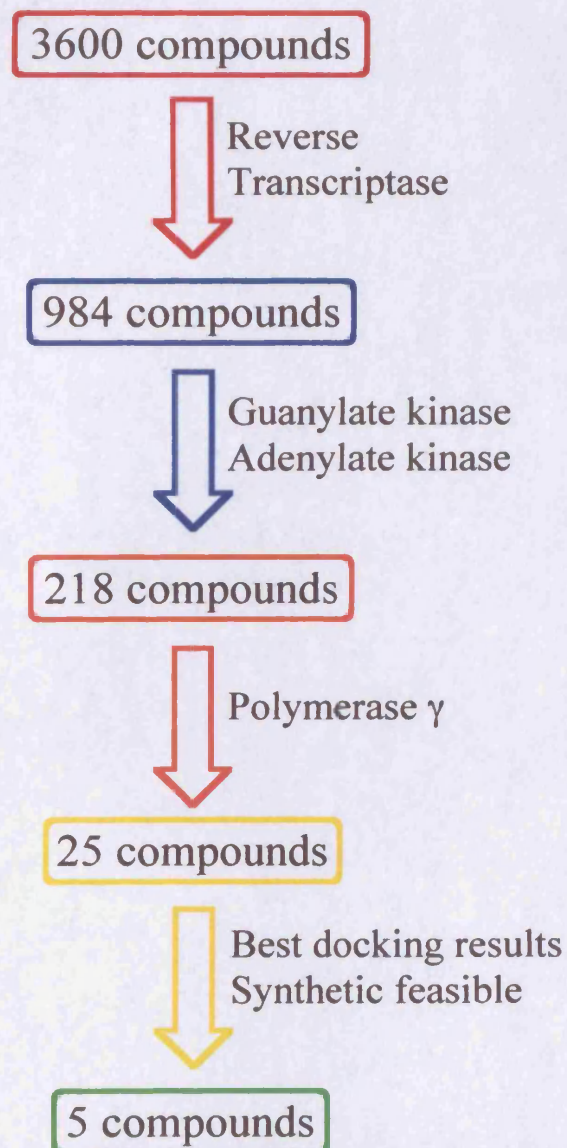
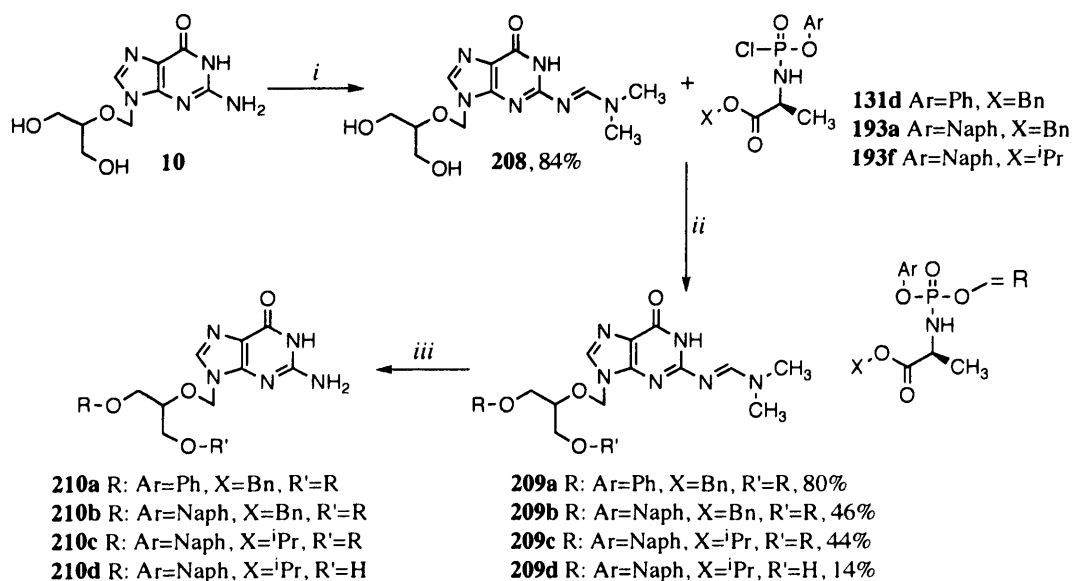


Fig. 8.15: summary of the ACV derivatives virtual screening.

8.5 Chemistry

8.5.1 Synthesis of ganciclovir ProTides

The first compound to be selected was GCV as it showed the best results in the RT docking, but also good results with guanylate kinase and polymerase γ docking. Moreover, GCV is commercially available. For the synthesis of the ProTides the same procedure used for the ACV ProTides synthesis was followed (**Scheme 8.1**). The 2-amino functionality of the guanine base was protected using *N,N*-dimethylformamide dimethyl acetal followed by coupling with the appropriate phosphorochloridates **131d**, **193a**, **f** using t BuMgCl as a hydroxyl activator.¹³ However, the protected GCV was less soluble than the corresponding ACV derivatives; in fact, the addition of pyridine to dissolve the compound was necessary. The deprotection step was carried out by refluxing compounds **209a-d** in 2-propanol to obtain the final compounds **210a-d**.

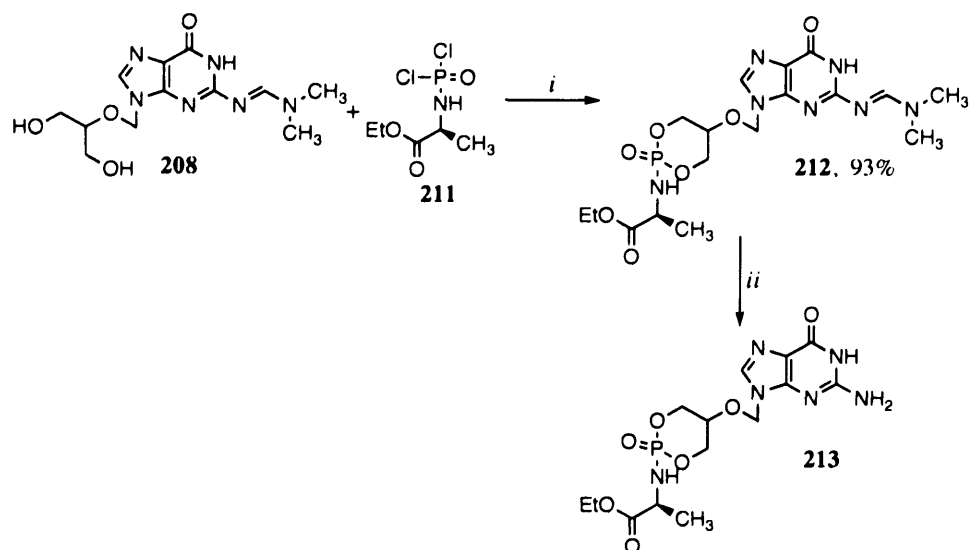


Reagents and Conditions: (i) *N,N*-dimethylformamide dimethyl acetal, DMF, rt, 29 h; (ii) t BuMgCl, anhydrous THF, anhydrous pyridine, rt, 20-84 h; (iii) 2-propanol, reflux, 24-80 h.

Scheme 8.1: synthesis of GCV ProTides.

Due to the presence of two hydroxyl groups the compounds were obtained as bis-ProTides. Only in the case of the 1-naphthyl-L-alaninyl-isopropyl derivative the mono-ProTide **210d** was isolated.

Scheme 8.2 reports the synthesis of a cyclic GCV derivative **213**, which does not have the aryl moiety.



Reagents and Conditions: (i) $t\text{-BuMgCl}$, anhydrous THF, anhydrous pyridine, rt, 40 h; (ii) 2-propanol, reflux, 9 h.

Scheme 8.2: synthesis of GCV cyclic ProTide.

Table 8.1 reports the summary of the GCV ProTides with the yield and the ^{31}P -NMR data.

Cpds	ProTide	Ar	Ester	CLogP	Yield	^{31}P -NMR
210a	Di	Ph	Bn	3.42	7%	3.86, 3.85, 3.80, 3.73, 3.52, 3.49, 3.41
210b	Di	Naph	Bn	5.77	3%	4.23, 4.08, 3.96, 3.93
210c	Di	Naph	$i\text{Pr}$	4.03	8%	4.29, 4.25, 4.23, 4.17
210d	Mono	Naph	$i\text{Pr}$	0.41	33%	4.40, 4.36, 4.34, 4.27
213	Cyclic	-	Et	- 0.69	11%	3.73, 2.89

Table 8.1: summary of GCV ProTides

8.5.1.1 Biological evaluation of ganciclovir ProTides

GCV ProTides have been evaluated for their ability to inhibit HIV-1 and HIV-2 and the results are shown in **Table 8.2**.

Cpds	ProTide	Ar	Ester	CLogP	Antiviral		Cytotoxicity
					Activity EC ₅₀		
					(μM)		
					HIV-1	HIV-2	CC ₅₀ ^b
210a	Di	Ph	Bn	3.42	>50	>50	159±82
210c	Di	Naph	ⁱ Pr	4.03	>100	>100	>100
210d	Mono	Naph	ⁱ Pr	0.41	>20	>20	35±4.2
GCV	-	-	-	-2.54	>250	>250	>250

a) effective concentration required to reduce virus plaque formation by 50%;

b) inhibitory concentration required to inhibit cell proliferation by 50%.

Table 8.2: anti-HIV activity for GCV ProTides.

Unfortunately, none of these compounds showed any anti-HIV activity. This could be due to a poor bioactivation of these ProTides with the consequent poor delivery of the monosphosphate.

An enzymatic study using carboxypeptidase Y (**Fig. 8.16**) has been performed using compound **210d** following the same procedure already described in Chap. 6 and 7.

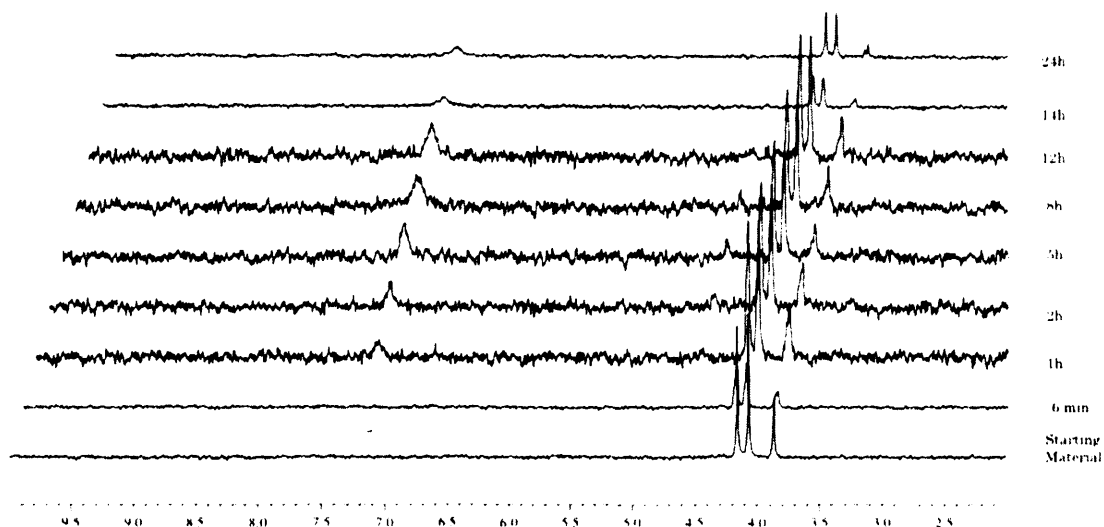
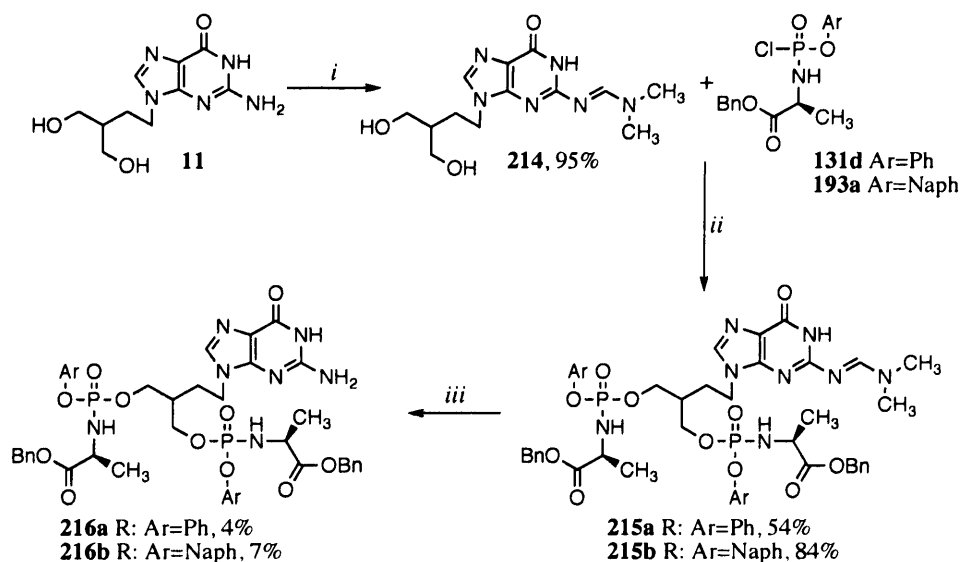


Fig. 8.16: carboxypeptidase-mediated cleavage of compound **210d**, monitored by ³¹P NMR.

The experiment shows a slow and partial conversion of the starting material ($\delta_P = \sim 4$) to the metabolised compound ($\delta_P = \sim 7$). The result from this experiment may support the fact that the poor activity found for these compounds may be due to a poor bioactivation.

8.5.2 Synthesis of penciclovir ProTides

The second derivative selected was PCV. For the synthesis of these ProTides the same synthetic procedure used for GCV was used. Firstly, PCV was protected with *N,N*-dimethylformamide dimethyl acetal giving compound **214** (Scheme 8.2). The coupling with the appropriate phosphorochloridate (**131d** or **193a**) was performed using $^t\text{BuMgCl}$ as a hydroxyl activator.¹³ The deprotection step was carried out by refluxing compounds **215a, b** in 2-propanol to obtain the final compounds **216a, b**.



Reagents and Conditions: (i) *N,N*-dimethylformamide dimethyl acetal, DMF, rt, 6 h; (ii) $^t\text{BuMgCl}$, anhydrous THF, rt, 8 h; (iii) 2-propanol, reflux, 39-72 h.

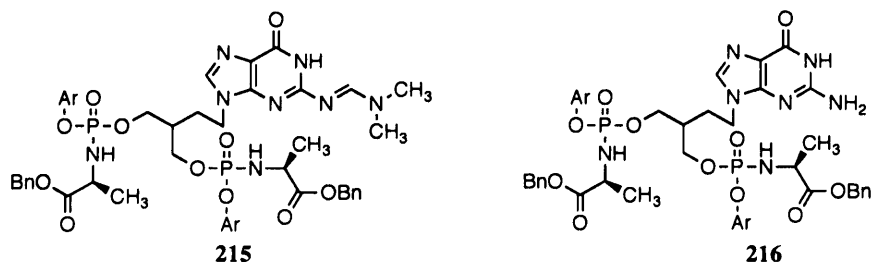
Scheme 8.2: synthesis of PCV ProTides.

The coupling reaction seems to work better compared to the GCV ProTides synthesis probably due to a slightly better solubility of PCV compared to GCV. However, poor yields have been obtained in the deprotection step due to degradation of the products. In this case, the protected PCV ProTides have also been biologically evaluated.

8.5.2.1 Biological evaluation of penciclovir ProTides

The biological evaluation of these compounds against HIV is currently underway. The compounds have been evaluated for their ability to inhibit VZV and HSV-1 and 2.

Table 8.3 reports the anti-VZV activity for PCV ProTides.



Cpds	Aryl	CLogP	Antiviral Activity EC ₅₀ (μM) ^a				Cytotoxicity (μM)	
			TK ⁺ VZV		TK ⁻ VZV		MCC ^b	CC ₅₀ ^c
			YS strain	OKA strain	07-1 strain	YS/R		
215a	Ph	4.04	6.9	32.2	29.3	28.5	>50	44.5
215b	Naph	6.38	8.1	10.9	8.6	11.5	50	20
216a	Ph	3.83	8.9	23.7	33	>50	>50	>100
216b	Naph	6.18	14.9	17.1	>50	>50	>50	>100
PCV	-	-2.72	2.2	0.99	12.2	24.5	>100	>100

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

c) cytotoxic concentration required to reduce cell growth by 50%.

Table 8.3. Antiviral activity against VZV for PCV ProTides including PCV as a control.

From these results, the phosphoramidate technology did not improve the activity versus VZV compared to the parent nucleoside. Interestingly, a loss of activity in TK-deficient strains has been found for the phosphoramidates compared to PCV, and this could be due to a poor activation for these compounds. Moreover, all the compounds have been obtained as a di-ProTide and this aspect may influence the activity especially in terms of bioactivation. Notably, the toxicity found for the protected compounds is in the range between 20-44.5 μM.

Table 8.4 reports the activity versus HSV-1 and 2. Unfortunately, the biological results show a total loss of activity against HSV for the ProTides compared to the parent compound. This lack of activity can be due to a poor activation of the ProTides in this specific assay, which could be due to the fact that the di-ProTide is not a good substrate for the enzymes involved in the bioactivation pathway.

Cpds	Aryl	CLogP	Antiviral Activity EC ₅₀ (μM) ^a			Cytotoxicity (μM)
			HSV-1	HSV-2	HSV-1 TK ⁻	MCC ^b
215a	Ph	4.04	>100	>100	>100	>100
215b	Naph	6.38	>100	>100	>100	>100
216a	Ph	3.83	>100	>100	>100	>100
216b	Naph	6.18	>100	>100	>100	>100
PCV	-	-2.72	0.6	0.8	4	>100

a) effective concentration required to reduce virus plaque formation by 50%;

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology;

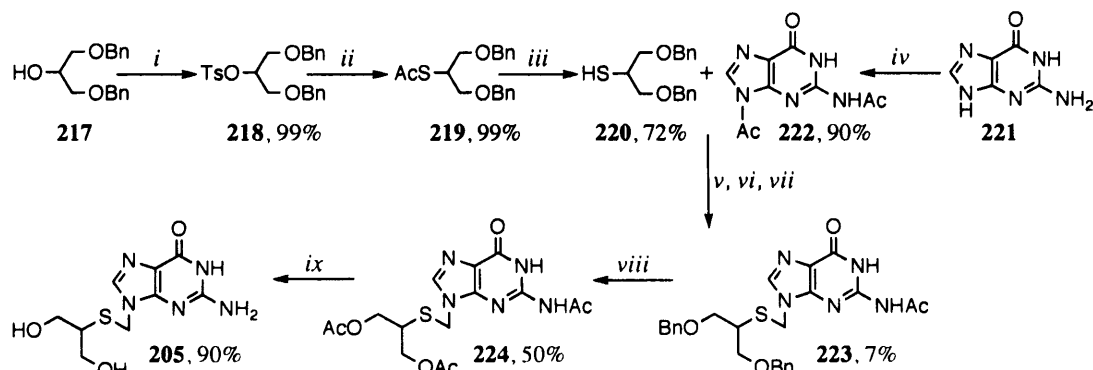
Table 8.4. Antiviral activity against HSV-1 and 2 for PCV ProTides including PCV as a control.

No activity (> 100 μM) for these compounds has been found against: vaccinia virus, vesicular stomatitis virus, feline corona virus, feline herpes virus (data not shown).

8.5.3 Synthesis of thio-gancyclovir derivative

The third compound selected from the modelling was the thio-derivative of GCV. The synthesis of this compound involves 9 steps following the procedure reported in the literature.¹⁴ The commercially available compound **217** is converted to the tosyl derivative **218** in quantitative yield, which is then converted to the thioacetyl derivative **219**. The deprotection of the acetyl group has been carried out using methanolic ammonia or sodium methoxide in methanol obtaining the first synthon **220** in 72% in both cases. The second synthon **222** was obtained by acetylation from the commercially available guanine **221** in acetic anhydride and dimethylacetamide.¹⁵ The coupling between the two synthons is a “one-pot-three-step” reaction involving firstly the attachment of the hydroxyl methylene group to the thio-group followed by acetylation of the primary alcohol and then coupling

with the base to obtain a mixture of N-7 and N-9 isomers. The desired compound **223** was obtained as a pure N-9 isomer after trituration using DCM and diethyl ether. The structure of the compound was confirmed by ^1H -NMR comparing the result with that reported in the literature.¹⁴

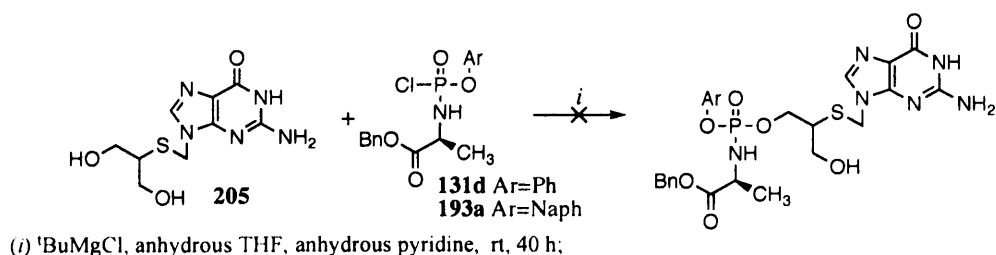


Reagents and Conditions: (i) *p*-Ts-Cl, anhydrous pyridine, rt, overnight; (ii) potassium thioacetate, anhydrous DMF, 90 °C, 2 h; (iii) MeOH/NH₃, rt, 16 h or MeONa, MeOH, rt, 1.5 h; (iv) acetic anhydride, dimethylacetamide, 160 °C, 7 h; (v) paraformaldehyde, HCl gas, 1,2-dichloroethane, 0 °C, 2 h; (vi) sodium acetate, anhydrous DMF, rt, 2 h; (vii) diacetylguanine, bis(4-nitrophenyl)phosphate, sulfolane, 100 °C, 6 h then rt, 17 h; (viii) acetic anhydride, BF₃·Et₂O, rt, 2.5 h; (ix) MeOH/NH₃, rt, 18 h.

Scheme 8.3: synthesis of the thio-GCV derivative.

The benzyl groups of **223** were converted to acetyl groups using acetic anhydride and borontrifluoride etherate to yield compound **224** which was then fully deprotected using methanolic ammonia to obtain the desired compound **205** in good yield.

An attempt to prepare the phosphoramidates of **205** was made starting from the unprotected nucleoside (**Scheme 8.4**).



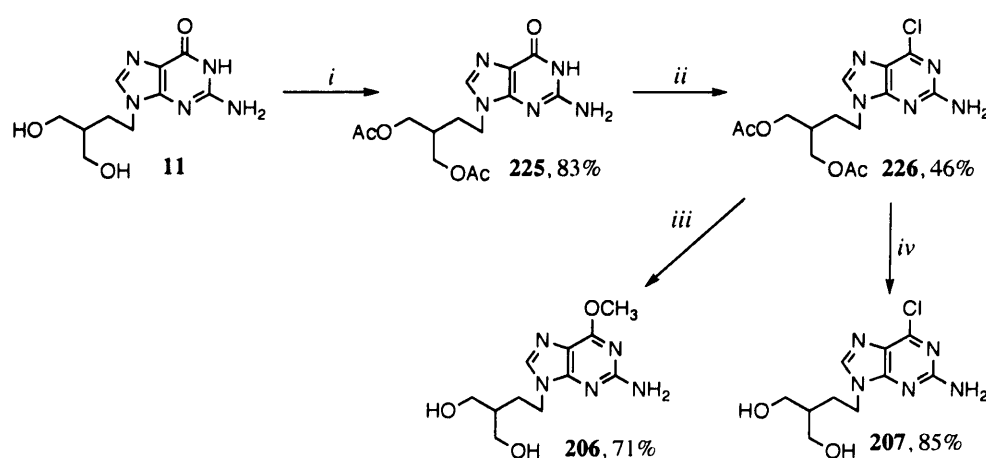
(i) $^i\text{BuMgCl}$, anhydrous THF, anhydrous pyridine, rt, 40 h;

Scheme 8.4: attempt to obtain thio-GCV derivative phosphoramidates.

Unfortunately, the direct coupling did not succeed, probably due to the poor solubility of the starting material. Due to time constraints, the synthesis of these derivatives using protected **205** was not investigated. However, compound **205** was biologically evaluated and it will be discussed later in this chapter.

8.5.4 Synthesis of 6-methoxy and 6-chloro penciclovir derivatives

For the synthesis of these two derivatives a procedure reported in the literature was followed.¹⁶ PCV was acetylated using acetic anhydride and DMAP as a catalyst to obtain compound **225** in good yield. The chlorination at the 6-position was achieved using POCl₃ in the presence of benzyltriethylammonium chloride and N,N-dimethylaniline to yield compound **226**. The deprotection of the acetyl group using sodium methoxide in methanol at 40 °C led also to the displacement of the 6-chloro obtaining compound **206**, whilst the use of methanolic ammonia at room temperature led only to the deprotection of the acetyl group obtaining compound **207**.

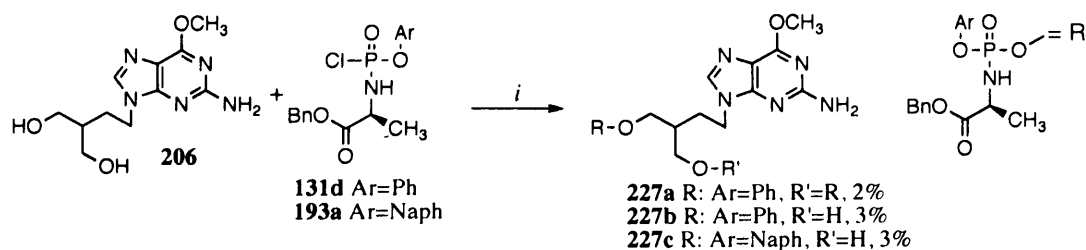


Reagents and Conditions: (i) acetic anhydride, DMAP, DMF, 45 °C, 1.5 h; (ii) POCl₃, benzyltriethylammonium chloride, N,N-dimethylaniline, acetonitrile, 70 °C, 1h; (iii) MeONa, MeOH, 40 °C, 6 h; (iv) MeOH/NH₃, rt, 6 h.

Scheme 8.4: synthesis of the 6-methoxy and 6-chloro PCV derivatives.

8.5.4.1 Synthesis of 6-methoxy penciclovir ProTides

The synthesis of 6-methoxy PCV ProTides was achieved without the need of the 2-amino protection as this compound was soluble in THF. Also in this case the coupling was performed using ^tBuMgCl as a hydroxyl activator (**Scheme 8.5**).¹³



(i) ^tBuMgCl, anhydrous THF, rt, 40 h.

Scheme 8.5: synthesis of 6-methoxy PCV ProTides.

In the case of the phenyl derivative the mono- and the di- ProTides were isolated, whilst for the 1-naphthyl derivative only the mono-ProTide was isolated.

In spite of being soluble in THF, compound **206** did not show a good reactivity. However, the desired compounds were isolated and the structure confirmed by NMR and mass spectroscopy.

Due to time constraints, it was not possible to synthesise a ProTide of compound **207**, however, the nucleoside was biologically evaluated (**Table 8.5**).

8.5.4.2 Biological evaluation

The biological evaluation of these compounds against HIV is currently underway. The compounds have been evaluated for their ability to inhibit HSV-1 and 2.

Table 8.5 reports the biological evaluation against HSV-1 and 2 TK-efficient, and versus HSV-1 TK-deficient strain.

Cpds	ProTide	Aryl	CLogP	Antiviral Activity EC ₅₀ (μM) ^a			Cytotoxicity (μM)
				HSV-1	HSV-2	HSV-1 TK ⁻	MCC ^b
205	-	-	-2.38	0.5	0.6	2.4	>100
206	-	-	-0.76	100	>100	>100	>100
207	-	-	-1.01	>100	>100	>100	>100
227a	Di	Ph	5.79	100	>100	100	>100
227b	Mono	Ph	2.51	58	45	58	>100
227c	Mono	Naph	3.39	38	20	20	>100
GCV	-	-	-2.54	0.03	0.03	0.03	>100
ACV	-	-	-2.42	0.4	0.4	10	>250

a) effective concentration required to reduce virus plaque formation by 50%.

b) minimum cytotoxic concentration that cause a microscopically detectable alteration of cell morphology.

Table 8.5: anti-HSV activity of thio-GCV derivatives, 6-chloro-PCV, 6-methoxy-PCV and its ProTides.

The biological results show some activity of the 6-methoxy-PCV Protides. In fact, whilst the parent nucleoside **206** did not show any anti-HSV activity the monoProTide derivatives **227b** and **227c** showed some activity, which is fully retained versus the TK-deficient strain. Interestingly, compound **205** showed a

good activity in submicromolar range confirming the results reported in the literature.¹⁴

8.6 Conclusion

A series of compounds have been designed and synthesised as potential anti-HIV agents. Unfortunately, in the case of GCV, although it was found one of the best RT-docked compounds, no activity against HIV has been observed. This lack of activity may be due to a lack in the bioactivation pathway, which is supported by the carboxypeptidase study.

For the other designed compounds, the anti-HIV evaluation is currently underway. Other antiviral assays have been performed for these compounds showing poor activity versus VZV and HSV in the case of PCV ProTides and slight improvement of activity for the **206** ProTides. It must be said that in the case of PCV, only the di-Protides were isolated and this aspect may influence the activity in terms of bioactivation and the delivery of the monophosphate.

8.7 References

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9. Experimental Part

9.1 General Experimental Details

Solvents and reagents

The following anhydrous solvents were bought from Aldrich with subseal stopper:

Chloroform (CHCl_3), dichloromethane (DCM), diethyl ether (Et_2O), N,N-dimethylformamide (DMF), N-methylimidazole (NMI), pyridine, tetrahydrofuran (THF), triethylamine (TEA). All reagents commercially available were used without further purification.

Thin Layer Chromatography (TLC)

Precoated, aluminium backed plates (60 F_{254} , 0.2 mm thickness, Merck) were visualized under both short and long wave ultraviolet light (254 nm and 366 nm). Preparative TLC plates (20x20 cm, 500-2000 μm) were purchased from Merck.

Column Chromatography (CC)

Column chromatography processes were carried out using silica gel supplied by Fisher (60A, 35-70 μm). Glass columns were slurry packed using the appropriate eluent and samples were applied either as a concentrated solution in the same eluent or pre-adsorbed on silica gel.

High Performance Liquid Chromatography (HPLC)

Analytical and semi-preparative HPLC were conducted by Varian Prostar (LC Work Station- Varian prostar 335 LC detector, Varian fraction collector (model 701), pro-star 210 solvent delivery system, using Varian Polaris C18-A (10 μm) as an analytic column and Varian Polaris C18-A (10 μm) as a semi-preparative column. The software used was Galaxie Chromatography Data System.

Nuclear Magnetic Resonance (NMR)

^1H -NMR (500 MHz), ^{13}C -NMR (125 MHz), ^{31}P -NMR (202 MHz) were recorded on a Bruker Avance 500MHz spectrometer at 25 °C. Spectra were calibrated to the residual signal of the deuterated solvent used. Chemical shifts are given in parts per million (ppm) and coupling constants (J) in Hertz.

The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), dt (doublet of triplet).

Mass Spectroscopy (MS)

High resolution mass spectroscopy was performed as a service by Birmingham University, using fast atom bombardment (FAB) and by Cardiff University, using electrospray (ES).

Elemental Analysis (CHN)

CHN microanalysis were performed as a service by the School of Pharmacy at the University of London and by MEDAC Ltd, Surrey.

9.2 Standard procedures

Standard procedure A: synthesis of dichlorophosphates

To a solution of phosphorus oxychloride (1.00 mol/eq) and the appropriate substituted phenol or naphthol (1.00 mol/eq) in anhydrous diethyl ether stirred under an argon atmosphere and was added dropwise at -78 °C anhydrous TEA (1.00 mol/eq). Following the addition, the reaction mixture was stirred at -78 °C for 30 min, then at room temperature overnight. The formation of the desired compound was monitored by ^{31}P NMR. The mixture was filtered under nitrogen and the corresponding filtrate reduced to dryness to give the crude product as an oil.

Standard Procedure B: synthesis of phosphorochloridates

To a stirred solution of the appropriate aryl dichlorophosphate (1.00 mol/eq) and the appropriate amino acid ester salt (1.00 mol/eq) in anhydrous DCM was added, dropwise at -78 °C under an argon atmosphere, anhydrous TEA (2.00 mol/eq). Following the addition the reaction mixture was stirred at -78 °C for 1 h, then at room temperature for 2 h. Formation of the desired compound was monitored by ^{31}P NMR. After this period the solvent was removed under reduced pressure and the residue triturated with dry diethyl ether. The precipitate was filtered under nitrogen and the solution was concentrated to give an oil. Most of the aryl phosphorochloridates synthesised were purified by flash column chromatography (eluting with ethyl acetate/petroleum ether = 60/40).

Standard Procedure C: synthesis of phosphoramidates ($^t\text{BuMgCl}$ method)

To a stirring suspension/solution of the appropriate nucleoside (1.00 mol/eq) in anhydrous THF was added dropwise under an argon atmosphere $^t\text{BuMgCl}$ (1.10 to 2.00 mol/eq) and the reaction mixture was stirred at room temperature for 30 min. Then was added dropwise a solution of the appropriate phosphorochloridate (1.10 to 4.00 mol/eq) in anhydrous THF. The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was purified by column chromatography eluting with DCM/MeOH in different proportions.

Standard Procedure D: synthesis of phosphoramidates (NMI method)

To a stirring solution of the appropriate nucleoside (1.00 mol/eq) and the appropriate phosphorochloridate (3.00 – 4.00 mol/eq) in anhydrous THF was added dropwise NMI (5-10 mol/eq) and the reaction was stirred at room temperature overnight. After this period the solvent was removed under reduced pressure. The residue was dissolved in DCM, washed with water (twice) and 0.5 N HCl (twice). The organic phase was dried over MgSO_4 , filtered, reduced to dryness and the crude purified by column chromatography eluting with DCM/MeOH in different proportions.

Standard enzymatic procedure using carboxypeptidase Y

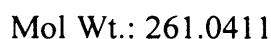
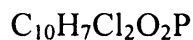
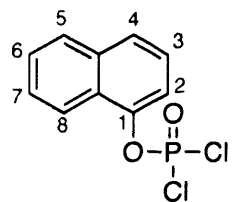
An appropriate phosphoramidate (~5 mg) was dissolved in d_6 -acetone (0.15 mL) and trizma buffer (pH = 7.6) (0.30 mL) and a ^{31}P -NMR experiment was recorded (starting material). Then a solution of carboxypeptidase Y (~0.1 mg) in trizma buffer (0.15 mL) was added and a ^{31}P -NMR experiment was performed recording the experiment every 7 or 15 min.

Molecular modelling

All molecular modelling studies were performed on a MacPro dual 2.66GHz Xeon running Ubuntu 8 using Molecular Operating Environment (MOE) 2008.10 and Plants 1.0. Hydrogen atoms were added to the crystal structure of proteins and minimised with MOE until a gradient of 0.05 Kcal mol⁻¹ Å⁻¹ was reached, using the MMFF94x forcefield. The partial charges were automatically calculated. Docking experiments were carried out using Plants with the default parameters. The results were examined with MOE.

9.3 Synthesis of phosphorodichloridates

Synthesis of 1-naphthyl dichlorophosphate [228]

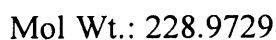
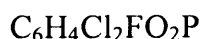
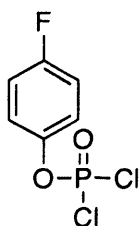


To a stirred solution of 1-naphthol (4.00 g, 27.74 mmol) in dry diethyl ether (60 mL), under an Argon atmosphere, were added POCl_3 (2.59 mL, 27.74 mmol) and dry TEA (3.87 mL, 27.74 mmol) was then added dropwise, at -78°C . Following the addition, after 30 min at -78°C , the reaction mixture was stirred at room temperature overnight. After ^{31}P NMR, the solvent was removed under reduced pressure and the residue was triturated with dry diethyl ether. The precipitate was filtered, and the organic phase was removed under reduced pressure to give an oil (95%, 6.91 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 3.72.

^1H -NMR (CDCl_3 , 500 MHz): δ 8.02-8.00 (1H, m, H-8), 7.81-7.80 (1H, m, H-5), 7.72-7.70 (1H, m, H-4), 7.54-7.45 (4H, m, H-2, H-3, H-6, H-7).

Synthesis of 4-fluorophenyldichlorophosphate [229]



To a stirred solution of 4-fluorophenol (1.83 g, 16.34 mmol) in dry diethyl ether (30 mL), under an Argon atmosphere, were added POCl_3 (16.34 mL, 16.34 mmol) and dry TEA (2.28 mL, 16.34 mmol) was then added dropwise, at -78°C . Following the addition, after 30 min at -78°C , the reaction mixture was stirred at room temperature overnight. After ^{31}P NMR, the solvent was removed under reduced pressure and the residue was triturated with dry diethyl ether. The precipitate was filtered, and the organic phase was removed under reduced pressure to give an oil (79%, 2.94 g).

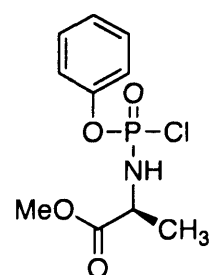
^{31}P -NMR (CDCl_3 , 202 MHz): δ 4.18.

^{19}F -NMR (CDCl_3 , 471 MHz): δ -114.28, -114.29.

^1H -NMR (CDCl_3 , 500 MHz): δ 7.33-7.27 (2H, m, Ph), 7.17-7.11 (2H, m, Ph).

9.4 Synthesis of phosphorochloridates

Synthesis of phenyl-(methoxy-L-alaninyl)-phosphorochloridate. [131a]



$\text{C}_{10}\text{H}_{13}\text{ClNO}_4\text{P}$

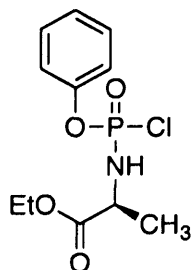
Mol Wt.: 277.6413

Prepared according to Standard Procedure B, from phenyldichlorophosphate (2.24 mL, 15.00 mmol), L-alanine methyl ester $\cdot\text{HCl}$ (2.09 g, 15.00 mmol), anhydrous TEA (4.20 mL, 30.00 mmol) and anhydrous DCM (80 mL). The reaction mixture was stirred at -78°C for 30 min, then at room temperature for 2.5 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 6/4 to give an oil (81%, 3.35 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 7.95, 7.66.

^1H -NMR (CDCl_3 , 500 MHz): δ 7.32-7.15 (5H, m, PhO), 4.42-4.34 (1H, m, NH), 4.17-4.08 (H, m, CHNH), 3.72, 3.70 (3H, 2s, CH_3O), 1.45-1.43 (3H, m, CHCH_3).

Synthesis of phenyl-(ethoxy-L-alaninyl)-phosphorochloridate. [131b]



$\text{C}_{11}\text{H}_{15}\text{ClNO}_4\text{P}$

Mol Wt.: 291.6679

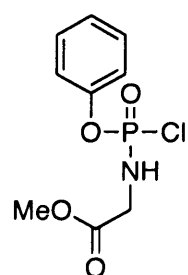
Prepared according to Standard Procedure B, from phenyldichlorophosphate (2.24 mL, 15.00 mmol), L-alanine ethyl ester $\cdot\text{HCl}$ (2.30 g, 15.00 mmol), anhydrous TEA (4.20 mL, 30 mmol) and anhydrous DCM (80 mL). The reaction mixture was stirred at -78°C for 30 min, then at room temperature for 3 h. The crude was purified by column

chromatography eluting with ethyl acetate/hexane = 6/4 to give an oil (75%, 3.29 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 8.03, 7.75, 7.71.

^1H -NMR (CDCl_3 , 500 MHz): δ 7.31-7.15 (5H, m, Ph), 4.20-4.02 (3H, m, OCH_2CH_3 , NHCH), 1.44 (3H, t, OCH_2CH_3), 1.25-1.16 (3H, m, CHCH_3).

Synthesis of phenyl-(methoxy-glyciny)-phosphorochloridate. [131c]



$\text{C}_9\text{H}_{11}\text{ClNO}_4\text{P}$

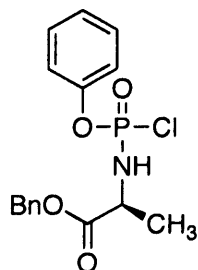
Mol Wt.: 263.6147

Prepared according to Standard Procedure B, from phenyldichlorophosphate (2.24 mL, 15.00 mmol), glycine methyl ester $\cdot\text{HCl}$ (1.88 g, 15.00 mmol), anhydrous TEA (4.20 mL, 30.00 mmol) and anhydrous DCM (80 mL). The reaction mixture was stirred at -78°C for 30 min, then at room temperature for 2 h. The crude was obtained as an oil (93%, 3.70 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 9.03.

^1H -NMR (CDCl_3 , 500 MHz): δ 7.28-7.12 (5H, m, PhO), 4.43 (1H, bs, NHCH_2), 3.84 (2H, d, NHCH_2), 3.72 (3H, s, OCH_3).

Synthesis of phenyl-(benzoxy-L-alaniny)-phosphorochloridate. [131d]



$\text{C}_{16}\text{H}_{17}\text{ClNO}_4\text{P}$

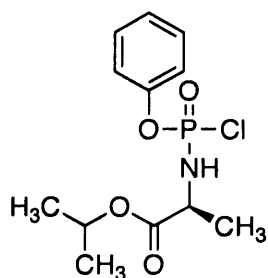
Mol Wt.: 353.7372

Prepared according to Standard Procedure B, using phenyldichlorophosphate (0.30 mL, 2.00 mmol), L-alanine benzyl ester tosylate (0.43 g, 2.00 mmol), anhydrous TEA (0.56 mL, 4.00 mmol) in anhydrous DCM (15 mL). The reaction mixture was stirred at -78°C for 1 h, then at room temperature for 3.5 h. The crude was obtained as an oil (87%, 0.62 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 7.86, 7.52.

^1H -NMR (CDCl_3 , 500 MHz): δ 7.33-7.28 (10H, m, PhO, OCH_2Ph), 5.15-5.13 (2H, m, OCH_2Ph), 4.18-4.13 (1H, m, CHNH), 1.46-1.44 (3H, m, CH_3).

Synthesis of phenyl-(isopropoxy-L-alaninyl)-phosphorochloridate. [131e]



$\text{C}_{12}\text{H}_{17}\text{ClNO}_4\text{P}$

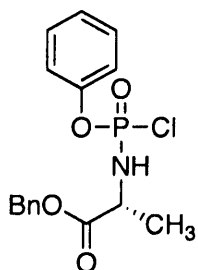
Mol Wt.: 305.6944

Prepared according to Standard Procedure B, from phenyldichlorophosphate (2.00 mL, 13.85 mmol), L-alanine isopropyl ester $\cdot\text{HCl}$ (2.24 g, 13.85 mmol), anhydrous TEA (3.73 mL, 26.77 mmol) and anhydrous DCM (50 mL). The reaction mixture was stirred at -78°C for 30 min, then at room temperature for 2 h. The crude was obtained as an oil (96%, 3.94 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 8.13, 7.75.

^1H -NMR (CDCl_3 , 500 MHz): δ 7.47-7.16 (5H, m, PhO), 5.18-4.98 (1H, m, COOCH), 4.41, 4.33 (1H, 2bs, NHCH), 4.21-4.09 (1H, m, NHCH), 1.53, 1.51 (3H, 2d, $J = 2.3$, CHCH_3), 1.35 – 1.27 (6H, m, $\text{COOCH}(\text{CH}_3)_2$).

Synthesis of phenyl-(benzoxy-D-alaninyl)-phosphorochloridate. [131f]



$\text{C}_{16}\text{H}_{17}\text{ClNO}_4\text{P}$

Mol Wt.: 353.7372

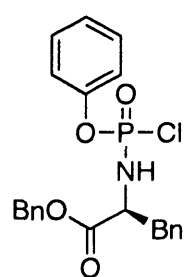
Prepared according to Standard Procedure B, using phenyldichlorophosphate (1.06 mL, 7.11 mmol), D-alanine benzyl ester tosylate (2.50 g, 7.11 mmol), anhydrous TEA (1.98 mL, 14.23 mmol) in anhydrous DCM (50 mL). The reaction mixture was stirred at -78°C for 1 h, then at room temperature for 3.5 h. The crude was purified by column

chromatography eluting with ethyl acetate/hexane = 7/3 to give an oil (84%, 2.11 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 7.86, 7.49.

^1H -NMR (CDCl_3 , 500 MHz): δ 7.47-7.22 (10H, m, PhO, OCH_2Ph), 5.25-5.23 (2H, 2s, OCH_2Ph), 4.32-4.20 (1H, m, CHNH), 1.56, 1.54 (3H, 2d, $J = 3.3$, $J = 2.9$ CH_3).

Synthesis of 1-phenyl-(benzoxy-L-phenylalaninyl)-phosphorochloridate. [131g]



$\text{C}_{22}\text{H}_{21}\text{ClNO}_4\text{P}$

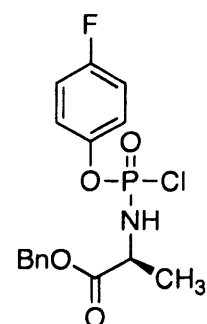
Mol Wt.: 429.8332

Prepared according to Standard Procedure B, from phenyldichlorophosphate (1.81 g, 8.57 mmol), L-phenylalanine benzyl ester $\cdot\text{HCl}$ (2.50 g, 8.57 mmol), anhydrous TEA (2.40 mL, 17.13 mmol) and anhydrous DCM (80 mL). The reaction mixture was stirred at -78°C for 30 min, then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 6/4 to give an oil (58%, 2.15 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 7.80, 7.77.

^1H -NMR (CDCl_3 , 500 MHz): δ 7.32-6.91 (15H, m, PhO, CHCH_2Ph , OCH_2Ph), 5.08-5.07 (2H, m, OCH_2Ph), 4.46-4.33 (1H, m, CHNH), 3.12-3.01 (2H, m, CHCH_2Ph).

Synthesis of 4-fluoro-phenyl-(benzoxy-L-alaninyl)-phosphorochloridate. [131h]


 $C_{16}H_{16}ClFNO_4P$

Mol Wt.: 371.7277

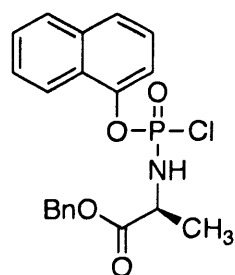
Prepared according to Standard Procedure B, using **229** (1.50 mL, 6.55 mmol), L-alanine benzyl ester tosylate (2.30 g, 6.55 mmol), anhydrous TEA (1.82 mL, 13.10 mmol) in anhydrous DCM (40 mL). The reaction mixture was stirred at -78 °C for 30 min, then at room temperature for 3 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 7/3 to give an oil (72%, 1.75 g).

 ^{31}P -NMR (CDCl₃, 202 MHz): δ 8.34, 8.02.

 ^{19}F -NMR (CDCl₃, 471 MHz): δ -116.29 (d, J = 3.1), -116.33 (d, J = 2.9).

 1H -NMR (CDCl₃, 500 MHz): δ 7.43-7.33 (5H, m, Ph), 7.26-7.20 (2H, m, Ph), 7.09-7.04 (2H, m, Ph), 5.24, 5.23 (2H, 2s, OCH₂Ph), 4.37 (1H, bs, CHNH), 4.32-4.20 (1H, m, CHNH), 1.55, 1.54 (3H, 2d, J = 5.0, J = 4.6, CHCH₃).

Synthesis of 1-Naphthyl(benzoxy-L-alaninyl)-phosphorochloridate. [193a]


 $C_{20}H_{19}ClNO_4P$

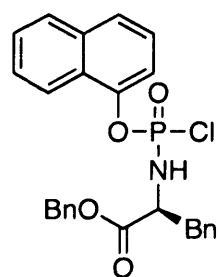
Mol Wt.: 403.7959

Prepared according to Standard Procedure B, **228** (6.91 g, 26.48 mmol), L-alanine benzyl ester tosylate (9.30 g, 26.48 mmol), anhydrous TEA (7.40 mL, 52.96 mmol) in anhydrous DCM (100 mL). The reaction mixture was stirred at -78 °C for 1 h, then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 5/5 to give an oil (72%, 7.68 g).

 ^{31}P -NMR (CDCl₃, 202 MHz): δ 8.14, 7.88.

$^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 7.99-7.25 (12H, m, Naph, OCH_2Ph), 5.15-5.07 (2H, m, CH_2Ph), 4.30-4.23 (1H, m, CHCH_3), 1.49-1.46 (3H, m, CHCH_3).

Synthesis of 1-Naphthyl(benzoxo-L-phenylalaninyl)-phosphorochloridate. [193b]



$\text{C}_{26}\text{H}_{23}\text{ClNO}_4\text{P}$

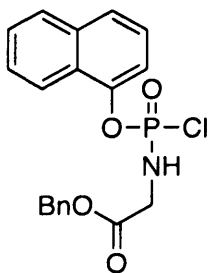
Mol Wt.: 479.8919

Prepared according to Standard Procedure B, from **228** (0.78 g, 3.00 mmol), L-phenylalanine benzyl ester $\cdot\text{HCl}$ (0.87 g, 3.00 mmol), anhydrous TEA (0.84 mL, 6.00 mmol) and anhydrous DCM (20 mL). The reaction mixture was stirred at -78°C for 30 min, then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 6/4 to give an oil (40%, 0.57 g).

$^{31}\text{P-NMR}$ (CDCl_3 , 202 MHz): δ 8.32, 8.19.

$^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 8.04-6.96 (17H, m, Naph, CHCH_2Ph , OCH_2Ph), 5.20-5.11 (2H, m, OCH_2Ph), 4.64-4.52 (1H, m, CHNH), 3.22-3.08 (2H, m, CHCH_2Ph).

Synthesis of 1-Naphthyl(benzoxo-glycinyl)-phosphorochloridate. [193c]



$\text{C}_{19}\text{H}_{17}\text{ClNO}_4\text{P}$

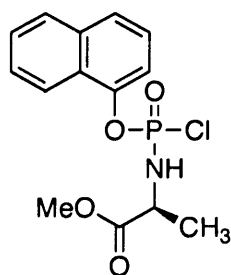
Mol Wt.: 389.7693

Prepared according to Standard Procedure B, **228** (0.78 g, 3.00 mmol), glycine benzyl ester $\cdot\text{HCl}$ (1.01 g, 3.00 mmol), anhydrous TEA (0.84 mL, 6.00 mmol) and anhydrous DCM (20 mL). The reaction mixture was stirred at -78°C for 30 min, then at room temperature for 2.5 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 6/4 to give an oil (73%, 0.85 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 8.90.

^1H -NMR (CDCl_3 , 500 MHz): δ 8.02-7.30 (12H, m, Naph, OCH_2Ph), 5.17 (2H, s, OCH_2Ph), 4.24-4.19 (1H, m, NHCH_2), 4.00-3.96 (2H, m, NHCH_2).

Synthesis of 1-Naphthyl-(methoxy-L-alaninyl)-phosphorochloridate. [193d]



$\text{C}_{14}\text{H}_{15}\text{ClNO}_4\text{P}$

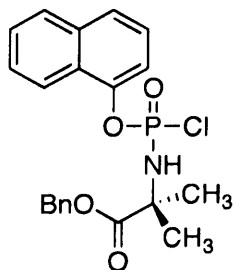
Mol Wt.: 327.7000

Prepared according to Standard Procedure B, **228** (2.00 g, 7.66 mmol), L-alanine methyl ester $\cdot\text{HCl}$ (1.07 g, 7.66 mmol), anhydrous TEA (2.14 mL, 15.32 mmol) in anhydrous DCM (45 mL). The reaction mixture was stirred at -78°C for 1 h, then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 6/4 to give an oil (61%, 1.54 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 8.14, 7.88.

^1H -NMR (CDCl_3 , 500 MHz): δ 8.01-7.35 (7H, m, Naph), 4.33-4.27 (1H, m, NH), 4.26-4.20 (1H, m, CHNH), 3.74, 3.69 (3H, 2s, CH_3O), 1.50-1.46 (3H, m, CHCH_3).

Synthesis of 1-Naphthyl(benzoxo-dimethylglycinyl)-phosphorochloridate. [193e]



$\text{C}_{21}\text{H}_{21}\text{ClNO}_4\text{P}$

Mol Wt.: 417.8225

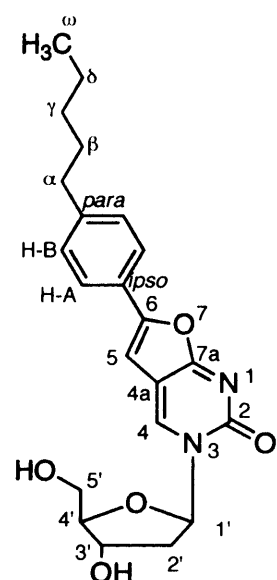
Prepared according to the Standard Procedure B, using **228** (1.53 g, 5.88 mmol) and dimethylglycine benzyl ester tosylate (2.15 g, 5.88 mmol), anhydrous TEA (4.59 mL, 32.94 mmol) and anhydrous DCM (50 mL). The reaction was stirred at room temperature for 2 h. The crude product was obtained as an oil (33%, 0.80 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 5.78.

^1H -NMR (CDCl_3 , 500 MHz): δ 8.03-7.28 (12H, m, Naph, OCH_2Ph), 5.16 (2H, s, OCH_2Ph), 1.76, 1.70 (6H, 2s, $\text{C}(\text{CH}_3)_2$).

9.5 Synthesis of 5'-derivatives of BCNA lead compound

Synthesis of 3-(2'-deoxy- β -D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one. [86f]



$C_{22}H_{26}N_2O_5$

Mol Wt.: 398.4522

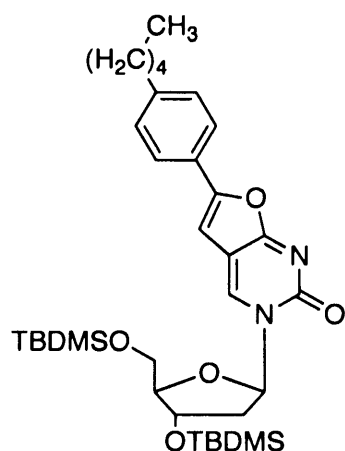
To a stirred solution of **121** (3.18 g, 9.00 mmol) in anhydrous DMF (46 mL), were added: 4-*n*-pentylphenylacetylene (5.26 mL, 27.00 mmol), tetrakis(triphenylphosphine) palladium(0) (1.04 g, 0.90 mmol), copper (I) iodide (0.34 g, 1.80 mmol) and DIPEA (3.14 mL, 18.00 mmol) and the reaction mixture was stirred at room temperature, under an Argon atmosphere overnight. After this period were added copper (I) iodide (0.34 g, 1.80 mmol)

and anhydrous TEA (46 mL) and the reaction mixture was stirred at 85 °C for 6 h. The solvent was then removed in vacuo, and the resulting residue was dissolved in DCM and the solution was stirred at room temperature overnight. The precipitate obtained was filtered and washed with DCM, to give a white solid (63%, 2.25 g).

$^1\text{H-NMR}$ (DMSO, 500 MHz): δ 8.84 (1H, s, H-4), 7.74 (2H, d, $J = 8.2$, H_a), 7.33 (2H, d, $J = 8.2$, H_b), 7.21 (1H, s, H-5), 6.20 (1H, t, H-1'), 5.29 (1H, d, 3'-OH), 5.16 (1H, t, 5'-OH), 4.26 (1H, m, H-3'), 3.94 (1H, m, H-4'), 3.72 (1H, m, H-5'), 3.64 (1H, m, H-5'), 2.63 (2H, t, α -CH₂), 2.42 and 2.11 (2H, 2m, H-2'), 1.60 (2H, m, β -CH₂), 1.36-1.25 (4H, m, γ -CH₂, δ -CH₂), 0.87 (3H, t, CH₃).

$^{13}\text{C-NMR}$ (DMSO, 126 MHz): δ 13.87 (CH₃), 21.89, 30.35, 30.80, 34.88 (C₄H₈), 41.23 (C-2'), 60.68 (C-5'), 69.53 (C-3'), 87.56 (C-1'), 88.14 (C-4'), 98.67 (C-5), 106.88 (C-4a), 124.54 (C-H_b), 125.87 (*ipso*-C), 128.99 (C-H_a), 137.82 (C-4), 144.07 (*para*-C), 153.75 (C-6), 153.90 (C-2), 171.03 (C-7a).

Synthesis of 3-(2-deoxy-3,5-di-tert-butylidimethylsilyl- β -D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one. [122]



$C_{34}H_{54}N_2O_5Si_2$

Mol Wt.: 626.9740

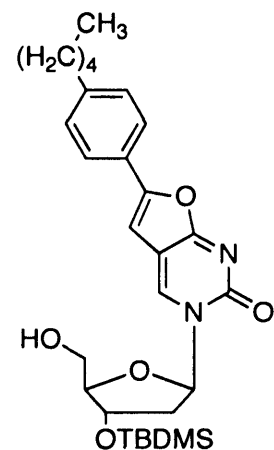
To a solution of **86f** (1.15 g, 2.90 mmol) in dry pyridine (30 mL) were added TBDMSCl (0.96 g, 6.38 mmol) and imidazole (0.43 g, 6.38 mmol) and the reaction mixture was stirred at room temperature for 2h. After this period the solvent was removed in vacuo

and the residue was dissolved in DCM. The organic phase was washed with 0.5 M aqueous solution of citric acid. The organic phase was dried over $MgSO_4$, concentrated to give a pale yellow solid (95%, 1.72 g).

1H -NMR ($CDCl_3$, 500 MHz): δ 8.58 (1H, s, H-4), 7.60 (2H, d, H_a), 7.18 (2H, d, H_b), 6.52 (1H, s, H-5), 6.28-6.26 (1H, m, H-1'), 4.34-4.31 (1H, m, H-3'), 3.97-3.94 (2H, m, H-4', H-5'), 3.77-3.74 (1H, m, H-5'), 2.60-2.55 (3H, m, $H_{a-2'}$, α - CH_2), 2.14-2.09 (1H, m, $H_{b-2'}$), 1.59-1.53 (2H, m, CH_2), 1.29-1.23 (4H, m, $2 \times CH_2$), 0.88 (9H, s, $C(CH_3)_3$), 0.82-0.81 (12H, m, CH_3 , $C(CH_3)_3$), 0.10 (3H, s, Si- CH_3), 0.07 (3H, s, Si- CH_3), 0.01 (3H, s, Si- CH_3), 0.00 (3H, s, Si- CH_3).

^{13}C -NMR ($CDCl_3$, 126 MHz): δ -5.40, -5.35, -4.92, -4.50 (Si- CH_3), 13.98 (ω - CH_3), 17.94, 18.42 ($C(CH_3)_3$), 22.49 (γ - CH_2), 25.71, 26.00 ($C(CH_3)_3$), 30.91, 31.41 (β - CH_2 , δ - CH_2), 35.81 (α - CH_2), 42.64 (C-2'), 61.89 (C-5'), 70.18 (C-3'), 88.06, 88.09 (C-1', C-4'), 96.71 (C-5), 107.83 (C-4a), 124.92 (C- H_b), 125.96 (*ipso*-C), 128.98 (C- H_a), 135.81 (C-4), 144.97 (*para*-C), 154.64 (C-6), 155.78 (C-2), 171.65 (C-7a).

Synthesis of 3-(2-deoxy-3-tert-butyldimethylsilyl-β-D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one. [123]



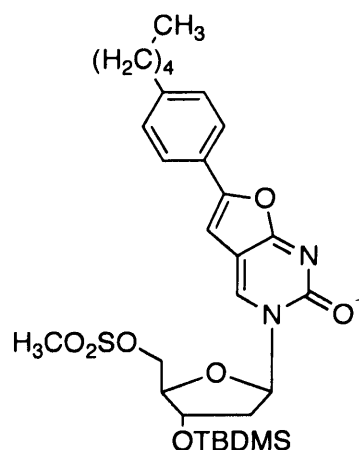
$C_{28}H_{40}N_2O_5Si$

Mol Wt.: 512.7131

To a solution of **122** (1.65 g, 2.64 mmol) in THF (40 mL) and cold water (5 mL) was added, dropwise at 0 °C, cold trifluoroacetic acid (5 mL) and the reaction mixture was stirred at 0 °C for 2 h. After this period the reaction mixture was neutralised with $NaHCO_3$, concentrated and the residue triturated with diethyl ether. The suspension was filtered and the solid washed with diethyl ether to give a white solid (83%, 1.12 g).

1H -NMR ($CDCl_3$, 500 MHz): δ 8.54 (1H, s, H-4), 7.57 (2H, d, H_a), 7.15 (2H, d, H_b), 6.60 (1H, s, H-5), 6.23 (1H, t, H-1'), 4.42-4.38 (1H, m, H-3'), 4.01-3.96 (2H, m, H-4', H-5'), 3.81-3.78 (1H, m, H-5'), 2.56-2.49 (3H, m, H_a -2', α -CH₂), 2.28-2.23 (1H, m, H_b -2'), 1.60-1.52 (2H, m, β -CH₂), 1.29-1.23 (4H, m, δ -CH₂, γ -CH₂), 0.83-0.81 (12H, m, CH₃, C(CH₃)₃), 0.00 (6H, s, Si-(CH₃)₂).

Synthesis of 3-(2-deoxy-3-tert-butyldimethylsilyl-5-mesyl-β-D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one. [124]



$C_{29}H_{42}N_2O_7SSi$

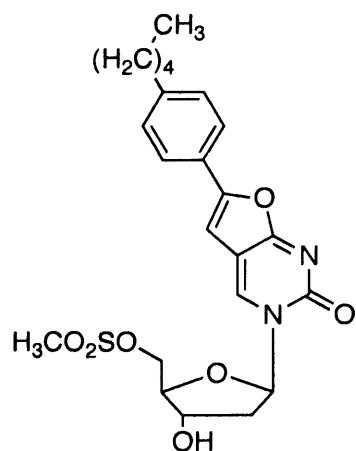
Mol Wt.: 590.2482

To a solution of **123** (1.02 g, 2.00 mmol) in dry pyridine (20 mL) was added dropwise, at 0 °C, methanesulfonyl chloride (0.23 mL, 3.00 mmol) and the reaction mixture was stirred at room temperature overnight, then methanesulfonyl chloride (0.23 mL,

3.00 mmol) was added and the reaction mixture was stirred for 7 h at room temperature. The mixture was quenched with water and the precipitate was filtered to give a yellow solid (66%, 0.78 g).

¹H-NMR (CDCl₃, 500 MHz): δ 8.34 (1H, s, H-4), 7.58 (2H, d, J = 6.9, H_a), 7.16 (2H, d, J = 6.9, H_b), 6.60 (1H, s, H-5), 6.23 (1H, dd, J = 6.6, H-1'), 4.53 (1H, m, H-3'), 4.33-4.28 (2H, m, H-5'), 4.09 (1H, m, H-4'), 3.02 (3H, s, CH₃), 2.59 (1H, m, H-2'), 2.55 (2H, t, J = 7.5, α-CH₂), 2.17 (1H, m, H-2'), 1.55 (2H, m, β-CH₂), 1.27-1.22 (4H, m, δ-CH₂, γ-CH₂), 0.82 (9H, s, C(CH₃)₃), 0.80 (3H, t, CH₃), 0.00 (6H, s, Si(CH₃)₂).

Synthesis of 3-(2-deoxy-5-mesyl-β-D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one. [125]



C₂₃H₂₈N₂O₇S

Mol Wt.: 476.1617

To a solution of **124** (0.93 g, 1.58 mmol) in THF (12 mL) and water (12 mL) was added trifluoroacetic acid (3 mL), and the reaction mixture was stirred at room temperature for 2h, then trifluoroacetic acid (1 mL) was added. After 30 minutes trifluoroacetic acid (1 mL) was added and the reaction mixture was stirred for further 1h. After this period the solvent was removed under reduced pressure and the crude was purified by column chromatography (gradient elution of DCM/MeOH = 97/3 then 95/5) to give a pale yellow solid (70%, 0.60 g).

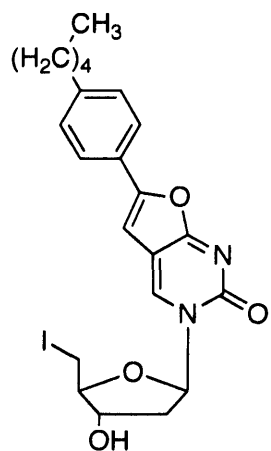
¹H-NMR (DMSO, 500 MHz): δ 8.55 (1H, s, H-4), 7.75 (2H, d, J = 8.2, H_a), 7.34 (2H, d, J = 8.2, H_b), 7.16 (1H, s, H-5), 6.26 (1H, t, J = 6.4, H-1'), 5.56 (1H, d, J = 4.4, 3'-OH), 4.54-4.42 (2H, m, H-5'), 4.30-4.26 (1H, m, H-3'), 4.18-4.16 (1H, m, H-4') 3.31 (3H, s, CH₃), 2.62 (2H, t, J = 7.6, α-CH₂), 2.49-2.41 (1H, m, H_a-2'), 2.26-2.17 (1H, m, H_b-2'), 1.64-1.56 (2H, m, β-CH₂), 1.36-1.23 (4H, m, δ-CH₂, γ-CH₂), 0.87 (3H, t, J = 7.0, ω-CH₃).

^{13}C -NMR (DMSO, 126 MHz): δ 13.86 ($\omega\text{-CH}_3$), 21.88 (C- γ), 30.33 (C- β), 30.79 (C- δ), 34.88 (C- α), 36.87 (SO_2CH_3), 40.27 (C-2'), 69.17 (C-5'), 69.80 (C-3'), 84.38 (C-4'), 87.86 (C-1'), 98.52 (C-5), 107.20 (C-4a), 124.59 (C- H_b), 125.77 (*ipso*-C), 128.99 (C- H_a), 137.57 (C-4), 144.16 (*para*-C), 153.68 (C-6), 154.11 (C-2), 171.14 (C-7a).

EI MS= 499.1520 (M+Na).

Anal. Calcd for $\text{C}_{23}\text{H}_{28}\text{N}_2\text{O}_7\text{S}$: C, 57.97; H, 5.92; N, 5.88. Found: C, 57.98; H, 5.90; N, 5.79.

Synthesis of 3-(2,5-dideoxy-5-iodo- β -D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one. [126]



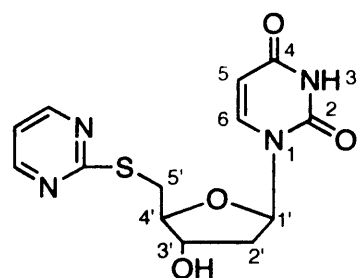
$\text{C}_{22}\text{H}_{25}\text{IN}_2\text{O}_4$

Mol Wt.: 508.0859

To a solution of **125** (0.10 g, 0.21 mmol), in acetone (3 mL) was added sodium iodide (0.25 g, 1.68 mmol) and the reaction mixture was stirred at 65 °C for 3.5 h, then at room temperature overnight, then at 70 °C for 7h. The solvent was removed and the residue purified by column chromatography eluting DCM/MeOH = 93/7, to give a pale yellow solid. (61%, 0.065g).

^1H -NMR (DMSO 500 MHz): δ 8.60 (1H, s, H-4), 7.75 (2H, d, J = 8.3, H_a), 7.34 (2H, d, J = 8.3, H_b), 7.22 (1H, s, H-5), 6.26 (1H, t, H-1'), 5.57 (1H, 3'-OH), 4.21 (1H, bs. H-3'), 4.06-4.03 (1H, m, H-4'), 3.57-3.55 (2H, m, H-5') 2.63 (2H, t, J = 7.6, $\alpha\text{-CH}_2$), 2.47-2.43 (1H, m, H-2'), 2.30-2.24 (1H, m, H-2'), 1.63-1.57 (2H, m, $\beta\text{-CH}_2$), 1.35-1.25 (4H, m, $\delta\text{-CH}_2$, $\gamma\text{-CH}_2$), 0.87 (3H, t, CH_3).

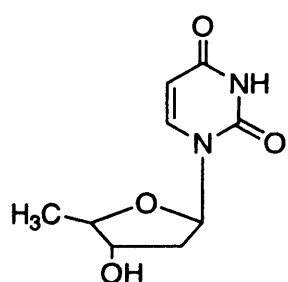
^{13}C -NMR (DMSO, 126 MHz): δ 7.27 (C-5'), 13.87 (CH_3), 21.89, 30.34, 30.79, 34.88 (C_4H_8), 40.06 (C-2'), 72.93 (C-3'), 86.73 (C-1'), 87.88 (C-4'), 98.63 (C-5), 107.23 (C-4a), 124.58 (C- H_b), 125.78 (*ipso*-C), 129.03 (C- H_a), 137.38 (C-4), 144.07 (*para*-C), 153.70 (C-6), 154.11 (C-2), 171.15 (C-7a).

Synthesis of 5'-(pyrimidin-2-ylthio)-2'-deoxyuridine. [128]C₁₃H₁₄N₄O₄S

Mol Wt.: 322.3397

To a solution of **127** (3.00 g, 13.46 mmol) in dry acetonitrile (60 mL) were added 2-mercapto-pyrimidine (1.84 g, 16.43 mmol) and N,N-dimethylformamide dioneopentylacetal (8 mL) and the reaction mixture was stirred under reflux for 4 h. The solvent was then removed under reduced pressure and the residue was purified by column chromatography eluting with DCM/MeOH = 9/1, to give a yellow solid (84%, 3.56 g).

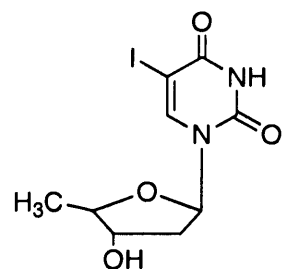
¹H-NMR (DMSO 500 MHz): δ 11.32 (1H, s, NH), 8.65 (2H, d, J = 5.1, pyrimidine), 7.67 (1H, d, J = 8.1, H-6), 7.24 (1H, t, J = 5.0, pyrimidine), 6.17 (1H, t, H-1'), 5.66 (1H, d, J = 8.1, H-5), 5.41 (1H, d, 3'-OH), 4.24-4.23 (1H, m, H-3'), 4.01-3.98 (1H, m, H-4'), 3.46-3.43 (2H, m, H-5'), 2.28-2.23 (1H, m, H-2'_a), 2.14-2.10 (1H, m, H-2'_b).

Synthesis of 2',5'-dideoxyuridine. [129]C₉H₁₂N₂O₄

Mol Wt.: 212.2026

To a solution of **128** (2.32 g, 7.21 mmol) in ethanol (110 mL) was added raney-nickel (13 g) and the reaction mixture was stirred at 80 °C for 1 h. After this period the suspension was filtered through celite and the catalyst was washed with hot ethanol. The clear solution was concentrated under reduced pressure to give a yellow solid (63%, 1.50 g).

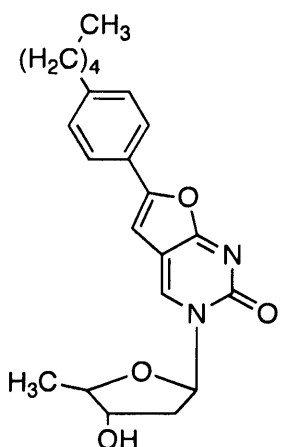
¹H-NMR (DMSO 500 MHz): δ 11.28 (1H, bs, NH), 7.59 (1H, d, J = 8.0, H-6), 6.09 (1H, t, H-1'), 5.64 (1H, d, J = 8.0, H-5), 5.26 (1H, bs, OH), 3.96-3.95 (1H, m, H-3'), 3.83-3.78 (1H, m, H-4'), 2.19-2.14 (1H, m, H_a-2'), 2.12-2.07 (1H, m, H_b-2'), 1.24 (3H, d, H-5').

Synthesis of 5-iodo-2',5'-dideoxyuridine. [130]C₉H₁₁IN₂O₄

Mol Wt.: 338.0991

To a solution of **129** (0.42 g, 1.96 mmol) in acetonitrile (20 mL) were added iodine (0.30 g, 1.18 mmol) and cerium (IV) ammonium nitrate (CAN) (0.54 g, 0.98 mmol) and the reaction mixture was stirred at reflux for 1.5 h. After this period the reaction was quenched with saturated solution of NaHSO₃ then concentrated. The residue was dissolved in ethyl acetate and washed with brine (twice), dried under MgSO₄ and concentrated under reduced pressure to give a pale yellow solid (83%, 0.55 g).

¹H-NMR (DMSO 500 MHz): δ 11.74 (1H, bs, NH), 7.98 (1H, d, H-6), 6.07 (1H, t, H-1'), 5.29 (1H, d, OH), 4.04-4.00 (1H, m, H-3'), 3.90-3.86 (1H, m, H-4'), 2.37-2.31 (1H, m, H_a-2'), 2.16-2.11 (1H, m, H_b-2'), 1.31 (3H, d, H-5').

Synthesis of 3-(2',5'-dideoxy-β-D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one. [111]C₂₂H₂₆N₂O₄

Mol Wt.: 382.4528

To a stirred solution of **130** (1.36 g, 4.04 mmol) in dry DMF (25 mL), were added: 4-*n*-pentylphenylacetylene (2.36 mL, 12.12 mmol), tetrakis(triphenylphosphine) palladium(0) (0.47 g, 0.40 mmol), copper (I) iodide (0.15 g, 0.81 mmol) and DIPEA (1.41 mL, 8.08 mmol) and the reaction mixture was stirred at room temperature, under an Argon atmosphere, overnight. After this period, were added copper (I) iodide (0.15 g, 0.81 mmol) and anhydrous TEA (25 mL) and the reaction mixture was stirred at 85 °C for 7 h. After cooling the reaction mixture was concentrated under reduced pressure and the residue purified by column chromatography eluting with DCM/MeOH = 94/6, to give a brown solid (63%, 0.93 g).

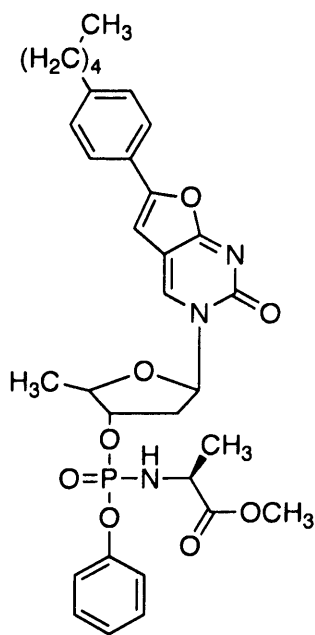
^1H -NMR (DMSO, 500 MHz): δ 8.57 (1H, s, H-4), 7.78 (2H, d, $J = 8.2$, H_a), 7.38 (2H, d, $J = 8.2$, H_b), 7.24 (1H, s, H-5), 6.19 (1H, t, H-1'), 5.36 (1H, d, 3'-OH), 4.07-4.02 (2H, m, H-3', H-4'), 2.68 (2H, t, α -CH₂), 2.47-2.45 (1H, m, H_a -2'), 2.24-2.20 (1H, m, H_b -2'), 1.67-1.64 (2H, m, β -CH₂), 1.41 (3H, d, H-5'), 1.38-1.32 (4H, m, δ -CH₂, γ -CH₂), 0.92 (3H, t, CH₃).

^{13}C -NMR (DMSO, 126 MHz): δ 13.86 (CH₃), 18.71 (C-5'), 21.89, 30.34, 30.79, 34.88 (C₄H₈), 40.39 (C-2'), 74.03 (C-3'), 83.09 (C-1'), 87.51 (C-4'), 98.83 (C-5), 107.11 (C-4a), 124.51 (C-H_b), 125.85 (*ipso*-C), 129.01 (C-H_a), 137.60 (C-4), 144.08 (*para*-C), 153.74 (C-6), 153.81 (C-2), 171.08 (C-7a).

^{13}C -NMR (CDCl₃, 126 MHz): δ 13.99 (ω -CH₃), 19.34 (C-5'), 22.49, 30.89, 31.42, 35.81 (C₄H₈), 41.23, 43.05 (C-2'), 75.13 (C-3'), 83.68 (C-4'), 88.63 (C-1'), 97.06 (C-5), 108.49 (C-4a), 124.94 (C-H_b), 125.77 (*ipso*-C), 128.76, 129.02 (C-H_a), 135.17 (C-4), 145.13 (*para*-C), 154.97 (C-6), 156.10 (C-2), 171.59 (C-7a).

9.6 Synthesis of 5'-deoxy-3'-phosphoramidate of BCNA lead compound

Synthesis of 3-(2',5'-dideoxy-β-D-ribofuranosyl-3-[phenyl-(methoxy-L-alaninyl)] phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one. [132a]



$C_{32}H_{38}N_3O_8P$

Mol Wt.: 623.6332

Prepared according to Standard Procedure D, from **21** (0.20 g, 0.52 mmol) in anhydrous THF (5 mL), **49a** (0.58 g, 2.09 mmol) in anhydrous THF (2 mL), NMI (0.42 mL, 5.23 mmol) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 97/3. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 99/1 then 98/2) to give a white solid (13%, 0.041 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.40, 2.66.

^1H -NMR (MeOD, 500 MHz): δ 8.42, 8.41 (1H, 2s, H-4), 7.57 (2H, d, Ph), 7.28 (2H, t, OPh), 7.17-7.08 (5H, m, Ph, OPh), 6.89, 6.88 (1H, 2s, H-5), 6.11, 6.07 (1H, 2t, H-1'), 4.80-4.78, 4.75-4.73 (2H, 2m, H-3'), 4.35-4.33, 4.30-4.28 (2H, 2m, H-4'), 3.90-3.87 (1H, m, CHCH₃), 3.60, 3.57 (3H, 2s, COOCH₃), 2.89-2.85, 2.81-2.76 (1H, 2m, H_a-2'), 2.53 (2H, t, α-CH₂), 2.36-2.30, 2.27-2.21 (1H, 2m, H_b-2'), 1.56-1.50 (2H, m, β-CH₂), 1.37, 1.34 (3H, 2d, H-5'), 1.27-1.19 (7H, m, γ-CH₂, δ-CH₂, CHCH₃), 0.80 (3H, t, ω-CH₃).

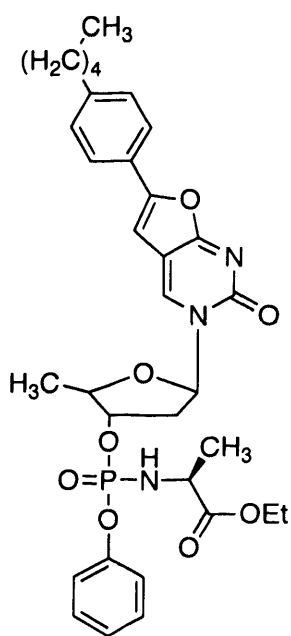
^{13}C -NMR (MeOD, 126 MHz): δ 14.39, 15.46 (2s, ω-CH₃), 19.15, 19.33 (2s, C-5'), 20.16 (d, $J_{\text{C-P}} = 7.4$, CHCH₃), 20.35 (d, $J_{\text{C-P}} = 7.0$, CHCH₃), 23.57, 32.12, 32.61, 36.76 (C₄H₈), 40.34, 40.37 (2s, C-2'), 51.55, 51.74 (2s, CHCH₃), 52.77, 52.81 (2s, COOCH₃), 81.78 (d, $J_{\text{C-P}} = 5.0$, C-3'), 82.24 (d, $J_{\text{C-P}} = 5.5$, C-3'), 83.85 (d, $J_{\text{C-P}} = 5.7$, C-4'), 83.94 (d, $J_{\text{C-P}} = 6.6$, C-4'), 90.00 (C-1'), 98.93, 98.95 (2s, C-5), 110.27 (C-4a), 121.41, 121.45, 121.59, 121.63, 125.94, 126.27, 126.33,

127.23, 130.16, 130.85, 130.89 (C-H_a, C-H_b, 'ipso'-C, PhO), 138.06 (C-4), 146.37 (para-C), 152.18 ('ipso' PhO), 156.32 (C-6), 157.38 (C-2), 173.08 (C-7a), 175.62 (COOCH₃).

EI MS= 646.23 (M+Na).

Anal. Calcd for C₃₂H₃₈N₃O₈P: C, 61.63; H, 6.14; N, 6.74. Found: C, 61.38; H, 6.31; N, 6.45.

Synthesis of 3-(2',5'-dideoxy-β-D-ribofuranosyl-3-[phenyl-(ethoxy-L-alanyl)] phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydro-furo-[2,3 *d*]pyrimidin-2-one. [132b-d]



C₃₃H₄₀N₃O₈P

Mol Wt.: 637.6598

Prepared according to Standard Procedure D, from **21** (0.20 g, 0.52 mmol) in anhydrous THF (5 mL), **50b** (0.61 g, 2.09 mmol) in anhydrous THF (2 mL), NMI (0.42 mL, 5.23 mmol) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2 then 97/3. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 99/1 then 98/2) to give a white solid (total yield 22%, 0.072 g). **132b** (9.6%, 0.032 g),

132c (6%, 0.020 g), **132d** (6%, 0.020 g).

132b:

³¹P-NMR (MeOD, 202 MHz): δ 3.47, 2.73.

¹H-NMR (MeOD, 500 MHz): δ 8.43, 8.42 (1H, 2s, H-4), 7.59-7.58 (2H, m, Ph), 7.27 (2H, t, OPh), 7.17-7.08 (5H, m, Ph, OPh), 6.89, 6.88 (1H, 2s, H-5), 6.12, 6.08 (1H, 2t, H-1'), 4.80-4.77, 4.75-4.73 (1H, 2m, H-3'), 4.36-4.34, 4.30-4.28 (1H, 2m, H-4'), 4.06, 4.00 (2H, 2q, COOCH₂CH₃), 3.88-3.84 (1H, m, CHCH₃), 2.88-2.84, 2.81-2.77 (1H, 2m, H_a-2'), 2.53 (2H, t, α-CH₂), 2.37-2.32, 2.27-2.22 (1H, 2m, H_b-2'), 1.56-1.50 (2H, m, β-CH₂), 1.37, 1.34 (3H, 2d, H-5'), 1.28-1.23

(7H, m, γ -CH₂, δ -CH₂, CHCH₃), 1.15, 1.11 (3H, 2t, COOCH₂CH₃), 0.80 (3H, t, ω -CH₃).

¹³C-NMR (MeOD, 126 MHz): δ 14.34, 14.49 (ω -CH₃, OCH₂CH₃), 19.05, 19.29 (2s, C-5'), 20.18 (d, J_{C-P} = 7.4, CHCH₃), 20.41 (d, J_{C-P} = 7.0, CHCH₃), 23.56, 32.15, 32.58, 36.74 (C₄H₈), 40.32, 40.35 (2s, C-2'), 51.64, 51.84 (2s, CHCH₃), 62.34, 62.38 (2s, OCH₂CH₃), 81.63 (d, J_{C-P} = 5.0, C-3'), 82.22 (d, J_{C-P} = 5.6, C-3'), 83.78 (d, J_{C-P} = 5.7, C-4'), 83.94 (d, J_{C-P} = 6.6, C-4'), 90.01, 90.06 (2s, C-1'), 98.92, 98.96 (2s, C-5), 110.30 (C-4a), 121.39, 121.43, 121.58, 121.61, 125.96, 126.25, 126.31, 127.29, 130.19, 130.83, 130.87 (C-H_a, C-H_b, 'ipso'-C, PhO), 138.13 (C-4), 146.43 (para-C), 152.15, 152.20 ('ipso' PhO), 156.76, 156.80 (2s, C-6), 157.47 (C-2), 173.15 (C-7a), 174.87, 175.62 (2s, COOCH₂CH₃).

EI MS= 660.25 (M+Na).

Anal. Calcd for C₃₃H₄₀N₃O₈P·H₂O: C, 60.45; H, 6.46; N, 6.41. Found: C, 60.76; H, 6.67; N, 6.10.

132c:

³¹P-NMR (MeOD, 202 MHz): δ 3.48.

¹H-NMR (MeOD, 500 MHz): δ 8.45 (1H, s, H-4), 7.61 (2H, d, Ph), 7.28 (2H, t, OPh), 7.19 (2H, d, Ph), 7.14-7.09 (3H, m, OPh), 6.92 (1H, s, H-5), 6.13 (1H, t, H-1'), 4.80-4.75 (1H, m, H-3'), 4.31-4.29 (1H, m, H-4'), 4.01 (2H, q, COOCH₂CH₃), 3.88-3.84 (1H, m, CHCH₃), 2.90-2.85 (1H, m, H_a-2'), 2.55 (2H, t, α -CH₂), 2.38-2.33 (1H, m, H_b-2'), 1.58-1.52 (2H, m, β -CH₂), 1.35 (3H, d, H-5'), 1.29-1.22 (7H, m, γ -CH₂, δ -CH₂, CHCH₃), 1.11 (3H, t, COOCH₂CH₃), 0.81 (3H, t, ω -CH₃).

¹³C-NMR (MeOD, 126 MHz): δ 14.34, 14.48 (ω -CH₃, OCH₂CH₃), 19.04 (C-5'), 20.18 (d, J_{C-P} = 7.4, CHCH₃), 23.56, 32.15, 32.58, 36.74 (C₄H₈), 40.34 (d, J_{C-P} = 4.3, C-2'), 51.84 (CHCH₃), 62.34 (OCH₂CH₃), 81.63 (d, J_{C-P} = 5.0, C-3'), 83.77 (d, J_{C-P} = 5.7, C-4'), 90.02 (C-1'), 98.97 (C-5), 110.30 (C-4a), 121.58, 121.61, 125.96, 126.31, 127.30, 130.20, 130.83 (C-H_a, C-H_b, 'ipso'-C, PhO), 138.14 (C-4), 146.44 (para-C), 152.15 ('ipso' PhO), 156.80 (C-6), 157.46 (C-2), 173.16 (C-7a), 175.23 (COOCH₂CH₃).

EI MS= 660.25 (M+Na).

Anal. Calcd for $C_{33}H_{40}N_3O_8P \cdot 2H_2O$: C, 58.83; H, 6.58; N, 6.24. Found: C, 58.62; H, 6.82; N, 5.13.

132d:

^{31}P -NMR (MeOD, 202 MHz): δ 2.74.

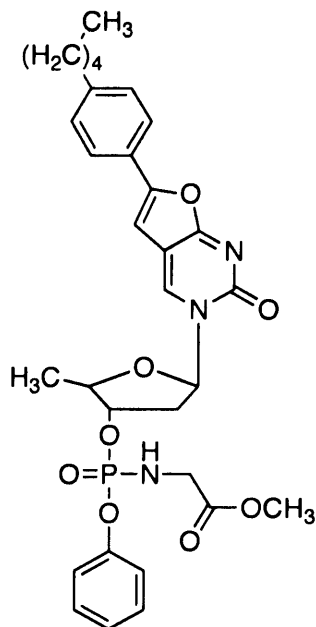
1H -NMR (MeOD, 500 MHz): δ 8.44 (1H, s, H-4), 7.61 (2H, d, Ph), 7.20-7.16 (4H, m, Ph, OPh), 7.12-7.09 (1H, m, OPh), 7.14-7.0 (1H, m, H-4'), 4.06 (2H, q, $COOCH_2CH_3$), 3.88-3.85 (1H, m, $CHCH_3$), 2.82-2.77 (1H, m, H_a-2'), 2.55 (2H, t, $\alpha-CH_2$), 2.28-2.22 (1H, m, H_b-2'), 1.56-1.52 (2H, m, $\beta-CH_2$), 1.38 (3H, d, H-5'), 1.29-1.22 (7H, m, $\gamma-CH_2$, $\delta-CH_2$, $CHCH_3$), 1.16 (3H, t, $COOCH_2CH_3$), 0.81 (3H, t, $\omega-CH_3$).

^{13}C -NMR (MeOD, 126 MHz): δ 14.34, 14.49 ($\omega-CH_3$, OCH_2CH_3), 19.29 (C-5'), 20.41 (d, $J_{C-P} = 7.0$, $CHCH_3$), 23.56, 32.15, 32.58, 36.74 (C_4H_8), 40.34 (d, $J_{C-P} = 3.9$, C-2'), 51.64 ($CHCH_3$), 62.38 (OCH_2CH_3), 82.22 (d, $J_{C-P} = 5.6$, C-3'), 83.94 (d, $J_{C-P} = 6.6$, C-4'), 90.06 (C-1'), 98.92 (C-5), 110.30 (C-4a), 121.39, 121.43, 125.97, 126.25, 127.30, 130.19, 130.87 (C- H_a , C- H_b , 'ipso'-C, PhO), 138.12 (C-4), 146.42 (para-C), 152.15 ('ipso' PhO), 156.77 (C-6), 157.47 (C-2), 173.17 (C-7a), 174.91 ($COOCH_2CH_3$).

EI MS= 660.24 (M+Na).

Anal. Calcd for $C_{33}H_{40}N_3O_8P \cdot H_2O$: C, 60.45; H, 6.46; N, 6.41. Found: C, 60.96; H, 6.69; N, 5.71.

Synthesis of 3-(2',5'-dideoxy- β -D-ribofuranosyl-3-[phenyl-(methoxy-glyni-nyl)] phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydro-furo [2,3-*d*]pyrimidin-2-one. [132e]



$C_{31}H_{36}N_3O_8P$

Mol Wt.: 609.6066

Prepared according to Standard Procedure D, from **21** (0.20 g, 0.52 mmol) in anhydrous THF (5 mL), **50c** (0.55 g, 2.09 mmol) in anhydrous THF (2 mL), NMI (0.42 mL, 5.23 mmol) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 97/3. The product was purified by preparative TLC (eluting gradient DCM/MeOH = 99/1 then 98/2), then by preparative reverse phase HPLC

(gradient elution of H_2O/CH_3CN = from 100/0 to 0/100 in 30 min) to give a white solid (3%, 0.010 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.42, 4.21.

1H -NMR (MeOD, 500 MHz): δ 8.45, 8.45 (1H, 2s, H-4), 7.61 (2H, d, Ph), 7.28 (2H, t, OPh), 7.19 (2H, d, Ph), 7.17-7.09 (3H, m, OPh), 6.92, 6.92 (1H, 2s, H-5), 6.13-6.09 (1H, m, H-1'), 4.85-4.80 (1H, m, H-3'), 4.42-4.37, 4.32-4.28 (1H, 2m, H-4'), 3.70-3.65 (2H, m, $NHCH_2$), 3.61-3.60 (3H, 2s, $COOCH_3$), 2.87-2.77 (1H, m, H_a -2'), 2.55 (2H, t, α - CH_2), 2.35-2.24 (1H, 2m, H_b -2'), 1.58-1.52 (2H, m, β - CH_2), 1.40, 1.35 (3H, 2d, H-5'), 1.31-1.20 (4H, m, γ - CH_2 , δ - CH_2), 0.81 (3H, t, ω - CH_3).

^{13}C -NMR (MeOD, 126 MHz): δ 14.35 (ω - CH_3), 19.11, 19.26 (2s, C-5'), 23.56, 32.15, 32.59, 36.75 (C_4H_8), 40.35 (C-2'), 43.65 ($NHCH_2$), 52.62 ($COOCH_3$), 81.84 (d, J_{C-P} = 6.0, C-3'), 82.09 (d, J_{C-P} = 5.4, C-3'), 83.86 (d, J_{C-P} = 4.7, C-4'), 83.92 (d, J_{C-P} = 6.9, C-4'), 89.99, 90.04 (C-1'), 98.93 (C-5), 121.44, 121.48, 121.54, 121.58, 125.96, 126.33, 127.28, 130.18, 130.86, 130.89 (PhO, Ph), 138.11

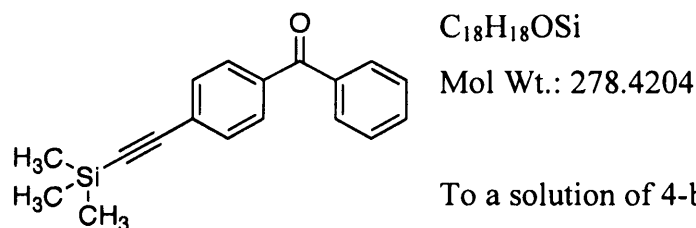
(C-4), 146.41 (para-C), 152.15 ('ipso' PhO), 156.77 (C-6), 157.43 (C-2), 173.14 (C-7a), 174.09 (COOCH₃).

EI MS= 660.24 (M+Na).

HPLC (H₂O/CH₃CN from 100/0 to 0/100 in 30 min): Rt 22.67, 22.97 min.

9.7 Synthesis of benzophenone derivative of BCNA

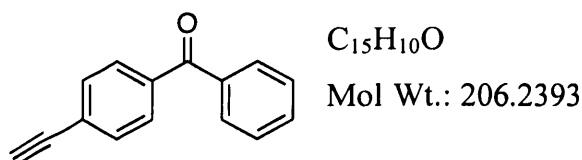
Synthesis of 4-(trimethylsilyl)ethynyl-benzophenone [150]



To a solution of 4-bromo-benzophenone **149** (2.50 g, 9.57 mmol) in anhydrous DMF (30 mL) were added: ethynyltrimethylsilane (4.00 mL, 28.72 mmol), tetrakis-(triphenylphosphine) palladium(0) (1.11 g, 0.96 mmol), Cu(I)I (0.37 g, 1.91 mmol), anhydrous DIPEA (3.33 mL, 19.15 mmol) and the reaction mixture was stirred at room temperature overnight. After this period, the solvent was removed and the residue triturated with acetone and methanol and the filtered. The organic phase was concentrated and further triturated with methanol and filtered. The filtrate was concentrated to give the desired compound, which was used without any further purification. (quantitative, 2.66 g).

¹H-NMR (DMSO, 500 MHz): δ 7.75-7.56 (9H, m, Ph), 0.26 (9H, s, 3xCH₃).

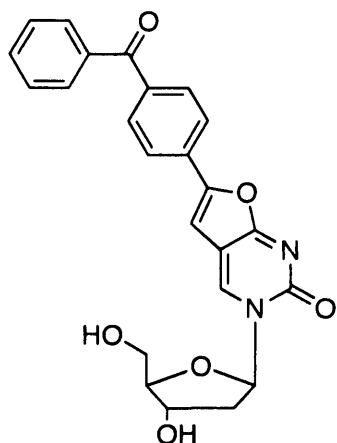
Synthesis of 4-ethynyl-benzophenone [151]



To a suspension of **150** (2.65 g, 9.57 mmol) in anhydrous methanol (50 mL) was added MeONa (0.62 g, 11.49 mmol) and the reaction mixture was stirred at room temperature for 1 h. Then the reaction mixture was neutralized with amberlite, filtered and concentrated to give a black oil (95%, 1.97 g).

¹H-NMR (DMSO, 500 MHz): δ 7.75-7.55 (9H, m, Ph), 4.48 (1H, s, CH).

Synthesis of 3-(2'-deoxy- β -D-ribofuranosyl)-6-(4-*n*-benzoylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one. [143]



$C_{24}H_{20}N_2O_6$

Mol Wt.: 432.4254

To a stirred solution of **121** (1.06 g, 3.00 mmol) in anhydrous DMF (15 mL), were added: **151** (1.81 g, 8.80 mmol), tetrakis-(triphenylphosphine) palladium(0) (0.35 g, 0.30 mmol), Cu(I)I (0.11 g, 0.60 mmol) and anhydrous DIPEA (1.04 mL, 6.00 mmol) and the reaction mixture was stirred at room temperature, under an argon

atmosphere overnight. After this period were added Cu(I)I (0.11 g, 0.60 mmol) and anhydrous TEA (15 mL) and the reaction mixture was stirred at 85 °C for 8 h. The solvent was then removed and the residue was triturated with DCM and the solution was stirred at room temperature overnight. The precipitate obtained was filtered and washed with DCM, to give a white solid as pure compound which was filtered through silica gel eluting with DCM/MeOH = 90/10 (59%, 0.77 g).

$^1\text{H-NMR}$ (DMSO, 500 MHz): δ 8.96 (1H, s, H-4), 8.01 (2H, d, $J = 8.3$, Ph), 7.86 (2H, d, $J = 8.3$, Ph), 7.77 (2H, d, $J = 7.2$, Ph), 7.71 (1H, t, $J = 7.4$, Ph), 7.59 (2H, t, $J = 7.6$, Ph), 7.50 (1H, s, H-5), 6.19 (1H, t, $J = 6.0$, H-1'), 5.30 (1H, d, $J = 4.3$, 3'-OH), 5.18 (1H, t, $J = 5.2$, 5'-OH), 4.27 (1H, dd, $J = 9.5, 4.3$, H-3'), 3.97-3.95 (1H, m, H-4'), 3.77-3.69 (1H, m, H-5'), 3.69-3.60 (1H, m, H-5'), 2.48-2.39 (1H, m, H-2'), 2.20-2.07 (1H, m, H-2').

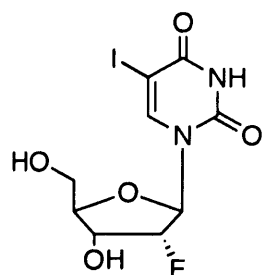
$^{13}\text{C-NMR}$ (DMSO, 126 MHz): δ 41.24 (C-2'), 60.61 (C-5'), 69.44 (C-3'), 87.78 (C-1'), 88.22 (C-4'), 102.27 (C-5), 106.52 (C-4a), 124.44, 128.60, 129.55, 130.46, 131.97, 132.75 (Ph), 136.90, 136.92 (ipso-carbonyl), 139.27 (C-4), 152.33 (C-6), 153.70 (C-2), 171.05 (C-7a), 194.92 (C=O).

EI MS= 433.1414 (M+H) and 496.1508 (M+MeCN Na^+).

Anal. Calcd for $C_{24}H_{20}N_2O_6$: C, 66.66; H, 4.66; N, 6.48. Found: C, 66.26; H, 4.43; N, 6.28.

9.8 Synthesis of 2'-fluoro derivative of BCNA lead compound

Synthesis of 5-iodo-2'-deoxy-2'- α -fluoro-uridine [159]



$C_9H_{10}FIN_2O_5$

Mol Wt.: 372.0890

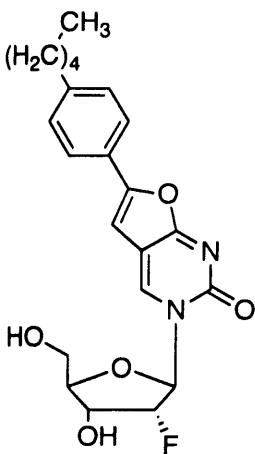
To a solution of 2'- α -fluoro-2'-deoxy-uridine **158** (2.00 g, 8.12 mmol) in anhydrous acetonitrile (50 mL) were added iodine (1.24 g, 4.87 mmol) and CAN (2.23 g, 4.06 mmol) and the reaction mixture was stirred under reflux for 1h. After this period, the reaction mixture was quenched with a saturated solution of $Na_2S_2O_3$ and then concentrated. The residue was dissolved in ethyl acetate and washed with brine (twice). The organic phase was dried over $MgSO_4$, concentrated to give a pale yellow solid (79%, 2.40 g).

^{19}F -NMR (DMSO, 471 MHz): δ -202.14.

1H -NMR (DMSO, 500 MHz): δ 11.71 (1H, s, NH), 8.52 (1H, s, H-6), 5.86 (1H, d, J = 16.9, H-1'), 5.58 (1H, d, J = 6.3, 3'-OH), 5.36 (1H, s, 5'-OH), 5.08 (0.5H, s, H-2'), 4.98 (0.5H, s, H-2'), 4.17 (1H, d, H-3'), 3.89 (1H, d, J = 7.5, H-4'), 3.81 (1H, d, J = 10.5, H-5'), 3.60 (1H, d, J = 10.5, H-5').

^{13}C -NMR (DMSO, 126 MHz): δ 58.50 (C-5'), 66.73 (d, J_{C-F} = 16.3, C-3'), 69.04 (C-5), 83.00 (C-4'), 87.39 (d, J_{C-F} = 34.1, C-1'), 95.12 (d, J_{C-F} = 184.3, C-2'), 144.66 (C-6), 149.94 (C-2), 160.53 (C-4).

Synthesis of 3-(2'- α -fluoro-2'-deoxy- β -D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [155]



$C_{22}H_{25}FN_2O_5$

Mol Wt.: 416.4427

To a solution of **159** (1.50 g, 4.03 mmol) in anhydrous DMF (20 mL) were added: 4-*n*-pentylphenylacetylene (2.36 mL, 12.09 mmol), tetrakis (0.47 g, 0.40 mmol), copper (I) iodide (0.15 g, 0.81 mmol) and DIPEA (1.40 mL, 8.06 mmol) and the reaction mixture was stirred at room temperature, under an Argon atmosphere overnight. After this period were added copper (I) iodide (0.15 g, 0.81 mmol) and anhydrous TEA (20 mL) and the reaction mixture was stirred at 85 °C for 7.5 h. The solvent was then removed in vacuo, and the resulting residue was triturated with methanol, filtered and washed with methanol and DCM to obtain a black solid (pure by NMR). The organic phase was concentrated and the residue purified by column chromatography, eluting with DCM/MeOH = 96/4. The compound was obtained as a dark solid which was combined with the previous precipitate and filtered through a silica gel column, eluting with DCM/MeOH = 96/4 to give a black solid. The solid obtained was washed with acetone, filtered and the solid was further washed with petroleum ether to give a white-grey solid (42%, 0.70 g).

^{19}F -NMR (DMSO, 471 MHz): δ -201.19.

^1H -NMR (DMSO, 500 MHz): δ 8.94 (1H, s, H-4), 7.75 (2H, d, J = 8.1, H_a), 7.32 (2H, d, J = 8.1, H_b), 7.22 (1H, s, H-5), 6.02 (1H, d, J = 17.0, H-1'), 5.60 (1H, d, J = 6.6, 3'-OH), 5.42 (1H, t, J = 4.9, 5'-OH), 5.00 (1H, dd, J = 3.7, J_{H-F} = 52.6, H-2'), 4.26-4.20 (1H, m, H-3'), 3.95 (1H, dd, J = 12.4, 2.9, H-4'), 3.77-3.67 (2H, m, H-5'), 2.61 (2H, t, J = 7.6, α -CH₂), 1.65-1.61 (2H, m, β -CH₂), 1.42-1.18 (4H, m, γ -CH₂, δ -CH₂), 0.86 (3H, t, J = 6.9, ω -CH₃).

^{13}C -NMR (DMSO, 126 MHz): δ 13.84 (CH₃), 21.88, 30.33, 30.79, 34.88 (C₄H₈), 58.42 (C-5'), 66.31 (d, J_{C-F} = 16.3, C-3'), 83.04 (C-4'), 89.54 (d, J_{C-F} = 34.0, C-1'), 94.16 (d,

$J_{C-F} = 185.7$, C-2'), 98.56 (C-5), 107.19 (C-4a), 124.58 (C-H_b), 125.77 (*ipso*-C), 128.96 (C-H_a), 137.77 (C-4), 144.14 (*para*-C), 153.69 (C-6), 154.12 (C-2), 171.27 (C-7a).

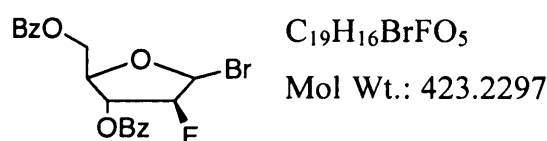
EI MS= 416.1738 (M^+).

Anal. Calcd for $C_{22}H_{25}FN_2O_5 \cdot 0.5H_2O$: C, 62.11; H, 6.16; N, 6.58. Found: C, 61.73; H, 6.15; N, 6.41.

Synthesis of 2-deoxy-1- α -bromo-2- β -fluoro-3,5-di-*O*-benzoyl-d-ribofuranose

Synthesis of 2-deoxy-1- α -bromo-2- β -fluoro-3,5-di-*O*-benzoyl-d-ribofuranose

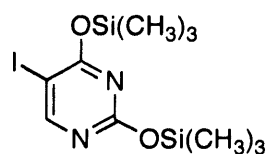
[161]



To a solution of **160** (2.00 g, 4.31 mmol) in anhydrous DCM (9 mL) was added dropwise under Ar atmosphere a 33%wt solution of HBr in acetic acid (1.54 mL, 9.04 mmol) and the reaction mixture was stirred at rt overnight. After this period, 33%wt solution of HBr in acetic acid (0.77 mL, 4.50 mmol) was added and the reaction mixture was stirred at rt for further 7h. Then the reaction mixture was quenched with saturated solution of $NaHCO_3$. The organic phase was washed with saturated solution of $NaHCO_3$ (twice) and then dried over $MgSO_4$, then concentrated to give a colourless oil (95%, 1.73g).

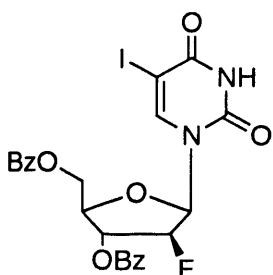
^{19}F -NMR ($CDCl_3$, 471 MHz): δ -165.93.

1H -NMR ($CDCl_3$, 500 MHz): δ 8.18-8.04 (4H, m, Bz), 7.69-7.43 (6H, m, Bz), 6.66 (1H, d, $J = 12.2$, H-1'), 5.57 (1H, dd, $J = 3.2$, $J_{H-F} = 22.0$, H-2'), 4.87-4.72 (4H, m, H-3', H-4', H-5').

Synthesis of 2,4-bis-*O*-(trimethylsilyl)-5-iodouracil [163]C₁₀H₁₉IN₂O₂Si₂

Mol Wt.: 382.3455

To a suspension of **162** (0.49 g, 2.04 mmol) in anhydrous acetonitrile (10 mL) were added ammonium sulphate (0.027 g, 0.2 mmol) and hexamethyldisilazane (0.48 mL, 2.30 mmol) and the reaction mixture was stirred at reflux for 22h. After this period, hexamethyldisilazane (0.96 mL, 4.08 mmol) was added and the reaction mixture was stirred at reflux for further 4h and then concentrated. The crude was used in the next step without purification.

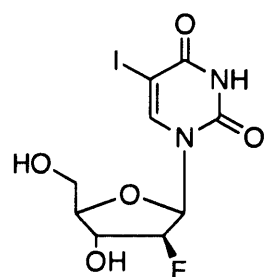
Synthesis of 1-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-D-arabino-furanosyl)-5-iodouracil [164]C₂₃H₁₈FIN₂O₇

Mol Wt.: 580.3011

To a solution of **161** (3.50 g, 8.27 mmol) in DCM (65 mL) and **163** (8.27 mmol) in acetonitrile (15 mL) was added NaI (0.99 g, 6.62 mmol) and the reaction mixture was stirred at rt for 6 days. After this period the suspension was filtered and the solid was washed with water and DCM to give the desired compound as a white solid (51%, 2.45 g).

¹⁹F-NMR (DMSO, 471 MHz): δ -199.13.

¹H-NMR (DMSO, 500 MHz): δ 8.08-7.99 (5H, m, H-6, Bz), 7.74-7.66 (2H, m, Bz), 7.60-7.52 (4H, m, Bz), 6.31 (1H, dd, J = 3.7, J_{H-F} = 19.4, H-1'), 5.71 (1H, ddd, J = 4.8, 1.3, J_{H-F} = 20.3, H-3'), 5.54 (1H, ddd, J = 3.8, 1.4, J_{H-F} = 50.7, H-2'), 4.81-4.71 (2H, m, H-5'), 4.63 (1H, dd, J = 8.3, 4.3, H-4').

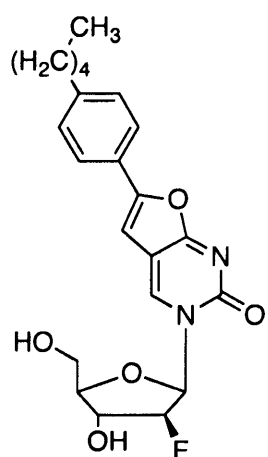
Synthesis of 5-iodo-2'-β-fluoro-2'-deoxyuridine [165]C₉H₁₀FIN₂O₅

Mol Wt.: 372.0890

To a stirring solution of **164** (2.40 g, 4.14 mmol) in anhydrous methanol (60 mL) was added NaOMe (0.49 g, 9.70 mmol) and the reaction mixture was stirred at room temperature for 1 h. After this period, the reaction was neutralized with amberlite, filtered and concentrated to give the desired product, which was used in the following step without further purification.

¹⁹F-NMR (DMSO, 471 MHz): δ -198.65.

¹H-NMR (DMSO, 500 MHz): δ 8.21 (1H, s, H-6), 6.08 (1H, dd, J = 4.5, J_{H-F} = 13.9, H-1'), 5.93 (1H, bs, 3'-OH), 5.27-5.25 (1H, m, 5'-OH), 5.14-5.11 (1H, m, H-2'), 5.04-4.99 (1H, m, H-2'), 4.29-4.19 (1H, m, H-3'), 3.81-3.78 (1H, m, H-4'), 3.71-3.65 (1H, m, H-5'), 3.61-3.56 (1H, m, H-5').

Synthesis of 3-(2'-α-fluoro-2'-deoxy-β-D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [156]C₂₂H₂₅FN₂O₅

Mol Wt.: 416.4427

To a solution of **165** (1.54 g, 4.14 mmol) in anhydrous DMF (20 mL) were added: 4-*n*-pentylphenylacetylene (2.40 mL, 12.41 mmol), tetrakis (0.48 g, 0.41 mmol), copper (I) iodide (0.16 g, 0.83 mmol) and anhydrous DIPEA (1.44 mL, 8.27 mmol) and the reaction mixture was stirred at room temperature, under an argon atmosphere overnight. After this period were added copper (I) iodide (0.16 g, 0.83 mmol) and anhydrous TEA (20 mL) and the reaction mixture was stirred at 85 °C for 8 h. The solvent was then removed in vacuo and the residue was

trituted with DCM and stirred at room temperature for 2h and then filtered and washed with DCM to give a white solid (45%, 0.77 g). A sample was filtered through silica gel for biological testing.

^{19}F -NMR (DMSO, 471 MHz): δ -197.85.

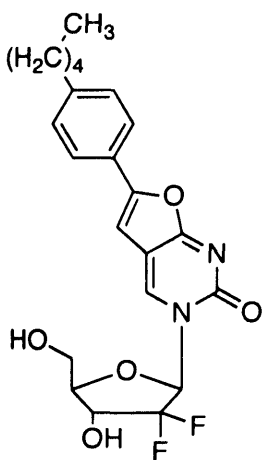
^1H -NMR (DMSO, 500 MHz): δ 8.73 (1H, s, H-4), 7.74 (2H, d, J = 8.3, Ph), 7.33 (2H, d, J = 8.3, Ph), 7.22 (1H, s, H-5), 6.25, 6.22 (1H, 2d, J = 3.7, $J_{\text{H-F}}$ = 17.1, H-1'), 6.07 (1H, d, J = 4.6, 3'-OH), 5.28 (1H, t, J = 5.8, 5'-OH), 5.24, 5.14 (1H, 2dd, J = 3.6, 2.3, $J_{\text{H-F}}$ = 52.0, H-2'), 4.29, 4.26 (1H, 2dd, J = 6.1, 4.3, $J_{\text{H-F}}$ = 18.2, H-3'), 3.99 (1H, q, H-4'), 3.71-3.63 (2H, m, H-5'), 2.62 (2H, t, α -CH₂), 1.59 (2H, quintet, β -CH₂), 1.35-1.24 (4H, m, γ -CH₂, δ -CH₂), 0.86 (3H, t, CH₃).

^{13}C -NMR (DMSO, 126 MHz): δ 13.85 (CH₃), 21.87 (CH₂), 30.32 (β -CH₂), 30.78 (CH₂), 34.87 (α -CH₂), 60.13 (C-5'), 73.08 (d, $J_{\text{C-F}}$ = 24.5 Hz, C-3'), 85.22 (C-4'), 86.15 (d, $J_{\text{C-F}}$ = 16.6 Hz, C-1'), 94.66 (d, $J_{\text{C-F}}$ = 191.5 Hz, C-2') 98.60 (C-5), 107.14 (C-4a), 124.61 (Ph), 125.70 (*ipso*-C), 128.99 (Ph), 138.70 (C-4), 144.20 (*para*-C), 153.47 (C-6), 154.20 (C-2), 171.30 (C-7a).

EI MS= 416.1749 (M⁺).

HPLC = H₂O/AcCN from 100/0 to 0/100 in 30 min = retention time 23.81 min.

Synthesis of 3-(2'-di-fluoro-2'-deoxy- β -D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [157]



C₂₂H₂₄F₂N₂O₅

Mol Wt.: 434.4332

To a solution of **166** (0.18g, 0.47 mmol) in anhydrous DMF (5 mL) were added: 4-*n*-pentylphenylacetylene (0.27 mL, 1.41 mmol), tetrakis (0.055 g, 0.047 mmol), copper (I) iodide (0.018 g, 0.047 mmol) and DIPEA (0.16 mL, 0.94 mmol) and the reaction mixture was stirred at room temperature, under an Argon atmosphere

overnight. After this period were added copper (I) iodide (0.018 g, 0.047 mmol) and anhydrous TEA (5 mL) and the reaction mixture was stirred at 85 °C for 7.5 h. The solvent was then removed in vacuo and the residue was purified by column chromatography, eluting gradient of DCM, then DCM/MeOH = 98/2, then 96/4. The compound was obtained as a brown-dark solid, which was triturated with acetone and petroleum ether then filtered, and the solid was washed with acetone and petroleum ether to give a white-pale yellow solid (60%, 0.075 g).

^{19}F -NMR (DMSO, 471 MHz): δ -116.84.

^1H -NMR (DMSO, 500 MHz): δ 8.75 (1H, s, H-4), 7.76 (2H, d, J = 8.2, H_a), 7.34 (2H, d, J = 8.2, H_b), 7.23 (1H, s, H-5), 6.35 (1H, d, J = 6.5, H-1'), 6.33-6.30 (1H, m, 3'-OH), 5.43 (1H, t, J = 5.3, 5'-OH), 4.36-4.18 (1H, m, H-3'), 3.99-3.95 (1H, m, H-4'), 3.91-3.85 (1H, m, H-5'), 3.75-3.69 (1H, m, H-5'), 2.63 (2H, t, J = 7.6, α -CH₂), 1.68-1.52 (2H, m, β -CH₂), 1.40-1.21 (4H, m, γ -CH₂, δ -CH₂), 0.87 (3H, t, J = 7.0, CH₃).

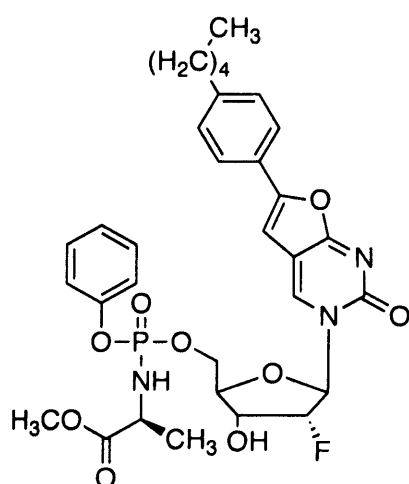
^{13}C -NMR (DMSO, 126 MHz): δ 13.85 (CH₃), 21.88, 30.32, 30.79, 34.90 (C₄H₈), 58.62 (C-5'), 68.14 (t, $J_{\text{C-F}}$ = 22.4, C-3'), 81.11 (C-4'), 85.14 (t, $J_{\text{C-F}}$ = 31.2, C-1'), 98.37 (C-5), 107.94 (C-4a), 120.84, 122.91 (C-2'), 124.75 (C-H_b), 125.56 (*ipso*-C), 129.01 (C-H_a), 137.26 (C-4), 144.44 (*para*-C), 153.68 (C-6), 154.87 (C-2), 171.53 (C-7a).

EI MS= 435.1731 (M+H).

Anal. Calcd for C₂₂H₂₄F₂N₂O₅·0.5H₂O: C, 59.59; H, 5.68; N, 6.32. Found: C, 59.38; H, 5.59; N, 6.25.

9.9 Synthesis of 2'-fluoro derivatives on BCNA lead compound ProTides

Synthesis of 3-(2'- α -fluoro-2'-deoxy- β -D-ribofuranosyl-5-[phenyl-(methoxy-L-alaninyl)] phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [169a]



$C_{32}H_{37}FN_3O_9P$

Mol Wt.: 657.6230

Prepared according to Standard Procedure D, from **155** (0.20 g, 0.48 mmol) in anhydrous THF (5 mL) and anhydrous pyridine (5 mL), **131a** (0.67 g, 2.40 mmol) in anhydrous THF (2 mL), NMI (0.19 mL, 2.40 mmol) and the reaction mixture was stirred at room temperature overnight. After this period were added **131a** (0.40 g,

1.44 mmol) in anhydrous THF (2 mL) and NMI (0.11 mL, 1.44 mmol) and the reaction mixture was stirred at room temperature for further 7 h. After this period the solvent was removed and the residue dissolved in DCM. The organic phase was washed with a 0.5 M aqueous solution of citric acid (twice) and water (twice), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 97/3 to give a white solid which was further purified by preparative TLC (gradient elution of DCM/MeOH = 99/1, then 98/2, then 96/4) to give a white solid (5%, 0.015 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.90, 3.83.

^{19}F -NMR (DMSO, 471 MHz): δ -203.40, -203.72.

1H -NMR (MeOD, 500 MHz): δ 8.72, 8.71 (1H, 2s, H-4), 7.66-7.59 (2H, m, Ph), 7.43-7.17 (7H, m, Ph), 6.87, 6.75 (1H, 2s, H-5), 6.16 (0.5H, d, J = 8.3, H-1'), 6.13 (0.5H, d, J = 8.5, H-1'), 5.09 (1H, dd, J = 3.1, J_{H-F} = 52.1, H-2'), 4.79-4.64 (1H, m, H-5'), 4.57-4.42 (1H, m, H-4'), 4.40-4.27 (2H, m, H-3', H-5'), 4.08-3.99 (1H, m, $CHCH_3$), 3.68 (3H, s,

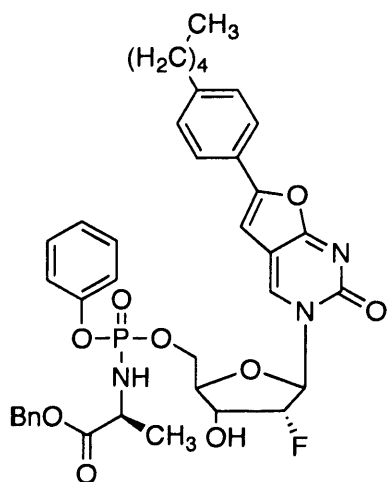
OCH₃), 2.66 (2H, t, *J* = 7.7, α-CH₂), 1.73-1.60 (2H, m, β-CH₂), 1.44-1.28 (7H, m, γ-CH₂, δ-CH₂, CHCH₃), 0.93 (3H, t, *J* = 7.0, CH₃).

¹³C-NMR (MeOD, 126 MHz): δ 14.35 (CH₃), 20.34 (d, *J*_{C-P} = 7.4, CHCH₃), 20.53 (d, *J*_{C-P} = 6.4, CHCH₃), 23.56, 32.14, 32.59, 36.74 (C₄H₈), 51.55, 51.77 (CHCH₃), 52.81, 52.85 (2s, OCH₃), 65.64 (d, *J*_{C-P} = 2.8, C-5'), 65.68 (d, *J*_{C-P} = 2.0, C-5'), 68.80 (d, *J*_{C-P} = 16.5, C-4'), 68.93 (d, *J*_{C-P} = 16.2, C-4'), 82.56, (d, *J*_{C-P} = 8.3, C-3') 82.63 (d, *J*_{C-P} = 8.3, C-3'), 92.26, 92.54 (2d, *J*_{C-F} = 34.8, C-1'), 94.30, 95.80 (2d, *J*_{C-F} = 187.7, C-2'), 98.96, 98.99 (2s, C-5), 110.45 (C-4a), 121.42, 121.50, 121.51, 121.53, 121.55, 123.94, 125.90, 126.02, 126.40, 127.12, 127.14, 130.09, 130.17, 130.20, 130.59, 130.72, 130.93, 131.00, (PhO, Ph), 138.52, 138.54 (C-4), 146.49, 146.50 (2s, *para*-C), 152.00, 152.05 (2s, 'ipso' PhO), 156.59 (C-6), 157.45, 157.49 (C-2), 173.36 (C-7a), 175.31 (d, *J*_{C-P} = 5.2, COOCH₃), 175.58 (d, *J*_{C-P} = 4.5, COOCH₃).

EI MS= 658.2330 (M+H).

HPLC = H₂O/AcCN from 100/0 to 0/100 in 30 min = retention time 20.43 min.

Synthesis of 3-(2'-α-fluoro-2'-deoxy-β-D-ribofuranosyl-5-[phenyl-(benzoxy-L-alaninyl)] phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [169b]



C₃₈H₄₁FN₃O₉P

Mol Wt.: 733.7190

Prepared according to Standard Procedure D, from **155** (0.17 g, 0.40 mmol) in anhydrous THF (5 mL) and anhydrous pyridine (5 mL), **131d** (0.70 g, 2.00 mmol) in anhydrous THF (2 mL), NMI (0.16 mL, 2.00 mmol) and the reaction mixture was stirred at room temperature overnight. After this period were added **131d** (0.42 g, 1.20

mmol) in anhydrous THF (2 mL) and NMI (0.09 mL, 1.20 mmol) and the reaction mixture was stirred at room temperature for further 24 h. Then NMI (0.09 mL, 1.20 mmol) was added and the reaction mixture stirred at room temperature for further 7 h.

After this period the solvent was removed and the residue dissolved in DCM. The organic phase was washed with a 0.5 M aqueous solution of citric acid (twice) and water (twice), dried over MgSO_4 and concentrated. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 97/3 to give a white solid which was further purified by preparative TLC (gradient elution of DCM/MeOH = 99/1, then 98/2, then 96/4) to give a white solid (7%, 0.020 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.94, 3.71.

^{19}F -NMR (DMSO, 471 MHz): δ -200.55, -200.94.

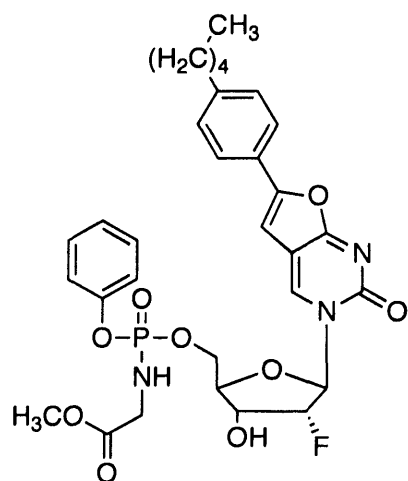
^1H -NMR (MeOD, 500 MHz): δ 8.70, 8.67 (1H, 2s, H-4), 7.70-7.60 (2H, m, Ph), 7.47-7.18 (12H, m, Ph, PhO, CH_2Ph), 6.86, 6.79 (1H, 2s, H-5), 6.14 (0.5H, d, $J = 9.3$, H-1'), 6.11 (0.5H, d, $J = 10.0$, H-1'), 5.18-5.06 (2H, m, CH_2Ph), 5.00-4.93 (1H, m, H-2'), 4.71-4.51 (1H, m, H-5'), 4.50-4.41 (1H, m, H-5'), 4.36-4.23 (2H, m, H-3', H-4'), 4.16-4.02 (1H, m, CHCH_3), 2.68 (2H, t, $J = 7.4$, $\alpha\text{-CH}_2$), 1.73-1.55 (2H, m, $\beta\text{-CH}_2$), 1.48-1.27 (7H, m, $\gamma\text{-CH}_2$, $\delta\text{-CH}_2$, CHCH_3), 0.93 (3H, t, $J = 6.6$, CH_3).

^{13}C -NMR (MeOD, 126 MHz): δ 14.35 (CH_3), 20.28 (d, $J_{\text{C-P}} = 7.7$, CHCH_3), 20.51 (d, $J_{\text{C-P}} = 6.3$, CHCH_3), 23.56, 32.15, 32.58, 36.74 (C_4H_8), 51.68, 51.94 (CHCH_3), 65.59 (d, $J_{\text{C-P}} = 5.0$, C-5'), 65.80 (d, $J_{\text{C-P}} = 5.4$, C-5'), 68.03 (CH_2Ph), 68.71, 68.85 (C-4'), 82.52, 82.58 (2s, C-3'), 92.23, 92.51 (2d, $J_{\text{C-F}} = 24.4$, C-1'), 95.27, 95.77 (2d, $J_{\text{C-F}} = 188.7$, C-2'), 99.00, 99.02 (2s, C-5), 110.46 (C-4a), 121.45, 121.49, 121.53, 121.56, 125.93, 126.37, 126.42, 127.14, 129.14, 129.18, 129.32, 129.57, 130.19, 130.93, 130.98, (PhO, CH_2Ph , Ph), 137.17 ('ipso' OCH_2Ph), 138.53 (C-4), 146.50 (*para*-C), 152.03 ('ipso' PhO), 156.58 (C-6), 157.45 (C-2), 173.34 (C-7a), 174.95 (COOCH_3).

EI MS= 734.2616 (M+H) 756.2448 (M+Na).

HPLC = $\text{H}_2\text{O}/\text{MeOH}$ 20/80 isocratic = retention time 28.08, 30.03 min.

Synthesis of 3-(2'- α -fluoro-2'-deoxy- β -D-ribofuranosyl-5-[phenyl-(methoxyglycyl)] phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [169c]



$C_{31}H_{35}FN_3O_9P$

Mol Wt.: 643.5965

Prepared according to Standard Procedure D, from **155** (0.20 g, 0.48 mmol) in anhydrous THF (10 mL), **131c** (0.25 g, 0.96 mmol) in anhydrous THF (2 mL), NMI (0.08 mL, 0.96 mmol) and the reaction mixture was stirred at room temperature overnight. After this period were added anhydrous pyridine (2 mL), **131c** (0.25 g,

0.96 mmol) in anhydrous THF (2 mL) and NMI (0.08 mL, 0.96 mmol) and the reaction mixture was stirred at room temperature for further 7 h. Then were added **131c** (0.38 g, 1.44 mmol) in anhydrous THF (2 mL) and NMI (0.11 mL, 1.44 mmol) and the reaction mixture was stirred at room temperature for 16 h. After this period the solvent was removed and the residue dissolved in DCM. The organic phase was washed with a 0.5 M aqueous solution of citric acid (twice) and water (twice), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 97/3 to give a white solid (15%, 0.046 g).

^{31}P -NMR (MeOD, 202 MHz): δ 5.24, 4.95.

^{19}F -NMR (MeOD, 471 MHz): δ -203.47, -203.56.

1H -NMR (MeOD, 500 MHz): δ 8.71, 8.69 (1H, 2s, H-4), 7.61-7.56 (2H, m, Ph), 7.49-7.34 (2H, m, Ph), 7.34-7.17 (5H, m, Ph), 6.84, 6.73 (1H, 2s, H-5), 6.13 (1H, d, $J = 17.0$, H-1'), 5.14, 5.04 (1H, 2t, $J = 3.8$, $J_{H-F} = 52.0$, H-2'), 4.78-4.68 (1H, m, H-5'), 4.59-4.47 (1H, m, H-4'), 4.41-4.28 (2H, m, H-3', H-5'), 3.89-3.80 (2H, m, $NHCH_2$), 3.68 (3H, 2s, OCH_3), 2.64 (2H, t, $J = 7.7$, α - CH_2), 1.71-1.59 (2H, m, β - CH_2), 1.46-1.28 (4H, m, γ - CH_2 , δ - CH_2), 0.93 (3H, t, $J = 7.0$, CH_3).

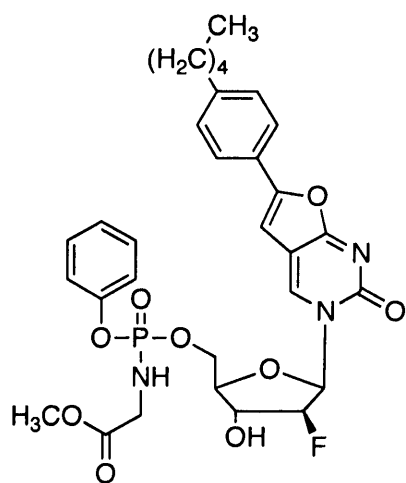
^{13}C -NMR (MeOD, 126 MHz): δ 14.38 (CH_3), 23.57, 32.11, 32.62, 36.75 (C_4H_8), 43.76 (CH_2NH), 52.69, 52.71 (2s, OCH_3), 65.63 (d, $J_{\text{C-P}} = 5.0$, C-5'), 65.72 (d, $J_{\text{C-P}} = 4.8$, C-5'), 68.75 (d, $J_{\text{C-P}} = 11.7$, C-4'), 68.89 (d, $J_{\text{C-P}} = 11.7$, C-4'), 82.57, 82.64 (2s, C-3'), 92.17, 92.44 (2d, $J_{\text{C-F}} = 34.8$, C-1'), 95.09 (d, $J_{\text{C-F}} = 187.8$, C-2'), 98.98 (C-5), 110.42 (C-4a), 121.51, 121.55, 125.87, 125.90, 126.43, 127.08, 130.14, 130.97, 131.02 (PhO, Ph), 138.50, 138.56 (C-4), 146.41 (*para*-C), 152.00, 152.06 (2s, 'ipso' PhO), 156.77 (C-6), 157.35, 157.42 (C-2), 172.97 (d, $J_{\text{C-P}} = 4.5$, COOCH_3), 173.03 (C-7a), 173.27 (d, $J_{\text{C-P}} = 1.8$, COOCH_3).

EI MS= 644.2202 (M+H) and 667.2043 (M+Na).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 100/0 to 0/100 in 30 min = retention time 27.59 min.

= $\text{H}_2\text{O}/\text{MeOH}$ 20/80 isocratic = retention time 5.88 min.

Synthesis of 3-(2'- β -2'-deoxy- β -D-ribofuranosyl-5-[phenyl-(methoxy-glyciny)]phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [170]



$\text{C}_{31}\text{H}_{35}\text{FN}_3\text{O}_9\text{P}$

Mol Wt.: 643.5965

Prepared according to Standard Procedure D, from **156** (0.20 g, 0.48 mmol) in anhydrous THF (10 mL) and anhydrous pyridine (2 mL), **131c** (0.63 g, 2.40 mmol) in anhydrous THF (5 mL), NMI (0.20 mL, 2.40 mmol) and the reaction mixture was stirred at room temperature overnight. After this period were added **131c** (0.63 g,

2.40 mmol) in anhydrous THF (5 mL) and NMI (0.20 mL, 2.40 mmol) and the reaction mixture was stirred at room temperature for further 24 h. After this period the solvent was removed and the residue dissolved in DCM. The organic phase was washed with a 0.5 M aqueous solution of HCl (twice) and water (twice), dried over MgSO_4 and concentrated. The residue was purified by column chromatography gradient elution of $\text{DCM}/\text{MeOH} = 98/2$ then 97/3 to give a white solid which was further purified by preparative TLC

(gradient elution of DCM/MeOH = 99/1, then 98/2, then 96/4) to give a white solid (4%, 0.012 g).

^{31}P -NMR (MeOD, 202 MHz): δ 5.26, 5.15.

^{19}F -NMR (DMSO, 471 MHz): δ -200.08.

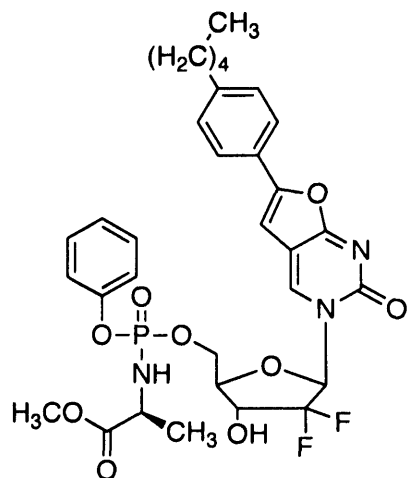
^1H -NMR (MeOD, 500 MHz): δ 8.66, 8.64 (1H, 2s, H-4), 7.70-7.16 (9H, m, Ph, PhO), 6.88, 6.86 (1H, 2s, H-5), 6.41, 6.37 (1H, 2t, $J = 3.5$, $J_{\text{H-F}} = 18.5$, H-1'), 5.31-5.30 (0.5H, m, H-2'), 5.21-5.20 (0.5H, m, H-2'), 4.49-4.32 (4H, m, H-3', H-4', H-5'), 3.86-3.81 (2H, m, NHCH_2), 3.68 (3H, 2s, OCH_3), 2.67 (2H, t, $J = 8.3$, $\alpha\text{-CH}_2$), 1.70-1.64 (2H, m, $\beta\text{-CH}_2$), 1.42-1.28 (4H, m, $\gamma\text{-CH}_2$, $\delta\text{-CH}_2$), 0.93 (3H, t, $J = 7.3$, CH_3).

^{13}C -NMR (MeOD, 126 MHz): δ 14.36 (CH_3), 23.56, 32.14, 32.59, 36.75 (C_4H_8), 43.70 (CH_2NH), 52.57, 52.66 (2s, OCH_3), 66.92 (C-5'), 75.25 (d, $J_{\text{C-P}} = 10.9$, C-4'), 75.46 (d, $J_{\text{C-P}} = 10.6$, C-4'), 85.24, 85.31 (2s, C-3'), 89.06 (d, $J_{\text{C-F}} = 13.7$, C-1'), 89.16 (d, $J_{\text{C-F}} = 14.3$, C-1'), 96.16 (d, $J_{\text{C-F}} = 194.4$, C-2'), 98.89, 98.91 (C-5), 110.12 (C-4a), 121.56, 121.60, 121.64, 121.67, 121.70, 121.74, 125.78, 125.96, 126.21, 126.35, 127.17, 130.19, 130.66, 130.89, 130.90 (PhO, Ph), 139.75, 139.78 (C-4), 146.47 (*para*-C), 152.18 (2s, 'ipso' PhO), 156.46 (C-6), 157.46 (C-2), 172.94 (COOCH_3), 173.26 (C-7a), 173.28 (COOCH_3).

EI MS= 666.2001 (M+Na).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 95/5 to 0/100 in 30 min = retention time 28.60 min.

Synthesis of 3-(2'-di-fluoro-2'-deoxy-β-D-ribofuranosyl-5-[phenyl-(methoxy-L-alaninyl)] phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [171a] and [171b]



$C_{32}H_{36}F_2N_3O_9P$

Mol Wt.: 675.6135

Prepared according to Standard Procedure D, from **157** (0.20 g, 0.46 mmol) in anhydrous THF (10 mL), **131a** (0.64 g, 2.30 mmol) in anhydrous THF (5 mL), NMI (0.18 mL, 2.30 mmol) and the reaction mixture was stirred at room temperature overnight. After this period were added **131a** (0.64 g, 2.30 mmol) in anhydrous THF

(5 mL) and NMI (0.18 mL, 2.30 mmol) and the reaction mixture was stirred at room temperature for further 8 h. Then NMI (0.09 mL, 1.20 mmol) was added and the reaction mixture stirred at room temperature for further 24 h. After this period the solvent was removed and the residue dissolved in DCM. The organic phase was washed with a 0.5 M aqueous solution of HCl (twice) and water (twice), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography gradient elution of DCM/MeOH = 100/0 then 98/2 then 97/3 to give a white solid which was further purified by preparative TLC (gradient elution of DCM/MeOH = 99/1, then 98/2, then 96/4) to give a white solid as a mix of two diastereoisomer (0.005 g) and as a single diastereoisomer (0.004 g).

171a

^{31}P -NMR (MeOD, 202 MHz): δ 3.98, 3.96.

^{19}F -NMR (DMSO, 471 MHz): δ -118.46, -118.83, -118.93, -119.44, -120.23.

1H -NMR (MeOD, 500 MHz): δ 8.55, 8.51 (1H, 2s, H-4), 7.81-7.65 (2H, m, Ph), 7.48-7.16 (7H, m, Ph), 6.96, 6.88 (1H, 2s, H-5), 6.48-6.44 (1H, m, H-1'), 4.68-4.46 (2H, m, H-5'), 4.43-4.31 (1H, m, H-4'), 4.31-4.24 (1H, m, H-3'), 4.04-3.92 (1H, m, $CHCH_3$), 3.68, 3.67 (3H, 2s, OCH_3), 2.69 (2H, t, J = 7.6, α - CH_2), 1.71-1.65 (2H, m, β - CH_2), 1.43-1.30 (7H, m, γ - CH_2 , δ - CH_2 , $CHCH_3$), 0.94 (3H, t, J = 6.9, CH_3).

^{13}C -NMR (MeOD, 126 MHz): δ 14.35 (CH_3), 21.00 (d, $J_{\text{C-P}} = 7.8$, CHCH_3), 21.06 (d, $J_{\text{C-P}} = 7.0$, CHCH_3), 23.55, 32.15, 32.57, 36.76 (C_4H_8), 50.04 (CHCH_3), 52.79 (OCH_3), 65.76 (C-5'), 69.82, 70.04 (2s, C-4'), 82.86, 82.92 (2s, C-3'), 94.47 (C-1'), 98.86, 98.89 (2s, C-5), 110.87 (C-4a), 121.32, 121.52, 121.56, 126.06, 126.09, 126.22, 126.44, 126.48, 127.01, 129.59, 129.83, 130.23, 130.40, 130.94, 131.03, 132.51, 132.64 (C-2', PhO, Ph), 138.38 (C-4), 145.11, 146.70 (2s, *para*-C), 150.97 ('ipso' PhO), 156.66 (C-6), 158.01 (C-2), 173.56 (C-7a), 174.50 (COOCH_3).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 95/5 to 0/100 in 40 min = retention time 29.72, 33.48 min.

171b

^{31}P -NMR (MeOD, 202 MHz): δ 3.89.

^{19}F -NMR (DMSO, 471 MHz): δ -118.54.

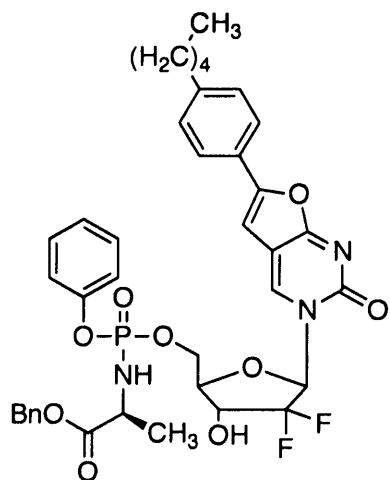
^1H -NMR (MeOD, 500 MHz): δ 8.54 (1H, s, H-4), 7.71-7.69 (2H, m, Ph), 7.45-7.19 (7H, m, Ph), 6.83 (1H, 2s, H-5), 6.56-6.38 (1H, m, H-1'), 4.66-4.51 (1H, m, H-5'), 4.50-4.41 (1H, m, H-5'), 4.40-4.29 (1H, m, H-4'), 4.29-4.20 (1H, m, H-3'), 4.08-3.98 (1H, m, CHCH_3), 3.69 (3H, s, OCH_3), 2.69 (2H, t, $J = 7.7$, $\alpha\text{-CH}_2$), 1.73-1.58 (2H, m, $\beta\text{-CH}_2$), 1.46-1.26 (7H, m, $\gamma\text{-CH}_2$, $\delta\text{-CH}_2$, CHCH_3), 0.94 (3H, t, $J = 7.0$, CH_3).

^{13}C -NMR (MeOD, 126 MHz): δ 14.35 (CH_3), 20.45 (d, $J_{\text{C-P}} = 6.6$, CHCH_3), 23.56, 32.15, 32.58, 36.75 (C_4H_8), 50.04 (CHCH_3), 52.78 (OCH_3), 65.56 (C-5'), 68.87 (C-4'), 81.04 (C-3'), 94.47 (C-1'), 98.72 (C-5), 110.81 (C-4a), 121.44, 121.48, 121.53, 121.57, 126.05, 126.24, 127.00, 129.55, 129.83, 130.23, 130.82, 130.98, 132.63 (C-2', PhO, Ph), 138.38 (C-4), 146.72 (*para*-C), 156.66 (C-6), 158.02 (C-2), 173.56 (C-7a), 174.50 (COOCH_3).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 95/5 to 0/100 in 40 min = retention time 34.41 min.

= $\text{H}_2\text{O}/\text{MeOH}$ 20/80 isocratic = 23.53.

Synthesis of 3-(2'-di-fluoro-2'-deoxy- β -D-ribofuranosyl-5-[phenyl-(benzoxy-L-alaninyl)] phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [171c]



$C_{38}H_{40}F_2N_3O_9P$

Mol Wt.: 751.7095

Prepared according to Standard Procedure D, from **157** (0.20 g, 0.46 mmol) in anhydrous THF (12 mL), **131d** (0.81 g, 2.30 mmol) in anhydrous THF (5 mL), NMI (0.18 mL, 2.30 mmol) and the reaction mixture was stirred at room temperature overnight. After this period were added **131d** (0.81 g, 2.30 mmol) in anhydrous THF (5 mL) and

NMI (0.18 mL, 2.30 mmol) and the reaction mixture was stirred at room temperature for further 8 h. Then NMI (0.09 mL, 1.20 mmol) was added and the reaction mixture stirred at room temperature overnight. After this period the solvent was removed and the residue dissolved in DCM. The organic phase was washed with a 0.5 M aqueous solution of citric acid (twice) and water (twice), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography gradient elution of DCM/MeOH = 100/0 then 98/2 then 96/4 to give a white solid which was further purified by preparative HPLC (isocratic MeOH/H₂O = 80/20) to give a white solid (4%, 0.012 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.99, 3.79.

^{19}F -NMR (DMSO, 471 MHz): δ -117.93, -118.23, -118.44, -118.74, -119.61.

1H -NMR (MeOD, 500 MHz): δ 8.52, 8.49 (1H, 2s, H-4), 7.66 (2H, d, J = 7.9, Ph), 7.40-7.17 (12H, m, Ph, PhO, CH_2Ph), 6.85, 6.79 (1H, 2s, H-5), 6.44 (1H, dd, J = 14.8, 7.4, H-1'), 5.21-5.10 (2H, m, CH_2Ph), 4.65-4.52 (1H, m, H-5'), 4.51-4.38 (1H, m, H-5'), 4.39-4.29 (1H, m, H-4'), 4.27-4.16 (1H, m, H-3'), 4.16-4.04 (1H, m, $CHCH_3$), 2.67 (2H, t, J = 7.5, α -CH₂), 1.72-1.60 (2H, m, β -CH₂), 1.47-1.28 (7H, m, γ -CH₂, δ -CH₂, $CHCH_3$), 0.93 (3H, t, J = 7.0, CH₃).

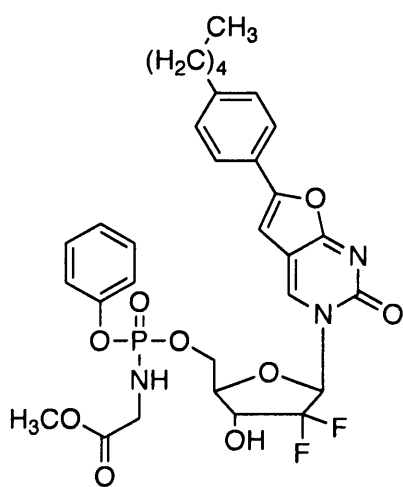
^{13}C -NMR (MeOD, 126 MHz): δ 14.36 (CH_3), 20.26 (d, $J_{\text{C-P}} = 7.7$, CHCH_3), 20.43 (d, $J_{\text{C-P}} = 6.9$, CHCH_3), 23.56, 32.14, 32.58, 36.75 (C_4H_8), 51.71, 51.93 (CHCH_3), 65.55 (d, $J_{\text{C-P}} = 6.1$, C-5'), 65.62 (d, $J_{\text{C-P}} = 5.8$, C-5'), 68.02, 68.05 (2s, CH_2Ph), 70.75, 70.91 (2s, C-4'), 81.03 (C-3'), 87.68 (C-1'), 98.75, 98.78 (2s, C-5), 110.80 (C-4a), 121.51, 121.54, 121.55, 121.59, 126.05, 126.41, 126.98, 129.12, 129.20, 129.35, 129.58, 130.21, 130.91, 130.97, (C-2', PhO, CH_2Ph , Ph), 137.17 ('ipso' OCH_2Ph), 138.25, 138.35 (2s, C-4), 146.68 (*para*-C), 152.07 ('ipso' PhO), 156.62 (C-6), 157.94 (C-2), 173.49 (C-7a), 174.66, 174.96 (COOCH_2Ph).

EI MS= 752.2555 (M+H), 774.2372 (M+Na).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 95/5 to 0/100 in 40 min = retention time 38.96 min.

$\text{H}_2\text{O}/\text{MeOH}$ 20/80 isocratic = retention time 33.48, 35.43 min.

Synthesis of 3-(2'-di-fluoro-2'-deoxy- β -D-ribofuranosyl-5-[phenyl-(methoxyglycyl)] phosphate)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one [171d]



$\text{C}_{31}\text{H}_{34}\text{F}_2\text{N}_3\text{O}_9\text{P}$

Mol Wt.: 661.5869

Prepared according to Standard Procedure D, from **157** (0.20 g, 0.46 mmol) in anhydrous THF (10 mL), **131c** (0.61 g, 2.30 mmol) in anhydrous THF (5 mL), NMI (0.18 mL, 2.30 mmol) and the reaction mixture was stirred at room temperature overnight. After this period were added **131c** (0.61 g, 2.30 mmol) in anhydrous THF

(5 mL) and NMI (0.18 mL, 2.30 mmol) and the reaction mixture was stirred at room temperature for further 8 h. Then NMI (0.09 mL, 1.20 mmol) was added and the reaction mixture stirred at room temperature for further 24 h. After this period the solvent was removed and the residue dissolved in DCM. The organic phase was washed with a 0.5 M aqueous solution of HCl (twice) and water (twice), dried over MgSO_4 and concentrated. The residue was purified by column chromatography gradient elution of DCM/MeOH =

98/2 then 97/3 to give a white solid which was further purified by preparative TLC (gradient elution of DCM/MeOH = 99/1, then 98/2, then 96/4) to give a white solid (14%, 0.042 g).

^{31}P -NMR (MeOD, 202 MHz): δ 5.42, 4.92.

^{19}F -NMR (DMSO, 471 MHz): δ -118.13, -118.65.

^1H -NMR (MeOD, 500 MHz): δ 8.55, 8.52 (1H, 2s, H-4), 7.70-7.59 (2H, m, Ph), 7.48-7.17 (7H, m, Ph, PhO), 6.88, 6.79 (1H, 2s, H-5), 6.45 (1H, d, J = 7.0, H-1'), 4.73-4.63 (1H, m, H-5'), 4.60-4.48 (1H, m, H-5'), 4.47-4.30 (1H, m, H-4'), 4.30-4.23 (1H, m, H-3'), 3.89-3.82 (2H, m, NHCH_2), 3.70 (3H, 2s, OCH_3), 2.64 (2H, t, J = 7.6, α - CH_2), 1.69-1.59 (2H, m, β - CH_2), 1.45-1.26 (4H, m, γ - CH_2 , δ - CH_2), 0.92 (3H, t, J = 7.0, CH_3).

^{13}C -NMR (MeOD, 126 MHz): δ 14.39 (CH_3), 23.57, 32.10, 32.61, 36.76 (C_4H_8), 43.72 (CH_2NH), 52.72 (OCH_3), 65.55 (C-5'), 70.90 (C-4'), 80.97 (C-3'), 87.40, 87.52 (C-1'), 98.76, 98.79 (2s, C-5), 110.80, 110.83 (2s, C-4a), 121.53, 121.56, 121.60, 123.49, 126.01, 126.03, 126.44, 126.47, 126.92, 126.94, 129.57, 130.18, 130.86, 130.96, 131.00, 132.61 (C-2', PhO, Ph), 138.32, 138.44 (C-4), 146.63 (*para*-C), 152.03, 152.09 (2s, 'ipso' PhO), 156.64 (C-6), 157.88, 157.94 (2s, C-2), 173.04, 173.08 (2s, C-7a), 173.47 (COOCH_3).

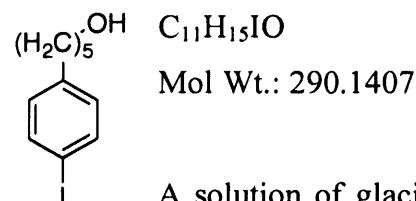
EI MS= 662.2073 (M+H), 684.1885 (M+Na).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 100/0 to 0/100 in 40 min = retention time 35.12 min.

= $\text{H}_2\text{O}/\text{MeOH}$ 20/80 isocratic = retention time 21.44 min.

9.10 Synthesis of the terminal fluoro derivative of BCNA lead compound

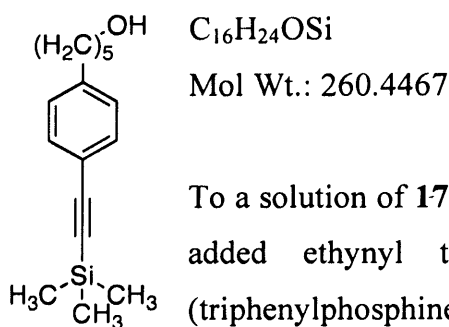
Synthesis of 5-(4-iodophenyl)pentan-1-ol [174]



A solution of glacial acetic acid (13.5 mL) and iodine (2.50 g, 8.81 mmol) was heated at 100 °C then were added conc. H_2SO_4 (1.4 mL) and **173** (3.37 mL, 20 mmol). After was added dropwise a suspension of sodium iodate (0.99g, 5.0 mmol) in water (3 mL) in 30 minutes and the reaction mixture was stirred at 105 °C for 1h (monitored by TLC). The reaction was poured in ice/water and quenched with a saturated solution of NaHCO_3 . The aqueous solution was extracted with DCM. The organic phase was then dried over MgSO_4 , concentrated. The crude of reaction was dissolved in a solution of NaOH (2.0 g) in methanol (20mL) and water (20 mL) and the reaction mixture was stirred at 75 °C for 1.5 h. After cooling, the reaction mixture was extracted with DCM, the organic phase was dried over MgSO_4 , concentrated to give a brown oil as a mixture of ortho and para desired compound (94%, 5.45 g).

$^1\text{H-NMR}$ (DMSO, 500 MHz): δ 7.63-7.61 (2H, s, Ph), 7.02 (1H, d, Ph), 4.35-4.31 (1H, m, OH), 3.42-3.36 (2H, m, $\omega\text{-CH}_2$), 2.54-2.50 (2H, m, $\alpha\text{-CH}_2$), 1.57-1.49 (2H, m, $\beta\text{-CH}_2$), 1.47-1.23 (4H, m, $\gamma\text{-CH}_2$, $\delta\text{-CH}_2$).

Synthesis of 5-(4-((trimethylsilyl)ethynyl)phenyl)pentan-1-ol. [175]

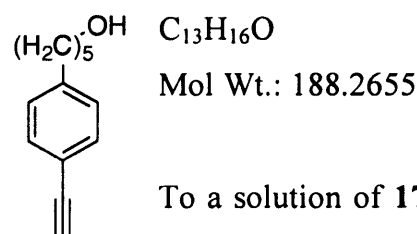


To a solution of **174** (2.50 g, 8.62 mmol) in anhydrous DMF (30 mL) were added ethynyl trimethylsilyl (3.58 mL, 25.85 mmol), tetrakis-(triphenylphosphine) palladium(0) (0.99g, 0.86 mmol), copper (I) iodide

(0.33 g, 1.72 mmol) and DIPEA (3.00 mL, 17.23 mmol) and the reaction mixture was stirred at room temperature overnight. After this period the solvent was removed and the residue purified by column chromatography eluting petroleum ether/ ethyl acetate = 8/2, to give a brown oil (53%, 1.18 g).

$^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 7.26-7.24 (2H, d, $J = 8.2$, Ph), 6.97 (2H, d, $J = 8.2$, Ph), 3.52-3.48 (2H, m, $\omega\text{-CH}_2$), 2.51-2.44 (2H, m, $\alpha\text{-CH}_2$), 1.56-1.41 (4H, m, $2\times\text{CH}_2$), 1.28-1.22 (1H, m, $\gamma\text{-CH}_2$), 0.12 (9H, s, $3\times\text{CH}_3$).

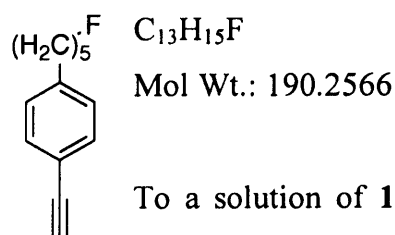
Synthesis of 5-(4-ethynylphenyl)pentan-1-ol. [176]



To a solution of **175** (1.08 g, 4.16 mmol) in anhydrous MeOH (20 mL) was added dropwise under an argon atmosphere a solution of sodium methoxide (0.27 g, 5.0 mmol) in anhydrous MeOH (5 mL) and the reaction mixture was stirred at room temperature for 1h. After this period the solution was neutralised with amberlite 120, filtered and then concentrated to give a brown oil. (99%, 0.77 g).

$^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 7.34 (2H, d, $J = 8.1$, Ph), 7.07 (2H, d, $J = 8.1$, Ph), 3.59-3.56 (2H, m, $\omega\text{-CH}_2$), 2.97 (1H, s, CH), 2.56 (2H, t, $J = 7.7$, $\alpha\text{-CH}_2$), 1.62-1.51 (4H, m, $2\times\text{CH}_2$), 1.37-1.30 (2H, m, $\gamma\text{-CH}_2$).

Synthesis of 1-ethynyl-4-(5-fluoropentyl)benzene. [177]



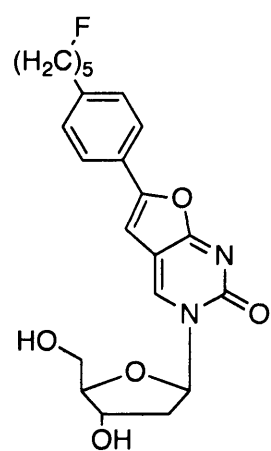
To a solution of **176** (0.71 g, 3.76 mmol) in anhydrous DCM (10 mL) was added dropwise at 0 °C under an argon atmosphere (diethylamino)sulfur

trifluoride (DAST) (1.5 mL, 11.28 mmol). After the addition the solution was stirred at 0 °C for 5 min then at room temperature for 2h. After this period, the reaction mixture was placed in an ice bath and quenched with a saturated solution of NaHCO₃. The solution was then extracted with DCM and the organic phase washed with water (twice) and saturated solution of NaHCO₃ (once). The organic phase was dried over MgSO₄, concentrated and the residue purified by column chromatography eluting petroleum ether/ethyl acetate = 9/1, to give a brown oil (39%, 0.28 g).

¹⁹F-NMR (CDCl₃, 471 MHz): δ -218.20.

¹H-NMR (CDCl₃, 500 MHz): δ 7.33 (2H, d, J = 8.1, Ph), 7.05 (2H, d, J = 8.1, Ph), 4.42-4.39 (1H, m, ω-CH_a), 4.32-4.30 (1H, m, ω-CH_b), 2.95 (1H, s, CH), 2.55 (2H, t, α-CH₂), 1.67-1.57 (4H, m, 2xCH₂), 1.40-1.35 (2H, m, γ-CH₂).

Synthesis of 3-(2'-deoxy-β-D-ribofuranosyl)-6-(4-*n*-F-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one. [172]



C₂₂H₂₅FN₂O₅

Mol Wt.: 416.4427

To a stirred solution of **121** (0.25 g, 0.69 mmol) in anhydrous DMF (5 mL), were added: **177** (0.26 g, 1.39 mmol), tetrakis-(triphenylphosphine) palladium(0) (0.08 g, 0.069 mmol), copper (I) iodide (0.026 g, 0.14 mmol) and DIPEA (0.24 mL, 1.39 mmol) and the reaction mixture was stirred at room temperature, under an argon atmosphere overnight. After this period were added copper (I) iodide (0.026 g, 0.14 mmol) and anhydrous TEA (5 mL) and the reaction mixture was stirred at 85 °C for 7.5 h. The solvent was then removed in vacuo and the residue purified by column chromatography (gradient elution of DCM/MeOH = 98/2 then 96/4 then 90/10, to give a pale yellow solid (50%, 0.29 g).

¹⁹F-NMR (DMSO, 471 MHz): δ -216.66.

$^1\text{H-NMR}$ (DMSO, 500 MHz): δ 8.84 (1H, s, H-4), 7.75 (2H, d, $J = 8.2$, H_a), 7.34 (2H, d, $J = 8.3$, H_b), 7.21 (1H, s, H-5), 6.20 (1H, t, $J = 6.1$, H-1'), 5.29 (1H, d, $J = 4.4$, 3'-OH), 5.16 (1H, t, $J = 5.2$, 5'-OH), 4.48 (1H, t, $J = 6.1$, $\omega\text{-H}_a$), 4.39 (1H, t, $J = 6.1$, $\omega\text{-H}_b$), 4.28-4.25 (1H, m, H-3'), 3.95-3.93 (1H, m, H-4'), 3.75-3.69 (1H, m, $\text{H}_a\text{-5'}$), 3.67-3.62 (1H, m, $\text{H}_b\text{-5'}$), 2.65 (2H, t, $J = 7.6$, $\alpha\text{-CH}_2$), 2.44-2.39 (1H, m, $\text{H}_a\text{-2'}$), 2.14-2.07 (1H, m, $\text{H}_b\text{-2'}$), 1.73-1.59 (4H, m, $\beta\text{-CH}_2$, $\delta\text{-CH}_2$), 1.41-1.35 (2H, m, $\gamma\text{-CH}_2$).

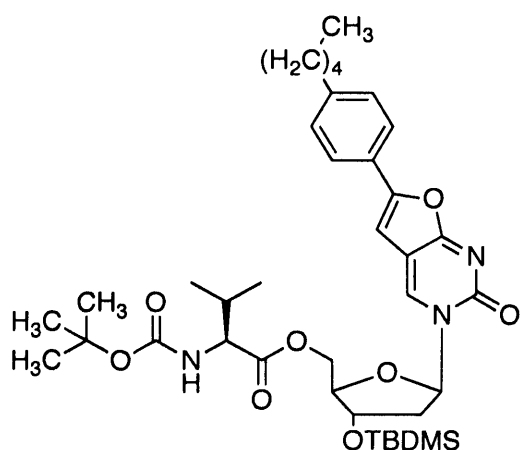
$^{13}\text{C-NMR}$ (DMSO, 126 MHz): δ 24.26, 24.30 (C- γ), 29.52, 29.67 (C- β), 30.22 (C- δ), 34.79 (C- α), 41.23 (C-2'), 60.67 (C-5'), 69.54 (C-3'), 83.06, 84.35 (C- ω), 87.56 (C-4'), 88.14 (C-1'), 98.69 (C-5), 106.87 (C-4a), 124.55 (C- H_b), 125.93 (*ipso*-C), 129.02 (C- H_a), 137.83 (C-4), 143.84 (para-C), 153.75 (C-6), 153.87 (C-2), 171.02 (C-7a).

EI MS= 439.164 (M+Na).

Anal. Calcd for $\text{C}_{22}\text{H}_{25}\text{FN}_2\text{O}_5 \cdot \frac{1}{2}\text{H}_2\text{O}$: C, 62.11; H, 6.16; N, 6.58. Found: C, 61.91; H, 6.18; N, 6.93.

9.11 Synthesis of FV100

Synthesis of 3-(2-deoxy-3-tert-butyldimethylsilyl- β -D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one 5'-boc-L-valyl ester [183]



$C_{38}H_{57}N_3O_8Si$

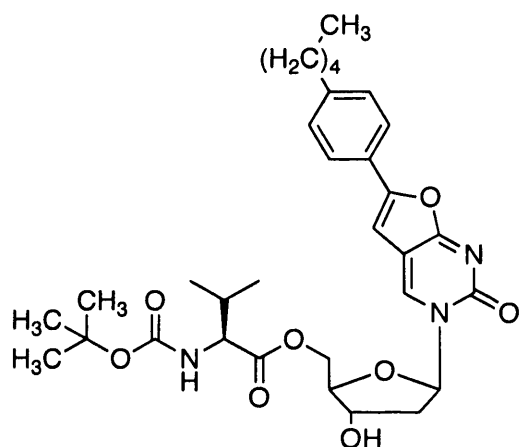
Mol Wt.: 711.9600

To a solution of **123** (0.98 g, 1.92 mmol) in anhydrous DCM (25 mL) were added at room temperature under an argon atmosphere boc-L-valine (0.83 g, 3.83 mmol), DCC (0.98 g, 4.80 mmol) and

DMAP (0.05 g, 0.38 mmol) and the reaction mixture was stirred at room temperature overnight. After this period the suspension was filtered and the organic phase was concentrated. The residue was triturated with DCM and filtered. The organic phase was concentrated to give a white solid contaminated with boc-L-valine and used as a crude in the next step.

1H -NMR (DMSO, 500 MHz): δ 8.55 (1H, s, H-4), 7.71 (2H, d, $J = 8.1$, Ph), 7.33 (2H, d, $J = 8.3$, Ph), 7.27 (1H, d, $J = 8.0$, NHCH), 7.20 (1H, s, H-5), 6.20 (1H, t, $J = 6.1$, H-1'), 4.46-4.35 (2H, m, H-3', H-4'), 4.32-4.25, 4.18-4.12 (2H, 2m, H-5'), 3.98-3.92 (1H, m, NHCH), 2.63 (2H, $J = 7.6$, α -CH₂), 2.47-2.40, 2.36-2.28 (2H, 2m, H-2'), 2.06-1.96 (1H, m, CH(CH₃)₂), 1.65-1.55 (2H, m, β -CH₂), 1.39 (9H, COO(CH₃)₃), 1.36-1.24 (4H, m, γ -CH₂, δ -CH₂), 0.91-0.83 (18H, m, ω -CH₃, C(CH₃)₃, CH(CH₃)₂), 0.09 (6H, 2s, Si(CH₃)₂).

Synthesis of 3-(2-deoxy- β -D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one 5'-Boc-L-valyl ester



$C_{32}H_{43}N_3O_8$

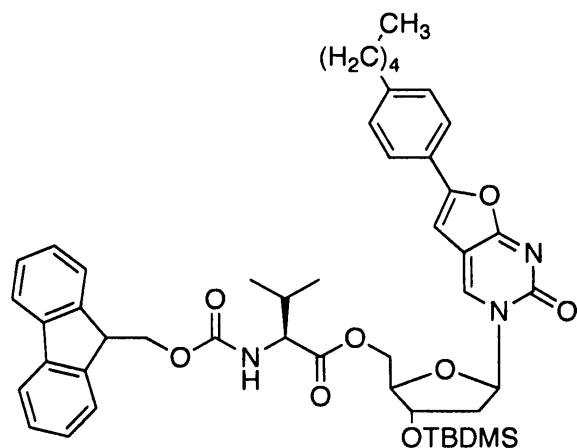
Mol Wt.: 597.6991

To a solution of **183** (0.25 g, 0.35 mmol) in THF (4 mL) and water (1 mL) was added dropwise TFA (1 mL) and the reaction mixture was stirred at room temperature for 7 h. After this period the reaction mixture was quenched with

saturated solution of sodium bicarbonate and concentrated. The residue was purified by column chromatography eluting with DCM/MeOH = 97/3 to give a white solid (55%, 0.11 g).

$^1\text{H-NMR}$ (DMSO, 500 MHz): δ 8.54 (1H, s, H-4), 7.71 (2H, d, $J = 8.1$, Ph), 7.33 (2H, d, $J = 8.2$, Ph), 7.26 (1H, d, $J = 7.8$, NHCH), 7.18 (1H, s, H-5), 6.23 (1H, t, $J = 6.3$, H-1'), 5.47 (1H, d, $J = 4.0$, 3'-OH), 4.39-4.28 (H, m, H-5'), 4.27-4.21 (1H, m, H-3'), 4.18-4.14 (1H, m, H-4'), 3.96-3.92 (1H, m, NHCH), 2.62 (2H, $J = 7.6$ Hz, $\alpha\text{-CH}_2$), 2.49-2.41, 2.27-2.12 (2H, 2m, H-2'), 2.06-1.92 (1H, m, $\text{CH}(\text{CH}_3)_2$), 1.66-1.54 (2H, m, $\beta\text{-CH}_2$), 1.39 (9H, $\text{COO}(\text{CH}_3)_3$), 1.36-1.22 (4H, m, $\gamma\text{-CH}_2$, $\delta\text{-CH}_2$), 0.94-0.82 (9H, m, $\omega\text{-CH}_3$, $\text{CH}(\text{CH}_3)_2$).

Synthesis of 3-(2-deoxy-3-tert-butyltrimethylsilyl-β-D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one 5'-Boc-L-valyl ester [184]



$C_{48}H_{59}N_3O_8Si$

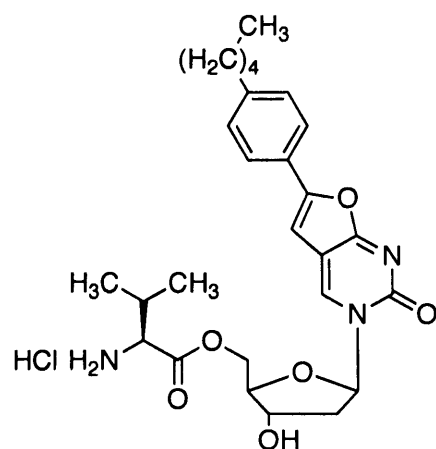
Mol Wt.: 834.0829

To a solution of **123** (1.46 g, 2.85 mmol) in anhydrous DCM (35 mL) were added at room temperature under an argon atmosphere Fmoc-L-valine (1.93 g, 5.70 mmol), DCC

(1.47 g, 7.13 mmol) and DMAP (0.07 g, 0.57 mmol) and the reaction mixture was stirred at room temperature overnight. After this period the suspension was filtered and the organic phase was concentrated. The residue was triturated with DCM and filtered. The organic phase was concentrated to give a white solid contaminated with Fmoc-L-valine and used as a crude in the next step.

1H -NMR (DMSO, 500 MHz): δ 8.50 (1H, s, H-4), 7.93-7.82 (4H, m, Ph, NHCH), 7.81-7.59 (4H, m, Ph), 7.45-7.21 (5H, m, Ph), 7.13 (1H, s, H-5), 6.18 (1H, t, J = 6.2, H-1'), 4.45-4.13 (8H, m, H-3', H-4', H-5', Fmoc-CHCH₂, NHCH), 2.60 (2H, J = 7.5, α -CH₂), 2.45-2.37, 2.33-2.24 (2H, 2m, H-2'), 2.18-1.93 (1H, m, CH(CH₃)₂), 1.65-1.53 (2H, m, β -CH₂), 1.38-1.19 (4H, m, γ -CH₂, δ -CH₂), 0.96-0.81 (18H, m, ω -CH₃, C(CH₃)₃, CH(CH₃)₂), 0.07 (6H, s, Si(CH₃)₂).

Synthesis of 3-(2-deoxy- β -D-ribofuranosyl)-6-(4-*n*-pentylphenyl)-2,3-dihydrofuro [2,3-*d*]pyrimidin-2-one 5'-L-valyl ester HCl salt; FV-100 [119·HCl]

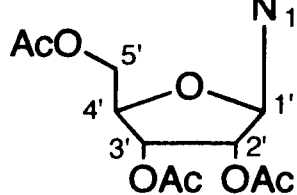


$C_{27}H_{36}ClN_3O_6$

Mol Wt.: 534.0442

To a solution of **184** (1.02 g, 1.23 mmol) in anhydrous DCM (10 mL) was added piperidine (0.61 mL, 6.15 mmol) and the reaction mixture was stirred at room temperature for 3.5 h. After this period, was added at 0 °C 20% HCl/IPA to pH = 2-3, and the reaction mixture was stirred at room temperature for 1 day. A precipitate, corresponding to the desired compound, was formed and it was filtered. The organic phase was concentrated and the residue triturated with MeOH and diethyl ether to give the desired compound as a white solid (23%, 0.13 g).

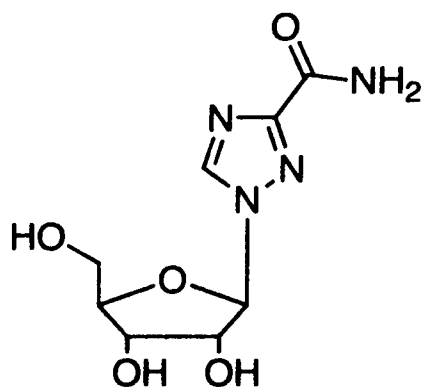
$^1\text{H-NMR}$ (DMSO, 500 MHz): δ 8.57 (1H, s, H-4), 8.50 (3H, bs, NH_3^+), 7.75 (2H, d, $J = 8.2$, Ph), 7.34 (2H, d, $J = 8.3$, Ph), 7.22 (1H, s, H-5), 6.25 (1H, t, $J = 6.2$, H-1'), 5.57 (1H, d, $J = 4.4$, 3'-OH), 4.52-4.44 (2H, m, H-5'), 4.33-4.23 (1H, m, H-3'), 4.17-4.10 (1H, m, H-4'), 3.93 (1H, d, $J = 4.9$, NH_3CH), 2.63 (2H, $J = 7.6$, $\alpha\text{-CH}_2$), 2.48-2.39, 2.32-2.22 (2H, 2m, H-2'), 2.22-2.13 (1H, m, $\text{CH}(\text{CH}_3)_2$), 1.63-1.55 (2H, m, $\beta\text{-CH}_2$), 1.41-1.23 (4H, m, $\gamma\text{-CH}_2$, $\delta\text{-CH}_2$), 0.99 (3H, t, $J = 6.5$, $\text{CH}(\text{CH}_3)_2$), 0.96 (3H, t, $J = 6.9$, $\text{CH}(\text{CH}_3)_2$), 0.87 (3H, t, $J = 6.9$, $\omega\text{-CH}_3$).



A round bottom flask containing a mixture of **189** (5.00 g, 15.73 mmol), **190** (2.00 g, 15.73 mmol) and bis(p-nitrophenyl)phosphate (0.10 g) was placed in a preheated oil bath at 170 °C under vacuum for 25 minutes. After cooling, to 70 °C, ethyl acetate and saturated solution of NaHCO₃ were added and the aqueous layer was extracted with ethyl acetate. The combined ethyl acetate extracts were washed with a saturated solution of NaHCO₃, water and brine. The organic phase was dried over MgSO₄ and concentrated. The residue was dissolved in ethanol (15 mL) and methanol (8 mL), heated until dissolved, then filtered over cotton and cooled at 0 °C for 4 h. The suspension was filtered to give a white solid (52%, 3.15 g).

¹H-NMR (CDCl₃ 500 MHz): δ 8.95 (1H, s, H-5), 6.37 (1H, d, H-1'), 5.70-5.68 (1H, m, H-2'), 5.55-5.53 (1H, m, H-3'), 4.44-4.35 (2H, m, H-4', H-5'), 4.12-4.08 (1H, m, H-5'), 3.88 (3H, s, OCH₃), 2.09 (6H, 2s, Ac), 2.03 (3H, s, Ac).

Synthesis of ribavirin (1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide). [50]



C₈H₁₂N₄O₅

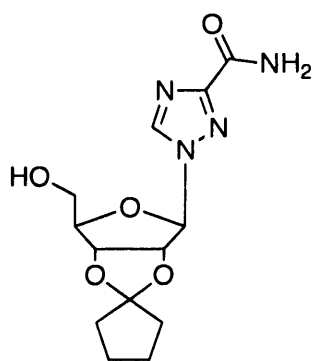
Mol Wt.: 244.2047

191 (3.10 g, 8.04 mmol) was dissolved in freshly prepared methanolic ammonia (20 mL). The reaction mixture was stirred at room temperature for 20 h. After this period the solution was concentrated and the residue dissolved in ethanol and water. The volume of the ethanol solution was reduced by heating and the hot

ethanol solution on cooling provided colourless crystals which was filtered, the filtrate washed with acetone, dried under vacuum to give a white solid (87%, 1.70 g).

¹H-NMR (DMSO 500 MHz): δ 8.89 (1H, s, H-5), 7.85 (1H, s, NH), 7.64 (1H, s, NH), 5.81 (1H, d, H-1'), 5.59 (1H, d, 2'-OH), 5.21 (1H, d, 3'-OH), 4.94 (1H, t, 5'-OH), 4.37-4.34 (1H, m, H-2'), 4.16-4.12 (1H, m, H-3'), 3.96-3.94 (1H, m, H-4'), 3.65-3.61 (1H, m, H-5'), 3.52-3.48 (1H, m, H-5').

Synthesis of 2',3'-O,O-cyclopentylidene ribavirin. [192]



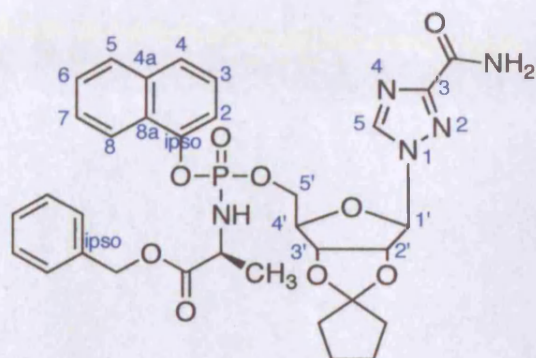
C₁₃H₁₈N₄O₅

Mol Wt.: 310.3058

To a solution of **50** (0.25 g, 1.03 mmol) in dry cyclopentanone (15 mL) was added 70% perchloric acid (0.10 mL) and the reaction mixture was stirred at room temperature for 1.5 h. Then the reaction was quenched with NaHCO₃ and the solution concentrated. The residue was purified by column chromatography gradient elution of DCM/MeOH = 9/1 then 8/2, to give a white solid (90%, 0.30 g).

¹H-NMR (DMSO 500 MHz): δ 8.80 (1H, s, H-5), 7.84 (1H, s, NH), 7.64 (1H, s, NH), 6.22 (1H, d, H-1'), 5.15-5.14 (1H, m, H-2'), 4.97-4.95 (1H, t, 5'-OH), 4.87-4.86 (1H, m, H-3'), 4.27-4.24 (1H, m, H-4'), 3.51-3.46 (1H, m, H-5'), 3.43-3.37 (1H, m, H-5'), 1.95-1.92 (2H, t, CH₂), 1.76-1.73 (2H, m, CH₂), 1.66-1.61 (4H, m, 2xCH₂).

Synthesis of 2',3'-O,O-cyclopentylidene-ribavirin-5'-[1-naphthyl(benzoxo-L-alaninyl)] phosphate. [194a]



$C_{33}H_{36}N_5O_9P$

Mol Wt.: 677.6408

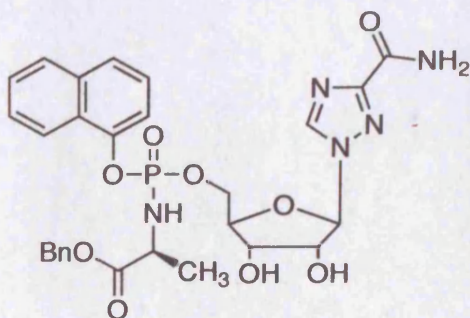
Prepared according to Standard Procedure C, from **192** (0.20 g, 0.64 mmol) in anhydrous THF (6 mL), t BuMgCl (1.0 M THF solution, 1.30 mL,

1.30 mmol), **193a** (0.29 g, 0.71 mmol) in anhydrous THF (6 mL), and the reaction mixture was stirred at room temperature overnight. After this period the solution was concentrated and the residue purified by column chromatography eluting with DCM/MeOH = 98/2, to give a white solid (32%, 0.14 g).

^{31}P -NMR ($CDCl_3$, 202 MHz): δ 3.44, 3.17.

1H -NMR ($CDCl_3$, 500 MHz): δ 8.20, 8.19 (1H, 2s, H-5), 7.97-7.18 (12H, m, Naph, OCH_2Ph), 5.94 and 5.85 (1H, 2d, H-1'), 5.03-5.00 (2.5H, m, OCH_2Ph , H-2' of one diastereoisomer), 4.78-4.76 (0.5H, m, H-3' of one diastereoisomer), 4.69-4.67 (0.5H, m, H-2' of one diastereoisomer), 4.51-4.49 (0.5H, m, H-3' of one diastereoisomer), 4.47-4.44 (1H, m, H-4'), 4.25-4.20 (1H, m, H-5'), 4.19-4.11 (1H, m, H-5'), 4.07-4.02, 4.00-3.90 (1H, 2m, $CHCH_3$), 1.92-1.87 (2H, m, CH_2), 1.67-1.52 (6H, m, 3x CH_2), 1.30, 1.27 (3H, 2d, $CHCH_3$).

Synthesis of ribavirin-5'-[1-naphthyl(benzoxo-L-alaninyl)] phosphate. [195a]



$C_{28}H_{30}N_5O_9P$

Mol Wt.: 611.5397

A solution of **194a** (0.14 g, 0.20 mmol) in 60% formic acid (8 mL) was stirred at room temperature overnight. After this period the

solution was concentrated and the residue purified by column chromatography gradient elution of DCM/MeOH = 96/4 then 95/5, to give a white solid (28%, 0.035 g).

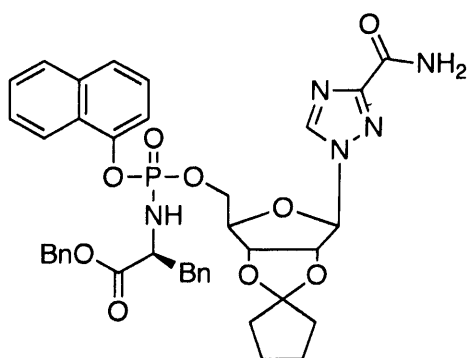
^{31}P -NMR (MeOD, 202 MHz): δ 4.21, 4.07.

^1H -NMR (MeOD, 500 MHz): δ 8.56, 8.54 (1H, 2s, H-5), 8.01-7.99 (1H, m, H-8 Naph), 7.77-7.75 (1H, m, H-6 Naph), 7.64-7.62 (1H, m, H-2 Naph), 7.43-7.15 (9H, m, Naph, OCH_2Ph), 5.84, 5.82 (1H, 2d, H-1'), 4.97-4.89 (2H, m, PhCH_2), 4.40-4.38 (1H, m, H-2'), 4.35-4.17 (4H, m, H-3', H-4', H-5'), 3.98-3.89 (1H, m, CHCH_3), 1.19-1.16 (3H, m, CHCH_3).

^{13}C -NMR (MeOD, 126 MHz): δ 20.23 (d, $J_{\text{C-P}} = 7.4$, CH_3), 20.40 (d, $J_{\text{C-P}} = 6.4$, CH_3), 51.72, 51.82 (CHCH_3), 67.67 (d, $J_{\text{C-P}} = 5.3$, C-5'), 67.91, 67.93 (2s, OCH_2Ph), 68.06 (d, $J_{\text{C-P}} = 5.6$, C-5') 71.68, 71.80 (2s, C-3'), 76.18, 76.22 (2s, C-2'), 84.61 (d, $J_{\text{C-P}} = 8.0$, C-4'), 84.69 (d, $J_{\text{C-P}} = 9.6$, C-4'), 93.79, 93.83 (2s, C-1'), 116.17 (d, $J_{\text{C-P}} = 3.2$, C-2 Naph), 116.35 (d, $J_{\text{C-P}} = 3.3$, C-2 Naph) 122.68, 122.81, 125.92, 125.98, 126.50, 127.48, 127.50, 127.75, 127.88, 128.80, 128.83, 129.24, 129.26, 129.52, 129.54 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH_2Ph), 136.27, 137.20 (C-4a Naph, 'ipso' OCH_2Ph), 146.64, 146.68 (2s, C-5), 147.90, 147.96 (2s, 'ipso' Naph), 158.62 (C-3), 163.22 (CONH_2), 174.59 (d, $J_{\text{C-P}} = 5.2$, COOCH_2Ph), 174.87 (d, $J_{\text{C-P}} = 5.1$, COOCH_2Ph).
EI MS= 634.17 (M+Na).

Anal. Calcd for $\text{C}_{28}\text{H}_{30}\text{N}_5\text{O}_9\text{P}$: C, 54.99; H, 4.94; N, 11.45. Found: C, 54.74; H, 5.41; N, 10.40.

Synthesis of 2',3'-O,O-cyclopentylidene-ribavirin-5'-[1-naphthyl(benzoxo-L-phenylalaninyl)] phosphate. [194b]



$\text{C}_{39}\text{H}_{40}\text{N}_5\text{O}_9\text{P}$

Mol Wt.: 753.7368

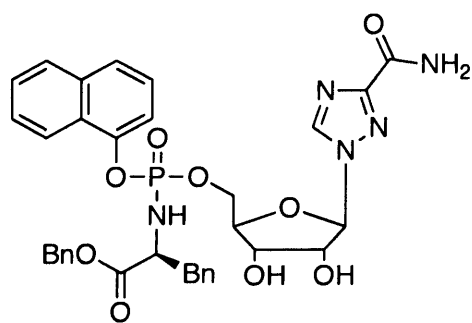
Prepared according to Standard Procedure C, from **192** (0.20 g, 0.64 mmol) in anhydrous THF (6 mL), $^t\text{BuMgCl}$ (1.0 M THF solution,

1.30 mL, 1.30 mmol), **193b** (0.34 g, 0.71 mmol) in anhydrous THF (7 mL), and the reaction mixture was stirred at room temperature overnight. After this period the solution was concentrated and the residue purified by column chromatography eluting DCM/MeOH = 98/2, to give a white solid (44%, 0.22 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 3.30, 3.22.

^1H -NMR (CDCl_3 , 500 MHz): δ 8.17, 8.13 (1H, 2s, H-5), 7.90-6.83 (17H, m, Naph, OCH_2Ph , CHCH_2Ph), 5.91, 5.86 (1H, 2d, H-1'), 4.96-4.94 (2H, m, OCH_2Ph), 4.92-4.90, 4.73-4.71 (1H, 2m, H-2'), 4.64-4.63, 4.51-4.49 (1H, 2m, H-3'), 4.41-4.38 (1H, m, H-4'), 4.30-4.23 (1H, m, CHCH_2Ph) 4.08-4.01, 3.90-3.83 (1H, 2m, H-5'), 3.81-3.76, 3.67-3.63 (1H, m, H-5'), 2.90-2.82 (2H, m, CH_2Ph), 1.95-1.87 (2H, m, CH_2), 1.72-1.55 (6H, m, $3\times\text{CH}_2$).

Synthesis of ribavirin-5'-[1-naphthyl(benzoxo-L-phenylalaninyl)] phosphate. [195b]



$\text{C}_{34}\text{H}_{34}\text{N}_5\text{O}_9\text{P}$
Mol Wt.: 687.6356

A solution of **194b** (0.14 g, 0.203 mmol) in 60% formic acid (8 mL) was stirred at room temperature overnight. After this period the

solution was concentrated and the residue purified by column chromatography gradient elution of DCM/MeOH = 96/4 then 95/5, to give a white solid (28%, 0.035 g).

^{31}P -NMR (MeOD , 202 MHz): δ 3.97, 3.83.

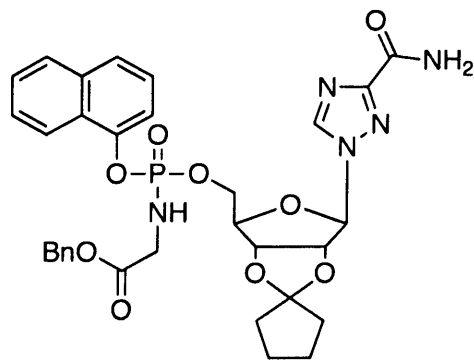
^1H -NMR (MeOD , 500 MHz): δ 8.64, 8.63 (1H, 2s, H-5), 8.04, 8.00 (1H, 2d, H-8 Naph), 7.88-7.85 (1H, m, H-6 Naph), 7.68-7.65 (1H, m, H-2 Naph), 7.54-7.46 (2H, m, H-5, H-7 Naph), 7.34-7.07 (12H, m, Naph, OCH_2Ph , CHCH_2Ph), 5.93-5.92 (1H, m, H-1'), 5.04-4.96 (2H, m, OCH_2Ph), 4.58-4.55 (1H, m, H-2'), 4.34-4.29 (1H, m, H-3'), 4.25-4.17 (2H, m, H-4', NHCH), 4.14-3.97 (2H, m, H-5'), 3.05-3.02, 2.89-2.80 (2H, 2m, CHCH_2Ph).

^{13}C -NMR (MeOD, 126 MHz): δ 40.88 (d, $J_{\text{C-P}} = 10.3$, CHCH_2Ph), 40.95 (d, $J_{\text{C-P}} = 7.4$, CHCH_2Ph), 57.91, 58.06 (2s, NHCH), 67.53 (d, $J_{\text{C-P}} = 5.5$, C-5'), 67.85 (d, $J_{\text{C-P}} = 5.5$, C-5'), 67.94, 68.00 (2s, OCH_2Ph), 71.70, 71.79 (2s, C-3'), 76.17, 76.24 (2s, C-2'), 84.57 (d, $J_{\text{C-P}} = 8.1$, C-4'), 84.70 (d, $J_{\text{C-P}} = 8.4$, C-4'), 93.80, 93.86 (2s, C-1'), 116.06 (d, $J_{\text{C-P}} = 3.2$, C-2 Naph), 116.10 (d, $J_{\text{C-P}} = 3.2$, C-2 Naph), 122.77, 122.80, 125.86, 125.89, 126.48, 126.51, 127.46, 127.72, 127.80, 127.84, 127.89, 127.92, 128.75, 128.81, 129.26, 129.28, 129.40, 129.46, 129.49, 129.52, 130.52, 130.57 (C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH_2Ph , CHCH_2Ph), 136.21, 136.25, 136.94, 137.95, 138.00 ('ipso' CHCH_2Ph , 'ipso' OCH_2Ph), 146.58 (C-5), 147.82, 147.87 ('ipso' Naph), 158.59 (C-3), 163.22 (CONH_2), 173.68 (d, $J_{\text{C-P}} = 3.2$, COOCH_2Ph), 173.86 (d, $J_{\text{C-P}} = 3.6$, COOCH_2Ph).

EI MS= 710.20 (M+Na).

Anal. Calcd for $\text{C}_{34}\text{H}_{34}\text{N}_5\text{O}_9\text{P}$: C, 59.39; H, 4.98; N, 10.18. Found: C, 59.38; H, 5.03; N, 9.98.

Synthesis of 2',3'-O,O-cyclopentylidene-ribavirin-5'-[1-naphthyl(benzoxylglycyl)] phosphate. [194c]



$\text{C}_{32}\text{H}_{34}\text{N}_5\text{O}_9\text{P}$

Mol Wt.: 663.6142

Prepared according to Standard Procedure C, from **192** (0.20 g, 0.64 mmol) in anhydrous THF (6 mL), $^t\text{BuMgCl}$ (1.0 M THF solution, 1.30 mL, 1.30 mmol), **193c** (0.28 g, 0.71

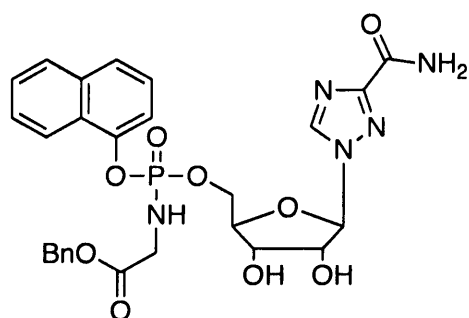
mmol) in anhydrous THF (6 mL), and the reaction mixture was stirred at room temperature overnight. After this period the solution was concentrated and the residue purified by column chromatography eluting with $\text{DCM}/\text{MeOH} = 98/2$, to give a white solid (15%, 0.066 g).

^{31}P -NMR (CDCl_3 , 202 MHz): δ 4.39, 4.22.

^1H -NMR (CDCl_3 , 500 MHz): δ 8.20, 8.17 (1H, 2s, H-5), 7.99-7.19 (12H, m, Naph, OCH_2Ph), 5.94, 5.82 (1H, 2d, H-1'), 5.14-5.11 (0.5H, m, H-2' of one

diastereoisomer), 5.04-5.00 (2H, m, OCH_2Ph) 4.84-4.83 (0.5 H, m, H-3' of one diastereoisomer), 4.58-4.56 (0.5 H, m, H-2' of one diastereoisomer), 4.52-4.15 (3.5 H, m, H-3' of one diastereoisomer, H-4', H-5'), 3.86, 3.65 (2H, m, NHCH_2), 1.93-1.56 (8H, m, $4\times\text{CH}_2$).

Synthesis of ribavirin-5'-[1-naphthyl(benzoxo-glyciny)] phosphate. [195c]



$\text{C}_{27}\text{H}_{28}\text{N}_5\text{O}_9\text{P}$

Mol Wt.: 597.5131

A solution of **194c** (0.065 g, 0.098 mmol) in 60% formic acid (6 mL) was stirred at room temperature overnight. After this period the

solution was concentrated and the residue purified by column chromatography eluting with $\text{DCM}/\text{MeOH} = 95/5$, to give a white solid (17%, 0.010 g).

^{31}P -NMR (MeOD, 202 MHz): δ 5.34, 5.27.

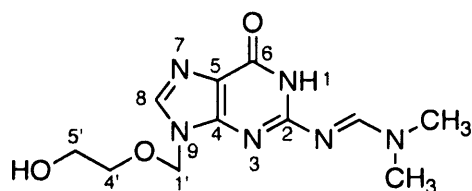
^1H -NMR (MeOD, 500 MHz): δ 8.55, 8.52 (1H, 2s, H-5), 8.03-7.95 (1H, m, H-8 Naph), 7.77-7.74 (1H, m, H-6 Naph), 7.57-7.56 (1H, m, H-2 Naph) 7.42-7.13 (8H, m, Naph, OCH_2Ph), 5.85, 5.80 (1H, 2d, H-1'), 4.99-4.97 (2H, m, OCH_2Ph), 4.45-4.44 (0.5 H, m, H-2' of one diastereoisomer), 4.39-4.16 (4.5 H, m, H-2' of one diastereoisomer, H-3', H-4', H-5'), 3.73-3.65 (2H, m, NHCH_2).

^{13}C -NMR (MeOD, 126 MHz): δ 44.01, 44.05 (2s, NHCH_2), 67.76 (d, $J_{\text{C-P}} = 5.1$, C-5'), 67.92 (COOCH_2Ph), 68.06 (d, $J_{\text{C-P}} = 5.4$, C-5'), 71.74, 71.91 (2s, C-2'), 76.22 (C-3'), 84.80, 84.84 (2s, C-4'), 93.78 (C-1'), 116.24, 116.39, 122.69, 122.75, 125.99, 126.04, 126.51, 127.46, 127.54, 127.76, 128.82, 129.32, 129.36, 129.55 (C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH_2Ph), 136.27, 137.15 (C-4a Naph, 'ipso' OCH_2Ph), 146.70, 146.78 (C-5), 147.88, 147.97 ('ipso' Naph), 158.62 (C-3), 163.22 (CONH_2), 172.34 (COOCH_2Ph).

EI MS= 620.15 (M+Na).

9.13 Synthesis of acyclovir ProTides

Synthesis of N²-DMF acyclovir (*N'*-(9-((2-hydroxyethoxy)methyl)-6-oxo-6,9-dihydro-1*H*-purin-2-yl)-*N,N*-dimethylformimidamide). [200]

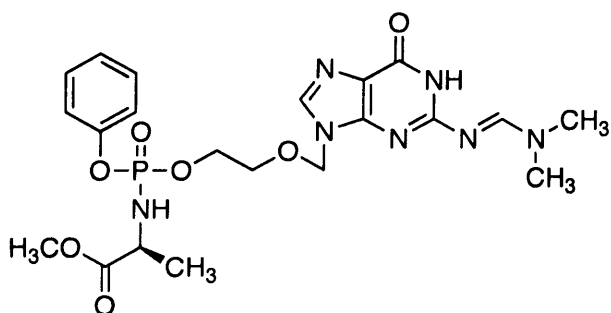
C₁₁H₁₆N₆O₃

Mol Wt.: 280.2831

To a suspension of **9** (1.00 g, 4.44 mmol) in dry DMF (20 mL) was added *N,N*-dimethylformamide dimethyl acetal (2.96 mL, 22.2 mmol) and the reaction mixture was stirred at room temperature for 1 day. After this period the suspension was filtered, and the solid was washed with diethyl ether to give a white solid (97%, 1.20 g).

¹H-NMR (DMSO 500 MHz): δ 11.30 (1H, s, NH), 8.58 (1H, s, CHN(CH₃)₂), 7.94 (1H, s, H-8), 5.45 (2H, s, H-1'), 4.65 (1H, t, OH), 3.52-3.49 (4H, m, H-4', H-5'), 3.17, 3.04 (6H, 2s, N(CH₃)₂).

Synthesis of N²-DMF acyclovir-[1-phenyl-(methoxy-L-alaninyl)] phosphate. [201a]

C₂₁H₂₈N₇O₇P

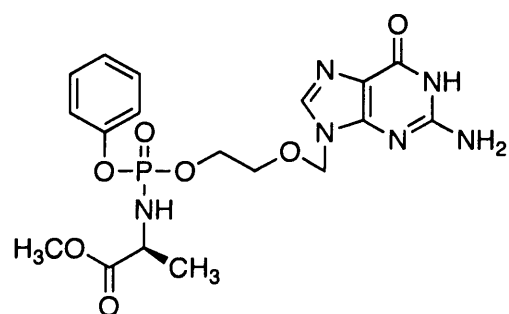
Mol Wt.: 521.4635

Prepared according to Standard Procedure C, from **200** (0.40 g, 1.44 mmol) in anhydrous THF (15 mL), ^tBuMgCl (1.0 M THF solution, 2.88 mL, 2.88 mmol), **131a** (1.20 g, 4.32 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 96/4 then 94/6, to give a white solid (36%, 0.27 g).

³¹P-NMR (MeOD, 202 MHz): δ 3.76, 3.53.

^1H -NMR (MeOD, 500 MHz): δ 8.60 (1H, s, $\text{NCHN}(\text{CH}_3)_2$), 7.88, 7.86 (1H, 2s, H-8), 7.27-7.04 (3H, m, PhO), 6.97-6.90 (2H, m, PhO), 5.47, 5.45 (2H, 2s, H-1'), 4.15-4.12 (1H, m, H-5' of one diastereoisomer), 4.10-4.07 (1H, m, H-5' of one diastereoisomer), 3.85-3.78 (1H, m, CHCH_3), 3.74-3.66 (2H, m, H-4'), 3.58, 3.57 (3H, 2s, COOCH_3), 3.05, 3.01 (6H, 2s, $\text{N}(\text{CH}_3)_2$), 1.15-1.14 (3H, s, CHCH_3).

Synthesis of acyclovir-[1-phenyl-(methoxy-L-alaninyl)] phosphate. [199a]



$\text{C}_{18}\text{H}_{23}\text{N}_6\text{O}_7\text{P}$

Mol Wt.: 466.3850

A solution of **201a** (0.27 g, 0.52 mmol) in 2-propanol (10 mL) was stirred under reflux for 40 h. The solvent was then

removed under reduced pressure and the residue was purified by column chromatography eluting with DCM/MeOH = 95/5. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 95/5 then 94/6 then 92/8) to give a white solid (13%, 0.032 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.72, 3.55.

^1H -NMR (MeOD, 500 MHz): δ 7.87-7.85 (1H, 2s, H-8), 7.36-7.33 (2H, m, PhO), 7.20-7.16 (3H, m, PhO), 5.50, 5.47 (2H, 2s, H-1'), 4.27-4.17 (2H, m, H-5'), 3.97-3.90 (1H, m, CHCH_3), 3.84-3.78 (2H, m, H-4'), 3.69, 3.67 (3H, 2s, COOCH_3), 1.35-1.31 (3H, m, CHCH_3).

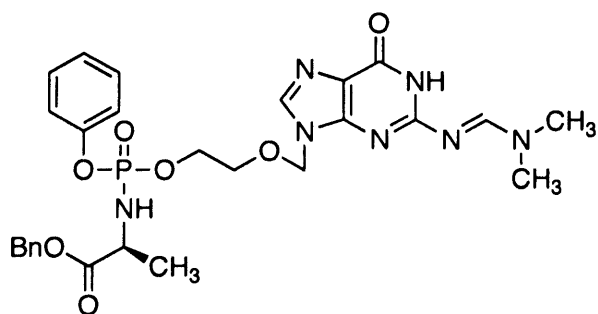
^{13}C -NMR (MeOD, 125 MHz): δ 20.36 (d, $J_{\text{C-P}} = 7.0$, CH_3) 20.44 (d, $J_{\text{C-P}} = 6.4$, CH_3), 51.45, 51.52 (CHCH_3), 52.72, 52.77 (COOCH_3), 67.01 (d, $J_{\text{C-P}} = 5.3$, C-5'), 67.11 (d, $J_{\text{C-P}} = 5.3$, C-5'), 69.43 (d, $J_{\text{C-P}} = 5.3$, C-4'), 69.48 (d, $J_{\text{C-P}} = 5.3$, C-4'), 73.68 (C-1'), 117.52, 121.10, 121.14, 121.32, 121.40, 121.42, 121.44, 121.45, 121.54, 121.58, 123.89, 126.07, 130.10, 130.25, 130.71 (C-5, PhO), 139.79 (C-8), 152.17, 152.22, 152.24 (C-4), 155.69 (C-2), 159.41 (C-6), 175.46 (d, $J_{\text{C-P}} = 5.4$, COOCH_3), 175.58 (d, $J_{\text{C-P}} = 4.9$, COOCH_3).

EI MS = 489.1267 (M+Na).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 100/0 to 0/100 in 20 min = retention time 9.71 min.

= $\text{H}_2\text{O}/\text{MeOH}$ 40/60 isocratic = retention time 5.56 min, 5.72 min.

Synthesis of N²-DMF acyclovir-[1-phenyl-(benzoxy-L-alaninyl)] phosphate. [201b]

C₂₇H₃₂N₇O₇P

Mol Wt.: 597.5594

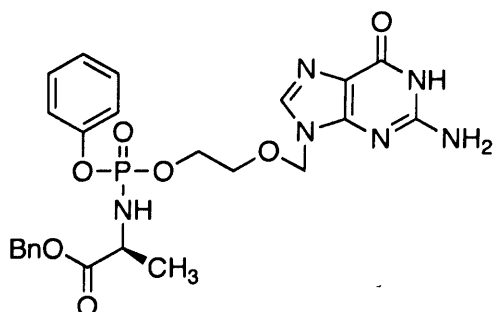
Prepared according to Standard Procedure C, from **200** (0.40 g, 1.44 mmol) in anhydrous THF (15 mL),

^tBuMgCl (1.0 M THF solution, 2.88 mL, 2.88 mmol), **131d** (1.53 g, 4.32 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography eluting with DCM/MeOH = 95/5, to give a white solid (44 %, 0.38 g).

³¹P-NMR (MeOD, 202 MHz): δ 3.84, 3.47.

¹H-NMR (MeOD, 500 MHz): δ 8.54 (1H, s, NCHN(CH₃)₂), 7.80, 7.77 (1H, 2s, H-8), 7.26-7.14 (7H, m, PhO, OCH₂Ph), 7.05-7.00 (3H, m, PhO, OCH₂Ph), 5.41, 5.38 (2H, 2s, H-1'), 5.01, 4.99 (2H, 2s, OCH₂Ph), 4.08-4.05 (1H, m, H-5' of one diastereoisomer), 4.04-4.00 (1H, m, H-5' of one diastereoisomer), 3.86-3.81 (1H, m, CHCH₃), 3.66-3.61 (2H, m, H-4'), 3.03, 3.02 (3H, 2s, N(CH₃)₂), 2.97 (3H, s, N(CH₃)₂), 1.22-1.18 (3H, m, CHCH₃).

Synthesis of acyclovir-[1-phenyl-(benzoxy-L-alaninyl)] phosphate. [199b]

C₂₄H₂₇N₆O₇P

Mol Wt.: 542.4809

A solution of **201b** (0.379 g, 0.63 mmol) in 2-propanol (15 mL) was stirred under reflux for 40 h. The solvent was then

removed under reduced pressure and the residue was purified by column chromatography gradient elution of DCM/MeOH = 95/5 then 90/10. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 96/4 then 94/6) to give a white solid (14%, 0.048 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.80, 3.50.

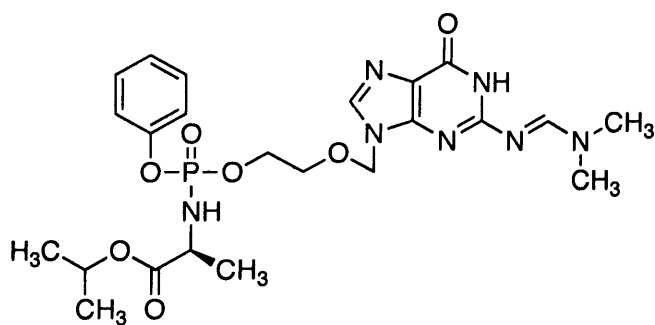
^1H -NMR (MeOD, 500 MHz): δ 7.74-7.71 (1H, 2s, H-8), 7.23-7.14 (7H, m, PhO, OCH_2Ph), 7.06-7.01 (3H, m, PhO, OCH_2Ph), 5.32, 5.29 (2H, 2s, H-1'), 5.00-4.99 (2H, 2s, OCH_2Ph), 4.04-3.99 (2H, m, H-5'), 3.89-3.82 (1H, m, CHCH_3), 3.62-3.59 (2H, m, H-4'), 1.22, 1.21 (3H, 2d, CHCH_3).

^{13}C -NMR (MeOD, 125 MHz): δ 20.34 (d, $J_{\text{C-P}} = 7.6$, CH_3) 20.43 (d, $J_{\text{C-P}} = 6.3$, CH_3), 51.61, 51.73 (2s, CHCH_3), 67.01 (d, $J_{\text{C-P}} = 5.3$, C-5'), 67.10 (d, $J_{\text{C-P}} = 5.5$, C-5'), 67.97, 67.98 (2s, OCH_2Ph), 69.39 (d, $J_{\text{C-P}} = 3.9$, C-4'), 69.45 (d, $J_{\text{C-P}} = 3.9$, C-4'), 73.76 (C-1'), 117.30, 121.39, 121.43, 121.47, 121.51, 126.08, 126.10, 129.19, 129.33, 129.36, 129.53, 129.58, 129.61, 130.19, 130.73 (C-5, PhO, OCH_2Ph), 137.27 ('ipso' OCH_2Ph), 139.75 (C-8), 152.11, 152.17 (C-4), 155.73 (C-2), 159.32 (C-6), 174.77 (d, $J_{\text{C-P}} = 5.2$, COOCH_2Ph), 174.91 (d, $J_{\text{C-P}} = 4.6$, COOCH_2Ph).

EI MS= 565.1581 (M+Na).

Anal. Calcd for $\text{C}_{24}\text{H}_{27}\text{N}_6\text{O}_7\text{P}$: C, 53.14; H, 5.02; N, 15.49. Found: C, 53.01; H, 4.90; N, 15.10.

Synthesis of N^2 -DMF acyclovir-[1-phenyl-(isopropoxy-L-alaninyl)] phosphate. [201c]



$\text{C}_{23}\text{H}_{32}\text{N}_7\text{O}_7\text{P}$

Mol Wt.: 549.5166

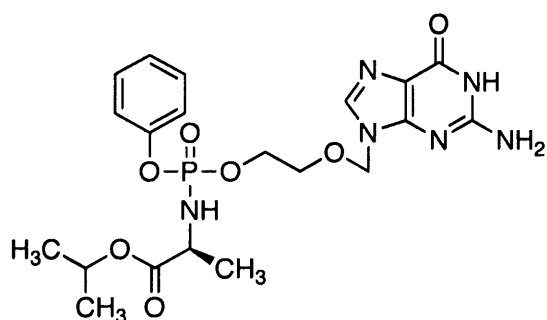
Prepared according to Standard Procedure C, from **200** (0.40 g, 1.43 mmol) in anhydrous THF

(15 mL), $^t\text{BuMgCl}$ (1.0 M THF solution, 2.86 mL, 2.86 mmol), **131e** (1.31 g, 4.28 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 97/3 then 96/4, to give a white solid (59 %, 0.54 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.88, 3.65.

^1H -NMR (MeOD, 500 MHz): δ 8.72 (1H, s, $\text{NCHN}(\text{CH}_3)_2$), 7.96, 7.94 (1H, 2s, H-8), 7.36-7.32 (2H, m, PhO), 7.20-7.17 (3H, m, PhO), 5.59, 5.57 (2H, 2s, H-1'), 5.08-4.92 (1H, m, $\text{COOCH}(\text{CH}_3)_2$), 4.29-4.19 (2H, m, H-5'), 3.91-3.80 (3H, m, CHCH_3 , H-4'), 3.21, 3.14 (6H, 2s, $\text{N}(\text{CH}_3)_2$), 1.41-1.01 (9H, m, $\text{COOCH}(\text{CH}_3)_2$, CHCH_3).

Synthesis of acyclovir-[1-phenyl-(isopropoxy-L-alaninyl)] phosphate. [199c]



$\text{C}_{20}\text{H}_{27}\text{N}_6\text{O}_7\text{P}$

Mol Wt.: 494.4381

A solution of **201c** (0.53 g, 0.98 mmol) in 2-propanol (18 mL) was stirred under reflux for 72 h. The solvent was

then removed under reduced pressure and the residue was purified by column chromatography with DCM/MeOH = 95/5. The appropriate fractions were collected, the solvent removed under reduced pressure and the residue was triturated with water and the precipitate filtered to give a white solid (7%, 0.035 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.80, 3.65.

^1H -NMR (MeOD, 500 MHz): δ 7.86-7.84 (1H, 2s, H-8), 7.36-7.33 (2H, m, PhO), 7.20-7.17 (3H, m, PhO), 5.50, 5.47 (2H, 2s, H-1'), 5.00-4.94 (1H, m, $\text{COOCH}(\text{CH}_3)_2$), 4.29-4.16 (2H, m, H-5'), 3.92-3.86 (1H, m, CHCH_3), 3.84-3.79 (2H, m, H-4'), 1.34, 1.31 (3H, 2d, $J = 7.1$, $J = 6.7$, CHCH_3), 1.25-1.22 (6H, m, $\text{COOCH}(\text{CH}_3)_2$).

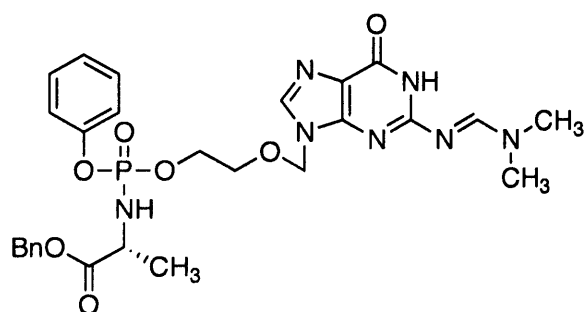
^{13}C -NMR (MeOD, 125 MHz): δ 20.39 (d, $J_{\text{C-P}} = 7.0$, CHCH_3) 20.52 (d, $J_{\text{C-P}} = 6.4$, CHCH_3), 21.89 ($\text{CH}(\text{CH}_3)_2$), 21.96 (d, $J_{\text{C-P}} = 3.0$, $\text{CH}(\text{CH}_3)_2$), 51.68, 51.77 (CHCH_3), 67.01 (d, $J_{\text{C-P}} = 5.6$, C-5'), 67.08 (d, $J_{\text{C-P}} = 5.5$, C-5'), 69.40 (d, $J_{\text{C-P}} = 6.1$, C-4'), 69.46 (d, $J_{\text{C-P}} = 6.2$, C-4'), 70.13, 70.17 ($\text{COOCH}(\text{CH}_3)_2$), 73.67 (C-1'), 117.54 (C-5), 121.42 (d, $J_{\text{C-P}} = 4.9$, PhO), 121.47 (d, $J_{\text{C-P}} = 4.8$, PhO), 126.07 (d, $J_{\text{C-P}} = 2.4$, PhO), 130.72 (C-5, PhO), 139.75 (C-8), 152.20 (d, $J_{\text{C-P}} = 4.3$, C-4), 152.26 (d, $J_{\text{C-P}} = 4.2$, C-4), 153.44 ('ipso' Ph), 155.71 (C-2), 159.41 (C-6), 174.55 (d, $J_{\text{C-P}} = 5.5$, $\text{COOCH}(\text{CH}_3)_2$), 174.69 (d, $J_{\text{C-P}} = 5.0$, $\text{COOCH}(\text{CH}_3)_2$).

EI MS= 517.1588 (M+Na)

HPLC = H₂O/AcCN from 100/0 to 0/100 in 20 min = retention time 11.27 min.

= H₂O/MeOH 40/60 isocratic = retention time 7.40 min.

Synthesis of N²-DMF acyclovir-[1-phenyl-(benzoxy-D-alaninyl)] phosphate. [201d]



C₂₇H₃₂N₇O₇P

Mol Wt.: 597.5594

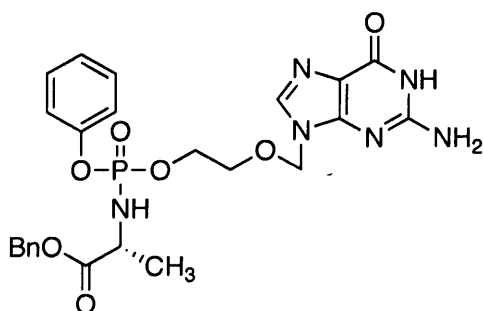
Prepared according to Standard Procedure C, from **200** (0.40 g, 1.44 mmol) in anhydrous THF (15 mL),

^tBuMgCl (1.0 M THF solution, 2.86 mL, 2.86 mmol), **131f** (1.51 g, 4.32 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography eluting with DCM/MeOH = 95/5, to give a white solid (57 %, 0.49 g).

³¹P-NMR (MeOD, 202 MHz): δ 3.84, 3.46.

¹H-NMR (MeOD, 500 MHz): δ 8.70 (1H, s, NCHN(CH₃)₂), 7.95, 7.92 (1H, 2s, H-8), 7.43-7.09 (10H, m, PhO, OCH₂Ph), 5.55, 5.52 (2H, 2s, H-1'), 5.18-5.06 (2H, m, OCH₂Ph), 4.22-4.09 (2H, m, H-5'), 4.03-3.94 (1H, m, CHCH₃), 3.82-3.74 (2H, m, H-4'), 3.18-3.11 (3H, m, N(CH₃)₂), 1.36-1.26 (3H, m, CHCH₃).

Synthesis of acyclovir-[1-phenyl-(benzoxy-D-alaninyl)] phosphate. [199d]



C₂₄H₂₇N₆O₇P

Mol Wt.: 542.4809

A solution of **201d** (0.48 g, 0.80 mmol) in 2-propanol (20 mL) was stirred under reflux for 48 h. The solvent was then

removed under reduced pressure and the residue was purified by column chromatography gradient elution of DCM/MeOH = 96/4 then 94/6 (25%, 0.11 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.80, 3.50.

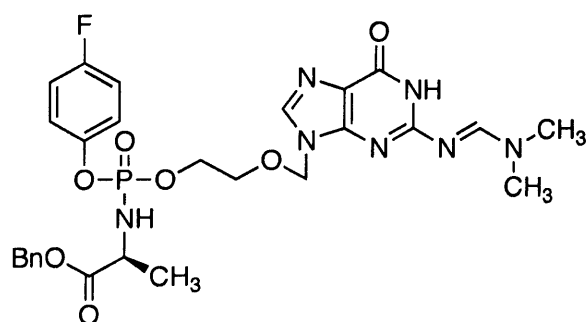
^1H -NMR (MeOD, 500 MHz): δ 7.83, 7.80 (1H, 2s, H-8), 7.38-7.25 (7H, m, PhO, OCH_2Ph), 7.19-7.11 (3H, m, PhO, OCH_2Ph), 5.44, 5.41 (2H, 2s, H-1'), 5.13, 5.12 (2H, 2s, OCH_2Ph), 4.20-4.10 (2H, m, H-5'), 4.02-3.95 (1H, m, CHCH_3), 3.76-3.69 (2H, m, H-4'), 1.35, 1.32 (3H, 2d, $J = 7.2$, CHCH_3).

^{13}C -NMR (MeOD, 125 MHz): δ 20.34 (d, $J_{\text{C-P}} = 6.8$, CH_3) 20.43 (d, $J_{\text{C-P}} = 6.6$, CH_3), 51.60, 51.73 (2s, CHCH_3), 67.01 (d, $J_{\text{C-P}} = 5.5$, C-5'), 67.10 (d, $J_{\text{C-P}} = 5.4$, C-5'), 67.97, 67.98 (2s, OCH_2Ph), 69.34 (d, $J_{\text{C-P}} = 3.7$, C-4'), 69.40 (d, $J_{\text{C-P}} = 3.7$, C-4'), 73.69 (C-1'), 117.55, 121.39, 121.43, 121.48, 121.51, 126.10, 126.23, 129.33, 129.36, 129.57, 129.60, 130.29, 130.59, 130.73 (C-5, PhO, OCH_2Ph), 137.29, 137.59 ('ipso' OCH_2Ph), 139.75 (C-8), 152.13, 152.17 (C-4), 155.68 (C-2), 159.46 (C-6), 174.76 (d, $J_{\text{C-P}} = 5.0$, COOCH_2Ph), 174.91 (d, $J_{\text{C-P}} = 4.6$, COOCH_2Ph).

EI MS= 565.1571 (M+Na)

Anal. Calcd for $\text{C}_{24}\text{H}_{27}\text{N}_6\text{O}_7\text{P}\cdot\text{H}_2\text{O}$: C, 51.43; H, 5.22; N, 14.99. Found: C, 51.90; H, 5.03; N, 14.67.

Synthesis of N^2 -DMF acyclovir-[1-*p*-fluoro-phenyl-(benzoxy-L-alaninyl)] phosphate. [201e]



$\text{C}_{27}\text{H}_{31}\text{FN}_7\text{O}_7\text{P}$

Mol Wt.: 615.5499

Prepared according to Standard Procedure C, from **200** (0.40 g, 1.43 mmol) in anhydrous THF (15 mL),

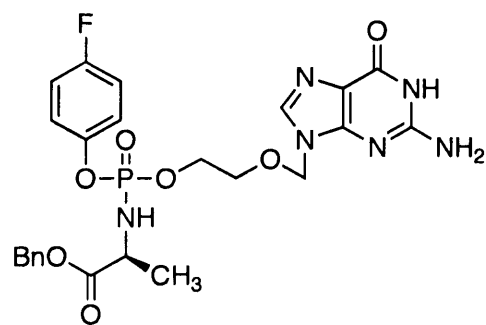
$t\text{BuMgCl}$ (1.0 M THF solution, 2.86 mL, 2.86 mmol), **131h** (1.60 g, 4.28 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 95/5, to give a white solid (52 %, 0.88 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.08, 3.76.

^{19}F -NMR (CDCl_3 , 471 MHz): δ -120.16 (d, $J = 2.3$), -120.18 (d, $J = 2.3$).

^1H -NMR (MeOD, 500 MHz): δ 8.71 (1H, s, $\text{NCHN}(\text{CH}_3)_2$), 7.97, 7.94 (1H, 2s, H-8), 7.40-7.28 (6H, m, Ph), 7.22-7.11 (3H, m, PhO), 5.60-5.50 (2H, m, H-1'), 5.19-5.06 (2H, m, OCH_2Ph), 4.20-4.15 (2H, m, H-5'), 3.99-3.95 (1H, m, CHCH_3), 3.80-3.75 (2H, m, H-4'), 3.19, 3.71 (3H, 2s, $\text{N}(\text{CH}_3)_2$), 3.12 (3H, s, $\text{N}(\text{CH}_3)_2$), 1.36-1.28 (3H, m, CHCH_3).

Synthesis of acyclovir-[1-*p*-fluoro-phenyl-(benzoxo-L-alaninyl)] phosphate. [199e]



$\text{C}_{24}\text{H}_{26}\text{FN}_6\text{O}_7\text{P}$

Mol Wt.: 560.4714

A solution of **201e** (0.458 g, 0.73 mmol) in 2-propanol (20 mL) was stirred under reflux for 24 h. The solvent was then

removed under reduced pressure and the residue was purified by column chromatography with $\text{DCM}/\text{MeOH} = 95/5$ to give a white solid (16%, 0.068 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.02, 3.78.

^{19}F -NMR (CDCl_3 , 471 MHz): δ -120.20, -120.22.

^1H -NMR (MeOD, 500 MHz): δ 7.84, 7.81 (1H, 2s, H-8), 7.39-7.29 (5H, m, Ph), 7.19-7.11 (2H, m, Ph), 7.06-7.02 (2H, m, Ph), 5.47, 5.44 (2H, 2s, H-1'), 5.15, 5.14 (2H, 2s, OCH_2Ph), 4.21-4.11 (2H, m, H-5'), 4.01-3.96 (1H, m, CHCH_3), 3.79-3.71 (2H, m, H-4'), 1.37, 1.34 (3H, 2d, $J = 7.1$, $J = 7.2$, CHCH_3).

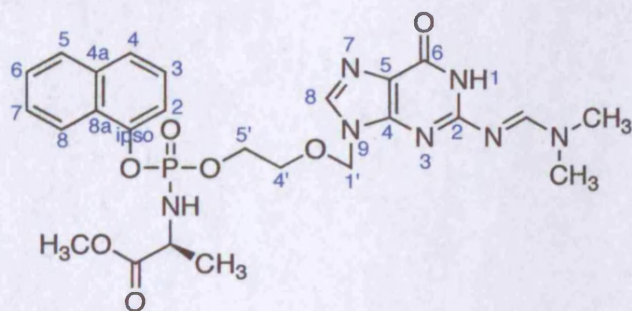
^{13}C -NMR (MeOD, 125 MHz): δ 20.30 (d, $J_{\text{C-P}} = 7.1$, CH_3) 20.40 (d, $J_{\text{C-P}} = 6.5$, CH_3), 51.60, 51.72 (2s, CHCH_3), 67.04 (d, $J_{\text{C-P}} = 5.5$, C-5'), 67.12 (d, $J_{\text{C-P}} = 5.5$, C-5'), 67.97 (OCH_2Ph), 69.32 (d, $J_{\text{C-P}} = 4.0$, C-4'), 69.38 (d, $J_{\text{C-P}} = 4.1$, C-4'), 73.76 (C-1'), 116.96, 117.15, 117.55, 122.99, 123.04, 123.06, 123.07, 123.10, 123.14, 128.00, 128.27, 129.35, 129.36, 129.38, 129.57, 129.60, (C-5, PhO, OCH_2Ph), 137.28 ('ipso' OCH_2Ph), 139.74 (C-8), 153.41 (C-4), 155.71 (C-2),

159.42 (C-6), 160.15, 162.09 (F-Ph), 174.71 (d, $J_{C-P} = 5.2$, COOCH_2Ph), 174.83 (d, $J_{C-P} = 4.6$, COOCH_2Ph).

EI MS= 583.1479 (M+Na).

Anal. Calcd for $\text{C}_{24}\text{H}_{26}\text{FN}_6\text{O}_7\text{P}\cdot\text{H}_2\text{O}$: C, 49.83; H, 4.88; N, 14.53. Found: C, 49.96; H, 4.57; N, 14.38.

Synthesis of N^2 -DMF acyclovir-[1-naphthyl(methoxy-L-alaninyl)] phosphate. [201f]



$\text{C}_{25}\text{H}_{30}\text{N}_7\text{O}_7\text{P}$

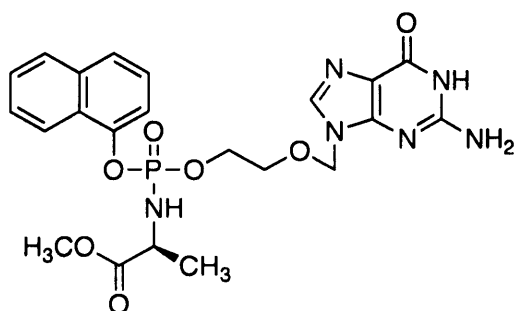
Mol Wt.: 571.5222

Prepared according to Standard Procedure C, from **200** (0.40 g, 1.44 mmol) in anhydrous THF

(15 mL), $t\text{BuMgCl}$ (1.0 M THF solution, 2.88 mL, 2.88 mmol), **193d** (1.41 g, 4.32 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 96/4 then 94/6, to give a white solid (36%, 0.43 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.09, 3.99.

^1H -NMR (MeOD, 500 MHz): δ 8.41 (1H, s, $\text{NCHN}(\text{CH}_3)_2$), 8.01-7.95 (1H, m, H-8 Naph), 7.79-7.78 (1H, m, H-6 Naph), 7.73-7.71 (1H, m, H-2 Naph), 7.55, 7.53 (1H, 2s, H-8), 7.43-7.39 (2H, m, Naph), 7.33-7.18 (2H, m, Naph), 5.38, 5.36 (2H, 2s, H-1'), 4.20-4.13 (2H, m, H-5'), 3.89-3.82 (1H, m, CHCH_3), 3.72-3.66 (2H, m, H), 3.48, 3.45 (3H, 2s, COOCH_3), 2.94, 2.90 (6H, 2s, $\text{N}(\text{CH}_3)_2$), 1.18-1.14 (3H, m, CHCH_3).

Synthesis of acyclovir-[1-naphthyl(methoxy-L-alaninyl)] phosphate. [199f]C₂₂H₂₅N₆O₇P

Mol Wt.: 516.4437

A solution of **201f** (0.41 g, 0.72 mmol) in 2-propanol (15 mL) was stirred under reflux for 72 h. The solvent was then

removed under reduced pressure and the residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 92/8. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 98/2 then 96/4 then 95/5) then by preparative reverse phase HPLC (gradient elution of H₂O/CH₃CN = from 100/0 to 0/100 in 45 min) to give a white solid (7%, 0.028 g).

³¹P-NMR (MeOD, 202 MHz): δ 4.07, 4.05.

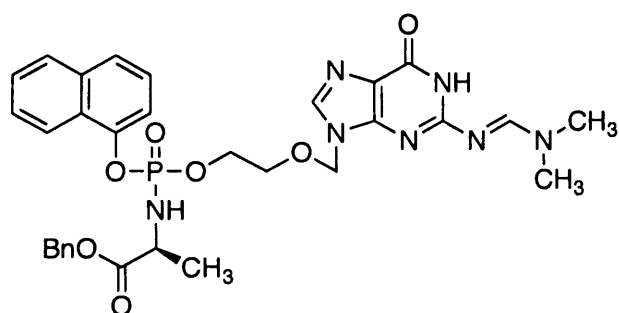
¹H-NMR (MeOD, 500 MHz): δ 8.14-8.12 (1H, m, H-8 Naph), 7.88-7.87 (1H, m, H-6 Naph), 7.82, 7.81 (1H, 2s, H-8), 7.71-7.70 (1H, m, H-2 Naph), 7.56-7.51 (2H, m, H-5 Naph, H-7 Naph), 7.45-7.39 (2H, m, H-3 Naph, H-4 Naph), 5.44, 5.42 (2H, 2s, H-1'), 4.30-4.28 (1H, m, H-5' of one diastereoisomer), 4.27-4.24 (1H, m, H-5' of one diastereoisomer), 4.06-3.98 (1H, m, CHCH₃), 3.84-3.82 (1H, m, H-4' of one diastereoisomer), 3.80-3.79 (1H, m, H-4' of one diastereoisomer), 3.63, 3.59 (3H, 2s, COOCH₃), 1.34-1.30 (3H, m, CHCH₃).

¹³C-NMR (MeOD, 125 MHz): δ 20.38 (d, J_{C-P} = 7.3, CH₃), 20.50 (d, J_{C-P} = 6.5, CH₃), 51.59 (CHCH₃), 52.67, 52.76 (COOCH₃), 67.24 (d, J_{C-P} = 5.6, C-5'), 67.31 (d, J_{C-P} = 5.1, C-5'), 69.39 (d, J_{C-P} = 7.4, C-4'), 69.47 (d, J_{C-P} = 7.6, C-4'), 73.68 (C-1'), 116.23, 116.25, 116.28, 117.56, 122.69, 122.74, 125.90, 126.51, 127.42, 127.44, 127.75, 127.89, 127.94, 128.81, 128.83 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 136.26 (C-4a Naph), 139.69 (C-8), 148.00, 148.06 ('ipso' Naph), 153.38 (C-4), 155.80 (C-2), 159.57 (C-6), 175.47 (d, J_{C-P} = 5.2, COOCH₃), 175.60 (d, J_{C-P} = 4.4, COOCH₃).

EI MS = 539.1410 (M+Na).

Anal. Calcd for C₂₂H₂₅N₆O₇P·H₂O: C, 50.29; H, 4.99; N, 15.99. Found: C, 50.18; H, 4.37; N, 13.34.

Synthesis of N²-DMF acyclovir-[1-naphthyl(benzoxo-L-alaninyl)] phosphate. [201h]



C₃₁H₃₄N₇O₇P

Mol Wt.: 647.6181

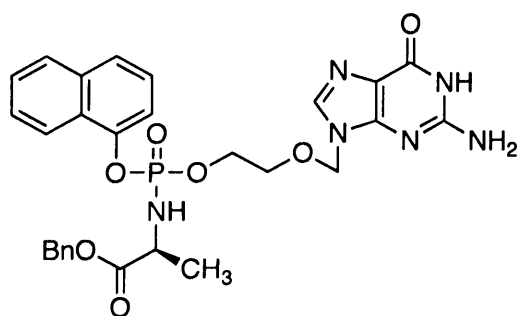
Prepared according to Standard Procedure C, from **200** (0.30 g, 1.08 mmol) in anhydrous THF

(10 mL), ^tBuMgCl (1.0 M THF solution, 2.16 mL, 2.16 mmol), **193a** (1.31 g, 3.25 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography, eluting with DCM/MeOH = 95/5, to give a white solid (17 %, 0.12 g).

³¹P-NMR (MeOD, 202 MHz): δ 4.18, 3.92.

¹H-NMR (MeOD, 500 MHz): δ 8.47, 8.46 (1H, 2s, NCHN(CH₃)₂), 8.01-7.98 (1H, m, H-8 Naph), 7.78-7.74 (2H, m, H-8, H-6 Naph), 7.56, 7.55 (1H, m, H-2 Naph), 7.41-7.12 (9H, m, Naph, OCH₂Ph), 5.37-5.36 (2H, 2s, H-1'), 5.00-4.93 (2H, m, OCH₂Ph), 4.14-4.06 (2H, m, H-5'), 3.96-3.88 (1H, m, CHCH₃), 3.88-3.59 (2H, m, H-4'), 2.95-2.93 (6H, m, N(CH₃)₂), 1.20-1.17 (3H, m, CHCH₃).

Synthesis of acyclovir-[1-naphthyl(benzoxo-L-alaninyl)] phosphate. [199h]



C₂₈H₂₉N₆O₇P

Mol Wt.: 592.5396

A solution of **201h** (0.10 g, 0.16 mmol) in 2-propanol (5 mL) was stirred under reflux for 2 days. The solvent was then

removed under reduced pressure and the residue was purified by column chromatography eluting with DCM/MeOH = 96/4. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 99/1, then 98/2, then 96/4) to give a white solid (35%, 0.032 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.13, 3.96.

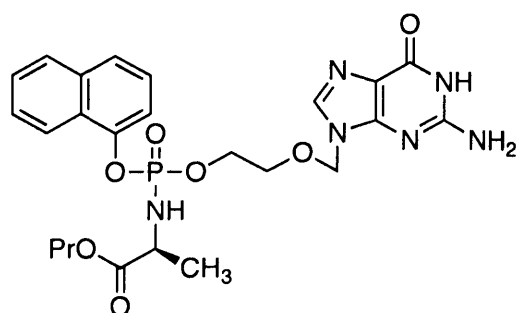
^1H -NMR (MeOD, 500 MHz): δ 8.01-7.99 (1H, m, H-8 Naph), 7.77-7.75 (1H, m, H-6 Naph), 7.67, 7.64 (1H, 2s, H-8), 7.58-7.13 (10H, m, Naph, OCH_2Ph), 5.28, 5.25 (2H, 2s, H-1'), 4.99-4.94 (2H, m, OCH_2Ph), 4.12-4.06 (2H, m, H-5'), 3.97-3.93 (1H, m, CHCH_3), 3.64-3.59 (2H, m, H-4'), 1.24-1.20 (3H, m, CHCH_3).

^{13}C -NMR (MeOD, 125 MHz): δ 20.32 (d, $J_{\text{C-P}} = 7.6$, CH_3), 20.43 (d, $J_{\text{C-P}} = 6.6$, CH_3), 51.76, 51.81 (2s, CHCH_3), 67.20 (d, $J_{\text{C-P}} = 5.6$, C-5'), 67.28 (d, $J_{\text{C-P}} = 4.9$, C-5'), 67.95, 67.98 (2s, OCH_2Ph), 69.34 (d, $J_{\text{C-P}} = 7.7$, C-4'), 69.40 (d, $J_{\text{C-P}} = 8.1$, C-4'), 73.65 (C-1'), 116.26, 116.29, 116.35, 122.69, 122.80, 125.92, 126.51, 127.20, 127.42, 127.46, 127.74, 128.81, 128.83, 129.27, 129.33, 129.52, 129.57 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH_2Ph), 136.26, 137.23 (C-4a Naph, 'ipso' OCH_2Ph), 139.69 (C-8), 147.98, 148.04 ('ipso' Naph, C-4), 152.44 (C-2), 159.39 (C-6), 174.61, 174.88 (2s, COOCH_2Ph).

EI MS= 615.17 (M+Na).

Anal. Calcd for $\text{C}_{28}\text{H}_{29}\text{N}_6\text{O}_7\text{P}\cdot\text{H}_2\text{O}$: C, 55.08; H, 5.12; N, 13.76. Found: C, 55.81; H, 4.91; N, 12.78.

Synthesis of acyclovir-[1-naphthyl(n-propoxy-L-alaninyl)] phosphate. [199g]



$\text{C}_{24}\text{H}_{29}\text{N}_6\text{O}_7\text{P}$

Mol Wt.: 544.4968

A solution of **201h** (0.78 g, 1.20 mmol) in n-propanol (28 mL) was stirred under reflux for 18 h. The solvent was then

removed under reduced pressure and the residue was purified by column chromatography eluting with DCM/MeOH = 95/5, to give a white solid (3%, 0.020g).

^{31}P -NMR (DMSO, 202 MHz): δ 4.09, 4.01.

^1H -NMR (DMSO, 500 MHz): δ 10.68 (1H, bs, NH), 8.16-8.12 (1H, m, H-8 Naph), 8.01-7.99 (1H, m, H-6 Naph), 7.86, 7.85 (1H, 2s, H-8), 7.79-7.50 (5H, m,

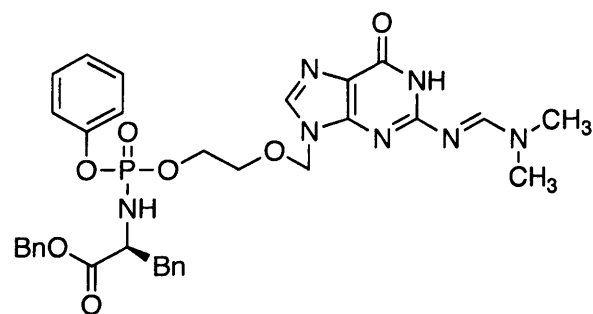
Naph), 6.55 (2H, bs, NH₂), 6.20-6.14 (1H, m, NHCH), 5.41, 5.40 (2H, 2s, H-1'), 4.22-4.17 (2H, m, H-5'), 4.00-3.93 (3H, m, CHCH₃, OCH₂CH₂CH₃), 3.77-3.74 (2H, m, H-4'), 1.61-1.50 (2H, m, OCH₂CH₂CH₃), 1.31-1.27 (3H, m, CHCH₃), 0.90-0.84 (3H, m, OCH₂CH₂CH₃).

¹³C-NMR (DMSO, 125 MHz): δ 10.07 (CH₃CH₂CH₂), 19.65, 19.72, 19.77 (CHCH₃), 21.36, 21.39 (2s, CH₃CH₂CH₂), 49.81, 49.89 (2s, CHCH₃), 64.87, 65.11, 65.14 (C-5'), 65.86, 65.90 (2s, OCH₂CH₂CH₃), 67.57, 67.63 (2, C-4'), 71.84 (C-1'), 114.76, 116.48, 121.45, 121.51, 124.08, 125.64, 125.99, 126.04, 126.17, 126.24, 126.61, 127.64, 127.96, 128.32 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 134.21 (C-4a Naph), 137.57 (C-8), 146.46, 146.51 ('ipso' Naph), 151.36 (C-4), 153.88 (C-2), 156.73 (C-6), 173.13, 173.17, 173.32 (COOCH₂CH₂CH₃).

EI MS= 567.17 (M+Na).

Anal. Calcd for C₂₄H₂₉N₆O₇P·H₂O: C, 51.25; H, 5.55; N, 14.94. Found: C, 51.40; H, 5.40; N, 14.37.

Synthesis of N²-DMF acyclovir-[1-phenyl-(benzoxy-L-phenylalaninyl)] phosphate. [201i]



C₃₃H₃₆N₇O₇P

Mol Wt.: 673.6554

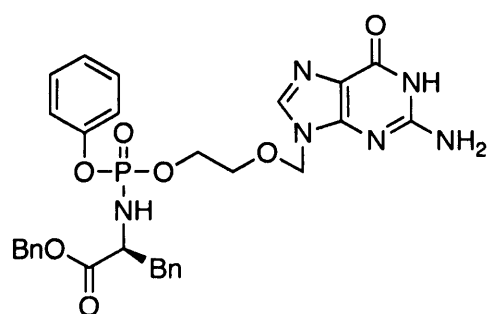
Prepared according to Standard Procedure C, from **200** (0.40 g, 1.44 mmol) in anhydrous THF (15 mL),

^tBuMgCl (1.0 M THF solution, 2.88 mL, 2.88 mmol), **131g** (1.25 g, 2.88 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 95/5, to give a white solid (59 %, 0.58 g).

³¹P-NMR (MeOD, 202 MHz): δ 3.39, 3.36.

$^1\text{H-NMR}$ (MeOD, 500 MHz): δ 8.48, 8.47 (1H, 2s, $\text{NCHN}(\text{CH}_3)_2$), 7.74, 7.73 (1H, 2s, H-8), 7.19-6.86 (15H, m, PhO, COOCH_2Ph , CHCH_2Ph), 5.33, 5.32 (2H, 2s, H-1'), 4.95-4.90 (2H, m, COOCH_2Ph), 4.03-3.96 (1H, m, CHCH_2Ph), 3.91-3.86 (0.5H, m, H-5' of one diastereoisomer), 3.81-3.67 (1.5H, m, H-5'), 3.54-3.48 (2H, m, H-4'), 2.96-2.87 (7H, m, $\text{N}(\text{CH}_3)_2$, CHCH_2Ph of one diastereoisomer), 2.76-2.71 (1H, m, CHCH_2Ph of one diastereoisomer).

Synthesis of acyclovir-[1-phenyl-(benzoxy-L-phenylalaninyl)] phosphate. [199i]



$\text{C}_{30}\text{H}_{31}\text{N}_6\text{O}_7\text{P}$

Mol Wt.: 618.5769

A solution of **201i** (0.49 g, 0.73 mmol) in 2-propanol (20 mL) was stirred under reflux for 64 h. The solvent was then removed

under reduced pressure and the residue was purified by column chromatography with DCM/MeOH = 95/5. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 98/2 then 96/4) to give a white solid (14%, 0.054 g).

$^{31}\text{P-NMR}$ (MeOD, 202 MHz): δ 3.41, 3.31.

$^1\text{H-NMR}$ (MeOD, 500 MHz): δ 7.65-7.64 (1H, 2s, H-8), 7.19-6.87 (15H, m, PhO, COOCH_2Ph , CHCH_2Ph), 5.25, 5.24 (2H, 2s, H-1'), 4.98-4.95 (2H, m, COOCH_2Ph), 4.06-3.97 (1H, m, CHCH_2Ph), 3.89-3.84 (0.5H, m, H-5' of one diastereoisomer), 3.80-3.64 (1.5H, m, H-5'), 3.48 (2H, bs, H-4'), 2.97-2.90 (1H, m, CHCH_2Ph of one diastereoisomer), 2.78-2.73 (1H, m, CHCH_2Ph of one diastereoisomer).

$^{13}\text{C-NMR}$ (MeOD, 125 MHz): δ 40.91 (d, $J_{\text{C-P}} = 7.2$, CHCH_2Ph), 40.96 (d, $J_{\text{C-P}} = 6.8$, CHCH_2Ph), 57.77, 57.91 (2s, CHCH_2Ph), 66.87 (C-5'), 68.02, 68.03 (2s, COOCH_2Ph), 69.25, 69.32 (C-4'), 73.64 (C-1'), 117.55, 121.30, 121.34, 121.37, 121.41, 126.00, 127.93, 129.37, 129.52, 129.54, 129.57, 130.62, 130.65, 130.68 (C-5, PhO, OCH_2Ph , CHCH_2Ph), 137.03, 138.02, 138.08 ('ipso' CHCH_2Ph ,

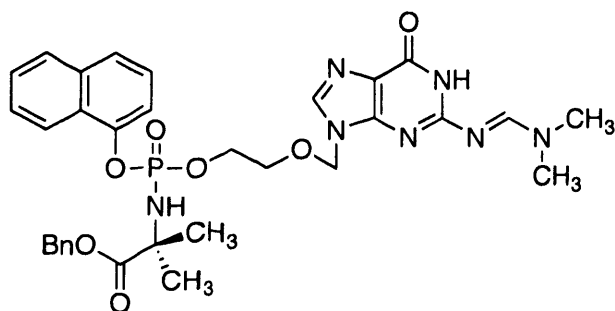
'ipso' OCH_2Ph), 139.73 (C-8), 152.08 (C-4), 155.65 (C-2), 159.41 (C-6), 173.85 (COOCH_2Ph).

EI MS= 641.1886 ($\text{M}+\text{Na}$).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 100/0 to 0/100 in 20 min = retention time 14.56 min.

= $\text{H}_2\text{O}/\text{MeOH}$ 40/60 isocratic = retention time 22.52, 24.61 min.

Synthesis of N^2 -DMF acyclovir-[1-naphthyl(benzoxymethyl)dimethylglycyl]phosphate. [201j]



$\text{C}_{32}\text{H}_{36}\text{N}_7\text{O}_7\text{P}$

Mol Wt.: 661.6447

Prepared according to Standard Procedure C, from **200** (0.20 g, 0.72 mmol) in anhydrous THF

(10 mL), $^t\text{BuMgCl}$ (1.0 M THF solution, 0.86 mL, 0.86 mmol), **193e** (0.80 g, 1.92 mmol) and the mixture reaction was stirred at room temperature overnight. Then $^t\text{BuMgCl}$ (1.0 M THF solution, 0.58 mL, 0.58 mmol), was added, and after 3 h the solution was concentrated. The residue was purified by column chromatography eluting with DCM/MeOH = 93/7, to give a white solid (35%, 0.17 g).

^{31}P -NMR (MeOD, 202 MHz): δ 2.55.

^1H -NMR (MeOD, 500 MHz): δ 8.59 (1H, s, $\text{NCHN}(\text{CH}_3)_2$), 8.15 (1H, d, J = 7.9 Hz, H-8 Naph), 7.88 (1H, s, H-6 Naph), 7.86 (1H, s, H-8), 7.67 (1H, d, J = 8.2 Hz, H-2 Naph), 7.55-7.26 (9H, m, Naph, OCH_2Ph), 5.48 (2H, s, H-1'), 5.17-5.08 (2H, m, OCH_2Ph), 4.28-4.17 (2H, m, H-5'), 3.79-3.70 (2H, m, H-4'), 3.09, 3.07 (6H, 2s, $\text{CHN}(\text{CH}_3)_2$), 1.51, 1.49 (6H, 2s, $\text{NHC}(\text{CH}_3)_2$).

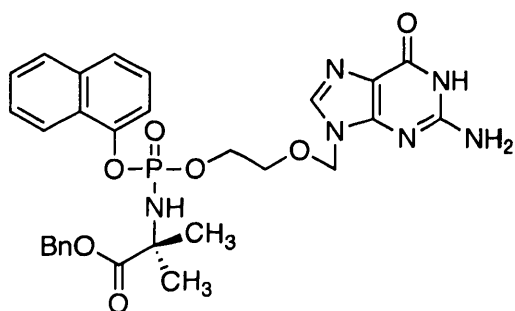
^{13}C -NMR (MeOD, 125 MHz): δ 27.46 (d, $J_{\text{C-P}}$ = 4.8, CH_3), 27.71 (d, $J_{\text{C-P}}$ = 6.9, CH_3), 35.30, 41.41 (2s, $\text{N}(\text{CH}_3)_2$), 58.08 ($\text{C}(\text{CH}_3)_2$), 67.20 (d, $J_{\text{C-P}}$ = 5.7, C-5'), 68.23 (OCH_2Ph), 69.43 (d, $J_{\text{C-P}}$ = 7.3, C-4'), 73.67 (C-1'), 116.31, 117.37 120.33, 122.95 125.77, 126.47, 127.30, 127.70, 127.89, 128.48, 128.82, 129.29, 129.55, 130.89 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH_2Ph), 136.25, 137.33 (C-4a Naph, 'ipso' OCH_2Ph),

140.61 (C-8), 148.08, 148.14 ('ipso' Naph, C-4), 152.45 (C-2), 159.37 (C-6), 159.85 (CH-N(CH₃)₂) 176.56 (d, J_{C-P} = 3.3, COOCH₂Ph).

EI MS= 662.2499 (M+H).

Anal. Calcd for C₃₂H₃₆N₇O₇P·H₂O: C, 56.55; H, 5.64; N, 14.43. Found: C, 56.86; H, 5.05; N, 13.93.

Synthesis of acyclovir-[1-naphthyl(benzyloxy-dimethylglycyl)] phosphate. [199j]



C₂₉H₃₁N₆O₇P

Mol Wt.: 606.5662

A solution of **201j** (0.17 g, 0.26 mmol) in 2-propanol (10 mL) was stirred under reflux for 96 h. The solvent was then

removed under reduced pressure and the residue was purified by column chromatography eluting with DCM/MeOH = 96/4. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 98/2 then 96/4) to give a white solid (23%, 0.037 g).

³¹P-NMR (MeOD, 202 MHz): δ 2.52.

¹H-NMR (MeOD, 500 MHz): δ 8.20-8.13 (1H, m, H-8 Naph), 7.87 (1H, dd, J = 6.2, 3.2 Hz, H-6 Naph), 7.76 (1H, s, H-8), 7.68 (1H, d, J = 8.2 Hz, H-2 Naph), 7.55-7.49 (2H, m, Naph, OCH₂Ph), 7.46-7.25 (7H, m, Naph, OCH₂Ph), 5.38 (2H, s, H-1'), 5.16-5.08 (2H, m, OCH₂Ph), 4.20-4.17 (2H, m, H-5'), 3.71-3.69 (2H, m, H-4'), 1.52, 1.50 (6H, 2s, NHC(CH₃)₂).

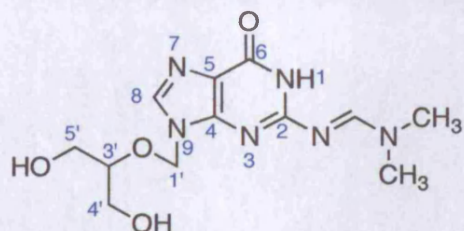
¹³C-NMR (MeOD, 125 MHz): δ 27.45 (d, J_{C-P} = 4.6, CH₃), 27.72 (d, J_{C-P} = 6.8, CH₃), 58.10 (C(CH₃)₂), 67.21 (d, J_{C-P} = 5.7, C-5'), 68.25 (OCH₂Ph), 69.48 (d, J_{C-P} = 7.5, C-4'), 73.61 (C-1'), 116.34, 117.55, 122.97, 125.76, 126.50, 127.30, 127.68, 127.93, 128.78, 129.26, 129.31, 129.54 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH₂Ph), 136.26, 137.32 (C-4a Naph, 'ipso' OCH₂Ph), 139.69 (C-8), 148.09, 148.15 ('ipso' Naph, C-4), 155.62 (C-2), 159.36 (C-6), 176.59 (d, J_{C-P} = 3.6, COOCH₂Ph).

EI MS= 607.2048 (M+Na).

Anal. Calcd for $C_{29}H_{31}N_6O_7P \cdot H_2O$: C, 55.77; H, 5.33; N, 13.46. Found: C, 55.83; H, 5.15; N, 12.92.

9.14 Synthesis of ganciclovir ProTides

Synthesis of N²-DMF ganciclovir (N²-(9-[(1,3-dihydroxypropan-2-yloxy)methyl]-6-oxo-6,9-dihydro-1H-purin-2-yl)-N,N-dimethylformimidamide). [208]



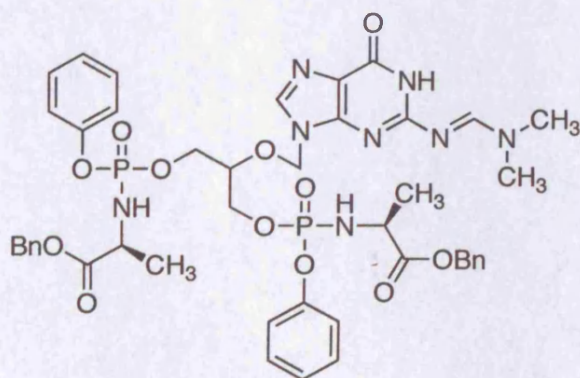
C₁₂H₁₈N₆O₃

Mol Wt.: 310.3091

To a suspension of **10** (0.50 g, 1.96 mmol) in anhydrous DMF (10 mL) was added *N,N*-dimethylformamide dimethyl acetal (1.30 mL, 9.79 mmol) and the reaction mixture was stirred at room temperature for 22 h. After this period was added *N,N*-dimethylformamide dimethyl acetal (0.65 mL, 4.90 mmol) and the suspension was stirred at room temperature for 7 h. The suspension was then filtered, and the solid was washed with diethyl ether to give a white solid (84%, 0.51 g).

¹H-NMR (DMSO 500 MHz): δ 11.31 (1H, s, NH), 8.57 (1H, s, 1H), 7.93 (1H, s, H-8), 5.54 (2H, s, H-1'), 4.64 (2H, bs, 2xOH), 3.66-3.60 (1H, m, H-3'), 3.49-3.43 (4H, m, H-4', H-5'), 3.16, 3.04 (6H, 2s, N(CH₃)₂).

Synthesis of N²-DMF ganciclovir-[1-phenyl(benzyloxy-L-alaninyl)] diphosphate [209a]



C₄₄H₅₀N₈O₁₂P₂

Mol Wt.: 944.8617

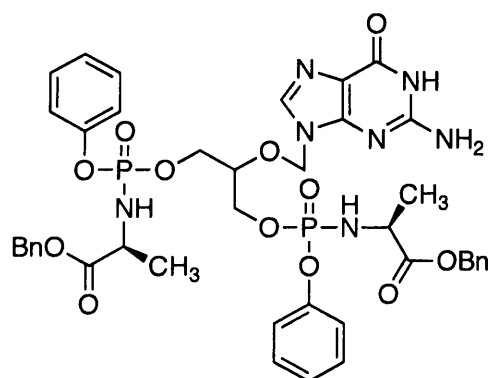
Prepared according to Standard Procedure C, from **208** (0.23 g, 0.74 mmol) in anhydrous THF (15 mL) and anhydrous pyridine (5 mL)

^tBuMgCl (1.0 M THF solution, 1.50 mL, 1.50 mmol), **131d** (0.78 g, 2.22 mmol) in anhydrous THF (6 mL) and the reaction mixture was stirred at room temperature overnight. After this period were added: ^tBuMgCl (1.0 M THF

solution, 0.75 mL, 0.75 mmol) and **131d** (1.31 g, 3.70 mmol) in anhydrous THF (6 mL) and the reaction mixture was stirred at room temperature for 24h. Then the residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2, then 95/5, to give a white product: (80%, 0.60 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.91, 3.52, 3.39.

Synthesis of ganciclovir-[1-phenyl(benzoyl-L-alaninyl)] diphosphate. [210a]



$\text{C}_{41}\text{H}_{45}\text{N}_7\text{O}_{12}\text{P}_2$

Mol Wt.: 889.7832

A solution of **209a** (0.60 g, 0.64 mmol) in 2-propanol (20 mL) was stirred under reflux for 40 h. After this period the solvent was removed and the residue

purified by column chromatography gradient elution of DCM/MeOH = 98/2, then 96/4, then 95/5, and then further purified by preparative reverse phase HPLC (gradient elution of $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ = from 100/0 to 0/100 in 35 min) to give a white solid (7%, 0.040 g).

^{31}P -NMR (MeOD, 202 MHz): δ 3.86, 3.85, 3.80, 3.73, 3.52, 3.49, 3.41.

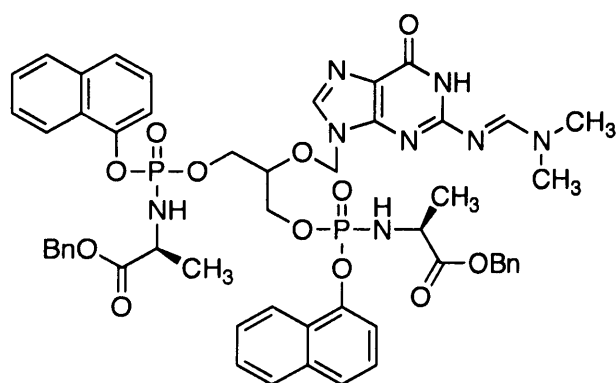
^1H -NMR (MeOD, 500 MHz): δ 7.80-7.72 (1H, m, H-8), 7.41-7.26 (12H, m, PhO, OCH_2Ph), 7.22-7.09 (8H, m, PhO, OCH_2Ph), 5.51-5.36 (2H, m, H-1'), 5.18-5.10 (4H, m, 2x OCH_2Ph), 4.24-3.90 (7H, m, H-3', H-4', H-5', 2x CHCH_3), 1.38-1.30 (6H, m, 2x CHCH_3).

^{13}C -NMR (MeOD, 125 MHz): δ 20.38, 20.43, 20.49 (CHCH_3), 51.60, 51.63, 51.75 (CHCH_3), 66.32, 66.36, 66.40, 66.44, 68.01, 68.03, (C-4', C-5'), 72.74, 72.85, 72.98 (C-1'), 76.94, 77.00, 77.07 (C-3'), 117.62, 121.40, 121.43, 121.46, 121.51, 121.55, 126.15, 126.20, 128.01, 128.28, 129.25, 129.28, 129.33, 129.36, 129.38, 129.60, 129.62, 129.64, 130.79, 130.83, 137.26 (C-5, PhO, OCH_2Ph), 139.61, 139.66 (C-8), 152.09, 153.29 ('ipso' Ph and OCH_2Ph , C-4), 155.82 (C-2), 159.55 (C-6), 174.67, 174.75, 174.93 (COOCH_2Ph).

EI MS= 912.25 (M+Na).

Anal. Calcd for $C_{41}H_{45}N_7O_{12}P_2 \cdot H_2O$: C, 54.25; H, 5.22; N, 10.80. Found: C, 54.10; H, 4.97; N, 10.53.

Synthesis of N²-DMF-ganciclovir-[1-naphthyl(benzoxo-L-alaninyl)] di-phosphate [209b]



$C_{52}H_{54}N_8O_{12}P_2$

Mol Wt.: 1044.9791

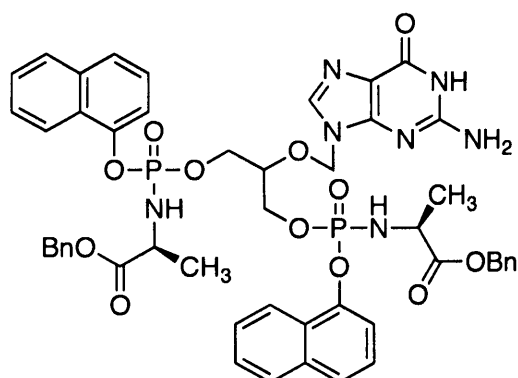
Prepared according to Standard Procedure C, from **208** (0.30 g, 0.97 mmol) in anhydrous THF (15 mL), ^tBuMgCl (1.0 M THF

solution, 1.93 mL, 1.93 mmol), **193a** (1.17 g, 2.90 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature for 20 h. Then the residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2, then 95/5, to give a white product: (46%, 0.47 g).

³¹P-NMR (MeOD, 202 MHz): δ 4.30, 4.23, 4.04, 4.00, 3.93.

¹H-NMR (MeOD, 500 MHz): δ 8.63-8.40 (1H, m, NCHN(CH₃)₂), 8.16-8.03 (2H, m, H-8 Naph), 7.90-7.74 (3H, m, H-8, Naph), 7.71-7.60 (2H, m, Naph), 7.55-7.17 (18H, m, Naph, Ph), 5.50-5.31 (2H, m, H-1'), 5.16-4.99 (4H, m, 2xCH₂Ph), 4.35-3.97 (7H, m, H-3', H-4', H-5', 2xCHCH₃), 3.34-3.32 (6H, m, N(CH₃)₂), 1.34-1.26 (6H, m, 2xCHCH₃).

Synthesis of ganciclovir-[1-naphthyl(benzyloxy-L-alaninyl)] di-phosphate [210b]



$C_{49}H_{49}N_7O_{12}P_2$

Mol Wt.: 989.9006

A solution of **209b** (0.40 g, 0.38 mmol) in 2-propanol (15 mL) was stirred under reflux for 70 h. After this period the solvent was removed and the residue

purified by column chromatography gradient elution of DCM/MeOH = 95/5, then 93/7, and then further purified by preparative TLC (gradient elution of DCM/MeOH = 98/2 then 96/4 then 95/5) to give a white solid (3%, 0.010 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.23, 4.08, 3.96, 3.93.

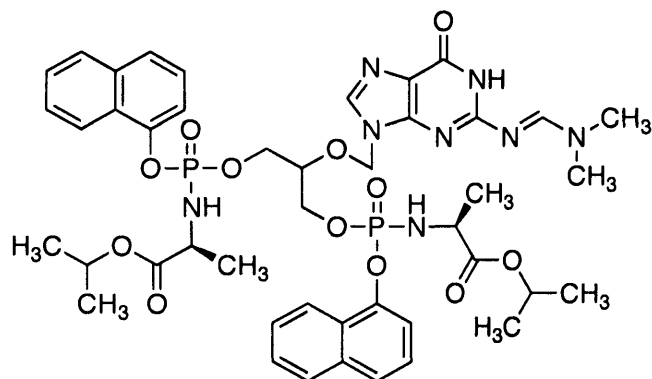
1H -NMR (MeOD, 500 MHz): δ 8.17-8.00 (2H, m, H-8 Naph), 7.93-7.79 (2H, m, Naph), 7.75-7.60 (3H, m, H-8, Naph), 7.54-7.16 (18H, m, Naph, Ph), 5.46-5.17 (2H, m, H-1'), 5.14-4.95 (4H, m, 2xCH₂Ph), 4.31-3.94 (7H, m, H-3', H-4', H-5', 2xCHCH₃), 1.41-1.22 (6H, m, 2xCHCH₃).

^{13}C -NMR (MeOD, 125 MHz): δ 20.33 (d, J_{C-P} = 7.4, CHCH₃), 20.45 (d, J_{C-P} = 7.2, CHCH₃), 51.76, 51.84 (CHCH₃), 65.24, 66.46, 66.50, 66.55, 66.59 (C-4', C-5'), 67.98, 68.01, 68.03 (CH₂Ph), 72.63, 72.77, 72.91 (C-1'), 76.73, 76.80, 76.84, 76.90 (C-3'), 116.35, 116.38, 116.42, 116.44, 116.49, 116.52, 117.61, 122.63, 122.66, 122.80, 126.03, 126.06, 126.55, 127.56, 127.79, 127.89, 128.00, 128.84, 128.86, 129.18, 129.21, 129.26, 129.28, 129.31, 129.33, 129.52, 129.58 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH₂Ph), 136.26, 137.15, 137.20 (C-4a Naph, 'ipso' OCH₂Ph), 139.53 (C-8), 147.88, 147.94 ('ipso' Naph, C-4), 153.19 (C-2), 159.25, 159.85 (C-6), 174.64, 174.93 (2s, COOCH₂Ph).

EI MS = 990.3035 (M+H).

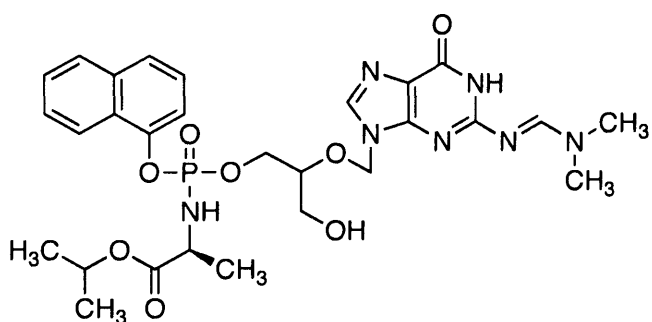
HPLC = H₂O/MeOH 40/60 isocratic = retention time 4.71 min.

Synthesis of N²-DMF-ganciclovir-[1-naphthyl(isopropoxy-L-alaninyl)] di-phosphate [209c] and N²-DMF-ganciclovir-[1-naphthyl(isopropoxy-L-alaninyl)] di-phosphate. [209d]



C₄₄H₅₄N₈O₁₂P₂

Mol Wt.: 948.8935



C₂₈H₃₆N₇O₈P

Mol Wt.: 629.6013

Prepared according to Standard Procedure C, from **208** (0.25 g, 0.81 mmol) in anhydrous THF (15 mL), ^tBuMgCl (1.0 M THF solution, 1.61 mL, 1.61 mmol), **193f** (0.86 g, 2.42 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. After this period were added: pyridine (5 mL), ^tBuMgCl (1.0 M THF solution, 1.61 mL, 1.61 mmol) and **193f** (1.60 g, 4.49 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature for 3 days. Then the residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2, then 95/5, to give two products: **[209c]** (44%, 0.34 g), **[209d]** (14%, 0.071 g).

DiProTide: [209c]

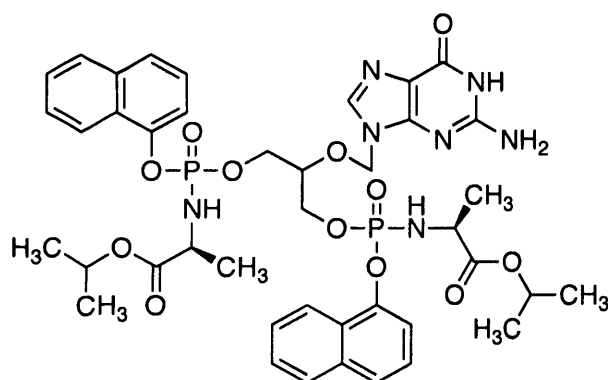
³¹P-NMR (MeOD, 202 MHz): δ 4.04 (bs).

MonoProTide: [209d]

³¹P-NMR (MeOD, 202 MHz): δ 4.19 (bs).

^1H -NMR (MeOD, 500 MHz): δ 8.74-8.68 (1H, m, $\text{NCHN}(\text{CH}_3)_2$), 8.17-7.36 (8H, m, H-8, Naph), 5.73-5.70 (2H, m, H-1'), 4.99-4.80 (1H, bs, $\text{COOCH}(\text{CH}_3)_2$), 4.39-4.05 (3H, m, H-3', H-5'), 3.97-3.88 (1H, m, CHCH_3), 3.70-3.56 (2H, m, H-4'), 3.20, 3.12 (6H, s, $\text{N}(\text{CH}_3)_2$), 1.33-1.28 (3H, m, CHCH_3), 1.22-1.16 (6H, m, $\text{COOCH}(\text{CH}_3)_2$).

Synthesis of ganciclovir-[1-naphthyl(isopropoxy-L-alaninyl)] diphosphate. [210c]



$\text{C}_{41}\text{H}_{49}\text{N}_7\text{O}_{12}\text{P}_2$

Mol Wt.: 893.8150

A solution of **209c** (0.33 g, 0.35 mmol) in 2-propanol (10 mL) was stirred under reflux for 80 h. After this period the solvent was

removed and the residue purified by column chromatography gradient elution of DCM/MeOH = 95/5, then 94/6, then 93/7, to give a white solid (8%, 0.025 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.29, 4.25, 4.23, 4.17.

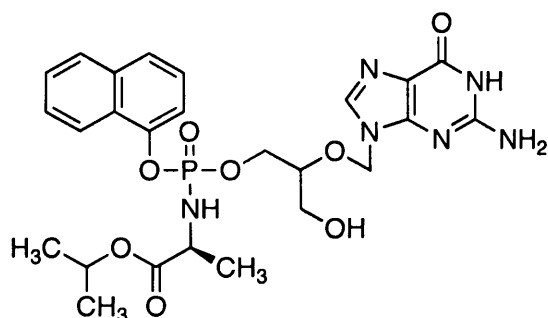
^1H -NMR (MeOD, 500 MHz): δ 8.17-8.05 (2H, m, H-8 Naph), 7.85 (2H, m, H-6 Naph), 7.75-7.71 (1H, m, H-8), 7.68-7.34 (10H, m, Naph), 5.41-5.37 (2H, m, H-1'), 4.99-4.87 (2H, m, $2\times\text{CH}(\text{CH}_3)_2$), 4.27-4.14 (5H, m, H-3', H-4', H-5'), 4.01-3.91 (2H, m, $2\times\text{CHCH}_3$), 1.36-1.26 (6H, m, $2\times\text{CHCH}_3$), 1.21-1.11 (12H, m, $2\times\text{COOCH}(\text{CH}_3)_2$).

^{13}C -NMR (MeOD, 125 MHz): δ 20.49, 20.56, 20.61, 20.64, 20.69 (CHCH_3), 21.90, 22.00, 22.02 ($\text{CH}(\text{CH}_3)_2$), 51.87 (CHCH_3), 66.51, 66.55, 66.66, 66.70 (C-4', C-5'), 70.23, 70.30, 70.32 ($\text{COOCH}(\text{CH}_3)_2$), 72.91 (C-1'), 76.94 (C-3'), 116.13, 116.42, 122.67, 122.70, 122.79, 126.03, 126.55, 127.54, 127.80, 127.86, 128.87 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 136.26 (C-4a Naph), 138.42 (C-8), 147.92, 147.98, 150.02 ('ipso' Naph, C-4), 155.64 (C-2), 159.44 (C-6), 174.72 (COOCH_2Ph).

EI MS= 916.3 (M+Na).

Anal. Calcd for $C_{41}H_{49}N_7O_{12}P_2$: C, 55.09; H, 5.53; N, 10.97. Found: C, 54.62; H, 5.56; N, 10.60.

Synthesis of ganciclovir-[1-naphthyl(isopropoxy-L-alaninyl)] diphosphate. [210d]



$C_{25}H_{31}N_6O_8P$

Mol Wt.: 574.5228

A solution of **209d** (0.070 g, 0.11 mmol) in 2-propanol (4 mL) was stirred under reflux for 24 h. After this period the solvent was removed and the residue purified by preparative reverse phase HPLC (gradient elution of H_2O/CH_3CN = from 100/0 to 0/100 in 35 min) to give a white solid (33%, 0.021 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.40, 4.36, 4.34, 4.27.

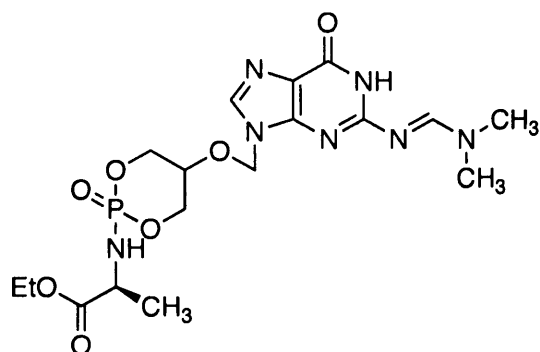
1H -NMR (MeOD, 500 MHz): δ 8.20-8.06 (1H, m, Naph), 7.90-7.85 (1H, m, Naph), 7.83-7.79 (1H, m, H-8), 7.75-7.66 (1H, m, Naph), 7.59-7.49 (2H, m, Naph), 7.49-7.36 (2H, m, Naph), 5.51-5.45 (2H, m, H-1'), 5.03-4.94 (1H, m, $CH(CH_3)_2$), 4.39-4.11 (2H, m, H-5'), 4.05-3.91 (2H, m, H-3', $CHCH_3$), 3.65-3.47 (2H, m, H-4'), 1.40-1.29 (3H, m, $CHCH_3$), 1.26-1.14 (6H, m, $COOCH(CH_3)_2$).

^{13}C -NMR (MeOD, 125 MHz): δ 20.46 (d, J_{C-P} = 6.7, $CHCH_3$), 20.57 (d, J_{C-P} = 6.7, $CHCH_3$), 20.68 (d, J_{C-P} = 6.6, $CHCH_3$), 21.90 (d, J_{C-P} = 2.3, $CH(CH_3)_2$), 21.99 (d, J_{C-P} = 2.9, $CH(CH_3)_2$), 51.83 ($CHCH_3$), 61.78, 61.92 (2s, C-4'), 67.36 (d, J_{C-P} = 5.4, C-5'), 67.45 (d, J_{C-P} = 5.7, C-5'), 70.17 ($COOCH(CH_3)_2$), 70.26 (d, J_{C-P} = 5.6, $COOCH(CH_3)_2$), 72.89, 73.06, (C-1'), 78.95 (d, J_{C-P} = 7.3, C-3'), 79.21 (d, J_{C-P} = 7.6, C-3'), 116.31, 116.34, 116.37, 116.40, 117.48, 117.56, 122.70, 122.74, 122.83, 125.98, 126.55, 127.50, 127.78, 127.80, 127.88, 127.93, 128.85 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 136.27 (C-4a Naph), 139.70, 139.75 (C-8), 147.97, 148.05 ('ipso' Naph, C-4), 155.72 (C-2), 159.46 (C-6), 174.52 ($COOCH_2Ph$).

EI MS= 597.1832 (M+Na).

Anal. Calcd for $C_{25}H_{31}N_6O_8P \cdot H_2O$: C, 50.68; H, 5.61; N, 14.18. Found: C, 50.75; H, 5.44; N, 13.89.

Synthesis of N²-DMF-ganciclovir-[ethoxy-L-alaninyl] cyclic phosphate. [212]



$C_{17}H_{26}N_7O_7P$

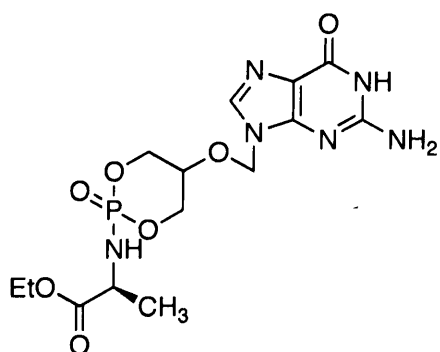
Mol Wt.: 471.4048

To a stirring solution of **208** (0.30 g, 0.97 mmol) in anhydrous THF (10 mL) and anhydrous pyridine (5 mL) was added ^tBuMgCl (1.0 M THF solution,

2.00 mL, 2.00 mmol) and the reaction mixture was stirred at room temperature for 30 min. After this period, a solution of **211** (0.45 g, 1.93 mmol) in anhydrous THF (5 mL) was added and the reaction mixture was stirred at room temperature for 24 h. After this period, anhydrous pyridine (5 mL) and ^tBuMgCl (1.0 M THF solution, 2.00 mL, 2.00 mmol) were added and the reaction mixture was stirred at room temperature for 16 h. After this period the solvent was removed and the residue purified by column chromatography gradient elution of DCM, then DCM/MeOH = 98/2, 92/8 then 80/20, to give a white product: (93%, 0.43 g).

³¹P-NMR (MeOD, 202 MHz): δ 3.53, 2.44.

Synthesis of ganciclovir-[ethoxy-L-alaninyl] cyclic phosphate. [213]



$C_{14}H_{21}N_6O_7P$

Mol Wt.: 416.3263

A solution of **212** (0.43 g, 0.91 mmol) in 2-propanol (20 mL) was stirred at reflux for 9 h. After this period the solvent was removed and the residue purified by column chromatography

gradient elution of DCM/MeOH = 97/3, 94/6, 90/10 then 85/15 to give a white solid, which was further purified by preparative TLC (gradient elution of DCM/MeOH = 97/3 then 95/5) to give a white solid (11%, 0.040 g).

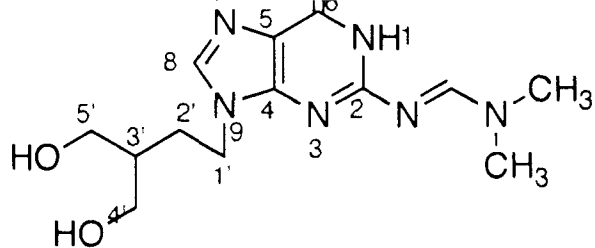
^{31}P -NMR (MeOD, 202 MHz): δ 3.73, 2.89.

^1H -NMR (MeOD, 500 MHz): δ 7.91 (1H, s, H-8), 5.70-5.55 (2H, m, H-1'), 4.63-4.30 (5H, m, H-3', H-4', H-5'), 4.26-4.10 (2H, m, CH_2CH_3), 3.93-3.76 (1H, m, CHCH_3), 1.45-1.32 (3H, m, CH_2CH_3), 1.31-1.24 (3H, m, CHCH_3).

^{13}C -NMR (MeOD, 125 MHz): δ 14.46, 14.49 (CH_2CH_3), 20.43 (d, $J_{\text{C-P}} = 6.4$, CHCH_3), 20.51 (d, $J_{\text{C-P}} = 6.6$, CHCH_3), 50.93, 51.34 (CHCH_3), 62.29, 62.38 (CH_2CH_3), 69.89, 69.92, 69.94, 69.97, 70.26, 70.31, 70.42, 70.46, 70.51, 70.85, 70.90 (C-3', C-4', C-5'), 71.26, 71.42 (C-1'), 117.49 (C-5), 139.69 (C-8) 155.82 (C-2), 159.40 (C-6), 175.02 (d, $J_{\text{C-P}} = 5.8$, $\text{COOCH}_2\text{CH}_3$), 175.11 (d, $J_{\text{C-P}} = 5.5$, $\text{COOCH}_2\text{CH}_3$).

EI MS= 417.12 (M+H) and 439.11 (M+Na).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ 95/5 to 0/100 in 30 min = retention time 7.75 min.



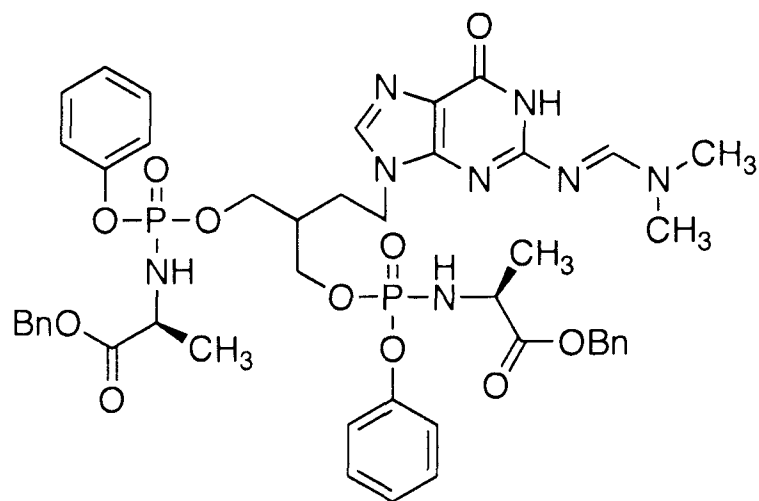
Mol Wt.: 308.3363

To a suspension of **11** (3.00 g, 11.85 mmol) in anhydrous DMF (40 mL) was added N,N

dimethylformamidedimethylacetal (7.90 mL, 59.23 mmol) and the reaction mixture was stirred at room temperature for 6 h. After this period, the solvent was removed and the residue was triturated with Et₂O and filtered. The solid was washed with Et₂O to give a white solid (95%, 3.45 g).

¹H-NMR (DMSO, 500 MHz): δ 11.17 (1H, bs, NH), 8.56 (1H, s, N=CHN), 7.81 (1H, s, H-8), 4.09 (2H, t, J = 7.3, H-1'), 3.49-3.35 (4H, m, H-4', H-5'), 3.16, 3.04 (6H, s, 2xCH₃), 1.76 (2H, dd, J = 14.1, 7.0, H-2'), 1.46 (1H, dt, J = 12.0, 5.9, H-3').

Synthesis of N²-DMF-penciclovir-[1-phenyl-(benzoxy-L-alaninyl)] diphosphate [215a]



C₄₅H₅₂N₈O₁₁P₂

Mol Wt.: 942.8889

Prepared according to Standard Procedure C, from **214** (0.40 g, 1.30 mmol) in anhydrous THF (15 mL), ^tBuMgCl (1.0 mL, 1.30 mmol), ^tBuMgCl in THF solution, 2.60 mL, 2.60 mmol), **1** (0.92 g, 2.60 mmol) in anhydrous THF (8 mL) and the reaction mixture was stirred at room temperature for 8 h. Then the solvent was removed and the residue purified

column chromatography gradient elution of DCM/MeOH = 98/2, then 96/4 then 94/6 then 90/10 then 80/20 to give a white solid (54%, 0.67 g). A sample was further purified by preparative TLC gradient elution of DCM/MeOH = 98/2 then 95/5 to give a white solid.

^{31}P -NMR (MeOD, 202 MHz): δ 4.01, 3.96, 3.46, 3.43, 3.37.

^1H NMR (MeOD, 500 MHz): δ 8.67 (1H, s, N=CHN), 7.78 (1H, m, H-8), 7.37-7.24 (14H, m, PhO, PhCH₂), 7.24-7.11 (6H, m, PhO, PhCH₂), 5.15-5.07 (4H, m, 2xCH₂Ph), 4.21-3.92 (8H, m, H-1', H-4', H-5', 2xCHCH₃), 3.09, 3.06 (6H, 2s, N(CH₃)₂), 1.91-1.74 (3H, m, H-2', H-3'), 1.39-1.28 (6H, m, 2xCHCH₃).

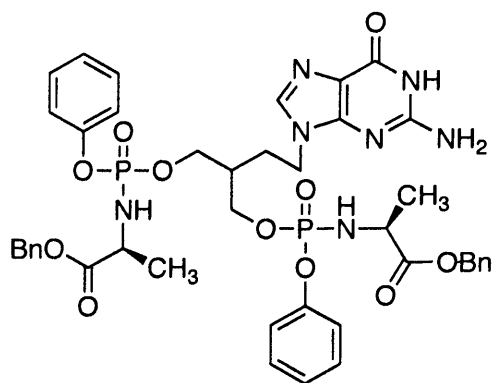
^{13}C NMR (MeOD, 126 MHz): δ 20.34 (d, $J_{\text{C-P}} = 3.2$, CH₃), 20.39 (d, $J_{\text{C-P}} = 3.6$, CH₃), 28.94, 29.02, 29.10 (C-2'), 35.34 (NCH₃), 38.36 (C-3'), 41.51, 41.52 (NCH₃), 41.76, 41.85 (2s, C-1'), 51.66, 51.81 (2s, CHCH₃), 66.85, 66.90, 66.98, 67.02 (C-4', C-5'), 67.94, 67.96 (2s, CH₂Ph), 121.42, 121.46, 121.49, 121.54, 121.58, 121.62, 126.20, 129.23, 129.24, 129.27, 129.28, 129.30, 129.31, 129.34, 129.36, 129.38, 129.54, 129.60, 129.63, 130.13, 130.81, 130.82 (PhO, CH₂Ph), 137.26 ('ipso' OCH₂Ph), 140.38 (C-8), 152.17, 155.23 (C-2, C-4), 158.94 (C-6), 159.84 (N=CHN), 174.68 (d, $J_{\text{C-P}} = 4.5$, COOCH₂Ph), 174.96 (d, $J_{\text{C-P}} = 3.7$, COOCH₂Ph).

EI MS = 943.3314 (M+H), 965.3083 (M+Na)

HPLC = H₂O/AcCN from 95/5 to 0/100 in 30 min = retention time 23.09 min.

= H₂O/MeOH 30/70 isocratic = retention time 12.68 min.

Synthesis of penciclovir-[1-phenyl-(benzoxyl-L-alaninyl)] diphosphate. [216a]



C₄₂H₄₇N₇O₁₁P₂

Mol Wt.: 887.8104

A suspension of **215a** (0.60 g, 0.64 mmol) in 2-propanol (30 mL) was stirred at 95 °C for 39 h. After this period the solvent was removed and the residue purified by column chromatography

gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6 to give a white solid which was further purified by preparative TLC (gradient elution of DCM/MeOH = 98/2, then 96/4, then 95/5) to give a white solid (0.026 g) corresponding to the desired compound and (4%, 0.020 g) corresponding to the pure starting material.

^{31}P -NMR (MeOD, 202 MHz): δ 3.90, 3.86, 3.43, 3.34.

^1H NMR (MeOD, 500 MHz): δ 7.67 (1H, m, H-8), 7.40-7.25 (14H, m, PhO, PhCH_2), 7.23-7.11 (6H, m, PhO, PhCH_2), 5.17-5.08 (4H, m, $2\times\text{CH}_2\text{Ph}$), 4.20-3.93 (8H, m, H-1', H-4', H-5', $2\times\text{CHCH}_3$), 1.91-1.69 (3H, m, H-2', H-3'), 1.36 (3H, t, $J = 2.2$ Hz, CHCH_3) 1.35 (3H, t, $J = 2.6$ Hz, CHCH_3).

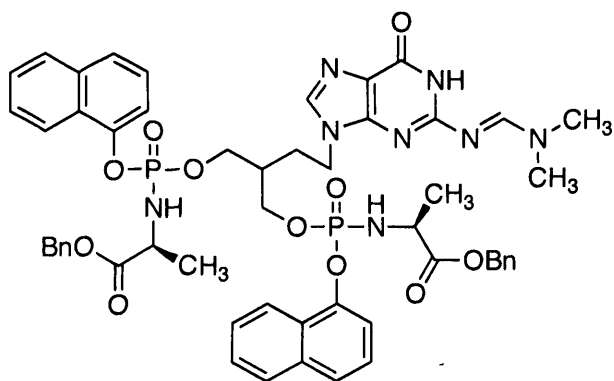
^{13}C NMR (MeOD, 126 MHz): δ 20.37, 20.42 (2s, CHCH_3), 28.99, 29.05 (2s, C-2'), 38.42 (C-3'), 41.79, 41.87 (C-1'), 51.68, 51.81 (2s, CHCH_3), 66.97, 67.02 (C-4', C-5'), 67.58, 67.97 (2s, CH_2Ph), 121.45, 121.51, 121.55, 121.59, 121.63, 126.15, 129.21, 129.26, 129.30, 129.33, 129.34, 129.37, 129.51, 129.59, 129.61, 130.05, 130.80 (PhO, CH_2Ph), 137.26 ('ipso' OCH_2Ph), 152.16, 155.24 (C-2, C-4), 174.73, 174.99 (2s, COOCH_2Ph).

EI MS = 888.2908 (M+H), 910.2741 (M+Na).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 95/5 to 0/100 in 30 min = retention time 24.68 min.

= $\text{H}_2\text{O}/\text{MeOH}$ 30/70 isocratic = retention time 4.55 min.

Synthesis of N^2 -DMF-penciclovir-[1-naphthyl(benzoxymethyl-L-alaninyl)] diphosphate. [215b]



$\text{C}_{53}\text{H}_{56}\text{N}_8\text{O}_{11}\text{P}_2$

Mol Wt.: 1043.0063

Prepared according to Standard Procedure C, from **214** (0.40 g, 1.30 mmol) in anhydrous THF (15 mL), $t\text{-BuMgCl}$ (1.0 M THF solution, 2.60 mL,

2.60 mmol), **193a** (1.05 g, 2.60 mmol) in anhydrous THF (8 mL) and the reaction mixture was stirred at room temperature for 8 h. Then the solvent was removed and the

residue purified by column chromatography gradient elution of DCM/MeOH = 98/2, then 97/3 then 96/4 to give a white solid (20%, 0.28 g). A sample was further purified by preparative TLC gradient elution of DCM/MeOH = 98/2 then 95/5 to give a white solid.

^{31}P -NMR (MeOD, 202 MHz): δ 4.43, 4.42, 4.37, 4.34, 4.06, 3.97, 3.95, 3.88.

^1H NMR (MeOD, 500 MHz): δ 8.52-8.44 (1H, m, N=CHN), 8.16-8.06 (2H, m, H-8 Naph), 7.90-7.77 (2H, m, H-6 Naph), 7.70-7.57 (3H, m, H-8, Naph), 7.52-7.19 (18H, m, NaphO, Ph), 5.11-5.00 (4H, m, 2xCH₂Ph), 4.20-3.87 (8H, m, H-1', H-4', H-5', 2xCHCH₃), 3.02-2.92 (6H, m, N(CH₃)₂), 1.90-1.48 (3H, m, H-2', H-3'), 1.37-1.29 (6H, m, 2xCHCH₃).

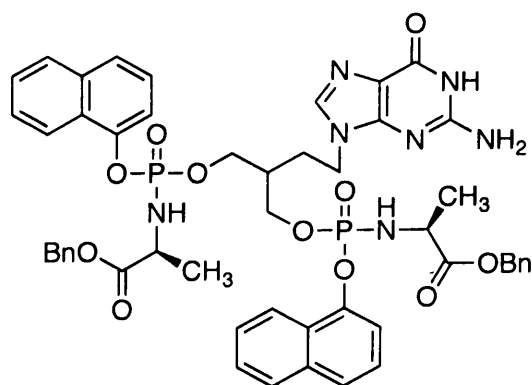
^{13}C NMR (MeOD, 126 MHz): δ 20.34, 20.40 (2s, CHCH₃), 28.83, 28.99, 29.08 (C-2'), 35.25 (NCH₃), 38.37 (C-3'), 41.39, 41.44 (NCH₃), 41.64, 41.72 (2s, C-1'), 51.80, 51.88 (2s, CHCH₃), 66.92, 67.10, 67.26 (C-4', C-5'), 67.96 (CH₂Ph), 116.48, 116.60, 116.71, 122.68, 122.86, 126.05, 126.09, 126.51, 127.48, 127.52, 127.79, 128.89, 128.92, 129.18, 129.27, 129.33, 129.58 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH₂Ph), 136.23, 137.16 (C-4a Naph, 'ipso' OCH₂Ph), 140.12 (C-8), 147.95 ('ipso' Naph, C-4), 152.06 (C-2), 158.83 (C-6), 159.72 (N=CHN), 174.68, 175.00 (2s, COOCH₂Ph).

EI MS = 1043.3613 (M⁺).

HPLC = H₂O/AcCN from 95/5 to 0/100 in 30 min = retention time 26.35 min.

= H₂O/MeOH 20/80 isocratic = retention time 9.23 min.

Synthesis of penciclovir-[1-naphthyl(benzoxo-L-alaninyl)] diphosphate. [216b]



C₅₀H₅₁N₇O₁₁P₂

Mol Wt.: 987.9278

A suspension of **215b** (0.23 g, 0.22 mmol) in 2-propanol (15 mL) was stirred at 95 °C for 72 h. After this period the solvent was removed and

the residue purified by column chromatography gradient elution of DCM/MeOH = 99/1, then 98/2, then 96/4 to give a white solid which was further purified by preparative TLC (gradient elution of DCM/MeOH = 98/2, then 96/4, then 95/5) to give a white solid (7%, 0.015 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.32, 4.26, 4.24, 3.98, 3.94, 3.85.

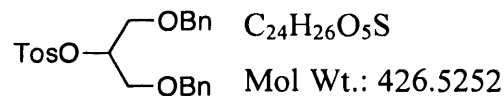
^1H NMR (MeOD, 500 MHz): δ 8.18-8.06 (2H, m, H-8 Naph), 7.88-7.78 (2H, m, H-6 Naph), 7.72-7.59 (2H, m, Naph), 7.53-7.16 (19H, m, H-8, NaphO, Ph), 5.10-5.04 (4H, m, 2xCH₂Ph), 4.16-3.99 (6H, m, H-1', H-4', H-5'), 3.91-3.76 (2H, 2xCHCH₃), 1.67-1.41 (3H, m, H-2', H-3'), 1.33-1.30 (6H, m, 2xCHCH₃).

^{13}C NMR (MeOD, 126 MHz): δ 20.34, 20.39 (2s, CHCH₃), 28.98 (C-2'), 38.45 (C-3'), 41.60, 41.65, 41.73 (C-1'), 51.81, 51.89 (2s, CHCH₃), 67.07, 67.16 (C-4', C-5'), 67.97 (CH₂Ph), 116.41, 116.50, 116.64, 116.75, 117.54, 122.65, 122.70, 122.73, 122.85, 126.02, 126.52, 127.50, 127.74, 127.77, 127.94, 128.86, 128.88, 129.21, 129.29, 129.32, 129.52, 129.54, 129.57 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH₂Ph), 136.25, 137.16 (C-4a Naph, 'ipso' OCH₂Ph), 139.29 (C-8), 147.97, 148.03 ('ipso' Naph, C-4), 155.16 (C-2), 159.39 (C-6), 174.77, 175.02 (2s, COOCH₂Ph).

HPLC = H₂O/AcCN from 95/5 to 0/100 in 30 min = retention time 25.35 min.

9.16 Synthesis of thio-ganciclovir ProTides

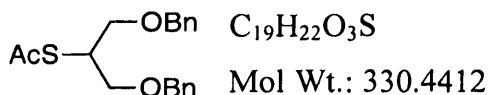
Synthesis of (1,3-di-*O*-benzyl-2-*O*-*p*-toluenesulfonyl)glycerol. [218]



To a solution of **217** (25.78 g, 93.63 mmol) in anhydrous pyridine (90 mL) was added *p*-toluenesulfonyl chloride (22.31 g, 117.03 mmol) and the reaction mixture was stirred at room temperature overnight. After this period the solvent was removed and the residue dissolved in DCM. The organic phase was washed with water (twice), with a saturated solution of Na₂CO₃ (twice), dried over MgSO₄, filtered and concentrated to give a clear oil (99%, 39.90 g).

¹H NMR (CDCl₃, 500 MHz): δ 7.80 (2H, d, J = 8.3, tosylate), 7.39-7.27 (10H, m, Ph), 7.26-7.20 (2H, m, Ph), 4.78 (1H, p, J = 5.1, CH), 4.46 (4H, q, J = 11.9, CH₂Ph), 3.73-3.64 (4H, m, 2xCH₂), 2.41 (3H, s, CH₃).

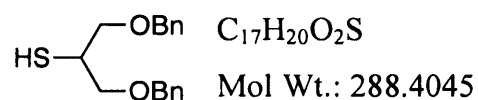
Synthesis of 1,3-bis(benzyloxy)-2-(acetylthio)propane. [219]



To a solution of **218** (39.90 g, 93.55 mmol) in anhydrous DMF (70 mL) was added potassium acetate (14.42 g, 126.29 mmol) and the reaction mixture was stirred at 90 °C for 2 h. After this period, the reaction mixture was allowed to cool down and it was diluted with DCM. The organic phase was washed with water (5 times), saturated solution of Na₂CO₃ (3 times), and 10% aqueous solution of HCl (3 times), then dried over MgSO₄. After filtration, the solvent was removed to give a dark/brown oil (99%, 30.50 g).

^1H NMR (CDCl_3 , 500 MHz): δ 7.42-7.26 (10H, m, Ph), 4.56 (4H, s, $2\times\text{CH}_2\text{Ph}$), 4.02-3.96 (1H, m, CH), 3.74 (2H, dd, $J = 9.8, 4.9$, CH_2CH), 3.69 (2H, dd, $J = 9.7, 6.4$, CH_2CH), 2.37 (3H, s, CH_3).

Synthesis of 1,3-bis(benzyloxy)-2-propanethiol. [220]

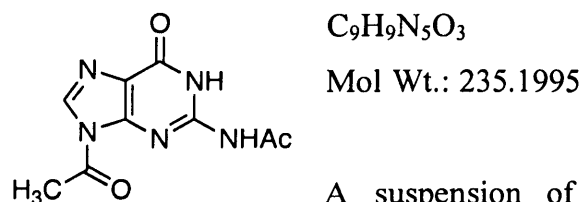


Procedure A: A solution of **219** (10.33 g, 31.26 mmol) in methanolic ammonia (70 mL) was stirred at room temperature for 16 h. After this period, the solvent was removed and the residue filtered through silica gel eluting with DCM to give a red/brown oil (72%, 6.52 g).

Procedure B: to a solution of **219** (30.90 g, 93.51 mmol) in anhydrous MeOH (350 mL) was added sodium methoxide (6.06 g, 112.21 mmol) and the reaction mixture was stirred at room temperature for 1.5 h. After this period, the solution was neutralised with amberlite, filtered and concentrated to give a brown/red oil, which was filtered through silica gel eluting with DCM to give a red/brown oil (73%, 19.58 g).

^1H NMR (CDCl_3 , 500 MHz): δ 7.44-7.29 (10H, m, Ph), 4.58 (4H, s, $2\times\text{CH}_2\text{Ph}$), 3.69 (4H, d, $J = 5.8$, $2\times\text{CH}_2\text{CH}$), 3.27-3.19 (1H, m, CH), 1.97 (1H, d, $J = 8.2$, SH).

Synthesis of diacetylguanine. [222]

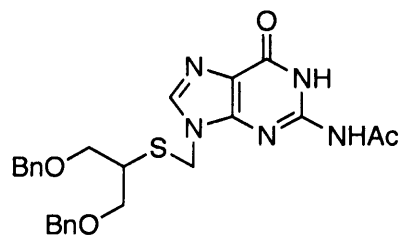


A suspension of guanine **221** (15.00 g, 99.25 mmol) in dimethylacetamide (120 mL) and acetic anhydride (25 mL) and the reaction mixture was stirred at 160 °C for 7 h. Then the reaction mixture was allowed to cool down and the precipitate formed was filtered and washed with ethanol. A second precipitate was

formed and it was filtered and washed with ethanol to give a pale brown solid (90%, 21.01 g).

^1H NMR (DMSO, 500 MHz): δ 11.98 (2H, bs, 2xNH), 8.45 (1H, s, H-8), 2.82 (3H, s, CH_3), 2.22 (3H, s, CH_3).

Synthesis of N^2 -acetyl-9- $\{[1,3\text{-bis}(\text{benzyloxy})\text{-2-propylthio}]\text{methyl}\}$ guanine. [223]



$\text{C}_{25}\text{H}_{27}\text{N}_5\text{O}_4\text{S}$

Mol Wt.: 493.5780

HCl gas (generated in situ) was bubbled into a stirred mixture of **220** (19.50, 67.61 mmol) and paraformaldehyde (4.06 g, 135.25 mmol) in 1,2-dichloroethane at 0 °C for 2 h. Then, the reaction mixture was dried over MgSO_4 and then concentrated.

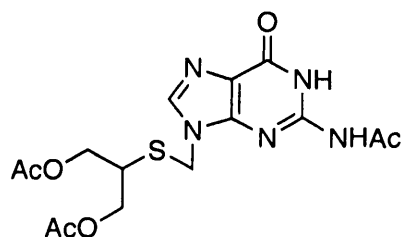
The resulting oil was dissolved in anhydrous DMF (40 mL) and sodium acetate (11.09 g, 135.25 mmol) was added and the reaction mixture was stirred at room temperature for 2 h. The suspension was filtered and washed with DCM. The filtrate was further diluted with DCM and washed with water (3 times), saturated solution of Na_2CO_3 (3 times) and 10% aqueous solution of HCl (3 times), then dried over MgSO_4 and concentrated.

The resulting oil was dissolved in sulfolane (100 mL) and were added **222** (15.90 g, 67.61 mmol) and bis(4-nitrophenyl) phosphate (0.30 g) and the reaction mixture was stirred at 100 °C for 6 h then at room temperature for 17 h. After this period, the resulting mixture was diluted with DCM and filtered through celite and the resulting solution was filtered through silica gel gradient elution of DCM then DCM/MeOH = 98/2 then 95/5. The residue was further purified by column chromatography eluting with DCM/MeOH = 97/3 to give a mixture of N-7 and N-9 isomer as an oil. This mixture was further purify by column chromatography gradient elution of DCM then DCM/MeOH = 98/2 then 96/4 to obtain a mixture of N-7 and N-9 isomer as an oil. This crude was dissolved in a minimum amount of DCM and triturated with Et_2O and filtered. The solid was washed with Et_2O (3 times) to give N-9 isomer as a white solid (7%, 2.20 g).

^1H NMR (DMSO, 500 MHz): δ 12.04 (1H, bs, NH), 11.70 (1H, bs, NH), 8.09 (1H, s, H-8), 7.40-7.17 (10H, m, Ph), 5.31 (s, 2H, H-1'), 4.56-4.30 (4H, m, 2xCH₂Ph), 3.64-3.46 (4H, m, H-4', H-5'), 3.39-3.29 (1H, m, H-3'), 2.17 (3H, s, CH₃).

^{13}C NMR (DMSO, 126 MHz): δ 23.75 (CH₃), 44.13 (C-1'), 45.36 (C-3'), 69.86 (C-4', C-5'), 72.07 (CH₂Ph), 120.21 (C-5), 127.30, 127.40, 128.17 (Ph), 138.10 (*ipso*-Ph), 139.31 (C-8), 147.85 (C-2), 148.37 (C-4), 154.77 (C-6), 173.51 (COOCH₃).

Synthesis of N²-acetyl-9-[(1,3-diacetoxy-2-propylthio)methyl]guanine. [224]



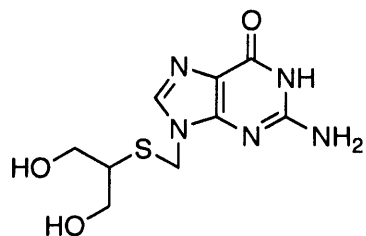
C₁₅H₁₉N₅O₆S

Mol Wt.: 397.4063

To a suspension of **223** (1.40 g, 2.84 mmol) in acetic anhydride (30 mL) was added dropwise at 0 °C boron trifluoride etherate (1.00 mL, 7.94 mmol) and the reaction mixture was stirred at room temperature for 2.5 h. After this period, the solvent was removed and the residue dissolved in a small amount of DCM and triturated with Et₂O and filtered. The solid was purified by column chromatography gradient elution of DCM then DCM/MeOH = 97/3 then 95/5 to give a pink solid (50%, 0.57 g).

^1H NMR (DMSO, 500 MHz): δ 12.06 (1H, bs, NH), 11.69 (1H, bs, NH), 8.11 (1H, s, H-8), 5.33 (2H, s, H-1'), 4.18-4.06 (4H, m, H-4', H-5'), 3.46 (1H, p, J = 6.0, H-3'), 2.20 (3H, s, NHCOOCH₃), 1.96 (6H, s, 2xCOOCH₃).

^{13}C NMR (DMSO, 126 MHz): δ 20.39 (COOCH₃), 23.78 (NHCOOCH₃), 43.19 (C-3'), 43.79 (C-1'), 63.12 (C-4', C-5'), 120.25 (C-5), 139.26 (C-8), 147.92 (C-2), 148.36 (C-4), 154.78 (C-6), 169.99 (COOCH₃), 173.51 (NHCOOCH₃).

Synthesis of 9-[(1,3-dihydroxy-2-propylthio)methyl]guanine. [205] $\text{C}_9\text{H}_{13}\text{N}_5\text{O}_3\text{S}$

Mol Wt.: 271.2962

A suspension of **224** (0.52 g, 1.31 mmol) in methanolic ammonia (15 mL) was stirred at room temperature for 18 h.

After this period the solvent was removed under reduced pressure and the residue triturated with DCM to give a white solid (90%, 0.32 g).

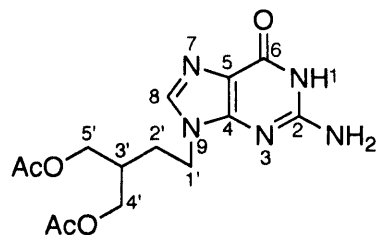
^1H NMR (DMSO, 500 MHz): δ 10.56 (1H, bs, NH), 7.78 (1H, s, H-8), 6.47 (2H, bs, NH_2), 5.15 (2H, s, H-1'), 4.76 (2H, t, $J = 5.4$, OH), 3.52 (4H, t, $J = 5.5$, H-4', H-5'), 2.89 (1H, p, $J = 5.7$, H-3').

^{13}C NMR (DMSO, 126 MHz): δ 42.87 (C-1'), 49.71 (C-3'), 61.21 (C-4', C-5'), 116.52 (C-5), 136.98 (C-8), 150.92 (C-2), 153.62 (C-4), 156.71 (C-6).

EI MS = 271.0737 (M^+), 272.0813 ($\text{M}+\text{H}$).

9.17 Synthesis of 6-methoxy and 6-chloro penciclovir derivatives

Synthesis of 9-(4-acetoxy-3-acetoxymethylbut-1-yl)guanine. [225]



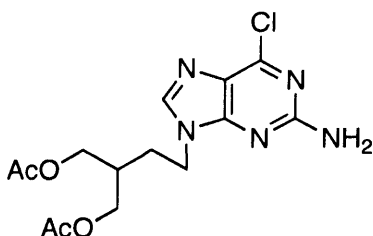
$C_{14}H_{19}N_5O_5$

Mol Wt.: 337.3312

A mixture of **11** (2.00, 7.90 mmol) in anhydrous DMF (15 mL) was added to a mixture of DMAP (0.05 g, 0.40 mmol) in acetic anhydride (1.70 mL, 18.16 mmol) and the reaction mixture was stirred at 45 °C for 1.5 h. After this period, the mixture was allowed to cool down and 2-propanol (30 mL) was added and the reaction mixture was stirred at 0 °C for 1.5 h. The suspension was filtered and the solid was washed 2-propanol (2 x 5mL). The wet crystals were added to ethyl acetate (20 mL), heated at 70 °C and cooled to room temperature. The solid formed was filtered and washed with ethyl acetate to give a white solid (83%, 2.20 g).

^1H NMR (DMSO, 500 MHz): δ 10.52 (1H, bs, NH), 7.71 (1H, s, H-8), 6.40 (2H, bs, NH_2), 4.11-3.92 (6H, m, H-1', H-4', H-5'), 2.00 (6H, s, CH_3), 1.97-1.88 (1H, m, H-3'), 1.85-1.75 (2H, m, H-2').

Synthesis of 9-(4-acetoxy-3-acetoxymethylbut-1-yl)-2-amino-6-chloropurine. [226]



$C_{14}H_{18}ClN_5O_4$

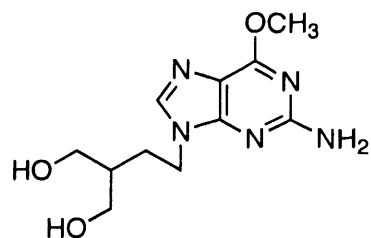
Mol Wt.: 355.7768

A mixture of **225** (2.12 g, 6.28 mmol) and benzyltriethylammonium chloride (2.86 g, 12.57 mmol) in acetonitrile (15 mL) was cooled to 0 °C and added to a mixture of *N,N*-dimethylaniline (0.40 mL, 3.14 mmol) and POCl_3 (2.60 mL, 28.28 mmol) and the reaction mixture was stirred at 70 °C 1 h. After this period, the solvent was removed and the residue dissolved in DCM. The organic phase was washed with saturated solution of NaHCO_3 (twice) and

water (once), dried over MgSO_4 and concentrated. To the residue was added methanol (4 mL) and water (2 mL) and the mixture was stirred at 0 °C for 1.5 h. The suspension was filtered and the solid washed with 75% aqueous methanol to give a white solid (46%, 1.04 g).

^1H NMR (DMSO, 500 MHz): δ 8.16 (1H, s, H-8), 6.87 (2H, bs, NH_2), 4.14 (2H, t, J = 7.1, H-1'), 4.02 (4H, d, J = 5.6, H-4', H-5'), 1.99 (6H, s, COOCH_3), 1.96-1.90 (1H, m, H-3'), 1.90-1.83 (2H, m, H-2').

Synthesis of 9-(4-hydroxy-3-hydroxymethylbut-1-yl)-2-amino-6-methoxypurine. [206]



$\text{C}_{11}\text{H}_{17}\text{N}_5\text{O}_3$

Mol Wt.: 267.2844

To a suspension of **226** (0.35 g, 1.04 mmol) in anhydrous methanol (10 mL) was added sodium methoxide (0.30 g, 4.16 mmol) and the reaction mixture was stirred at 40 °C for 6 h. After this period, the reaction mixture was neutralized with amberlite, filtered and concentrated. The crude was purified by column chromatography eluting with DCM/MeOH = 9/1 to give a white solid (71%, 0.20 g).

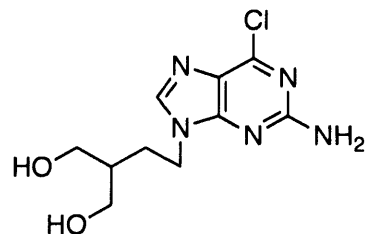
^1H NMR (DMSO, 500 MHz): δ 7.86 (1H, s, H-8), 6.37 (2H, bs, NH_2), 4.42 (2H, t, J = 5.1, OH), 4.07 (2H, t, J = 7.4, H-1'), 3.96 (3H, s, OCH_3), 3.47-3.40, 3.39-3.34 (4H, 2m, H-4', H-5'), 1.80-1.69 (2H, m, H-2'), 1.51-1.39 (1H, m, H-3').

^{13}C NMR (DMSO, 126 MHz): δ 28.56 (C-2'), 40.75 (C-3'), 41.04 (C-1'), 53.05 (OCH_3), 61.29 (C-4', C-5'), 113.78 (C-5), 139.66 (C-8), 154.08 (C-2), 159.68 (C-4), 160.57 (C-6).

EI MS= 268.14 (M+H).

HPLC = $\text{H}_2\text{O}/\text{AcCN}$ from 95/5 to 0/100 in 30 min = retention time 6.52 min.

= $\text{H}_2\text{O}/\text{MeOH}$ from 95/5 to 0/100 in 40 min = retention time 12.79 min.

Synthesis of 9-(4-hydroxy-3-hydroxymethylbut-1-yl)-2-amino-6-chloropurine. [207]C₁₀H₁₄ClN₅O₂

Mol Wt.: 271.7035

A solution of **226** (0.18 g, 0.51 mmol) in methanolic ammonia (10 mL) was stirred at room temperature for 6 h.

After this period the solvent was removed and the residue purified by column chromatography eluting with DCM/MeOH = 90/10 to give a white solid (85%, 0.12 g).

¹H NMR (DMSO, 500 MHz): δ 8.16 (1H, s, H-8), 6.88 (2H, bs, NH₂), 4.43 (2H, t, J = 5.2, OH), 4.12 (2H, t, J = 7.4, H-1'), 3.47-3.41, 3.38-3.33 (4H, 2m, H-4', H-5'), 1.82-1.74 (2H, m, H-2'), 1.51-1.41 (1H, m, H-3').

¹³C NMR (DMSO, 126 MHz): δ 28.33 (C-2'), 40.77 (C-3'), 41.45 (C-1'), 61.29 (C-4', C-5'), 123.33 (C-5), 143.18 (C-8), 149.23 (C-2), 154.04 (C-4), 159.69 (C-6).

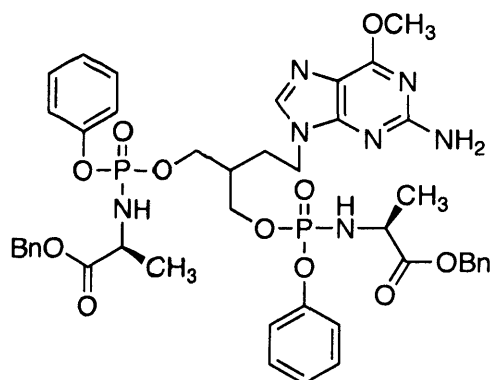
EI MS= 271.08 (M⁺).

HPLC = H₂O/AcCN from 95/5 to 0/100 in 30 min = retention time 10.71 min.

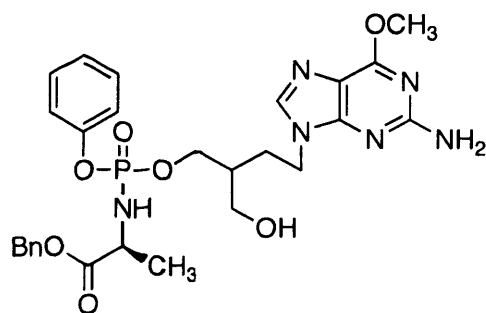
= H₂O/MeOH from 95/5 to 0/100 in 40 min = retention time 12.33 min.

9.18 Synthesis of 6-methoxy penciclovir ProTides

Synthesis of 9-(4-hydroxy-3-hydroxymethylbut-1-yl)-2-amino-6-methoxypurine-[1-phenyl(benzoy-L-alaninyl)] diphosphate [227a] and 9-(4-hydroxy-3-hydroxymethylbut-1-yl)-2-amino-6-methoxypurine-[1-phenyl(benzoy-L-alaninyl)] phosphate. [227b]

 $C_{43}H_{49}N_7O_{11}P_2$

Mol Wt.: 901.8370

 $C_{27}H_{33}N_6O_7P$

Mol Wt.: 584.5607

Prepared according to Standard Procedure C, from **206** (0.13 g, 0.49 mmol) in anhydrous THF (10 mL), t BuMgCl (1.0 M THF solution, 0.58 mL, 0.58 mmol), **131d** (0.20 g, 0.58 mmol) in anhydrous THF (5 mL) and the reaction mixture was stirred at room temperature overnight. Then were added t BuMgCl (1M solution in THF, 0.50 mL, 0.50 mmol) and **131d** (0.20 g, 0.58 mmol) in anhydrous THF (5 mL) and the reaction mixture was stirred at room temperature for further 24 h. After this period the solvent was removed and the residue was purified by column chromatography gradient elution of DCM, then DCM/MeOH = 98/2, 96/4 to give compound **227a** as a white solid (2%, 0.01 g). An impure fraction was further purified by preparative TLC to give **227b** as a white solid (3%, 0.01 g).

227a

³¹P-NMR (MeOD, 202 MHz): δ 3.91, 3.86, 3.83, 3.44, 3.41, 3.40, 3.32.

¹H NMR (MeOD, 500 MHz): δ 7.84-7.71 (1H, m, H-8), 7.40-7.24 (14H, m, PhO, PhCH₂), 7.23-7.08 (6H, m, PhO, PhCH₂), 5.14-5.09 (4H, m, 2xCH₂Ph), 4.20-3.97 (8H, m, H-1', H-4', H-5', 2xCHCH₃), 3.37 (3H, s, OCH₃), 2.05-1.67 (3H, m, H-2', H-3'), 1.39-1.27 (6H, m, 2xCHCH₃).

¹³C NMR (MeOD, 126 MHz): δ 20.34 (d, J_{C-P} = 3.7, CHCH₃), 20.40 (d, J_{C-P} = 3.5, CHCH₃), 28.83, 28.86 (2s, C-2'), 38.48 (d, J_{C-P} = 6.2, C-3'), 38.57 (d, J_{C-P} = 7.8, H-3'), 41.91, 41.97 (C-1'), 49.87 (OCH₃), 51.65, 51.68 (CHCH₃), 66.92, 66.98, 67.02, 67.11 (4s, C-4', C-5'), 67.90, 67.96 (CH₂Ph), 115.25 (C-5), 121.45, 121.50, 121.51, 121.55, 121.58, 121.59, 121.64, 126.13, 126.17, 129.16, 129.27, 129.32, 129.33, 129.35, 129.58, 129.60, 130.79, 131.11 (PhO, CH₂Ph), 137.25 ('ipso' OCH₂Ph), 140.96 (C-8), 152.15, 152.21, 155.05 (C-2, C-4), 161.82 (C-6), 174.73, 174.99 (2s, COOCH₂Ph).

HPLC = H₂O/AcCN from 95/5 to 0/100 in 30 min = retention time 23.49 min.

227b

³¹P-NMR (MeOD, 202 MHz): δ 4.06, 4.00, 3.59.

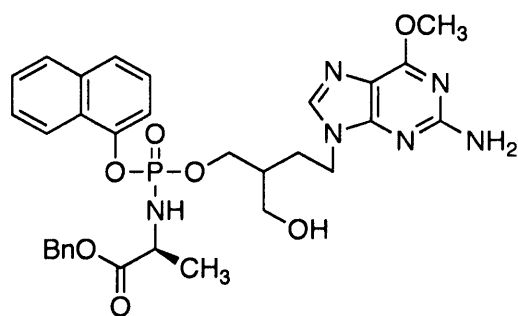
¹H NMR (MeOD, 500 MHz): δ 7.85-7.79 (1H, m, H-8), 7.38-7.27 (7H, m, PhO, PhCH₂), 7.23-7.13 (3H, m, PhO, PhCH₂), 5.15-5.12 (2H, m, CH₂Ph), 4.24-3.99 (5H, m, H-1', H-5', CHCH₃), 3.60-3.48 (2H, m, H-4'), 3.37 (3H, s, OCH₃), 1.92-1.73 (3H, m, H-2', H-3'), 1.40-1.33 (3H, m, CHCH₃).

¹³C NMR (MeOD, 126 MHz): δ 20.36 (d, J_{C-P} = 4.4, CHCH₃), 20.43 (d, J_{C-P} = 2.8, CHCH₃), 29.34, 29.36 (2s, C-2'), 40.32 (d, J_{C-P} = 3.0, C-3'), 40.42 (d, J_{C-P} = 7.4, C-3'), 42.37, 42.39 (C-1'), 48.96 (OCH₃), 51.65, 51.68 (CHCH₃), 62.27, 62.32 (C-4'), 67.80, 67.84, 67.86, 67.91 (C-5', CH₂Ph), 115.27 (C-5), 121.48, 121.51, 121.54, 121.57, 121.61, 126.09, 126.12, 129.28, 129.31, 129.34, 129.57, 130.74 (PhO, CH₂Ph), 137.25 ('ipso' OCH₂Ph), 141.04 (C-8), 152.22, 152.25, 152.28, 152.31 (C-2, C-4), 161.82, 162.72 (C-6), 174.72 (d, J_{C-P} = 5.0, COOCH₂Ph), 175.00 (d, J_{C-P} = 4.6, COOCH₂Ph).

HPLC = H₂O/AcCN from 95/5 to 0/100 in 30 min = retention time 16.41 min.

= H₂O/MeOH 40/60 isocratic = retention time 9.60 min, 10.44 min.

Synthesis of 9-(4-hydroxy-3-hydroxymethylbut-1-yl)-2-amino-6-methoxypurine-[1-naphthyl(benzoxo-L-alaninyl)] phosphate. [227c]



$C_{31}H_{35}N_6O_7P$

Mol Wt.: 634.6194

Prepared according to Standard Procedure C, from **206** (0.13 g, 0.49 mmol) in anhydrous THF (10 mL), t BuMgCl (1.0 M THF solution, 0.58

mL, 0.58 mmol), **193a** (0.23 g, 0.58 mmol) in anhydrous THF (5 mL) and the reaction mixture was stirred at room temperature overnight. Then were added t BuMgCl (1M solution in THF, 0.50 mL, 0.50 mmol) and **193a** (0.23 g, 0.58 mmol) in anhydrous THF (5 mL) and the reaction mixture was stirred at room temperature for further 24 h. After this period the solvent was removed and the residue was purified by column chromatography gradient elution of DCM, then DCM/MeOH = 98/2, 96/4 to give a white solid which was further purified by preparative TLC to give DM1739 as a white solid (3%, 0.01 g).

^{31}P -NMR (MeOD, 202 MHz): δ 4.15, 4.44, 4.50.

1H -NMR (MeOD, 500 MHz): δ 8.19-8.12 (1H, m, H-8 Naph), 7.90-7.81 (1H, m, H-6 Naph), 7.81-7.62 (2H, m, H-8, Naph), 7.56-7.23 (9H, m, PhO, $PhCH_2$), 5.16-5.03 (2H, m, CH_2Ph), 4.29-3.98 (5H, m, H-1', H-5', $CHCH_3$), 3.53-3.42 (2H, m, H-4'), 3.37 (3H, s, OCH_3), 1.81-1.58 (3H, m, H-2', H-3'), 1.47-1.34 (3H, m, $CHCH_3$).

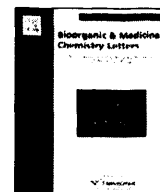
^{13}C NMR (126 MHz, MeOD) δ 20.37 (d, J_{C-P} = 3.0, $CHCH_3$), 20.45 (d, J_{C-P} = 3.8, $CHCH_3$), 29.29, 29.35 (2s, C-2'), 40.38 (d, J_{C-P} = 4.2, C-3'), 40.44 (d, J_{C-P} = 5.8, C-3'), 42.28, 42.32, 42.36 (C-1'), 49.86 (OCH_3), 51.82, 51.87 ($CHCH_3$), 62.24, 62.31 (C-4'), 67.90, 67.97, 68.03, 68.08 (C-5', CH_2Ph), 115.23 116.49, 116.29, 116.35, 122.69, 122.80, 125.92, 126.51, 127.20, 127.42, 127.45, 127.73, 128.85, 129.26, 129.30, 129.32, 129.53, 129.56, 130.79 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph; OCH_2Ph), 136.26, 137.20 (C-4a Naph, 'ipso' OCH_2Ph),

140.89 (C-8), 148.11 ('ipso' Naph, C-4,) 152.92 (C-2, C-4), 161.78, 162.73 (C-6), 174.76, 174.80 (COOCH₂Ph).

HPLC = H₂O/AcCN from 95/5 to 0/100 in 30 min = retention time 18.43 min.

Appendix I: Publications

1. McGuigan, C.; Derudas, M.; Bugert, J. J.; Andrei, G.; Snoeck, R.; Balzarini, J. Successful kinase bypass with new acyclovir phosphoramidate prodrugs. *Bioorg Med Chem. Lett.* **2008**, *18*, 4364-4367.
2. Lisco, A.; Vanpouille, C.; Tchesnokov, E. P.; Grivel, J. C.; Biancotto, A.; Brichacek, B.; Elliott, J.; Fromentin, E.; Shattock, R.; Anton, P.; Gorelick, R.; Balzarini, J.; McGuigan, C.; Derudas, M.; Götte, M.; Schinazi, R. F.; Margolis, L. Acyclovir is activated into a HIV-1 reverse transcriptase inhibitor in herpesvirus-infected human tissues. *Cell Host Microbe* **2008**, *4*, 260-270.
3. Tchesnokov, E. P.; Obikhod, A.; Massud, I.; Lisco, A.; Vanpouille, C.; Brichacek, B.; Balzarini, J.; McGuigan, C.; Derudas, M.; Margolis, L.; Schinazi, R. F.; Götte, M. Mechanisms associated with HIV-1 resistance to acyclovir by the V75I mutation in reverse transcriptase. *J. Biol. Chem.* **2009**, *284*, 21496-21504.
4. Derudas, M.; Carta, D.; Brancale, A.; Vanpouille, C.; Lisco, A.; Margolis, L.; Balzarini, J.; McGuigan, C. The application of phosphoramidate prodrug technology to acyclovir confers anti-HIV inhibition. *J. Med. Chem.* **2009**, *52*, 5520-5530.
5. McGuigan, C.; Derudas, M.; Quintiliani, M.; Andrei, G.; Snoeck, R.; Henson, G.; Balzarini, J. Fluorosugar analogues of the highly potent anti-VZV BCNAs. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6264-6267.



Successful kinase bypass with new acyclovir phosphoramidate prodrugs

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ABSTRACT

Novel phosphoramidates of acyclovir have been prepared and evaluated in vitro against acyclovir-sensitive and -resistant herpes simplex virus (HSV) types 1 and 2 and varicella-zoster virus (VZV). Unlike the parent nucleoside these novel phosphate prodrugs retain antiviral potency versus the ACV-resistant virus strain, suggesting an efficient bypass of the viral thymidine kinase.

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Herpes simplex virus infection is often well managed by the use of acyclovir, (ACV, **1**), its prodrug valacyclovir, or related compounds. The widespread use of (**1**) has led to the emergence of SV strains that are resistant to this drug.

Resistance appears uncommon in immunocompetent patients; Morfin¹ reports a prevalence below 1%. A more recent study in the Netherlands reports a prevalence of 0.27% in this population.² However, resistance is significantly more common in immunocompromised patients. Stranska et al.² report 7% and Morfin cites 5%. Notably, the proportion of resistant isolates rises to 30% in patients receiving allogeneic bone marrow transplants. Three separate mechanisms of resistance to (**1**) have been considered to occur; a loss of viral thymidine kinase (TK) activity, an altered TK substrate specificity and an alteration of viral DNA polymerase.³ Given the non-essential nature of the viral TK and the importance of the viral polymerase, it is unsurprising that the great majority of resistant isolates correspond to deletion/inactivation of the TK gene.⁴ One approach to manage TK-related resistance is to use agents not requiring HSV TK for activation, such as cidofovir or foscarnet. However, they may carry a risk of increased toxicity. Another approach would be to bypass the dependence of (**1**) on HSV TK by using a suitable phosphate prodrug, or ProTide. Several such methods now exist, such as the cyclosal approach,⁵ ester-based methods like SATE⁶ and phosphoramidate diesters.⁷ Our group has developed an aryloxy phosphoramidate triester approach,⁸ which has been

recently successfully applied to abacavir for HIV⁹ and 4'-substituted nucleosides for Hepatitis C Virus (HCV).¹⁰

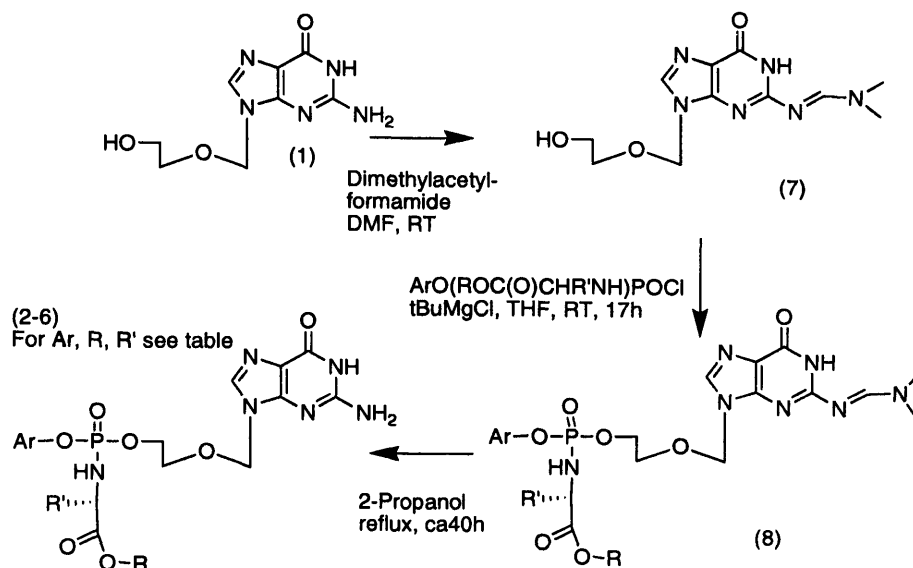
We have previously reported the application of this method to ACV (**1**) and the results indicated that the approach failed.¹¹ Thus, the ProTide (**2**) derived from (**1**) was found to be poorly active versus HSV2 (EC₅₀ ≥ 100 μM) unlike (**1**) itself. Compound (**2**) was roughly equi-active as (**1**) versus VZV and slightly more active versus human cytomegalovirus (HCMV) but there was no clear therapeutic advantage. However we have recently reported a new generation of phosphoramidate protides in which the aryl moiety is a bicyclic system such as 1-naphthyl.¹²

We were keen to explore the application of the naphthyl ProTide methodology to (**1**) for two reasons. Firstly the observation that naphthyl for phenyl can give a potency boost and secondly that these compounds may have a significant lipophilicity enhancement over prior structures. This may be particularly important in the case of (**1**) where the inherent lipophilicity is rather low, and first generation protides may be insufficiently lipophilic for efficient passive diffusion into cells. Indeed, ClogP¹³ estimates on (**2**) indicate a figure of ca. −0.8; although significantly more lipophilic than (**1**) this is somewhat lower than what may be viewed as optimal. Thus, in addition to examining naphthyl phosphates we also sought more lipophilic esters than the methyl ester (**2**) previously reported.

Compounds were prepared from (**1**) as shown in Scheme 1.

In order to improve the solubility of ACV we protected the guanine base using *N,N*-dimethylformamide dimethyl acetal, to give (**7**). The coupling with the appropriate phosphorochloridate

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Scheme 1.

was performed using *tert*-butyl magnesium chloride as a hydroxyl activator, to give blocked compounds of type (8).¹⁴ The DMF-protecting group was then removed by refluxing in 2-propanol (40–72 h). Owing to the chirality of the phosphorus, all of these compounds have been isolated and tested as a mixture of two diastereoisomers. Their structures have been demonstrated by NMR (³¹P, ¹H and ¹³C), mass spectroscopy and elemental analysis.¹⁵

The target compounds were first evaluated by plaque assay for their ability to inhibit the replication of ACV-sensitive and ACV-resistant HSV2 in Vero cells (Table 1).¹⁶

As can be seen from Table 1, while the previously reported phenyl methylalanine phosphate (2) is poorly active, being ca 7-fold less active than (1), in marked contrast to (1) it does retain full potency versus the resistant strain. This implies that (2) does function as a monophosphate prodrug as intended, but with low efficiency, particularly in the nucleoside-sensitive assay. By comparison, the naphthyl phosphate (3) is roughly equi-active with (1) versus TK-competent virus and notably retains full potency versus ACV-resistant HSV-2. The simplest explanation of this is that the ACV-resistant HSV-2 mutant is TK deficient and that (3) is TK-independent, strongly implying a successful thymidine kinase bypass. Notably, ClogP calculations on (3) indicate a significant enhancement over (1) to a figure of ca. 2 which may be regarded as near optimal. Indeed the 'mixed' compounds (4) and (5) have lower ClogP values and are less active in the HSV-2 TK⁺ assay, but retain good activity in the HSV-2 TK⁻ assay. In a further assay in HEL cells we evaluated the samples against both TK⁺ and TK⁻ HSV-1 and HSV-2 with data shown in Table 2.

Similar data emerge here, with (3) being particularly active and retaining significant activity in the TK⁻ assay. In this case compound (4) shows a similar profile, while (5) is less active. This implies that the ester moiety (benzyl in (3) and (4), versus methyl in (2) and (5)) is more important than the aryl moiety. Notably, the amino acid-modified compound (6) which has Phe in place of Ala, is poorly active in this assay, particularly versus the resistant virus. This is despite what might be regarded as a near-optimal lipophilicity for (6) (Table 1) and points to the importance of the amino acid moiety for activity.

One concern with bypassing the HSV-TK might be of enhanced cytotoxicity and loss of antiviral selectivity. However, the MCC data on this series (Table 2) do not reveal a significant toxicity. If, as appears likely, the viral TK is being bypassed, there must still be some element of viral specificity at another stage, most likely at the polymerase level.

In another assay we examined this family of prodrugs against kinase-competent and kinase deficient VZV (in HEL cells) with data shown in Table 3. As noted in this table, unlike ACV (1) several of the agents retain good potency in the TK⁻ VZV assays, notably compound (4) which essentially retains full potency. Interestingly (4) is also non-toxic while (3) does have some toxicity here.

Thus, compound (4) emerges as particularly active in a range of assays. It retains full activity versus all resistant viral strains, HSV-1, -2 and VZV, being low or sub- μ M in most cases, and non-toxic.

In conclusion, we report the successful application of the ProTide approach to acyclovir. The naphthyl and phenyl benzyl alanine ProTides are fully active in vitro against ACV-resistant

Table 1
Anti-HSV-2 activity of ProTides

Compound	Ar	R	Amino acid	ClogP	EC ₅₀ ^a (μ M)	
					HSV2-HG32 (ECACC 158)	HSV2-ACVR (ECACC 513)
1	—	—	—	−2.42	6 \pm 1.3	>100
2	Ph	Me	Ala	−0.82	43.1 \pm 10.6	18.7 \pm 7.2
3	1-Nap	Bn	Ala	2.06	9.8 \pm 2.2	14.5 \pm 6.6
4	Ph	Bn	Ala	0.89	20.3 \pm 6.6	15.5 \pm 7.4
5	1-Nap	Me	Ala	0.35	40.4 \pm 13.4	15 \pm 6.2
6	Ph	Bn	Phe	2.31	20.3 \pm 6.6	33.2 \pm 3.9

^a Values are means of three experiments, with standard deviations given, in Vero cells.

Compound	Ar	R	Amino acid	EC ₅₀ ^a /μM			MCC ^b (μM)
				HSV-1 (Kos)	HSV-2 (G)	HSV-1 TK-Kos ACV ^R	
1	—	—	—	0.4	0.2	50	>250
2	Ph	Me	Ala	20	15	80	>100
3	1-Nap	Bn	Ala	2	1.4	11	>100
4	Ph	Bn	Ala	0.9	1.4	8	>100
5	1-Nap	Me	Ala	16	10.4	80	>100
6	Ph	Bn	Phe	17	8	>100	>100

¹ MCC, minimal cytotoxic concentration that causes a microscopically visible alteration of cell morphology.

Compound	EC ₅₀ ^a (μM)				MCC ^b (μM)	CC ₅₀ ^c (μM)
	OKA TK ⁺	YS TK ⁺	07/1TK ⁻	YS/RTK ⁻		
1	2.5	2.9	61	43	>500	1350
2	19	20	24	16	>100	162
3	7.2	3.3	6.9	ND	>50	20
4	0.72	1.0	1.8	0.59	>50	>100
5	7.6	10.9	22	6.1	>50	>100
6	5.2	6.6	8.4	10.0	>50	86.9

^c CC₅₀, 50% cytostatic concentration that inhibits cell proliferation by 50%.

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8. (d, CH₃, J_{C-P} = 7.63) 20.43 (d, CH₃, J_{C-P} = 6.61), 51.76, 51.81 (2s, CHCH₃), 67.20 (d, C-4', J_{C-P} = 5.58), 67.28 (d, C-4', J_{C-P} = 4.91) 67.95, 67.98 (2s, OCH₂Ph), 69.34 (d, C-3', J_{C-P} = 7.72), 69.40 (d, C-3', J_{C-P} = 8.14), 73.65 (C-1), 116.26, 116.29, 116.35, 122.69, 122.80, 125.92, 126.51, 127.20, 127.42, 127.46, 127.74, 128.81, 128.83, 129.27, 129.33, 129.52, 129.57 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH₂Ph), 136.26, 137.23 (C-4a Naph, 'ipso' OCH₂Ph), 139.69 (C-8), 147.98, 148.04 ('ipso' Naph, C-4), 152.44 (C-2), 159.39 (C-6), 174.61, 174.88 (COOCH₂Ph). EI MS = 615.17 (M+Na).
- 16.. Biological methods: Table 1. Vero cells (ECACC #84113001) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine

serum, 300 µg/ml L-glutamine, 105 IU/ml penicillin and 100 µg/ml streptomycin and grown in 24-well plates to density. Appropriate wells were preincubated for 30 minutes with drug (prediluted in DMEM without additives), and kept growing in the appropriate amount of drug over the course of the experiment. HSV 2 strain (100 pfu) HG32 (ECACC # 158) and HSV 2 ACR (ECACC # 513) were inoculated per well, adsorbed for 45 min and then overlaid with 1.2% Avicel RC-591 (Camida Ltd.) suspended in DMEM. After 3 days the overlay was removed and the cells stained with crystal violet, the plates were scanned and the plaques counted. All assays were run in quadruplicate on each plate and plaques were counted as averages of four assays. EC50 was expressed as averages with standard deviation of 3 experiments. Tables 2 and 3. The antiviral assays were based on inhibition of virus-induced cytopathicity in human embryonic lung (HEL) fibroblasts

[herpes simplex virus type 1 (HSV-1) (KOS and KOS-R) and herpes simplex virus type 2 (HSV-2) (G)]. Confluent HEL cell cultures in 96-well microtiter plates were inoculated with 100 CCID₅₀ of virus (1 CCID₅₀ being the virus dose to infect 50% of the cell cultures). After 1-hour virus adsorption period, residual virus was removed, and the cells were incubated in the presence of serial dilutions of the test compounds. Viral cytopathicity was recorded within 48 h. For varicella-zoster virus [VZV (wild-type Oka and YS and TK⁻ deficient 07/1 and YS-R)], the inhibition of plaque formation was recorded. Confluent HEL cells in 96-well microtiter plates were infected with 20 plaque forming units (PFU)/well. After 2-hours incubation, residual virus was removed and the cells were incubated in the presence of the compounds. Virus plaque formation was recorded after 5 days.¹⁷

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Acyclovir Is Activated into a HIV-1 Reverse Transcriptase Inhibitor in Herpesvirus-Infected Human Tissues

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SUMMARY

For most viruses, there is a need for antimicrobials that target unique viral molecular properties. Acyclovir (ACV) is one such drug. It is activated into a human herpesvirus (HHV) DNA polymerase inhibitor exclusively by HHV kinases and, thus, does not suppress other viruses. Here, we show that ACV suppresses HIV-1 in HHV-coinfected human tissues, but not in HHV-free tissue or cell cultures. However, addition of HHV-6-infected cells renders these cultures sensitive to anti-HIV ACV activity. We hypothesized that such HIV suppression requires ACV phosphorylation by HHV kinases. Indeed, an ACV monophosphorylated prodrug bypasses the HHV requirement for HIV suppression. Furthermore, phosphorylated ACV directly inhibits HIV-1 reverse transcriptase (RT), terminating DNA chain elongation, and can trap RT at the termination site. These data suggest that ACV anti-HIV-1 activity may contribute to the response of HIV/HHV-coinfected patients to ACV treatment and could guide strategies for the development of new HIV-1 RT inhibitors.

INTRODUCTION

HIV-1 infection is usually accompanied by infection with other microbes, HIV-1 copathogens, which either pre-exist in the human body or invade it de novo. The replication of HIV-1 copathogens is frequently promoted in HIV-1-infected hosts, and their suppression is often beneficial for the clinical course of HIV disease (Corey, 2007; Jacobson and Mills, 1988). Human herpesvirus (HHV) infections are commonly associated with HIV-1. In particular, herpes simplex virus 2 (HSV-2) infection is associated

with an increase in HIV-1 transmission (Cohen, 2004; Freeman et al., 2006) and worsens the clinical course of HIV disease. HSV-2 reactivation may lead to increased plasma HIV-1 levels, thereby adversely affecting survival rates (Corey et al., 2004; Schacker et al., 2002).

Acyclovir (ACV) is a guanosine nucleoside analog particularly active against HSV-2 as well as against the other α -HHVs (HSV-1 and VZV) (Elion, 1983). It also inhibits, although with lower potency, the replication of the β -HHVs (CMV, HHV-6, and HHV-7) and of the γ -HHVs (EBV and HHV-8) (De Clercq et al., 2001).

The mechanism of HHV suppression by ACV is well understood. ACV is phosphorylated in HHV-infected cells by viral-encoded kinases. The resulting monophosphate derivative (ACV-MP) is then converted into ACV triphosphate (ACV-TP) by cellular enzymes and is subsequently incorporated by the viral DNA polymerase into the nascent viral DNA chain, causing its obligate termination (Reardon and Spector, 1989). Generally, the sensitivity of different HHVs to ACV is determined by the extent of its phosphorylation by HHV kinase and by the rate of incorporation of ACV-TP into the viral DNA chain (De Clercq et al., 2001). The mechanism described above explains why ACV does not directly affect viruses other than HHVs, including HIV-1. Nevertheless, early clinical trials indicated that ACV treatment may result in survival benefits in cohorts of HIV-1-infected patients with a high incidence of clinically identifiable HHV (Ioannidis et al., 1998). In agreement with these data, recent trials showed that, in HSV-2-coinfected individuals, the suppression of HSV-2 by ACV is accompanied by reduction of the plasmatic, genital, and rectal HIV-1 load (Baeten et al., 2007; Delany et al., 2007; Dunne et al., 2008; Nagot et al., 2007; Zuckerman et al., 2007). This decrease was attributed to the suppression of HSV-2-mediated inflammation indirectly reducing HIV-1 load.

Here, we report on the direct activity of ACV on HIV-1 and, specifically, on the inhibition of HIV-1 reverse transcriptase

(RT) by ACV-TP. We demonstrate that, consequently, ACV is an anti-HIV-1 agent in human lymphoid, rectal, and genital tissues, which are widely infected with one or more HHVs that are able to phosphorylate ACV.

RESULTS

ACV Suppresses HIV Replication in Human Lymphoid Tissue Coinfected Ex Vivo with HSV-2

In this study, we used an ex vivo system that preserves the cytoarchitecture of human tissues and supports replication of various viruses without exogenous stimulation (Glushakova et al., 1995).

Initially, we inoculated tissues with HIV-1_{LAI.04} and HSV-2 (strain G). Both viruses readily replicated in tissues. As expected, ACV at a concentration of 30 μ M suppressed HSV-2 replication in both HSV-2 singly infected and HIV-1 coinfecting tissues (by $97 \pm 1.8\%$ and $93 \pm 5.8\%$, respectively, $p = 3 \times 10^{-5}$, $p = 5 \times 10^{-4}$, $n = 4$). However, in these HSV-2-coinoculated tissues, ACV also suppressed HIV-1 replication by $62 \pm 4.2\%$ ($p = 2 \times 10^{-3}$, $n = 4$) (Figure 1A).

ACV Suppresses HIV Replication in Singly Infected Human Lymphoid Tissue Ex Vivo

Surprisingly, ACV was also found to suppress HIV-1 replication in tonsillar tissues from 38 donors that were not coinoculated with HSV-2 (Figures 1B–1D).

In HIV-1 singly infected tissues, the suppression of HIV-1 replication, measured as p24_{gag} accumulation in culture medium, was dose dependent with a 50% effective concentration (EC₅₀) of 3.1 μ M (95% confidence interval: 1.85–5.24) (Figure 1B). HIV-1 replication, evaluated by the measurement of HIV-1 proviral DNA in tissue blocks at day 12 postinfection, was reduced by 94.8% (interquartile range [IQR] 65.9%–99.8%) in blocks of tonsillar tissues treated with ACV at the concentration of 30 μ M compared with donor-matched untreated blocks. The median viral load was reduced by ~ 1.3 log₁₀, from 3108 gag DNA copies per 10⁴ cells in untreated tissues (3.49 log₁₀; IQR 2.99–4.01) to 162 gag DNA copies per 10⁴ cells in ACV-treated tissues (2.27 log₁₀; IQR 0.09–3.07, $p < 10^{-4}$, $n = 27$) (Figure 1C). Consistently, ACV significantly decreased the amount of p24_{gag} released into the culture medium on average by $80 \pm 4\%$ ($p < 7 \times 10^{-7}$, $n = 38$) in human tonsillar tissues (Figure 1D). The average cumulative production of p24_{gag} in the culture medium of tonsillar tissues was 46.4 ± 8 ng/ml and 16.6 ± 4.6 ng/ml, respectively, in tissues untreated and treated with 30 μ M ACV.

To avoid the possible confounding effects of contaminating products in commercial ACV, we used ACV from three sources and found that all of them similarly suppressed HIV ($p \geq 0.45$, $n = 4$). Suppression of HIV-1 replication by ACV was not associated with T cell depletion, as evidenced from the similar numbers of total T cells (CD4⁺CD3⁺ and CD8⁺CD3⁺, $p \geq 0.5$, $n = 3$) and their subsets of activated (CD25⁺, HLA-DR⁺, or CD38), naive (CD45RA⁺CD62L⁺), or nonnaive (CD45RA⁺CD62L⁺) ($p \geq 0.16$, $n = 3$) cells. Moreover, ACV antiviral activity was restricted to HSV-2 and HIV-1 since, as expected, ACV did not reduce vaccinia virus replication in tonsillar tissue ex vivo (data not shown).

ACV Suppresses Replication of CCR5- and CXCR4-Tropic HIV-1 Variants in Various Human Tissues

To test whether ACV suppressed HIV-1 replication in tissues other than tonsils, we used explants of tissues that also play an important role in HIV transmission and pathogenesis in vivo: lymph nodes and cervicovaginal and colorectal tissues. ACV suppressed HIV-1 replication, measured by p24_{gag} accumulation in culture medium, in lymph nodes by $70 \pm 13.9\%$ ($p = 2 \times 10^{-3}$, $n = 7$), in colorectal tissues by $72.6 \pm 9\%$ ($p = 2 \times 10^{-2}$, $n = 3$), and in cervical tissues by $60.1 \pm 3.5\%$ ($p = 7 \times 10^{-3}$, $n = 3$). ACV treatment of ex vivo human lymph nodes reduced the average cumulative production of p24_{gag} in culture medium from 67.3 ± 28.1 ng/ml to 15.4 ± 6.6 ng/ml, in ex vivo human colorectal tissues from 7.2 ± 6.8 ng/ml to 1.3 ± 1.2 ng/ml ($n = 3$), and in ex vivo human cervicovaginal tissues from 3.8 ± 1.7 ng/ml to 1.4 ± 0.5 ng/ml ($n = 3$) (Figure 2A).

Furthermore, we evaluated the efficiency of ACV in suppressing different HIV-1 variants. We inoculated ex vivo lymphoid tissues with CCR5- and CXCR4-tropic HIV-1 variants X4_{LAI.04}, R5_{SF162}, R5_{BaL}, and R5_{AD8}. As shown in Figure 2B, ACV suppressed replication of all of these isolates in tonsillar tissues with similar efficiency (by 88%, 95%, 89%, and 94%, respectively, as assessed from p24_{gag} production).

Coinfection with Different Endogenous Human Herpesviruses Is Associated with the Anti-HIV Effect of ACV

To test whether suppression of HIV by ACV is related to the antiherpetic activity of this drug, we measured the presence of various HHVs in the tonsillar tissues used in the present work (Figure 2C). Real-time PCR analysis revealed that all 27 tonsillar tissues that were tested for the presence of HHVs were negative for HSV-1, HSV-2, HHV-3 (VZV), and HHV-8 but were infected with HHV-4 (EBV), -5 (CMV), -6, and -7 in various combinations (Figure 2C). CMV was present in 15% of tissues; EBV, in 52%; HHV-7, in 89%; and HHV-6, in all but one tissue (96%).

Similar to what was observed in immunocompromised patients (Lusso and Gallo, 1994), HHV load was increased in tissues ex vivo. There was a 5-fold increase in the median HHV-6 load at day 12 in culture, from 22.4 DNA copies per 10⁴ cells at the time of surgery (IQR 1.8–57.7) to 116.6 DNA copies per 10⁴ cells (IQR 33.7–808, $p = 3 \times 10^{-4}$, $n = 26$). After ACV treatment, the median HHV-6 load at day 12 was reduced to 55.1 DNA copies per 10⁴ cells (IQR 21.3–334.6, $p = 10^{-2}$, $n = 26$), demonstrating that ACV significantly suppressed HHV-6 replication. The sensitivity of HHV-6 to ACV was further evaluated in separate experiments in which ex vivo tonsillar tissues were inoculated with HHV-6B (PL-1 strain). In these experiments, ACV suppressed HHV-6B replication in a dose-dependent manner with an EC₅₀ of ~ 27 μ M. We found that ACV reduced the total production of HHV-6B in culture medium on average by $56.2 \pm 11.4\%$ ($52.2 \pm 12.4 \times 10^8$ DNA copies per ml versus $22.5 \pm 9.4 \times 10^8$ DNA copies per ml in control and ACV-treated tissues, respectively; $p = 3 \times 10^{-3}$, $n = 3$).

HHV-7, HHV-4, and HHV-5 median loads in tonsillar tissues were, respectively, 9.23 (IQR 0.51–56.81), 4.71 (IQR 0.06–448.3), and 0.1 (IQR 0.06–0.1) DNA copies per 10⁴ cells at the time of surgery and 21.87 (IQR 0.9–219.7, $n = 24$), 31.16

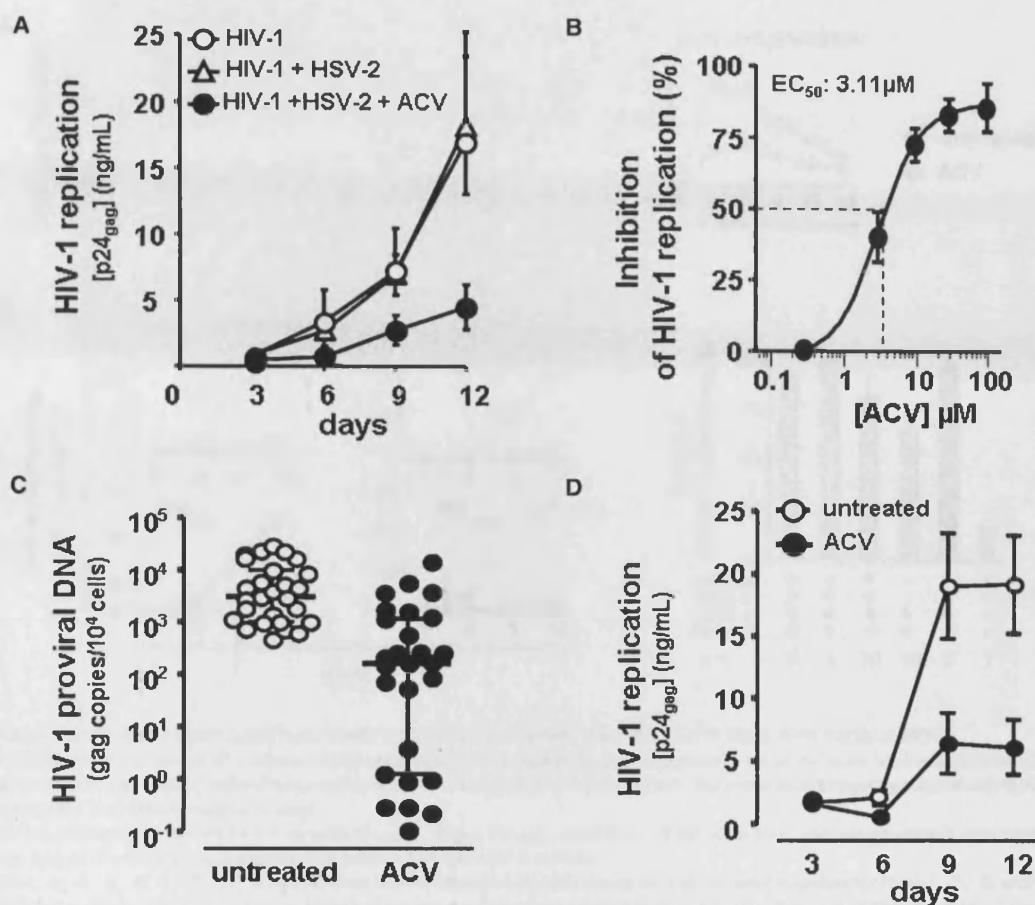


Figure 1. ACV Suppresses HIV Infection in Human Tonsillar Tissues

(A) Blocks of human tonsillar tissue were coinoculated *ex vivo* with HSV-2 strain G and X4_{LAI.04} and treated or not with ACV (30 μM). HIV-1 replication was monitored by measuring p24_{gag} accumulated in culture media over 3 day periods. Presented are means ± SEM of the results with tissues from 4 to 17 donors. For each donor, each data point represents pooled viral release from 27 tissue blocks. Note that ACV suppresses HIV-1 in HSV-2-coinfected tissues.

(B) HIV-1 replication was measured in tonsillar blocks infected with X4_{LAI.04} as in (A). ACV was added at the concentrations of 0.3, 3, 10, 30, and 100 μM, and its anti-HIV activity was evaluated from the suppression of viral replication compared with donor-matched HIV-infected tissues not treated with ACV. The 50% effective concentration (EC₅₀) was estimated by fitting the data to four-parameter logistic regression and was estimated to be 3.1 μM (95% confidence interval: 1.85–5.24). Presented are means ± SEM of the results with tissues from three to seven donors. Note that ACV suppresses HIV-1 replication in the tonsillar tissues in a dose-dependent manner.

(C) HIV-1 proviral DNA load in tissue blocks at day 12 postinfection was measured by real-time PCR. Presented are medians and interquartile ranges of the results (n = 27). Note that ACV efficiently reduces HIV-1 proviral DNA load in blocks of tonsillar tissue.

(D) HIV-1 replication was monitored as in (A). Presented are means ± SEM of the results (n = 38). Note that ACV efficiently suppresses HIV-1 replication in the tonsillar tissues tested.

(IQR 3.63–260.9, n = 14), and 388 (IQR 175.9–2416, n = 4) copies per 10⁴ cells after 12 days of culture.

In all tissues infected with different combinations of HHVs, ACV suppressed HIV-1 replication with a similar efficiency (Figure 2C). HHV-6 was the only HHV that was present in all combinations. Moreover, it seems that the suppression of HIV-1 by ACV is related to the amount of HHV-6. In tissue blocks in which ACV inhibited HIV-1 replication by more than 50%, the median HHV-6 load on day 12 in culture was significantly higher than in the tissues in which ACV suppressed HIV-1 replication by less than 50%: 131.1 DNA copies per 10⁴ cells (IQR 36.1–1154, n = 23) versus 21.6 DNA copies per 10⁴ cells (IQR 0.6–77.4, n = 4; p = 4 × 10^{−2}), respectively. Furthermore, we documented a correlation between the ACV-mediated suppression

of HIV-1 replication, as measured by p24_{gag} release, and the level of HHV-6 in tissues where the HIV-1 inhibition was suboptimal (between 0 and 99%; r = 0.43, p = 0.03, n = 25).

Although several HHVs, such as HHV-6, are ubiquitous and transmitted early in childhood, we identified a tissue in which there was no detectable HHV. In agreement with our hypothesis, in this tissue, ACV suppression of HIV-1 (at the concentration of 30 μM) was negligible (16%) (Figure 2C).

Coinfection with HHVs Is Necessary and Sufficient for the Anti-HIV Effect of ACV

To prove this claim, we performed experiments with MT4 cells, an HHV-uninfected T cell line that efficiently supports the replication of HIV-1_{LAI.04}. We found that ACV did not suppress

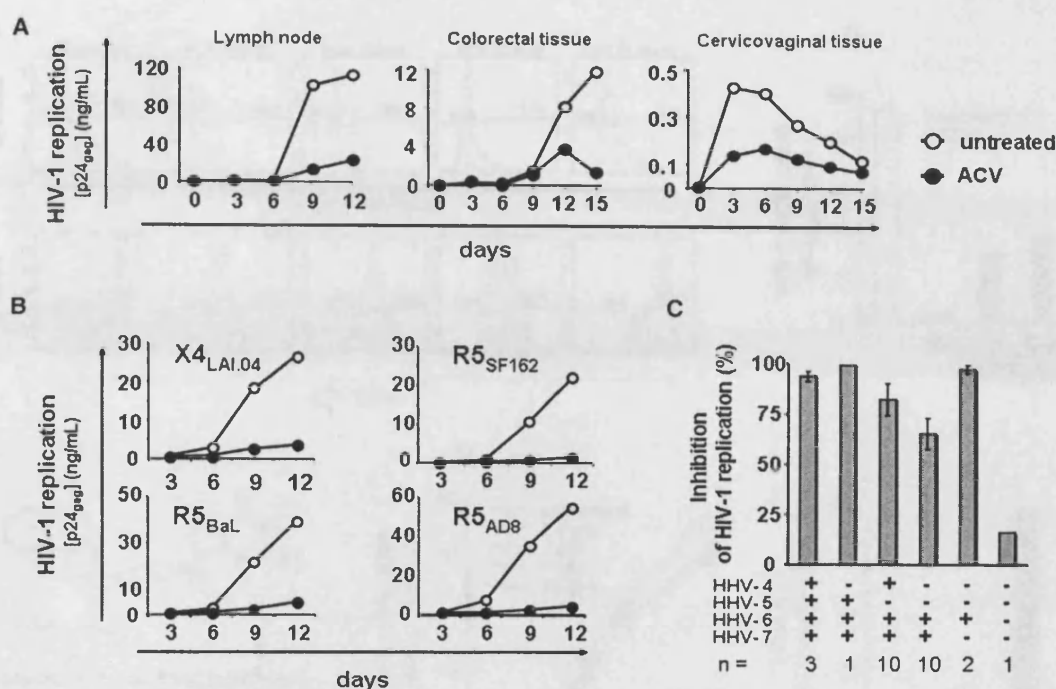


Figure 2. ACV Suppresses Replication of Different HIV-1 Variants in Human Tissues Coinfected with Various HHVs

(A) HIV-1 replication in blocks of human lymph nodes and colorectal and cervicovaginal tissues was monitored as in Figure 1A. For each type of tissue, the graph represents a typical result of three to seven experiments performed with tissues from different donors. Note that ACV efficiently suppresses replication of HIV-1 in lymph nodes and colorectal and cervicovaginal tissues.

(B) Examples of ACV suppression of different HIV-1 variants (X4_{LAI.04}, R5_{BaL}, R5_{SF162}, and R5_{AD8}). Each data point represents pooled viral release from 27 tissue tonsillar blocks. Note that ACV efficiently suppresses replication of all four HIV-1 variants.

(C) Presence of HHV-1, -2, -3, -4, -5, -6, -7, and -8 by real-time PCR in blocks of tonsillar tissue. All tissues were negative for HHV-1, -2, -3, and -8. Presented are means \pm SEM of the results with tonsils from *n* donors. Note that there are no significant differences in the level of ACV suppression of HIV-1 replication in tissues infected with various HHVs.

HIV-1_{LAI.04} ($EC_{50} > 250 \mu M$) in these cultures, in agreement with earlier observations (Barral et al., 2003). To test whether HHV infection is sufficient for the anti HIV-1 effect of ACV, we added various amounts of HHV-6B-infected MT4 cells to HHV-free MT4 cultures infected with HIV-1_{LAI.04}. As expected, ACV suppressed HHV-6. After 3 days of culture, the fraction of HHV-6B-infected cells was reduced in a dose-dependent manner, as measured by flow cytometry (Figure 3A). Importantly, in these cultures, ACV suppressed HIV replication as well, as evaluated by the number of p24_{gag}⁺ T cells and p24_{gag} release into the culture medium (EC_{50} of $\sim 50 \mu M$) (Figures 3A and 3B). In these experiments, 9% of HHV-6B-infected cells were sufficient to suppress HIV-1_{LAI.04} replication by $\sim 70\%$ (Figure 3A). We found that, in agreement with earlier publications (De Clercq et al., 2001), the concentration that corresponds to the EC_{50} in these experiments did not affect cell viability (50% cytotoxic concentration $> 250 \mu M$).

To further prove that ACV phosphorylation is required for HIV-1 inhibition, we synthesized the monophosphorylated ACV prodrug acyclovir-(1-naphthyl [methoxy-L-alaninyl]) phosphoramidate (Cf2649) (Figure 3C). This compound bypasses the requirement of HHVs for the activation of ACV since it is already monophosphorylated. Indeed, when applied to (HHV-free) MT-4 cells, Cf2649, in contrast to nonphosphorylated ACV ($EC_{50} > 250 \mu M$), suppressed HIV-1 replication with an EC_{50} of $\sim 3 \mu M$

(Figures 3D and 3E). Similar results were obtained with another HHV-free T cell line (CEM) (data not shown).

In conclusion, we demonstrated that neither in HHV-free tissue nor in HHV-free cell lines does ACV suppress HIV-1 infection. Reconstitution of the cell line system with HHV-6-infected cells or bypassing the HHV-kinases requirement by applying an ACV monophosphate derivative makes HHV-free systems susceptible to HIV suppression by ACV.

ACV-TP Inhibits HIV-1 RT

Since we demonstrated that the anti-HIV-1 activity of ACV requires HHV-mediated activation and that phosphorylated derivatives of ACV are produced in HHV-infected tissues (see Supplemental Data and Figure S1 available online), we hypothesized that ACV-TP, into which ACV is ultimately converted (Elion, 1983), interferes with the activity of the HIV-1 RT and directly suppresses HIV-1 replication.

We tested whether ACV-TP suppresses RT by measuring the polymerizing activity of RT from lysed HIV-1 using an exogenously added HIV-1 template. We observed a dose-dependent inhibition of HIV-1 RT activity by ACV-TP, while we noted no suppression of HIV-1 RT activity by ACV itself, even at high concentrations (Figure 4A).

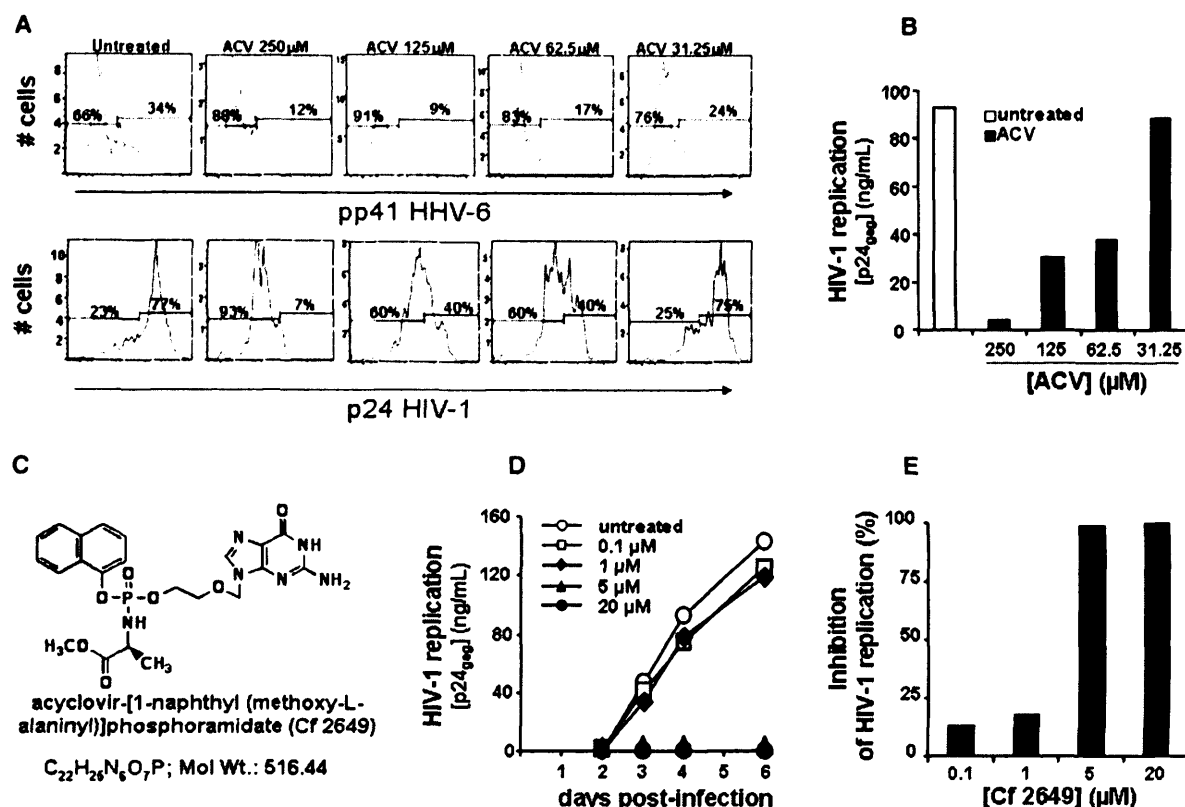


Figure 3. ACV Suppresses HIV-1 in HHV-Free MT-4 Cell Cultures Either in the Presence of HHV-6-Infected Cells or as Monophosphorylated Prodrug

(A) HIV-1_{LAI 04}-infected MT4 cells were cocultured with HHV-6B-infected MT4 cells in a ratio of 10:1. Presented are the distributions of HHV-6B (upper panel) and HIV-1_{LAI 04}-infected cells (lower panel), as measured by flow cytometry at day 3 post-HIV-1 infection, in cultures untreated or treated with various concentrations of ACV. Note that the fraction of both HHV-6B- and HIV-1-infected cells is reduced in a dose-dependent manner by ACV treatment.

(B) HIV-1_{LAI 04}-infected MT4 cells were cocultured with HHV-6B-infected MT4 cells. HIV-1 replication was monitored as in Figure 1A. Note that the replication of HIV-1 in HHV-6B/HIV-1-infected cocultures is suppressed by ACV treatment in a dose-dependent manner.

(C) The compound acyclovir-[1-naphthyl (methoxy-L-alaninyl)] phosphoramidate (Cf2649) has been synthesized as described in the Supplemental Data.

(D) HIV-1_{LAI 04}-infected HHV-free MT4 cells were treated with various concentrations of the ACV monophosphorylated prodrug Cf2649. Presented are the kinetics of HIV-1_{LAI 04} replication, monitored by measuring p24_{gag} accumulated in culture media at days 2, 3, 4, and 6 postinfection. Presented data are representative of two experiments. Note that compound Cf2649 suppresses the replication of HIV-1_{LAI 04} in a dose-dependent manner.

(E) HIV-1_{LAI 04}-infected MT4 cells not infected with any HHVs were treated with various concentrations of the compound Cf2649. The cumulative release of p24_{gag} into culture media over 6 days of culture treated with various concentrations of the compound Cf2649 is presented as a fraction of the p24_{gag} production in the untreated cultures. Presented data are representative of two independent experiments. Note that compound Cf2649 suppresses the replication of HIV-1_{LAI 04} in a dose-dependent manner.

Since ACV-TP is a guanosine-5'-triphosphate analog, we investigated whether it acts as a nucleotide RT inhibitor by testing whether dGTP prevents ACV-TP inhibition of RT. We found that, at ACV-TP concentrations of 3.38 μ M or 33.8 μ M, HIV-1 RT inhibition was inversely dependent on the dGTP concentrations. At the highest concentration of ACV-TP tested, no competition with dGTP was observed (Figure 4B). Since ACV-TP lacks an additional hydroxyl group (present in dGTP and essential for DNA chain polymerization), we further hypothesized that the above-described suppression of HIV-1 RT by ACV-TP is similar to its suppression of HSV DNA polymerase, namely by incorporation into the nascent HIV DNA resulting in its chain termination (Reardon and Spector, 1989).

To prove this hypothesis, we used a gel-based assay (Marchand and Gotte, 2003; Marchand et al., 2007) and found that HIV-

1 RT incorporated ACV-TP into the DNA primer as efficiently as the natural substrate dGTP. The efficiency of single-nucleotide incorporation events (k_{cat}/K_m) under steady-state conditions for dGTP and ACV-TP was 14.7 μ M⁻¹min⁻¹ and 14.0 μ M⁻¹min⁻¹, respectively (Table S1). Furthermore, ACV-TP incorporation resulted in complete DNA chain termination, as shown in Figures 5A and 5B. A primer that contained the natural dGMP at its 3' end was successfully extended in the presence of dTTP, which is the substrate for the following three consecutive template positions (Figure 5B, left panel). In contrast, DNA synthesis was effectively blocked when the primer was terminated with ACV-MP. Even the relatively high concentration of 50 μ M of dTTP did not permit its incorporation (Figure 5B, right panel). These data suggest that the mechanism of action of ACV is similar to the currently approved anti-HIV nucleoside reverse transcriptase inhibitors (NRTIs).

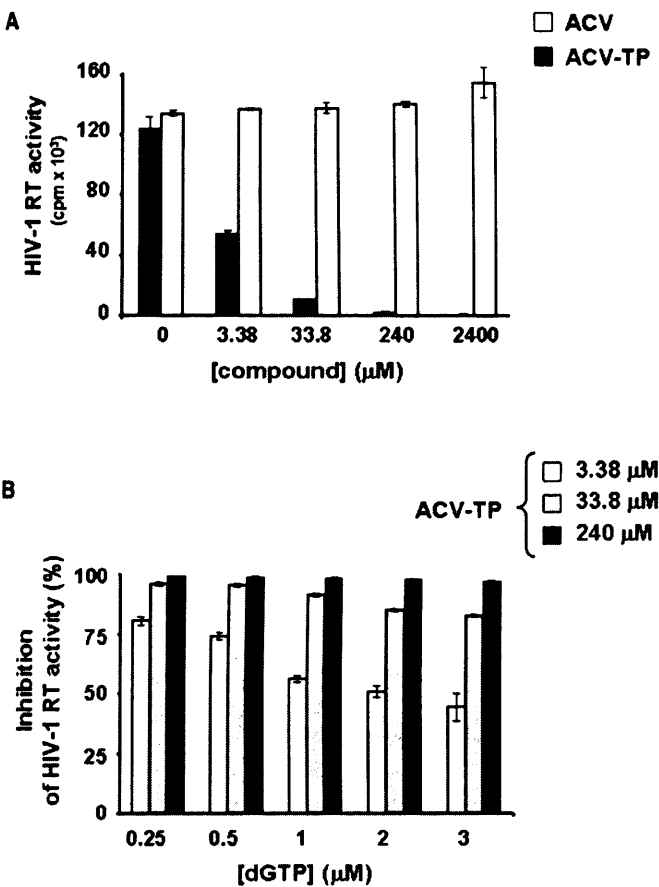


Figure 4. ACV-TP Inhibits HIV-1 RT in Exogenous Template Reverse Transcriptase Assays
(A) Exogenous template reverse transcriptase assays were performed in the presence of various concentrations of ACV or ACV-TP as described in the Experimental Procedures. Presented are means \pm SEM of the results of two experiments performed in duplicates. Note that ACV-TP inhibits HIV-1 RT in a dose-dependent manner.
(B) The dependence of the RT inhibition by ACV-TP on the concentration of dGTP was evaluated using an exogenous template reverse transcriptase assay. dGTP was used at the indicated concentrations. The reactions were performed in the presence of the indicated concentrations of ACV-TP. Presented are means \pm SEM of the results of two experiments performed in duplicate. Note that inhibition of HIV-1 RT activity by ACV-TP is inversely dependent on the concentration of dGTP.

DNA chain termination by NRTIs is not irreversible but can be subjected to phosphorolytic excision of the incorporated drug by HIV RT (Meyer et al., 1998). Binding of the next complementary nucleotide following the DNA chain terminator can lead to the formation of a dead-end complex (DEC). In this complex, HIV RT is trapped in a conformation that blocks the excision reaction. Thus, to test whether ACV-MP-terminated DNA is accompanied with the formation of a dead-end complex, we evaluated the inhibition of ACV-MP phosphorolytic excision from the 3' end of the primer by HIV-1 RT. The excision of a DNA chain terminator requires the presence of pyrophosphate (PPi) or pyrophosphate donor molecules (such as ATP). We found that HIV-1 RT is capable of excising the incorporated ACV-MP in the presence of physiologically relevant concentrations of ATP (data not shown)

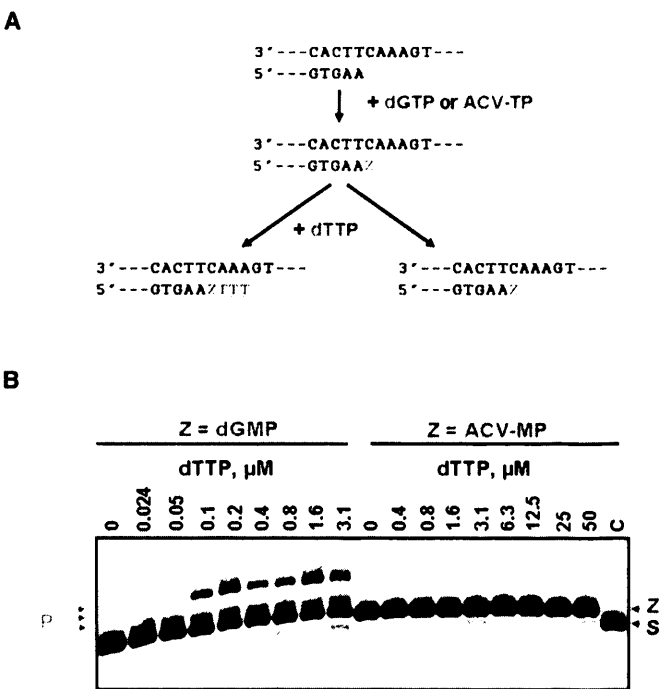


Figure 5. Chain Termination with ACV-MP
(A) The reaction scheme shows the relevant region of the primer and template and two possible outcomes of the primer elongation. Elongation of the primer in the presence of dGTP or ACV-TP is indicated by the "Z" in red, which refers to the incorporated monophosphate dGMP or ACV-MP, respectively. Incorporation events following position Z with dTTP are in blue and underlined.
(B) The primer (S, lane C) was initially elongated to incorporate dGMP or ACV-MP, respectively, at the 3' end referred to as Z. Incubation with increasing concentrations of the next nucleotide dTTP resulted in three nucleotide incorporation events with the dGMP-terminated primer, which are labeled as products (P). Note that the ACV-MP-terminated primer is not extended, which shows that the inhibitor acts as a chain terminator.

as demonstrated for certain NRTIs. However, to analyze whether the ACV-terminated primer permits formation of a dead-end complex, the ATP-dependent excision reaction was assayed in the presence of increasing concentrations of the next complementary nucleotide. We found that the next complementary nucleotide caused 50% inhibition of ACV-MP excision (IC₅₀) at a concentration of ~26 μM (Figures 6A and 6B). These data strongly suggest the formation of a dead-end complex in ACV-MP-incorporated templates. The higher IC₅₀ (26 μM versus 3.1 μM) in comparison to reactions conducted with the control DNA chain terminator ddGMP was probably due to the acyclic nature of ACV-MP. In general, the excision reaction was inefficient; only 10%–15% of the terminated primer strands were rescued for continuation of DNA synthesis at low concentrations of the next complementary nucleotide.
To provide additional direct evidence of dead-end complex formation, we employed site-specific footprinting that allowed us to determine the position of HIV-1 RT on its primer/template at single-nucleotide resolution (Marchand and Gotte, 2003; Marchand et al., 2007). Nucleotide binding and formation of a dead-end complex can only occur in the posttranslocated conformation in which the nucleotide-binding site of HIV-1 RT has

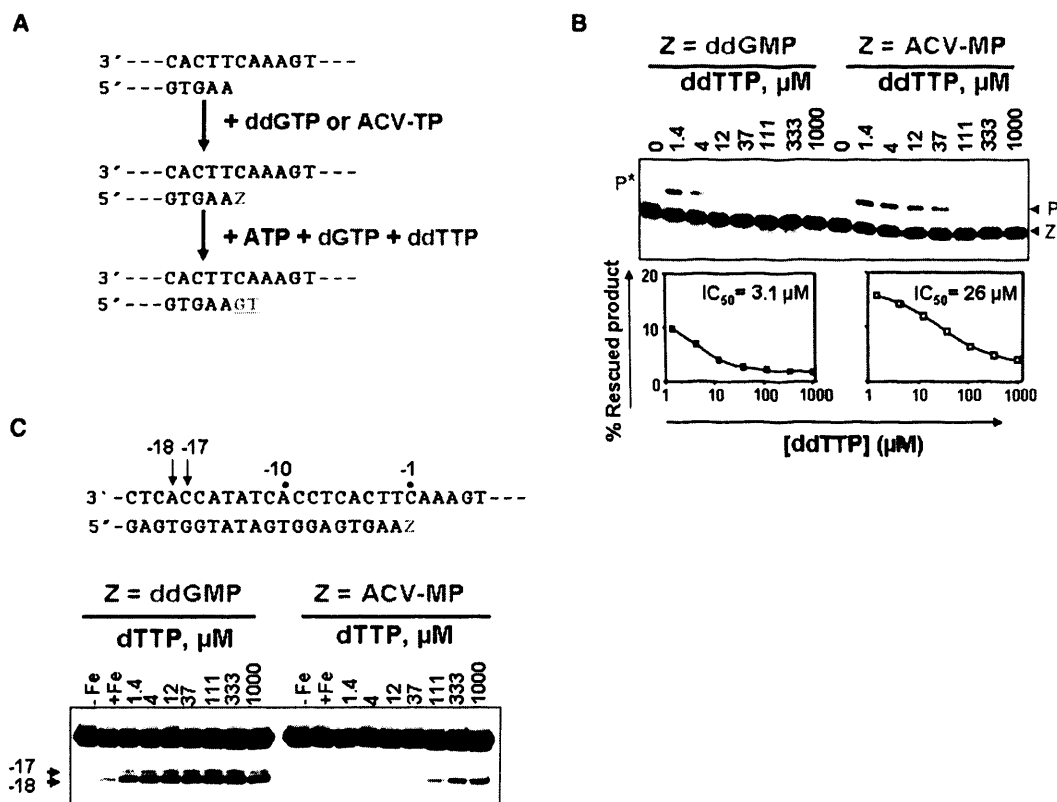


Figure 6. Inhibition of ATP-Dependent Excision in the Presence of the Next Nucleotide Substrate

ATP-dependent excision was monitored in a combined excision/rescue assay as previously described in the Supplemental Experimental Procedures.

(A) The reaction scheme shows the relevant region of the primer and template. Extension of the primer in the presence of ddGTP or ACV-TP is indicated by the "Z" in red. The excision of a DNA chain terminator requires the presence of ATP as pyrophosphate donor. The 3'-ultimate nucleotide (Z) was excised with ATP, and the simultaneous presence of dGTP and ddTTP (in blue) allowed the rescue of DNA synthesis.

(B) The combined excision/rescue reaction was studied with ddGMP and ACV-MP-terminated primers. Z refers to the terminated primer and P to the rescued product. The asterisk indicates dGMP misincorporation in the absence of the correct ddTTP substrate. Quantification of excision/rescue reactions for ddGMP and ACV-MP are plotted. The concentration of the next complementary nucleotide required to inhibit 50% of the reaction is calculated on the basis of these two curves. Note that ACV-MP-terminated primer excision/rescue reaction is inhibited due to dead-end complex formation.

(C) Incorporation of ACV-TP produces a dead-end complex with HIV-1 RT. Presented is site-specific footprinting of HIV-1 RT with ddGMP- and ACV-MP-terminated primers in the presence of increasing concentrations of the next complementary nucleotide. Lanes -Fe and +Fe show control reactions in the absence and presence of divalent Fe^{2+} ions that cause site-specific cleavage on the labeled template. The arrows and the sequence underneath the gel show the position of the oxidative cleavage on the template strand at positions -17 and -18, which are indicative for post- and pretranslocated complexes, respectively. Note that, for ACV-MP-terminated primer, no cleavage occurs at low next complementary nucleotide concentrations, indicating that the complex between RT and terminated primer is fragile, whereas, at higher concentrations of the next complementary nucleotide, RT is blocked in a dead-end complex.

been cleared. In this conformation, the RT has moved a single nucleotide further downstream relative to the pretranslocated state. Our footprints are based on oxidative cleavage on the template strand at positions -17 and -18, which are indicative of post and pretranslocated complexes, respectively. We compared footprints of complexes with ddGMP- and ACV-MP-terminated primers in the presence of increasing concentrations of the next complementary nucleotide (Figure 6C). Low concentrations of the next complementary nucleotide are sufficient to stabilize the posttranslocated complex when the primer was terminated with ddGMP, proving the formation of a dead-end complex. A similar pattern is seen with ACV-MP, although the concentration of the next nucleotide required to stabilize the complex is higher. Oxidative cleavage in case of ACV-MP did not occur in the absence of or at low concentrations of the next complementary nucleotide. These findings suggest that, at the low next comple-

mentary nucleotide concentration, the complex between RT and terminated primer formed at the site of the ACV-MP termination is relatively fragile—perhaps prone to dissociation. In contrast, at high concentration of the next complementary nucleotide, RT can be trapped in a dead-end complex. The dissociation constant for ACV-terminated primers in the presence of a high concentration of the next nucleotide (111 μM) is increased ~ 3 -fold (Figure S2).

Thus, these data explain why the excision of ACV-MP is generally inefficient at both low and high concentrations of the next complementary nucleotide. At low concentrations, the complex between RT and ACV-MP is relatively unstable, while, at higher concentrations, a DEC is formed.

To prove that ACV targets HIV-1 RT in human tissues as in cell-free assays, we tested two pairs of HIV isogenic strains that are resistant to some of the currently approved NRTIs. The isolate

AZT.4x containing the mutations at D67N, K70R, T215Y, and K219Q express complete resistance to AZT and resulted to be as sensitive to ACV in infected tissues as its parental HIV-1 variant (Figure S3A). However, an HIV-1 isolate carrying the M184V mutation (that confers resistance to Lamivudine) was less sensitive to ACV than its parental strain (Figure S3B). The EC_{50} for the M184V isolate was approximately four times higher than that for the parental HIV-1 isolate. Thus, the evidence that a specific isolated mutation of one amino acid in RT can reduce the sensitivity of an HIV-1 isolate to ACV further proves that HIV-1 RT is targeted and suppressed by ACV in human tissues, in agreement with the data on ACV suppression of HIV-1 RT in cell-free systems.

DISCUSSION

Growing epidemics of HIV-1 infection, especially in countries with limited resources, and the emergence of drug-resistant viruses make it necessary to find ever new safe, efficient, and inexpensive strategies against this virus. Strategies as effective and safe as those developed against several other human pathogens such as HSV, which can be efficiently treated with ACV, have yet to be developed against HIV. The antitherpetic specificity of ACV is primarily based on the unique ability of HHV-encoded kinases to phosphorylate ACV to its monophosphate derivative, which is subsequently converted into the antivirally active ACV-TP (Reardon and Spector, 1989). Consistent with its highly restricted antitherpetic activity, ACV is not currently used as a direct HIV-1 inhibitor.

Our findings demonstrate (1) the direct inhibitory effect of ACV-TP, but not of ACV itself, on isolated HIV-1 RT in a cell-free system, (2) the suppression of HIV-1 replication by ACV in human tissues and in T cell lines if and only if they carry HHV that phosphorylate ACV, and (3) the ability of a phosphorylated ACV prodrug to bypass the requirement of HHV to suppress HIV. Thus, we provide definitive experimental evidence of inhibition of HIV-1 RT activity by phosphorylated ACV and demonstrate that ACV phosphorylation occurring in HHV-infected human tissues transforms this widely used antitherpetic drug into an HIV-1 inhibitor.

The direct suppression of HIV-1 RT activity by ACV-TP was demonstrated in two different cell-free assays, one using recombinant HIV-1 RT and the other using RT extracted from lysed HIV-1 virions. We showed that ACV-TP is incorporated into the nascent viral DNA chain with a level of efficiency similar to that of its natural equivalent dGTP. Incorporation of ACV-TP results in the termination of reverse transcription, while the excision of the incorporated ACV-MP from the DNA chain is partially inhibited because of the fragility of the complex between HIV-1 RT and the terminated DNA chain or because of the formation of a dead-end complex. These properties of ACV resemble those of certain approved anti-HIV NRTIs (Marchand and Gotte, 2003; Meyer et al., 1999), and we showed here that ACV-TP, a non-phosphonate acyclic nucleoside analog, inhibits HIV-1 RT.

To further confirm that, in HIV-1-infected cells, ACV-triggered HIV-1 suppression is mediated by RT, we identified a pair of isogenic HIV-1 strains differing only by one amino acid (M184V) in RT but having different sensitivities to ACV in ex vivo human tissues. Future studies on selection of ACV-resistant HIV-1 isolates

and testing of various multidrug-resistant HIV-1 isolates for ACV sensitivity will reveal the exact set of RT mutations associated with reduced sensitivity to ACV and the rate of their evolution. Identification of ACV-resistant mutants does not, in general, exclude the use of ACV against HIV-1 variants resistant to other NRTIs; for instance, ACV efficiently suppresses the replication of AZT-resistant HIV-1.

As demonstrated in a cell-free system, to inhibit HIV-1 RT, ACV has to be converted into ACV-TP. In human tissues, such conversion requires HHV infection. Accordingly, we found that, in the presence of HHVs that are capable of phosphorylating ACV, HIV-1 was inhibited in ACV-treated tonsils, lymph nodes, and cervico-vaginal and colorectal tissues, where the critical events of HIV-1 pathogenesis and transmission occur in vivo.

Various HHVs, including the ubiquitous HHV-6 detected in all but one tissue ex vivo, may mediate HIV-1 suppression by ACV. The level of HHV-6 replication may be essential. In tonsillar tissues, in which ACV inhibited HIV replication by more than 50%, the median HHV-6 load was significantly higher than in the tissues in which ACV suppressed HIV replication by less than 50%. Moreover, in tissues where the HIV-1 inhibition was suboptimal, there was a correlation between the ACV-mediated suppression of HIV-1 replication and the level of HHV-6. These results demonstrate again the critical role of HHVs in ACV-mediated suppression of HIV-1 and indicate the importance of HHV-6 in mediating HIV-1 suppression by ACV in our ex vivo tissue system.

HHV-6 and HHV-7 are ubiquitous viruses, and, therefore, the probability of finding an HHV-free tissue is very low. Nevertheless, by testing tissues from multiple donors, we identified one tonsillar tissue that was not infected by any HHV. ACV did not inhibit HIV-1 replication in this tissue.

However, to further demonstrate that HHVs are necessary and sufficient for the anti-HIV effect of ACV, we used the HHV-uninfected MT4 cell line. Consistent with the proposed mechanism based on HHV-mediated activation, ACV did not inhibit HIV replication in this HHV-free cell line. However, when HHV-6-infected cells were added to the HIV-1-infected cultures, ACV became an HIV-1 suppressor. This effect was dependent on both the concentration of ACV and the fraction of HHV-6-infected cells. Apparently, in HHV reconstituted MT4 cultures, ACV that is phosphorylated in HHV-6-infected cells is transferred to HIV-1-infected cells, since the majority of these cells were not coinfecting with HHV-6. These results are in full agreement with the published data on the transfer of phosphorylated ACV between cells (Burrows et al., 2002; Degreve et al., 1999). In tissues, transfer of phosphorylated ACV between cells is facilitated by specialized contacts (Nicholas et al., 2003). These and probably other factors (e.g., rapid proliferation and large endogenous dNTP pools, as well as a high level of HIV-1 replication) may contribute to a lower sensitivity of HIV-infected cell lines to ACV compared with integral tissues.

To further demonstrate the necessity of ACV activation for HIV suppression, we synthesized an already monophosphorylated (activated) ACV in which the phosphate is masked by lipophilic groups. In contrast to nonphosphorylated ACV, this prodrug suppressed HIV-1 in HHV-free cultures of MT4 or CEM cells. Although enzymatic reactions mediating ACV prodrug conversion into its active form have been described (Congiatu et al., 2007),

the entire process of this conversion remains to be elucidated. Nevertheless, our results provide strong evidence that, upon phosphorylation, ACV suppresses HIV-1 replication in cells. Importantly, these experiments also demonstrate the feasibility of designing a new class of anti-HIV compounds. However, unlike ACV (with a proven safety record and exhaustively studied pharmacokinetics), ACV prodrug effects in various systems have to be evaluated in order to form conclusions on their potential clinical use.

In summary, the following mechanism seems to be responsible for ACV suppression of HIV-1 in human tissues *ex vivo*, the majority of which carry one or several HHVs, including HHV-6: ACV is monophosphorylated by herpesviral enzymes in HHV-infected cells and then further converted to ACV-TP, which suppresses HIV by inhibiting HIV-1 RT, similarly to other NRTIs.

Our results suggest that ACV may be therapeutically beneficial for various HIV-1-infected patients, since the majority of humans are already infected with HHV-6, often together with other HHVs that activate ACV at least during reactivation. In particular, in immunocompromised patients for whom HHV replication is frequent, those HHVs that are not completely suppressed by ACV (e.g., HHV-6) can continuously generate phosphorylated ACV derivatives. The incomplete inhibition of HHV-6 by ACV confirmed in our experiments is consistent with the much higher K_i of ACV-TP for HHV-6 DNA polymerase (UL69) compared with that of HSV-2 or HSV-1 DNA polymerase (Bapat et al., 1989).

However, clinical trials are needed to test whether replication of HHVs—in particular of HHV-6, which is typically maintained in various organs, including the intestines and the vagina, even in immunocompetent individuals (De Bolle et al., 2005)—would be sufficient to suppress HIV-1 in ACV-treated individuals. Also, clinical trials should reveal whether the range of ACV concentrations used in our study to suppress HIV-1 is clinically relevant. Although ACV penetration efficiency and drug clearance were unknown for *ex vivo* tissues, the calculated EC_{50} of 3.1 μ M was in the range of what was reported *in vivo*: a dose of 1 g of oral valacyclovir per day results in a plasma peak concentration of 29.5 μ M, a minimum concentration in serum of 3 μ M, and a plasma concentration time curve of 89 μ M/h ACV (Lycke et al., 2003; Soul-Lawton et al., 1995). Moreover, the therapeutic dose of the orally administered ACV prodrug valacyclovir, depending on clinical indications, can be increased to as much as 3 g per day.

Recent clinical trials performed so far are in agreement with our *ex vivo* results and demonstrated that ACV is efficient in suppressing HIV in HSV-2-coinfected individuals (Baeten et al., 2007; Delany et al., 2007; Dunne et al., 2008; Nagot et al., 2007; Zuckerman et al., 2007). Valacyclovir treatment, at the dose of 1 g per day, reduced the HIV-1 plasma load in these individuals by 50%–70%, an effect comparable to that reported here for human tissues infected with other HHVs. This HIV-1 viral load reduction was clinically beneficial (Corey, 2007) and similar to that reported for AZT or stavudine monotherapy (~70%) (Delta Coordinating Committee and Delta Virology Committee, 1999; Katzenstein et al., 2000; Rey et al., 1998). In contrast to the established ACV activity in reducing HIV load in HSV-2 coinfected patients, recent trials failed to demonstrate that HSV-2 suppressive therapy prevents acquisition of HIV-1 (Celum et al., 2008; Cohen, 2007; Lisco and Vanpouille, 2008; Watson-Jones et al.,

2008). However, none of the approved NRTIs widely used for therapy was yet developed into an efficient preventive drug when used alone.

Our results provide new insights into the effect of ACV in HIV-1-infected patients. In all previous trials, the effect of ACV on HIV-1 was considered to be indirect and due to the suppression of HSV-2-mediated inflammation. Here, we demonstrate that ACV directly suppresses HIV-1 RT in HHV-coinfected tissue. This effect depends on the levels of HHVs, which were not evaluated in these clinical trials or in previous *in vitro* studies (Resnick et al., 1986). Obviously, the results obtained in tissues *ex vivo* should be extrapolated to the *in vivo* situation with caution. However, the reliance on lymphotropic HHVs to create the active HIV suppressor at the site of HIV replication may, in principle, solve the critical pharmacological problem of drug delivery.

In conclusion, our data on HIV-infected tissues coinfected with various HHVs suggest that ACV may be used to decrease the HIV load in both the peripheral blood and the genital compartments of patients infected with one or several HHVs, including the highly prevalent HHV-6 (Campadelli-Fiume et al., 1999). Although the magnitude of HIV-1 suppression by ACV as well as by the currently approved NRTIs is too low to be used in monotherapy, it is sufficient to be an important part of drug cocktails.

In general, the combination of ACV with an endogenous HHV infection to suppress HIV may constitute a new principle of anti-HIV therapy—a “binary weapon” in which one inert component is converted by another endogenous component into an active therapeutic compound. In the case of ACV, its exceptionally low toxicity and the low cost of ACV and related drugs that have been safely used in humans for more than 30 years, as well as their existing formulations as pills and creams, make them potentially applicable for HIV treatment, possibly in combination with other drugs. New targeted clinical trials will test whether ACV and its derivatives can be used for this new purpose in line with a popular trend to identify new uses for old drugs (Chong and Sullivan, 2007).

EXPERIMENTAL PROCEDURES

Tissue and Cell Culture

Tonsillar tissues from routine surgery were obtained from the Children's National Medical Center (Washington, DC). Lymph nodes and colorectal and cervicovaginal tissues were obtained either from routine surgery or from cadavers through the National Disease Research Interchange (Philadelphia, PA). All tissues were obtained according to IRB-approved protocols. Tissues were dissected into 2 mm³ blocks and cultured as described earlier (Fletcher et al., 2006; Glushakova et al., 1995; Grivel et al., 2007). MT4 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium with 10% heat-inactivated FCS.

Viral Infections

Tissue inoculation with HIV-1 and HHV-6B was performed as described earlier (Grivel et al., 2007). For further details, see the Supplemental Experimental Procedures.

ACV and ACV Monophosphorylated Prodrug Treatment

ACV pharmaceutical formulations for intravenous infusion (Bedford Laboratories, Bedford, OH; NDC 55390-612-10) were diluted in distilled water and used at the indicated concentrations. A different pharmaceutical formulation for intravenous infusion (American Pharmaceutical Partners, Schaumburg, IL; NDC 63323-325-10) and a commercial preparation of ACV (Sigma-Aldrich, St. Louis, MO) were tested for HIV-1 suppression in matched tonsillar tissues

from four donors, with similar results. Synthesis of the monophosphorylated ACV prodrug acyclovir-(1-naphthyl [methoxy-L-alaninyl]) phosphoramidate (Cf2649) is described in the Supplemental Experimental Procedures. Compounds were added to the culture medium 12 hr prior to HIV-1 infection and again at each culture medium change.

Real-Time PCR

The HHV load and the HIV-1 proviral load in tissues were determined by measurement of the number of viral DNA copies. DNA from two blocks of tissue was extracted with the QIAamp kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. For further details on real-time PCR TaqMan assay, primers, and probes sequences, see the Supplemental Experimental Procedures.

Exogenous Template RT Assay

Exogenous template HIV-1 RT assays were performed as described previously (Gorelick et al., 1990), with modifications specified in the Supplemental Experimental Procedures.

Steady-State Kinetics, ATP-Dependent Excision, and Site-Specific Footprinting

These assays were performed as described previously (Gotte et al., 1998; Marchand and Gotte, 2003; Marchand et al., 2007) with modifications specified in the Supplemental Experimental Procedures.

Statistical Analysis

Each datum point is the result of analysis of sets of 9 to 27 tissue blocks derived from each of *n* donors, where *n* is indicated in the text. Since the absolute level of HIV-1 replication varied from donor to donor, for every experiment, we compared these levels using data from matched control blocks as the basis of normalization. This allowed us to pool results obtained from tissues from different donors. We analyzed these data using a two-tailed paired Student's *t* test. Because the distribution of the numbers of HHV-6 and HIV-1 (proviral) DNA equivalents failed the Kolmogorov-Smirnov normality test, we used distribution-free nonparametric methods (Wilcoxon Match-Pairs, Signed-Ranks Test, and Mann-Whitney U test) to evaluate the significance of the differences between various experimental groups. However, when these data were log₁₀ transformed, the normality was achieved and we applied parametric methods (paired or unpaired Student's *t* test). The statistical significance of differences between data from various experimental groups evaluated after transformation was similar to that evaluated with nonparametric methods applied to non-transformed results. Depending on the type of statistical analysis, the pooled data are presented either as means \pm standard error of the mean (SEM) or as median and interquartile range (IQR). All of the hypothesis tests were two-tailed, and a *p* value of ≤ 0.05 defined statistical significance.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at <http://www.cellhostandmicrobe.com/cgi/content/full/4/3/260/DC1/>.

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Mechanisms Associated with HIV-1 Resistance to Acyclovir by the V75I Mutation in Reverse Transcriptase*

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It has recently been demonstrated that the anti-herpetic drug acyclovir (ACV) also displays antiviral activity against the human immunodeficiency virus type 1 (HIV-1). The triphosphate form of ACV is accepted by HIV-1 reverse transcriptase (RT), and subsequent incorporation leads to classical chain termination. Like all approved nucleoside analogue RT inhibitors (NRTIs), the selective pressure of ACV is associated with the emergence of resistance. The V75I mutation in HIV-1 RT appears to be dominant in this regard. By itself, this mutation is usually not associated with resistance to currently approved NRTIs. Here we studied the underlying biochemical mechanism. We demonstrate that V75I is also selected under the selective pressure of a monophosphorylated prodrug that was designed to bypass the bottleneck in drug activation to the triphosphate form (ACV-TP). Pre-steady-state kinetics reveal that V75I discriminates against the inhibitor at the level of catalysis, whereas binding of the inhibitor remains largely unaffected. The incorporated ACV-monophosphate (ACV-MP) is vulnerable to excision in the presence of the pyrophosphate donor ATP. V75I compromises binding of the next nucleotide that can otherwise provide a certain degree of protection from excision. Collectively, the results of this study suggest that ACV is sensitive to two different resistance pathways, which warrants further investigation regarding the detailed resistance profile of ACV. Such studies will be crucial in assessing the potential clinical utility of ACV and its derivatives in combination with established NRTIs.

clinic to treat infections caused by herpes simplex virus 1 and 2 (HSV-1 and HSV-2) (1–3). As its valyl prodrug, and to a lesser extent as parent ACV, it is also used in treating varicella zoster infections. The drug is an acyclic guanosine analogue that needs to be selectively converted to its triphosphate form (ACV-TP) that is accepted by the viral polymerase and acts as a chain terminator. The herpesviruses provide the kinases that generate the monophosphate (ACV-MP), whereas cellular enzymes are required to synthesize the triphosphate form (4–6). ACV-TP competes with intracellular dGTP pools for incorporation. Once incorporated, it acts as a chain terminator because of the lack of a structural equivalent of the 3'-hydroxyl group of the sugar moiety of a natural nucleotide (7, 8). The next complementary nucleotide, immediately downstream of the ACV-terminated 3'-end of the primer, can still bind to the HSV DNA polymerase and triggers formation of a stable dead-end complex (DEC) (9).

It has recently been demonstrated that under certain conditions ACV also exhibits antiviral activity against the human immunodeficiency virus type 1 (HIV-1) (10, 11). ACV was shown to suppress HIV-1 replication in human tissues co-infected with HIV-1 and human herpesviruses (10). The latter provide the viral kinase that facilitates production of ACV-MP. This bottleneck in the production of the active antiviral agent can also be bypassed with a monophosphorylated prodrug (CF2648) (Fig. 1, left), that shows anti-HIV activity in herpesvirus-free cells (4, 12, 13). Cell-free assays revealed HIV-1 reverse transcriptase (RT) as the target (10, 11). ACV-TP binds to the nucleotide binding site, and the incorporated ACV-MP causes DNA chain termination after the release of pyrophosphate (PPi). Like most other nucleoside analogues (14–16), the incorporated ACV-MP can be excised from the 3'-end of the primer in the presence of PPi or the PPi-donor ATP (10). This reaction can reduce the overall inhibitory effect; however, the removal of the chain terminator can be blocked through formation of a DEC (17). DEC formation depends critically on the chemical nature of the inhibitor (18). High concentrations (>100 μ M) of the next nucleotide are required to form a DEC with a primer

Acyclovir (ACV)⁴ (Fig. 1, right) was developed decades ago as one of the first selective antiviral agents, and it is still used in the

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⁴ The abbreviations used are: ACV, acyclovir; ACV-MP, ACV monophosphate; ACV-TP, ACV triphosphate; HIV-1, human immunodeficiency virus, type 1; HSV-1, herpes simplex virus, type 1; RT, reverse transcriptase; NRTI, nucle-

oside analogue RT inhibitor; DEC, dead-end complex; PPi, pyrophosphate; AZT, zidovudine; TAM, thymidine analogue-associated mutation; PFA, foscarnet; DTT, dithiothreitol; WT, wild type.

CF2648

Acyclovir

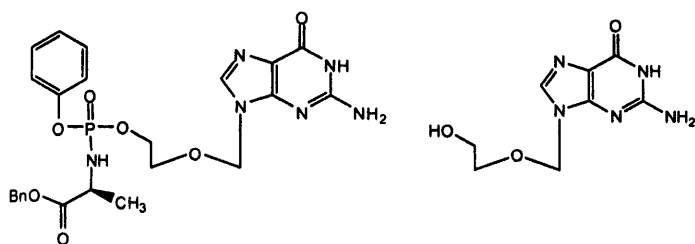


FIGURE 1. Structures of ACV and the monophosphorylated prodrug CF2648.

terminated with zidovudine (AZT), whereas submicromolar concentrations are often sufficient to form a DEC with ddNTPs (16). ACV-MP shows a behavior in the middle of the spectrum; $\sim 25 \mu\text{M}$ concentrations of the next nucleotide inhibits excision by 50% (10).

In vitro selection experiments revealed that ACV drug pressure is linked to the emergence of mutation V75I in the RT gene (11). A similar change, *i.e.* V75T, has earlier been associated with resistance to stavudine (19). Mutations M184V and T69N are other previously known resistance-conferring mutations that emerged under the selective pressure of ACV; however, V75I outgrew the culture over protracted periods of time, suggesting that this mutation is strongly associated with ACV resistance. HIV variants containing V75I showed marked increases in 50% effective antiviral concentrations (EC_{50}), which confirms the selection experiments (11).

Here we studied the underlying biochemical mechanism of HIV resistance to ACV associated with V75I. Two major mechanisms of resistance to nucleoside analogue RT inhibitors (NRTIs) have been described (20–24). The first mechanism is based on substrate discrimination. In this case the mutant enzyme can selectively diminish binding and/or incorporation of the nucleotide analogue, whereas the properties of the natural counterpart remain largely unaffected. M184V that confers high level resistance to 2',3'-dideoxy-3'-thiacytidine is a prominent example in this regard. The second major resistance mechanism associated with NRTIs is based on excision. In this case, the mutant enzyme can increase the rate of excision of the incorporated inhibitor. Thymidine analogue-associated mutations (TAMs) were shown to be able to recruit ATP as a PPi donor and increase excision of incorporated AZT-MP (17). In this study we demonstrate that V75I discriminates against ACV-TP at the level of incorporation. Excision of the incorporated nucleotide is also increased when compared with wild type RT; however, the effect is less pronounced, as seen with the excision of ACV-MP against a background of TAMs. V75I does not provide further protection from excision through DEC formation. Collectively, the data suggest that ACV is vulnerable to both major resistance mechanisms.

EXPERIMENTAL PROCEDURES

Enzymes and Nucleic Acids—Heterodimeric reverse transcriptase p66/p51 was expressed and purified as described (25). Mutant enzymes were generated through site-directed mutagenesis using the Stratagene QuikChange kit according to the manufacturer's protocol. TAM2 refers to HIV1-RT con-

taining the following substitutions: D67N, K70R, T215F, and K219Q. Oligodeoxynucleotides used in this study were chemically synthesized and purchased from Invitrogen and from Integrated DNA Technologies. The following sequence was used as template T50A6: 5'-CCAATATTCAACATCAAGGC-TTGATGAAACTTCACTCCACTATACCACTC. The underlined nucleotides are the portion of the templates annealed to the primer. The following primer was used in this study: P1, 5'-GAGTGGTATAGTGGAGTGAA.

Synthesis of ACV-TP—ACV (1.5 mmol) was dissolved in 200 μl of dry 1,3-dimethyl-2-oxohexahydropyrimidine *N,N'*-dimethylpropylene urea with 12–15 molecular sieves under nitrogen and stirred for 24 h. The mixture was chilled with an ice-water bath and stirred for 1 h followed by slow addition of 3 eq of phosphorus oxychloride and stirring for an additional 25 min. A solution of tributylammonium pyrophosphate (4 eq) in 200 μl of *N,N'*-dimethylpropylene urea and tributylamine (15 eq) was simultaneously added to the reaction. After 45 min the reaction was quenched with ice-cold water, and then it was slowly brought to the room temperature. The reaction was washed with chloroform, and the aqueous layer was collected and co-evaporated with deionized water three times. The residue was resuspended in 100 μl of deionized water and purified on an ion-exchange column by high performance liquid chromatography $\lambda_{(\text{max})} = 253$. To reduce the amount of excess salt, the final product was co-evaporated with water 5 times, giving total yield of ACV-TP (NH_3)₄ of 18% with purity $\geq 95\%$. The molecular weight of the ACV-TP was confirmed by liquid chromatography-tandem mass spectrometry; m/z ($M+1$) 466 \rightarrow 152 (26).

Synthesis of CF2648—The ACV prodrug CF2648 was prepared by the coupling of suitably base-protected ACV with the appropriate phosphorochloridate reagent under anhydrous conditions followed by base deprotection, according to procedures we have previously reported (27).

Competition between ACV-TP and dGTP—DNA synthesis was monitored with 5'-end-labeled primers unless otherwise indicated. 150 nM DNA/DNA (T50A6/P1) was incubated with 30 nM HIV-1 RT in a buffer containing 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, constant concentrations of dGTP (1 μM) and ddTTP (2 μM) or dNTP mix (1 μM) and increasing concentrations of ACV-TP. Nucleotide incorporation was initiated by the addition of MgCl_2 to a final concentration of 10 mM, and the reactions were allowed to proceed for 5 min. The reactions were stopped by the addition of 3 reaction volumes of formamide containing traces of bromophenol blue and xylene cyanol. The samples were then subjected to 15% denaturing PAGE followed by phosphorimaging. Inhibitory concentrations of ACT-TP required to inhibit DNA synthesis at position +2 by 50% (IC_{50}) was determined by normalizing the product fraction formed at position +2 in the presence of ACV-TP to the corresponding value in the absence of inhibitor. Data points were fit to a sigmoidal dose response (variable slope) function using GraphPad Prism (Version 5.0).

ATP-dependent Excision—The T50A6/P1 DNA/DNA substrate was extended by a single nucleotide to generate an oligonucleotide with ACV-MP at the 3'-end. The extended primer was gel-purified and annealed with template T50A6. 50 nM sub-

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strate was then incubated with 500 nM HIV-1 RT in a buffer containing 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 10 mM MgCl₂, and 3.5 mM ATP (pyrophosphatase-treated). Aliquots were taken at different time points and analyzed as described.

Site-specific Footprinting—In preparation of the footprinting experiments, template T50A6 was 5'-end-labeled and heat-annealed with primer P1. 50 nM DNA/DNA hybrid was incubated with 750 nM HIV-RT for 10 min in a reaction mixture containing sodium cacodylate, pH 7 (120 mM), NaCl (20 mM), DTT (0.5 mM), MgCl₂ (10 mM), and 25 μM ddGTP or 5 μM ACV-TP in a final volume of 15 μl. In control reactions ddGTP and ACV-TP were either omitted or substituted with 100 μl phosphonoformic acid (PFA or foscarnet). After complete complex formation and/or nucleotide incorporation, increasing concentrations of dTTP were added to the reactions followed by an incubation of 5 min at 37 °C. For the actual footprinting, complexes were treated with 0.1 mM ammonium iron (II) sulfate hexahydrate (28). The reactions were allowed to proceed for 5 min and were processed and analyzed as described.

DEC Formation—DNA synthesis at template position +1 was conducted in a similar fashion as described above except that a chain terminator (ddGTP, 25 μM or ACV-TP, 2.5 μM) was incorporated at this position. A time course of incorporation of the chain terminator at position +1 in the absence or in the presence of increasing concentrations of the nucleotide at the following position, +2, was monitored. The reactions were processed and analyzed as described. Slopes of the linear portion of product formation illustrate the velocities of the reaction, which when normalized to the enzyme concentration, determine the turnover number (k_{cat}). Inhibition of DNA synthesis by DEC formation is illustrated by a decrease in k_{cat} .

Pre-steady-state Kinetics for Nucleotide Incorporation—Nucleotide incorporation under single-turnover conditions was monitored using a rapid quench-flow instrument (KinTek RQF-3). Reactions involved rapid mixing of a solution containing preincubated 100 nM DNA/DNA template/primer hybrid with 500 nM HIV-1 RT in a buffer consisting of 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, and 10 mM MgCl₂ at 37 °C with a solution of the same buffer composition except that template/primer and RT were substituted with a given concentration of dGTP or ACV-TP. Nucleotide incorporation was monitored at time points of 0.015, 0.025, 0.05, 0.075, 0.1, 0.2, 0.3, 0.5, and 1 s. The reactions were processed and analyzed as described. Data points from time courses were fit by nonlinear regression (GraphPad Prism (Version 5.0)) to a single exponential equation, $[product] = A(1 - \exp(-k_{observed}t))$, where A represents the amplitude, and $k_{observed}$ is the first order rate constant for dGTP or ACV-TP incorporation. $k_{observed}$ were replotted versus increasing concentrations of dGTP or ACV-TP to determine the respective $k_{observed}$. Data points were fit to a hyperbolic function, $k_{observed} = k_{pol}[dNTP]/(K_{d,dNTP} + [dNTP])$, where k_{pol} is the maximum first-order rate constant for dGMP or ACV-MP incorporation, and $K_{d,dNTP}$ is the equilibrium dissociation constant for the interaction of dGTP or ACV-TP with the RT-template/primer complex.

Selection of Resistance—MT-4 cells were obtained from the NIH AIDS Research and Reference Reagent Program and infected with HIV-1_{LAI.04} in the absence or presence of CF2648.

TABLE 1
Inhibitory activity of ACV ProTide CF2648 against HIV-1_{LAI.04} in MT-4 cells

	WT	CF2648-selected virus	-Fold increase
EC ₅₀ ± S.E. (μM) ^a	3.4 ± 2.8	51 ± 2.3	15

^a EC₅₀, effective concentration that inhibits 50% of HIV-1_{LAI.04} replication ± S.E.

Every 3–5 days, 3% of the culture supernatant was used to infect fresh cells. Cultures were maintained in the absence or presence of gradually escalating concentrations of CF2648. HIV-1 replication in infected cultures was assessed by measuring p24_{gag} from culture supernatants, as previously described (10). Phenotypic resistance of viruses serially passed in the absence or in the presence of the CF2648 was evaluated in MT-4 cell cultures using drug concentrations ranging from 0 to 150 μM. The effective concentration that inhibits 50% replication was calculated by fitting the data points to a sigmoidal dose-response curve using GraphPad Prism (Version 4.0).

Genotyping of the Drug-exposed HIV-1 Strains—Viral RNA was extracted from plasma using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). HIV-1 RNA was reverse-transcribed into cDNA, and a 2878-bp nucleotide fragment encompassing protease and reverse transcriptase was amplified in an outer PCR using SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen) and outer primers AV190-1 and CR1 (29). A 2853-bp nucleotide fragment was amplified using Expand High Fidelity PCR System (Roche Diagnostics) and the inner primers AV190-2 and CR2. Amplification products were separated on a 1% agarose gel and visualized by ethidium bromide staining. PCR products were purified with Microspin S-400 (GE Healthcare). Sequencing was performed using the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit as described before (29). The reactions were run on an ABI3100 Genetic Analyzer, and analysis was performed using Sequence Analysis Version 3.7 and SeqScape version 2.0 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

RESULTS

Selection of Resistance with CF2648—The V75I mutation in HIV-1 RT was shown to emerge rapidly under the selective pressure of ACV (11). The poor production of ACV-MP and, in turn, the poor production of ACV-TP is a possible factor that diminishes the efficacy of ACV and facilitates the selection of resistance. It is currently unknown whether monophosphorylated prodrugs can prevent or significantly delay the development of resistance. To address this problem, we attempted to select for resistance with the monophosphorylated prodrug CF2648. HIV-1 was propagated in MT-4 cells in the presence of increasing concentrations of CF2648. The antiviral activity of CF2648 after 39 serial passages (~120 days) in the presence of the compound was reduced relative to the control propagated in the absence of the drug. Sequencing of HIV-1 RT revealed the emergence of V75I, and phenotypic resistance measurements revealed a 15-fold increase in the EC₅₀ value (Table 1). Overall, the data suggest that the selection of V75I in the RT enzyme may not be prevented with prodrugs that bypass the first phosphorylation step. Given the potential relevance of this

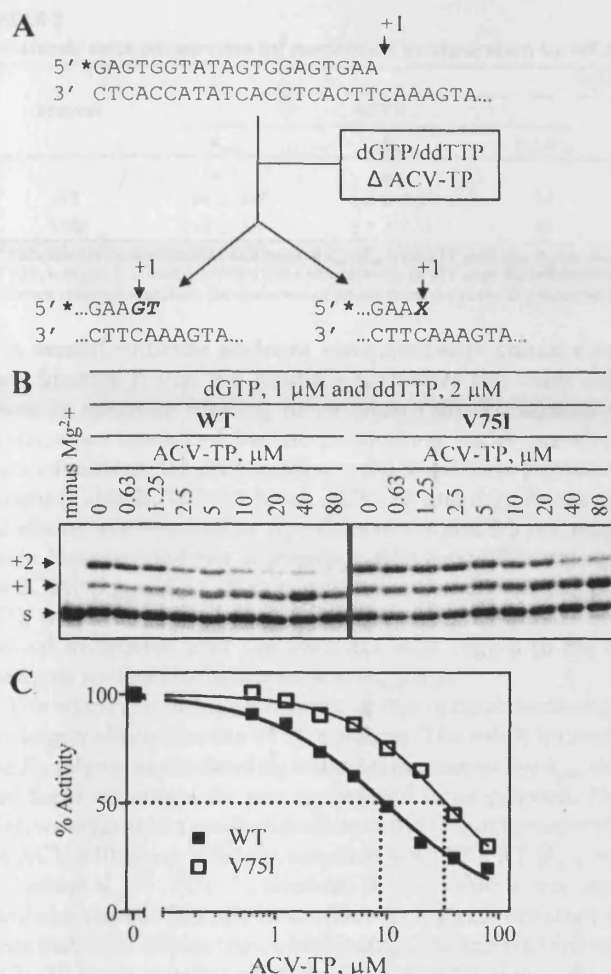


FIGURE 2. Competition between ACV-TP and dGTP. *A*, reaction scheme. Nucleotide incorporation was monitored at positions +1 and +2. Letters in *bold italic* illustrate the incorporated nucleotide(s). *X* illustrates an incorporated ACV-MP. The *asterisk* points to the 5'-end radiolabeled primer. *B*, two nucleotide incorporation events in the presence of constant concentrations of dGTP and ddTTP and increasing concentrations of ACV-TP. ACV-mediated inhibition of DNA synthesis is monitored at position +2, and corresponding arrows illustrate the migration pattern of non-extended substrate and extended primers. *C*, graph of data shown under *B*. Dotted lines illustrate a shift in IC_{50} .

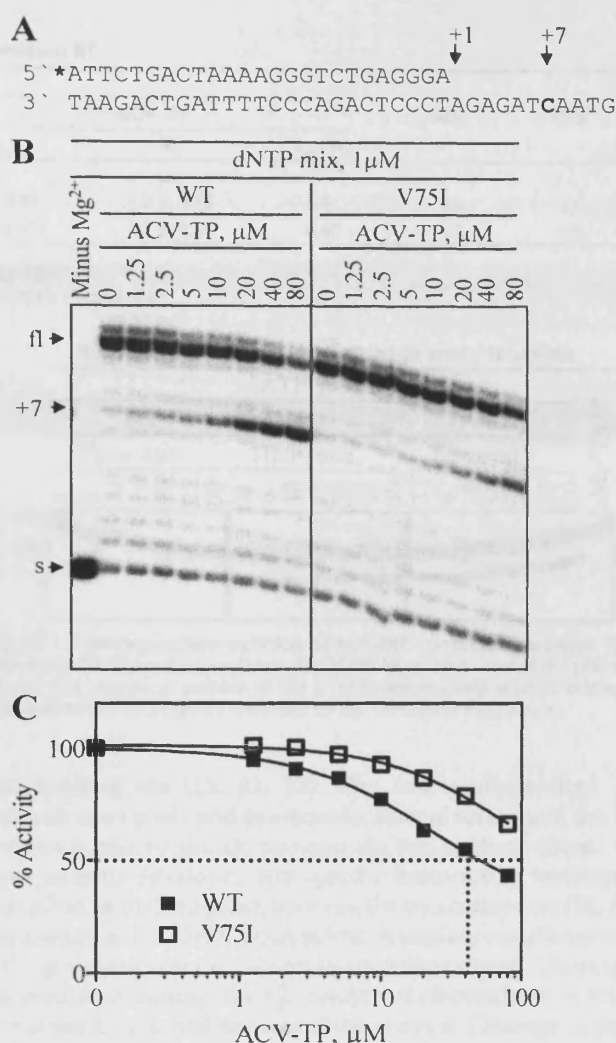


FIGURE 3. Competition between ACV-TP and dGTP during later stages of DNA synthesis. *A*, DNA/DNA primer/template substrate used in the experiment. ACV-MP incorporation was monitored at position +7. The *asterisk* points to the 5'-end radiolabeled primer. *B*, multiple nucleotide incorporation events in the presence of constant concentration of dNTP mix and increasing concentrations of ACV-TP. Arrows illustrate the migration pattern of non-extended substrate, extended primers by seven nucleotides, and full-length (fl) product. *C*, graph of data shown under *B*. Dotted lines point to 50% product formation; however, we were unable to accurately determine IC_{50} values under these conditions. Incorporation of ACV-MP is generally diminished when compared with the sequence used in Fig. 2.

mutation in clinical settings, we studied the underlying biochemical mechanisms.

Discrimination against ACV-TP—We initially studied substrate discrimination as a possible mechanism for resistance. Classic protocols that address this problem include steady-state kinetics that measure efficiencies of incorporation of the inhibitor and its natural counterpart in separate experiments. However, with this approach we were unable to detect significant differences between purified wild type (WT) RT and the mutant enzyme containing V75I (data not shown). We considered two potential problems associated with this assay. First, incorporation of the inhibitor was measured in the absence of the natural counterpart dGTP. Therefore, we devised an assay that monitors incorporation of ACV-MP in the presence of both ACV-TP and dGTP (Fig. 2*A*). The primer/template was designed to monitor incorporation of ACV-MP at the next

template position ($n + 1$) after the 3'-end of the primer (n). Incorporation of ACV-MP causes chain termination, whereas incorporation of dGMP allows the addition of a second nucleotide that is provided as a "stop nucleotide," which limits DNA synthesis and reduces complexity of the assay. The natural dGTP was provided at a constant concentration of 1 μM, and ACV-TP was provided at varying concentrations (Fig. 2*B*). We found that the concentrations required to diminish dinucleotide extensions by 50% were significantly higher with the mutant enzyme, which suggests that the V75I mutant increases discrimination against the inhibitor (Fig. 2*C*). We obtained essentially the same result with a different primer/template sequence that allowed us to monitor ACV-MP incorporation at template position +7 (Fig. 3).

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TABLE 2

Pre-steady state parameters for nucleotide incorporation by WT and V75I mutant RT

Enzyme	Substrate						SEL ^a	RES ^b
	dGTP			ACV-TP				
	k_{pol} s^{-1}	K_d μM	k_{pol}/K_d	k_{pol} s^{-1}	K_d μM	k_{pol}/K_d		
WT	66 ± 3.0 ^c	1.3 ± 0.16	51	14 ± 0.39	2.2 ± 0.18	6.4	8.0	1.0
V75I	114 ± 2.9	3.7 ± 0.18	31	1.5 ± 0.071	3.8 ± 0.43	0.40	78	9.8

^a SEL, selectivity, is calculated as a ratio of k_{pol}/K_d for dGTP over k_{pol}/K_d for ACV-TP.

^b RES, resistance, is calculated as a ratio of selectivity of WT over the selectivity of V75I mutant RT.

^c Errors reported represent the deviation of points from the curve fit generated by GraphPad Prism (Version 5.0).

A second common problem associated with classic steady-state kinetics is that the multiple turnovers can mask differences in substrate binding or catalysis (30). To address this problem we measured kinetic parameters under pre-steady-state conditions, which provides at the same time mechanistic insight (Table 2). WT RT binds ACV-TP and dGTP with similar affinity as evidenced by K_d values of 2.2 and 1.3 μM , respectively. However, the rate of incorporation is significantly higher for dGMP ($k_{\text{pol}} = 66 \text{ s}^{-1}$) as compared with ACV-TP ($k_{\text{pol}} = 14 \text{ s}^{-1}$), which translates to an 8-fold selective advantage of the natural nucleotide over the inhibitor with regard to the efficiency of nucleotide incorporation (k_{pol}/K_d).

The efficiency of incorporation of the natural nucleotide is not largely affected by the V75I mutation. The subtle increase in the K_d value is neutralized by a subtle increase in the k_{pol} value, and these variations are not considered to be relevant. However, we measured a marked decline in the rate of incorporation for ACV-MP when V75I is compared with WT RT ($k_{\text{pol}} = 1.5 \text{ s}^{-1}$ versus $k_{\text{pol}} = 14 \text{ s}^{-1}$), whereas the K_d value is not significantly increased. The selective effect on the rate constant suggests that V75I affects the catalytic step. Nucleotide binding of ACV-TP is not significantly changed. Overall, the data point to a 10-fold increase in discrimination against the inhibitor at single nucleotide resolution.

Excision of ACV-MP—An increased rate of excision is yet another possible mechanism that could help to explain the resistance phenotype associated with V75I. We devised a primer that was terminated with ACV-MP and studied the efficiency of the ATP-dependent excision reaction with WT RT, V75I, and a mutant enzyme that contains four TAMs (D67N, K70R, T215F, and K219Q). This particular combination represents the TAM2 pathway. Like other combinations of TAMs, this cluster facilitates the recruitment of ATP as a PPi donor. In agreement with previous reports, excision with WT RT is inefficient (Fig. 4). However, V75I appears to increase the efficiency of ACV-MP excision over time, and the TAM-containing enzyme shows further significant increases. Thus, increases in excision of the incorporated ACV-MP may be considered as another possible factor that contributes to ACV resistance.

Positioning of RT on ACV-terminated Primers—The precise positioning of RT on its primer/template and, in turn, enzymatic function can be influenced by the chemical nature of the 3'-end of the primer (31, 32). Before nucleotide incorporation, the enzyme needs to translocate a single position farther downstream, which liberates the nucleotide binding site from the 3'-end of the primer (28, 33, 34). In contrast, excision can only take place when the 3'-end of the primer occupies the nucleo-

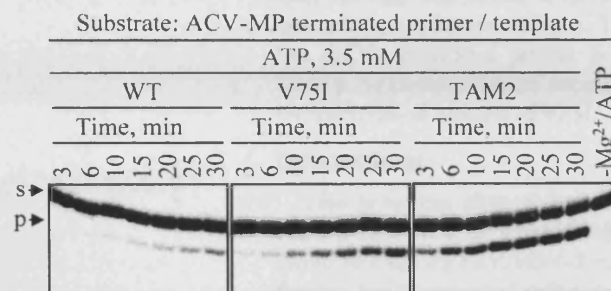


FIGURE 4. ATP-dependent excision of ACV-MP-containing primers. Time course of ATP-dependent excision of ACV-MP from the 3'-end of the primer is shown. The migration pattern of the 5'-end radioactively-labeled primer (s) and reaction product (p) is illustrated by the corresponding arrows.

tide binding site (15, 21, 33). The two conformations are referred to as post- and pre-translocational states, and the RT enzyme is able to shuttle between the two conformations. We have recently developed site-specific footprinting techniques that allow us to distinguish between the two complexes (28, 32). Incubation of RT-DNA/DNA primer/template complexes with Fe^{2+} generates specific cuts on the template strand. These cuts are mediated through the RT-associated ribonuclease H active site at the C-terminal domain of the enzyme. Cleavage at position -17 is indicative for post-translocated complexes, whereas cleavage at position -18 is indicative for pre-translocated complexes. Here we compared footprints obtained with WT RT and V75I in complex with either ddGMP- or ACV-terminated primers (Fig. 5). Complexes with the bound nucleotide can only exist in the post-translocational state, whereas complexes with the PPI-analogue PFA exist pre-translocation (see controls). In an attempt to gradually stabilize the complex (DEC formation), we increased concentrations of the next complementary nucleotide, *i.e.* dTTP, which correlated with an increase in signal intensity. Complexes with WT RT and ddGMP-terminated primers exist predominantly in the post-translocated state even in the absence of nucleotide substrate. The signal is increased in the presence of increasing concentrations of the next nucleotide, which confirms formation of a DEC in the post-translocational state. In contrast, corresponding complexes with ACV-terminated primers are difficult to identify. Much higher concentrations of the next nucleotide are required to trap the complex in the post-translocational state. We obtained very similar results with the V75I mutant. The trend is the same, although the overall band intensity appears slightly diminished when compared with WT RT. Overall, these findings suggest that DEC formation is compromised with ACV-MP, and the

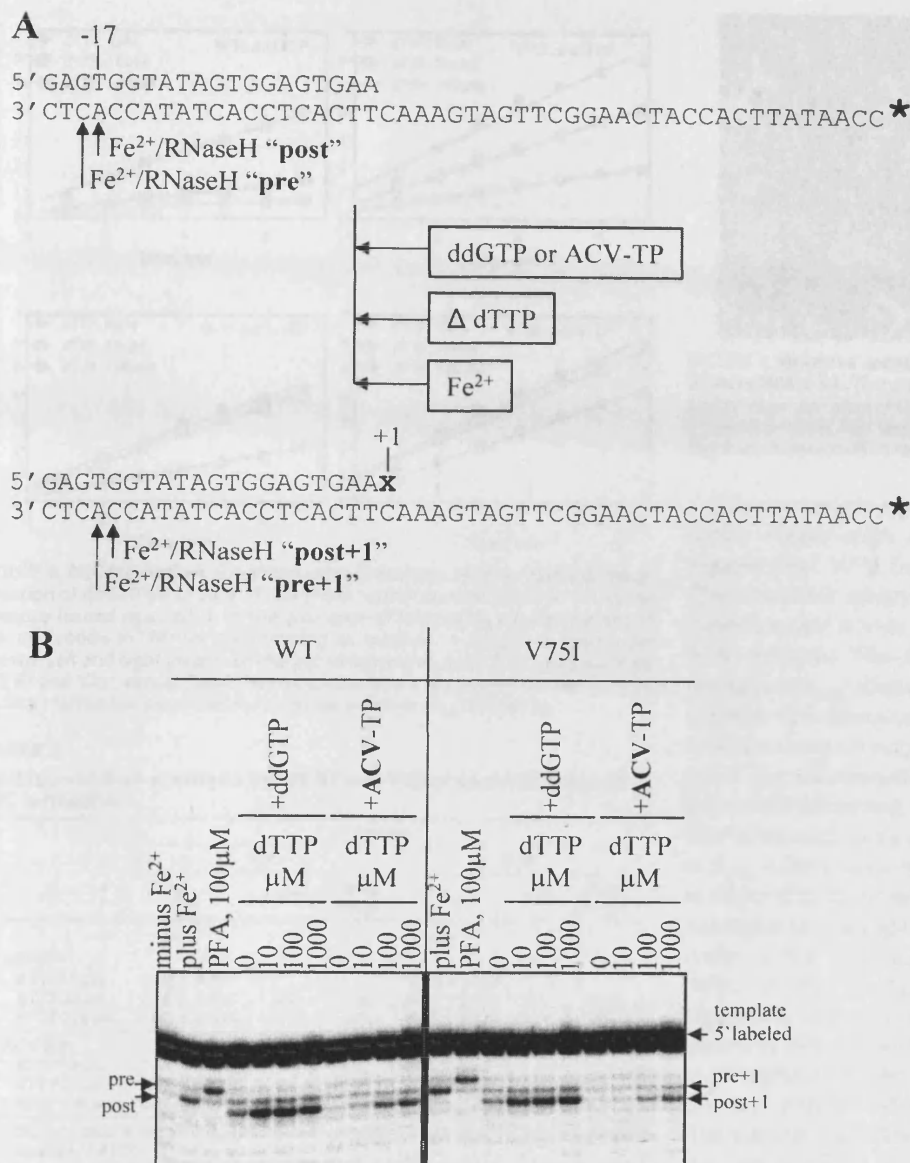


FIGURE 5. Site-specific footprinting of HIV1 RT-DNA substrate complexes. A, reaction scheme. Complexes were treated with Fe^{2+} after incorporation of ACV-MP or ddGMP. The additional presence of PFA or dTTP provides conditions to trap the pre- or post-translocated complex, respectively. The asterisk illustrates a 5'-end radioactively labeled template. B, footprinting patterns with ddGMP- or ACV-MP-terminated primers. *minus* Fe^{2+} represents a control experiment in the absence of Fe^{2+} . *plus* Fe^{2+} represents treatment of binary complexes with Fe^{2+} before nucleotide incorporation. PFA 100 μM represents the footprint in the presence of 100 μM PFA that traps the pre-translocated complex.

V75I mutation may further contribute to this effect. The diminished signal points to increased complex dissociation.

DEC Formation—To compare and quantify the ability of WT RT and V75I to form a DEC, we measured the turnover after incorporation of ACV-MP and ddGMP, respectively. The simultaneous inclusion of the next nucleotide at different concentrations allows formation of the ternary DEC (Fig. 6). In this set-up, increases in the turnover number (k_{cat}) correlate inversely with DEC formation (Table 3). WT RT shows a decline in the turnover with ddGMP-terminated primers and increasing concentration of the next nucleotide. DEC formation is ~8-fold enhanced at concentrations of 100 μM dTTP

when compared with the control in the absence of the nucleotide substrate. Under the same conditions DEC formation is compromised with ACV-terminated primers (only 2-fold increases with 100 μM dTTP), and V75I further enhances this effect. These findings suggest that DEC formation is likely to be insignificant under physiologically relevant conditions with dNTP concentrations below 100 μM . Thus, the ACV-terminated primer is unlikely to be protected from excision in the mutational context of V75I.

DISCUSSION

The potential clinical benefit of ACV or the prodrug version valacyclovir in treating HIV/HSV-2 co-infection has been tested in large clinical trials (35–37). HIV co-infection with HSV-2 can exacerbate disease progression (38, 39). In support of this notion, several trials have been shown that ACV-mediated suppression of HSV-2 is associated with reductions in HIV viral load (36, 40). In contrast, other trials failed to show that anti-herpetic therapy prevents infection with HIV-1 (41, 42). Two subsequent studies demonstrated that ACV can also directly inhibit HIV-1 replication by targeting the RT enzyme (10, 11). However, the development of resistance can compromise the antiviral activity in clinical settings. Three different mutations in HIV-1 RT were shown to emerge under the selective pressure of ACV *in vitro*, M184V, T69N, and V75I (11). Experiments with constructs that were generated by site-directed mutagenesis confirmed the selection experiments and revealed that each of the three

mutations can reduce susceptibility to ACV. V75I shows by far the strongest effect in this regard. Moreover, here we demonstrate that V75I is likewise selected in the presence of a monophosphorylated prodrug derivative of ACV that was developed to bypass the bottleneck in the metabolic conversion of ACV to ACV-TP. Although other mutations independent of or in conjunction with V75I may also contribute to HIV-1 resistance to ACV or its prodrugs, here we focused on the characterization of the effect of V75I on the function of RT.

Residue Val-75 is located in close proximity to template position $n + 1$ opposite the incoming nucleotide (Fig. 7) (43). Thus, a direct effect of a mutation at this position on binding and/or

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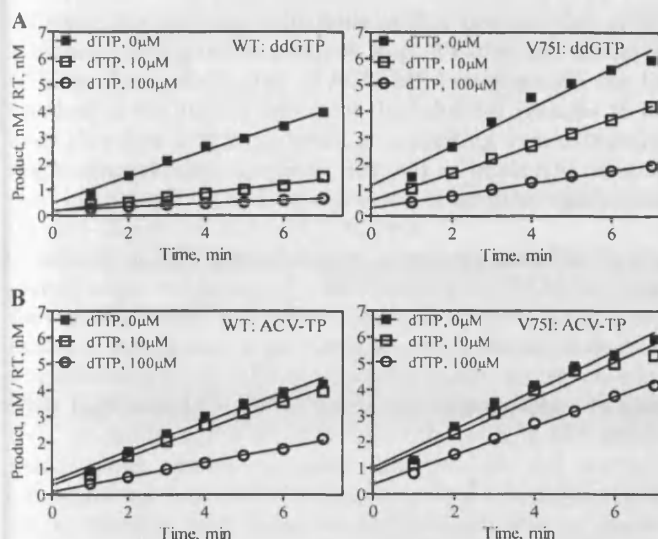


FIGURE 6. DEC formation. Graphical representations of time course of incorporation of ddGTP (A) or ACV-TP (B) chain terminators at position +1 in the absence (closed squares) or in the presence of increasing concentrations of the nucleotide (dTTP) for the binding at position +2 (open symbols) are shown. Left and right graphs of the panel represent ddGTP incorporation by WT RT and V75I, respectively. Slopes of the linear portion of the normalized product formation illustrate the turnover number (k_{cat}) (Table 3).

TABLE 3

Inhibition of DNA synthesis by WT RT and V75I mutant RT through DEC formation

	Enzyme					
	WT			V75I		
	k_{cat}	DEC ^a	-Fold change ^b	k_{cat}	DEC	-Fold change
	min^{-1}			min^{-1}		
ddGTP ^c						
dTTP 0 μM	0.46 ± 0.057 ^e			0.77 ± 0.078		
dTTP 10 μM	0.16 ± 0.014	2.9	1.0	0.51 ± 0.021	1.5	1.9
dTTP 100 μM	0.060 ± 0.0057	7.7	1.0	0.23 ± 0.014	3.3	2.0
ACV-MP						
dTTP 0 μM	0.63 ± 0.028			0.64 ± 0.15		
dTTP 10 μM	0.54 ± 0.0071	1.2	1.0	0.65 ± 0.078	1.0	1.2
dTTP 100 μM	0.27 ± 0.014	2.3	1.0	0.55 ± 0.021	1.2	1.9

^a DEC formation is calculated as a ratio of k_{cat} in the absence of dTTP over k_{cat} in the presence of dTTP.

^b -Fold change reflects differences in DEC formation by WT RT over V75I mutant RT.

^c The 3'-end of the primer was terminated in the presence of ddGTP or ACV-TP to prevent incorporation of the next nucleotide during DEC formation.

^d Next complementary nucleotide.

^e S.D. were determined on the basis of at least three independent experiments.

incorporation of nucleoside analogues is not evident. V75I is part of the "Q151M cluster" that is associated with multiple resistance to NRTIs (44, 45). However, in this context, V75I does not appear to contribute to the resistance phenotype; the mutation rather compensates for enzymatic deficits that are introduced by Q151M (46). A related mutation, *i.e.* V75T, has been associated with low level (3–4-fold) resistance to stavudine, which is a 2',3'-unsaturated thymidine analogue and, therefore, structurally distinct from ACV (19, 46). The increase of approximately 1–2 orders of magnitude in resistance to ACV is by far the strongest effect that has been reported for changes at this position (Ref. 11 and this study). We consider three complementary mechanisms that help to explain the resistant phenotype.

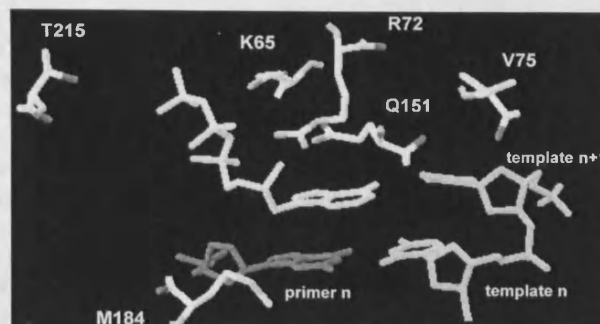


FIGURE 7. Relative location of Val-75 and critical other amino acid residues in HIV-1 RT. The crystal structure of HIV-1 RT in complex with tenofovir and primer/template (PDB code 1t05) (62). Tenofovir is shown in cyan, primer position is in red, and template positions *n* and *n* + 1 are highlighted in green. Residues implicated in resistance are labeled (see "Discussion").

Discrimination against ACV-TP—Competition experiments under steady-state conditions and pre-steady-state kinetics suggest that V75I further discriminates against the inhibitor. The selective advantage for the natural nucleotide over the inhibitor that is seen with WT RT is 10-fold increased with the V75I mutant. The difference can be assigned to equivalent changes in k_{pol} values. Thus, the V75I mutation appears to compromise the chemical step, whereas affinities to substrate and inhibitor remain largely unchanged. This result is unexpected given the considerable distance between Val-75 and the active site or the incoming nucleotide (Fig. 7). The V75I change may, therefore, indirectly affect the catalytic step. Specific decreases in k_{pol} values have been reported for other NRTI resistance-associated mutations (47, 48). K65R and Q151M are important examples in this regard (47–50). Lys-65 is located in close proximity to the γ -phosphate of the incoming nucleotide, which indicates that changes at this position may affect its proper alignment with the attacking 3'-end of the primer. The side chains of Gln-151 and also Arg-72 are seen in the vicinity of the α -phosphate of the incoming nucleotide, which suggests a direct role in the catalytic step (51, 52). By extension, changes at this position can directly compromise catalysis at the center of the reaction. It is, therefore, conceivable that changes at position Val-75 can affect the precise positioning of one of the two residues or both, which could help explain the observed decreases in k_{pol} values. The backbone of Val-75 is in contact distance with the backbone of Gln-151, and subtle structural alterations at Val-75, *i.e.* V75I, can impact on the positioning of Arg-72, both part of the flexible β 3- β 4 hairpin loop that traps the incoming nucleotide. Regardless of the precise mechanism, the effect of V75I on ACV-MP incorporation appears to be indirectly mediated through other amino acids. In contrast, classic NRTI-associated mutations, such as M184V, K65R, and Q151M, discriminate against the inhibitor directly at the active site.

Previous other studies have shown that changes at Val-75 can also affect the affinity to the substrate. V75T showed increases in K_d values for stavudine without affected k_{pol} (46). Moreover, mispair extension experiments with the V75I mutant enzyme showed likewise significant increases in K_d values without affecting k_{pol} (53). Together the results suggest that changes at position Val-75 can affect nucleotide incorporation through

different mechanisms. Mutations at this position can affect substrate binding or the catalytic step. Whether the effects of V75I on the catalytic step of ACV-MP incorporation can be ascribed to the acyclic nature of the inhibitor remains to be seen. However, in this context it is interesting to note that the acyclic phosphonate tenofovir, which is an important component in currently used drug regimens, is sensitive against the Q151M cluster that includes V75I (54).

Excision of ACV-MP—Excision of incorporated NRTIs is a second major mechanism for NRTI resistance. TAMs increase the excision of AZT-MP when compared with WT RT (15, 16); however, the reaction is not restricted to thymidine analogues. The efficiency of the ATP-dependent excision reaction is relatively high with AZT-MP at the 3'-end of the primer. In contrast, ddNMPs and 2',3'-dideoxy-3'-thiacytidine-MP terminated primer strands are poor substrates for the reaction, although these nucleotides analogues are not completely resistant to excision (55). Here we demonstrate that a mutant enzyme containing four TAMs can also markedly increase excision of ACV-MP. Of note, purine analogues are usually poorly excised when compared with their pyrimidine counterparts, which indicates that the base moiety is an important determinant for the reaction (56). However, the acyclic purine analogues tenofovir (32) and ACV-MP are both efficiently removed from the primer terminus, suggesting that the increased flexibility of the acyclic linker between the base and the phosphate or phosphonate of these compounds can facilitate the reaction.

The V75I mutation shows an intermediate phenotype when the excision of ACV-MP is compared with WT RT and TAMs. However, the mechanism associated with such an increase in efficiency of the excision reaction is likely to be different as described for TAMs. TAMs were shown to facilitate binding of ATP in a catalytically competent fashion (15). The aromatic side chains of T215Y or T215F, *i.e.* the hallmark for AZT resistance, are implicated in stacking interaction with the base moiety of ATP, which facilitates its binding in an orientation that allows its use as a PPi donor (Fig. 7). In contrast, the position of Val-75 in close proximity to template position $n + 1$ and the nature of the amino acid substitution do not support such interaction. The mutation may indirectly affect the excision reaction in a similar manner as proposed for the incorporation of ACV-MP; however, such an interaction would be counterproductive. Thus, we also consider an alternative interpretation. Excision of ACV-MP with ATP generates the ACV-(5')-tetraphospho-(5')-A (ACVp4A) that is eventually released from the complex. The tetraphosphate can be used as a substrate (16, 57), resulting in re-incorporation of ACV-MP. At this level, the V75I mutant has a disadvantage over WT RT, which leads to an accumulation of the excised product. In contrast, TAM-containing enzymes do not significantly affect the efficiency of incorporation of nucleotide analogues (58–60).

DEC Formation—The formation of a DEC with the next complementary nucleotide after the chain terminator at the 3'-end of the primer has two major consequences. First, the bound nucleotide stabilizes the complex, which translates in a diminished turnover of the reaction under steady-state conditions (61). This experimental set-up allowed us to compare and

to quantify DEC formation of WT RT with mutant V75I RT. ACV-MP generally diminishes DEC formation when compared with ddGTP, and V75I increased this effect. A similar effect has been observed when comparing tenofovir with ddATP, suggesting that the acyclic linker can negatively influence nucleotide binding (32). V75I may further affect DEC formation through structural alterations at template position $n + 1$ that is complementary to the next nucleotide. Of note, our data show at the same time that neither the ACV-terminated primer nor the V75I mutation affect the distribution of pre- and post-translocated complexes.

The second major consequence of a stable DEC is that the 3'-end of the primer is protected from excision (16). The nucleotide traps the complex in its post-translocational state, which prevents excision to occur (33). This observation is of potential biological relevance considering that WT RT can excise ACV-MP, although the reaction is inefficient.

Conclusion—The results of this study suggest that discrimination against the inhibitor at the level of incorporation is the dominant mechanism associated with ACV resistance conferred by V75I. Protection against excision is compromised through diminished formation of a ternary DEC, and TAMs can directly increase efficiency of excision of ACV-MP. Thus, ACV appears to be vulnerable to both major NRTI-associated resistance pathways. It is, therefore, essential to characterize the detailed resistance profile of this compound to better assess its potential clinical utility in combination with established antiretrovirals. The use of ACV might be compromised in persons who were recently infected with resistant HIV variants. In addition, the clinical use of ACV may cause the emergence of resistance mutations that can decrease susceptibility to established antiretroviral agents.

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The Application of Phosphoramidate ProTide Technology to Acyclovir Confers Anti-HIV Inhibition

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Recently, it has been reported that phosphorylated acyclovir (ACV) inhibits human immunodeficiency virus type 1 (HIV-1) reverse transcriptase in a cell-free system. To deliver phosphorylated ACV inside cells, we designed ACV monophosphorylated derivatives using ProTide technology. We found that the L-alanine derived ProTides show anti-HIV activity at noncytotoxic concentrations; ester and aryl variation was tolerated. ACV ProTides with other amino acids, other than L-phenylalanine, showed no detectable activity against HIV in cell culture. The inhibitory activity of the prodrugs against herpes simplex virus (HSV) types -1 and -2 and thymidine kinase-deficient HSV-1 revealed different structure–activity relationships but was again consistent with successful nucleoside kinase bypass. Enzymatic and molecular modeling studies have been performed in order to better understand the antiviral behavior of these compounds. ProTides showing diminished carboxypeptidase lability translated to poor anti-HIV agents and vice versa, so the assay became predictive.

Introduction

Human immunodeficiency virus (HIV^a) belongs to the retroviridae family and causes the acquired immunodeficiency syndrome (AIDS). A variety of different compounds have been developed for the treatment of HIV, and currently 25 drugs have been approved for clinical use including nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PI), a viral fusion inhibitor (FI), a CCR-5 coreceptor inhibitor, and a viral integrase (IN) inhibitor.¹

Because of the rapid development of drug resistance as well as to the toxicity shown by these drugs,² novel anti-HIV agents are needed. Diverse structures are sought to address the constant threat of viral resistance.

In this context, recently it has been reported how the antiherpetic drug acyclovir (ACV, **1**, Figure 2) inhibits HIV upon human herpesvirus (HHV) coinfection in tissue cultures.³ This activity was found to be correlated with the phosphorylation of the parent drug to the monophosphate form mediated by HHV-encoded kinase(s). HIV does not encode an enzyme that recognizes ACV as a substrate for this activation (phosphorylation) step, hence the need for the HHV coinfection for activity. The subsequent phosphorylations to the di- and triphosphate derivatives may be mediated by cellular guanosine monophosphate kinase and nucleoside diphosphate kinase, respectively.^{4,5} In its triphosphate form,

ACV inhibits HIV RT acting as a chain terminator.³ Following these results, it is evident that the anti-HIV activity can only occur upon ACV monophosphate (ACV-MP) formation which requires HHV coinfection. ACV-MP itself can not be used as efficient anti-HIV chemotherapeutic agent to bypass the first limiting phosphorylation step because of its instability in biological media and its poor efficiency of diffusion through intact cell membranes. A suitable strategy to overcome these limitations would consist of masking the negative charges of the monophosphate with lipophilic groups. In this regard, the phosphoramidate ProTide technology has been developed and successfully applied to a range of nucleosides of antiviral and anticancer interest.^{6–9} The structural motif of this approach consists of masking the nucleoside monophosphate with an aryl moiety and an amino acid ester. Cell entry then apparently occurs by passive diffusion. Once inside the cell, the phosphoramidate prodrug is activated and converted to the monophosphorylated ACV (Figure 1).¹⁰ The first step involves an enzymatic hydrolysis of the amino acid ester moiety mediated by an esterase- or carboxypeptidase-type enzyme followed by spontaneous cyclization and subsequent spontaneous displacement of the aryl group and opening of the unstable ring mediated by water. The last step before release of the ACV monophosphate involves a hydrolysis of the P–N bond mediated by a phosphoramidase-type enzyme. The phosphoramidate ProTide approach has been already successfully applied to ACV, demonstrating its ability to bypass the thymidine kinase deficiency of HSV-1 and -2 and varicella zoster virus strains resistant to ACV.¹¹

In this paper, we present the synthesis and initial biological evaluation of a novel series of ACV ProTides (Figure 2). The ProTide moiety has three different changeable parts: the aryl moiety, the amino acid, and the ester. In the first part of this study, we have chosen L-alanine as the

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^aAbbreviations: ACV, acyclovir; HIV, human immunodeficiency virus; HSV, herpes simplex virus; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitor; FI, fusion inhibitor; HHV, human herpes virus; ACVMP, acyclovir 5'-monophosphate; DMF, N,N-dimethylformamide.

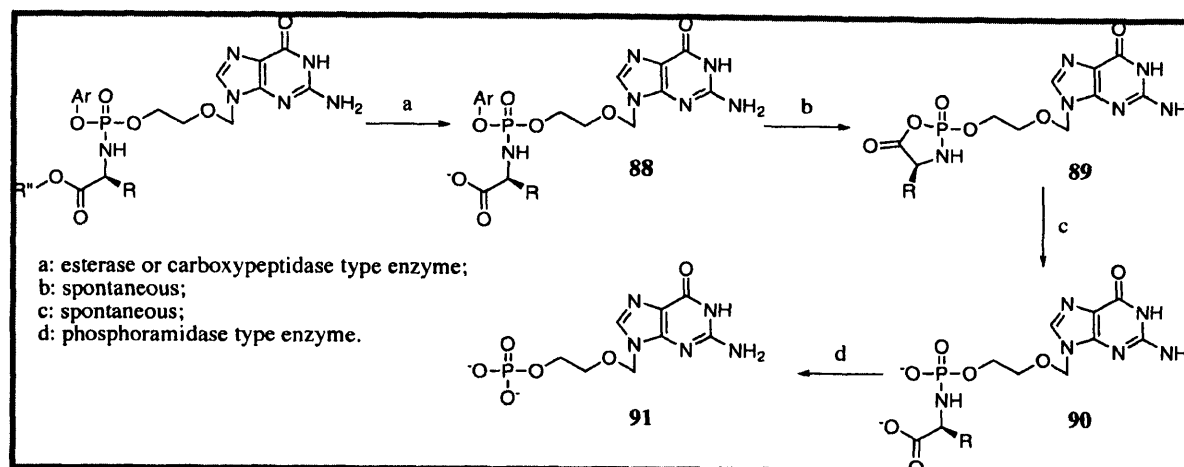


Figure 1. Proposed activation pathway of the acyclovir ProTides.

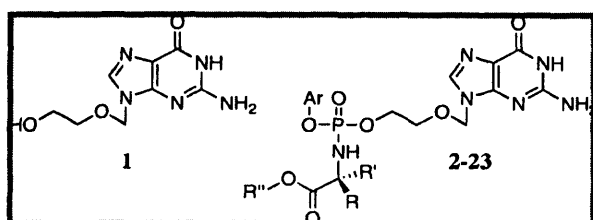


Figure 2. ACV and its ProTides.

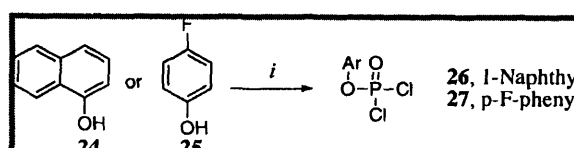
amino acid, as it has shown previously an optimal biological profile,¹² varying the other two components. For the aryl moiety we considered phenol, naphthol, and *p*-fluorophenol and as the ester moiety methyl, ethyl, *n*-propyl, *iso*-propyl, *tert*-butyl, and benzyl and combinations thereof. All of these combinations allowed us to extensively vary the LogP for these compounds and to study how this variation can influence the antiviral activity. Moreover, it has been previously reported how the substitutions can influence the bioactivation of the ProTide; for example, naphthol has shown an enhancement of activity against a panel of cancer cell lines for phosphoramidates⁶ and the *tert*-butyl ester showed a lack of biological activity due to the poor bioactivation of the bulky moiety. Following the results for these derivatives, different amino acids have been considered including L-valine, L-leucine, L-isoleucine, L-proline, glycine, and the non-natural D-alanine, D-valine, and dimethylglycine. Moreover, some intermediate-protected (*N*₂-DMF)-ACV ProTides have been biologically evaluated.

Chemistry

The compounds have been synthesized following the procedure reported by Uchiyama¹³ using *tert*-butylmagnesium chloride (*t*-BuMgCl) as a coupling reagent and using THF as a solvent in most of the cases.

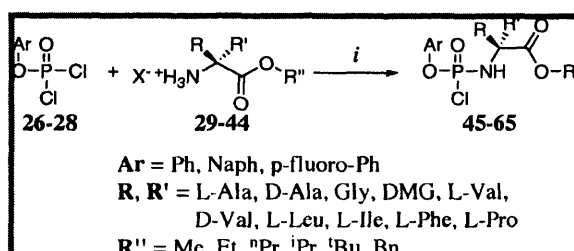
Aryl phosphorodichlorophosphates **26** and **27** have been synthesized, coupling respectively 1-naphthol (**24**) or *p*-fluorophenol (**25**) with POCl₃ in the presence of Et₃N (1 equiv) (Scheme 1), while phenyl dichlorophosphate (**28**) was commercially available. The coupling with the appropriate amino acid ester salt (**29–44**) has been performed in the presence of Et₃N (2 equiv) (Scheme 2), giving the final product (**45–65**) as an oil which was, in most of the cases, purified by column chromatography.

Scheme 1^a



^a Reagents and conditions: (i) POCl₃, anhydrous TEA, anhydrous Et₂O, –78 °C, 1 h then rt, overnight.

Scheme 2^a

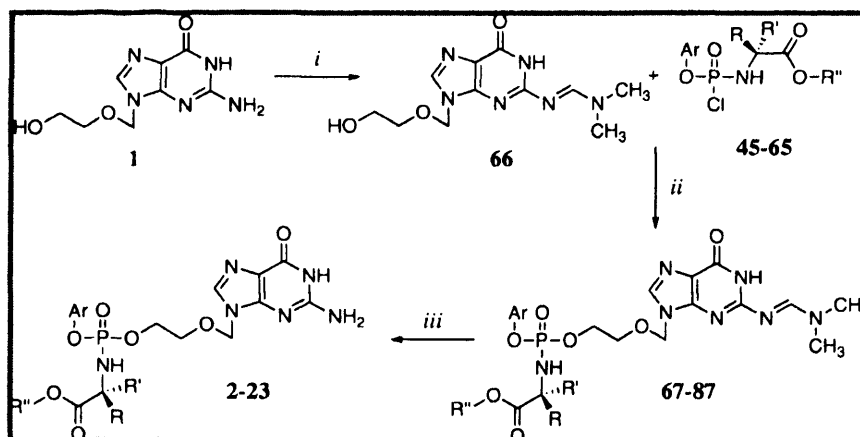


^a Reagents and conditions: (i) anhydrous TEA, anhydrous DCM, –78 °C, 30 min to 1 h, then rt, 30 min to 4 h.

To improve the solubility of ACV in THF, used as ideal solvent for the coupling reaction, the 2-amino was protected using dimethylformamide dimethyl acetal (Scheme 3). However, compound **66** is not completely soluble in THF but the solubility was improved sufficiently to carry out the reaction. The final coupling of the nucleoside was performed using an excess of the appropriate phosphorochloridate (1.50–4.00 equiv) in the presence of *t*-BuMgCl (2 equiv). Because of the reactivity problem, the use of *N*-methylimidazole (NMI), following the Van Boom procedure,¹⁴ was used for the synthesis of the L-proline (**22**) and glycine (**23**) derivatives. Moreover, a mixture of THF/pyridine (3/2) was used as a solvent to improve the solubility of *N*₂-DMF-ACV.

The deprotection of the dimethylformyl DMF derivative was initially carried out by refluxing the compound in 1-propanol (Scheme 3). However, because of a transesterification during the synthesis of **2**, obtaining compound **3**, the solvent was changed to 2-propanol, obtaining the desired compounds (**2**, **4–23**).

All the compounds were obtained as a mixture of two diastereoisomers confirmed by the presence of two peaks in the ³¹P NMR, with the exception of the glycine and

Scheme 3^a

^a Reagents and conditions: (i) dimethylformamide dimethyl acetal, anhydrous DMF, rt, 1 day; (ii) ^tBuMgCl, THF, rt, overnight or NMI, THF/pyridine = 3/2, rt, overnight; (iii) 1-propanol, reflux, for 18 h or 2-propanol, reflux, 24–96 h.

Table 1. Anti-HSV Activity for ACV and Its Protides

compds	aryl	amino acid	ester	CLogP	antiviral activity EC ₅₀ ^a (μM)			cytotoxic/cytostatic activity (μM)	
					HSV-1	HSV-2	HSV-1 TK ⁻	MCC ^b (Hel)	IC ₅₀ ^c (Hel)
2	Naph	L-Ala	Bn	2.06	2 ± 0	1.4 ± 0.8	10 ± 2.1	≥ 20	20
3	Naph	L-Ala	ⁿ Pr	1.41	5.5 ± 2.1	1.9 ± 1.6	16 ± 5.7	≥ 50	68
4	Naph	L-Ala	Me	0.35	16 ± 5.7	10 ± 2.1	79 ± 29	> 50	> 100
5	Naph	L-Ala	Et	0.88	32 ± 25	9.5 ± 0.7	32 ± 18	> 150	> 100
6	Naph	L-Ala	^t Bu	1.59	> 100	50	> 100	> 50	> 100
7	Naph	L-Ala	ⁱ Pr	1.19	15 ± 7.1	10 ± 0	≥ 45	> 50	> 100
8	Ph	L-Ala	Me	-0.82	20 ± 0	16 ± 5.7	79 ± 29	> 100	ND
9	Ph	L-Ala	Bn	0.89	8 ± 5.7	4 ± 0	15 ± 7.1	> 50	91
10	Ph	L-Ala	ⁱ Pr	0.02	10 ± 0	8.5 ± 0.7	27 ± 25	> 50	> 100
11	<i>p</i> -F-Ph	L-Ala	Bn	1.11	0.9 ± 0.1	0.5 ± 0	1.5 ± 0.7	> 100	ND
12	Ph	D-Ala	Bn	0.89	2 ± 0	1.4 ± 0.8	23 ± 16	> 100	ND
13	Naph	DMG	Bn	2.37	2.4 ± 0	1.6 ± 1.1	3.2 ± 1.1	> 50	> 100
14	Ph	DMG	Bn	1.20	1.4 ± 0.85	0.8 ± 0.0	5.5 ± 2.1	> 100	ND
15	Ph	Phe	Bn	2.31	17 ± 4.2	8 ± 5.7	≥ 100	> 50	87
16	Ph	L-Val	Bn	1.82	2 ± 0	0.85 ± 0.2	7.5 ± 6.4	> 100	ND
17	Naph	L-Val	Me	1.28	> 100	> 100	> 100	> 100	ND
18	Naph	L-Val	Et	1.81	51 ± 9.2	32 ± 18	42 ± 3.5	> 100	ND
19	Naph	D-Val	Me	1.28	> 100	> 100	> 100	> 100	ND
20	Ph	L-Leu	Bn	2.35	0.8 ± 0.07	0.7 ± 0	1.4 ± 0.8	> 100	ND
21	Ph	L-Ile	Bn	2.35	1.1 ± 0.4	1.1 ± 0.4	1.4 ± 0.8	> 100	ND
22	Ph	L-Pro	Bn	2.82	> 100	> 100	> 100	> 100	> 100
23	Ph	Gly	Bn	0.58	3	0.8	9	> 100	ND
ACV (I)				-2.42	0.4	0.2	50	> 100	ND

^a 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%. ^b Minimal cytotoxic concentration, or compound concentration required to cause a microscopically visible alteration of cell morphology. ^c 50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%. ND = not determined

dimethylglycine derivatives, due to the absence of a chiral center, and L-proline, for which we were able to isolate only one diastereoisomer.

Biological Results

Anti-HSV Activity. The activity of the compounds were evaluated against three different strains of HSV including HSV-1 (KOS), HSV-2 (G), and thymidine kinase-deficient HSV-1 (ACV^R).

Native ACV showed submicromolar (EC₅₀: 0.4 μM and 0.2 μM) activity against respectively HSV-1 and HSV-2 (Table 1) but was inactive against TK-deficient HSV-1 (EC₅₀: 50 μM). The ACV ProTides did not show increased activity against HSV-1 and HSV-2 compared to the parent compound. Only two compounds (11 and 20), respectively the *p*-fluorophenyl-L-Ala-OBn and the phenyl-L-Leu-OBn

derivatives, showed anti-HSV activity in the submicromolar range, while the majority of the compounds showed an activity in the range of ca. 1–30 μM. Compound 6 having the bulky *t*-butyl group as ester moiety did not show any marked activity (≥ 50 μM) against the HSV strains. ACV has been evaluated against thymidine kinase-deficient HSV-1 showing a dramatic loss of activity (> 100-fold) (EC₅₀: 50 μM). Interestingly, several of the ProTides showed significant retention of activity, demonstrating a successful bypass of the first phosphorylation step (i.e., compounds 11, 13, 20, 21). Notably, none of the ACV ProTides showed appreciable cytostatic/cytotoxic activity despite the potential loss of antiviral selectivity that could follow from viral nucleoside kinase bypass.

Anti-HIV Aactivity. The ACV ProTides have also been evaluated against HIV-1 and HIV-2 in CEM and against

Table 2. Anti-HIV Activity of the ACV ProTides and ACV

compds	aryl	amino acid	ester	antiviral activity EC ₅₀ ^a (μM)			cytostatic activity (μM)		
				HIV-1 CEM	HIV-2 CEM	HIV-1 MT-4	IC ₅₀ ^b CEM	CC ₅₀ ^c (MT-4)	IC ₅₀ ^b (MT-4)
2	Naph	L-Ala	Bn	15 ± 14	8.9 ± 6.3	0.8	17	ND	> 150
3	Naph	L-Ala	ⁿ Pr	6.6 ± 5.6	24 ± 30	10	22	ND	ND
4	Naph	L-Ala	Me	10 ± 7.9	13 ± 6.4	4.7 ± 2.1	57	> 150	18.7 ± 3.2
5	Naph	L-Ala	Et	12 ± 9.8	42 ± 13	1.7 ± 0.8	32 ± 7.8	> 150	12 ± 5.3
6	Naph	L-Ala	^t Bu	> 100	> 100	> 150	> 100	> 150	> 150
7	Naph	L-Ala	ⁱ Pr	6.2 ± 5.4	12 ± 0.71	5.4	36 ± 15	> 150	72.5
8	Ph	L-Ala	Me	17 ± 4.6	26 ± 8.5	15	67 ± 7.8	ND	ND
9	Ph	L-Ala	Bn	16 ± 14	11 ± 4.9	5.7 ± 1.6	42 ± 11	> 150	33.8 ± 10.6
10	Ph	L-Ala	ⁱ Pr	> 100	> 100	> 150	> 100	> 150	> 150
11	p-F-Ph	L-Ala	Bn	> 20	> 20	ND	76 ± 13	ND	ND
12	Ph	D-Ala	Bn	> 250	> 250	ND	≥ 250	ND	ND
13	Naph	DMG	Bn	≥ 100	79 ± 30	ND	> 100	ND	ND
14	Ph	DMG	Bn	> 100	> 100	7	> 100	> 150	> 150
15	Ph	Phe	Bn	26 ± 11	34 ± 24	16	42	ND	ND
16	Ph	L-Val	Bn	> 50	> 50	ND	≥ 100	ND	ND
17	Naph	L-Val	Me	> 100	> 100	ND	> 100	ND	ND
18	Naph	L-Val	Et	> 100	> 100	ND	> 100	ND	ND
19	Naph	D-Val	Me	> 100	> 100	ND	> 100	ND	ND
20	Ph	L-Leu	Bn	> 20	> 20	0.8	> 20	17	> 150
21	Ph	L-Ile	Bn	> 20	> 20	ND	ND	ND	ND
22	Ph	L-Pro	Bn	> 20	> 20	ND	> 100	ND	ND
23	Ph	Gly	Bn	> 100	> 100	ND	> 100	ND	ND
77 ^d	Naph	DMG	Bn	> 20	> 20	15	40 ± 2.8	45	140
78 ^d	Ph	DMG	Bn	> 100	> 100	70	> 100	> 150	> 150
81 ^d	Naph	L-Val	Me	> 100	> 100	> 150	> 100	> 150	> 150
86 ^d	Ph	L-Pro	Bn	> 20	> 20	30	45 ± 0.0	90	> 150
ACV (I)				> 250	> 250	> 250	> 250	> 250	> 250

^a 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%. ^b 50% Cytotoxic concentration, or compound concentration required to decrease the viability of the cell cultures by 50%. ^c 50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%. ND = not determined. ^d N₂-DMF-ACV.

HIV-1 in MT-4 cell cultures and in HIV-infected tonsillar tissues *ex vivo* (Table 2).

While parent ACV was inactive, in most of the L-alanine derivatives (2–11), with the exception of 6, 10, and 11, showed activity (EC₅₀) in a range of 6.2–17 μM against HIV-1 (CEM) and in a range of 8.9–42 μM against HIV-2 (CEM) (Table 2). Similar results were obtained when these compounds were applied to the HIV-1-infected MT-4 cell cultures. In these cells, parental ACV was inactive whereas HIV was suppressed by all the tested compounds with EC₅₀'s in the range of 0.8–30 μM, with the exception of 6, 10, 78, and 81 (Table 2). The antiviral activity of the ACV ProTides was confirmed in HIV-infected tonsillar tissue *ex vivo*. The compounds 4, 5, and 9 suppressed HIV replication with EC₅₀'s in a range of 0.1–0.6 μM (data not shown). These findings indicate that the ACV ProTide approach can also be successfully applied to HIV-infected tissue.

No clear-cut structure–activity relationship could be observed with regard to the nature of the aryl moiety nor the alaninyl ester moiety in terms of eventual antiviral activity of the ProTide derivatives. The lack of activity obtained for the *t*-butyl analogue (6) is in agreement with the previous report and with the enzymatic experiment to be discussed later. All of these compounds showed an antiproliferative effect (on CEM or MT-4 cell cultures) in a range between 17 and 76 μM.

With regard to the amino acid modifications, we found that, besides the L-alanine ProTides, only the phenylalanine derivative (15) had activity against HIV-1 (26 μM) and HIV-2 (34 μM). All other derivatives, including D-alanine (12), dimethylglycine (13 and 14), L-valine (16–18), D-valine (19), L-leucine (20), L-isoleucine (21), L-proline (22), and glycine

(23), did not show appreciable activity in CEM. These results are in agreement with previous reports for other nucleosides, in which the substitution of the L-alanine with different natural L-amino acids gave loss (~10- to 100-fold) of antiviral activity.¹⁵ However, in the case of dimethylglycine, this result is quite surprising as this variation led usually to a retention of anti-HIV activity compared to the L-alanine derivatives.¹⁶

Interestingly, in MT-4 cell cultures, the amino acid modification seems to be tolerated, in fact compounds 14 (DMG) and 20 (L-Leu), showed respectively an anti-HIV-1 activity of 7 and 0.8 μM.

Moreover, in MT-4, the N₂-DMF-protected ACV ProTides (77, 78, and 86) showed activity against HIV-1 (EC₅₀'s: 15, 70, and 30, respectively), indicating that this kind of substitution may be tolerated. However, in the case of CEM, these compounds did not show any inhibitory activity.

From these results, it is possible to conclude that the amino acid L-alanine is optimal for the anti-HIV activity of the ACV ProTides. Neither D-alanine nor glycine can efficiently substitute for L-alanine nor can bulkier amino acids.

In contrast to the structural requirement for anti-HIV activities, the anti-HSV activity tolerates liberal amino acid variation. This may reflect different substrate specificities and/or different intracellular levels of the necessary activating enzymes. It should indeed be noticed that the HIV assays are performed in rapid proliferating lymphocyte cell cultures (generation time ~ 24 h), whereas the antiherpetic assays are carried out in confluent fibroblast monolayer (nonproliferating) cell cultures. Thus, the different cell-type and cell-cycle conditions between both assay models can result in different prodrug activation modalities that may

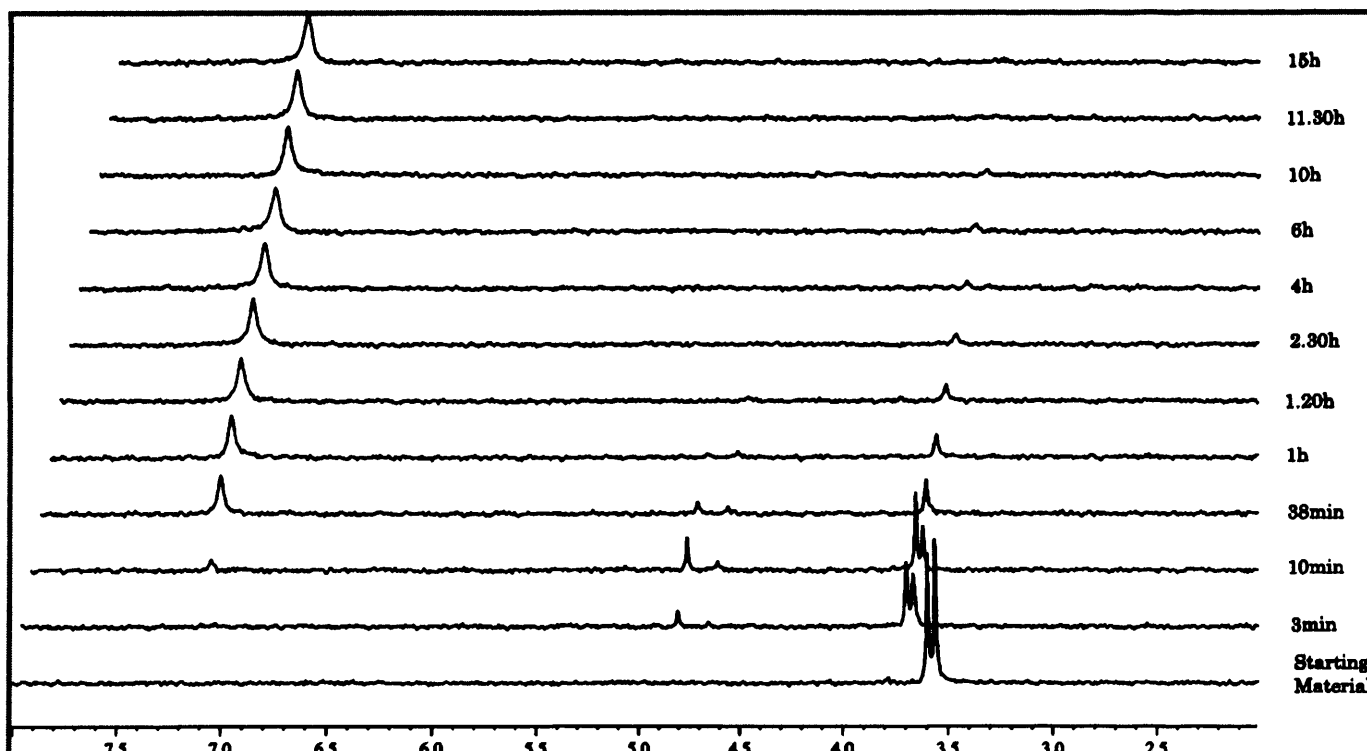


Figure 3. Carboxypeptidase-mediated cleavage of compound 9, monitored by ^{31}P NMR.

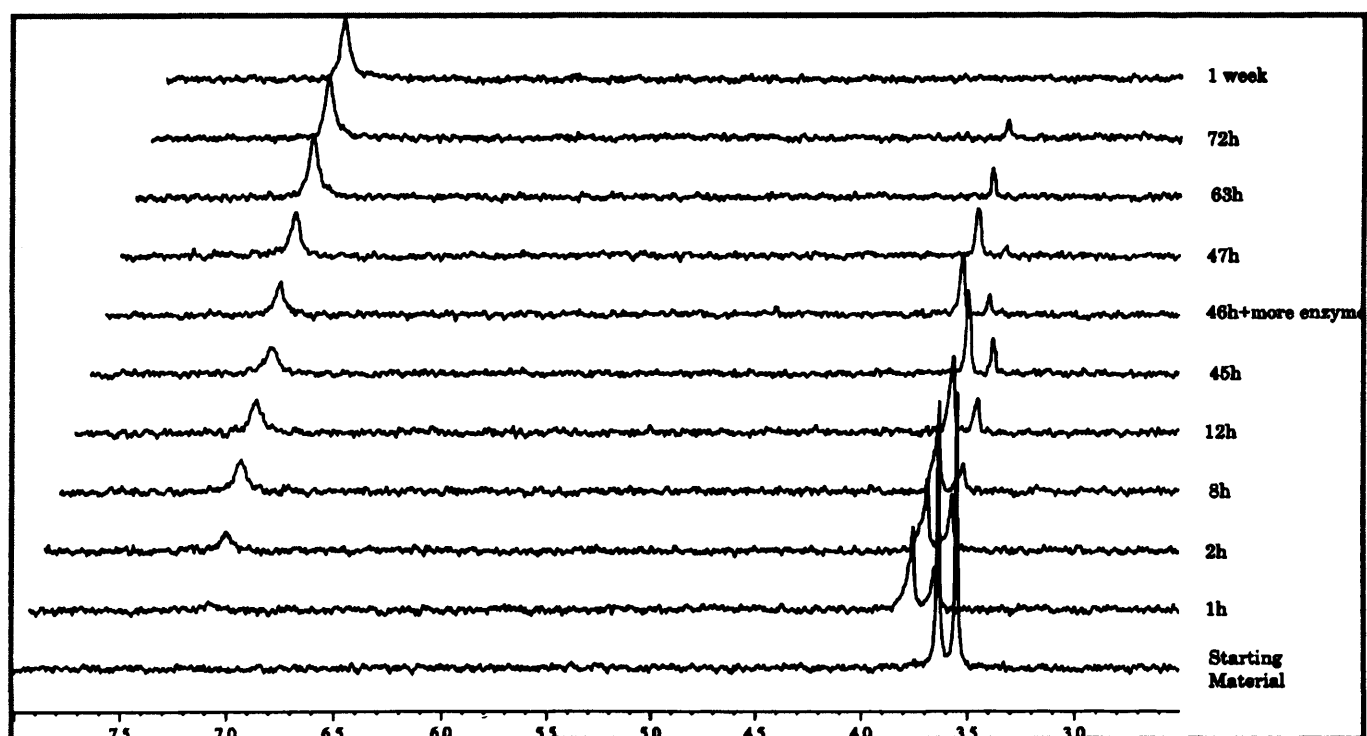


Figure 4. Carboxypeptidase-mediated cleavage of compound 10, monitored by ^{31}P NMR.

explain the differential antiviral activities of the ACV ProTides.

Enzymatic Studies. The mechanism of activation of the ProTides involves a first enzymatic activation step mediated by carboxypeptidase-type enzyme(s), which hydrolyze the ester of the amino acid moiety (Figure 1).

To probe the activation of the ACV ProTides to the monophosphorylated form inside cells, we performed an

enzymatic study using carboxypeptidase Y following the conversion by ^{31}P NMR. Of three different L-alanine derivatives (9, 6, and 10), the first one is active vs HIV and the second and third compounds are inactive against HIV, as well as the inactive L-valine 17 and D-valine derivatives 19 that have been considered for these experiments. The assay has been carried out by dissolving the compounds in acetone- d_6 and trizma buffer (pH = 7.6), incubating with the enzyme

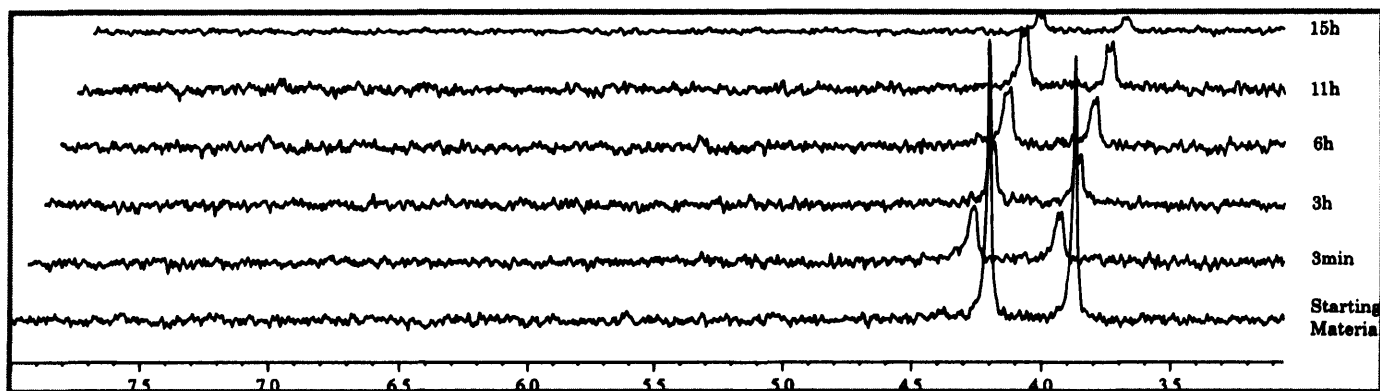


Figure 5. Carboxypeptidase-mediated cleavage of compound 6, monitored by ^{31}P NMR.

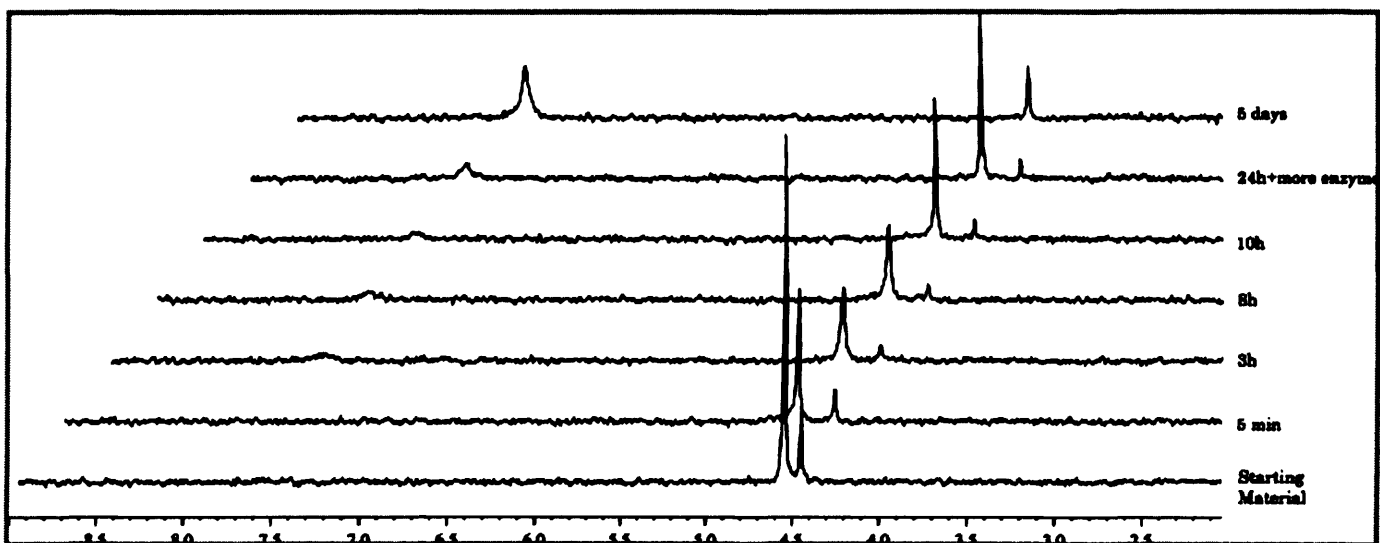


Figure 6. Carboxypeptidase-mediated cleavage of compound 17, monitored by ^{31}P NMR.

and recording a blank for each sample before the addition of the enzyme.

In the case of the phenyl benzylalanine compound **9** (Figure 3), the experiment showed a fast hydrolysis of the starting material ($\delta_{\text{P}} = 3.65$ and 3.60) to the intermediate type **88** (Figure 1) ($\delta_{\text{P}} = 4.85$ and 4.70), noting the presence of the two diastereoisomers. This intermediate is then processed to a compound of type **90** ($\delta_{\text{P}} = 7.10$) through the putative intermediate **89**, which is not detected by ^{31}P NMR. The half-life for **9** is 17 min. In the case of the isopropyl ester analogue **10** (Figure 4), the experiment showed a slow conversion of the starting material to **90** with a half-life of 46 h. This is ca. 150 times slower than **9**. This result is in accordance with the inactivity of **10** against HIV (Table 2). Notably one of the two diastereoisomers seems to be faster converted compared to the other one.

Compound **6**, the naphthyl *t*-butyl alanine analogue (Figure 5), showed no conversion at all presumably due to the presence of the *tert*-butyl ester, which is too bulky to be processed by the enzyme. This observation is in agreement with the lack of antiviral activity for this compound.

In the case of the L-valine derivative **17** (Figure 6), the experiment showed, as already demonstrated for compound **10**, a slow conversion to the compound of type **90**, with a half-life of 72 h. The D-valine derivative **19** (Figure 7) was not processed due to the presence of the non-natural amino acid, which seems not to be recognized by the enzyme.

Also, CEM cell extracts have been prepared to examine the rate of hydrolysis of the antivirally active **9** and **4** and the inactive **6** derivatives. Whereas **9** and **4** were efficiently hydrolyzed within a short time period ($>95\%$ conversion of **9** and 65% conversion of **4** within 1 h of incubation), **6** proved entirely stable after a 120 min incubation period (Figure 8). These observations are in agreement with the antiviral data and demonstrate that CEM cell-associated enzymes can efficiently convert methyl and benzyl esters of the ACV ProTides but not *tert*-butyl esters. Tonsil extracts were also found to efficiently hydrolyze **9** and **4**, with the same preference profile of **9** over **4** as found for the CEM cell extracts (data not shown).

These experiments support the need of activation of ACV ProTide in order to deliver the ACV monophosphate metabolite. The enzymatic data correlate well with the *in vitro* anti-HIV data and may support the role of carboxypeptidase Y in the ProTide activation in the lymphocyte cell cultures.

Stability Studies of ACV ProTide. Two different stability studies of compound **9** using human serum and pH 1.0 buffer have been conducted. In the case of human serum, **9** was dissolved in DMSO and D_2O and human serum was added. The experiment was conducted at 37°C and monitored by ^{31}P NMR. In Figure 9 are reported ^{31}P NMR spectra 10 min after the addition of the serum and after 12 h. For a better resolution, both original spectra and deconvoluted ones have

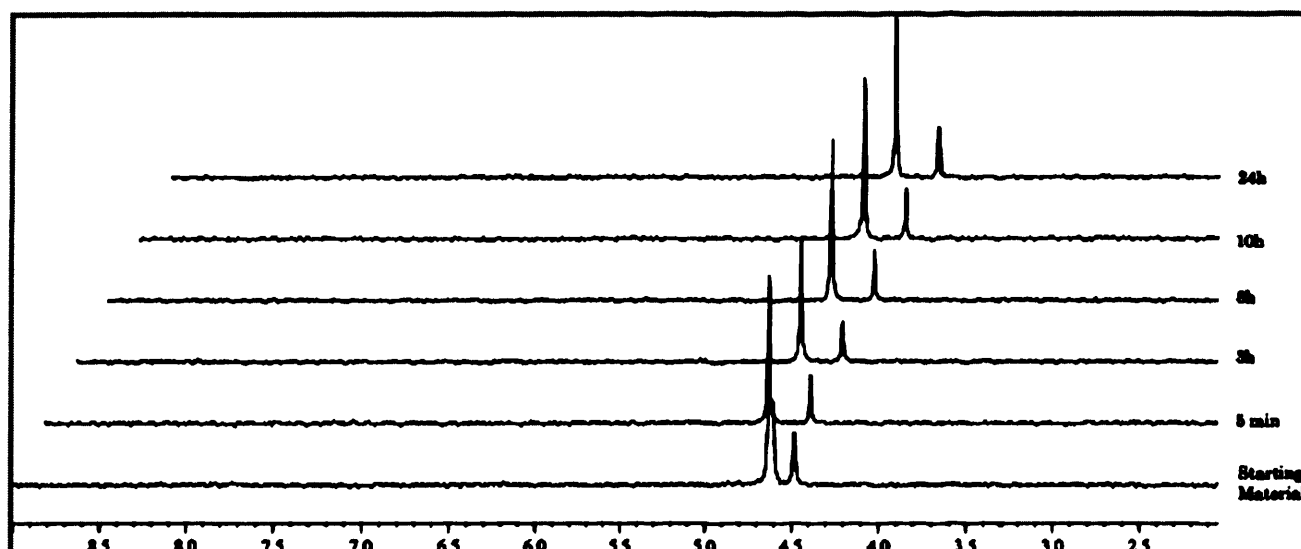


Figure 7. Carboxypeptidase-mediated cleavage of compound 19, monitored by ^{31}P NMR.

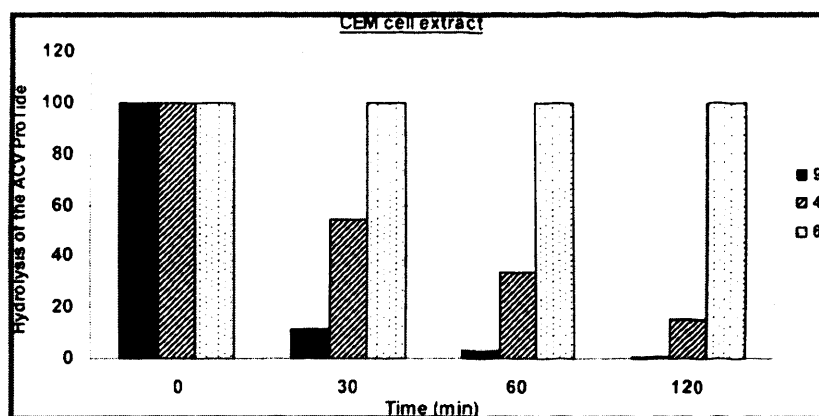


Figure 8. Stability of ACV ProTides in crude CEM cell extracts as a function of incubation time.

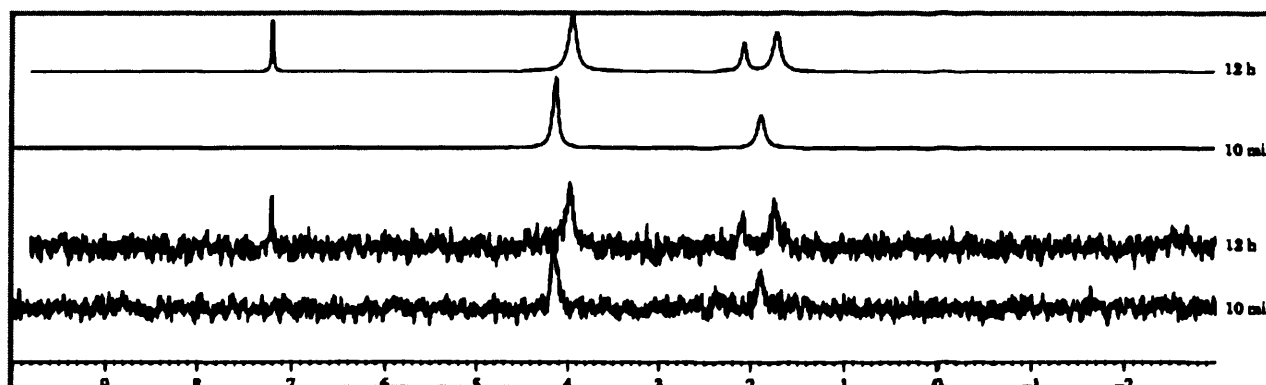


Figure 9. Stability of compound 9 in human serum, monitored by ^{31}P NMR.

been reported. The spectra show that the ACV ProTide is stable under these conditions. In fact, after 12 h, 56% of the compound is still present. The spectra also show the formation of the compound type **90** and the formation of a peak at $\delta_{\text{P}} = 1.90$, which may correspond to the monophosphate form. The peak at $\delta_{\text{P}} = 2.25$ corresponds to the human serum that in the first experiment is overlapping with the peak at $\delta_{\text{P}} = 1.90$.

In the case of the stability in acid, a pH of 1.0 was used. Compound **9** was dissolved in MeOD, and the buffer was

added. The experiment was conducted at 37 °C and monitored by ^{31}P NMR. The experiment showed a good stability of the compound (see Figure 12 in the Supporting Information) having an half-life of 11 h. Notably, the formation after 5 h of a peak at $\delta_{\text{P}} = -0.25$, which should correspond to the monophosphate form was observed.

Molecular Modeling-1: Carboxypeptidase Y Enzyme. To better understand the enzymatic results obtained using carboxypeptidase Y, molecular modeling studies using a crystal structure of the enzyme have been performed.¹⁷ The

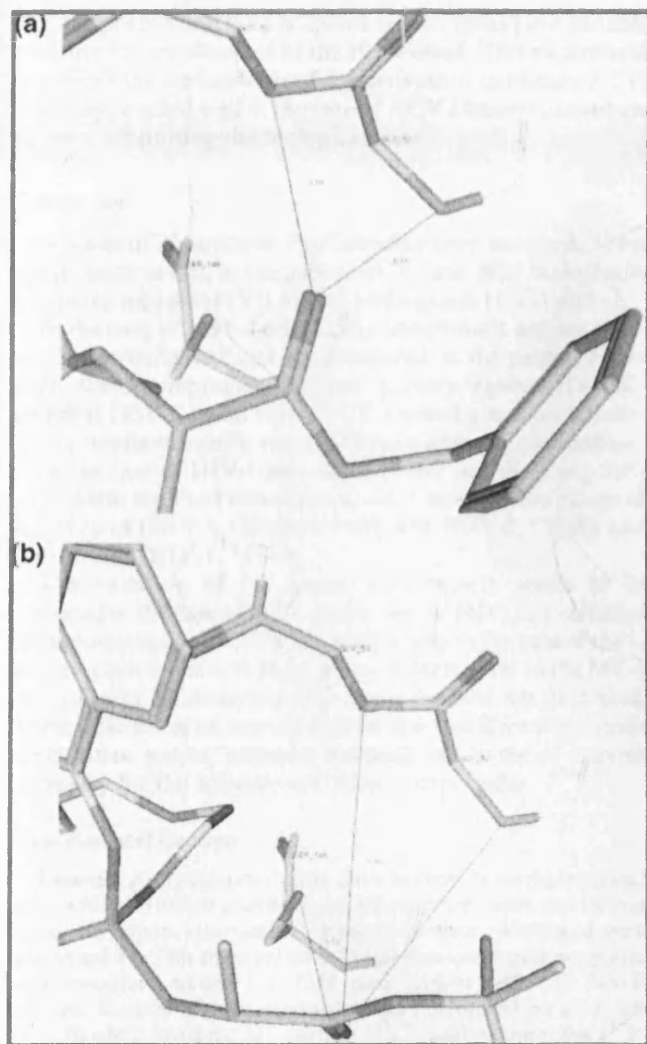


Figure 10. (a) Docking of compound **9** within the catalytic site of carboxypeptidase Y enzyme. (b) Docking of compound **6** within the catalytic site of carboxypeptidase Y enzyme.

putative mechanism of action involves an attack from the Ser146 to the carbonyl of the ester, which is coordinated with the NH from Gly52 and Gly53.¹⁸

The processed compound **9** showed a positive interaction with the active site of the enzyme for both phosphate diastereoisomers (shown only the *R_p* diastereoisomer) (Figure 10a). In particular, the carbonyl moiety is in a suitable position for the nucleophilic attack from the catalytic Ser146, with the NH from Gly52 and Gly53 correctly placed to stabilize the tetrahedral intermediate. This result is in accordance with the enzymatic result for this compound. In the case of the inactive compound **6**, the carbonyl is not in a favorable position, pointing away from Gly52 and Gly53, probably due to the presence of the bulky *tert*-butyl, which may influence the interaction with enzyme resulting in a poor activation (reported only the *S_p*-diastereoisomer, Figure 10b). The docking of compound **10**, which showed a faster hydrolysis of one diastereoisomer compared to the other one, showed interesting results. In fact, the two diastereoisomers docked in a different way. The *R*-diastereoisomer showed a preferable position for the carbonyl moiety, while in the case of the *S*-diastereoisomer the position of carbonyl group is different and it is not able to coordinate

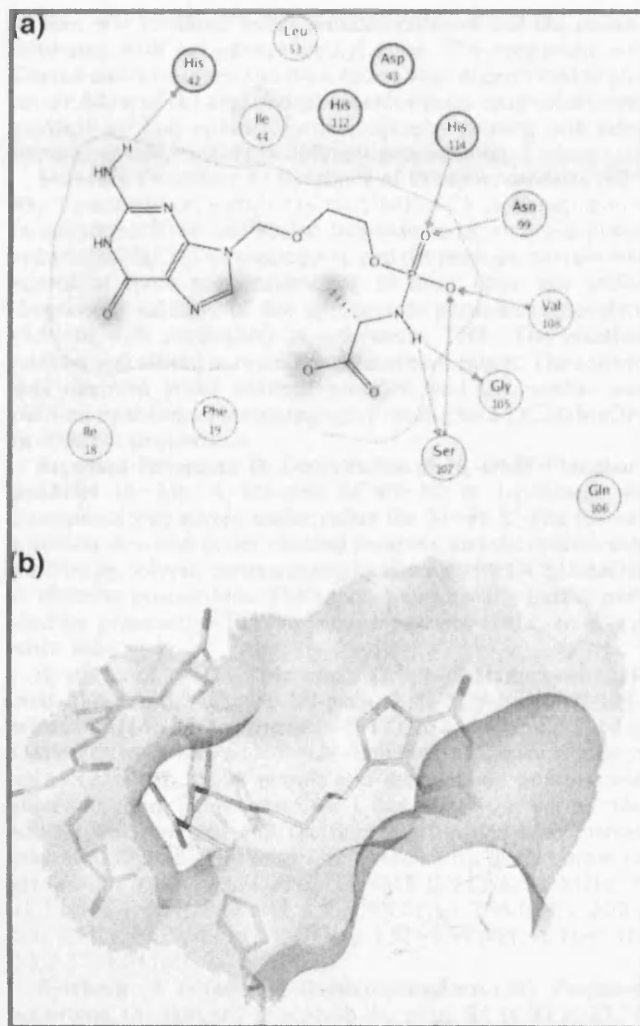


Figure 11. (a) Interactions of **90** (L-Ala) with the active site of human Hint-1. (b) Docking of compound **90** (L-Ala) ACV-MP phosphoramidate within the catalytic site of human HINT (I) enzyme.

with the Gly52 and Gly53. This result supports the fact that one of them is faster metabolized, presumably the *R_p*, than the other one, and this is due to a different binding in the catalytic site of the enzyme. In the case of the valine derivatives, none of them showed a suitable pose in the active site of the enzyme.

Molecular Modeling-2: Human Hint Enzyme. As shown in the enzymatic experiment on **9**, the first step of activation proceeds well and leads to compound **90**, which needs to be further converted in order to release the monophosphate form **91**. The last step of the activation of the ProTide involving the cleavage of the P–N bond is not well-known, and it is considered to be mediated by a phosphoramidase-type enzyme called Hint, belonging to the HIT superfamily.¹⁹ A molecular modeling study using human hint enzyme 1, cocrystallized with adenosine monophosphate, has been performed in order to investigate this last step of activation. The catalytic activity of this enzyme is due to the presence of three histidines, which interact with the substrate, and to the presence of a serine, which binds the nitrogen of the amino acid, protonating the nitrogen, and favoring P–N bond cleavage (Figure 11a). From Figure 11b, it is clear to see how the compound binds correctly in the active site of the

enzyme positioning the phosphate moiety (pink) in a suitable position for the cleavage of the P–N bond. This experiment suggests that the last step of the activation to release ACV-MP may proceed well in the case of ACV alaninyl phosphate *in vivo*, supporting the biological data.

Conclusion

A series of 22 acyclovir ProTides has been reported. These compounds as well as the parent ACV have been biologically evaluated against HSV-1 and -2 and against HIV-1 and -2.

In the case of HSV-1 and -2, the compounds did not show any improvement of activity compared to the parent. However, these compounds retained activity against the TK-deficient HSV-1 strain while ACV showed a loss on activity. These results showed a successful thymidine-kinase bypass.

In the case of HIV-1 and -2, ACV did not show any activity, while the ProTides show a good activity in a range of 6.2–17 μ M (HIV-1, CEM), 8.9–42 μ M (HIV-2, CEM), and 0.8–30 μ M (HIV-1, MT-4).

The variation of the amino acid moiety seems to be tolerated in the case of HSV. In the case of HIV, this variation is less tolerated, showing good results only in the case of the L-alanine derivatives and phenylalanine derivative. In the MT-4 cell cultures, dimethylglycine, and L-leucine are tolerated. These differences on activity may be due to different substrate specificities and/or different intracellular levels of enzyme necessary for the activation of these compounds.

Experimental Section

General. Anhydrous solvents were bought from Aldrich and used without further purification. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on silica gel 60-F254 precoated aluminum plates and visualized under UV (254 nm) and/or with ^{31}P NMR spectra. Column chromatography was performed on silica gel (35–70 μ M). Proton (^1H), carbon (^{13}C), and phosphorus (^{31}P) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 $^\circ\text{C}$. Spectra were autocalibrated to the deuterated solvent peak and all ^{13}C NMR and ^{31}P NMR were proton-decoupled. High resolution mass spectra was performed as a service by Birmingham University using electrospray (ES). CHN microanalysis were performed as a service by the School of Pharmacy at the University of London. Purity ($\geq 95\%$) of all final products was assured by a combination of microanalysis, and HPLC, with additional characterization in every case by: ^1H , ^{13}C , and ^{31}P NMR, and HRMS.

Standard Procedure A: Synthesis of Dichlorophosphates (26, 27). To a solution of phosphorus oxychloride (1.00 mol/equiv) and the appropriate substituted phenol or naphthol (1.00 mol/eq) in anhydrous diethyl ether, stirred under an argon atmosphere, and added dropwise at -78°C under an argon atmosphere anhydrous TEA (1.00 mol/equiv). Following the addition, the reaction mixture was stirred at -78°C for 30 min and then at room temperature overnight. Formation of the desired compound was monitored by ^{31}P NMR. The mixture was filtered under nitrogen and the corresponding filtrate reduced to dryness to give the crude product as an oil.

Standard Procedure B: Synthesis of Phosphorochloridates (45–65). To a stirred solution of the appropriate aryl dichlorophosphate **26–28** (1.00 mol/equiv) and the appropriate amino acid ester salt **29–44** (1.00 mol/equiv) in anhydrous DCM was added dropwise at -78°C under an argon atmosphere, anhydrous TEA (2.00 mol/equiv). Following the addition the reaction mixture was stirred at -78°C for 30 min to 1 h and then at room temperature for 30 min to 3.5 h. Formation of the desired compound was monitored by ^{31}P NMR. After this period, the

solvent was removed under reduced pressure and the residue triturated with anhydrous diethyl ether. The precipitate was filtered under nitrogen and the solution was concentrated to give an oil. Most of the aryl phosphorochloridates synthesized were purified by flash column chromatography (eluting with ethyl acetate/petroleum ether in different proportions).

Standard Procedure C: Synthesis of Phosphoramidates (67–85). To a stirring suspension of N_2 -DMF-ACV (1.00 mol/equiv) in anhydrous THF was added dropwise under an argon atmosphere $^t\text{BuMgCl}$ (2.00 mol/equiv), and the reaction mixture was stirred at room temperature for 30 min. Then was added dropwise a solution of the appropriate phosphorochloridate (1.50 to 4.00 mol/equiv) in anhydrous THF. The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the residue was purified by column chromatography eluting with DCM/MeOH in different proportions.

Standard Procedure D: Deprotection of N_2 -DMF-Phosphoramidates (2–23). A solution of **67–87** in 1-propanol or 2-propanol was stirred under reflux for 24–96 h. The solvent was then removed under reduced pressure, and the residue was purified by column chromatography eluting with DCM/MeOH in different proportions. The product was usually further purified by preparative TLC or semipreparative HPLC to give a white solid.

Synthesis of N^2 -DMF Acyclovir (*N*-(9-((2-Hydroxyethoxy)-methyl)-6-oxo-6,9-dihydro-1*H*-purin-2-yl)-*N,N*-dimethylformamide) (66). To a suspension of **1** (1.00 g, 4.44 mmol) in dry DMF (20 mL) was added *N,N*-dimethylformamide dimethyl acetal (2.96 mL, 22.20 mmol) and the reaction mixture was stirred at room temperature for 1 day. After this period, the solvent was removed and the residue triturated with diethyl ether and filtered. The solid was washed with diethyl ether to give a white solid (97%, 1.20 g). ^1H NMR (DMSO, 500 MHz): δ 11.30 (1H, s, NH), 8.58 (1H, s, $\text{CHN}(\text{CH}_3)_2$), 7.94 (1H, s, H-8), 5.45 (2H, s, H-1'), 4.65 (1H, t, OH), 3.52–3.49 (4H, m, H-4', H-5'), 3.17, 3.04 (6H, 2s, $\text{N}(\text{CH}_3)_2$).

Synthesis of 1-Naphthyl Dichlorophosphate (26). Prepared according to standard procedure A, using **24** (4.00 g, 27.74 mmol) in anhydrous diethyl ether (60 mL), POCl_3 (2.59 mL, 27.74 mmol), and anhydrous TEA (3.87 mL, 27.74 mmol). After ^{31}P NMR, the solvent was removed under reduced pressure and the residue was triturated with anhydrous diethyl ether. The precipitate was filtered, and the organic phase was removed under reduced pressure to give an oil (95%, 6.91 g). ^{31}P NMR (CDCl_3 , 202 MHz): δ 3.72. ^1H NMR (CDCl_3 , 500 MHz): δ 8.02–8.00 (1H, m, H-8), 7.81–7.80 (1H, m, H-5), 7.72–7.70 (1H, m, H-4), 7.54–7.45 (4H, m, H-2, H-3, H-6, H-7).

Synthesis of N^2 -DMF-(benzoxo-*L*-alaninyl)-phosphorochloridate (45). Prepared according to standard procedure B, **26** (6.91 g, 26.48 mmol), *L*-alanine benzyl ester tosylate **29** (9.30 g, 26.48 mmol), and anhydrous TEA (7.40 mL, 52.96 mmol) in anhydrous DCM (100 mL). The reaction mixture was stirred at -78°C for 1 h, then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 5/5 to give an oil (72%, 7.68 g). ^{31}P NMR (CDCl_3 , 202 MHz): δ 8.14, 7.88. ^1H NMR (CDCl_3 , 500 MHz): δ 7.99–7.25 (12H, m, Naph, OCH_2Ph), 5.15–5.07 (2H, m, CH_2Ph), 4.30–4.23 (1H, m, CHCH_3), 1.49–1.46 (3H, m, CHCH_3).

Synthesis of N^2 -DMF-acyclovir-[1-naphthyl(benzoxo-*L*-alaninyl)] Phosphate (67). Prepared according to standard procedure C, from **66** (0.30 g, 1.07 mmol) in anhydrous THF (10 mL), $^t\text{BuMgCl}$ (1.0 M THF solution, 2.14 mL, 2.14 mmol), **45** (1.31 g, 3.25 mmol) in anhydrous THF (10 mL), and the reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography, eluting with DCM/MeOH = 95/5, to give a white solid (17%, 0.12 g). ^{31}P NMR (MeOD, 202 MHz): δ 4.18, 3.92. ^1H NMR (MeOD, 500 MHz): δ 8.47, 8.46 (1H, 2s, $\text{NCHN}(\text{CH}_3)_2$), 8.01–7.98 (1H, m, H-8 Naph), 7.78–7.74 (2H, m, H-8, H-6 Naph), 7.56, 7.55 (1H, m,

H-2 Naph), 7.41–7.12 (9H, m, Naph, OCH_2Ph), 5.37–5.36 (2H, 2s, H-1'), 5.00–4.93 (2H, m, OCH_2Ph), 4.14–4.06 (2H, m, H-5'), 3.96–3.88 (1H, m, CHCH_3), 3.88–3.59 (2H, m, H-4'), 2.95–2.93 (6H, m, $\text{N}(\text{CH}_3)_2$), 1.20–1.17 (3H, m, CHCH_3).

Synthesis of Acyclovir-[1-naphthyl(benzyloxy-L-alaninyl)] Phosphate (2). A solution of **67** (0.10 g, 0.16 mmol) in 2-propanol (5 mL) was stirred under reflux for 2 days. The solvent was then removed under reduced pressure, and the residue was purified by column chromatography eluting with DCM/MeOH = 96/4. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 99/1, then 98/2, then 96/4) to give a white solid (35%, 0.032 g). ^1H NMR (MeOD, 500 MHz): δ 8.01–7.99 (1H, m, H-8 Naph), 7.77–7.75 (1H, m, H-6 Naph), 7.67, 7.64 (1H, 2s, H-8), 7.58–7.13 (10H, m, Naph, OCH_2Ph), 5.28, 5.25 (2H, 2s, H-1'), 4.99–4.94 (2H, m, OCH_2Ph), 4.12–4.06 (2H, m, H-5'), 3.97–3.93 (1H, m, CHCH_3), 3.64–3.59 (2H, m, H-4'), 1.24–1.20 (3H, m, CHCH_3). ^{13}C NMR (MeOD, 125 MHz): δ 20.32 (d, $J_{\text{C-P}}$ = 7.63, CHCH_3), 20.43 (d, $J_{\text{C-P}}$ = 6.61, CHCH_3), 51.76, 51.81 (2s, CHCH_3), 67.20 (d, $J_{\text{C-P}}$ = 5.58, C-5'), 67.28 (d, $J_{\text{C-P}}$ = 4.91, C-5'), 67.95, 67.98 (2s, OCH_2Ph), 69.34 (d, $J_{\text{C-P}}$ = 7.72, C-4'), 69.40 (d, $J_{\text{C-P}}$ = 8.14, C-4'), 73.65 (C-1'), 116.26, 116.29, 116.35, 122.69, 122.80, 125.92, 126.51, 127.20, 127.42, 127.46, 127.74, 128.81, 128.83, 129.27, 129.33, 129.52, 129.57 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph, OCH_2Ph), 136.26, 137.23 (C-4a Naph, "ipso" OCH_2Ph), 139.69 (C-8), 147.98, 148.04 ("ipso" Naph, C-4), 152.44 (C-2), 159.39 (C-6), 174.61, 174.88 (2s, COOCH_2Ph). EI MS = 615.1735 (M + Na). Anal. Calcd for $\text{C}_{28}\text{H}_{29}\text{N}_6\text{O}_7\text{P} \cdot 0.5\text{H}_2\text{O}$: C, 55.91; H, 5.03; N, 13.97. Found: C, 55.81; H, 4.91; N, 13.78.

Antiviral Activity Assays. The compounds were evaluated against the following viruses: HSV-1 strain KOS, thymidine kinase-deficient (TK^-) HSV-1 KOS strain resistant to ACV (ACV^r), HSV-2 strain G, HIV-1 strain IIIB/Lai, and HIV-2 strain ROD. The antiviral, other than anti-HIV, assays were based on inhibition of virus-induced cytopathicity or plaque formation in human embryonic lung (HEL) fibroblasts. Confluent cell cultures in microtiter 96-well plates were inoculated with 100 CCID₅₀ of virus (1 CCID₅₀ being the virus dose required to infect 50% of the cell cultures). After a 1–2 h adsorption period, residual virus was removed and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Antiviral activity was expressed as the EC_{50} or effective compound concentration required to reduce virus-induced cytopathicity by 50%.

Human CEM cell cultures ($\sim 3 \times 10^5$ cells mL^{-1}) were infected with 100 CCID₅₀ HIV-1(IIIB) or HIV-2(ROD) per mL and seeded in 200 μL well microtiter plates, containing appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, CEM giant cell formation was examined microscopically.

MT-4 cells (1×10^4 cells per mL) were suspended in fresh culture medium and infected with 10 μL (0.7 ng of p24) of X4_{LAI.04} viral stock per mL of cell suspension. Infected cell suspensions were then transferred to microplate wells, mixed with 1 mL of medium containing the test compound at an appropriate dilution and further incubated at 37 °C. After 3 days, p24 production was measured in the MT-4 cell culture supernatants. The EC_{50} corresponded to the compound concentration required to suppress the production of p24 in the virus-infected MT-4 cell cultures by 50%. Viability in MT-4 cell cultures were evaluated using a nucleocounter automated cell counting system (Chemometec, Denmark). Total number of cells and number of dead cells in the cultures untreated and treated with ACV ProTides were enumerated using a propidium iodide-based assay according to the manufacturers' protocol.

Data were collected and analyzed using Nucleoview software (Chemometec, Denmark).

Human tonsils obtained under an IRB-approved protocol were dissected into ~ 2 mm blocks and cultured on collagen rafts at the medium–air interface. Tissues were inoculated ex vivo with X4_{LAI.04} (~ 0.5 μg of p24_{gag} per block) and treated with ACV ProTides at concentrations ranging from 0.1 to 10 μM . The culture medium was changed every 3 days, and ACV ProTides were replenished. For each compounds' concentration HIV-1 release was quantified by measurements of p24_{gag} accumulated over 3-day periods in the culture media bathing 18 tissue blocks. The EC_{50} corresponded to the compound concentration required to suppress by 50% the production of p24.

Preparation of CEM and Tonsil Cell Extracts and Analysis of ProTide Conversion. Exponentially growing CEM cells or tonsil tissues were washed twice with PBS. Then, cells and tissues were suspended in PBS, and extracts were made in a Precellys-24 homogenizer (Berlin Technologies, Montigny-en-Bretonneux, France) (tonsils) or by a Hielscher-Ultrason Technology (CEM cells) (Germany). The extracts were cleared by centrifugation (10 min, 15000 rpm) and frozen at -20 °C before use. Ten micromolar solutions of **9**, **4**, and **6** were added to the crude cell and tissue extracts (100 μL) and incubated for 30, 60, and 120 min at 37 °C. At each time point, 20 μL of the incubation mixtures were withdrawn and added to 30 μL cold methanol to precipitate the proteins. After centrifugation, the supernatants were subjected to HPLC analysis on a reverse phase C18 column (Merck) to separate the parent ACV ProTides from their hydrolysis products that may be formed during the incubation process. Data were plotted as percent of disappearance of the intact parent ACV ProTide from the incubation mixture.

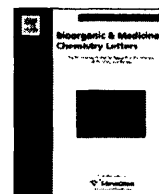
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Supporting Information Available: Preparative methods, spectroscopic and analytical data on target compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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2'-Fluorosugar analogues of the highly potent anti-varicella-zoster virus bicyclic nucleoside analogue (BCNA) Cf 1743

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Fluorosugars

ABSTRACT

We report the preparation of 2'- α -F, 2'- β -F and 2',2'-difluoro analogues of the leading anti-varicella zoster virus (VZV) pentyphenyl BCNA Cf 1743. VZV thymidine kinase showed the highest phosphorylating capacity for the β -fluoro derivative, that retained equal antiviral potency as the parent compound. In contrast, the α -fluoro- and 2',2'-difluoro BCNA derivatives were markedly less (~100-fold) antivirally active.

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In 1999 we first reported the anti-VZV activity of the bicyclic nucleoside analogue family now known as the BCNAs.¹ We subsequently reported nanomolar activity and exclusive anti-VZV selectivity for the pentyphenyl BCNA (Fig. 1, 1).²

As its 5'-valyl Pro-Drug FV100 (Fig. 1, 2) human phase 2 clinical trials for VZV shingles have recently commenced.³

We have reported extensively on the structure–activity relationships surrounding this family of potent antivirals.⁴ In general, there is little tolerance for structural modifications; indeed we recently reported that the corresponding carbocycle, a modification often tolerated amongst antiviral nucleosides, is very poorly active in this case.⁵ Also, the arabinosyl BCNA was considerably less inhibitory than its parental 2'-deoxyribose BCNA.⁶

There have been a number of cases where 2'-modification, in particular 2'-fluorination of bioactive nucleosides leads to enhancements in the biological activity profile, notably, the anti-cancer agent gemcitabine with a 2',2'-difluoro pattern⁷ and Pharmasset's anti-HCV agent PSI6130 (2'-deoxy-2'- α -fluoro-2'- β -C-methyl cytidine).⁸ Therefore, we were interested to prepare various 2'-fluorinated analogues of the parent BCNA (1) (Fig. 2).

The desired 2'-fluoro-substituted BCNAs were prepared via sugar base coupling of protected 5-iodouracil to the appropriate fluoro sugar followed by construction of the BCNA base.

Thus, as shown in Scheme 1, 5-iodouracil (3) was silylated to give (4) and 1,3,5-tri-O-benzoyl-2-deoxy-2- β -fluororibose (5) was converted to its 1-bromo analogue (6) using HBr/AcOH,⁹ and these reagents were allowed to couple using HMDS and ammonium sulfate to give mixed anomers of the protected nucleoside (7).⁹

The two isomers were separated by filtration to give the pure β -anomer as a white solid 7 in a yield of 51%. The deprotection of the benzoyl groups was performed using sodium methoxide and the corresponding unprotected compound 8 converted to its *p*-pentyphenyl BCNA (9) by standard methods^{1,2} in a yield of 45% from (7). Compound (9) showed spectroscopic (¹H NMR and ¹³C NMR) and analytical data entirely as expected including a ¹⁹F NMR peak at –198 ppm and other data (high resolution mass spectra and HPLC) confirmed its structure and purity.¹⁰ Similarly prepared by analogous methods were the α -fluoro analogue (10) and the 2',2'-difluoro BCNA derivative (11). Compound (10) was prepared starting from the commercially available 2'- α -F-2'-deoxyuridine which was iodinated at the 5-position and then coupled with the phenyl acetylene under standard method. Compound (11) was prepared starting from commercially available 2-deoxy-

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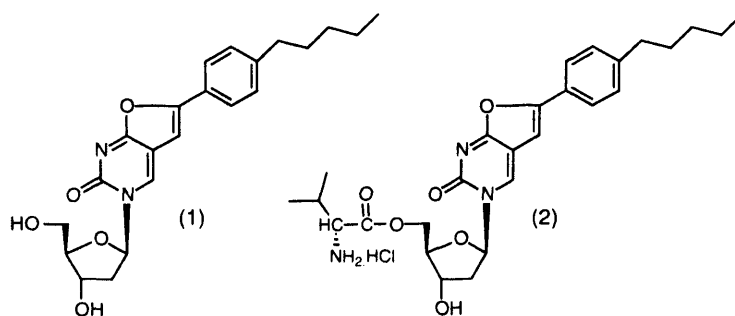


Figure 1.

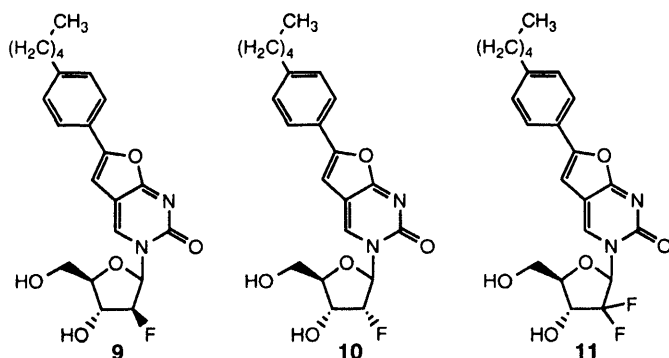


Figure 2.

Table 1

Compound	2'-Fluoro position	VZVTK IC ₅₀ /μM
1	–	3.3
9	β-Fluoro	38
10	α-Fluoro	2.7
11	Difluoro	~13

the phosphorylation of 1 μM [CH₃-³H]thymidine by purified recombinant VZV TK.

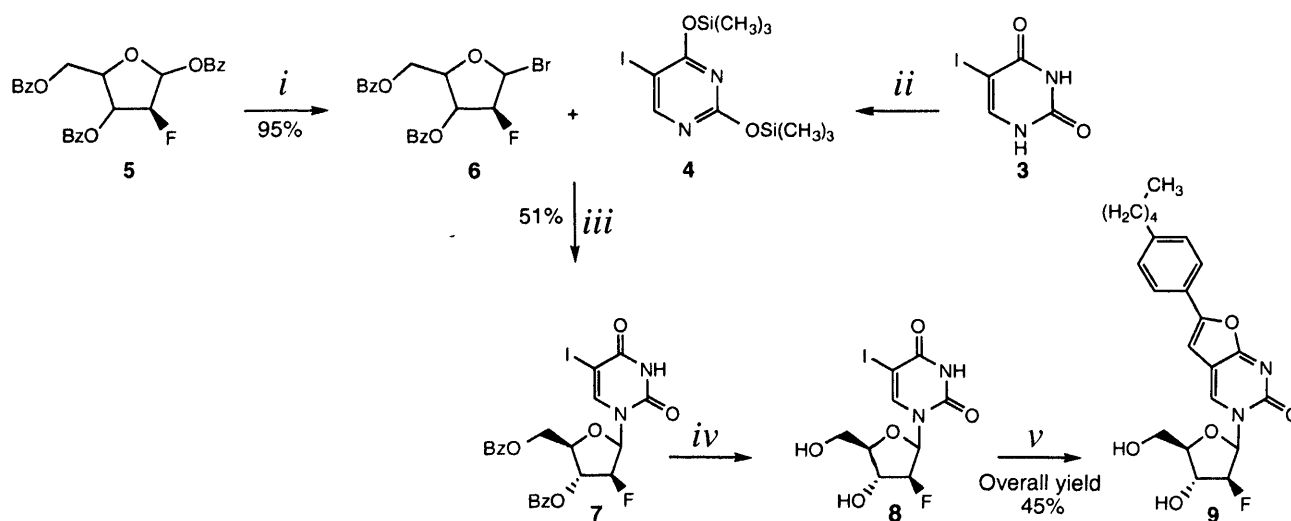
It is notable from Table 1 that the α-fluoro analogue (**10**) retains low μM potency as an inhibitor of VZV TK-catalysed dThd phosphorylation. The β-fluoro derivative **9** was at least 10-fold less inhibitory. The 2',2'-difluoro analogue **11** showed an IC₅₀ value in between **9** and **10** (Table 1).

We then measured substrate activity of the BCNA derivative for VZV TK at different compound concentrations by determining both *K_m* and *V_{max}* values for each compound (Table 2, SI).

Compared with the parent compound **1** (Cf 1743), the three fluoro derivatives were endowed with *K_m* values that were somewhat (up to threefold for compound **10**) lower than for compound **1**. Whereas the *V_{max}* value for **9** was ~2.5-fold higher and for **11** was 1.5-fold lower than noticed for **1**, the *V_{max}* for the α-fluoro derivative **10** was markedly lower than observed for the parent compound (6–7-fold). As a result, the phosphorylating capacity (*V_{max}*/*K_m*) of the enzyme proved highest for the β-fluoro derivative **9** and lowest for the α-fluoro derivative **10** (Table 2).

2,2-difluoro-*D*-erythro-pentafuranos-1-ulose-3,5-dibenzoate which was reduced to benzoylated lactol using LiAl(O-*t*Bu)₃H and then mesylated. The crude mesylate was coupled with silyl-protected 5-iodouracil in dichloroethane and the β-anomer was obtained by precipitation from the organic solvent after the work-up. Deprotection of the benzoyl groups by sodium methoxide in methanol provided the desired nucleoside.

Given the crucial requirement for the BCNAs to be 5'-phosphorylated by VZV thymidine kinase (TK) for their antiviral activity¹¹ we first probed their interaction with VZV TK.¹² Thus, in Table 1 we show the 50% inhibitory concentrations (IC₅₀) of (**1**) and (**9–11**) for



Scheme 1. Reagents and conditions: (i) HBr in acetic acid, DCM, rt, 22 h; (ii) hexamethyldisilazane, ammonium sulfate, acetonitrile, 70 °C, 5 h; (iii) NaI, DCM, acetonitrile, rt, 1 week; (iv) MeONa, MeOH, 1 h, rt; (v) 4-*n*-pentylphenylacetylene, tetrakis (triphenylphosphine)Pd(0), CuI, DIPEA, DMF, rt, overnight, then CuI, TEA, 85 °C, 8 h.

Table 2
Kinetic values of the 2'-fluoro-substituted test compounds

Compound	K_m (μ M)	V_{max} (μ mol/ μ g protein/h)	V_{max}/K_m
1	1.58	22	14
9	1.11	50	45
10	0.47	3.4	7.2
11	0.95	14	14

The kinetic values shown in Table 2 were derived from the Lineweaver–Burk diagrams based on the data shown in Supplementary data.

Table 3

Compound	VZV EC ₅₀ ^a (μ M)			MCC ^b (μ M)
	OKA	YS	TK-07-1	
1	0.0097	—	>20	≥50
9	0.007	0.011	>50	>50
10	0.75	—	>20	>50
11	1.5	6.7	>50	≥50

^a 50% Effective concentration, or compound concentration required to reduce viral plaque formation by 50% in the VZV-infected human embryonic (HEL) cell cultures.

^b Minimal cytotoxic concentration, or compound concentration that results in a microscopical alteration of HEL cell morphology.

When tested in vitro against two strains of TK-competent VZV¹³ (Table 3) we found that (**10**) was moderately antivirally active, being ca. 100 times less active than (**1**). The 2',2'-difluoro BCNA (**11**) was also markedly less active than the parent drug **1**. By contrast, the β fluoro derivative (**9**) was highly active, being comparable to (**1**) in its anti-VZV activity. As previously shown,^{1,2} all compounds lost activity versus VZV TK- deficient virus strains, confirming their need for VZV TK-mediated activation (phosphorylation).

As noticed before for other BCNA derivatives,¹¹ phosphorylation by VZV TK proved necessary, but not sufficient to display potent antiviral efficacy. In fact, the parent compound **1** showed an equal capacity for phosphorylation to that of compound **11** (difluoro-derivative) but proved ≥100-fold more antivirally active than **11**. Instead, the β -fluoro derivative **9** could be ~3.5-fold better phosphorylated than **1**, but was found equally antivirally active as **1**. Thus, there is no correlation between antiviral potency and VZV TK affinity (substrate) properties indicating that other factors are important for eventual antiviral action.¹¹

Thus, in conclusion, we report the synthesis of the α - and β -mono-2'-fluoro analogues **10** and **9** of the potent anti-VZV BCNA (**1**) and also the 2',2'-difluoro BCNA **11**. Only the 2'- β -fluoro analogue retains full low-nanomolar potency versus VZV in cell culture.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.116.

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- Procedure for the preparation of (9).** Synthesis of 5-iodo-2'- β -fluoro-2'-deoxyuridine (**8**). To a stirring solution of (**7**) (2.40 g, 4.14 mmol) in anhydrous methanol (60 mL) was added NaOMe (0.49 g, 9.70 mmol) and the reaction mixture was stirred at room temperature for 1 h. After this period, the reaction was neutralized with Amberlite, filtered and concentrated to give the desired product, which was in the following step without further purification. Synthesis of 3-(2'- β -fluoro-2'-deoxy- β -D-ribofuranosyl)-6-(4-n-pentylphenyl)-2,3-dihydrofuro [2,3-d]pyrimidin-2-one (**9**). To a solution of (**8**) (1.56 g, 4.14 mmol) in anhydrous DMF (20 mL) were added: 4-n-pentylphenylacetylene (2.40 mL, 12.41 mmol), tetrakis triphenylphosphine palladium(0) (0.48 g, 0.41 mmol), copper(I) iodide (0.16 g, 0.83 mmol) and DIPEA (1.44 mL, 8.27 mmol) and the reaction mixture was stirred at room temperature, under an Argon atmosphere overnight. After this period were added copper(I) iodide (0.16 g, 0.83 mmol) and anhydrous TEA (20 mL) and the reaction mixture was stirred at 85 °C for 8 h. The solvent was then removed in vacuo and the residue was triturated with DCM and stirred at room temperature for 2 h. The solid was filtered and washed with DCM to give the desired compound as light brown solid (0.78 g, 45%). A sample of this compound was further purified by filtration through silica gel for testing. ¹⁹F NMR (DMSO-d₆, 471 MHz): δ -197.85. ¹H NMR (DMSO-d₆, 500 MHz): δ 8.73 (1H, s, H-4), 7.74 (2H, d, J = 8.25 Hz, Ph), 7.33 (2H, d, 8.25 Hz, Ph), 7.22 (1H, s, H-5), 6.25, 6.22 (1H, d, J = 3.70 Hz, J_{H-F} = 17.10 Hz, H-1'), 6.07 (1H, d, J = 4.60 Hz, 3'-OH), 5.28 (1H, t, J = 5.80 Hz, 5'-OH), 5.24, 5.14 (1H, ddd, J = 2.35 Hz, J = 3.65, J_{H-F} = 52 Hz, H-2'), 4.29, 4.26 (1H, ddd, J = 4.30 Hz, J = 6.10, J_{H-F} = 18.16 Hz, H-3'), 3.99 (1H, q, H-4'), 3.71–3.63 (2H, m, H-5'), 2.62 (2H, t, α -CH₂), 1.59 (2H, qn, β -CH₂), 1.35–1.24 (4H, m, 2 \times CH₂), 0.86 (3H, t, CH₃). ¹³C NMR (DMSO-d₆, 126 MHz): δ 13.85 (CH₃), 21.87 (CH₂), 30.32 (β -CH₂), 30.78 (CH₂), 34.87 (α -CH₂), 60.13 (C-5'), 73.08 (d, J_{C-F} = 24.49 Hz, C-3'), 85.22 (C-4'), 86.15 (d, J_{C-F} = 16.64 Hz, C-1'), 94.66 (d, J_{C-F} = 191.53 Hz, C-2') 98.60 (C-5), 107.14 (C-4a), 124.61 (Ph), 125.70 (ipso-C), 128.99 (Ph), 138.70 (C-4), 144.20 (para-C), 153.47 (C-6), 154.20 (C-2), 171.30 (C-7a). EI MS = 416.1749 (M⁺). HPLC = H₂O/CH₃CN from 100/0 to 0/100 in 30 min = retention time 23.81 min. 3-(2'- α -Fluoro-2'-deoxy- β -D-ribofuranosyl)-6-(4-n-pentylphenyl)-2,3-dihydrofuro [2,3-d]pyrimidin-2-one (**10**). ¹⁹F NMR (DMSO-d₆, 471 MHz): δ -201.19. ¹H NMR (DMSO-d₆, 500 MHz): δ 8.94 (1H, s, H-4), 7.75 (2H, H_a) -7.32 (2H, H_b) (J = 8.10), 7.22 (1H, s, H-5), 6.02 (1H, d, J = 17.0, H-1'), 5.60 (1H, d, J = 6.60, 3'-OH), 5.42 (1H, t, J = 4.90, 5'-OH), 5.00 (1H, dd, J = 3.70, J_F = 52.65, H-2'), 4.26–4.20 (1H, m, H-3'), 3.95 (1H, dd, J = 12.4, 2.9, H-4'), 3.77–3.67 (2H, m, H-5'), 2.61 (2H, t, J = 7.6, α -CH₂), 1.65–1.61 (2H, m, β -CH₂), 1.42–1.18 (4H, m, g/d-CH₂), 0.86 (3H, t, J = 6.90, CH₃). ¹³C NMR (DMSO-d₆, 126 MHz): δ 13.84 (CH₃), 21.88, 30.33, 30.79, 34.88 (C₄H₈), 58.62 (C-5'), 68.14 (t, J_{C-F} = 22.40, C-3'), 81.11 (C-4'), 85.14 (t, J_{C-F} = 31.2, C-1'), 98.37 (C-5), 107.94 (C-4a), 120.84, 122.91 (C-2'), 124.75 (C-H_a), 125.56 (ipso-C), 129.01 (C-H_b), 137.26 (C-4), 144.44 (para-C), 153.68 (C-6), 154.87 (C-2), 171.53 (C-7a). EI MS = 435.1731 (M+H). Anal. Calcd for C₂₂H₂₄F₂N₂O₅·0.5H₂O: C, 59.59; H, 5.68; N, 6.32. Found: C, 59.38; H, 5.59; N, 6.25.
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- Procedure of the VZV thymidine kinase experiments.** The IC₅₀ of the test compounds against phosphorylation of [CH₃-³H] dThd as the natural substrate by VZV TK was determined under the following reaction conditions: the standard reaction mixture (50 μ L) contained 50 mM Tris-HCl, pH8.0, 2.5 mM MgCl₂, 10 mM dithiothreitol, 2.5 mM ATP, 10 mM NaF, 1.0 mg/mL bovine serum albumin, 1 μ M [CH₃-³H] dThd (0.1 μ Ci), an appropriate amount of test compound and 5 μ L milli Q water. The reaction was started by the addition of

enzyme, and incubated at 37 °C for 30 min, and the reaction was terminated by spotting an aliquot of 45 µL onto DE-81 discs (Whatman, Maidstone, England). After 15 min, the discs were washed for three times 5 min in 1 mM HCOONH₄ while shaking, followed by 5 min in ethanol (70%). Finally, the filters were dried and assayed for radioactivity in a toluene-based scintillant. The IC₅₀ was defined as the drug concentration required to inhibit 1 µM thymidine phosphorylation by 50%. Thymidine kinase assays to evaluate the test compounds as a substrate for the enzyme were performed as follows: the standard reaction mixture contained 50 mM Tris HCl pH 8, 2.5 mM MgCl₂, 10 mM dithiothreitol, 2.5 mM ATP, 10 mM NaF, 10 µL milli Q water, test compound at various concentrations (0.5, 2, 5 and 12.5 µM) dissolved in DMSO and 5 µL of an appropriate amount (1.5 pg) of protein VZV TK in a total reaction mixture of 50 µL. The reaction mixture was incubated at 37 °C for 60 min, and the reaction was terminated by transferring the contents into 150 µL ice-cold methanol, followed after 10 min by centrifugation at 12,000g. The resulting samples were injected on a Waters HPLC to separate and quantify the nucleoside and 5'-monophosphates of the BCNAs. HPLC analysis to separate and quantitate the lipophilic reaction products was done on a Merck (Darmstadt, Germany) LiChroCART 125-4 RP column (5 µm) using the following gradient (flow 1 mL/min): 2 min at 98% NaH₂PO₄ (Acros, New Jersey, USA) 50 mM + heptanesulfonic acid 5 mM pH 3.2 (buffer) (Sigma, St. Louis, MO) and 2% acetonitrile (ACN) (Biosolve, Valkenswaard, The Netherlands); 6 min linear gradient to 80% buffer and 20% ACN; 2 min linear

gradient to 75% buffer and 25% ACN; 10 min linear gradient to 65% buffer and 35% ACN; 10 min linear gradient to 50% buffer and 50% ACN; 10 min isocratic flow; 5 min linear gradient to 98% buffer and 2% ACN; 5 min equilibration at the same conditions. Metabolites of the BCNAs were determined by fluorescence detection (excitation at 340 nm and emission at 415 nm). Retention times of BCNA nucleoside and 5'-monophosphate derivatives were as follows: **1** (Cf 1743): 30.2 and 22.0 min; **9** (Cf 2852): 31.8 and 24.0 min; **10** (Cf 2792): 31.3 and 22.7 min; **11** (Cf 2819): 32.8 and 24.5 min, respectively.

13. Procedure of the anti-VZV experiments in HEL cell cultures. The laboratory wild-type VZV strain OKA and the thymidine kinase-deficient VZV strain 07/1 were used. The OKA strain was supplied by Dr. M. Takahashi, Osaka University, Osaka, Japan. The YS strain was isolated from vesicular fluid of a patient with varicella and the TK-deficient 07/1 strain was isolated after exposure of BVaraU to VZV (YS)-infected cell cultures (Sakuma, Antimicrob. Agents Chemother. 1984, 25, 742). Confluent HEL cell cultures grown in 96-well microtiter plates were inoculated with VZV at an input of 20 PFU per well. After a 2-h incubation period, residual virus was removed and varying concentrations of the test compounds were added (in duplicate). Antiviral activity was expressed as the 50%-effective concentration required to reduce viral plaque formation after 5 days by 50% as compared with untreated controls. Cytotoxicity was expressed as the minimum cytotoxic concentration (MCC) or the compound concentration that causes a microscopically detectable alteration of cell morphology.