Cardiff School of Pharmacy and Pharmaceutical Sciences

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Design, Synthesis and Biological Evaluation of Acyclic Nucleotide Prodrugs as Potential Antiviral Agents

A Thesis submitted to Cardiff University for the degree of

Philosophiae Doctor

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ABSTRACT

Acyclovir (ACV) is an acyclic guanine nucleoside analogue used in the treatment of herpes simplex virus (HSV) and varicella zoster virus (VZV) infections. The human herpes virus-encoded thymidine kinase (HHV-TK) phosphorylates ACV generating ACV monophosphate, which is then converted to the active form ACV triphosphate by cellular kinases.
The ProTide approach, causing the direct release of the monophosphate form into the cell, allows bypass of the first phosphorylation step of nucleoside analogues. In previous studies acyclovir ProTides were found to be active *in vitro* against human immunodeficiency virus (HIV), demonstrating a successful release of ACV monophosphate into the cell.

In this work, an extensive study of structure-activity relationship was carried out varying the masking groups of the ACV ProTide. Subsequently, several substituents were considered on specific position of the guanine base and side chain of ACV resulting in the synthesis of the aryl phosphoramidate derivatives of ganciclovir, penciclovir, 6-O-alkyl acyclovir, 8-bromoacyclovir, and 8-methylacyclovir.
These derivatives were evaluated *in vitro* for their antiviral activity against HSV, VZV, human cytomegalovirus (HCMV), and HIV.
Enzymatic and molecular modeling studies were carried out in order to investigate the bioactivaton of the phosphoramidate derivatives synthesised in this work and correlate these results with their biological activity.

Finally, the ProTide approach was also applied to cidofovir resulting in the formation of the phosphonoamidate derivative of cyclic cidofovir. The *in vitro* antiviral activity of this compound against herpes viruses and poxviruses is reported, as well as investigation of the mechanism of activation, using enzymatic and molecular modeling studies.

Publication

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Abbreviation & Acronyms

AA	amino acid
ABC	abacavir
ACN	acetonitrile
ACV	acyclovir
Ac_2O	acetic anhydride
ADA	adenosine deaminase
Ala	alanine
AMP	adenosine monophosphate
ANP	acyclic nucleoside phosphonate
Ara-C	cytarabine
Ara-G	9-β-d-arabinofuranosylguanine
AZT	azidothymine
B	nucleobase
Bn	benzyl
BTEA	benzyltriethylammonium chloride
BVDU	brivudin
BVU	(*E*)-5-(2-bromovinyl)-uracil
CatA	cathepsin A
CC_{50}	50% cytotoxic concentration
CCR5	C-C chemokine receptor type 5
CD4	cluster of differentiation 4
cHPMPC	cyclic 1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine
cHx	cyclohexyl
Cl	chlorine
CMLV	camelpox virus
CN	ciano
CPY	carboxypeptidase Y
CPXV	cowpox virus

*Cyclo*Sal	cyclosaligenyl triester
Cyt	cytosine
cCMP	cyclic cytosine monophosphate
cPrPMEDAP	9-(2-phosphonomethoxyethyl)-N^6-cyclopropyl-2,6,diaminopurine
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DCMC	N,N-dicyclohexyl-4-morpholinecarboxamidine
ddI	2'-3'-dideoxyinosine
DMA	*N*,*N*-dimethylaniline
DMAP	dimethylaminopyridine
DMF	dimethylformamide
DMG	2,2-dimethylglycine
DMSO	dimethylsulfoxide
DMTr	dimethoxytrityl
DNA	deoxyribonucleic acid
DP	diphosphate
d4T	2',3'-didehydro-2',3'-dideoxythimidine
EPV	Epstain-Barr virus
EC_{50}	50% effective concentration
Et	Ethyl
EtO	ethoxide
FDA	Food and Drug Administration
FTC	(-)-β'-2'-3'dideoxy-5-fluoro-3'-thiacytidine
GCV	ganciclovir
Gly	glycine
GTP	guanosine triphosphate
IDU	idoxouridine
HCMV	human cytomegalovirus
HDP	hexadecyloxypropyl
HAART	highly active antiretroviral therapy

HEL	human embryonic lung
HBV	hepatitis B virus
HCV	hepatitis C virus
HMDS	hexamethyldisiloxane
HHV	human herpes virus
Hint	histidine triad nucleoside-binding protein
His	histidine
HIT	histidine triad
HIV	human immunodeficiency virus
hPEPT1	human peptide-specific intestinal transporter
HPLC	high performance liquid chromatography
i-Bu	isobutyryl
IC_{50}	50% inhibitory concentration
ID_{50}	50% inhibitory dose
PMPA	9-[(2-phosphonylmethoxy)propyl]-adenine
HEL	human embryonic lung
HPMPC	1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine
HPMPC-pp	1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine diphosphate
HSV	herpes simplex virus
Ile	isoleucine
*i*Pr	isopropil
Leu	leucine
MCC	microscopically visible alteration of cell morphology
Me	methyl
MeOH	methanol
MeO	methoxide
min	minutes
MP	monophosphate
mRNA	messanger RNA
MTT	monomethoxytrityl

Naph	naphthyl
NBS	*N*-bromosuccinamide
N.D.	not determined
NMI	N-methylimidazole
NMR	nuclear magnetic resonance
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NtRTI	nucleotide reverse transcriptase inhibitor
NO_2	Nitro
Nuc	nucleoside
ODE	octadecyloxyethyl
^{31}P	phosphorus isotope 31
PCV	penciclovir
Ph	phenyl
Phe	phenylalanine
PK	protein kinase
PLE	pig liver esterase
PMEA	9-[2-(Phosphonylmethoxy)ethyl]-adenine
POC	isopropoxycarbonyl
POM	pyvaloyloxymethyl
PPL	porcine pancreatic lipase
PRK	primary rabbit kidney
Pro	proline
ProTide	nucleotide prodrug
Pyr	pyridine
$(PyS)_2$	2,2'-dithiophyridine
RNA	ribonucleic acid
RT	reverse transcriptase
SATE	S-acyl-2-thioethyl
Ser	serine

SDTE	S-(2-hydroxyethylsulfidyl)-2-thioethy
TBA	tetrabutylammonium
*t*Bu	tert-butyl
*t*BuCH$_2$	neopentyl
*t*BuMgCl	ter-butylmagnesium chloride
TEA	triethylamine
Tetrakis	tetrakistrisphenylphosphine palladium
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMSBr	trimethylsilyl bromide
TosCl	para-toluensulfonic chloride
Tr	trityl
TsOH	para-toluensulfonic acid
Trizma	2-amino-2-hydroxymethylpropane-1,3-diol
TK	thymidine kinase
TLC	thin layer chromatography
TP	triphosphate
VZV	varicella zoster virus
Val	valine
VV	vaccinia virus
3TC	(-)-β'-3'-thia-2',3'-dideoxycytidine
3-Et-Ph	*meta*-ethyl-phenyl
4-Et-Ph	*para*-ethyl-phenyl

Chapter 1. Introduction

1.1 Viruses

Viruses are submicroscopic agents that infect animals, plants, insects, as well as unicellular organisms, such as protozoa, bacteria, fungi, and yeasts. Despite their self-replicating capacity, they are not considered living organisms. The essence of viruses consists in a small fragment of nucleic acid (DNA or RNA) enclosed in a protein shell. Lacking the machinery that synthesises the viral proteins, they need a host-cell to duplicate themselves. For this reason, they are obligate parasites.[1]

1.1.1 Viruses Structure

Virus structure has evolved in order to ensure the propagation of its genome. Despite several differences among virus families, some features are often present (fig.1.1):[2]

- Genome and functional proteins such as enzymes
- Capsid
- Envelope
- Receptors

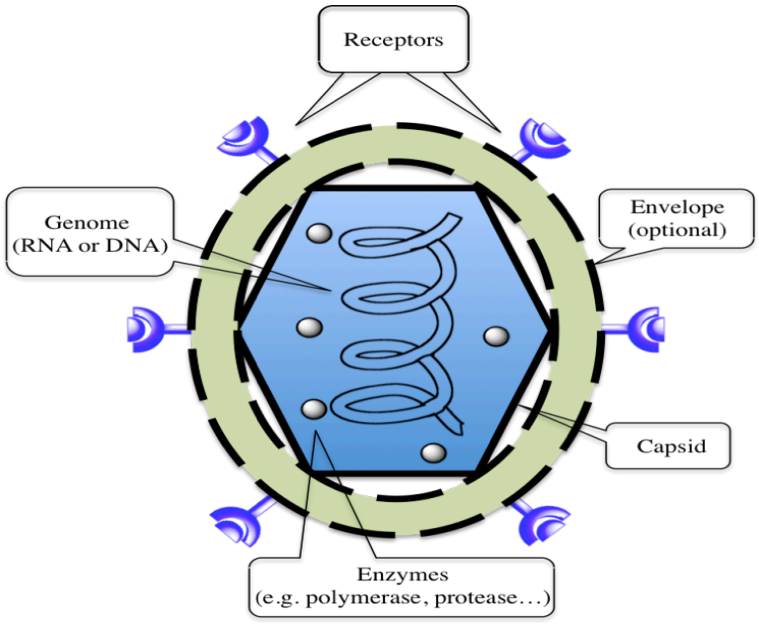


Figure 1.1. Generic virus structure

Genome

The genome is a nucleic acid that contains the information needed for replication of viruses. Depending on the virus, the genome is either DNA or RNA. Its size does not allow viruses to encode for all the machinery required for their replication. For this reason, viruses have evolved as obligate parasites: they must use proteins of host cell to complete their life cycle[1]. The viral genome encodes for capsid subunits, receptors that protrude from the virus surface and are required to initiate the entry phase of viral infection, and enzymes that are involved either in viral genome transcription and replication or in the maturation of viral proteins.[2]

Capsid

The protein shell (capsid), which encloses the viral genome and proteins, is constituted of many identical subunits by self-assembly to produce a symmetrical structure. Considering the small size of viral nucleic acid and the limited number of proteins that can be encoded, this is an example of genetic efficiency developed by viruses. The purpose of the capsid is to protect its contents from the external environment and to release its contents inside the host cell.[1]

Envelope

Some viruses are surrounded by a phospholipid bilayer, containing transmembrane proteins, called the envelope. This additional coating is acquired by budding through cellular membranes of the host cell. Its presence or absence constitutes the distinction between enveloped and non-enveloped viruses. It greatly affects the initial phase of virus infection, the entry phase, and the release phase.[2]

1.1.2 Virus lifecycle

The lifecycle of a virus is commonly constituted by the following phases (fig. 1.2):[1, 2]

1. Entry
2. Uncoating
3. Viral protein biosynthesis
4. Replication of the viral genome

5. Assembly

6. Maturation and release

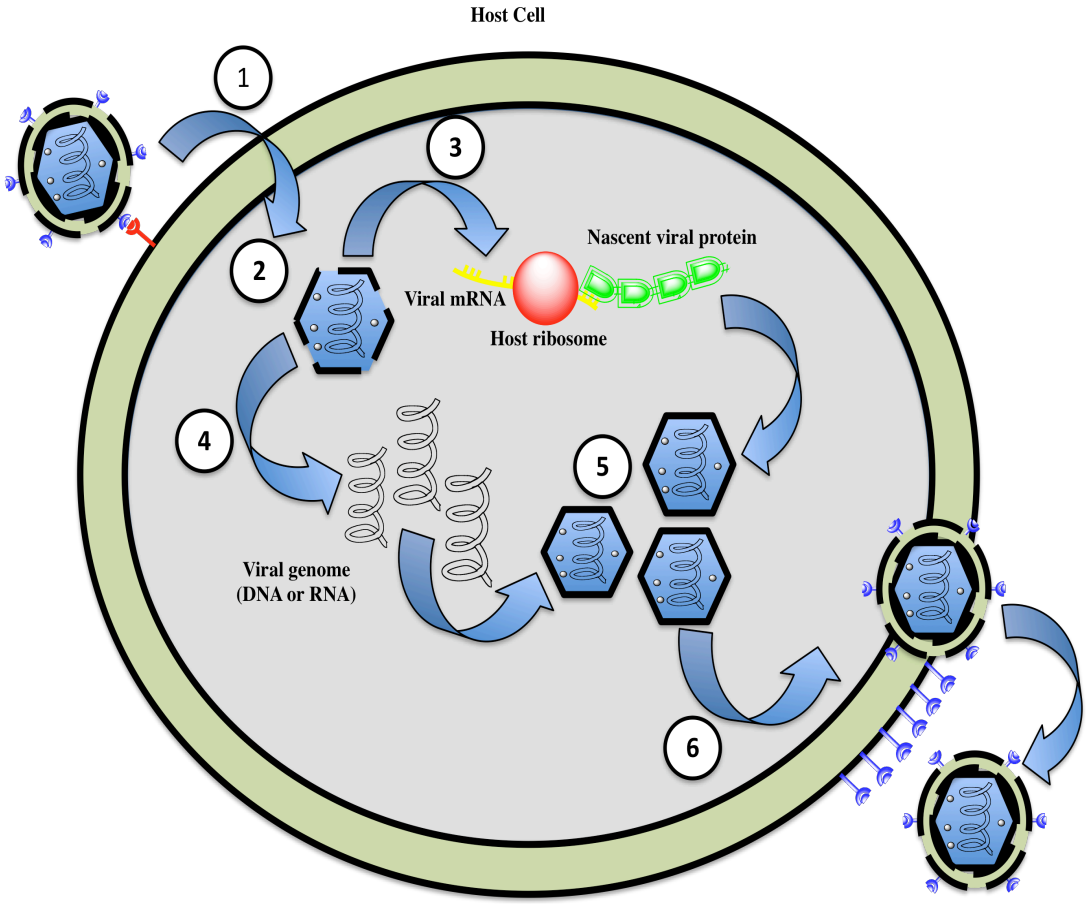


Figure 1.2. Generic virus life cycle: (1) entry; (2) uncoating; (3) viral protein biosynthesis; (4) replication of the viral genome; (5) assembly; (6) maturation and release

Entry and uncoating

The entry phase starts with the binding of virus-coded proteins on the surface of viral particle to specific proteins, carbohydrates, or lipid on the surface of the host cell.[2] This event triggers the release of viruses into the host cell.[2] The specificity of such interaction defines which type of cell is the target of virus infection. The pathway by which a virus passes through the plasma membrane differs from enveloped to non-enveloped viruses. The enveloped viruses are released inside the host cell by fusion of the envelope with either the plasma membrane or the membrane of endosomes, in which the virus is enclosed after the receptor-mediated endocytosis.[1] The non-enveloped viruses can either release their genome directly inside the cell through the formation of a membrane pore or induce endocytosis of the whole viral particle.[1]

Once inside the cell, the following step is the uncoating, the removal of the capsid, through which the viral genome is made available to be transcribed and duplicated.

Transcription, translation, and replication of the viral genome

After the cell entry and uncoating, the virus life cycle differs from DNA to RNA viruses.

DNA viruses are translocated into the nucleus, where the viral genome is transcribed into messanger RNA (mRNA) by host cell polymerase and duplicated by either cellular DNA polymerase (e.g. polyomaviruses) or virally encoded DNA polymerase (e.g herpes viruses).[1] The viral mRNA is decoded by the ribosome of the host cell to produce the virus proteins.[2]

In the case of RNA viruses, the genome either acts as mRNA and is translated immediately by the host ribosomes into proteins or requires a RNA-dependent RNA polymerase, which is carried into the host cell directly by the infecting virus, to produce the viral mRNA.[2] The replication of the virus genome is carried out by a virus-encoded RNA polymerase and generally does not require translocation into the cell nucleus.[2]

Retroviruses are a particular kind of RNA viruses that use the virus-encoded reverse transcriptase enzyme to convert the viral genome into a DNA molecule which is integrated into the chromosomes of the host cell.[2]

Assembly and maturation

The following phase of virus infection is the assembly of viral particles. In this process viral nucleic acid and proteins, which are required to start a new cycle of viral infection, are packaged into the capsid.[1] Once the viral particle has been assembled, the maturation of viruses is achieved by means of viral proteases, which cleave structural proteins in specific sites affording infectivity to the nascent viral particles.[1]

Release

The mature viral particles are released in the extracellular environment either by lysis of the host cell or exocytosis. In this phase enveloped viruses acquire their external phospholipid bilayer by budding through the cell membranes.[1]

1.2 Antiviral therapy

Viral infections cause several diseases worldwide. In many cases the immune system of the host is able to defeat the viral infection by producing antibodies, but in other cases viruses can evade such defensive systems and cause an infection that can be either symptomatic or asymptomatic.[3]

Vaccines have always been valid weapon in the prevention of viral infection by stimulating the immune system of a potential host to produce antibodies against a specific virus by means of the exposure to its antigens. However, their use in the antiviral therapy is prophylactic and their administration is generally ineffective when the virus has already infected the host.[1]

In order to keep viral infections under control, antiviral agents have been developed for those viruses that are able to overcome the natural defences of the host or when a vaccine is not available. In 1959, idoxouridine (IDU) was the first nucleoside analogue to be described as an antimetabolite that interferes with the polymerization of herpes viruses DNA.[4] In the late 1970s, Acyclovir represented a significant breakthrough in antiviral drug development due to its high selectivity in inhibiting herpes viruses infections and low toxicity.[4] After that, the research was further stimulated by the pandemic disease of acquired immunodeficiency syndrome (AIDS) and the need of new antiviral drugs against its etiological agent: the human immunodeficiency virus (HIV).[4] Nowadays, anti-HIV therapy has made a fatal disease like AIDS manageable.[5] Despite these successes, there are still some viral diseases for which an appropriate antiviral therapy is missing. For example, the standard of care of Hepatitis C virus (HCV) is ribavirin with interferon but this therapy is successful in only half of the population worldwide.[6] Moreover, the activity of antiviral agents is generally specific against one or few viruses.

In general, an ideal target of the antiviral therapy is a virus-encoded protein. It should be essential for the viral replication or at least its inhibition should lead to the formation of non-infective viral particles. Moreover, the antiviral agent should exhibit a high affinity for the viral protein, but not for the cellular counterpart, in order to have low toxicity. The ratio between antiviral activity and toxicity define the selectivity index of an antiviral agent.[6]

One of the main issues of the treatment of viral infections is their capacity to develop drug resistance. This is due to mutations of the genes that encode for the target protein, which is no longer recognised by the antiviral agent.[1, 6] The exposure to an antiviral drug induces a selection pressure that allows the survival of only mutant strains that are drug resistant. Mutations occur naturally during genome replication. They are more frequent in the case of RNA viruses than in DNA viruses, because their polymerase is less accurate.[1, 6] The higher the mutation rate, the more rapidly the resistance can develop. For this reason, the highly active antiretroviral therapy (HAART), which is currently applied for the treatment of HIV infection, is constituted by the combination of three or more different drugs in order to counteract the fast development of drug resistance.[1, 5, 6] Conversely, drug resistance is less likely to develop against antiviral agents that target host functions. In principle, a host protein could be an antiviral target if it is more important for the viral life cycle than for host functions.[1] An example of this approach is given by the antiviral drug Maraviroc, which inhibits the entry phase of HIV by binding the co-receptor CCR5 on the surface of CD4+ T-Helper lymphocytes (target cells of HIV).[5, 7] Obviously, targeting a host protein is inherently more likely to result in toxicity.

Considering the virus lifecycle, the potential targets of the inhibition of virus replication are virus attachment and entry, virus uncoating, viral genome replication, virus maturation, and virus release.[6] Most of the antiviral drugs on the market block the viral genome replication.[8-10] Being the main focus of this thesis, this class of antiviral agents will be discussed more extensively.

1.2.1 Inhibition of viral genome replication

The viral genome replication is generally mediated by virus-encoded polymerases. These enzymes, similarly to cellular counterpart, mediate the polymerisation of nucleic acid by insertion of monophosphate nucleosides (nucleotides) in the growing chain.[1] The natural nucleosides in DNA are deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine (fig. 1.3); in RNA the same nucleobases are bound to ribose sugar affording adenosine, guanosine, and cytidine; the only exception is thymine that is replaced by uracil affording uridine.[11]

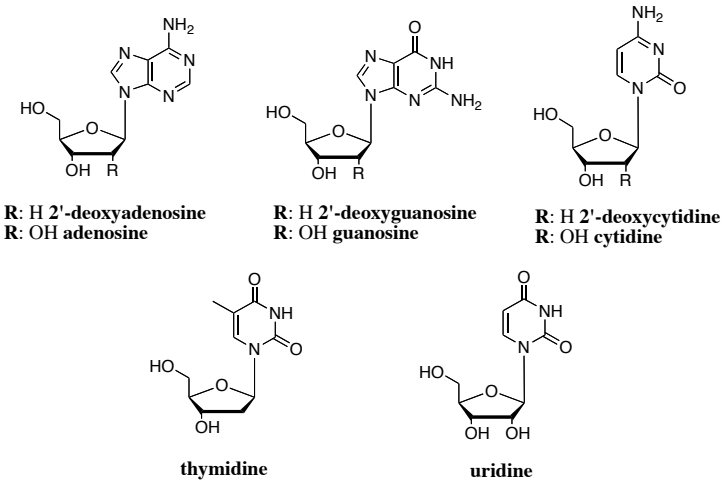


Figure 1.3. Structure of natural (deoxy)nucleosides

In the nucleic acid strand, nucleosides are bound together by a phosphodiester bond between the 3'-hydroxyl group of a residue and the 5'-hydroxyl group of the following one (fig. 1.4).[11]

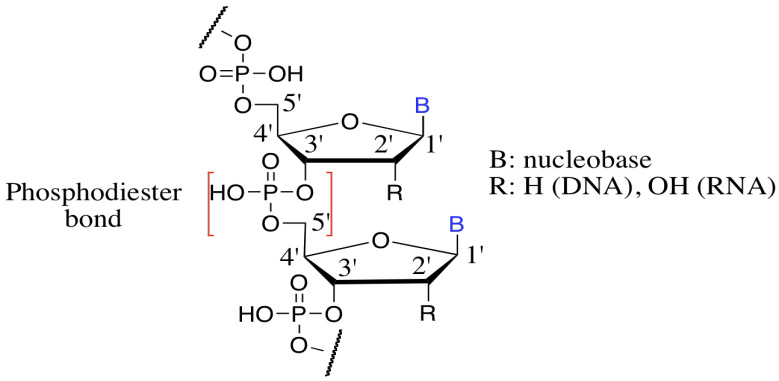


Figure 1.4. Phosphodiester bond of nucleic acid

DNA consists of two nucleic acid strands, while RNA is constituted by only one strand. Figure 1.5 depicts the base pairing between the two strands of DNA: the hydrogen bonds lead adenine to interact with thymine and guanine with cytosine.[11] According to this principle, knowing the sequence of nucleotides of one strand of DNA, we can deduce the complementary sequence on the other strand.

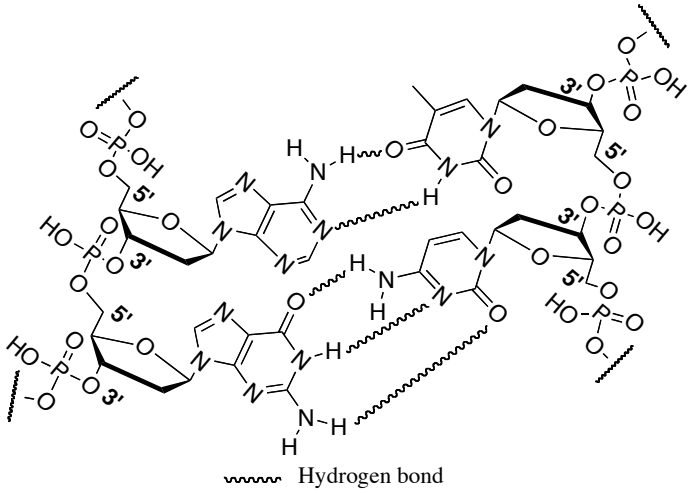


Figure 1.5. Base pairing of DNA

The elongation of the nucleic acid, is catalysed by the polymerase, DNA or RNA, in 5'-3' direction using one strand as a template and the mentioned principle of the base pairing. This means that the 3'-hydroxyl group of the terminal nucleotide residue in the growing nucleic acid strand attacks the phosphate in 5' position of the incoming nucleoside triphosphate allowing its insertion with elimination of the pyrophosphate group (fig. 1.6). [11]

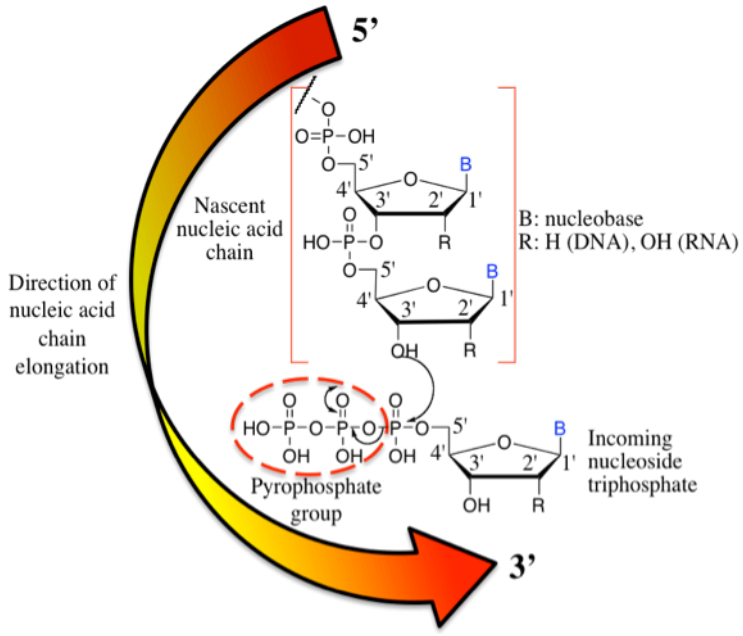


Figure 1.6. Polymerase-mediated insertion of nucleotides into the growing nucleid acid chain.

The antiviral agents that mimic the natural nucleosides are defined as nucleoside analogues. They are inhibitors of viral polymerase and compete with the natural nucleoside triphosphates for insertion into the growing nucleic acid strand, often

resulting in the termination of its elongation. Due to their mechanism of action they must be phosphorylated to the triphosphate form in order to exert their activity, so a nucleoside analogue is a prodrug of nucleoside triphosphate. A high phosphorylation rate is essential in order to achieve good antiviral activity. Currently, the antiviral drugs belonging to this class have been approved for the treatment of herpes viruses, HIV, hepatitis B virus (HBV), and Hepatitis C virus (HCV).[6, 8-10]

Non-nucleoside inhibitors of viral polymerase are another class of antiviral agents that exert their activity on the viral polymerase through a different mechanism of action in comparison to the antimetabolic nucleosides. At the moment foscarnet and the non-nucleoside inhibitors of reverse transcriptase (NNRTI) are the only antiviral drugs belonging to this class that have been approved for the treatment of herpesviruses and HIV, respectively. [1, 6, 8-10]

1.2.1.1 Herpesvirus DNA polymerase inhibitors

The *Herpesviridae* or herpes viruses are a family of DNA viruses. The members that have been identified as human pathogens are: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpes virus 6 (HHV-6), human herpes virus 7 (HHV-7), and human herpes virus 8 known as Kaposi's sarcoma-associated herpes virus (HHV-8).[1] The viral-encoded DNA polymerase carries out the replication of their genome.

Vidarabine, idoxuridine, and trifluridine are anti-herpes viruses agents that have been superseded in late 1970s by acyclovir (ACV), due to its better profile in terms of potency and selectivity.[12] Nowadays, ACV is still the first choice for the treatment of HSV and VZV infections. Ganciclovir (GCV) and penciclovir (PCV) are analogues of ACV.[12] Other drugs, which are currently approved for the treatment of herpes viruses, are the acyclic nucleoside phosphonate cidofovir (*S*-HPMPC) and the non-nucleoside foscarnet.[13] Brividun (BVDU) is a deoxyuridine analogue that has not been approved in the UK and in the USA, but it has been commercialized in various countries in Europe and in Japan. [6,10,14] All these antiviral agents, illustrated in fig. 1.7, are inhibitors of the DNA polymerase encoded by herpes viruses.[10]

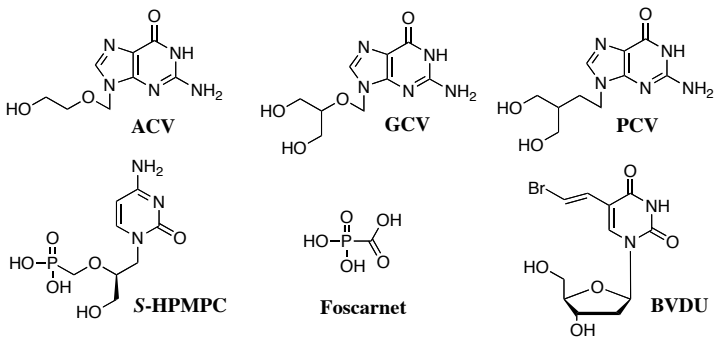


Figure 1.7. Structure of herpes viruses DNA polymerase inhibitors

Acyclovir (ACV)

ACV is an acyclic guanine nucleoside analogue that lacks the 3'-hydroxyl on the side chain (fig. 1.8).[15]

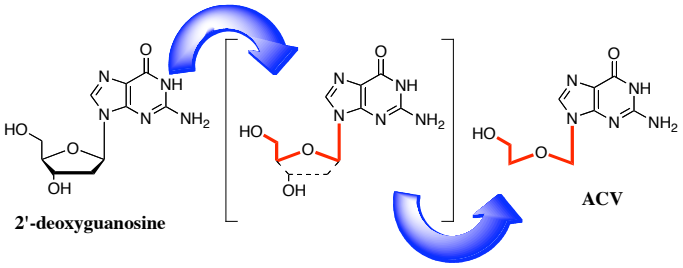


Figure 1.8. Comparison of the structures of ACV and 2'-deoxyguanosine showing the lack of the 3'-hydroxyl group

In 1982 the FDA approved ACV for the treatment of herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and varicella zoster virus (VZV). In order to improve its poor oral bioavailability (10-30%),[6] the L-valinyl ester derivative valacyclovir (Valtrex®) was synthesised (fig. 1.9).[16] This prodrug is completely metabolised to ACV by hydrolase enzymes during the first-pass intestinal and hepatic metabolism. The oral administration of valacyclovir increases the relative bioavailability of ACV by 3- to 5-fold. This is thought to result from the capacity of Valacyclovir to bind the intestinal and renal peptide transporters.[17]

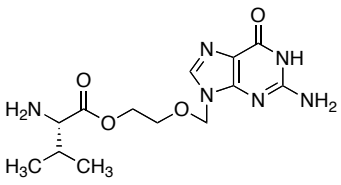


Figure 1.9. Structure of valaciclovir

In order to inhibit the herpes virus DNA polymerase, ACV must be phosphorylated to its triphosphate form.[15] The first phosphorylation step is mediated by the human herpes virus-encoded thymidine kinase (HHV TK), which has 200 times greater affinity for ACV in comparison to the cellular kinase.[15] Accordingly, the concentration of phosphorylated ACV is much higher (30 to 100 times) in HSV and VZV-infected cells than the concentration detected in uninfected cells. The monophosphate derivative (ACV-MP) is converted to ACV diphosphate (ACV-DP) and subsequently ACV triphosphate (ACV-TP) by cellular kinases (fig. 1.10).[18, 19] The triphosphate form inhibits the viral polymerase 10-30 times more potently than the cellular counterpart.[15, 20]

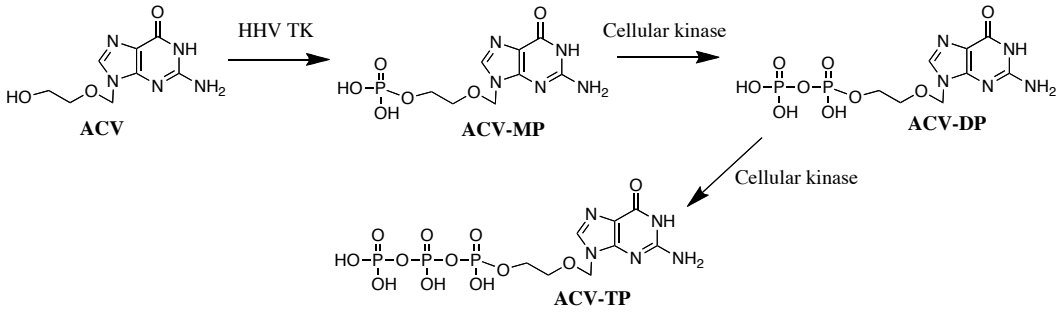


Figure 1.10. Phosphorylation steps of ACV

Acyclovir triphosphate is not only a competitive inhibitor of the viral polymerase, but it is also incorporated as a substrate into the nascent viral DNA chain (fig. 1.11).[21, 22]

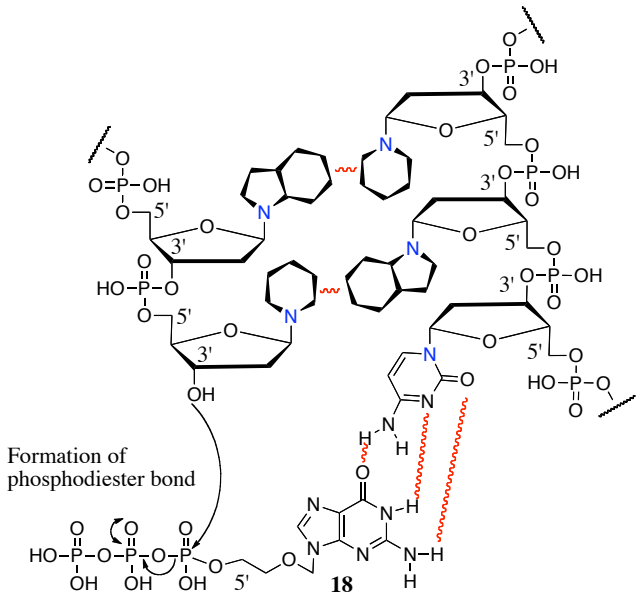


Figure 1.11. Insertion of ACV-TP into the nascent viral DNA

Due to its lack of the 3'-hydroxyl group, ACV-TP acts as an obligate chain terminator preventing further elongation of the nucleic acid strand trapping the polymerase in a so called "dead-end-complex", thus causing the inactivation of the replication machinery (fig. 1.12).

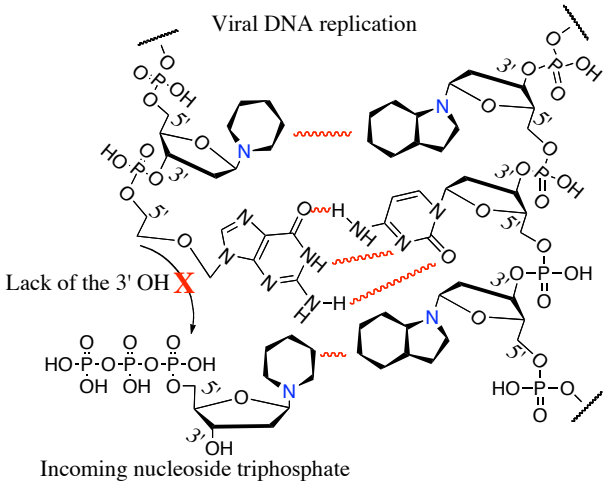


Figure 1.12. Termination of viral DNA elongation following ACV-TP incorporation

The selectivity for the virus-encoded TK and the viral polymerase account for the high therapeutic index of ACV.[6] The treatment of herpes virus infections with ACV only rarely leads to development of resistance in immunocompetent patients. Several surveys revealed the low incidence of ACV-resistant strain of HSV (below 1%). This percentage increases in immunocompromised patients (5%) and particularly in patients receiving allogenic bone marrow transplant (30%).[23] The mechanisms through which resistance to ACV develops are:[24, 25]

- Total lack of the viral TK (TK deficient strains)
- Impaired production or low activity of the viral TK (TK lower producer)
- Modification of the viral TK with loss of binding affinity for ACV (TK altered)
- Modification of the viral DNA polymerase with loss of binding affinity for ACV-TP

Resistance to ACV involving the viral TK is more common than an alteration of the polymerase. This is related to the fact that the TK enzyme is not strictly necessary for the successful completion of viral replication, while the mutation of the viral polymerase results in highly attenuated strains.[1]

Ganciclovir (GCV)

GCV is an analogue of ACV from which it differs due to the presence of an additional hydroxymethylene group on the acyclic side chain that mimics the 3'-hydroxyl group of the sugar moiety of natural nucleosides (fig 1.13).

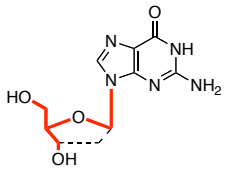


Figure 1.13 Structure of GCV showing the analogy with 2'-deoxyguanosine

GCV is active against HSV and HCMV;[26] it has been approved for the treatment of HCMV infections in immunocompromised patients and the prevention of HCMV disease in transplant patients. Ganciclovir inhibits HSV and HCMV DNA polymerase; the active form is ganciclovir triphosphate. It shares a similar pathway of activation with ACV. The first phosphorylation step is mediated by the viral TK in HSV, while in HCMV this process is catalysed by a viral protein kinase (PK), which is encoded by the gene UL97.[27] The lack of phosphorylation by this viral protein kinase is the reason for which ACV does not exert any activity against this member of herpes viruses [26]. Phosphorylation of GCV to the diphosphate and triphosphate forms is mediated by cellular kinases.[13] Similarly to ACV-TP, GCV triphosphate (GCV-TP) is a competitive inhibitor of deoxyguanosine triphosphate incorporation into the growing nucleic acid strand and also a substrate of viral DNA polymerase. However, it is not an obligate chain terminator: after GCV incorporation the insertion of an additional nucleotide is allowed before the termination of the DNA chain elongation.[28] This is due to the presence of the hydroxymethylene group of GCV, which, as already mentioned, mimics the 3'-hydroxyl group.[29] The main issue of this antiviral agent is its toxicity due to a lower selectivity for viral DNA polymerase in comparison to ACV: GCV-TP inhibits in some extent also cellular DNA polymerase. This is predictive of the bone marrow suppression (neutropaenia, anaemia, and thrombocytopaenia) that GCV induces.[13]

Mechanisms of resistance involve modification of TK in HSV and PK in HCMV leading to lack of phosphorylation of GCV to the monophosphate form (GCV-MP). As already seen for ACV, the incidence of viral DNA polymerase alteration is far more likely.[27] The oral bioavailability of Ganciclovir averages 6-9%.[6] This percentage

increases up to 60% after adminstration of its valyl ester derivative valganciclovir (fig. 1.14). The mechanism of metabolisation to Ganciclovir is the same of Valaciclovir.[13]

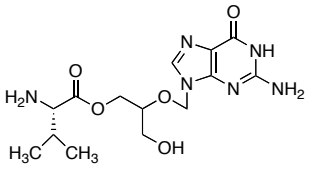


Figure 1.14. Structure of valganciclovir

Penciclovir (PCV)

PCV is the carbo analogue of GCV, where a methylene group replaces the ether moiety on the side chain. Similarly to ACV, this compound is active against HSV and VZV.[30] Moreover, PCV has showed inhibitory activity of EBV and HBV replication.[10]

As already seen for ACV and GCV, penciclovir triphosphate (PCV-TP) is an inhibitor of HSV and VZV DNA polymerase.[31] HSV or VZV TK mediates the first phosphorylation step, and subsequent phosphorylation steps are mediated by cellular kinases. In particular, PCV-TP is less potent than ACV-TP as an inhibitor of viral DNA polymerase. However, due to a more efficient phosphorylation by both viral and cellular kinases, it reaches a higher concentration inside the host cell over that of ACV-TP.[32] The long intracellular half-life ($t_{1/2}$= 17-20 h) of PCV-TP accounts for its prolonged antiviral activity.[6] TK deficient strains, which are ACV-resistant, show cross-resistance to PCV.[33] The low oral bioavailability (<5%) of PCV is considerably increased (ca 75%) by its prodrug famciclovir (fig. 1.15) that consists in the diacetyl derivative of 6-deoxypenciclovir.[34] The bioactivation pathway of famciclovir to PCV involves the hydrolytic cleavage of one acetyl group in the intestine, followed by the cleavage of the second acetyl group in the liver.[35] Finally, xantine oxidase or aldehyde oxidase catalyze the oxidation of the 6-position.[10, 36] Oral famciclovir, topical penciclovir, and intravenous PCV have been approved for the treatment of HSV and VZV infections.[10]

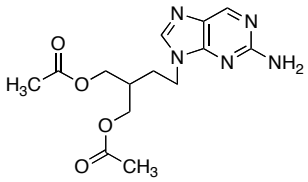


Figure 1.15. Structure of famciclovir

Cidofovir (S-HPMPC)

Cidofovir is the *S*-enantiomer of 1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (*S*-HPMPC); the reference to its chirality is important, since the *R*-enantiomer is not as effective as the *S*-enantiomer.[37, 38] Like adefovir and tenofovir, which are inhibitors of HIV reverse transcriptase and will be discussed in the following paragraphs,[39] *S*-HPMPC is an acyclic nucleoside phosphonate (ANP). This class of compounds bears a phosphonate group that mimics the phosphate moiety of normal nucleotides. For this reason, ANPs are able to deliver an isoster of the monophosphate form of nucleoside analogues directly into the cell.[37] The replacement of the phosphoric ester bond with a methylene bridge in the phosphate moiety confers stability against the hydrolytic activity of phosphatase enzymes, which dephosphorylate normal nucleotides and prevent their use as antiviral agents.[37] The antiviral spectrum of *S*-HPMPC is broad, including several DNA viruses, such as all eight human herpes viruses, papillomavirus, polyomavirus, poxviruses (vaccinia virus, cowpox virus, variola virus, etc.), and adenovirus.[39] Intravenous cidofovi*r* (Vistide®) has been approved for the treatment of CMV retinitis in HIV-infected patients.[10]

Cidofovir exerts antiviral activity by means of its diphosphate metabolite (*S*-HPMPC-pp), which is competitive inhibitor of deoxycitydine triphosphate incorporation into the viral DNA chain, akin to triphosphate nucleoside analogues.[37] Both phosphorylation steps are mediated by cellular kinases (fig. 1.16).[40]

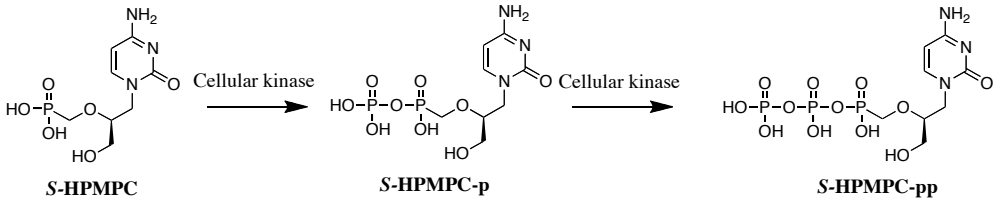


Figure 1.16. Bioactivation pathway of Cidofovir

In addition to the competitive inhibition of viral DNA polymerase, *S*-HPMPC-pp acts

also as a substrate. However, it is not an obligate chain terminator due to the presence of the hydroxymethylene group on the acyclic side chain, which mimics the 3'-hydroxyl group of natural nucleosides. Only the insertion of two consecutive molecules of *S*-HPMPC into the growing nucleic acid strand leads to the termination of its polymerisation.[41] The lack of involvement of virus-encoded proteins in the phosphorylation process explains the antiviral activity of *S*-HPMPC against thymidine kinase deficient-strains of HSV and VZV.[37]

At physiological pH the phosphonate group is negatively charged and this accounts for the extreme low oral bioavailability of *S*-HPMPC that requires intravenous administration. However, the long intracellular half-life ($t_{1/2}$= 87 h) of the phosphocoline metabolite, which slowly releases the antiviral agent as a sort of intracellular reservoir, allows the application of an infrequent dosing regimen.[6] Differently from the others ANPs, no approved prodrugs of *S*-HPMPC possessing an increased oral biovailability are available. The alkoxyalkyl esters of Cidofovir are under preclinical investigation and will be discussed more extensively later.

The main dose-limiting side effect of cidofovir is nephrotoxicity due to its accumulation in the renal proximal tubules.[37, 39]

The internal cyclisation of the hydroxymethylene residue with the phosphonate group generates cyclic cidofovir (*S*-cHPMPC), which has been shown to be a prodrug of *S*-HPMPC (fig 1.17). It exhibits similar antiviral activity and lower nephrotoxicity, due to its reduced uptake in the renal proximal tubule cells.[42, 43] The cellular cyclic cytosine monophosphate (cCMP) phosphodiesterase was found able to cleave the phosphodiester bond generating cidofovir.[44]

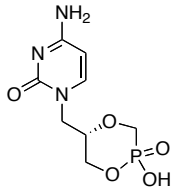
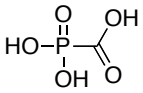
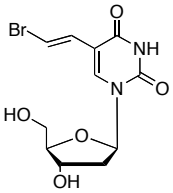


Figure 1.17. Structure of *S*-cHPMPC

Foscarnet and brivudin



Foscarnet (phosphonoformic acid) is a non-nucleoside inhibitor of viral polymerase.[45] It has been approved for the treatment of HCMV infections, particularly in immunocompromised patients, and acyclovir-resistant HSV infections. Moreover, foscarnet has shown inhibitory activity against HIV.[14] Foscarnet structurally is a pyrophosphate analogue and its mechanism of action involves reversible binding to the pyrophosphate binding site of the viral polymerase. This event blocks the cleavage of the pyrophosphate group of the incoming deoxynucleoside triphosphate into the growing DNA chain by the viral polymerase during the viral genome replication.[14] This results in termination of viral DNA polymerisation. Due to this mechanism of action, foscarnet is active against HSV TK mutants and VZV UL97 mutants that show resistance to the antimetabolite nucleosides. The major dose-limiting side effect of foscarnet is nephrotoxicity. Due to its ionisation at physiological pH the oral bioavailability is poor and it requires intravenous administration.[6]



Brivudin or (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) is a nucleoside inhibitor of HSV and VZV DNA polymerase.[46] Its triphosphate form is both a competitive inhibitor and substrate of viral DNA polymerase. The incorporation of BVDU in the viral genome destabilizes the DNA chain leading to termination of its polymerisation.[47] Thymidine phosphorylase metabolises BVDU to (E)-5-(2-bromovinyl)-2'-uracil (BVU) and 2-deoxyribose-1-phosphate. BVU is a potent inhibitor of the enzyme involved in pyrimidine metabolism. This enzyme is responsible for the metabolism of 5-fluorouracil and its inhibition increases the antiproliferative activity and toxicity of this anticancer agent, leading to fatalities in several cases.[47]

1.2.1.2 Reverse transcriptase inhibitors

Reverse transcriptase (RT) is a particular kind of polymerase that is able to produce copies of DNA using RNA as template. This enzyme mediates the replication of HIV and HBV genomes and differs consistently from the cellular counterpart, suggesting an ideal target of antiviral therapy. Most of the approved antiviral agents that inhibit the RT of HIV and HBV are nucleoside antimetabolites and are defined nucleoside reverse transcriptase inhibitors (NRTIs);[8] some of them belong to the class of acyclic nucleoside phosphonates (adefovir and tenofovir) and are defined nucleotide reverse transcriptase

inhibitors (NtRTIs).[8] As already mentioned, a group of non-nucleoside reverse transcriptase inhibitors (NNRTIs) have been approved for the treatment of HIV.[8] They exert their activity by binding to an allosteric pocket of RT that is distinct from the catalytic pocket where the NRTIs carry out the inhibition of viral genome replication.[1]

NRTIs: anti-HIV agents

In 1987, zidovudine (3'-azido-2',3'-dideoxythymidine, AZT) was the first NRTI to be approved by the FDA for the treatment of HIV.[48, 49] It is a 2',3'-dideoxythymidine analogue where the hydroxyl group in 3' position of the sugar moiety is replaced by an azido group (fig. 1.18). The resistance to AZT and its toxicity lead to the development of alternative anti-HIV drugs. The other NRTIs, which have been approved as anti-HIV agents, are (fig. 1.18): 2',3'-didehydro-2',3'-dideoxythymidine (Stavudine, d4T), 2'-3'dideoxyinosine (Didanosine, ddI),[50] (1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopenten-1-methanol (Abacavir, ABC),[51] (-)-β'-3'-thia-2',3'-dideoxycytidine (Lamivudine, 3TC),[52] and (-)-β'-2'-3'dideoxy-5-fluoro-3'-thiacytidine (Emtricitabine, FTC).[53]

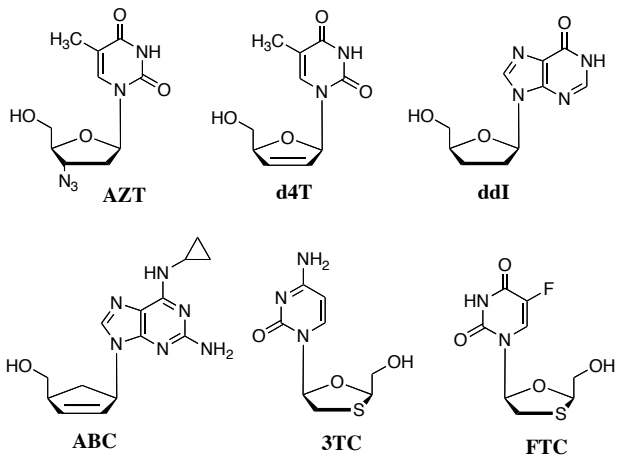
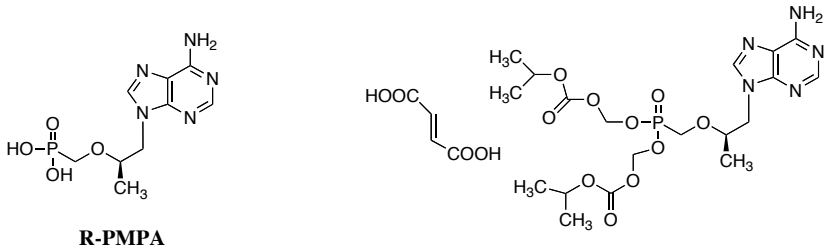


Figure 1.18. Structures of NRTIs

Also tenofovir, *R*-9-(2-phosphonylmethoxypropyl)adenine (*R*-PMPA), is an inhibitor of RT.[37] It differs from the others nucleoside analogues in being a NtRTI that belongs to the class of ANPs (fig. 1.19). Differently from *S*-HPMPC, the *R*-enantiomer is more potent than the *S*-enantiomer.[54] Its prodrug Tenofovir disoproxil fumarate, which will be further discussed later, has been approved for the treatment of HIV by the FDA under the name of Viread®.[8,39,55] Tenofovir is also used in combination with either emtricitabine (Truvada®) or emtricitabine and efavirenz (Atripla®) for the treatment of HIV infections.[8]



R-PMPA **Tenofovir disoproxil fumarate**

Figure 1.19. Structure of tenofovir and its diisoproxil fumarate prodrug

Similarly to the inhibitors of herpesviruses DNA polymerase, the triphosphate of NRTIs and the diphosphate of tenofovir are the active forms that act as competitive inhibitors and substrates of RT.[3,5] Since they lack the hydroxyl group in 3' position, these compounds are obligate chain terminators. In contrast to herpesviruses, HIV does not encoded for enzymes that can phosphorylate nucleoside analogues. The formation of their active form is completely carried out by the cellular kinases. This lack of selectivity accounts for the accumulation of the active form in all dividing cells and not just in infected cells.[1,6] A good affinity for the cellular kinases is essential in order to achieve an effective concentration of the nucleoside analogue triphosphate into the cell. In particular, the phosphorylation to the monophosphate form usually is the rate-limiting step. The only well known exception is given by the bioactivation of AZT where the second phosphorylaton is a rate-limiting step.[56] Similarly to *S*-HPMPC, *R*-PMPA bypasses the first phosphorylation step by releasing the monophosphate nucleoside analogue directly into the cell.[37]

Despite the good selectivity for RT, the inhibition of mitochondrial polymerase-γ is the main reason of NRTIs toxicity.[6] However, the development of 3TC and its analogue FTC has overcome this problem by means of an unusual sugar moiety: its L-configuration, instead of the natural D-configuration, and the atom of sulphur in position 3 account for the great decrease of the inhibitory activity exerted on the cellular polymerase.[1, 57] Although the efficacy against RT is lower in comparison to AZT, their therapeutic index is 100 times higher. Another important issue of these antiviral agents is the development of cross-resistance due to the error prone nature of RT.[1, 6]

NRTIs: anti-HBV agents

HBV reverse transcriptase is very similar to HIV reverse transcriptase.[1] For this reason the anti-HIV drugs lamivudine and tenofovir are also active against HBV and they have been approved for the treatment of HBV infections, particularly in co-infected HIV

patients where they can exert their activity on both viruses.[8,37,58] Adefovir, 9-[2-(phosphonylmethoxy)ethyl]adenine (PMEA), is an analogue of tenofovir and is active against HIV, HBV, and also herpesviruses (fig. 1.20).[59] Its mechanism of action is analogous to that of tenofovir: its diphosphate form (akin to triphosphate nucleoside analogues) targets the viral polymerase inhibiting the elongation of the DNA chain.[37] Its oral prodrug Adefovir dipivoxil (fig. 1.20), which will be further discussed later, has been approved for the treatment of HBV infections under the name of Hepsera®.[6,8,60]

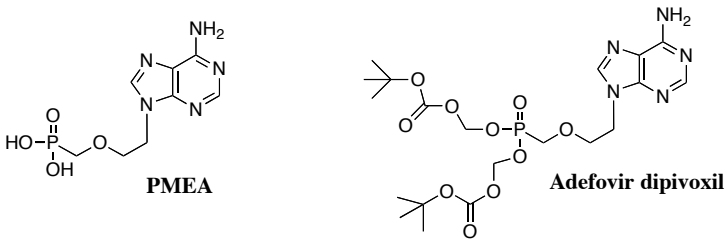


Figure 1.20. Structure of adefovir and its produg adefovir dipivoxil

Entecavir and telbivudine are nucleoside analogues that selectively target the HBV reverse trascriptase and have been approved for the treatment of this infection (fig. 1.21).[61,62]

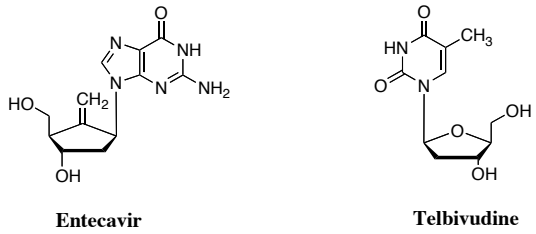


Figure 1.21. Structure of entecavir and telbivudine

NNRTIs

Nevirapine, efavirenz, delaviridine, etravirine, and rilpivirine are NNRTIs (fig. 1.22), which have been approved for the treatment of HIV infections by the FDA.[8] These agents are non-competitive inhibitors of the HIV-RT.[5] They bind to a hydrophobic pocket distinct from the active site and not essential for the function of RT. This interaction induces a conformational change in the enzymatic structure that greatly reduces the activity of RT.[63] The binding site is virus strain specific, so NNRTIs are active only against HIV-1. For this same reason, their cytotoxicity is low, as they do not interact with human polymerases.[63] The main issue of this class of antiviral agents,

particularly in the first generation (Nevirapine, Delaviridine, Efavirenz), is the rapid development of resistance due to a modification of the binding site.[1, 6] For this reason, NNRTIs are never used in monotherapy, they are combined with others antiretroviral drugs in order to enhance efficacy and to avoid resistance.[63, 64]

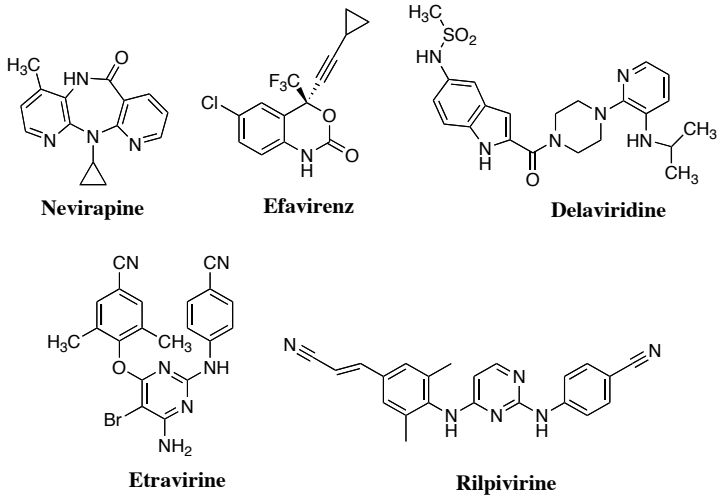


Figure 1.22. Structure of NNRTIs

1.2.1.3 HIV integrase inhibitors

HIV integrase catalyses the integration of the viral genome into the host cell DNA.[1] It is an essential and exclusive enzyme of HIV life cycle. Due to these features HIV integrase is an ideal target of antiviral therapy.[3] However the only FDA-approved HIV integrase inhibitor is raltegravir (fig. 1.23). It is only used in combination with other anti-HIV drugs.[65]

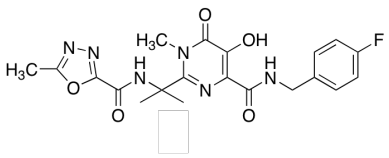


Figure 1.23. Structure of raltegravir

1.2.1.4 Ribavirin

Ribavirin (Virazole®) is a nucleoside analogue with antiviral activity that ranges *in vitro* and *in vivo* from DNA to RNA viruses.[66] However, it has been approved only for the treatment of respiratory syncytial virus infection and, in combination with pegylated interferon-α, for hepatitis C virus infection; both of them are RNA viruses.[9] Structurally, ribavirin differs from the majority of nucleoside analogues in having a

normal sugar unit (D-Ribose) attached to a triazole group that mimics a natural purine nucleobase (fig. 1.24).

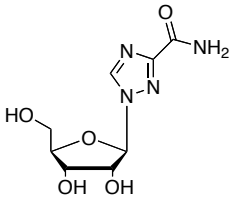


Figure 1.24. Structure of ribavirin

Its mechanism of action has not been fully clarified yet. Several studies showed that ribavirin has various sites where it can exert its antiviral activity. Host cell kinases mediate the phosphorylation of Ribavirin to the mono-, di-, and triphosphate forms.[67] The monophosphate derivative is a competitive inhibitor of inosine monophosphate dehydrogenase that is involved in *de novo* guanosine triphosphate (GTP) biosynthesis.[68, 69] In principle, lowering the cellular GTP pools affects the viral genome replication and the activity of others viral GTP-dependent enzymes, which rely on the host cell's source of GTP. Moreover, this effect may promote the incorporation of ribavirin triphosphate into the viral nucleic acid.[70] This event induces the so called error 'catastrophe', which is an accumulation of mutations over the edge of an error threshold that leads to the production of a non functional viral genome.[71] In influenza virus infection, the triphosphate form of ribavirin shows inhibition of the RNA polymerase.[72] In Dengue virus infection, it inhibits the viral 2'-*O*-methyl transferase, while the monophosphate derivative inhibits the viral guanylyl transferase.[73] Finally, ribavirin was found to exert an immunomodulatory activity in HCV and HBV patients.[74, 75]

1.3 Pronucleotides

The abundance of nucleoside analogues among the antiviral agents points at the relevance of viral polymerases as target of antiviral drug therapy.[8-10] The main features that make an ideal target of this enzyme are:[1]

- The difference of structure with the cellular DNA polymerase, which is crucial in order to achieve selectivity.
- The importance of its function for the viral life cycle
- Its druggability, due to the small molecular weight of its substrates

As already described in the paragraph on antiviral agents, nucleoside analogues need to be phosphorylated to the triphosphate form in order to exert their inhibitory activity on the viral polymerase; their success as antiviral agents depends not only on the affinity and selectivity for the viral polymerase but also on the rate of phosphorylation that provides the therapeutic concentration of the active form into host cells. On account of this, the release of the monophospate derivative of nucleoside analogues directly into infected cells has always been a target for antiviral drug development, because bypassing the first phosphorylation step has several advantages. First of all, this is the rate-limiting step of the metabolic activation for most nucleoside analogues, so nucleotides are supposed to show an improvement in activity in cases where parent nucleosides are inactive due to poor first intracellular phosphorylation step.[6,76,77] In many cases, this is followed by an extension of antiviral spectrum, or an altered biological profile in some cases. For example, acyclovir triphosphate shows inhibitory activity against HIV and the pronucleotide of BVDU has proliferative activity against colon cancer.[78,79] Therefore, nucleotides retain activity against certain mutant virus strains that develop resistance to the parent nucleosides (e.g TK^- HSV and VZV).[80]
Nevertheless, nucleotides themselves are not ideal drug candidates. There are some disadvantages that do not allow their administration as such. Due to the negative charge on the phosphate moiety at physiological pH, they have poor membrane permeability and bioavailability.[76,80] Another disadvantage of nucleotides is their instability in biological media as they are rapidly dephosphorylated by phosphatase enzymes.[80]

As already discussed in the paragraphs regarding cidofovir, tenofovir, and adefovir, ANPs are resistant to the dephosphorylating activity of extracellular phosphatases. However, the therapeutic application of ANPs is limited by the negative charge of the phosphonate group that does not allow a significant cellular uptake or good bioavailability due to poor membrane permeability of these compounds.[37, 38]

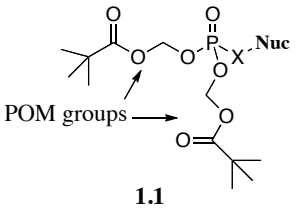
In order to overcome this issue, several pronucleotide approaches have been developed by masking the phosphate group with lipophilic moieties that allow the passive diffusion through cellular membranes.[38, 76, 80-83] Once inside the cell, the pronucelotide is metabolised releasing the monophosphate form. Approaches that make use of this strategy are:

- Bis(POM) and bis(POC) pronucleotides

- Bis(SDTE) and bis(SATE) pronucleotides
- Cyclosal approach
- Phospholipid conjugates
- Peptide conjugates
- Hepdirect approach
- Phosphoramidate diester pronucleotides: the Wagner approach
- Aryl Phosphoramidate triester pronucleotides: the ProTide approach
- Phosphorodiamidate pronucleotides

1.3.1 Bis(POM) and Bis(POC) pronucleotides

The bis(POM) phosphoester derivatives of nucleoside analogues (**1.1**) mask the negative charge of the phosphate (**X**: O) or phosphonate moiety (**X**: CH_2) by means of two pivaloyloxymethyl (POM) groups (figure 1.25).[80, 84]

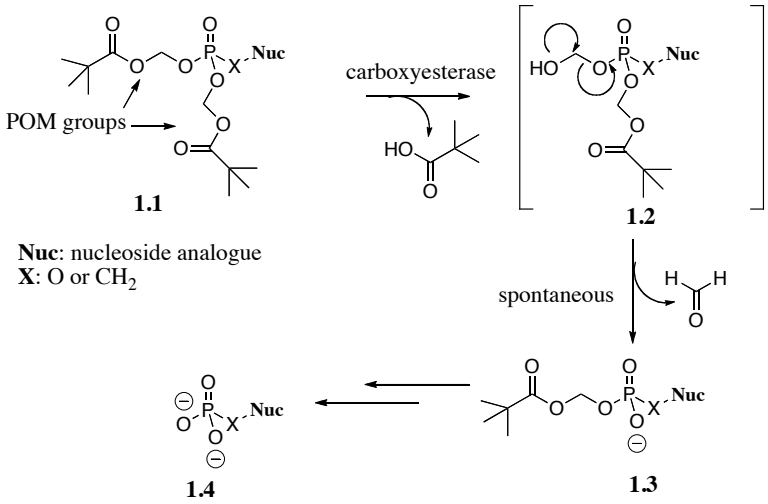


1.1

Nuc: nucleoside analogue
X: O or CH_2

Figure 1.25. Generic structure of Bis(POM) pronucleotides

The intracellular mechanism of bioactivation involves the esterase-mediated cleavage of both POM-groups in two steps (scheme 1.1).[85] In the first step the carboxyesterase-mediated cleavage of one of the two POM groups affords the hydroxymethyl phosphoester **1.2**. This highly reactive intermediate spontaneously dissociates to give the phosphoester **1.3** by elimination of formaldehyde. The cleavage of the second POM group follows the same mechanism and results in the release of the 5'-monophosphate (**X**: O) or monophosphonate (**X**: CH_2) form (**1.4**) of the nucleoside analogue.



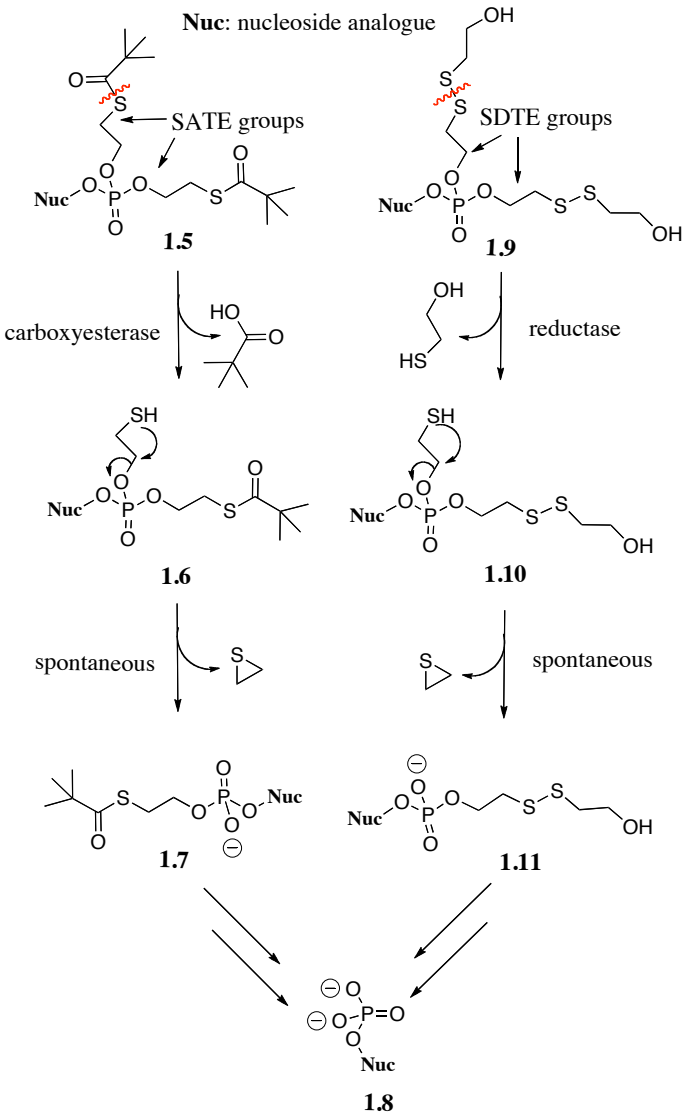
Scheme 1.1. Bioactivation pathway of Bis(POM) pronucleotides

The bis(POM) prodrug of Adefovir (Adefovir dipivoxil) improves the poor oral bioavailability of the parent compound up to 30-60% and has been approved in 2002 for the treatment of HBV infections.[6]

In the bis(POC) phosphotriester analogues the pivaloyl groups are replaced by isopropoxycarbonyl groups. Similarly to the bioactivation mechanism of bis(POM), the elimination of the masking group is catalysed by carboxyesterase enzymes and results in the release of the monophosphate form of the nucleoside analogue together with isopropanol, CO_2 and formaldehyde. Although this approach avoids the formation of potentially toxic pivalic acid, it still generates formaldehyde. The application of the bis(POC) technology to tenofovir resulted in the synthesis of tenofovir disoproxyl fumarate that showed a 16- to 35- fold increase of the anti-HIV activity and no significant toxicity on account of the improvement of its cellular uptake.[86]

1.3.2 Bis(SDTE) and Bis(SATE) pronucleotides

The bis-[S-(2-hydroxyethylsulfidyl)-2-thioethyl] [bis(SDTE)] and bis-(S-acyl-2-thioethyl) [bis(SATE)] phosphotriesters were first described by Imbach and Grosselin.[87] Similarly to bis(POM) and bis(POC) approaches, their mechanism of activation is mediated by enzymes (scheme 1.2).



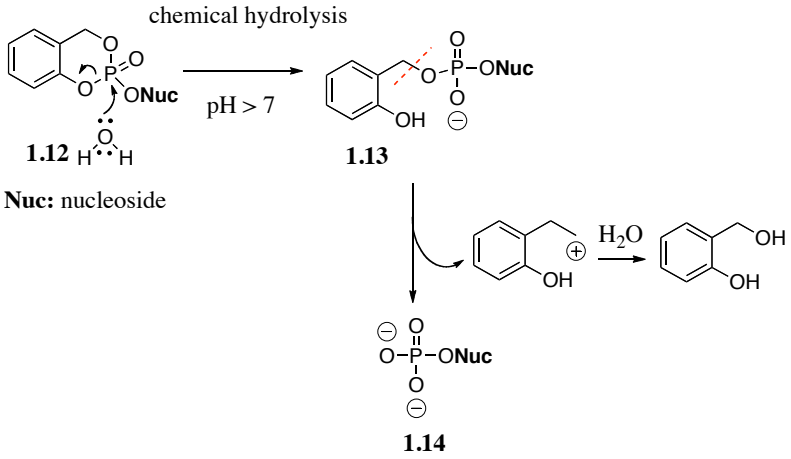
Scheme 1.2. Bioactivation pathway of bis(SATE) and bis(SDTE) phosphotriesters

In the bioactivation of bis(SATE) phosphotriester derivatives (**1.5**), a carboxyesterases enzyme carries out the initial cleavage of the thioester bond of one of the two SATE groups with release of pivalic acid and the corresponding phosphothioester **1.6**. Then, the spontaneous elimination of episulfide affords the intermediate phosphodiester **1.7**. The elimination of the second S-acyl-2-thioethyl group follows the same pathway and leads to the release of the 5'-monophosphate of the nucleoside analogue (**1.8**). The bioactivation pathway of bis(SDTE) analogues (**1.9**) is slightly different. The initial step is carried out by a reductase enzyme that cleaves the disulfide bond releasing thioethanol and the reactive intermediate thioethyltriester **1.10**. The spontaneous elimination of episulfide affords the intermediate phosphodiester **1.11**, which is metabolised to the 5'-monophosphate derivative **1.8** by a further reductase-mediated cleavage. Alternatively, phosphodiesterase enzyme can catalyse the hydrolysis of the

second masking group leading to the formation of the 5'-monophosphate product.[80] Examples of the application of these approaches are the bis(SATE) phosphotriester analogues of ACV,[88] AZT,[89] and d4T.[90] All of them are successful in the intracellular release of the monophosphate of the parent nucleoside, and thus bypass the first phosphorylation step. In particular, the bis(SATE) pronucleotide of ACV extends the antiviral activity of ACV on HBV.

1.3.3 The cyclosal approach

The cyclosaligenyl phosphotriester derivatives of nucleoside analogues bear a salicyl alcohol molecule on the phosphate or phosphonate group. In contrast to other strategies, which mask the negative charge of the phosphate moiety by means of a biolabile group, the cyclosal approach involves chemical hydrolysis of the lipophilic masking unit (scheme 1.3).[91]



Scheme 1.3. Chemical hydrolysis of a generic Cyclosal pronucleotide

The release of the nucleoside 5'-monophosphate **1.14** from the phosphotriester derivative **1.12** proceeds in two consecutive steps. The first step is the selective hydrolysis of the phenolic ester bond, which is the most labile of the three P-O bonds due to the delocalisation of the negative charge of the phenoxide ion in the aromatic ring. Then, the spontaneous cleavage of the benzylic ester bond (**1.13**) results in the release of the nucleoside 5'-monophosphate **1.14**. The cyclosal approach has been applied to many nucleoside analogues, such as ACV,[92] BVDU,[93] AZT,[94] d4T,[95] and the acyclic nucleoside phosphonate PMEA.[96] This approach presents some limits that interfere with the efficacious release of nucleotide analogues in the intracellular compartment:[80]

- The efflux of the cyclosal derivatives through the membrane cell decreases their intracellular concentration
- The masking group is cleaved also in the extracellular compartment due to its low hydrolytic stability

In order to overcome these issues, second and third generations of cyclosal triesters bearing an esterase-cleavable function (lock-in cyclosal pronucleotides) were developed. In the second generation of cyclosal derivatives the cleavage of the ester group is mediated by cellular esterase and makes the metabolite more polar.[97] Such an increase in polarity traps the phosphotriester derivative in the intracellular compartment, where it is bioactivated through the same mechanism of the first generation derivatives. This strategy was applied to the synthesis of various ester-functionalised derivatives of cycloSal-d4TMP (fig. 1.26).[98] However, the chemical hydrolysis that affords d4TMP is very slow.

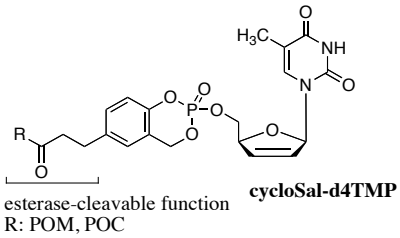


Figure 1.26. Lock-in cyclosal pronucleotide (2nd generation)

In the third generation, the esterase–cleavable function bears electron-donating or weak electron-withdrawing groups. Once inside the cell, the esterase-mediated cleavage of this substituent leads to a decrease in the hydrolysis stability of the cyclosal derivative that favors the elimination of the masking group on the phosphate moiety. The rational of this technology is to trigger selectively the release of the nucleotide analogue in the intracellular compartment: the idea of trapping the compound into the cell is applied at the nucleotide level. Following this strategy, diacyloxymethyl-*cyclo*Sal-d4TMPs and 5-(1-acetoxyvinyl)-*cyclo*Sal-d4TMPs were synthesised (fig. 1.27).[99, 100]

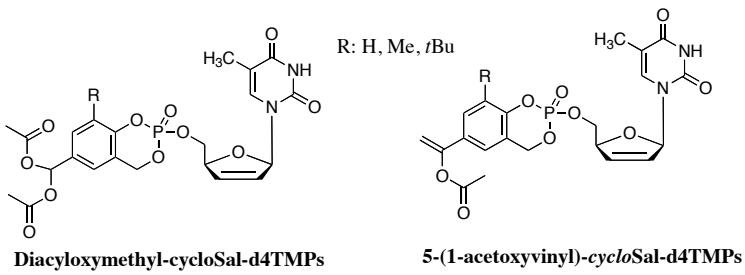


Figure 1.27. Structure of cyclosal phosphotriester of nucleoside analogues (3rd generation)

1.3.4 Phospholipid conjugates

This technology has been developed by Hostetler and coworkers to improve the oral bioavailability and cellular uptake of acyclic nucleoside phosphonates.[101] It consists of the esterification of the phosphonate group with an alkoxyalkyl chain that masks one of the two negative charges (**1.15**). The rational of this substitution is its resemblance to lysophosphatidylcholine (**1.16**), which easily crosses the cellular membrane and allows the cellular uptake of this lipophilic precursor (fig. 1.28).[101] In particular the best results have been obtained using hexadecyloxypropyl (HDP) and octadecyloxyethyl (ODE) esters. Inside the cell, the alkoxyalkyl chain of phospholipid moiety is selectively cleaved by the phospholipase C with release of the acyclic nucleoside phosphonate. The absence of this enzyme in plasma or pancreatic secretions avoids the metabolisation of these mimetic phospholipid derivatives during the oral absorption and prior to reach tissues.

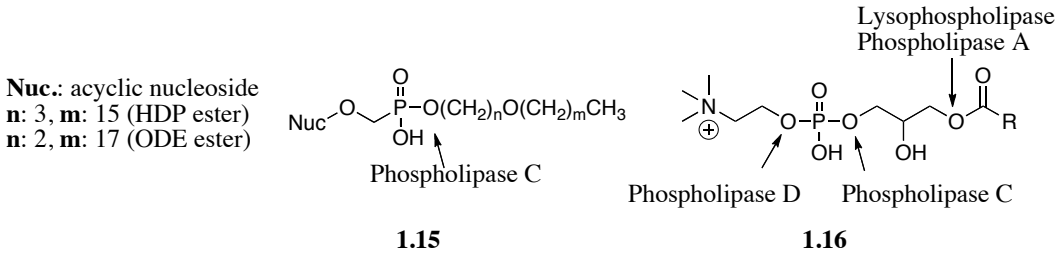
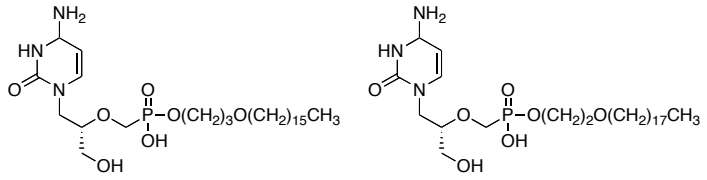


Figure 1.28. Generic structure of alkoxyalkyl ester of acyclic nucleoside phosphonate (1.15) in comparison to lysophosphatidylcholine (1.16)

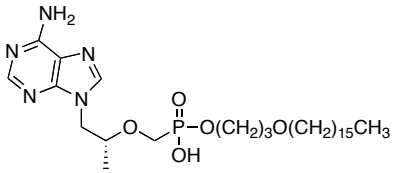
The application of this approach to cidofovir afforded the alkoxyalkyl ester derivatives HDP-(*S*)-HPMPC and ODE-(*S*)-HPMPC, which exhibited a striking increase in potency against several double stranded DNA viruses in comparison to the parent nucleoside (fig.1.29).[101]



HDP-(*S*)-HPMPC **ODE-(*S*)-HPMPC**

Figure 1.29. Structures of hexadecyloxypropyl (HDP) and octadecyloxyethyl (ODE) esters of *S*-HPMPC

The activity *in vitro* against HCMV and others herpes viruses has shown 2.5-to 4-log increases in potency (depending on the antiviral assay used).[102] These compounds were also found to be more active than cidofovir against several poxviruses, such as smallpox, cowpox, monkeypox, and vaccinia viruses.[103] The oral adminstration of such phospholipid conjugates was as effective as parental cidofovir in the treatment of CMV infections in murine models. They were also found to be effective in animal models of cowpox and vaccinia virus infections.[104] Another important achievement of this approach is the lack of nephrotoxicity, which is the main dose limiting toxicity of intravenous cidofovir.[6] The alkoxyalkyl ester derivative of cidofovir is not recognised by the transport mechanism that causes the accumulation of cidofovir in the kidney.[105] HDP-(*S*)-HPMPC, under the name of CMX001, completed Phase I clinical trials as oral drug for the treatment of HCMV and small pox infections. Phase II trials are currently in progress. This same approach has been applied to the cyclic derivative of cidofovir and several others acyclic nucleoside phosphonate including tenofovir and adefovir.[101] Recently, the hexadecyloxypropyl ester derivative of tenofovir (HDP-(*R*)-PMPA, fig. 1.30) has entered in Phase I clinical trials as an oral drug, with the name CMX157, for the treatment of HIV infections.



HDP-(*R*)-PMPA

Figure 1.30. Structure of the hexadecyloxypropyl ester of tenofovir

Despite the striking improvement of antiviral activity afforded by this technology, the poor water solubility of these lipophilic precursors is still an issue of this approach. Recently, Krečmerová *et al*. tried to address this issue by synthesising ester derivatives of cidofovir and adefovir bearing hydroxyl groups or oxygen atoms on the aliphatic

chain.[106] In general, these modifications resulted in a decrease or loss of activity due to inefficient cellular uptake or a lack of metabolism mediated by phospholipase C.

1.3.5 Peptide conjugates

In order to improve the oral bioavailability of acyclic nucleoside phosphonates, McKenna and co-workers have developed a technology that conjugates a dipeptide or a single amino acid with the phosphonate group.[38] Similarly to valacyclovir, the rational of this approach is to improve the bioavailability of the parent drug by targeting the human peptide-specific intestinal transporter (hPEPT1), which is present in the gastrointestinal tract. The amino acidic moiety is cleaved during the first-pass metabolism. The L-Val-L-Ser-OMe dipeptide derivative of cyclic cidofovir (fig. 1.31) showed a 8-fold increase in the oral bioavailability of the parent drug.[107]

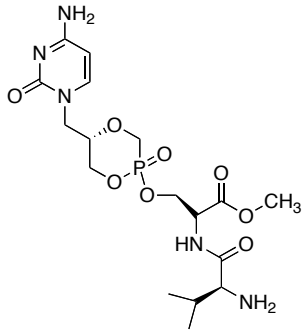


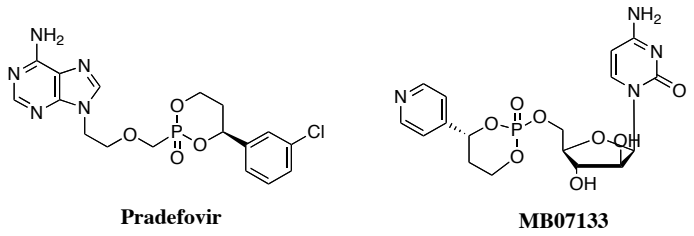
Figure 1.31. Structure of the L-Val-L-Ser-OMe dipeptide derivative of *S*-cHPMPC

However, further studies showed that such dipeptide conjugates are not transported, although they are recognised, by hEPT1.[108] Steric hindrance or polarity of the amino acid moiety can be accounted for the lack of hEPT1-mediated transport of these peptidic conjugates, thus suggesting that a different transporter is involved in their absorption.

1.3.6 HepDirect approach

The cyclic 1-aryl-1,3-propanyl esters of phosphate or phosphonate nucleoside analogues are a class of pronucleotides developed by Metabasis and called HepDirect prodrugs. The aim of this approach is to treat the infections that affect the liver by delivering the drug specifically in this organ and thus decreasing the exposure to the other organs. This goal is achieved by coupling the parent drug with a masking group that is selectively recognized and cleaved by the cytocrome P450 (isoenzyme CYP3A4) expressed predominantly in the liver.[109] Pradefovir (fig. 1.32) is the HepDirect prodrug of

Adefovir and it has been developed to treat HBV infections.[110] The analogous prodrug of cytarabine monophosphate (MB07133, fig. 1.32) has been developed for the treatment of hepatocellular carcinoma.[81]



Pradefovir **MB07133**

Figure 1.32. Hepedirect prodrugs

1.3.7 Phosphoramidate diester pronucleotides: the Wagner approach

This approach has been developed by Wagner *et al*. to deliver the monophosphate form of nucleoside analogues into the cell. The cellular uptake is favored by masking one of the phosphate charges with an amino acid ester.[80] This strategy is a variant of the ProTide approach, developed by McGuigan *et al*., that masks both negative charges of the phosphate group. The unmasked negative charge improves the water solubility and the stability in human blood of these compounds in comparison to the phosphotriester analogues.[80] This class of pronucleotides differs from the other approaches with regard to the lack of chirality at the phosphorus. A phosphoramidase-type enzyme (Hint-1) mediates the cleavage of the amino acid moiety with release of the monophosphate derivative of the nucleoside analogue.[80] The application of this approach afforded the phosphoramidate derivatives of AZT, 1-β-arabinofuranosylcytosine, and 5-fluoro-2'-deoxyuridine (fig. 1.33).[111, 112] Despite the improvement of antiviral activity *in vitro*, *in vivo* these derivatives have poor bioavailability and degrade to the parent nucleoside in the gastro intestinal tract.

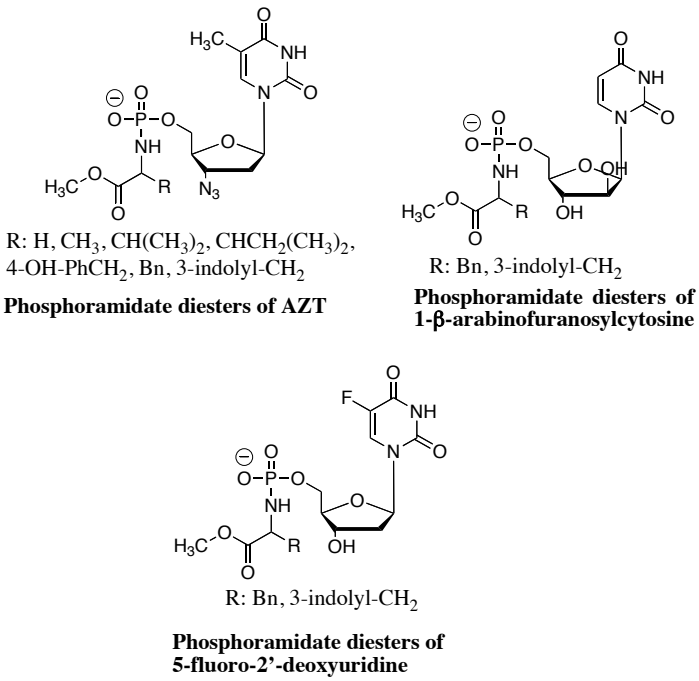


Figure 1.33. Structures of phosphoramidate diesters of nucleoside analogues

1.3.8 Aryl phosphoramidate triester pronucleotides: the ProTide approach

This approach has been developed by McGuigan *et al*. with the aim of allowing the intracellular delivery of lipophilic precursors of nucleotide analogues by passive diffusion of the cellular membrane.[82] As depicted in figure 1.34, the aryl phosporamidate triester of nucleoside analogues mask both negative charges of the phosphate group by means of aryl and amino acid moieties.

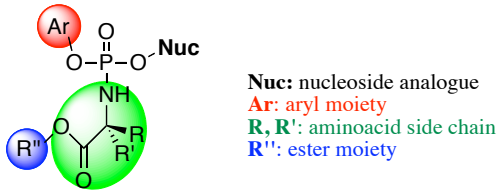


Figure 1.34. Structural motifs of aryloxy phosphoramidate ProTide

In 1992 this technology was applied for the first time to the anti-HIV agent zidovudine.[113] These derivatives exhibited anti-HIV activity in kinase deficient cell lines, proving the successful intracellular release of zidovudine monophosphate.[114] Since then, the ProTide approach has been applied to several nucleoside analogues, such as 2',3'-dideoxyuridine,[115] stavudine,[116] abacavir,[117] 4'-azidouridine,[118] brivudin,[78] the anticancer agent 5-fluorouridine, [119] as well as the acyclic phosphonate nucleosides tenofovir and adefovir.[120, 121] Recently, the aryl phosphoramidate pronucleotides

INX-189, developed by Inhibitex/BMS, PSI-7977, developed by Pharmasset/Gilead, and the hybrid SATE/phosphoramidate IDX184, developed by Idenix, all entered clinical trials for the treatment of HCV infections (fig. 1.35).[122-124]

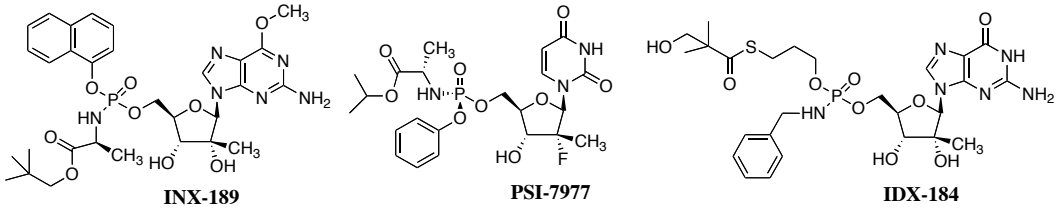
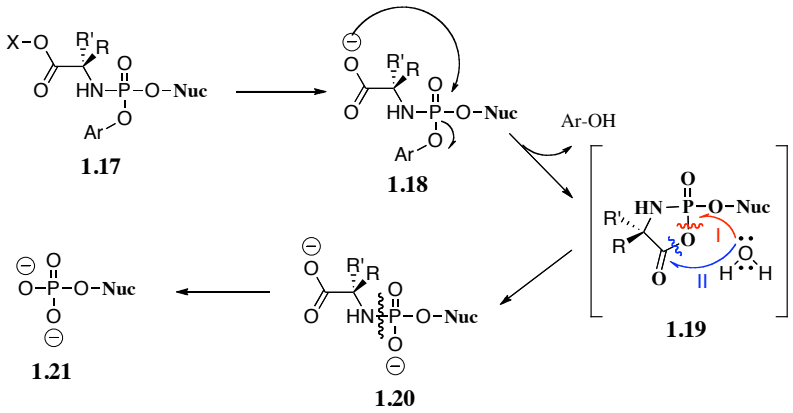


Figure 1.35. Structure of phosphoramidate pronucleotides in clinical trials as candidate for the treatment of HCV infection

The by-pass of the first phosphorylation step was achieved also in the case of acyclovir,[125] providing the anti-HIV activity of acyclovir triphosphate.[126] This topic will be discussed more extensively in the next chapter. The aryl phosphoramidate derivatives of N-acetylglucosamine were the first application of the ProTide approach on non-nucleoside agents.[127] They were shown to have enhanced anti-osteoarthritic activity compared to their respective parent compounds.

Proposed mechanism of activation of ProTides.

The putative bioactivation mechanism of ProTides (**1.17**) is depicted in scheme 1.4.[128-130]



Scheme 1.4. Putative bioactivation pathway of aryloxy phosphoramidate pronucleotides

Once inside the cell, the first activation step is the hydrolysis of the carboxylic ester moiety that affords the metabolite **1.18**, a carboxyesterase enzyme is thought to mediate this cleavage.[129] Recently, Gilead Science has performed mechanistic studies on the aryl phosphonamidate derivative of tenofovir GS-7340 showing that cathepsin A is the carboxypeptidase involved in this reaction.[131] This cleavage is followed by an internal

nucleophilic attack of the carboxylate on the phosphorus atom with displacement of the aryl group and transient formation of a five-member cyclic intermediate **1.19**. The instability of such intermediate does not allow its isolation. This cyclic anhydride is rapidly hydrolysed to the corresponding phosphoramidate derivative **1.20**. The nucleophilic attack can take place either on the phosphorus centre (attack I) or on the carboxylate group (attack II). Recent *in silico* studies suggested that the former might be favored over the latter.[132] The last step is cleavage of the P-N bond with delivery of the nucleoside monophosphate (**1.21**).[129] Human Hint-1 (histidine triad nucleoside-binding protein), a phosphoramidase-type enzyme that belongs to the HIT (histidine triad) superfamily, is presumed to mediate this cleavage.[78, 133]

Generic correlation structure – activity of ProTides

An extensive study concerning the aryl, amino acid, and ester moieties was performed with the aim to identify the substituents that afford the optimal biological activity.[82] Figure 1.36 summarises the results of such investigation.

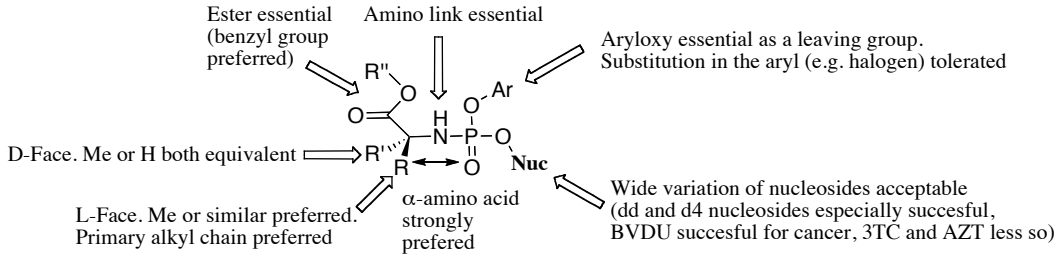


Figure 1.36. SAR of aryloxy phosphoramidate pronucleotides

Ester moiety

The hydrolysis of the ester moiety is determinant for the bio-activation of the phosphoramidate. Even though the rate of the cleavage in the esterase studies does not correlate to the antiviral activity, the lability of the ester bond is necessary (but not sufficient alone) to achieve high biological activity. Different esters have been evaluated: linear (methyl, ethyl, n-propyl, pentyl), branched (*tert*-butyl, neopentyl, 2-propyl), cyclic (cyclohexyl), and aromatic (benzyl, naphthyl). In particular, the t-butyl ester derivatives are often poorly active due to the lack of cleavage of the ester bond as its branched chain is too bulky to be processed by the carboxyesterase enzyme, while in many cases, the more lipophilic benzyl ester derivatives have exhibited the highest potency. Clearly, the more lipophilic the compound is, the better the cellular membrane diffusion will be, but the steric hindrance of these moieties must be taken into

consideration in terms of capacity of the enzymes to process the pronucleotide properly.

Aryl moiety

The elimination of the aryl group is not mediated by any enzyme, but it is the result of intramolecular cyclisation. For this reason the ability of this moiety to act as a good leaving group is essential for the successful metabolism of the ProTide. This is also the reason for which the development of the phosphoramidate technology has moved from the alkyl and haloakyl moieties to the aryl moiety (phenyl and 1-naphthol), which is a better leaving group due to its ability to delocalise the negative charge. Further studies performed on the aryl substitution of d4T phosphoramidates have shown that mild electron-withdrawing substituents (Cl, COOMe) lead to an increase of potency by enhancement of the leaving group ability of the aryl moiety in the formation of the aminoacyl intermediate, whilst strong electron-withdrawing groups (*p*-CN, *p*-NO_2) cause a decrease of activity.

Amino acid moiety

The α-amino acid is essential for the successful bio-activation of phosphoramidates. Early studies have evaluated the replacement of the P-N bond by a P-O bond in lactyl and glycolyl analogues of AZT and d4T phosphoramidate showing a significant or complete loss of activity.[82] Its replacement with n-alkylamine chains led to a complete loss of antiviral activity of AZT and d4T phosphoramidates. In general, the distance between the nitrogen and the carboxylic acid in the amino acid moiety is important for the activity: extending the chain length (e.g. β-amino acid) in d4T phosphoramidates resulted in poor activity.[134] This study showed that in these derivatives the ester moiety is hydrolysed, but the intracellular cyclisation does not take place. A recent *in silico* study showed that the formation of the six-membered ring intermediate is not favored.[132] An extensive study that correlates the amino acid structure of d4T phosphoramidates with the antiviral activity was performed.[135] From this study it was demonstrated that L-alanine provides the best results. The one exception being phosphoramidates of N-acetyl-glucosamine, where the L-Proline derivatives gave the best activity. Dimethylglycine derivatives retain the good antiviral activity exhibited by the L-alanine analogues. Surprisingly, the loss of the methyl group in the glycine phosphoramidate derivatives of d4T leads to a 60- 70- fold decrease of potency in comparison to the L-alanine analogues. Bulky L-amino acids (e.g. L-valine, L-leucine, and L-isoleucine) are

not usually tolerated giving reduced activity. According to the study performed by Gilead Science on the hydrolysis rate of the ester moiety of nucleoside phosphonoamidate, Cathepsin A is not able to hydrolyse phosphoramidate with hindered side chains.[131] Concerning the stereochemistry, unnatural D-amino acids are poorly active; this is probably due to the bad interaction with the enzymes involved in the bioactivation pathway. The D-alaninyl derivative of N-acetyl-glucosamine phosphoramidate is an exception to this trend.

Phosphorus stereochemistry

The unsymmetrical substitution introduces a chiral center in the phosphorus atom that, together with the other chiral centers of the molecule, causes the formation of a mixture of diastereomers. In some cases, one of the diastereomers has more potent biological activity than the other due to stereoselectivity of the enzymes involved in the bioactivation pathway. For example, the anti-HCV agent PSI-7977 (fig. 1.37) is the S_p diastereomer and exhibits a greater ability to produce higher intracellular concentration of triphosphate metabolite in comparison to the R_p diastereomer thus making it more active.[123] The correlation between absolute configuration at the phosphorus centre and the relative potency of the antiviral agent has not been clarified yet.

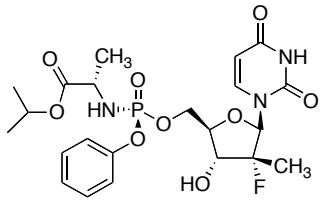


Figure 1.37 Structure of PSI-7977

1.3.9 Phosphorodiamidate pronucleotides

The phoshorodiamidate pronucleotide approach makes it possible to overcome some limits of the ProTide approach: the release of phenol or naphthol, which might be toxic, and the formation of mixture of diasteroisomers that arises from the chirality of the phosphate centre. Such phosphate prodrug motifs were first applied to AZT by McGuigan *et al.*,[82] but at that time their development was not further pursued due to the lack of improvement of the biological activity and the poor chemical yield. Since then, this approach has been applied by Gilead for the development of diamidate prodrugs of acyclic nucleoside phosphonates, such in the anticancer agent GS-9191 (fig. 1.38),

which is in Phase I clinical trial for the topical treatment of HPV lesions.[81, 82, 136]

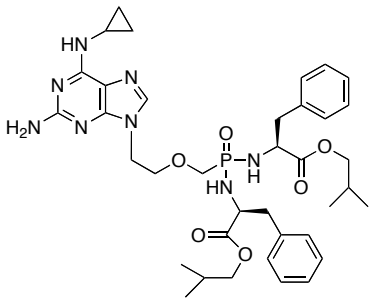
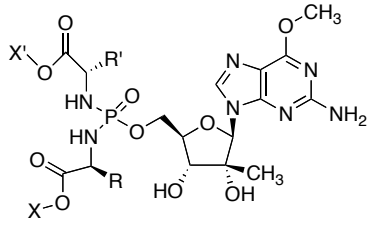


Figure 1.38. Structure of the phosphonodiamidate derivative GS-9191

More recently, phosphorodiamidates of 6-O-methy-2'-*C*-methyl guanosine (fig. 1.39) were described by McGuigan *et al*. as good candidates for clinical studies in the treatment of HCV infections.[137]



R, R': H, Me, iPr, $CH_2CH(CH_3)_2$, $CH_3CHCH_2CH_3$, Bn, CH_2COOH, CH_2PhOH, $CH_2CH_2SCH_3$

X, X': Me, Et, nPr, iPr, But, Pnt, CH_2tBu, cbu, cPnt, cHx, Bn

Figure 1.39. Phosphorodiamidates of 6-O-methyl-2'-*C*-methyl guanosinee

The mechanism of bioactivation is analogous to the one already described for the aryl phosphoroamidate triester pronucleotides: the carboxyesterase-mediated cleavage of one or both the ester moieties leads to intracellular cyclization with elimination of the other amino acid moiety and subsequent formation of the phosphoramidate monoester, then the same pathway follows.[137]

1.3.10 Summary of pronucleotide technologies

Table 1 gives a summary of the strategies that allow the delivery of nucleotide analogues into the cell.

Table 1.1. Pronucleotide approaches

Pronucleotide Approach	Bioactivation Pathway	In Clinical Trials/Approved
Bis(POM)/Bis(POC)	Esterase cleavage	Yes
Bis(SDTE)/Bis(SATE)	Esterase/Reductase cleavage	No
Cyclosal	Chemical hydrolys	No
Phosphoramidate diester	Phosphoramidase cleavage	No
Phospholipid conjugate	Phospholipase-C cleavage	Yes
Peptide conjugates	Phosphatase cleavage	No
Hepdirect	Cytochrome P_{450}	Yes
Arylphosphoramidate ProTide	Esterase, phosphoramidase cleavage	Yes
Phosphorodiamidate	Esterase, phosphoramidase cleavage	Yes

1.4 Aim of work

The ProTide approach has proved to be effective in delivering the monophosphate form of acyclovir, thus bypassing the first phosphorylation step mediated by the human herpes virus-encoded thymidine kinase (HHV-TK) and achieving *in vitro* antiviral activity also against HIV.[125, 126]

The aim of this work is:

- to further investigate the application of the ProTide approach to acyclovir by modifying the aryl, amino acid, and ester moieties;
- to extend this technology to other acyclic nucleosides by introducing different substituents at the guanine base and side chain of acyclovir.

As summarised in figure 1.40, in the following chapters the synthesis and *in vitro* antiviral activity of novel aryl phosphoramidate derivatives of acyclovir and its derivatives 6-O-alkyl acyclovir, 8-bromoacyclovir, 8-methylacyclovir, penciclovir, and ganciclovir will be presented. Enzymatic and modelling studies are also presented in order to investigate the mechanism of activation of ProTides.

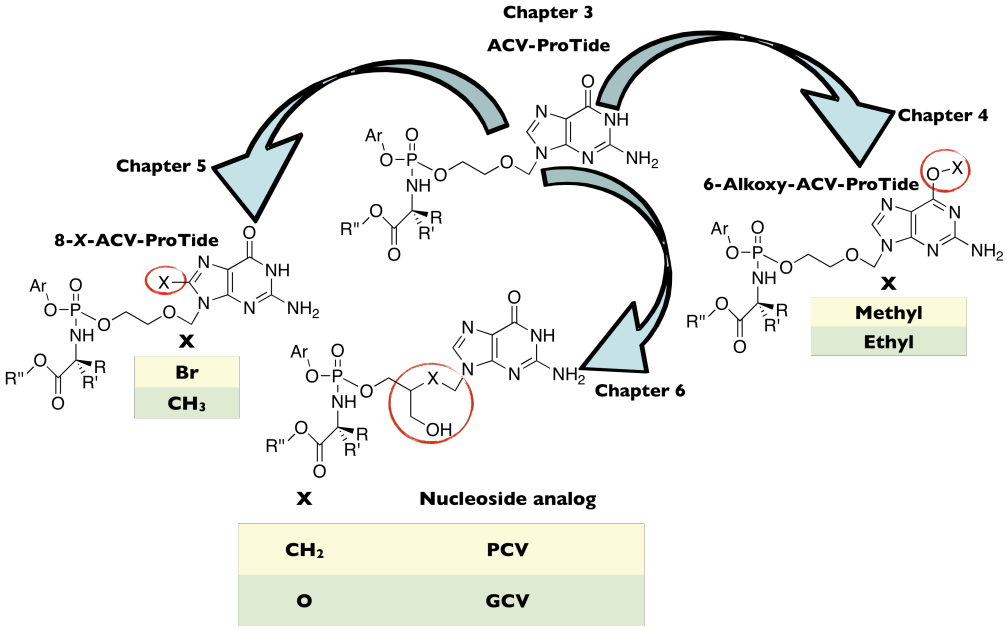


Figure 1.40. Outline of the thesis

Furthermore, in this work the synthesis and *in vitro* biological activity of phosphonoamidate derivatives of cidofovir were evaluated (chapter 7).

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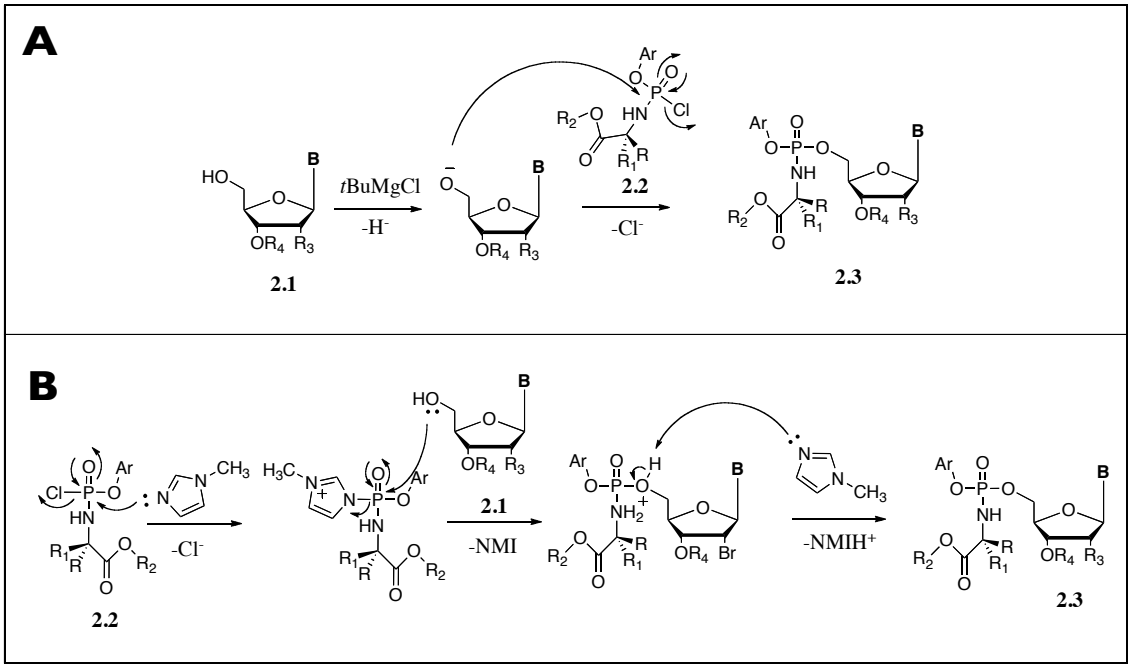
Chapter 2. Synthetic route of ProTides

2.1 Synthesis of aryl phosphoramidate derivatives of nucleoside analogues

The synthesis of ProTides (**2.3**) can be achieved by the coupling reaction of the aryl amino acid ester phosphorochloridate **2.2** with the nucleoside analogue **2.1** through two different synthetic approaches (scheme 2.1A and B).



Scheme 2.1. General synthesis of ProTides: (A) Uchyiama approach, (B) van Boom approach. R, R_1: amino acid side chain; R_2: alkyl, aryl; R_3: H, OH, O-protecting groups; R_4: H, O-protecting group

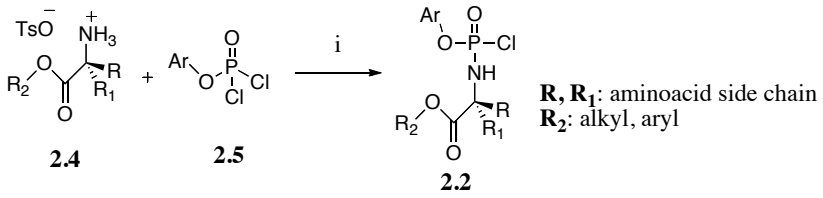
One method has been developed by Uchiyama *et al*. and is based on the deprotonation of the hydroxy group in the 5' position of the nucleoside analogue **2.1** using *tert*-butyl magnesium chloride (*t*BuMgCl) as strong base (scheme 2.3A).[1] The deprotonation affords the 5'-alkoxide group, which is more nucleophilic than the 5'-hydroxy group and this allows it to attack the phosphorus of the phosphorochloridate **2.2**. Considering that the pK_a of nucleoside hydroxyl groups are around 15 and the pK_a of the conjugate acid of *t*BuMgCl is 45,[2] both the secondary hydroxy groups in the 2' and 3' position and the primary hydroxy group in the 5' position of the unprotected nucleoside analogue deprotonate in the presence of *t*BuMgCl. For this reason, the hydroxy groups in the 2' and 3' position must be protected in order to obtain exclusively the 5'-monophosphate analogue **2.3.**

Another method that is applied in our lab for the synthesis of ProTides, has been developed by van Boom *et al*. and utilises *N*-methylimidazole (NMI) as reagent (scheme 2.3B).[3] In this case, NMI ($pK_a \approx 17$) does not act as a base, because it is not strong enough to deprotonate the hydroxy group. However, NMI is presumably able to attack the phosphorochloridate **2.2** displacing the chloride.[4] The corresponding intermediate delocalises a positive charge at the NMI moiety, which acts as a very good leaving group, thus increasing the reactivity towards nucleophiles. The phosphorylation of the unprotected nucleoside **2.1** occurs mainly at the primary hydroxy group in the 5' position probably due to steric hindrance of the phosphorous provided by the imidazolium group.

Starting from a diastereomeric mixture of aryl amino acid ester phosphorochloridate, both approaches generally afford the aryl phoshoramidate derivative of nucleoside analogue as mixture of diastereomers meaning that the coupling reaction is not stereospecific.[5]

2.2 Synthesis of aryl amino acid ester phosphorochloridates

The phosphorochloridate **2.2** is obtained by reaction of the appropriate amino acid ester salt **2.4** and the aryl phosphorodichloridate **2.5** in the presence of triethylamine (scheme 2.2).[6]



2.4 **2.5** **2.2**

Reagents and conditions: (i) TEA, DCM, -78 ºC, then rt, 2 h

Scheme 2.2 General synthesis of aryl amino acid ester phosphorochloridates

Table 2.1 summarises the aryl amino acid ester phosphorochloridates **2.2a-p** synthesised in this work, including the ^{31}P NMR chemical shifts (δ) in $CDCl_3$, and the isolated yields.

Table 2.1. Aryl amino acid phosphorochloridates 2.2a-p synthesised.
(AA: amino acid moiety; R": ester moiety; Ar: aryl moiety)

Cpd	AA	R''	Ar	Yield	^{31}P NMR δ (ppm)
2.2a	L-Alanine	Bn	Ph	86%	7.52, 8.05
2.2b	L-Alanine	Bn	1-Naph	87%	7.52, 7.86
2.2c	L-Alanine	*t*Bu	Ph	64%	7.81, 8.20
2.2d	L-Alanine	CH_2*t*Bu	Ph	Quantitative	7.66, 8.19
2.2e	L-Alanine	CH_2*t*Bu	1-Naph	63%	7.90, 8.21
2.2f	Glycine	Bn	Ph	90%	8.75
2.2g	2,2-Dimethylglycine	Bn	Ph	90%	5.43
2.2h	2,2-Dimethylglycine	Bn	1-Naph	Quantitative	5.83
2.2i	L-Valine	Bn	Ph	77%	8.89, 9.37
2.2j	L-Valine	Bn	1-Naph	Quantitative	9.26, 9.72
2.2k	L-Leucine	Bn	Ph	93%	8.10, 8.34
2.2l	L-Leucine	Bn	1-Naph	65%	8.31, 8.56
2.2m	L-Isoleucine	Bn	Ph	Quantitative	8.61, 9.01
2.2n	L-Isoleucine	Bn	1-Naph	70%	8.98, 9.40
2.2o	L-Proline	Bn	Ph	Quantitative	7.72, 7.78
2.2p	L-Phenylalanine	Bn	1-Naph	60%	8.16, 8.29

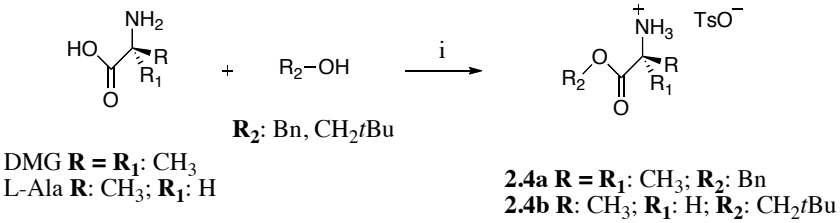
Chiral amino acids (e.g. L-alanine) afforded the relative phosphorochloridates as a mixture of two diastereomers (*SS*p and *SR*p) that displayed two peaks in the ^{31}P NMR spectrum. The phosphorochloridates of achiral amino acid (e.g. glycine and 2,2-dimethylglycine) were obtained as a mixture of enatiomers (*S*p and *R*p) and displayed only one peak in the ^{31}P NMR spectrum.

2.2.1 Synthesis of amino acid esters

Some of the amino acid ester salts used in this work for the synthesis of the phosphorochloridates **2.2a-p** are not commercially available, so their synthesis was required.

The tosylate salt of 2,2-dimethylglycine benzyl ester (**2.4a**) and L-alanine neopentyl ester (**2.4b**) were prepared by reaction of the amino acid with the appropriate alcohol in toluene and in the presence of *para*-toluenesulphonic acid (TsOH) (scheme 2.3).[6] A Dean-Stark apparatus was used in order to remove the water produced by the

condensation reaction. The desired products were obtained in high yield by ether-induced crystallization from the crude of reaction.

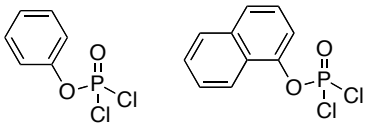


Reagents and conditions: (i) TsOH, toluene, 110 ºC, 12 h

Scheme 2.3. Synthesis of the tosylate salt of 2,2-dimethylglycine benzyl ester (2.4a) and L-alanine neopentyl ester (2.4b)

2.2.2 Synthesis of aryl phosphorodichloridates

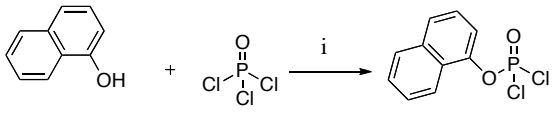
The phenyl phosphorodichloridate (**2.5a**, fig. 2.1) is commercially available, while the naphthyl phosphorodichloridate (**2.5b**, fig. 2.1) is not.



2.5a **2.5b**

Figure 2.1. Structures of phenyl phosphorodichloridate (2.5a) and naphthyl phosphorodichloridate (2.5b).

The synthesis of **2.5b** was carried out by treatment of naphthol with phosphorus oxychloride in the presence of triethylamine (TEA) (scheme 2.4).[6] The crude of reaction was filtrated and used in the next step without any further purification.



2.5b

Reagents and conditions: (i) TEA, Et_2O, -78 ºC, then rt, 2 h

Scheme 2. 4 Synthesis of naphthyl phosphorodichloridate (2.5b)

2.4 Bibliography

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Chapter 3. Aryl phosphoramidate derivatives of acyclovir

3.1 Introduction to the application of the ProTide approach to acyclovir

As already discussed in chapter 1, acyclovir (ACV) is an acyclic guanine nucleoside analogue (fig 3.1) that has been approved for the treatment of HSV and VZV infections.[1] This antimetabolite nucleoside exerts inhibitory activity on the viral DNA polymerase by means of its triphosphate form (ACV-TP). The selectivity for the virus-encoded TK, which is responsible for the formation of ACV monophosphate (ACV-MP), and the viral polymerase accounts for the high therapeutic index of ACV.[2]

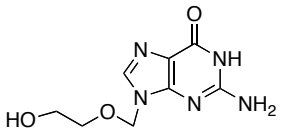


Figure 3.1. Structure of ACV

The development of drug resistance to ACV mainly involves a partial or complete loss of the virus-encoded TK activity. Despite the fact that ACV has been used in the treatment of herpes virus infections for more than 30 years, the incidence of ACV-resistant infections is not a risk among immunocompetent patients. However, this risk is a concern for immunocompromised patients and particularly in patients receiving allogenic bone marrow transplant.[3]

The release of ACV-MP directly into the cell bypasses the HSV- and VZV-TK-mediated phosphorylation of ACV affording activity also against TK-deficient strains of HSV and VZV. For this reason, in our group the ProTide approach was previously applied to the synthesis of aryl phosphoramidates of ACV, which were evaluated *in vitro* against ACV-sensitive and ACV-resistant strains of HSV and VZV.[4] In particular, the L-alanine benzyl ester derivatives **3.1a** and **3.1b** (fig. 3.2) exhibited retention of activity against TK-deficient strains of HSV and VZV in the micromolar range, proving a successful bypass of the first phosphorylation step by releasing ACV-MP.

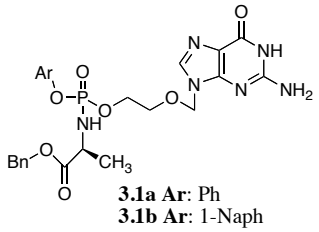


Figure 3.2 L-alanine benzyl ester derivatives 3.1a and 3.1b

These compounds were also found significantly active against HIV in human T-lymphocyte CEM and MT-4 cells (table 3.1).[5]

Table 3.1. Anti-HIV and cytostatic activity of L-alanine benzyl ester derivatives 3.1a-b and ACV in CEM and MT-4 cell cultures (from ref. 5)

	Antiviral Activity			Cytotoxic/Cytostatic Activity		
	EC_{50} (μM) [a]			IC_{50} [b] (μM)		CC_{50} [c] (μM)
Cps	HIV-1 (CEM)	HIV-2 (CEM)	HIV-1 (MT-4)	CEM	MT-4	MT-4
3.1a	16 ± 14	11 ± 4.9	5.7 ± 1.6	42 ± 11	33.8 ± 10.6	> 150
3.1b	15 ± 14	8.9 ± 6.3	0.8	17	> 150	ND
ACV	> 250	> 250	> 250	> 250	> 250	> 250

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%; [b] 50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%; [c] 50% Cytotoxic concentration, or compound concentration that induces 50% cell death in the cultures

This result was not totally unexpected considering that early clinical trials pointed out the survival benefits of ACV treatment in HIV patients co-infected with HHV.[6] At first the mechanism of action of ACV in inhibiting HIV was not clear. Recent trials, which showed reduction of HIV load in HSV-2 co-infected individuals following ACV treatment, argued that the suppression of the inflammation mediated by HSV-2 could be accountable for HIV load decrease.[7, 8] However, studies *in vitro* proved that acyclovir triphosphate inhibits HIV-1 RT and HSV co-infection is necessary for the suppression of HIV-1 replication by ACV (EC_{50}= 3.1 μM in tonsillar tissues co-infected ex-vivo with HSV-2).[9, 10] The bioactivation mechanism of ACV accounts for the need of HSV-TK activity in order to have inhibitory activity against HIV-1 RT. This finding is in agreement with results obtained *in vitro* with ACV ProTides.

Compounds **3.1a** and **3.1b** are considered the lead compounds for ACV ProTide series and they will be reported as references for the antiviral activity of the novel aryl phosphoramidate derivatives of ACV discussed in the following paragraphs.

3.2 Design of novel aryl phosphoramidate derivatives of acyclovir: modification of aryl, amino acid, and ester moieties.

In order to improve the antiviral activity of the L-Alanine benzyl ester derivatives **3.1a** and **3.1b**,[5] novel aryl phosphoramidates of ACV were designed and synthesised varying the aryl, amino acid and ester moieties (**3.2**, fig. 3.3).

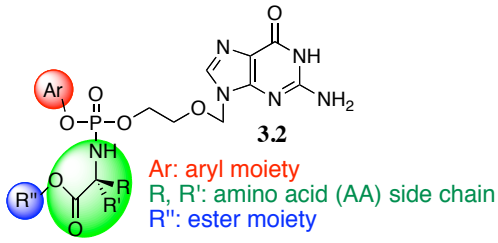


Figure 3.3. General structure of ACV ProTides

As already discussed in chapter 1, the capacity of the masking groups to be good leaving groups or to adopt the right conformation in order to interact properly with the enzymes involved in the bioactivation pathway of ProTides is crucial for the successful release of the monophosphate form of nucleoside analogues.[11] For this reason, the present work is focused on the study of the structure-activity relationships of the masking groups of ACV ProTides (tab. 3.2).

In the first instance, the ACV ProTides **3.2a-f** containing the phenyl group as aryl moiety, the benzyl group as ester moiety, and different amino acids were considered. Hydrophobic amino acids were selected considering to balance the liphophilicity and the steric hindrance of the amino acid side chain. The increase in the lipophilicity is meant to improve the cellular uptake of ProTides. However, it must be considered that bulky aliphatic side chains are more likely to cause loss of activity due to the lack of interaction with the enzymes involved in the bioactivation pathway of ProTides.[11] The non-natural amino acid 2,2-dimethylglycine **3.2b** was considered in order to evaluate the influence of the amino acid stereochemistry on the activity. Then, the replacement of the phenyl group with the naphthyl **3.2g-j** was evaluated with the aim of improving the lipophilicity and leaving group ability of the aryl moiety. In addition, the biological evaluation of the elsewhere reported derivatives **3.2p** and **q**, containing *meta*- and *para*-ethyl-phenyl groups at the aryl moiety respectively, is included in this work due to the similar lipophilicity and lower steric hindrance of these substituents in comparison to the naphthyl group.[12] Finally, in addition to the benzyl different esters such as neopentyl

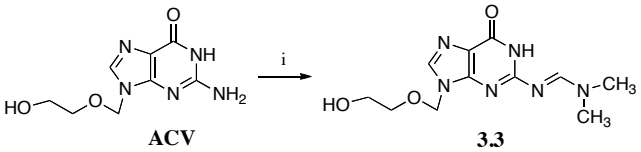
and *tert*-butyl were also evaluated (**3.2k** and **l**). The biological evaluation of the previously reported L-valine methyl and ethyl ester derivatives **3.2n** and **o** is included in this work.[13]

Table 3.2. Aryl (Ar), amino acid (AA) and ester (R") substituents of ACV ProTides 3.2 a-q

Cps	Ar	AA	R"
3.2a	Ph	Gly	Bn
3.2b	Ph	DMG	Bn
3.2c	Ph	L-Val	Bn
3.2d	Ph	L-Leu	Bn
3.2e	Ph	L-Ile	Bn
3.2f	Ph	L-Pro	Bn
3.2g	1-Naph	L-Val	Bn
3.2h	1-Naph	L-Leu	Bn
3.2i	1-Naph	L-Ile	Bn
3.2j	1-Naph	L-Phe	Bn
3.2k	Ph	L-Ala	tBu
3.2l	Ph	L-Ala	CH_2-*t*Bu
3.2m	1-Naph	L-Ala	CH_2-*t*Bu
3.2n[13]	1-Naph	L-Val	Me
3.2o[13]	1-Naph	L-Val	Et
3.2p[12]	3-Et-Ph	L-Ala	Bn
3.2q[12]	4-Et-Ph	L-Ala	Bn

3.3 Synthesis of ACV ProTides.

Based on the previous experience in our group,[14] ACV was protected at the amino group using *N*,*N*-dimethylformamide dimethyl acetal in order to improve its poor solubility in THF (scheme 3.1),[15] which is the solvent commonly used for the synthesis of ProTides.

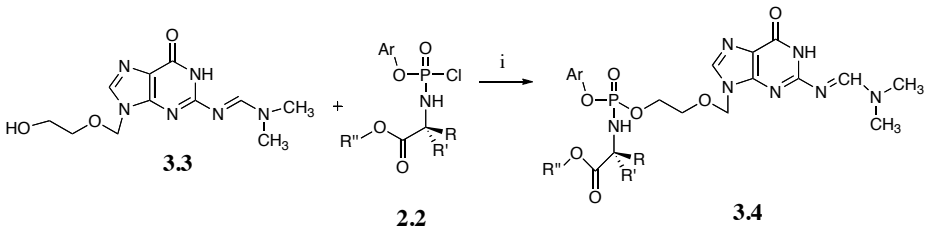


ACV **3.3**

Reagent and conditions: (i) *N*,*N*-dimethylformamide dimethyl acetal, DMF, rt, 24 h

Scheme 3.1. Protection of ACV

For most of the ACV ProTides synthesised, coupling reaction of protected ACV (**3.3)** with the appropriate aryl amino acid ester phosphorochloridate (**2.2**) in anhydrous THF was carried out in the presence of *tert*-Butyl magnesium chloride (*t*BuMgCl), according to the Uchiyama approach described in chapter 2, and afforded the protected derivatives of ACV ProTide (**3.4**, scheme 3.2).[16] However, in the case of glycine and proline derivatives this approach was not successful, so their synthesis was carried out in anhydrous THF/Pyridine mixture (3:2) in the presence of N-methylimidazole (NMI) according to the Van Boom procedure (scheme 3.2).[17]

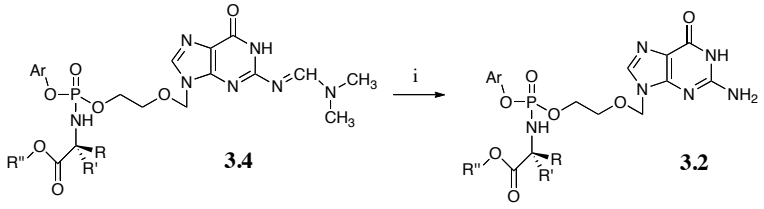


3.3 **2.2** **3.4**

Reagents and conditions: (i) anhydrous THF, *t*BuMgCl, rt, 12-14 h;
or anhydrous THF/Pyr, NMI, rt, 12-14 h

Scheme 3.2. Generic synthesis of ACV ProTides

The DMF-protecting group was removed by refluxing **3.4** in isopropyl alcohol (*i*PrOH) obtaining the desired compounds **3.2** (scheme 3.3).



3.4 **3.2**

Reagents and conditions: (i) *i*PrOH, reflux, 48-62 h

Scheme 2.3. N^2-DMF protecting group removal

Table 3.3 summarises structures, ^{31}P NMR chemical shifts (δ) in deuterated methanol, and isolated yields of the ACV ProTides synthesised. All the ACV ProTides containing

chiral amino acid display two peaks at the ^{31}P NMR spectra corresponding to the two diastereomeric configurations, except the L-Proline derivative, which was isolated as single diastereomer. This was likely due to the stereospecificity of the coupling reaction, giving only one peak at the ^{31}P NMR spectrum.

Table 3.3. ACV ProTides synthesised.
(Ar: aryl moiety; AA: amino acid moiety; R": ester moiety)

Protected Cps	Ar	AA	R"	Coupling Yield	Final Cps	^{31}P NMR δ (ppm)	Deprotection Yield
3.4a	Ph	Gly	Bn	81%	**3.2a**	4.77	2%
3.4b	Ph	DMG	Bn	93%	**3.2b**	2.13	14%
3.4c	Ph	L-Val	Bn	57%	**3.2c**	4.27, 4.51	8%
3.4d	Ph	L-Leu	Bn	65%	**3.2d**	3.60, 4.05	8%
3.4e	Ph	L-Ile	Bn	45%	**3.2e**	4.13, 4.42	3%
3.4f	Ph	L-Pro	Bn	16%	**3.2f**	1.68	4%
3.4g	1-Naph	L-Val	Bn	60%	**3.2g**	4.79, 4.90	10%
3.4h	1-Naph	L-Leu	Bn	65%	**3.2h**	4.04, 4.94	21%
3.4i	1-Naph	L-Ile	Bn	47%	**3.2i**	4.63, 4.82	17%
3.4j	1-Naph	L-Phe	Bn	50%	**3.2j**	3.77, 3.86	16%
3.4k	Ph	L-Ala	tBu	75%	**3.2k**	3.71, 3.81	22%
3.4l	Ph	L-Ala	CH_2tBu	85%	**3.2l**	3.59, 3.80	14%
3.4m	1-Naph	L-Ala	CH_2tBu	53%	**3.2m**	4.09, 4.15	22%

The yields of the coupling reactions may suggest that the phosphorylation of **3.3** with the phosphorochloridate **2.2** is negatively affected by the steric hindrance of the amino acid side chain. The instability of the P-N bond does not allow the removal of the DMF-protecting group in basic conditions, but instead requires milder conditions, which afforded poor yields at the deprotection step.

3.4 Biological evaluation of acyclovir ProTides

The newly synthesised ACV ProTides **3.2a-m** and the previously reported **3.2n-q** were evaluated for their activity against HSV, VZV, HCMV in human embryonic lung (HEL) cell cultures. The antiviral activity of ACV ProTides was evaluated also against HIV in human T-lymphocyte cell cultures (CEM and MT-4 cells). In the case of the antiviral assays performed in HEL cell cultures against the herpes viruses, the minimum

concentration required to cause a microscopically visible alteration of cell morphology was measured and considered as a parameter of cytotoxicity. The cytostatic activity of these compounds on HEL and MT-4 cell cultures is also reported. In the case of the MT-4 cells, the compound concentration that induces 50% cell death in the cultures was evaluated too. The results of these assays will be presented in the next paragraphs.

These derivatives were evaluated also against feline herpes virus, feline corona virus, influenza A and B viruses, para-influenza-3-virus, vescicular stomatitis, respiratory syncitial virus, punta toro virus, but no activity was detected.

Also the protected intermediates of the ACV ProTides **3.4b**, **d**, **f**, **g**, **h**, **i**, **k**, and **m** were evaluated for their activity against HSV, VZV, HCMV, and HIV. Most of them were found to be inactive. Only the 2,2-dimethylglycine and the L-proline benzyl ester derivatives **3.4b** and **3.4f** exhibited moderate to poor activity against HIV-1 in MT-4 cells (EC_{50}= 30-70 μM). These compounds resulted significantly less active in comparison to the unprotected ACV ProTides **3.2**, indicating a detrimental effect of the N^2-DMF protecting group on their antiviral activity.

3.4.1 Anti-HSV activity

Three different strains of HSV were used to evaluate the anti-HSV activity of the ACV ProTides **3.2a-j** in HEL cells: HSV-1 (KOS), HSV-2 (G), and thymidin kinase-deficient (TK^-) HSV-1 (KOS). The minimum concentration required to cause a microscopically visible alteration of cell morphology (MCC) was found to be above 100 μM for all the compounds synthesised.

Modification of the aryl and amino acid moieties

Table 3.4 reports the anti-HSV activity of ACV ProTides **3.2a-j** containing phenyl and naphthyl as aryl moieties, benzyl group as the ester, and different amino acids. The data include ACV and the lead compounds **3.1a** and **b** as references.[4]

Table 3.4. Anti-HSV activity of ACV ProTides 3.2a-j in HEL cell cultures
(Ar: aryl moiety; AA: amino acid moiety; R": ester moiety)

				Antiviral Activity EC_{50} [a] (μM)		
Cps	**Ar**	**AA**	**R"**	**HSV-1**	**HSV-2**	**TK$^-$ HSV-1**
3.2a	Ph	Gly	Bn	3	0.8	9
3.2b	Ph	DMG	Bn	1.4 ± 0.8	0.8 ± 0.1	5.5 ± 2.1
3.2c	Ph	L-Val	Bn	2 ± 0.1	0.9 ± 0.2	7.5 ± 6.4
3.2d	Ph	L-Leu	Bn	0.8 ± 0.1	0.7 ± 0.1	1.4 ± 0.8
3.2e	Ph	L-Ile	Bn	1.1 ± 0.4	1.1 ± 0.4	1.4 ± 0.8
3.2f	Ph	L-Pro	Bn	>100	>100	>100
3.2g	1-Naph	L-Val	Bn	0.7	3	2.5
3.2h	1-Naph	L-Leu	Bn	1.2	1.1	1.3
3.2i	1-Naph	L-Ile	Bn	1.2	1.5	3
3.2j	1-Naph	L-Phe	Bn	>20	>20	>20
ACV	-	-	-	0.4	0.2	50
3.1a	Ph	L-Ala	Bn	8 ± 5.7	4 ± 0	15 ± 7.1
3.1b	1-Naph	L-Ala	Bn	2 ± 0	1.4 ± 0.8	10 ± 2.1

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

ACV exhibited activity against HSV-1 and HSV-2 in the sub-micromolar range (EC_{50}= 0.4 and 0.2 μM respectively) but markedly lost the activity against TK$^-$-HSV-1 (EC_{50}= 50 μM). Lead compounds **3.1a** and **3.1b**, did not improve the antiviral activity of ACV showing micromolar activity against HSV-1 and HSV-2. However, as already mentioned, they retained their activity against TK$^-$-HSV-1, demonstrating a successful by-pass of the first phosphorylation step.[5]

The majority of the novel ACV ProTides **3.2a-j** were found active against HSV-1 and HSV-2 (EC_{50}: 0.7-3 μM) and showed retention of activity against TK-deficient HSV-1 in contrast to ACV, demonstrating successful bypass of the first phosphorylation step.

More specifically:

- Glycine and 2,2-dimethylglycine derivatives **3.2a** and **b** exhibited retention of activity when compared with the lead compounds **3.1a** and **b**.
- In disagreement with the common structure-activity relationship of the

ProTides, showing that the replacement of L-alanine with bulky amino acids leads to a significant loss of activity,[11] ACV ProTides containing L-valine (**3.2c** and **3.2g**), L-leucine (**3.2d** and **3.2h**), and L-isoleucine (**3.2e** and **3.2i**) as amino acid moiety displayed anti-HSV activity in the micromolar range. In particular, L-leucine and L-isoleucine derivatives of the phenyl series (**3.2d** and **3.2e)** showed slight improvement of activity against HSV-1 (EC_{50}= 0.8-1.1 μM), HSV-2 (EC_{50}= 0.7-1.1 μM), and TK-deficient HSV-1 (EC_{50}= 1.4 ± 0.8 μM) in comparison to the analogous lead compound **3.1a**.

- The L-proline and L-phenylalanine derivatives (**3.2f** and **3.2j**) exhibited a complete loss of the anti-HSV activity.

- The increase of lipophilicity, achieved through the replacement of the phenyl with the naphtyl group in the aryl moiety, did not improve the anti-HSV activity.

Evaluation of meta- and para-ethyl-phenyl susbstituents

Table 3.5 shows the anti-HSV activity of the elsewhere reported L-alanine benzyl ester derivatives of ACV ProTides bearing *meta*- or *para*-ethyl substituted phenyl group as aryl moiety (**3.2p-q**).[12] As already mentioned, these substituents were considered due to their similar lipophilicity and lower steric hindrance when compared with the naphthyl group. ACV and the lead compounds **3.1a** and **b** are reported as the reference compounds.

Table 3.5. Anti-HSV activity of ACV ProTides 3.2p-q in HEL cell cultures
(Ar: aryl moiety; AA: amino acid moiety; R": ester moiety)

				Antiviral activity EC_{50} [a] (μM)		
Cps	**Aryl**	**AA**	**R"**	**HSV-1**	**HSV-2**	**TK$^-$ HSV-1**
3.2p[12]	4-Me-Ph	L-Ala	Bn	32.5	32.5	47.5
3.2q[12]	4-Et-Ph	L-Ala	Bn	32.5	32.5	45
ACV	-	-	-	2	1	250
3.1a	Ph	L-Ala	Bn	8 ± 5.7	4 ± 0	15 ± 7.1
3.1b	1-Naph	L-Ala	Bn	2 ± 0	1.4 ± 0.8	10 ± 2.1

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

The data show a marked decrease of potency of compounds **3.2p** and **q** against HSV-1 and HSV-2 in comparison to ACV and the analogous ProTides containing the unsubstituted phenyl (**3.1a)** or naphthyl group (**3.1b)** as aryl moiety. This antiviral activity was retained against TK$^-$-HSV-1, proving that this series of ACV ProTide is able to deliver the monophosphate form of ACV into the cell.

Modification of the ester moiety

Table 3.6 shows the anti-HSV activity of different ester derivatives of ACV ProTide such as the L-alanine *tert*-butyl and neopentyl ester derivatives **3.2k-m**, as well as the previously reported L-valine methyl and ethyl ester derivatives **3.2n** and **o**, which have been included in this work. The analogous benzyl ester derivatives **3.1a**, **3.1b** and **3.2g** have been reported for comparison.

Table 3.6. Anti-HSV activity of ACV ProTides 3.2k-o in HEL cell cultures
(Ar: aryl moiety; AA: amino acid moiety; R": ester moiety)

				Antiviral Activity EC_{50} [a] (μM)		
Cps	**Ar**	**AA**	**R"**	**HSV-1**	**HSV-2**	**TK$^-$ HSV-1**
3.2k	Ph	L-Ala	*t*Bu	>100	>100	>100
3.2l	Ph	L-Ala	CH_2*t*Bu	3	12	2.5
3.2m	1-Naph	L-Ala	CH_2*t*Bu	1.3	1.7	1.5
3.2n[13]	1-Naph	L-Val	Me	>100	>100	>100
3.2o[13]	1-Naph	L-Val	Et	51 ± 9.2	32 ± 18	42 ± 3.5
ACV	-	-	-	0.4	0.2	50
3.1a	Ph	L-Ala	Bn	8 ± 5.7	4 ± 0	15 ± 7.1
3.1b	1-Naph	L-Ala	Bn	2 ± 0	1.4 ± 0.8	10 ± 2.1
3.2g	1-Naph	L-Val	Bn	0.7	3	2.5

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

The inactivity of the *tert*-butyl ester derivative **3.2k** confirms the early study of structure-activity relationship conducted on aryl phosphoramidates of stavudine (d4T) showing that the *tert*-butyl ester derivative was the least potent of the series.[11]

In contrast, the neopentyl ester analogues **3.2l** and **3.2m** showed antiviral activity in the micromolar range against HSV-1, HSV-2, and TK-deficient HSV-1.

Finally, the data show that in the case of L-valine derivatives the replacement of the benzyl (**3.2g**) with methyl (**3.2n**) and ethyl (**3.2o**) esters led to a significant loss of antiviral activity. This result is in agreement with the previous studies showing that L-alanine methyl ester derivative of ACV ProTide was poorly active against HSV.[4] As previously reported, the unsuccessful ester cleavage mediated by the cellular carboxyesterase may account for such a lack of activity.[5]

3.4.2 Anti-VZV activity

The antiviral activity of ACV protides **3.2a-d**, **3.2f-g**, **3.2k-l**, and **3.2o-r** against TK-positive (TK^+) and TK-deficient (TK^-) strains of VZV in HEL cell cultures is reported in table 3.7. The data include ACV as reference compound. The lead compounds **3.1a** and **b** are reported for comparison.[4]

Table 3.7. Anti-VZV of ACV ProTides 3.2a-d, f-g, k-l, and o-r in HEL cell cultures
(Ar: aryl moiety; AA: amino acid moiety; R": ester moiety)

				Antiviral Activity EC_{50}[a] (μM)			
Cps	**Ar**	**AA**	**R"**	**TK^+ VZV strains**		**TK^- VZV strains**	
				YS	**OKA**	**07-1**	**YS/R**
3.2a	Ph	Gly	Bn	8.1	7.4	10.4	8.5
3.2b	Ph	DMG	Bn	5.8	5.9	3.7	1.6
3.2c	Ph	L-Val	Bn	2.6	6.8	7.8	0.3
3.2d	Ph	L-Leu	Bn	N.D.[b]	1.6	5.3	2.6
3.2f	Ph	L-Pro	Bn	N.D.	>50	>50	N.D.[b]
3.2g	1-Naph	L-Val	Bn	0.7	0.7	1.7	1.4
3.2k	Ph	L-Ala	*t*Bu	N.D.[b]	35	N.D.[b]	>50
3.2l	Ph	L-Ala	CH_2*t*Bu	1.1	2.1	3.3	3
3.2o	1-Naph	L-Val	Me	N.D.[b]	>50	>50	N.D.[b]
3.2p	1-Naph	L-Val	Et	N.D.[b]	26	23.7	N.D.[b]
3.2q	3-Et-Ph	L-Ala	Bn	1.5	1	4	2.9
3.2r	4-Et-Ph	L-Ala	Bn	1.5	1.4	3.2	2.7
ACV	-	-	-	1	4.17	73.6	94.3
3.1a	Ph	L-Ala	Bn	0.72	1.0	1.8	0.59
3.1b	1-Naph	L-Ala	Bn	7.2	3.3	6.9	N.D

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%; [b]not determined

ACV was found active against TK^+-VZV in the micromolar range; such activity was markedly decreased against the TK^- strains. The lead compounds, **3.1a** and **b**, exhibited good antiviral activity against both TK^+ and TK^- strains of VZV.[4] In general, the newly synthesised ACV ProTides retained this activity, proving the successful release of ACV monophosphate inside the cell.

In agreement with the major results obtained against HSV, L-valine and L-leucine benzyl ester derivatives (**3.2c**, **3.2d**, **3.2g**) were found active against VZV in the micromolar range retaining the antiviral activity of the lead compounds **3.1a** and **b**. The L-Proline derivative **3.2f** confirmed to be inactive, as already observed in the anti-HSV assay. The L-alanine neopentyl ester derivative **3.2l** retained the anti-VZV activity in comparison to the benzyl ester analogue **3.1a**. The L-valine methyl and ethyl ester derivatives **3.2o** and **3.2p** were found to be inactive or poorly active.

In contrast to the results obtained against HSV, the *meta* and *para*-ethyl-phenyl derivatives **3.2q** and **3.2-r** did not show the same marked decrease of the anti-VZV activity in comparison to the L-alanine benzyl ester analogues **3.1a** and **3.1b**.

The minimum concentration required to cause a microscopically visible alteration of HEL cell morphology (MCC) was found to be above 100 μM for all the compounds evaluated in the antiviral assay against VZV.

3.4.3 Anti-HCMV activity

Table 3.8 reports the antiviral activity of ACV ProTides **3.2a-d**, **3.2f-g**, **3.2k-l**, and **3.2o-r** against HCMV in HEL cell cultures. GCV is included as reference compound. The L-alanine benzyl ester derivatives **3.1a** and **b** are reported for comparison.[18] The minimum concentration required to cause a microscopically visible alteration of HEL cell morphology (MCC) was above 100 μM for all the compounds tested against HCMV.

Table 3.8. Anti-HCMV activity of ACV ProTides 3.2a-d, f-g, k-l, and o-r in HEL cell cultures
(Ar: aryl moiety; AA: amino acid moiety; R": ester moiety)

				Antiviral Activity EC_{50}[a] (μM)	
Cps	**AR**	**AA**	**R"**	**AD-169 strain**	**Davis strain**
3.2a	Ph	Gly	Bn	33.8	11.0
3.2b	Ph	DMG	Bn	11	4.3
3.2c	Ph	L-Val	Bn	4	2
3.2d	Ph	L-Leu	Bn	2.7	1
3.2f	Ph	L-Pro	Bn	>50	>50
3.2g	1-Naph	L-Val	Bn	2	0.9
3.2k	Ph	L-Ala	*t*Bu	>50	>50
3.2l	Ph	L-Ala	CH_2-*t*Bu	14.3	6.1
3.2o	1-Naph	L-Val	Me	50	20
3.2p	1-Naph	L-Val	Et	12.6	4.3
3.2q	3-Et-Ph	L-Ala	Bn	9.3	6.7
3.2r	4-Et-Ph	L-Ala	Bn	16.2	4.1
GCV	**-**	**-**	**-**	6.7	8.3
3.1a	Ph	L-Ala	Bn	12.6	2.9
3.1b	1-Naph	L-Ala	Bn	2.1	1.8

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

All the ACV ProTides, except for the L-proline benzyl and L-alanine *tert*-butyl ester derivatives **3.2f** and **3.2k**, exhibited good to moderate anti-HCMV activity. Considering that ACV is not active against HCMV due to the lack of phosphorylation,[19] this result confirms that the ProTide approach is effective in releasing the ACV monophosphate inside the cell. In particular, the 2,2-dimethylglycine, L-valine and L-leucine benzyl ester derivatives **3.2b**, **3.2d** and **3.2g** showed activity against HCMV comparable to GCV and the lead compounds **3.1a** and **3.1b**. In contrast to the anti-HSV and anti-VZV assays, the L-valine ethyl ester derivative **3.2p** displayed good anti-HCMV activity (EC_{50}= 4.3-12.6 μM), while the L-valine methyl ester derivative **3.2o** was poorly active (EC_{50}= 20-50 μM).

3.4.4 Cytostatic activity of ACV ProTides in HEL cell cultures

The cytostatic activity in HEL cell cultures of ACV protides **3.2a-d**, **3.2f-g**, **3.2k-l**, and **3.2o-r** is reported in table 3.9.

ACV is included as reference compound. The antiproliferative activity of L-alanine benzyl ester derivatives **3.1a** and **3.1b** on HEL cells is reported for comparison.[4]

Table 3.9 Cytostatic activity of ACV ProTides 3.2a-d, f-g, k-l, and o-r on HEL cell cultures
(Ar: aryl moiety; AA: amino acid moiety; R": ester moiety)

				Cytostatic Activity IC_{50}[a] (μM)
Cps	**AR**	**AA**	**R"**	**HEL**
3.2a	Ph	Gly	Bn	>100
3.2b	Ph	DMG	Bn	>100
3.2c	Ph	L-Val	Bn	100
3.2d	Ph	L-Leu	Bn	100
3.2f	Ph	L-Pro	Bn	>100
3.2g	1-Naph	L-Val	Bn	74
3.2k	Ph	L-Ala	*t*Bu	>100
3.2l	Ph	L-Ala	CH_2-*t*Bu	43.3
3.2o	1-Naph	L-Val	Me	>100
3.2p	1-Naph	L-Val	Et	>100
3.2q	3-Et-Ph	L-Ala	Bn	45.4
3.2r	4-Et-Ph	L-Ala	Bn	47.3
ACV	**-**	**-**	**-**	559
3.1a	Ph	L-Ala	Bn	>100
3.1b	1-Naph	L-Ala	Bn	20

[a] 50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%

Most of the ACV ProTides did not exert antiproliferative activity on HEL cells below 100 μM. Some of them showed moderate cytostatic activity (IC_{50}: 43.3-74 μM). However, this result was not completely unexpected considering that the lead compound **3.1b** exhibited similar effect (IC_{50}: 20 μM), as previously reported in the literature.[4]

3.4.5 Anti-HIV activity

The antiviral activity of ACV ProTides **3.2b**, **3.2d**, **3.2g-i**, and **3.2k-m** was evaluated against HIV-1 in CEM and MT-4 cell cultures and against HIV-2 in CEM cell cultures. Table 3.10 reports the anti-HIV-1 and cytostatic activity in MT-4 cell cultures of the selected ACV ProTides. The data include ACV as reference compound and the previously reported L-alanine benzyl ester derivatives **3.1a** and **3.1b** for comparison.[5]

Table 3.10. Anti-HIV-1 and cytostatic activty of ACV ProTides in MT-4 cell cultures.
(Ar: aryl moiety; AA: amino acid moiety; R": ester moiety)

				Antiviral Activity EC_{50}[a] (μM)	Cytostatic Activity IC_{50}[b] (μM)
Cps	**Ar**	**AA**	**R"**	**HIV-1**	**MT-4**
3.2b	Ph	DMG	Bn	7	>150
3.2d	Ph	L-Leu	Bn	0.8	17
3.2g	1-Naph	L-Val	Bn	10	48
3.2h	1-Naph	L-Leu	Bn	2.5	21
3.2i	1-Naph	L-Ile	Bn	4	21
3.2k	Ph	L-Ala	*t*Bu	>150	>150
3.2l	Ph	L-Ala	CH_2*t*Bu	4	>150
3.2m	1-Naph	L-Ala	CH_2*t*Bu	0.7	70
ACV	-	-	-	>250	>250
3.1a	Ph	L-Ala	Bn	5.7 ± 1.6	33.8 ± 10.6
3.1b	1-Naph	L-Ala	Bn	0.8	> 150

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%; [b] 50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%

As known, ACV does not exert any activity against HIV-1 and HIV-2 in the absence of herpes virus-encoded thymidine kinase.[9]

The antiviral activity of the newly synthesised ACV ProTides against HIV-1 in MT-4 cells suggests that the ACV monophosphate was released into the cell bypassing the first phosphoryation step of ACV. The 2,2-dimethylglycine benzyl ester derivative **3.2b** was found active in the micromolar range (EC_{50}= 7 μM) showing retention of activity in comparison to **3.1a** and **3.1b**. The ACV ProTides containing bulky amino acids such as L-Leucine (**3.2d**, **3.2h**), L-Valine (**3.2g**) and L-isoleucine (**3.2i**) displayed good to moderate activity (EC_{50}= 0.8-10 μM). However, these compounds showed also

moderate cytostatic activity (IC_{50}= 21-48 μM). As observed against the herpes viruses, the L-alanine *tert*-butyl ester derivative **3.2k** was inactive against HIV-1 in MT-4 cells. While, the L-alanine neopentyl ester derivatives **3.2l** and **3.2m** retained the activity diplayed by the lead compounds **3.1a** and **3.1b**.

The concentration that induces 50% cell death in the cultures was above 150 μM for all the ACV ProTides tested against HIV-1 in MT-4 cells.

Surprisingly, all these compounds were found to be totally inactive against HIV-1 and HIV-2 in CEM cell cultures, where the previously reported L-alanine benzyl ester derivatives **3.1a** and **3.1b** exhibited anti-HIV activity in the micromolar range (table 3.1).

3.4.6 Summary of the biological activity of ACV ProTides

The biological evaluation of the ACV ProTides presented in this work strongly suggests that most of them successfully bypass the first phosphorylation of ACV, releasing the ACV monophosphate into the cell. This hypothesis is supported by the activity of these compounds against TK-deficient strains of HSV and VZV as well as against HCMV and HIV-1 in MT-4 cells.

The glycine and 2,2-dimethylglycine benzyl ester derivatives **3.2a** and **3.2b** showed retention of activity against the herpes viruses when compared to the analogous L-alanine benzyl ester derivatives **3.1a** and **3.1b** (tab. 3.4 and 3.7). Compound **3.2b** showed similar result also against HIV-1 in MT-4 cell (tab 3.10).

In disagreement with the common structure-activity relationships of ProTides, the aryl phosphoramidate derivatives of ACV **3.2c-e** and **3.2g-l** containing benzyl ester of bulky amino acids such as L-valine, L-leucine, and L-isoleucine exhibited good antiviral activity against both herpes viruses and HIV-1 in MT-4 cells with general retention of activity in comparison to the lead compounds **3.1a** and **3.1b** (tab. 3.4, 3.7, 3.8, 3.10). The flexibility of the acyclic chain of ACV may allow these derivatives to assume the right conformation in order to interact properly with the different enzymes involved in the activation pathway of the ProTides.

The complete lack of activity of the L-proline derivative **3.2f** has already been described in the application of the ProTide approach to the other nucleoside analogues.[11] Whether the steric hinderance or the lack of the free NH are accountable

for the missed bioactivation of L-proline derivatives has not been clarified yet.[5]
The inactivity of the L-phenylalanine derivative **3.2j** is most probably correlated to the steric hindrance of the amino acid moiety that interferes with the carboxyesterase- or phosphoramidase-mediated cleavages.[5]

The increase of lipophilicity afforded by the replacement of the phenyl group at the aryl moiety with the naphthyl did not improve the potency of the antiviral activity (tab. 3.4). The introduction of *meta*- and *para*-ethyl-phenyl led to a marked decrease of the anti-HSV activity of **3.2p** and **3.2q** in comparison to **3.1a** and **3.1b** (tab 3.5). Similar decrease was not observed against VZV (tab 3.7). In a previous study, it was proved that the benzyl ester of **3.2p** and **3.2q** is successfully cleaved by the carboxypeptidase Y suggesting that these compounds can release ACV monophosphate into the cell according to the putative mechanism of activation of ProTides described in chapter 1.[12]

The replacement of the benzyl ester of the L-alanine derivatives **3.1a** and **3.1b** with the *tert*-butyl group caused a complete loss of antiviral activity in all the biological assays presented in this work (tab 3.6-3.8 and 3.10). This result confirms previous studies showing that the *tert*-butyl ester derivatives are not successfully processed according to the putative mechanism of activation of ProTides.[5, 11] Differently, the L-alanine neopentyl ester derivatives **3.2l** and **3.2m** were found active against both the herpes viruses and HIV-1 in MT-4 cells (tab. 3.6-3.9) showing a general retention of the antiviral activity when compared to the analogous benzyl ester derivatives **3.1a** and **3.1b**. In comparison to the *tert*-butyl group, the additional methylene group of the neopentyl ester must be crucial in order to properly accommodate this moiety into the active site of the carboxyesterase.
The previously reported L-valine methyl and ethyl ester derivatives **3.2n** and **3.2o** were found inactive or poorly active against HSV and VZV presumably due to the poor cleavage mediated by the carboxyesterase (tab. 3.6-3.7).[5] However, compound **3.2o** showed also good antiviral activity against HCMV (EC_{50}= 4.3-12.6 μM, tab. 3.8).

Some of the ACV ProTides presented in this work exerted moderate antiproliferative activity on Hel cell (IC_{50}= 45.4-74 μM, tab 3.9) and MT-4 cell cultures (IC_{50}= 17-70 μM, tab. 3.10). These results may indicate that the application of the ProTide approach could induce the release of cytotoxic concentration of ACV triphosphate into the cell.

The lack of activity of the newly synthesised ACV ProTides against HIV-1 and HIV-2 in CEM cell cultures is in contrast with all the biological results presented so far. However, it is in agreement with previous studies of the application of the ProTide approach to different nucleoside analogues showing that the replacement of the L-alanine with other amino acids leads to the loss of the antiviral activity (10- to 100-fold), particularly in the case of bulky amino acids.[11] The inactivity of the 2,2-dimethylglycine derivative is surprising, considering that this amino acid generally involves retention of the antiviral activity displayed by the L-alanine analogues. In comparison to the antiviral assays performed in HEL and MT-4 cell cultures, the lack of anti-HIV activity of the ACV ProTides in CEM cell cultures could be explained by a difference in terms of substrate affinity or intracellular levels of the enzymes involved in the intracellular bioactivation of ProTides.

3.5 Enzymatic and modelling studies on ACV ProTides

With the aim to understand the difference of antiviral activity among the novel ACV ProTides presented in the previous paragraphs, enzymatic and molecular modelling studies were performed.

3.5.1 Study of the carboxyesterase activity

According to the putative bioactivation mechanism of ProTides described in chapter 1, the cleavage of the ester moiety leads to the loss of the aryl moiety with formation of the phosphoramidate monoester intermediate, which is cleaved by a phosphoramidase-type enzyme affording the monophosphate derivative of nucleoside analogues.[11] As already mentioned, cathepsin A (catA, EC 3.4.16.5) has been indicated as the main intracellular enzyme responsible for the cleavage of the ester moiety of ProTides.[20] Unfortunately, human cathepsin A is not commercially available. However, it has been shown that this enzyme has a high degree of amino acid sequence homology with the yeast carboxypeptidase Y (CPY, EC 3.4.16.1), which is commercially available. The two enzymes have similar structure and similar substrate specificities.[21,22] The formation of the phosphoramidate monoester as a product of the enzymatic ester hydrolysis has been extensively investigated in our group monitoring the reaction by ^{31}P NMR.[18] The phosphoramidate monoester was also synthesised by chemical hydrolysis of a ProTides in the presence of triethylamine confirming that the chemical shift of the phosphorus is

around 7-8 ppm in the ^{31}P NMR spectrum.[23]

In order to evaluate whether the ester group of the ACV ProTides presented in this work can be hydrolyzed according to the putative bioactivation mechanism of ProTides, enzymatic studies have been performed using CPY.

In particular, compounds **3.2b**, **3.2d**, **3.2f**, and **3.2j** were selected with the aim to investigate the effect of the amino acid side chain hindrance on the carboxypeptidase Y activity and whether the difference of their antiviral activity can be correlated to the efficiency of the enzymatic reaction. Compounds **3.2k** and **3.2l** were chosen in order to study the enzymatic hydrolysis of the *tert*-butyl and neopentyl ester groups and correlate the activity of the CPY with their biological activity.

General procedure

Following the procedure developed in our lab to study the CPY-mediated hydrolysis of aryl phosphoramidate derivatives,[18] ACV ProTide was dissolved in acetone-D6 and Trizma buffer (pH 7.6), then ^{31}P NMR spectrum of the blank was recorded. The compound was incubated with CPY for 14h and the enzymatic reaction was monitored by ^{31}P NMR analysis.

The proposed pathway of the enzymatic reaction is depicted in figure 3.4.

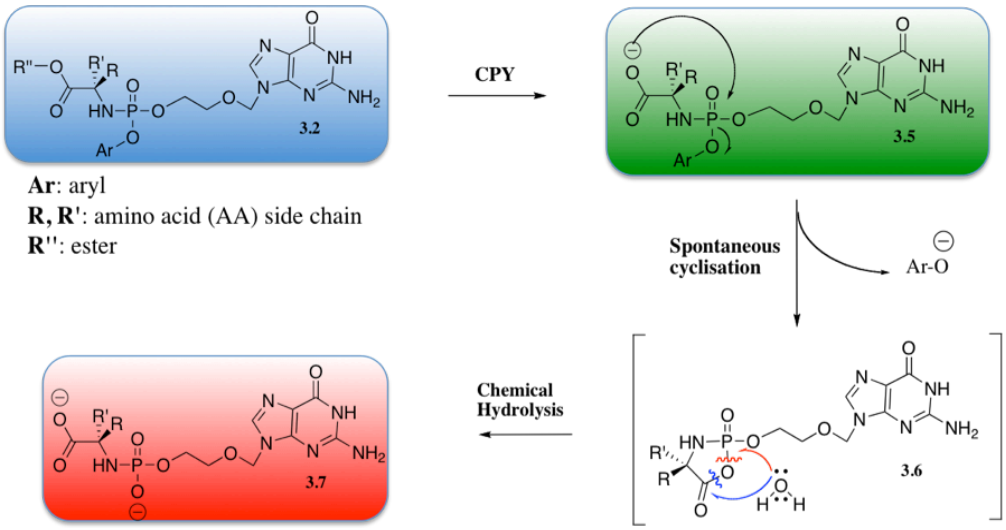


Figure 3.4. Proposed mechanism of ester cleavage of ACV ProTides mediated by CPY

Except for the 2,2-dimethylglycine and L-proline derivatives **3.2b** and **3.2f**, the starting material **3.2** shows two peaks around 4-5 ppm in the ^{31}P NMR spectrum that correlate to

the two diastereomeric configurations. The hydrolysis of the ester affords compound **3.5** (δ≈6 ppm) that leads through intracellular cyclisation to the formation of the unstable intermediate **3.6**. The nucleophilic attack of water, either on the phosphorus or on the carbonyl group, opens the ring affording the phosphoramidate monoester **3.7**. As already reported, this species gives rise to only one peak around 7-8 ppm in the ^{31}P NMR spectrum indicating the loss of chirality of the phosphorus.[23]

Enzymatic study results-1: amino acid modification

In the case of compound **3.2b** (δ_P= 2.31 ppm, fig. 3.5A) and **3.2d** (δ_P= 3.74, 3.91 ppm, fig. 3.5B)**,** the hydrolysis of the ester moiety occurred in less than 6 min from exposure to CPY.

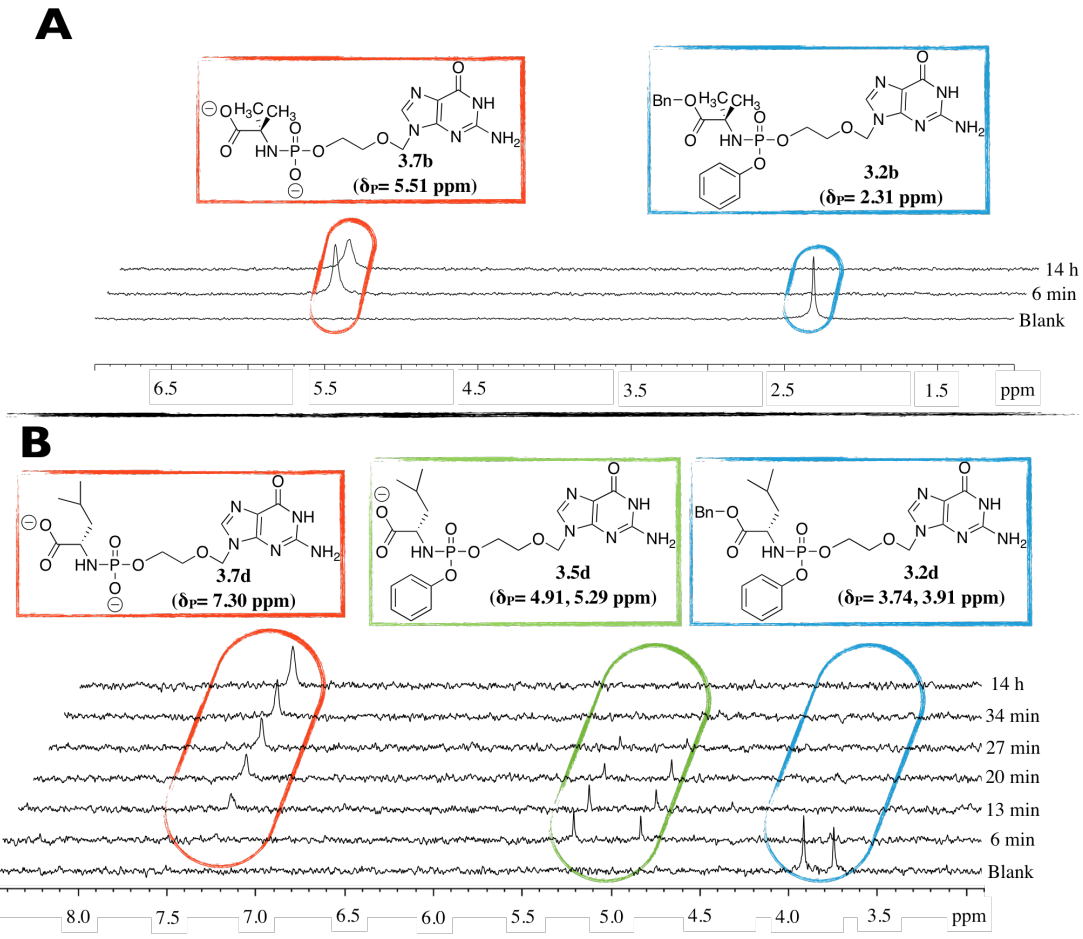


Figure 3.5. ^{31}P NMR spectra (acetone-D6, 202 MHz) at different time of the enzymatic study performed on compound (A) 3.2b and (B) 3.2d

The formation of the intermediate resulting from the cleavage of the benzyl group of **3.2b** was not observed before the formation of the final product **3.7b** (δ_P= 5.51 ppm, fig. 3.5A).

In the case of **3.2d**, the intermediate **3.5d** (δ_P= 4.91, 5.29 ppm, fig. 3.5B) was converted to the corresponding phosphoramidate monoester **3.7d** (δ_P= 7.30 ppm, fig. 3.5B) approximately by 50% after 13 min from exposure to CPY. The conversion of **3.2d** was complete in less than 30 min. The fast cleavage of the benzyl ester of compound **3.2d** proves that the hindrance of the L-leucine side chain is well tolerated by the carboxyesterase, confirming the biological activity seen with this compound.

Similarly, the inactivity of compounds **3.2f** (δ_P= 1.73 ppm, fig. 3.6A) and **3.2j** (δ_P= 3.77, 3.86 ppm, fig. 3.6B) has been confirmed by the incapacity of CPY to cleave the benzyl ester in presence of L-proline and L-phenylalanine amino acids, proving the detrimental effect of their hindrance on the bioactivation of these aryl phosphoramidate derivatives of ACV.

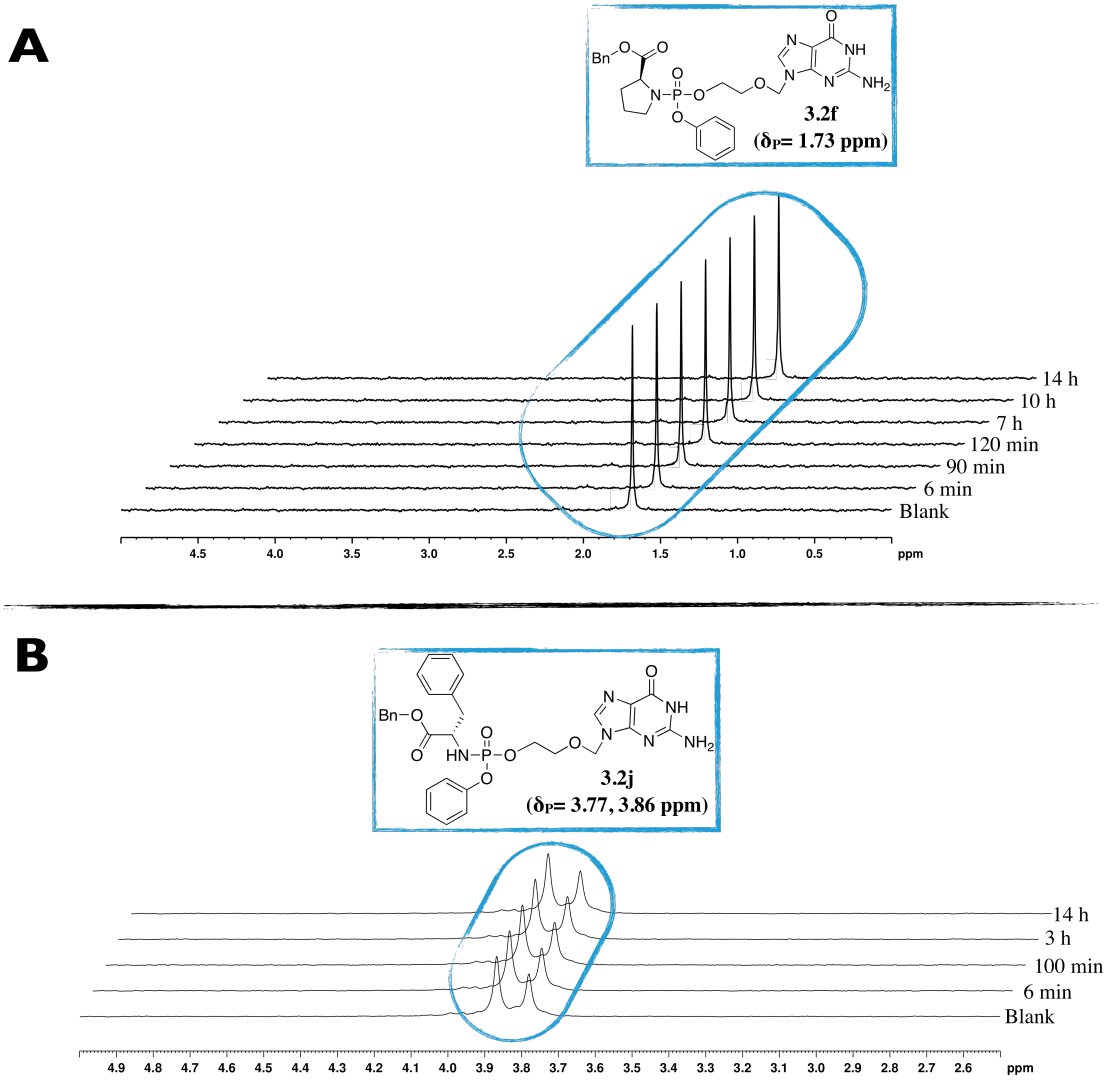


Figure 3.6. ^{31}P NMR spectra (acetone-D6, 202 MHz) at different time of the enzymatic study performed on compound (A) 3.2f and (B) 3.2j

Enzymatic study results-2: ester modification

Figure 3.7 shows the ^{31}P NMR spectra of compound **3.2k** (δ_P= 3.77, 3.82 ppm) over the 14 h incubation period with CPY. As expected from the previous studies and considering the inactivity of this compound in the antiviral assays, the enzyme was probably not able to cleave the *tert*-butyl ester group.

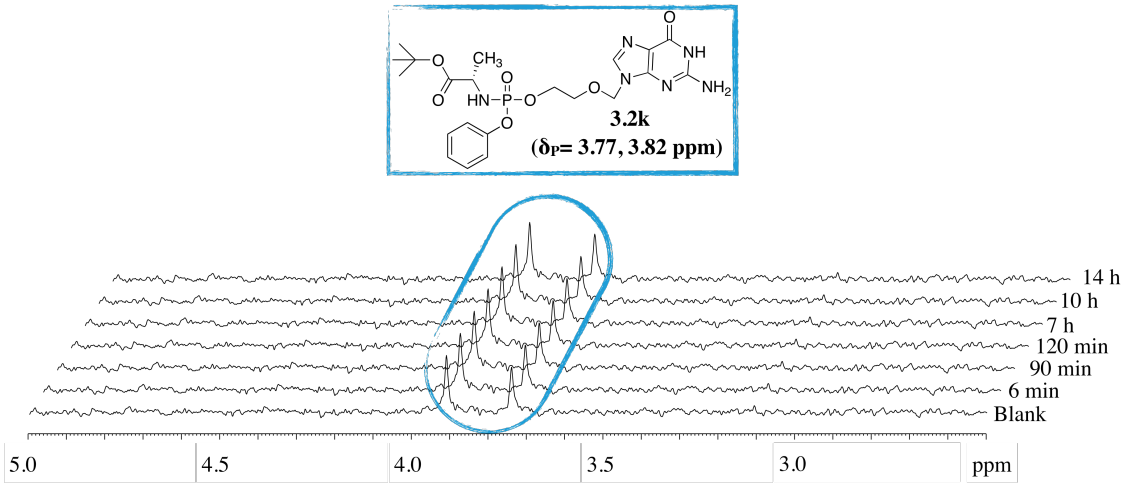


Figure 3.7. ^{31}P NMR spectra (acetone-D6, 202 MHz) at different time of the enzymatic study performed on compound 3.2k

In the case of compound **3.2l** (δ_P= 3.81, 3.92 ppm, fig. 3.8), the enzymatic hydrolysis of the neopentyl ester afforded the intermediate **3.5l** (δ_P= 4.75, 4.89 ppm, fig. 3.8), which then formed the phosphoramidate monoester **3.7l** (δ_P= 7.19 ppm, fig 3.8) by loss of the phenyl group, as previously described in figure 3.4. After 90 min from the exposure to CPY, the diastereomer correlated to the upfield peak in the ^{31}P NMR spectrum was almost completely processed, while 56% of the other diastereomer was still unreacted. After a 5 h period incubation, **3.2l** was completely converted to **3.7l**. This result, indicating that CPY is able to hydrolyse the neopentyl ester more efficiently than the *tert*-butyl ester, could justify the retention of antiviral activity against HSV, VZV, HCMV and HIV-1 in MT-4 cells found for this compound in comparison to L-alanine benzyl ester derivative **3.1a** and **3.1b**.

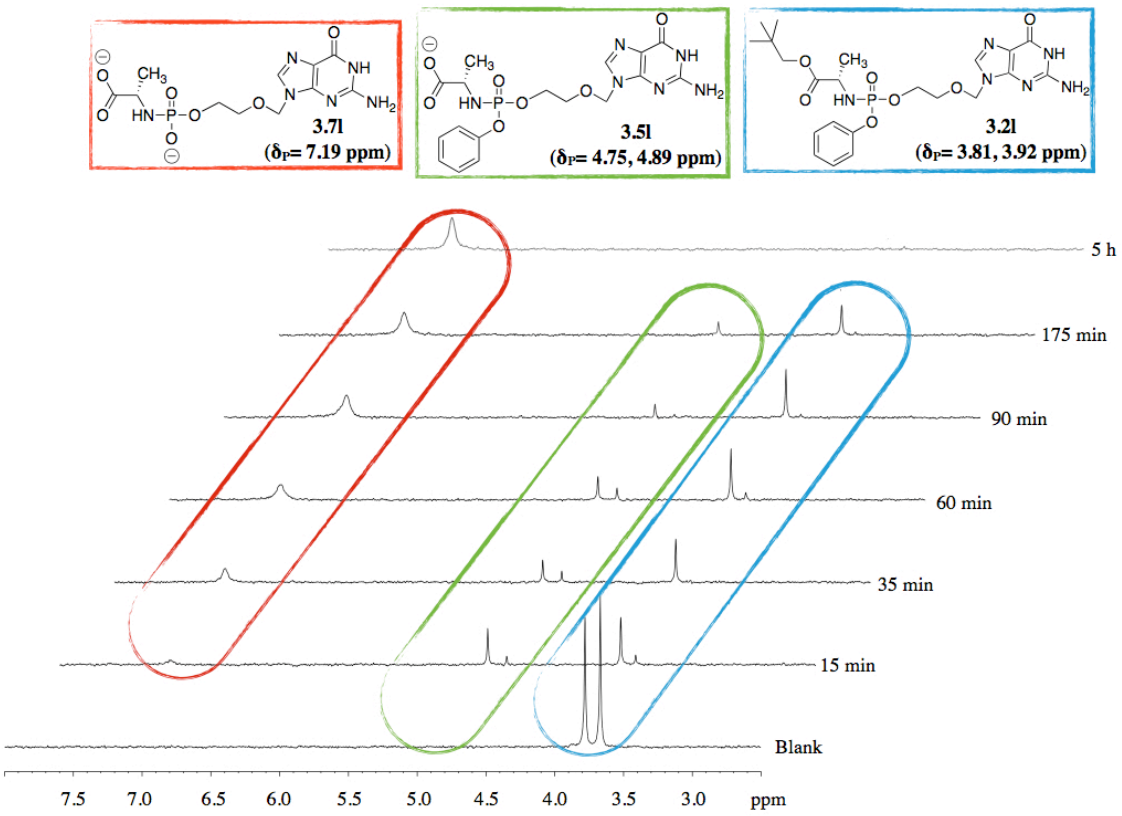


Figure 3.8. ^{31}P NMR spectra (acetone-D6, 202 MHz) at different time of the enzymatic study performed on compound 3.2l

3.5.2 Molecular modelling studies

Modelling studies were carried out in order to further investigate the ester hydrolysis of ACV ProTides by CPY and the processing of the correlated phosphoramidate monoester derivatives by human Hint-1.

Docking of ACV ProTides within the active site of carboxypeptidase Y

Using docking techniques, the interaction of ACV ProTides with the active site of the CPY was modelled on the crystal structure of the enzyme (protein data bank, 1YSC).[24] As depicted in figure 3.9, the mechanism of action of the ester cleavage within the catalytic site of this enzyme involves the coordination of the NH of the glycine 52 (Gly52) and glycine 53 (Gly53) residues with the carbonyl group of the ester, which undergoes the nucleophilic attack of the serine residue 146 (Ser146).[25]

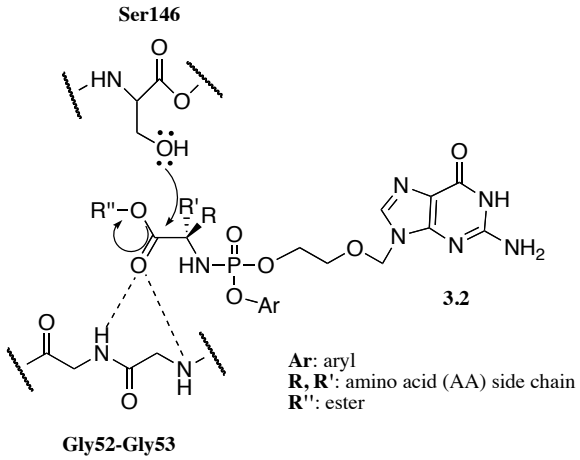


Figure 3.9. Mechanism of cleavage of the ester moiety of generic ACV ProTide 3.2 within the active site of CPY

Compound **3.2d**, **3.2f**, and **3.2j** were selected for this study in order to confirm the results obtained in the enzymatic experiments that suggest the poor interaction of the CPY with the ACV Protide containing L-proline and L-phenylalanine as amino acid moiety, while L-leucine is well tolerated. At the same time, the difference in terms of ester cleavage efficiency between the two diastereomers of the L-alanine neopentyl ester derivative **3.2l** was investigated.

CPY docking results-1: amino acid hindrance investigation

The docking study of compound **3.2d** within the catalytic site of CPY showed a positive interaction for both diastereomers. As depicted in figure 3.10 for the *R*p diastereomer, the guanine base (in blue) is projected outside the pocket of the active site, the phenyl group (yellow) on the phosphate (purple) is on the right pocket, while the benzyl ester (red) is on the left pocket.

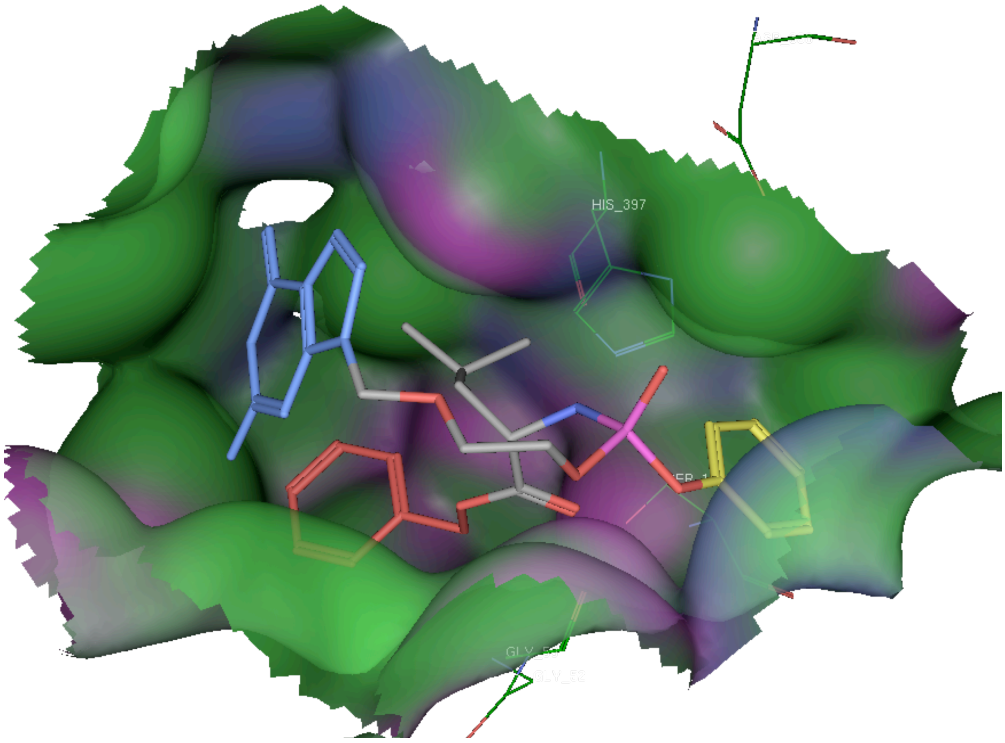


Figure 3.10. Docking of 3.2d (*Rp* diasteromer) within the catalytic site of CPY.
Guanine base (blue), phenyl group (yellow), phosphorus (purple), benzyl ester (red).

The ester moiety is properly orientated to interact with the amino acid residues (Gly52, Gly53, and Ser146) involved in its cleavage (fig 3.11).

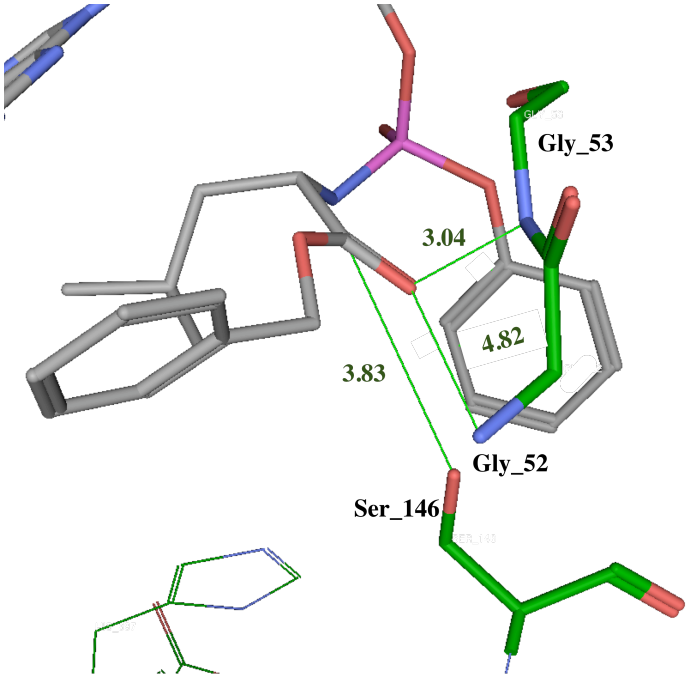


Figure 3.11. Detail of the docking of 3.2d within the active site of CPY showing the orientation and the distance in angstroms of the carboxyl group toward the glycine and serine residues (Gly52, Gly53, and Ser146) responsible for its cleavage.

The same study was performed on compound **3.2f** and **3.2j** proving the detrimental effect of the L-proline and L-phenylalanine on this interaction. In particular, the restriction imposed by the pyrrolidine ring of L-proline forces the carbonyl group of the ester moiety to adopt an orientation that does not favour its coordination with Gly52 and Gly53 residues (figure 3.12, *S*p diastereomer shown).

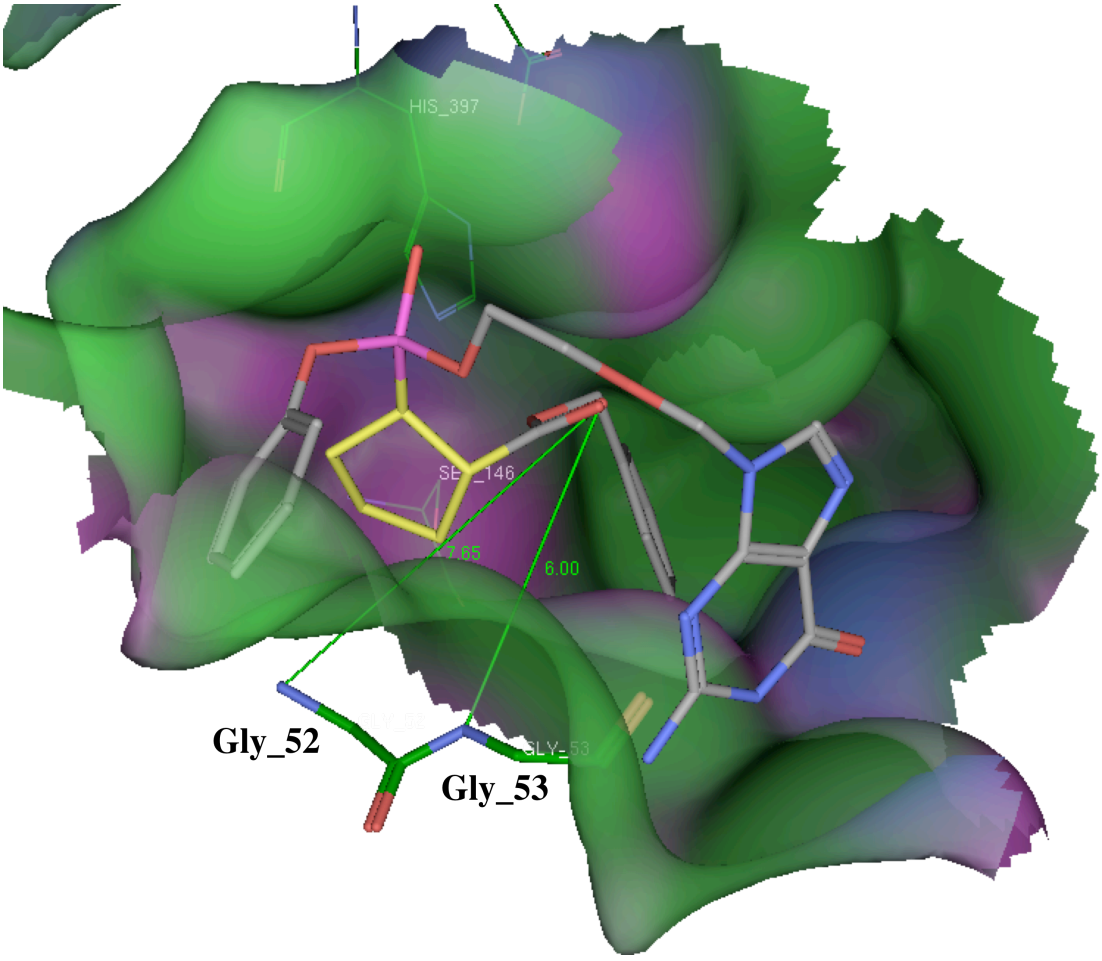


Figure 3.12. Docking of 3.2f (*S*p diastereomer) within the catalytic site of CPY.
The pyrrolidine ring of L-Proline is shown in yellow.

In the case of compound **3.2j**, the hindrance of the phenylalanine side chain has a similar effect on the orientation of the carbonyl group of the ester moiety (fig. 3.13).

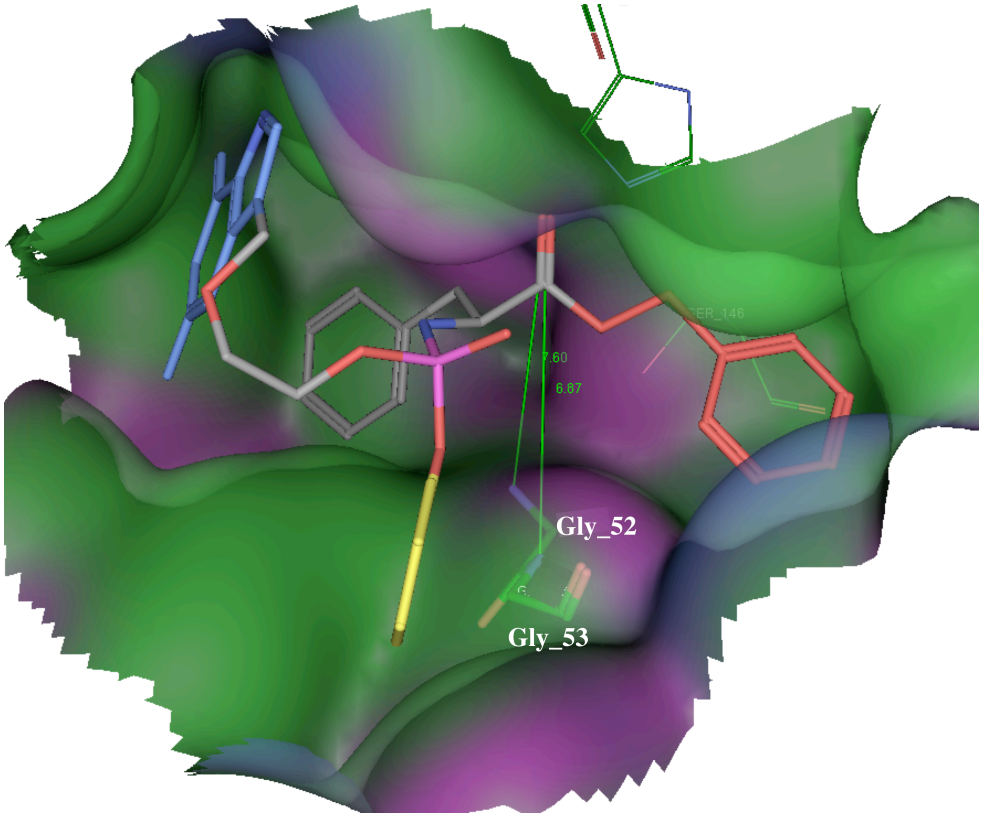


Figure 3.13. Docking of compound 3.2j (*R*p diastereomer) within the catalytic site of CPY.

CPY docking results-2: neopentyl ester investigation.

Figure 3.14 shows the docking of both phosphate diastereomers (*R*p and *S*p) of compound **3.2j** within the catalytic site of CPY.

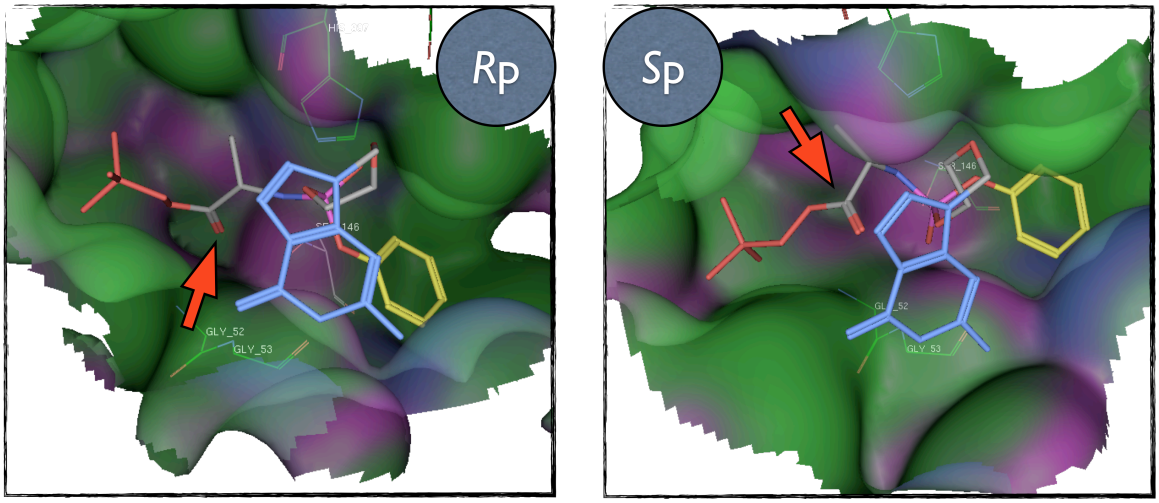


Figure 3.14 Docking of 3.2l within the catalytic site of CPY. The red arrows indicate the position of the carbonyl group.

In the *R*p configuration, the carbonyl group of the neopentyl ester, indicated by the red arrow in figure 3.14, is orientated in opposite direction in comparison to the orientation adopted in the *S*p configuration. As depicted in figure 3.15, this affects the interaction with Gly52, Gly53, and Ser146 residues. In particular, the carbonyl group of the *R*p diastereomer appears to be properly orientated in order to be attacked by the residue of Ser146 and coordinated by the NH of the glycine residues, while the orientation in the *S*p configuration is different and a positive interaction with these residues is less favoured.

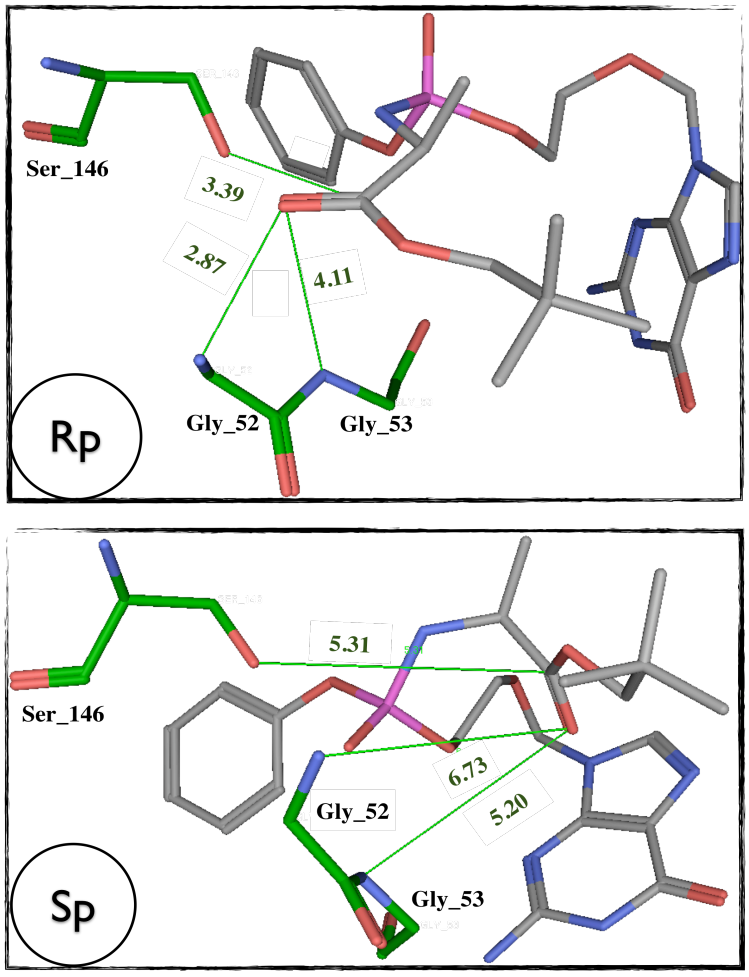


Figure 3.15. Detail of the docking of 3.2l (*R*p and *S*p diastereomers) within the active site of CPY showing the orientation and the distance in angstroms of the carboxyl group toward the glycine and serine residues (Gly52, Gly53, and Ser146) responsible for its cleavage.

In conclusion, the result of this study suggests that the *S*p may be the configuration of the diasteroisomer that is metabolised less efficiently by the CPY in the enzymatic experiment performed on **3.2l** (fig. 3.8). This hypothesis also implies that the *S*p diastereomer is correlated to the downfield signal in the ^{31}P NMR spectrum, while the *R*p diastereomer is correlated to the upfield signal.

Docking of the phosphoramidate monoester derivatives of ACV within the active site of human Hint-1

According to the putative bioactivation mechanism of ProTides, the phosphoramidate monoester derivative of nucleoside analogue **3.7** is considered to be a substrate of human Hint-1 enzyme that may hydrolyse the phosphorus-nitrogen bond, releasing ACV monophosphate (ACV-MP).[26, 27] As already described in chapter 1, human Hint-1 is a phosphoramidase-type enzyme belonging to the HIT superfamily and its catalytic activity is based on three histidine residues. The proposed mechanism of action of this enzyme, depicted in figure 3.16, involves the nucleophilic attack of the hisitidine 112 residue (shown in blue) to the phosphorus with the elimination of the amino acid moiety, which is favoured by the protonation mediated by a serine residue. Finally, the nucleophilic attack of a molecule of water to the phosphorus release ACV-MP and protonate the serine residue.[28]

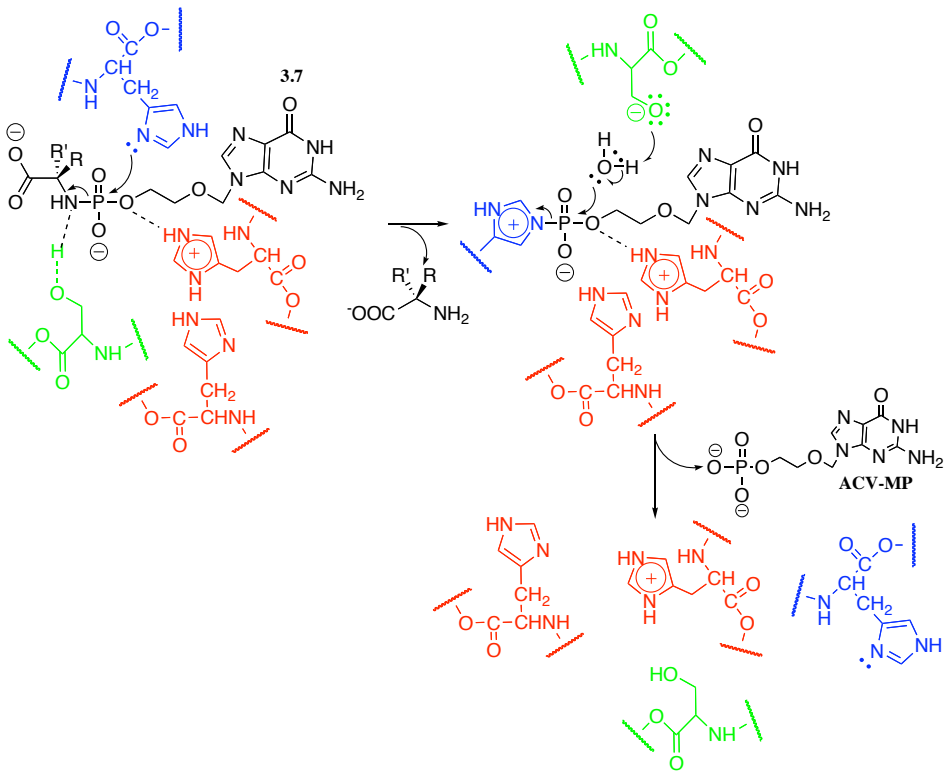


Figure 3.16. Proposed mechanism of action of human Hint-1 (form ref. 26)

In order to investigate the effect of the amino acid side chain hindrance on the human Hint-1 activity, docking studies of the metabolites **3.7b** and **3.7d** (fig. 3.17) were performed using a co-crystallised structure of the enzyme with adenosine monophosphate (AMP).

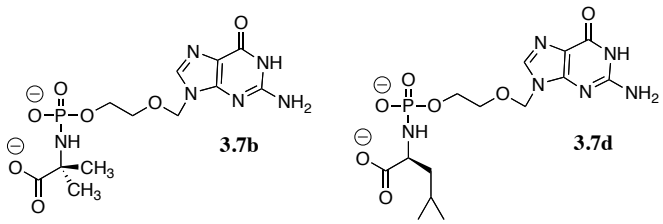


Figure 3.17. ACV phosphoramidates 3.7b and 3.7d

Figure 3.18 shows that **3.7b** binds in the catalytic site of human Hint-1. The guanine base and the side chain of acyclovir are well positioned in the enzymatic pocket. The phosphate moiety (purple) is slightly moved from the position adopted by the phosphate group of AMP (yellow),

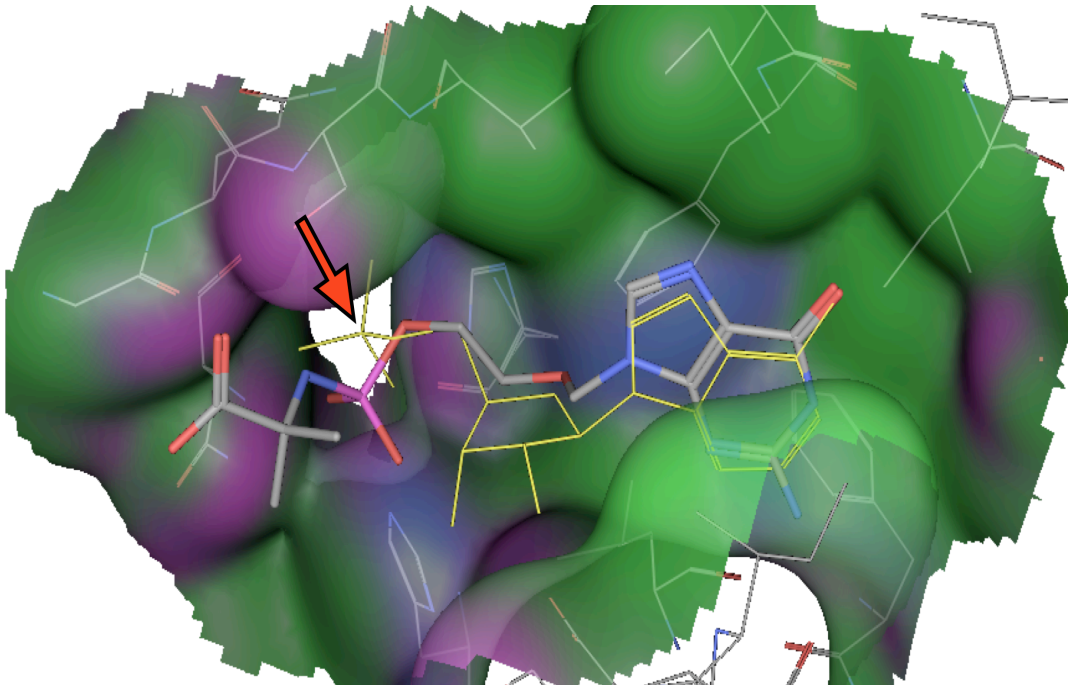


Figure 3.18. Superimposition of acyclovir phosphoramidate 3.7b with AMP (yellow) within the active site of huma Hint-1. The red arrow indicates the position of the phosphate group of AMP.

However, figure 3.19 shows that the histidine and serine residues (His51, His112, His114, and Ser107) are in a suitable position to catalyse the cleavage of the P-N bond according to the mechanism of action previously described. This result is in agreement with the antiviral activity exhibited by the parent compound **3.2b** and confirms the capacity of this compound to release ACV-MP inside the cell.

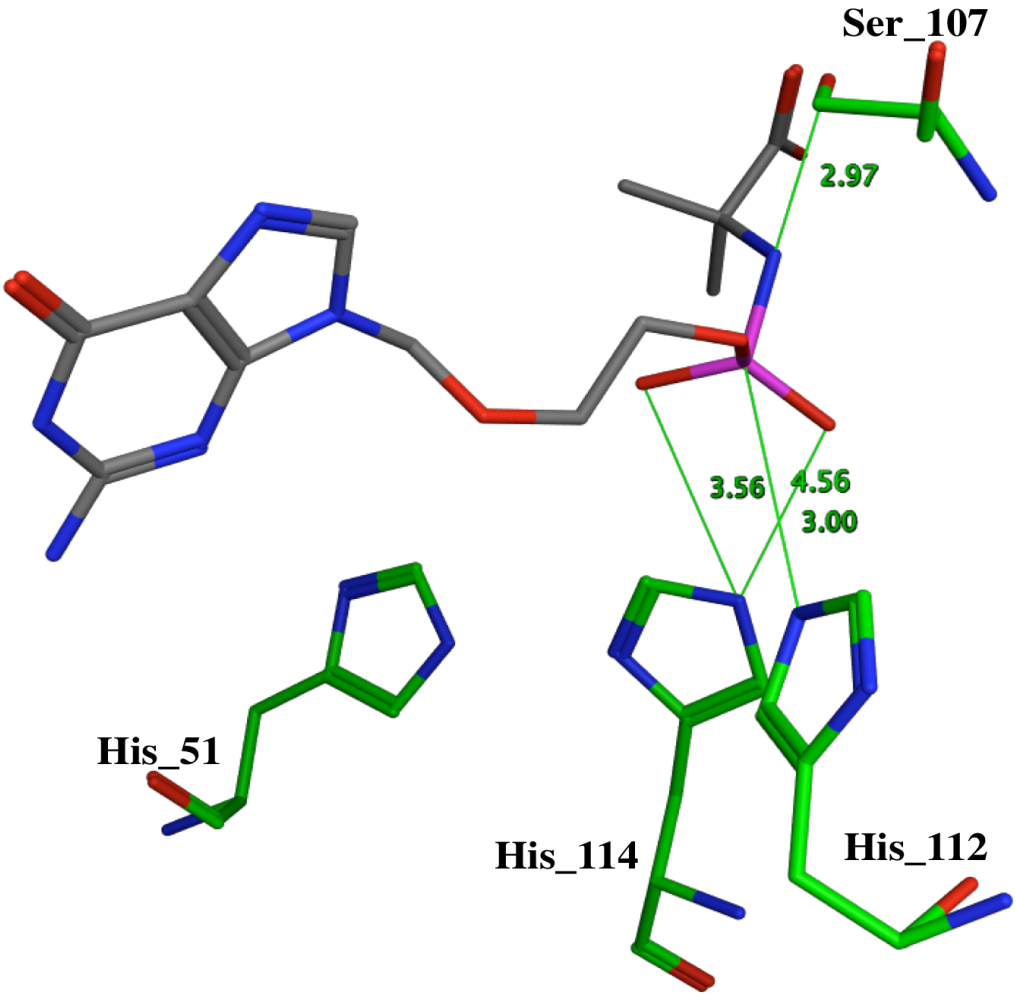


Figure 3.19. Detail of the docking of 3.7b within the active site of human Hint-1 showing the orientation and the distance in angstroms of the P-N bond (purple and blue) toward the histidine and the serine residues (His-51, His-112, His 114, and Ser107) responsible for its cleavage.

Similarly, the modelling study performed on compound **3.7d** (fig. 3.20) showed that this compound interact positively with the active site of the human Hint-1 confirming that the hindrance of L-leucine side chain is well tolerated by this enzyme, as suggested by the results of the antiviral assays.

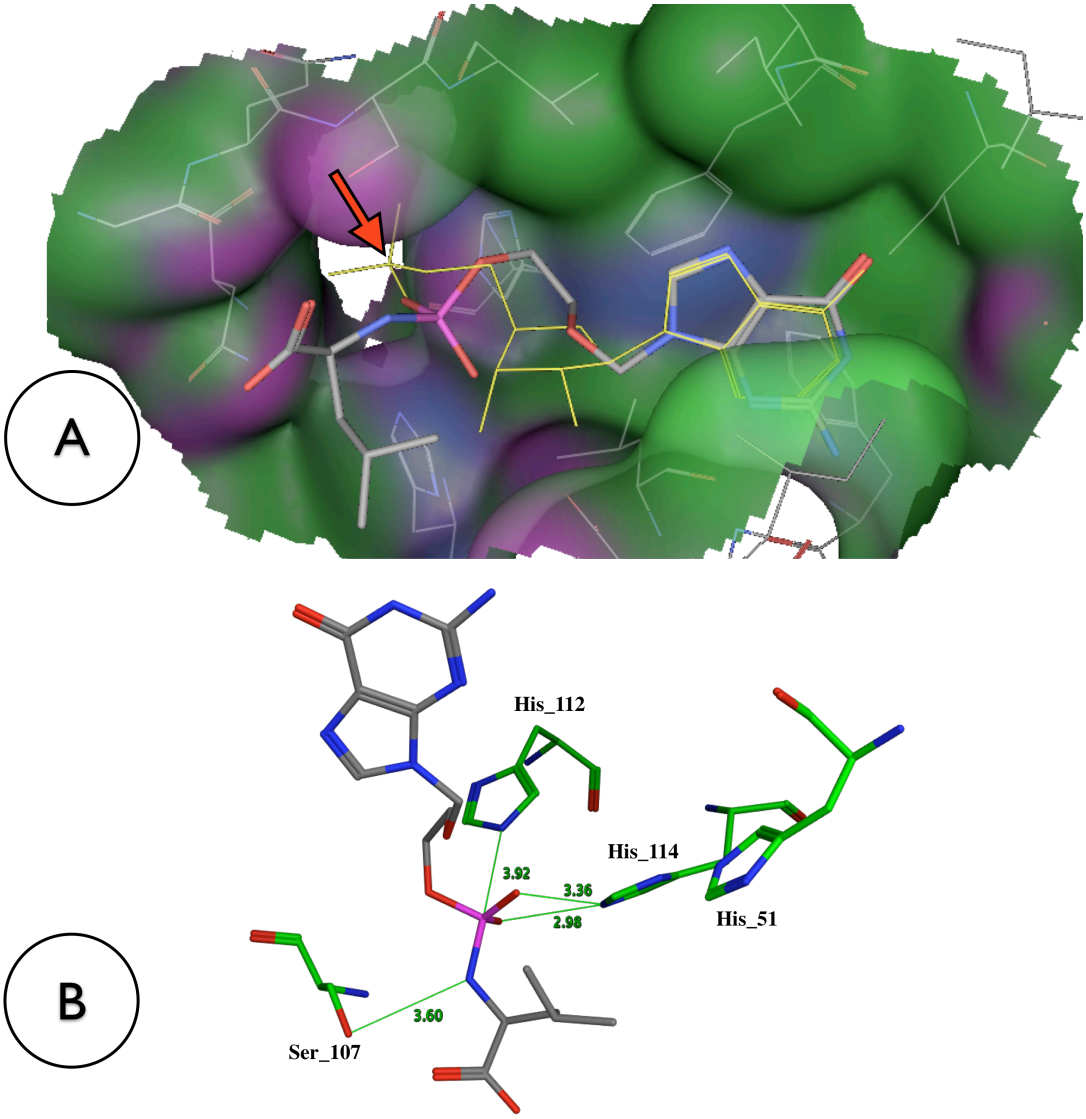


Figure 3.20 A: superimposition of 3.7d with AMP (yellow) within the active site of human Hint-1 enzyme. The red arrow indicates the position of the phosphate group of AMP. B: Detail of the docking of 3.7d within the active site of human Hint-1 showing the orientation and the distance in angstroms of the P-N bond (purple and blue) toward the histidine and the serine residues (His-51, His-112, His 114, and Ser107) responsible for its cleavage.

3.6 Conclusions

An extensive study of structure-activity relationship of ACV ProTide was conducted in this work by evaluating the effect of the modification of the aryl, amino acid, and ester moieties on the antiviral activity of compounds **3.2a-q** against HSV, VZV, HCV, and HIV. The retention of antiviral activity of the L-valine, L-leucine, and L-isoeucine benzyl ester derivatives **3.2c-e** and **3.2g-i** against HSV, VZV, HCV, and HIV-1 in MT-4 cells, in comparison to the L-alanine benzyl ester derivatives **3.1a** and **3.1b**, indicates that ACV monophosphate is released also in the presence of bulky masking groups. The L-alanine neopentyl ester derivatives **3.2l** and **3.2m** resulted to be as effective as the

analogous benzyl ester derivatives **3.1a** and **3.1b.** The increase of lipophilicity by replacement of the phenyl with the naphthyl group at the aryl moiety did not afford any improvement of activity. However, concern has arisen from the potential toxicity of some ACV ProTides due to their cytostatic effects observed on HEL and MT-4 cell cultures.

The lack of activity of all the ACV ProTides synthesised in this work against HIV-1 and HIV-2 in CEM cell points at the L-alanine benzyl ester as the masking group of choice in the synthesis of the novel acyclic nucleoside ProTides, which will be discussed in the subsequent chapters.

3.7 Bibliography

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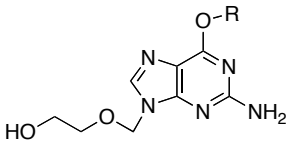
Chapter 4. Aryl phosphoramidate derivatives of 6-*O*-alkyl acyclovir

4.1 Introduction

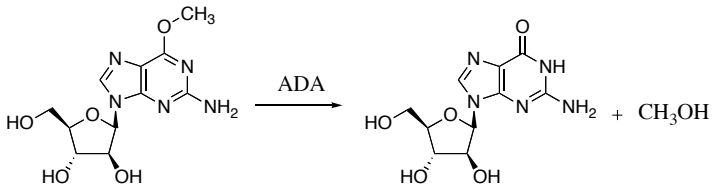
In this study alkoxy groups were introduced in the 6 position of ACV in order to investigate the effect of the nucleobase modification on the antiviral activity. Figure 4.1 depicts the structure of 6-*O*-methyl ACV (**4.1a**) and 6-*O*-ethyl ACV (**4.1b**).



4.1a R: CH_3
4.1b R: CH_2CH_3

Figure 4.1 Structure of 6-*O*-methyl ACV (4.1a) and 6-*O*-ethyl ACV (4.1b)

The 6-*O*-alkyl-2-aminopurine nucleosides have already been described in the literature as prodrugs of guanosine analogues.[1, 2] In particular, nelarabine (fig. 4.2) is the 6-methoxy prodrug of the anti-proliferative agent 9-β-D-arabinofuranosylguanine (Ara-G, fig. 4.2).[1] In 2005 nelarabine was approved for the treatment of T-cell lymphoblastic lymphoma and T-cell acute lymphoblastic leukemia. The endogenous adenosine deaminase (ADA) was identified as the enzyme responsible for the conversion of nelarabine to Ara-G *in vivo* (fig 4.2).[1]



Nelarabine **Ara-G**

Figure 4.2. Structures and metabolism of nelarabine and Ara-G

Recently, the ProTide approach has been applied to 6-*O*-methyl-2'-*C*-methyl guanosine affording the "double prodrug" INX-189 developed by the Inhibitex/BMS (fig. 4.3).[2] As already mentioned in chapter 1, INX-189 is in phase 2 clinical trials for the treatment of HCV infections.

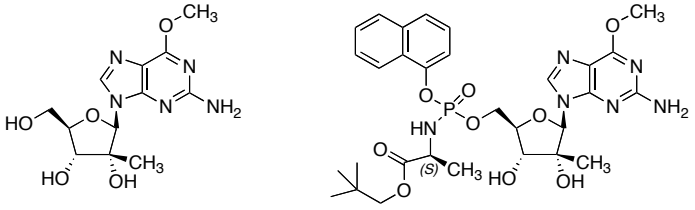


Figure 4.3. Structures of INX-189 and the parent nucleoside 6-*O*-methyl-2'-C-methyl guanosine

In vitro, the anti-HCV activity of INX-189 was 500-fold greater, compared to the parent nucleoside.[2] The improvement in antiviral activity of INX-189 has been observed also in comparison to ProTides of 2'-C-methyl guanosine, suggesting that the increase in compound lipophilicity, which is conferred by the 6-methoxy group, could account for the enhancement of cellular uptake of INX-189.[2-4] The carboxypeptidase Y study indicates that INX-189 is successfully bioactivated according to the putative mechanism of activation of ProTides.[2] It has been reported that in order to exert antiviral activity against HCV *in vitro*, INX-189 must be deaminated by most probably adenylate deaminase, which exerts the same activity as adenosine deaminase at the nucleotide level.[4] Further studies *in vitro* were reported showing that INX-189 is converted to the monophosphate form before the 6-methoxy group is removed (fig. 4.4).[5]

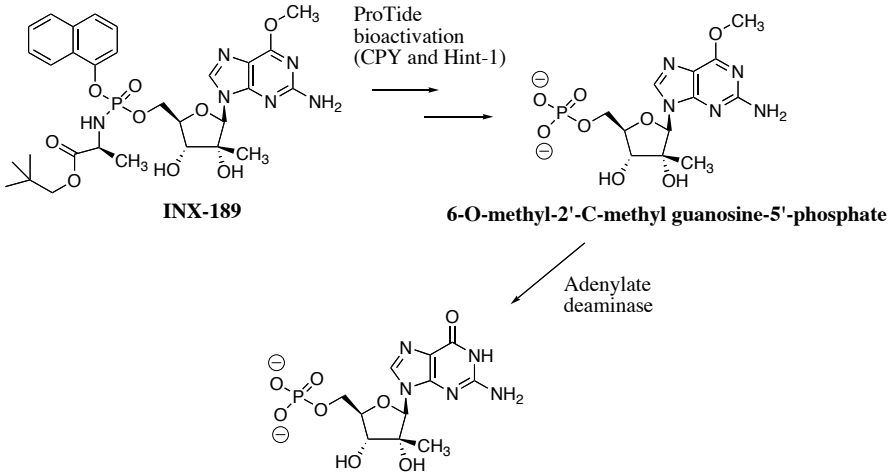


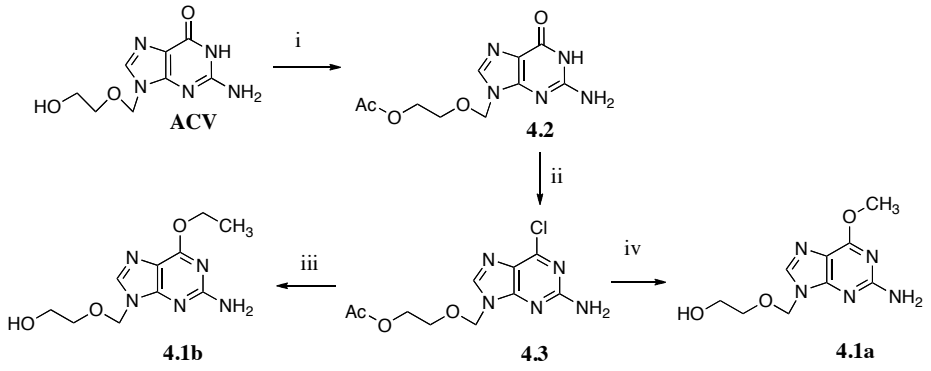
Figure 4.4. Proposed metabolic pathway of INX-189

Considering the successful application of the ProTide approach to 6-*O*-methyl-2'-C-methyl guanosine, it appeared reasonable in this study to synthesise the phosphoramidate derivatives of 6-*O*-methyl ACV and 6-*O*-ethyl ACV in order to

evaluate the possibility of improving the antiviral activity of the ACV ProTides reported in chapter 3.

4.2 Synthesis of 6-*O*-methyl ACV and 6-*O*-ethyl ACV

The synthesis of compounds **4.1a** and **4.1b** was achieved following the procedure described in scheme 4.1. As previously described in the literature for the synthesis of penciclovir, ACV was acetylated using acetic anhydride (Ac_2O) and 4-dimethylaminopyridine (DMAP) as a catalyst to afford compound **4.2**.[6] The chlorination at the 6-position was carried out using phosphorus oxychloride ($POCl_3$), benzyltriethylammonium chloride (BTEA) and *N*,*N*-dimethylaniline (DMA) to afford compound **4.3**. According to the procedure reported in the literature for the synthesis of 6-*O*-methyl-2'-C-methyl guanosine,[4] the reaction of compound **4.3** with sodium methoxide (MeONa) in anhydrous methanol (MeOH) at room temperature led to the elimination of the acetyl group and the displacement of the 6-chloro affording compound **4.1a**. Following the same strategy, the reaction of compound **4.3** with sodium ethoxide (EtONa) in anhydrous ethanol (EtOH) at 40 °C afforded compound **4.1b.**



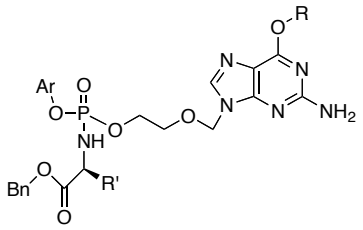
(i) Ac_2O, DMAP, DMF, 45 °C, 1.5h; (ii) $POCl_3$, BTEA, DMA, ACN, 70 °C, 1h;
(iii) MeONa, anhydrous MeOH, rt; (iv) EtONa, anhydrous EtOH, 40 ° C, 4 h

Scheme 4.1. Synthesis of 6-*O*-methyl ACV and 6-*O*-ethyl ACV

4.3 Design of 6-*O*-methyl and 6-*O*-ethyl ACV ProTides

Considering the results obtained from the evaluation of the ACV ProTides as antiviral agents, phenyl and naphthyl L-alanine benzyl ester phosphoramidate derivatives **4.4a-d** were synthesised (fig. 4.5). The L-leucine benzyl ester derivative **4.4e** was synthesised as well (fig.4.5). Considering that the analogous phosphoramidate of ACV (**3.2d)** is

effective in the release of its monophosphate form inside the cell, also in this case the hindrance of L-leucine side chain was expected to be well tolerated by enzymes involved in the bioactivation pathway of ProTides.

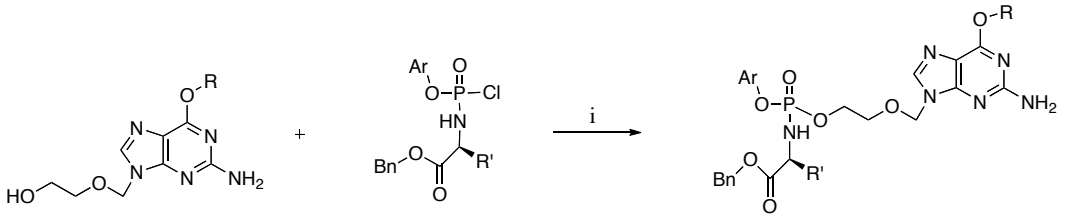


4.4a R: CH_3; **AR:** Ph; **R'**: CH_3
4.4b R: CH_3; **AR:** 1-Naph; **R'**: CH_3
4.4c R: CH_2CH_3 ; **AR:** Ph; **R'**: CH_3
4.4d R: CH_2CH_3 ; **AR:** 1-Naph; **R'**: CH_3
4.4e R: CH_3; **AR:** Ph; **R'**: $CH_2CH(CH_3)_2$

Figure 4.5 Structure of aryl phosphoramidate derivatives 4.4a-e

4.3.1 Synthesis of 6-*O*-methyl and 6-*O*-ethyl ACV ProTides

Following the procedure described in chapter 3 for the synthesis of ACV ProTides, the coupling of compounds **4.1a** and **4.1b** with the appropriate phosphochloridate **2.2** was performed in the presence of *t*BuMgCl and afforded compounds **4.4a-e** (scheme 4.2).[7] All the compounds were obtained as mixtures of two diastereomers.



4.1a R: CH_3
4.1b R: CH_2CH_3

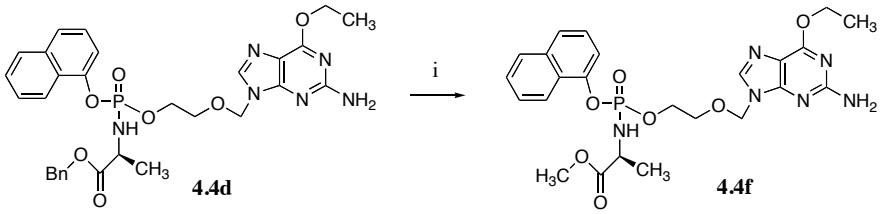
2.2a AR: Ph; **R'**: CH_3
2.2b AR: 1-Naph; **R'**: CH_3
2.2k AR: Ph; **R'**: $CH_2CH(CH_3)_2$

4.4a R: CH_3; **AR:** Ph; **R'**: CH_3
4.4b R: CH_3; **AR:** 1-Naph; **R'**: CH_3
4.4c R: CH_2CH_3 ; **AR:** Ph; **R'**: CH_3
4.4d R: CH_2CH_3 ; **AR:** 1-Naph; **R'**: CH_3
4.4e R: CH_3; **AR:** Ph; **R'**: $CH_2CH(CH_3)_2$

Reagents and conditions: (i) Anhydrous THF, *t*BuMgCl, rt, 12-18h

Scheme 4.2. Synthesis of aryl phosphoramidate derivatives 4.4a-e

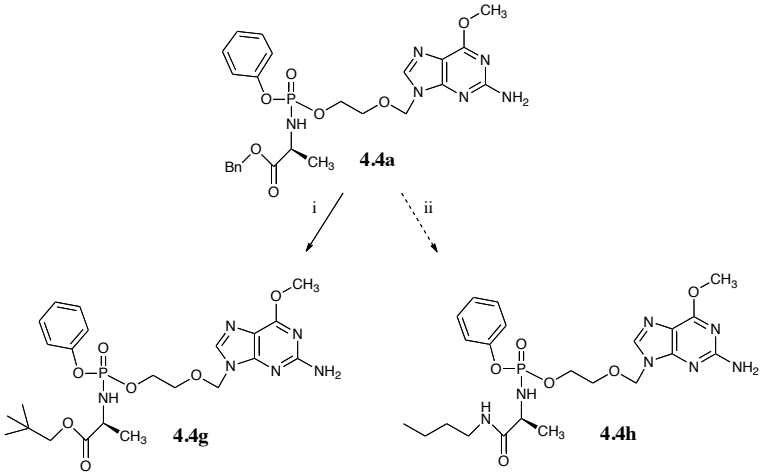
In the case of compound **4.4d**, a transesterification reaction occurred during chromatographic purification, presumably due to a residue of the unreacted *t*BuMgCl and methanol that was used as solvent during the absorption of the sample on the silica. This reaction afforded compound **4.4f** (scheme 4.3).



Reagents and conditions: (i) MeOH, *t*BuMgCl, rt

Scheme 4.3 Transesterification reaction of compound 4.4d

In order to prove that *t*BuMgCl was responsible of this novel transesterification reaction of the ProTides, neopentyl alcohol was added to a THF solution of compound **4.4a** with a slight excess of *t*BuMgCl (1.1 eq). After 2 h, compound **4.4g** was obtained in 70% yield (scheme 4.4). According to this result, the transesterifcation reaction of compound **4.4a** using *n*-butylamine was performed in the attempt to obtain compound **4.4h**, but no detectable traces of product were obtained (scheme 4.4).



Reagents and conditions: (i) neopentyl alcohol, *t*BuMgCl, rt, 2h;
(ii) *n*-butylamine, *t*BuMgCl, rt, 2h

Scheme 4.4 Transesterification reaction of compound 4.4a

In the case of the neopentyl alcohol, *t*BuMgCl ($pK_a = 45$)[8] deprotonates the alcoholic group ($pK_a = 18$)[8] affording the corresponding neopentyloxy anion that acts as a nucleophile in the replacement of the benzyl ester of compound **4.4a**. The amino group of *n*-butylamine is a weak acid ($pK_a = 40$)[8] and might not be strong enough in terms of nucleophilicity in order to displace the benzyl group of compound **4.4a**. It must be pointed out that this study has been performed at room temperature and that an increase of temperature could overcome the lack of reactivity. Also further alcohols could be pursued to evaluate the extent of this reaction.

Table 4.1 summarises the aryl phosphoramidates of 6-*O*-methyl and 6-*O*-ethyl ACV synthesised.

Table 4.1. 6-*O*-methyl and 6-*O*-ethyl ACV ProTides 4.4a-g
(O^6-R: O^6-alkyl substituent; Ar: aryl group; AA: amino acid; R'': ester group)

Cps	O^6-R	Ar	AA	R''	^{31}P NMR	Yield
4.4a	MeO	Ph	L-Ala	Bn	3.76, 3.47	78%
4.4b	MeO	1-Naph	L-Ala	Bn	4.11, 3.93	60%
4.4c	EtO	Ph	L-Ala	Bn	3.76, 3.47	53%
4.4d	EtO	1-Naph	L-Ala	Bn	4.11, 3.94	30%
4.4e	MeO	Ph	L-Leu	Bn	4.03, 3.58	40%
4.4f	EtO	1-Naph	L-Ala	Me	4.05, 4.04	16%[b]
4.4g	MeO	Ph	L-Ala	CH_2tBu	3.76, 3.47	70%[b]

[b] Transesterification product

The synthesis of the phosphoramidate derivatives **4.4a-e** was carried out without protecting the 2-amino group of the parent nucleosides, as previously described for the synthesis of ACV ProTides, due to increased solubility in THF afforded by 6-alkoxy substituents. Not needing the deprotection step after the coupling reaction greatly improved the overall yield. As already described in the synthesis of the ACV ProTides, the hindrance of the L-leucine side chain negatively affects the coupling reaction. Formation of the transesterification side-product **4.4f** accounts for the poor yield of compound **4.4d** (scheme 4.3).

4.3 Biological evaluation of 6-*O*-methyl ACV, 6-*O*-ethyl ACV and their phosphoramidate derivatives

The 6-alkoxy derivatives of ACV **4.1a**, **4.1b**, and their phosphoramidate derivatives **4.4a-g** were evaluated for their antiviral activity against HSV, VZV, HCMV, and HIV. The antiproliferative activity of these compounds on several cell cultures was also evaluated.

4.3.1 Anti-HSV activity

Table 4.2 reports the antiviral activity of **4.1a**, **4.1b** and their phosphoramidates derivatives **4.1a-g** against HSV-1, HSV-2, and thymidine kinase-deficient (TK^-) HSV-1

in human embryonic lung (HEL) cells. The data include ACV and the L-alanine benzyl ester derivatives of ACV ProTide (**3.1a** and **3.1b**)[9] as reference compounds.

Table 4.2. Anti-HSV activity of 4.1a, 4.1b and their phosphoramidate derivatives in HEL cells
(R: O^6-alkyl substituent; Ar: aryl group; AA: amino acid; R'': ester group)

					Antiviral Activity EC_{50} [a] (μM)		
Cps	**O^6-R**	**Ar**	**AA**	**R''**	**HSV-1**	**HSV-2**	**TK^- HSV-1**
4.1a	MeO	-	-	-	73	73	100
4.4a	MeO	Ph	L-Ala	Bn	0.7	0.6	2.5
4.4b	MeO	1-Naph	L-Ala	Bn	0.4	0.4	2
4.4e	MeO	Ph	L-Leu	Bn	15	6.5	65
4.4g	MeO	Ph	L-Ala	CH_2tBu	0.8	0.8	1.4
4.1b	EtO	-	-	-	>100	>100	>100
4.4c	EtO	Ph	L-Ala	Bn	0.8	0.8	2
4.4d	EtO	1-Naph	L-Ala	Bn	0.4	0.4	2
4.4f	EtO	1-Naph	L-Ala	Me	1	1	1
ACV	-	-	-	-	0.4	0.2	>250
3.1a	-	-	-	-	8 ± 5.7	4 ± 0	15 ± 7.1
3.1b	-	-	-	-	2 ± 0	1.4 ± 0.8	10 ± 2.1

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

The antiviral activity observed for **4.1a** and **4.1b** show that the introduction of the alkoxy group in the 6 position of ACV nucleobase is detrimental for the anti-HSV activity. However, all the phosphoramidate derivatives of **4.1a** and **4.1b** showed good to moderate activity against HSV- 1 and HSV-2 (EC_{50}= 0.4-15 μM). Most of them exhibited retention of this activity against TK^- HSV-1. These results indicate that the bypass of the first phosphorylation step is crucial for the anti-HSV activity of the 6-*O*-alkyl ACV ProTides. It is also suggested that into the cell compounds **4.1a** and **4.1b** are neither phosphorylated by the HSV-TK to afford the monophosphate form nor dealkylated by the cellular adenosine deaminase to afford ACV.

The L-alanine derivatives **4.4a-d** and **4.4g** exhibited retention of the anti-HSV activity in comparison to the analogous derivatives of ACV ProTides **3.1a** and **3.1b**.[9] In contrast to the results obtained in the case of the ACV Protides described in chapter 3, the replacement of L-alanine (**4.4a**) with L-Leucine (**4.4b**) in the amino acid moiety of 6-*O*-alkyl ACV ProTides led to the partial loss of activity, particularly against TK^- HSV-1

(EC_{50}= 65 μM). This result suggests that the hindrance of the L-leucine side chain may not be well tolerated by enzymes involved in the bioactivation pathway. Moreover, the methyl ester derivative **4.4f** showed good antiviral activity against HSV-1, HSV-2, TK^--HSV-1 (EC_{50}= 1 μM), while the analogous ProTide of ACV were markedly less active (EC_{50}= 10-16 μM against HSV-1 and HSV-2, EC_{50}= 79 ± 29 μM against TK^- HSV-1).[9] Finally, the concentration required to cause a microscopically visible alteration of cell morphology (MCC) was above 100 μM for all compounds synthesised.

4.3.2 Anti-VZV activity

Compounds **4.1a, 4.1b**, and their phosphoramidate derivatives **4.4a-g** were evaluated for their activity against TK-positive (TK^+) and TK-deficient (TK^-) strains of VZV in HEL cells (table 4.3). These data include ACV and the L-alanine benzyl ester derivatives of ACV ProTides (**3.1a** and **3.1b**)[10] as reference compounds.

Table 4.3. Anti-VZV activity of 4.1a, 4.1b and their phosphoramidate derivatives in HEL cells
(R: O^6-alkyl substituent; Ar: aryl group; AA: amino acid; R'': ester group)

					Antiviral Activity EC_{50} [a] (μM)			
Cps	**O^6-R**	**Ar**	**AA**	**R''**	**TK^+ VZV strains**		**TK^- VZV strains**	
					YS	**OKA**	**07-1**	**YS/R**
4.1a	MeO	-	-	-	-	>50	>50	-
4.4a	MeO	Ph	L-Ala	Bn	0.5	2.2	1.4	0.9
4.4b	MeO	1-Naph	L-Ala	Bn	-	1.3	8.9	-
4.4e	MeO	Ph	L-Leu	Bn	0.6	0.3	2.77	1.2
4.4g	MeO	Ph	L-Ala	CH_2tBu	0.1	0.2	0.2	0.3
4.1b	EtO	-	-	-	-	>50	>50	-
4.4c	EtO	Ph	L-Ala	Bn	0.3	0.4	2.6	2.2
4.4d	EtO	1-Naph	L-Ala	Bn	1.55	1.78	5.47	6.4
4.4f	EtO	1-Naph	L-Ala	Me	0.2	0.1	0.3	0.2
ACV	-	-	-	-	-	1.2	75	93
3.1a	-	Ph	L-Ala	Bn	0.7	1.0	1.8	0.59
3.1b	-	1-Naph	L-Ala	Bn	7.2	3.3	6.9	N.D

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

When compared to ACV, compounds **4.1a** and **4.1b** exhibited a complete loss of activity against VZV, as already observed in the anti-HSV assay.

The phosphoramidate derivatives **4.4a-g** were active against both TK^+ and TK^- strains of VZV in the micromolar and subicromolar range (EC_{50}= 0.1-8.9 μM). These data show that the phosphoramidate derivatives **4.4a-g** successfully bypassed the VZV-TK-mediated phosphorylation, as proved by the activity against TK^- VZV. Moreover, all these compounds exhibited retention of activity in comparison to ACV ProTides **3.1a** and **3.1b.**

No cytotoxic effects were detected by microscopic analysis of HEL cells morfology (MCC > 100 μM)

4.3.3 Anti-HCMV activity

Table 4.4 reports the anti-HCMV activity in HEL cells of **4.1a**, **4.1b** and their phosphoramidates derivatives **4.4a**, **4.4f**, **4.4g**. GCV is reported as reference compound.

Table 4.4. Anti-HCMV activity of 4.1a, 4.1b and their derivatives 4.4a, 4.4f, 4.4g in HEL cells
(R: O^6-alkyl substituent; Ar: aryl group; AA: amino acid; R'': ester group)

					Antiviral Activity EC_{50} [a] (μM)	
Cps	**O^6-R**	**Ar**	**AA**	**R''**	**AD-169 strain**	**Davis strain**
4.1a	MeO	-	-	-	-	>50
4.1b	EtO	-	-	-	-	>50
4.4a	MeO	Ph	L-Ala	Bn	17.5	11.3
4.4f	EtO	1-Naph	L-Ala	Me	4.2	2.4
4.4g	MeO	Ph	L-Ala	CH_2tBu	1.6	1.3
GCV	-	-	-	-	8.3	3.1

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

The data regarding the nucleoside analogues **4.1a** and **4.1b** confirm their inactivity.

The phosphoramidates derivatives **4.4a**, **4.4f**, and **4.4g** showed anti-HCMV activity comparable to that of GCV.
The concentration of these compounds required to cause a microscopically visible alteration of cell morphology (MCC) was above 100 μM.

4.3.4 Antiproliferative activity on HEL cell cultures of 6-*O*-methyl ACV, 6-*O*-ethyl ACV and their phosphoramidate derivatives

The cytostatic activity on HEL cell cultures of compounds **4.1a, 4.1b**, and their phosphoramidate derivatives **4.4a-g** is reported in table 4.5.

These data include ACV and the L-alanine benzyl ester derivatives of ACV ProTides (**3.1a** and **3.1b**) as reference compounds.[10]

Table 4.5. Cytostatic activity on HEL cell cultures of 4.1a, 4.1b and their phosphoramidate derivatives 4.4a-g. (R: O^6-alkyl substituent; Ar: aryl group; AA: amino acid; R'': ester group)

					Cytostatic Activity IC_{50} [b] (μM)
Cps	**O^6-R**	**Ar**	**AA**	**R''**	**HEL Cells**
4.1a	MeO	-	-	-	>100
4.4a	MeO	Ph	L-Ala	Bn	1.9
4.4b	MeO	1-Naph	L-Ala	Bn	0.7
4.4e	MeO	Ph	L-Leu	Bn	0.9
4.4g	MeO	Ph	L-Ala	CH_2tBu	1.4
4.1b	EtO	-	-	-	>100
4.4c	EtO	Ph	L-Ala	Bn	2.1
4.4d	EtO	1-Naph	L-Ala	Bn	1.3
4.4f	EtO	1-Naph	L-Ala	Me	4.2
ACV	-	-	-	-	1770
3.1a	-	Ph	L-Ala	Bn	>100
3.1b	-	1-Naph	L-Ala	Bn	20

[a] 50% inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%

Despite the fact that the concentration required to cause a microscopically visible alteration of cell morphology (MCC) was above 100 μM, the aryl phosphoramidate derivatives **4.4a-g** exhibited marked antiproliferative activity on HEL cells (IC_{50}= 0.7-4.2 μM). It must be pointed out that this effect was not detected in the antiviral assays previously described, where confluent monolayers of HEL cells, which are not proliferating, were used. Indeed, the cytostatic activity on HEL cell was measured using non-confluent and highly proliferating cell cultures. This difference is indicative of a different cellular metabolism and must be taken into account when the results of the antiviral assays are compared with the cytostatic activity obtained in this assay. In

comparison to the ACV ProTides **3.1a** and **3.1b**, an increased cellular uptake of the 6-*O*-alkyl ACV ProTides **4.4a-g** may account for the formation of ACV triphosphate at concetrations that could exert inhibitory activity also on the DNA polymerase of HEL cells. Alternatively, it might be assumed that 6-*O*-alkyl ACV triphosphate could act itself as inhibitor of cellular DNA polymerase. However, no study has been reported in the literature that could support this hypothesis.

4.3.5 Antiproliferative activity of 6-*O*-methyl ACV, 6-*O*-ethyl ACV and their phosphoramidate derivatives on murine leukemia, murine mammary carcinoma, human T-lymphocyte, and human cervix carcinoma cell cultures.

Considering the cytostatic activity exerted on HEL cells, the inhibitory effect of the phosphoramidates derivatives **4.4a-f** was evaluated on the proliferation of murine leukemia cells (L1210), murine mammary carcinoma cells (FM3A), human T-lymphocyte cells (CEM) and human cervix carcinoma cells (HeLa) (tab. 4.6). In addition the antiproliferative activity of the parent nucleosides **4.1a** and **4.1b** is reported.

Table 4.6. Antiproliferative activity of compounds 4.1a, 4.1b and their phosphoramidate derivatives
(R: O^6-alkyl substituent; Ar: aryl group; AA: amino acid; R'': ester group)

					IC_{50} [a] (μM)			
Cps	**O^6-R**	**Ar**	**AA**	**R''**	**L1210**	**FM3A**	**CEM**	**HeLa**
4.1a	Me	-	-	-	> 500	> 500	> 500	> 500
4.4a	Me	Ph	L-Ala	Bn	0.88 ± 0.41	54 ± 14	17 ± 4	31 ± 3
4.4b	Me	1-Naph	L-Ala	Bn	1.9 ± 1.1	6.2 ± 4.0	11 ± 2	N.D.
4.4e	Me	Ph	L-Leu	Bn	1.7 ± 1.0	26 ± 6	29 ± 11	16 ± 3
4.1b	Et	-	-	-	349 ± 126	> 500	> 500	> 500
4.4c	Et	Ph	L-Ala	Bn	2.8 ± 1.6	25 ± 3	31 ± 6	22 ± 1
4.4d	Et	1-Naph	L-Ala	Bn	5.6 ± 1.1	49 ± 4	16 ± 5	16 ± 1
4.4f	Et	1-Naph	L-Ala	Me	3.2 ± 0.9	172 ± 2	41 ± 9	23 ± 11

[a] 50% inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%

Consistent with the previous results, compounds **4.1a** and **4.1b** did not show inhibitory activity on cell proliferation. As expected, the phosphoramidate derivatives **4.4a-f** had antiproliferative effect. This activity was particularly marked in murine leukemia cells (L1210). The selectivity for this cell line was striking in the case of compound **4.4a**.

These results confirm the cytotoxicity of the aryl phosphoramidate derivatives **4.4a-g** and suggest it to be very cell dependent.

4.3.6 Anti-HIV activity

Table 4.6 reports the anti-HIV-1 and cytotoxic activity in MT-4 cell cultures of **4.1a**, **4.1b** and their aryl phosphoramidate derivatives **4.4a-d** and **4.4g**. These data include ACV as reference. The previously reported L-alanine benzyl ester derivatives of ACV ProTide (**3.1a** and **3.1b**)[9] are reported for comparison. The anti-HIV activity in CEM cell cultures of these compounds is currently under evaluation.

Table 4.7 Anti-HIV-1, cytostatic and cytotoxic activity in MT-4 cells of 4.1a, 4.1b and their derivatives 4.4a-d and 4.4g. (R: O^6-alkyl substituent; Ar: aryl group; AA: amino acid; R'': ester group)

					Antiviral Activity	Cytostatic/Cytotoxic Activity	
Cps	**O^6-R**	**Ar**	**AA**	**R''**	EC_{50}[a] (μM)	IC_{50}[b] (μM)	CC_{50}[c] (μM)
					HIV-1	**MT-4 cells**	
4.1a	Me	-	-	-	>150	>150	>150
4.4a	Me	Ph	L-Ala	Bn	0.4	2	20
4.4b	Me	1-Naph	L-Ala	Bn	0.6	6	>150
4.4g	Me	Ph	L-Ala	CH_2tBu	1.3	20	>150
4.1b	Et	-	-	-	>150	>150	>150
4.4c	Et	Ph	L-Ala	Bn	1.2	6.2	50
4.4d	Et	1-Naph	L-Ala	Bn	0.7	2	4
ACV	-	-	-	-	>250	>250	>250
3.1a	-	Ph	L-Ala	Bn	5.7 ± 1.6	33.8 ± 10.6	> 150
3.1b	-	1-Naph	L-Ala	Bn	0.8	N.D.[d]	> 150

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%; [b] 50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%; [c] 50% Cytotoxic concentration, or compound concentration that induces 50% cell death in the culture; [d] N.D.: not determined

As expected compounds **4.1a** and **4.1b** did not exert any inhibitory activity against HIV-1.

The phosphoramidates derivatives **4.4a-d** and **4.4g** showed antiviral activity in the micromolar and sub-micromolar range (EC_{50}= 0.4-1.3 μM). As already described in the previous paragraphs, these compounds exerted antiproliferative activity on MT-4 cells (IC_{50}= 2-20 μM) and, except **4.4b** and **4.4g**, induced 50% cell death in the culture at a concentration in the micromolar range (CC_{50}= 4-50 μM).

4.3.7 Summary of the antiviral and antiproliferative activitiy of the 6-*O*-methyl and 6-*O*-ethyl acyclovir and their phosphoramidate derivatives.

The biological results of 6-*O*-methyl ACV (**4.1a)** and 6-*O*-ethyl ACV (**4.1b)** showed a significant or complete loss of antiviral activity in comparison to ACV. Considering the biological activity exerted by the phosphoramidate derivatives **4.4a-g**, these results suggest that the parent nucleosides **4.1a** and **4.1b** were neither phosphorylated to afford the monophosphate form nor dealkylated by the cellular adenosine deaminase to afford ACV.

The antiviral activity of the phosphoramidate derivatives **4.4a-g** shows that the application of the ProTide approach to the parent nucleosides **4.1a** and **4.1b** was successful in the bypass of the HHV-TK-mediated phosphorylation and suggests that the monophosphate form was released into the cell according to the putative mechanism of ProTide bioactivation. The studies reported in the literature on the phosphoramidates of 6-*O*-methyl-2'-C-methyl guanosine suggest that ProTides of 6-*O*-alkyl ACV may release ACV monophosphate.[2,4] However, it has not been clarified yet whether the triphosphate form of 6-*O*-methyl and 6-*O*-ethyl ACV are inhibitors of DNA polymerase. An antiviral assay that combines the exposure to the phosphoramidate derivatives **4.4a-g** with deoxycoformycin-5'-phosphate, inhibitor of adenilate deaminase and N^6-methyl-AMP/dAMP aminohydrolase, may confirm whether or not the ProTides of 6-*O*-methyl and 6-*O*-ethyl ACV derivative are 'double prodrugs' of ACV triphosphate.[4, 11-13]

Unfortunately, the phosphoramidate derivatives **4.4a-g** exhibited marked cytostatic activity on HEL cell cultures and also on MT-4 cell cultures. Moreover, the antiproliferative activity of these compounds was observed also on L1210, FM3A, CEM, and HeLa cell cultures (tab 4.6). Inhibition of cellular DNA polymerase may account for this activity. No evidence has been reported in the literature that could account for the inhibitory activity of 6-*O*-alkyl ACV derivatives on cellular enzymes causing such cytotoxic effects.

4.4 Study of the adenosine deaminase activity on the 6-*O*-methyl and 6-*O*-ethyl ACV

Compounds **4.1a** and **4.1b** were incubated with adenosine deaminase (ADA) enzyme over a period of 20 h in order to prove whether the 6-alkoxy derivatives of ACV could be a prodrug of ACV triphosphate. The enzymatic reaction was monitored by recording UV spectra every 10 minutes (fig 4.6B). A UV spectrum of acyclovir was also recorded for comparison (fig 4.6A). As expected, the ADA assay performed on compound **4.1a** showed no conversion within the incubation period of 20 h. Similar results were obtained for compound **4.1b.**

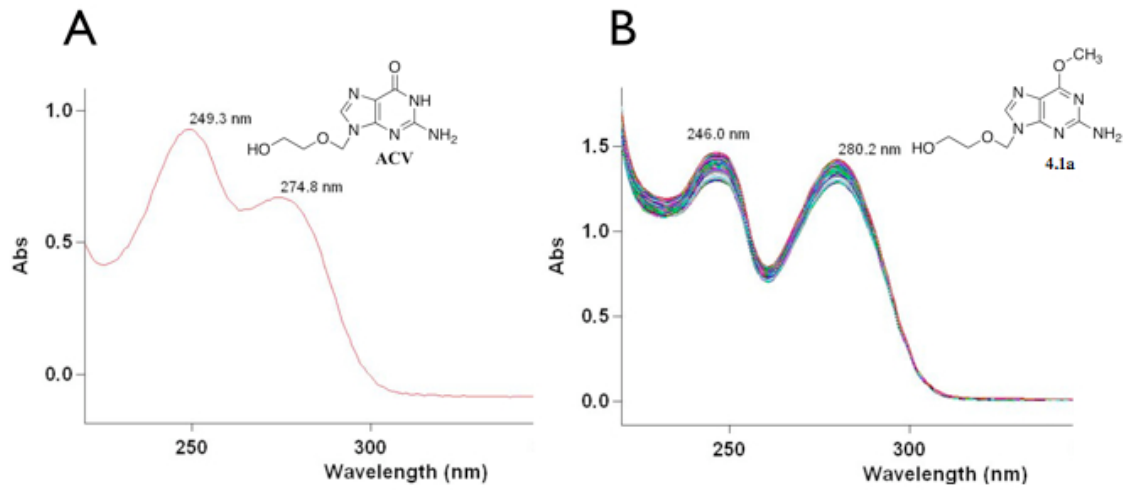


Figure 3.6. (A) UV spectrum of acyclovir; (B) Adenosine deaminase mediated deamination of compound 4.1a followed by UV spectroscopy

The lack of conversion of compounds **4.1a** and **4.1b** to ACV could explain the inactivity of these compounds.

Assuming that inside the cell the ProTides of 6-*O*-methyl and 6-*O*-ethyl ACV release acyclovir monophosphate, these results suggest that the adenylate deaminase may exhibit broader substrate specificity in comparison to ADA or that the conversion is catalysed by a different type of deaminase such as N^6-methyl-AMP/dAMP aminohydrolase that has been proved to be involved in the deamination of several 6-*O*-alkyl-2-aminopurine nucleotides.[14]

4.5 Study of the carboxyesterase activity

In order to investigate the ester cleavage of the phosphoramidate derivative **4.4a** and confirm the successful release of the monophosphate form according to the putative mechanism of activation of ProTides, an enzymatic study has been performed using CPY. The enzymatic assay was carried out following the procedure described for ACV ProTides in chapter 3.

Figure 4.7 depicts the ^{31}P NMR spectra of the enzymatic reaction over 14 h period of incubation.

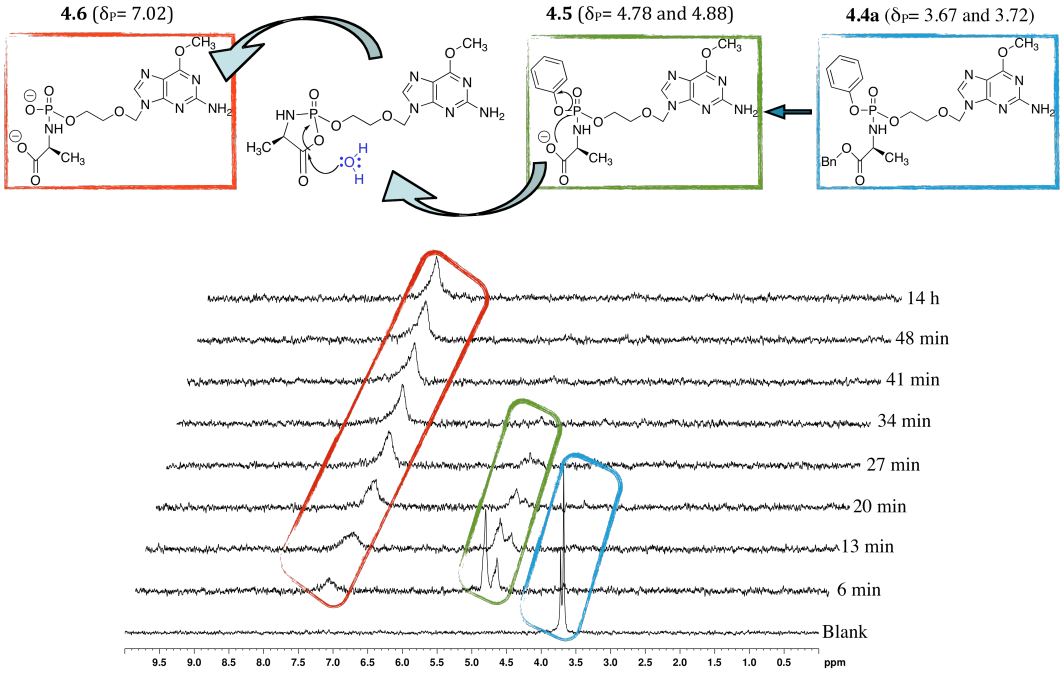


Figure 4.7. ^{31}P NMR spectra (acetone-D6, 202 MHz) at different time of the enzymatic study performed on compound 4.4a

The hydrolysis of compound **4.4a** (δ_P= 3.67, 3.72 ppm) occured in less than 6 min from the exposure to carboxypeptidase Y affording the intermediate **4.5** (δ_P= 4.78 and 4.88 ppm). After 13 min from exposure to CPY, the intermediate **4.5** was converted to the corresponding phosphoramidate monoester **4.6** (δ_P= 6.81 ppm) by 50%. The conversion was complete in less than 34 min from the exposure to the enzyme. The fast cleavage of the benzyl ester of compound **4.4a** proves that the hindrance of the 6-methoxy substituent is well tolerated by CPY, suggesting an efficient ester cleavage.

4.6 Docking study of the phosphoramidate derivatives of 6-*O*-methyl and 6-*O*-ethyl ACV within the active site of human Hint-1 enzyme

As already mentioned, a study showed that INX-189, the ProTide of 6-*O*-methyl-2'-C-methyl guanosine, is converted to the monophosphate form before that the 6-methoxy group is removed (fig. 4.4).[5]

The modeling study of compounds **4.6** and **4.7** (fig. 4.8) within the active site of human Hint-1 co-crystallised with adenosine monophosphate (AMP) was performed using docking techniques in order to investigate whether the phosphoramidate monoester of 6-*O*-methyl and 6-*O*-ethyl ACV could be successfully processed by this enzyme.

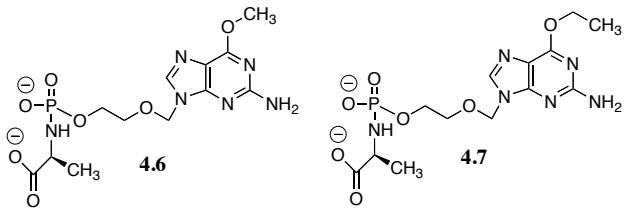


Figure 4.8. Phosphoramidate monoester derivatives of 6-*O*-methyl ACV (4.6) and 6-*O*-ethyl ACV (4.7)

The docking study of compound **4.6** (fig. 4.9) shows that it binds in the enzymatic pocket of the human Hint-1 interacting in a similar way to AMP (green).

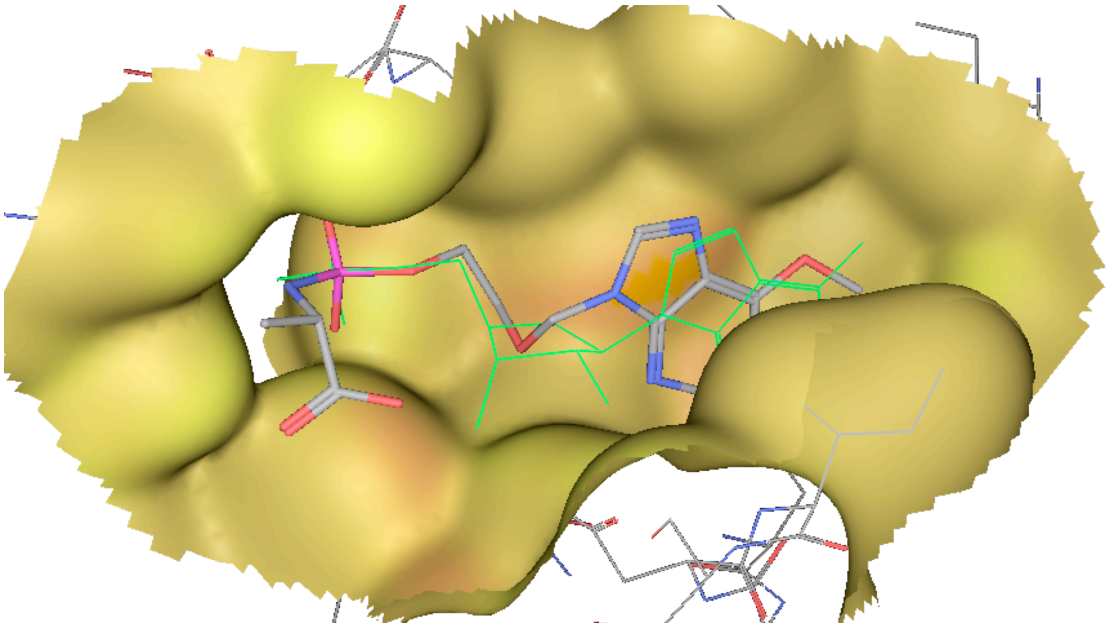


Figure 4.9. Superimposition of compound 4.6 with AMP (green) within the active site of human Hint-1 enzyme.

Figure 4.10 shows that the histidine and serine residues (His51, His112, and His114, Ser107) are in a suitable position to catalyze the cleavage of the P-N bond according to the mechanism of action of the human Hint-1 described in chapter 3.[15] This result suggests that **4.6** is successfully processed to the monophosphate in the cell.

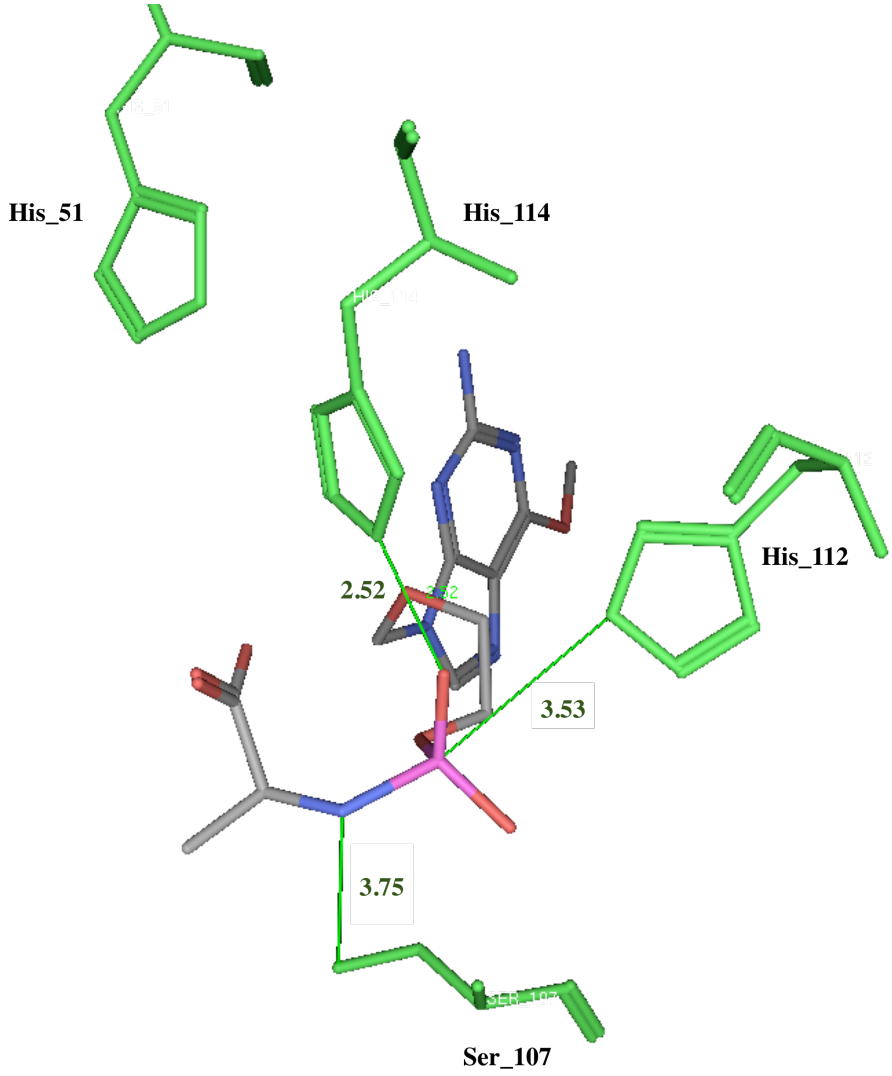


Figure 4.10. Detail of the docking of 4.6 within the active site of human Hint-1 showing the orientation and the distance in angstroms of the P-N bond (purple and blue) toward the histidine and the serine residues (His-51, His-112, His 114, and Ser_107) responsible for its cleavage (from ref 15)

Figure 4.11 shows that compound **4.7** binds in the catalytic site of the human Hint-1. The guanine base and the side chain are well positioned in the enzymatic pocket.

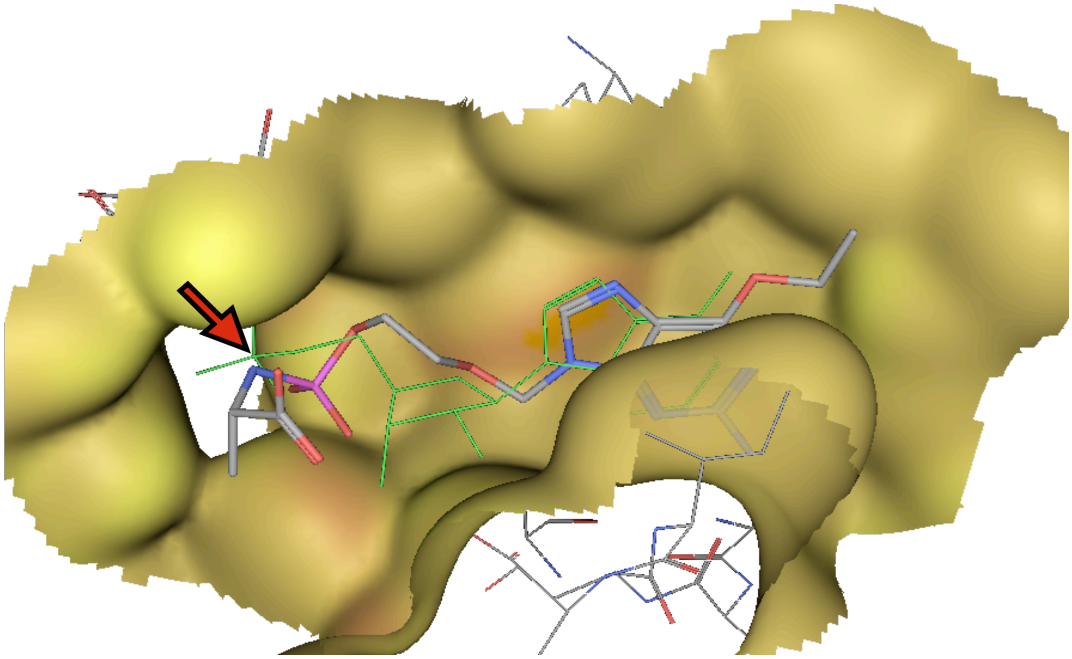


Figure 4.11. Superimposition of compound 4.7 with AMP (green) within the active site of human Hint-1 enzyme. The red arrow indicates the position of the phosphate group of AMP

Althought the phosphate moiety (purple) is slightly moved from the position adopted by the phosphate group of AMP (green), figure 4.12 shows that the histidine and serine residues (His51, His112, and His114, Ser107) are in a suitable position to catalyze the cleavage of the P-N bond.

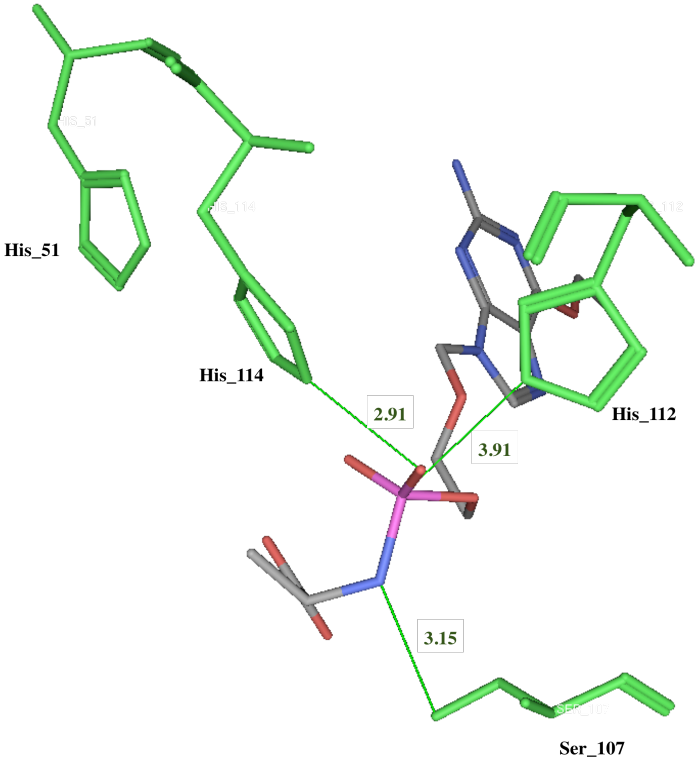


Figure 4.12. Detail of the docking of 4.7 within the active site of human Hint-1 showing the orientation and the distance in angstroms of the P-N bond (purple and blue) toward the histidine and the serine residues (His-51, His-112, His 114, and Ser_107) responsible for its cleavage

4.7 Conclusions

The application of the ProTide approach to 6-*O*-methyl and 6-*O*-ethyl ACV was successful in the intracellular release of the monophosphate form inside the cell conferring biological activity to the parent nucleosides. The results of the enzymatic and modeling studies supported the hypothesis that the aryl phosphoramidate derivatives of 6-*O*-methyl and 6-*O*-ethyl ACV were processed according to the putative mechanism of ProTide activation. However it has not been confirmed whether these compounds are dual prodrugs of ACV triphosphate.

Unfortunately, the introduction of the alkoxy group in the 6 position of acyclovir led to the rise in cytotoxicity clearly indicating the unsuitability of this strategy as a way to improve the antiviral activity of ACV Protides.

4.8 Bibliography

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Chapter 5. Aryl phosphoramidate derivatives of 8-bromo- and 8-methylacyclovir

5.1 Introduction

As already described in chapter 1, the high therapeutic index of ACV as an antiviral agent is guaranteed by its high selectivity for the herpes virus DNA polymerase and by the fact that its active form, ACV triphosphate, is generated at effective concentrations in HSV and VZV infected cells, which contain the virally encoded TK required for the first phosphorylation step.[1] The application of the ProTide approach to ACV, allowing the release of the monophosphate form directly into the cell, exposes also the uninfected cells to ACV triphosphate thus possibly decreasing its antiviral selectivity.[2] Indeed, some of the ACV ProTides described in chapter 3 had moderate cytostatic activity. Also the 6-O-alkyl ACV ProTides, described in chapter 4, were found to be cytotoxic.

Following these results, a modification of the structure of ACV was investigated in order to obtain phosphoramidate derivatives that could improve the antiviral activity of ACV without exerting cytotoxic effects. According to a previous study, the acyclic guanine nucleoside analogues such as 8-bromo-, 8-methyl-, and 8-iodoacyclovir (**5.1-5.3**, fig 5.1) display good antiviral activity against HSV with an improved therapeutic index when compared to ACV.[3]

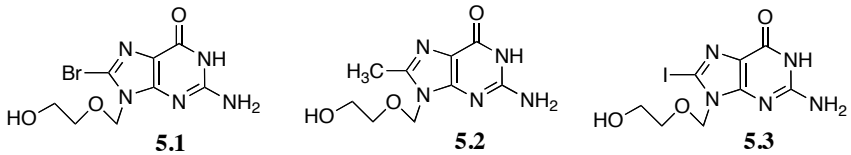


Figure 5.1. Structures of 8-bromo-, 8-methyl-, and 8-iodoacyclovir derivatives

Table 5.1 contains the antiviral and the antimetabolic activity, as well as the antiviral index, reported in the literature for ACV and compounds **5.1-5.3**.[3] The antimetabolic activity of the acyclic guanine nucleoside analogues has been evaluated by measuring the dose of compound required to inhibit the incorporation of 2'-deoxythymidine (dThd) or 2'-deoxyuridine (dUrd) into the DNA of primary rabbit kidney (PRK) cell cultures by 50% (ID_{50}).[4, 5] The antiviral index is the ratio of the ID_{50} of the host cell DNA synthesis and the ID_{50} of the anti-HSV activity. All the three derivatives **5.1-5.3** have a better antiviral index when compared to ACV, with **5.2** being the best compound.

Table 5.1 Antiviral activity, antimetabolic activity and antiviral index of 8-bromo-, 8-methyl-, and 8-iodoacyclovir. X: substituent in C-8 of ACV nucleobase

		Antiviral Activity in PRK Cell Cultures ID_{50}[a] (μg/ml)		Antimetabolic Activity in PRK Cell Cultures ID_{50}[b] (μg/ml)		
Cps	**X**	**HSV-1 (KOS)**	**HSV-2 (G)**	**dThd incorp**	**dUrd incorp**	**Antiviral Index [c]**
5.1	**Br**	**0.5**	**0.5**	**340**	**225**	**450**
5.2	**CH_3**	**0.5**	**0.5**	**>400**	**>400**	**>800**
5.3	**I**	**0.4**	**0.4**	**>350**	**>250**	**625**
ACV	**-**	**0.05**	**0.04**	**12**	**13**	**300**

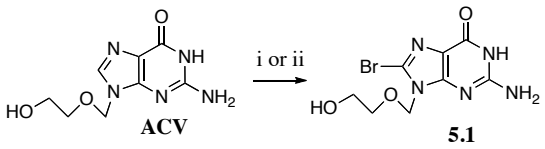
[a] 50% Inhibitory dose or concentration required to reduce cytopathogenicity by 50%; [b] 50% Inhibitory $dose_{50}$ or concentration required to reduce [^{3}H-methyl]dThd or [^{3}H-1',2']dUrd incorporation into cellular DNA by 50%; [c] Ratio of ID_{50} for dThd or dUrd incorporation (whatever was lowest) to ID_{50} for HSV-1, HSV-2 (whatever was lowest)

With the aim of identifying possible modifications of ACV that could improve the inhibitory activity and selectivity of the triphosphate form against HIV-RT, the introduction of a methyl or a halogen subsituent in the C-8 position of the guanine base of ACV was evaluated in an *in silico* study previously performed in our lab.[6] For this purpose, virtual screening was carried out by applying the docking technique to HIV-RT, adenylate kinase, guanylate kinase, and cellular DNA polymerase γ. The docking study within the active site of HIV-RT showed that substitution at the 8 position of the guanine base of the acyclic guanine nucleoside analogues with a halogen atom or a methyl group is permitted.

Considering these previous reports, it appeared reasonable to synthesise the phosphoramidate derivatives of **5.1** and **5.2**.

5.2 Synthesis of 8-bromo- and 8-methylacyclovir

According to the procedure described in the literature,[7] the bromination of ACV was achieved using *N*-bromosuccinamide (NBS) in acetic acid (CH_3CO_2H) affording compound **5.1** in 40% yield (procedure i, scheme 5.1). Alternatively, ACV was brominated on the C-8 position of the guanine base using a saturated solution of bromine in water (Br_2/H_2O). Compound **5.1** was obtained in 80% yield (procedure ii, scheme 5.1).[3]



Reagents and conditions:
(i) NBS, CH_3CO_2H, rt, 20 h (**yield:** 40%)
(ii) sature Br_2/H_2O, H_2O, 0° C, 2h (**yield:** 80%)

Scheme 5.1. Synthesis of 8-bromoacyclovir

In the H-NMR spectrum of **5.1** (fig 5.2B) the absence of the peak at 7.82 ppm corresponding to the hydrogen on the C-8 of ACV (fig 5.2A) is one of the parameters that indicates the effective substitution at this position.

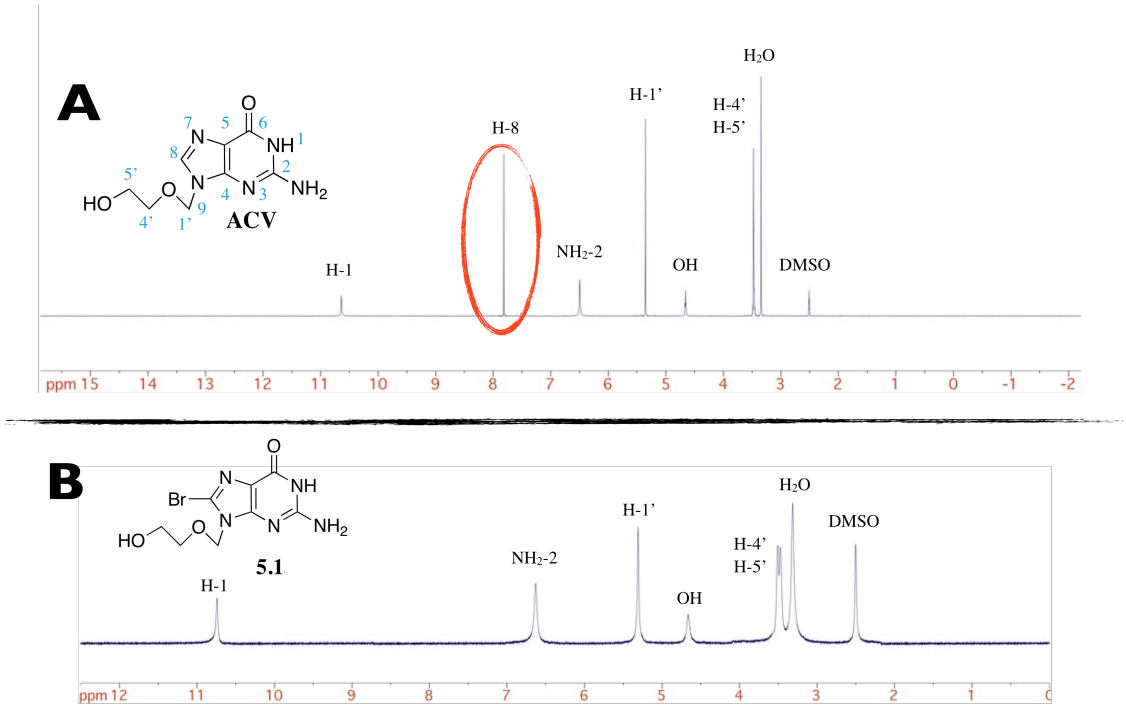


Figure 5.2. H-NMR spectra (DMSO, 500 MHz) of (A) ACV and (B) 8-bromoacyclovir

Furthermore the substitution of the hydrogen with bromine is also suggested in the ^{13}C-NMR spectrum, as the unsubstituted C-8 of ACV gives a negative signal (fig 5.3A), while its substitution with bromine results in a positive signal and in a characteristic upfield shift from 137 to 121 ppm due to the heavy halogen atom effect (fig. 5.3B).[8]

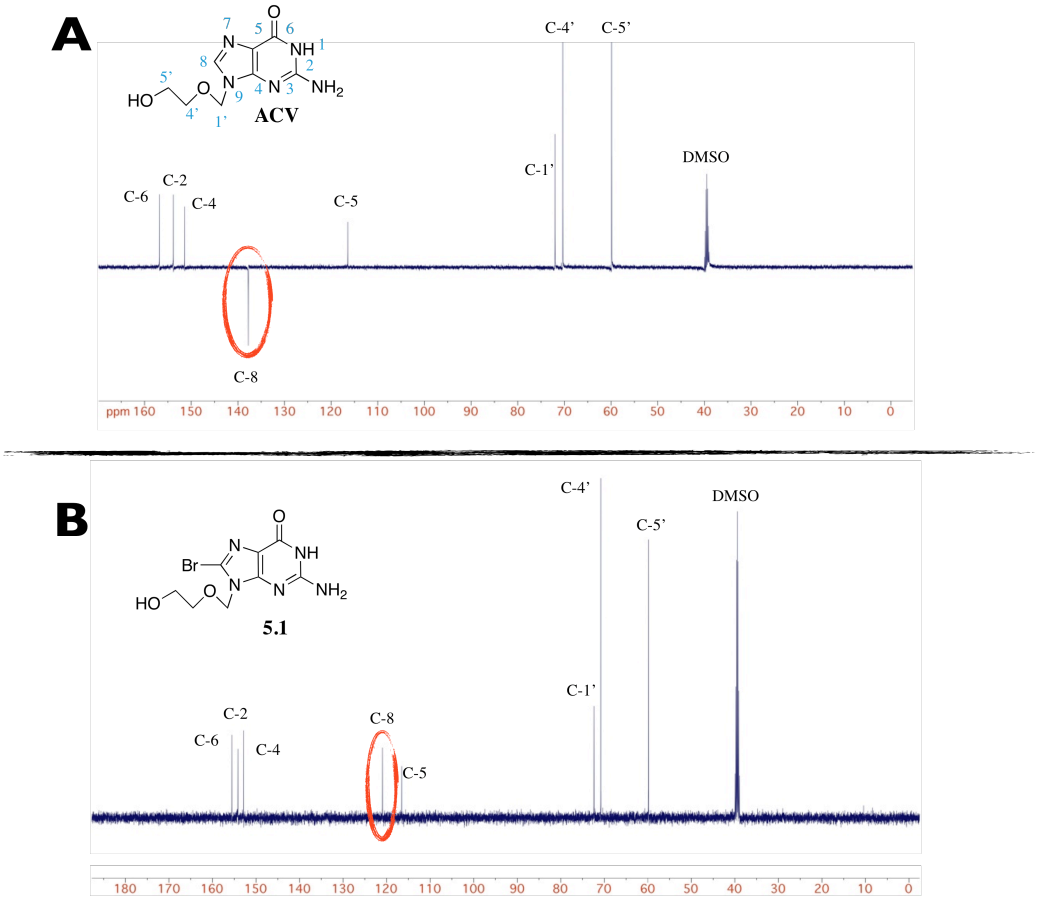
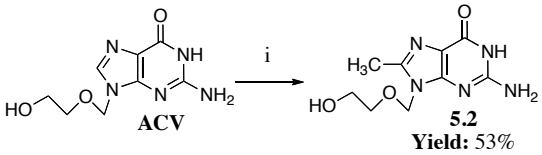


Figure 5.3. ^{13}C-NMR spectra (DMSO, 125 MHz) of (A) ACV and (B) 8-bromoacyclovir

According to the procedure described in the literature,[3, 9] the free-radical methylation of ACV with *tert*-butylhydroperoxide (*t*BuOOH) in the presence of ferrous sulfate ($FeSO_4$) and sulfuric acid (H_2SO_4) afforded compound **5.2** in 53% yield (scheme 5.2).



Reagents and conditions: (i) H_2SO_4 1M, $FeSO_4$*$7H_2O$, *t*BuOOH, 30-60 min, rt

Scheme 5.2. Free-radical methylation of ACV

The introduction of the methyl group on the C-8 of ACV is evident in the H-NMR spectrum from the presence of a singlet integrating for 3 protons at 2.36 ppm, while the peak at 7.82 ppm, corresponding to the hydrogen on the C-8 of ACV is not visible (fig. 5.4A). The presence of the methyl group on the C-8 is also recognised in the ^{13}C-NMR spectrum where a peak at 13.34 ppm is present (fig 5.4B). Moreover, the signal of the C-8 changes from negative to positive due to the effect of the substitution and a

downfield shift of this signal is seen when the ^{13}C-NMR spectra of ACV and **5.2** are compared (fig 5.4B).

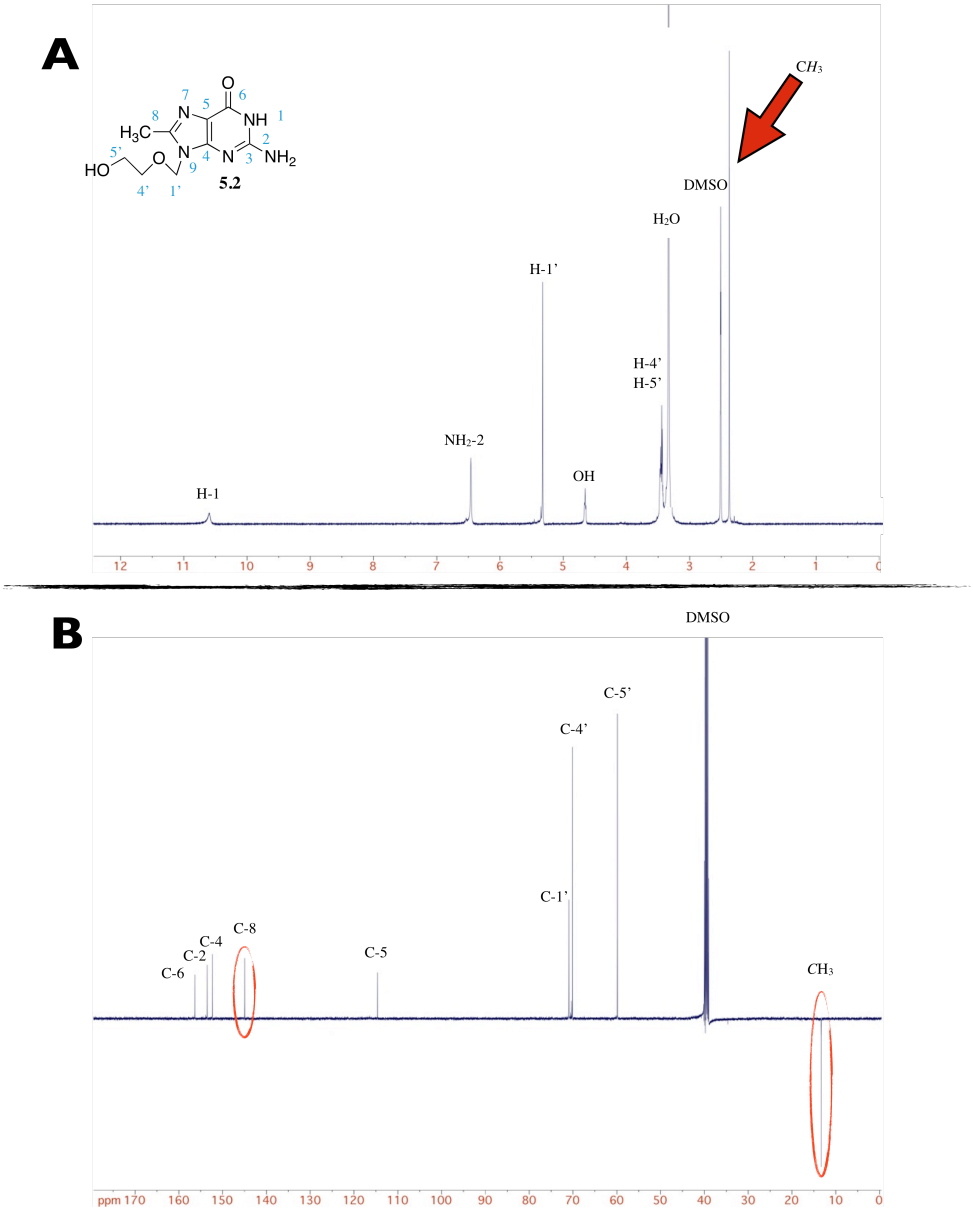
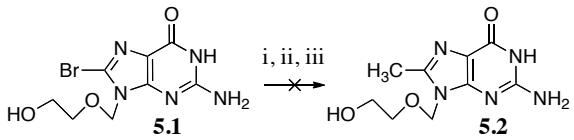


Figure 5.4. (A) H-NMR spectrum (DMSO, 500 MHz) and (B) ^{13}C-NMR spectrum (DMSO, 125 MHz) of 8-methylacyclovir

In order to improve the yield of **5.2** a different synthetic approach was followed. In the literature the cross coupling reaction of 8-bromoadenosine with trimethylaluminum ($AlMe_3$) has been reported to afford very good yield of 8-methyladenosine.[10] According to the procedure described in the literature,[10] the protection of **5.1** with the trimethylsilyl group was performed in 1,4-dioxane using hexamethyldisiloxane (HMDS) in the presence of a catalytic amount of ammonium sulphate ($(NH_4)_2SO_4$) under reflux conditions in argon atmosphere (i, scheme 5.3). The crude of the reaction, without any

purification, was redissolved in anhydorus THF and refluxed for 2 h in the presence of tetrakis ($Pd(PPh_3)_4$, 0.1 eq) and $AlMe_3$ (2 eq) (ii, scheme 5.3). No formation of the desired product of the cross coupling reaction with $AlMe_3$ was detected and the further desilylation was not carried out (iii, scheme 5.2).

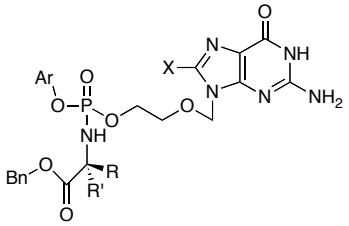


Reagents and conditions: (i) HMDS, $(NH_4)_2SO_4$, 1-4 dioxane, 110 °C, 2-3 h; (ii) $Pd(PPh_3)_4$, $AlMe_3$, anhydrous THF, 70 °C, 18 h; (iii) NH_4Cl, MeOH, 65 °C, 2 h

Scheme 5.3. Palladium-catalyzed cross coupling reaction of compound 8-bromoacyclovir with trimethylalluminium

5.3 Design of 8-bromo- and 8-methylacyclovir ProTides

In order to apply the ProTide approach to the acyclic guanosine analogues **5.1** and **5.2**, compounds **5.4a-d** and **5.5** were synthesised (fig 5.2). On the basis of the biological results of the ACV Protides described in chapter 3, the phosphoramidate derivatives **5.4a-d** and **5.5** were synthesized using L-Alanine benzyl ester as amino acid group and phenyl or naphthyl groups as aryl moiety. The phosphoramidate derivatives of ACV containing L-alanine as amino acid moiety were indeed the only ProTides active against HIV-1 and HIV-2 in CEM cell cultures with, in particular, the L-alanine benzyl ester derivatives exhibiting the best antiviral activity.[2] Compound **5.4c** was also synthesised in order to evaluate whether the 2,2-dimethylglycine benzyl ester derivative could retain the antiviral activity of the L-alanine benzyl ester phosphoramidate analogues **5.4a-b**. Finally, the L-Valine benzyl ester derivative **5.4d** was also prepared in order to evaluate whether the bulky side chain is well tolerated by the enzymes involved in the bioactivation of the ProTides.



5.4a X: Br; **Ar:** Ph; **R**: CH_3; **R'**: H
5.4b X: Br; **Ar:** 1-Naph; **R**: CH_3; **R'**: H
5.4c X: Br; **Ar:** 1-Naph; **R**: CH_3; **R'**: CH_3
5.4d X: Br; **Ar:** 1-Naph; **R**: $CH(CH_3)_2$; **R'**: H
5.5 X: CH_3; **Ar:** Ph; **R**: CH_3; **R'**: H

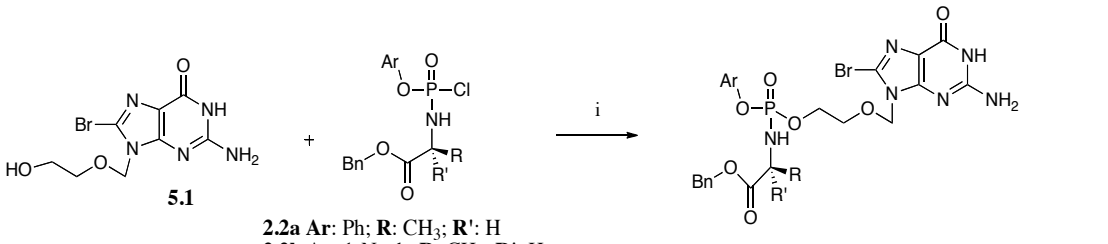
Figure 4.5. Structure of phosphoramidate derivatives 5.4a-d and 5.5

5.3.1 Synthesis of 8-bromo- and 8-methylacyclovir ProTides

The synthesis of 8-bromoacyclovir ProTides **5.4a-d** was performed following two different routes:

- coupling reaction of **5.1** with the appropriate phosphorochloridate **2.2** affording compounds **5.4a-c**;
- bromination of ACV ProTide **3.2g** affording compound **5.4d**.

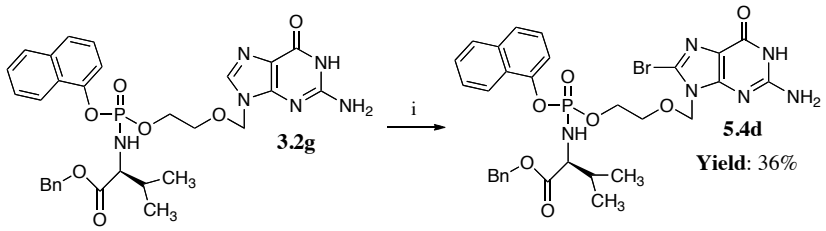
According to the procedure described in chapter 3 for the synthesis of ACV ProTides, the coupling reactions of compounds **5.1** with the freshly prepared phosphorochloridates **2.2a, 2.2b** and **2.2g** were performed in the presence of *t*BuMgCl in anhydrous THF affording compounds **5.4a-c** in 18-21% yield (scheme 5.4).



Reagents and conditions: (i) anhydrous THF, *t*BuMgCl, rt, 12-18h

Scheme 5.4. Synthesis of the phosphoramidate derivatives 5.4a-c

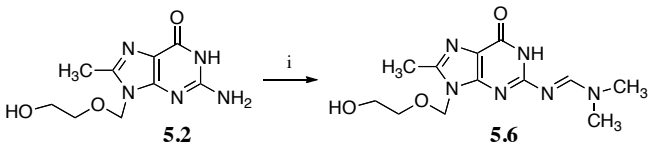
Alternatively, compound **5.4d** was synthesised by bromination of the previously reported aryl phosphoramidate derivative of ACV **3.2g** using NBS in THF (scheme 5.5).[7]



Reagents and conditions: (i) NBS, MeOH, rt. 20 h

Scheme 5.5. Synthesis of the phosphoramidate derivative 5.4d by bromination of the ACV ProTide 3.2g

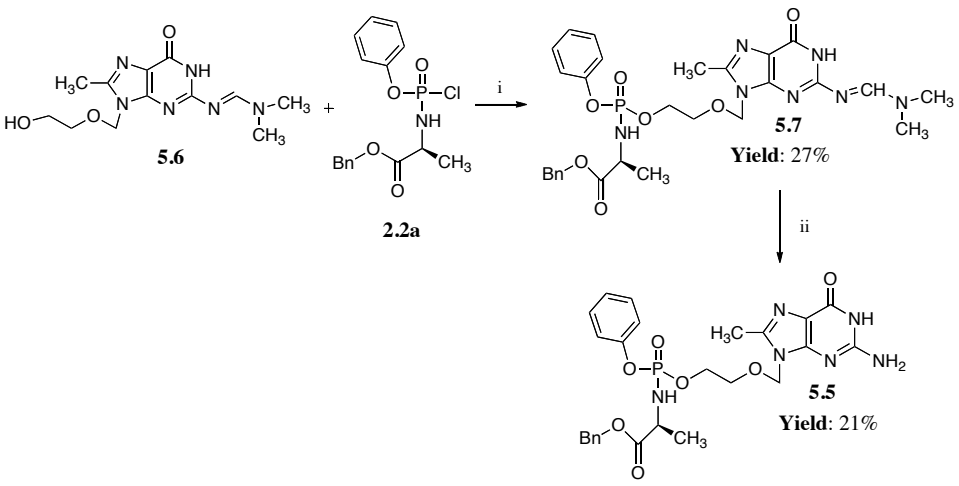
In the case of the synthesis of 8-methylacyclovir ProTide **5.5** the guanine base of **5.2** was protected using *N*,*N*-dimethyl formamide dimethyl acetal with the aim to improve the solubility in THF and affording the N^2-dimethylformamidine derivative **5.6** (scheme 5.6).[11]



Reagents and conditions: (i) *N*,*N*-dimethyl formamide dimethyl acetal , anhydrous DMF, rt, 24 h

Scheme 5.6. Synthesis of the N^2-dimethylformamidine derivative 5.6

The coupling reaction of compound **5.6** with the phosphorochloridate **2.2a** in anhydrous THF was performed in the presence of *t*BuMgCl and afforded compound **5.7** as mixture of two diastereomers in 27% yield (scheme 5.7). The dimethylformyl group was removed by refluxing **5.7** in 2-propanol (*i*PrOH) obtaining the desired compound **5.5** in 21% yield (scheme 5.7).



Reagents and conditions: (i) anhydrous THF, *t*BuMgCl, rt, 12h; (ii) *i*PrOH, reflux, 62 h.

Scheme 5.7. Synthesis of the phosphoramidate derivative 5.5

Table 4.2 summarises the ProTides of 8-bromo- and 8-methylacyclovir synthesised.

Table 4.2. ProTides of 8-bromo- and 8-methylacyclovir
(X: substituent in C-8; AA: amino acid)

Cps	X	Aryl	AA	^{31}P NMR (ppm)	Yield
5.4a	Br	Ph	L-Ala	3.72, 3.46	18%
5.4b	Br	1-Naph	L-Ala	4.01, 3.92	21%
5.4c	Br	1-Naph	DMG	2.52	20%
5.4d	Br	1-Naph	L-Val	4.82, 4.74	36%[a]
5.5	Me	Ph	L-Ala	3.77, 3.48	6%[b]

[a] direct bromination of the ACV ProTide **3.4g**; [b] overall yield of 2-steps synthesis

The low overall yield of compound **5.5** (6%) is due to the additional deprotection step of the N^2-dimethylformamidine derivative **5.7** required to obtain the final compound. In order to avoid the cleavage of the P-N bond, the removal of the N^2-dimethylformamide group was performed under mild conditions, which are responsible for the incomplete deprotection of **5.7** and thus the low yield of the reaction (21%, scheme 4.6). Interestingly, the use of NBS as source of bromine in THF proved to be a suitable approach to introduce a bromine atom in the C-8 position of the guanine base of ACV ProTides.

5.4 Biological evaluation of 8-bromoacyclovir, 8-methylacyclovir, and their phosphoramidate derivatives

The acyclic guanine nucleoside analogues **5.1**, **5.2**, and their phosphoramidate derivatives **5.4a-d** and **5.5** were evaluated for their antiviral activity against HSV, VZV, HCMV, and HIV.

5.4.1 Anti-HSV activity

Table 5.3 reports the antiviral activity of the phosphoramidate derivatives **5.4a-d** and **5.5** against HSV-1, HSV-2, and TK$^-$-HSV-1 in HEL cells. The anti-HSV activity of the parent nucleosides **5.1** and **5.2** was also evaluated and compared with the values reported in the literature (tab 5.1).[3] ACV is included as a reference compound.

Table 5.3 Anti-HSV activity of 5.1, 5.2 and their phosphoramidate derivatives in HEL cell cultures
(X: substituent in C-8; AA: amino acid)

				Antiviral Activity EC_{50}[a] (μM)		
Cps	**X**	**Aryl**	**AA**	**HSV-1**	**HSV-2**	**TK⁻ HSV-1**
5.1	Br	-	-	11	16	>100
5.4a	Br	Ph	L-Ala	79	48	>100
5.4b	Br	1-Naph	L-Ala	>100	>100	>100
5.4c	Br	Ph	DMG	60	50	>100
5.4d	Br	1-Naph	L-Val	>100	>100	>100
5.2	Me	-	-	8	4	>100
5.5	Me	Ph	L-Ala	46	42	>100
ACV	-	-	-	0.24	0.14	120

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

Compounds **5.1** and **5.2** exhibited activity against HSV-1 and HSV-2 in the micromolar range. The decrease of anti-HSV activity of compounds **5.1** and **5.2** in comparison to ACV (EC_{50}= 0.14-0.24 μM) is consistent with the data reported in the literature (tab. 4.1).[3] Moreover **5.1** and **5.2**, as well as ACV, did not retain activity against TK⁻ HSV-1, showing that the 8-substituted derivatives of ACV require phosphorylation by HSV TK.

The phosphoramidate derivatives **5.4a**, **5.4c**, and **5.5**, bearing the phenyl group as aryl moiety, (EC_{50}= 42-79 μM) showed a significant loss of activity against HSV-1 and HSV-2 in comparison to the parent nucleosides **5.1** and **5.2**. The naphthyl derivatives **5.4b** and **5.4d** were inactive against HSV-1 and HSV-2. None of the phosphoramidate derivatives **5.4a-d** and **5.5** exerted antiviral activity against TK⁻ HSV-1 below 100 μM.

The concentration required to cause a microscopically visible alteration of cell morphology (MCC) was found above 100 μM for all the compounds synthesised.

5.4.2 Anti-VZV activity

The antiviral activity of compounds **5.1, 5.2**, and their phosphoramidate derivatives **5.4a-d** and **5.5** against TK-positive (TK^+) and TK-deficient (TK^-) strains of VZV in HEL cells are reported in table 5.4. ACV is included as reference compound.

Table 5.4. Anti-VZV activity of 5.1, 5.2 and their phosphoramidate derivatives in HEL cell cultures
(X: substituent in C-8; AA: amino acid)

				Antiviral Activity EC_{50}[a] (μM)			
				TK^+ VZV strains		TK^- VZV strains	
Cps	**X**	**Aryl**	**AA**	**YS**	**OKA**	**07-1**	**YS/R**
5.1	Br	-	-	-	>50	>50	-
5.4a	Br	Ph	L-Ala	-	>50	>50	-
5.4b	Br	1-Naph	L-Ala	-	>50	>50	-
5.4c	Br	Ph	DMG	-	>50	>50	-
5.4d	Br	1-Naph	L-Val	-	>50	>50	-
5.2	Me	-	-	-	40.4	>100	-
5.5	Me	Ph	L-Ala	-	40.2	>100	-
ACV	-	-	-	-	1.16	75	93

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%;

Compound **5.1** was found inactive against TK^+ strains of VZV (EC_{50}> 50 μM), while compound **5.2** showed weak activity (EC_{50}= 40.4 μM). As expected from data reported in the literature,[12] ACV was active against TK^+ VZV in the micromolar range (EC_{50}= 1.16 μM). These results could reflect a different substrate specificity of the VZV-TK compared with the HSV-TK. Indeed, the characterisation of the crystal structure of VZV-TK has highlighted some differences in the conformation of the active site between both enzymes that suggest different substrate selectivity.[13] None of these compounds showed activity against TK^- strains of VZV.

Similarly to the parent nucleoside **5.1**, the phosphoramidate derivatives **5.4a-d** were inactive against TK^+ and TK^- VZV. The phosphoramidate derivative **5.5** retained the antiviral activity of the parent nucleoside **5.2** against TK^+ VZV (EC_{50}= 40.6 μM) and was inactive against TK^- VZV.

The concentration required to cause a microscopically visible alteration of cell morphology (MCC) was above 100 μM for all the compounds synthesised.

5.4.3 Anti-HCMV activity

Table 5.5 reports the antiviral activity of compounds **5.1**, **5.2**, and their phosphoramidate derivatives **5.4a-d** and **5.5** against HCMV in HEL cells. Ganciclovir is reported as a reference compound.

Table 5.5. Anti-HCMV activity of 5.1, 5.2, and their phosphoramidate derivatives in HEL cells
(X: substituent in C-8; AA: amino acid)

				Antiviral Activity EC_{50}[a] (μM)	
Cps	**X**	**Aryl**	**AA**	**AD-169 strain**	**Davis strain**
5.1	Br	-	-	>50	>50
5.4a	Br	Ph	L-Ala	N.D[b]	>50
5.4b	Br	1-Naph	L-Ala	N.D[b]	>50
5.4c	Br	Ph	DMG	N.D[b]	>50
5.4d	Br	1-Naph	L-Val	N.D[b]	>50
5.2	Me	-	-	>20	>100
5.5	Me	Ph	L-Ala	>100	>100
GCV	-	-	-	8.3	3.1

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%; [b] N.D.: not determined

Compounds **5.1** and **5.2** were inactive against HCMV. This result is consistent with the lack of anti-HCMV activity of ACV as it is not phoshorylated by the virally encoded protein kinase, which instead is able to phosphorylate GCV.[14, 15]

The phosphoramidate derivatives **5.4a-d** and **5.5** were inactive against HCMV.

The antiviral assay performed against HCMV has also shown that none of the compound synthesised altered cell morphology at concentrations below 100 μM.

5.4.4 Cytostatic activity on HEL cell cultures of 8-bromoacyclovir, 8-methylacyclovir and their phosphoramidate derivatives

The acyclic guanine nucleoside analogues **5.1**, **5.2**, and their phosphoramidate derivatives **5.4a-d** and **5.5** were evaluated for their antiproliferative activity on HEL cells. The concentration required to reduce cell growth by 50% was above 100 μM for all the compounds synthesised.

5.4.5 Anti-HIV activity

Compounds **5.1**, **5.2**, and the L-alanine benzyl ester phoshoramidate derivatives **5.4a-b** and **5.5** were evaluated for their activity against HIV-1 in CEM cell and MT-4 cell cultures and against HIV-2 in CEM cell cultures.

Table 5.6 reports the anti-HIV-1 activity and cytostatic activity in MT-4 cell cultures of **5.1**, **5.2**, and their phosphoramidate derivatives **5.4a-b** and **5.5**. ACV and the previously reported L-alanine benzyl ester derivatives of ACV ProTides (**3.1a** and **3.1b**)[2] are included as reference compounds.

Table 5.6. Anti-HIV-1 and cytostatic activity in MT-4 cell cultures of compounds 5.1, 5.2, and their L-alanine benzyl ester derivatives 5.4a-b and 5.5. (X: substituent in C-8; AA: amino acid)

				Antiviral Activity EC_{50}[a] (μM)	Cytostatic Activity IC_{50}[b] (μM)
Cps	**X**	**Aryl**	**AA**	**HIV-1**	**MT-4 cells**
5.1	Br	-	-	>150	>150
5.4a	Br	Ph	L-Ala	>150	>150
5.4b	Br	1-Naph	L-Ala	>150	>150
5.2	Me	-	-	>150	>150
5.5	Me	Ph	L-Ala	>150	>150
ACV	-	-	-	>250	>250
3.1a	-	Ph	L-Ala	5.7 ± 1.6	33.8 ± 10.6
3.1b	-	1-Naph	L-Ala	0.8	N.D.[c]

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%; [b] 50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%; [c] N.D.: not determined

Compounds **5.1** and **5.2** were inactive against HIV-1 in MT-4 cells. Since the *in silico* study previously performed in our lab on several derivatives of ACV as potential anti-HIV agent suggested that 8-methyl- and 8-bromoacyclovir triphosphate may be inhibitors of HIV-RT,[16] this result may indicate that the lack of anti-HIV activity of compounds **5.1** and **5.2**, similarly to ACV,[17] may be explained by the poor conversion of the 8-substituted ACV derivatives to the monophosphate form. However it must be pointed out that no *in vitro* studies have been reported in the literature regarding the inhibition of HIV-RT by 8-methylacyclovir triphosphate and 8-bromoacyclovir triphosphate.

The phosphoramidate derivatives **5.4a-b** and **5.5** were inactive against HIV-1 in MT-4 cell cultures.

None of the compound synthesised exerted antiprioliferative activity against MT-4 cell.

The results of the anti-HIV-1 and anti-HIV-2 assays performed in CEM cell culture confirm the lack of anti-HIV activity of compounds **5.1**, **5.2** and their L-alanine benzyl ester phosphoramidate derivatives **5.4a-b** and **5.5**, which exhibited antiviral activity (EC_{50}) above 250 μM.

5.4.6 Summary of the biological activity of 8-bromo-, 8-methylacyclovir and their phosphoramidate derivatives

The results of the antiviral assay performed on 8-bromo- and 8-methyacyclovir confirm the selective antiviral activity of these compounds against HSV-1 and HSV-2. The poor formation of the monophosphate derivative may account for the lack of activity against VZV, HCMV, and HIV.
The lack of antiviral activity of the phosphoramidate derivatives **5.4a-d** and **5.5** against TK^--HSV-1 strongly suggests that the monophosphate form of 8-bromo- and 8-methylacyclovir was not successfully released into the cell. Most probably, the antiviral activity displayed by the phenyl derivatives **5.4a**, **5.4c**, and **5.5** against HSV-1 and HSV-2 was due to the intracellular release of the parent nucleoside. The results of the antiviral assays performed against VZV, HCMV, and HIV confirm this hypothesis.

5.5 Enzymatic and modeling studies

Considering the putative mechanism of activation of ProTides described in chapter 1, enzymatic and molecular modelling studies were performed in order to further investigate the biological activity of the phosphoramidate derivatives **5.4a-d** and **5.5** and evaluate whether their inactivity could be attributed to poor conversion to the monophosphate form.

5.5.1 Study of the carboxyesterase activity

The enzymatic study of compounds **5.4a** and **5.5** was performed using carboxypeoptidase Y (CPY) in order to investigate the first step in the bioactivation pathway of ProTides. The enzymatic assay was carried out following the procedure described in chapter 3. ^{31}P NMR spectra were recorded at 30 minutes intervals in order to monitor the enzymatic reaction.

Figure 5.6 depicts the ^{31}P NMR spectra of the enzymatic reaction performed on compound **5.4a** over a 60 min period of incubation.

The hydrolysis of compound **5.4a** (δ_P= 3.75 and 3.91 ppm) occurred in less than 30 min after exposure to CPY, affording the intermediate **5.8** (δ_P= 4.71 and 4.86 ppm). After this period, the intermediate **5.8** was already converted to the phosphoramidate monoester **5.9** (δ_P= 7.27 ppm) by 70%. After a 60 min period of incubation, the conversion to **5.9** was complete.

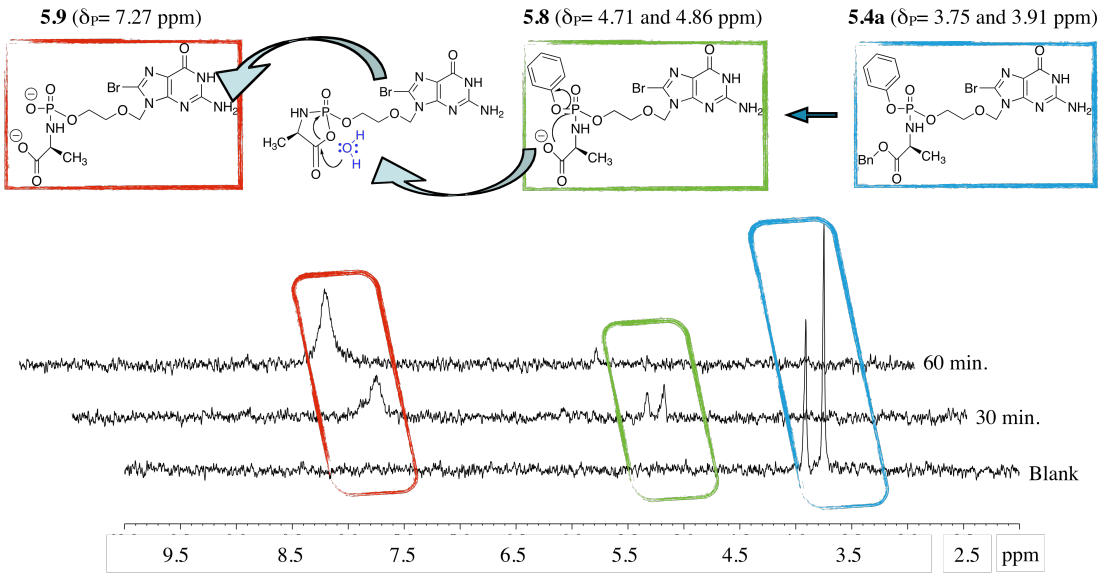


Figure 5.6 ^{31}P NMR spectra (acetone-D6, 202 MHz) of the enzymatic study performed on compound 5.4a

The ^{31}P NMR spectra of the enzymatic reaction performed on compound **5.5** over a 30 min period of incubation is depicted in figure 5.7.

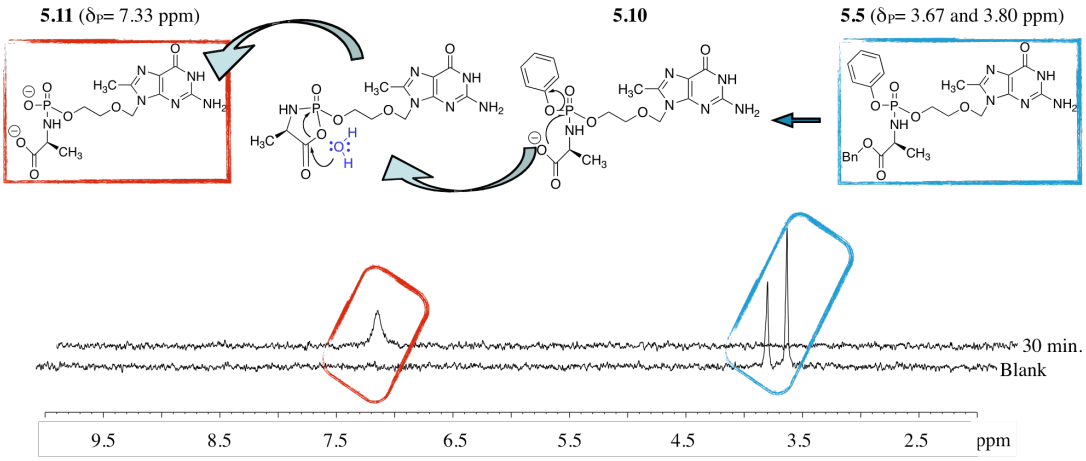


Figure 5.7 ^{31}P NMR spectra (acetone-D6, 202 MHz) of the enzymatic study performed on compound 5.5

The hydrolysis of compound **5.5** (δ_P= 3.67 and 3.80 ppm) occurred in less then 30 min from the exposure to the CPY (fig. 5.7). Due to rapid processing, the ^{31}P NMR signal

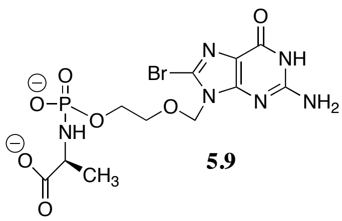
corresponding to intermediate **5.10** was not detected and only the formation of the final product **5.11** (δ_P= 7.33 ppm) was observed.

The enzymatic study performed on compounds **5.4a** and **5.5** indicates that the benzyl ester is successfully cleaved by CPY, according to the putative mechanism of activation of ProTides.[18] Thus the lack of antiviral activity probably cannot be correlated to a poor cleavage of the benzyl ester for these derivatives.

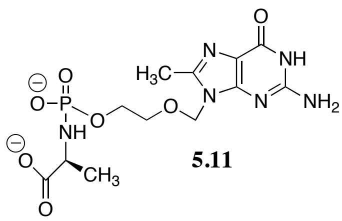
5.5.2 Docking study of the phosphoramidate derivatives of 8-bromo- and 8-methyl-acyclovir within the active site of human Hint-1 enzyme

According to the putative mechanism of activation of ProTides previously described in chapter 1, human Hint-1 is considered to be responsible for the release of the free monophosphate form of nucleoside analogues by cleaving the P-N bond of phosphoramidate derivatives.[19, 20]

In order to further investigate the bioactivation of the L-alanine benzyl ester phosphoramidate derivatives **5.4a** and **5.5**, the modeling study of compounds **5.9** and **5.11** (fig. 5.8) within the active site of the human Hint-1 was performed using docking techniques.



5.9



5.11

Figure 5.8. Structures of the L-alanine phosphoramidate monoester derivatives 5.9 and 5.11

Figure 5.9 shows the docking study of compound **5.9** within the active site of the human Hint-1 co-crystallised with AMP (shown in yellow).

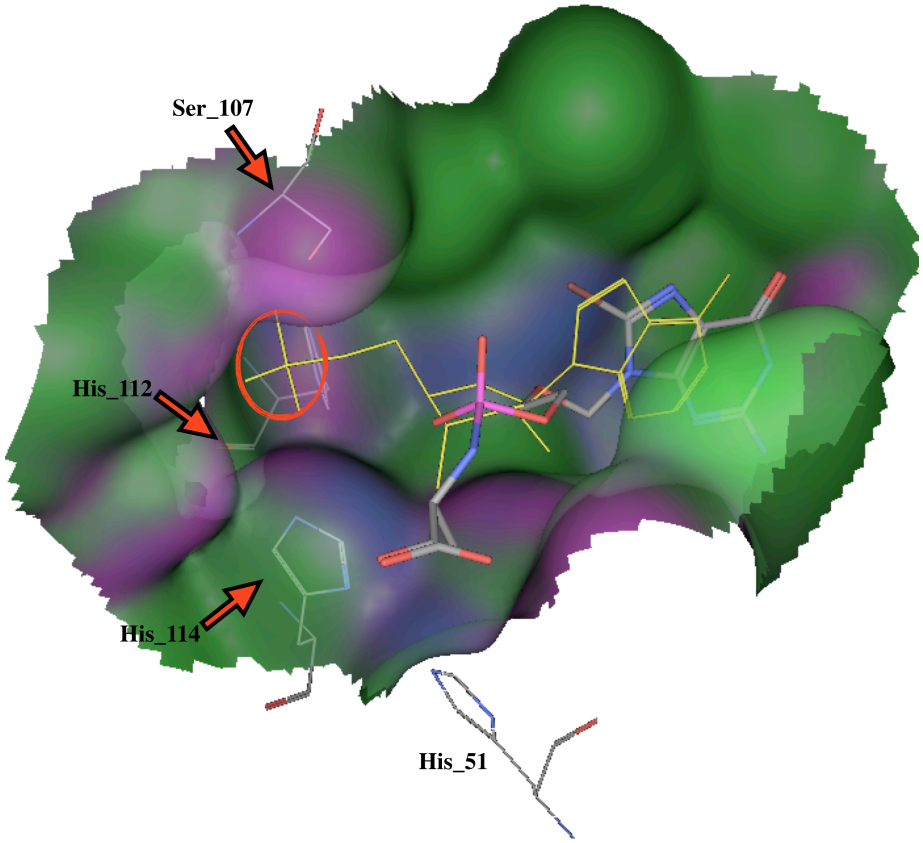


Figure 5.9. Superimposition of compound 5.9 with AMP (yellow) within the active site of human Hint-1 enzyme. The red circle indicates the phosphate moiety of AMP. The red arrows indicate the position of the residues of serine 107 (Ser_107), hisitidine 112 (His_112), and histidine 114 (His_114) involved in the cleavage of the P-N bond of compound 5.9 according to the mechanism of action of the human Hint-1 enzyme (from ref. 20)

Despite a slight difference in comparison to the position adopted by AMP, the guanine base of compound **5.9** fits well into the enzymatic pocket. Instead, the orientation of the side chain of 8-bromoacyclovir places the phosphate moiety (shown in purple) in a totally different position when compared to the phosphate group of AMP (highlighted by the red circle). As result of the placement outside the enzymatic pocket, the P-N bond is not in a suitable position to interact with serine and histidine residues (indicated by the red arrows in fig. 5.9), which are responsible for its cleavage according to the mechanism of action of human Hint-1 ezyme.[20]

The docking study of compound **5.11** (fig. 5.10) shows a similar result: the phosphate moiety (purple) is placed in a position that does not allow a proper interaction with the serine and histidine residues (red arrows in fig 4.6) involved in the cleavage of the P-N bond.

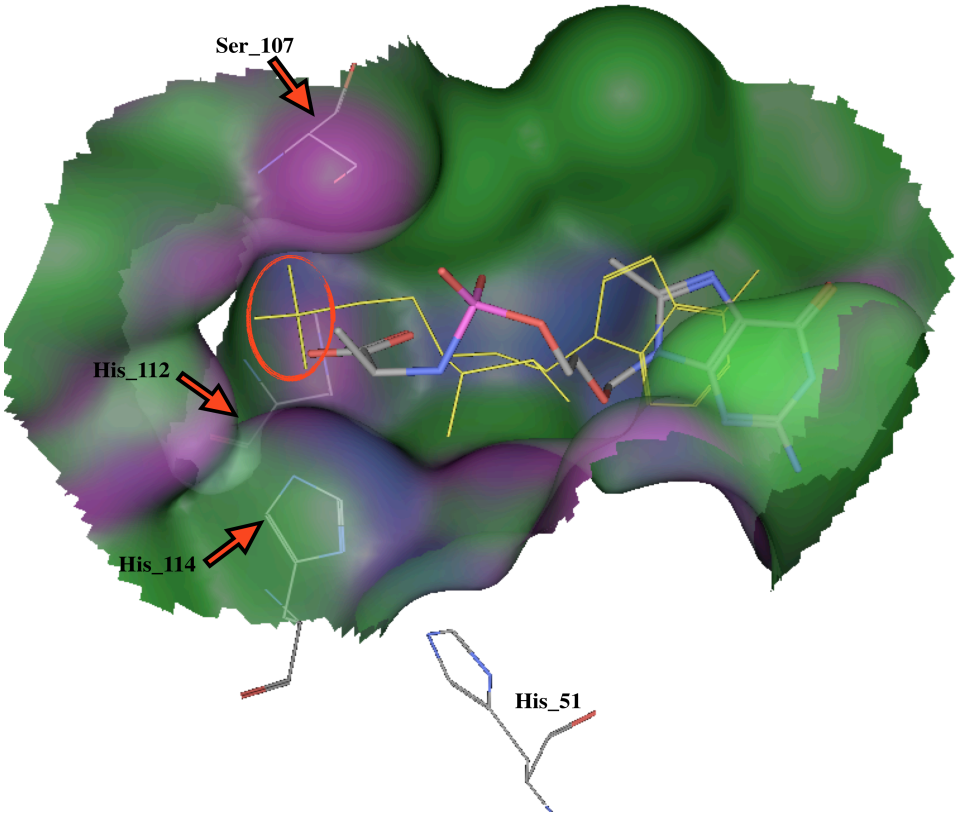


Figure 5.10. Superimposition of compound 5.11 with AMP (yellow) within the active site of human Hint-1 enzyme. The red circle highlights the phosphate moiety of AMP. The red arrows indicate the position of the residues of serine 107 (Ser_107), hisitidine 112 (His_112), and histidine 114 (His_114) involved in the cleavage of the P-N bond of compound 5.11 according to the mechanism of action of the Hint enzyme (from ref. 20)

In conclusion the modeling studies of compound **5.9** and **5.11** within the active site of the human Hint-1 enzyme strongly suggests that the inactivity of compounds **5.4a** and **5.5** may be due to the inefficacy of the phosphoramidase enzyme to cleave the P-N bond and release the monophosphate.

5.6 Conclusions

In order to overcome the decrease in selectivity induced by the application of the ProTide approach to ACV and the potential cytotoxic effect related to the inhibition of the cellular DNA replication, a series of phosphoramidate derivatives of 8-methyl- and 8-bromoacyclovir were synthesised and evaluated for their activity against HSV, VZV, HCMV, and HIV. Unfortunately, the phosphoramidate derivatives **54a-d** and **5.5** were found either poorly active or completely inactive. The enzymatic studies indicated that these compounds are successfully processed by carboxypeptidase Y. However modeling studies suggest that the metabolites of 8-methyl- and 8-bromoacyclovir ProTides may be poor substrates for human Hint-1, resulting in missed cleavage of the P-N bond.

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Chapter 6. Aryl phosphoramidate derivatives of ganciclovir and penciclovir

6.1 Introduction to the application of the ProTide approach to ganciclovir and penciclovir

As described in chapter 1, ganciclovir (GCV) and penciclovir (PCV) are analogues of acyclovir (ACV), from which they differ for the presence of an additional hydroxymethylene group on the acyclic side chain that mimics the 3' hydroxyl group of natural nucleosides (fig. 6.1).[1] Moreover, PCV differs from GCV and ACV for the replacement of the oxygen on the side chain with a methylene group. These compounds are anti-herpes virus agents and share a similar mechanism of activation with ACV, leading to the formation of the triphosphate form that inhibits the viral DNA polymerase.[1]

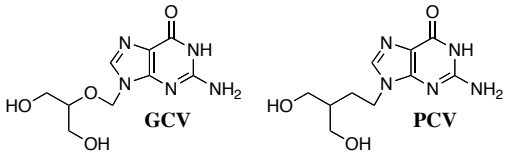
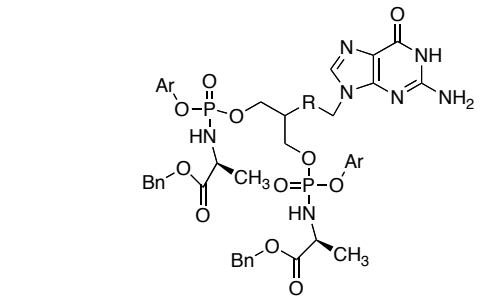
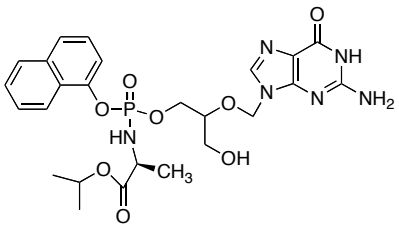


Figure 6.1. Structures of GCV and PCV

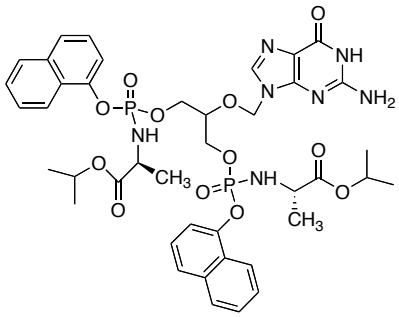
The application of the ProTide approach to GCV and PCV has been previously investigated in our group on the basis of the virtual screening carried out on several derivatives of ACV triphosphate as potential inhibitors of HIV-RT.[2] In particular, the *in silico* study pointed out that GCV and PCV triphosphate could be good candidates as inhibitors of this enzyme. Following the procedure already described for the synthesis of ACV ProTides,[3] several aryl phosphoramidate derivatives of GCV and PCV were previously prepared in our lab.[4] When phenyl and naphthyl L-alanine benzyl ester phosphorochloridates were used, the bisphosphate prodrugs (bis-ProTide) **6.1a-d** were exclusively obtained (fig. 6.2).[4] Only in the case of the L-alanine isopropyl ester derivative of GCV it was possible to isolate the mono-ProTide **6.2**, together with the bis-ProTide **6.3** (fig. 6.2).[4]



6.1a R: CH_2 **Ar**: Ph
6.1b R: CH_2 **Ar**: 1-NaPh
6.1c R: O **Ar**: 1-Naph
6.1d R: O **Ar**: 1-Naph



6.2



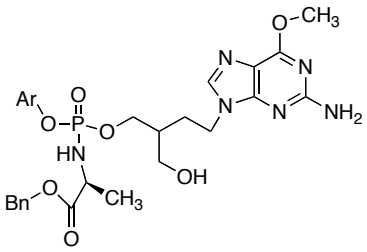
6.3

Figure 6.2. Structures of GCV and PCV ProTides 6.1a-d, 6.2, and 6.3 previously prepared in our lab (from ref. 4)

The bis-ProTides derivatives of PCV **6.1a-b** exhibited a significant loss of activity against TK-positive strains of VZV (EC_{50}= 6.9-32.2 μM) in comparison to the parent nucleoside (EC_{50}= 0.9-2.2 μM) and they were inactive against TK-deficient strains of VZV.[5] This result may suggest that either the bisphosphate prodrugs are not successfully bioactivated or they release the parent nucleoside.

The ProTides **6.1c-d**, **6.2**, and **6.3** were evaluated for their activity against HIV but found inactive.[4] The inactivity of the mono-ProTide **6.2** may be due to the poor cleavage of the ester moiety in the bioactivation pathway, as suggested by the enzymatic study performed on this compound using carboxypeptidase Y.[4]

In the same study also the application of the ProTide approach to the 6–methoxy derivative of PCV was described and the synthesis of ProTides **6.4a** and **6.4b** was reported (fig 6.3).[4]



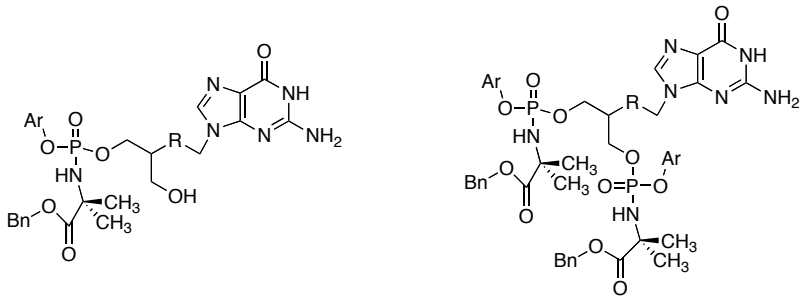
6.4a Ar: Ph
6.4b Ar: 1-NaPh

Figure 6.3. Structure of the 6-methoxy-PCV ProTides 6.4a and 6.4b previously prepared in our lab (from ref. 4)

These compounds exhibited poor activity against TK-positive strains of HSV (EC_{50}= 38-58 μM against HSV-1 and 20-45 μM against HSV-2) and retention of this activity against TK-deficient strains of HSV-1 (EC_{50}= 20-58 μM), while the parent nucleoside was completely inactive against both strains of HSV.

6.2 Design of novel aryl phosphoramidate derivatives of GCV and PCV

Considering the results obtained in the evaluation of ACV ProTides (chapter 3), compounds **6.5a-d** (fig 6.4) were synthesised in order to investigate the antiviral activity of the phenyl and naphthyl 2,2-dimethylglycine benzyl ester phosphoramidate derivatives of GCV and PCV. The bisphosphate derivatives **6.6a-d** were also obtained (fig 6.4).

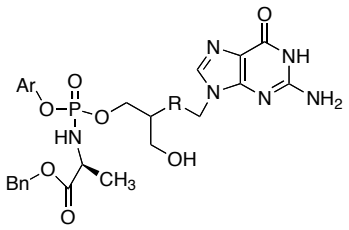


6.5a R: CH_2 **Ar**: Ph
6.5b R: CH_2 **Ar**: 1-NaPh
6.5c R: O **Ar**: Ph
6.5d R: O **Ar**: 1-Naph

6.6a R: CH_2 **Ar**: Ph
6.6b R: CH_2 **Ar**: 1-NaPh
6.6c R: O **Ar**: Ph
6.6d R: O **Ar**: 1-Naph

Figure 6.4. Structure of phenyl and naphthyl 2,2-dimethylglycine benzyl ester phosphoramidate derivatives 6.5a-d (mono-ProTides) and 6.6a-d (bis-ProTides)

Phenyl and naphthyl L-alanine benzyl ester phosphoramidate derivatives **6.7a-d** (fig 6.5) were synthesised in order to evaluate the antiviral activity of the mono-ProTides of GCV and PCV and compare their activity with that of the analogous bis-ProTides **6.1a-d** previously prepared in our lab (fig. 6.2).

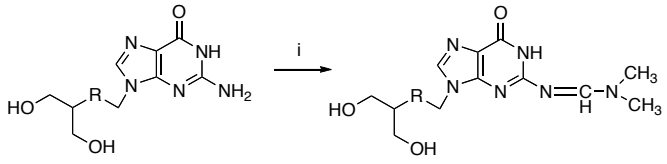


6.7a R: CH_2 **Ar**: Ph
6.7b R: CH_2 **Ar**: 1-NaPh
6.7c R: O **Ar**: Ph
6.7d R: O **Ar**: 1-Naph

Figure 6.5. Structure of phenyl and naphthyl L-alanine benzyl ester phosphoramidate derivatives 6.7a-d

6.2.1 Synthesis of the 2,2-dimethylglycine benzyl ester derivatives of GCV and PCV ProTides

The synthesis of the phenyl and naphthyl 2,2-dimethylglycine benzyl ester phosphoramidate derivatives of GCV and PCV was carried out following the same procedure described in chapter 3 for the synthesis of ACV ProTides. The protection of the guanine base with *N*,*N*-dimethyl formamide dimethyl acetal afforded the N^2-dimethylformamidine (N^2-DMF) derivatives **6.8a** and **6.8b** (scheme 6.1).[6]



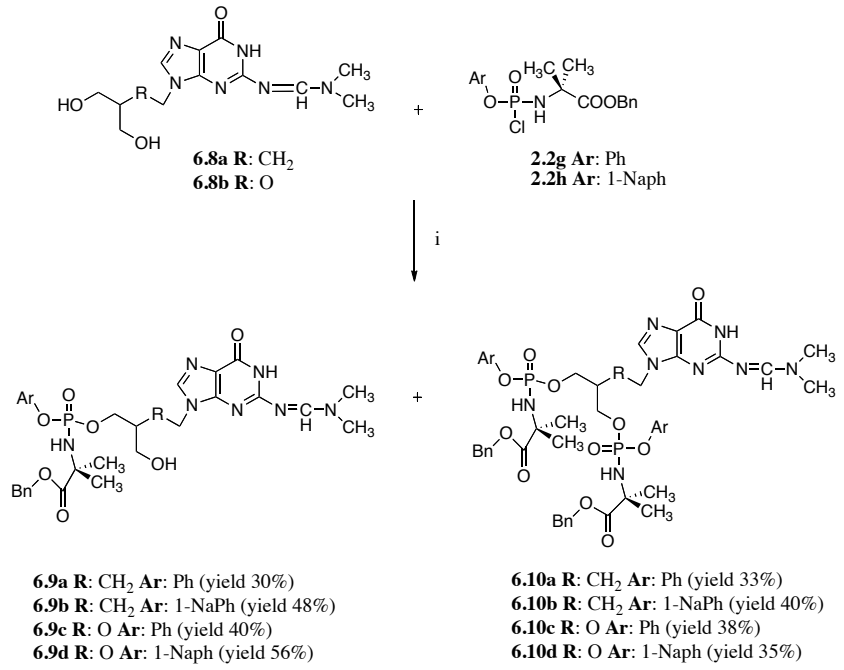
PCV R: CH_2
GCV R: O

6.8a R: CH_2
6.8b R: O

Reagents and conditions: (i) *N*,*N*-dimethyl formamide dimethyl acetal , anhydrous DMF, rt, 24 h

Scheme 6.1 N^2-dimethylformamidine derivatives of GCV and PCV

The coupling reaction of compounds **6.8a** and **6.8b** with an excess of the appropriate phosphorochloridate **2.2** in THF was performed in the presence of *t*BuMgCl and afforded the monophosphate derivatives **6.9a-d** in 30-56% yield along with the bisphosphate derivatives **6.10a-d** in 33-40% yield (scheme 6.2).[7] All these products were obtained as mixtures of diastereomers.

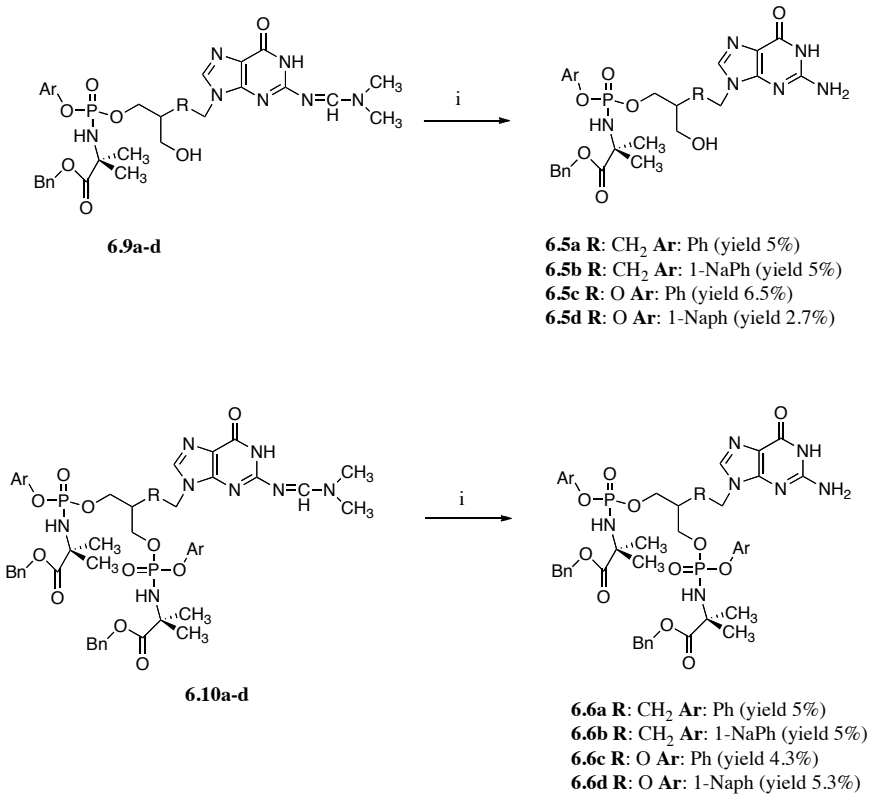


Reagents and conditions: (i) *t*BuMgCl, anhydrous THF, rt, on

Scheme 6.2. Synthesis of the phenyl and naphthyl 2,2-dimethylglycine benzyl ester phosphoramidate derivatives 6.9a-d (mono-ProTides) and 6.10a-d (bis-ProTides)

The yields of the coupling reactions reported in scheme 6.2 show that the monoProTides **6.9a-d** and the bis-ProTides **6.10a-d** are obtained in comparable amounts. These results are in contrast with the findings previously reported in our group showing that under the same conditions the synthesis of the L-alanine benzyl ester derivatives of GCV and PCV ProTides afforded only the bis-ProTide derivatives **6.1a-d** (fig. 6.2).[4]

The N^2-DMF group was removed in 2-propanol (*i*PrOH) under reflux conditions obtaining the desired compounds **6.5a-d** and **6.6-d** in 2.7-6.5% yield (scheme 6.3).



6.9a-d

6.5a R: CH_2 **Ar**: Ph (yield 5%)
6.5b R: CH_2 **Ar**: 1-NaPh (yield 5%)
6.5c R: O **Ar**: Ph (yield 6.5%)
6.5d R: O **Ar**: 1-Naph (yield 2.7%)

6.10a-d

6.6a R: CH_2 **Ar**: Ph (yield 5%)
6.6b R: CH_2 **Ar**: 1-NaPh (yield 5%)
6.6c R: O **Ar**: Ph (yield 4.3%)
6.6d R: O **Ar**: 1-Naph (yield 5.3%)

Reagents and conditions: (i) *i*PrOH, reflux, 24-72 h.

Scheme 6.3. Deprotection of N_2-DMF derivatives 6.9a-d and 6.10a-d

6.2.2 Synthesis of the L-alanine benzyl ester derivatives of GCV and PCV mono-ProTides

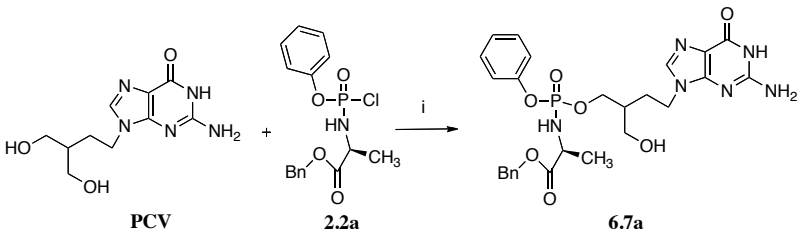
In this work, different synthetic approaches were evaluated in order to obtain the L-alanine benzyl ester derivatives of GCV and PCV mono-ProTides (**6.7a-d**, fig. 6.5).

As described in the previous paragraph, the use of an excess of aryl amino acid ester phosphorochloridate (2-4 eq) and *t*BuMgCl (2 eq) can be the reason why both hydroxyl groups of GCV and PCV can be phosphorylated in the coupling reaction.

In preliminary studies, the use of a lower amount (1.1 eq) of the freshly prepared phenyl and naphthyl L-alanine benzyl ester phosphorochloridates (**2.2a** and **2.2b**) in the coupling reaction with the N^2-DMF derivatives of GCV (**6.8a)** and PCV **(6.8b)** was evaluated. Unfortunately, the reaction did not afford any product indicating that the excess of the phosphorochloridate is required to carry out the reaction in these conditions. The same reaction was repeated using 2 eq of phosphorochloridate and decreasing the amount of *t*BuMgCl from 2 to 1.1 eq in order to achieve the deprotonation of only one of the two hydroxyl groups of GCV and PCV and drive the

reaction toward the formation of the mono-ProTide. Also in this case no product was obtained.

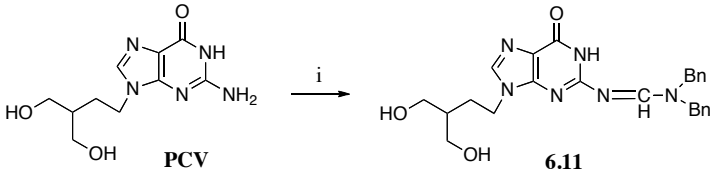
Following this unsuccessful result, pyridine was used as solvent to improve the solubility of the parent nucleoside. The phosphorylation of the N^2-DMF derivatives **6.8a** and **6.8b** was performed in pyridine using 2-4 eq of the appropriate phosphorochloridate (**2.2a** and **2.2b**) and in the presence of *t*BuMgCl (2 eq). However, this approach was not successful. The same reaction was performed using unprotected PCV affording compound **6.7a** in 5% yield as a mixture of diastereomers (scheme 6.4).



Reagents and conditions: (i) *t*BuMgCl, anhydrous Pyr, rt, 12-14 h

Scheme 6.4. Coupling reaction of PCV in pyridine

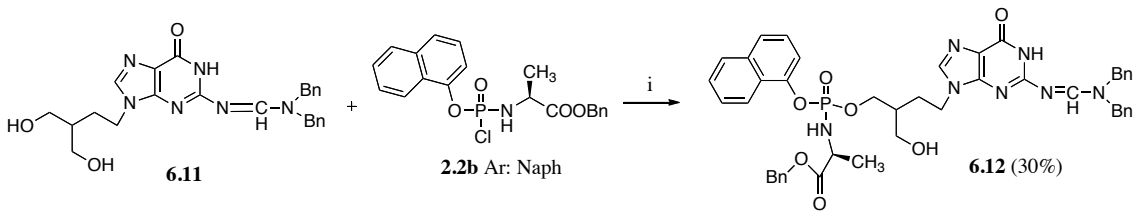
As alternative approach, the solubility of the parent nucleoside in THF was improved using a different protective group. For this purpose, the 2-amino group of PCV was protected using *N,N*-dibenzylformamide dimethylacetal affording compound **6.11** (scheme 6.5), which showed higher solubility in THF in comparison to compound **6.8a**.[8]



Reagents and conditions: (i) freshly prepared *N,N*-dibenzylformamide dimethyl acetal, anhydrous ACN, 45 ºC, 24 h

Scheme 6.5. Protection of PCV with the *N,N*-dibenzylformamide group

The coupling reaction of **6.11** with the phosphorochloridate **2.2b** (2 eq) was performed in THF and in the presence of *t*BuMgCl (2 eq) affording compound **6.12** in 30% yield as a mixture of diastereomers (scheme 6.6). No traces of the bis-ProTide analogue were detected. This result indicates that the solubility of the parent nucleoside is a crucial factor for the synthesis of the mono-ProTide derivative as the main product of the coupling reaction with aryl amino acid ester phosphorochloridates.

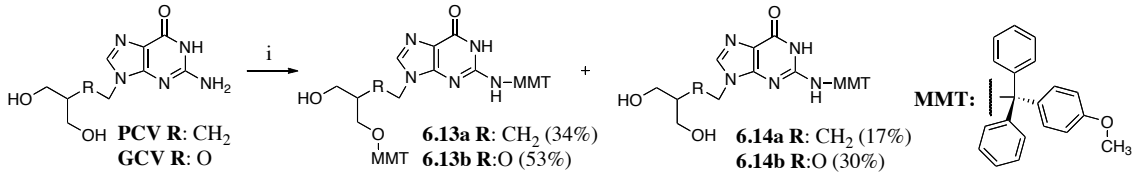


Reagents and conditions: (i) *t*BuMgCl, anhydrous THF, rt, 12-14 h

Scheme 6.6. Coupling reaction of *N,N*-dibenzyl-formamide-protected PCV with the phosphorochloridate 2.2b.

According to the procedure described in the literature, trifluoroacetic acid aqueous (50% V/V) was used to remove the *N,N*-dibenzylformamide group from compound **6.12**.[9] However, degradation of the starting material was observed indicating that under these conditions this approach is not suitable for the synthesis of ProTides. Milder conditions were applied to the elimination of the protective group. The reflux reaction in 2-propanol did not afford any product after 72 h.

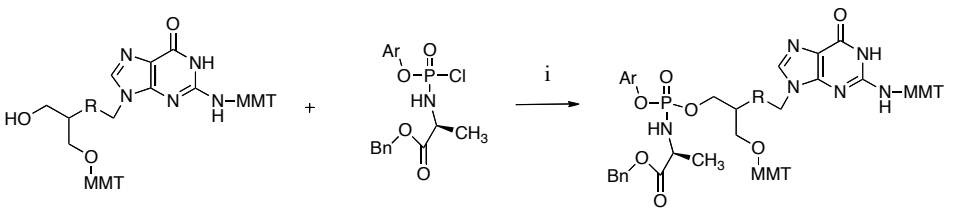
In order to improve the solubility in THF and make only one of the two hydroxyl groups on the side chain available for phosphorylation, the protection of GCV and PCV with the mono-methoxytrityl group (MMT) was evaluated. Following the procedure described in the literature, GCV and PCV were dissolved in DMF and reacted with mono-methoxytrityl chloride (MMT-Cl) in the presence of TEA and a catalytic amount of DMAP (scheme 6.7).[10] The reaction yielded two different products for each parent compound: the more lipophilic (**6.13a** and **6.13b**) bearing two protecting groups at the 2-amino position and at one of the two hydroxyl group on the side chain; and the less lipophilic (**6.14a** and **6.14b**) bearing the MTT group only at the 2-amino position.



Reagents and conditions: (i) MTT-Cl, TEA, $DMAP_{(cat)}$, DMF, rt, 2h

Scheme 6.7. Protection of GCV and PCV with MMT-Cl

The coupling reaction of compound **6.13a** and **6.13b** with the appropriate phosphorochloridate (**2.2a** and **2.2b**) in THF was performed in the presence of *t*BuMgCl and afforded compounds **6.15a** and **6.15b** as mixtures of diastereomers in 40% and 38% yield, respectively (scheme 6.8).



6.13a R: CH_2
6.13b R: O

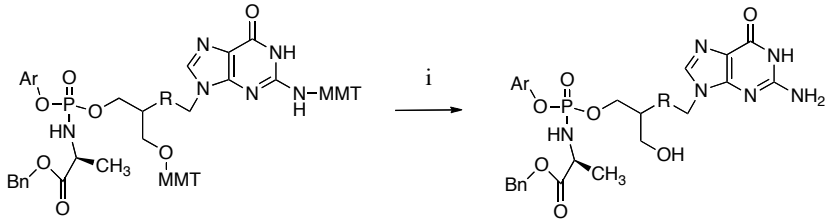
2.2a Ar: Ph
2.2b Ar: 1-Naph

6.15a R: CH_2 **Ar**: 1-Naph (40%)
6.15b R:O **Ar**: Ph (38%)

Reagents and conditions: (i) *t*BuMgCl, THF, rt, 12-14 h

Scheme 6.8. Coupling reaction of 6.13a and 6.13b with the phosphorochloridates 2.2a and 2.2b

The removal of the MTT group was performed using *para*-toluensulfonic acid (TsOH) at room temperature and afforded the desired compounds **6.7b** and **6.7c** in 30% and 28% yield, respectively (scheme 6.9).



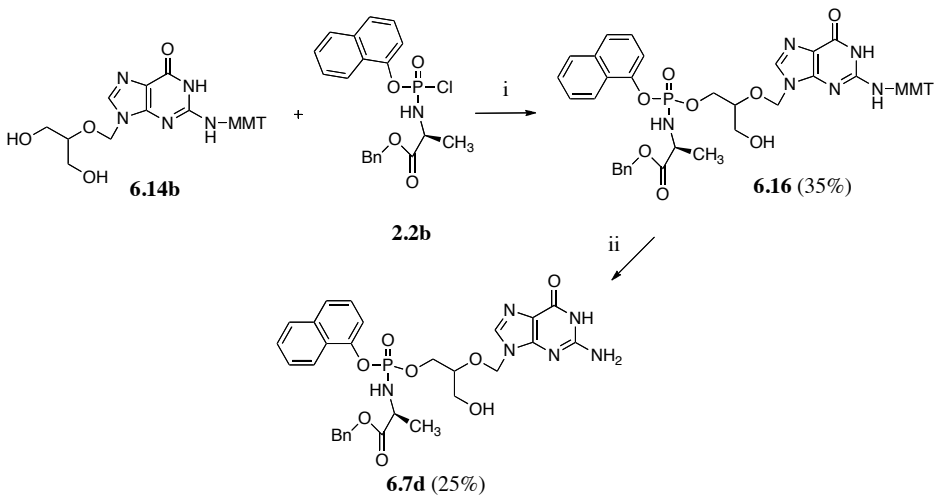
6.15a R: CH_2 **Ar**: 1-Naph
6.15b R:O **Ar**: Ph

6.7b R: CH_2 **Ar**: 1-Naph (30%)
6.7c R:O **Ar**: Ph (28%)

Reagents and conditions: (i) DCM/MeOH, TsOH, rt, 2h

Scheme 6.9 Detritylation reaction

Finally, a diastereomeric mixture of the protected mono-ProTide **6.16** was obtained in 35% yield by coupling reaction in THF of **6.14b** with the phosphorochloridate **2.2b** in the presence of *t*BuMgCl (scheme 6.10). This result shows that also compound **6.14b** is suitable for the synthesis of the mono-ProTides confirming the important role of the solubility of the parent nucleoside in the coupling reaction. The detritylation of **6.16** afforded the desired compound **6.7d** in 25 % yield (scheme 6.10).



Reagents and conditions: (i) *t*BuMgCl, THF, rt, 12-14 h; (ii) DCM/MeOH, TsOH, rt, 2h

Scheme 6.10. Coupling reaction of compound 6.14b with the phosphorochloridate 2.2b and detritylation reaction.

The coupling reaction of the phosphorochloridates **2.2a** and **2.2b** with the MMT-protected derivatives of GCV and PCV represents the first successful route to the synthesis of the mono-ProTides derivatives containing L-alanine benzyl ester as a masking group.

6.2.3 Summary of the GCV and PCV ProTides synthesised.

Table 6.1 reports a summary of the L-alanine and 2,2-dimethylglycine benzyl ester derivatives of GCV and PCV ProTides **6.5a-d**, **6.6a-d**, and **6.7a-d**, including the synthetic pathway, the yields of the coupling reaction (Coupl. Yield) and deprotection (Depr. Yield), the substitution pattern, and the ^{31}P NMR chemical shift (δ) of the final compounds in deuterated methanol.

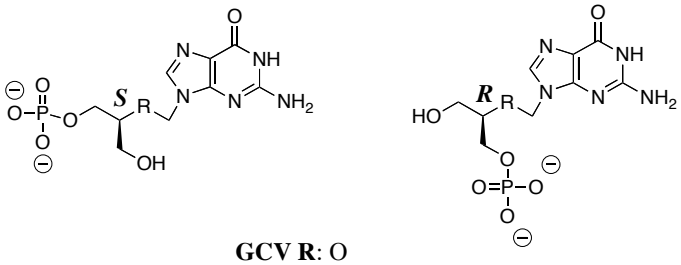
Table 6.1. GCV and PCV ProTides synthesised
(Ar: aryl moiety; AA: amino acid moiety;)

Starting material	Coupl. Yield	Depr. Yield	Final Cps	Ar	AA	Mono/Bis ProTide	^{31}P NMR δ (ppm)
N^2-DMF PCV	30%	5%	**6.5a**	Ph	DMG	Mono	2.37, 2.35
N^2-DMF PCV	48%	5%	**6.5b**	1-Naph	DMG	Mono	2.89, 2.85
N^2-DMF GCV	40%	6.5%	**6.5c**	Ph	DMG	Mono	2.26, 2.24
N^2-DMF GCV	56%	2.7%	**6.5d**	1-Naph	DMG	Mono	2.65, 2.69
N^2-DMF PCV	33%	5%	**6.6a**	Ph	DMG	Bis	2.23, 2.18, 2.17
N^2-DMF PCV	40%	5%`	**6.6b**	1-Naph	DMG	Bis	2.76, 2.70, 2.64, 2.57
N^2-DMF GCV	38%	4.3%	**6.6c**	Ph	DMG	Bis	2.18, 2.16, 2.11
N^2-DMF GCV	35%	5.3%	**6.6d**	1-Naph	DMG	Bis	2.63, 2.57, 2.53, 2.52
PCV[b]	5%	-	**6.7a**	Ph	L-Ala	Mono	4.12, 4.05, 3.61, 3.59
N^2,O-MMT PCV	40%	30%	**6.7b**	1-Naph	L-Ala	Mono	4.46, 4.44, 4.16, 4.13
N^2,O-MMT GCV	38%	28%	**6.7c**	Ph	L-Ala	Mono	3.92, 3.83, 3.63, 3.61
N^2-MMT GCV	35%	25%	**6.7d**	1-Naph	L-Ala	Mono	4.29, 4.24, 4.18, 4.09

Not only the protection of GCV and PCV with the monomethoxytrityl group allowed to obtain the mono-ProTide derivatives, but it also represented an improvement in terms of yield of the deprotection step when compared to N^2-DMF group.

6.3 Diastereospecific synthesis of the mono-phosphoramidate derivatives of GCV and PCV

Ganciclovir and penciclovir are prochiral molecules: the phosphorylation of one of the two hydroxyl groups generates a chiral center at the side chain affording the *S* and *R* enantiomers of GCV and PCV mono-phosphate (fig. 6.6).



GCV R: O
PCV R: CH_2

Figure 6.6. *S* and *R* enantiomers of GCV and PCV monophosphate

A Previous study pointed out the important role of stereochemistry in the intracellular activation of GCV showing that:

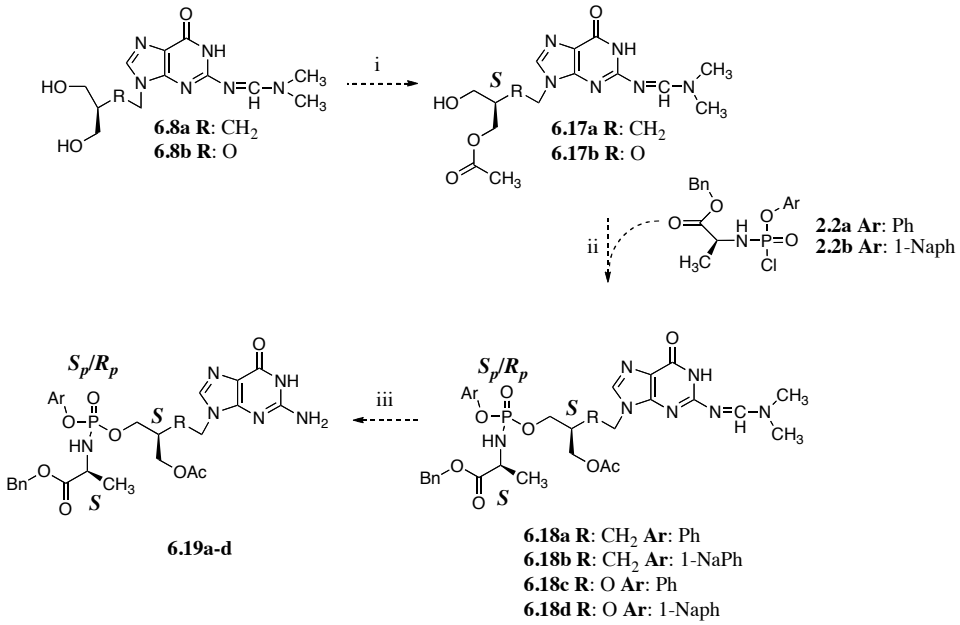
- HSV-TK phosphorylates GCV affording exclusively the *S* enantiomer of the monophosphate derivative.[11]
- The *S* enantiomers of GCV monophosphate reacts much faster than the *R* enantiomer in the phosphorylation step mediated by GMP kinase.[11]
- While the *S* enantiomer of GCV triphosphate displays potent inhibitory activity against HSV-1 DNA polymerase, the *R* enantiomer is a poor inhibitor of this enzyme.[11]

Considering these results, a pronucloetide approach that allows to release into the cell exclusively the *S* enatiomer of GCV monphosphate represents an improvement over the release of a mixture of the enatiomers.

The synthetic route that has been described in previous paragraphs for the preparation of GCV and PCV ProTides is not stereoselective meaning that inside the cell their activation may lead to the release of both the enantiomers of the monophosphate. According to the procedure described in the literature for the enantioselective synthesis of ganciclovir cyanoethyl phospharamidite derivative, a route aimed to obtain the diastereoselective synthesis of GCV and PCV ProTides was developed.[12]

Scheme 6.11 describes this route based on the enantioselective acetylation of the N^2-DMF derivatives of GCV and PCV (**6.8a** and **6.8b**) in the presence of porcine pancreatic lipase (PPL). As previously reported in the literature for the acetylation of the analogous isobutyryl derivative of GCV, this reaction was expected to afford exclusively the *S* enantiomer of **6.17a** and **6.17b**.[12] Thus, the coupling reaction of **6.17a** and **6.17b** with the phosphorochloridates **2.2a** and **2.2b** in the presence of *t*BuMgCl was

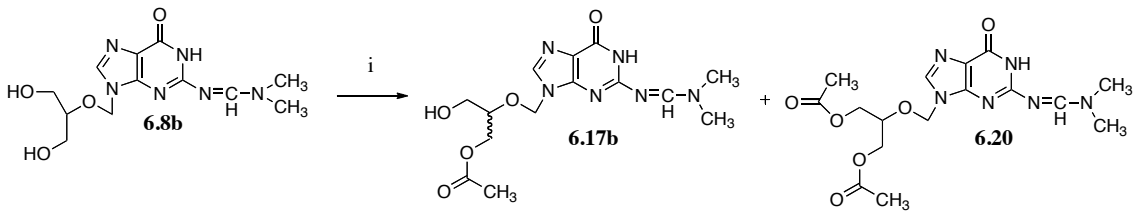
presumed to give rise to the formation of the diastereomers of **6.18a-d** with only the *S* configuration at the chiral carbon of the side chain, which is the desired configuration according to the studies reported in the literature about the stereoselective bioactivation of GCV.[11] The ^{31}P NMR analysis of the ProTides synthesised through this route is expected to give only two peaks corresponding to the two diastereomers obtained (*SS*p*S* and *SR*p*S*) and has been considered as an easy tool to evaluate the diastereselectivity of the whole synthetic approach. However, no information can be obtained about the configuration of the stereocenters by this analysis.



Reagents and conditions: (i) vinyl acetate, PPL, anhydrous pyridine/benzene, rt. 24-48 h
(ii) *t*BuMgCl, THF, rt, 12-14 h.; (iii) *i*PrOH, reflux, 24-72 h.

Scheme 6.11. Diastereoselective route of GCV and PCV ProTides with *S* configuration at the chiral carbon of the side chain

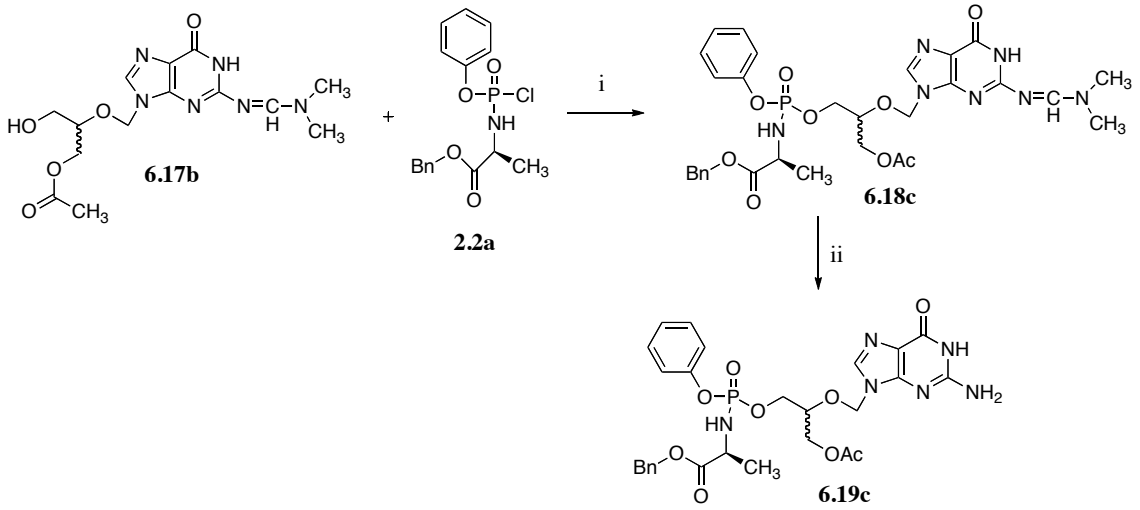
Following this route, the N^2-DMF derivative of GCV **6.8b** was reacted with vinyl acetate in the presence of PPL (scheme 6.12) affording the mono-acetylated derivative **6.17b** as main product (53%) along with a minor amount of the di-acetylated derivative **6.20** (8%).



Reagents and conditions: (i) vinyl acetate, PPL, anhydrous pyridine/benzene, rt, 48 h

Scheme 6.12. PPL-mediated acetylation of N^2-DMF derivative of GCV

The coupling reaction of compound **6.17b** with the freshly prepared phosphorochloridate **2.2a** was performed in the presence of *t*BuMgCl and afforded compound **6.18c** (scheme 6.13). The dimethylformamide group was removed in 2-propanol (*i*PrOH) under reflux conditions affording compound **6.19c** (scheme 6.13).



Reagents and conditions: (i) *t*BuMgCl, anhydrous THF, rt, 12-14 h.; (ii) *i*PrOH, reflux, 24-72 h.

Scheme 6.13 Coupling reaction of the mono-acetylated derivative of GCV 6.17b with the phosphorchloridate 2.2a

As depicted in figure 6.7, the ^{31}P NMR spectrum of compound **6.19c** in deuterated methanol showed 4 peaks corresponding to the 4 diastereomers with configuration *SS*p*S*, *SR*p*S*, *SS*p*R*, and *SR*p*R* at the stereocenters, demonstrating that the whole synthetic route was not diastereoselective as expected. This result may suggest that the PPL-mediated acetylation of compound **6.8b** (scheme 6.12) lacked the expected enantioselectivity. The formation of the diacetate derivative **6.20**, showed in scheme 6.12, strongly supports this hypothesis.

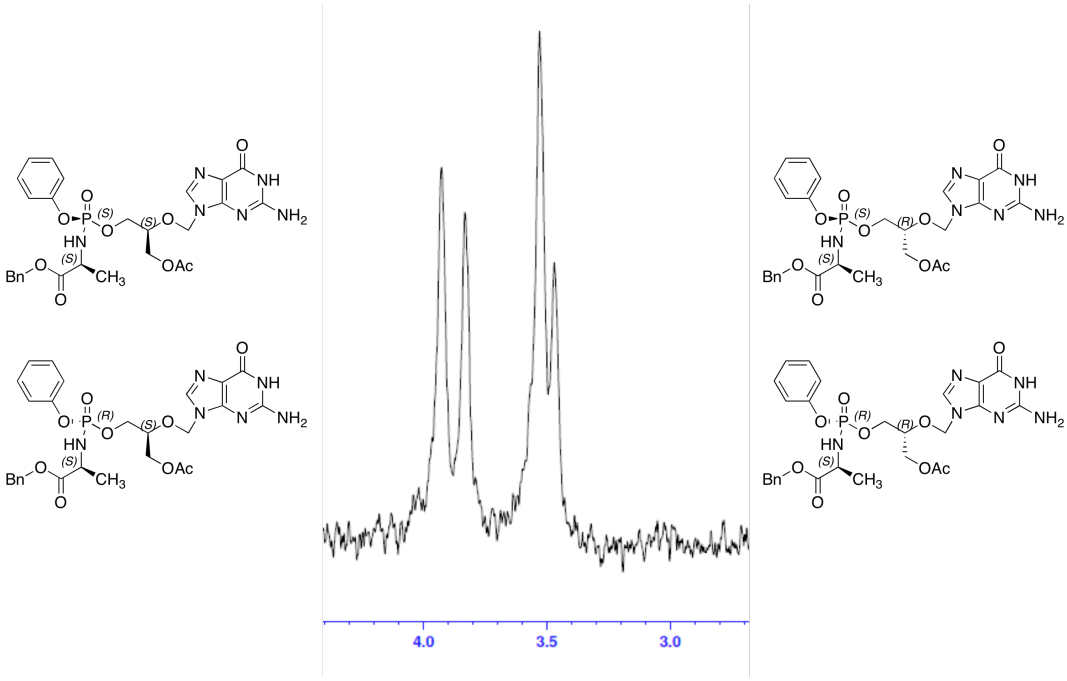
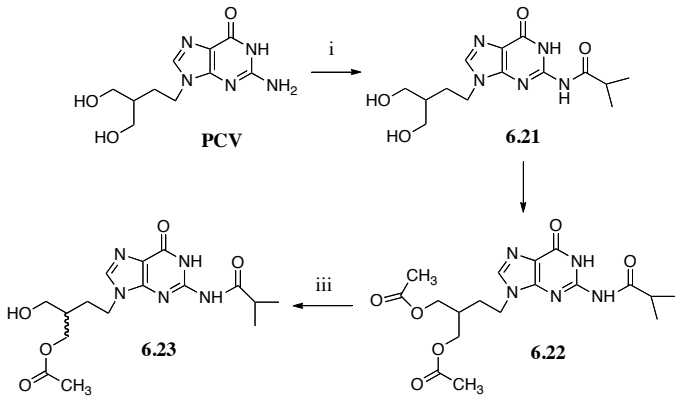


Figure 6.7. ^{31}P NMR spectrum (MeOD, 202 Mhz) and structures of the 4 diastereomers (*S*-*S*p-*S*, *S*-*R*p-*S*, *S*-*S*p-*R*, and *S*-*R*p-*R*) of compound 6.19c

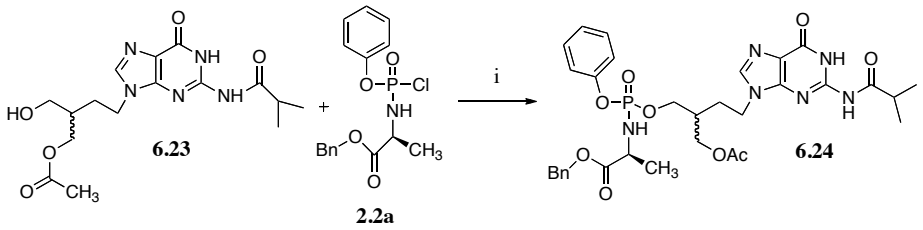
Due to this unsuccessful result, an alternative approach was applied to the diastereoselective synthesis of PCV ProTides. As previously reported in the literature for the enantioselective synthesis of cyclopropavir monophosphate, PCV was protected at the 2-amino group of the guanine base with the isobutyryl group (*i*-Bu) affording compound **6.21**, which was then converted to compound **6.22** by acetylation with acetic anhydride (Ac_2O) (scheme 6.14).[13] Deacetylation was performed using pig liver esterase (PLE) and afforded the mono-acetyl derivative **6.23** in 60% yield (scheme 6.14). As described in the literature, this reaction was expected to be enantioselective.[13]



Reagents and conditions: (i) *i*-BuCl, Pyr, from 0ºC to r.t., 16 h; (ii) Ac_2O, $DMAP_{(cat)}$, DMF, r.t., 1h; (iii) PLE, DMF, buffer phosphate (pH 7.00), rt, 2 h

Scheme 6.14 Synthesis of the mono-acetyl derivative of N^2-*i*BU-PCV (6.23)

Coupling reaction of compound **6.23** with the phosphorochloridate **2.2a** was performed in the presence of *t*BuMgCl and afforded compound **6.24** (scheme 6.15).



Reagents and conditions: (i) *t*BuMgCl, anhdrous THF/Pyr, rt, 12-14 h.

Scheme 6.15. Coupling reaction of 6.23 with the phosphorochloridate 2.2a

The ^{31}P NMR spectrum of compound **6.24** (fig. 6.8) showed two peaks corresponding to two of the four possible diastereomeric configurations. This result suggests that the PLE-mediated deacetylation of compound **6.22** was enantioselective (scheme 6.15).

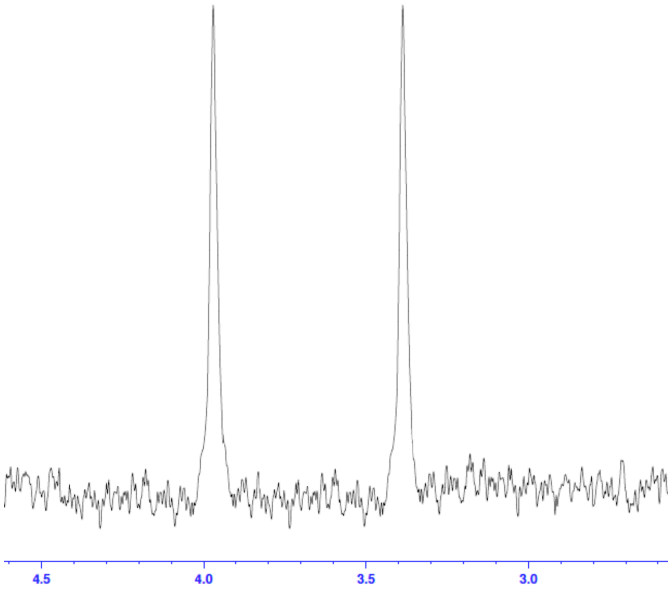
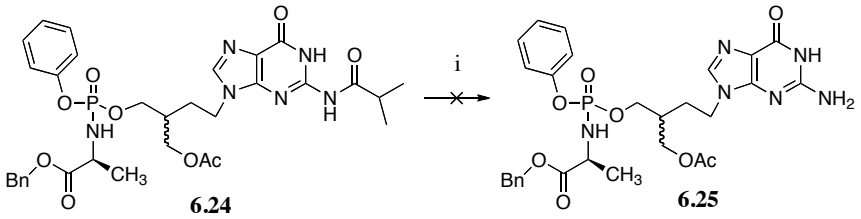


Figure 6.8. ^{31}P NMR spectrum (MeOD, 202 Mhz) of compound 6.24

The removal of the N^2-*i*Bu group was attempted according to the procedure described in the literature (scheme 6.16).[13] However, degradation of compound **6.24** occurred under these conditions and compound **6.25** was not obtained.



Reagents and conditions:.; (ia) Me_3SiCl, imidazole, Pyr, rt, 20 min; (ib) I_2, Pyr, rt, 16 h; (ic) NH_4OH, 50-60ºC, 16 h

Scheme 6.16. N^2-*i*Bu group deprotection of compound 6.24

In conclusion, the PPL-mediated acetylation of the N^2-DMF derivatives of GCV did not achieve the expected selectivity (scheme 6.12), while the diastereoselective synthesis of PCV ProTide **6.24** was successfully obtained by means of the PLE-mediated deacetylation of compound **6.22** (scheme 6.14). However the isobutyryl group proved to be unsuitable for the synthesis of ProTides. These results suggest that the route involving the PLE-mediated deacetylation must be preferred over that where PPL-mediated acetylation is used to achieve the diastereoselective synthesis of GCV and PCV ProTides.

6.4 Biological evaluation of GCV and PCV ProTides

The aryl phosphoramidate derivatives of GCV and PCV synthesised in this work were evaluated for their antiviral activity against HSV, VZV, HCMV, and HIV. The cytostatic activity on HEL and MT-4 cell cultures was also evaluated, as well as the concentration required to cause a microscopically visible alteration of HEL cell morphology and the concentration that induces 50% cell death in MT-4 cell cultures.

6.4.1 Anti-HSV activity

Table 6.2 reports the antiviral activity of compounds **6.5a-d**, **6.6a-d**, and **6.7a-d** against HSV-1, HSV-2, and TK^--HSV-1 in HEL cell cultures. The data include ACV and GCV as references. Moreover, the anti-HSV activity of the L-alanine benzyl ester derivatives of ACV ProTides (**3.1a** and **3.1b**) are reported for comparison.[3]

Table 6.2. anti-HSV activity of GCV and PCV ProTides 6.5a-d, 6.6a-d, and 6.7a-d in HEL cells
(Nuc: nucleoside; AA: amino acid moiety)

					Antiviral Activity EC_{50} [a] (μM)		
Cps	**Nuc**	**Aryl**	**AA**	**Mono/Bis**	**HSV-1**	**HSV-2**	**TK$^-$ HSV-1**
6.5a	PCV	Ph	DMG	Mono	29	39	100
6.5b	PCV	1-Naph	DMG	Mono	75	51.5	100
6.5c	GCV	Ph	DMG	Mono	0.1	0.3	0.1
6.5d	GCV	1-Naph	DMG	Mono	0.6	0.8	1.4
6.6a	PCV	Ph	DMG	Bis	>100	>100	>100
6.6b	PCV	1-Naph	DMG	Bis	>20	>20	>20
6.6c	GCV	Ph	DMG	Bis	1.45	2.5	1.5
6.6d	GCV	1-Naph	DMG	Bis	0.8	0.9	1.6
6.7a	PCV	Ph	L-Ala	Mono	12	17.3	100
6.7b	PCV	1-Naph	L-Ala	Mono	72.5	79	100
6.7c	GCV	Ph	L-Ala	Mono	0.55	3	3
6.7d	GCV	1-Naph	L-Ala	Mono	0.4	0.5	3
ACV	-	-	-	-	0.1	0.2	112
GCV	-	-	-	-	0.1	0.03	100
3.1a	ACV	Ph	L-Ala	Mono	8 ± 5.7	4	15 ± 7.1
3.1b	ACV	1-Naph	L-Ala	Mono	2	1.4 ± 0.8	10 ± 2.1

[a] 50% Effective concentration or compound concentration required to inhibit virus-induced cytopathicity by 50%

Similarly to ACV, GCV exhibited activity against HSV-1 and HSV-2 in the submicromolar range (EC_{50}= 0.1 and 0.03 μM respectively) and it lost activity against TK$^-$ strain of HSV-1 (EC_{50}= 100 μM).

All the novel derivatives of GCV ProTides (**6.5c-d**, **6.6c-d**, and **6.7c-d)** showed activity against HSV-1 and HSV-2 in the micromolar and sub-micromolar range (EC_{50}= 0.1-3 μM). The retention of antiviral activity against TK$^-$ HSV-1 indicates the successful bypass of the HSV-TK-mediated phosphorylation step. The 2,2-dimethylglycine benzyl ester derivatives of GCV ProTides **6.5c** and **6.5d** exhibited retention of anti-HSV activity when compared to the L-alanine benzyl ester analogues **6.7c** and **6.7d**. The antiviral activity of **6.6c** and **6.6d** against TK$^-$ HSV-1 (EC_{50}= 1.5-1.6 μM) also suggests that the bis-ProTide derivatives of GCV successfully bypassed the first phosphorylation step mediated by the HSV-TK. This result is in contrast to the

study of the analogous L-alanine benzyl ester derivatives of GCV bis-ProTides **6.1c** and **6.1d** previously reported in our group (fig 6.2).[4]

In comparison to the previously reported L-alanine benzyl ester derivatives of ACV ProTides (**3.1a** and **3.1b**),[3] all the GCV ProTides presented in this work (**6.5c-d**, **6.6c-d**, and **6.7c-d)** displayed retention of activity against HSV-1, HSV-2, and a slight improvement of potency against TK^- HSV-1 (EC_{50}= 0.15-3 μM).

Surprisingly, the PCV ProTides **6.5a-b**, **6.6a-b**, and **6.7a-b** showed a significant or complete loss of the anti-HSV activity in comparison to the analogous GCV derivatives. The antiviral activity of these compounds against HSV-1 and HSV-2 indicates that the phenyl derivatives **6.5a** and **6.7a** were more active than the naphthyl derivatives **6.5b** and **6.7b**. However, the significant decrease of potency against TK^- HSV-1, which is comparable to that exhibited by ACV and GCV, strongly suggests that these derivatives of PCV ProTides did not release the phosphate form inside the cell and their anti-HSV activity was due to the release of the parent nucleoside instead.

The concentration required to cause a microscopically visible alteration of cell morphology (MCC) was above 100 μM for all the compounds synthesised, indicating low cytotoxicity.

6.4.2 Anti-VZV activity

Compounds **6.5a-c**, **6.6b-d**, and **6.7b-d** were evaluated for their antiviral activity against TK-positive (TK^+) and TK-deficient (TK^-) strains of VZV in HEL cell cultures (table 6.3). ACV and the previously reported L-alanine benzyl ester derivatives of ACV ProTides **3.1a** and **3.1b** are reported as reference compounds.[14]

Table 6.3. Anti-VZV activity of GCV and PCV ProTides 6.5a-c, 6.6b-d, and 6.7b-d in HEL cells
(Nuc: nucleoside; AA: amino acid moiety)

					Antiviral Activity EC_{50}[a] (μM)			
Cps	**Nuc**	**Aryl**	**AA**	**Mono/Bis**	**TK^+ VZV strains**		**TK^- VZV strains**	
					YS	**OKA**	**07-1**	**YS/R**
6.5a	PCV	Ph	DMG	Mono	>50		>50	
6.5b	PCV	1-Naph	DMG	Mono	23.3		>50	
6.5c	GCV	Ph	DMG	Mono	6.40	7.3	3.3	2.4
6.6b	PCV	1-Naph	DMG	Bis	>50	>50	>50	>50
6.6c	GCV	Ph	DMG	Bis	8.8	21.7	8.3	12.5
6.6d	GCV	1-Naph	DMG	Bis	12.1	13.6	7.2	20
6.7b	PCV	1-Naph	L-Ala	Mono	16.2	9.3	>50	N.D.
6.7c	GCV	Ph	L-Ala	Mono	N.D.	2.7	4.8	N.D.
6.7d	GCV	1-Naph	L-Ala	Mono	N.D.	9.5	8.2	N.D.
ACV	**-**	**-**	**-**	**-**	1	4.2	73.6	94.3
3.1a	**ACV**	Ph	L-Ala	**-**	0.7	1.0	1.8	0.59
3.1b	**ACV**	1-Naph	L-Ala	**-**	7.2	3.3	6.9	N.D

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

The phosphoramidate derivatives of GCV **6.5c**, **6.6c-d**, and **6.7c-d** exhibited antiviral activity against TK^+-VZV and TK^--VZV in the micromolar range, retaining the activity of the L-alanine benzyl ester derivatives of ACV ProTides **3.1a** and **3.1b**.[14] These results show that the application of the ProTide approach to GCV allowed bypass of the first phosphorylation step mediated by the virus-encoded thymidine kinase also in the case of VZV. Moreover, the effective bioactivation of the bis-ProTide derivatives **6.6c** and **6.6d**, which was already observed in the anti-HSV assay, was confirmed also against VZV**.**

PCV ProTides **6.5b** and **6.7b** were moderately active against TK^+-VZV (EC_{50}= 9.3-23.3 μM), while compounds **6.5a** and **6.6b** were completely inactive (EC_{50}> 50 μM). None of the PCV ProTides showed antiviral activity below 50 μM against TK^--VZV.

The concentration required to cause a microscopically visible alteration of cell morphology (MCC) were above 100 μM for all the compounds synthesised confirming the lack of cytotoxicity, which was already observed in the anti-HSV assays.

6.4.3 Anti-HCMV activity

Table 6.4 reports the antiviral activity of GCV and PCV ProTides **6.5a-d**, **6.6a-d**, and **6.7a-d** against HCMV in HEL cell cultures. Ganciclovir is included as reference compound.

Table 5.4. Anti-HCMV activity of GCV and PCV ProTides 6.5a-d, 6.6a-d, and 6.7a-d in HEL cells
(Nuc: nucleoside; AA: amino acid moiety)

					Antiviral Activity EC_{50} [a] (μM)	
Cps	**Nuc**	**Aryl**	**AA**	**Mono/Bis**	**AD-169 strain**	**Davis strain**
6.5a	PCV	Ph	DMG	Mono	>50	>50
6.5b	PCV	1-Naph	DMG	Mono	>50	>50
6.5c	GCV	Ph	DMG	Mono	38.5	15.7
6.5d	GCV	1-Naph	DMG	Mono	6.6	9.8
6.6a	PCV	Ph	DMG	Bis	>50	>50
6.6b	PCV	1-Naph	DMG	Bis	>50	>50
6.6c	GCV	Ph	DMG	Bis	50	23.5
6.6d	GCV	1-Naph	DMG	Bis	45	26
6.7a	PCV	Ph	L-Ala	Mono	>50	>50
6.7b	PCV	1-Naph	L-Ala	Mono	>50	>50
6.7c	GCV	Ph	L-Ala	Mono	6.84	4.0
6.7d	GCV	1-Naph	L-Ala	Mono	11.7	8.9
GCV	-	-	-	-	9.2	9.4

[a] 50% Effective concentratio n, or compound concentration required to inhibit virus-induced cytopathicity by 50%

GCV ProTides **6.5c-d**, **6.6c-d**, and **6.7c-d** showed good to moderate activity against HCMV (EC_{50}: 4-45 μM) in comparison to the parent nucleoside. The 2,2-dimethylgycine benzyl ester derivative **6.5d** showed retention of activity in comparison to the analogous L-alanine benzyl ester derivative **6.7d**, while compound **6.5c** exhibited a significant decrease of potency (EC_{50}: 38.5-15.7 μM). Also the bis-ProTide derivatives of GCV **6.6c** and **6.6d** exhibited a decrease in potency (EC_{50}: 26-50 μM) in comparison to the mono-ProTide analogues **6.5c** and **6.5d**. PCV ProTides **6.5a-b**, **6.6a-b**, and **6.7a-b** resulted completely inactive against HCMV. Also in this case none of the compounds evaluated resulted cytotoxic (MCC > 100 μM).

6.4.4 Cytostatic activity of GCV and PCV ProTides on HEL cell cultures

All the phosphoramidate derivatives of GCV and PCV synthesised in this work were evaluated for their antiproliferative activity on HEL cell cultures. The concentration required to reduce cell growth by 50% was above 100 μM for all the compounds evaluated confirming the lack of cytotoxicity, as already indicated by the microscopy analysis of cell morphology performed during the antiviral assays previously described.

6.4.5 Anti-HIV activity

Table 6.5 reports the antiviral and cytostatic activity of the GCV and PCV ProTides **6.5b-d**, **6.6c-d**, and **6.7a-d** against HIV-1 in MT-4 cell cultures. ACV and the L-alanine derivatives of ACV ProTides **3.1a** and **3.1d** are included as reference compounds.[3]

Table 6.5. Anti-HIV and cytostatic activity in MT-4 cell cultures of GCV and PCV ProTides
(Nuc: nucleoside; AA: amino acid moiety)

					Antiviral Activity EC_{50}[a] (μM)	Cytostatic Activity IC_{50}[b] (μM)
Cps	**Nuc**	**Aryl**	**AA**	**Mono/Bis**	**HIV-1**	**MT-4 Cells**
6.5b	PCV	1-Naph	DMG	Mono	95	100
6.5c	GCV	Ph	DMG	Mono	> 150	> 150
6.5d	GCV	1-Naph	DMG	Mono	10	26
6.6c	GCV	Ph	DMG	Bis	10	13
6.6d	GCV	1-Naph	DMG	Bis	> 150	> 150
6.7a	PCV	Ph	L-Ala	Mono	10	> 150
6.7b	PCV	1-Naph	L-Ala	Mono	21	100
6.7c	GCV	Ph	L-Ala	Mono	17	20
6.7d	GCV	1-Naph	L-Ala	Mono	8	100
ACV	-	-	-	-	>250	>250
3.1a	ACV	Ph	L-Ala	-	5.7 ± 1.6	33.8 ± 10.6
3.1b	ACV	1-Naph	L-Ala	-	0.8	>150

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%; [b] 50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%.

The results of the anti-HIV assay performed in MT-4 cell cultures show that the L-alanine benzyl ester derivatives of GCV ProTides **6.7c** and **6.7d** were active against HIV-1 in the micromolar range (EC_{50}= 8-17 μM). Compound **6.7c** exhibited

antiproliferative activity on the MT-4 cells (IC_{50}= 20 μM). However, similar cytostatic activity was observed also in the case of the ACV ProTide **3.1a** (IC_{50}= 33.8 ± 10.6 μM).[3] Surprisingly, the 2,2-dimethylglycine benzyl ester derivative of GCV ProTides **6.5c** resulted inactive, while the analogous derivative bearing the naphthyl group as aryl moiety (**6.5d**) exerted moderate antiviral activity against HIV-1 (EC_{50}= 10 μM), but also cytostatic activity (IC_{50}= 26 μM). Similarly, the bis-ProTide derivative **6.6d** was inactive, while compound **6.6c** exhibited antiviral and cytostatic activity.

PCV ProTides **6.7a** and **6.7b** showed antiviral activity against HIV-1 in MT-4 cell cultures comparable to that of the analogous derivatives of GCV ProTides **6.7c** and **6.7d** and weak or no inhibition of cell proliferation. These results suggests that compounds **6.7a** and **6.7b** successfully released PCV monophosphate inside MT-4 cells. The lack of bioactivation of the dimethylglycine benzyl ester derivative of PCV ProTides **6.5b** is indicated by the significant loss of anti-HIV-1 activity (EC_{50}= 95 μM) in comparison to the analogous L-alanine benzyl ester derivative **6.7b** (EC_{50}= 21 μM).

The concentration that induces 50% cell death in the MT-4 cell cultures was above 150 μM for all the compounds synthesised.

The anti-HIV activity in CEM cell cultures of GCV and PCV ProTides is currently under evaluation.

6.4.6 Summary of the biological activity of the novel aryl phosphoramidate derivatives of GCV and PCV

The results of the anti-HIV activity of the newly synthesised GCV and PCV ProTides **6.5a-d**, **6.6a-d**, and **6.7a-d** afford structure activity relationships significantly different from that implied by their antiviral activity against HSV, VZV, and HCMV. As claimed in the case of ACV ProTides, the differences in cell-type and cell cycle between the antiviral assays may affect the bioactivation pathway of ProTides.

The data on anti-herpes virus activity, in particular against TK^- strains of HSV and VZV, show that the application of the ProTide approach to GCV successfully achieved the bypass of the phosphorylation step mediated by the virus-encoded kinase. This result strongly suggests that the mono-ProTides **6.5c-d**, **6.7c-d**, and surprisingly also the bis-ProTides **6.6c-d** may be converted inside the cell according to the putative mechanism of bioactivation of ProTides.[15]

In comparison to the GCV ProTides presented in this work, the loss of antiviral activity of the analogous derivatives of PCV **6.5a-b**, **6.6a-b**, and **6.7a-b** against the herpes viruses suggests that the replacement of the ether group at the side chain of the nucleoside moiety with a methylene group may lead to the lack of bioactivation of these phosphoramidate derivatives.

Also the 6-alkoxy derivatives of PCV ProTides **6.3a** and **6.3b**, which have been previously reported by our group (fig. 6.3),[4] exhibited poor activity against HSV-1 (EC_{50}= 38-58 μM) and HSV-2 (EC_{50}= 20-45 μM). However, they showed retention of activity against TK-deficient strains of HSV-1 (EC_{50}= 20-58 μM), while the analogous PCV ProTides **6.7a** and **6.7b** presented in this work were completely inactive (EC_{50}= 100 μM). Assuming that the 6-alkoxy derivatives of PCV Protides are prodrugs of PCV triphosphate and are deaminated after the release of the monophosphate form, as reported for the 6-methoxy derivative of the 2'-C-methyl-guanosine ProTide,[16] this result suggests that **6.3a** and **6.3b** may be bioactivated more efficiently than **6.7a** and **6.7b**.

The data of the anti-HIV-1 assay performed in MT-4 cell cultures suggest that the L-alanine benzyl ester derivatives of both GCV and PCV ProTides **6.7a-d** successfully bypass the first phosphorylation step by releasing their monophasphate form. This result confirms the findings of the *in silico* study previously performed in our group, which indicated PCV and GCV triphosphate as potential anti-HIV agents.[2] However, in the case of the PCV ProTide **6.7a** and **6.7b** these results are in contrast to the data on the antiviral activity against HSV, VZV, and HCMV.

The results of the anti-HIV activity of the 2,2-dimethylglycine benzyl ester derivatives of GCV ProTides **6.5c-d** and **6.6c-d** are in contrast with those obtained against the herpes viruses. Indeed, **6.5c**, and **6.6d** were inactive, while **6.5d** and **6.6c** exerted antiproliferative activity on MT-4 cells (IC_{50}= 13-26 μM) comparable to their anti-HIV-1 activity (EC_{50}= 10 μM). Thus, the correlation of the anti-HIV activity with the replacement of L-alanine with 2,2-dimethylglycine at the amino acid moiety is not clear. The poor antiviral activity of the 2,2-dimethylglycine benzyl ester derivative of PCV Protide **6.5b** observed in the anti-herpes virus assays was confirmed also against HIV-1 in MT-4 cells (EC_{50}= 95 μM). This result suggests that in MT-4 cell cultures the replacement of the L-alanine with 2,2-dimethylglycine at the amino acid moiety may

lead to a significant decrease of anti-HIV activity. These results further support the notion that the ProTide motif need to be tuned on a case-by-case basis.

6.5 Enzymatic and modeling studies

Enzymatic and molecular modelling studies were performed in order to investigate the difference of antiviral activity among the GCV and PCV ProTides presented in this work.

6.5.1 Study of the carboxyesterase activity

The enzymatic study of selected derivatives of GCV and PCV ProTides was performed using carboxypeoptidase Y (CPY) with the aim to evaluate whether the difference of their antiviral activity can be correlated to the activity of this enzyme. The enzymatic assay was carried out following the procedure described for the ACV ProTides (chapter 3). The L-alanine benzyl ester phosphoramidate derivatives of PCV **6.7b** and GCV **6.7c** were chosen in order to study the effect of the nucleoside on the activity of CPY. The same assay was performed on the 2,2-dimethylglycine benzyl ester phosphoramidate of GCV **6.5c** in order to evaluate the effect of the amino acid moiety replacement on the activity of CPY by comparison with the enzymatic study performed on compound **6.7c**.

The ^{31}P NMR spectra of the enzymatic reaction performed on compound **6.7b** over the 14 h period of incubation is depicted in figure 6.9.

According to the putative mechanism of ProTides activation, the hydrolysis of the benzyl ester of **6.7b** (δ_P= 3.95, 4.00, and 4.16 ppm) afforded the intermediate **6.26** (δ_P= 5.59 and 5.67 ppm), which was converted to the corresponding phosphoramidate monoester **6.27** (δ_P= 7.49 ppm). Compound **6.7b** was hydrolyzed by 50% after a 40 min incubation period. The conversion of **6.7b** to **6.27** was not complete after 14 h from exposure to the enzyme and 30% of the starting material was still unreacted after this period, indicating that compound **6.7b** is slowly converted by CPY.

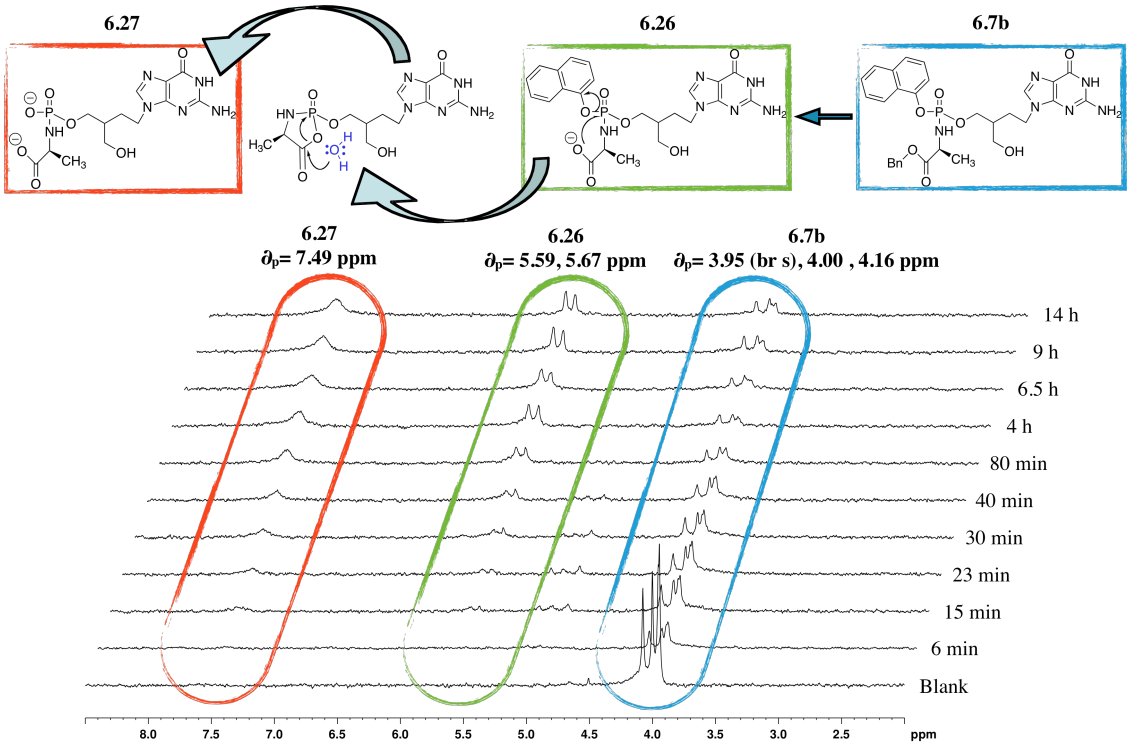


Figure 6.9. ^{31}P NMR spectra (acetone-D6, 202 MHz) at different time of the enzymatic study performed on compound 6.7b

The ^{31}P NMR spectra of the enzymatic reaction performed on compound **6.7c** over the 18 h period of incubation is depicted in figure 6.10.

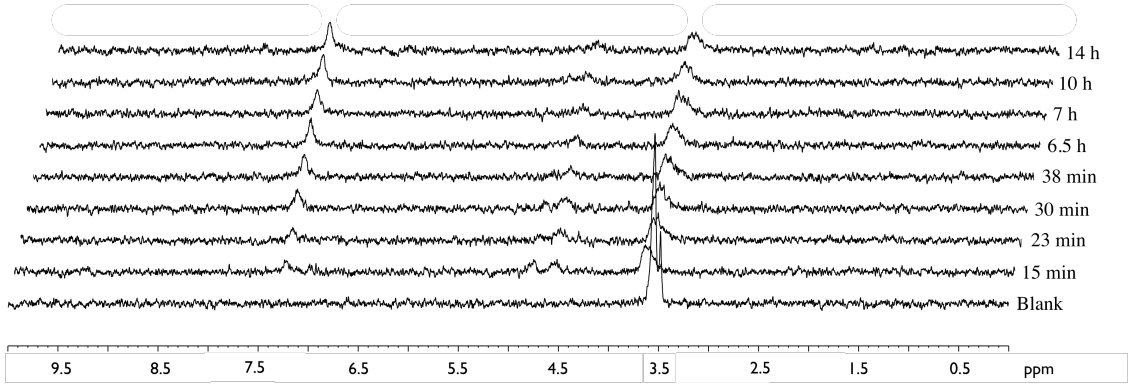


Figure 6.10. ^{31}P NMR spectra (acetone-D6, 202 MHz) at different time of the enzymatic study performed on compound 6.7c

Due to the background noise the ^{31}P NMR spectra showed in figure 6.10 are not very clear. For this reason the spectra have been deconvoluted (Lorentz-Gauss deconvolution, Bruker TOPSPIN 2.1). Deconvolution is a mathematical processing of the spectrum. It allows to show only the peaks that stands out significantly from the noise. The advantage of this procedure is that much cleaner spectra are obtained. However, some peaks may be mistaken for noise and not considered in the

deconvoluted spectrum. Figure 6.11 shows the deconvoluted ^{31}P NMR spectra of the enzymatic reaction performed on compound **6.7c**. In comparison to the original spectra (fig. 6.10) the appearance of the signal around 4.5 ppm after 15 min from the exposure to CPY is clearer

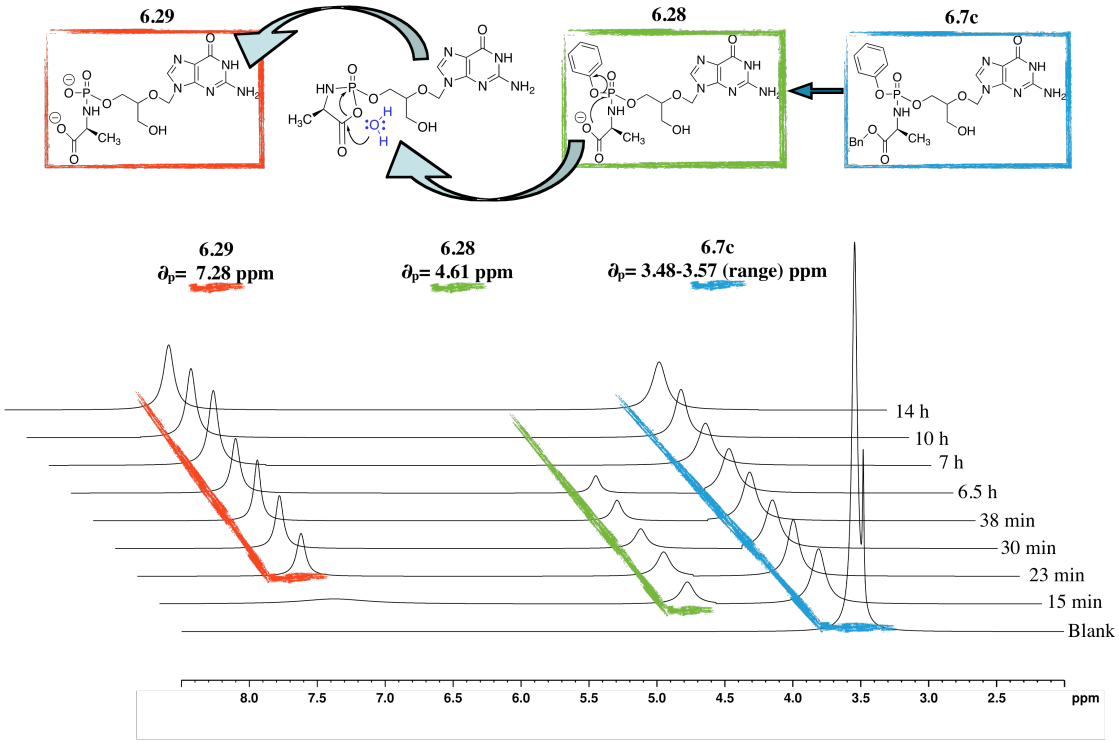


Figure 6.11. Deconvoluted ^{31}P NMR spectra (acetone-D6, 202 MHz) at different time of the enzymatic study performed on compound 6.7c

According to the putative mechanism of ProTides activation, the benzyl ester cleavage of compound **6.7c** (δ_P= 3.48-3.57 ppm,) afforded the intermediate **6.28** (δ_P= 4.61 ppm), which was converted to the corresponding phosphoramidate monoester **6.29** (δ_P= 7.28 ppm) (fig 6.11). Also in this case the catalytic activity of CPY was slow, as demonstrated by the fact that the conversion of compound **6.7c** to compound **6.29** was not complete after the 14 h incubation period resulting in 40% of **6.7c** unreacted. This result is surprising considering that ACV ProTides with comparable antiviral activity were converted very quickly by CPY, as described in chapter 3.

The ^{31}P NMR spectra of the enzymatic reaction performed on compound **6.5c** over a 14 h period of incubation is showed in figure 6.12.

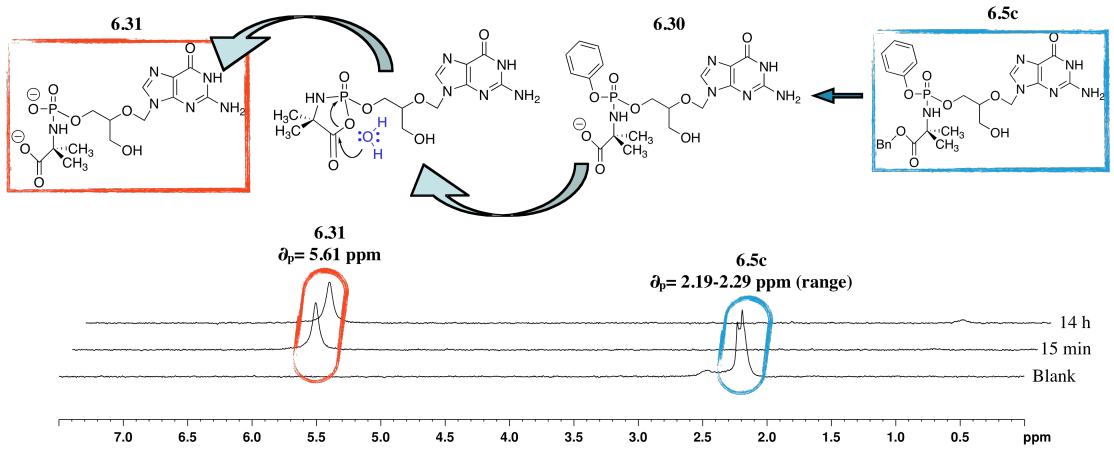


Figure 6.12. ^{31}P NMR spectra (acetone-D6, 202 MHz) at different time of the enzymatic study performed on compound 6.5c

The hydrolysis of compound **6.5c** (δ_p= 2.19-2.29 ppm, broad signal) occurred in less then 15 minutes from the exposure to CPY (fig. 6.12). According to the putative mechanism of activation of ProTides, the formation of the intermediate **6.30** resulting from the cleavage of the benzyl group of **6.5c** was not observed before the formation of the final product **6.31** (δ_p= 5.61 ppm, broad signal). The fast conversion of compound **6.5c** is in agreement with the results of the enzymatic studies performed on the analogous ACV Protide **3.2b**, which was hydrolysed by CPY in less than 6 minutes and exhibited similar antiviral activity against HSV, VZV, and HCMV, as described in chapter 3. The lack of antiviral activity of compound **6.5c** against HIV-1 in MT-4 cell cultures suggest that a different substrate affinity could occur either at the level of the enzyme responsible for the ester cleavage or at the level of the phosphoramidase enzyme responsible for the P-N bond cleavage.

6.5.2 Docking study of L-alanine derivatives of GCV and PCV ProTides

The modeling study of the phosphoramidate monoesters **6.27** and **6.29** (fig. 6.13) into the active site of the human Hint-1 enzyme co-crystallised with adenosine monophosphate (AMP) was performed with the aim to further investigate the bioactivation of the L-alanine benzyl ester phosphoramidates of GCV and PCV according to the putative mechanism of activation of ProTides.[15, 17] Both the diastereomers of **6.27** and **6.29** were evaluated for their ability to fit into the active site of the human Hint-1 enzyme.

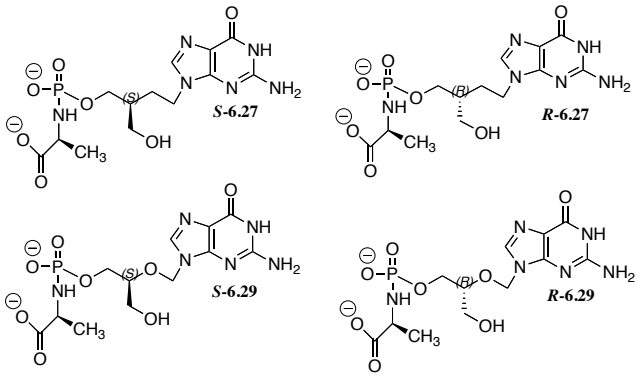


Figure 6.13 Structure of the diastereomers of compound 6.27 and 6.29

The docking study of compound **6.29** is shown in figure 6.14**.**

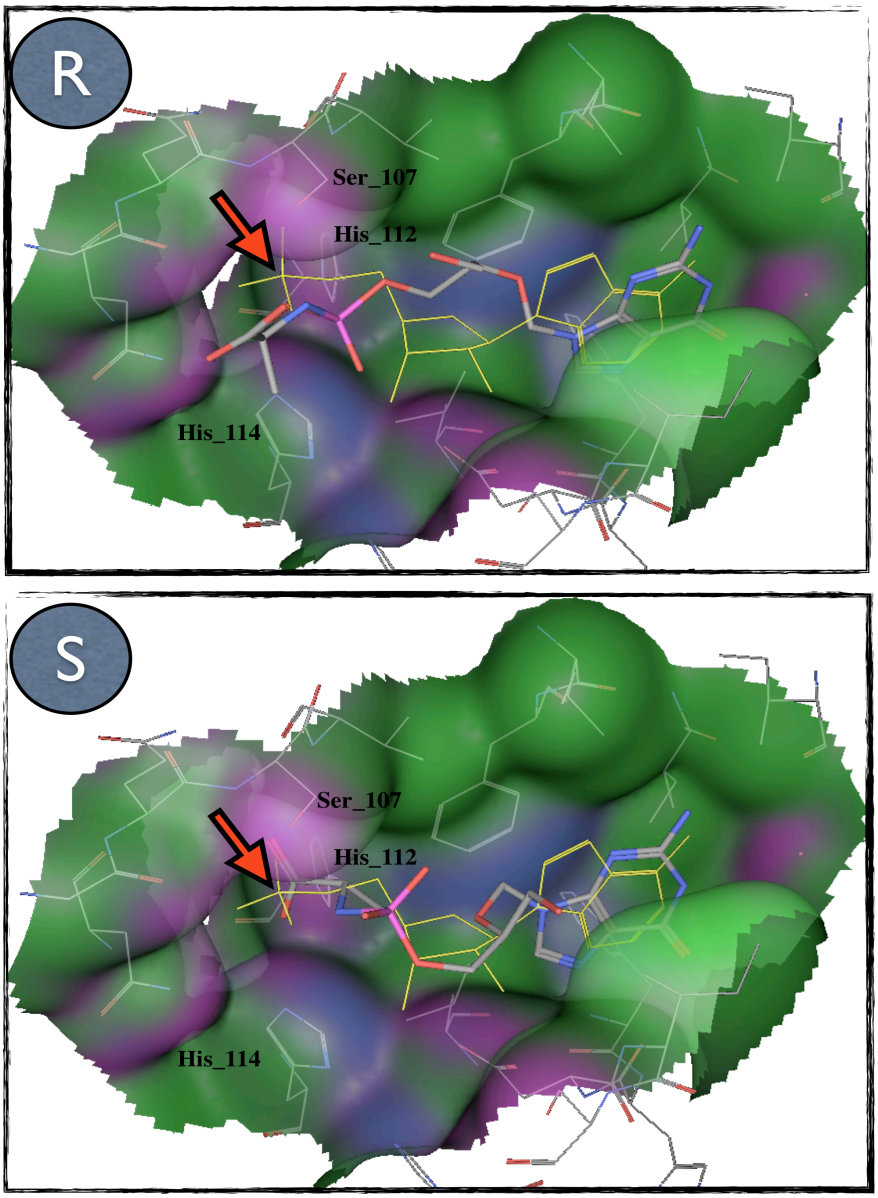


Figure 6.14. Superimposition of the diastereomers (R and S) of compound 6.29 with AMP (yellow) within the active site of human Hint-1 enzyme. The red arrows indicate the position of the phosphate group of AMP. The phosphate group of 6.29 is shown in purple. The position in the catalytic pocket of histidine and serine residues (His_112, His_114, and Ser_107) responsible for the cleavage of the P-N bond is also shown

Both the diastereomers (***R*-6.29** and ***S*-6.29**) bind quite well in the active site of the human Hint-1 enzyme. The guanine base and the side chain are well positioned in the enzymatic pocket. However, the phosphate moiety (purple) is moved from the position adopted by the phosphate group of AMP (yellow), as shown by the red arrow in figure 6.14. Nevertheless, the P-N bond is in a suitable position to be cleaved by interaction with the histidine and serine residues of the active site according to the proposed mechanism of action of the human Hint-1 described in chapter 3.[17, 18]
This result is in agreement with the antiviral activity found for compounds **6.7c** and **6.7d** suggesting that the L-alanine derivatives of GCV Protides are able to bypass the first phosphorylation step by releasing the monophosphate form of the parent nucleoside.

Figure 6.15 shows the docking study of both the diastereomers of compound **6.27** (***R*-6.27** and ***S*-6.27**). The guanine base and the side chain are well positioned in the enzymatic pocket, as shown by the superimposition with AMP (yellow). Similarly to compound **6.27**, the phosphate moiety (purple) is moved from the position adopted by the phosphate group of AMP. However, in this case the amino acid moiety has an orientation that appears to favour the interaction of the serine residue 107 (red circle) with the phosphate group (purple) rather than the amino group (blue) of compound **6.27**. Considering that the serine residue 107 is supposed to protonate the amino group in order to favour the elimination of the amino acid moiety,[17] this study suggests that **6.27** does not interact properly with active site of the human Hint-1 enzyme resulting in the missed release of the monophosphate form. This result is strongly supported by the inactivity of compounds **6.7a** and **6.7b** against HSV, VZV, and HCMV. However, the anti-HIV activity exhibited by these compound in MT-4 cell cultures is in contrast to this hypothesis. This may suggest that in MT-4 cells a different substrate affinity of the phosphoramidase enzyme responsible for the cleavage of the P-N bond could be accountable for the anti-HIV activity exerted by the L-alanine benzyl ester derivatives of PCV ProTides **6.7a** and **6.7b**.

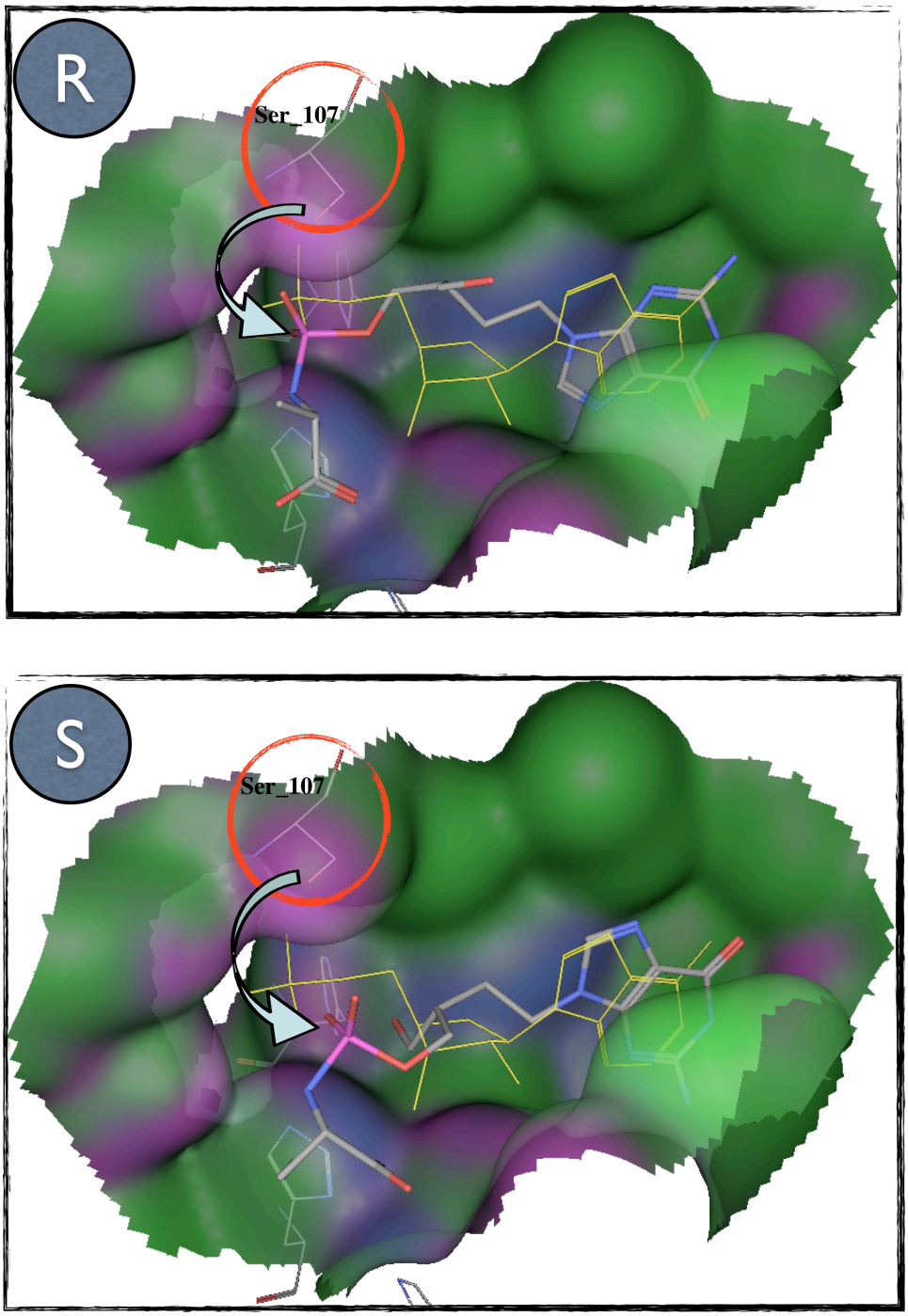


Figure 6.15. Superimposition of the diastereomers (R and S) of compound 6.27 with AMP (yellow) within the active site of human Hint-1 enzyme. The red circles indicate the serine residue 107. The phosphate group of 6.27 is shown in purple.

6.6 Conclusions

Novel L-alanine and 2,2-dimethylglycine benzyl ester derivatives of GCV and PCV Protides were synthesised in this work. In particular, a synthetic route was developed in order to achieve the phosphorylation of only one of the two primary hydroxyl groups of PCV and GCV side chain. The application of this route resulted in the synthesis of the L-alanine benzyl ester derivatives **6.7a-d**. Moreover the use of the monomethoxytrityl

group for the protection of GCV and PCV improved the yield at the deprotection step (25-30%) in comparison to that usually achieved with the N^2-DMF group (4-6%).

According to the results of the antiviral assays performed against the herpes viruses, the aryl phosphoramidate derivatives of GCV **6.5c-d**, **6.6c-d**, and **6.7c-d** were successfully bioactivated in the cell exerting antiviral activity against HSV, VZV, and HCMV. By contrast, the application of the ProTide approach to PCV did not succeed in bypassing the first phosphorylation step. This result is confirmed by the modelling study of the L-alanine phosphoramidate monoester derivatives of PCV and GCV **6.27** and **6.29** within the active site of the human Hint-1 enzyme, showing that in the case of the PCV derivative **6.27** the P-N bond is not in a suitable position for the cleavage.

The anti-HIV-1 assay performed in MT-4 cell cultures afforded contrasting results. The L-alanine benyl ester derivatives of GCV and PCV ProTides **6.7a-d** showed activity against HIV-1 (EC_{50}: 8-17 μM). This result confirms the findings of the virtual screening previously performed in our group on the derivatives of ACV showing that GCV and PCV triphosphate are good candidates as inhibitors of the HIV-1 RT. This result also suggests that a different substrate affinity of the enzymes involved in the bioactivation of ProTides may occur in the MT-4 cells.

6.7 Bibliography

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Chapter 7. Phosphonoamidate prodrugs of cidofovir

7.1 Introduction

Cidofovir (*S*-HPMPC), tenofovir (*R*-PMPA), and adefovir (PMEA) are acyclic nucleoside phosphonates (ANPs) that exert broad antiviral activity (fig. 7.1).[1-3]

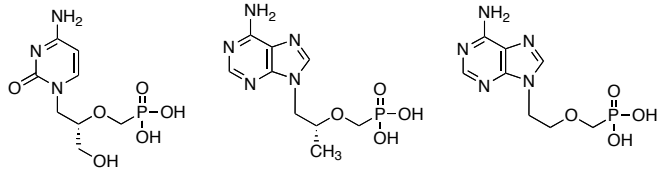


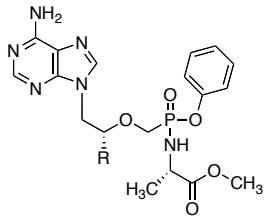
Figure 7.1. Structures of the ANPs cidofovir, tenofovir, and adefovir

In particular, cidofovir is active against a wide variety of DNA viruses including all the herpes viruses, poxviruses, polyomavirus, papillomavirus, and adenovirus.[2] Adefovir is active against herpes viruses, retroviruses, and hepadnaviruses displaying the broadest antiviral spectrum among the series of ANPs.[2] The antiviral spectrum of tenofovir is limited to retroviruses and hepadnaviruses.[2]

This class of nucleotide analogues are characterised by the replacement of the sugar moiety with an acyclic chain and by the presence of a phosphonate group that mimics the 5'-phosphate of natural nucleotides and confers resistance against extracellular phosphatases.[3] Inside the cell the ANPs are phosphorylated by cellular kinases to the diphosphate form, which in similarity to the triphosphate form of the antiviral nucleoside analogues inhibits the replication of the viral genome.[3]

As mentioned in chapter 1, in order to overcome the poor membrane permeability of the ANPs, due to the negative charge of the phosphonate group, several oral prodrugs of this class of compounds were synthesised.[4-7]

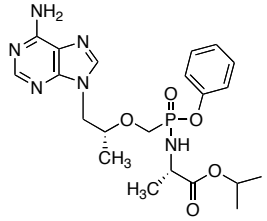
In particular, the ProTide approach was applied to tenofovir and adefovir in a previous study carried out in our group by Ballatore resulting in the formation of the phenyl L-alanine methyl ester phosphonoamidate derivatives **7.1** and **7.2** (fig. 7.2) that exhibited improved anti-HIV activity *in vitro* in comparison to the parent ANPs.[8]



7.1 R: CH_3
7.2 R: H

Figure 7.2. Structure of the phenyl L-alanine methyl ester phosphonamidate derivatives 7.1 and 7.2

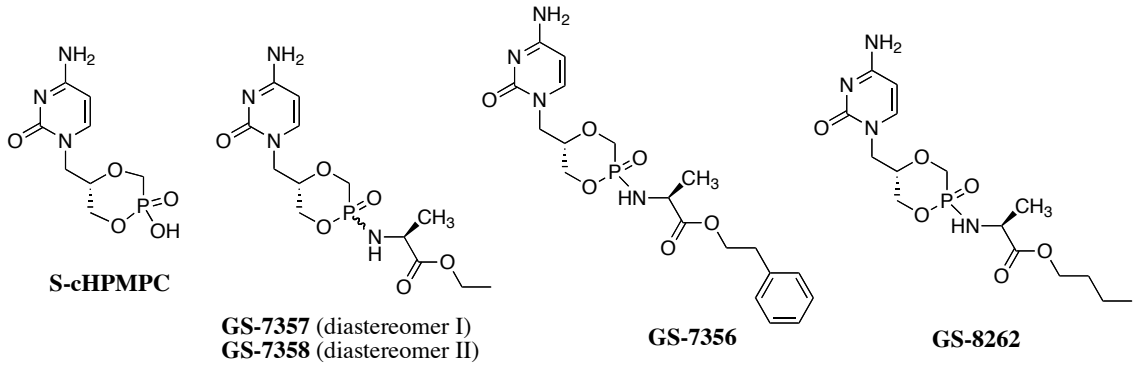
Also Gilead-Science investigated the aryl phosphonoamidate derivatives of ANPs extensively resulting in the synthesis of the prodrug of tenofovir GS-7340 (fig 7.3), which proved to be 500 to 1000-fold more potent than tenofovir against HIV *in vitro*. GS-7340 completed phase 1 clinical trials for the treatment of HIV infections.[9]



GS-7340

Figure 7.3. Structure of the phosphonoamidate prodrug GS-7340

Moreover, Gilead-Science has also applied similar approach to the synthesis of phosphonoamidate derivatives of cyclic cidofovir (*S*-cHPMPC) obtaining the L-alanine ethyl (GS-7357 diastereomer I, GS7358 diastereomer II), phenylethyl (GS-7356), and butyl (GS-8262) ester derivatives (fig 7.4), which were evaluated for their activity against vaccinia virus (VV) and cowpox virus (CPXV).[10] The two diastereomers of the L-alanine ethyl ester derivative (GS-7357 and GS7358) showed retention of the anti-poxvirus activity when compared to *S*-HPMPC, meanwhile compounds GS-7356 and GS-8262 were found to be 5- to 7-fold more potent than *S*-HPMPC.



S-cHPMPC

GS-7357 (diastereomer I)
GS-7358 (diastereomer II)

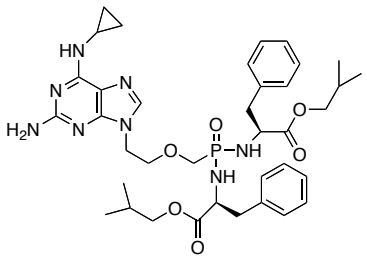
GS-7356

GS-8262

Figure 7.4. Structures of cyclic cidofovir and the L-alanine phosphonoamidate derivatives

As already mentioned in chapter 1, *S*-cHPMPC was proved to exert antiviral activity as prodrug of *S*-HPMPC.[11, 12] A study reported in the literature indicated that the cellular cyclic cytidine monophosphate (cCMP) phosphodiesterase is the intracellular enzyme responsible for the release of *S*-HPMPC by cleavage of the phosphodiester bond of *S*-cHPMPC.[13] However, no studies regarding the metabolism of the phosphonoamidate derivatives of *S*-cHPMPC were reported.

More recently, the phosphonodiamidate pronucleotide approach, which shares a similar mechanism of activation with ProTides,[14] have been applied to the antiproliferative agent 9-(2-phosphonomethoxyethyl)-N^6-cyclopropyl-2,6,diaminopurine (cPrPMEDAP) by Gilead-Science, resulting in the synthesis of the phosphonodiamidate derivative GS-9191 (fig 7.5), now in phase 1 clinical trials for the topical treatment of human papillomavirus-induced lesions.[15]



GS-9191

Figure 7.5. Structure of the phosphonodiamidate derivative GS-9191

These studies show that the amidate derivatives of ANPs succesfully overcome the poor membrane permeability of the parent drugs. For this reason, the purpose of this study is the synthesis of the amidate derivatives of *S*-HPMPC and *S*-cHPMPC and the evaluation of their biological activity.

7.2 Design of amidate derivatives cidofovir and cyclic cidofovir

As a first example of the synthesis of diamidate prodrugs of *S*-HPMPC the L-alanine benzyl ester phosphonodiamidate **7.3** was planned (fig 7.6). L-alanine was chosen as masking group on the basis of the results obtained in our lab on the anti-HIV activity of adefovir and tenofovir ProTides.[8] The benzyl ester was selected on the basis of studies reported in chapter 3 on acyclovir ProTides.

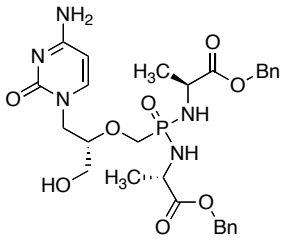


Figure 7.6. Structure of the phosphonodiamidate 7.3

The phosphonoamidates GS-7356 and GS-8262 (fig. 7.4) were proved to be more effective against VV and CPXV than *S*-HPMPC also suggesting that the amidate derivatives of *S*-cHPMPC successfully improve the cellular uptake of cidofovir.[10] The synthesis of the phosphonoamidate derivative of *S*-cHPMPC **7.4** was thus also considered (fig. 7.7).

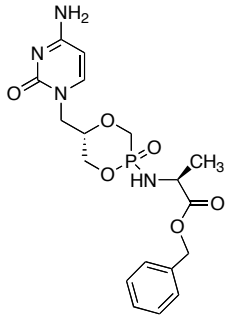
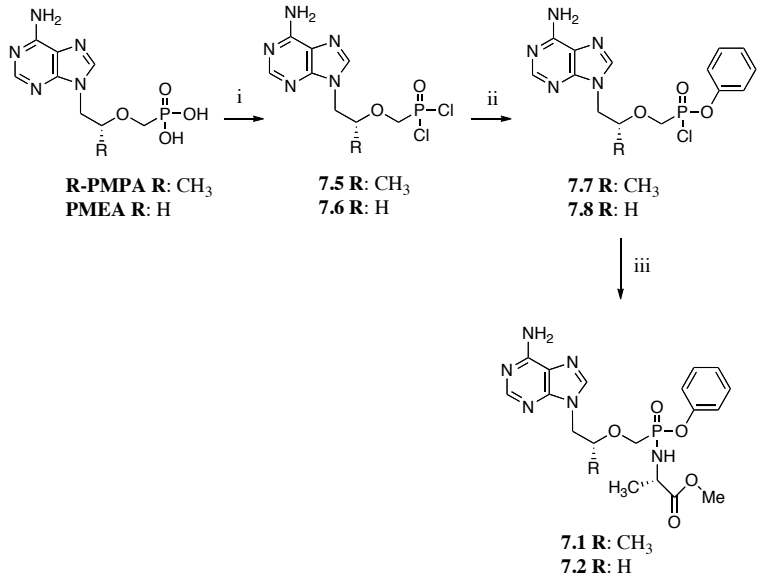


Figure 7.7. Structure of the phosphonoamidate 7.4.

7.2.1 Synthesis of the diamidate derivatives of cidofovir

The synthesis of adefovir and tenofovir ProTides was previously developed in our lab.[8] It is based on the replacement of the hydroxyl groups at the phosphonate moiety with chlorine atoms, which then act as leaving groups in the nucleophilic substitutions of the phosphorochloridate intermediates **7.5-7.8** with the aryloxy and the amino acid ester, affording the aryl phosphonamidate derivatives **7.1** and **7.2** (scheme 7.1). However, this methodology afforded the ProTides in extremely low yield (5%).



R-PMPA R: CH_3
PMEA R: H

7.5 R: CH_3
7.6 R: H

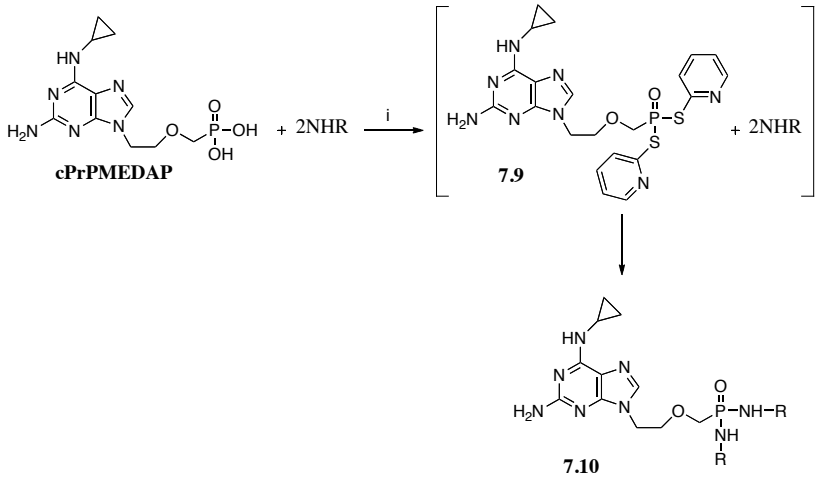
7.7 R: CH_3
7.8 R: H

7.1 R: CH_3
7.2 R: H

Reagent and conditions: (i) $SOCl_2$, anhydrous Pyr, anhydrous DCM, 30 min., rt; (ii) PhOH, TEA, anhydrous DCM, -78 °C, then rt, 16 h; (iii) L-ala-O-Me, TEA, anhydrous DCM, -78 °C, then rt, 16 h

Scheme 7.1. Synthetic route of amidate derivatives of tenofovir and adefovir described by Ballatore *et al.* (from ref. 8)

An alternative synthetic route has been reported for the synthesis of the aryl phosphonoamidate and phosphonodiamidate derivatives of the cyclic nucleoside phosphonate GS-9148 and the antiproliferative agent cPrPMEDAP by Gilead-Science.[16, 17] The synthesis of a generic diamidate derivative of cPrPMEDAP, performed according to this strategy, is depicted in scheme 7.2. The free phosphonic acid is activated toward the nucleophilic attack of the amino derivative (NH_2R) by oxidation-reduction condensation with 2,2'-dithiophyridine ($(PyS)_2$) and triphenylphosphine (PPh_3) affording the S,S-di-2-pyridyl-dithiophosphonate intermediate **7.9**, where the pyridylthio group is replaced by the nucleophilic species leading to the product **7.10**.[18]

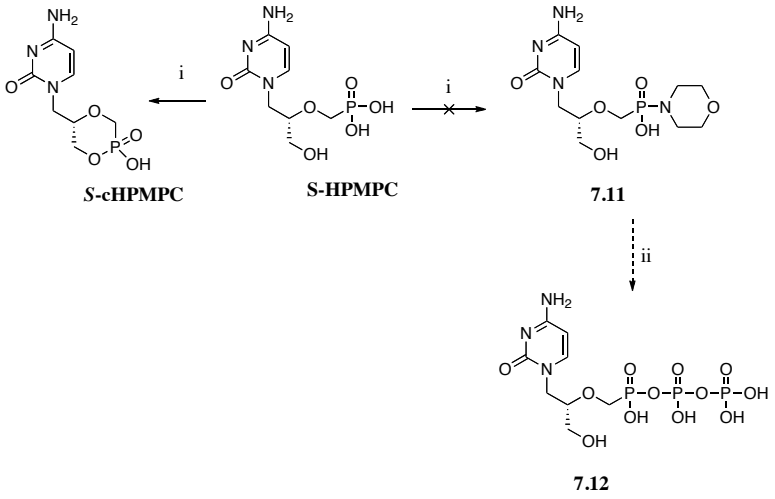


Reagents and conditions: (i) TEA, $(PyS)_2$, PPh_3, anhydrous Pyr, 60 °C, 16 h

Scheme 7.2. Synthetic route of the diamidate derivatives of cPrPMEDAP (7.10)

The yields of the reaction are variable (11-74%) and may depend on the nature of the amino acid,[19] but in general they were higher than the yields achieved through the Ballatore procedure. For this reason, we decide to apply this methodology also to the synthesis of **7.3**. However, using *S*-HPMPC as starting material the desired product was not obtained under these conditions.

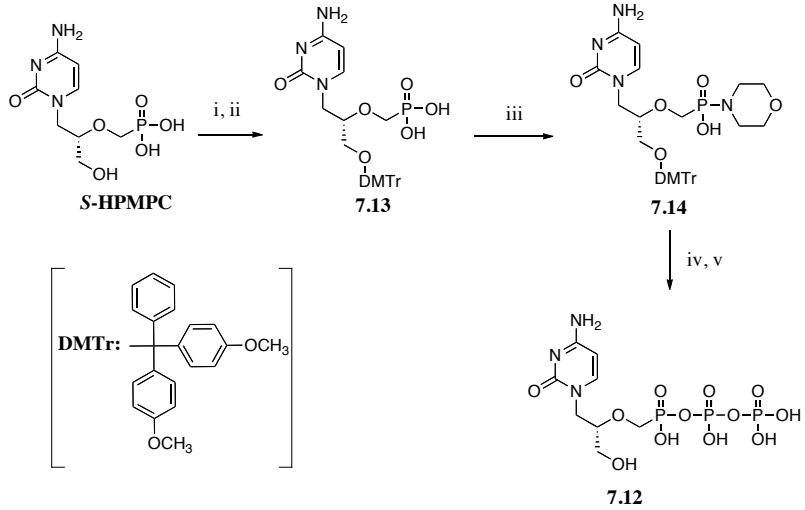
In a study previously reported, the synthesis of the morpholide derivative of *S*-HPMPC (**7.11**) was investigated as intermediate for the preparation of the diphosphoryl derivative (**7.12**).[20] Indeed, the standard procedure for the synthesis of triphosphate nucleoside analogues consists in conversion of the monophosphate derivative into its activate intermediate morpholide or imidazolide in the presence of 1,3-dicyclohexylcarbodiimide (DCC) as coupling regent followed by the reaction of the residue with inorganic diphosphate.[21,22] However, the synthesis of the morpholide derivative of *S*-HPMPC (**7.12**) was not succesful under these conditions and the internal cyclisation of the hydroxymethylene group with the phosphorus occurred affording instead the cyclic derivative of *S*-HPMPC (scheme 7.3).[20]



Reagents and conditions: (i) morpholine, DCC, 2-methylpropan-2-ol, reflux;
(ii) $(Bu_3NH)_2H_2P_2O_7$, DMSO

Scheme 7.3. Attempted synthesis of the diphosphoryl derivative of cidofovir (7.12) through the morpholide intermediate 7.11 resulting in the formation of cyclic cidofovir (from ref. 20)

The synthesis of the morpholide intermediate (**7.14)** and thus the diphosphate derivative **7.12** was accomplished by protecting the primary hydroxyl group of *S*-HPMPC with the dimethoxytrityl group (DMTr) (**7.13**) (scheme 7.4).[20]

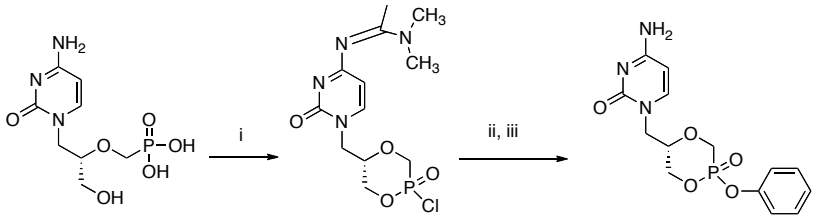


Reagents and conditions: (i) TBA, methanol, rt; (ii) DMMTr-Cl, DMSO, rt, 3 h; (iii) morpholine, DCC, 2-methylpropan-2-ol, reflux; (iv) $(Bu_3NH)_2H_2P_2O_7$, DMSO; (v) HCl

Scheme 7.4. Synthesis of the diphosphoryl derivative of cidofovir by means of hydroxymethylene tritylation (from ref. 20)

Moreover, during the synthesis of the phenyl ester derivative of *S*-cHPMPC (**7.16**) the phosphorochloridate **7.15** was obtained starting from cidofovir under the conditions of the Vilsmeier reaction (scheme 7.5).[23] This result confirms that the activation of the

phosphonic acid, by introduction of a good leaving group, induces intramolecular cyclisation with the hydroxymethylene group.

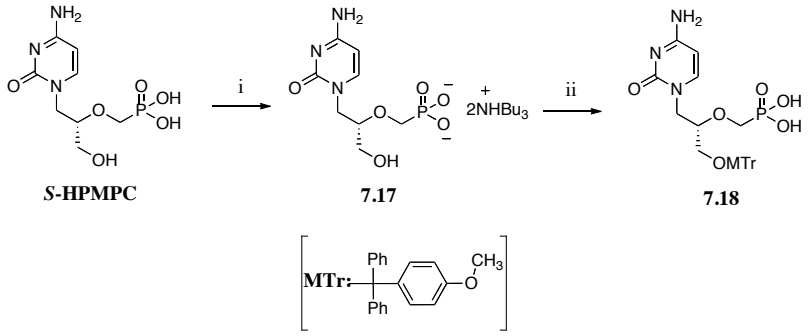


***S*-HPMPC** **7.15** **7.16**

Reagent and conditions : (i) $SOCl_2$, DMF, rt, 1 h; (ii) PhOH, NaH, DMF/THF, -78 ºC, then rt, 2h; (iii) AcOH, MeOH, rt

7.5. Synthesis of salicylate and aryl ester derivatives 198a-l (from ref. 23)

Considering these studies, it appeared necessary first to protect the hydroxymethylene group of the side chain of *S*-HPMPC in order to prepare the diamidate derivative **7.3**. Following a literature procedure,[20] the tritylation of the tributylammonium salt of *S*-HPMPC (**7.17**) afforded compound **7.18** in 32% yield as a free acid (scheme 7.6).

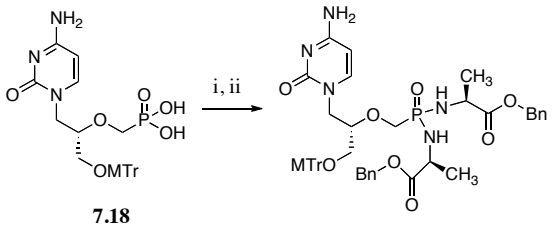


***S*-HPMPC** **7.17** **7.18**

Reagents and conditions: (i) TBA, methanol, rt; (ii) MTr-Cl, DMSO, rt, 3 h

Scheme 7.6. Tritylation of the primary hydroxyl group of *S*-HPMPC

The phosphonate **7.18** was treated with an excess of L-alanine benzyl ester (6 eq) in the presence of triethylamine (12 eq) in anhydrous pyridine at 60 °C under argon atmosphere. Addition of a solution of 2,2'-dithiopyridine (7 eq) and triphenylphosphine (7 eq) in pyridine at 60 °C afforded compound **7.19** in 20% yield (scheme 7.7).

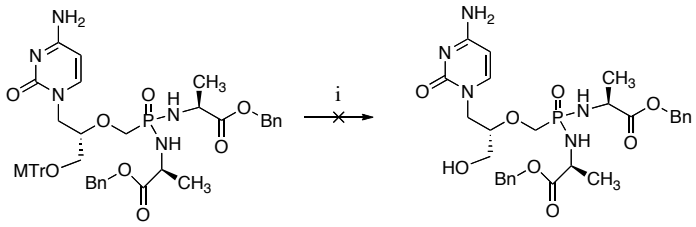


7.18 **7.19**

Reagents and conditions: (i) L-ala-O-Bn, TEA, anhydrous Pyr, 60 °C, 20 min; (ii) $(PyS)_2$, PPh_3, anhydrous Pyr, 60 °C, 16 h

Scheme 7.7 Synthesis of the diamidate derivative of *S*-HPMPC (7.19)

The detritylation of compound **7.19** was attempted using 5 equivalents of *p*-toluensulfonic acid (TsOH) in a mixture of dichloromethane and methanol at room temperature, as previously described for GCV and PCV (scheme 7.8). Unfortunately, the desired product **7.4** was not obtained. After 1 h the starting material had not reacted. An additional 2.5 equivalents of TsOH led to the degradation of **7.19**.

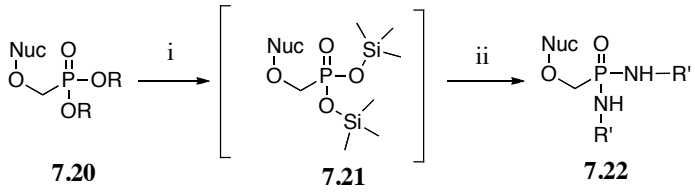


7.19 **7.4**

Reagents and conditions: (i) TsOH, DCM/MeOH, rt, 2 h

Scheme 7.8. Detritylation of compound 7.19

More recently, the improvement in the synthesis of diamidate prodrugs of ANPs was reported in the literature.[19] According to this strategy, the dialkyl phosphonate ester derivative of ANPs (**7.20)** was silylated with trimethylsilyl bromide (TMS-Br) affording the intermediate **7.21** (scheme 7.9), which was used in the next step without any purification. The reaction of the bis(trimethylsilyl) ester derivative **7.21** with 2,2'-dithiopyridine and triphenylphosphine in the presence of an excess of the amino acid ester (NH_2R) yielded the diamidate derivative of ANPs **7.22** (scheme 7.9) with improved yield (83-98%) when compared with those obtained with the same procedure starting from the free phosphonic acid (50-70%).

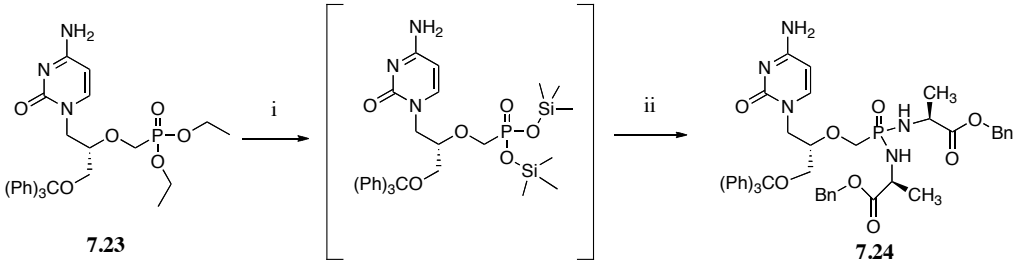


Nuc: acyclic nucleoside
R: ethyl. proyl

Reagents and conditions: (i) TMSBr, anhydrous ACN; (ii) NH_2R', TEA, anhydrous Pyr, 60 °C, 20 min; (iii) $(PyS)_2$, PPh_3, anhydrous Pyr, 60 °C, 16 h

Scheme 7.9. General synthetic route of diamidate prodrugs of ANPs starting from the dialkyl ester derivative

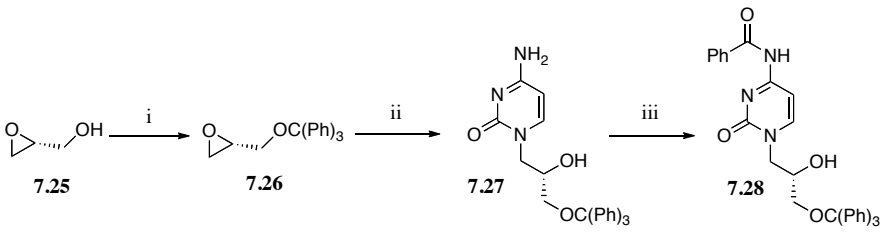
The application of this approach to the synthesis of the diamidate derivative of *S*-HPMPC **7.24** required (S)-N^1-[(3-triphenylmethoxy-2-diethylphosphonylmethoxy)-propyl)]cytosine (**7.23**) as starting material (scheme 7.10).



Reagents and conditions: (i) TMSBr, 2,6-lutidine, anhydrous ACN; (ii) L-ala-O-Bn, TEA, anhydrous Pyr, 60°C, 20 min; (iii) $(PyS)_2$, PPh_3, anhydrous Pyr, 60°C, 16 h

Scheme 7.10. General synthetic route of the diamidate derivative of *S*-HPMPC (7.24) starting from (S)-N^1-[(3-triphenylmethoxy-2-diethylphosphonylmethoxy)-propyl)]-cytosine (7.23)

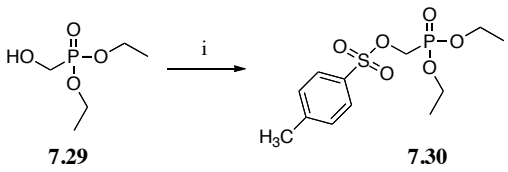
For this purpose, the synthesis of compound **7.23** was performed. Following the procedure described in the literature,[24, 25] (*R*)-glycidol (**7.25**) was protected using trityl chloride (TrCl) in the presence of TEA under argon atmosphere (i, scheme 7.11). The epoxy ring of the trityl derivative **7.26** was opened by reaction with cytosine (Cyt) and K_2CO_3 in DMF at 105 °C affording compound **7.27** in 71% yield (ii, scheme 7.11). The N^4-benzoyl derivative **7.28** was obtained in 83% yield protecting **7.27** with benzoic anhydride at 100 °C (iii, scheme 7.11).



Reagents and conditions: (i) TrCl, TEA, anhydrous DCM, rt, 3h; (ii) Cyt, K_2CO_3, anhydrous DMF, 105 °C, 5 h; (iii) Bz_2O, anhydrous Pyr/DMF, 100 °C, 3 h

Scheme 7.11. Synthesis of the intermediate 7.28

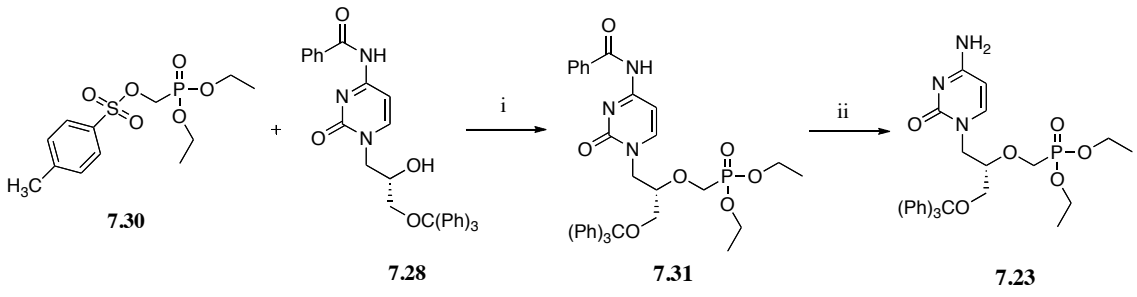
The tosylation of the diethyl(hydroxymethyl)phosphonate **7.29** was performed using *p*-toluensulfonyl chloride (TosCl) in the presence of DMAP and TEA and affording compound **7.30** in 61% yield (scheme 7.12).



Reagents and conditions: (i) ToSCl, DMAP, TEA, anhydrous DCM, 5 °C, 3 h

Scheme 7.12. Tosylation of diethyl-(hydroxymethyl)-phosphonate (7.29)

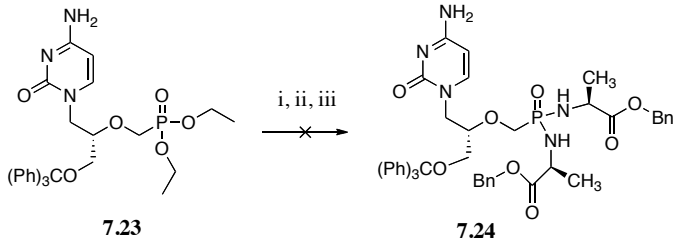
The coupling reaction of the tosyl derivative **7.30** with compound **7.28** in the presence of NaH at 5° C under argon atmosphere yielded the fully protected derivative of cidofovir **7.31** (i, scheme 7.13). Debenzoylation of **7.31** was performed using methanolic ammonia and afforded compound **7.23** in 95% yield (ii, scheme 7.13).



Reagents and conditions: (i) NaH, anhydrous DMF, 5 °C, 2 h; (ii) $NH_{3(g)}$, MeOH, rt, 3 h

Scheme 7.13 Synthesis of N^1-[(3-triphenylmethoxy-2-diethylphosphonylmethoxy)-propyl)]-cytosine (7.23)

According to the strategy previously described,[19, 26] the diethyl ester derivative **7.23** was silylated using trimethylsilyl bromide (TMSBr) in the presence of 2,6-lutidine under argon atmosphere (i, scheme 6.14). The crude of the reaction, without further purification, was treated with an excess of L-alanine benzyl ester (L-Ala-O-Bn) in anhydrous pyridine at 60 °C (ii, scheme 7.14), then a solution of 2,2'-dithiopyridine and triphenylphosphine in anhydrous pyridine was added (iii, scheme 7.14). Unfortunately the desired product (**7.24**) was not detected by mass spectrometry analysis of the crude of reaction.



7.23 **7.24**

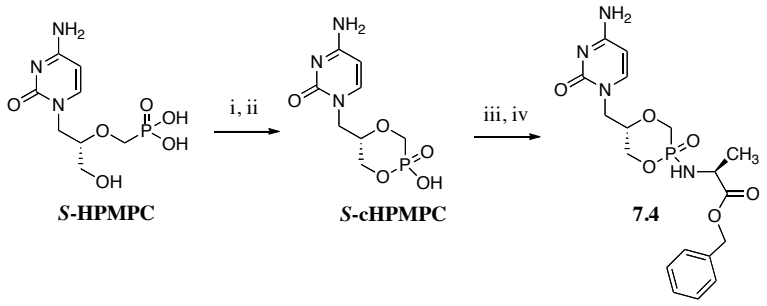
Reagents and conditions: (i) TMSBr, 2,6-lutidine, anhydrous ACN; (ii) L-ala-O-Bn, TEA, anhydrous Pyr, 60 °C, 20 min; (iii) $(PyS)_2$, PPh_3, anhydrous Pyr, 60 °C, 16 h

Scheme 7.14. Synthesis of the diamidate derivative of *S*-HPMPC through silylation of the diethyl ester derivative 7.23

In conclusion, the synthesis of the phosphonodiamidate **7.3** was not achieved perhaps due to the interference of the hydroxymethylene group of *S*-HPMPC and the unsuitability of its protection with the trityl group.

7.2.2 Synthesis of the amidate derivatives of cyclic cidofovir

Finally, the synthesis of the amidate derivative of *S*-cHPMPC (**7.4**) was performed. Following the procedure described in the literature,[27] the treatment of *S*-HPMPC with *N,N*-dicyclohexyl-4-morpholinecarboxamidine (DCMC) and 1,3-dicyclohexylcarbodiimide (DCC) afforded *S*-cHPMPC as dicyclohexyl-morpholinocarboxamidine salt in 50% yield (scheme 7.15). The reaction of *S*-cHPMPC with an excess of L-alanine benzyl ester in presence of 2,2'-dithiopyridine and triphenylphosphine in anhydrous pyridine at 60 °C afforded compound **7.4** in 30% yield as a mixture of two diastereomers (scheme 7.15).



Reagents and conditions: (i) DCMC, DMF, 12 h; (ii) DCC, Pyr, 100 °C, 16 h; (iii) L-ala-O-Bn, TEA, anhydrous Pyr, 60 °C, 20 min; (iv) $(PyS)_2$, PPh_3, anhydrous Pyr, 60 °C, 16 h

Scheme 7.15 Synthesis of the phosphonoamidate derivative 7.4

7.3 Biological evaluation of the amidate derivative of cyclic cidofovir

The amidate derivative of cyclic cidofovir **7.4** was evaluated for its activity against HSV, VZV, HCMV, and poxviruses such as vaccinia virus (VV), cowpox virus (CPXV), and camelpox virus (CMLV). The antirpoliferative activity of this compound on HEL cell cultures was also evaluated.

7.3.1 Anti-HSV activity

Table 7.1 reports the antiviral activity of **7.4** against HSV-1, HSV-2, and thymidine kinase-deficient (TK^-) HSV-1 in HEL cells. The anti-HSV activity of the parent compound, cyclic cidofovir (*S*-cHPMPC), was also evaluated. Cidofovir (*S*-HPMPC) and acyclovir (ACV) are included as reference compounds.

Table 7.1 Anti-HSV activity of *S*-cHPMPC and its phosphonamidate derivative 7.4 in HEL cells

	Antiviral Activity EC_{50}[a] (μM)		
Cps	**HSV-1**	**HSV-2**	**TK^- HSV-1**
7.4	0.3	0.4	0.2
***S*-cHPMPC**	0.8	1.2	0.5
***S*-HPMPC**	0.8	N.D.	0.6
ACV	0.5	0.4	>222

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

ACV, *S*-HPMPC and *S*-cHPMPC showed antiviral activity against HSV-1 and HSV-2 confirming the results reported in the literature.[3, 11, 28] As already described in chapter 1, inside the cell *S*-cHPMPC is converted to *S*-HPMPC by the cCMP phosphodiesterase-

mediated cleavage of its phosphodiester bond,[11] thus the antiviral activity of *S*-cHPMPC is due to the release of the diphosphate derivative of *S*-HPMPC as the active form.[11] Due to the presence of the phosphonate group, *S*-HPMPC and its prodrug *S*-cHPMPC, akin to monophosphate nucleoside analogues, do not require the HSV-TK-mediated phosphorylation to be bioactivated thus retaining activity also against TK⁻HSV-1 (EC_{50}= 0.20-0.65 μM), while ACV is completely inactive.

The phosphonoamidate derivative **7.4** exhibited anti-HSV activity in the submicromolar range (EC_{50}= 0.20-0.40 μM) retaining the activity of the parent compound *S*-cHPMPC.

The concentration required to cause a microscopically visible alteration of cell morphology (MCC) was found above 100 μM for **7.4**, as well as *S*-cHPMPC and *S*-HPMPC.

7.3.2 Anti-VZV activity

The antiviral activity of compounds **7.4** and its parent compound *S*-cHPMPC against TK-positive (TK^+) and TK-deficient (TK^-) strains of VZV are reported in table 7.2. ACV is included as a reference compound.

Table 7.2. Anti-VZV activity of *S*-cHPMPC and its phosphonamidate derivative 7.4 in HEL cells

	Antiviral Activity EC_{50}[a] (μM)			
	TK^+ VZV strains		**TK^- VZV strains**	
Cps	**YS**	**OKA**	**07-1**	**YS/R**
7.4	N.D.[c]	0.14	0.16	0.16
***S*-cHPMPC**	N.D.[c]	0.25	0.49	0.28
ACV	N.D.[c]	2.55	182	N.D.[c]

[a] 50% Effective concentration, or compound concentration required to reduce virus plaque formation by 50%

S-cHPMPC exhibited antiviral activity in the submicromolar range against both TK^+ and TK^- strains of VZV (EC_{50}= 0.25-0.49 μM), while ACV lost its activity against TK^- VZV (EC_{50}= 182 μM) due to the lack of virus-encoded TK required for phosphorylation to its monophosphate form.

The phosphonoamidate derivative **7.4** retained the anti-VZV activity of the parent compound (*S*-cHPMPC) against both TK^+ and TK^- strains (EC_{50}= 0.14-0.16 μM).

The minimum concentration of **7.4** required to cause a microscopically visible alteration of cell morphology was found to be above 100 μM

7.3.3 Anti-HCMV activity

Table 7.3 reports the antiviral activity of *S*-cHPMPC and its phosphonoamidate derivative **7.4** against AD-169 and Davis strains of HCMV in HEL cell cultures. GCV and *S*-HPMPC are included as reference compounds.

Table 7.3. Anti-HCMV activity of *S*-cHPMPC and its phosphonamidate derivative 7.4 in HEL cells

	Antiviral Activity EC_{50} [a] (μM)	
Cps	**AD-169 strain**	**Davis strain**
7.4	0.42	0.22
***S*-cHPMPC**	1.1	0.61
***S*-HPMPC**	0.97	0.69
GCV	11.38	4.45

[a] 50% Effective concentration, or compound concentration required to reduce virus plaque formation by 50%

In agreement with the studies reported in the literature,[3, 29] *S*-HPMPC and *S*-cHPMPC were active against HCMV displaying similar potency.

Compound **7.4** exerted anti-HCMV activity in the submicromolar range (EC_{50}= 0.22-0.42 μM) showing retention of activity in comparison to *S*-HPMPC and *S*-cHPMPC.

Also in this antiviral assay, microscopy analysis of the cell culture revealed that the minimum concentration of **7.4** required to cause alteration of cell morphology was above 100 μM.

7.3.4 Anti-poxvirus activity

The phosphonoamidate **7.4** and its parent compound *S*-cHPMPC were evaluated for their antiviral activity against Lederle and Western Reserve strains of vaccinia virus (VV), Brighton strain of cowpox virus (CPXV), and CMLV-1 strain of camelpox virusv (CMLV) in HEL cell cultures (table 7.4). Also *S*-HPMPC was evaluated for its anti-poxvirus activity and these results were compared with data reported in the literature.[30]

Table 7.4. Anti-poxvirus activity of *S*-cHPMPC and phosphonamidate derivative 7.4 in HEL cells

	Antiviral Activity EC_{50} [a] (μM)			
	VV		**CPXV**	**CMLV**
Cps	**Lederle strain**	**Western Reserve strain**	**Brighton strain**	**CMLV-1 strain**
7.4	0.94	2.0	2.38	2.15
***S*-cHPMPC**	2.57	10.10	4.52	4.10
***S*-HPMPC**	3.48	9.17	7.48	8.20

[a] 50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%

S-HPMPC exhibited anti-poxvirus activity in the micromolar range confirming the results reported in the literature,[30] *S*-cHPMPC retained the antiviral activity of *S*-HPMPC against VV, CPXV, and CMLV.

As already observed in antiviral assays against the herpes viruses, the phosphonoamidate derivative **7.4** exhibited retention of activity in comparison to *S*-HPMPC and *S*-cHPMPC.

7.3.5 Cytostatic activity of the amidate derivative of cyclic cidofovir on HEL cell cultures

The cytostatic activity on HEL cell cultures of **7.4** and its parent compound *S*-cHPMPC is reported in table 7.5.
These data include *S*-HPMPC, ACV, and GCV as reference compounds.

Table 7.5. Cytostatic activity of *S*-cHPMPC and its phosphonamidate derivative 7.4

	Cytostatic Activity IC_{50} [a] (μM)
Cps	**HEL Cells**
7.4	53
***S*-cHPMPC**	240
***S*-HPMPC**	330
ACV	>1778
GCV	520

[a] 50% Inhibitory concentration or compound concentration required to reduce cell proliferation by 50%

S-HPMPC, *S*-cHPMPC, ACV, and GCV exerted cytostatic activity only at high concentrations.

The amidate derivative of *S*-cHPMPC **7.4** exhibited moderate antiproliferative activity on HEL cell cultures (IC_{50}= 53 μM). As already described for the 6-alkoxy derivatives of ACV ProTide (chapter 4), it must be pointed out that the inhibition of cell growth was evaluated in non-confluent and highly proliferating HEL cells, while the antiviral assays previously described were performed on confluent resting HEL cells. It could be assumed that the higher metabolism of proliferating HEL cell may affect positively the bioactivation of **7.4**. Improvement in the bioactivation and cellular uptake, due to the masking of the negative charge of the phosphonate group, could account for the cytostatic activity of **7.4** detected in this assay.

7.3.6 Summary of the biological activity of the phosphonoamidate derivative of cyclic cidofovir

The results of the antiviral assays against herpes viruses (HSV, VZV, and HCMV) and poxivruses (VV, CPXV, CMLV) indicate that the phosphonoamidate derivative **7.4** released cyclic cidofovir, which was then converted inside the cell to cidofovir and its active form cidofovir diphosphate according to the metabolic pathway already described in the literature.[2,3,11,13] No significant improvement in the antiviral activity was observed compared to cidofovir and cyclic cidofovir. However, it must be pointed out that the improvement of the antiviral activity against poxvirus of analogous derivatives of cyclic cidofovir previously reported in the literature was observed in human foreskin fibroblast cell cultures, while the antiviral activity of **7.4** was evaluated in non-proliferating HEL cells.[10] The uptake, activation and conversion steps of this class of prodrugs may be dependent on the nature of the cell line and its metabolism, as also suggested by the cytostatic activity exerted by **7.4** on highly proliferating HEL cells (IC_{50}= 53 μM).

7.4 Enzymatic and modeling studies

Enzymatic and molecular modelling studies were performed in order to investigate the mechanism of activation of **7.4**.

7.4.1 Study of the carboxyesterase activity

In order to evaluate whether **7.4** could be processed following the same mechanism of bioactivation of phosphonoamidate prodrugs of ANPs,[9, 31] an enzymatic study was performed using carboxypeptidase Y (CPY) according to the procedure described in chapter 3.

Figure 7.8 depicts the ^{31}P NMR spectra and the proposed metabolic pathway of the enzymatic reaction performed on compound **7.4** over a 20 h period of incubation. The ^{31}P NMR spectra of compound **7.4** in deuterated acetone (acetone-d6) and phosphate buffer (pH= 7.6) showed two peaks at 17.80 and 18.98 ppm (blank, fig. 7.8A) corresponding to the two diastereomers (S*p* and R*p*). The mixture of diastereomers was not separated, so it was not possible to correlate the absolute configuration at the phosphorus center with the ^{31}P NMR signals. Normalisation of the integrals to the total sum of the spectrum was carried out on the data and used as an indicator for the relative abundance of the two diastereomers. The relative abundance in the blank of the diastereomers of **7.4** was 54% (δ_P = 17.80 ppm) and 46% (δ_P = 18.98 ppm) (fig. 7.8A). The ^{31}P NMR spectra at different time of the enzymatic study showed that:

- After 15 min from the exposure to the CPY, two peaks appeared at 18.39 ppm and 19.47 ppm. They may be tentatively correlated to the hydrolysis of the benzyl ester of compound **7.4** affording the intermediate **7.34** (fig. 7.8B).
- After 180 min from exposure to CPY, normalisation of the integrals to the total sum of the spectrum showed that the relative abundance of the two diastereomers of **7.4** was 17% (δ_P = 17.80 ppm) and 30% (δ_P = 18.98 ppm). Comparing this data with the relative abundance of the two diastereomers in the blank, the diastereomer corresponding to the signal at 17.80 ppm was found to be processed faster than the diastereomer corresponding to the signal at 18.98 ppm.

- After the 20 h period of incubation, the diastereomers of compound **7.4** with chemical shift at 17.80 ppm was completely processed, while 20% of the initial amount of the other (δ_P= 18.98 ppm) was still unreacted (fig. 7.8A).

The enzymatic reaction afforded two metabolites that displayed ^{31}P NMR signals at 17.17 ppm and 8.22 ppm, respectively (fig. 7.8A).

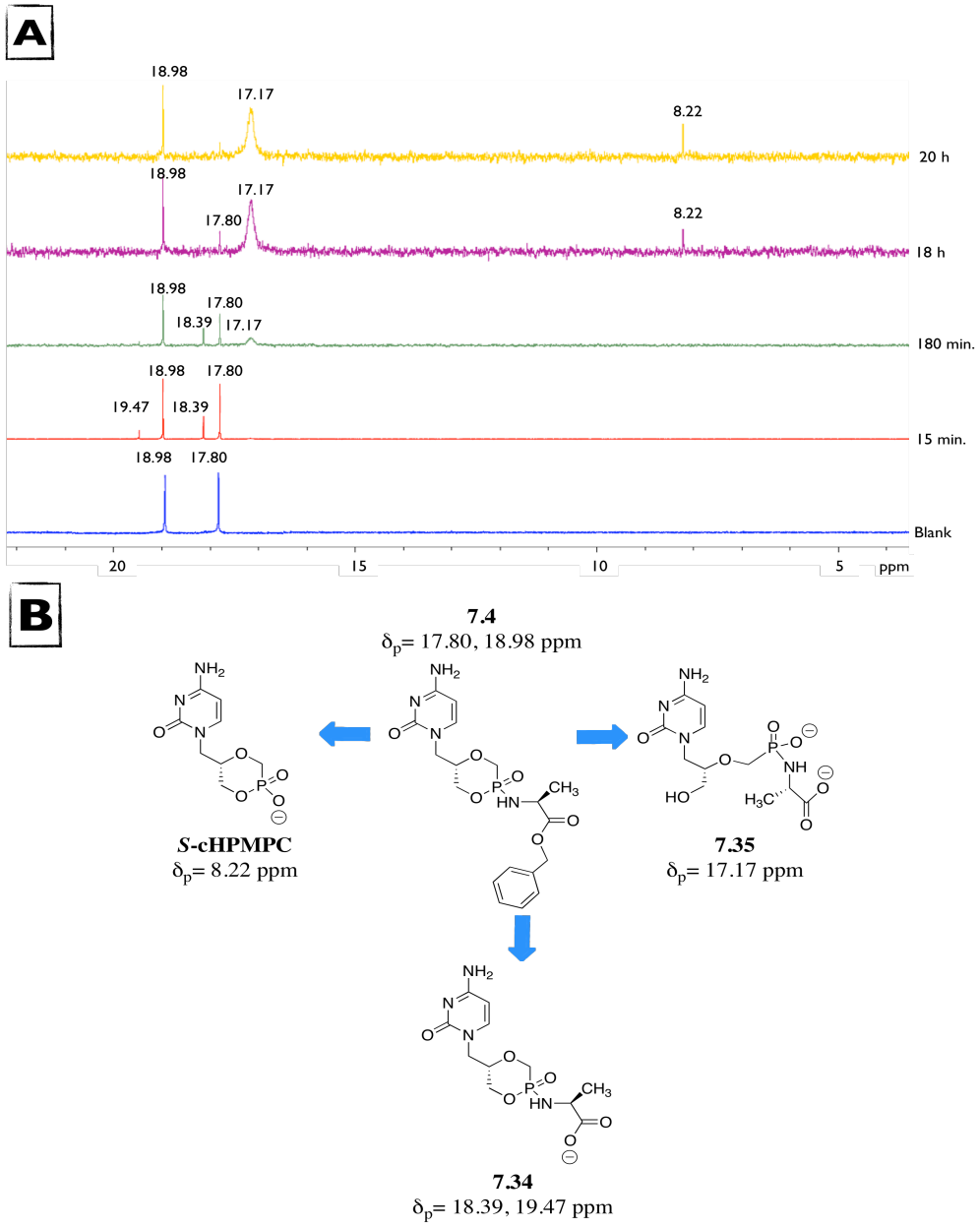


Figure 7.8. ^{31}P NMR spectra (acetone-D6, 202 MHz) and (B) proposed methabolic pathway of the enzymatic study performed on compound 7.4

After this period, the solution of the enzymatic reaction was treated with methanol, causing denaturation of the enzyme, and centrifuged.[32] The mass analysis performed on the supernatant liquid strongly suggests that one of the metabolites was *S*-cHPMPC (fig. 7.9). From the ^{31}P NMR experiment performed on *S*-cHPMPC in deuterated methanol,

this metabolite can be correlated to the signal at 8.22 ppm (fig. 7.8A). The mass analysis revealed also that the other metabolite with ^{31}P NMR signal at 17.17 ppm (fig. 7.8A) was the L-alanine conjugate of cidofovir (**7.35**), while the ^{31}P NMR signal at 18.98 (fig. 7.8A) is correlated to the unreacted diastereomer of **7.4** (fig. 7.9).

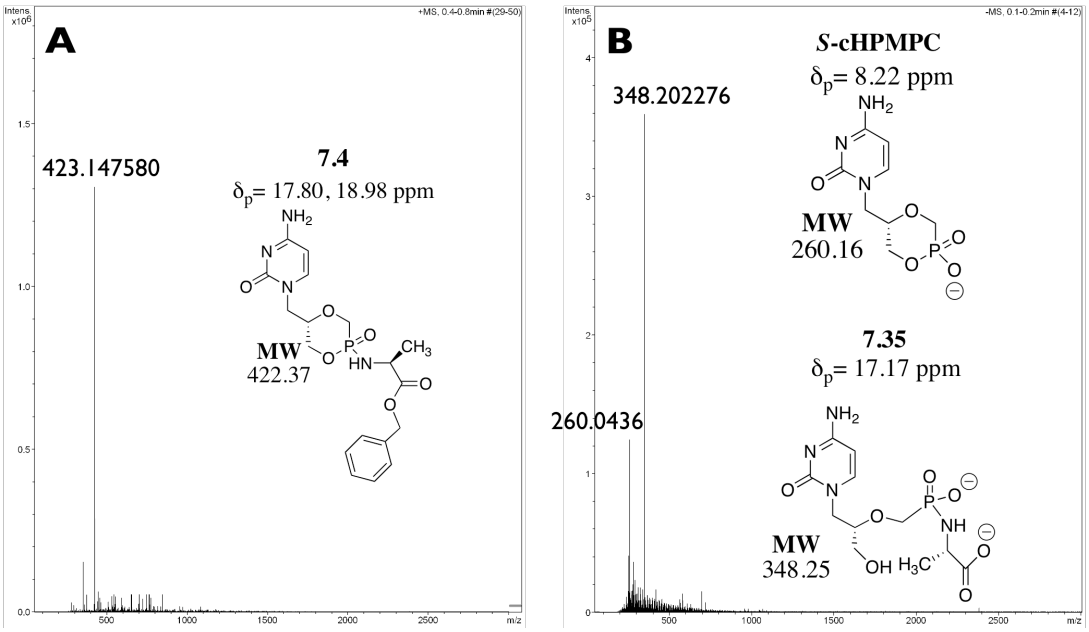
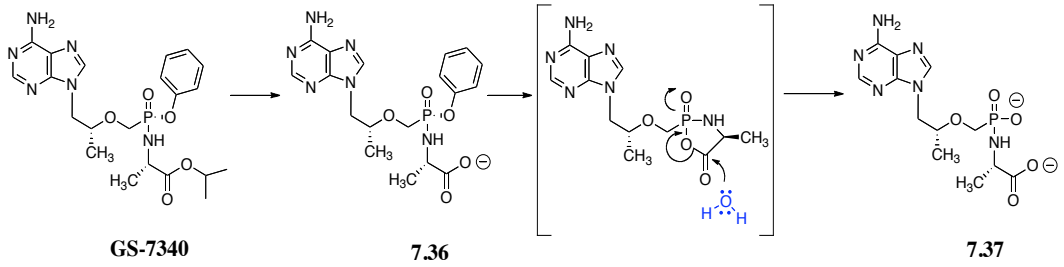


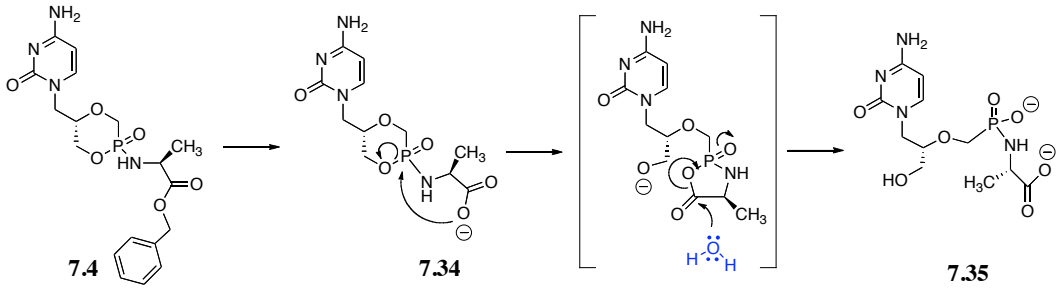
Figure 7.9. Mass analysis in positive mode (A) and negative mode (B) performed on the supernatant liquid of the enzymatic reaction after CPY denaturation with methanol and centrifugation

These results are in agreement to some extent with the metabolic study of the phosphonamidate prodrug GS-7340 reported in the literature showing that the cathepsin-A-mediated cleavage of the ester moiety, which affords the intermediate **7.36**, leads to formation of the metabolite **7.37** according to the putative mechanism of activation depicted in scheme 7.16.[9, 31, 33]



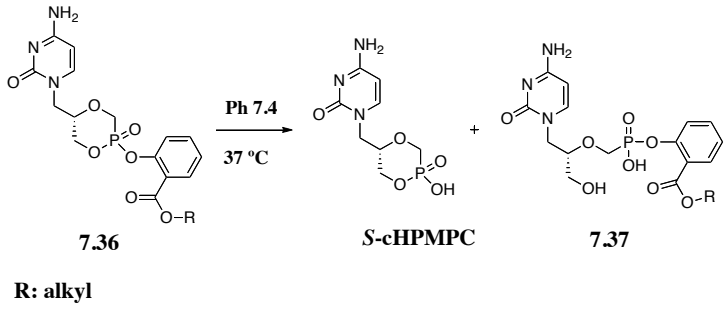
Scheme 7.16 Proposed mechanism of activation of GS-7340 mediated by cathepsin A (from ref.10, 32, 34)

It may be tentatively assumed that similar mechanism of reaction also occurs in the case of **7.4** in the presence of CPY leading to the formation of the metabolite **7.34** (scheme 7.17).



Scheme 7.17 Proposed mechanism of reaction of 7.4 mediated by CPY

The faster metabolism observed for one diastereomer over the other points out the role of the phosphorus stereochemistry in the bioactivation process. In analogy with this result, the half life of the *R*p diastereomer of phenyl L-alanine isopropyl ester derivative of tenofovir (GS-7339) in MT-2 cell cultures ($t_{1/2}$>1000 min.) has been reported to be longer in comparison to the *S*p diastereomer GS-7340 ($t_{1/2}$= 28.3 ± 7.4 min.) indicating a slower conversion to tenofovir as suggested by the 12-fold higher anti-HIV-1 activity of GS-7340 compared to that of GS-7339.[9] However, it must be taken into consideration that the stability study of the two diastereomers of salicylate phosphoester prodrugs of *S*-cHPMPC (**7.36**) in buffer solution at pH 7.4 and 37 °C, previously reported in the literature, showed that exocyclic and endocyclic hydrolysis of the P-O bond occur resulting in the formation of *S*-cHPMPC and the monoester derivative of *S*-HPMPC **7.37**, respectively (scheme 7.18).[23] Therefore, the chemical hydrolysis of the endocyclic P-O bond of compound **7.4** cannot be ruled out. This could also explain the formation of cyclic cidofovir detected during the enzymatic assay of **7.4** performed in the presence of CPY (fig 7.8).



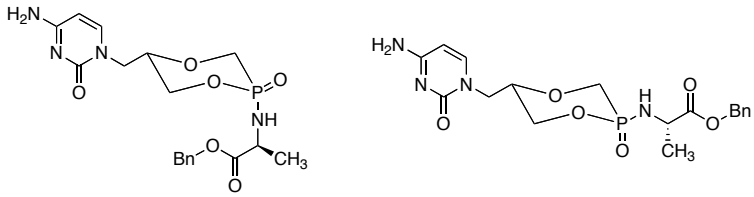
Scheme 7.18. Chemical hydrolysis of the salicylate phosphoester derivatives of cyclic cidofovir (7.36) at pH 7.4 and 37 ºC.

7.4.2 Docking of the L-alanine benzyl ester derivative of cyclic cidofovir within the active site of carboxypetidase Y.

A modeling study of **7.4** was performed using a crystal structure of CPY in order to investigate the interaction of the benzyl ester group with the residues of the catalytic site responsible for the carboxylester cleavage. Moreover this study meant to detect any difference of orientation of the ester moiety in an attempt to explain the lower reactivity of one diastereomer over the other in the enzymatic assay previously described (fig. 7.8).

The conformational study performed on salicylate and aryl phosphoester derivatives of *S*-cHPMPC previously described in the literature has shown that the 6-membered phosphonate ring of cyclic cidofovir adopts a chair conformation with the cytosine preferentially in an equatorial position.[23] However, it was also proved that in same cases the nucleobase of analogous compounds can be crystallised in axial position, and that in solution there could be an equilibrium between two different chair conformations where the nucleobase alternates between axial and equatorial position.[34]

The separation of the mixture of diastereomers was not achieved during the purification process of **7.4**. For this reason, it was not possible to carry out the studies of the crystal structure and NMR coupling constants required to assign the relative conformation of the phosphonate ring of each diastereomer. Thus, we assumed that the 6-membered phosphonate ring of **7.4** adopts a chair conformation with the cytosine in equatorial position placing the amino acid moiety at the phosphonate group in axial position in the case of the *R*p diastereomer (**7.4ax**) and in the equatorial position in the case of the *S*p diastereomer (**7.4eq**) (fig. 7.10), based on previous studies led on the salicylate and aryl phosphoester derivatives of *S*-cHPMPC.[23]



7.4ax (*R*p) **7.4eq (*S*p)**

Figure 7.10 Chair conformations of *R*p and *S*p diastereomers of 7.4

Figure 7.11 shows the docking of both phosphate diastereomers (*R*p and *S*p) of **7.4** within the active site of the CPY enzyme.

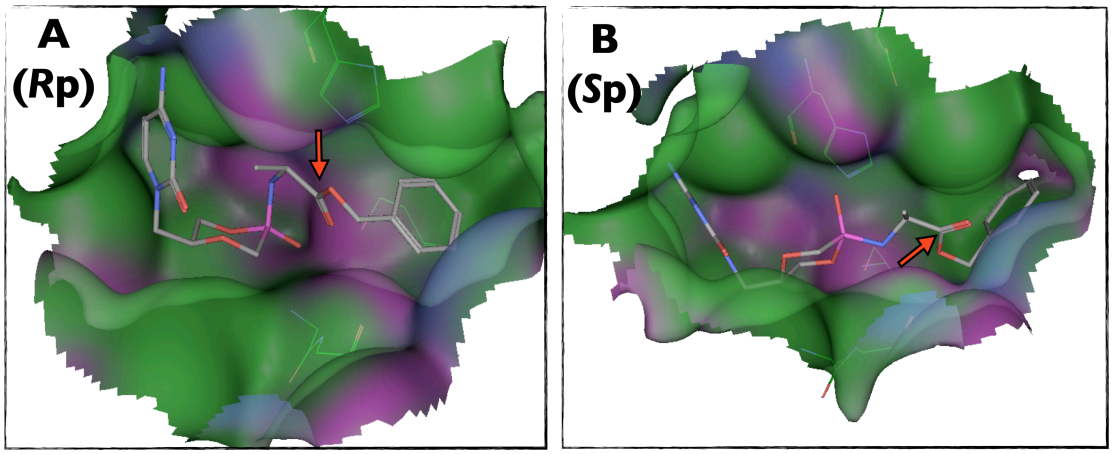


Figure 7.11 Docking of (A) *R*p and (B) *S*p diastereomers of 7.4 within the active site of CPY. The red arrows indicate the position of the carboxylester group

The carboxyl group of the *R*p diastereomer, indicated by the red arrow in figure 7.11A, is oriented in the opposite direction compared to the carboxyl group of *S*p diastereomer (fig. 7.11B) affecting the interaction with the glycine (Gly52 and Gly53) and serine (Ser146) residues, which are responsible for the benzyl ester cleavage, as already describe in chapter 3.[35] Indeed, in the case of the *R*p diastereomer the carboxyl group points toward the glycine and serine residues resulting in a suitable position for them to interact properly (fig 7.12A). By contrast, in the case of the *S*p diastereomer the orientation of the carboxyl group does not favour a positive interaction with these residues (fig 7.12B).

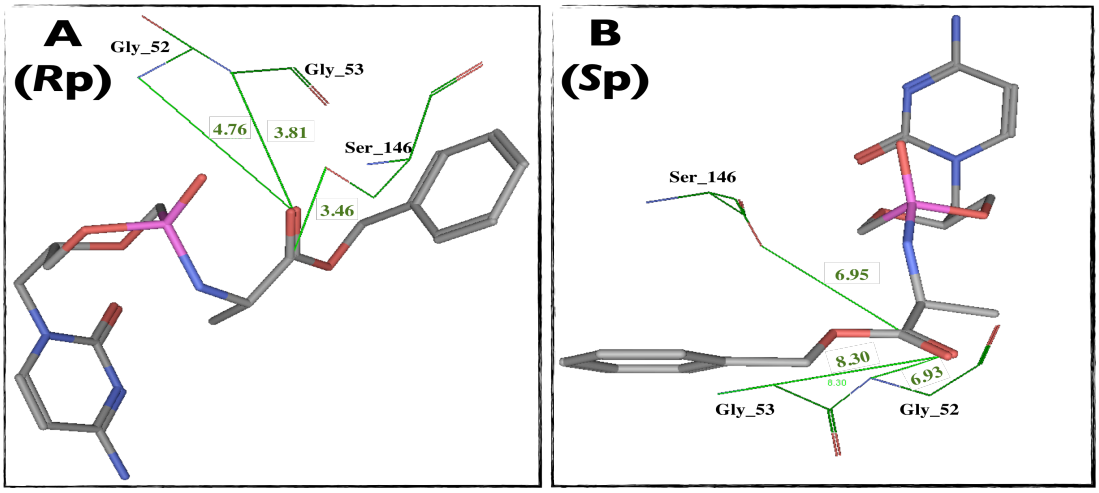


Figure 7.12 Detail of the docking of (A) *R*p and (B) *S*p diastereomers of 7.4 within the active site of the CPY enzyme showing the orientation and the distance in angstroms of the carboxyl group toward the glycine and the serine residues (Gly_52, Gly_53, and Ser_146) responsible for the benzyl ester cleavage.

Assuming that **7.4** is converted to **7.35** by carboxypeptidase Y according to the mechanism proposed in scheme 7.17, these results are in agreement with the enzymatic

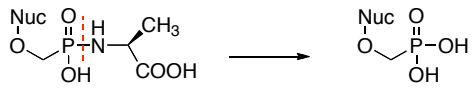
study previously described (fig. 7.8) showing that one of the two diastereomers of **7.4** is processed faster than the other. Moreover this study suggests that the *R*p diastereomer may be processed faster than the *S*p diastereomer.

7.4.3 Docking of the L-alanine conjugate of cidofovir within the active site of Hint-1 enzyme

According to the putative mechanism of activation of ProTides, the hydrolysis of the ester moiety of the aryl phosphoramidate derivatives, which leads to the elimination of the aryl group, is followed by the cleavage of the P—N bond.[36] In the case of phosphonoamidate and phosphonodiamidate derivatives of ANPs, this last step was investigated affording two possible mechanisms of reaction:[37]

- Chemical hydrolysis at acidic pH
- Enzymatic hydrolysis mediated by a phosphoramidase-type enzyme such as the human Hint-1

In particular, it was proved that the L-alanine conjugate of tenofovir **7.37** (matabolite of the phosphonoamidate derivative GS-7340 in the presence of cathepsin A) was converted to R-PMPA with a half life of 49 min at pH 4.5, while it was stable in the presence of human Hint-1 at pH 7.2 (scheme 7.19). Instead, the analogous L-alanine conjugate of cPrPMEDAP **7.38** was successfully converted by both chemical hydrolysis at acidic pH and human Hint-1-mediated cleavage of the P-N bond affording cPrPMEDAP as product and suggesting that both mechanisms of action could be involved in the conversion of the prodrug (scheme 7.19).[37]



7.37 Nuc: R-PMPA
7.38 Nuc: cPrPMEDAP

Nuc: R-PMPA
Nuc: cPrPMEDAP

Cps	Conditions	$T_{1/2}$ (min.)
7.37	pH 4.5	49
	Hint-1, pH7.2	Stable
7.38	pH 4.5	111
	Hint-1, pH7.2	154

Scheme 7.19 Half-lives ($t_{1/2}$) of nucleotide-amino acid conjugates 7.38 and 7.39 at pH 4.5 and in the presence of Hint-1 (from ref. 37)

The modelling study of the L-alanine conjugate of *S*-HPMPC (**7.35**) was performed within the active site of human Hint-1 enzyme co-crystallised with adenosine monophosphate (AMP) using docking techniques in order to evaluate whether this phosphoramidase-type enyme may be involved in the bioactivation of the phosphonoamidate of *S*-cHPMPC (fig 7.13).

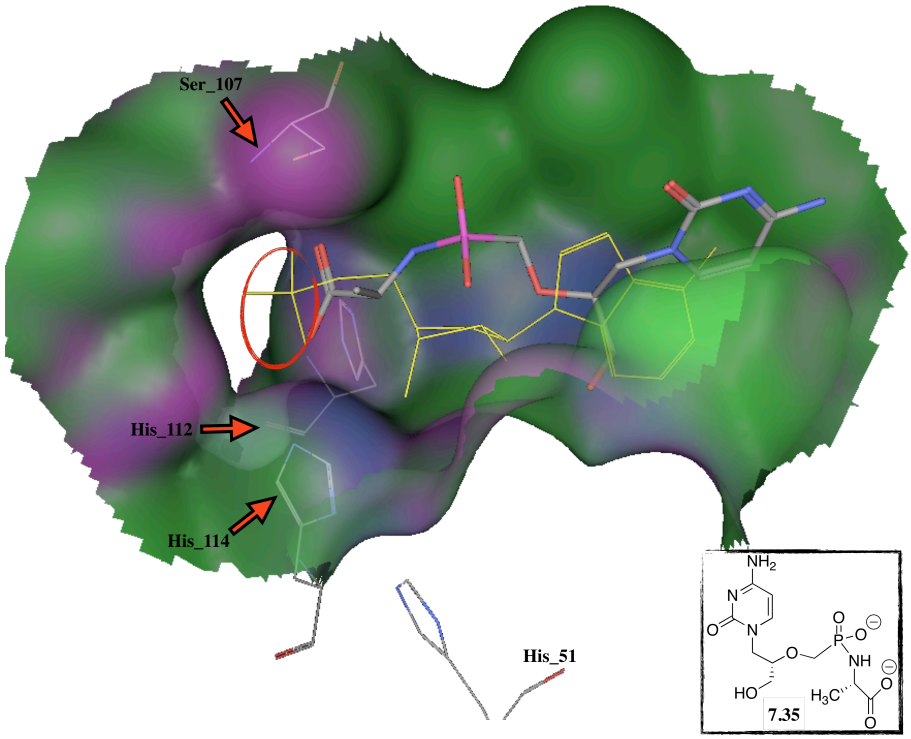


Figure 7.13 Superimposition of compound 7.35 with AMP (yellow) within the active site of human Hint-1 enzyme. The red circle highlights the phosphate moiety of AMP. The red arrows indicate the position of the residues of serine 107 (Ser_107), hisitidine 112 (His_112), and histidine 114 (His_114) involved in the cleavage of the P-N bond of compound 7.35 according to the mechanism of action of the Hint enzyme.[38]

The superimposition of **7.35** with AMP (shown in yellow in fig 7.13) within the enzymatic pocket of Hint-1 shows that the phosphorus of **7.35** (shown in purple in fig 7.13) is placed in a completely different position in comparison to the phosphate group of AMP (highlighted by the red circle in fig 7.13). As a result, the P-N bond of **7.35** is not in a suitable position to interact with the serine and histidine residues (indicated by the red arrows in fig. 7.13), which are responsible for its cleavage according to the mechanism of action of human Hint-1 enzyme.[38] The result of this study strongly suggests that **7.35** is a poor substrate for human Hint-1 and could be converted to cidofovir by chemical hydrolysis of the P-N bond at acidic pH, typical of lysosomes, as previously reported in the case of GS-7340.[37] In order to validate the modelling study, the docking of L-alanine conjugates of R-PMPA (**7.37**) and cPrPMEDAP (**7.38**) within

the active site of Hint-1 enzyme co-crystallised with AMP was carried out confirming the results previously reported in the literature (scheme 7.19).[37] In particular, the docking of the L-alanine conjugate of cPrPMEDAP (**7.38**) within the active site of human Hint-1 (fig. 7.14) shows that the nucleobase fits quite well into the enzymatic pocket adopting a position similar to that of AMP (shown in yellow), while the phosphonate moiety of **7.38** (purple) is moved from the position adopted by the phosphate group of AMP (highlighted by the red circle).

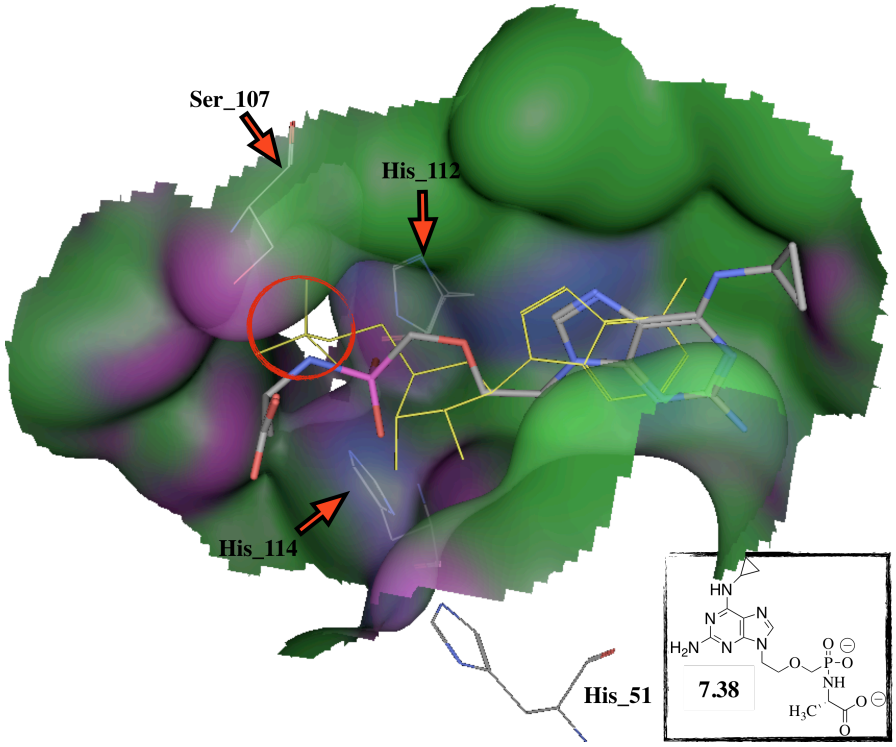


Figure 7.14 Superimposition of compound 7.38 with AMP (yellow) within the active site of human Hint-1 enzyme. The red circle highlights the phosphate moiety of AMP. The red arrows indicate the position of the residues of serine 107 (Ser_107), hisitidine 112 (His_112), and histidine 114 (His_114) involved in the cleavage of the P-N bond of compound 7.39 according to the mechanism of action of the Hint enzyme.[38]

Nevertheless, the P-N bond is in a suitable position to interact with serine and histidine residues, which are responsible for its cleavage, as also indicated more specifically in figure 7.15.

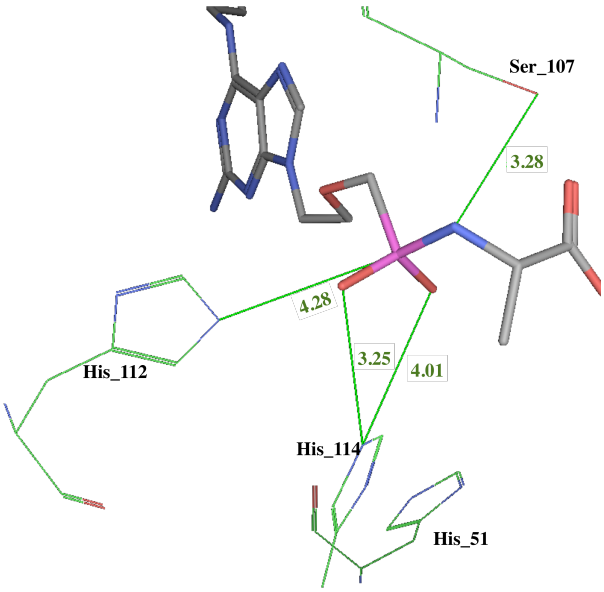


Figure 7.15. Detail of the docking of 7.38 within the active site of Hint-1 showing the orientation and the distance in angstroms of the P-N bond (purple and blue) toward the histidine and the serine residues (His-51, His-112, His 114, and Ser_107) responsible for its cleavage.

To the contrary, the phosphonate group of **7.37**, like in the case of **7.35**, is placed in a totally different position within the active site of human Hint-1 in comparison to AMP resulting in an unsuitable position to interact with the serine residue 107 and histidine residues 112 and 114 (fig. 7.16).

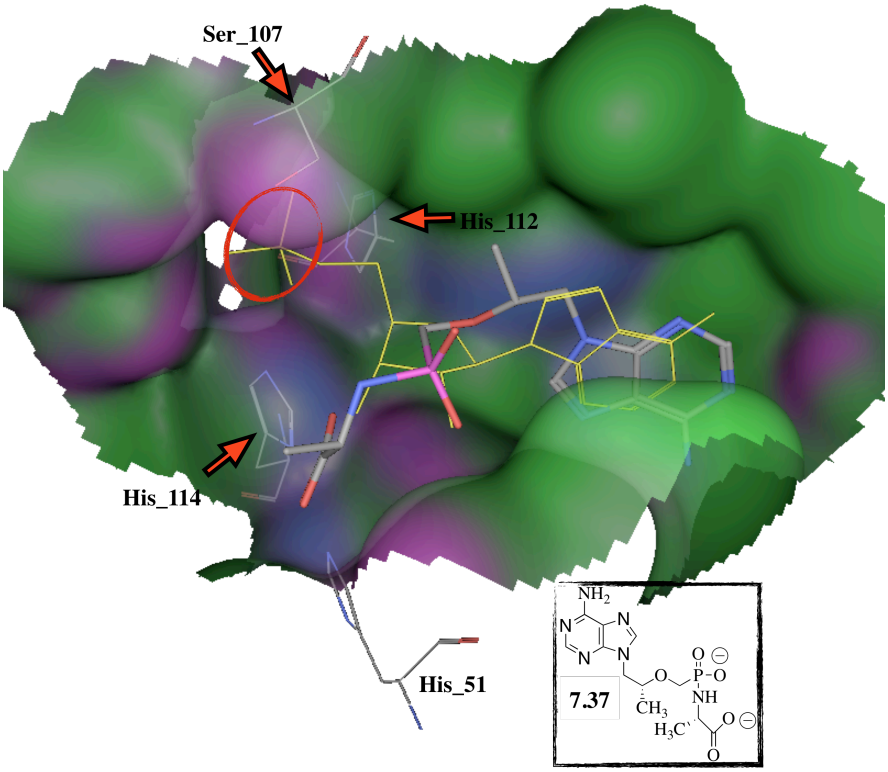


Figure 7.16. Superimposition of compound 7.37 with AMP (yellow) within the active site of human Hint-1 enzyme. The red circle highlights the phosphate moiety of AMP. The red arrows indicate the position of the residues of serine 107 (Ser_107), hisitidine 112 (His_112), and histidine 114 (His_114) involved in the cleavage of the P-N bond according to the mechanism of action of the Hint enzyme.[38]

7.5 Conclusion

The phosphonoamidate prodrugs of cidofovir were considered as a possible strategy to overcome the poor membrane permeability of the parent compound. The L-alanine benzyl ester derivative of cyclic cidofovir (**7.4**) was synthesised. Unfortunately the synthesis of the analogous phosphonodiamidate derivative of cidofovir (**7.3**) was not achieved. This result suggests that a different protective group of the hydroxymethylene side chain of cidofovir should be used in order to obtain the phosphonodiamidate derivatives of cidofovir.

The biological results show retention of antiviral activity of **7.4** against herpes viruses and poxviruses indicating the release of cidofovir inside the cell. No cytotoxic effects were observed during the antiviral assays by microscopy analysis of cell morphology. However, a moderate cytostatic activity was observed in proliferating HEL cells. This effect may be explained by improvement of cellular uptake of **7.4** in comparison to *S*-cHPMPC and *S*-HPMPC.

Enzymatic study of **7.4** was performed using carboxypeptidase Y and afforded the L-alanine conjugate of cidofovir (**7.35**) as main metabolite of the reaction. However, it is not clear whether the endocyclic cleavage occurs through the mechanism of reaction already described for the phosphoramidate and phosphonoamidate derivatives of nucleoside analogues or by chemical hydrolysis of the phosphoester bond, as previously described in the case of salicylate and aryl ester prodrugs of cyclic cidofovir. [23, 31, 33, 36, 37] The enzymatic study also showed also that one of the two diastereomers is converted faster than the other. The docking of **7.4** within the active site of CPY suggests that the diastereomer *R*p may interact better with the amino acid residues of the catalytic site than the diastereomer *S*p. The modelling study of **7.35** within the active site of human Hint-1 suggests that this metabolite is not converted to cidofovir by enzymatic hydrolysis of the P-N bond but by chemical hydrolysis at acidic pH as previously reported in the case of the aryl phosphonoamidate prodrug GS-7340.[37]

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Chapter 8. Main conclusions

The application of the ProTide approach to several acyclic nucleosides was evaluated in this work starting from acyclovir and varying the aryl, amino acid, and ester moieties, as well as specific positions of the side chain and guanine base.

ProTides bearing L-alanine have historically shown good results. For this reason, new projects have usually started from a family of L-alanine-derived ProTides. Indeed, the first ProTides of acyclovir reported in the literature were L-alanine derivatives.
Very importantly, the study of structure-activity relationship initially performed in this work on ACV ProTides showed that bulky amino acids such as L-valine, L-leucine, and L-isoleucne were well tolerated by the enzymes involved in their bioactivation pathway, as demonstrated by their antiviral activity against TK^- HSV and TK^- VZV, as well as HCMV and HIV-1 in MT-4 cells. These results further support the notion that the ProTide motif needs to be tuned on a case-by-case basis when applying this technology to a novel nucleoside. However, the lack of activity against HIV-1 and HIV-2 in CEM cells cultures of the aryl phosphoramidate derivatives of ACV synthesised in this work suggests that their activation might be dependent on the nature of the cell line.

The biological evaluation of 6-O-alkyl ACV ProTides as potential double prodrugs of ACV monophosphate showed that these derivatives were cytotoxic, perhaps the increase of their cellular uptake may account for these toxic effects.
The study of the aryl phosphoramidate derivatives of 8-bromoacyclovir, 8-methylacyclovir, ganciclovir and penciclovir showed that even small structural changes on the nucleoside moiety of ProTides can affect the processing of the phosphoramidate moiety, and thus the antiviral activity of these compounds. In particular, introduction of methyl group or bromine atom at the C-8 of the guanine base of ACV ProTides caused a significant loss of antiviral activity. The additional hydroxymethylene group on the side chain was well tolerated in the case of ganciclovir ProTides. The replacement of the oxygen on the side chain of ganciclovir with the methylene group of PCV affected negatively the antiviral activity of the ProTides derivatives against herpes viruses. However, PCV ProTides showed anti-HIV-1 activty in MT-4 cells comparable to that of GCV ProTides supporting the hypothesis that different substrate affinities of the enzymes involved in the bioactivation of PCV ProTides may occur in the MT-4 cells.

Collectively these results points out the necessity of evaluating the potential of each nucleoside and not relying on structural similarities for prediction of ProTides bioactivation.

Finally, the application of the phosphoramidate approach to cidofovir resulted in the synthesis of the L-alanine benzyl ester derivative of cyclic cidofovir, which showed retention of antiviral activity in comparison to the parent compound.

Chapter 9. EXPERIMENTAL PROCEDURES

9.1 General Experimental Details

Solvents and Reagents

All solvents and reagents commercially available were used without any further purification. The following solvents were purchased as anhydrous: chloroform (CHCl3), dichloromethane (DCM), diethyl ether (Et2O), *N*,*N*-dimethylformamide (DMF), pyridine, tetrahydrofyran (THF).All glassware was dried in the oven at 130°C for several hours and allowed to cool down.

Thin Layer Chromatography

The reactions were analysed by Thin Layer Chromatography (TLC) on commercially available Merck Kieselgel plates. Separated components were visualized using ultra violet light (245 and 366 nm). Preparative TLC plates (20x20 cm, 500-2000 μm) were purchased from Merck.

Column Chromatography (CC)

Column chromatography was performed using Silica gel (Fisher, 60A, 35-70μm) as stationery phase. Glass columns were packed in the appropriate eluent under gravity. Samples were applied as a concentrated solution in the same eluent, or pre absorbed onto silica gel. The fractions containing the product were analyzed by TLC then combined together and the solvent removed under vacuum.

NMR Spectroscopy

^{1}H, ^{13}C, ^{31}P were recorded on a Brucker Avance 500 spectrometer (500, 125, 202 MHz respectively) at 25 °C. Spectra were calibrated to the residual signal of the deuterated solvent used. Chemical shift are given in parts per million (ppm) and coupling costant (J) in Hertz. The following abbreviations are used in the NMR signals assignment: *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), *m* (multiplet), br s (broad singlet), $J_{C\text{-}P}$ (coupling between carbon and phosphorus), *J*gem (geminal coupling).

High Performance Liquid Chromatography (HPLC)

Analytical and semi-preparative experiments were ran on a Varian ProStar equipment (LC Work Station, Varian ProStar 335LC detector, Varian fraction collector model 701, ProStar 201 delivery system) using Varian Pursuit XRs 5C18 (150 x 4.6mm) as an analytical column and Varian Puruit XRs 5C18 (150 x 21.2 mm) as semi-preparative column. Used software was Galaxie Chromatography Data System.

Mass Spetroscopy (MS)

High and low resolution mass spectroscopy was performed as a service by CardiffUniversity, using electrospray (ES).

Elemental Analysis (CHN)

CHN microanalysis was performed as a service by MEDAC Ltd., Surrey.

UV Spectroscopy (UV)

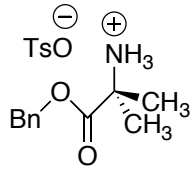
UV experiments were conducted using a Varian 50 Bio UV-Visisble spectrophotometer.

9.2. Experimental procedures for chapter 2: synthesis of aryl amino acid ester phosphorochloridates

Standard procedure A

To a stirred solution of the appropriate amino acid ester salt **2.4** (1 eq) and the appropriate aryl dichlorophosphate **2.5** (1 eq.) in anhydrous DCM was added dropwise at -78 °C anhydrous TEA (2 eq). Following the addition, the reaction mixture was stirred at -78 °C for 30 min, then at room temperature for 1 h. Formation of desired compound was monitored by ^{31}P NMR. After this period the solvent was removed under reduced pressure to give an oil. Most of the aryl phosphorochloridates synthesized were purified by flash column chromatography (eluting with ethyl acetate/petroleum ether= 70/30).

Synthesis of 2,2-Dimethylglycine benzyl ester tosylate [2.4a]

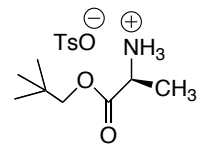


Chemical Formula: $C_{19}H_{25}NO_5S$
Molecular Weight: 379.4705

2,2-Dimethylglycine (1.02 g, 9.89 mmol), benzyl alcohol (15 mL) and *p*-toluenesulphonic acid (2.06 g, 10.83 mmol) were refluxed in toluene (13 mL) overnight using a Dean-Stark apparatus. After cooling to room temperature toluene was evaporated under reduced pressure. Upon standing (1 h) a solid formed from the crude oil and Et_2O was added to give the product as a white precipitate. The mixture was filtered and the white solid washed with Et_2O and dried under vacuum. (Yield: 80%, 2.90 g)

^{1}H NMR (500 MHz, CDCl3): δ 7.74 (2H, d, *J* = 8.1 Hz, Ts), 7.44-7.35 (5H, m, CH_2*Ph*), 7.10 (2H, d, *J* = 8.1 Hz, Ts), 5.12 (2H, s, OCH_2), 2.32 (3H, s, CH_3 (Ts)), 1.54 (6H, s, 2 x CH_3).

Synthesis of L-alanine neopentyl ester tosylate [2.4b]



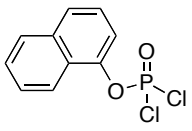
Chemical Formula: $C_{15}H_{25}NO_5S$
Molecular Weight: 331.4277

L-alanine (4 g, 44.89 mmol), neopentyl alcohol (25 mL) and *p*-toluenesulphonic acid (9.48 g, 49.84 mmol) were refluxed in toluene (60 mL) overnight using a Dean-Stark apparatus. After cooling to room temperature toluene was evaporated under reduced pressure. Upon standing (1 h) a solid formed from the crude

oil and Et_2O was added to give the product as a white precipitate. The mixture was filtered and the white solid washed with Et_2O and dried under vacuum. (Yield: 80%, 12g)

^{1}H NMR (500 MHz, MeOD): δ 7.73 (2H, d, *J* = 8.1, Ts), 7.26 (2H, d, *J* = 8.1, Ts), 4.16 (1H, q, *J* = 7.2 Hz, C*H*CH3), 4.03 (1H, d, *J*gem = 10.5 Hz, COOC*H*$_2$(CH$_3$)$_3$), 3.92 (1H, d, *J*gem = 10.5 Hz, COOC*H*$_2$(CH$_3$)$_3$), 2.39 (3H, s, CH$_3$ (Ts)), 1.58 (3H, d, *J* = 7.2 Hz, CHC*H*$_3$), 1.00 (9H, s, COOCH$_2$(C*H*$_3$)$_3$).

Synthesis of 1-naphthyl dichlorophosphate [2.5b]

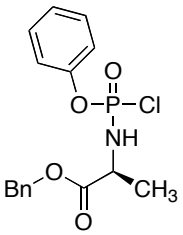


Chemical Formula: $C_{10}H_7Cl_2O_2P$
Molecular Weight: 331.4277

Phosphorus oxychloride (2.59 mL, 27.74 mmol) and 1-naphthol (4.00 g, 27.74 mmol) were stirred in anhydrous Et_2O under an argon atmosphere. Anhydrous TEA was added (3.87 mL, 27.74 mmol) at -78 ºC and after 30 minutes, the solution was allowed to warm to room temperature. After ^{31}P NMR, the solvent was removed under reduced pressure and the residue was triturated with anhydrous Et_2O to give the crude product as yellowish oil. (Crude yield 95%, 6.91 g).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.72. ^{1}H-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 phenyl-(benzyloxy-L-alaninyl) phosphorochloridate [2.2a]

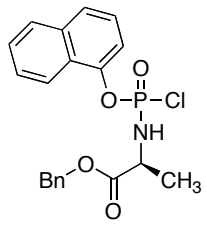


Chemical Formula: $C_{16}H_{17}ClNO_4P$
Molecular Weight: 353.7372

Prepared according to standard procedure A, using phenyl dichlorophosphate (1.27 mL, 8.50 mmol), L-alanine benzyl ester tosylate (3 g, 8.5 mmol), anhydrous TEA (2.36 mL, 17 mmol) in anhydrous DCM (70 mL). The reaction mixture was stirred at -78 ºC for 30 min., then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (86%, 2.57 g).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 8.05, 7.52. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.44-7.25 (10H, m, PhO, OCH$_2$P*h*), 5.24-5.22 (2H, m, OC*H*$_2$Ph), 4.30-4.21 (2H, m, C*H*NH, CHN*H*), 1.56-1.54 (3H, m, CHC*H*$_3$).

Synthesis of 1-naphthyl-(benzyloxy-L-alaninyl) phosphorochloridate [2.2b]

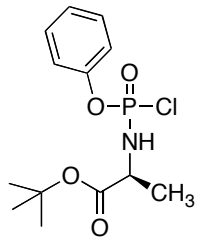


Chemical Formula: $C_{20}H_{19}ClNO_4P$
Molecular Weight: 403.7959

Prepared according to standard procedure A, using naphthyl dichlorophosphate (0.30 mL, 2 mmol), L-alanine benzyl ester hydrochloride (0.43 g, 2 mmol), anhydrous TEA (0.56 mL, 4 mmol) in anhydrous DCM (15 mL). The reaction mixture was stirred at -78 ºC 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. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.33-7.28 (10H, m, NaphO, OCH_2Ph), 5.15-5.13 (2H, m, OCH_2Ph), 4.18-4.13 (1H, m, CHNH), 1.46-1.44 (3H, m, $CHCH_3$).

Synthesis of phenyl-(*tert*-butoxy-L-alaninyl) phosphorochloridate [2.2c]

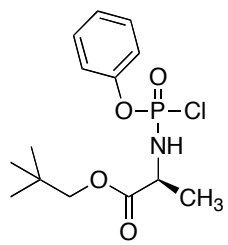


Chemical Formula: $C_{13}H_{19}ClNO_4P$
Molecular Weight: 319.721

Prepared according to standard procedure A, phenyl dichlorophosphate (3.95 mL, 26.54 mmol), L-alanine *tert*-butyl ester tosylate (4.8 g, 26.42 mmol), anhydrous TEA (7.36 mL, 52.84 mmol) in anhydrous DCM (80 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 30 min. The crude was purified by column chromatography eluting with ethyl acetate/hexane = 70/30 to give an oil (64%, 5.4 g).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 8.20, 7.81. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.41-7.38 (2H, m, PhO), 7.30-7.24 (3H, m, PhO), 4.35-4.24 (1H, m, NH), 4.14-4.01 (1H, m, $CHCH_3$), 1.52-1.49 (12H, m, $C(CH_3)_3$, $CHCH_3$).

Synthesis of phenyl-(neopentyloxy-L-alaninyl) phosphorochloridate [2.2d]



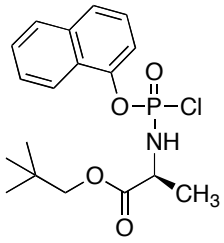
Chemical Formula: $C_{14}H_{21}ClNO_4P$
Molecular Weight: 333.7476

Prepared according to standard procedure A, from phenyl-dichlorophosphate (1.13 mL, 7.54 mmol), L-alanine neopentyl ester tosylate (2.5 g, 7.54 mmol), anhydrous TEA (2.10 mL, 15.08 mmol) and anhydrous DCM (45 mL). The reaction mixture was stirred at -78 ºC for 30

min, then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (quantitative yield , 2.48 g).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 7.66, 8.19. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.44-7.26 (5H, m, PhO), 4.42-4.37 (1H, m, NH), 4.34-4.21 (1H, m, C*H*CH_3), 3.97-3.93 (1H, m, $COOCH_2(CH_3)_3$ of one diasteroisomer), 3.90-3.86 (1H, m, $COOCH_2(CH_3)_3$ of one diasteroisomer), 1.58-1.55 (3H, m, $CHCH_3$), 0.99 (4.5H, s, $COOCH_2(CH_3)_3$ of one diastereomer), 0.98 (4.5H, s, $COOCH_2(CH_3)_3$ of one diastereomer.

Synthesis of phenyl-(neopentyloxy-L-alaninyl) phosphorochloridate [2.2e].

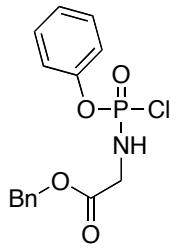


Chemical Formula: $C_{18}H_{23}ClNO_4P$
Molecular Weight: 383.8063

Prepared according to standard procedure A, from naphthyl dichlorophosphate (1.97 g, 7.54 mmol), L-alanine neopentyl ester tosylate (2.5 g, 7.54 mmol), anhydrous TEA (2.10 mL, 15.08 mmol) and anhydrous DCM (45 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 2 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (63%, 1.8 g).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 8.21, 7.90. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.11-7.45 (7H, m, NaphO), 4.48-4.44 (1H, m, NH), 4.38-4.35 (1H, m, C*H*CH_3), 3.99-3.84 (2H, m, $COOCH_2(CH_3)_3$), 1.62-1.58 (3H, m, $CHCH_3$), 0.99 (4.5H, s, $COOCH_2(CH_3)_3$ of one diastereomer), 0.98 (4.5H, s, $COOCH_2(CH_3)_3$ of one diastereomer.

Synthesis of phenyl-(benzyloxy-glicinyl) phosphorochloridate [2.2f].



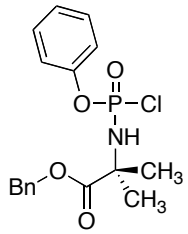
Chemical Formula: $C_{15}H_{15}ClN_4OP$
Molecular Weight: 339.7107

Prepared according to standard procedure A, from phenyl dichlorophosphate (2.21 mL, 14.81 mmol), glycine benzyl ester tosylate (5.00 g, 14.81 mmol), anhydrous TEA (4.20 mL, 30.00 mmol) and anhydrous DCM (100 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (90%, 4.50 g).

^{31}P NMR ($CDCl_3$, 202 MHz): δ 8.75. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.45-7.24 (10H, m, PhO, OCH_2Ph), 5.26 (2H, s, OCH_2Ph), 4.27-4.26 (1H, bs, NH), 4.04-3.91 (2H, m,

NHCH_2).

Synthesis of phenyl-(benzyloxy-dimethylglycinyl) phosphorochloridate [2.2g].

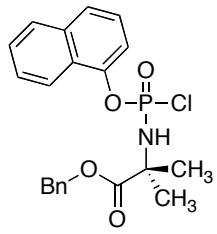


Chemical Formula: $C_{17}H_{19}ClNO_4P$
Molecular Weight: 367.7638

Prepared according to the standard procedure A, from phenyl dichlorophosphate (2.45 mL, 16.40 mmol) and 2,2-dimethylglycine benzyl ester tosylate (6.00 g, 16.4 mmol), anhydrous TEA (4.58 mL, 33.00 mmol) and anhydrous DCM (150 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (90%, 5.50 g).

^{31}P NMR ($CDCl_3$, 202 MHz): δ 5.43. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.41-7.23 (10H, m, PhO, OCH$_2$*Ph*), 5.24 (2H, s, OC*H*$_2$Ph), 4.70-4.68 (1H, bs, NH), 1.74, 1.72 (6H, 2s, C(CH$_3$)$_2$).

Synthesis of 1-naphthyl-(benzyloxy-dimethylglycinyl) phosphorochloridate [2.2h]

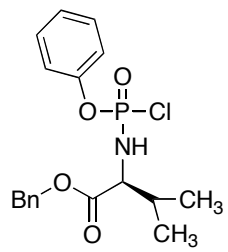


Chemical Formula: $C_{21}H_{21}ClNO_4P$
Molecular Weight: 417.8225

Prepared according to the standard procedure A, from naphthyl dichlorophosphate (1.53 g, 5.88 mmol) and 2,2-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 mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (quantitative yield, 2.7 g).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 5.83. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.13-7.33 (12H, m, Naph, OCH$_2$*Ph*), 5.25 (2H, s, OC*H*$_2$Ph), 1.80, 1.76 (6H, 2s, C(CH$_3$)$_2$).

Synthesis of phenyl-(benzyloxy-L-valinyl) phosphorochloridate [2.2i]



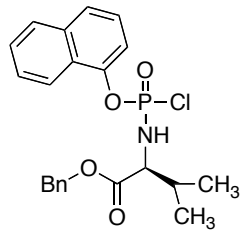
Chemical Formula: $C_{18}H_{21}ClNO_4P$
Molecular Weight: 381.7904

Prepared according to standard procedure A, from phenyl-dichlorophosphate (0.91 mL, 6.13 mmol), L-valine benzyl ester tosylate (2.33 g, 6.13 mmol), anhydrous TEA (1.71 mL, 12.26

mmol) and anhydrous DCM (50 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (77%, 1.80 g).

^{31}P NMR ($CDCl_3$, 202 MHz): δ 9.37, 8.89. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.42-7.23 (10H, m, PhO, OCH_2Ph), 5.24-5.23 (2H, m, OCH_2Ph), 4.08-3.96 (1H, m, NHC*H*), 2.24-2.17 (1H, m, C*H*(CH_3)$_2$), 1.05-1.01 (3H, m, CH(CH_3)$_2$), 0.94-0.91 (3H, m, CH(CH_3)$_2$).

Synthesis of 1-naphthyl-(benzyloxy-L-valinyl) phosphorochloridate [2.2j]

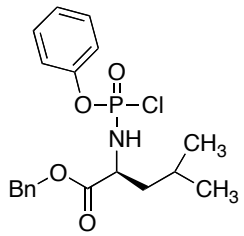


Chemical Formula: $C_{22}H_{23}ClNO_4P$
Molecular Weight: 431.8491

Prepared according to standard procedure A, from naphthyl dichlorophosphate (1.72 g, 6.58 mmol), L-valine benzyl ester tosylate (2.5 g, 6.58 mmol), anhydrous TEA (1.83 mL, 13.16 mmol) and anhydrous DCM (50 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (quantitative yield %, 2.8 g).

^{31}P NMR ($CDCl_3$, 202 MHz): δ 9.72, 9.26. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.09-7.31 (12H, m, Naph, OCH_2Ph), 5.24-5.16 (2H, m, OCH_2Ph), 4.29-4.20 (1H, m, NHC*H*), 2.26-2.18 (1H, m, C*H*(CH_3)$_2$), 1.05-1.02 (3H, m, CH(CH_3)$_2$), 0.96-0.92 (3H, m, CH(CH_3)$_2$)

Synthesis of phenyl-(benzyloxy-L-leucinyl) phosphorochloridate [2.2k].



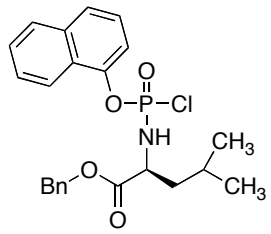
Chemical Formula: $C_{19}H_{23}ClNO_4P$
Molecular Weight: 395.8170

Prepared according to standard procedure A, from phenyl-dichlorophosphate (0.91 mL, 6.13 mmol), L-leucine benzyl ester tosylate (2.41 g, 6.13 mmol), anhydrous TEA (1.71 mL, 12.26 mmol) and anhydrous DCM (50 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (93%, 2.25 g).

^{31}P NMR ($CDCl_3$, 202 MHz): δ 8.34, 8.10. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.43-7.22 (10H, m, PhO, OCH_2Ph), 5.27-5.09 (2H, m, OCH_2Ph), 4.21-4.11 (1H, m, NHC*H*), 1.90-

1.75 (1H, m, C*H*$_2$CH(CH$_3$)$_2$), 1.71-1.59 (2H, m, C*H*$_2$C*H*(CH$_3$)$_2$), 0.98-0.95 (6H, m, CH$_3$).

Synthesis of Naphthyl-(benzyloxy-L-leucinyl) phosphorochloridate [2.2l].

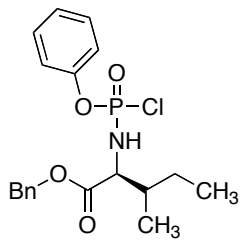


Chemical Formula: $C_{23}H_{25}ClNO_4P$
Molecular Weight: 445.8757

Prepared according to standard procedure procedure A, from naphthyl dichlorophosphate (1.6 g, 6.13 mmol), L-leucine benzyl ester tosylate (2.41 g, 6.13 mmol), anhydrous TEA (1.71 mL, 12.26 mmol) and anhydrous DCM (50 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (65%, 1.76 g).

^{31}P NMR (CDCl$_3$, 202 MHz): δ 8.56, 8.31. ^{1}H NMR (CDCl$_3$, 500 MHz): δ 8.03-7.28 (12H, m, Naph, OCH$_2$*Ph*), 5.27-5.09 (2H, m, OC*H*$_2$Ph), 4.21-4.11 (1H, m, NHC*H*), 1.90-1.75 (1H, m, C*H*$_2$CH(CH$_3$)$_2$), 1.71-1.59 (2H, m, C*H*$_2$C*H*(CH$_3$)$_2$), 0.98-0.95 (6H, m, CH$_3$).

Synthesis of phenyl-(benzyloxy-L-isoleucinyl) phosphorochloridate [2.2m].

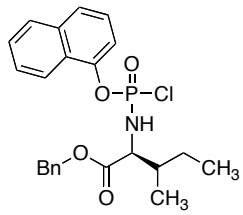


Chemical Formula: $C_{19}H_{23}ClNO_4P$
Molecular Weight: 395.8170

Prepared according to standard procedure procedure A, from phenyl dichlorophosphate (0.91 mL, 6.13 mmol), L-isoleucine benzyl ester tosylate (2.41 g, 6.13 mmol), anhydrous TEA (1.71 mL, 12.26 mmol) and anhydrous DCM (50 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (quantitative yield, 2.41 g).

^{31}P NMR (CDCl$_3$, 202 MHz): δ 9.01, 8.61. ^{1}H NMR (CDCl$_3$, 500 MHz): δ 7.45-7.22 (10H, m, PhO, OCH$_2$*Ph*), 5.27-5.19 (2H, m, OC*H*$_2$Ph), 4.11-4.00 (1H, m, NHC*H*), 1.52-1.40 (1H, m, NHCHC*H*, 1.25-1.16 (2H, m, CH(CH$_3$)*CH*$_2$CH$_3$), 0.94-0.75 (6H, m, CH$_3$).

Synthesis of Naphthyl-(benzyloxy-L-isoleucinyl) phosphorochloridate [2.2n].

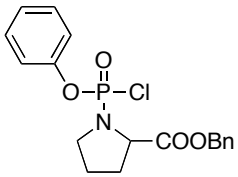


Chemical Formula: $C_{23}H_{25}ClNO_4P$
Molecular Weight: 445.8757

Prepared according to standard procedure A, from naphthyl dichlorophosphate (1.85 g, 7.1 mmol), L-isoleucine benzyl ester tosylate (2.8 g, 7.1 mmol), anhydrous TEA (1.97 mL, 14.2 mmol) and anhydrous DCM (50 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (70%, 2.26 g).

^{31}P NMR ($CDCl_3$, 202 MHz): δ 9.40, 8.98. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.09-7.29 (12H, m, Naph, OCH_2*Ph*), 5.25-5.23 (2H, m, OCH_2Ph), 4.20-4.17 (1H, m, NHC*H*), 1.54-1.42 (1H, m, NHCHC*H*), 1.26-1.14 (2H, m, $CHCH(CH_3)CH_2CH_3$), 1.00 (1.5 H, d, J = 6.8 Hz, $CHCH_3$ of one diasteroisomer), 0.97 (1.5H, d, J = 6.8 Hz, $CHCH_3$ of one diasteroisomer), 0.91 (3H, t, J = 7.4 Hz, CH_2CH_3).

Synthesis of phenyl-(benzyloxy-L-prolinyl) phosphorochloridate [2.2o].

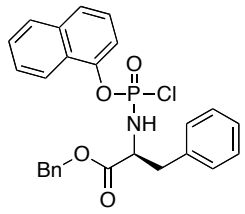


Chemical Formula: $C_{18}H_{19}ClNO_4P$
Molecular Weight: 379.7745

Prepared according to standard procedure A, from phenyl dichlorophosphate (3.09 mL, 20.68 mmol), L-proline benzyl ester tosyltate (5.00 g, 20.68 mmol), anhydrous TEA (5.76 mL, 41.36 mmol) and anhydrous DCM (100 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude product was obtained as an oil (quantitative, 7.85 g).

^{31}P NMR ($CDCl_3$, 202 MHz): δ 7.78, 7.72. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.40-7.19 (10H, m, PhO, OCH_2*Ph*), 5.25-5.15 (2H, m, OCH_2Ph), 4.58-4.55 (0.5H, m, NC*H* of one diastereoisomer), 4.49-4.45 (0.5H, m, NC*H*, of one diastereoisomer), 3.64-3.45 (2H, m, NCH_2), 2.31-2.21 (1H, m, $NCHCH_2$), 2.18-2.10 (1H, m, $NCHCH_2$), 2.09-1.92 (2H, m, $NCHCH_2CH_2$).

Synthesis of Naphthyl-(benzyloxy-L-phenylalaninyl) phosphorochloridate [2.2p].



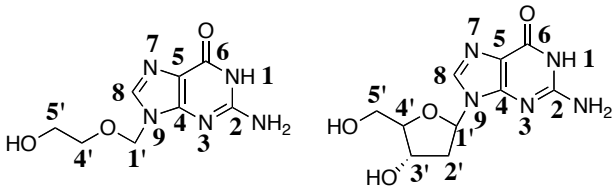
Chemical Formula: $C_{26}H_{23}ClNO_4P$
Molecular Weight: 479.8919

Prepared according to standard procedure A, from naphthyl dichlorophosphate (2.7 g, 10.28 mmol), L-Phenylalanine benzyl ester tosylate (3 g, 10.28 mmol), anhydrous TEA (2.86 mL, 20.56 mmol) and anhydrous DCM (60 mL). The reaction mixture was stirred at -78 ºC for 30 min, then at room temperature for 1 h. The crude was purified by column chromatography eluting with ethyl acetate/petroleum ether = 70/30 to give an oil (60%, 3 g).

^{31}P NMR ($CDCl_3$, 202 MHz): 8.29, 8.16. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.05-6.80 (17H, m, NaphO, $CHCH_2$*Ph*, OCH_2*Ph*), 5.19-4.97 (2H, m, OCH_2Ph), 4.64-4.52 (1H, m, C*H*NH), 3.22-3.10 (2H, m, $CHCH_2$Ph).

9.3. Experimental procedures for chapter 3: synthesis of acyclovir ProTides

Figure 9.1 reports the numbering assigned to different positions of ACV structure. In particular the side chain positions have been assigned in analogy to the structure of 2'-deoxyguanosine.



ACV **2'-deoxyguanosine**

Figure 9.1. Numbering of ACV structure and analogy with 2'-deoxyguanosine

Standard procedure B: Grignard (*t*ButMgCl) method

To a stirring suspension of N^2-DMF ACV **3.3** (1 eq.) in anhydrous THF was added dropwise, under argon atmosphere *t*ButMgCl, (2 eq.). The reaction mixture was stirred at room temperature for 30 min. Then, a solution of the appropriate aryl amino acid ester phosphorochloridate **2.2** (2 to 4 eq.) in anhydrous THF was added dropwise and the reaction mixture was stirred at room temperature overnight. The solvent was

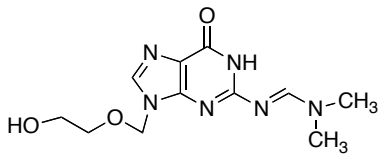
removed under reduced pressure and the residue was purified by column chromatography eluting with DCM/MeOH in different proportions.

Standard procedure C: *N*-methylimidazole (NMI) method

To a stirring suspension of **3.3** (1 eq.) and the appropriate aryl amino acid ester phosphorochloridate **2.2** (3 to 4 eq.) in anhydrous THF/pyridine mixture (3/2) was added dropwise under an argon atmosphere NMI (5-10 eq.). The reaction mixture was stirred at room temperature overnight. 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.

Synthesis of N^2-DMF-acyclovir:

N^2-(*N*,*N*-dimethylformamidine)-9-[(2-hydroxyethoxy)methyl]guanine [3.3]

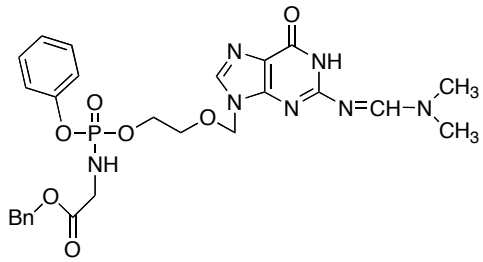


Chemical Formula: $C_{11}H_{16}N_6O_3$
Molecular Weight: 280.2831

To a suspension of ACV in anhydrous DMF (70 ml) was added *N*,*N*-dimethylformamide dimethyl acetale (11.75 ml, 88.8 mmol) and the reaction mixture was stirred at room temperetature for 24 h. After this period the suspension was filtered, and the solid was washed with diethyl ether to give a white solid (92%, 4.55g).

^{1}H NMR (DMSO, 500 MHz): δ 11.30 (1H, s, NH), 8.58 (1H, s, *CH*N$(CH_3)_2$), 7.94 (1H, s, H-8), 5.45 (2H, s, H-1'), 4.65 (1H, br s, OH), 3.53-3.49 (4H, m, H-4', H-5'), 3.18-3.04 (6H, 2s, N$(CH_3)_2$).

Synthesis of N^2-DMF-acyclovir-[phenyl(benzyloxyglicinyl)] phosphate [3.4a].



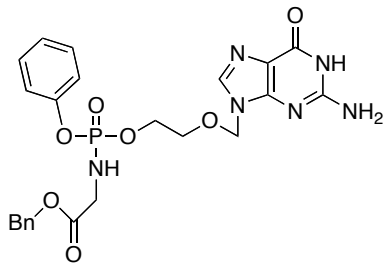
Chemical Formula: $C_{26}H_{30}N_7O_7P$
Molecular Weight: 583.5329

Prepared according to standard procedure C using **3.3** (1.00 g, 3.57 mmol) in a 3/2 mixture of THF/pyridine (50 mL), **2.2f** (3.64 g, 10.70 mmol) dissolved in anhydrous THF (10 mL) and NMI (0.85 ml, 10.70 mmol). The reaction mixture was stirred at room temperature

overnight. The solvent was removed under reduced pressure. The residue was purified by column chromatography, gradient elution of DCM/MeOH (98/2, then 97/3, then 96/4, then 95/5) to give a white solid (81%, 1.69 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.78 ^{1}H NMR (MeOD, 500 MHz): δ 8.70 (1H, s, NC*H*N(CH_3)$_2$), 7.92 (1H, s, H-8), 7.39-7.28 (7H, m, PhO, OCH_2*Ph*), 7.20-7.15 (3H, m, PhO, OCH_2*Ph*), 5.53 (2H, s, H-1'), 5.16 (2H, s, O*C*H_2Ph), 4.29-4.17 (2H, m, H-5'), 3.80-3.74 (4H, m, H-4', NHCH_2), 3.17, 3.11 (6H, 2s, N(CH_3)$_2$).

Synthesis of acyclovir-[phenyl(benzyloxyglicinyl)] phosphate [3.2a].

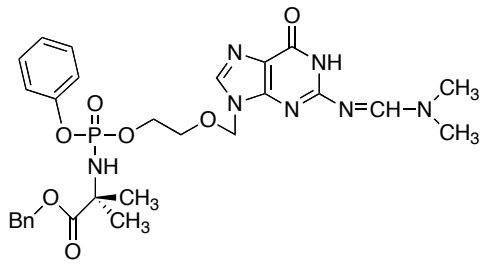


Chemical Formula: $C_{23}H_{25}N_6O_7P$
Molecular Weight: 528.4544

A solution of **3,4a** (1.69 g, 2.89 mmol) in isopropanol (60 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 = 98/2, then 96/4, then 94/6. The product was purified by preparative reverse phase HPLC (gradient elution of H_20/MetOH from 100/0 to 20/80 in 5 min, isocratic 20/80 for 10 min, from 20/80 to 0/100 in 10 min, isocratic 0/100 for 5 min) to give a white solid (2%, 0.031 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.77. ^{1}H NMR (MeOD, 500 MHz): δ 7.81 (1H, s, H-8), 7.37-7.31 (7H, m, PhO, OCH_2*Ph*), 7.18-7.16 (3H, m, PhO, OCH_2*Ph*), 5.44 (2H, s, H-1'), 5.17 (2H, s, O*C*H_2Ph), 4.26-4.16 (2H, m, H-5'), 3.80 (1H, s, NHCH_2), 3.77-3.76 (3H, m, H-4', NHCH_2). ^{13}C NMR (MeOD, 126 MHz): δ 43.91 ($NHCH_2$), 67.11 (d, J_{C-P} = 5.40 Hz, C-5'), 67.95 (O*C*H_2Ph), 69.37 (d, J_{C-P} = 7.40 Hz, C-4'), 73.66 (C-1'), 117.57, 121.43, 121.47, 126.10, 129.37, 129.43, 129.58, 130.73 (C-5, PhO, OCH_2*Ph*), 137.23 ('ipso' OCH_2*Ph*), 139.74 (C-8), 152.31 (d, J_{C-P} = 6.00 Hz, *ipso*PhO), 153.23 (C-4), 155.67 (C-2), 159.39 (C-6), 172.31 (d, J_{C-P} = 4.90 Hz, *C*OOCH_2Ph). EI MS= 529.1617 (M+H). Elemental analysis calculated for $C_{23}H_{25}N_6O_7P{\cdot}H_2O$: C, 50.55; H, 4.98; N, 15.38. Found: C, 50.97; H, 4.73; N, 15.31.

Synthesis of N^2-DMF-acyclovir-[phenyl-(benzyloxydimethylglicinyl)] phosphate [3.4b].

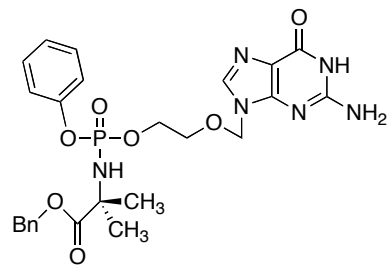


Chemical Formula: $C_{28}H_{34}N_7O_7P$
Molecular Weight: 611.5860

Prepared according to standard procedure B, from **3.3** (0.50 g, 1.78 mmol) in anhydrous THF (20 mL), *t*ButMgCl (1.0 M THF solution, 3.70 mL, 3.70 mmol), **2.2g** (2.01 g, 5.34 mmol) in anhydrous THF (10 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2, then 96/4 to give a white solid (93%, 1.02 g).

^{31}P NMR (MeOD, 202 MHz): δ 2.17. ^{1}H NMR (MeOD, 500 MHz): δ 8.69 (1H, s, NC*H*N$(CH_3)_2$), 7.91 (1H, s, H-8), 7.40-7.25 (7H, m, PhO, OCH_2*Ph*), 7.17-7.15 (3H, m, PhO, OCH_2*Ph*), 5.53 (2H, s, H-1'), 5.19-5.06 (2H, m, O*C*H_2Ph), 4.18-4.12 (2H, m, H-5'), 3.77-370 (2H, m, H-4'), 3.18 (3H, s, N$(CH_3)_2$), 3.12 (3H, s, N$(CH_3)_2$), 1.48, 1.47 (6H, 2s, C$(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.42 (d, J_{C-P} = 6.60 Hz, CH_3), 27.58 (d, J_{C-P} = 6.60 Hz, CH_3), 35.38 (NCHN(*C*$H_3)_2$), 41.51 (NCHN(*C*$H_3)_2$), 57.98 (*C*$(CH_3)_2$), 67.02 (d, J_{C-P} = 5.60 Hz, C-5'), 68.20 (O*C*H_2Ph), 69.43 (d, J_{C-P} = 7.40 Hz, C-4'), 73.69 (C-1'), 120.35, 121.47, 121.51, 125.99, 129.31, 129.58, 130.68 (C-5, PhO, OCH_2*Ph*), 137.36 ('ipso' OCH_2*Ph*), 140.62 (C-8), 152.28, 152.33 (*ipso*PhO, C-4), 159.37 (C-2), 159.90 (N*C*HN$(CH_3)_2$), 176.51 (d, J_{C-P} = 3.70 Hz, *C*OOCH_2Ph). EI MS= 612.2316 (M+H). Anal. Calcd for $C_{28}H_{34}N_7O_7P{\cdot}0.5H_2O$: C, 54.19; H, 5.68; N, 15.80. Found: C, 54.16; H, 5.61; N, 15.70.

Synthesis of acyclovir [phenyl(benzyloxy-dimethylglicinyl)] phosphate [3.2b].

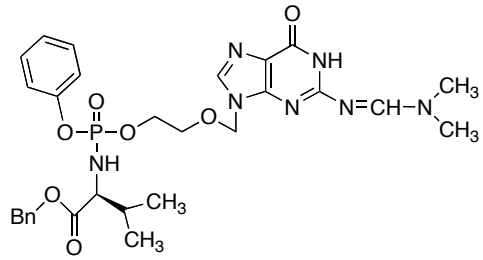


Chemical Formula: $C_{25}H_{29}N_6O_7P$
Molecular Weight: 556.5075

A solution of **3.4b** (1.02 g, 1.65 mmol) in isopropanol (50 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 (98/2, then 96/4, then 94/6). The residue was then

triturated with ether, filtered and washed with water to give a white solid (14%, 0.13 g). ^{31}P NMR (MeOD, 202 MHz): δ 2.13. ^{1}H NMR (MeOD, 500 MHz): δ 7.81 (1H, s, H-8), 7.39-7.29 (7H, m, PhO, OCH_2*Ph*), 7.25-7.14 (3H, m, PhO, OCH_2*Ph*), 5.43 (2H, s, H-1'), 5.17-5.09 (2H, m, OCH_2Ph), 4.15-4.12 (2H, m, H-5'), 3.74-3.69 (2H, m, H-4'), 1.50, 1.49 (6H, 2s, $C(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.37 (d, J_{C-P} = 4.80 Hz, $C(CH_3)_2$), 27.57 (d, J_{C-P} = 6.60 Hz, $C(CH_3)_2$), 57.99 ($C(CH_3)_2$), 67.01 (d, J_{C-P} = 5.70 Hz, C-5'), 68.22 (OCH_2Ph), 69.46 (d, J_{C-P} = 7.50 Hz, C-4'), 73.61 (C-1'), 117.52, 121.34, 121.38, 121.47, 121.51, 125.96, 129.06, 129.14, 129.29, 129.34, 129.52, 129.56, 130.06, 130.66 (C-5, PhO, OCH_2*Ph*), 137.36 ('ipso' OCH_2*Ph*), 139.74 (C-8), 152.31 (d, J_{C-P} = 7.30, *ipso*PhO), 153.45 (C-4), 155.68 (C-2), 176.54 (d, J_{C-P} = 3.80, *C*$OOCH_2$Ph). EI MS= 557.1891 (M+H). HPLC = H_2O/ACN from 100/0 to 0/100 in 30 min = retention time 16.37 min; H_2O/MeOH 100/0 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 10 min = retention time 18.19 min.

Synthesis of N^2-DMF-acyclovir-[phenyl(benzyloxy-L-valinyl)] phosphate [3.4c].

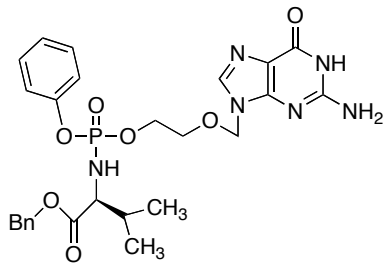


Chemical Formula: $C_{29}H_{36}N_7O_7P$
Molecular Weight: 625.6126

Prepared according to Standard Procedure C, from **3.3** (0.40 g, 1.43 mmol) in anhydrous THF (20 mL), *t*BuMgCl (1.0 M THF solution, 2.86 mL, 2.86 mmol), **2.2i** (1.63 g, 4.29 mmol) in anhydrous THF (10 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6, to give a white solid (57%, 0.51 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.52, 4.18. ^{1}H NMR (MeOD, 500 MHz): δ 8.68 (1H, s, NC*H*N$(CH_3)_2$), 8.03, 7.97 (1H, 2s, H-8), 7.38-7.11 (10H, m, PhO, OCH_2*Ph*), 5.52, 5.50 (2H, 2s, H-1'), 5.10, 5.03 (2H, 2s, OCH_2Ph), 4.16 (2H, s, H-5'), 3.76-3.64 (3H, m, NHC*H*, H-4'), 3.15, 3.10 (6H, 2s, $N(CH_3)_2$), 2.03-1.99 (0.5H, m, C*H*$(CH_3)_2$ of one diastereoisomer), 1.93-1.91 (0.5H, m, C*H*$(CH_3)_2$ of one diastereoisomer), 0.86-0.84 (6H, m, $CH(CH_3)_2$).

Synthesis of acyclovir-[phenyl(benzyloxy-L-valinyl)] phosphate [3.2c].

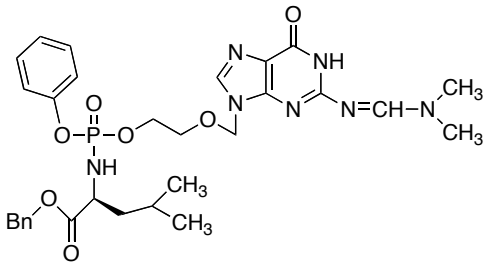


Chemical Formula: $C_{26}H_{31}N_6O_7P$
Molecular Weight: 570.5341

A solution of **3.4c** (0.51 g, 0.81 mmol) in isopropanol (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 (98/2 then 96/4 then 94/6) to give a white solid which was further purified by preparative reverse phase HPLC (gradient elution of H_20/CH_3CN from 100/0 to 0/100 in 30 min) to give a white solid (8%, 0.037 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.51, 4.27. ^{1}H NMR (MeOD, 500 MHz): δ 7.82-7.80 (1H, 2s, H-8), 7.37-7.30 (7H, m, PhO, OCH_2*Ph*), 7.19-7.14 (3H, m, PhO, OCH_2*Ph*), 5.45-5.43 (2H, 2s, H-1'), 5.14-5.13 (2H, m, OCH_2Ph), 4.16-4.14 (2H, m, H-5'), 3.74-3.66 (3H, m, H-4', NHC*H*), 2.05-2.01 (1H, m, NHCHC*H*), 0.91-0.85 (6H, m, $CH(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 18.15, 18.31 (2s, $CH(CH_3)_2$), 19.47, 19.51 (2s, $CH(CH_3)_2$), 33.07 (d, J_{C-P} = 7.20 Hz, $CH(CH_3)_2$) 33.25 (d, J_{C-P} = 7.20 Hz, $CH(CH_3)_2$), 61.93, 61.97 (2s, NH*C*H), 67.05 (d, J_{C-P} = 5.80 Hz, C-5'), 67.13 (d, J_{C-P} = 5.80 Hz, C-5'), 67.82 (OCH_2Ph), 69.36 (d, J_{C-P} = 4.70 Hz, C-4'), 69.42 (d, J_{C-P} = 4.70 Hz, C-4'), 73.63 (s, C-1'), 117.56, 121.34, 121.38, 121.48, 121.51, 126.00, 126.07, 129.39, 129.41, 129.56, 129.57, 129.62, 130.69 (C-5, PhO, OCH_2*Ph*), 137.23 ('ipso' OCH_2*Ph*), 139.71 (C-8), 152.22 (d, J_{C-P} = 4.70 Hz, *ipso*PhO), 152.28 (d, J_{C-P} = 4.70 Hz, *ipso*PhO), 153.39 (C-4) 155.68 (C-2), 159.41 (C-6), 173.95 (d, J_{C-P} = 3.50, $COOCH_2$Ph), 174.08 (d, J_{C-P} = 3.50, $COOCH_2$Ph). EI MS= 593.1895 (M+Na). HPLC = H_2O/ACN from 100/0 to 0/100 in 30 min = retention time 17.29 min; H_2O/MeOH 100/0 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 10 min = retention time 20.09, 20.47 min.

Synthesis of N^2-DMF-acyclovir-[phenyl(benzyloxy-L-leucinyl)] phosphate [3.4d].



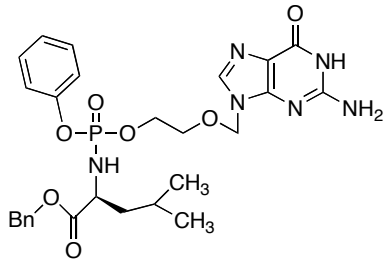
Chemical Formula: $C_{30}H_{38}N_7O_7P$
Molecular Weight: 639.6392

Prepared according to Standard Procedure C, from **3.3** (0.40 g, 1.43 mmol) in anhydrous THF (20 mL), *t*BuMgCl (1.0 M THF solution, 2.86

mL, 2.86 mmol), **2.2k** (2.25 g, 5.68 mmol) in anhydrous THF (10 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 95/5, to give a white solid (65%, 0.59 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.10, 3.57. ^{1}H NMR (MeOD, 500 MHz): δ 8.69 (1H, s, NC*H*N(CH_3)$_2$), 7.95, 7.93 (1H, 2s, H-8), 7.44-6.98 (10H, m, PhO, OCH_2*Ph*), 5.56-5.49 (2H, m, H-1'), 5.12-5.11 (2H, m, OCH_2Ph), 4.21-4.07 (2H, m, H-5'), 3.99-3.83 (1H, m, NHC*H*), 3.76-3.74 (2H, m, H-4'), 3.16 (3H, s, N(CH_3)$_2$), 3.11 (3H, s, N(CH_3)$_2$), 1.70-1.61 (1H, m, $CH_2CH(CH_3)_2$), 1.54-1.40 (2H, m, $CH_2CH(CH_3)_2$), 0.91-0.76 (6H, m, $CH_2CH(CH_3)_2$).

Synthesis of acyclovir-[phenyl(benzyloxy-L-leucinyl)] phosphate [3.2d].



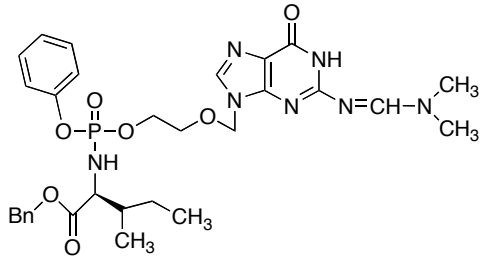
Chemical Formula: $C_{27}H_{33}N_6O_7P$
Molecular Weight: 584.5607

A solution of **3.4d** (0.59 g, 0.93 mmol) in isopropanol (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 = 98/2 then 96/4 then 94/6. The product was purified by preparative reverse phase HPLC (gradient elution of H_20/CH_3CN from 100/0 to 0/100 in 30 min) to give a white solid (8%, 0.042 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.05, 3.60. ^{1}H NMR (MeOD, 500 MHz): δ 7.82, 7.79 (1H, 2s, H-8), 7.38-7.37 (7H, m, PhO, OCH_2*Ph*), 7.19-7.15 (3H, m, PhO, OCH_2*Ph*), 5.45, 5.42 (2H, 2s, H-1'), 5.12, 5.13 (2H, 2s, OCH_2Ph), 4.16-4.07 (2H, m, H-5'), 3.95-3.88 (1H, m, NHC*H*) 3.74-3.71 (2H, m, H-4'), 1.73-1.66 (0.5H, m, $CH_2CH(CH_3)_2$ of one diastereoisomer), 1.62-1.46 (2.5H, m, $CH_2CH(CH_3)_2$ of one diastereoisomer, $CH_2CH(CH_3)_2$), 0.91-0.80 (6H, m, $CH_2CH(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 21.75, 22.01, 23.12, 23.20 (4s, $CH_2CH(CH_3)_2$), 25.40, 25.55 (2s, $CH(CH_3)_2$), 43.84 (d, J_{C-P} = 7.40 Hz, $CH_2CH(CH_3)_2$), 44.05 (d, J_{C-P} = 7.40 Hz, $CH_2CH(CH_3)_2$), 54.48, 54.70 (2s, NHCH), 67.03 (d, J_{C-P} = 1.80 Hz, C-5'), 67.07 (d, J_{C-P} = 1.80 Hz, C-5'), 67.87, 67.89 (2s, OCH_2Ph), 69.35 (d, J_{C-P} = 2.40 Hz, C-4'), 69.41 (d, J_{C-P} = 2.40 Hz, C-4'), 73.64 (s, C-1'), 117.54, 121.26, 121.30, 121.51, 121.54, 125.99, 126.09, 129.36, 129.38, 129.47, 129.57, 129.59, 130.69, 130.71 (C-5, PhO, OCH_2*Ph*), 137.27 ('ipso' OCH_2*Ph*),

139.70, 139.74 (2s, C-8), 152.22 (d, J_{C-P} = 2.10 Hz, *ipso*PhO), 152.27 (d, J_{C-P} = 2.10 Hz, *ipso*PhO), 153.56 (C-4), 155.66 (C-2), 159.37, 159.38 (2s, C-6), 174.85 (d, J_{C-P} = 2.80 Hz, *C*OOCH$_2$Ph), 175.09 (d, J_{C-P} = 2.80 Hz, *C*OOCH$_2$Ph). EI MS= 607.2051 (M+Na). Anal. Calcd for $C_{27}H_{33}N_6O_7P \cdot H_2O$: C, 53.82; H, 5.85; N, 13.95. Found: C, 54.16; H, 5.62; N, 13.67.

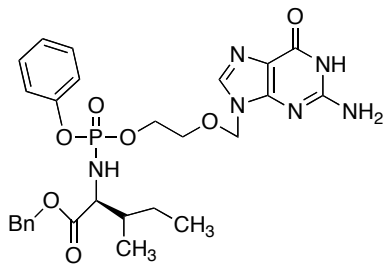
Synthesis of N^2-DMF-Acyclovir-[phenyl(benzyloxy-L-isoleucinyl)] phosphate [3.4e].



Chemical Formula: $C_{30}H_{38}N_7O_7P$
Molecular Weight: 639.6392

Prepared according to Standard Procedure C, from **3.3** (0.49 g, 1.75 mmol) in anhydrous THF (20 mL), *t*BuMgCl (1.0 M THF solution, 2.86 mL, 2.86 mmol), **2.2m** (2.41 g, 6.09 mmol) in anhydrous THF (10 mL). The reaction mixture was stirred at room temperature overnight. After this period *t*BuMgCl (1.0 M THF solution, 2. mL, 2 mmol) was added and the reaction mixture was stirred at room temperature for 6 h. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 95/5, to give a white solid (45%, 0.50 g). ^{31}P NMR (MeOD, 202 MHz): δ 4.44, 4.05. ^{1}H NMR (MeOD, 500 MHz): δ 8.69 (1H, s, NC*H*N(CH$_3$)$_2$), 7.98-7.97 (1H, m, H-8), 7.33-7.16 (10H, m, PhO, OCH$_2$*Ph*), 5.53-5.52 (2H, 2s, H-1'), 5.14-5.06 (2H, m, OC*H*$_2$Ph), 4.20-4.11 (2H, m, H-5'), 3.83-3.65 (3H, m, NHC*H*, H-4'), 3.15-3.10 (6H, 2s, N(C*H*$_3$)$_2$), 1.78-1.71 (1H, m, C*H*(CH$_3$)CH$_2$CH$_3$), 1.52-1.36 (1H, m, CH(CH$_3$)C*H*$_2$CH$_3$), 1.16-1.07 (1H, m, CH(CH$_3$)C*H*$_2$CH$_3$), 0.86-0.80 (6H, m, CH(C*H*$_3$)CH$_2$C*H*$_3$).

Synthesis of acyclovir-[phenyl(benzyloxy-L-isoleucinyl)] phosphate [3.2e].



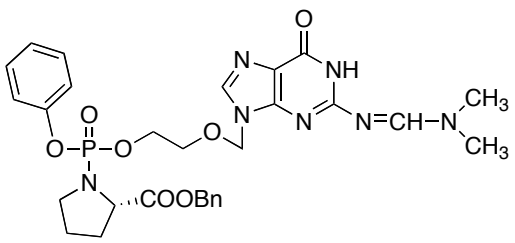
Chemical Formula: $C_{27}H_{33}N_6O_7P$
Molecular Weight: 584.5607

A solution of **3.4e** (0.50 g, 0.78 mmol) in isopropanol (20 mL) was stirred under reflux for 62 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 94/6. The product was purified

by preparative reverse phase HPLC (gradient elution of H_20/CH_3CN from 100/0 to 0/100 in 30 min) to give a white solid (3%, 0.015 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.42, 4.13. ^{1}H NMR (MeOD, 500 MHz): δ 7.82-7.80 (1H, 2s, H-8), 7.39-7.30 (7H, m, PhO, OCH_2*Ph*), 7.19-7.14 (3H, m, PhO, OCH_2*Ph*), 5.45-5.44 (2H, 2s, H-1'), 5.16-5.09 (2H, m, OCH_2Ph), 4.17-4.12 (2H, m, H-5'), 3.79-3.77 (1H, m, NHC*H*), 3.75-3.73 (2H, m, H-4'), 1.78-1.72 (1H, m, NHCHC*H*), 1.49-1.39 (1H, m, $CHCH_2CH_3$), 1.19-1.08 (1H, m, $CHCH_2CH_3$), 0.88-0.80 (6H, m, $CH(CH_3)CH_2CH_3$). ^{13}C NMR (MeOD, 126 MHz): δ 11.52, 11.55, 15.84, 15.88 (4s, $CH(CH_3)CH_2CH_3$), 25.80, 25.90 (2s, $CH(CH_3)CH_2CH_3$), 39.86 (d, J_{C-P} = 6.90 Hz, $CH(CH_3)CH_2CH_3$), 40.01 (d, J_{C-P} = 6.90 Hz, $CH(CH_3)CH_2(CH_3)$, 60.72, 60.86 (2s, NHCH), 67.05 (d, J_{C-P} = 5.00 Hz, C-5'), 67.10 (d, J_{C-P} = 5.00 Hz, C-5'), 67.81 (OCH_2Ph), 69.36 (d, J_{C-P} = 5.50 Hz, C-4'), 69.42 (d, J_{C-P} = 5.50 Hz, C-4'), 73.63, 73.65 (2s, C-1'), 117.54, 121.32, 121.36, 121.50, 121.54, 126.00, 126.08, 129.40, 129.41, 129.55, 129.57, 129.65, 129.66, 130.68, 130.69 (C-5, PhO, OCH_2*Ph*), 137.21 ('ipso' OCH_2*Ph*), 139.72 (C-8), 152.22 (d, J_{C-P} = 1.60 Hz, *ipso*PhO), 152.27 (d, J_{C-P} = 1.60 Hz, *ipso*PhO), 153.21 (C-4), 155.67 (C-2), 159.38 (C-6), 173.91 (d, J_{C-P} = 3.00 Hz, $COOCH_2$Ph), 174.07 (d, J_{C-P} = 3.00 Hz, $COOCH_2$Ph). EI MS= 607.2044 (M+Na). HPLC: H_2O/ACN from 100/0 to 0/100 in 20 min = retention time 15.00 min.

Synthesis of N^2-DMF-acyclovir-[phenyl(benzyloxy-L-prolinyl)] phosphate [3.4f].



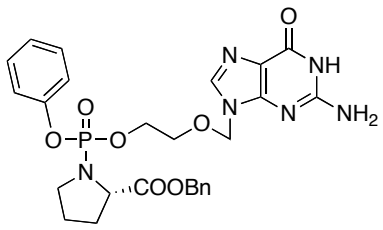
Chemical Formula: $C_{29}H_{34}N_7O_7P$
Molecular Weight: 623.5967

Prepared according to standard procedure C using **3.3** (1.00 g, 3.57 mmol) in a 3/2 mixture of THF/pyridine (50 mL), **2.2o** (4.07 g, 10.71 mmol) in anhydrous THF (10 mL) and NMI (0.85 mL, 10.70 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. 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 (16%, 0.35 g).

^{31}P NMR (MeOD, 202 MHz): δ 1.73. ^{1}H NMR (MeOD, 500 MHz): δ 8.68 (1H, s, $NCHN(CH_3)_2$), 7.94 (1H, s, H-8), 7.38-7.26 (7H, m, PhO, OCH_2*Ph*), 7.19-7.13 (3H, m, PhO, OCH_2*Ph*), 5.60-5.50 (2H, m, H-1'), 5.16-5.08 (2H, m, OCH_2Ph), 4.31-4.22 (2H, m, H-5', NC*H*), 4.19-4.14 (1H, m, H-5'), 3.79-3.78 (2H, m, H-4'), 3.27-3.19 (2H, m, NCH_2), 3.17 (3H, s, $N(CH_3)_2$), 3.10 (3H, s, $N(CH_3)_2$) 2.15-2.07 (1H, m, $NCHCH_2$),

1.98-1.91 (1H, m, NCHCH_2), 1.89-1.73 (2H, m, NCHCH$_2$CH_2). ^{13}C NMR (MeOD, 126 MHz): δ 26.18 (d, J_{C-P} = 8.90 Hz, NCHCH$_2$*C*H$_2$CH$_2$), 32.19 (d, J_{C-P} = 8.90 Hz, NCH*C*H$_2$), 35.36, 41.49 (2s, NCHN(*C*H$_3$)$_2$), 48.09 (d, J_{C-P} = 4.50 Hz, N*C*H$_2$), 62.17 (d, J_{C-P} = 7.00 Hz, N*C*H), 67.08 (d, J_{C-P} = 5.00 Hz, C-5'), 67.99 (O*C*H$_2$Ph), 69.49 (d, J_{C-P} = 7.20 Hz, C-4'), 73.73 (C-1'), 120.38, 121.17, 121.21, 126.18, 129.34, 129.60, 130.87 (C-5, PhO, OCH$_2$*Ph*), 137.26 ('ipso' OCH$_2$*Ph*), 140.68 (C-8), 152.03 (d, J_{C-P} = 6.50 Hz, *ipso*PhO), 154.54 (C-4), 159.41 (C-2), 159.55 (N*C*HN(CH$_3$)$_2$), 160.23 (C-6), 174.84 (*C*OOCH$_2$Ph). EI MS= 624.2338 (M+H). Elemental analysis Calculated for $C_{29}H_{34}N_7O_7P \cdot H_2O$: C, 54.29; H, 5.66, N, 15.28. Found: C, 54.63; H, 5.44; N, 15.23

Synthesis of acyclovir-[phenyl(benzyloxy-L-prolinyl)] phosphate [3.2f].



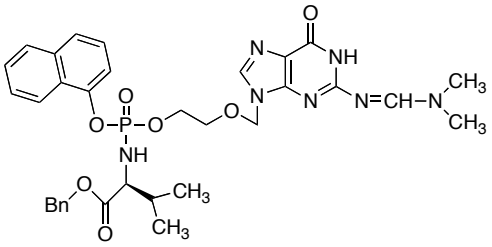
Chemical Formula: $C_{26}H_{29}N_6O_7P$
Molecular Weight: 568.5182

A solution of **3.4f** (0.35 g, 0.56 mmol) in isopropanol (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 (98/2, then 96/4, then 94/6). The product was purified by preparative reverse phase HPLC (gradient eluition of H_20/MeOH from 100/0 to 20/80 in 5 min, isocratic 20/80 for 10 min, from 20/80 to 0/100 in 10 min, isocratic 0/100 for 5 min) to give a white solid as a pure diastereoisomer (4%, 0.020 g).

^{31}P NMR (MeOD, 202 MHz): δ 1.68. ^{1}H NMR (MeOD, 500 MHz): δ 7.84 (1H, m H-8), 7.38-7.27 (7H, m, PhO, OCH$_2$*Ph*), 7.22-7.12 (3H, m, PhO, OCH$_2$*Ph*), 5.49-5.44 (2H, m, H-1'), 5.17-5.11 (2H, m, OCH_2Ph), 4.32-4.21 (2H, m, NCH, H-5'), 4.18-4.10 (1H, m, H-5'), 3.80-3.71 (2H, m, H-4'), 3.31-3.22 (2H, m, CHNCH_2), 2.15-2.11 (1H, m, CH$_2$NCHCH_2CH$_2$), 1.99-197 (1H, m, CH$_2$NCHCH_2CH$_2$), 1.91-1.76 (2H, m, CH$_2$NCHCH$_2$*C*H$_2$). ^{13}C NMR (MeOD, 126 MHz): δ 26.18 (d, J_{C-P} = 8.90 Hz, NCHCH$_2$*C*H$_2$), 32.20 (d, J_{C-P} = 4.60 Hz, NCH*C*H$_2$), 48.12 (d, J_{C-P} = 4.60 Hz, CHN*C*H$_2$), 62.17 (d, J_{C-P} = 7.00 Hz, N*C*H), 67.08 (d, J_{C-P} = 5.00 Hz, C-5'), 68.03 (O*C*H$_2$Ph), 69.46 (d, J_{C-P} = 7.40 Hz, C-4'), 73.66 (C-1'), 117.60, 121.18, 121.22, 126.16, 129.36, 129.40, 129.59, 130.85 (C-5, PhO, OCH$_2$*Ph*), 137.25 ('ipso' OCH$_2$*Ph*), 139.75 (C-8), 152.05 (d, J_{C-P} = 6.70 Hz, *ipso*PhO), 153.34 (C-4), 155.74 (C-2), 159.50 (C-6), 174.84 (*C*OOCH$_2$Ph). EI MS= 569.1917 (M+H). HPLC = H_2O/ACN from 100/0 to 0/100 in 30 min = retention time 16.57 min; H_2O/MeOH 100/0 to 20/80 in 5 min, 20/80 isocratic 10

min, then to 0/100 in 10 min = retention time 19.07 min.

Synthesis of N^2-DMF-acyclovir-[1-naphthyl(benzyloxy-L-valinyl)] phosphate [3.4g].

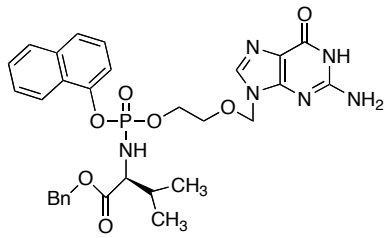


Chemical Formula: $C_{33}H_{38}N_7O_7P$
Molecular Weight: 675.6713

Prepared according to Standard Procedure B, from **3.3** (0.70 g, 2.5 mmol) in anhydrous THF (35 mL), ^{t}BuMgCl (1.0 M THF solution, 5 mL, 5 mmol), **2.2j** (2.16 g, 5 mmol) in anhydrous THF (15 mL). The reaction mixture was stirred at room temperature overnight. After this period the solvent was removed under reduced pressure. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6, to give a white solid (60%, 1 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.96, 4.73. ^{1}H NMR (MeOD, 500 MHz): δ 8.50 (1H, s, NC*H*N(CH_3)$_2$), 8.13-8.10 (1H, m, H-8 Naph), 7.97-7.82 (2H, m, H-8, H-6 Naph), 7.64-7.63 (1H, m, H-2 Naph), 7.5-7.42 (2H, m, H-5 Naph, H-7 Naph), 7.35-7.22 (7H, m, Naph, OCH$_2$P*h*), 5.44 (2H, 1s, H-1'), 5.02 (2H, 1s, OC*H*$_2$Ph), 4.25-4.20 (2H, m, H-5'), 3.74-3.70 (3H, m, NHC*H*, H-4'), 3.03-2.99 (6H, m, N(C*H*$_3$)$_2$), 2.02-1.95 (1H, m, C*H*(CH_3)$_2$), 0.84-0.81 (6H, m, CH(C*H*$_3$)$_2$). ^{13}C NMR (MeOD, 126 MHz): δ 18.38, 18.27 (2s, CH(*C*H$_3$)$_2$), 19.55, 19.60 (2s, CH(*C*H$_3$)$_2$), 33.07 (d, J_{C-P} = 7.20 Hz, *C*H(CH_3)$_2$) 33.23 (d, J_{C-P} = 7.20 Hz, *C*H(CH_3)$_2$), 35.34, 41.48 (2s, N(*C*H$_3$)$_2$), 62.03, 62.07 (2s, NH*C*H), 67.24 (d, J_{C-P} = 5.54 Hz, C-5'), 67.38 (d, J_{C-P} = 5.54 Hz, C-5'), 67.80 (s, O*C*H$_2$Ph), 69.40, 69.46 (2s, C-4'), 73.72 (s, C-1'), 116.13, 116.15, 116.33, 116.36, 120.35, 122.74, 122.86, 125.87, 125.92, 126.53, 127.42, 127.45, 127.78, 127.87, 128.84, 128.88, 129.35, 129.40, 129.53, 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, OCH$_2$*Ph*), 136.21 ('ipso' OCH$_2$*Ph*), 137.11, 137.14 (2s, C-8a Naph) 140.55 (s, C-8), 148.03 (d, J_{C-P} = 7.50 Hz, 'ipso' O*Naph*), 148.05 (d, J_{C-P} = 7.50 Hz, 'ipso' O*Naph*) 152.36 (s, C-4), 159.24 (s, C-2), 159.77 (s, *N*(CH_3)$_2$), 160.18, (s, C-6), 173.86 (d, J_{C-P} = 2.52 Hz, *C*OOCH$_2$Ph), 174.09 (d, J_{C-P} = 2.52 Hz, *C*OOCH$_2$Ph). EI MS= 676.26 ($M+H^+$). HPLC = H_2O/ACN from 90/10 to 0/100 in 20 min = retention time 15.23 min; H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min = retention time 12.45, 12.84 min.

Synthesis of acyclovir-[1-naphthyl(benzyloxy-L-valinyl)] phosphate [3.2g].

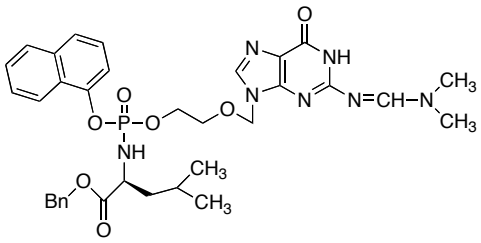


Chemical Formula: $C_{30}H_{33}N_6O_7P$
Molecular Weight: 620.5928

A solution of **3.4g** (0.9 g, 1.33 mmol) in isopropanol (60 mL) was stirred under reflux for 62 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 94/6) to give a white solid which was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (10%, 0.08 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.79, 4.90. ^{1}H NMR (MeOD, 500 MHz): δ 8.13-8.10 (1H, m, H-8 Naph), 7.89-7.87 (1H, m, H-6 Naph), 7.77 (1H, s, H-8), 7.71-7.68 (1H, m, H-2 Naph), 7.55-7.51 (2H, m, H-5 Naph, H-7 Naph), 7.45-7.25 (7H, m, Naph, OCH_2Ph), 5.39 (2H, 1s, H-1'), 5.05 (2H, 1s, OCH_2Ph), 4.24-4.19 (2H, m, H-5'), 3.78-3.73 (3H, m, NHC*H*, H-4'), 2.03-2.01 (1H, m, $CH(CH_3)_2$), 0.89-0.84 (6H, m, $CH(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 18.23, 18.32 (2s, $CH(CH_3)_2$), 19.48, 19.52 (2s, $CH(CH_3)_2$), 33.07 (d, J_{C-P} = 7.30 Hz, $CH(CH_3)_2$) 33.27 (d, J_{C-P} = 7.30 Hz, $CH(CH_3)_2$), 62.05, 62.09 (2s, NHCH), 67.26 (d, J_{C-P} = 5.54 Hz, C-5'), 67.37 (d, J_{C-P} = 5.54 Hz, C-5'), 67.83 (s, OCH_2Ph), 69.36 (d, J_{C-P} = 7.18 Hz, C-4'), 69.45 (d, J_{C-P} = 7.18 Hz, C-4'), 73.68, 73.64 (2s, C-1'), 116.19, 116.22, 116.35, 116.38, 117.54, 117.59, 122.75, 122.87, 125.87, 125.91, 126.51, 126.52, 127.40, 127.43, 127.74, 128.79, 128.83, 129.33, 129.37, 129.49, 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, OCH_2Ph), 136.26 ('ipso' OCH_2Ph), 137.13 (s, C-8a Naph) 139.69 (s, C-8), 148.03 (d, J_{C-P} = 7.56 Hz, 'ipso' O*Naph*), 148.06 (d, J_{C-P} = 7.56 Hz, 'ipso' O*Naph*) 153.36 (s, C-4), 155.56 (s, C-2), 159.37 (s, C-6), 173.96 (d, J_{C-P} = 2.52 Hz, $COOCH_2Ph$), 174.10 (d, J_{C-P} = 2.52 Hz, $COOCH_2Ph$). EI MS= 621.2204 ($M+H^+$). HPLC = H_2O/ACN from 90/10 to 0/100 in 20 min = retention time 14.19 min; H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min = retention time 11.59, 11.89 min.

Synthesis of N^2-DMF-acyclovir-[1-naphthyl(benzyloxy-L-leucinyl)] phosphate [3.4h].



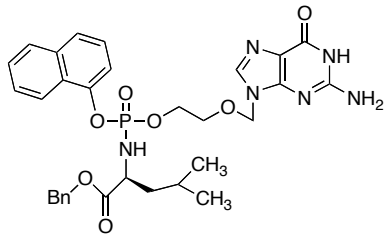
Chemical Formula: $C_{34}H_{40}N_7O_7P$
Molecular Weight: 689.6979

Prepared according to Standard Procedure B, from **3.3** (0.45 g, 1.60 mmol) in anhydrous THF (20 mL), *t*BuMgCl (1.0 M THF solution, 3.21 mL, 3.21 mmol), **2.2l** (1.43 g, 3.21 mmol) in anhydrous THF (5 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 96/4 to give a white solid (65%, 0.73 g).

^{31}P NMR (MeOD, 202 MHz): δ 4.39, 3.87. ^{1}H NMR (MeOD, 500 MHz): δ 8.51, 8.49 (1H, 2s, NC*H*N$(CH_3)_2$), 8.29, 8.28 (1H, 2s, H-8 Naph), 8.14, 8.11 (1H, m, H-6 Naph), 7.91, 7.89 (1H, 2s, H-8), 7.85-7.84 (1H, m, H-2 Naph), 7.78-7.77 (1H, m, H-5 Naph), 7.66, 7.64 (1H, m, H-7 Naph), 7.50-7.19 (7H, m, Naph, OCH_2Ph), 5.44, 5.41 (2H, 2s, H-1'), 5.06, 5.04 (2H, m, OCH_2Ph), 4.25-4.13 (2H, m, H-5'), 3.99-3.92 (1H, m, NHC*H*), 3.74-3.71 (2H, m, H-4'), 3.03, 3.02, 3.00, 2.99 (6H, 4s, N$(CH_3)_2$) 1.64-1.57 (0.5H, m, $CH_2CH(CH_3)_2$ of one diastereoisomer), 1.51-1.30 (2.5H, m, $CH_2CH(CH_3)_2$ of one diastereoisomer, $CH_2CH(CH_3)_2$), 0.80-0.66 (6H, m, $CH_2CH(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 22.74, 22.93, 23.08, 23.22 (4s, $CH_2CH(CH_3)_2$), 25.42, 25.60 (2s, $CH(CH_3)_2$), 35.39, 41.51 (2s, N$(CH_3)_2$), 43.81 (d, J_{C-P} = 7.68 Hz, $CH_2CH(CH_3)_2$), 44.03 (d, J_{C-P} = 7.68 Hz, $CH_2CH(CH_3)_2$), 54.74, 54.85 (2s, NHCH), 67.28 (d, J_{C-P} = 5.42 Hz, C-5'), 67.53 (OCH_2Ph), 67.88 (d, J_{C-P} = 5.42 Hz, C-5'), 69.43 (d, J_{C-P} = 3.49 Hz, C-4'), 69.49 (d, J_{C-P} = 3.49 Hz, C-4'), 73.76 (s, C-1'), 115.07, 116.06, 116.09, 116.43, 116.46, 122.72, 122.87, 123.32, 123.79, 125.89, 125.99, 126.39, 126.53, 126.88, 127.12, 127.49, 127.79, 127.81, 128.53, 128.88, 128.92, 129.19, 129.26, 129.39, 129.42, 129.47, 129.56, 129.60 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH_2Ph), 136.17, 136.26 (2s, 'ipso' OCH_2Ph), 137.17 137.20 (2s, C-8a Naph), 138.37 (C-8), 148.02 (d, J_{C-P} = 7.50, 'ipso' O*Naph*), 150.50 (d, J_{C-P} = 7.50, 'ipso' O*Naph*), 152.26, 152.58 (2s, C-4), 159.27 (s, C-2), 159.84 (s, *N*$(CH_3)_2$), 174.70 (d, J_{C-P} = 2.71 Hz, *C*$OOCH_2$Ph), 175.10 (d, J_{C-P} = 2.71 Hz, *C*$OOCH_2$Ph). EI MS= 712.26 ($M+Na^+$). HPLC = H_2O/ACN from 90/10 to 0/100 in 30 min, then 0/100 isocratic for 5 min = retention time 19.00 min; H_2O/MeOH 90/10 to 0/100 in 30 min,

then 0/100 isocratic for 5 min = retention time 26.85, 27.01 min.

Synthesis of acyclovir-[1-naphthyl(benzyloxy-L-leucinyl)] phosphate [3.2h]



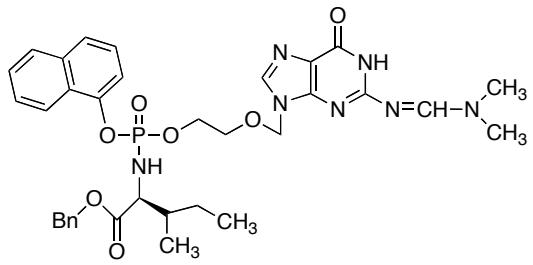
Chemical Formula: $C_{34}H_{40}N_7O_7P$
Molecular Weight: 689.6979

A solution of **3.4h** (0.73 g, 1.06 mmol) in isopropanol (20 mL) was stirred under reflux for 62 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 94/6. The product was purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (21%, 0.14g).

^{31}P NMR (MeOD, 202 MHz): 4.49, 4.04. ^{1}H NMR (MeOD, 500 MHz): δ 8.29, 8.28 (1H, 2s, H-8 Naph), 8.15, 8.11 (1H, m, H-6 Naph), 7.86, 7.85 (1H, 2s, H-8), 7.85-7.84 (1H, m, H-2 Naph), 7.79-7.78 (1H, m, H-5 Naph), 7.69-7.66 (1H, m, H-7 Naph), 7.62-7.20 (7H, m, Naph, OCH_2P*h*), 5.36, 5.33 (2H, 2s, H-1'), 5.06, 5.05 (2H, m, OC*H*$_2$Ph), 4.23-4.13 (2H, m, H-5'), 3.99-3.94 (1H, m, NHC*H*), 3.72-3.68 (2H, m, H-4'), 1.67-1.60 (0.5H, m, CH_2C*H*$(CH_3)_2$ of one diastereoisomer), 1.55-1.30 (2.5H, m, CH_2C*H*$(CH_3)_2$ of one diastereoisomer, C*H*$_2CH(CH_3)_2$), 0.82-0.67 (6H, m, $CH_2CH(C$*H*$_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 22.74, 22.95, 23.08, 23.22 (4s, $CH_2CH(C$*H*$_3)_2$), 25.42, 25.63 (2s, C*H*$(CH_3)_2$), 43.82 (d, J_{C-P} = 7.86, Hz C*H*$_2CH(CH_3)_2$), 44.08 (d, J_{C-P} = 7.86 Hz, C*H*$_2CH(CH_3)_2$), 54.75, 54.85 (2s, NH*C*H), 67.30 (d, J_{C-P} = 5.47 Hz, C-5'), 67.54 (OC*H*$_2$Ph), 67.92 (d, J_{C-P} = 5.47 Hz, C-5'), 69.42 (d, J_{C-P} = 7.44 Hz, C-4'), 69.48 (d, J_{C-P} = 7.44 Hz, C-4'), 73.73 (s, C-1'), 115.10, 115.12, 116.12, 116.15, 116.48, 116.50, 122.73, 122.88, 123.37, 123.79, 125.90, 125.99, 126.40, 126.56, 126.88, 127.12, 127.46, 127.49, 127.79, 128.52, 128.85, 128.89, 129.18, 129.26, 129.39, 129.40, 129.43, 129.45, 129.55, 129.59 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH_2*Ph*), 136.18, 136.26 (2s, 'ipso' OCH_2*Ph*), 137.17 137.23 (2s, C-8a Naph), 139.88 (C-8), 148.00 (d, J_{C-P} = 7.39 Hz, 'ipso' O*Naph*), 150.50 (d, J_{C-P} = 7.39 Hz, 'ipso' O*Naph*), 155.64 (C-4), 159.50 (s, C-2), 174.90 (d, J_{C-P} = 2.50, C*O*OCH_2Ph), 175.10 (d, J_{C-P} = 2.50, C*O*OCH_2Ph). EI MS= 657.22 ($M+Na^+$). HPLC = H_2O/ACN from 100/0 to 0/100 in 20 min, then 0/100 isocratic for 5 min = retention time min 15.00; H_2O/MeOH 90/10 to 0/100 in 25 min, then 0/100 isocratic for 5 min =

retention time 22.91 min.

Synthesis of N^2-DMF-acyclovir-[1-naphthyl(benzyloxy-L-isoleucinyl)] phosphate [3.4i].

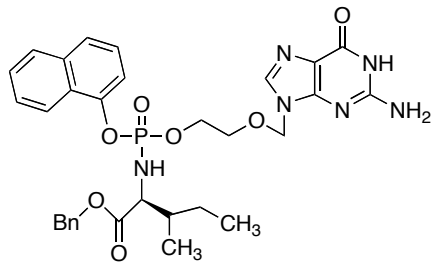


Chemical Formula: $C_{34}H_{40}N_7O_7P$
Molecular Weight: 689.6979

Prepared according to Standard Procedure B, from **3.3** (0.40 g, 1.43 mmol) in anhydrous THF (20 mL), *t*BuMgCl (1.0 M THF solution, 2.85 mL, 2.85 mmol), and **2.2n** (1.27 g, 2.85 mmol) in anhydrous THF (5 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 to give a white solid (47%, 0.47 g). ^{31}P NMR (MeOD, 202 MHz): δ 4.87, 4.56. ^{1}H NMR (MeOD, 500 MHz): δ 8.55 (1H, bs, NC*H*N$(CH_3)_2$), 8.13, 8.11 (1H, 2s, H-8 Naph), 7.88-7.85 (2H, m, H-6 Naph, H-8), 7.67-7.65 (1H, m, H-2 Naph), 7.52-7.46 (2H, m, H-5 Naph, H-7 Naph), 7.43-7.24 (7H, m, Naph, OCH_2Ph), 5.46 (2H, bs, H-1'), 5.08-4.96 (2H, m, OCH_2Ph), 4.29-4.16 (2H, m, H-5'), 3.83-3.74 (3H, m, H-4', NHC*H*), 3.06, 3.04 (3H, 2s, N$(CH_3)_2$), 3.03 (3H, bs, N$(CH_3)_2$) 1.74-1.69 (1H, m, C*H*$(CH_3)CH_2CH_3$), 1.44-1.35 (1H, m, CH$(CH_3)CH_2CH_3$), 1.12-1.03 (1H, m, CH$(CH_3)CH_2CH_3$), 0.81 (3H, m, CH$(CH_3)CH_2CH_3$), 0.76-0.73 (3H, m, CH$(CH_3)CH_2CH_3$). ^{13}C NMR (MeOD, 126 MHz): δ 11.55, 11.64 (2s, CH$(CH_3)CH_2CH_3$), 15.84, 15.94 (2s, CH$(CH_3)CH_2CH_3$), 25.89, 25.95 (2s, CH$(CH_3)CH_2CH_3$), 35.35 (N$(CH_3)_2$), 39.91 (d, J_{C-P} = 7.08 Hz, *C*H$(CH_3)CH_2CH_3$), 39.99 (d, J_{C-P} = 7.08 Hz, *C*H$(CH_3)CH_2(CH_3)$, 41.49 (N$(CH_3)_2$), 60.84, 60.96 (2s, NH*C*H), 67.26 (d, J_{C-P} = 5.44 Hz, C-5'), 67.37 (d, J_{C-P} = 5.44 Hz, C-5'), 67.80 (OCH_2Ph), 69.42, 69.48 (2s, C-4'), 73.73 (2s, C-1'), 116.11, 116.14, 116.39, 116.41, 120.36, 122.75, 122.88, 125.88, 125.95, 126.50, 126.53, 127.43, 127.46, 127.78, 127.79, 128.85, 128.88, 129.37, 129.42, 129.53, 129.58, 129.62 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH_2Ph), 136.23 ('ipso' OCH_2Ph), 137.11 137.14 (2s, C-8a Naph), 140.56 (C-8), 148.00 (d, J_{C-P} = 7.26 Hz, 'ipso' O*Naph*), 148.04 (d, J_{C-P} = 7.26 Hz, 'ipso' O*Naph*), 152.39 (C-4), 159.27 (C-2), 159.80 (N$(CH_3)_2$), 160.19 (C-6), 173.80 (d, J_{C-P} = 2.75 Hz, *C*OOCH_2Ph), 174.00 (d, J_{C-P} = 2.75 Hz, *C*OOCH_2Ph). EI MS= 712.267 ($M+Na^+$). HPLC = H_2O/ACN from 90/10 to 0/100 in 30 min, then 0/100 isocratic for 5 min = retention time 19.00 min; H_2O/MeOH

90/10 to 0/100 in 30 min, then 0/100 isocratic for 5 min = retention time 24.24, 24.61 min.

Synthesis of acyclovir-[1-naphthyl(benzyloxy-L-isoleucinyl)] phosphate [3.2i].



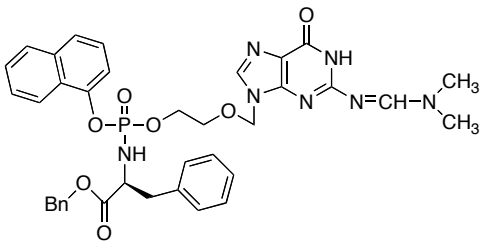
Chemical Formula: $C_{34}H_{40}N_7O_7P$
Molecular Weight: 689.6979

A solution of **3.4i** (0.44 g, 0.64 mmol) in isopropanol (20 mL) was stirred under reflux for 62 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 94/6. The product was purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (17%, 0.07g).

^{31}P NMR (MeOD, 202 MHz): δ 4.82, 4.63. ^{1}H NMR (MeOD, 500 MHz): δ 8.16-8.11 (1H, m, H-8 Naph), 7.88-7.87 (1H, 2s, H-6 Naph), 7.77 (bs, H-8), 7.70-7.68 (1H, m, H-2 Naph), 7.54-7.50 (2H, m, H-5 Naph, H-7 Naph), 7.43-7.20 (7H, m, Naph, OCH_2Ph), 5.38 (2H, bs, H-1'), 5.09-4.98 (2H, m, OCH_2Ph), 4.24-4.19 (2H, m, H-5'), 3.84-3.81 (1H, m, NHC*H*), 3.75-3.73 (2H, m, H-4'), 1.77-1.70 (1H, m, $CH(CH_3)CH_2CH_3$), 1.47-1.37 (1H, m, $CH(CH_3)CH_2CH_3$), 1.14-1.05 (1H, m, $CH(CH_3)CH_2CH_3$), 0.87-0.85 (3H, m, $CH(CH_3)CH_2CH_3$), 0.79-0.74 (3H, m, $CH(CH_3)CH_2CH_3$). ^{13}C NMR (MeOD, 126 MHz): δ 11.50, 11.59 (2s, $CH(CH_3)CH_2CH_3$), 15.79, 15.87 (2s, $CH(CH_3)CH_2CH_3$), 25.90, 25.97 (2s, $CH(CH_3)CH_2CH_3$), 39.92 (d, J_{C-P} = 7.12 Hz, $CH(CH_3)CH_2CH_3$), 40.06 (d, J_{C-P} = 7.12 Hz, $CH(CH_3)CH_2(CH_3)$, 60.84, 60.98 (2s, NH*C*H), 67.27 (d, J_{C-P} = 5.46 Hz, C-5'), 67.36 (d, J_{C-P} = 5.46 Hz, C-5'), 67.83 (OCH_2Ph), 69.38 (d, J_{C-P} = 7.24 Hz, C-4'), 69.47 (d, J_{C-P} = 7.24 Hz, C-4'), 73.66, 73.70 (2s, C-1'), 116.17, 116.19, 116.40, 116.42, 117.53, 117.57, 122.75, 122.88, 123.10, 123.85, 125.88, 125.94, 126.26, 126.52, 126.87, 127.00, 127.42, 127.44, 127.76, 128.81, 128.84, 129.36, 129.39, 129.43, 129.51, 129.52, 129.62 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH_2Ph), 136.28 ('ipso' OCH_2Ph), 137.11 137.12 (2s, C-8a Naph), 139.71 (C-8), 148.00 (d, J_{C-P} = 8.03 Hz, 'ipso' O*Naph*), 153.38 (C-4), 155.64, 155.65 (2s, C-2), 159.38 (C-6), 173.90 (d, J_{C-P} = 3.48 Hz, $COOCH_2Ph$), 174.10 (d, J_{C-P} = 3.48 Hz, $COOCH_2Ph$). EI MS= 657.22 ($M+Na^+$). HPLC = H_2O/ACN from 100/0 to 0/100 in 20 min, then 0/100 isocratic for 5 min = retention time min 14.92; H_2O/MeOH

90/10 to 0/100 in 25 min, then 0/100 isocratic for 5 min = retention time 22.77, 23.00 min.

Synthesis of N^2-DMF-acyclovir-[phenyl(benzyloxy-L-phenylalaninyl)] phosphate [3.4j]

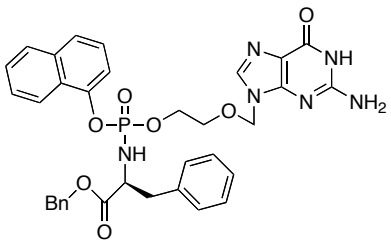


Chemical Formula: $C_{37}H_{38}N_7O_7P$
Molecular Weight: 723.7141

Prepared according to Standard Procedure B, from **3.3** (0.7 g, 2.5 mmol) in anhydrous THF (30 mL), ^{t}BuMgCl (1.0 M THF solution, 5 mL, 5 mmol), and **2.2p** (2.4 g, 5 mmol) in anhydrous THF (3 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4, then 94/6 to give to give a white solid (50%, 0.72 g).

^{31}P-NMR (MeOD, 202 MHz): δ 3.85, 3.73. ^{1}H-NMR (MeOD, 500 MHz): δ 8.41, 8.39 (1H, 2s, NC*H*N$(CH_3)_2$), 8.26, 8.24 (1H, 2s, H-8 Naph), 8.04, 8.01 (1H, m, H-6 Naph), 7.87, 7.85 (1H, 2s, H-8), 7.71-7.70 (1H, m, H-2 Naph), 7.57-7.56 (1H, m, H-5 Naph), 7.45, 6.82 (13H, m, Naph, OCH_2Ph, $CHCH_2Ph$), 5.34, 5.33 (2H, 2s, H-1'), 4.99-4.93 (2H, m, $COOCH_2Ph$), 4.23-4.14 (1H, m, $CHCH_2Ph$), 4.07-3.95 (2H, m, H-5'), 3.61-3.57 (2H, m, H-4'), 3.02-2.99 (6H, m, $N(CH_3)_2$), 2.92-2.69 (2H, m, $CHCH_2Ph$).

Synthesis of acyclovir-[phenyl(benzyloxy-L-phenylalaninyl)] phosphate [3.2j].



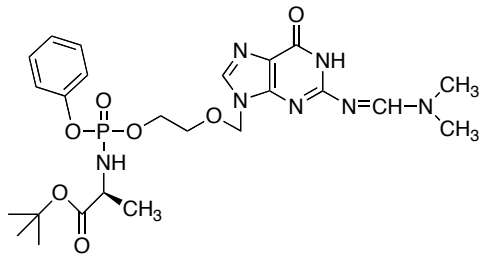
Chemical Formula: $C_{34}H_{33}N_6O_7P$
Molecular Weight: 668.6356

A solution of **3.4j** (0.34 g, 0.47 mmol) in isopropanol (20 mL) was stirred under reflux for 62 h. The solvent was then removed under reduced pressure and the residue was purified by flash 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 reverse phase HPLC (gradient elution of H_20/CH_3CN from 90/10 to 0/100 in 25 min) to give a white solid (16%, 0.05 g).

^{31}P-NMR (MeOD, 202 MHz): δ 3.86, 3.77. ^{1}H-NMR (MeOD, 500 MHz): δ 8.05, 8.00 (1H, 2s, H-8 Naph), 7.85, 7.84 (1H, m, H-6 Naph), 7.73 (1H, bs, H-8), 7.66-7.64 (1H, m, H-2 Naph), 7.52-7.45 (2H, m, H-5 Naph, H-7 Naph), 7.34, 7.08 (12H, m, Naph,

OCH_2Ph, $CHCH_2Ph$), 5.31, 5.30 (2H, 2s, H-1'), 5.05-4.98 (2H, m, $COOCH_2Ph$), 4.25-4.18 (1H, m, $CHCH_2Ph$), 4.00-3.75 (2H, m, H-5' of one diastereoisomer), 3.59 (2H, br s, H-4'), 3.08-3.01 (1H, m, $CHCH_2Ph$ of one diastereoisomer), 2.89-2.81 (1H, m, $CHCH_2Ph$ of one diastereoisomer). ^{13}C-NMR (MeOD, 125 MHz): δ 40.91 (d, J_{C-P} = 8.14 Hz, $CHCH_2Ph$), 40.98 (d, J_{C-P} = 8.14 Hz, $CHCH_2Ph$), 57.89, 58.08 (2s, $CHCH_2Ph$), 67.01 (d, J_{C-P} = 5.53 Hz, C-5'), 67.08 (d, J_{C-P} = 5.53 Hz, C-5'), 68.01, 68.06 (2s, $COOCH_2Ph$), 69.28, 69.33 (2s, C-4'), 73.64 (C-1'), 116.15, 116.18, 116.21, 122.80, 122.83, 125.88, 126.54, 127.42, 127.44, 127.73, 127.91, 127.94, 128.78, 128.81, 129.32, 129.47, 129.50, 129.52, 129.54, 130.55, 130.66 (C-5, NaphO, OCH_2Ph, $CHCH_2Ph$), 136.21, 136.23(2s, 'ipso' $CHCH_2Ph$), 136.93, 136.98 (2s, 'ipso' OCH_2Ph), 137.95 138.09 (2s, C-8a Naph), 139.69 (C-8), 147.90 (d, J_{C-P} = 7.25 Hz, 'ipso' O*Naph*), 148.00 (d, J_{C-P} = 7.25 Hz, 'ipso' O*Naph*), 153.32 (C-4), 155.62 (C-2), 159.38 (C-6), 173.86 (d, J_{C-P} = 3.53 Hz, $COOCH_2Ph$), 173.94 (d, J_{C-P} = 3.53 Hz, $COOCH_2Ph$). EI MS= 691.21 ($M+Na^+$), 707.18 ($M+K^+$). HPLC = H_2O/ACN from 100/0 to 30/70 in 10 min, then 30/70 isocratic for 10 min, from 30/70 to 0/100 in 5 min, then 0/100 isocratic for 5 min = retention time min 15.35; H_2O/MeOH 90/10 to 0/100 in 25 min, then 0/100 isocratic for 5 min = retention time 22.77, 23.00 min.

Synthesis of N^2-DMF-acyclovir-[1-phenyl(*tert*-butoxy-L-alaninyl)] phosphate [3.4k].

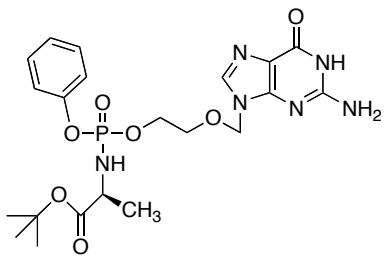


Chemical Formula: $C_{24}H_{34}N_7O_7P$
Molecular Weight: 563.5432

Prepared according to Standard Procedure B, from **3.3** (0.40 g, 1.43 mmol) in anhydrous THF (15 mL), *t*BuMgCl (1.0 M THF solution, 2.86 mL, 2.86 mmol), **2.2c** (1.37 g, 4.32 mmol) in anhydrous THF (10 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by flash column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4, to give a white solid (75%, 0.60 g). ^{31}P-NMR (MeOD, 202 MHz): δ 3.91, 3.73. ^{1}H-NMR (MeOD, 500 MHz): δ 8.63 (1H, s, $NCHN(CH_3)_2$), 7.95, 7.93 (1H, 2s, H-8), 7.34-7.31 (2H, m, PhO), 7.20-7.15 (3H, m, PhO), 5.56, 5.55 (2H, 2s, H-1'), 4.28-4.20 (2H, m, H-5'), 3.85-3.76 (3H, m, H-4', $CHCH_3$), 3.17, 3.08 (3H, 2s, $N(CH_3)_2$), 3.07, 3.06 (3H, 2s, $N(CH_3)_2$), 1.43-1.42 (9H, m, $C(CH_3)_3$), 1.31-1.26 (3H, m, $CHCH_3$). ^{13}C-NMR (MeOD, 125 MHz): δ 20.63 (d, J_{C-P} =

7.03 Hz, CH*C*H$_3$), 20.74 (d, J_{C-P} = 7.03 Hz, CH*C*H$_3$), 28.31 (C(*C*H$_3$)$_3$), 35.46, 41.63 (2s, N(*C*H$_3$)$_2$), 52.14, 52.26 (2s, *C*HCH$_3$), 67.07 (d, J_{C-P} = 6.12 Hz, C-5'), 67.12 (d, J_{C-P} = 6.12 Hz, C-5'), 69.49 (d, J_{C-P} = 3.74 Hz, C-4'), 69.55 (d, J_{C-P} = 3.74 Hz, C-4'), 73.81 (C-1'), 82.63, 82.66 (2s, *C*(CH$_3$)$_3$), 120.29, 121.43, 121.47, 121.49, 121.53, 126.11, 130.78 (C-5, PhO), 140.67 (C-8), 152.22 (d, J_{C-P} = 5.30 Hz, '*ipso*'PhO), 152.27 (d, J_{C-P} = 5.30 Hz, '*ipso*'PhO), 153.85 (C-4), 159.33 (C-2), 159.92 (*C*HN(*C*H$_3$)$_2$), 160.19 (C-6), 174.21 (d, J_{C-P} = 5.90 Hz, *C*OOC(CH$_3$)$_3$), 174.40 (d, J_{C-P} = 5.90 Hz, *C*OOC(CH$_3$)$_3$). EI MS = 564.23 (M+H), 586.22 (M+Na).

Synthesis of acyclovir-[phenyl(*tert*-butoxy-L-alaninyl)] phosphate [3.2k].

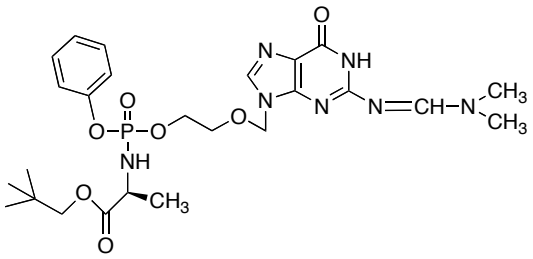


Chemical Formula: $C_{21}H_{29}N_6O_7P$
Molecular Weight: 508.4647

A solution of **3.4k** (0.50 g, 0.89 mmol) in isopropanol (25 mL) was stirred under reflux for 50 h. After this period the solvent was removed under reduced pressure and the residue was purified by flash column chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 98/2 then 96/4 then 92/8) to give a white solid (22%, 0.10 g).

^{31}P-NMR (MeOD, 202 MHz): δ 3.81, 3.71. ^{1}H-NMR (MeOD, 500 MHz): δ 7.87, 7.86 (1H, 2s, H-8), 7.34-7.31 (2H, m, PhO), 7.26-7.14 (3H, m, PhO), 5.48, 5.46 (2H, 2s, H-1'), 4.26-4.21 (2H, m, H-5'), 3.83-3.80 (3H, m, H-4', C*H*CH$_3$), 1.44-1.42 (9H, m, C(C*H*$_3$)$_3$), 1.33-1.29 (3H, m, CHC*H*$_3$). ^{13}C-NMR (MeOD, 125 MHz): δ 20.61 (d, J_{C-P} = 6.70 Hz, CH*C*H$_3$), 20.73 (d, J_{C-P} = 6.70 Hz, CH*C*H$_3$), 28.29 (C(*C*H$_3$)$_3$), 52.18, 52.28 (2s, *C*HCH$_3$), 67.08 (d, J_{C-P} = 5.50 Hz, C-5'), 67.14 (d, J_{C-P} = 5.50 Hz, C-5'), 69.45 (d, J_{C-P} = 6.63 Hz, C-4'), 69.51 (d, J_{C-P} = 6.63 Hz, C-4'), 73.79 (C-1'), 82.81, 82.87 (2s, *C*(CH$_3$)$_3$), 117.44, 121.37, 121.41, 121.15, 121.49, 121.62, 121.66, 124.02, 124.62, 124.92, 126.16, 130.12, 130.20, 130.22, 130.30, 130.34, 130.80, (C-5, PhO), 139.98 (C-8), 152.14, 152.20 (2s, C-4), 153.44 ('*ipso*'PhO), 155.70 (C-2), 159.55 (C-6), 174.44 (d, J_{C-P} = 5.30 Hz, *C*OOC(CH$_3$)$_3$), 174.55 (d, J_{C-P} = 5.30 Hz, *C*OOC(CH$_3$)$_3$). EI MS = 509.19 (M+H). HPLC = H$_2$O/MeOH from 100/0 to 30/70 in 5 min, then 30/70 isocratic for 10 min, from 30/70 to 0/100 in 5 min, then 0/100 isocratic for 5 min = retention time 12.64 min.

Synthesis of N^2-DMF-acyclovir-[phenyl(neopentoxy-L-alaninyl)] phosphate [3.4l].

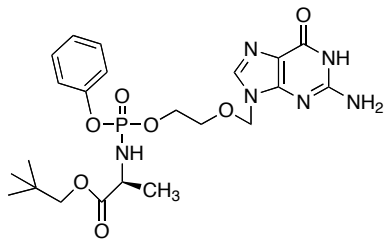


Chemical Formula: $C_{25}H_{36}N_7O_7P$
Molecular Weight: 577.5698

Prepared according to Standard Procedure B, from **3.3** (0.50 g, 1.78 mmol) in anhydrous THF (25 mL), *t*BuMgCl (1.0 M THF solution, 3.56 mL, 3.56 mmol), **2.2d** (1.19 g, 3.56 mmol) in anhydrous THF (5 mL). The reaction mixture was stirred at room temperature overnight. After this period the solvent was removed under reduced pressure. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6, to give a white solid (85%, 0.88 g).

^{31}P-NMR (MeOD, 202 MHz): δ 3.87, 3.59. ^{1}H-NMR (MeOD, 500 MHz): δ 8.65, 8.60 (1H, 2s, NC*H*N(CH_3)$_2$), 7.92 (1H, bs, H-8), 7.32 (2H, bs, PhO), 7.17 (3H, m, PhO), 5.54, 5.51 (2H, 2s, H-1'), 4.25-4.21 (2H, m, H-5'), 3.95 (1H, bs, C*H*CH_3), 3.82-3.75 (4H, m, H-4', COOC*H*$_2$(CH_3)$_3$), 3.22-3.14 (3H, m, N(C*H*$_3$)$_2$), 3.08-3.04 (3H, m, N(C*H*$_3$)$_2$) 1.34-1.33 (3H, m, CHC*H*$_3$), 0.92-0.90 (9H, m, COOCH$_2$(C*H*$_3$)$_3$).

Synthesis of acyclovir-[phenyl(neopentoxy-L-alaninyl)] phosphate [3.2l].



Chemical Formula: $C_{22}H_{31}N_6O_7P$
Molecular Weight: 522.4913

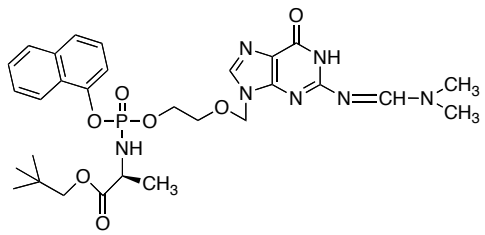
A solution of **3.4l** (0.88 g, 1.52 mmol) in isopropanol (30 mL) was stirred under reflux for 62 h. The solvent was then removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH (98/2 then 96/4 then 94/6) to give a white solid which was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (14%, 0.11 g).

^{31}P-NMR (MeOD, 202 MHz): δ 3.80, 3.59. ^{1}H-NMR (MeOD, 500 MHz): δ 7.90, 7.88 (1H, 2s, H-8), 7.36-7.33 (2H, m, PhO), 7.21-7.17 (3H, m, PhO), 5.56, 5.50 (2H, 2s, H-1'), 4.24-4.19 (2H, m, H-5'), 4.01-3.95 (1H, m, C*H*CH_3), 3.87-3.77 (4H, m, H-4', COOC*H*$_2$(CH_3)$_3$), 1.39 (1.5H, d, J= 2.52, CHC*H*$_3$ of one diasteroisomer), 1.35 (1.5H, d, J= 2.52, CHC*H*$_3$ of one diasteroisomer), 0.96-0.95 (9H, m, COOCH$_2$(C*H*$_3$)$_3$).

^{13}C NMR (MeOD, 126 MHz): δ 20.56 (d, J_{C-P} = 6.90 Hz, CH*C*H$_3$) 20.70 (d, J_{C-P} = 6.90

Hz, CH*C*H_3), 26.73, 26.84 (2s, COOCH$_2$(*C*H_3)$_3$), 51.63, 51.73 (2s, *C*HCH$_3$), 67.03 (d, J_{C-P} = 5.63 Hz, C-5'), 67.12 (d, J_{C-P} = 5.63 Hz, C-5'), 69.41 (d, J_{C-P} = 7.17 Hz, C-4'), 69.49 (d, J_{C-P} = 7.17 Hz, C-4'), 73.75 (C-1'), 75.40, 75.42 (2s, COO*C*H$_2$(CH$_3$)$_3$), 117.27, 121.40, 121.43, 121.47, 121.50, 122.23, 126.06, 126.10, 127.08, 127.77, 130.14, 130.43, 130.72, 130.88 (C-5, PhO), 139.73 (C-8), 152.20 (d, J_{C-P} = 2.80 Hz, '*ipso*'PhO), 152.25 (d, J_{C-P} = 2.80 Hz, '*ipso*'PhO), 153.73 (s, C-4), 155.73 (C-2), 159.23 (s, C-6), 175.01 (d, J_{C-P} = 5.64 Hz. *C*OOCH$_2$(CH$_3$)$_3$), 175.16 (d, J_{C-P} = 5.64 Hz, *C*OOCH$_2$(CH$_3$)$_3$). EI MS= 523.2061 (M+H$^+$). HPLC = H$_2$O/ACN from 90/10 to 0/100 in 20 min = retention time 11.87 min; H$_2$O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min = retention time 11.40 min.

Synthesis of *N*^{2}-DMF-acyclovir-[1-naphthyl(neopentoxy-L-alaninyl)] phosphate [3.4m].



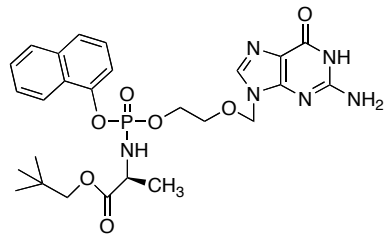
Chemical Formula: $C_{29}H_{38}N_7O_7P$
Molecular Weight: 627.6285

Prepared according to Standard Procedure B, from **3.3** (0.45 g, 1.61 mmol) in anhydrous THF (20 mL), *t*BuMgCl (1.0 M THF solution, 3.21 mL, 3.21 mmol), **2.2e** (1.20 g, 3.21 mmol) in anhydrous THF (5 mL). The reaction mixture was stirred at room temperature overnight. After this period the solvent was removed under reduced pressure. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6, to give a white solid (53%, 0.54 g).

^{31}P-NMR (MeOD, 202 MHz): δ 4.21, 4.05. ^{1}H-NMR (MeOD, 500 MHz): δ 8.56, 8.60 (1H, 2s, NC*H*N(CH$_3$)$_2$), 8.16-8.12 (1H, m, H-8 Naph), 7.92-7.89 (1H, m, H-6 Naph), 7.82, 7.80 (1H, 2s, H-8), 7.70-7.68 (1H, m, H-2 Naph), 7.54-7.52 (2H, m, H-5 Naph, H-7 Naph), 7.47, 7.37 (2H, m, H-3 Naph, H-4 Naph), 5.53, 5.51 (2H, 2s, H-1'), 4.34-4.26 (2H, m, H-5'), 4.08-4.01 (1H, m, C*H*CH$_3$), 3.86-3.84 (2H, m, H-4'), 3.80-3.70 (2H, m, COOC*H*$_2$(CH$_3$)$_3$), 3.12, 3.11 (3H, 2s, N(C*H*$_3$)$_2$), 3.08-3.07 (3H, 2s, N(C*H*$_3$)$_2$), 1.35, 1.33 (3H, 2d, *J*= 7.11 Hz, CHC*H*$_3$), 0.92-0.91 (9H, m, COOCH$_2$(C*H*$_3$)$_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.71 (d, J_{C-P} = 7.00 Hz, CH*C*H$_3$) 20.83 (d, J_{C-P} = 7.00 Hz, CH*C*H$_3$), 26.80, 26.82 (2s, COOCH$_2$C(*C*H$_3$)$_3$), 32.34, 32.37 (2s, COOCH$_2$*C*(CH$_3$)$_3$), 35.40, 41.56 (2s, N(*C*H$_3$)$_2$), 51.77, 51.82 (2s, *C*HCH$_3$), 67.32 (d, J_{C-P} = 5.80 Hz, C-5'), 67.36 (d, J_{C-P} = 5.80 Hz, C-5'), 69.47 (d, J_{C-P} = 6.76 Hz, C-4'), 69.52 (d, J_{C-P} = 6.76 Hz, C-4'), 73.74

(C-1'), 75.39, 75.42 (2s, COO*C*H_2C(CH_3)$_3$), 116.24, 116.26, 116.32, 116.34, 120.36, 122.73, 122.81, 125.95, 126.56, 127.45, 127.47, 127.80, 128.88, 128.91 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph), 136.23 (C-8a Naph), 140.57 (C-8), 148.00 (d, J_{C-P} = 1.51 Hz, 'ipso' O*Naph*), 148.10 (d, J_{C-P} = 1.63 Hz, 'ipso' O*Naph*) 152.38 (C-4), 159.23 (s, C-2), 159.79 (*C*HN(CH_3)$_2$), 160.17 (C6) 174.90 (d, J_{C-P} = 5.33 Hz, *C*OOCH$_2$C(CH_3)$_3$), 175.10 (d, J_{C-P} = 5.33 Hz, *C*OOCH$_2$C(CH_3)$_3$). EI MS= 650.252 ($M+Na^+$). HPLC = H_2O/ACN from 90/10 to 0/100 in 30 min, then 0/100 isocratic for 5 min = retention time 17.21 min; H_2O/MeOH 90/10 to 0/100 in 30 min, then 0/100 isocratic for 5 min = retention time 25.05 min.

Synthesis of acyclovir-[1-naphthyl(neopentoxy-L-alaninyl)] phosphate [3.2m]



Chemical Formula: $C_{26}H_{33}N_6O_7P$
Molecular Weight: 572.5500

A solution of **3.4m** (0.54 g, 0.86 mmol) in isopropanol (20 mL) was stirred under reflux for 62 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 94/6. The product was purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (22%, 0.11g). ^{31}P-NMR (MeOD, 202 MHz): δ 4.15, 4.09. ^{1}H-NMR (MeOD, 500 MHz): δ 8.14-8.12 (1H, 2s, H-8 Naph), 7.87-7.85 (1H, m, H-6 Naph), 7.81, 7.79 (1H, 2s, H-8), 7.69-7.68 (1H, 2s, H-2 Naph), 7.58-7.50 (2H, m, H-5 Naph, H-7 Naph), 7.46-7.35 (2H, m, H-3 Naph, H-4 Naph), 5.42, 5.40 (2H, 2s, H-1'), 4.32-4.29 (1H, m, H-5'), 4.28-4.24 (1H, m, H-5'), 4.11-4.03 (1H, m, C*H*CH_3), 3.83-3.70 (4H, m, H-4', COOC*H*$_2$(CH_3)$_3$), 1.35, 1.38 (3H, 2d, *J*= 7.10 Hz, CHC*H*$_3$), 0.92-0.90 (9H, m, COOCH$_2$(C*H*$_3$)$_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.71 (d, J_{C-P} = 7.00 Hz CH*C*H$_3$) 20.86 (d, J_{C-P} = 7.00 Hz, CH*C*H$_3$), 26.77, 26.79 (2s, COOCH$_2$C(*C*H$_3$)$_3$), 32.31, 32.35 (2s, COOCH$_2$*C*(CH_3)$_3$), 51.80, 51.84 (2s, *C*HCH$_3$), 67.31 (d, J_{C-P} = 6.12 Hz, C-5'), 67.37 (d, J_{C-P} = 6.12 Hz, C-5'), 69.40 (d, J_{C-P} = 6.97 Hz, C-4'), 69.50 (d, J_{C-P} = 6.97 Hz, C-4'), 73.77 (C-1'), 75.41, 75.46 (2s, COO*C*H$_2$(CH_3)$_3$), 116.28, 116.30, 116.36, 116.38, 117.47, 117.50, 122.71, 122.79, 125.96, 126.56, 127.46, 127.77, 128.50, 128.86 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-4a Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph), 136.23 (C-4a Naph), 139.77 (C-8), 147.99, 148.00 ('ipso' O*Naph*), 153.37 (C-4), 155.64 (s, C-2), 159.57 (C6) 175.10 (d, J_{C-P} = 5.47 Hz, *C*OOCH$_2$C(CH_3)$_3$), 175.20 (d, J_{C-P} = 5.47 Hz,

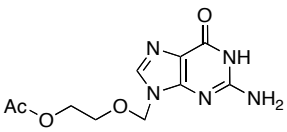
*C*OOCH$_2$C(CH$_3$)$_3$). EI MS= 595.211 (M+Na$^+$). HPLC = H$_2$O/ACN from 100/0 to 0/100 in 20 min, then 0/100 isocratic for 5 min = retention time 13.77 min; H$_2$O/MeOH 90/10 to 0/100 in 25 min, then 0/100 isocratic for 5 min = retention time 21.29, 21.41 min.

9.4. Experimental procedures for chapter 4: synthesis of 6-O-methyl and 6-O-ethyl acyclovir ProTides

Standard procedure D

To a stirring solution of 6-O-alkyl acyclovir **4.1a** and **4.1b (**1 eq.) in anhydrous THF was added dropwise under argon atmosphere *t*ButMgCl (2 eq.). The reaction mixture was stirred at room temperature for 30 min. Then, a solution of the appropriate aryl amino acid ester phosphorochloridate **2.2** (2 to 4 eq.) in anhydrous THF was added dropwise and 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.

Synthesis of 9-[(2-Acetoxy-ethoxy)methyl]guanine [4.2]

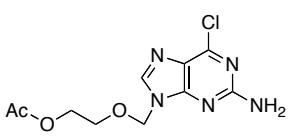


Chemical Formula: $C_{10}H_{13}N_5O_4$
Molecular Weight: 267.2413

Acetic anhydride (4.20 mL, 44.40 mmol) was added dropwise to a suspension of ACV (4 g, 17.76 mmol), TEA (5 mL, 35.87 mmol), and DMAP (0.22 g, 1.77mmol) in ACN (250 mL). The reaction was stirred at room temperature for 4 h. Methanol was added to quench the reaction and the mixture was filtered. The resulting solid was washed with Et$_2$O and dried (4.35 g, 92%).

^{1}H NMR (DMSO, 500 MHz): δ 10.63 (1H, s, NH), 7.82 (1H, s, H-8), 6.50 (2H, bs, NH$_2$), 5.36 (2H, s, H-1'), 4.09-4.07 (2H, m, H-5'), 3.68-3.66 (2H, m, H-4'), 1.97 (3H, s, CH$_3$).

Synthesis of 9-[(2-Acetoxy-ethoxy)methyl]-2-amino-6-chloropurine [4.3]



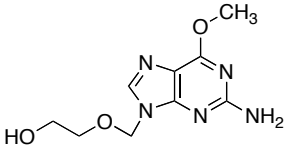
Chemical Formula: $C_{10}H_{12}ClN_5O_3$
Molecular Weight: 285.6870

A mixture of **4.2** (2g, 7.48 mmol) and bennzyltriethylammonium chloride (3.42 g, 14.96 mmol) in ACN (24 mL) was cooled to 0° C and added to a mixture of N,N-dimethylaniline (1.04 mL, 8.22 mmol) and

$POCl_3$ (3.44 mL, 37.4 mmol). The reaction mixture was stirred in a preheated oil bath at 85 °C for 20 min. The solvent was removed. The yellow foam was dissolved in $CHCl_3$ and vigorously stirred with crushed ice for 30 min. The layers were separated and the aqueous phase was extracted with $CHCl_3$. Crushed ice was added to the combined organic phase, which was washed (ice/H_2O 3 times, 5% Na_2CO_3/H_2O once) and dried over Na_2SO_4 and concentrated. The residue was purified by column chromathography, gradient elution of DCM/MeOH (98/2, then 96/4, then 95/5) to give a white solid (47%, 0.5 g).

^{1}H NMR ($CHCl_3$, 500 MHz): δ 7.88 (1H, s, H-8), 5.47 (2H, s, H-1'), 4.14-4.11 (2H, m, H-5'), 3.71-3.68 (2H, m, H-4'), 1.96 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 20.73 (CH_3), 62.78 (C-4'), 67.59 (C-5'), 72.67 (C-1'), 124.70 (C-5), 142.17 (H-8), 151.36 (C-6), 154.09 (C-4), 159.66 (C-2), 170.79 (*C*OOCH$_3$). EI MS= 286.07 (M+H).

Synthesis of 6-*O*-methyl acyclovir: 9-[(2-hydroxyethoxy)methyl]-2-amino-6-methoxypurine [4.1a]

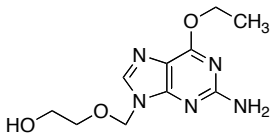


Chemical Formula: $C_9H_{13}N_5O_3$
Molecular Weight: 239.2312

To a stirring suspension of **4.3** (1.5 g; 5.25 mmol) in anhydrous MeOH (18 mL) was added at 0 °C NaOMe (1.48 g, 27 mmol) in anhydrous MeOH (25% P/P). The mixture was stirred at room temperature for 24 h then quenched by addition of Amberlite (H^+). The mixture was then filtered and methanol was removed under reduced pressure. The residue was purified by column chromatography gradient elution DCM/MeOH (98/2, then 96/4, then 94/6, then 92/8) to give a white solid (0.8 g, 68%).

^{1}H NMR (MeOH, 500 MHz): δ 7.98 (1H, s, H-8), 5.56 (2H, s, H-1'), 4.08 (3H, s, OCH_3), 3.66-3.63 (4H, m, H-4', H-5'). ^{13}C NMR (MeOH, 126 MHz): δ 54.26 (OCH_3), 61.94 (C-4'), 71.96 (C-5'), 73.94 (C-1'), 115.08 (C-5), 141.27 (C-8), 155.25 (C-4), 162.18 (C-2), 162.80 (C-6). EI MS= 239.09 (M+H). HPLC = H_2O/ACN from 100/0 to 0/100 in 20 min = retention time 6.60 min; H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min = retention time 5.91 min.

Synthesis of 6-*O*-ethyl acyclovir: 9-[2-hydroxyethoxy)methyl[-2-amino-6-ethoxypurine [4.1b]

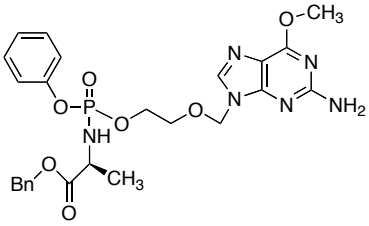


Chemical Formula: $C_{10}H_{15}N_5O_3$
Molecular Weight: 253.2578

To a stirring suspension of **4.3** (1.7 g; 5.95 mmol) in anhydrous methanol (20 mL) was added at 0 °C NaOEt (1.82 g, 26.78 mmol) in anhydrous methanol (25% P/P). The mixture was stirred at 40 °C for 4 h then quenched by addition of Amberlite (H^+). The mixture was then filtered and methanol was removed under reduced pressure. The residue was purified by column chromatography gradient elution DCM/Methanol (98/2, then 96/4, then 94/6, then 92/8) to give a white solid (0.55 g, 36%).

^{1}H NMR (MeOH, 500 MHz): δ 7.98 (1H, s, H-8), 5.56 (2H, s, H-1'), 4.56 (2H, q, J= 7.09 Hz, OC*H*$_2$CH$_3$), 3.66-3.63 (4H, m, H-4', H-5'), 1.46 (3H, t, J= 7.09 Hz, OCH$_2$C*H*$_3$). ^{13}C NMR (MeOH, 126 MHz): δ 14.82 (OCH$_2$*C*H$_3$), 61.95 (O*C*H$_2$CH$_3$), 63.59 (C-4'), 71.95 (C-5'), 73.91 (C-1'), 115.09 (C-5), 141.19 (H-8), 155.28 (C-4), 162.18 (C-2), 162.46 (C-6). EI MS= 254.13 (M+H). HPLC= H$_2$O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min = retention time 7.15, 8.64 min.

Synthesis of 6-*O*-methyl acyclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [4.4a]



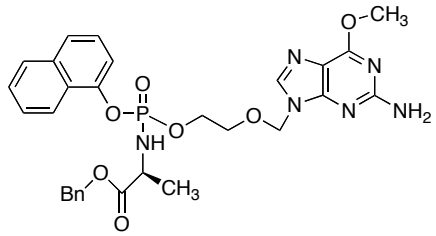
Chemical Formula: $C_{25}H_{29}N_6O_7P$
Molecular Weight: 556.5075

Prepared according to Standard Procedure D, from **4.1a** (0.40 g, 1.68 mmol) in anhydrous THF (20 mL), *t*BuMgCl (1.0 M THF solution, 3.36 mL, 3.36 mmol), **2.2a** (1.78 g, 5.04 mmol) in anhydrous THF (10 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6. The product was purified by preparative reverse phase HPLC (gradient elution of H$_2$O/MeOH 90/10 to 40/60 in 5 min, 60/40 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (733 mg, 78%).

^{31}P NMR (MeOD, 202 MHz): δ 3.76, 3.47. ^{1}H NMR (MeOD, 500 MHz): δ 7.94-7.91 (1H, m, H-8), 7.35-7.28 (7H, m, PhO, OCH$_2$*Ph*), 7.18-7.12 (3H, m, PhO, OCH$_2$*Ph*),

5.52-5.48 (2H, m, H-1'), 5.13-5.12 (2H, m, OC*H*$_2$Ph), 4.18-4.11 (2H, m, H-5'), 4.06-4.05 (3H, 2s, OCH$_3$), 3.99-3.95 (1H, m, NHC*H*), 3.76-3.71 (2H, m, H-4'), 1.35-1.31 (3H, m, CHC*H*$_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.30 (d, J_{C-P} = 6.3 Hz, CH*C*H$_3$), 20.38 (d, J_{C-P} = 6.3 Hz, CH*C*H$_3$), 51.58, 51.70 (2s, NH*C*H), 54.24 54.26 (2s, OCH$_3$), 66.98 (d, J_{C-P} = 5.04 Hz, C-5'), 67.08 (d, J_{C-P} = 5.04 Hz, C-5'), 67.94 (O*C*H$_2$Ph), 69.38 (d, J_{C-P} = 6.30, C-4'), 69.40 (d, J_{C-P} = 6.30, C-4'), 73.71 (C-1'), 115.10, 121.39, 121.43, 121.47, 121.50, 126.03, 126.06, 129.32, 129.34, 129.37, 129.57, 129.59, 129.88, 130.69 (C-5, PhO, OCH$_2$*Ph*), 137.28 ('ipso' OCH$_2$*Ph*), 141.17, 141.20 (2s, C-8), 152.13 (d, J_{C-P} = 5.04 Hz, 'ipso'PhO), 152.24 (d, J_{C-P} = 5.04 Hz, 'ipso'PhO), 153.24 (C-4) 155.26 (C-2), 162.20, 162.79 (2s, C-6), 174.73 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph), 174.88 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph). EI MS= 579.17 (M+Na). HPLC = H$_2$O/ACN from 100/0 to 0/100 in 20 min = retention time 12.00 min; H$_2$O/MeOH 90/10 to 40/60 in 5 min, 40/60 isocratic 10 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 14.35, 15.39 min.

Synthesis of 6-*O*-methyl acyclovir-[1-naphthyl(benzyloxy-L-alaninyl)] phosphate [4.4b]



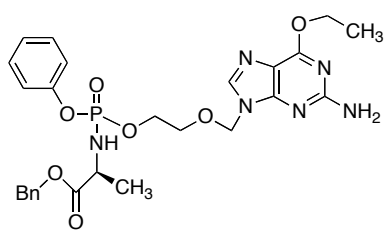
Chemical Formula: $C_{29}H_{31}N_6O_7P$
Molecular Weight: 606.5662

Prepared according to Standard Procedure D, from **4.1a** (0.30 g, 1.26 mmol) in anhydrous THF (15 mL), *t*BuMgCl (1.0 M THF solution, 2.52 mL, 2.52 mmol), **2.2b** (1.02 g, 2.52 mmol) in anhydrous THF (5 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4. The product was purified by preparative reverse phase HPLC (gradient elution of H$_2$O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (0.46 g, 60%).

^{31}P NMR (MeOD, 202 MHz): δ 4.11, 3.93. ^{1}H NMR (MeOD, 500 MHz): δ 8.10-8.08 (1H, m, H-8 Naph), 7.88-7.86 (1H, 2s, H-6 Naph), 7.85-7.84 (1H, m, H-8), 7.67-7.66 (1H, m, H-2 Naph), 7.52-7.47 (2H, m, H-5 Naph, H-7 Naph), 7.42-7.23 (7H, m, Naph, OCH$_2$P*h*), 5.45,5.41 (2H, 2s, H-1'), 5.10-5.00 (2H, m, OC*H*$_2$Ph), 4.23-4.16 (2H, m, H-5'), 4.09-4.04 (1H, m, NHC*H*), 4.02, 4.01 (3H, 2s, OCH$_3$), 3.74-3.70 (2H, m, H-4'), 1.33 (1.5H of one diasteroisomer, d, J= 7.08 Hz, CHC*H*$_3$), 1.30 (1.5H of one

diasteroisomer, d, J= 7.08 Hz, CHC*H*$_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.35 (d, J_{C-P} = 6.3 Hz, CH*C*H$_3$), 20.46 (d, J_{C-P} = 6.3 Hz, CH*C*H$_3$), 51.73, 51.79 (2s, NH*C*H), 54.26 (OCH$_3$), 67.21 (d, J_{C-P} = 6.3 Hz, C-5'), 67.28 (d, J_{C-P} = 6.3 Hz, C-5'), 67.93, 67.96 (O*C*H$_2$Ph), 69.35 (d, J_{C-P} = 7.56 Hz, C-4'), 69.41 (d, J_{C-P} = 7.56 Hz, C-4'), 73.70 (C-1'), 115.12, 116.25, 116.27, 116.34, 116.36, 122.71, 122.82, 125.89, 125.91, 126.52, 126.52, 127.42, 127.44, 127.73, 127.90, 127.95, 128.80, 128.82, 129.27, 129.32, 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, OCH$_2$*Ph*), 136.23 ('ipso' OCH$_2$*Ph*), 137.18, 137.22 (2s, C-8a Naph) 141.11, 141.14 (2s, C-8), 147.96, 148.02 (2s, 'ipso' O*Naph*), 155.21 (C-4), 162.15 (s, C-2), 162.75 (C-6), 174.74 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph), 174.91 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph). EI MS= 607.20 (M+H), 629.19 (M+Na). HPLC= H$_2$O/ACN from 100/0 to 0/100 in 20 min = retention time 16.55 min; H$_2$O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 10 min, 0/100 isocratic 5 min = retention time 12.31, 12.85 min.

Synthesis of 6-*O*-ethyl acyclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [4.4c]



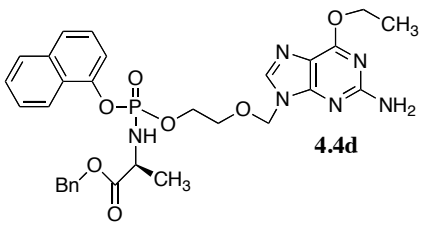
Chemical Formula: $C_{26}H_{31}N_6O_7P$
Molecular Weight: 570.5341

Prepared according to Standard Procedure D, from **4.1b** (0.20 g, 0.79 mmol) in anhydrous THF (10 mL), *t*BuMgCl (1.0 M THF solution, 1.58 mL, 1.58 mmol), **2.2a** (0.56 g, 1.58 mmol) in anhydrous THF (5 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, 96/4 then 94/6. The product was further purified by preparative reverse phase HPLC (gradient elution of H$_2$O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (241 mg, 53%).

^{31}P NMR (MeOD, 202 MHz): δ 3.76, 3.47. ^{1}H NMR (MeOD, 500 MHz): δ 7.92-7.89 (1H, 2s, H-8), 7.35-7.26 (7H, m, PhO, OCH$_2$*Ph*), 7.15-7.12 (3H, m, PhO, OCH$_2$*Ph*), 5.49, 5.46 (2H, 2s, H-1'), 5.11 (2H, OC*H*$_2$Ph), 4.54-4.49 (2H, m, OC*H*$_2$CH$_3$), 4.19-4.10 (2H, m, H-5'), 4.00-3.93 (1H, m, NHC*H*), 3.74-3.70 (2H, m, H-4'), 1.42 (3H, t, J= 7.09 Hz, OCH$_2$C*H*$_3$), 1.34 (1.5H of one diasteroisomer, d, J= 7.00 Hz, CHC*H*$_3$), 1.31 (1.5H of one diasteroisomer, d, J= 7.00 Hz, CHC*H*$_3$). ^{13}C NMR (MeOD, 126 MHz): δ 14.82 (OCH$_2$*C*H$_3$), 20.37 (d, J_{C-P} = 6.3 Hz, CH*C*H$_3$), 20.45 (d, J_{C-P} = 6.3 Hz, CH*C*H$_3$), 51.58,

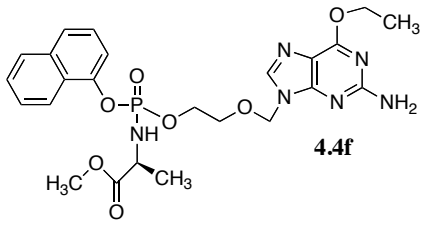
51.71 (2s, NH*C*H), 63.63 (O*C*$H_2$$CH_3$), 66.99 (d, J_{C-P} = 5.04 Hz, C-5'), 67.09 (d, J_{C-P} = 5.04 Hz, C-5'), 67.95 (O*C*H_2Ph), 69.36 (d, J_{C-P} = 7.56 Hz, C-4'), 69.39 (d, J_{C-P} = 7.56 Hz, C-4'), 73.71 (C-1'), 115.13, 121.41, 121.45, 121.49, 121.53, 126.05, 126.08, 129.33, 129.37, 129.59, 129.61, 129.72, 130.71, 130.69 (C-5, PhO, OCH$_2$*Ph*), 137.28 ('ipso' OCH$_2$*Ph*), 141.16 (C-8), 152.11, 152.17 (2s, 'ipso'PhO), 153.41 (C-4), 155.27 (C-2), 162.16, 162.43 (2s, C-6), 174.74 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph), 174.88 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph). EI MS= 571.29 (M+H), 593.27 (M+Na). HPLC = H_2O/ACN 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 7.51 min; H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 10.36 min.

Synthesis of 6-*O*-ethyl acyclovir-[1-naphthyl(benzyloxy-L-alaninyl)] phosphate [4.4d] and 6-*O*-ethyl acyclovir-[1-naphthyl(methoxy-L-alaninyl)] phosphate [4.4f]



4.4d

Chemical Formula: $C_{30}H_{33}N_6O_7P$
Molecular Weight: 620.5928



4.4f

Chemical Formula: $C_{24}H_{29}N_6O_7P$
Molecular Weight: 544.4968

Prepared according to Standard Procedure D, from **4.1b** (0.20 g, 0.79 mmol) in anhydrous THF (10 mL), *t*BuMgCl (1.0 M THF solution, 1.58 mL, 1.58 mmol), **2.2b** (0.64 g, 1.58 mmol) in anhydrous THF (2 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6 to give two products: **4.4d** and its transesterification product **4.4f**. The products were further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min) to give a white solid [(**4.4d**) 0.15 g, 30%, (**4.4f**) 0.07 g, 16%].

[4.4d]

^{31}P NMR (MeOD, 202 MHz): δ 4.11, 3.94. ^{1}H NMR (MeOD, 500 MHz): δ 8.10-8.09 (1H, m, H-8 Naph), 7.88-7.85 (2H, m, H-8, H-6 Naph), 7.68-7.66 (1H, m, H-2 Naph), 7.53-7.48 (2H, m, H-5 Naph, H-7 Naph), 7.41-7.23 (7H, m, Naph, OCH$_2$P*h*), 5.45, 5.42

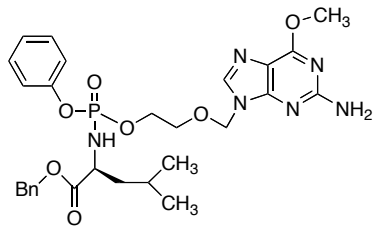
(2H, 2s, H-1'), 5.05, 5.03 (2H, 2s, OC*H*$_2$Ph), 4.52-4.47 (2H, m, OC*H*$_2$CH$_3$), 4.23-4.16 (2H, m, H-5'), 4.09-4.02 (1H, m, NHC*H*), 3.75-3.70 (2H, m, H-4'), 1.42 (1.5 H, t, J= 7.09 Hz, OCH$_2$C*H*$_3$), 1.41 (1.5 H, t, J= 7.09 Hz, OCH$_2$C*H*$_3$), 1.33 (1.5H of one diasteroisomer, d, J= 7.12 Hz, CHC*H*$_3$), 1.30 (1.5H of one diasteroisomer, d, J= 7.12 Hz, CHC*H*$_3$). ^{13}C NMR (MeOD, 126 MHz): δ 14.68 (OCH$_2$*C*H$_3$), 20.33 (d, J_{C-P} = 7.56 Hz, CH*C*H$_3$), 20.45 (d, J_{C-P} = 7.56 Hz, CH*C*H$_3$) 51.73, 51.79 (2s, NH*C*H), 63.59 (O*C*H$_2$CH$_3$), 67.23 (d, J_{C-P} = 5.41 Hz, C-5'), 67.30 (d, J_{C-P} = 5.41 Hz, C-5'), 67.95, 67.93 (O*C*H$_2$Ph), 69.34 (d, J_{C-P} = 6.3 Hz, C-4'), 69.41 (d, J_{C-P} = 6.3 Hz, C-4'), 73.69 (C-1'), 115.14, 116.25, 116.28, 116.35, 116.37, 122.72, 122.82, 125.88, 125.91, 126.23, 126.53, 127.42, 127.44, 127.72, 127.86, 127.91, 127.95, 128.80, 128.82, 129.27, 129.33, 129.52, 129.57, 129.65, 129.73, 129.90 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH$_2$*Ph*), 136.24 ('ipso' OCH$_2$*Ph*), 137.18, 137.22 (2s, C-8a Naph) 141.06, 141.09 (2s, C-8), 147.96, 148.02 (2s, 'ipso' O*Naph*), 155.23 (C-4), 162.15 (s, C-2), 162.42 (C-6), 174.74 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph), 174.91 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph). EI MS= 621.02 (M+H), 642.99 (M+Na). HPLC = H$_2$O/ACN 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 8.56 min; H$_2$O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 15.56, 16.65 min.

[4.4f]

^{31}P NMR (MeOD, 202 MHz): δ 4.05, 4.04. ^{1}H NMR (MeOD, 500 MHz): δ 8.11-8.09 (1H, m, H-8 Naph), 7.91-7.90 (1H, 2s, H-8), 7.87-7.85 (1H, m, H-6 Naph), 7.68-7.67 (1H, m, H-2 Naph), 7.54-7.50 (2H, m, H-5 Naph, H-7 Naph), 7.42-7.37 (2H, m, H-3 Naph, H-4 Naph), 5.49, 5.47 (2H, 2s, H-1'), 4.53-4.48 (2H, m, OC*H*$_2$CH$_3$), 4.31-4.22 (2H, m, H-5'), 4.04-3.96 (1H, m, NHC*H*), 3.75-3.70 (2H, m, H-4'), 3.61, 3.58 (3H, 2s, OCH$_3$), 1.42 (1.5 H, t, J= 7.09 Hz, OCH$_2$C*H*$_3$), 1.41 (1.5 H, t, J= 7.09 Hz, OCH$_2$C*H*$_3$), 1.32 (1.5H of one diasteroisomer, d, J= 7.14 Hz, CHC*H*$_3$), 1.28 (1.5H of one diasteroisomer, d, J= 7.14 Hz, CHC*H*$_3$). ^{13}C NMR (MeOD, 126 MHz): δ 14.68 (OCH$_2$*C*H$_3$), 20.40 (d, J_{C-P} = 7.56 Hz, CH*C*H$_3$), 20.51 (d, J_{C-P} = 7.56 Hz, CH*C*H$_3$) 51.78 (NH*C*H), 52.70, 52.78 (2s, OCH$_3$), 63.60 (O*C*H$_2$CH$_3$), 67.25 (d, J_{C-P} = 6.30 Hz, C-5'), 67.31 (d, J_{C-P} = 6.30 Hz, C-5'), 69.41 (d, J_{C-P} = 7.56 Hz, C-4'), 69.48 (d, J_{C-P} = 7.56 Hz, C-4'), 73.73 (C-1'), 115.14, 116.22, 116.25, 116.27, 122.71, 122.75, 125.88, 126.53, 127.43, 127.73, 127.87, 127.92, 128.81 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5

Naph, C-6 Naph, C-7 Naph, C-8 Naph), 136.23 (C-8a Naph) 141.11 (C-8), 147.97, 148.03 (2s, 'ipso' O*Naph*), 155.23 (C-4), 162.16 (s, C-2), 162.42 (C-6), 175.44 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph), 174.61 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph). EI MS= 545.26 (M+H), 567.25 (M+Na). HPLC = H_2O/ACN 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 9.36 min; H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 6.88 min.

Synthesis of 6-*O*-methyl acyclovir-[phenyl(benzyloxy-L-leucinyl)] phosphate [4.4e]

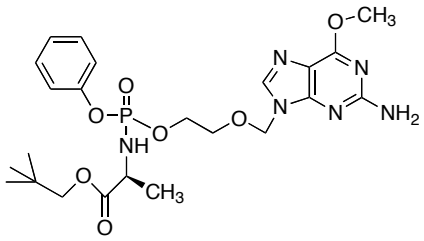


Chemical Formula: $C_{28}H_{35}N_6O_7P$
Molecular Weight: 598.5873

Prepared according to Standard Procedure D, from **4.1a** (0.06 g, 0.25 mmol) in anhydrous THF (10 mL), *t*BuMgCl (1.0 M THF solution, 0.5 mL, 0.5 mmol), **2.2k** (0.198 g, 0.5 mmol) in anhydrous THF (2 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (60 mg, 40%). ^{31}P NMR (MeOD, 202 MHz): δ 4.03, 3.58. ^{1}H NMR (MeOD, 500 MHz): δ 7.94-7.91 (1H, 2s, H-8), 7.36-7.28 (7H, m, PhO, OCH$_2$*Ph*), 7.19-7.14 (3H, m, PhO, OCH$_2$*Ph*), 5.51, 5.49 (2H, 2s, H-1'), 5.11(2H, OC*H*$_2$Ph), 4.20-4.09 (2H, m, H-5'), 4.06 (3H, OCH$_3$), 3.96-3.86 (1H, m, NHC*H*), 3.74-3.71 (2H, m, H-4'), 1.74-1.65 (1H, m, CH$_2$C*H*(CH$_3$)$_2$), 1.60-1.48 (2H, m, C*H*$_2$CH(CH$_3$)$_2$), 0.89 (3H, d, J= 6.64 Hz, CH$_2$CH(C*H*$_3$)$_2$), 0.80 (3H, d, J= 6.64 Hz, CH$_2$CH(C*H*$_3$)$_2$). ^{13}C NMR (MeOD, 126 MHz): δ 21.74, 22.00, 23.11, 23.20 (4s, CH$_2$CH(*C*H$_3$)$_2$), 25.39, 25.54 (2s, CH$_2$*C*H(CH$_3$)$_2$), 43.84 (d, J_{C-P} = 7.84 Hz, *C*H$_2$CH(CH$_3$)$_2$), 44.06 (d, J_{C-P} = 7.84 Hz, *C*H$_2$CH(CH$_3$)$_2$), 54.25 (OCH$_3$), 54.56, 54.69 (2s, NH*C*H), 67.05 (d, J_{C-P} = 5.04 Hz, C-5'), 67.06 (d, J_{C-P} = 5.04 Hz, C-5'), 67.85, 67.88 (2s, O*C*H$_2$Ph), 69.41 (d, J_{C-P} = 7.29 Hz, C-4'), 69.42 (d, J_{C-P} = 7.29 Hz, C-4'), 73.71 (C-1'), 115.09, 121.25, 121.29, 121.51, 121.55, 125.96, 126.07, 129.35, 129.37, 129.45, 129.48, 129.56, 130.67 (C-5, PhO, OCH$_2$*Ph*), 137.28 ('ipso' OCH$_2$*Ph*), 141.16, 141.20 (2s, C-8), 152.19, 152.24 (2s, 'ipso'PhO), 153.42 (C-4), 155.26 (C-2), 162.20, 162.78 (2s, C-6), 174.81 (d, J_{C-P} = 2.76 Hz, *C*OOCH$_2$Ph), 175.14

(d, J_{C-P} = 2.76 Hz, *C*$OOCH_2Ph$). EI MS= 599.05 (M+H), 621.03 (M+Na). HPLC = H_2O/ACN 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 10 min, 0/100 isocratic 5 min = retention time 8.50 min; H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 15.12, 16.03 min.

Synthesis of 6-*O*-methyl acyclovir-[1-naphthyl(neopentyloxy-L-alaninyl)] phosphate [4.4g]



Chemical Formula: $C_{23}H_{33}N_6O_7P$
Molecular Weight: 536.5179

Neopentyl alcohol (0.05 g, 0.55mmol) was added to a solution of **4.4a** (0.15 g, 0.27 mmol) and *t*BuMgCl (sol THF 1 M, 0.3 ml, 0.3mmol) in THF (3 mL). The mixture was stirred for 2h at room temperature. The solvent was removed and the the residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6 to give a solid which was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 40/60 in 5 min, 60/40 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (100 mg, 70%).

^{31}P NMR (MeOD, 202 MHz): δ 3.76, 3.47. ^{1}H NMR (MeOD, 500 MHz): δ δ 7.90, 7.88 (1H, 2s, H-8), 7.36-7.33 (2H, m, PhO), 7.21-7.17 (3H, m, PhO), 5.56, 5.50 (2H, 2s, H-1'), 4.18-4.11 (2H, m, H-5'), 4.06 (3H, OCH_3), 3.99-3.95 (1H, m, NHC*H*), 3.86-3.76 (4H, m, H-4', $COOCH_2C(CH_3)_3$), 1.39 (1.5H, d, J= 2.52 Hz, $CHCH_3$ of one diasteroisomer), 1.35 (1.5H, d, J= 2.52 Hz, $CHCH_3$ of one diasteroisomer) 0.96-0.95 (9H, m, $COOCH_2C(CH_3)_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.57 (d, J_{C-P} = 6.97 Hz, $CHCH_3$), 20.71 (d, J_{C-P} = 6.97 Hz, $CHCH_3$), 26.74 ($C(CH_3)_3$), 32.36 ($C(CH_3)_3$), 51.63, 51.72 (2s, $CHCH_3$), 54.25 (OCH_3), 67.05 (d, J_{C-P} = 5.51 Hz, C-5'), 67.14 (d, J_{C-P} = 5.51 Hz, C-5'), 69.42 (d, J_{C-P} = 6.97 Hz, C-4'), 69.46 (d, J_{C-P} = 6.97 Hz, C-4'), 73.75 (C-1'), 75.40 ($COOCH_2C(CH_3)_3$), 115.10, 121.41, 121.45, 121.48, 121.52, 126.04, 126.08, 130.71 (C-5, PhO), 141.19, 141.22 (2s, C-8), 152.19 ('ipso'PhO), 153.41 (C-4) 155.29 (C-2), 162.22, 162.79 (2s, C-6), 174.99 (d, J_{C-P} = 5.53 Hz, $COOCH_2C(CH_3)_3$), 175.14 (d, J_{C-P} = 5.53 Hz, $COOCH_2C(CH_3)_3$). EI MS= 559.20 (M+Na). HPLC = H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 13.25, 13.57 min.

9.5. Experimental procedures for chapter 5: synthesis of 8-bromo- and 8-methylacyclovir ProTides

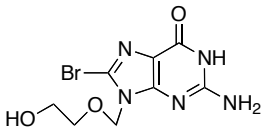
Standard procedure E: *t*BuMgCl method

To a stirring suspension of the appropriate nucleoside (1 eq.) in anhydrous THF was added dropwise under argon atmosphere *t*ButMgCl (2 eq.). The reaction mixture was stirred at room temperature for 30 min. Then, a solution of the appropriate aryl amino acid ester phosphorochloridate **2.2** (2 to 4 eq.) in anhydrous THF was added dropwise and 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 F: bromination of ACV PRoTides

N-Bromosuccinimide (1 eq) was added to a solution of the appropriate ACV Protide **3.2** (1 eq) in methanol. The mixture was stirred at room temperature overnight. The solvent was then removed under reduced pressure and the residue was purified by column chromatography eluting with DCM/MeOH in different proportions.

Synthesis of 8-bromoacyclovir: 9-[(2-hydroxyethoxy)methyl]-8-bromoguanine [5.1]



Chemical Formula: $C_8H_{10}BrN_5O_3$
Molecular Weight: 304.1007

Method 1

N-Bromosuccinimide (0.45 g, 2.5 mmol) was added to a solution of ACV (0.54 g, 2.4 mmol) in acetic acid (7 ml) and the mixture was stirred at room temperature for 20 h. The solution was diluted with water. The precipitated was filtered and washed with water to give a white solid (40%, 0.30 g).

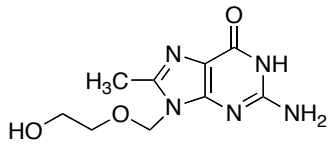
Method 2

A saturated solution of bromine in water was added slowly to a stirring solution of ACV (0.45 g, 2 mmol) in 100 mL of H_2O until the color of Br_2 persisted in solution. This solution was allowed to stand at 0 °C for 2 h. The separated solid was filtered and

recrystallised from EtOH/H_2O to give a white solid (80%, 0.50g).

^{1}H NMR (DMSO, 500 MHz): δ 10.74 (1H, s, NH), 6.63 (2H, s, NH_2), 5.31 (2H, s, H-1'), 4.66 (1H, bs, OH), 3.51-3.47 (4H, m, H-4', H-5'). ^{13}C NMR (MeOD, 126 MHz): δ 59.82 (C-5'), 70.78 (C-4'), 72.36 (C-1'), 116.57 (C-5), 120.85 (C-8), 152.83 (C-4), 154.15 (C-2), 155.50 (C-6). EI MS= 302.9965 (M+H). HPLC = H_2O/AcCN from 90/10 to 0/100 in 20 min = retention time 3.32 min; H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min = retention time 5.36 min.

Synthesis of 8-methylacyclovir: 9-[(2-hydroxyethoxy)methyl]-8-bromoguanine [5.2]

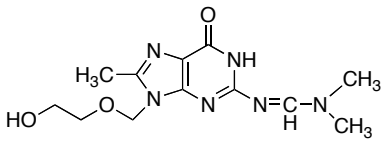


Chemical Formula: $C_9H_{13}N_5O_3$
Molecular Weight: 239.2312

A stirred solution of ACV (0.5 g, 2.22 mmol) and $FeSO_4$*7H_2O (2 g, 7.25 mmol) in 125 mL of H_2SO_4/H_2O (1 M) was deoxygenated thoroughly (O_2-freeN_2) and treated slowly with a mixture of *tert*-butyl hydroperoxide (2.5 g, 27.74 mmol) in 12.5 mL of H_2O over 30 min. The resulting solution was stirred for an additional 30min and adjusted to neutral pH with 1M NaOH/H_2O. A fine brown precipitate was removed by centrifugation and the supernatant solution was evaporated under reduced pressure. The residue was purified by reverse phase column chromatography. Gradient elution with H_2O/ACN (10/0, then 7/3) gave an approximately equal mixture of starting material and product (TLC, *i*/PrOH/ H_2O/conc. NH_3 (aq), 7:2:1). These fractions were combined and evaporated and the residue was resubmitted to the above reaction conditions. TLC now indicated complete conversion of ACV to **5.2**. Purification by reverse phase column chromatography gave a white solid (56%, 0.3 g).

^{1}H NMR (DMSO, 500 MHz): δ 10.60 (1H, bs, NH), 6.46 (2H, s, NH_2), 5.32 (2H, s, H-1'), 4.65 (1H, br s, OH), 3.47-3.42 (4H, m, H-4', H-5'), 2.37 (3H, s, CH_3). ^{13}C NMR (MeOD, 126 MHz): δ 13.34 (CH_3), 59.87 (C-5'), 70.11 (C-4'), 70.93 (C-1'), 114.64 (C-5), 144.64 (C-8), 152.34 (C-4), 153.52 (C-2), 156.34 (C-6). EI MS= 240.1052 (M+H). HPLC= H_2O/AcCN from 100/0 to 90/10 in 5 min, 90/10 isocratic 5 min, then to 0/100 in 5 min, 0/100 isocratic 5 min = retention time 7.87 min; H_2O/MeOH 100/0 to 0/100 in 20 min, 0/100 isocratic 5 min = retention time 7.13 min.

Synthesis of N^2-DMF-8-methylacyclovir: N^2-(*N,N*-dimethylformamidine)-(9-[(2-hydroxyethoxy)methyl]-8-methylguanine [5.6]

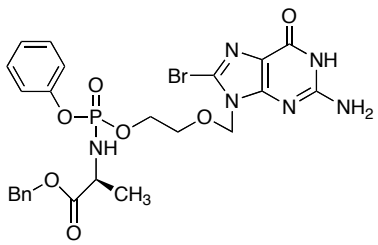


Chemical Formula: $C_{12}H_{18}N_6O_3$
Molecular Weight: 294.3097

To a suspension of **5.2** (0.3g, 1.25 mmol) in dry DMF (10 ml) was added *N,N*-dimethylformamide dimethyl acetale (0.83 mL, 6.25 mmol). The reaction mixture was stirred at room temperetature for 24 h. After this period the suspension was filtered, and the solid was washed with ET_2O to give a white solid (68%, 0.25 g).

^{1}H NMR (MeOH, 500 MHz): δ 8.61 (1H, s, *CH*N$(CH_3)_2$), 5.58 (2H, s, H-1'), 3.67-3.60 (4H, m, H-4', H-5'), 3.24, 3.15 (6H, 2s, N$(CH_3)_2$), 2.55 (3H, s, CH_3).

Synthesis of 8-bromoacyclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [5.4a]



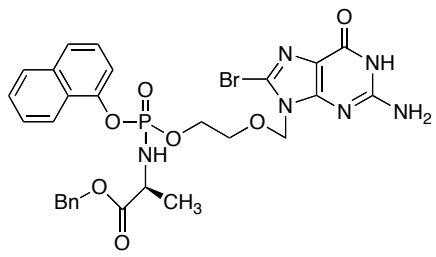
Chemical Formula: $C_{24}H_{26}BrN_6O_7P$
Molecular Weight: 621.3770

Prepared according to Standard Procedure E, from **5.1** (0.40 g, 1.43 mmol) in anhydrous THF (15 mL), *t*BuMgCl (1.0 M THF solution, 2.86 mL, 2.86 mmol), **2.2a** (1.53 g, 4.32 mmol) in anhydrous THF (10 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/AcCN from 90/10 to 0/100 in 20 min) to give a white solid (18%, 0.15 g).

^{31}P-NMR (MeOD, 202 MHz): δ 3.72, 3.46. ^{1}H-NMR (MeOD, 500 MHz): δ 7.36-7.29 (7H, m, PhO, OCH_2*Ph*), 7.19-7.14 (3H, m, PhO, OCH_2*Ph*), 5.44, 5.40 (2H, 2s, H-1'), 5.15-5.13 (2H, 2s, O*C*H_2Ph), 4.18-4.14 (2H, m, H-5'), 4.02-3.99 (1H, m, C*H*CH_3), 3.78-3.76 (2H, m, H-4'), 1.37-1.31 (3H, m, CHCH_3). ^{13}C-NMR (MeOD, 126 MHz): δ 20.35 (d, J_{C-P} = 7.02 Hz, CH*C*H_3), 20.40 (d, J_{C-P} = 7.02 Hz, CH*C*H_3), 51.58, 51.71 (2s, *C*HCH_3), 66.92 (d, J_{C-P} = 6.30 Hz, C-5'), 66.98 (d, J_{C-P} = 6.30 Hz, C-5'), 67.95, 67.97 (2s, O*C*H_2Ph), 69.61 (d, J_{C-P} = 6.31 Hz, C-4'), 69.66 (d, J_{C-P} = 6.31 Hz, C-4'), 73.75 (C-1'), 117.93 (C-5), 121.41, 121.45, 121.48, 121.51, 123.61, 126.08, 127.04, 129.32, 129.35, 129.57, 129.59, 130.22, 130.71 (C-8, PhO, OCH_2*Ph*), 137.27 ('ipso' OCH_2P*h*), 152.13, 152.19 (2s, 'ipso'PhO), 154.88 (C-4), 155.84 (C-2), 158.30 (C-6), 174.71 (d,

J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph), 174.87 (d, J_{C-P} = 5.04 Hz, *C*OOCH$_2$Ph). EI MS= 643.0697 (M+Na). HPLC= H$_2$O/AcCN from 90/10 to 0/100 in 20 min = retention time 12.40 min.

Synthesis of 8-bromoacyclovir-[1-naphthyl(benzyloxy-L-alaninyl)] phosphate [5.4b].

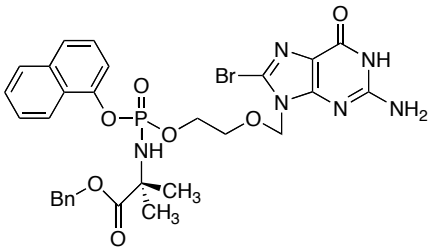


Chemical Formula: $C_{28}H_{28}BrN_6O_7P$
Molecular Weight: 671.4357

Prepared according to Standard Procedure E, from **5.1** (0.30 g, 0.98 mmol) in anhydrous THF (10 mL), ^{t}BuMgCl (1.0 M THF solution, 1.96 mL, 1.96 mmol), **2.2b** (0.79 g, 1.96 mmol) in anhydrous THF (3 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6. The product was further purified by preparative reverse phase HPLC (gradient elution of H$_2$O/AcCN from 90/10 to 0/100 in 20 min) to give a white solid (21%, 0.14 g).

^{31}P-NMR (MeOD, 202 MHz): δ 4.01, 3.92. ^{1}H-NMR (MeOD, 500 MHz): δ 8.15-8.12 (1H, m, H-8 Naph), 7.88-7.87 (1H, m, H-6 Naph), 7.70-7.68 (1H, m, H-2 Naph), 7.53-7.51 (2H, m, H-5 Naph, H-7 Naph), 7.46-7.25 (7H, m, Naph, OCH$_2$P*h*), 5.36, 5.33 (2H, 2s, H-1'), 5.11-5.05 (2H, m, OC*H*$_2$Ph), 4.25-4.18 (2H, m, H-5'), 4.12-4.06 (1H, m, C*H*CH$_3$), 3.78-3.74 (2H, m, H-4'), 1.36-1.33 (3H, m, CHC*H*$_3$). ^{13}C-NMR (MeOD, 126 MHz): δ 20.39 (d, J_{C-P} = 7.84 Hz, CH*C*H$_3$), 20.45 (d, J_{C-P} = 7.84 Hz, CH*C*H$_3$), 51.73, 51.81 (2s, *C*HCH$_3$), 67.17 (d, J_{C-P} = 4.85 Hz, C-5'), 67.21 (d, J_{C-P} = 4.85 Hz, C-5'), 67.95, 67.99 (2s, O*C*H$_2$Ph), 69.65 (d, J_{C-P} = 7.79 Hz, C-4'), 69.70 (d, J_{C-P} = 7.79 Hz, C-4'), 73.74 (C-1'), 116.30, 116.32, 116.38, 116.41, 117.93, 122.74, 122.85, 123.55, 123.57, 125.95, 126.54, 127.44, 127.48, 127.75, 128.81, 128.85, 129.28, 129.30, 129.34, 129.53, 129.58, 130.22, 130 (C-5, C-8, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH$_2$*Ph*), 136.26 ('ipso' OCH$_2$*Ph*), 137.18 137.24 (2s, C-8a Naph), 148.00 (d, J_{C-P} = 7.38 Hz, 'ipso' O*Naph*), 154.81, 154.84 (2s, C-4), 155.83 (C-2), 158.30, 158.32 (C-6), 174.70 (d, J_{C-P} = 4.39 Hz, *C*OOCH$_2$Ph), 174.90 (d, J_{C-P} = 4.39 Hz, *C*OOCH$_2$Ph). EI MS= 693.119 (M+Na). HPLC= H$_2$O/AcCN from 90/10 to 0/100 in 20 min = retention time 12.40 min.

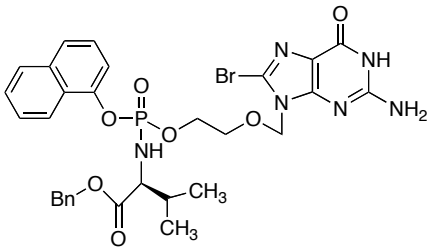
Synthesis of 8-bromoacyclovir-[1-naphthyl(benzyloxy-dimethylglycinyl)] phosphate [5.4c]



Chemical Formula: $C_{29}H_{30}BrN_6O_7P$
Molecular Weight: 685.4623

Prepared according to Standard Procedure E, from **5.1** (0.25 g, 0.83 mmol) in anhydrous THF (15 mL), ^{t}BuMgCl (1.0 M THF solution, 1.7 mL, 1.7 mmol), **2.2h** (0.67 g, 1.7 mmol) in anhydrous THF (5 mL). The reaction mixture was stirred at room temperature overnight. The residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4, to give a white solid (20%, 0.11 g). ^{31}P-NMR (MeOD, 202 MHz): δ. 2.52. ^{1}H-NMR (MeOD, 500 MHz): δ 8.18-8.16 (1H, m, H-8 Naph), 7.87-7.85 (1H, m, H-6 Naph), 7.68-7.67 (1H, m, H-2 Naph), 7.53-7.51 (2H, m, H-7 Naph, H-5 Naph), 7.46-7.25 (7H, m, Naph, OCH_2*Ph*), 5.33 (2H, s, H-1'), 5.16-5.08 (2H, m, OCH_2Ph), 4.20-4.17 (2H, m, H-5'), 3.74-3.71 (2H, m, H-4'), 1.53, 1.51 (6H, 2s, $NHC(CH_3)_2$). ^{13}C-NMR (MeOD, 126 MHz): δ 27.46 (d, J_{C-P} = 4.65 Hz, CH_3), 27.76 (d, J_{C-P} = 4.65 Hz, CH_3), 58.09 ($C(CH_3)_2$), 67.16 (d, J_{C-P} = 5.63 Hz, C-5'), 68.25 (OCH_2Ph), 69.81 (d, J_{C-P} = 7.65 Hz, C-4'), 73.74 (C-1'), 116.35, 116.38, 117.93, 122.99, 123.54, 125.76, 126.51, 127.31, 127.68, 127.90, 127.95, 128.78, 129.26, 129.54 (C-5, C-8, 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_2*Ph*), 136.25, 137.31 (C-4a Naph, 'ipso' OCH_2*Ph*), 148.11 (d, J_{C-P} = 7.61, 'ipso' Naph), 154.82 (C-4), 155.62 (C-2), 158.34 (C-6), 176.57 (d, J_{C-P} = 3.55 Hz, *C*$OOCH_2$Ph). EI MS= 707.1128 (M+Na). HPLC = H_2O/AcCN from 90/10 to 0/100 in 20 min = retention time 14.80 min; H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min = retention time 12.27 min.

Synthesis of 8-bromoacyclovir-[1-naphthyl(benzyloxy-L-valinyl)] phosphate [5.4d].



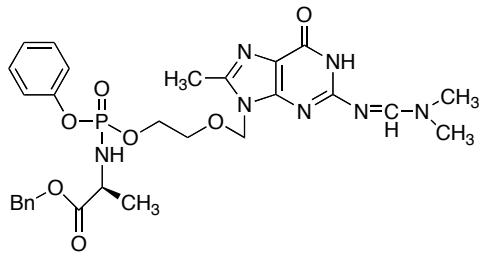
Chemical Formula: $C_{30}H_{32}BrN_6O_7P$
Molecular Weight: 699.4888

Prepared according to standard procedure F, from N-Bromosuccinimide ($7.7*10^{-3}$ g, $0.43*10^{-1}$ mmol) and **3.2g** ($2.7*10^{-2}$ g, $0.44*10^{-1}$ mmol) in MeOH (6 ml). The mixture was stirred at room temperature overnight. The solvent was then removed under reduced pressure and the residue was purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10

to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (36%, $0.11*10^{-1}$ g).

^{31}P-NMR (MeOD, 202MHz): δ 4.74, 4.82. ^{1}H NMR (MeOD, 500 MHz): δ 8.17-8.14 (1H, m, H-8 Naph), 7.89-7.87 (1H, s, H-6 Naph), 7.70-7.68 (1H, m, H-2 Naph), 7.55-7.50 (2H, m, H-5 Naph, H-7 Naph), 7.46-7.37 (2H, m, H-3 Naph, H-4 Naph), 7.33-7.25 (5H, m, OCH_2Ph), 5.36 (2H, 1s, H-1'), 5.05 (2H, 1s, OCH_2Ph), 4.28-4.18 (2H, m, H-5'), 3.81-3.75 (3H, m, NHC*H*, H-4'), 2.06-1.99 (1H, m, $CH(CH_3)_2$), 0.89-0.86 (6H, m, $CH(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 18.27, 18.32 (2s, $CH(CH_3)_2$), 19.48, 19.54 (2s, $CH(CH_3)_2$), 33.09 (d, J_{C-P} = 7.56 Hz, $CH(CH_3)_2$) 33.27 (d, J_{C-P} = 7.56 Hz, $CH(CH_3)_2$), 62.03, 62.07 (2s, NHCH), 67.18 (d, J_{C-P} = 5.55 Hz, C-5'), 67.34 (d, J_{C-P} = 5.55 Hz, C-5'), 67.82 (s, OCH_2Ph), 69.69 (d, J_{C-P} = 7.23 Hz, C-4'), 69.75 (d, J_{C-P} = 7.23 Hz, C-4'), 73.75, 73.78 (2s, C-1'), 116.20, 116.23, 116.38, 117.94, 122.79, 122.91, 123.54, 125.88, 125.92, 126.54, 127.41, 127.44, 127.74, 127.88, 128.78, 128.83, 129.32, 129.36, 129.49, 129.54, 129.56 (C-5, C-8 C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH_2Ph), 136.27 ('ipso' OCH_2Ph), 137.13 (C-8a Naph), 148.03 (d, J_{C-P} = 3.78 Hz, 'ipso' O*Naph*), 148.08 (d, J_{C-P} = 3.78 Hz, 'ipso' O*Naph*) 154.83 (C-4), 155.80 (C-2), 158.28 (C-6), 173.90 (d, J_{C-P} = 2.52 Hz, $COOCH_2Ph$), 174.09 (d, J_{C-P} = 2.52 Hz, $COOCH_2Ph$). EI MS= 699.1307 ($M+H^+$). HPLC = H_2O/AcCN from 90/10 to 0/100 in 20 min = retention time 16.48 min; H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min = retention time 13.44 min., 14.09 min.

Synthesis of N^2-DMF-8-methylacyclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [5.7]

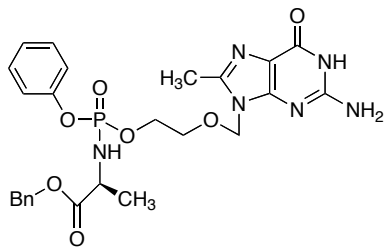


Chemical Formula: $C_{28}H_{34}N_7O_7P$
Molecular Weight: 611.5860

Prepared according to Standard procedure E, from **5.6** (0.25 g, 0.85 mmol) in anhydrous THF (8 mL), ^{t}BuMgCl (1.0 M THF solution, 1.7 mL, 1.7 mmol), **2.2a** (0.6 g, 1.7 mmol) in anhydrous THF (2 mL). The reaction mixture was stirred at room temperature overnight. After this period the solvent was removed under reduced pressure. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 to give a white solid (27%, 0.14 g).

^{31}P-NMR (MeOD, 202 MHz): δ 3.87, 3.51. ^{1}H-NMR (MeOD, 500 MHz): δ 8.69 (1H, bs, NC*H*N(CH_3)$_2$), 7.34-7.29 (7H, m, PhO), 7.19-7.14 (3H, m, PhO), 5.53 (2H, s, H-1'), 5.13, 5.12 (2H, 2s, OC*H*$_2$Ph), 4.20-4.12 (2H, m, H-5'), 3.99-3.94 (1H, m, C*H*CH_3), 3.74-3.69 (2H, m, H-4'), 3.17, 3.11 (6H, 2s, N(C*H*$_3$)$_2$), 2.51, 2.49 (3H, 2s, CH_3-8), 1.36, 1.34 (1.5H, d, J = 6.30, CHC*H*$_3$ of one diasteroisomer), 1.33, 1.31 (1.5H, d, J = 6.30 CHC*H*$_3$ of one diasteroisomer). ^{13}C NMR (MeOD, 126 MHz): δ 13.60 (CH_3-8), 20.35 (d, J_{C-P} = 7.30 Hz, CH*C*H_3), 20.42 (d, J_{C-P} = 7.30 Hz, CH*C*H_3), 35.38, 41.51 (2s, N(*C*H_3)$_2$), 51.60, 51.75 (2s, *C*HCH$_3$), 66.97 (d, J_{C-P} = 5.5 Hz, C-5'), 67.04 (d, J_{C-P} = 5.5 Hz, C-5'), 67.95 (O*C*H$_2$Ph), 69.04 (d, *J* = 6.4 Hz, C-4'), 69.09 (d, J_{C-P} = 6.4, C-4'), 72.46 (C-1'), 118.57, 121.38, 121.42, 121.46, 121.50, 126.10, 126.12, 129.33, 129.34, 129.60, 130.75, 130.77 (C-5, PhO, OCH$_2$*Ph*), 137.28 ('ipso' OCH$_2$*Ph*), 149.57 (C-8), 152.14, 152.19 (2s, 'ipso'PhO), 153.21 (C-4), 154.33 (C-2), 156.91 (C-6), 159.77 (N*C*HN(CH_3)$_2$), 174.65 (d, J_{C-P} = 5.2 Hz, *C*OOCH$_2$Ph), 174.85 (d, J_{C-P} = 5.2 Hz, *C*OOCH$_2$Ph). EI MS= 634.21 (M+Na).

Synthesis of 8-methylacyclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [5.5]



Chemical Formula: $C_{25}H_{29}N_6O_7P$
Molecular Weight: 556.5075

A solution of **5.7** (0.14 g, 0.81 mmol) in isopropanol (10 mL) was stirred under reflux for 62 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 94/6. The product was further purified by preparative TLC plate DCM/MeOH 98/2 then 96/4 then 94/6) to give a white solid (21%, 0.027 g).

^{31}P NMR (MeOD, 202 MHz): δ 3.77, 3.48. ^{1}H NMR (MeOD, 500 MHz): δ 7.39-7.14 (10H, m, OCH$_2$*Ph*) 5.43-5.40 (2H, 2s, H-1'), 5.14-5.13 (2H, 2s, OC*H*$_2$Ph), 4.18-4.11 (2H, m, H-5'), 4.02-3.94 (1H, m, C*H*CH_3), 3.71-3.66 (2H, m, H-4'), 2.47, 2.45 (3H, 2s, CH_3-8), 1.37, 1.35 (1.5H, d, J = 6.30 Hz, CHC*H*$_3$ of one diasteroisomer), 1.34, 1.32 (1.5H, d, J = 6.30 Hz CHC*H*$_3$ of one diasteroisomer).^{13}C NMR (MeOD, 126 MHz): δ 13.45 (CH_3-8), 20.31 (d, J_{C-P} = 7.21 Hz, CH*C*H_3), 20.39 (d, J_{C-P} = 7.21 Hz, CH*C*H_3), 51.70, 51.85 (2s, *C*HCH$_3$), 66.95 (d, J_{C-P} = 5.55 Hz, C-5'), 67.03 (d, J_{C-P} = 5.55 Hz, C-5'), 67.97 (O*C*H$_2$Ph), 69.05 (d, J_{C-P} = 5.79 Hz, C-4'), 69.12 (d, J_{C-P} = 5.79 Hz, C-4'), 72.37 (C-1'), 115.68, 121.38, 121.42, 121.46, 121.49, 122.08, 124.11, 126.08, 126.10, 128.90, 129.02, 129.36, 129.42, 129.58, 130.02, 130.72 (C-5, PhO, OCH$_2$*Ph*), 137.27,

137.69 (2s, 'ipso' OCH_2*Ph*), 148.60 (C-8), 152.14, 152.19 (2s, 'ipso'PhO), 153.25 (C-4), 154.42 (C-2), 155.31 (C-6), 174.70 (d, J_{C-P} = 5.47 Hz, *C*$OOCH_2$Ph), 174.90 (d, J_{C-P} = 5.47 Hz, *C*$OOCH_2$Ph). EI MS= 579.17 (M+Na). HPLC = H_2O/ACN from 100/0 to 0/100 in 15 min, then 0/100 isocratic 5 min = retention time 10.23 min; H_2O/MeOH 90/10 to 0/100 in 25 min, then 0/100 isocratic 5 min = retention time 18.03, 18.29 min.

9.5. Experimental procedures for chapter 5: synthesis of penciclovir and ganciclovir ProTides

Figure 9.2 reports the numbering assigned to the different position in PCV and GCV structures.

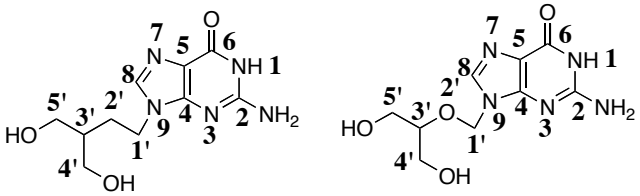


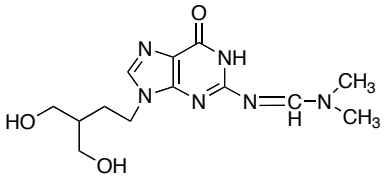
Figure 9.2. Numbering of GCV and PCV structures

Standard procedure G

To a stirring solution/suspension of the appropriate nucleoside (1 eq.) in anhydrous THF or pyridine was added dropwise under argon atmosphere *t*ButMgCl (2 to 6 eq). The reaction mixture was stirred at room temperature for 30 min. Then, a solution of the appropriate aryl amino acid ester phosphorochloridate **2.2** (2 to 4 eq.) in anhydrous THF was added dropwise and 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.

9.5.1 Synthesis of 2,2-dimethylglycine benzyl ester derivatives of penciclovir ProTides 6.5a-b and 6.6a-b

Synthesis of N^2-DMF-penciclovir:
***N*²-(*N*,*N*-dimethylformamidine)-9-[(4-dihydroxy-3-hydroxymethylbut-1-ylpropan-2-yl]-guanine [6.8a].**

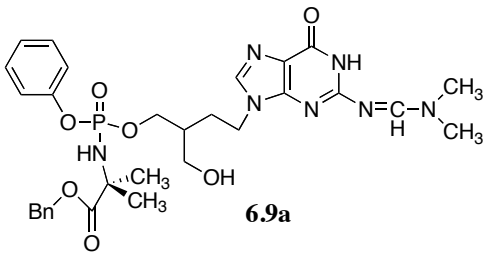


Chemical Formula: C13H20N6O3
Molecular Weight: 308.3363

To a suspension of PCV (2.5 g, 9.8 mmol) in anhydrous DMF (35 ml) was added *N*,*N*-dimethylformamide dimethyl acetal (6.5 mL, 49 mmol) and the reaction mixture was stirred at room temperature overnight. After this period the suspension was filtered and the solid was washed with diethyl ether to give a white solid (90%, 2.7 g).

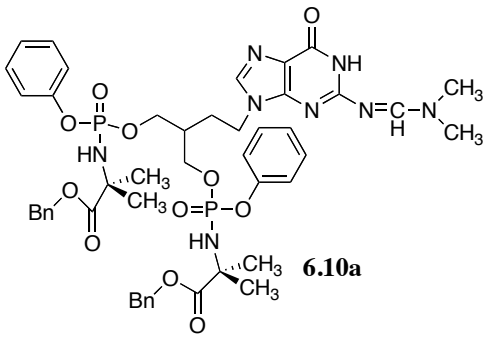
^{1}H-NMR (DMSO, 500 MHz): δ 11.20 (1H, bs, NH), 8.56 (1H, s, C*H*N$(CH_3)_2$), 7.81 (1H, s, H-8), 4.09 (2H, t, J = 7.3 Hz, H-1'), 3.47-3.37 (4H, m, H-4', H-5'), 3.16, 3.04 (6H, 2s, N$(CH_3)_2$), 1.79-1.74 (2H, m, H-2'), 1.47 (1H, m, H-3').

Synthesis of *N*²-DMF-penciclovir-[phenyl(benzyloxy-dimethylglicinyl)] phosphate [6.9a] and *N*²-DMF-penciclovir-[phenyl(benzyloxy-dimethylglicinyl)] diphosphate [6.10a] .



6.9a

Chemical Formula: $C_{30}H_{38}N_7O_7P$
Molecular Weight: 639.6392



6.10a

Chemical Formula: $C_{47}H_{56}N_8O_{11}P_2$
Molecular Weight: 970.9421

Prepared according to standard procedure G from **6.8a** (0.6 g, 1.95 mmol) in anhydrous THF (30 mL) *t*BuMgCl (1.0 M THF solution, 3.89 mL, 3.89 mmol), **2.2g** (1.43 g, 3.9 mmol) in anhydrous THF (5 mL). The reaction mixture was stirred at room temperature overnight. After this period *t*BuMgCl (1.0 M THF solution, 2.0 mL, 2.0 mmol), **2.2g** (0.77 g, 2.1 mmol) in anhydrous THF (2 mL) were added and the reaction mixture was

stirred at room temperature for 6 h. The solvent was removed under reduced pressure. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8, to give two products: **[6.9a]** (30%, 0.38 g) and **[6.10a]** (33%, 0.61 g).

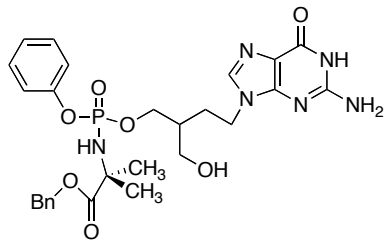
[6.9a]

^{31}P-NMR (MeOD, 202 MHz): δ 2.07, 1.49. ^{1}H-NMR (MeOD, 500 MHz): δ 8.72 (1H, s, NC*H*N(CH$_3$)$_2$), 7.75, 7.70 (1H, 2s, H-8), 7.39-7.28 (7H, m, PhO, OCH$_2$*Ph*), 7.20-7.14 (3H, m, PhO, OCH$_2$*Ph*), 5.13-5.11 (2H, m, H-5'), 4.16-4.00 (4H, m, H-1', H-5'), 3.08, 2.99 (6H, 2s, N(C*H*$_3$)$_2$) 3.54-3.53 (2H, m, H-4'), 1.80-1.75 (3H, m, H-2', H-3'), 1.50 (6H, br s, C(C*H*$_3$)$_2$).

[6.10a]

^{31}P-NMR (MeOD, 202 MHz): δ 2.28, 2.22 (br s). ^{1}H-NMR (MeOD, 500 MHz): δ 8.65 (1H, s, NC*H*N(CH$_3$)$_2$), 7.84 (1H, bs, H-8), 7.31-7.20 (20H, m, 2 x PhO, 2 x OCH$_2$*Ph*), 5.10-5.08 (4H, m, 2 x OC*H*$_2$Ph), 4.17-4.09 (6H, m, H-1', H-4', H-5'), 3.01-3.00 (6H, m, N(C*H*$_3$)$_2$) 1.88-1.74 (3H, m, H-2', H-3'), 1.52-1.47 (12H, m, 2 x C(CH$_3$)$_2$).

Synthesis of penciclovir-[phenyl(benzyloxy-dimethylglicinyl)] phosphate [6.5a]



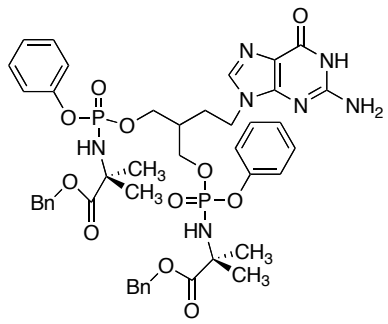
Chemical Formula: $C_{27}H_{33}N_6O_7P$
Molecular Weight: 584.5607

A solution of **6.9a** (0.15 g, 0.23 mmol) in isopropanol (10 mL) was stirred under reflux for 24 h. After this period the solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8 then 90/10. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 40/60 in 5 min, 40/60 isocratic 15 min, then to 0/100 in 5 min) to give a white solid (5.2%, 0.07*10^{-1} g).

^{31}P-NMR (MeOD, 202 MHz): δ 2.37, 2.35. ^{1}H-NMR (MeOD, 500 MHz): δ 7.70, 7.69 (1H, 2s, H-8), 7.38-7.27 (7H, m, PhO, OCH$_2$*Ph*), 7.20-7.14 (3H, m, PhO, OCH$_2$*Ph*), 5.15, 5.14 (2H, 2s, H-5'), 4.19-4.07 (4H, m, H-1', H-5'), 3.54-3.53 (2H, m, H-4'), 1.85-1.72 (3H, m, H-2', H-3'), 1.49 (6H, br s, C(C*H*$_3$)$_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.43 (d, J_{C-P} = 4.95 Hz, C(*C*H$_3$)$_2$), 27.49 (d, J_{C-P} = 4.95 Hz, C(*C*H$_3$)$_2$), 27.66 (d, J_{C-P} = 7.06 Hz, C(*C*H$_3$)$_2$), 27.73 (d, J_{C-P} = 7.06 Hz, C(*C*H$_3$)$_2$), 29.43, 29.52 (2s, C-2'), 40.19 (d,

J_{C-P} = 7.56 Hz, C-3'), 40.28 (d, J_{C-P} = 7.56 Hz, C-3'), 42.33, 42.25 (2s, C-1'), 62.25, 62.29 (2s, C-4'), 67.74 (d, J_{C-P} = 6.3 Hz, C-5'), 67.91 (d, J_{C-P} = 6.3 Hz, C-5'), 68.19 (br s, O*C*H_2Ph), 117.59, 121.54, 121.58, 121.59, 121.63, 126.01, 129.23, 129.27, 129.57, 130.68 (C-5, PhO, OCH$_2$*Ph*), 137.37 ('ipso' OCH$_2$*Ph*), 139.54 (C-8), 152.37, 152.43 (2s, 'ipso' OPh), 153.11 (C4), 155.24 (C-2), 159.45 (C-6), 176.62 (bs, *C*OOCH$_2$Ph). EI MS= 607.21 (M+Na). HPLC = H_2O/ACN from 90/10 to 0/100 in 25 min = retention time 12.07 min; H_2O/MeOH 90/10 to 40/60 in 5 min, 40/60 isocratic 15 min, then to 0/100 in 5 min = retention time 12.35 min.

Synthesis of penciclovir-[phenyl(benzyloxy-dimethylglicinyl)] diphosphate [6.6a]



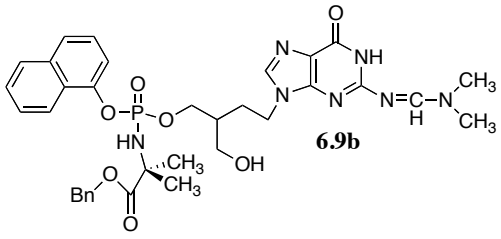
Chemical Formula: $C_{44}H_{51}N_7O_{11}P_2$
Molecular Weight: 915.8636

A solution of **6.10a** (0.60 g, 0.62 mmol) in isopropanol (20 mL) was stirred under reflux for 62 h. After this period the solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 100/0, then 98/2, then 97/3. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 15 min, then to 0/100 in 5 min) to give a white solid (5%, 0.03g).

^{31}P-NMR (MeOD, 202 MHz): δ 2.23, 2.18, 2.17. ^{1}H-NMR (MeOD, 500 MHz): δ 7.65, 7.63 (1H, 2s, H-8), 7.34-7.26 (14H, m, PhO, OCH$_2$*Ph*), 7.19-7.13 (6H, m, PhO, OCH$_2$*Ph*), 5.15-5.09 (4H, m, 2 x OC*H*$_2$Ph), 4.09-4.07 (4H, m, H-4', H-5'), 4.02-3.98 (2H, m, H-1'), 1.88-1.82 (1H, m, H-3'), 1.76-1.65 (2H, m, H-2') 1.49 (12H, br s, 2x C(C*H*$_3$)$_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.47 (d, J_{C-P} = 5.04 Hz, C(*C*H$_3$)$_2$), 27.56 (d, J_{C-P} = 5.04 Hz, C(*C*H$_3$)$_2$), 27.71 (d, J_{C-P} = 6.30 Hz, C(*C*H$_3$)$_2$), 27.81 (d, J_{C-P} = 6.30 Hz, C(*C*H$_3$)$_2$), 29.01, 29.05 (2s, C-2'), 38.38 (d, J_{C-P} = 7.56 Hz, C-3'), 38.50 (d, J_{C-P} = 7.56 Hz, C-3'), 41.81, 41.85 (2s, C-1'), 66.90, 66.94, 67.08, 67.13 (C-4', C-5'), 68.19 (s, O*C*H$_2$Ph), 117.56, 121.35, 121.52, 121.56, 121.58, 121.62, 121.66, 126.05, 129.19, 129.21, 129.24, 129.28, 129.58, 130.74 (C-5, PhO, OCH$_2$*Ph*), 137.36 ('ipso' OCH$_2$*Ph*), 139.49 (C-8), 152.29, 152.35 (2s, 'ipso' OPh), 153.16 (C4), 155.23 (C-2), 159.44 (C-6), 176.56, 176.58, 176.59 (3s, *C*OOCH$_2$Ph), EI MS= 938.31 (M+Na); H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 15 min, then to 0/100 in 5 min =

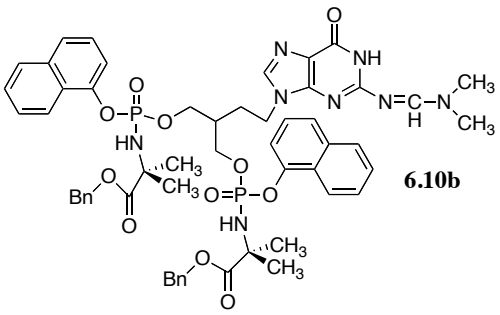
retention time 16.28 min.

Synthesis of N^2-DMF-penciclovir-[1-naphthyl(benzyloxy-dimethylglicinyl)] phosphate [6.9b] and N^2-DMF-penciclovir-[1-naphthyl(benzyloxy-dimethylglicinyl)] diphosphate [6.10b].



6.9b

Chemical Formula: $C_{34}H_{40}N_7O_7P$
Molecular Weight: 689.6979



6.10b

Chemical Formula: $C_{55}H_{60}N_8O_{11}P_2$
Molecular Weight: 1071.0594

Prepared according to standard procedure G from **6.8a** (0.6 g, 1.95 mmol) in anhydrous THF (35 ml), *t*BuMgCl (1.0 M THF solution, 3.89 mL, 3.89 mmol), **2.2h** (1.43 g, 3.9 mmol) in anhydrous THF (10 ml) and the reaction mixture was stirred at room temperature overnight. After this period *t*BuMgCl (1.0 M THF solution, 2.0 mL, 2.0 mmol), **2.2h** (0.9 g, 2.1 mmol) in anhydrous THF (2 mL) were added and the reaction mixture was stirred at room temperature for 4 h. The solvent was removed under reduced pressure. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8, to give two products: **[6.9b]** (48%, 0.66 g) and **[6.10b]** (40%, 0.54 g,).

[6.9b]

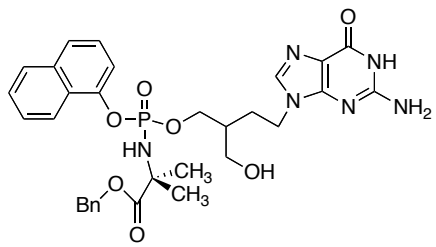
^{31}P-NMR (MeOD, 202 MHz): δ 2.44 (br s). ^{1}H-NMR (MeOD, 500 MHz): δ 8.65 (1H, s, NC*H*N$(CH_3)_2$), 8.15-8.09 (1H, m, H-8 Naph), 7.89-7.70 (2H, m, H-6-Naph, H-8), 7.65-7.55 (1H, m, H-2 Naph), 7.50-7.40 (2H, m, H-5 Naph, H-7 Naph), 7.30-7.21 (7H, NaphO, OCH_2*Ph*), 5.15-5.07 (2H, m, OCH_2Ph), 4.06-3.98 (4H, m, H-1', H-5'), 3.37-3.25 (2H, m, H-4'), 2.80-2.75 (6H, 2s, N$(CH_3)_2$), 1.75-1.64 (1H, m, H-3'), 1.56-1.50 (8H, m, H-2', C$(CH_3)_2$).

[6.10b]

^{31}P-NMR (MeOD, 202 MHz): δ 2.82, 2.74, 2.59. ^{1}H-NMR (MeOD, 500 MHz): δ 8.34-8.29 (1H, m, NC*H*N$(CH_3)_2$), 8.18-8.13 (2H, m, 2 x H-8 Naph), 7.77-7.70 (2H, m, 2 x H-

6 Naph), 7.67-7.52 (3H, m, H-8, 2 x H-2 Naph), 7.47-7.16 (18H, m, Naph, 2 x OCH_2*Ph*) 5.11-5.04 (4H, m, 2 x OCH_2Ph), 4.15-4.00 (4H, m, H-4', H-5'), 3.91-3.79 (2H, m, H-1'), 2.89-2.81 (6H, m, $N(CH_3)_2$), 1.79-1.75 (1H, m, H-3'), 1.53-1.47 (14H, m, H-2', 2 x $C(CH_3)_2$).

Synthesis of penciclovir-[phenyl(benzyloxy-dimethylglicinyl)] phosphate [6.5b]

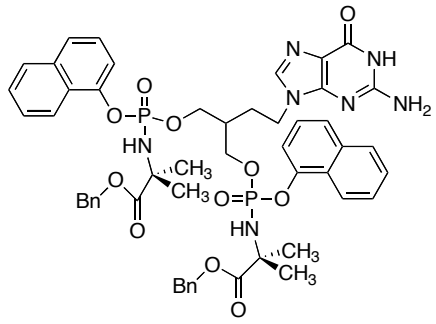


Chemical Formula: $C_{31}H_{35}N_6O_7P$
Molecular Weight: 634.6194

A solution of **6.9b** (0.66 g, 0.95 mmol) in isopropanol (20 mL) was stirred under reflux for 24 h . After this period the solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8 then 90/10. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 80/20 in 5 min, 80/20 isocratic 15 min, then to 0/100 in 5 min) to give a white solid (5%, $0.3*10^{-1}$ g).

^{31}P-NMR (MeOD, 202 MHz): δ 2.89, 2.85. ^{1}H-NMR (MeOD, 500 MHz): δ 8.18-8.13 (1H, m, H-8 Naph), 7.84-7.79 (1H, m, H-6 Naph), 7.66,7.64 (1H, 2s, H-8), 7.62-7.59 (1H, m, H-2 Naph), 7.49-7.40 (2H, m, H-5 Naph, H-7 Naph), 7.37-7.21 (7H, NaphO, OCH_2*Ph*), 5.1-5.04 (2H, m, OCH_2Ph), 4.06-4.00 (4H, m, H-1', H-5'), 3.37-3.20 (2H, m, H-4'), 1.79-1.70 (1H, m, H-3'), 1.56-1.50 (8H, m, H-2', $C(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.50 (d, J_{C-P} = 4.77 Hz, $C(CH_3)_2$), 27.57 (d, J_{C-P} = 4.77 Hz, $C(CH_3)_2$), 27.86 (d, J_{C-P} = 7.28 Hz, $C(CH_3)_2$), 27.96 (d, J_{C-P} = 7.28 Hz, $C(CH_3)_2$), 29.35, 29.53 (2s, C-2'), 40.21 (d, J_{C-P} = 7.48 Hz, C-3'), 40.31 (d, J_{C-P} = 7.48 Hz, C-3'), 42.16, 42.26 (2s, C-1'), 62.21, 62.25 (2s, C-4'), 67.85 (d, J_{C-P} = 6.07 Hz, C-5'), 68.08 (d, J_{C-P} = 6.07 Hz, C-5'), 68.25 (bs, OCH_2Ph), 116.55, 116.58, 116.71, 116.73, 117.55, 123.02, 125.84, 125.87, 126.48, 127.29, 127.68, 127.69, 127.97, 128.02, 128.06, 128.81, 129.23, 129.25, 129.27, 129.55 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH_2*Ph*), 136.25 ('ipso' OCH_2*Ph*), 137.34 (C-8a Naph) 139.45 (C-8), 148.20, 148.26 (2s, 'ipso' O*Naph*), 153.03 (C-4), 155.20 (C-2), 159.46 (C-6), 176.69, 176.72 (2s, *C*$OOCH_2$Ph). EI MS= 635.28 (M+H). HPLC = H_2O/ACN from 90/10 to 0/100 in 25 min = retention time 13.87 min; H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 5 min, then to 0/100 in 5 min= retention time 8.55 min.

Synthesis of penciclovir-[1-naphtyl-(benzyloxy-dimethylglicinyl)]diphosphate [6.6b]



Chemical Formula: $C_{52}H_{55}N_7O_{11}P_2$
Molecular Weight: 1015.9809

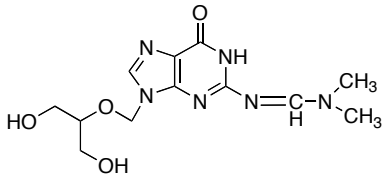
A solution of **6.10b** (0.54 g, 0.50 mmol) in isopropanol (20 mL) was stirred under reflux for 62 h. After this period the solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 100/0, 98/2, then 97/3. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 25 min, then to 0/100 in 5 min) to give a white solid (5%, $0.25*10^{-1}$ g).

^{31}P-NMR (MeOD, 202 MHz): δ 2.76, 2.70, 2.64, 2.57. ^{1}H-NMR (MeOD, 500 MHz): δ 8.18-8.13 (2H, m, 2 x H-8 Naph), 7.84-7.79 (2H, m, 2 x H-6 Naph), 7.65,7.64 (1H, 2s, H-8), 7.62-7.59 (2H, m, 2 x H-2 Naph), 7.49-7.18 (18H, m, Naph, 2 x OCH_2*Ph*), 5.12-5.03 (4H, m, 2 x OCH_2Ph), 4.09-4.07 (4H, m, H-4', H-5'), 4.02-3.98 (2H, m, H-1'), 1.78-1.72 (1H, m, H-3'), 1.52-1.47 (14H, m, H-2', 2 x $C(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.48 (d, J_{C-P} = 7.48 Hz, $C(CH_3)_2$), 27.54 (d, J_{C-P} = 7.48 Hz, Hz $C(CH_3)_2$), 27.59 (d, J_{C-P} = 7.56, $C(CH_3)_2$), 27.89 (d, J_{C-P} = 7.56, $C(CH_3)_2$), 27.97 (d, J_{C-P} = 7.56 Hz, $C(CH_3)_2$), 28.07 (d, J_{C-P} = 7.56 Hz, $C(CH_3)_2$), 28.88, 28.72 (2s, C-2'), 38.32 (d, J_{C-P} = 7.56 Hz, C-3'), 38.48 (d, J_{C-P} = 7.56 Hz, C-3'), 41.72, 41.67 (2s, C-1'), 66.88, 66.97, 67.01, 67.28 (C-4', C-5'), 68.21 (OCH_2Ph), 116.46, 116.48, 116.66, 116.69, 116.77, 116.80, 117.53, 122.96, 123.02, 123.07, 123.10, 125.83, 125.88, 125.92, 126.50, 126.52, 126.54, 127.35, 127.37, 127.68, 127.70, 127.72, 128.84, 129.17, 129.19, 129.22, 129.25 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH_2*Ph*), 136.22, 136.25 ('ipso' OCH_2*Ph*), 137.28, 137.31 (2s, C-8a Naph) 139.25, 139.31 (2s, C-8), 148.14, 148.18 (2s, 'ipso' O*Naph*), 153.00 (C-4), 155.15 (C-2), 159.43 (C-6), 176.62, 176.64 (2s, *C*$OOCH_2$Ph). EI MS= 1038.34 (M+Na). HPLC**=**H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 5 min, then to 0/100 in 5 min, then 0/100 for 5= retention time 30.76, 31.97 min.

9.5.2 Synthesis of 2,2-dimethylglycine benzyl ester derivatives of ganciclovir ProTides 6.5c-d and 6.5c-d

Synthesis of N^2-DMF-ganciclovir:

N^2-(*N,N*-dimethylformamidine)-9-[(1,3-dihydroxypropan-2-yloxy)methyl]-guanine [6.8b].

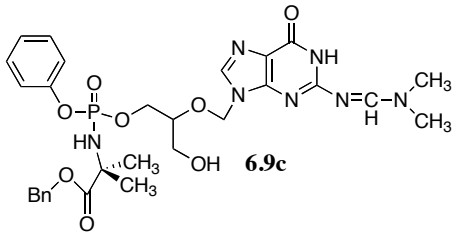


Chemical Formula: $C_{12}H_{18}N_6O_3$
Molecular Weight: 310.3091

To a suspension of GCV (2 g, 7.8 mmol) in anhydrous DMF (35 ml) was added *N,N*-dimethylformamide dimethyl acetal (5.2 mL, 39 mmol) and the reaction mixture was stirred at room temperature overnight. After this period the suspension was filtered and the solid was washed with diethyl ether to give a white solid (95% 2.3 g).

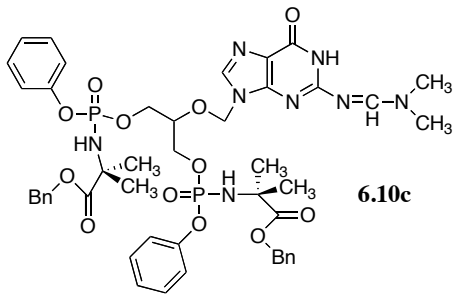
^{1}H-NMR (DMSO, 500 MHz): δ 11.33 (1H, bs, NH), 8.58 (1H, s, C*H*N$(CH_3)_2$), 7.96 (1H, s, H-8), 5.54 (2H, s, H-1'), 4.64 (2H, bs, 2XOH), 3.66-3.60 (1H, m, H-3'), 3.49-3.43 (4H, m, H-4', H-5'), 3.17, 3.05 (6H, 2s, N$(CH_3)_2$).

Synthesis of N^2-DMF-ganciclovir-[1-phenyl(benzyloxydimethylglicinyl)] phosphate [6.9c] and N^2-DMF ganciclovir-[1-phenyl(benzyloxydimethylglicinyl)] diphosphate [6.10c]



6.9c

Chemical Formula: $C_{29}H_{36}N_7O_8P$
Molecular Weight: 641.612



6.10c

Chemical Formula: $C_{46}H_{54}N_8O_{12}P_2$
Molecular Weight: 972.9149

Prepared according to standard procedure G from **6.8b** (0.4 g, 1.3 mmol) in anhydrous mixture of THF (30 mL) and pyridine (10 mL), *t*BuMgCl (1.0 M THF solution, 2.6 mL, 2.6 mmol), and **2.2g** (1.43 g, 3.9 mmol) in anhydrous THF (5 mL) and the reaction mixture was stirred at room temperature overnight. After this period *t*BuMgCl (1.0 M THF solution, 2.6 mL, 2.6 mmol) ws added and the reaction mixture was stirred at room temperature for 6 h. The solvent was removed under reduced pressure. The residue was

purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8, to give two products: **[6.9c]** (40%, 0.33 g), **[6.10c]** (38%, 0.47 g).

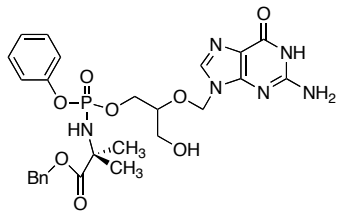
[6.9c]

^{31}P-NMR (MeOD, 202 MHz): δ 1.72 (br s). ^{1}H-NMR (MeOD, 500 MHz): δ 8.70 (1H, s, NC*H*N(CH$_3$)$_2$), 7.82-7.81 (1H, m, H-8), 7.40-7.30 (7H, m, PhO, OCH$_2$*Ph*), 7.20-7.15 (3H, m, PhO, OCH$_2$*Ph*), 5.55, 5.52 (2H, 2s, H-1'), 5.19-5.11 (2H, m, OC*H*$_2$Ph), 4.45-4.20 (1H, m, H-3'), 4.07-4.01 (1H, m, H-5'), 3.99-3.94 (1H, m, H-5'), 3.60-3.47 (2H, m, H-4'), 2.80-2.75 (6H, 2s, N(C*H*$_3$)$_2$), 1.49 (6H, br s, C(C*H*$_3$)$_2$).

[6.10c]

^{31}P-NMR (MeOD, 202 MHz): δ 1.91 (br s). ^{1}H-NMR (MeOD, 500 MHz): δ 8.70-8.64 (1H, m, NC*H*N(CH$_3$)$_2$), 7.70 (1H, bs, H-8), 7.40-7.30 (14H, m, PhO, OCH$_2$*Ph*), 7.19-7.15 (6H, m, PhO, OCH$_2$*Ph*), 5.45-5.42 (2H, m, H-1'), 5.20-5.15 (4H, m, OC*H*$_2$Ph), 4.18-4.01 (5H, m, H-3', H-4', H-5'), 2.90-2.82 (6H, 2s, N(C*H*$_3$)$_2$) 1.50 (12H, br s, 2 x C(C*H*$_3$)$_2$)

Synthesis of ganciclovir-[phenyl(benzyloxydimethylglicinyl)] phosphate [6.5c]



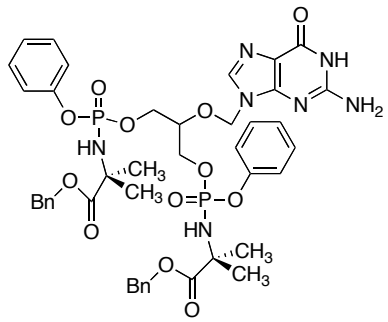
Chemical Formula: C26H31N6O8P
Molecular Weight: 586.5335

A solution of **6.9c** (0.33 g, 0.52 mmol) in isopropanol (10 mL) was stirred under reflux for 24 h. After this period the solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min) to give a white solid (6.5%, 0.02 g).

^{31}P-NMR (MeOD, 202 MHz): δ 2.26, 2.24. ^{1}H-NMR (MeOD, 500 MHz): δ 7.82, 7.81 (1H, 2s, H-8), 7.37-7.31 (7H, m, PhO, OCH$_2$*Ph*), 7.19-7.15 (3H, m, PhO, OCH$_2$*Ph*), 5.52, 5.51 (2H, 2s, H-1'), 5.16-5.12 (2H, m, OC*H*$_2$Ph), 4.20-4.14 (1H, m, H-3'), 4.09-4.04 (1H, m, H-5'), 3.94-3.90 (1H, m, H-5'), 3.60-3.47 (2H, m, H-4'), 1.49 (6H, br s, C(C*H*$_3$)$_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.39 (d, J_{C-P} = 6.3 Hz, C(*C*H$_3$)$_2$), 27.61 (d, J_{C-P} = 6.3 Hz, C(*C*H$_3$)$_2$), 27.63 (d, J_{C-P} = 6.3 Hz, C(*C*H$_3$)$_2$), 61.95, 61.99 (2s, C -4'), 67.15 (d, J_{C-P} = 5.54 Hz, C -5'), 67.19 (d, J_{C-P} = 5.54 Hz, C -5'), 68.21, 68.20 (2s,

O*C*H_2Ph), 72.96, 73.07 (2s, C-1') 79.23 (d, J_{C-P} = 5.29 Hz, C-3'), 79.30 (d, J_{C-P} = 5.29 Hz, CH_2*C*HCH_2), 115.24, 115.85, 117.55, 121.51, 121.55, 122.00, 126.01, 128.00, 129.25, 129.27, 129.57, 130.68, 130.70 (C-5, PhO, OCH$_2$*Ph*), 137.38 ('ipso' OCH$_2$*Ph*), 139.76 (s, C-8), 152.25 (d, J_{C-P} = 2.56 Hz, 'ipso' OPh), 152.31 (d, J_{C-P} = 2.56 Hz, 'ipso' OPh), 153.32 (C4), 155.65 (C-2), 159.34 (C-6), 176.54 (d, J_{C-P} = 3.78 Hz, *C*OOCH$_2$Ph), 176.57 (d, J_{C-P} = 3.78 Hz, *C*OOCH$_2$Ph). EI MS= 587.2022 (M+H$^+$). HPLC = H_2O/AcCN from 90/10 to 0/100 in 20 min = retention time 10.96 min; H_2O/MeOH 90/10 to 30/70 in 5 min, 30/70 isocratic 10 min, then to 0/100 in 5 min = retention time 9.67 min.

Synthesis of ganciclovir-[phenyl(benzyloxydimethylglicinyl)] diphosphate [6.6c]



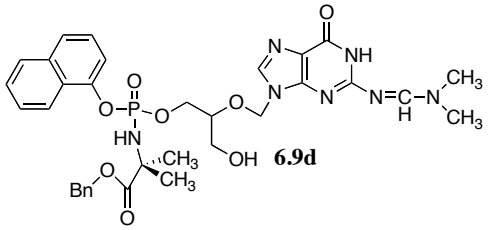
Chemical Formula: $C_{43}H_{49}N_7O_{12}P_2$
Molecular Weight: 917.8364

A solution of **6.10c** (0.47 g, 0.49 mmol) in isopropanol (15 mL) was stirred under reflux for 62 h. After this period the solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 30/70 in 5 min, 20/80 isocratic 15 min, then to 0/100 in 5 min) to give a white solid (4.3%, 0.02 g).

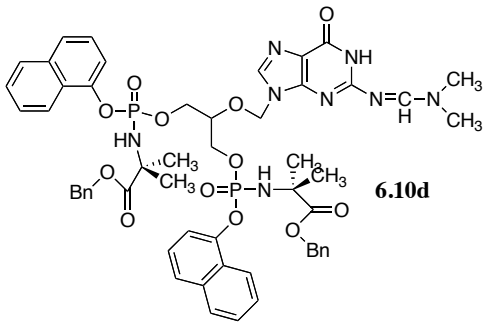
^{31}P-NMR (MeOD, 202 MHz): δ 2.18, 2.16, 2.11. ^{1}H-NMR (MeOD, 500 MHz): δ 7.76 (1H, bs, H-8), 7.37-7.27 (14H, m, PhO, OCH$_2$*Ph*), 7.18-7.14 (6H, m, PhO, OCH$_2$*Ph*), 5.43 (2H, br s, H-1'), 5.16-5.10 (4H, m, OC*H*$_2$Ph), 4.17-4.04 (5H, m, H-3', H-4', H-5'), 1.48 (12H, br s, 2 x C(C*H*$_3$)$_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.43 (d, J_{C-P} = 3.64 Hz, C(*C*H$_3$)$_2$), 27.65 (d, J_{C-P} = 3.64 Hz, C(*C*H$_3$)$_2$), 27.70 (d, J_{C-P} = 3.64 Hz, C(*C*H$_3$)$_2$), 66.38, 66.42, 66.47, 66.52 (C-4', C-5'), 68.22 (bs, O*C*H$_2$Ph), 72.66, 72.79 (2s, C-1') 77.96, 77.05 (2s, C-3'), 117.58, 121.50, 121.53, 121.57, 126.06, 129.23, 129.24, 129.29, 129.59, 130.72, 130.75 (C-5, PhO, OCH$_2$*Ph*), 137.36 ('ipso' OCH$_2$*Ph*), 139.66, 139.68 (s, C-8), 152.21 (d, J_{C-P} = 7.56 Hz, 'ipso' OPh), 152.22 (d, J_{C-P} = 7.56 Hz, 'ipso' OPh), 153.32 (C4), 155.65 (C-2), 159.30 (C-6), 176.52 (s, J_{C-P} = 3.78, *C*OOCH$_2$Ph), 176.54 (s, J_{C-P} = 2.52 Hz, *C*OOCH$_2$Ph). EI MS= 918.3033 (M+H$^+$). HPLC = H_2O/AcCN from 90/10 to 0/100 in 20 min = retention time 16.88 min; H_2O/MeOH 90/10 to 30/70 in 5

min, 20/80 isocratic 15 min, then to 0/100 in 5 min = retention time 14.65 min.

Synthesis of N^2-DMF-ganciclovir-[1-naphthyl(benzyloxydimethylglicinyl)] phosphate [6.9d] and N^2-DMF-ganciclovir-[1-naphthyl(benzyloxy-dimethylglicinyl)] diphosphate [6.10d]



Chemical Formula: $C_{33}H_{38}N_7O_8P$
Molecular Weight: 691.6707



Chemical Formula: $C_{54}H_{58}N_8O_{12}P_2$
Molecular Weight: 1073.0322

Prepared according to standard procedure G from **6.8b** (0.5 g, 1.6 mmol) in anhydrous THF (30 mL), *t*BuMgCl (1.0 M THF solution, 3.2 mL, 3.2 mmol), **2.2h** (1.67 g, 4.14 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. After this period the solvent was removed under reduced pressure. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8, to give two products: **6.9d** (56%, 0.62 g), **6.10d** (35%, 0.6 g).

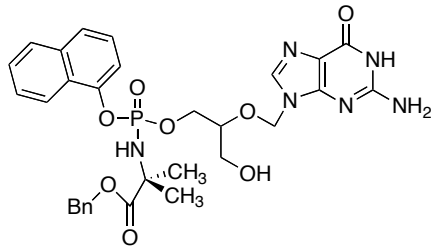
[6.9d]

^{31}P-NMR (MeOD, 202 MHz): δ 2.31 (br s). ^{1}H-NMR (MeOD, 500 MHz): δ 8.60 (1H, s, NC*H*N$(CH_3)_2$, 7.78-7.81 (1H, m, H-8), 7.40-7.35 (7H, m, PhO, OCH_2*Ph*), 7.20-7.14 (3H, m, PhO, OCH_2*Ph*), 5.57-5.50 (2H, m, H-1'), 5.15-5.12 (2H, m, OCH_2Ph), 4.20-4.14 (1H, m, H-3'), 4.07-4.00 (1H, m, H-5'), 3.91-3.87 (1H, m, H-5'), 3.60-3.47 (2H, m, H-4'), 2.74-2.64 (6H, 2 s, N$(CH_3)_2$), 1.52 (6H, br s, C$(CH_3)_2$).

[6.10d]

^{31}P-NMR (MeOD, 202 MHz): δ 2.70, 2.66, 2.62, 2.60. ^{1}H-NMR (MeOD, 500 MHz): δ 8.47, 8.46 (1H, 2s, NC*H*N$(CH_3)_2$, 8.13-8.10 (2H, m, 2x H-8 Naph), 7.85 (2H, bs, 2 x H-6 Naph), 7.68-7.65 (3H, m, H-8, 2 x H-2 Naph), 7.51-7.24 (18H, m, Naph, 2 x OCH_2P*h*), 5.32-5.28 (2H, m, H-1'), 5.11-5.08 (4H, m, OCH_2Ph), 4.25-4.07 (5H, m, H-3', H-4', H-5'), 2.90-2.88 (6H, m, N$(CH_3)_2$), 1.48-1.43 (12H, m, 2 x C$(CH_3)_2$)

Synthesis of ganciclovir-[1-naphthyl(benzyloxydimethylglicinyl)] phosphate [6.5d]

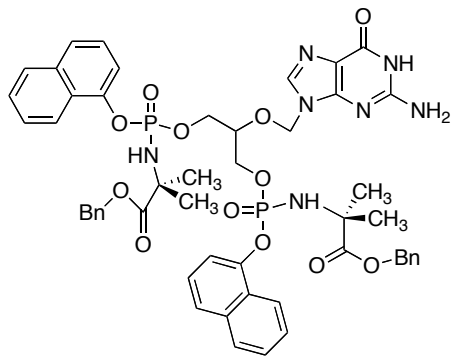


Chemical Formula: C30H33N6O8P
Molecular Weight: 636.5922

A solution of **6.9d** (0.62 g, 0.9 mmol) in isopropanol (20 mL) was stirred under reflux for 62 h. After this period the solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8. The product was further purified by preparative TLC (gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6) to give a white solid (2.7%, $0.15*10^{-1}$ g).

^{31}P-NMR (MeOD, 202 MHz): δ 2.65, 2.69. ^{1}H-NMR (MeOD, 500 MHz): δ 8.19-8.14 (1H, m, H-8 Naph), 7.89-7.87 (1H, 2s, H-6 Naph), 7.76 (1H, s, H-8), 7.71-7.68 (1H, m, H-2 Naph), 7.54-7.52 (2H, m, H-5 Naph, H-7 Naph), 7.47-7.27 (7H, m, Naph, OCH_2Ph), 5.42, 5.43 (2H, 2s, H-1'), 5.17-5.10 (2H, m, OCH_2Ph), 4.26-4.20 (1H, m, H-5'), 4.15-4.10 (1H, m, H-5'), 3.93-3.88 (1H, m, H-3'), 3.57-3.45 (2H, m, H-4'), 1.53-1.51 (6H, m, $C(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.47 (d, J_{C-P} = 5.20 Hz, $C(CH_3)_2$), 27.75 (d, J_{C-P} = 5.20 Hz, $C(CH_3)_2$), 27.80 (d, J_{C-P} = 5.20 Hz, $C(CH_3)_2$), 61.93, 61.96 (2s, C-4'), 67.34 (d, J_{C-P} = 5.76 Hz, C-5'), 67.38 (d, J_{C-P} = 5.76 Hz, C-5'), 68.24 (OCH_2Ph), 72.96, 73.06 (2s, C-1') 79.23 (d, J_{C-P} = 7.90 Hz, C-3'), 79.29 (d, J_{C-P} = 7.90 Hz, C-3'), 116.41, 116.44, 116.47, 117.54, 122.98, 125.82, 126.51, 127.36, 127.71, 127.91, 127.91, 127.96, 128.80, 129.23, 129.25, 129.55 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH_2Ph), 136.26 ('ipso' OCH_2Ph), 137.34 (C-8a Naph), 139.69 (C-8), 148.10 (d, J_{C-P} = 7.56, 'ipso' O*Naph*), 148.13 (d, J_{C-P} = 7.56, 'ipso' O*Naph*), 153.25 (C4), 155.62 (C-2), 159.31 (C-6), 176.60 (d, J_{C-P} = 3.35 Hz, $COOCH_2Ph$), 176.62 (d, J_{C-P} = 3.35 Hz, $COOCH_2Ph$). EI MS= 659.20 (M+Na). HPLC = H_2O/AcCN from 90/10 to 0/100 in 20 min = retention time 13.71 min; H_2O/MeOH from 90/10 to 20/80 in 5 min, 20/80 isocratic 10 min, then to 0/100 in 5 min = retention time 9.99 min.

Synthesis of ganciclovir-[1-naphthyl(benzyloxydimethylglicinyl)] diphosphate [6.6d]



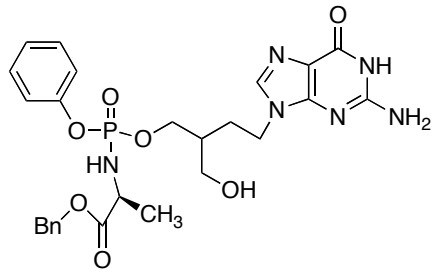
Chemical Formula: $C_{51}H_{53}N_7O_{12}P_2$
Molecular Weight: 1017.9537

A solution of **6.10d** (0.6 g, 0.56 mmol) in isopropanol (20 mL) was stirred under reflux for 62 h. After this period the solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 to give a white solid (5.3%, $0.3*10^{-1}$ g).

^{31}P-NMR (MeOD, 202 MHz): δ 2.63, 2.57, 2.53, 2.52. ^{1}H-NMR (MeOD, 500 MHz): δ 8.13-8.10 (2H, m, 2 x H-8 Naph), 7.85 (2H, bs, 2 x H-6 Naph), 7.68-7.65 (3H, m, H-8, 2 x H-2 Naph), 7.51-7.24 (18H, m, Naph, 2 x OCH_2Ph), 5.32-5.28 (2H, m, H-1'), 5.11-5.08 (4H, m, OCH_2Ph), 4.25-4.07 (5H, m, H-3', H-4', H-5'), 2.90-2.88 (6H, m, $N(CH_3)_2$), 1.48-1.43 (6H, m, $C(CH_3)_2$). ^{13}C NMR (MeOD, 126 MHz): δ 27.47 (br s, $C(CH_3)_2$), 27.79, 27.82, 27.85, 27.88, ($C(CH_3)_2$), 66.44, 66.49, 66.52, 66.57, 66.67, 66.64 (C-4', C-5'), 68.23 (br s, OCH_2Ph), 72.66, 72.79 (2s, C-1') 77.10, 77.03 (2s, C-3'), 116.39, 116.42, 116.44, 116.51, 116.53, 117.56, 122.93, 122.98, 123.50, 125.87, 126.53, 127.21, 127.42, 127.70, 127.73, 127.89, 127.94, 128.81, 129.19, 129.24, 129.54, 129.76 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, OCH_2Ph), 136.24, 136.26 (2s, 'ipso' OCH_2Ph), 137.29, 137.31 (2s, C-8a Naph), 139.53 (C-8), 147.99, 148.05 (2s, 'ipso'O*Naph*), 153.23 (C4), 155.59 (C-2), 159.26 (C-6), 176.57, 176.59 (2s, $COOCH_2Ph$). EI MS= 1018.34 ($M+H^+$). HPLC = H_2O/ACN from 90/10 to 0/100 in 40 min = retention time 34.05 min; H_2O/MeOH 90/10 to 20/80 in 5 min, 20/80 isocratic 30 min, then to 0/100 in 5 min = retention time 35.52 min.

9.5.3 Synthesis of L-alanine benzyl ester derivatives of penciclovir ProTides [6.7a-b]

Synthesis of penciclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [6.7a]

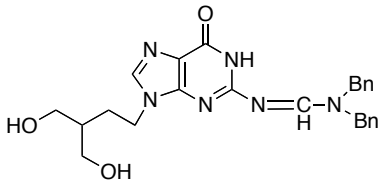


Chemical Formula: $C_{51}H_{53}N_7O_{12}P_2$
Molecular Weight: 1017.9537

Prepared according to standard procedure G, from penciclovir (0.3 g, 1.18 mmol) in anhydrous pyridine (20 mL), *t*BuMgCl (1.0 M THF solution, 2.36mL, 2.36 mmol), **2.2a** (0.8 g, 2.36 mmol) in anhydrous THF (2.5 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6, then 92/8. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 40/60 in 5 min, 40/60 isocratic 15 min, then to 0/100 in 5 min) to give a white solid (5%, 0.035 g) .

^{31}P-NMR (MeOD, 202 MHz): δ 4.12, 4.05, 3.61, 3.59. ^{1}H-NMR (MeOD, 500 MHz): δ 8.01-7.99 (1H, m, H-8), 7.37-7.27 (7H, m, PhO, OCH_2*Ph*), 7.21-7.15 (3H, m, PhO, OCH_2*Ph*), 5.15, 5.14 (2H, 2s, OCH_2Ph), 4.20-4.11 (4H, m, H-1', H-5'), 4.04-4.01 (1H, m, *CH*CH_3), 3.57-3.51 (2H, m, H-4'), 1.89-1.75 (3H, m, H-2', H-3'), 1.39-1.36 (3H, m, $CHCH_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.39, 20.44 ($CHCH_3$), 29.28, 29.32, 29.37, 29.39, 29.46 (C-2'), 40.20 (C-3'), 42.77, 42.81 (C-1'), 51.67, 51.70 (NH*C*H), 62.21, 62.25, 62.32 (C-4'), 67.70, 67.74, 67.79 (C-5'), 67.98, 68.00 (2S, OCH_2Ph), 120.84, 121.47, 121.52, 121.55, 121.59, 121.63, 126.17, 128.01, 128.28, 129.27, 129.33, 129.37, 130.32, 130.78, 131.06, 131.10 (C-5, PhO, OCH_2*Ph*), 137.26 ('ipso' OCH_2*Ph*), 141.05 (C-8), 152.21, 152.27 ('ipso' OPh), 153.11 (C4), 155.20, 152.24, 152.6 (C-2, C-3), 155.68 (C-6), 174.82, 175.07 (2s, *C*$OOCH_2$Ph). EI MS = 571.24 (M+H). HPLC = H_2O/ACN from 90/10 to 0/100 in 25 min, then 0/100 isocratic 5 min = retention time 16.88 min; H_2O/MeOH from 90/10 to 0/100 in 25 min, then 0/100 isocratic 5 min = retention time 23.35 min.

Synthesis of N^2-(*N*,*N*-dibenzylformamidine)-penciclovir: N^2-(*N*,*N*-dibenzylformamidine)-9-[(4-dihydroxy-3-hydroxymethylbut-1-ylpropan-2-yl]-guanine [6.11]

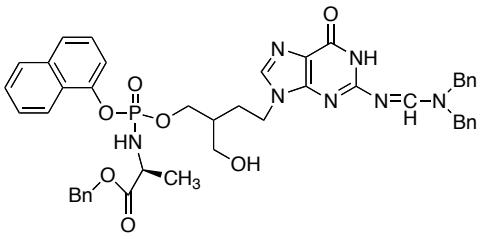


Chemical Formula: $C_{25}H_{28}N_6O_3$
Molecular Weight: 460.5282

Freshly distilled dibenzylamine (7.5 g, 38.1 mmol) and *N*,*N*-dimethylformamide dimethylacetale (1.7 mL, 12.8 mmol) were refluxed in ACN (15 mL) for 24 h. Anhydrous toluene (15 mL) was added and the solvent was removed under reduced pressure. The crude residue in anhydrous ACN (10 mL) was added to PCV (1.06 g, 4.2 mmol) in ACN (20 mL). The solution was stirred for 24 h at 45 °C and precipitated in ET_2O (65 mL). The solid was filtrated and purified by column chromatography, gradient elution of DCM/MeOH = 98/2 to 80/20, to give a white solid (52%, 1 g).

^{1}H-NMR (DMSO, 500 MHz): δ 11.41 (1H, bs, NH), 8.99 (1H, s, C*H*N(Bn)$_2$), 7.85 (1H, s, H-8), 7.42-7.31(10H, m, Bn), 4.62, 4.58 (2H, 2s, NC*H*$_2$Ph), 4.43 (2H, t, J = 5.23 Hz, H-1'), 3.49-3.38 (4H, m, H-4', H-5'), 1.81-1.76 (2H, m, H-2'), 1.51-1.46 (1H, m, H-3').

Synthesis of N^2-(*N*,*N*-dibenzylformamidine)-penciclovir-[1-naphthyl(benzyloxy-L-alaninyl)] phosphate [6.12]



Chemical Formula: $C_{45}H_{46}N_7O_7P$
Molecular Weight: 827.8632

Prepared according to standard procedure G from **6.11** (0.13 g, 0.28 mmol) in anhydrous mixture of THF (6 mL) and pyridine (2 mL), *t*BuMgCl (1.0 M THF solution, 0.56 mL, 0.56 mmol), and **2.2b** (0.23 g, 0.56 mmol) in anhydrous THF (3 mL) and the reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6 then 92/8, to give a white solid (30%, 0.07 g).

^{31}P-NMR (MeOD, 202 MHz): δ 4.52, 4.64, 4.19, 4.09. ^{1}H-NMR (MeOD, 500 MHz): δ 9.05 (1H, s, NC*H*N(*CH*$_2$Ph)$_2$), 8.28-8.27 (1H, m, H-8 Naph), 8.14-8.13 (1H, m, H-6 Naph), 7.78-7.13 (21H, m, H-8, Naph, OCH$_2$*Ph*, N(CH$_2$*Ph*)$_2$), 5.04-5.02 (2H, m, OC*H*$_2$Ph), 4.61-4.58 (2H, m, NC*H*$_2$Ph), 4.42-4.37 (2H, m, NC*H*$_2$Ph), 4.11-4.03 (6H,

m, H-1', H-5', C*H*CH_3), 3.51-3.45 (2H, m, H-4'), 1.78-1.66 (2H, m, H-2'), 1.31-1.28 (1H, m, H-3'), 1.19 (3H, d, J= 6.86 Hz, CHC*H*$_3$).

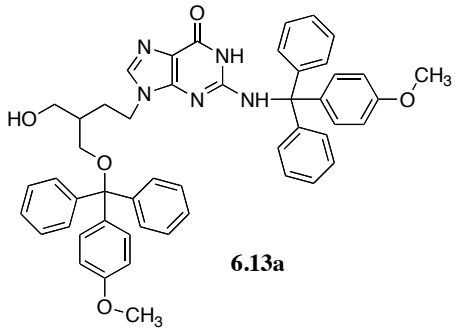
Synthesis of N^2,*O*-bis(MTT)-penciclovir:

(N^2-(monomethoxytrity1)-9-[3-(hydroxymethyl)-4-[(monomethoxytrityl)oxy]but-l-yl]guanine) [6.13a]

and

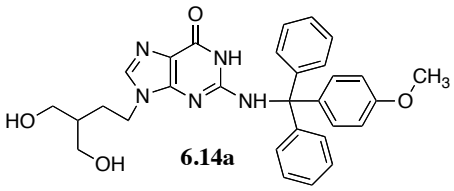
N^2-MTT-penciclovir:

(N^2-(monomethoxytrity1)-9-[4-hydroxy-3-(hydroxymethyl)but-1-yl]guanine) [6.14a]



6.13a

Chemical Formula: $C_{50}H_{47}N_5O_5$
Molecular Weight: 797.9387



6.14a

Chemical Formula: $C_{30}H_{31}N_5O_4$
Molecular Weight: 525.5982

A solution of penciclovir (4.05 g, 16 mmol), monomethoxytrityl chloride (10.9 g, 35 mmol), anhydrous triethylamine (6.7 mL) and 4-dimethylaminopyridine (40 mg) in anhydrous DMF (50 mL) was stirred at room temperature for 2 h. The reaction was quenched with MeOH and the solvent was removed. The residue was taken up in ethyl acetate and the solution washed with aqueous $NaHCO_3$ and water. The solution was dried ($MgSO_4$) and the solvent removed. The residue was purified by column chromatography on silica gel eluting with $CHCL_3$/MeOH (gradient elution = 100/0, then 98/2, then 96/4, then 94/6) The first product to elute was **6.13a** (4.4 g, 34%). The second product to elute was **6.14a** (1.4 g, 17%).

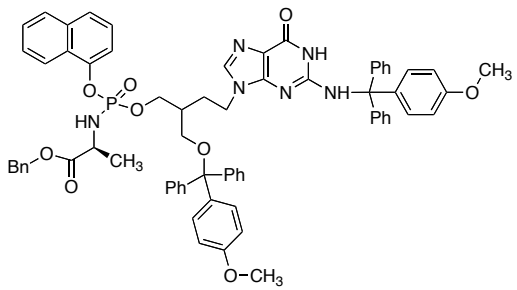
[6.13a]

^{1}H-NMR (DMSO, 500 MHz): δ 10.50 (1H, bs, NH), 7.55 (1H, s, N-2), 7.44 (1H, s, H-8), 7.4-6.7 (28H, m, Ph), 4.35 (1H, t, J = 4.8 Hz, OH), 3.74 (3H, s, CH_3O), 3.66 (3H, s, CH_3O), 3.42 (2H, t, J=6.7 Hz, H-1'), 3.40-3.10 (2H, m, H-5'), 2.90-2.70 (2H, m, H-4'), 1.43 (1H, m, H-3'), 1.24 (2H, m, H-2').

[6.13a]

^{1}H-NMR (DMSO, 500 MHz): δ 10.49 (1H, bs, NH), 7.56 (1H, s, N-2), 7.54 (1H, s, H-8), 7.35-6.86 (14H, m, Ph), 4.28 (2H, t, J = 5.2 Hz, 2 X OH), 3.72 (3H, s, CH_3O), 3.52 (2H, t, J=6.6 Hz, H-1'), 3.3-3.1 (4H, m, 2 X CH_2OH), 1.25 (3H, m, H-2', H-3').

Synthesis of *N*^{2},*O*-bis(MTT)-penciclovir-[1-naphtyl(benzyloxy-L-alaninyl)] phosphate [6.15a]

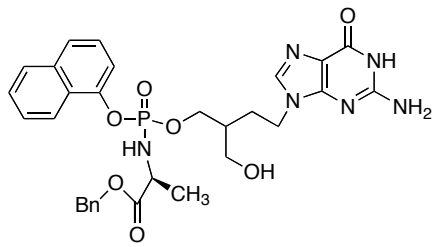


Chemical Formula: $C_{70}H_{65}N_6O_9P$
Molecular Weight: 1165.2737

Prepared according to standard procedure G from **6.13a** (0.6 g, 0.75 mmol) in anhydrous THF (15 mL) *t*BuMgCl (1.0 M THF solution, 1.5 mL, 1.5 mmol), **2.2b** (0.6 g, 1.5 mmol) in anhydrous THF (5 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was purified by column chromatography, gradient elution of DCM/MeOH = 100/0 then 98/2, to give a white solid (40%, 0.36 g,).

^{31}P-NMR (DMSO, 202 MHz): δ 3.99, 3.85, 3.81, 3.75. ^{1}H-NMR (DMSO, 500 MHz): δ 10.51 (1H, bs, NH-1), 8.09-8.03 (1H, m, H-8 Naph), 7.95-7.94 (1H, m, H-6 Naph), 7.73-7.71 (1H, m, H-8), 7.56 (1H, bs, NH-2), 7.46-6.74 (38H, m, Naph, OCH_2Ph, 2 x Tr), 5.13-4.95 (2H, m, OCH_2Ph), 4.03-3.80 (5H, m, H-1', *CH*CH_3, H-5'), 3.72 (3H, s, OCH_3), 3.65 (3H, s, OCH_3), 2.73-2.71 (2H, m, H-4'), 1.52-1.47 (3H, m, H-2', H-3'), 1.28-1.24 (3H, m, CHCH_3). EI MS = 1187.39 (M+Na).

Synthesis of penciclovir-[1-naphtyl(benzyloxy-L-alaninyl)] phosphate [6.7b]



Chemical Formula: $C_{30}H_{33}N_6O_7P$
Molecular Weight: 620.5928

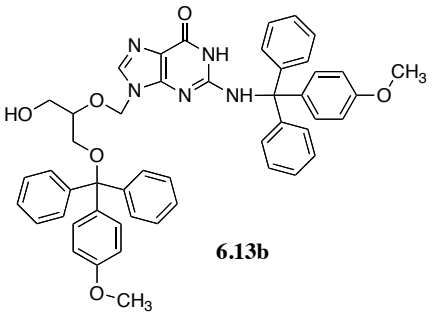
A solution of **6.15a** (0.164 g, 0.14 mmol) in CH_2Cl_2/MeOH mixture 7:3 (10 mL) was treated with *p*-toluensulfonic acid (0.133 g, 0.7 mmol). The mixture was stirred at room temperature for 2 h. After this period the solvent was removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6 then, 92/8, then 90/10). The product was further purified by preparative reverse phase HPLC

(gradient elution of H_2O/MeOH 90/10 to 80/20 in 5 min, 80/20 isocratic 15 min, then to 0/100 in 5 min) to give a white solid (30%, 0.027 g).

^{31}P-NMR (MeOD, 202 MHz): δ 4.46, 4.44, 4.16, 4.13. ^{1}H-NMR (MeOD, 500 MHz): δ 8.17-8.15 (1H, m, H-8 Naph), 7.87-7.86 (1H, m, H-6 Naph), 7.70-7.67 (1H, m, H-8), 7.63-7.58 (1H, m, H-2 Naph), 7.52-7.50 (2H, m, H-5 Naph, H-7 Naph), 7.47-7.26 (7H, NaphO, PhO), 5.13, 5.09 (2H, m, OC*H*$_2$Ph), 4.23-4.11 (3H, m, H-5', C*H*CH$_3$), 4.03-3.94 (1H, m, H-1'), 3.49-3.43 (2H, m, H-4'), 1.76-1.62 (3H, m, H-2', H-3'), 1.38 (3H, bs, CHC*H*$_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.39, 20.42, 20.44, 20.48, 20.50 (CH*C*H$_3$), 29.44, 29.54 (C-2'), 40.27, 40.31, 40.40 (C-3'), 42.25, 42.29 (C-1'), 51.83, 51.87, 51.93 (NH*C*H), 62.24, 62.31 (H-4'), 67.82, 67.86, 67.94, 67.99, 68.07 (H-5', O*C*H$_2$Ph), 116.47, 116.50, 116.60, 116.69, 116.71, 117.56, 119.99, 122.73, 122.76, 122.85, 122.88, 123.15, 125.46, 126.03, 126.51, 126.99, 127.42, 127.45, 127.75, 128.02, 128.27, 128.43, 128.84, 128.87, 129.25, 129.30, 129.33, 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, OCH$_2$*Ph*), 136.27 ('ipso' OCH$_2$*Ph*), 137.20 (C-8a Naph) 139.48 (C-8), 148.05, 148.11 (2s, 'ipso' O*Naph*), 153.04 (C-4), 155.21 (C-2), 159.42 (C-6), 174.84, 175.04 (2s, *C*OOCH$_2$Ph). EI MS = 621.22 (M+H). HPLC: H_2O/ACN from 90/0 to 0/100 in 30 min, then 0/100 isocratic 5 min = retention time 12.93 min

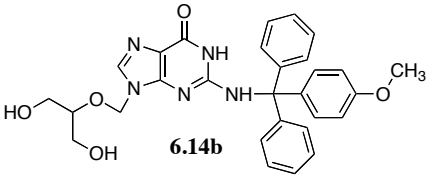
9.5.4 Synthesis of L-alanine benzyl ester derivatives of ganciclovir ProTides 6.7c-d

Synthesis of N^2,*O*-bis(MTT)-ganciclovir:
(N^2-(monomethoxytrityl)-9-[(1-monomethoxytrityloxy-3-hydroxypropan-2-yloxy)methyl]guanine) [6.13a]
and
N^2-MTT-ganciclovir:
(N^2-Monomethoxytrityl-9-[(1,3-dihydroxypropan-2-yloxy)methyl]guanine) [6.14b]



6.13b

Chemical Formula: $C_{49}H_{45}N_5O_6$
Molecular Weight: 799.9115



6.14b

Chemical Formula: $C_{29}H_{29}N_5O_5$
Molecular Weight: 527.5711

A solution of GCV (0.5 g, 1.96 mmol), monomethoxytrityl chloride (1.33 g, 4.32 mmol), triethylamine (0.82 mL, 5.88 mmol) and 4-dimethylaminopyridine (5 mg) in DMF (5 mL) was stirred at room temperature for 2 h. The reaction was quenched with MeOH and the solvent was removed. The residue was taken up in ethyl acetate and the solution washed with aqueous $NaHCO_3$ and water. The solution was dried ($MgSO_4$) and the solvent removed. The residue was purified by column chromatography on silica gel using a gradient elution of DCM/MeOH (98/2, then 96/4, then 94/6). The first product to elute was **6.13b** (53%, 0.84 g). The second product to elute was **6.14b** (30% 0.3 g)

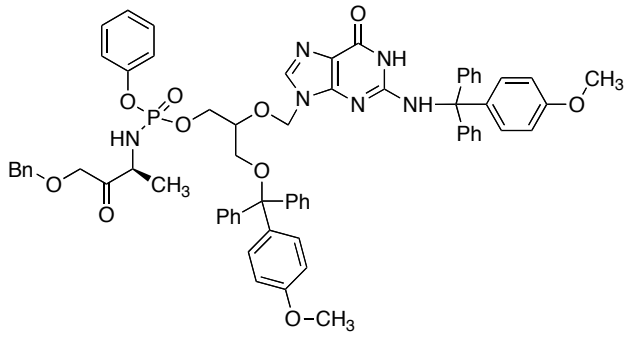
[6.13b]

^{1}H-NMR (MeOH, 500 MHz): δ 7.79 (1H, s, H-8), 7.32-6.73 (28H, m, Ph), 5.06 (2H, s, H-1'), 3.82 (3H, s, CH_3O), 3.66 (3H, s, CH_3O), 3.57-3.54 (1H, m, H-3'), 3.28-3.18 (2H, m, H-5'), 2.87-2.79 (2H, m, H-4')

[6.14b]

^{1}H-NMR (MeOH, 500 MHz): δ 7.74 (1H, s, H-8), 7.38-6.87 (14H, m, Ph), 3.80 (3H, s, CH_3O), 3.66-3.60 (1H, m, H-3'), 3.49-3.43 (4H, m, H-4', H-5').

Synthesis of N^2,*O*-bis(MTT)-ganciclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [6.15b]

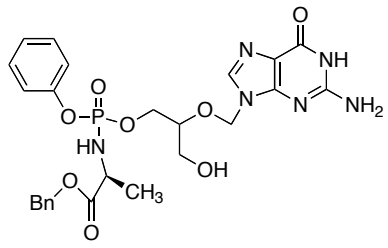


Chemical Formula: $C_{66}H_{63}N_6O_{10}P$
Molecular Weight: 1131.2144

Prepared according to standard procedure G from **6.13b** (0.45 g, 0.58 mmol) in anhydrous THF (10 mL) *t*BuMgCl (1.0 M THF solution, 1.12 mL, 1.12 mmol), **2.2a** (0.45 g, 1.27 mmol) in anhydrous THF (2 mL) and the reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was purified by column chromatography, gradient elution of DCM/MeOH = 100/0 then 98/2, to give a white solid (38%, 0.247 g,).

^{31}P-NMR (MeOD, 202 MHz): δ 3.32, 3.30, 3.27, 3.16. ^{1}H-NMR (MeOD, 500 MHz): δ 7.74-7.73 (1H, m, H-8), 7.34-6.70 (38H, m, PhO, OCH_2*Ph*, 2 x Tr), 5.17-4.99 (2H, m, OCH_2Ph), 4.95-4.89 (2H, m, H-1'), 3.79-3.60 (8H, m, *CH*CH_3, H-3', 2X OCH_3) 3.17-3.07 (2H, m, H-5'), 2.81-2.78 (2H, m, H-4'), 1.85, 1.72 (3H, m, CHCH_3).

Synthesis of ganciclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [6.7c]



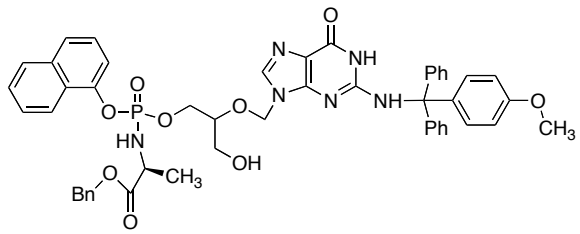
Chemical Formula: $C_{25}H_{29}N_6O_8P$
Molecular Weight: 572.5069

A solution of **6.15b** (0.11 g, 0.1 mmol) in CH_2Cl_2/MeOH mixture 7:3 (5 mL) was treated with *p*-toluensulfonic acid (0.09 g, 0.48 mmol). The mixture was stirred at room temperature for 2 h. After this period the solvent was removed under reduced pressure and the residue was purified by preparative TLC plate chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6. The product was further purified by preparative reverse phase HPLC (gradient elution of H_2O/MeOH 90/10 to 80/20 in 5 min, 80/20 isocratic 15 min, then to 0/100 in 5 min) to give a white solid (28%, $1.7*10^{-2}$ g).

^{31}P-NMR (MeOD, 202 MHz): δ 3.92, 3.83, 3.63, 3.61. ^{1}H-NMR (MeOD, 500 MHz): δ 7.84-7.79 (1H, m, H-8), 7.40-7.30 (7H, m, PhO, OCH_2*Ph*), 7.21-7.13 (3H, m, PhO,

OCH_2*Ph*), 5.54-5.50 (2H, m, H-1'), 5.17-5.14 (2H, m, OC*H*$_2$Ph), 4.23-3.91 (4H, m, H-5', H-3', C*H*CH_3), 3.61-3.47 (2H, m, H-4'), 1.38-1.31 (3H, m, C*H*$_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.50 (d, J_{C-P} = 7.1 Hz, CH*C*H_3), 20.40 (d, J_{C-P} = 7.1 Hz, CH*C*H_3), 20.37 (d, J_{C-P} = 7.1 Hz, CH*C*H_3), 51.62, 51.75 (*C*HCH_3), 61.91, 61.99, 62.02 (C-4'), 67.31 (d, J_{C-P} = 5.9 Hz, C-5'), 67.25 (d, J_{C-P} = 5.9 Hz, C-5'), 67.20 (d, J_{C-P} = 5.9 Hz, C-5'), 67.97, 68.00, 68.03 (O*C*H_2Ph), 73.07, 73.11, 79.13 (C-1') 79.24 (d, J_{C-P} = 7.56, C-3'), 79.25 (d, J_{C-P} = 5.6 Hz, C-3'), 79.30 (d, J_{C-P} = 5.6 Hz, C-3'), 117.55, 121.41, 121.44, 121.47, 121.49, 121.51, 121.53, 121.54, 126.09, 126.12, 126.15,129.27, 129.32, 129.33, 129.35, 129.58, 129.61, 129.63, 130.76 (C-5, PhO, OCH_2*Ph*), 137.30 ('ipso' OCH_2*Ph*), 139.8 (s, C-8), 152.12, 152.17, 152.19, 152.23 (4s, 'ipso'OPh), 153.30 (C-4), 155.68 (C-2), 159.37 (C-6), 174.92 (d, J_{C-P} = 5.4 Hz, 1C), 174.82 (d, J_{C-P} = 5.4 Hz, *C*$OOCH_2$Ph), 174.72 (d, J_{C-P} = 5.4 Hz, *C*$OOCH_2$Ph). EI MS= 595.162 (M+Na). HPLC = H_2O/ACN from 100/0 to 0/100 in 15 min, then 0/100 isocratic 5 min = retention time 9.29 min; H_2O/MeOH from 100/0 to 0/100 in 15 min, then 0/100 isocratic 5 min = retention time 13.18 min.

Synthesis of N^2-MTT-ganciclovir-[1-naphthyl(benzyloxy-L-alaninyl)] phosphate [6.16]

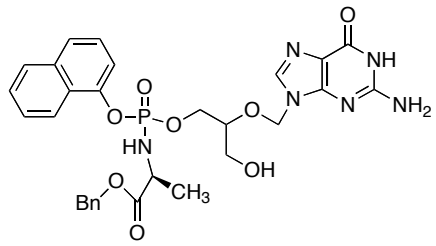


Chemical Formula: $C_{48}H_{45}N_6O_8P$
Molecular Weight: 864.8801

Prepared according to standard procedure G, from **6.14b** (0.24 g, 0.46 mmol) in anhydrous THF (8 mL) ^{t}BuMgCl (1.0 M THF solution, 0.92 mL, 0.92 mmol), **2.2b** (0.37 g, 0.92 mmol) in anhydrous THF (2.5 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was purified by column chromatography, gradient elution of DCM/MeOH = 100/0, 98/2, then 96/2, to give a white solid (35%, 0.146 g).

^{31}P-NMR (MeOD, 202 MHz): δ 3.58, 3.52, 3.48, 3.35. ^{1}H-NMR (MeOD, 500 MHz): δ 8.19-8.11 (1H, m, H-8 Naph), 7.91-7.89 (1H, m, H-6 Naph), 7.69-7.68 (1H, m, H-8), 7.66-6.75 (m, 24H, Naph, OCH_2*Ph*, Tr), 5.1-5.03 (m, 2H, OC*H*$_2$Ph), 4.95-4.91 (2H, m, H-1'), 4.04-3.72 (3H, m, *CH*CH_3, H-5'), 3.67 (3H, s, OCH_3), 3.52-3.44 (1H, m, H-3'), 3.29-3.08 (2H, m, H-4'), 1.34-1.30 (3H, m, CHC*H*$_3$).

Synthesis of ganciclovir-[1-phenyl(benzyloxy-L-alaninyl)] phosphate. [6.7d]



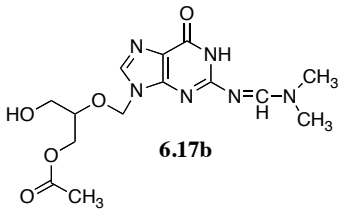
Chemical Formula: $C_{29}H_{31}N_6O_8P$
Molecular Weight: 622.5656

A solution of **6.16** (0.09 g, 0.1 mmol) in CH_2Cl_2/MeOH 7:3 (5 mL) was treated with *p*-toluensulfonic acid (0.09 g, 0.48 mmol). The mixture was stirred at room temperature for 2 h. After this period the solvent was removed under reduced pressure and the residue was purified by preparative TLC plate chromatography, gradient elution of DCM/MeOH = 98/2 then 96/4 then 94/6, to give a white solid (25%, $1.5*10^{-2}$ g).

^{31}P-NMR (MeOD, 202 MHz): δ 4.29, 4.24, 4.18, 4.09. ^{1}H-NMR (MeOD, 500 MHz): δ 8.16-8.09 (m, 1H, H-8 Naph), 7.90, 7.80 (1H, 2s, H-6 Naph), 7.79-7.75 (1H, m, H-8), 7.72-7.70 (1H, m, H-2 Naph), 7.54-7.24 (12H, m, Naph, OCH_2*Ph*), 5.46-5.42 (2H, m, H-1'), 5.13-5.07 (2H, m, OCH_2Ph), 4.29-4.06 (4H, m, H-5', H-3' $CHCH_3$), 3.97-3.90 (2H, m, H-4'), 1.38-1.31 (3H, m, $CHCH_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.38 (d, *J* = 6.7 Hz, $CHCH_3$), 20.43 (d, *J* = 6.7 Hz, $CHCH_3$), 20.53 (d, *J* = 6.7 Hz, $CHCH_3$), 51.80, 51.85 ($CHCH_3$), 61.84, 61.94, 61.97 (C-4'), 67.43 (d, *J* = 5.6 Hz, C-5'), 67.49 (d, *J* = 5.6 Hz, C-5'), 67.53 (d, *J* = 5.6 Hz, C-5'), 67.97, 68.01, 68.04 (OCH_2Ph), 72.96, 73.00, 73.08, 79.10 (4s, C-1'), 79.11 (d, J_{C-P} = 7.8 Hz, C-3'), 79.25 (d, J_{C-P} = 7.8 Hz, C-3'), 79.31 (d, J_{C-P} = 7.8 Hz, C-3'), 116.34, 116.36, 116.39, 116.42, 116.45, 116.47, 122.70, 122.74, 122.47, 125.99, 126.01, 126.55, 127.50, 127.53, 127.79, 128.87, 129.22, 129.27, 129.30, 129.32, 129.34, 129.54, 129.58, 129.60 (C-5, PhO, OCH_2*Ph*), 136.30, 137.23 ('ipso' OCH_2*Ph*, C4aNaph), 139.73 (C-8), 147.98, 148.03 (2s, 'ipso'OPh), 153.27 (C-4), 155.65 (C-2), 159.34 (C-6), 174.74 (d, J_{C-P} = 4.7 Hz, $COOCH_2$Ph), 174.86 (d, J_{C-P} = 4.7 Hz, $COOCH_2$Ph), 174.96 (d, J_{C-P} = 4.7 Hz, $COOCH_2$Ph). EI MS= 645.187 (M+Na). HPLC = H_2O/ACN from 100/0 to 0/100 in 15 min, then 0/100 isocratic 5 min = retention time 10.49 min; H_2O/MeOH from 100/0 to 0/100 in 15 min, then 0/100 isocratic 5 min = retention time 13.93 min.

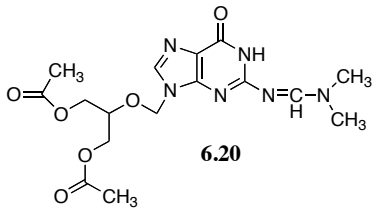
9.5.5 Synthesis of ganciclovir ProTide 6.19c

Synthesis of N^2-DMF-O-monoacetyl-ganciclovir:
N^2-(*N*,*N*-dimethylformamidine)-9-[(1-acetoxy-3-hydroxypropan-2-yloxy)methyl]-guanine) [6.17b]
and
N^2-DMF-*O*,*O*-diacetyl-ganciclovir:
N^2-(*N*,*N*-dimethylformamidine)-9-[(1,3-diacetoxy-propan-2-yloxy)methyl]-guanine) [6.20]



6.17b

Chemical Formula: $C_{14}H_{20}N_6O_5$
Molecular Weight: 352.3458



6.20

Chemical Formula: $C_{16}H_{22}N_6O_6$
Molecular Weight: 394.3825

A suspension of **6.8b** (0.3 g, 0.97 mmol) in a mixture of pyridine (50 mL) and benzene (25 mL) was treated with vinyl acetate (7.46 mL, 80.76 mmol) and porcine pancreatic lipase (1.46 g, 33'407 units). This suspension was allowed to stir at room temperature. After 24 h, TLC on silica of mixture reaction showed an incomplete acetylation of the starting material. An additional portion of porcine pancreatic lipase (0.48 g, 11'135 units) was added and the suspension was allowed to stir for an additional 24 h. At this point TLC of mixture reaction showed monoacetylated product along with traces of diacetylated product. The crude of the reaction was filtered and the solvent was removed under vacuum. The residue was purified by flash column chromatography, gradient elution of $CHCl_3$/MeOH = 98/2, then 95/5, then 92/8, to give **6.17b** as a white solid (53%, 0.180 g) **6.20** as a white solid (0.03 g, 8%).

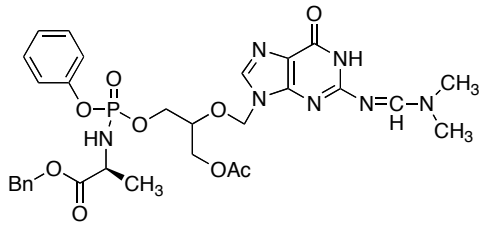
6.17b

^{1}H-NMR (MeOH, 500 MHz): δ 8.67 (1H, s, C*H*N$(CH_3)_2$), 7.95 (1H, s, H-8), 5.63 (2H, s, H-1'), 4.22-4.01 (1H, dd, *J* = 11.22, 3.13, H-4'), 4.05-3.98 (2H, m, H-3', H-4'), 3.64-3.56 (2H, m, H-5'), 3.23, 3.11 (6H, 2s, N$(CH_3)_2$), 1.91 (3H, br s, COC*H*$_3$).

6.20

^{1}H-NMR (MeOH, 500 MHz): δ 8.73 (1H, s, C*H*N$(CH_3)_2$), 7.98 (1H, bs, H-8), 5.62 (2H, s, H-1'), 4.30-4.05 (5H, m, H-3', H-4', H-5'), 3.27, 3.16 (6H, 2s, N$(CH_3)_2$), 1.95 (6H, br s, 2 x COC*H*$_3$).

Synthesis of N^2-dimethylformamidine-O-monoacetyl-ganciclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [6.18c]

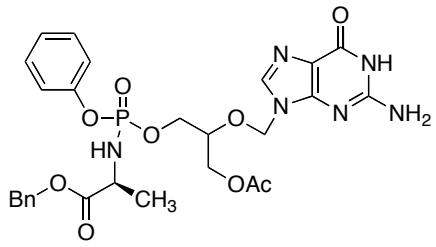


Chemical Formula: $C_{30}H_{36}N_7O_9P$
Molecular Weight: 669,6221

Prepared according to standard procedure G, from **6.17b** (0.18 g, 0.51 mmol) in anhydrous THF (10 mL), *t*BuMgCl (1.0 M THF solution, 3 mL, 3 mmol), **2.2a** (0.36 g, 1.02 mmol) in anhydrous THF (2.5 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. 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 (68%, 0.24 g,).

^{31}P-NMR ($CDCl_3$, 202 MHz): δ 3.93, 3.88, 3.47, 3.42. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.68-8.62 (1H, m, NC*H*N$(CH_3)_2$), 7.76-7.75 (1H, m, H-8), 7.39-6.81 (10H, m, Ph, OCH$_2$*Ph*), 5.53-5.37 (2H, m, H-1'), 4.95-4.84 (2H, m, OC*H*$_2$Ph), 4.23-3.98 (6H, m, H-3', H-4', H-5', C*H*CH_3), 3.14, 3.02 (6H, 2s, N(C*H*$_3)_2$), 1.92 (bs, 3H COC*H*$_3$), 1.41-1.38 (3H, m, C*H*$_3$).

Synthesis of O-monoacetyl-ganciclovir-[phenyl(benzyloxy-L-alaninyl)] phosphate [6.19c]



Chemical Formula: $C_{27}H_{31}N_6O_9P$
Molecular Weight: 614.5436

A solution of **6.18c** (0.24 g, 0.35 mmol) in isopropanol (15 mL) was stirred under reflux for 62 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 94/6) to give a white solid (31%, 0.07g).

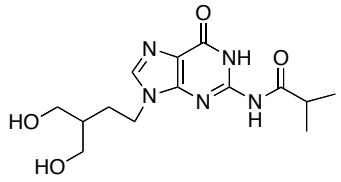
^{31}P-NMR (MeOD, 202 MHz): δ 3.93, 3.83, 3.53, 3.47. ^{1}H-NMR (MeOD, 500 MHz): δ 7.84-7.80 (1H, m, H-8); 7.43-7.17 (10H, m, Ph, OCH$_2$*Ph*), 5.51-5.49 (2H, m, H-1'),

5.18-5.14 (2H, m, OCH_2Ph), 4.18-4.11 (5H, m, H-3', H-4', H-5'), 4.01-3.98 (1H, m, CHCH$_3$), 1.94- 1.90 (3H, m, COCH_3), 1.38-1.31 (3H, m, CHCH_3).

9.5.6 Synthesis of penciclovir ProTide 6.24

Synthesis of N^2-isobutyryl penciclovir:

***N*^{2}-isobutyryl-9-(4-hydroxy-3-hydroxymethylbut-1yl)-guanine [6.21]**

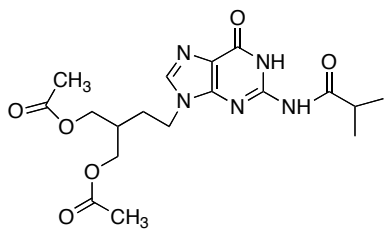


Chemical Formula: $C_{14}H_{21}N_5O_4$
Molecular Weight: 323.3476

Isobutyryl chloride (2.23 mL, 21.13 mmol) was added dropwise at 0 °C to a suspension of penciclovir (1 g, 3.95 mmol) suspended in pyridine (50 mL). The mixture was stirred at room temperature for 16 h. After this period, the reaction was quenched with methanol (5 mL) and the solvent was removed under reduced pressure. The residue was dissolved in pyridine/methanol mixture (2:1, 30 mL) and the pH was adjusted to 12.5 with 2M NaOH. The mixture was kept at room temperature for 10 min. The pH was adjusted to 7.0 with 2 M HCl and the solvent was removed under vacuum. The residue was purified by flash chromatography, gradient elution of DCM/MeOH = 95/5, then 90/10, the 85/15), to give a white solid (78%, 1 g). ^{1}H-NMR (MeOH, 500 MHz): δ 7.99 (1H, s, H-8), 4.28 (2H, t, J = 7.49 Hz, H-1'), 3.64 (2H, dd, *J* = 10.9 Hz, 5.9 Hz, H-5'), 3.59 (2H, dd, *J* = 10.9 Hz, 5.9 Hz, H-4'), 2.74 (1H, septet, *J* = 6.86 Hz, *CH*(CH$_3$)$_2$), 1.95 (2H, m, H-2'), 1.64 (1H, m, H-3'), 1.25 (6H, d, *J* = 6.86 Hz, CH(*CH$_3$*)$_2$).

Synthesis of *N*^{2}-isobutyryl-O,O-diacetyl-penciclovir:

***N*^{2}-isobutyryl-9-(4-Acetoxy-3-Acetoxymethylbut-1yl)-guanine [6.22]**



Chemical Formula: $C_{18}H_{25}N_5O_6$
Molecular Weight: 407.4210

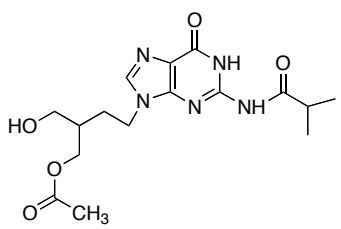
A mixture of **6.21** (1 g, 3.09 mmol), DMAP (0.05 g, 0.4 mmol) and acetic anhydride (2.1 mL, 22.47 mmol) in DMF (80 mL) was stirred for 1 h at room temperature. Solvent was removed under vacuum and the crude was purified by flash chromatography, gradient elution of DCM/MeOH = 98/2, then 96/4) to give a white solid (0.9 g, 71%).

^{1}H-NMR (MeOH, 500 MHz): δ 7.99 (1H, s, H-8), 4.29 (2H, t, J = 7.03 Hz, H-1'), 4.13

(4H, d, J = 5.35 Hz, H-4', H-5'), 2.74 (1H, septet, *J* = 6.87 Hz, *CH*(CH_3)$_2$), 2.06-1.98 (9H, m, H-2', H-3', 2X COC*H*$_3$), 1.25 (6H, d, *J* = 6.87 Hz, CH(*CH*$_3$)$_2$).

Synthesis of N^2-isobutyryl-O-monoacetyl-penciclovir :

N^2-isobutyryl-9-(4-hydroxy-3-Acetoxymethylbut-1yl)-guanine [6.23]

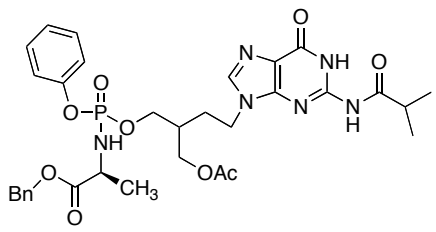


Chemical Formula: $C_{16}H_{23}N_5O_5$
Molecular Weight: 365.3843

A mixture of diacetate **6.22** (0.32 g, 0.79 mmol) in DMF (30 mL) and buffer phosphate (pH 7.00, 300 mL) was treated with and porcine liver esterase (1.44 g, 27'360 units). The mixture was stirred for 2 h at room temperature, then lyophilized. The residue was sonicated with DCM/MeOH (3:1, 3X 100 mL) and the insoluble portion was filtered off. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography using gradient elution of DCM/MeOH = 98/2, then 95/5, then 90/10 to give the monoacetate **6.23** (60%, 0.173 g) as a white solid and unreacted starting material **6.22** (0.15 g, 18%).

^{1}H-NMR (MeOH, 500 MHz): δ 7.98 (1H, s, H-8), 4.26 (2H, t, J = 7.25 Hz, H-1'), 4.16-4.08 (2H, m, H-4'), 3.63-3.60 (2H, m, H-5'), 2.77-2.72 (1H, m, *CH*(CH_3)$_2$), 2.04 (3H, m, C*H*$_3$CO), 2.01-1.87 (2H, m, H-2'), 1.84-1.79 (1H, m, H-3'), 1.24 (6H, d, *J* = 6.84 Hz, CH(*CH*$_3$)$_2$).

Synthesis of N^2-isobutyryl-O-monoacetyl-penciclovir-[phenyl(benzyloxy-L-alaninyl)]-phosphate. [6.24]



Chemical Formula: $C_{32}H_{39}N_6O_9P$
Molecular Weight: 682.6606

Prepared according to standard procedure G from **6.24** (0.93 g, 0.25 mmol) in a mixture of anhydrous THF (6 mL) and anhydrous pyridine (2 mL), *t*BuMgCl (1.0 M THF solution, 1.5 mL, 1.5 mmol), **2.2a** (0.18 g, 0.5 mmol) in anhydrous THF (1.5 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was purified by column chromatography, gradient elution of DCM/MeOH = then 98/2, then 96/4, then 94/6, to give a white solid (40%, 0.07 g).

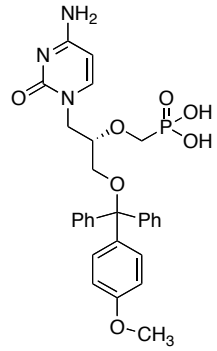
^{31}P-NMR (MeOH, 202 MHz): δ 3.95, 3.39. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.96-7.93 (1H, m, H-8); 7.33-7.29 (7H, m, PhO, OCH_2*Ph*), 7.19-7.16 (3H, m, PhO, OCH_2*Ph*),

5.13-5.12 (2H, m, OC*H*$_2$Ph), 4.28-3.97 (6H, m, H-1', H-5', H-4', C*H*CH$_3$), 2.77-2.68 (1H, m, *CH*(CH$_3$)$_2$), 2.04-1.84 (6H, m, H-2', H-3', C*H*$_3$CO), 1.38-1.35 (3H, m, CHC*H*$_3$), 1.25-1.20 (6H, m, CH(*CH*$_3$)$_2$).

9.6. Experimental procedures for chapter 6: synthesis of phosphonoamidate derivatives of *S*-HPMPC

9.6.1 Synthesis of the L-alanine benzyl ester phosphonodiamidate derivative of *S*-HPMPC [7.19]

Synthesis of (S)-1-{3-[(4-Methoxytrityl)oxy]-2-(phosphonomethoxy)propyl} cytosine [7.18]

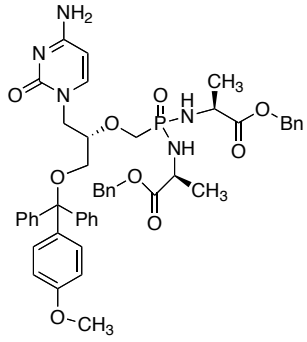


Chemical Formula: $C_{28}H_{30}N_3O_7P$
Molecular Weight: 551.5275

To a suspension of *S*-HPMPC dihydrate (free acid, 0.16 g, 0.5 mmol) in methanol (15 mL), tributylamine (1.6 g, 10 mmol) was added. The suspension of the free acid easily dissolved to a clear solution of tributyl-ammonium salt. The solvent was removed under reduced pressure. The residue, co-evaporated with acetonitrile, was dissolved in DMSO and 4-methoxytrityl chloride (0.46 g, 1.5 mmol) was added. The reaction mixture was stirred for 3 h. Proceeding of the reaction was monitored by TLC in propan-1-ol-ammonia-water (11:7:2). Tributylamine and DMSO were extracted down with Et_2O; the product was collected by filtration, recrystallized from ethyl acetate and dried in vacuo (32%, 0.09 g).

^{31}P NMR (DMSO, 202 MHz): δ 15.40. ^{1}H NMR (DMSO, 500 MHz): δ 7.55 (1H, d, *J* = 7.27 Hz, H-6), 7.43-6.90 (14 H, m, C*Ph*$_3$), 5.63 (1H, d, *J* = 7.27 Hz, H-5), 4.00 (1H, dd, *J* = 12.0 Hz, 7.5 Hz, H-1'); 3.83 (1H, m, H-2'), 3.71 (3H, s, MTr), 3.64 (1H, dd, *J* = 12.4 Hz, 10.0 Hz, CH$_2$P), 3.63 (1H, dd, *J* = 12.0 Hz, 7.5 H-1'), 3.40 (1H, dd, *J* = 12.4 Hz, 10.0 Hz CH$_2$P), 3.10 (1H, dd, *J* = 10.5 Hz, 4.0 H-3'), 2.95 (1H, dd, *J* = 10.5 Hz, 4.0 Hz, H-3'). EI MS= 445.12 (M+Na$^+$).

Synthesis of (*S*)-1-{[3-(4-methoxytrityl)-oxy]-2-{[bis(benzyloxy-L-Alaninyl)]-phosphonomethoxy}-propyl}-cytosine [7.19]

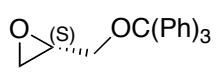


Chemical Formula: $C_{48}H_{52}N_5O_9P$
Molecular Weight: 873.9283

Compound **7.18** (0.08 g, 0.15 mmol) and the para-toluene-sulphate salt of L-alanine-O-benzyl ester (0.31 g, 0.89 mmol) were mixed in anhydrous pyridine (5 mL). Triethylamine (0.25 mL, 1.8 mmol) was added and the resultant mixture was stirred at 60 °C under nitrogen. In a separate flask, 2-2'dithiodipyridine (0.27 g, 1.05 mmol) and PPH_3 (0.23 g, 1.05 mmol) were dissolved in anhydrous pyridine (2 mL), and the resultant yellow solution was stirred for 20 minutes. The solution was then added to the 60 °C solution of **7.18** in one portion. The combined mixture was stirred at 60 °C under nitrogen for 16 h to give a clear yellow solution. The solvent was removed under reduced pressure. The resultant oil was dissolved in CH_2Cl_2 and purified by flash column chromatography, gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6. The product was purified by preparative TLC (gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6) to give a white solid (20%, $0.25*10^{-1}$g).
^{31}P NMR (MeOD, 202 MHz): δ 23.36. ^{1}H NMR (MeOD, 500 MHz): δ 7.48-6.88 (25 H, m, H-6, OC*H*$_2$*Ph*, C*Ph*$_3$), 5.74 (1H, d, *J* = 7.21 Hz, H-5), 5.16-5.05 (4H, m, 2 x OC*H*$_2$Ph), 4.10 (1H, dd, *J* = 13.66 Hz, 4.06 Hz, H-1'), 4.02-3.97 (2H, m, 2X *CH*CH$_3$), 3.84-3.71 (7H, m, H-1', H-2', OCH_2P, OCH_3), 3.30-3.27 (1H, m, H-3'), 3.15-3.12 (1H, m, H-3'), 1.36 (6H, d, *J* = 7.17 Hz, 2 x CHC*H*$_3$). EI MS= 896.34 ($M+Na^+$). HPLC = H_2O/MeOH from 100/0 to 20/80 in 5 min, then 20/80 isocratic for 10 min, then from 20/80 to 0/100 in 5 min, then 0/100 isocratic for 5 min = retention time 17.88 min.

9.6.2 Synthesis of the diethyl ester derivative of *S*-HPMPC [7.23]

Synthesis of (*S*)-Tritylglycidol [7.26]



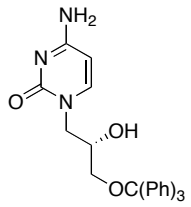
Chemical Formula: $C_{22}H_{20}O_2$
Molecular Weight: 316.3930

Tritylchloride (18 g, 64.5 mmol) was dissolved in CH_2Cl_2 (50 mL) under nitrogen, cooled down to around 0 °C and treated with TEA (13 mL, 94 mmol). After an hour of stirring at 0°C, a solution of (R)-Glycidol (5 g, 67.5 mmol) in CH_2Cl_2 (15 mL) was

added over 45 min. The resulting solution was allowed to warm to room temperature and was stirred for 3 h. It was then filtered and the filtrate was washed with water and brine. The organic phase was dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was crystallized to give the desired compound as an off-white powder (60%, 12 g).

^{1}H NMR (DMSO, 500 MHz): δ 7.42-7.40 (6H, m, Tr), 7.36-7.33 (6H, m, Tr), 7.29-7.26 (3H, m, Tr), 3.28 (1H, dd, *J* = 10.89 Hz, 2.58 Hz, CH_2OTr), 3.17-3.14 (1H, m, C*H*CH_2OTr), 2.91 (1H, dd, *J* = 10.89, 2.58 Hz, CH_2OTr), 2.71 (1H, dd, *J* = 4.65, 2.70 Hz, C*H*$_2$CHCH_2OTr), 2.56 (1H, dd, *J* = 5.07 Hz, 2.67 Hz, C*H*$_2$CHCH_2OTr).

Synthesis of (*S*)-N^1-(3-triphenylmethoxy-2-hydroxy)-propyl)-cytosine [7.27]

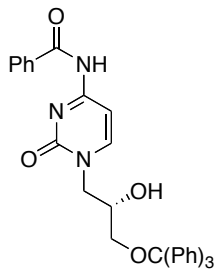


Chemical Formula: $C_{26}H_{25}N_3O_3$
Molecular Weight: 427.4950

Cytosine (1.68 g, 15.1 mmol) and K_2CO_3 (2.1 g, 15.3 mmol) in DMF (30 mL) were stirred for 1h at 105 °C under nitrogen. Compound **7.26** (4.77 g, 15 mmol) was dissolved in DMF (30 mL) and added to the solution of cytosine. According to TLC of reaction mixture, the alkylation reaction was complete after 5 h at 105 °C. Solvent was removed under reduced pressure and **7.27** was obtained by precipitation from ethyl ether (71%, 4.55 g).

^{1}H NMR (MeOH, 500 MHz): δ 7.38 (1H, d, *J* = 7.22 Hz, H-6), 7.49-7.48 (6H, m, Tr), 7.30-7.27 (6H, m, Tr), 7.23-7.20 (3H, m, Tr), 5.75 (1H, m, H-5), 4.19-4.12 (2H, m, H-1', H-2'), 3.57 (1H, d, *J* = 13.16 Hz, 7.87 Hz, H-1'), 3.18-3.12 (2H, m, H-3').

Synthesis of (*S*)-N^1-(3-triphenylmethoxy-2-hydroxy)-propyl)-N^4-benzoyl-cytosine [7.28].



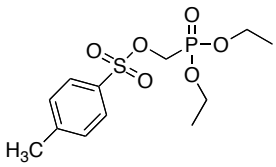
Chemical Formula: $C_{33}H_{29}N_3O_4$
Molecular Weight: 531.6011

Benzoic anhydride (2.91 g, 12.90 mmol) and **7.27** (4.59 g, 10.80 mmol) were added to an anhydrous mixture of pyridine (46.50 mL) and DMF (24 mL). The reaction was carried out under nitrogen at 100 °C for 3 h. Solvent was removed under reduced pressure and the residue was dissolved in 100 mL of CH_2CL_2, followed by aqueous work up using 35 mL saturated solution $NaHCO_3$ solution twice. The organic phase was dried over Na_2SO_4 and solvent removed under vacuum. The residue was further purified by flash

column chromatography, gradient elution DCM/MeOH = 100/0, then 98/2, to give a brown solid (82.5%, 4.74 g).

^{1}H NMR ($CDCl_3$ 500 MHz): δ 8.11 (1H, d, *J* = 7.10 Hz, H-6), 7.94-7.93 (2H, m, Bz), 7.68-7.23 (19H, m, H-5, Bz, Tr), 4.52 (1H, dd, *J* = 13.81 Hz, 4.09 Hz H-1'), 4.41-4.24 (1H, m, H-2'), 3.80 (1H, d, *J* = 13.81 Hz, 4.09 Hz, H-1'), 3.32-3.27 (1H, m, H-3'), 3.21-3.18 (1H, m, H-3').

Synthesis of diethyl-[(tosyloxy)methyl]-phoshonate [7.30].

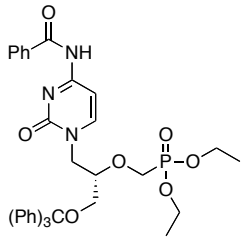


Chemical Formula: $C_{12}H_{19}O_6PS$
Molecular Weight: 322.3144

A two-necked, 250 mL, round bottom flask was charged under argon with CH_2Cl_2 (20 mL), diethyl-(hydroxymethyl)-phosphonate (5.04 g, 30 mmol) in CH_2Cl_2 (5 mL), DMAP (0.6 g), TEA (6.30 mL, 45.20 mmol). *P*-toluensulfonyl-chloride (8.04, 42.17 mmol) was dissolved in CH_2Cl_2 (20 mL) and added at 5 °C via a dropping funnel to the diethyl-(hydroxymethyl)-phosphonate. The reaction mixture stirred at 5 °C for 3 h. The organic phase was washed with H_2O and dried over Na_2SO_4. Solvent was removed under vacuum and the residue was purified by flash column chromatography, elution of chloroform:acetone (2:1), to give a clear yellow oil (61%, 5.90 g).

^{31}P NMR ($CDCl_3$ 500 MHz): δ 15.13. ^{1}H NMR ($CDCl_3$ 202 MHz): δ 7.76 (2H, d, *J* = 8.33 Hz, TsH), 7.34 (2H, d, *J* = 8.33 Hz, TsH), 4.16, 4.14 (2H, 2s, CH_2P), 4.13-4.07 (4H, m, 2 x O*C*H_2CH$_3$), 2.42 (3H, s, CH_3-Ts), 1.28 (6H, t, *J*= 7.32 Hz, 2 x OCH$_2$C*H*$_3$).

(S)-N^1-[(3-triphenylmethoxy-2-diethylphosphonylmethoxy)-propyl)]-N^4-benzoylcytosine [7.31].



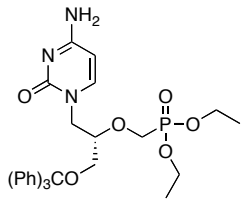
Chemical Formula: $C_{38}H_{40}N_3O_7P$
Molecular Weight: 681.7139

Compound **7.28** (1g, 1.88 mmol) was dissolved in anhydrous DMF (2.7 mL) and transferred to a solution of **7.29** (0.91 g, 2.8 mmol) in anhydrous DMF (2.77 mL). The reaction mixture was cooled down to 5 °C before 3-fold excess of NaH (0.23 g, 60% dispersion in oil, 5.64 mmol) was added under argon. The reaction mixture was stirred under argon for 2 h at 5 °C. The residue was dissolved in ethyl acetate and the excess of NaH was eliminated by dropwise addition of ethyl acetate (H_2O saturated) until no further evolvement of

hydrogen gas could be detected. An additional 100 mL of ethyl acetate was added to the reaction mixture followed by 5 mL of H_2O-saturated ethyl acetate. The organic phase was washed with 10 mL H_2O and 20 mL saturated $NaHCO_3$ solution and the organic phase was dried over Na_2SO_4. Solvent was removed under reduced pressure. The residue was purified by flash column chromatography, eluting with DCM/MeOH = 100:0 then 98:2, to give a white solid (62%, 0.80 g).

^{31}P NMR (MeOD, 202 MHz): δ 21.65. ^{1}H NMR (MeOD, 500 MHz): δ 8.00-7.98 (2H, m, Bz), 7.90 (1H, d, *J* = 7.28 Hz, H-6), 7.56-7.22 (19 H, m, H-5, Bz, C*Ph*$_3$), 4.36 (1H, dd, *J* = 13.31 Hz, 3.83 Hz, H-1'); 4.20-4.17 (1H, m, H-2'); 4.13-4.06 (1H, m, CH_2P), 3.98-3.93 (1H, m, CH_2P), 3.79-3.72 (1H, m, H-1'); 3.49 (4H, q, *J* = 7.27 Hz, 2 x OC*H*$_2$CH$_3$), 3.23-3.20 (1H, m, H-3'); 3.17-3.15 (1H, m, H-3'), 1.37 (6H, t, *J* = 7.27 Hz, 2 x OCH_2C*H*$_3$). EI MS= 720.22 ($M+K^+$).

(S)-N^1-[(3-triphenylmethoxy-2-diethylphosphonylmethoxy)-propyl)]-cytosine [7.23].



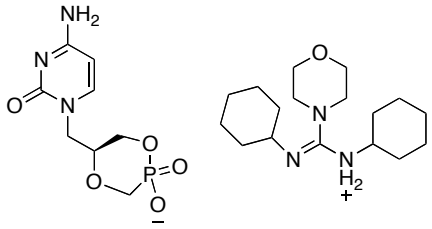
Chemical Formula: $C_{31}H_{36}N_3O_6P$
Molecular Weight: 577.6078

A stirring solution of **7.31** (0.22 g, 0.32 mmol) in MeOH (10 mL) was saturated with NH_3 gas in a sealed tube at 0 °C. The solution was stirred for 3 h at room temperature. Solvent was removed under vacuum. The residue was purified by flash column chromatography, gradient elution of DCM/MeOH = 100:0, then 98:2, then 96/4), to give a white solid (95%, 0.175 g).

^{31}P NMR (MeOD, 202 MHz): δ 21.57. ^{1}H NMR (MeOD, 500 MHz): δ 7.50-7.47 (5H, m, C*Ph*$_3$); 7.43 (1H, d, *J* = 7.25 Hz, H-6), 7.36-7.24 (10H, m, C*Ph*$_3$); 5.76 (1H, d, *J* = 7.25 Hz, H-5); 4.20 (1H, dd, *J* = 13.53 Hz, 3.93 Hz, H-1'), 4.13-4.09 (2H, m, H-2', CH_2P); 3.81-3.75 (1H, m, CH_2P); 3.63 (4H, q, *J* = 7.05 Hz, 2 x OC*H*$_2$CH$_3$); 3.61-3.57 (1H, m, H-1'); 3.18-3.11 (2H, m, H-3'); 3.17-3.15 (1H, m, H-3'); 1.20 (6H, t, *J* = 7.05 Hz, 2 x OCH_2C*H*$_3$).

9.6.2 Synthesis of the L-alanine benzyl ester phosphonoamidate derivative of *S*-cHPMPC [7.4]

Synthesis of the dicyclohexyl-morpholinocarboxamidine salt of 1-[((S-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine [S-cHPMPC].

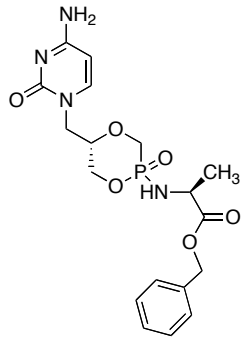


Chemical Formula: $C_{25}H_{43}N_6O_6P$
Molecular Weight: 554.6193

To a stirred suspension of S-HPMPC*$2H_2O$ (0.3 g, 1.07 mmol) in DMF (7.5 Ml) was added N,N-dicyclohexyl-4-morpholinecarboxamidine (0.31 g, 1.07 mmol). The reaction mixture was stirred for 12 h at room temperature, during which the S-HPMPC dissolved. This solution was then added slowly to a hot pyridine solution (7.5 Ml) of 1,3 dicyclohexyl carbodiimide (0.5 g, 2.42 mmol). The mixture was stirred at 100 °C for 16 h, cooled to room temperature and filtered, and the filtrate was concentrated under vacuum. The solvent was then removed under reduced pressure and the residue was purified by column chromatography, gradient elution of DCM/MeOH from 90/10 to 80/20, to give the dicyclohexyl-morpholinocarboxamidine (DCMC) salt of *S*-cHPMPC as a white solid (50%, 0.3 g).

^{31}P NMR (MeOD, 202 MHz): δ 7.30. ^{1}H NMR (MeOD, 500 MHz): δ 7.53 (1H, d, *J* = 7.26 Hz, H-6), 5.86 (1H, d, *J* = 7.26 Hz, H-5), 4.25-4.21 (1H, m, OC*H*$_2$P), 4.15-4.10 (1H, m, OC*H*$_2$P), 4.04-4.00 (2H, m, H-1'), 3.92-3.87 (1H, m, H-2'), 3.77-3.75 (4H, m, C*H*$_2$OC*H*$_2$ (DCMC)), 3.67-3.60 (2H, m, H-3'), 3.46-3.45 (4H, m, C*H*$_2$NC*H*$_2$ (DCMC), 3.37-3.33 (2H, m, C*H*N of (DCMC), 1.96,-1.18 (20H, m, cHx (DCMC)). ^{13}C NMR (MeOD, 126 MHz): δ 26.22 (cHx (DCMC)), 26.38 (cHx (DCMC)), 34.48 (cHx (DCMC)), 49.89 (*C*H$_2$N*C*H$_2$ (DCMC)), 50.68 (C-1'), 56.08 (C*H*N (DCMC)), 67.31 (d, $J_{c\text{-}p}$ = 144.07 Hz, O*C*H$_2$P), 67.38 (*C*H$_2$O*C*H$_2$ (DCMC)), 70.84 (d, $J_{c\text{-}p}$ = 6.67 Hz, C-3'), 75.85 (d, $J_{c\text{-}p}$ = 3.71 Hz, C-2'), 95.49 (C-5), 148.61 (C-6), 159.03 (C-2), 159.37 (N*C*(NH)N (DCMC)), 168.11 (C-4).

Benzyl-O-[(5S)-5-[(4-Amino-2-oxopyrimidin-1(2H)-yl)-methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl]-(L)-alaninate [7.4]



Chemical Formula: $C_{18}H_{23}N_4O_6P$
Molecular Weight: 422.3722

The dicyclohexyl-morpholinocarboxamidine salt of S-cHPMPC (0.113 g, 0.2 mmol) and para-toluenesulphate salt of L-alanine-O-benzyl ester (0.41 g, 1.2 mmol) were mixed in anhydrous pyridine (5 mL). Triethylamine (0.3 mL, 2.4 mmol) was added and the resultant mixture was stirred at 60 °C under nitrogen. In a separate flask, 2-2'dithiodipyridine (0.368 g, 1.40 mmol) and PPH_3 (0.306 g, 1.40 mmol) were dissolved in anhydrous pyridine (2 mL). The resultant yellow solution was stirred for 20 minutes. The solution was then added to the 60 °C solution of S-cHPMPC in one portion. The combined mixture was stirred at 60 °C under nitrogen for 16 h to give a clear yellow solution. The solvent was removed under reduced pressure. The resultant oil was dissolved in CH_2Cl_2 and purified by flash column chromatography, gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6. The product was further purified by preparative TLC (gradient elution of DCM/MeOH = 98/2, then 96/4, then 94/6) to give a white solid (30%, $0.25*10^{-1}$g).

^{31}P NMR (MeOD, 202 MHz): δ 17.76, 16.69. ^{1}H NMR (MeOD, 500 MHz): δ 7.50, 7.49 (1H, 2d, *J* = 7.80 Hz H-6), 7.42-7.32 (5H, m, OCH_2*Ph*), 5.86, 5.85 (1H, 2d, *J* = 7.80 Hz, H-5), 5.20-5.18 (2H, m, OCH_2Ph), 4.43-3.20 (2H, m, H-3'), 4.11-3.97 (3H, m, $CHCH_3$, H-2', OCH_2P), 3.86-3.73 (2H, m, OCH_2P, H-1'), 3.66-3.62 (1H, m, H-1'), 1.46, 1.40 (3H, 2d, *J* = 7.15 Hz, $CHCH_3$). ^{13}C NMR (MeOD, 126 MHz): δ 20.43 (d, J_{C-P} = 5.84 Hz $CHCH_3$) 20.73 (d, J_{C-P} = 5.84 Hz, $CHCH_3$), 50.08, 50.40 (C-1'), 50.48, 50.70 (2s, $CHCH_3$), 65.89 (d, J_{c-p} = 131.79 Hz, OCH_2P), 67.11 (d, J_{c-p} = 131.79 Hz, OCH_2P), 68.08 (OCH_2Ph), 71.13 (d, J_{C-P} = 6.64 Hz, C-3'), 71.62 (d, J_{C-P} = 6.64 Hz, C-3'), 75.00 (d, J_{C-P} = 5.19 Hz, C-2'), 75.40 (d, J_{C-P} = 5.19 Hz, C-2'), 95.48, 95.66 (2s, C-5), 129.37, 129.60 (2s OCH_2*Ph*), 137.28, 137.32 ('ipso' OCH_2*Ph*), 148.27, 148.68 (2s, C-6), 158.68, 158.71 (C-2), 167.87, 167.97 (C-4), 174.80 (d, J_{C-P} = 3.30 Hz, $COOCH_2$Ph), 175.10 (d, J_{C-P} = 3.30 Hz, $COOCH_2$Ph). EI MS= 445.12 ($M+Na^+$). HPLC = H_2O/MeOH from 90/10 to 0/100 in 20 min, then 0/100 isocratic for 5 min = retention time 13.11 min.

9.7 Enzymatic assays

Carboxypeptidase Y assay: standard procedure

The appropriate ProTide (5 mg) was dissolved in acetone-*d6* (150 μL) and Trizma buffer (pH 7.6, 300 μL) was added. A ^{31}P NMR spectrum was recorded at 25 ºC (64, 128 or 264 scans). Carboxypeptidase Y (ca. 0.1 mg) in the Trizma buffer (150 μL) was added to the solution of the ProTide and ^{31}P NMR spectra were recorded at regular intervals overnight (usually 128 or 264 scans, repeated every 6-15 min for 14 h, 25 ºC).

Adenosine deaminase

A stock solution of adenosine deaminase was prepared by dissolving 0.50 mg of adenosine deaminase in 1.0 mL of phosphate buffer (pH 7.5, 0.05 M). A solution of the appropriate nucleosides in phosphate buffer (pH 7.5, 44 μM) was similarly prepared. The assay was performed by transferring 1.0 mL of nucleoside stock solution and recording UV spectrum at 25 °C over the range 400 to 220 nm, then 5-30 μL of the enzyme solution was added and UV spectra were recorded at 10 min intervals over a period of 20 h.

9.8 Molecular modeling

The modeling studies presented in this work were performed on MacPro dual 2.66GHz Xeon running Ubuntu 9.10. The crystal structures were downloaded from PDB website (http://www.rcsb.org/). Hydrogen atoms were added to the protein, using Molecular Operating Enviroment (MOE) 2008.10 and minimized, keeping all the heavy atoms fixed until a rmsd gradient of 0.05 kcal mol-1 Å-1 was reached (MOE). Ligand structures were built with MOE and minimized using the MMFF94X force field until a rmsd gradient of 0.05 kcal mol-1 Å-1 was reached. The docking simulations were performed using PLANTS. Cathepsin Y binding site: center 47.758, -2.019, 35.82, radius 10 Å, cluster RMSD 2, cluster structures 10. Hint-1 binding site: center 10.8, 12.4, 14.4, radius 12 Å, cluster RMSD 2, cluster structures 10.

Appendix I: Publication

Derudas, M.; Carta, D.; Brancale, A.; Vanpouille, C.; Lisco, A.; Margolis, L.; Balzarini, J.; McGuigan, C. The application of phosphoramidate protide technology to acyclovir confers anti-HIV inhibition. *J. Med. Chem.* **2009,** 52, 5520-5530.

5520 J. Med. Chem. 2009, 52, 5520–5530
DOI: 10.1021/jm9007856

Journal of Medicinal Chemistry Article

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 retroviradae 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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[a] Abbreviations: 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.

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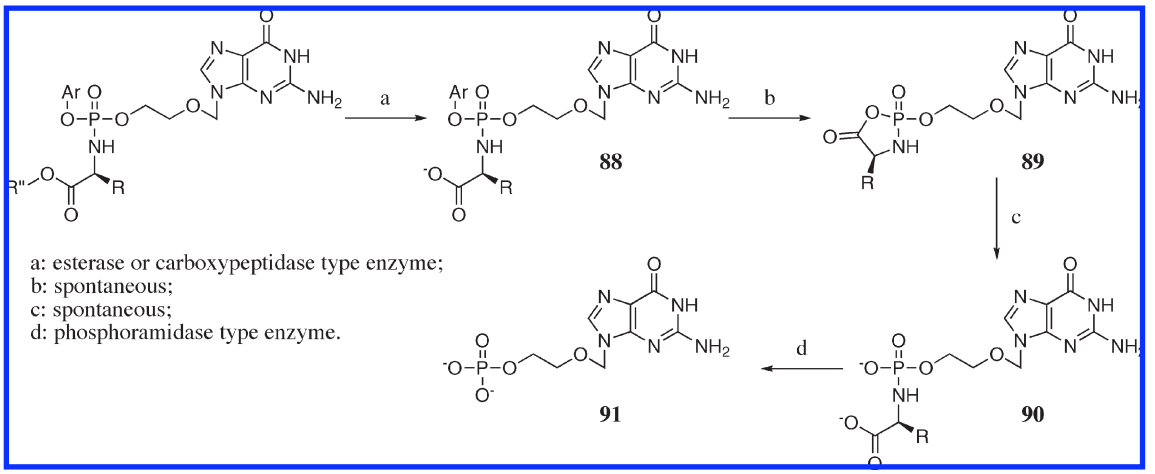


Figure 1. Proposed activation pathway of the acyclovir ProTides.

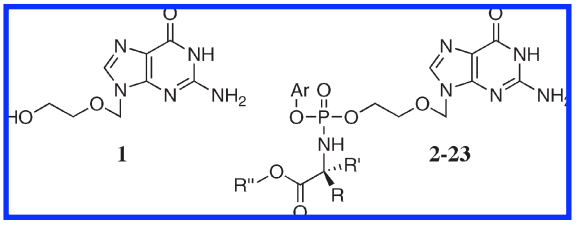


Figure 2. ACV and its ProTides.

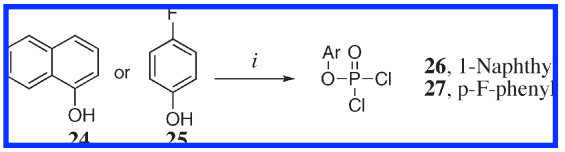
amino acid, as it has shown previously an optimal biological profile,[12] varying the other two components. For the aryl moiety we considered phenol, naphthol, and *p*-fluorophenol and as the ester moiety methyl, ethyl, *n*-propyl, *iso*-propyl, *tert*-butyl, and benzyl and combinations thereof. All 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 phosphoramidates[6] 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 Uchiyama[13] using *tert*-butylmagnesium chloride (^{t}BuMgCl) as a coupling reagent and using THF as a solvent in most of the cases.

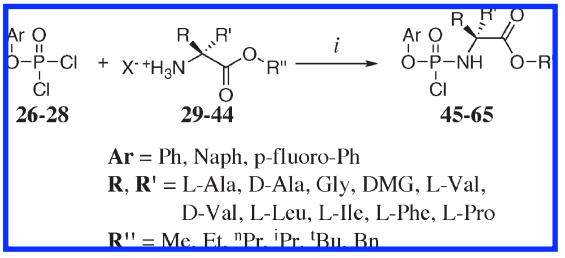
Aryl phosphorodichlorophosphates **26** and **27** have been synthesized, coupling respectively 1-naphthol (**24**) or *p*-fluorophenol (**25**) with $POCl_3$ in the presence of Et_3N (1equiv) (Scheme 1), while phenyl dichlorophosphate (**28**) was commercially available. The coupling with the appropriate amino acid ester salt (**29**−**44**) has been performed in the presence of Et_3N (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) $POCl_3$, anhydrous TEA, anhydrous Et_2O, −78 °C, 1 h then rt, overnight.

Scheme 2[a]



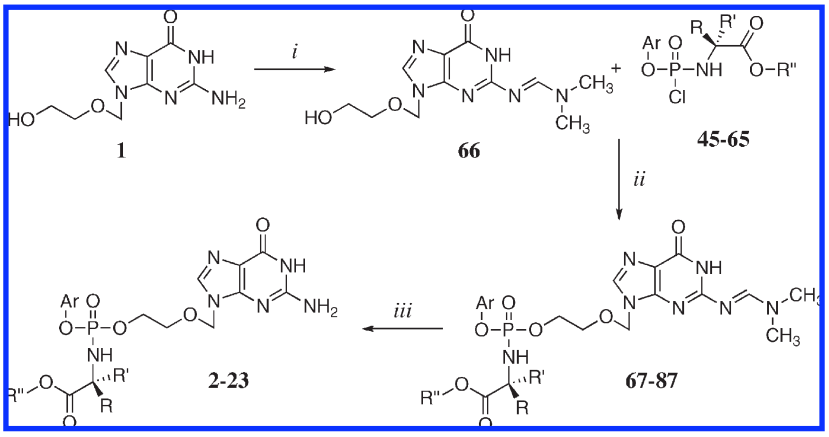
[a] Reagents and conditions: (i) anhydrous TEA, anhydrous DCM, −78 °C, 30 min to 1 h, then rt, 30 min to 4 h.

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 ^{31}P 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) ^{t}BuMgCl, 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

					antiviral activity EC_{50}[a] (μM)			cytotoxic/cytostatic activity (μM)	
compds	aryl	amino acid	ester	CLogP	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	p-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 (**1**)				−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 (EC_{50}: 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 (EC_{50}: 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) (EC_{50}: 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

				antiviral activity EC_{50}[a] (μM)			cytostatic activity (μM)		
compds	aryl	amino acid	ester	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 (**1**)				>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] N_2-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 (EC_{50}) 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 EC_{50}'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 EC_{50}'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 N_2-DMF-protected ACV ProTides (**77**, **78**, and **86**) showed activity against HIV-1 (EC_{50}'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

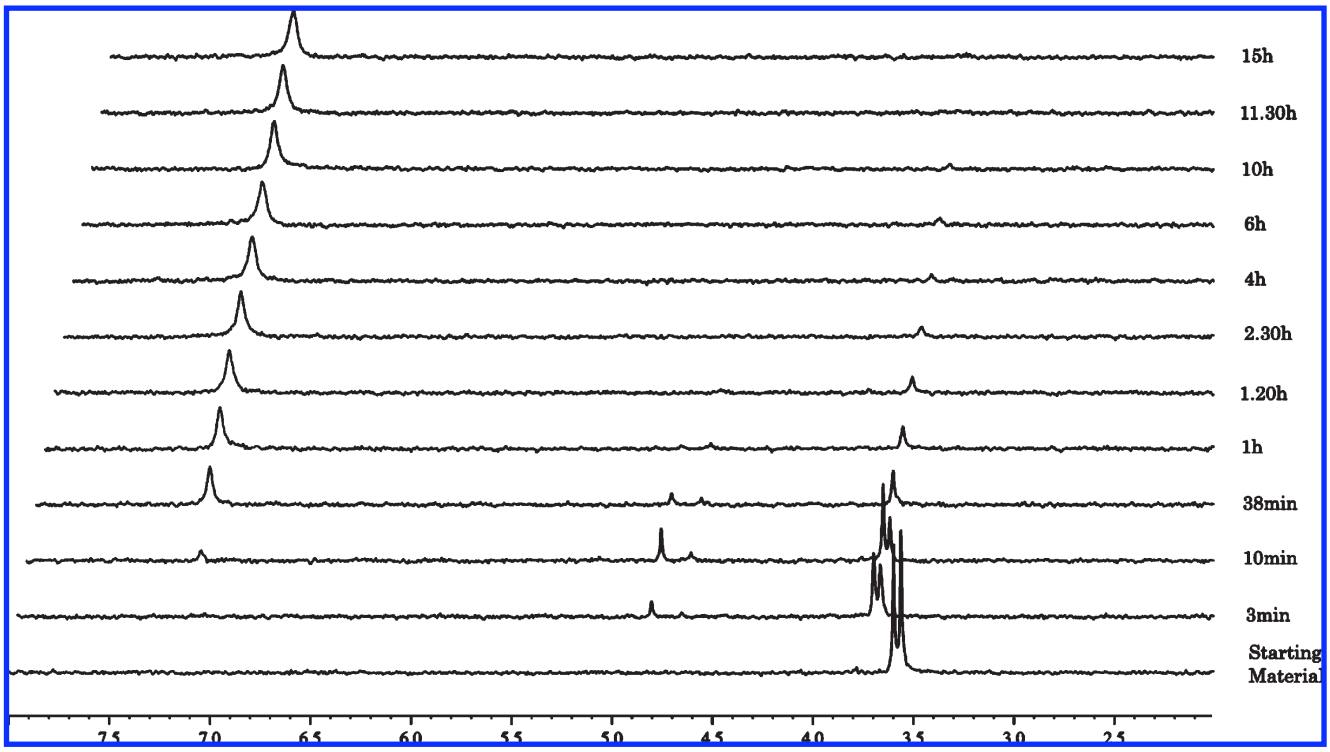


Figure 3. Carboxypeptidase-mediated cleavage of compound **9**, monitored by ^{31}P NMR.

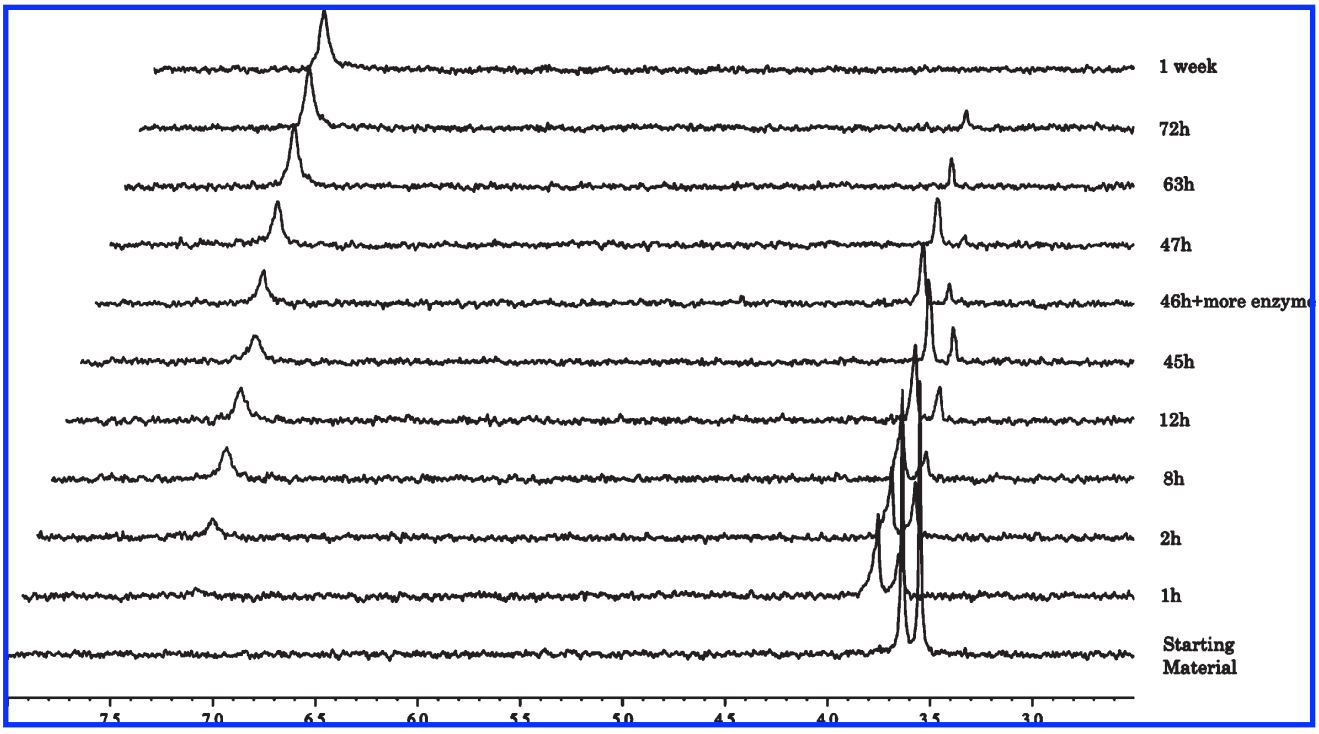


Figure 4. Carboxypeptidase-mediated cleavage of compound **10**, monitored by ^{31}P NMR.

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

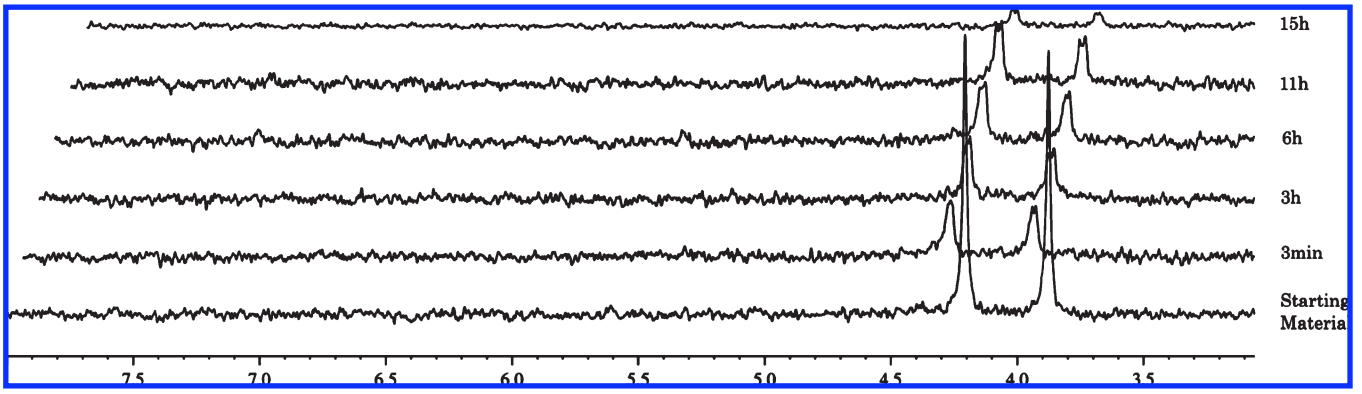


Figure 5. Carboxypeptidase-mediated cleavage of compound **6**, monitored by ^{31}P NMR.

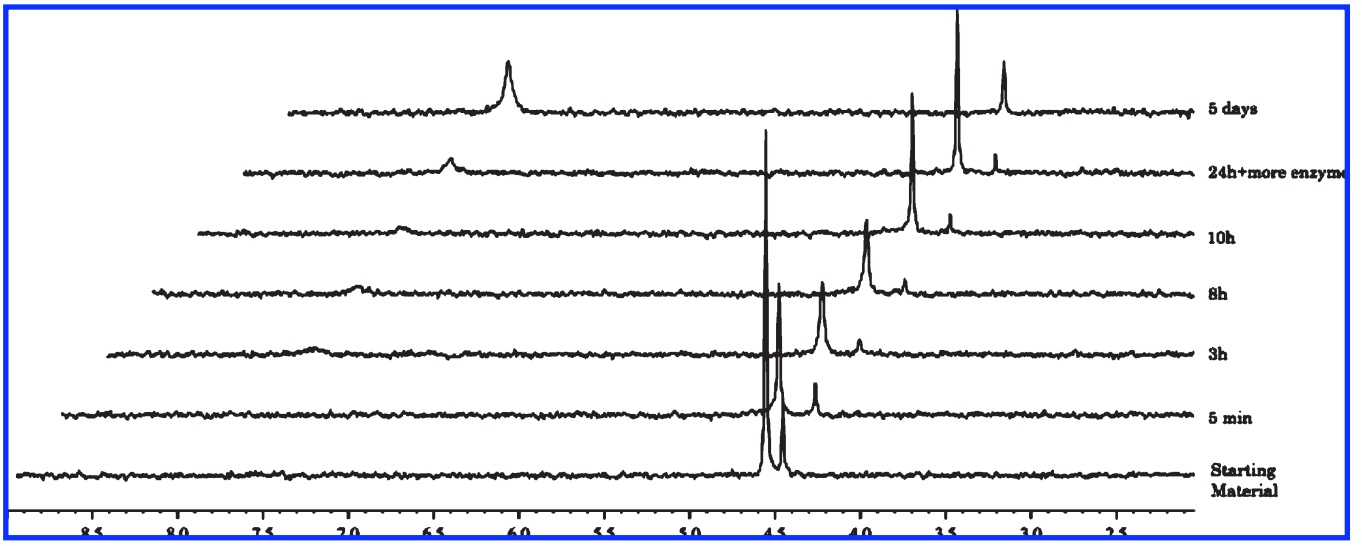


Figure 6. Carboxypeptidase-mediated cleavage of compound **17**, monitored by ^{31}P NMR.

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 (δ_P = 3.65 and 3.60) to the intermediate type **88** (Figure 1) (δ_P = 4.85 and 4.70), noting the presence of the two diastereoisomers. This intermediate is then processed to a compound of type **90** (δ_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

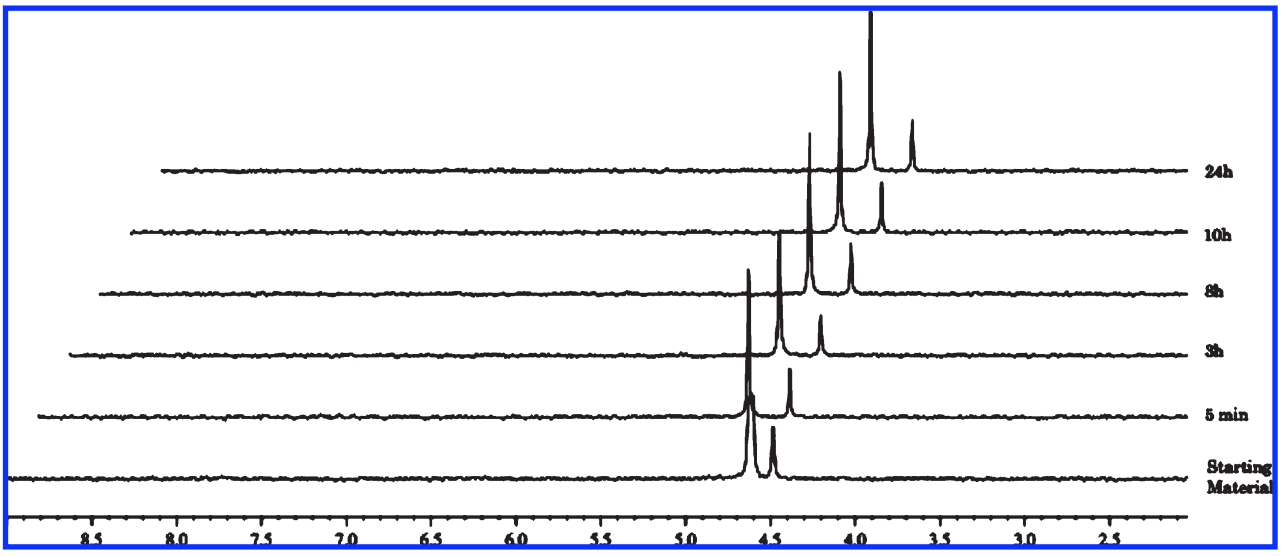


Figure 7. Carboxypeptidase-mediated cleavage of compound **19**, monitored by ^{31}P NMR.

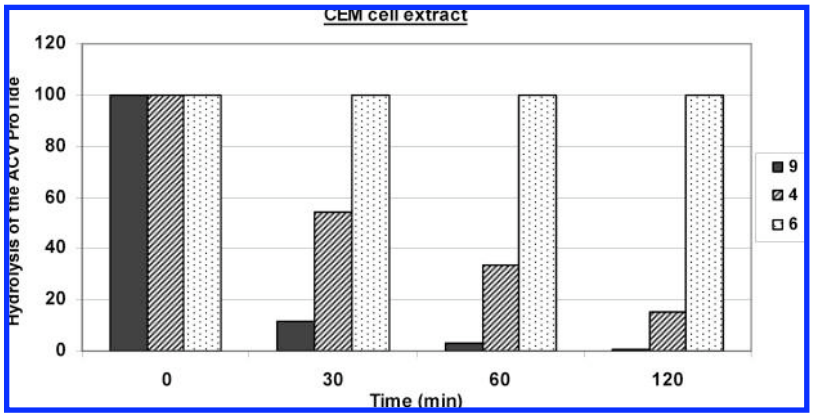


Figure 8. Stability of ACV ProTides in crude CEM cell extracts as a function of incubation time.

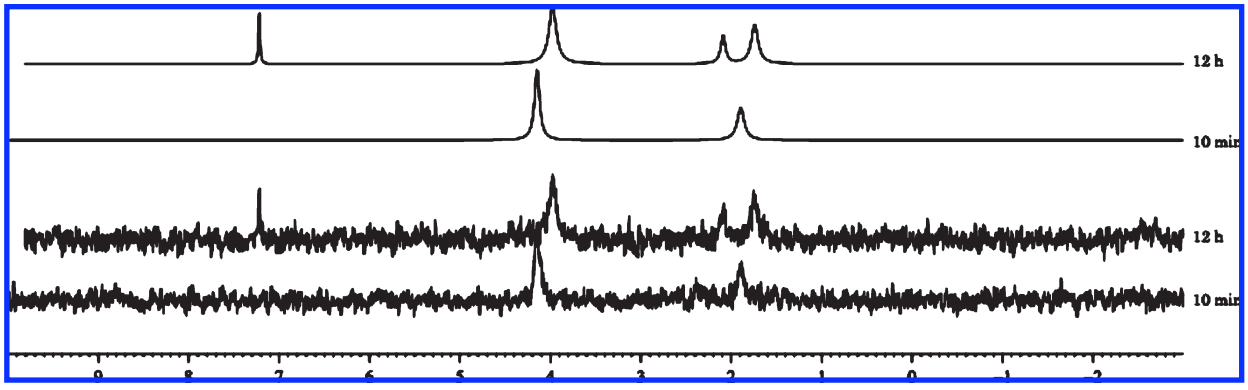


Figure 9. Stability of compound **9** in human serum, monitored by ^{31}P NMR.

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 δ_P = 1.90, which may correspond to the monophosphate form. The peak at δ_P = 2.25 corresponds to the human serum that in the first experiment is overlapping with the peak at at δ_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 δ_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

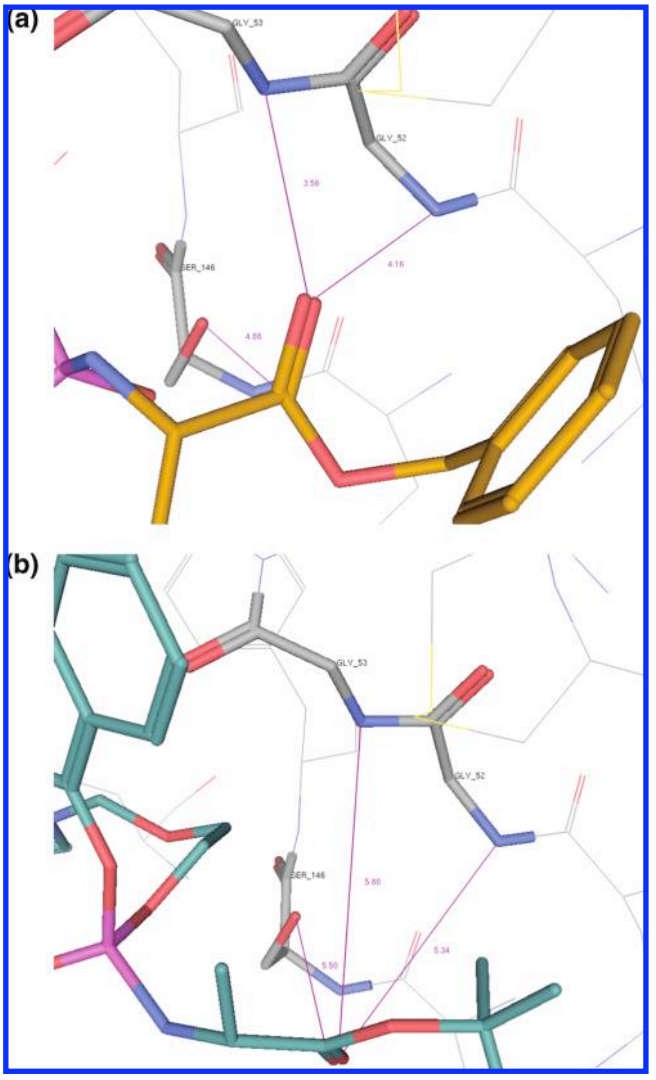


Figure 10. (a) Docking of compound **9** within the catalytic site of carboxypeptidase Y enzyme. (b) Docking of compound **6** within the catalytic site of carboxypeptidase Y enzyme.

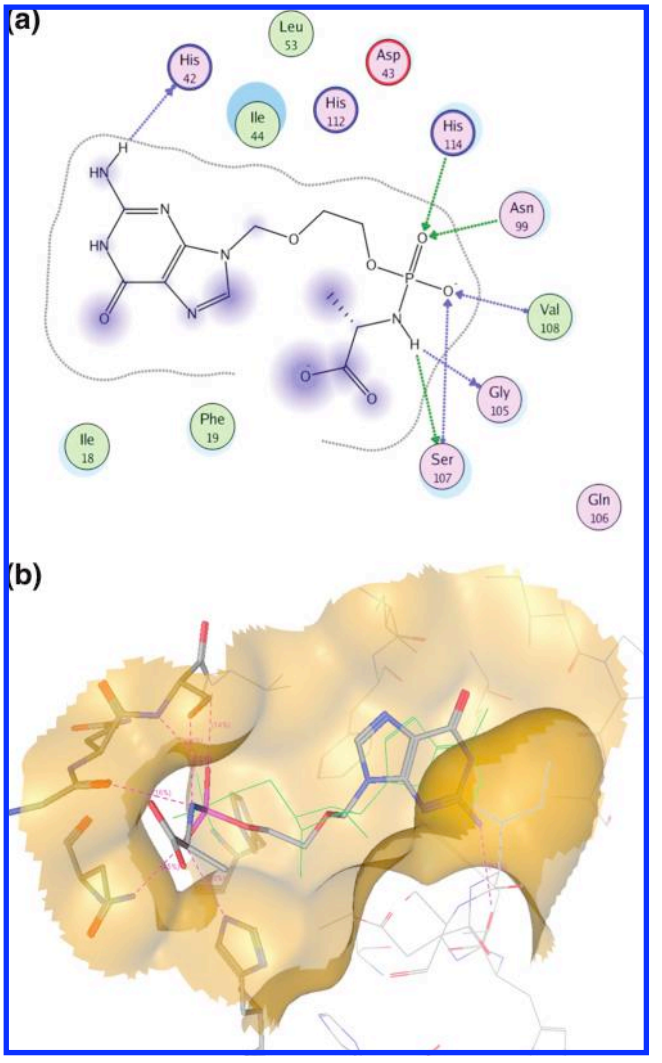


Figure 11. (a) Interactions of **90** (L-Ala) with the active site of human Hint-1. (b) Docking of compound **90** (L-Ala) ACV-MP phosphoramidate within the catalytic site of human HINT (I) enzyme.

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 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 μM (HIV-1, CEM), 8.9−42 μM (HIV-2, CEM), and 0.8−30 μM (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 μM). Proton (^{1}H), carbon (^{13}C), and phosphorus (^{31}P) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 °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 (≥95%) of all final products was assured by a combination of microanalysis, and HPLC, with additional characterization in every case by: H, C, and 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}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*-dimethylformimidamide) (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). ^{1}H NMR (DMSO, 500 MHz): δ 11.30 (1H, s, NH), 8.58 (1H, s, C*H*N(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, N(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. ^{1}H 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 1-Naphthyl(benzoxy-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. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.99−7.25 (12H, m, Naph, OC*H*$_2$Ph), 5.15−5.07 (2H, m, C*H*$_2$Ph), 4.30−4.23 (1H, m, C*H*CH$_3$), 1.49−1.46 (3H, m, CHC*H*$_3$).

Synthesis of N^2-DMF-acyclovir-[1-naphthyl(benzoxy-L-alaninyl)] Phosphate (67). Prepared according to standard procedure C, from **66** (0.30 g, 1.07 mmol) in anhydrous THF (10 mL), ^{t}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. ^{1}H NMR (MeOD, 500 MHz): δ 8.47, 8.46 (1H, 2s, NC*H*N(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, N(CH_3)$_2$), 1.20−1.17 (3H, m, CHCH_3).

Synthesis of Acyclovir-[1-naphthyl(benzoxy-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). ^{31}P NMR (MeOD, 202 MHz): δ 4.13, 3.96. ^{1}H 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$_2$*Ph*), 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_{C-P} = 7.63, CHCH_3), 20.43 (d, J_{C-P} = 6.61, CHCH_3), 51.76, 51.81 (2s, *C*HCH$_3$), 67.20 (d, J_{C-P} = 5.58, C-5′), 67.28 (d, J_{C-P} = 4.91, C-5′), 67.95, 67.98 (2s, OCH$_2$Ph), 69.34 (d, J_{C-P} = 7.72, C-4′), 69.40 (d, J_{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$_2$*Ph*), 136.26, 137.23 (C-4a Naph, "ipso" OCH$_2$*Ph*), 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, *C*OOCH$_2$Ph). EI MS = 615.1735 (M + Na). Anal. Calcd for $C_{28}H_{29}N_6O_7P\cdot0.5H_2O$: 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 (1CCID50 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 ($\sim3\times10^5$ cells mL^{-1}) were infected with 100 CCID50 HIV-1(III$_B$) 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 homogenizator (Berlin Technologies, Montigny-en-Bretonneux, France) (tonsils) or by a Hielscher-Ultrasound 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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