# Virtual screening of acyclovir derivatives as potential antiviral agents: design, synthesis and biological evaluation of new acyclic nucleoside ProTides

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[§]This work is dedicated to the memory of Prof. Chris McGuigan, a great colleague and scientist, invaluable source of inspiration and love for research

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**Abstract**

Following our findings on the anti-human immunodeficiency virus (HIV) activity of acyclovir (ACV) phosphate prodrugs, we herein report the ProTide approach applied to a series of acyclic nucleosides aimed at the identification of novel and selective antiviral, in particular anti-HIV agents. Acyclic nucleoside analogues used in this study were identified through a virtual screening using HIV-reverse transcriptase (RT), adenylate/guanylate kinase, and human DNA polymerase. A total of thirty-nine new phosphate prodrugs were synthesised and evaluated against HIV-1 (in *in vitro* and in *ex-vivo* human tonsillar tissue system) and human herpes viruses. Several ProTide compounds showed substantial potency against HIV-1 at low micromolar range while the parent nucleosides were not effective. Also, pronounced inhibition of herpesvirus replication was observed. A carboxypeptidase-mediated hydrolysis study was performed for a selection of compounds to assess the formation of putative metabolites and support the biological activity observed.

## Introduction

HIV infection currently affects over 36 million people worldwide and caused about 35 million deaths.[1] The rate of new HIV infections is still increasing, especially in third-world countries.[1] Twenty-six anti-HIV drugs are currently available and they act by interacting with different targets essential for the viral replication, such as RT, protease, integrase, or the fusion/entry step.[2] The current antiretroviral therapy significantly improved the life expectancy of HIV-infected individuals by reducing the viral load and postponing the insurgence of AIDS. Despite their success these drugs carry some limitations, such as the onset of drug resistance and adverse side-effects. Whilst the first problem has been addressed using a cocktail of different antiretroviral drugs, the second one still dramatically impacts the patients' compliance.

Several years ago we discovered that ACV, a gold-standard drug against herpes simplex virus (HSV) and varicella zoster virus (VZV) infections, can act as a new potential anti-HIV agent. ACV inhibited HIV in the presence of human herpesviruses (HHV) and this activity was attributed to the conversion of ACV to its monophosphate form by the HHV-encoded kinases; further phosphorylation steps provided the active triphosphate form of ACV able to inhibit HIV-RT.[3]

To circumvent the required first phosphorylation step of ACV, that requires HHV kinase activity, a series of phosphate prodrugs, so called ProTides, were synthesised. These compounds showed anti-HIV activity in the absence of HHV infection, demonstrating their nucleoside kinase independence.[4-5] These findings were also supported in a different study, where ACV phosphate prodrugs showed a full retention of antiviral activity against HSV-1 and VZV thymidine kinase (TK)-deficient strains.[6] Unfortunately, these compounds proved to be somewhat cytotoxic in some assays.[4] To overcome this issue and to better understand the full potential of acyclic nucleoside analogues as new anti-HIV agent, we performed a virtual screening on a library of ACV

derivatives. We then synthesised and biologically evaluated a series of different acyclic nucleosides and their corresponding ProTides and investigated their mechanism of bioactivation using a well-established enzymatic method.

## Results and Discussion

### Molecular modelling: virtual screening

The objective of this study was to increase the specificity of the compounds for HIV-1 RT and reduce the affinity for the cellular DNA polymerase, while still retaining substrate action for adenylate and guanylate kinases. A virtual screening using docking techniques was performed on HIV-1 RT, adenylate or guanylate kinase, and a model of DNA polymerase γ. The docking using nucleoside kinases involved in the first step of drug phosphorylation were not considered since the phosphoramidate ProTide approach will be applied to the selected compounds.

Firstly, a database of 3600 compounds was built considering modifications at the base and at the acyclic sugar moieties (Figure 1), including: simple and branched acyclic sugars (brown); oxygen, methylene or sulphur at the 2'-position (yellow); substitution at the C-8-position of the base including: hydrogen, methyl, fluorine, bromine or chlorine (red); substitution at the C-7-position of the base including: nitrogen or carbon (hydrogen or methyl substituted) (blue); substitution at the C-6-position of the base including: carbonyl, amine, chlorine, methoxide, ethoxide, phenoxy, methylamine, or cyclopropylamine (magenta); substitution at the 2-NH position of the base including: hydrogen, methyl, ethyl, phenyl or benzyl (green).

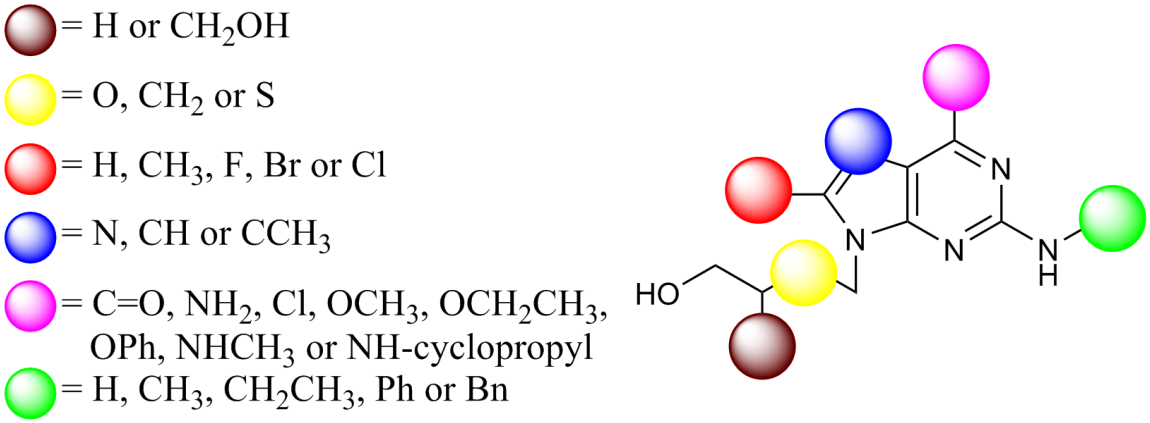



**Figure 1.** Acyclic nucleoside derivatives.

Based on the modification at the 6-position of the nucleobase, the database was divided in two groups: 6-carbonyl derivatives (450 compounds), which showed base-pairing with cytidine; and 6-O-modified and 6-N-modified derivatives (3150 compounds) which showed better base pairing with thymidine (Figure 2).

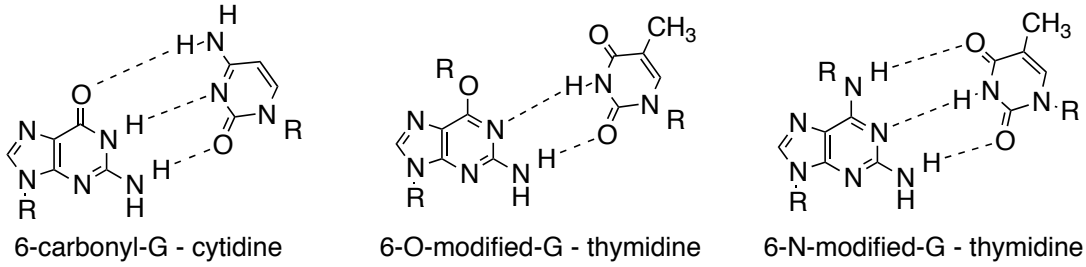



**Figure 2.** Base-pairing of the 6-carbonyl and 6-modified guanine derivatives.

The first screening was performed on HIV-RT using a crystal structure of the enzyme co-crystallised with tenofovir diphosphate (PDB: 1T05).[7] The original structure was used for the 6-modified ACV derivatives, while the substitution of a thymine by a cytidine was necessary for the 6-carbonyl derivatives. All the compounds were docked in their triphosphate form and the

stereochemistry, where present, was taken into account. For this screening, a root mean square deviation (RMSD) < 1 Å between the base of the compounds docked and the base of co-crystallised tenofovir diphosphate was considered leading to a total of 984 compounds. Interestingly, it was observed that branched sugar derivatives were well-tolerated due to the presence of a pocket in the proximity of amino acid residues Tyr115 and Phe116, which allowed the accommodation of a substitution in the acyclic sugar moiety (Figure 3). Moreover, the presence of a cleft that allows a potential substitution at the 2-amino position of the base was observed. In addition, substitutions at the 8-position of the base, especially with halogens, were well-tolerated.

Interestingly, the compound that showed the best interactions (RMSD < 0.18 Å) was ganciclovir (GCV)-TP (Figure 3), drug of choice for human cytomegalovirus (HCMV) infections.

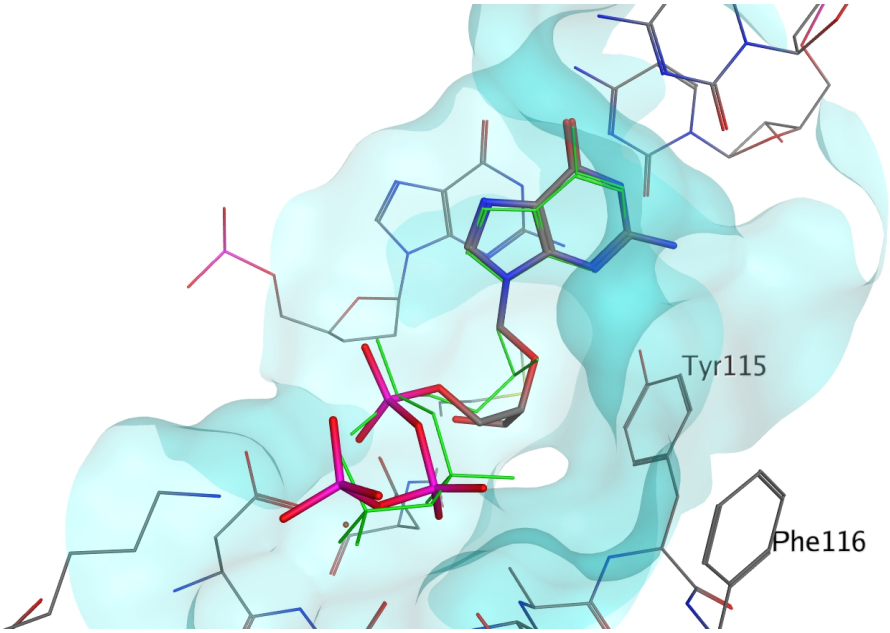



**Figure 3.** Docking pose of GCV triphosphate overlapped with tenofovir diphosphate (green) in the catalytic site of HIV-RT.

For the second screening human adenylate kinase (PDB: 2C95)[8] or mouse guanylate kinase (PDB: 1LVG, 88% human homology)[9] were considered for the 6-modified and for the 6-carbonyl analogues respectively. For these docking experiments, two different RMSDs were considered: the first one regarding the position of the base (<1 Å) and the second one considering the position of the phosphate moiety (<3 Å). These two values allowed a good position of the base and a reasonable position for the phosphate moiety, which, however, in some cases, was slightly moved compared to the crystallized compound. Similar to the results obtained for the HIV-RT docking, branched sugar derivatives showed the best docking results in both series. In addition, the halogen substitution at the 8-position for the 6-carbonyl series was well-tolerated. This second screening led to a total of 218 compounds, with the 6-carbonyl derivatives being preferred over the 6-modified ones.

The last part of the virtual screening included the use of human DNA polymerases. It is known that the toxicity of nucleoside analogues, especially HIV-1 RT inhibitors, is usually due to interactions with these enzymes.[10-13] The subtypes of human DNA polymerase mainly involved in the cytotoxicity are α and γ, with the latter being able to incorporate antiviral nucleotide analogues, which then act as a DNA chain terminators and consequently show cytotoxicity.[14-17] As the crystal structures for both human DNA polymerases have not yet been solved, a model for the human polymerase γ that covers the polymerase active site with the highest degree of homology with T7 DNA polymerase[18] has been used for the final step of our virtual screening.

In this case, the results were analysed visually, excluding all the compounds that showed good interactions with the catalytic site of the enzyme. A total of twenty-five compounds were identified, and amongst these, five compounds were selected which displayed the best scoring for

all the three docking experiments and that were easily accessible from a synthetic point of view. A summary of the virtual screening is depicted in Figure 4.

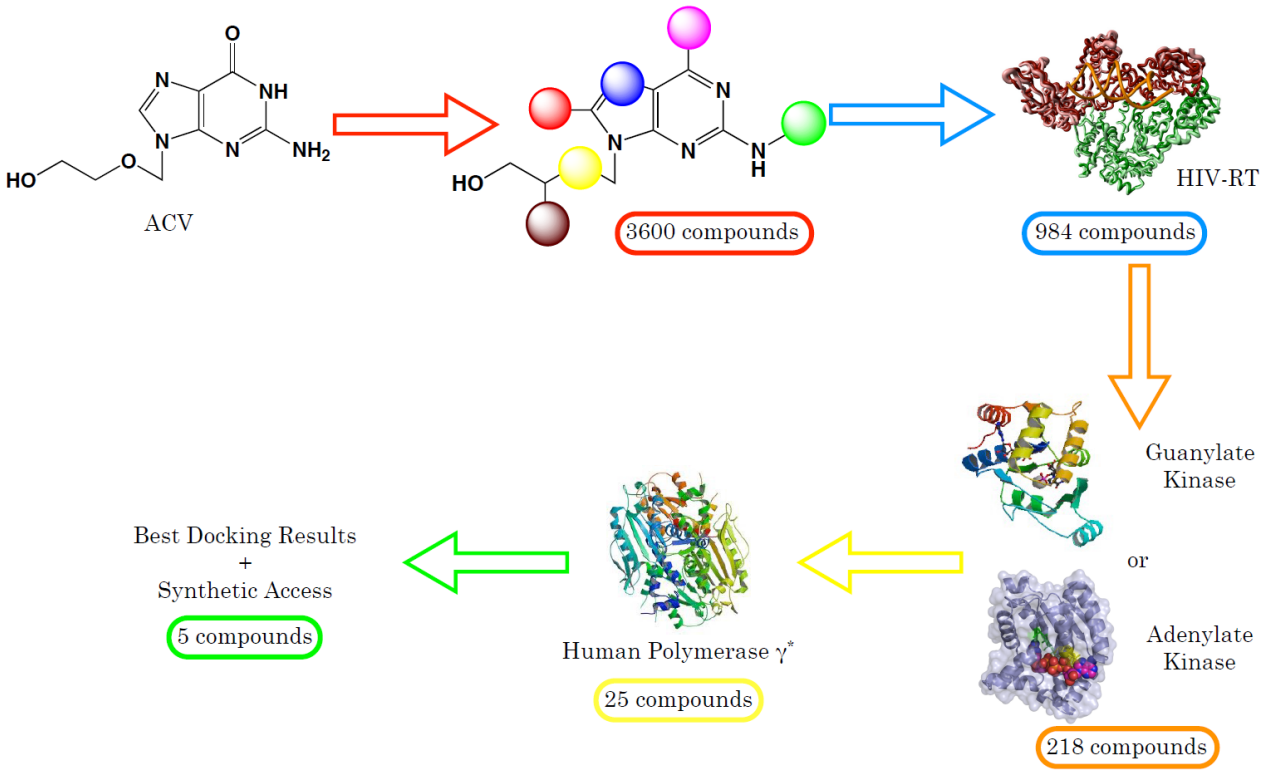



**Figure 4.** Summary of the virtual screening using HIV-RT, adenylate or guanylate kinases, and human polymerase γ.

Figure 5 shows the five compounds selected from the virtual screening, which are: ganciclovir (**1**), penciclovir (**2**), 2'-thio derivative of ganciclovir (**3**), 6-chloropenciclovir (**4**), and 6-methoxypenciclovir (**5**).

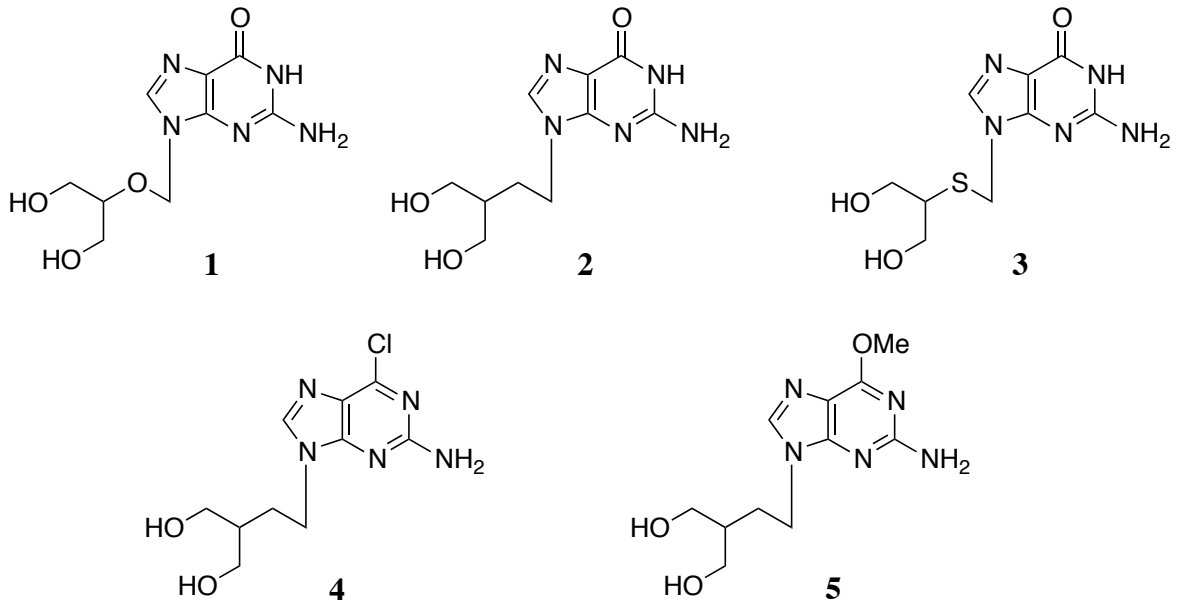



**Figure 5.** Selected acyclic nucleoside analogues from the virtual screening.

## Chemistry

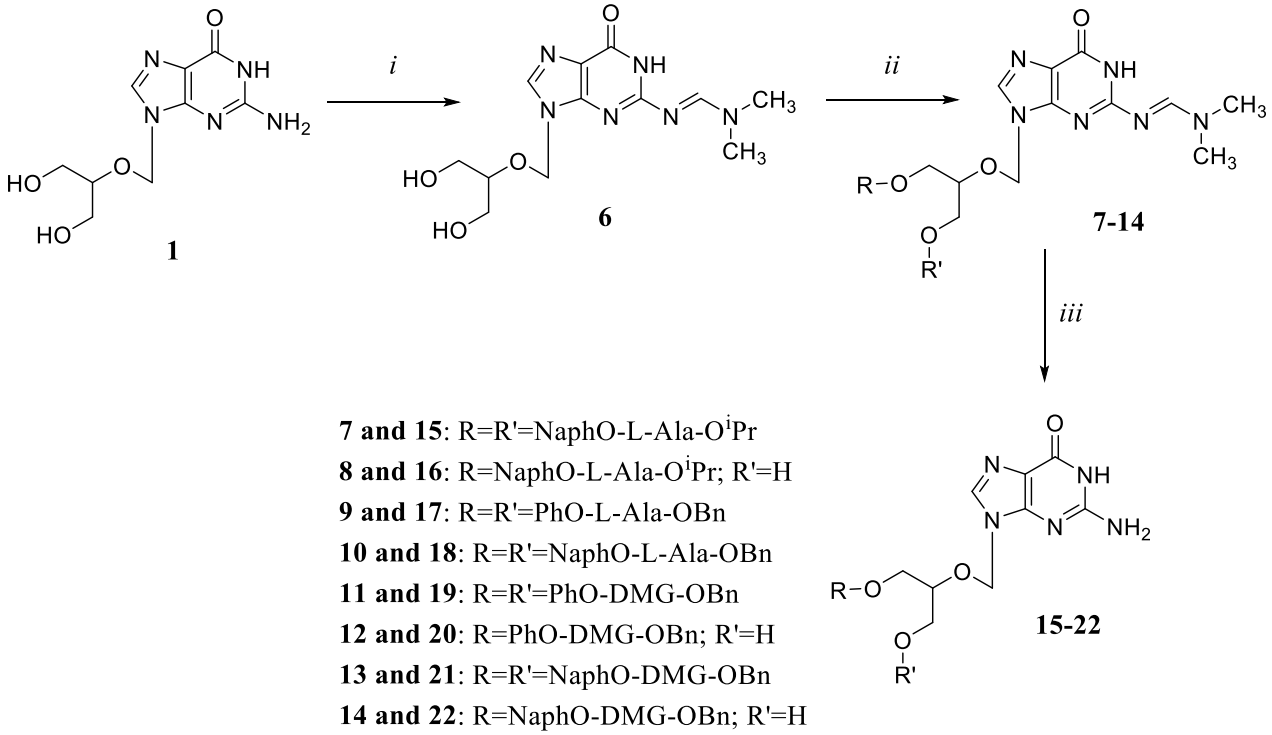
The desired phosphoramidates were synthesised using tert-butylmagnesium chloride ($^t$BuMgCl) as a coupling reagent, following the procedure reported by Uchiyama,[19] which is widely used for the synthesis of ProTides. The desired phosphorochloridates were prepared according to our established method by coupling the appropriate aryl dichlorophosphate with the appropriate amino acid ester to yield the desired compound as an oil.[4]

Synthesis of ProTides of nucleoside analogue **1**.

For the synthesis of ganciclovir **1** ProTides (Scheme 1), the 2-amino functionality of the guanine base was protected using N,N-dimethylamino formamide according to the literature.[4] The coupling with the appropriate phosphorochloridate (2-8 eq) was performed using an excess of $^t$BuMgCl (2-3 mol/eq) in a mixture of THF and pyridine. The presence of the extra hydroxyl group in the molecule played a crucial role in the synthesis of the desired compounds resulting in some cases

only in the isolation of the bisphosphate prodrug (**9** and **10**). The dimethylformamidine group was removed by refluxing in 2-propanol for 24-80 h yielding the desired compounds **15-22**.

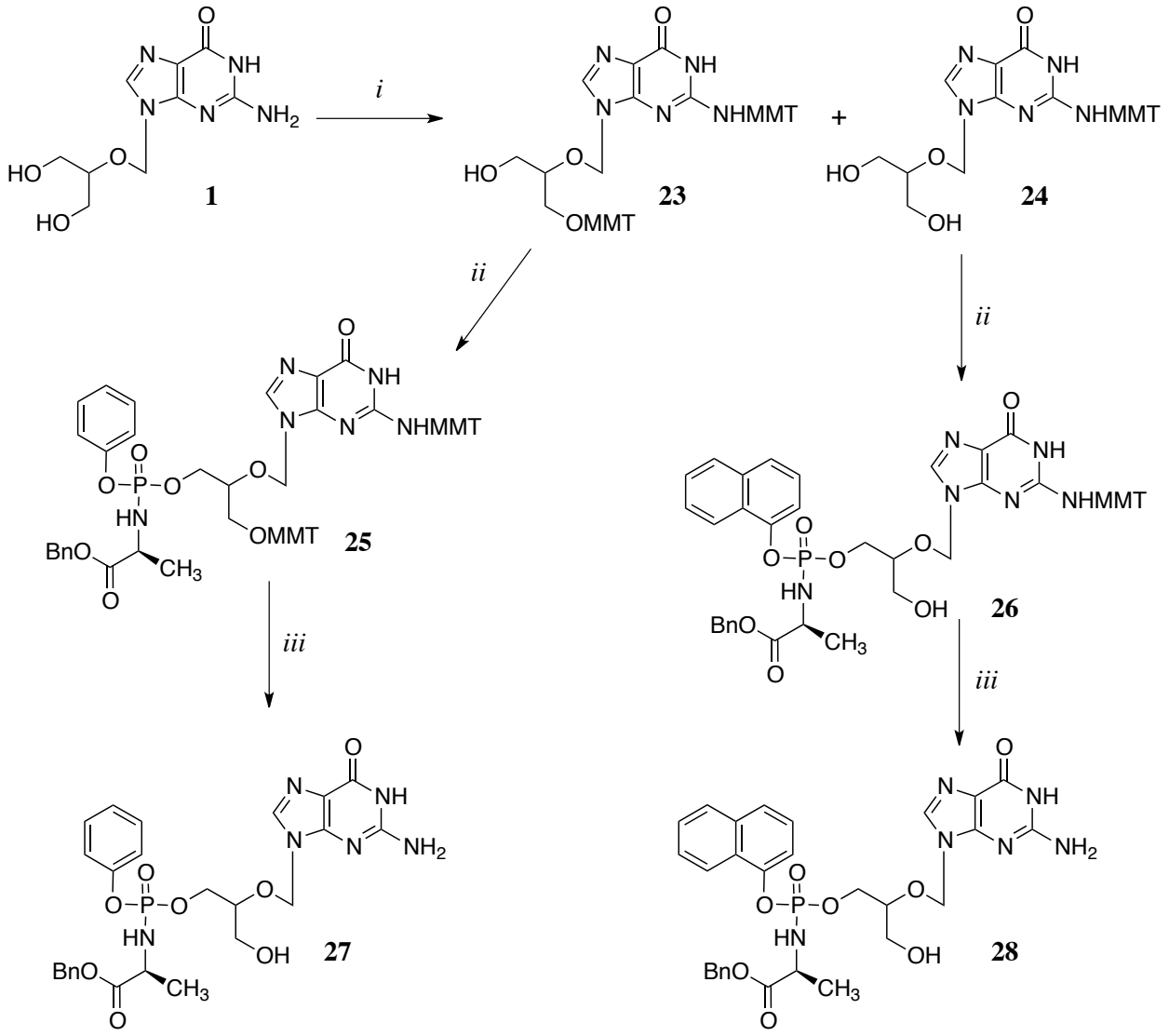
**Scheme 1**




Reagents and conditions: (*i*) dimethylformamide dimethyl acetal, anhydrous DMF, rt, 1 day; (*ii*) appropriate phosphorochloridate, $^{t}$BuMgCl, anhydrous THF/pyridine, rt, 24-84 h; (*iii*) 2-propanol, reflux, 24-80 h.

To overcome the issue related to the bis-phosphorylated side-product, the monomethoxytrityl (MMT) of **1** was synthetized using monomethoxytrityl chloride (2.2 eq), triethylamine (3 eq) and DMAP (cat.) in DMF. The reaction held two different products: **23** bearing two protecting groups at the 2-amino position and at the 3'-hydroxyl position and **24** bearing the monomethoxytrityl group only at the 2-amino position.

**Scheme 2**




**Scheme 2**. Reagents and conditions: (*i*) MMT-Cl, TEA, DMAP, anhydrous DMF, rt, 2 h; (*ii*) appropriate phosphorochloridate, $^{t}$BuMgCl, anhydrous THF, rt, overnight; (*iii*) DCM/MeOH, PTSA, rt, 2 h.

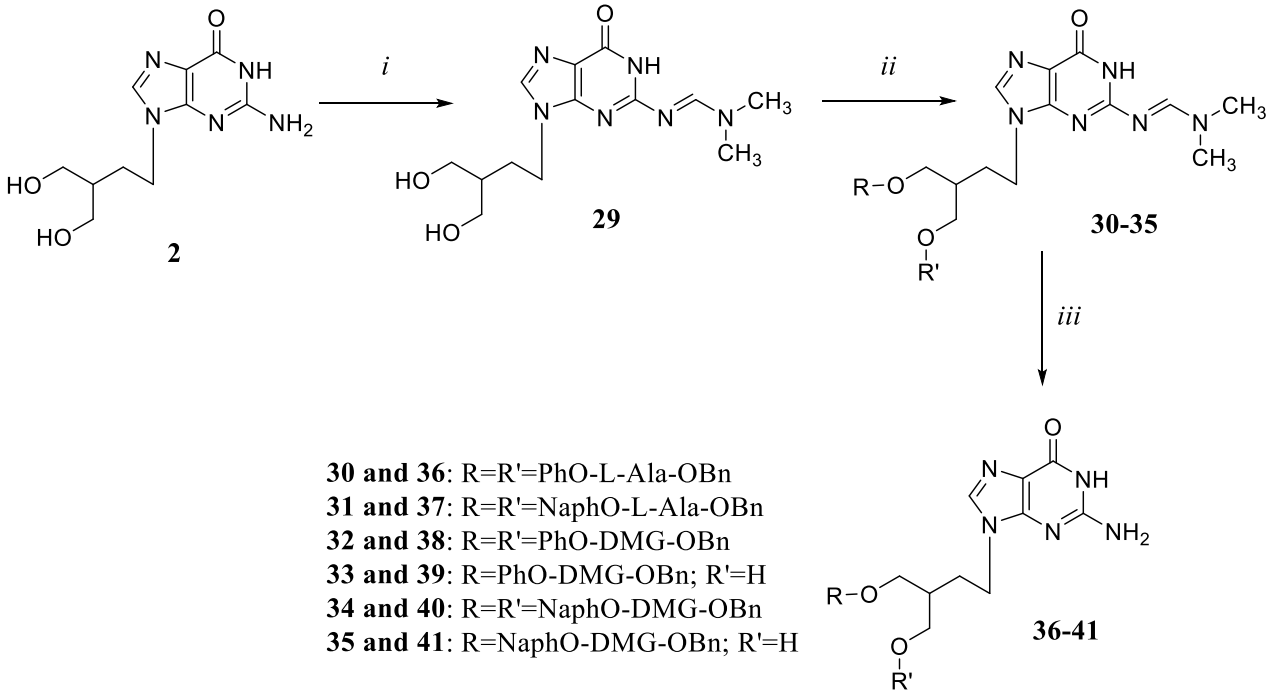
Compound **23** was then reacted with phenyl(benzoxy-L-alaninyl)-phosphorochloridate (2 eq) in the presence of $^{t}$BuMgCl (2 eq) to yield the desired compound **25**; compound **24** was reacted with 1-naphthyl(benzoxy-L-alaninyl)-phosphorochloridate under the same condition giving the desired mono-phosphorylated derivative **26**. The deprotection of the monomethoxytrityl group was

performed using an excess of *para*-toluene sulfonic acid in DCM/MeOH yielding the desired compounds **27** and **28**.

Synthesis of ProTides of nucleoside analogue **2**

The same synthetic approach was applied for the synthesis of compound **2** ProTides. Initially, the 2-amino functionality of the guanine base was protected under the same conditions seen previously to give compound **29** in excellent yield. Unfortunately, the coupling with the appropriate phosphorochloridate resulted in the isolation of only the bis-phosphorylated compounds **30** and **31** for L-alaninyl derivatives; while both bis- (**32** and **34**) and mono-phosphorylated (**33** and **35**) derivatives were obtained when dimethylglycine phosphorochloridates were used. The protecting group was removed by refluxing in 2-propanol to give the final compounds (**36-41**) in low yield due to degradation of the products.

**Scheme 3**

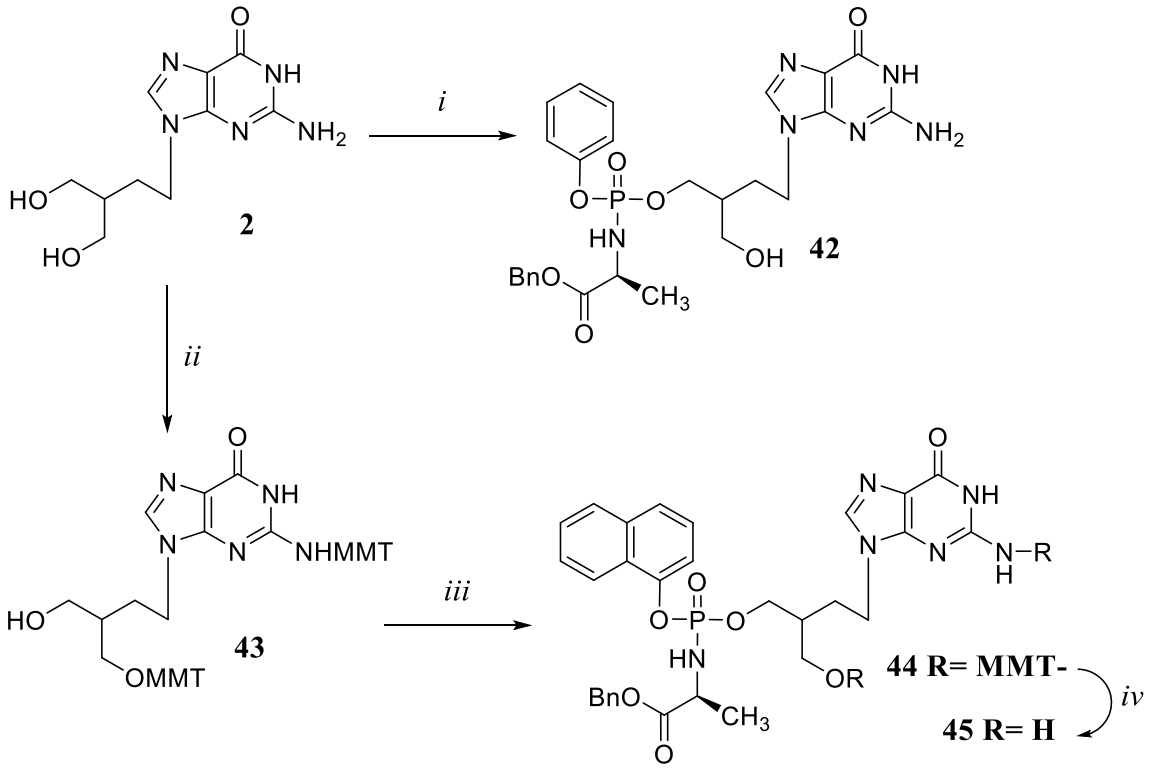



**30 and 36**: R=R'=PhO-L-Ala-OBn
**31 and 37**: R=R'=NaphO-L-Ala-OBn
**32 and 38**: R=R'=PhO-DMG-OBn
**33 and 39**: R=PhO-DMG-OBn; R'=H
**34 and 40**: R=R'=NaphO-DMG-OBn
**35 and 41**: R=NaphO-DMG-OBn; R'=H

Reagents and conditions: (*i*) dimethylformamide dimethyl acetal, anhydrous DMF, rt, 6 h; (*ii*) appropriate phosphorochloridate, $^{t}$BuMgCl, anhydrous THF/pyridine, rt, 8-16 h; (*iii*) 2-propanol, reflux, 24-72 h.

To overcome the issue related to the deprotection step, **2** was directly reacted with phenyl(benzoxy-L-alaninyl)-phosphorochloridate yielding compound **42** in very low yield (5%). Alternatively, compound **2** was converted to the bis-MMT derivative **43** and subsequently reacted with the appropriate phosphorochloridate to give **44**. The deprotection step was carried out using *para*-toluene sulfonic acid affording the desired compound **45**.

**Scheme 4**




Reagents and conditions: (*i*) appropriate phosphorochloridate, $^{t}$BuMgCl, anhydrous pyridine, rt overnight; (*ii*) MMT-Cl, TEA, DMAP, anhydrous DMF, rt, 2 h; (*iii*) appropriate phosphorochloridate, $^{t}$BuMgCl, anhydrous THF, rt, overnight; (*iv*) DCM/MeOH, PTSA, rt, 2 h.

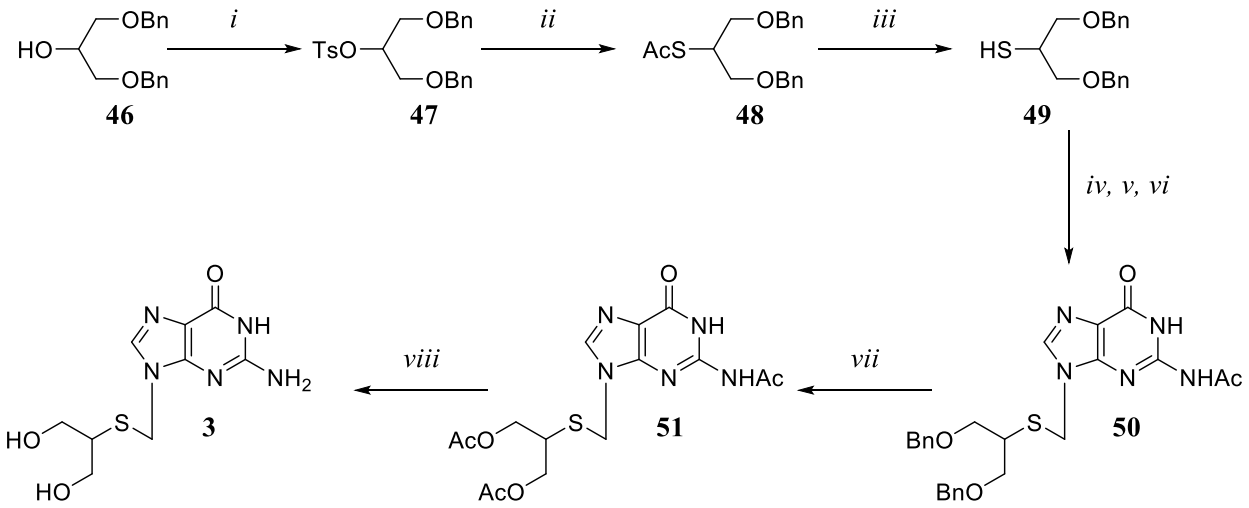
The protection of both **1** and **2** with the para-methoxytrityl group proved to be an effective approach for the synthesis of monophosphorylated derivatives, having a milder deprotection step, which led to a higher overall yield.

Synthesis of compound **3** and its ProTides.

The synthesis of **3** was performed adapting a reported procedure.[20] Compound **46**, easily made by reacting epichlorohydrin with benzyl alcohol in the presence of sodium hydride and tetra-n-butyl ammonium iodide, was reacted with tosyl chloride to give derivative **47**, which was then converted into its thio-acetyl derivative **48** by heating with potassium thioacetate in anhydrous DMF. The deprotection of the acetyl group was performed either in methanolic ammonia or using sodium methoxide in methanol affording compound **49** in comparable yield. A three-steps coupling

reaction between diacetyl-guanine and **48** gave the desired compound **50**; the N-7 isomer was also isolated during the purification step. The benzyl protecting groups were then converted into acetyl groups, using acetic anhydride and $BF_3OEt_2$ to yield **51**, which was then fully deprotected using methanolic ammonia to obtain the desired compound **3**.

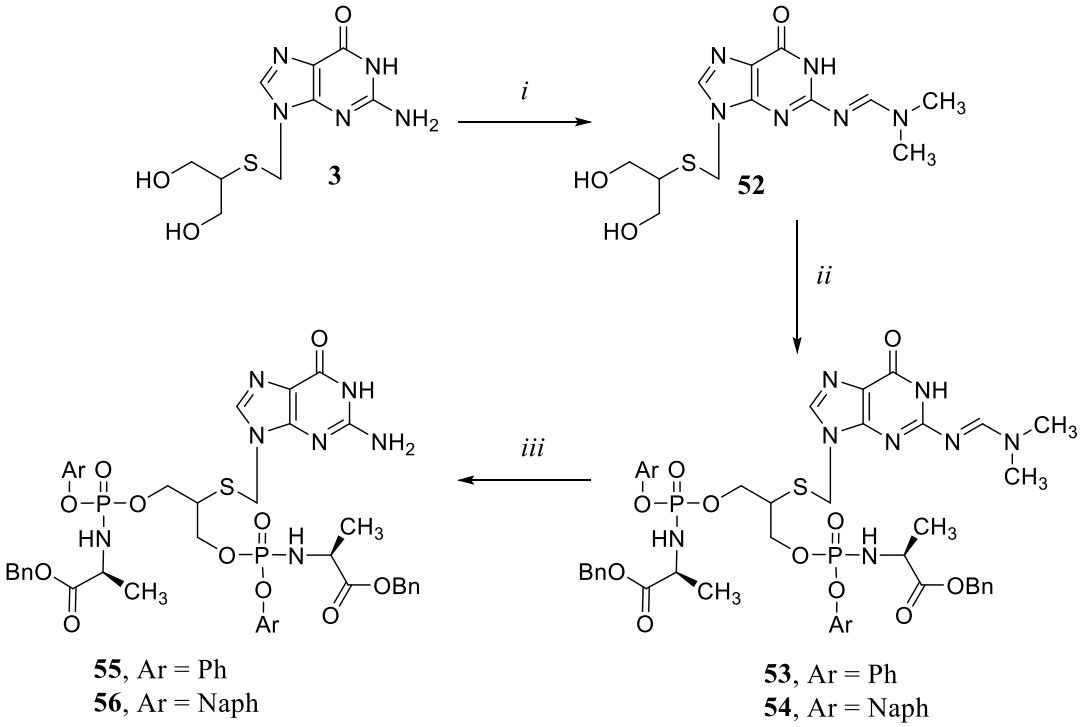
**Scheme 5**




Reagents and conditions: (*i*) *para*-toluene sulfonyl chloride, anhydrous pyridine, rt overnight; (*ii*) potassium thioacetate, anhydrous DMF, 90 °C, 2 h; (*iii*) $MeOH/NH_3$, rt, 16 h; or MeONa, anhydrous MeOH, rt, 1.5 h; (*iv*) paraformaldehyde, HCl gas, anhydrous 1,2-dichloroethane, 0 °C, 2 h; (*v*) sodium acetate, anhydrous DMF, rt, 2 h; (*vi*) diacetylguanine, bis(4-nitrophenyl)phosphate, sulfolane, 100 °C, 6 h then rt, 17 h; (*vii*) acetic anhydride, $BF_3{\cdot}OEt_2$, rt, 2.5 h; (*viii*) $MeOH/NH_3$, rt, 18 h.

Compound **3** was protected with N,N-dimethylformamide to give **52** in 88% yield followed by the coupling with the appropriate phosphorochloridate affording only the bis-phosphorylated derivatives **53** and **54**. The deprotection was only successful for compound **53** leading the desired compound **55**; whilst in the case of **54**, degradation of the starting material was observed (Scheme 6).

**Scheme 6**

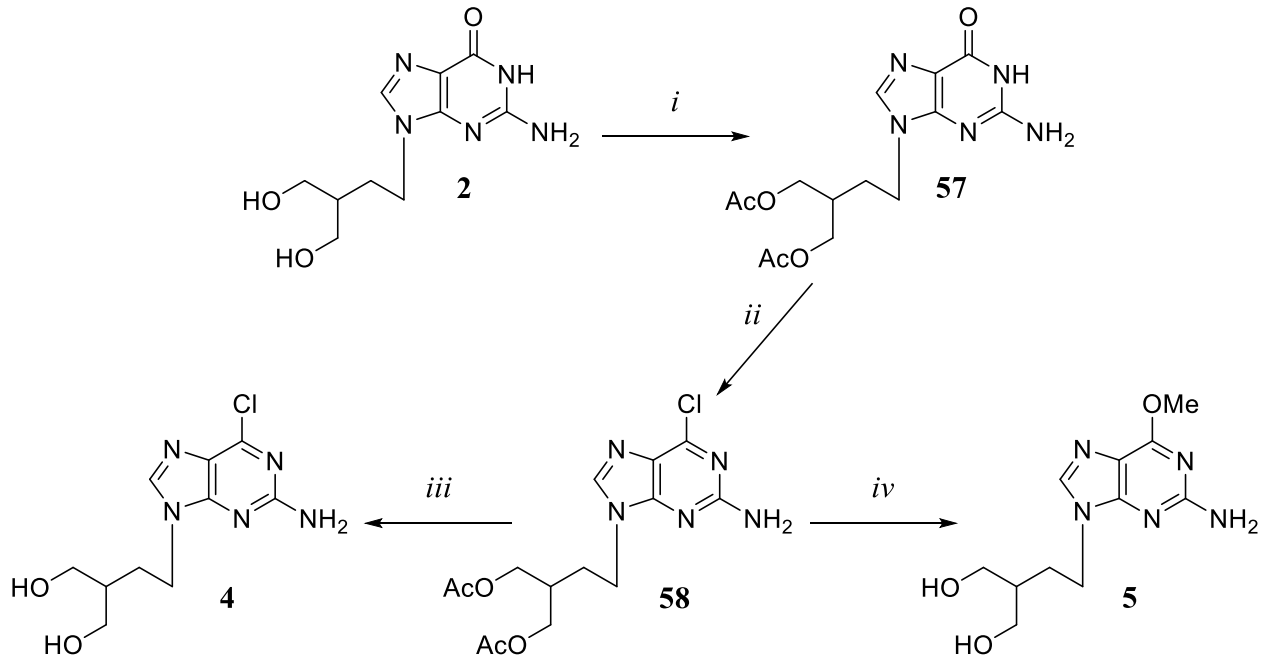



**Scheme 6**. Reagents and conditions: (*i*) dimethylformamide dimethyl acetal, anhydrous DMF, rt, overnight; (*ii*) appropriate phosphorochloridate, $^{t}$BuMgCl, anhydrous THF/pyridine, rt, 16 h; (*iii*) 2-propanol, reflux, 24-72 h.

Synthesis of nucleosides analogues **4** and **5**, and their ProTides.

Compounds **4** and **5** were prepared according to a procedure reported by Toori *et al*.[21] (Scheme 7). Compound **2** was acetylated using acetic anhydride and DMAP (cat.) to obtain **57** in good yield. Chlorination at the 6-position was achieved using $POCl_3$ in the presence of benzyltriethylammonium chloride and N,N-dimethylaniline to yield **58**. The deprotection of the acetyl group using methanolic ammonia at room temperature led only to the deprotection of the acetyl group, giving compound **4**; while the use of sodium methoxide in methanol at 40 °C afforded compound **5**.

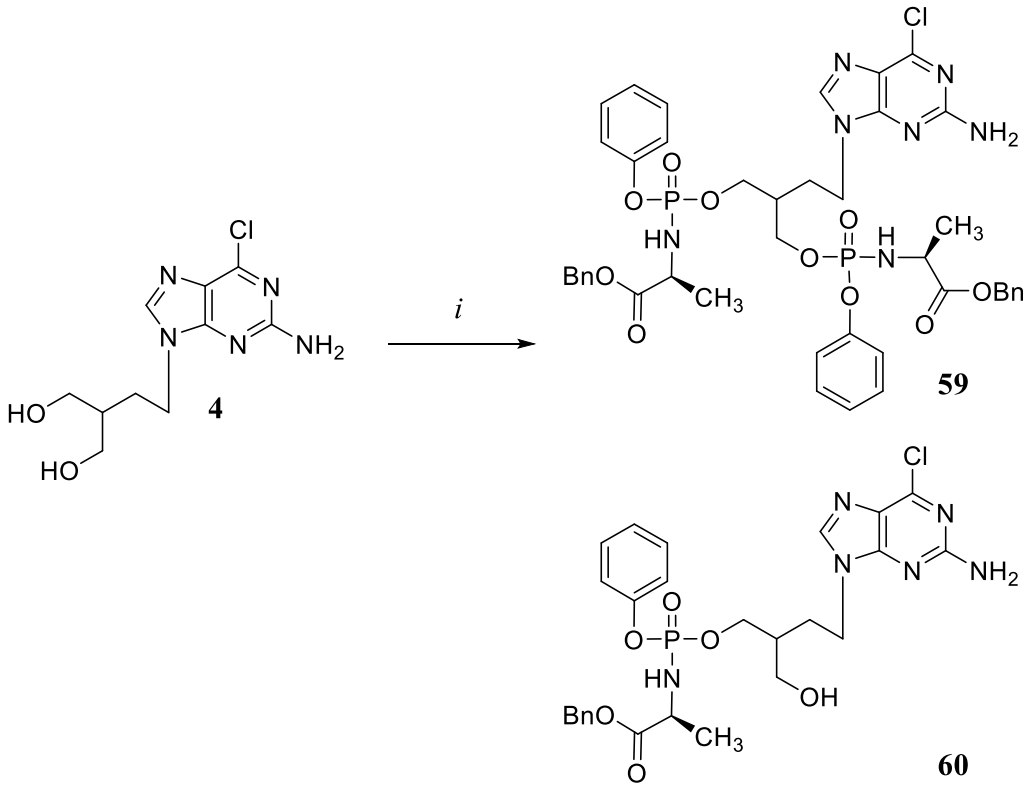
**Scheme 7**




Reagents and conditions: (*i*) acetic anhydride, DMAP, anhydrous DMF, 45 $^{o}$C, 1.5 h; (*ii*) $POCl_3$, benzyltriethylammonium chloride, *N*,*N*-dimethylaniline, anhydrous AcCN, 70 $^{o}$C, 1 h; (*iii*) MeOH/$NH_3$, rt, 6 h; (*iv*) MeONa, anhydrous MeOH, 40 $^{o}$C, 6 h.

Compounds **4** and **5** were coupled with the appropriate phosphorochloridate under the same conditions described above. The presence of the 6-chloro or 6-methoxy group greatly improved their solubility compared to the previous analogues with the subsequent improvement of their reactivity.

**Scheme 8**

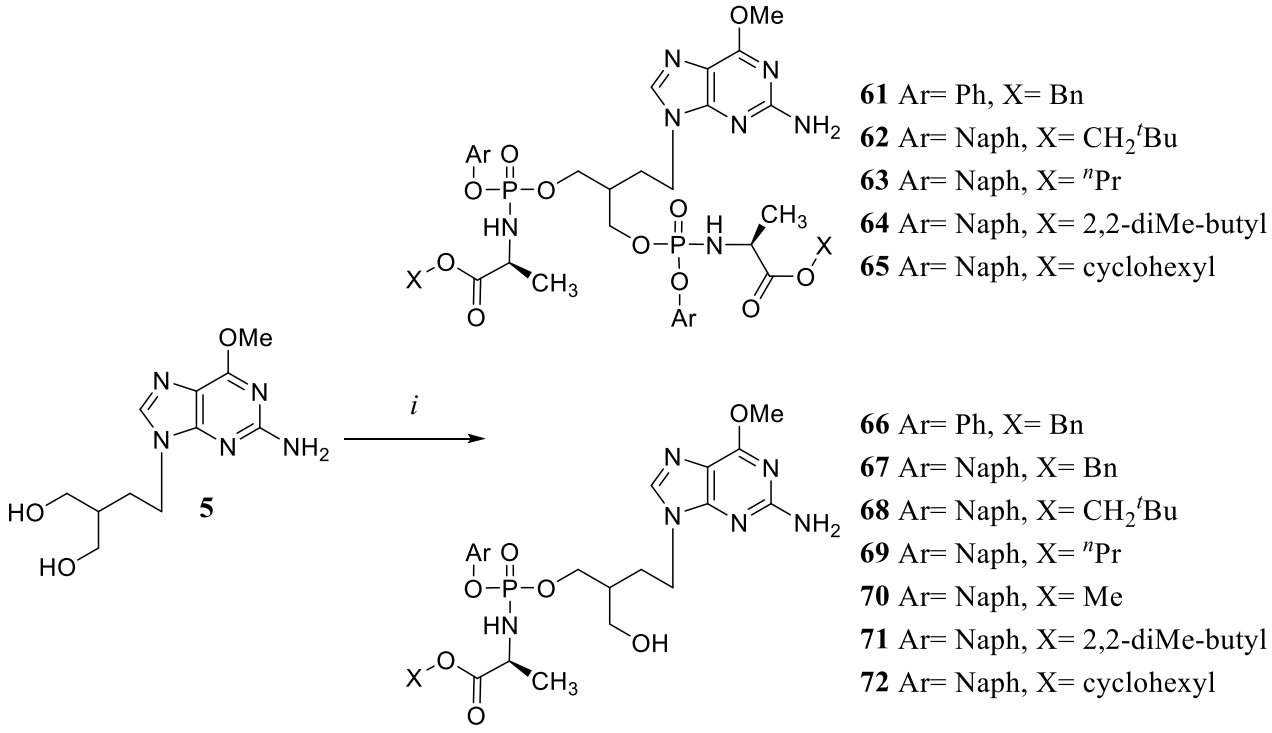



Reagents and conditions: (*i*) PhO-L-Ala-OBn phosphorochloridate, $^{t}$BuMgCl, anhydrous THF, rt, 24 h.

In the case of compound **4**, the monophosphorylated derivative **59** and the bisphosphorylated **60** were obtained in 9% and 24% yield respectively (Scheme 8).

In the case of compound **5**, given the previous success of 6-methoxy purine analogues in another antiviral program,[22] a wider range of ProTides was considered (Scheme 9). Monophosphorylated derivatives (**66-72**) were obtained most of the time together with the bisphosphorylated ProTides (**61-65**).

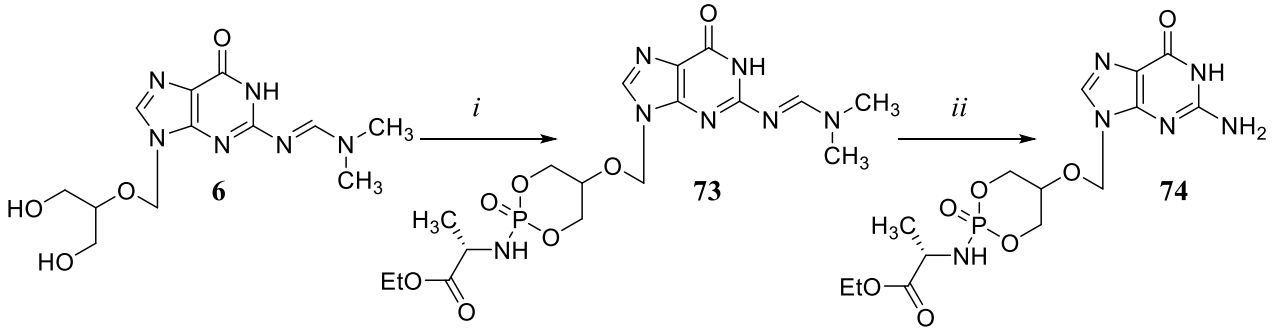
**Scheme 9**




Reagents and conditions: (*i*) appropriate phosphorochloridate, $^{t}$BuMgCl, anhydrous THF, rt, 40-48 h.

The monophosphorylated ProTides of all the parent nucleosides considered were obtained as an inseparable mixture of isomers due to the formation of a new chiral centre at the acyclic sugar side-chain.

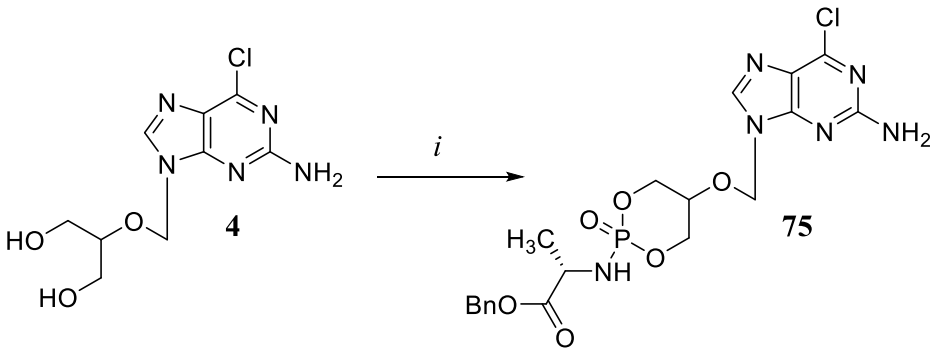
**Scheme 10**




Reagents and conditions: (*i*) Ethoxy-L-alaninyl dichlorophosphate, $^{t}$BuMgCl, anhydrous THF/pyridine, rt, 40 h; (*ii*) 2-propanol, reflux, 9 h.

To overcome this issue, the cyclic ProTides derivatives of **2** and **4** were prepared. Compound **6** was reacted with ethoxy-L-alaninyl dichlorophosphate using $^{t}$BuMgCl in THF/pyridine yielding compound **73** followed by deprotection in 2-propanol to afford compound **74** (Scheme 10).

**Scheme 11**

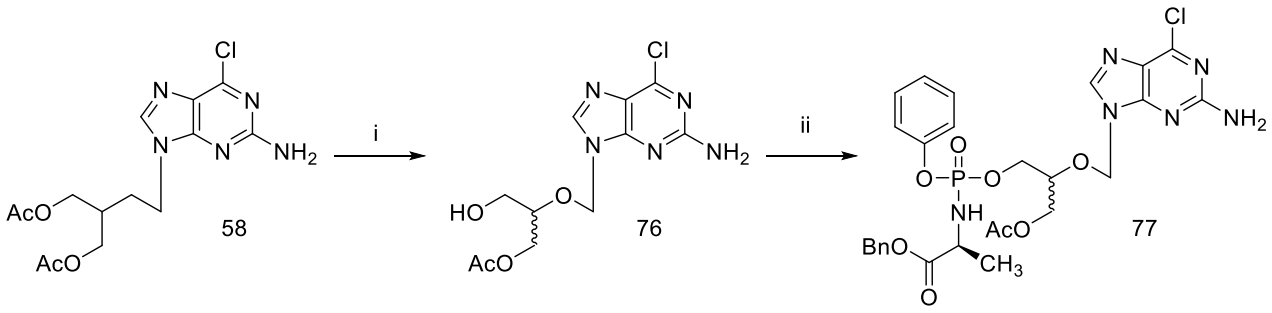



Reagents and conditions: (*i*) Benzoxy-L-alaninyl dichlorophosphate, $^{t}$BuMgCl, anhydrous THF, rt, 22 h.

Similarly, **4** was directly coupled with benzoxy-L-alaninyl phosphorochloridate providing the desired compound **75** (Scheme 11).

In an attempt to identify the formation of a metabolite in an enzymatic assay which will be discussed later, the monoacetylated derivative of **76** and its ProTide **77** were synthesised (Scheme 12). Treating **58** with ammonia gas generated *in situ* at 0 $^{\circ}$C gave the desired compound **76** in moderate yield. The synthesis of ProTide **77** was then performed using a standard procedure obtaining the desired compound as a mixture of four diasteroisomers, as clearly depicted in the corresponding $^{31}$P-NMR spectra.

**Scheme 12**




Reagents and conditions: (i) MeOH, $NH_3$ gas generated in situ, 0 $^oC$, 5.5h; (ii) phenyl-(benzoxy-L-alaninyl)-phosphochloridate, tBuMgCl, anhydrous THF, rt, 16 h.

**Biological Results**

**Anti-HIV activity**. The synthesised compounds were first tested for HIV-1 inhibition in MT-4 cell cultures (Table 1). While the parent nucleosides **1-5** were inactive against HIV-1, several of the ProTides showed anti-HIV activity, with the most potent being in the low μM range. Mono-ProTides (**16**, **20**, **22**, **27**, **28**) of compound **1** showed anti-HIV activity between 8 and >150 μM and an antiproliferative effect between 20 and >150 μM. In the case of bis-ProTides (**15**, **17**, **18**, **19**, and **21**) the anti-HIV activity varied between 8.5 and >150 μM and the antiproliferative effect between 8 and 150 $\mu$M. However, for the most active ones (i.e. **15, 17, 19** and **22**) the antiviral activity can be related to the relatively high cytotoxicity observed for these compounds. Penciclovir (**2**) mono-ProTides (**39**, **41**, **42**, and **45**) showed an anti-HIV activity in a range between 10-95 μM and cytotoxicity at ≥100 μM. The phenyl-L-alaninyl-*O*-benzyl derivative **42** was the most active in this series, with an $EC_{50}$= 10 μM and $IC_{50}$= >150 μM. Its therapeutic selectivity index (i.e. ratio $IC_{50}/EC_{50}$) was > 15. The substitution of the natural amino acid L-alanine with the non-natural amino acid dimethylglycine led to a >4-fold reduction of anti-HIV activity. As observed in the previous series, the bis-ProTides of **2** (**36-38**) and the 2-$NH_2$-DMF-protected

ProTides **30** and **31** were generally fairly cytotoxic (4-30 μM) and the antiviral activity observed may be due to the underlying cytotoxicity.

Similarly, the bis-ProTides **54** and **55** of nucleoside analogues **3** were cytotoxic (21 and 4 μM, respectively) which, as previously observed, may affect the apparent anti-HIV activity. The 2-$NH_2$-DMF protected ProTides (**30**, **31** and **54**) were tested because the HIV-RT active site showed the presence of a cleft in close proximity of the 2-amino position potentially allowing a better interaction with this enzyme.

Compound **60**, mono-ProTide of **4**, showed an anti-HIV activity in the low micromolar range (1 μM) and an $IC_{50}$ = 20 μM, while bis-ProTide **59** showed a 7-fold loss in antiviral activity while retaining cytotoxicity.

Mono-ProTides (**66**-**72**) of nucleoside **5** showed an $EC_{50}$ in a range between 1.1->150 μM, and an $IC_{50}$ in between 12-92 μM. It must be stated that the majority of the compounds showed a substantial antiproliferative effect and the observed anti-HIV activity may result from it, with the exception of compound **66**, which showed the best inhibitory activity ($EC_{50}$= 1.1 μM) as well as the best selectivity index = 21. This compound has similar biological profile as the best ACV ProTide reported in our previous study.[4] Interestingly, the substitution of the phenyl moiety with the naphthol led to a dramatic loss of activity (>20 fold), which was not observed in the previous series. Regarding the bis-ProTides (**61**-**65**), these compounds were found to be fairly cytotoxic.

The cyclic ProTides **74** and **75** were also evaluated in this study, showing an $EC_{50}$= 22 μM and $IC_{50}$= >150 μM (selectivity index > 7) for compound **74**, and $EC_{50}$= 85 μM and $IC_{50}$ of 150 μM for **75**. Acetyl protected nucleoside **76** did not show any anti-HIV activity or cytotoxicity ($EC_{50}$ and $IC_{50}$ > 150 μM), ProTide **77** showed an $EC_{50}$= 2 μM for HIV and an $IC_{50}$= 10 μM, data similar to its related compound **60**.

**Table 1.** Anti-HIV activity for synthesized ProTides and their related parent nucleosides in cell lines.

| | | | | | | | Antiviral activity $EC_{50}$[a] ($\mu$M) | Cytostatic/toxic activity ($\mu$M) | |
|---|---|---|---|---|---|---|---|---|---|
| **Cpds** | **Nucl** | **ProTide** | **Aryl** | **Amino Acid** | **Ester** | **cLogP** | **HIV-1 MT-4** | **$CC_{50}$[b] CEM** | **$IC_{50}$[c] MT-4** |
| **15** | **1** | Bis | Naph | L-Ala | $^i$Pr | 4.03 | 8.5 | >100 | 6 |
| **16** | **1** | Mono | Naph | L-Ala | $^i$Pr | 0.41 | 40 | 35±4.2 | 150 |
| **17** | **1** | Bis | Ph | L-Ala | Bn | 3.42 | 30 | 159±82 | >150 |
| **18** | **1** | Bis | Naph | L-Ala | Bn | 5.77 | ND | ND | >150 |
| **19** | **1** | Bis | Ph | DMG | Bn | 4.04 | 10 | 47 | 13 |
| **20** | **1** | Mono | Ph | DMG | Bn | 0.42 | >150 | >100 | >150 |
| **21** | **1** | Bis | Naph | DMG | Bn | 6.39 | >150 | >20 | >150 |
| **22** | **1** | Mono | Naph | DMG | Bn | 1.59 | 10 | >100 | 26 |
| **27** | **1** | Mono | Ph | L-Ala | Bn | 0.11 | 17 | ND | 20 |
| **28** | **1** | Mono | Naph | L-Ala | Bn | 1.28 | 8 | ND | 100 |
| **30**[d] | **2** | Bis | Ph | L-Ala | Bn | 4.04 | 9 | 21±0 | 10 |
| **31**[d] | **2** | Bis | Naph | L-Ala | Bn | 6.38 | 3.2 | 19±1 | 4 |
| **36** | **2** | Bis | Ph | L-Ala | Bn | 3.83 | 8 | 76±2 | 10 |
| **37** | **2** | Bis | Naph | L-Ala | Bn | 6.18 | ND | >10 | ND |
| **38** | **2** | Bis | Ph | DMG | Bn | 4.45 | 19 | 100 | 30 |
| **39** | **2** | Mono | Ph | DMG | Bn | 0.87 | 68 | 252±32 | >150 |
| **40** | **2** | Bis | Naph | DMG | Bn | 6.80 | >150 | >250 | >150 |
| **41** | **2** | Mono | Naph | DMG | Bn | 2.04 | 95 | 96±2 | 100 |
| **42** | **2** | Mono | Ph | L-Ala | Bn | 0.56 | 10 | >250 | >150 |
| **45** | **2** | Mono | Naph | L-Ala | Bn | 1.73 | 21 | 138±6 | 100 |
| **54**[d] | **3** | Bis | Naph | L-Ala | Bn | 6.14 | 9 | 17±2 | 4 |
| **55** | **3** | Bis | Ph | L-Ala | Bn | 3.59 | 24 | 77±21 | 21 |
| **59** | **4** | Bis | Ph | L-Ala | Bn | 5.54 | 7 | 14±3 | 20 |
| **60** | **4** | Mono | Ph | L-Ala | Bn | 2.27 | 1 | 33±8 | 20 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **61** | **5** | Bis | Ph | L-Ala | Bn | 5.79 | 12 | 19±7 | 20 |
| **62** | **5** | Bis | Naph | L-Ala | $CH_2$Bu | 8.43 | 8.2 | 4.6±0.6 | 5 |
| **63** | **5** | Bis | Naph | L-Ala | -Pr | 6.83 | 9.7 | 6.8±1.5 | 20 |
| **64** | **5** | Bis | Naph | L-Ala | 2,2-diMe-But | 9.49 | 5.6 | 7.2±1.9 | 9 |
| **65** | **5** | Bis | Ph | L-Ala | Cyclohexyl | 6.43 | 10 | 7.2±0.5 | 11 |
| **66** | **5** | Mono | Ph | L-Ala | Bn | 2.51 | 1.1 | 32±4 | 23 |
| **67** | **5** | Mono | Naph | L-Ala | Bn | 3.69 | 23 | 27±2 | 12 |
| **68** | **5** | Mono | Naph | L-Ala | $CH_2$Bu | 3.83 | 41 | 17±2 | 35 |
| **69** | **5** | Mono | Naph | L-Ala | -Pr | 3.03 | 11 | 17±5 | 13 |
| **70** | **5** | Mono | Naph | L-Ala | Me | 1.98 | 16 | 15±0 | 10 |
| **71** | **5** | Mono | Naph | L-Ala | 2,2-diMe-But | 4.36 | 24 | 18±2 | 17 |
| **72** | **5** | Mono | Ph | L-Ala | Cyclohexyl | 2.83 | >150 | 15±1 | 92 |
| **74** | **1** | Cyclic | - | L-Ala | Et | -0.69 | 22 | ND | >150 |
| **75** | **4** | Cyclic | | L-Ala | Bn | 1.55 | 85 | 165±90 | 150 |
| **77** | **4** | monoacetyl | Ph | L-Ala | Bn | 3.17 | 2.25 | 26±9 | 10 |
| **76** | **4** | monoacetyl | - | - | - | -0.60 | >150 | ≥250 | >150 |
| **1** | **-** | - | - | - | - | -2.54 | >50 | >100 | >50 |
| **2** | **-** | - | - | - | - | -2.72 | >150 | ND | >150 |
| **3** | **-** | - | - | - | - | -2.38 | >150 | >500 | >150 |
| **4** | **-** | - | - | - | - | -1.01 | >150 | 400±141 | >150 |
| **5** | **-** | - | - | - | - | -0.76 | >150 | 246±0 | >150 |

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

Next we tested a selection of ProTides and their respective parent nucleosides using *ex-vivo* human tonsillar tissues infected with HIV and HHV.[3] This system of histoculture offers major advantages over single cell cultures as it retains tissue cytoarchitecture and many important functional aspects of cell–cell interactions *in vivo*. In particular, cultures of human lymphoid tissue allow the testing of anti-HIV drugs upon HIV inoculation *ex vivo*. Lymphoid tissue explants support productive

HIV infection without exogenous cell activation, and retain the pattern of expression of key cell surface molecules relevant to HIV infection.[23] In this system, **1** proved to be fairly effective in inhibiting HIV at an $EC_{50}$= 5 μM. Compounds **28** and **74**, respectively the mono-ProTide and the cyclic-ProTide of **1**, showed retention of antiviral activity ($EC_{50}$= 11 and 4.6 μM, respectively) demonstrating that the delivery of the monophosphate form of **1** through a ProTide is as efficient as the phosphorylation mediated by the HHV-TK present in the tissue cultures. **2** displayed an $EC_{50}$= 11 μM *versus* HIV-1, result expected since this compound is a well-known anti-HSV drug and as such it is an efficient substrate for the HHV-thymidine kinase. This activity was also in accordance with the results obtained for its ProTide **42** in the MT4 assay ($EC_{50}$= 10 μM). An important result was obtained for compound **3** which lacked any anti-HIV activity ($EC_{50}$> 30 μM) in this assay, although it functions as a submicromolar anti-HSV compound (as reported in Table 3). Similar results were obtained for its bis-ProTide **55**, which did not inhibit HIV either. We can speculate that **3**, despite being a very close analogue of 1, is a poor substrate for HIV-RT and the inhibitory activity found in the MT-4 cell assay for **54** and **55** was just due to the underlying toxicity of these compounds.

Both compounds **4** and **5** were not effective in this assay ($EC_{50}$> 60 μM and > 30 μM, respectively), while their phosphate prodrugs were able to inhibit HIV in a low micromolar range: $EC_{50}$= 4 μM for mono-ProTide **60** and $EC_{50}$= 9 μM for cyclic ProTide **74** (both derivatives of nucleoside analogue **4**); and $EC_{50}$= 0.9 μM for **66** (mono-ProTide of **5**). The lack of activity reported for **4** and **5** is probably related to their poor conversion to the monophosphate form by TK-HHV. This assumption is supported by the lack of anti-HSV activity of these compounds (Table 3). Similar result was obtained for the monoacetylated derivative **76** ($EC_{50}$>10 μM), while its ProTide **77** showed an $EC_{50}$= 4 μM.

**Table 2.** Inhibitory Activity of ProTides against HIV-1 in human tonsillar tissues ex vivo.

| | | | | | | |
|---|---|---|---|---|---|---|
| **Cps** | **Nucl** | **ProTide** | **Aryl** | **Amino Acid** | **Ester** | **Anti-HIV Activity $EC_{50}$[a] (μM)** |
| **28** | **1** | Mono | Naph | L-Ala | Bn | 11 |
| **42** | **2** | Mono | Ph | L-Ala | Bn | >20 |
| **55** | **3** | Bis | Ph | L-Ala | Bn | >3 |
| **60** | **4** | Mono | Ph | L-Ala | Bn | 4 |
| **66** | **5** | Mono | Ph | L-Ala | Bn | 0.9 |
| **74** | **1** | Cyclic | - | L-Ala | Et | 4.6 |
| **75** | **4** | Cyclic | - | L-Ala | Bn | 9 |
| **77** | **MonoAcetyl 4** | Mono | Ph | L-Ala | Bn | 4 |
| **1** | **-** | - | - | - | - | 5 |
| **2** | **-** | - | - | - | - | 11 |
| **3** | **-** | - | - | - | - | >30 |
| **4** | **-** | - | - | - | - | >60 |
| **5** | **-** | - | - | - | - | >30 |
| **76** | **MonoAcetyl 4** | - | - | - | - | >10 |

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

**Anti-HSV activity.** The synthesized compounds were also evaluated for their ability to inhibit HSV-1, HSV-2, and a TK-deficient ($TK^-$) HSV-1 strain (ACV-resistant) in confluent virus-infected human embryonic lung fibroblasts (HEL cultures) (Table 3). Parent compound **1** showed antiviral activity ($EC_{50}$) of 0.03 μM, 0.04 μM and 4.3 μM against HSV-1, HSV-2 and HSV-1 $TK^-$, respectively. Similar to the anti-HIV activity in tissues *ex vivo*, its ProTides were found to be slightly less active compared to the parent nucleoside (with an activity against the HSV-1 KOS strain in the range of 0.09-2 μM for the mono-ProTides **16**, **20**, **22**, **27** and **28**, and in the range of 0.3-3 μM for the bis-ProTides **15**, **17**, **19** and **21**). Similar activity was obtained against HSV-2 with an $EC_{50}$ in the range of 0.3-3 μM for the mono-ProTides, and 0.9-2.5 μM for the bis-ProTides. As expected, while the parent nucleoside **1** showed a loss of activity of ~140-fold against HSV-1 $TK^-$, some of the ProTides (mono- and bis-) showed a good retention of the antiviral activity against this strain. In particular, compounds **18**, **19**, **20** and **22** showed a full retention of antiviral activity (<2.5 fold difference in $EC_{50}$ values between the HSV-1 $TK^+$ and $TK^-$ strains). While compounds **17**, **21** and **28** still show considerable activity against these two HSV-1 strains, compounds **15** and **16** displayed marginal activity against the $TK^-$ mutant virus. No cytotoxicity was observed for any of these compounds (MCC> 100 μM).

In spite of being at least 15-fold (for HSV-1) and 30-fold (for HSV-2) less potent than the parent nucleoside, the majority of these compounds were found as active as ACV, the current drug of choice for HSV infections.

Mono-ProTides **39**, **41**, **42**, and **45** showed a loss of antiherpes activity of ~16- to 150-fold against wild-type HSV-1, and ~27- to 132-fold against HSV-2 compared to the

parent nucleoside **2** ($EC_{50}$= 0.5, 0.6 and 4 μM against HSV-1, HSV-2 and HSV-1 $TK^-$ strain, respectively). In addition, no activity was found for the mono-ProTides of **2** against the HSV-1 $TK^-$ strain. Contrary to the results observed for derivatives of **1**, bis-ProTides of **2** (compounds **30**, **31**, **37**, **38**, and **40**) were found to be inactive against the three HSV strains. Similarly, compounds **54** and **55** (i.e. bis-ProTides of **3**) were found inactive ($EC_{50}$= >100 μM) against HSV-1 and HSV-2, while the parent compound **3** showed a sub-micromolar activity.

Compound **5** did not show any anti-HSV activity ($EC_{50}$>100 μM). Its mono-ProTide derivatives, with the exception of compound **70**, which showed a good to moderate inhibitory activity ($EC_{50}$= 9-38 μM and $EC_{50}$= 9-20 μM against HSV-1 and HSV-2, respectively), with retention of some activity against the $TK^-$ strain for compounds **66**, **67**, **68**, and **72**. Compound **68**, bearing the naphthyl as an aryl group, L-alanine as an amino acid and neopentyl as an ester, showed the best antiviral profile with an $EC_{50}$ of 9 μM, 9 μM and 15 μM against HSV-1, HSV-2 and HSV-1 $TK^-$, respectively. In this series, as already observed for derivatives of **2** and **3**, the bis-ProTides **61**, **62**, **63**, **64**, and **65** were found totally inactive ($EC_{50}$>100 μM).

**Table 3.** Anti-HSV activity of the synthesized ProTides and their corresponding parent nucleosides.

| | | | | | | Antiviral Activity $EC_{50}$ (μM)[a] | | | Cytotoxicity (μM) |
|---|---|---|---|---|---|---|---|---|---|
| **Cps** | **Nucl** | **ProTide** | **Aryl** | **Amino Acid** | **Ester** | **HSV-1 (KOS)** | **HSV-2 (G)** | **HSV-1 TK⁻ (KOS)** | **MCC[b]** |
| **15** | **1** | Bis | Naph | L-Ala | $^{i}$Pr | 1.2 ± 1.1 | 1.4 ± 0.8 | ≥73 ± 39 | ≥100 |
| **16** | **1** | Mono | Naph | L-Ala | $^{i}$Pr | 2 ± 0 | 3.0 ± 1.4 | ≥75 ± 35 | >100 |
| **17** | **1** | Bis | Ph | L-Ala | Bn | 3.0 ± 1.4 | 2.5 ± 2.1 | 14.5 ± 7.8 | >100 |
| **18** | **1** | Bis | Naph | L-Ala | Bn | 1.3 ± 1.1 | 2.5 ± 2.1 | 2.5 ± 2.1 | >100 |
| **19** | **1** | Bis | Ph | DMG | Bn | 1.5 ± 0.8 | 2.5 ± 2.1 | 1.5 ± 0.7 | ND |
| **20** | **1** | Mono | Ph | DMG | Bn | 0.09 ± 0.02 | 0.30 ± 0.28 | 0.15 ± 0.07 | >100 |
| **21** | **1** | Bis | Naph | DMG | Bn | 0.30 ± 0.28 | 0.90 ± 0.14 | 1.6 ± 0.6 | >100 |
| **22** | **1** | Mono | Naph | DMG | Bn | 0.60 ± 0.28 | 0.85 ± 0.07 | 1.4 ± 0.8 | >100 |
| **27** | **1** | Mono | Ph | L-Ala | Bn | 0.6 ± 0.4 | 3.0 ± 1.4 | 3.0 ± 1.4 | >100 |
| **28** | **1** | Mono | Naph | L-Ala | Bn | 0.4 ± 0 | 0.5 ± 0.1 | 3.0 ±1.4 | >100 |
| **30[c]** | **2** | Bis | Ph | L-Ala | Bn | >100 | >100 | >100 | >100 |
| **31[c]** | **2** | Bis | Naph | L-Ala | Bn | >100 | >100 | >100 | >100 |
| **36** | **2** | Bis | Ph | L-Ala | Bn | >100 | >100 | >100 | >100 |
| **37** | **2** | Bis | Naph | L-Ala | Bn | >100 | >100 | >100 | >100 |
| **38** | **2** | Bis | Ph | DMG | Bn | >100 | >100 | >100 | >100 |
| **39** | **2** | Mono | Ph | DMG | Bn | 29 ± 13 | 39 ± 27 | 100 ± 0 | >100 |
| **40** | **2** | Bis | Naph | DMG | Bn | >20 | >20 | >20 | 100 |
| **41** | **2** | Mono | Naph | DMG | Bn | 75 ± 35 | 52 ± 9 | >100 ± 0 | >100 |
| **42** | **2** | Mono | Ph | L-Ala | Bn | 8.0 ± 6.0 | 16 ± 6.0 | ≥100 | >100 |
| **45** | **2** | Mono | Naph | L-Ala | Bn | 73 ± 39 | 79 ± 30 | >100 ± 0 | >100 |
| **54[c]** | **3** | Bis | Naph | L-Ala | Bn | >100 | >100 | >100 | ≥100 |
| **55** | **3** | Bis | Ph | L-Ala | Bn | >100 | >100 | >100 | ≥100 |
| **59** | **4** | Bis | Ph | L-Ala | Bn | >100 | >100 | >100 | >100 |
| **60** | **4** | Mono | Ph | L-Ala | >100 | >100 | >100 | >100 | >100 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **61** | **5** | Bis | Ph | L-Ala | Bn | 100 | >100 | 100 | >100 |
| **62** | **5** | Bis | Naph | L-Ala | $CH_2tBu$ | >100 | >100 | >100 | ≥100 |
| **63** | **5** | Bis | Naph | L-Ala | iPr | >100 | >100 | >100 | ≥100 |
| **64** | **5** | Bis | Naph | L-Ala | 2,2-diMe-But | >100 | >100 | >100 | ≥100 |
| **65** | **5** | Bis | Ph | L-Ala | Cyclohexyl | >100 | >100 | >100 | >100 |
| **66** | **5** | Mono | Ph | L-Ala | Bn | 39 ± 27 | 29 ± 23 | 39 ± 27 | >100 |
| **67** | **5** | Mono | Naph | L-Ala | Bn | 38 | 20 | 20 | >100 |
| **68** | **5** | Mono | Naph | L-Ala | $CH_2tBu$ | 9.0 ± 1.4 | 9.0 ± 0 | 15 ± 7 | >100 |
| **69** | **5** | Mono | Naph | L-Ala | iPr | 14 ± 2 | 15 ± 8 | ≥20 | 100 |
| **70** | **5** | Mono | Naph | L-Ala | Me | >100 | >100 | >100 | ≥100 |
| **71** | **5** | Mono | Naph | L-Ala | 2,2-diMe-But | 11 ± 2 | 10 ± 1 | ≥20 | 100 |
| **72** | **5** | Mono | Ph | L-Ala | Cyclohexyl | 16 ± 6 | 16 ± 6 | 40 ± 28 | ≥100 |
| **74** | **1** | Cyclic | - | L-Ala | Et | ND | ND | ND | ND |
| **75** | **4** | Cyclic | - | L-Ala | Bn | >100 | >100 | >100 | >100 |
| **ACV** | **-** | - | - | - | - | 0.4 ± 0.5 | 0.4 ± 0.3 | 60 ± 82 | >250 |
| **1** | **-** | - | - | - | - | 0.03 ± 0.03 | 0.04 ± 0.03 | 4.3 ± 6.4 | >100 |
| **2** | **-** | - | - | - | - | 0.5 ± 0.2 | 0.6 ± 0.3 | 4.0 | >100 |
| **3** | **-** | - | - | - | - | 0.4 ± 0.1 | 0.5 ± 0.1 | 1.6 ± 1.1 | >100 |
| **4** | **-** | - | - | - | - | >100 | >100 | >100 | >100 |
| **5** | **-** | - | - | - | - | >100 | >100 | >100 | >100 |

[a]50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%. [b]Minimum cytotoxic concentration, or compound concentration required to cause a microscopically detectable alteration of normal cell morphology. [c]$N_2$-DMF-protected. ND = not determined.

**Anti-HCMV activity.** The synthesized compounds were also evaluated for their ability to inhibit two strains (AD-169 and Davis) of HCMV in confluent HEL cell cultures (Table 4). Compound **1** showed an $EC_{50}$ of 7.4 μM (AD-169 strain) and 7.3 μM (Davis strain); its mono-ProTides showed an $EC_{50}$ in a range between 6.6->50 μM (AD-169 strain) and in a range between 4.9-24 μM (Davis strain); the bis-ProTides showed a loss

of activity compared to the mono-ProTides. Compounds **22**, **27** and **28** were the most active of this series with $EC_{50}$'s comparable to that of the parent nucleoside. Compounds **2** and **3** and their ProTides showed poor, if any activity against HCMV ($EC_{50}$≥ 50 μM). The 6-methoxy-PCV **5** did not show any activity against HCMV ($EC_{50}$> 50 μM), while some mono- and bis-ProTides showed a moderate anti-HCMV activity, which, however, could be related to the cytotoxicity of these compounds ($CC_{50}$ 's in the range of 1.6 to ≥83 μM).

**Anti-VZV activity.** A biological evaluation against two strains (OKA and 07-1 $TK^-$) of VZV was also performed in monolayer HEL cell cultures (Table 4). Compounds **20** and **27** emerged as the most potent mono-ProTides of **1** with a high antiviral activity against VZV in the low μM range for both strains ($EC_{50}$= 7.3 μM against the $TK^+$ VZV strain Oka) and excellent retention of activity against the $TK^-$ VZV strain 07-1 ($EC_{50}$= 3.2-3.3 μM). These compounds were slightly less active than the current drug treatment of choice (ACV) against the $TK^+$-OKA strain. However, they showed to be at least ~20 fold more active against the ACV-resistant VZV $TK^-$ 07-1 strain.

Both mono- and bis-ProTides of **2** showed at least a 10-fold loss of anti-VZV activity compared to the parent compound and proved also poorly effective against the TK-deficient VZV strain. Similarly, poor activity was also found for ProTides **54** and **55**. None of the mono- and bis-ProTides of **4** and **5** displayed significant anti-VZV activity at non-toxic concentrations.

**Table 4.** Anti-HCMV and –VZV activity of the synthesized ProTides and their corresponding parent nucleosides

| | | | | | | Antiviral Activity $EC_{50}$ (μM)[a] | | | | Cytotoxicity (μM) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cpds** | **Nucl** | **ProTide** | **Aryl** | **Amino Acid** | **Ester** | **HCMV (AD-169)** | **HCMV (Davis)** | **TK+ VZV (OKA)** | **TK- VZV (07-1)** | **$CC_{50}$[b]** | **MCC[c]** |
| **15** | **1** | Bis | Naph | L-Ala | $^{i}$Pr | 16.3 ± 5.2 | 2.7 ± 1.5 | 4.2 ± 1.9 | 1.1 ± 0.4 | >50 | >100 |
| **16** | **1** | Mono | Naph | L-Ala | $^{i}$Pr | >50 ± 0 | 6.8 ± 4.6 | ≥23.5 ± 4.9 | ≥10.8 ± 13 | >50 | >100 |
| **17** | **1** | Bis | Ph | L-Ala | Bn | 44.7 | N.D. | 23.3 | 21.3 | >100 | >100 |
| **18** | **1** | Bis | Naph | L-Ala | Bn | 27.4 ± 10.4 | 11.2 ± 0.3 | 6.2 ± 1.0 | 4.1 ± 0.7 | >100 | >50 |
| **19** | **1** | Bis | Ph | DMG | Bn | 50 ± 0 | 23.6 ± 11.4 | 21.7 ± 7.7 | 8.3 ± 3.2 | 82 ± 6 | >50 |
| **20** | **1** | Mono | Ph | DMG | Bn | 38.6 ± 16.2 | 15.8 ± 6.0 | 7.3 ± 0.7 | 3.3 ± 1.4 | >100 | >50 |
| **21** | **1** | Bis | Naph | DMG | Bn | ≥44.9 ± 7.2 | 50 | 13.6 ± 4.5 | 7.2 ± 2.1 | >100 | >50 |
| **22** | **1** | Mono | Naph | DMG | Bn | 6.6 ± 4.8 | 4.9 ± 3.1 | 13.0 ± 5.3 | >50 | >100 | >50 |
| **27** | **1** | Mono | Ph | L-Ala | Bn | 7.9 ± 1.5 | 5.0 ± 1.4 | 7.3 ± 4.3 | 3.2 ± 1.4 | ≥81 ± 27 | >100 |
| **28** | **1** | Mono | Naph | L-Ala | Bn | 9.5 ± 3.1 | 6.5 ± 3.5 | 9.5 | 28.2 | N.D. | >100 |
| **30**[d] | **2** | Bis | Ph | L-Ala | Bn | >50 | >50 | 29.8 ± 3.5 | 28.6 ± 1.1 | 47 ± 3 | >50 |
| **31**[d] | **2** | Bis | Naph | L-Ala | Bn | >20 | >20 | 8.8 ± 2.9 | 11.7 ± 4.3 | 19 ± 1 | 50 |
| **36** | **2** | Bis | Ph | L-Ala | Bn | >50 | >50 | 16.3 ± 10.5 | 41.5 ± 12.0 | ≥100 | >50 |
| **37** | **2** | Bis | Naph | L-Ala | Bn | >50 | >50 | 13.5 ± 5.1 | >50 ± 0 | >100 | >50 |
| **38** | **2** | Bis | Ph | DMG | Bn | >50 | >50 | 10.3 ± 2.5 | 20.6 ± 9.5 | >100 | >50 |
| **39** | **2** | Mono | Ph | DMG | Bn | >50 | >50 | >50 | >50 | >50 | >50 |
| **40** | **2** | Bis | Naph | DMG | Bn | >50 | >50 | >50 | >50 | >100 | >50 |
| **41** | **2** | Mono | Naph | DMG | Bn | >50 | >50 | 20.9 ± 3.5 | >50 ± 0 | >100 | >50 |
| **42** | **2** | Mono | Ph | L-Ala | Bn | >50 | >50 | 2.6 ± 0.4 | 45.0 ± 7.1 | >100 | >50 |
| **45** | **2** | Mono | Naph | L-Ala | Bn | >50 | >50 | 9.3 ± 1.0 | >50 | >100 | >50 |
| **54**[d] | **3** | Bis | Naph | L-Ala | Bn | >50 | >50 | >20 | >20 | 30 | >50 |
| **55** | **3** | Bis | Ph | L-Ala | Bn | >50 | >50 | 23 | >50 | >100 | >50 |
| **59** | **4** | Bis | Ph | L-Ala | Bn | >100 | >100 | 44.0 ± 4.3 | 44.1 ± 2.7 | 4.0 ± 0 | >100 |
| **60** | **4** | Mono | Ph | L-Ala | Bn | >100 | >100 | 37.3 ± 8.8 | 42.9 ± 0.9 | 39 ± 2 | >100 |
| **61** | **5** | Bis | Ph | L-Ala | Bn | >50 | 37 | 22 | 20 | 25 | >50 |
| **62** | **5** | Bis | Naph | L-Ala | $CH_2$Bu | 15 | 20 | 4.3 | 7.7 | 2.5 | ≥20 |
| **63** | **5** | Bis | Naph | L-Ala | $^{n}$Pr | 32 | 40 | 6.1 | 8.1 | 4.9 | ≥50 |

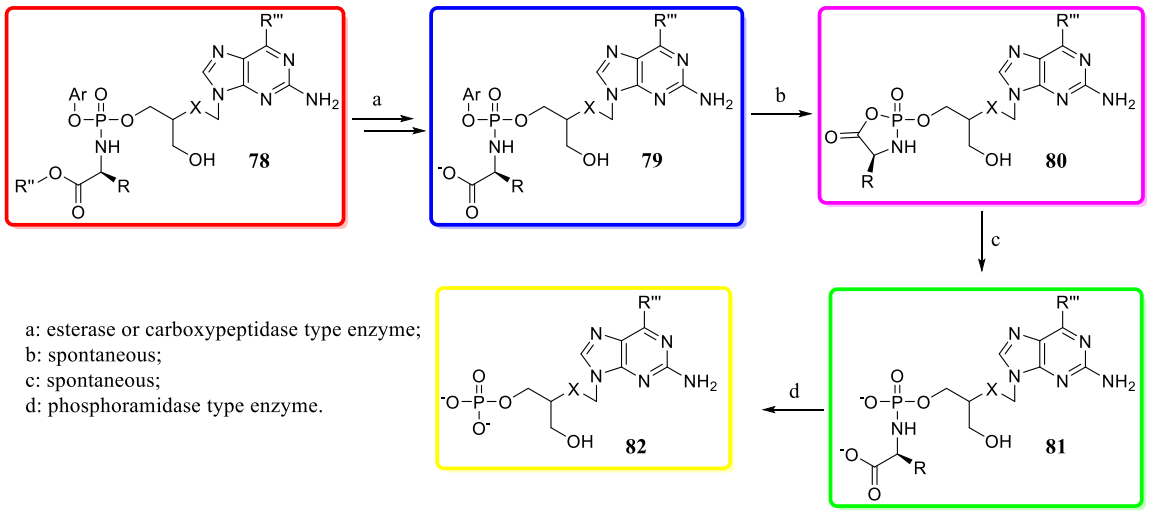
| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **64** | **5** | Bis | Naph | L-Ala | 2,2-diMe-But | >20 | >20 | 6.4 | 9.5 | 5.0 | 50 |
| **65** | **5** | Bis | Ph | L-Ala | Cyclohexyl | >20 | >20 | >5 | >5 | 1.6 | 50 |
| **66** | **5** | Mono | Ph | L-Ala | Bn | 31.6 ± 0 | 7.9 ± 1.1 | 17.1 ± 11.0 | 22.2 ± 5.7 | ≥83 ± 25 | >50 |
| **67** | **5** | Mono | Naph | L-Ala | Bn | >50 | 50 | 17 | 23 | 49 | >50 |
| **68** | **5** | Mono | Naph | L-Ala | $CH_2$Bu | 50 | 40 | 2.0 | 6.2 | 7.6 | >50 |
| **69** | **5** | Mono | Naph | L-Ala | $^n$Pr | 32 | 50 | 3.7 | 9.1 | 14.4 | ≥50 |
| **70** | **5** | Mono | Naph | L-Ala | Me | 32 | 14 | 13 | 25 | 37 | ≥50 |
| **71** | **5** | Mono | Naph | L-Ala | 2,2-diMe-But | 43 | >50 | 4.4 | 9.7 | 28 | ≥50 |
| **72** | **5** | Mono | Ph | L-Ala | Cyclohexyl | 32 | 20 | 5.0 | 9.4 | 7.4 | >50 |
| **74** | **1** | Cyclic | - | L-Ala | Et | N.D | N.D | N.D | N.D | N.D | N.D |
| **75** | **4** | Cyclic | - | L-Ala | Bn | N.D | N.D | N.D | N.D | N.D | N.D |
| **ACV** | **-** | - | - | - | - | ND | ND | 1.88 ± 0.99 | 73.0 ± 36.5 | 787 ± 367 | >400 |
| **1** | **-** | - | - | - | - | 7.4 ±2.1 | 7.3 ± 3.8 | N.D. | N.D. | 273 ± 121 | ≥350 |
| **2** | **-** | - | - | - | - | >50 | >50 | 0.62 ± 0.53 | 10.2 ± 2.8 | >100 | >100 |
| **3** | **-** | - | - | - | - | >50 | 40.8 ± 13.0 | 6.9 ± 2.2 | >50 | >100 | >50 |
| **4** | **-** | - | - | - | - | >50 | >50 | >50 | >50 | >100 | >50 |
| **5** | **-** | - | - | - | - | >50 | >50 | 22.8 ± 0 | >50 | >100 | >50 |

[a]50% Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%. [b]50% Cytostatic concentration, or compound concentration required to decrease the viability of the cell cultures by 50%. [c]Minimum cytotoxic concentration, or compound concentration required to cause a microscopically detectable alteration of normal cell morphology. [d]$N_2$-DMF-protected. ND = not determined.

**Enzymatic Studies: mechanism of bioactivation of ProTides**

It has earlier been observed that ProTides often enhance both solubility and permeability.[22] Therefore, the loss of biological activity of several of the compounds, might be related to other issues, including bioactivation. The putative mechanism of bioactivation of the phosphoramidate ProTide moiety involves two subsequent enzymatic steps (Scheme 13). The ester moiety of a general ProTide depicted as **78** (red) is cleaved by an esterase- or carboxypeptidase-type enzyme to give compound **79** (blue). The negatively charged oxygen attacks the phosphorous with the release of the aryl moiety forming the unstable mixed anhydride **80** (magenta), which is consequently opened by the water yielding compound **81** (green). The phosphorous-nitrogen bond of **81** is then cleaved by a phosphoramidase-type enzyme to release the monophosphate form **82** (yellow).

**Scheme 13.** Putative mechanism of bioactivation of ProTides.




The first bioactivation step for a selection of mono- and bis- ProTides as well as cyclic derivative **75** was investigated. This experiment involved the incubation of the appropriate compound with carboxypeptidase Y using $^{31}$P-NMR to follow the metabolism

of the starting material. For all the experiments a $^{31}$P-NMR of the starting material was registered prior to the addition of the enzyme, and then $^{31}$P-NMR spectra were registered every 5-10 min for a minimum period of 14 h. This experiment allowed understanding whether any lack of antiviral activity was due to a poor activation of the ProTide moiety with the subsequent low conversion to the monophosphate form.

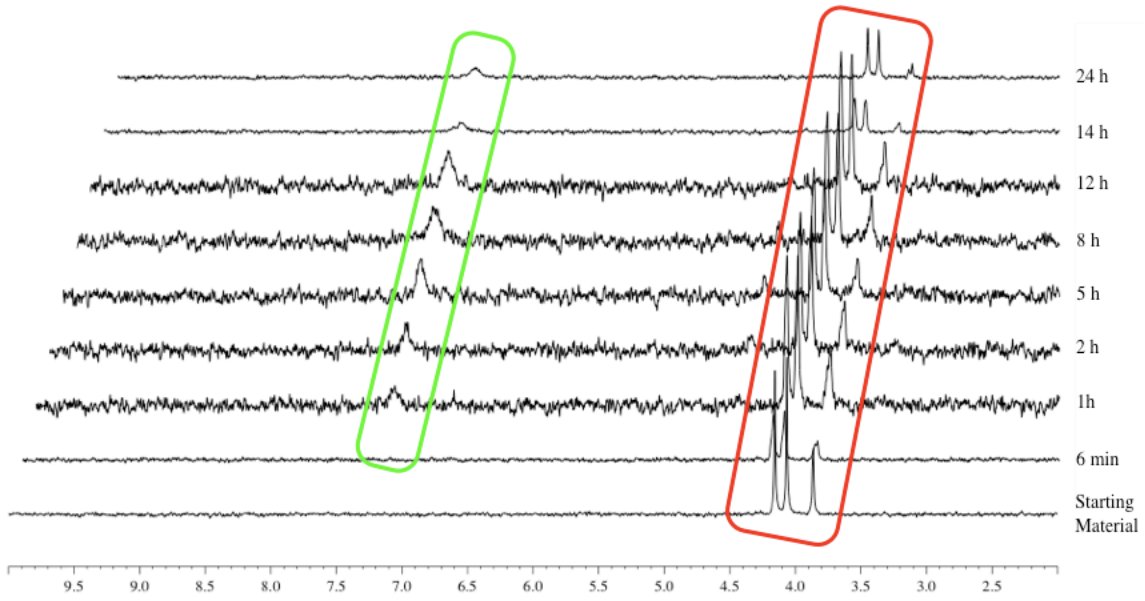



**Figure 6.** Carboxypeptidase-mediated cleavage of compound **16**, monitored by $^{31}$P-NMR.

Compound **16** (Figure 6, red) showed a slow and incomplete conversion to the metabolite **81** ($\delta_P$ = 7 ppm, green), which appeared within 1 h of incubation with the enzyme. After 24 h, the starting compound **16** was still well-detected. Therefore, a partial conversion of the starting material into the first metabolite could be noticed.

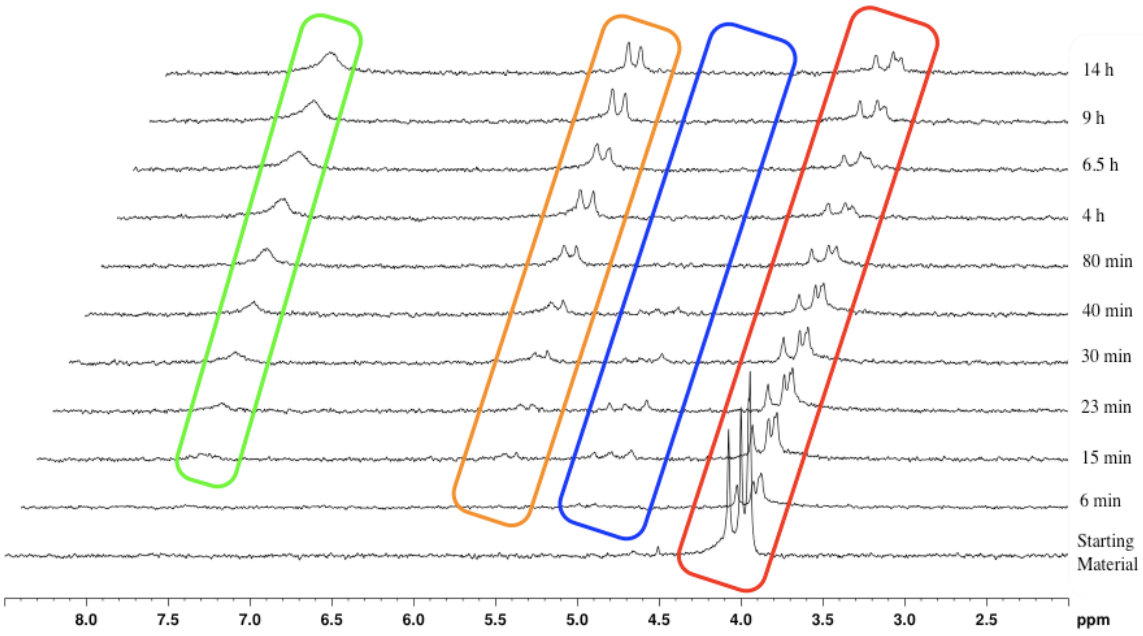



**Figure 7.** Carboxypeptidase-mediated cleavage of compound **45**, monitored by $^{31}$P-NMR.

Also for compound **45** (Figure 7), a slow and incomplete conversion of the starting material (red) to the metabolite **81** ($\delta_P$ = 7.5 ppm, green) was observed. The formation of the intermediate metabolite **79** ($\delta_P$ = 4.16, 4.08, 4.00 ppm, blue) was observed and its disappearance indicated its conversion to the desired metabolite **81**. Interestingly, the formation of an unknown species ($\delta_P$ = 5.67, 5.59 ppm, orange) was observed after 15 min and constantly increased with time.

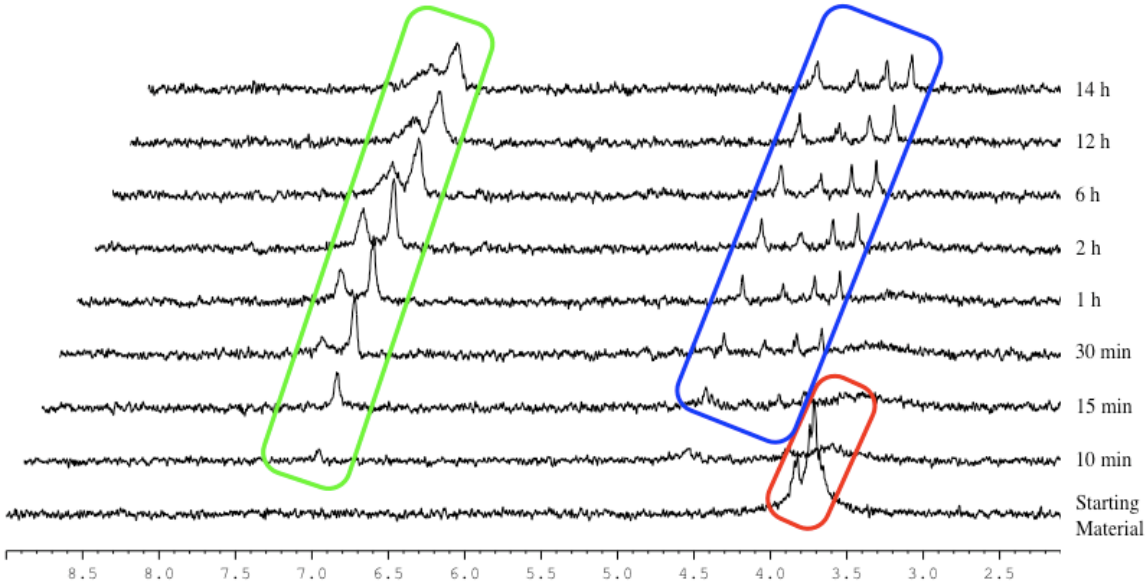



**Figure 8.** Carboxypeptidase-mediated cleavage of compound **63**, monitored by $^{31}$P-NMR.

Compound **63** (Figure 8, red), as an example of a bis-ProTide, showed a fast conversion to the first metabolite **79** (blue), which was then partially converted to the second metabolite **81** (green). Notably, the presence of two peaks ($\delta_P$ = 7.00, 7.20 ppm) reflect the presence of two phosphorus groups.

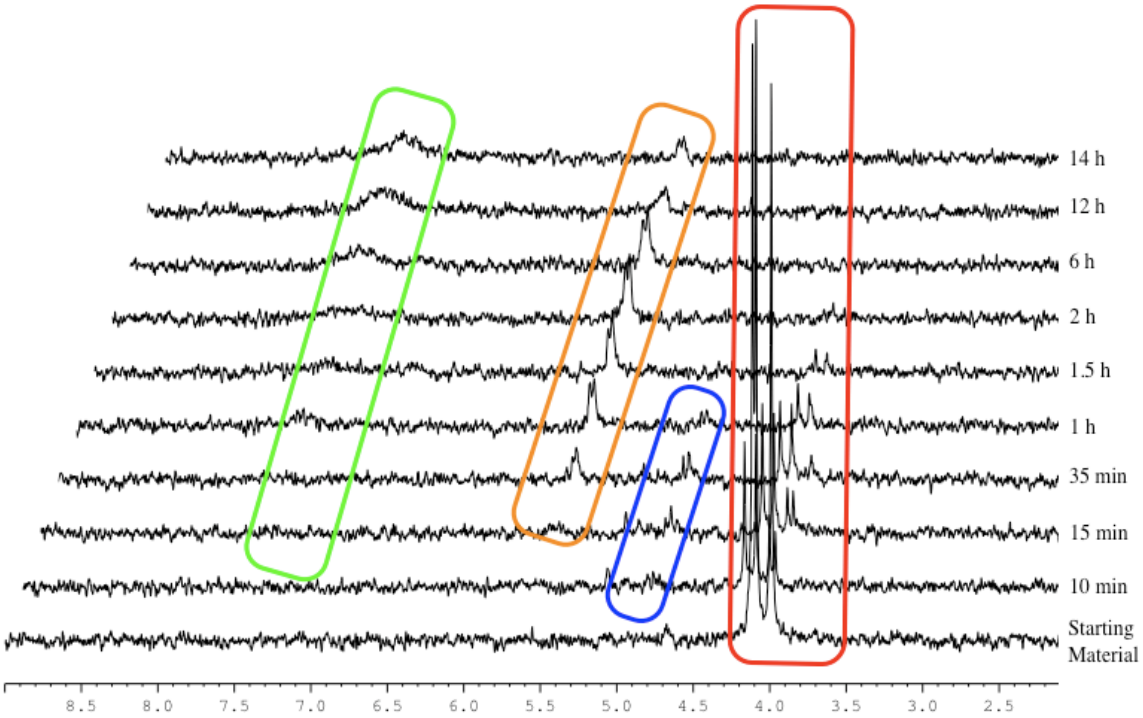



**Figure 9.** Carboxypeptidase-mediated cleavage of compound **69**, monitored by $^{31}$P-NMR.

As depicted in Figure 9, the mono-ProTide **69** showed a complete conversion of the starting material (red) within 2 h from the start of the experiment to the desired metabolite (green). The intermediate **79** (blue) was barely detectable indicating a fast conversion to metabolite **81**. As already observed for other mono-ProTides, an unknown species at $\delta_P$ = ~5.5 ppm (orange) did also appear in this experiment.

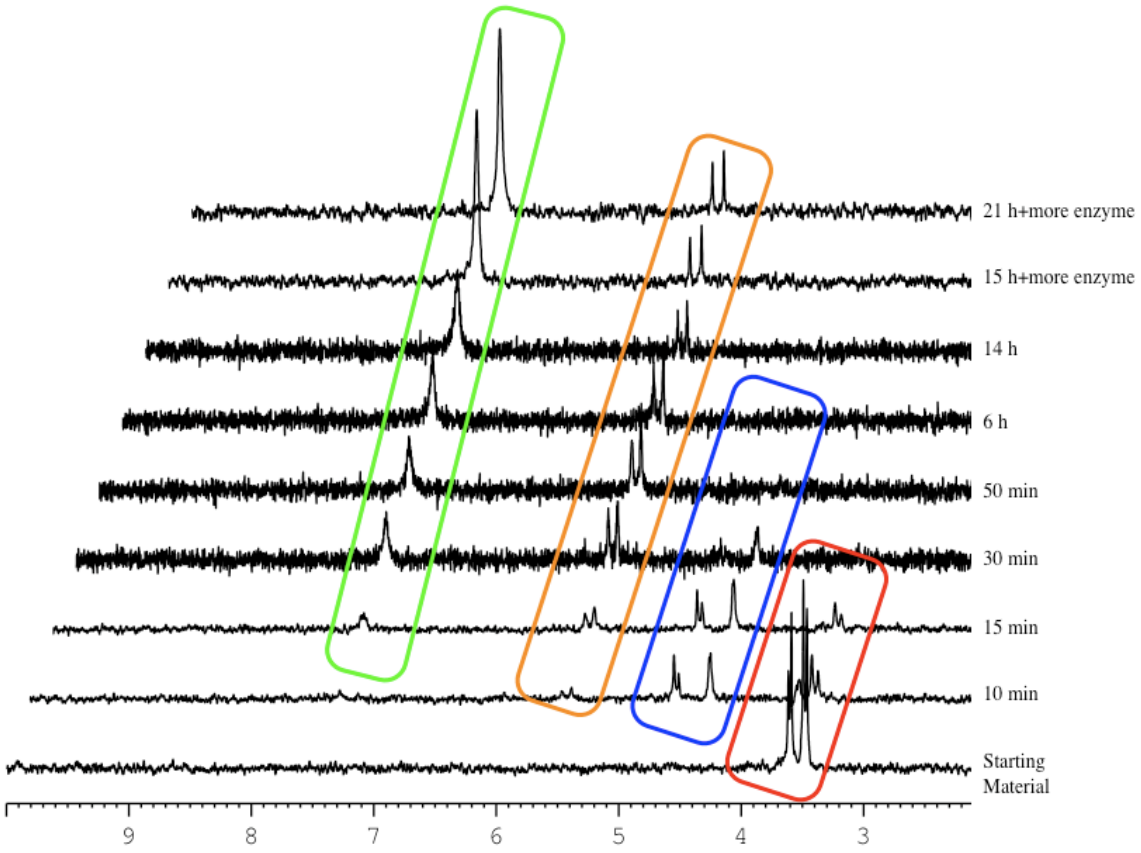


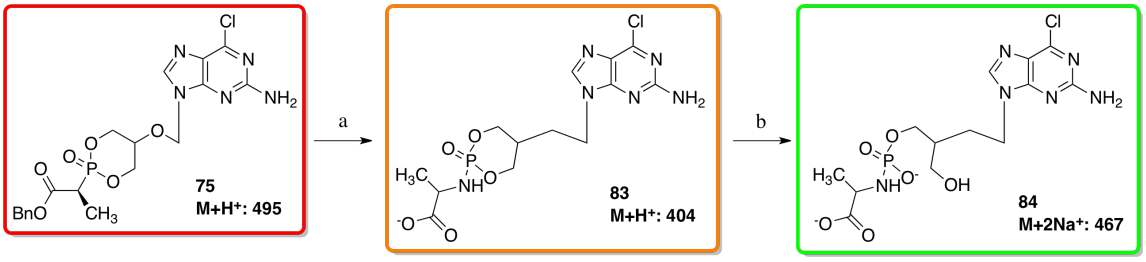

**Figure 10.** Carboxypeptidase-mediated cleavage of compound **60**, monitored by $^{31}$P-NMR.

Compound **60** (Figure 10, red) was completely converted within 30 min upon the addition of the enzyme to the desired metabolite ($\delta_P$ = ~7.0 ppm, green), through the intermediate **79** (blue). Also in this case, the species at $\delta_P$ = 5.5, 5.6 ppm (orange) was formed and increased in the first 30 minutes of the experiment and then appeared to remain constant for the whole duration of the experiment. In this experiment, it is easy to note two distinct peaks, which is usually related to the presence of a chiral phosphorous. In addition, this compound did not seem to be a substrate for the carboxypeptidase since the addition of two extra portions of enzyme did not have any effect on this compound.

The enzymatic studies performed showed a partial (compounds **16**, **45**, and **63**) to full (compounds **60** and **69**) conversion of the starting material to the desired metabolite **81** and the unknown species at 5-5.5 ppm. We hypothesise that this unknown species corresponds to the product of intramolecular cyclisation with the 3'-OH attacking the phosphorus center, and being in competition with the carboxylate released by the enzymatic cleavage.

To investigate and confirm this pathway, an enzymatic experiment was designed and performed using the cyclic derivative **75** (Scheme 14), and its conversion was monitored by $^{31}$P-NMR (Figure 11) as well as by mass spectroscopic analysis.

**Scheme 14.** Putative mechanism of bioactivation for compound **75**.



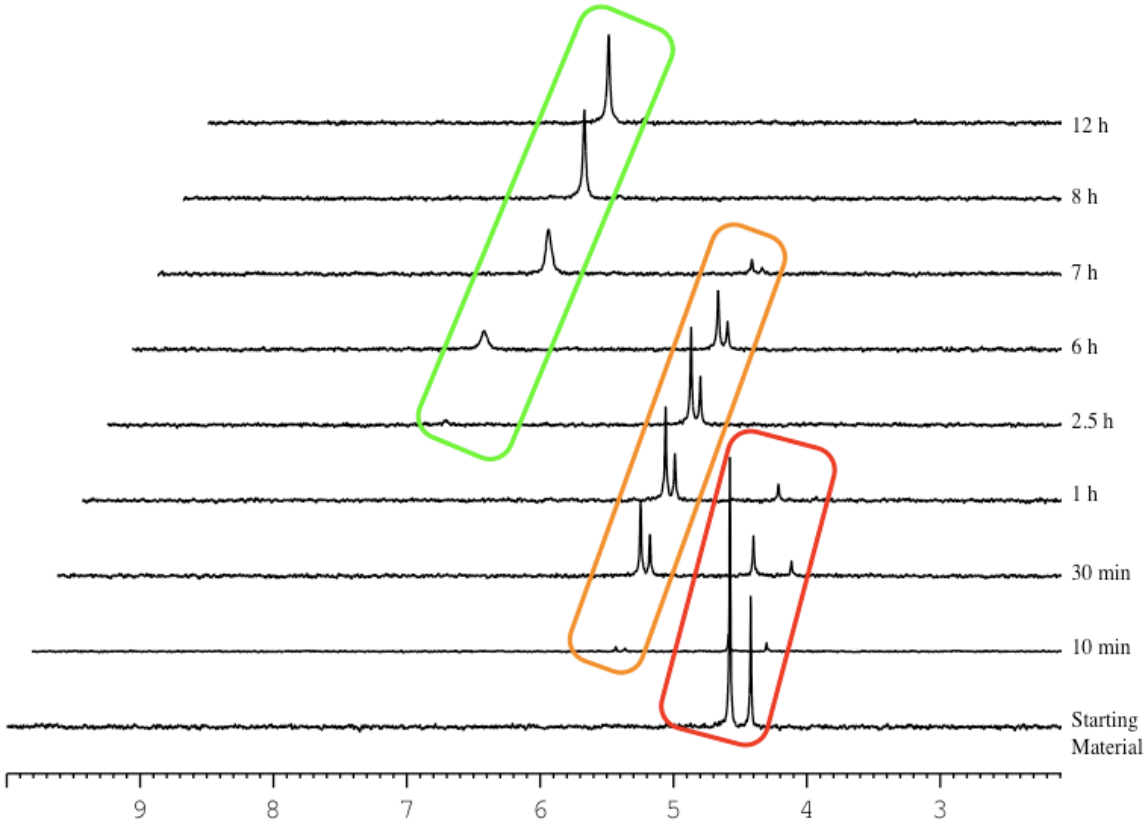



**Figure 11.** Carboxypeptidase-mediated cleavage of compound **75**, monitored by $^{31}$P-NMR.

Within 1 h, compound **75** (Figure 11, red) was completely metabolised to the first intermediate lacking the ester moiety ($\delta_P$ = 5.3, 5.4 ppm, orange) and having a similar chemical shift as the unknown species observed in the previous experiments. The identity of this compound was supported by mass spectroscopic analysis ($M+H^+$: 404). The conversion to the final metabolite **84** ($\delta_P$ = ~7.0 ppm, green) was observed after 2.5 h and it appeared to be the only species after 8 h. Mass spectroscopic analysis confirmed the formation of the metabolite **84** ($M+2Na^+$: 467). From these results, we can now confidently postulate that the formation of the peak at 5-5.5 ppm observed for the compounds analysed can be attributed to the formation of the corresponding cyclic intermediate, due to an intramolecular competition between the 3'-OH *versus* the

carboxylate, which did not lead to the formation of the desired compound indicated as **81** in Figure 6. Interestingly, intermediate **83** can still undergo attack from the carboxylate to the phosphorus followed by water-mediated opening of the mixed anhydride resulting in the formation of metabolite **84**.

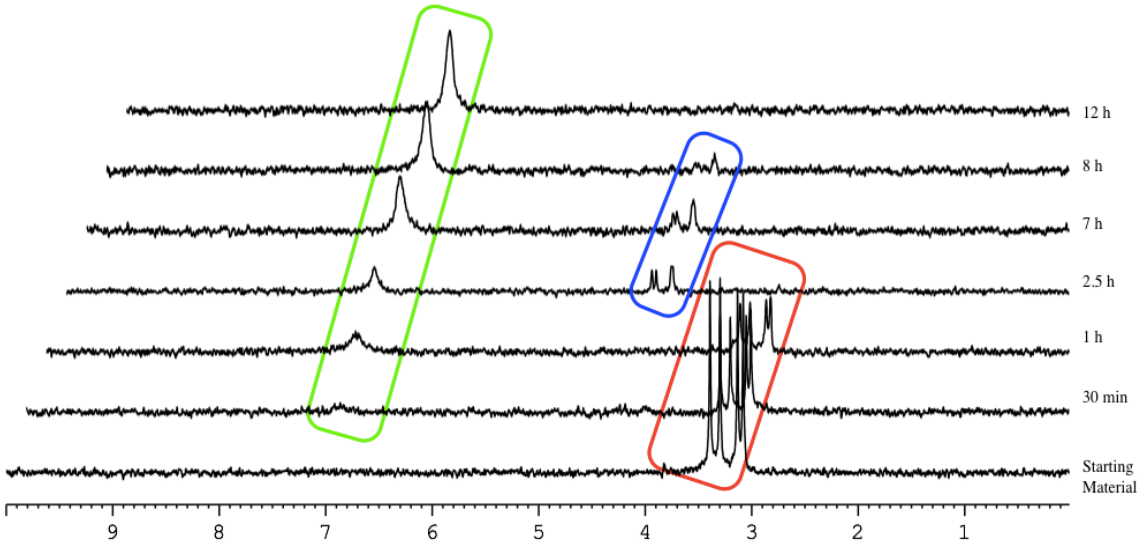



**Figure 12.** Carboxypeptidase-mediated cleavage of compound **77**, monitored by $^{31}$P-NMR.

To further support this hypothesis, the monoacetylated ProTide **77** was also studied (Figure 12). The compound proved to be an efficient substrate for the carboxypeptidase and it was quickly metabolised to the intermediate ($\delta_P$ = 4.35, 4.28, 4.16 ppm, blue) followed by spontaneous conversion to the desired metabolite ($\delta_P$ = ~7.0 ppm, green). As anticipated, the formation of an unknown species at 5-5.5 ppm was not observed supporting our hypothesis of the need of a free 3'-OH to form this metabolite.

It is worth to note the fast conversion of only one diastereoisomer, whilst the metabolism of the other three proceeded more slowly, although a full conversion was observed within 12 h. The difference in the rate of conversion for the different diastereoisomers had

already been observed previously.[4] Docking studies using the carboxypeptidase Y enzyme showed indeed the ability of the enzyme to discriminate between diasteroisomers.[4]

**Conclusion**

Previously we showed that the anti-herpetic drug ACV, an acyclic nucleoside analogue, has direct anti-HIV suppressive activity *ex vivo*[2] and *in vivo*.[24] The anti-HIV activity of ACV is dependent on its prior phophorylation by HHV-TK. To bypass this requirement, we developed a series of ACV ProTides that showed pronounced anti-HIV activity, low toxicity but somewhat cytostatic activity.[4] Here, to overcome this problem and to assess the potential of acyclic nucleoside analogues as anti-HIV drugs, we performed a virtual screening of a library of acyclic nucleoside derivatives. Docking experiments with a database of 3,600 compounds against three different enzymes (HIV RT, adenylate or guanylate kinase and a model of DNA polymerase γ) resulted in selecting 5 compounds that potentially were the strongest RT inhibitors and the weakest cellular DNA polymerase inhibitors. The five compounds selected were: ganciclovir (**1**), penciclovir (**2**), the 2'-thio derivative of ganciclovir (**3**), 6-chloropenciclovir (**4**), and 6-methoxypenciclovir (**5**). Altogether, 39 phosphate prodrugs were synthesised and assessed for their potency against HIV and three HHVs, namely HSV, VZV and HCMV. Most of the compounds were inhibitory against HIV with activity in the low μM range, but some toxicity was observed, except for compound **66**. Some of the mono- and bis-ProTides of 1 were active against HSV, VZV and HCMV, and were found to be equipotent to ACV, but not superior to their parent nucleoside ganciclovir **1**. Interestingly, they were also active against their TK-deficient counterparts, which is an

advantage over ACV. Mono- and bis-ProTide derivatives of compounds **2**, **3** and **5** did not display important acitivty against HHVs tested in this study.

Finally, we investigated the mechanism of bioactivation for these compounds using a well-established enzymatic assay. The majority of the compounds were found to be substrate for the first enzyme involved in the activation pathway to release the intermediate indicated in Scheme 13 as compound **81**. However, in some cases formation of a stable 3'-5'-cyclic-phosphate was also observed; the formation of this compound was supported by experiments depicted in Figure **11** and **12**.

**Experimental Section**

All anhydrous solvents were purchased from Sigma–Aldrich and amino acid esters from Novabiochem. All commercially available reagents were used without further purification. Thin Layer Chromatography (TLC): precoated aluminium-backed plates (60 F254, 0.2 mm thickness, Merck) were visualized under both short- and long-wave UV light (254 and 366 nm). Flash column chromatography was carried-out using silica gel supplied by Fisher (60A, 35–70 μm). Analytical and semipreparative High Performance Liquid Chromatography (HPLC) analysis was performed using either a ThermoScientific or a Varian Prostar 335 LC detector system using Varian Polaris C18-A (10 $\mu$M) as an analytical column and Varian Polaris C18-A (10 $\mu$M) as a semipreparative column. $^{1}$H-NMR (500 MHz), $^{13}$C-NMR (125 MHz), $^{31}$P-NMR (202 MHz) spectra were recorded on a Bruker Avance 500 MHz spectrometer at 25 °C. Chemical shifts (δ) are quoted in parts per million (ppm) relative to internal MeOD (δ 3.34 1H NMR, δ 49.86 13C NMR) and CDCl3 (δ 7.26 1H NMR, δ 77.36 13C NMR) or external 85% H3PO4 (δ 0.00 31P NMR). Coupling constants (J) are given in Hertz. The following abbreviations are used in

the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), qn (quintet), m (multiplet), bs (broad singlet), dd (doublet of doublet), dt (doublet of triplet). Low-resolution and high-resolution mass spectrometry was performed on a Bruker Daltonics microTof-LC system, as a service by the School of Chemistry at Cardiff University.Compound purity was assured by a combination of high field multinuclear NMR (H, C, P), elemental analysis and/or HPLC. Purity by the later was always always >95% with no detectable parent nucleoside for all final products.

**Chemistry**

**Standard Procedure A: Synthesis of Phosphoramidates**

To a stirring suspension of appropriate nucleoside (1.00 mol/equiv) in anhydrous THF, a 1.0M solution of $^{t}$BuMgCl (2.00 mol/equiv) in THF was added dropwise under an argon atmosphere and the reaction mixture was stirred at room temperature for 30 min. Then the appropriate phosphorochloridate (1.50 to 8.00 mol/equiv) was added dropwise a solution of in anhydrous THF. The reaction mixture was stirred at room temperature for 24-84 h. The solvent was removed under reduced pressure, and the residue was purified by column chromatography.

**Synthesis of N'-(9-{[(1,3-dihydroxypropan-2-yl)oxy]methyl}-6-oxo-6,9-dihydro-1H-purin-2-yl)-N, N-dimethylmethanimidamide (6).** To a suspension of **1** (0.50 g, 1.96 mmol) in anhydrous DMF (10 mL) was added *N*,*N*-dimethylformamide dimethyl acetal (1.30 mL, 9.79 mmol) and the reaction mixture was stirred at room temperature for 22 h. After this period was added *N*,*N*-dimethylformamide dimethyl acetal (0.65 mL, 4.90 mmol) and the

suspension was stirred at room temperature for 7 h. The suspension was then filtered, and the solid was washed with diethyl ether to give a colourless solid (84%, 0.51 g). $^{1}$H-NMR (DMSO 500 MHz): δ 11.31 (1H, s, NH), 8.57 (1H, s, 1H), 7.93 (1H, s, H-8), 5.54 (2H, s, H-1'), 4.64 (2H, bs, 2xOH), 3.66-3.60 (1H, m, H-3'), 3.49-3.43 (4H, m, H-4', H-5'), 3.16, 3.04 (6H, 2s, $N(CH_3)_2$).

**Synthesis of (*N*'-(9-[(1,3-dihydroxypropan-2-yloxy)me-thyl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl)-*N*,*N*-dimethylformimidamide)-[1-naphthyl(isopropoxy-L-alaninyl)] diphosphate (7) and N'-(9-{[(1,3-dihydroxypropan-2-yl)oxy]methyl}-6-oxo-6,9-dihydro-1H-purin-2-yl)-N, N-dimethylmethanimidamide-[1-naphthyl(isopropoxy-L-alaninyl)] phosphate (8).** Prepared according to Standard Procedure A, from **6** (0.25 g, 0.81 mmol) in anhydrous THF (15 mL), $^{t}$BuMgCl (1.0 M THF solution, 1.61 mL, 1.61 mmol), 1-naphthyl-(iso-propoxy-L-alaninyl)-phosphorochloridate (0.86 g, 2.42 mmol) in anhydrous THF (10 mL) and the reaction mixture was stirred at room temperature overnight. After this period pyridine (5 mL), $^{t}$BuMgCl (1.0 M THF solution, 1.61 mL, 1.61 mmol) and 1-naphthyl-(iso-propoxy-L-alaninyl)-phosphorochloridate (1.60 g, 4.49 mmol) in anhydrous THF (10 mL) were added and the reaction mixture was stirred at room temperature for 3 days. The residue was purified by column chromatography gradient elution of DCM/MeOH = 98/2, then 95/5, to give **7** as a colourless solid (44%, 0.34 g), $^{31}$P-NMR (MeOD, 202 MHz): δ 4.04 (bs). Compound **8** was obtained as a colourless solid (14%, 0.071 g) $^{31}$P-NMR (MeOD, 202 MHz): δ 4.19 (bs). $^{1}$H-NMR (MeOD, 500 MHz): δ 8.74-8.68 (1H, m, NC*H*$N(CH_3)_2$), 8.17-7.36 (8H, m, H-8, Naph), 5.73-5.70 (2H, m, H-1'), 4.99-4.80 (1H, bs, COOC*H*$(CH_3)_2$), 4.39-4.05 (3H, m, H-3', H-5'), 3.97-3.88 (1H, m, C*H*$CH_3$), 3.70-3.56

(2H, m, H-4'), 3.20, 3.12 (6H, s, $N(CH_3)_2$), 1.33-1.28 (3H, m, $CHCH_3$), 1.22-1.16 (6H, m, $COOCH(CH_3)_2$).

**Synthesis of 2-amino-9-{[(1,3-dihydroxypropan-2-yl)oxy]methyl}-6,9-dihydro-1H-purin-6-one-[1-naphthyl(isopropoxy-L-alaninyl)] diphosphate (15).** A solution of **7** (0.33 g, 0.35 mmol) in 2-propanol (10 mL) was stirred under reflux for 80 h. After this period the solvent was removed and the residue purified by column chromatography gradient elution of DCM/MeOH = 95/5, then 94/6, then 93/7, to give **15** as a colourless solid (8%, 0.025 g). $^{31}$P-NMR (MeOD, 202 MHz): δ 4.29, 4.25, 4.23, 4.17. $^{1}$H-NMR (MeOD, 500 MHz): δ 8.17-8.05 (2H, m, H-8 Naph), 7.85 (2H, m, H-6 Naph), 7.75-7.71 (1H, m, H-8), 7.68-7.34 (10H, m, Naph), 5.41-5.37 (2H, m, H-1'), 4.99-4.87 (2H, m, $2xCH(CH_3)_2$), 4.27-4.14 (5H, m, H-3', H-4', H-5'), 4.01-3.91 (2H, m, $2xCHCH_3$), 1.36-1.26 (6H, m, $2xCHCH_3$), 1.21-1.11 (12H, m, $2xCOOCH(CH_3)_2$). $^{13}$C-NMR (MeOD, 125 MHz): δ 20.49, 20.56, 20.61, 20.64, 20.69 ($CHCH_3$), 21.90, 22.00, 22.02 ($CH(CH_3)_2$), 51.87 ($CHCH_3$), 66.51, 66.55, 66.66, 66.70 (C-4', C-5'), 70.23, 70.30, 70.32 ($COOCH(CH_3)_2$), 72.91 (C-1'), 76.94 (C-3'), 116.13, 116.42, 122.67, 122.70, 122.79, 126.03, 126.55, 127.54, 127.80, 127.86, 128.87 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 136.26 (C-4a Naph), 138.42 (C-8), 147.92, 147.98, 150.02 ('ipso' Naph, C-4), 155.64 (C-2), 159.44 (C-6), 174.72 ($COOCH_2Ph$). EI MS= 916.3 (M+Na). Anal. Calcd for $C_{41}H_{49}N_7O_{12}P_2$: C, 55.09; H, 5.53; N, 10.97. Found: C, 54.62; H, 5.56; N, 10.60.

**Synthesis of 2-amino-9-{[(1,3-dihydroxypropan-2-yl)oxy]methyl}-**

**6,9-dihydro-1H-purin-6-one-[1-naphthyl(isopropoxy-L-alaninyl)]phosphate (16).** A solution of **8** (0.070 g, 0.11 mmol) in 2-propanol (4 mL) was stirred under reflux for 24 h. After this period the solvent was removed and the residue purified by preparative reverse phase HPLC (gradient elution of $H_2O/CH_3CN$= from 100/0 to 0/100 in 35 min) to give **16** as a colourless solid (33%, 0.021 g). $^{31}$P-NMR (MeOD, 202 MHz): δ 4.40, 4.36, 4.34, 4.27. $^{1}$H-NMR (MeOD, 500 MHz): δ 8.20-8.06 (1H, m, Naph), 7.90-7.85 (1H, m, Naph), 7.83-7.79 (1H, m, H-8), 7.75-7.66 (1H, m, Naph), 7.59-7.49 (2H, m, Naph), 7.49-7.36 (2H, m, Naph), 5.51-5.45 (2H, m, H-1'), 5.03-4.94 (1H, m, C*H*$(CH_3)_2$), 4.39-4.11 (2H, m, H-5'), 4.05-3.91 (2H, m, H-3', C*H*$CH_3$,), 3.65-3.47 (2H, m, H-4'), 1.40-1.29 (3H, m, CHC*H*$_3$), 1.26-1.14 (6H, m, COOCH(C*H*$_3$)$_2$). $^{13}$C-NMR (MeOD, 125 MHz): δ 20.46 (d, $J_{C-P}$ = 6.7 Hz, CH*C*$H_3$), 20.57 (d, $J_{C-P}$ = 6.7 Hz, CH*C*$H_3$), 20.68 (d, $J_{C-P}$ = 6.6 Hz, CH*C*$H_3$), 21.90 (d, $J_{C-P}$ = 2.3 Hz, CH(*C*$H_3$)$_2$), 21.99 (d, $J_{C-P}$ = 2.9 Hz, CH(*C*$H_3$)$_2$), 51.83 (*C*HCH$_3$), 61.78, 61.92 (2s, C-4'), 67.36 (d, $J_{C-P}$ = 5.4 Hz, C-5'), 67.45 (d, $J_{C-P}$ = 5.7 Hz, C-5'), 70.17 (COO*C*H(CH$_3$)$_2$), 70.26 (d, $J_{C-P}$ = 5.6 Hz, COO*C*H(CH$_3$)$_2$), 72.89, 73.06, (C-1'), 78.95 (d, $J_{C-P}$ = 7.3 Hz, C-3'), 79.21 (d, $J_{C-P}$ = 7.6 Hz, C-3'), 116.31, 116.34, 116.37, 116.40, 117.48, 117.56, 122.70, 122.74, 122.83, 125.98, 126.55, 127.50, 127.78, 127.80, 127.88, 127.93, 128.85 (C-5, C-2 Naph, C-3 Naph, C-4 Naph, C-5 Naph, C-6 Naph, C-7 Naph, C-8 Naph, C-8a Naph), 136.27 (C-4a Naph), 139.70, 139.75 (C-8), 147.97, 148.05 ('ipso' Naph, C-4), 155.72 (C-2), 159.46 (C-6), 174.52 (*C*OOCH$_2$Ph). EI MS= 597.1832 (M+Na). Anal. Calcd for $C_{25}H_{31}N_6O_8P\cdot H_2O$: C, 50.68; H, 5.61; N, 14.18. Found: C, 50.75; H, 5.44; N, 13.89.

**Synthesis of**

**9-[({1-hydroxy-3-[(4-methoxyphenyl)diphenylmethoxy]propan-2-yl}oxy)methyl]-2-{[(4-methoxyphenyl)diphenylmethyl]amino}-6,9-dihydro-1H-purin-6-one (23) and**

**9-{[(1,3-dihydroxypropan-2-yl)oxy]methyl}-2-{[(4-methoxyphenyl)diphenylmethyl]amino}-6,9-dihydro-1H-purin-6-one (24).** A solution of **1** (0.50 g, 1.96 mmol), monomethoxytrityl chloride (1.33 g, 4.32 mmol), triethylamine (0.82 mL, 5.88 mmol) and 4-(dimethylamino)pyridine (5 mg) in DMF (5 mL) was stirred at room temperature for 2 h. The reaction was then quenched with methanol 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 over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography gradient elution of DCM/MeOH (98/2, then 96/4, then 94/6) to give **23** as a colourless solid (53%, 0.84 g). $^1$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-4'), 3.28-3.18 (2H, m, C*H*$_2$OH), 2.87-2.79 (2H, m, C*H*$_2$OMMT). Compound **24** was obtained as a colourless solid (30% 0.30 g). $^1$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, $CH_2C$*H*$CH_2$), 3.49-3.43 (4H, m, C*H*$_2$CHC*H*$_2$).

**Synthesis of**

**9-[({1-hydroxy-3-[(4-methoxyphenyl)diphenylmethoxy]propan-2-yl}oxy)methyl]-2-{[(4-methoxyphenyl)diphenylmethyl]amino}-6,9-dihydro-1H-purin-6-one-[1-phenyl-(benzoxy-L-alaninyl)] phosphate (25).** Prepared according to standard procedure A, from **23** (0.45 g, 0.58 mmol) in anhydrous THF (10 mL), $^t$BuMgCl

(1.0 M THF solution, 1.12 mL, 1.12 mmol), phenyl-(benzoxy-L-alaninyl)-phosphorochloridate (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 and the residue was purified by column chromatography, gradient elution of DCM/MeOH = 100/0 then 98/2 to give **25** as a colourless solid (38%, 0.25 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, 2s, H-8), 7.34-6.70 (38H, m, PhO, $OCH_2Ph$, NH-C(P*h*)$_3$, O- C(P*h*)$_3$), 5.17-4.99 (2H, m, $OCH_2$Ph), 4.95-4.89 (2H, m, H-1'), 3.79-3.60 (8H, m, $CHCH_3$, H-3', 2X PhO$CH_3$) 3.17-3.07 (2H, m, $CH_2$OP), 2.81-2.78 (2H, m, $CH_2OC(Ph)_3$), 1.85, 1.72 (3H, m, $CHCH_3$).

**Synthesis of**

**9-{[(1,3-dihydroxypropan-2-yl)oxy]methyl}-2-{[(4-methoxyphenyl)diphenylmethyl]amino}-6,9-dihydro-1H-purin-6-one-[1-naphthyl-(benzoxy-L-alaninyl)] phosphate (26).** Prepared according to standard procedure A, from **24** (0.24 g, 0.46 mmol) in anhydrous THF (8 mL) $^{t}$BuMgCl (1.0 M THF solution, 0.92 mL, 0.92 mmol), 1-naphthyl-(benzoxy-L-alaninyl)-phosphorochloridate (0.37 g, 0.92 mmol) in anhydrous THF (2 mL) 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, gradient elution of DCM/MeOH = 100/0, 98/2, then 96/2 to give **26** as a colourless solid (35%, 0.15 g). $^{31}$P-NMR (MeOD, 202 MHz): δ 3.91, 3.93, 3.96. $^{1}$H-NMR (MeOD, 500 MHz): δ 8.19-8.15 (m, 0.5H, H-8 Naph), 8.11-8.08 (m, 0.5H, H-8 Naph), 7.91-7.89 (m, 1H, H-6 Naph), 7.69,-7.68 (m, 1H, H-8), 7.66-6.75 (m, 24H, PhO, $OCH_2Ph$, NH-C(P*h*)$_3$), 5.1, 5.08, 5.04, 5.03 (4s, 2H, $OCH_2$Ph), 4.95-4.91 (2H, m, H-1'),

4.04-3.72 (8H, m, *CH*$CH_3$, C*H*$_2$OH), 3.67 (3H, s, PhOC*H*$_3$), 3.52-3.44 (1H, m, H-3'), 3.29-3.08 (2H, m, $CH_2$OP), 1.34-1.30 (3H, m, CHC*H*$_3$).

**Synthesis of 2-amino-9-{[(1,3-dihydroxypropan-2-yl)oxy]methyl}-6,9-dihydro-1H-purin-6-one-[1-phenyl-(benzoxy-L-alaninyl)] phosphate (27).** *para*-toluene sulfonic acid (0.09 g, 0.48 mmol) was added to a solution of **25** (0.11 g, 0.1 mmol) in DCM (3.5 mL) and MeOH (1.5 mL). The mixture was stirred at room temperature for 4 h. After this period the solvent was removed under reduced pressure and the residue was purified by preparative TLC 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 80/20 in 5 min, 80/20 isocratic 15 min, then to 0/100 in 5 min) to give **27** as a colourless solid (28%, 0.02 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, 7.83 (0.5H, 2s, H-8), 7.80, 7.79 (0.5H, 2s, H-8) 7.40-7.30 (7H, m, PhO, OCH$_2$*Ph*), 7.21-7.13 (3H, m, PhO, OCH$_2$*Ph*), 5.54, 5.53, 5.51, 5.50 (2H, 4s, H-1'), 5.17, 5.16, 5.15, 5.14 (2H, 4s, 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, $J_{C-P}$ = 6.4 Hz, CH*C*$H_3$), 20.40 (d, $J_{C-P}$ = 6.4 Hz, CH*C*$H_3$), 20.37 (d, $J_{C-P}$ = 7.1 Hz, CH*C*$H_3$), 51.62, 51.75 (NH*C*H), 61.91, 61.99, 62.02 (3s, *C*$H_2$CHCH$_2$), 67.31 (d, $J_{C-P}$ = 5.9 Hz, $CH_2$CH*C*$H_2$), 67.25 (d, $J_{C-P}$ = 2.8 Hz, $CH_2$CH*C*$H_2$), 67.20 (d, $J_{C-P}$ = 5.7 Hz, $CH_2$CH*C*$H_2$), 67.97, 68.00, 68.03 (3s, O*C*$H_2$Ph), 73.07, 73.11, 79.13 (3s, C-1') 79.24 (d, $J_{C-P}$ = 7.6 Hz, $CH_2$*C*HCH$_2$), 79.25 (d, $J_{C-P}$ = 5.6 Hz, $CH_2$*C*HCH$_2$), 79.30 (d, $J_{C-P}$ = 7.4 Hz, $CH_2$*C*HCH$_2$), 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, C-4), 153.30 ('ipso'OPh), 155.68 (C-2), 159.37 (C-6), 174.92 (d, $J_{C-P}$ = 4.4 Hz, *C*OOCH$_2$Ph), 174.82 (d, $J_{C-P}$ = 5.4 Hz, *C*OOCH$_2$Ph), 174.72 (d, $J_{C-P}$ = 4.6 Hz, *C*OOCH$_2$Ph). EI MS= 595.162 (M+Na). HPLC = H$_2$O/ACN from 100/0 to 0/100 in 15 min, then 0/100 isocratic 5 min = retention time 9.29 min; H$_2$O/MeOH from 100/0 to 0/100 in 15 min, then 0/100 isocratic 5 min = retention time 13.18 min.

**Synthesis of 2-amino-9-{[(1,3-dihydroxypropan-2-yl)oxy]methyl}-6,9-dihydro-1H-purin-6-one-[1-naphthyl-(benzoxy-L-alaninyl)] phosphate (28).** *para*-toluene sulfonic acid (0.09 g, 0.48 mmol) was added to a solution of **26** (0.09 g, 0.1 mmol) in DCM (3.5 mL) and MeOH (1.5 mL). The mixture was stirred at room temperature for 4 h. After this period the solvent was removed under reduced pressure and the residue was purified by preparative TLC, gradient of DCM/MeOH (98/2 then 96/4 then 94/6) to give **28** as a colourless solid (25%, 0.015 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, PhO, OCH$_2$*Ph*), 5.46-5.42 (2H, m, H-1'), 5.13, 5.12, 5.08, 5.07 (2H, 4s, OC*H*$_2$Ph), 4.29-4.06 (4H, m, OC*H*$_2$C*H*CH$_2$O, CHCH$_3$), 3.97-3.90 (2H, m, OC*H*$_2$CHCH$_2$O), 1.38-1.31 (3H, m, CHC*H*$_3$). $^{13}$C-NMR (MeOD, 126 MHz): δ 20.38 (d, $J_{C-P}$ = 7.3 Hz, CH*C*H$_3$), 20.43 (d, $J_{C-P}$ = 6.7 Hz, CH*C*H$_3$), 20.53 (d, $J_{C-P}$ = 6.5 Hz, CH*C*H$_3$), 51.80, 51.85 (*C*HCH$_3$), 61.84, 61.94, 61.97 (3s, *C*H$_2$CHCH$_2$), 67.43 (d, *J* = 5.6 Hz, CH$_2$CH*C*H$_2$), 67.49 (d, *J* = 3.3 Hz, CH$_2$CH*C*H$_2$), 67.53 (d, *J* = 3.3 Hz, CH$_2$CH*C*H$_2$), 67.97, 68.01, 68.04 (3s, O*C*H$_2$Ph), 72.96, 73.00, 73.08, 79.10 (4s, C-1'), 79.11 (d, $J_{C-P}$ = 7.8 Hz, CH$_2$*C*HCH$_2$), 79.25 (d, $J_{C-P}$ = 6.0 Hz, CH$_2$*C*HCH$_2$), 79.31 (d, $J_{C-P}$ = 6.4 Hz, CH$_2$*C*HCH$_2$), 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, C-4), 153.27 ('ipso'OPh), 155.65 (C-2), 159.34 (C-6), 174.74 (d, $J_{C-P}$ = 4.7 Hz, *C*$OOCH_2$Ph), 174.86 (d, $J_{C-P}$ = 5.3 Hz, *C*$OOCH_2$Ph), 174.96 (d, $J_{C-P}$ = 4.5 Hz, *C*$OOCH_2$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.

**Synthesis of N'-{9-[4-hydroxy-3-(hydroxymethyl)butyl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-N,N-dimethylmethanimidamide-[ethoxy-L-alaninyl)] cyclic phosphate (73).** To a stirring solution of **6** (0.30 g, 0.97 mmol) in anhydrous THF (10 mL) and anhydrous pyridine (5 mL) was added $^t$BuMgCl (1.0 M THF solution, 2.00 mL, 2.00 mmol) and the reaction mixture was stirred at room temperature for 30 min. After this period, a solution of (ethoxy-L-alaninyl)-dichlorophosphate (0.45 g, 1.93 mmol) in anhydrous THF (5 mL) was added and the reaction mixture was stirred at room temperature for 24 h. After this period, anhydrous pyridine (5 mL) and $^t$BuMgCl (1.0 M THF solution, 2.00 mL, 2.00 mmol) were added and the reaction mixture was stirred at room temperature for 16 h. After this period the solvent was removed and the residue purified by column chromatography gradient elution of DCM, then DCM/MeOH = 98/2, 92/8 then 80/20, to give **73** as a colourless gum (93%, 0.43 g). $^{31}$P-NMR (MeOD, 202 MHz): δ 3.53, 2.44.

**Synthesis of 2-amino-9-[4-hydroxy-3-(hydroxymethyl)butyl]-6,9-**

**dihydro-1H-purin-6-one-[ethoxy-L-alaninyl)] cyclic phosphate (74).** A solution of **73** (0.43 g, 0.91 mmol) in 2-propanol (20 mL) was stirred at reflux for 9 h. After this period the solvent was removed and the residue purified by column chromatography gradient elution of DCM/MeOH = 97/3, 94/6, 90/10 then 85/15 to give a colourless solid, which was further purified by preparative TLC (gradient elution of DCM/MeOH = 97/3 then 95/5) to give **74** as a colourless solid (11%, 0.040 g). $^{31}$P-NMR (MeOD, 202 MHz): δ 3.73, 2.89. $^{1}$H-NMR (MeOD, 500 MHz): δ 7.91 (1H, s, H-8), 5.70-5.55 (2H, m, H-1'), 4.63-4.30 (5H, m, H-3', H-4', H-5'), 4.26-4.10 (2H, m, C*H*$_2$CH$_3$), 3.93-3.76 (1H, m, C*H*CH$_3$), 1.45-1.32 (3H, m, CH$_2$C*H*$_3$), 1.31-1.24 (3H, m, CHC*H*$_3$). $^{13}$C-NMR (MeOD, 125 MHz): δ 14.46, 14.49 (CH$_2$*C*H$_3$), 20.43 (d, $J_{C-P}$ = 6.4 Hz, CH*C*H$_3$), 20.51 (d, $J_{C-P}$ = 6.6 Hz, CH*C*H$_3$), 50.93, 51.34 (*C*HCH$_3$), 62.29, 62.38 (*C*H$_2$CH$_3$), 69.89, 69.92, 69.94, 69.97, 70.26, 70.31, 70.42, 70.46, 70.51, 70.85, 70.90 (C-3', C-4', C-5'), 71.26, 71.42 (C-1'), 117.49 (C-5), 139.69 (C-8) 155.82 (C-2), 159.40 (C-6), 175.02 (d, $J_{C-P}$ = 5.8 Hz, *C*OOCH$_2$CH$_3$), 175.11 (d, $J_{C-P}$ = 5.5 Hz, *C*OOCH$_2$CH$_3$). EI MS= 417.12 (M+H) and 439.11 (M+Na). HPLC = H$_2$O/AcCN 95/5 to 0/100 in 30 min = retention time 7.75 min.

**Synthesis of 2-((2-amino-6-chloro-9*H*-purin-9-yl)methoxy)-3-hydroxypropyl acetate (76).** A suspension of 6-chloro-diacetylated-penciclovir (1.17 g, 3.29 mmol) and anhydrous methanol (20 mL) was bubbled with ammonia gas (generated in situ by heating 35% ammonia solution at 50 °C) at 0 °C for 5.5 h. The solvent was then removed and the residue purified by column chromatography eluting with DCM/MeOH 95/5 to give **76** as a colourless solid (34%, 0.36 g). $^{1}$H-NMR (DMSO, 500MHz): δ 8.15 (1H, s, H-8), 6.87 (2H, s, NH$_2$), 4.15-4.12 (2H, t, J = 6.2 Hz, H-1'), 4.05-4.02 (2H, m, H-4'), 3.99-3.95 (2H, m, H-5'), 1.99 (3H, s, CH$_3$), 1.90-1.76 (2H, m, H-2'), 1.71-1.66 (1H, m,

H-3'). EI MS: 316.9 (M+H). HPLC = $H_2O$/AcCN from 95/5 to 0/100 in 30 min = retention time 8.64 min.

**Synthesis of 2 2-[2-(2-amino-6-chloro-9H-purin-9-yl)ethyl]-3-hydroxypropyl acetate-[1-phenyl(benzoxy-L-alaninyl)] phosphate (77).** Prepared according to Standard Procedure A, from **76** (0.070 g, 0.23 mmol) in anhydrous THF (5 mL), $^{t}$BuMgCl (1.0 M THF solution, 0.45 mL, 0.45 mmol) phenyl-(benzoxy-L-alaninyl)-phosphorochloridate (0.16 g, 0.45 mmol) in anhydrous THF (2 mL) and the reaction mixture was stirred at room temperature for 16 h. After this period, the solvent was removed and the residue purified by column chromatography gradient elution of DCM then DCM/MeOH 98/2, 97/3 to give **77** as a colourless solid (28%, 0.04 g). $^{31}$P-NMR (MeOD, 202MHz): δ 3.97, 3.90, 3.38. $^{1}$H-NMR (MeOD, 500MHz): δ 8.06 (0.5H, d, J = 8.1 Hz, H-8 of one diasteroisomer), 8.04 (0.5H, d, J = 4.9 Hz, H-8 of one diasteroisomer), 7.39-7.26 (7H, m, Ph, $CH_2Ph$), 7.23-7.13 (3H, m, Ph, $CH_2Ph$), 5.14, 5.13 (2H, 2s, $CH_2$-Ph), 4.23-3.96 (7H, m, H-1', H-4', H-5', $CHCH_3$), 2.02, 2.02 (3H, 2s, $COCH_3$), 2.00-1.81 (3H, m, H-2', H-3'), 1.38 (1.5H, d, J = 7.3 Hz, $CHCH_3$ of one diasteroisomer), 1.36 (1.5H, d, J = 8.1 Hz, $CHCH_3$ of one diasteroisomer); $^{13}$C-NMR (MeOD, 126MHz): δ 20.36 (d, $J_{C-P}$ = 6.4 Hz, $CHCH_3$), 20.73 ($COCH_3$), 29.04, 29.11 (C-2'), 37.38 (d, $J_{C-P}$ = 7.5 Hz, C-3'), 37.45 (d, $J_{C-P}$ = 8.0 Hz, C-3'), 42.33, 42.37, 42.42 (C-1'), 51.65, 51.82 ($CHCH_3$), 64.57 (C-4'), 64.62 (d, $J_{C-P}$ = 3.0 Hz, C-4'), 67.36, 67.39, 67.41, 67.43, 67.47 (C-5'), 67.95, 67.94 ($CH_2Ph$), 121.39, 121.41, 121.43, 121.45, 121.48, 121.53, 121.56 (Ph, $CH_2Ph$), 126.14 (C-5), 129.23, 129.30, 129.56, 130.77 (Ph, $CH_2Ph$), 137.26 (Ph), 144.47 (C-8), 151.57 (C-2), 155.30 (C-4), 161.58 (C-6), 172.68 ($COCH_3$), 174.72, 174.99 ($COOCH_2Ph$); EI MS:

631.1 (M+H) 653.0 (M+Na); HPLC = $H_2O$/AcCN from 95/5 to 0/100 in 30 min = retention time 18.56, 18.89 min.

**Antiviral Activity Assay**

HIV-1 inhibition by compounds was assessed in MT-4 cell cultures and in human tissue *ex vivo*.

*MT-4 cell cultures:* One million of MT-4 cells suspended in 100 uL of fresh culture medium were incubated with 100 μL (7 ng of p24) of HIV-1 $X4_{LAI.04}$ viral stock for 2h at 37 C. After incubation, cells were washed with 20 mL of PBS and resuspended in 10 mL of fresh medium. 1 mL of infected cell suspensions were then transferred to microplate wells and tested compounds were added at appropriate concentrations. Cell cultures were then further incubated for 3 days at 37 ºC. After 3 days, p24 production was measured in the MT-4 cell culture supernatants.

*Human tissue culture ex vivo*: Human tonsillar tissues were obtained from patients undergoing routine tonsillectomy at the Children's National Medical Center (Washington DC) under an IRB-approved protocol. Tissues were dissected into 2- to 3-$mm^3$ blocks and placed onto collagen sponge gels in culture medium at the air–liquid interface as described earlier.[23] Human tonsillar tissues (27 blocks of tissue from each of *n* donors for each experimental condition) were pretreated with compounds overnight and then infected with HIV-1 $X4_{LAI.04}$ (Rush University Virology Quality Assurance Laboratory, Chicago, IL). The tissue culture was kept for 12 days, and drugs were replenished after each medium change (every 3 days).

*Evaluation of antiviral activity of compounds*: We measured p24gag using a bead-based Luminex assay as described earlier.[25] We then evaluated the antiviral activity of each compound by measuring inhibition of human HIV-1 replication in MT-4 cell cultures and in human lymphoid tissues. For each compound, in MT-4 cell cultures or in lymphoid tissue *ex vivo*, HIV-1 inhibition at each single drug concentration was defined by the following formula: inhibition = $(1-R_{compounds}/R_{Ctl})x100$, where $R_{compounds}$ and $R_{Ctl}$ are the amounts of p24 accumulated in the medium in compound-treated cultures and in untreated cultures, respectively. The results are given as the concentration required to suppress viral replication by 50% ($[EC_{50}]$).

The compounds were evaluated against the following viruses: herpes simplex virus type 1 (HSV-1) strain KOS, thymidine kinase-deficient ($TK^-$) HSV-1 KOS strain resistant to ACV ($ACV^r$), herpes simplex virus type 2 (HSV-2) strains Lyons and G, varicella-zoster virus (VZV) strain Oka, $TK^-$ VZV strain 07-1, human cytomegalovirus (HCMV) strains AD-169 and Davis, and human immunodeficiency virus type 1 strain $III_B$. 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 $CCID_{50}$ of virus (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures) or with 20 plaque forming units (PFU) (VZV) in the presence of varying concentrations of the test compounds. Viral cytopathicity or plaque formation 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 $EC_{50}$ (50% effective concentration) or compound concentration required to reduce virus-induced cytopathogenicity or viral plaque

formation by 50%. The MCC (minimal cytotoxic concentration) values were determined as the compound concentration required to afford a microscopically visible alteration of HEL cell morphology.

**Cytostatic Activity Assays**

All assays were performed in 96-well microtiter plates. To each well were added (5–7.5) × $10^4$ CEM cells and a given amount of the test compound. The cells were allowed to proliferate for 72 h at 37 °C in a humidified $CO_2$-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The $IC_{50}$ (50% inhibitory concentration) was defined as the concentration of the compound that inhibited cell proliferation by 50%.

Viability assays in MT-4 cell cultures. Viability assays were performed in MT-4 cell cultures with the Nucleocounter automated cell counting system (ChemoMetec). 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). We defined $CC_{50}$ as the compound concentration that reduced the viability of the MT-4 by 50% and $IC_{50}$ as the compound concentration that inhibited MT-4 or CEM cell growth by 50%.

**Molecular Modelling**

All molecular modelling studies were performed on a MAC pro 2.66 GHz Quad-Core Intel Xeon, running Ubuntu. A database of 3600 compounds were built using Molecular Operating Environment (MOE)[26] and minimized using the MMFF94x force field before

docking. HIV-RT (PDB code: 1T05), human adenylate kinase (PDB: 2C95), mouse guanylate kinase (PDB: 1LVG) were downloaded from the RCSB Protein Data Bank and pre-processed using MOE. Hydrogen atoms were added to the crystal structures and minimised with MOE until a gradient of 0.05 Kcal $mol^{-1}$ $Å^{-1}$ was reached using MMFF94x force field. The partial charges were automatically calculated. An homology model of the human polymerase γ was used.[18] Docking simulations were performed using PLANTS[27] and docking results were analyzed with MOE.

## Associated Content

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Preparative methods, spectroscopic and analytical data on target compounds, enzymatic procedure (PDF) and Molecular formula strings (CSV).

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**Abbreviations Used**: HIV, human immunodeficiency virus; RT, reverse transcriptase; ACV, acyclovir; HSV, herpes simplex virus; VZV, varicella zoster virus; HHV, human herpesviruses; thymidine kinase, TK; RMSD, root mean square deviation; $^{t}$BuMgCl, tert-butylmagnesium chloride; Naph, naphthol; MMT, monomethoxytrityl; DMAP, 4-(N,N-dimethylamino)pyridine; PTSA, *para*-toluene sulfonic acid; cLogP: calculated logarithm of the octanol/water partition coefficient; HCMV, human cytomegalovirus.

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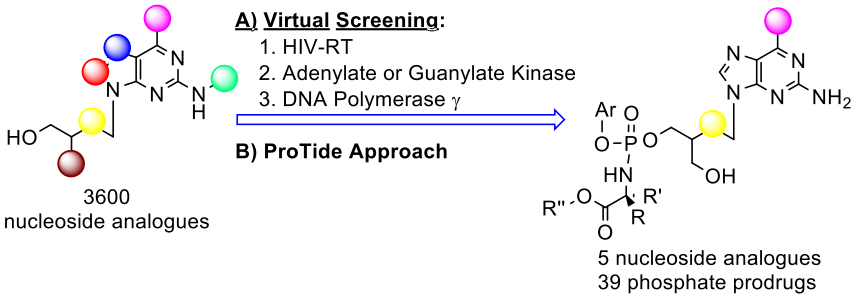
## TOC Graphic


A) Virtual Screening:
1. HIV-RT
2. Adenylate or Guanylate Kinase
3. DNA Polymerase γ
B) ProTide Approach
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NH2
5 nucleoside analogues
39 phosphate prodrugs