Design, Synthesis and Biological Evaluation of Phosphorodiamidate Prodrugs of Antiviral and Anticancer Nucleosides

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Abstract

We herein report the application of the phosphorodiamidate phosphate prodrug approach to a series of thirteen nucleoside analogues with antiviral or anticancer activity. Twenty-five symmetrical phosphorodiamidates were synthesised, bearing esterified L-Alanine (and in one case D-Alanine) in the prodrug moiety, each as single stereoisomer. The presence of an achiral phosphorus represents a potential advantage over the
phosphoramidate ProTide approach, where diastereoisomeric mixtures are routinely obtained, and different biological profiles may be expected from the diastereoisomers. Optimization of the synthetic pathway allowed us to identify two general methods depending on the particular nucleoside analogues. All the compounds were biologically evaluated in antiviral and anticancer assays and several showed improvement of activity compared to their parent nucleosides, as in the case of ddA, d4T, abacavir and acyclovir against HIV-1 and/or HIV-2. The biological results were supported by metabolism studies with carboxypeptidase Y monitored by $^{31}$P-NMR to investigate their bioactivation. This work further validates the phosphoramidate approach as a monophosphate prodrug motif with broad application in the antiviral and anticancer fields.
1. Introduction

Nucleoside analogues (NAs) play a pivotal role in antiviral and anticancer therapy.\textsuperscript{1,2} They are structurally related to the natural nucleosides bearing modifications at the base and/or at the sugar moieties, which, in most of the cases, confer selectivity \textit{versus} the desired targets.\textsuperscript{2} NAs are prodrugs and they need to be converted into their active species, which usually consists of their 5'-triphosphate form.\textsuperscript{3} Their bioactivation pathway often involves three consecutive phosphorylation steps starting from the parent nucleoside, which is converted to its mono-, di- and finally triphosphate form. Some NAs, such as abacavir or famciclovir for instance, require also additional bioactivation steps in order to display their biological activity.\textsuperscript{4,5} The first step of phosphorylation is usually considered to be the rate-limiting step in the bioactivation of NAs, with few exceptions, such as zidovudine for which the second phosphorylation may be rate limiting.\textsuperscript{6} After long-term treatment with NAs the activity of the nucleoside kinases involved in the first step of bioactivation may be decreased, therefore leading to drug resistance onset.\textsuperscript{7} Several monophosphate prodrug strategies are currently under investigations to overcome these issues,\textsuperscript{8} including the phosphoramidate ProTide approach developed in our group.\textsuperscript{9,10,11} More recently the phosphorodiamidate technology applied to 6-\textit{O}-alkyl-2'\textendash\textit{C}-methylguanosine was reported by us as a new promising approach for the delivery of monophosphates inside the cell. Several stability studies on the 6-\textit{O}-alkyl-2'\textendash\textit{C}-methylguanosine diamidates have shown a good stability profile under different conditions such as acid (pH = 2) and mild basic buffer (pH = 8.5-11), and in human
serum.\textsuperscript{12} This novel prodrug approach has been validated both in vitro and in vivo and some compounds are already under consideration for clinical studies.\textsuperscript{12} Similarly, several acyclic nucleoside phosphonate diamidate prodrugs showed a better biological profile compared to the parent compounds.\textsuperscript{13} In this approach two amino acid esters are introduced on the monophosphate moiety in order to mask the negative charges. As also in the case of the phosphoramidate diester approach of Wagner and colleagues,\textsuperscript{14} the phosphorus in the symmetrical diamidate prodrug is achiral, thus avoiding the presence of diastereoisomeric mixtures as in the case of the phosphoramidate ProTide derivatives. In fact, it has been reported how two diastereoisomers may interact differently with the enzymes involved in the bioactivation pathway, thus leading to different biological profiles.\textsuperscript{15} Moreover, the diamidate motif bears non-toxic and natural promoieties and obviate the need for a phenyl or naphthyl moiety. The putative bioactivation pathway of diamidate prodrugs, depicted in Scheme 1, is similar to the one reported for ProTides. The first step (a) may be mediated by an esterase or a carboxypeptidase-type enzyme, which is responsible for the cleavage of one of the two esters. This mechanism has been already described and supported by enzymatic experiments using $^{31}$P-NMR.\textsuperscript{12} The second step (b) involves an intramolecular attack of the carboxylate anion to the phosphorus with elimination of the second amino acid and formation of a five-membered ring (mixed anhydride intermediate). Spontaneous hydrolysis (c) of the cycle then leads to the formation of an intermediate bearing two negative charges. Finally, for the last step (d), a phosphoramidase-type enzyme cleaves the P-N bond to form the NA monophosphate.
Scheme 1: Putative bioactivation pathway of diamidate prodrugs: a) enzyme-mediated ester hydrolysis; b) spontaneous intracellular displacement; c) spontaneous hydrolysis; d) enzyme-mediated P-N bond cleavage.

We were keen to probe the scope of this new diamidate prodrug motif across several therapeutic arenas and for a broad range of NAs. In this context, we herein applied the diamidate approach to NAs with either antiviral or anticancer activity, and the novel prodrug compounds were evaluated for their biological activities. The NAs considered for this study are: 6-\(O\)-ethyl-2'-(deoxy-2'-\(\alpha\) -fluoro-2'-\(\beta\)-C-methyl)guanosine (1), stavudine (d4T, 2), 2',3'-dideoxyadenosine (ddA, 3), zidovudine (AZT, 4), lamivudine (3TC, 5), N-acetyl-lamivudine (N-acetyl-3TC, 6), 4'-azidouridine (4'-AzU, 7), 4'-azidocytidine (4'-AzC, 8), ribavirin (RBV, 9), acyclovir (ACV, 10), abacavir (ABC, 11), the bicyclic nucleoside analogue 12 (BCNA, also known as Cf1743) and acadesine (AICA, 13) (Figure 1).
Different synthetic conditions were necessary depending on solubility and reactivity issues of the parent nucleosides, and a total of twenty-five diamidates were synthesised.

**Figure 1:** NAs considered for this study.
Based on the previous work published on ProTides and diamidates, we selected L-alanine (L-Ala) as the amino acid of choice with benzyl and 2,2-dimethylpropyl as preferred ester moieties. For some derivatives, methyl and cyclohexyl esters were considered and, in one case, D-alanine (D-Ala) was used as the amino acid moiety.

2. Results and Discussion

2.1 Chemistry.

At first, we applied our previously reported successful methodology for the synthesis of anti-HCV 6-O-alkyl-2′-C-methylguanosine 5′-phosphorodiamidates\textsuperscript{12} to 6-O-ethyl-2′-deoxy-2′-α-fluoro-2′-β-C-methylguanosine 1, d4T 2, ddA 3, AZT 4, and 3TC 5. This procedure, called method A in this paper, is represented in Scheme 2.

Scheme 2. Synthetic method A to phosphorodiamidates 14-21. Reagents and conditions:
(a) anhydrous Et\textsubscript{3}N (1.0-1.2 mol/eq), anhydrous THF, room temperature, 30 min; then POCl\textsubscript{3} (1.0-1.2 mol/eq), -78 °C, 30 min; (b) amino acid ester p-TSA salt (3.0-5.0 mol/eq) in anhydrous CH\textsubscript{2}Cl\textsubscript{2}, room temperature; then anhydrous Et\textsubscript{3}N (5.0-10.0 mol/eq), -78 °C, then room temperature, 16-20 h.

In this strategy, the unprotected nucleoside dissolved in THF was treated with phosphorus oxychloride (1 equivalent) in the presence of triethylamine (1 equivalent) to generate a phosphorodichloridate intermediate (\textsuperscript{31}P NMR signal at ~7-8 ppm), which was
not isolated. Then, an excess of the appropriate amino acid ester as \( p \)-toluene sulfonate (\( p \)-TSA) salt (usually 5 equivalents) and triethylamine (5-10 equivalents) were added, leading after stirring at room temperature for 18-20 h to the desired phosphorodiamidates 14-21 (\( ^{31} \)P NMR signals at \( \delta_p \sim 11-14 \) ppm).

Interestingly, in the case of 3TC (5) both 5’-OH and 4-NH\(_2\) functional groups reacted yielding the tetradiamidate derivative 22, and the N-4-diamidate derivative 23.

![Scheme 3. Synthesis of 3TC phosphorodiamidates 22 and 23 with method A. Reagents and conditions: (a) anhydrous Et\(_3\)N (1.1 eq), POCl\(_3\) (1.1 eq), anhydrous THF, -78 °C to rt, 5 h; (b) L-alanine benzyl ester \( p \)-TSA salt (5 eq), anhydrous Et\(_3\)N (10 eq), anhydrous CH\(_2\)Cl\(_2\), -78 °C to rt, 20 h.](image)

However, when applied to the other NAs reported in this work, this method was not successful, probably due to a lack of solubility in the organic solvent used. Based on these findings, a modification of a synthetic approach reported by Yoshikawa \textit{et al.} for the synthesis of monophosphate species was used.\(^{16}\) This same method was previously reported for the synthesis of some phosphorodiamidates.\(^{17}\)
This second strategy, named method B, was used for the synthesis of diamidates of 3TC (5), N-acetyl 3TC (6), 4’-AzU (7), 4’-AzC (8), RBV (9), ACV (10), ABC (11), Cf1743 (12), and AICA (13) (Scheme 4).

**Scheme 4.** Synthetic method B to phosphorodiamidates 24-40. *Reagents and conditions:*

(a) POCl₃ (1.0 mol/eq), trimethylphosphate or triethylphosphate, -5 °C, 4-5 h, or 5 °C, 16 h; (b) amino acid ester *p*-TSA or HCl salt (5.0 mol/eq), anhydrous CH₂Cl₂, anhydrous Et₃N (10.0 mol/eq) or anhydrous DIPEA (5.0-10.0 mol/eq), -78 °C, 30 min, then room temperature, 16-45 h.

The reaction was carried out using either trimethylphosphate or triethylphosphate and POCl₃ to afford the corresponding intermediate dichlorophosphates following its formation by $^{31}$P-NMR ($\delta_p = \sim 7$). The addition of an excess of the appropriate amino acid ester salts in the presence of DIPEA or TEA yielded the desired phosphorodiamidates 24-40.

2.2 Biological results

The arylphosphoramidate ProTide technology as a monophosphate prodrug approach has proven to be a powerful tool in terms of enhancing the biological activity, by-passing certain mechanisms of resistance, as well as extending the biological profile of several NAs. Moreover, the increased lipophilicity of these prodrugs may also lead to a better delivery inside the cells by passive diffusion, thus favouring a greater uptake of the drug.
For instance, it has been reported how the application of the ProTide approach greatly enhanced the antiviral activity of the anti-hepatitis C virus (HCV) agent 2-amino-6-methoxy-9-\((2\text{-C-methyl-}\beta\text{-D-ribofuranosyl})\) purine,\(^{11}\) overcame the mechanisms of resistance for 5-fluoro-2'-deoxyuridine\(^{18}\) and extended the biological profile of BVdU and ACV from antitherpetic to anticancer and anti-human immunodeficiency virus (HIV) agents, respectively.\(^{19,20}\) In a similar context, the phosphorodiamidates reported here were tested \textit{versus} different cancer cell lines as well as versus a range of viruses with the aim to investigate the full potential of this phosphate prodrug moiety. To probe the delivery of the monophosphate inside the cells, thus by-passing the first step of phosphorylation, thymidine kinase-deficient (TK\(^-\)) mutant cancer cells and herpes virus strains were also used.

2.2.1 \textit{Anticancer activity}

Table 3 reports the anticancer activity for d4T (2), ddA (3), 3TC (5), N-acetyl-3TC (6), ABC (11), AICA (13) and their respective phosphorodiamidates \textit{versus} mouse lymphocytic leukemia cells (L1210), human T-lymphocyte cells (CEM), human cervical carcinoma cells (HeLa), for which TK\(^-\) mutant strains were also included (L1210-TK\(^-\), CEM-TK\(^-\), and HeLa-TK\(^-\)), colorectal adenocarcinoma cells (Caco-2) and human colon carcinoma cells (Colo-320). As expected, d4T itself did not show any antiproliferative activity \textit{versus} all the human cancer cell lines considered in this study. In the case of murine L1210, d4T showed cytostatic activity at \(~9\ \mu M\), while it was found ineffective (\(\geq 250 \mu M\) \textit{versus} L1210/TK\(^-\), which confirms its expected dependence on TK for phosphorylation for eventual biological activity. To the contrary, its phosphorodiamidates
16-18 showed antiproliferative activity versus all the cancer cell lines in a range between 8.9-47 μM for L1210, 73-96 μM for CEM, 96-116 μM for HeLa, 30-133 μM for Colo-320, while being poorly effective versus Caco-2 cell (135-241 μM). More importantly, all the compounds showed retention of activity against all the TK strains in a range between 7.4-25 μM versus L1210-TK, 37-54 μM vs CEM-TK, and 29-53 μM vs HeLa/TK strains. These results are strongly suggestive for the successful delivery of d4T monophosphate inside the cells, thus heading to their independence from TK activation.

As an alternative hypothesis, it could have been assumed that the similar antiproliferative activity observed for the d4T prodrugs against the L1210 TK cells versus wild-type L1210 cells could have been due to a direct effect of the prodrug or prodrug moieties such as the released benzyl part of the molecule. However, this is highly unlikely because in such a case, these prodrugs would have expected to display similar toxicities for the different prodrug molecules (i.e. 16, 22, 24, 25) and this had not been the case.

A similar trend was observed for 3TC (5) and its phosphorodiamidate 25; in fact while the parent nucleoside was found inactive versus all the cancer cell lines (> 250 μM) compound 25 showed a certain antiproliferative activity in a range between 38-116 μM, against all tested tumor cell lines including the TK mutant strains. As expected, the phosphorotetramidate 22 and the N-phosphorodiamidate 23 did not show any activity of particular interest.

In the case of ddA (3) and ABC (11) and their prodrugs, while the parent nucleosides did not show any potent antiproliferative activity (> 250 μM vs L1210, CEM, and HeLa for ddA, > 250 μM vs L1210 and CEM, and 170 μM vs HeLa for ABC), their prodrugs showed a great enhancement of the activity showing IC50 values in between 35-76 μM.
for the ddA derivative 19, and in the range of 19-54 µM for ABC phosphate prodrugs 34 and 35. Neither AICA (13) nor its prodrugs 38, 39 and 40 showed any activity, which may indicate a poor conversion of these prodrugs into the free monophosphate as it will be discussed.

2.2.2 Antiviral activity

One of the advantages of the monophosphate prodrug approach is the by-pass of the first phosphorylation step mediated by human and/or viral kinase enzymes. The monophosphate prodrugs here reported were tested for their antiviral activity versus different viruses.

2.2.2.1 Anti-HIV activity

d4T (2), ddA (3), AZT (4), 3TC (5), ACV (10), and ABC (11), and their respective phosphorodiamidates were tested against HIV-1 and HIV-2 in CEM or MT4 cells (Table 4). Compounds 16-18 showed a boost of their activity of ~2-4 fold vs HIV-1 and ~2-6 fold vs HIV-2 compared to their parent derivative d4T.

In the case of ddA a markedly greater boost in activity (72x vs HIV-1 and 57x vs HIV-2) was observed for its prodrug 19. AZT derivatives 20 and 21 were equipotent to the parent, while 3TC derivatives were significantly less effective, with the exception of N-phosphorodiamidate 23, which was surprisingly only two fold less active. In the case of ABC, compounds 34 and 35 showed an increased potency (15-41 fold) compared to the parent nucleoside.

In the case of ACV, a great improvement of its biological profile was observed. In fact, while ACV is not able to inhibit HIV-1 (EC50 >250 µM), both phosphorodiamidates 32
and 33 showed an inhibitory activity in the low micromolar range (2-4 μM), thus leading to a >60 fold boost in activity.

The positive results obtained may be due to a combination of both successful delivery of the monophosphate form of the parent nucleosides inside the cells as well as to an enhanced cellular uptake due to an increased lipophilicity. However, these positive features of the monophosphate prodrugs also led, in some cases, to a slightly increased cytotoxicity compared to the parent compounds. In the case of 3TC phosphoramidate 25, the poor anti-HIV activity may be due to a poor metabolic bioactivation of the phosphate prodrug moieties with the subsequent poor release of the free monophosphate form. In fact, the uptake and metabolic activation of the prodrugs is a multistep process for which it is currently not clear which enzymes are contributing to the eventual activity of the prodrugs. Not only the different type of enzymes involved in the drug conversion pathways have yet to be clarified but also their differential specificities related to the nature of the nucleoside and the prodrug part and their activity levels in the different cell systems are not exactly known. Without any doubt, such subtle differences are playing a role in the eventual biological activity and properties of these compounds.

2.2.2.2 Anti-HCV activity

The phosphorodiamidate phosphate prodrug applied to 6-O-alkyl-2’-C-methylguanosine showed outstanding results versus HCV both in vitro and in vivo.12 Driven by these encouraging results, a series of diamidates of compounds of interests versus HCV were prepared, including 6-O-ethyl-2’-deoxy-2’-α-fluoro-2’-β-C-methylguanosine (1), 4’-AzU (7), 4’-AzC (8), RBV (9), and ACV (10) (Table 5). Unfortunately, the majority of the
compounds showed a poor biological profile, with the exception of compounds 14 and 15 derived from compound 1. In fact, both compounds showed an inhibitory activity against HCV in the sub-micromolar range showing a great boost of activity compared to the parent guanosine nucleoside (EC$_{90}$ = 69.2 µM). Notably, the L-alanine derivative 14 gave a 4 fold improved activity compared to 15, which bears the non-natural amino acid D-alanine. Based on previous study, this finding may be the result of a lower conversion of the D-alanine based phosphate prodrug to the monophosphate, as a consequence of its lower affinity with the enzymes involved in the bioactivation pathway.

2.2.2.3 Anti-HSV-1 and 2 activity

AICA (13), ACV (10), Cf1743 (12) and their diamidates were then evaluated against human herpes simplex virus (HSV) type 1 and 2 and feline herpesvirus as reported in Table 6.

AICA (13), Cf1743 (12), and their respective phosphorodiamidate prodrugs 36, 38-40 were devoided of activity against human and feline herpes viruses (EC$_{50}$ > 100 µM) (Table 6). In the case of ACV, while the parent itself showed a submicromolar activity versus HSV-1 and 2, prodrugs 32 and 33 showed a loss (80-200 fold) of antiviral activity. However, when tested against HSV-1 TK$^-$, while ACV was 100 fold less effective (EC$_{50}$ = 23 µM), its diamidates retained their activity and were found to be equipotent to the parent compound thus indicating their TK independence. Indeed, since ACV obligatorily needs to be converted (activated) to its monophosphate derivative by HSV-1-encoded TK, it has markedly lowered antiviral activity against a HSV-1 TK$^-$ strain whereas its prodrugs have not. It has been ascertained that the prodrugs have no direct activity.
against purified herpetic DNA polymerase, excluding the possibility that the prodrugs might have activity as such against HSV-1 replication.

2.2.2.4 Other antiviral activity

Some of the phosphorodiamidate prodrugs were also examined for their inhibitory activity against a variety of other viruses, including vaccinia virus, vesicular stomatitis virus, influenza virus A (H1N1 and H3N2) and B, feline corona virus (FIPV), parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4, Punta Toro virus, respiratory syncytial virus and Coxsackie virus. None of them showed inhibitory activity nor cytotoxicity at 100 µM.

2.3 Enzymatic studies

As depicted in Scheme 1, the putative bioactivation pathway for the phosphorodiamidate phosphate moiety involves two enzymatic steps, mediated by an esterase or carboxypeptidase type enzyme (first step) and a phosphoramidase type enzyme (last step). We have reported the investigation of the first step of the bioactivation pathway for both ProTide$^{26}$ and diamidate$^{12}$ by incubating the desired compound with carboxypeptidase Y enzyme in $d_6$-acetone and Trizma buffer (pH = 7.6) and following the progress of the process through $^{31}$P-NMR. All the prodrugs tested, proved to be chemically robust in $d_6$-acetone and Trizma buffer (pH = 7.6) environment and in absence of carboxypeptidase Y. Fig. 2 and 3 report the first enzymatic cleavage for an ACV prodrug $^{32}$ (active compound) and for an AICA prodrug $^{40}$ (inactive compound) respectively. Compound $^{32}$ ($\delta_p = 13.66$) showed a fast metabolism to the first
intermediate lacking one ester moiety. The cleavage of only one ester was supported by the presence of two peaks at the $^{31}$P-NMR ($\delta_P = 14.30$ and 14.42), thus indicating the chirality at the phosphorus. After 30 minutes from the addition of the enzyme a new peak ($\delta_P = 7.16$) appeared, which corresponds to the metabolite lacking one amino acid and one ester, in agreement with our previously published results.\textsuperscript{11,26} Notably, compound 32 was fully converted in 7.5 h into its metabolite indicating that the first step in the bioactivation pathway proceeds well for ACV derivative. To the contrary, a poor metabolism was observed for compound 40. In fact, under the same condition used for compound 32, only a partial conversion into the desired metabolite ($\delta_P = 6.71$) was observed and after 24 h the starting material ($\delta_P = 14.20$) was still the predominant species.

From the two examples reported here, we can correlate the biological activity found for 32 and 40 and their bioactivation efficiency, thus supporting the need of an ester cleavage in the bioactivation pathway for this class of compounds. However intracellular activation of these prodrugs still needs to be confirmed.
Figure 2: carboxypeptidase-mediated cleavage of compound 32, monitored by $^{31}$P-NMR.
In conclusion, we report the synthesis and biological evaluation of twenty-five phosphorodiamidate prodrugs of known NAs. The key advantage of this nucleoside monophosphate prodrug approach is to overcome the chirality at the phosphorus center present in other monophosphate prodrug approaches, thus allowing the formation of single stereoisomers instead of diastereoisomeric mixtures. A broad anticancer and antiviral evaluation has been performed. Many of the compounds showed a good improvement and/or extension of their biological profile. A great improvement of activity was observed in the case of ddA, ABC and ACV phosphorodiamidates against HIV-1 and 2, while d4T prodrugs showed a good inhibitory activity versus different cancer cell
lines. To the contrary, AICA derivatives showed neither anticancer nor antiviral activity. A metabolic assays using carboxypeptidase Y was performed to support the importance of bioactivation of these compounds to exert their activity. In fact, when the compounds were slowly activated in our assays either a low or no activity was observed in the testing, whereas a rapid bioactivation resulted in an improved biological profile. The phosphorodiamidate approach we report shows considerable promise across the biological spectrum where NAs are active and we believe significantly augment the field of nucleotide prodrugs for use in drug discovery.
Table 3. Inhibitory effects on the proliferation of tumor cell lines in cell culture. IC$_{50} = 50\%$ inhibitory concentration.

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<th>Compd</th>
<th>Nucleoside</th>
<th>AA</th>
<th>R</th>
<th>L1210 IC$_{50}$ (µM)</th>
<th>L1210/TK IC$_{50}$ (µM)</th>
<th>CEM IC$_{50}$ (µM)</th>
<th>CEM/TK IC$_{50}$ (µM)</th>
<th>HeLa IC$_{50}$ (µM)</th>
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<th>Caco-2 IC$_{50}$ (µM)</th>
<th>Colo-320 IC$_{50}$ (µM)</th>
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<td>≥250</td>
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<td>&gt;250</td>
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$^a$Phosphorotetraamidate, $^b$N-Phosphorodiamidate. $^c$No dose response

Data represent the mean ± SD of at least 2 to 3 independent experiments.
### Table 4: Anti HIV-1 and HIV-2 activity (EC$_{50}$) in human T-lymphocyte CEM cells.

<table>
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<tr>
<th>Compd</th>
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<th>R</th>
<th>EC$_{50}$ (µM)</th>
<th>CC$_{50}$ (µM)</th>
</tr>
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<td>2</td>
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</tr>
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<td>17</td>
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<td>3</td>
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*Phosphorotetraamidate, *b*N-Phosphorodiamidate, *c*MT-4 cell.

Data represent the mean ± SD of at least 2 to 3 independent experiments.

### Table 5. HCV replicon assays (type 1b) in Huh 7 cells.

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<th>R</th>
<th>EC$_{50}$ (µM)</th>
<th>CC$_{50}$ (µM)</th>
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<td>I</td>
<td>L-Ala</td>
<td>CH$_2$Bu</td>
<td>0.05</td>
<td>73</td>
</tr>
<tr>
<td>15</td>
<td>I</td>
<td>D-Ala</td>
<td>CH$_2$Bu</td>
<td>0.23</td>
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<td>7</td>
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<td>&gt;100 ref. 21</td>
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<td>&gt;100</td>
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<td>ACV</td>
<td>L-Ala</td>
<td>CH$_2$Bu</td>
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Table 6. Antiviral activity of test compounds against herpes simplex virus type 1 and 2 and feline herpes virus and cytotoxicity (MCC) in HEL and CrFK cell cultures

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<th>AA</th>
<th>R</th>
<th>MCC (µM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM) HEL cell</th>
<th>Herpes simplex virus 1 (KOS)</th>
<th>Herpes simplex virus 2 (G)</th>
<th>Herpes simplex virus 1 TK (KOS)ACV&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Feline herpes virus</th>
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<td>CH&lt;sub&gt;2&lt;/sub&gt;Bu</td>
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</tbody>
</table>
4. Experimental Protocols

4.1 Chemistry

All anhydrous solvents were purchased from Sigma-Aldrich and amino acid esters from Novabiochem. All reagents commercially available were used without further purification. Thin Layer Chromatography (TLC): precoated aluminum 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 High Performance Liquid Chromatography (HPLC) analysis was performed using either a ThermoScientific or a Varian Prostar system. $^1$H NMR (500 MHz), $^{13}$C NMR (125 MHz), $^{31}$P NMR (202 MHz) and $^{19}$F NMR (470 MHz) spectra were recorded on a Bruker Avance 500 MHz spectrometer at 25 ºC. Chemical shifts ($\delta$) are quoted in parts per million (ppm) relative to internal MeOD ($\delta$ 3.34 $^1$H NMR, $\delta$ 49.86 $^{13}$C NMR) and CDCl$_3$ ($\delta$ 7.26 $^1$H NMR, $\delta$ 77.36 $^{13}$C NMR) or external 85% H$_3$PO$_4$ ($\delta$ 0.00 $^{31}$P 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 mass spectrometry was performed on a Bruker Daltonics microTof-LC system, as a service by the School of Chemistry at Cardiff University.

4.1.1 Standard procedure A: synthesis of phosphorodiamidates.

To a stirring solution/suspension of the desired nucleoside (1.00 mol/eq) in anhydrous THF, anhydrous Et$_3$N (1.00-1.20 mol/eq) was added under an argon atmosphere. After
stirring at room temperature for 30 min, POCl₃ (1.00-1.20 mol/eq) was added dropwise at -78 °C. The reaction mixture was stirred at -78 °C for 30 min and then allowed to warm to room temperature. Anhydrous CH₂Cl₂ and the appropriate amino acid ester salt (3.00-5.00 mol/eq) were added, followed dropwise addition of anhydrous Et₃N (5.00-10.00 mol/eq) at -78 °C. The reaction mixture was then stirred at room temperature. After this period, H₂O was added and the aqueous phase was extracted with CH₂Cl₂. The organic phase was washed with brine, dried over anhydrous Na₂SO₄ or MgSO₄, filtered and evaporated to dryness. The residue was purified by silica gel column chromatography using different eluent systems.

4.1.2 Standard procedure B: synthesis of phosphorodiamidates.

To a stirring solution of the desired nucleoside (1.00 mol/eq) in trimethylphosphate or triethylphosphate, POCl₃ (1.00 mol/eq) was added dropwise at -5 °C under an argon atmosphere. The reaction mixture was stirred at -5 °C for 4-5 h, or at 5 °C for 16 h. Anhydrous CH₂Cl₂ and the appropriate amino acid ester salt (5.0 mol/eq) were added, followed by dropwise addition of anhydrous Et₃N (10.0 mol/eq) at 0 °C, or anhydrous DIPEA (10.0 mol/eq) at -78 °C. The reaction mixture was stirred at -78 °C for 30 min and then at room temperature for 16-45 h. After this period, H₂O was added and the aqueous phase was extracted with CH₂Cl₂. The organic phase was washed with brine, dried over anhydrous Na₂SO₄ or MgSO₄, filtered and evaporated to dryness. The residue was purified by silica gel column chromatography using different eluent systems.
4.1.3 Synthesis of 4-Acetylamino-1-[\(\left(2'R,5'S\right)-2'-(\text{hydroxymethyl})-1,3\text{-oxathiolan-5'-yl}\)]-1,2-dihydropyrimidin-2-one (6).

Anhydrous DMF (20 mL) was added to (5) (5.0 g, 21.81 mmol) and the mixture was stirred under nitrogen atmosphere for 5 min at room temperature. Acetic anhydride (2.26 mL, 23.99 mmol) was added dropwise, and the mixture was stirred at room temperature overnight. Then, the solvent was removed under reduced pressure, and the residue was azeotroped with toluene. The crude solid was washed with diethyl ether (3 x 20 mL) to give the desired compound (6) as a white solid (82%, 4.84 g). \(^1\)H NMR (500 MHz, MeOD) \(\delta_H 8.59\) (d, \(J = 7.5\) Hz, 1H, H-6), 7.43 (d, \(J = 7.5\) Hz, 1H, H-5), 6.33 (dd, \(J = 5.3, 3.0\) Hz, 1H, H-1’), 5.36 (t, \(J = 3.6\) Hz, 1H, H-4’), 4.05 (dd, \(J = 12.6, 3.4\) Hz, 1H, H-5’), 3.95 (dd, \(J = 12.6, 3.9\) Hz, 1H, H-5’), 3.65 (dd, \(J = 12.4, 5.4\) Hz, 1H, H-2’), 3.27 (dd, \(J = 12.4, 3.0\) Hz, 1H, H-2’), 2.20 (s, 3H, COCH\(_3\)).

4.1.4 Synthesis of (2\(R\),3\(R\),4\(R\),5\(R\))-5-(2-amino-6-ethoxy-9H-purin-9-yl)-4-fluoro-2-(hydroxymethyl)-4-methyltetrahydrofuran-3-ol-5’-O-bis-(2,2-dimethylpropoxy-L-alaninyl)-phosphate(14).

Prepared according to standard procedure A, using (1) (0.15 g, 0.46 mmol) in anhydrous THF (3 mL), anhydrous Et\(_3\)N (0.06 mL, 0.46 mmol), and POCl\(_3\) (0.04 mL, 0.46 mmol). Anhydrous CH\(_2\)Cl\(_2\) (3 mL) and tosylate salt of 2,2-dimethylpropoxy-L-alanine (0.76 g, 2.29 mmol) were added, followed by dropwise addition of anhydrous Et\(_3\)N (0.64 mL, 4.58 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (0% to 3%) in CHCl\(_3\) to give the product (14) as an off white solid.
(9%, 0.03 g). $^1$H NMR (500 MHz, MeOD) $\delta$ 7.97 (s, 1H, $H-8$), 6.17 (d, $J_{H-F}= 19.0$ Hz, 1H, $H-1'$), 4.57-4.53 (m, 3H, OCH$_2$CH$_3$ and $H-3'$), 4.44-4.36 (m, 2H, $H-5'$), 4.23-4.19 (m, 1H, $H-4'$), 3.99-3.95 (m, 2H, 2x CHCH$_3$), 3.84, 3.82, 3.72, 3.66 (2AB, $J_{AB}= 10.5$ Hz, 4H, 2x OCH$_2$C(CH$_3$)$_3$), 1.45 (t, $J= 7.3$ Hz, 3H, OCH$_2$CH$_3$), 1.39 (d, $J= 7.1$ Hz, 3H, CHCH$_3$), 1.35 (d, $J= 7.1$ Hz, 3H, CHCH$_3$), 1.23 (d, $J_{H-F}= 20.0$ Hz, 3H, CCH$_3$), 0.94 (s, 9H, OCH$_2$C(CH$_3$)$_3$), 0.92 (s, 9H, OCH$_2$C(CH$_3$)$_3$).

$^{13}$C NMR (125 MHz, MeOD) $\delta$ 175.65, 175.60 (2d, $J_{C-P}= 6.3$ Hz, CO), 162.46 (C-6), 161.99 (C-2), 154.45 (C-4), 139.42 (C-8), 115.71 (C-5), 102.01 (d, $J_{C-F}= 180.0$ Hz, C-2'), 90.83 (d, $J_{C-F}= 39.0$ Hz, C-1'), 81.92 (d, $J_{C-P}= 7.5$ Hz, C-4'), 75.38, 75.36 (OCH$_2$C(CH$_3$)$_3$), 74.04 (d, $J_{C-F}= 18.0$ Hz, C-3'), 66.15 (d, $J_{C-P}= 3.8$ Hz, C-5'), 63.58 (OCH$_2$CH$_3$), 51.11 (CHCH$_3$), 32.32, 32.28 (OCH$_2$C(CH$_3$)$_3$), 26.80, 26.77 (OCH$_2$C(CH$_3$)$_3$), 21.11, 20.98 (CHCH$_3$), 16.89 (d, $J_{C-F}= 25.0$ Hz, 2'CCH$_3$), 14.88 (OCH$_2$CH$_3$). $^{31}$P NMR (202 MHz, MeOD) $\delta$ 13.98. $^{19}$F NMR (470 MHz, MeOD) $\delta$ -162.26. MS (ES+) $m/z$: 712.31 (M + Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 90/10 to 0/100 in 30 min, flow= 1mL/min, $\lambda= 254$ nm, $t_R= 20.63$ min.

4.1.5 Synthesis of (2R,3R,4R,5R)-5-(2-amino-6-ethoxy-9H-purin-9-yl)-4-fluoro-2-(hydroxymethyl)-4-methyltetrahydrofuran-3-ol-5'-O-bis-(2,2-dimethoxypropoxy-D-alaninyl)-phosphate (15).

Prepared according to standard procedure A, using (1) (0.15 g, 0.46 mmol) in anhydrous THF (2.4 mL), anhydrous Et$_3$N (0.08 mL, 0.55 mmol), and POC$_3$ (0.05 mL, 0.55 mmol). Anhydrous CH$_2$Cl$_2$ (3.5 mL) and tosylate salt of 2,2-dimethoxypropoxy-D-alanine (0.76 g, 2.30 mmol) were added, followed by dropwise addition of anhydrous Et$_3$N (0.64 mL,
4.60 mmol). The reaction mixture was stirred at room temperature overnight. After work-up, the crude residue was purified by silica gel column chromatography eluting with CHCl₃/MeOH (97.5/2.5) to give the product (15) as an off white solid (7%, 0.02 g). ³H NMR (500 MHz, CDCl₃) δH 7.76 (s, 1H, H-8), 6.04 (d, 1H, J_H-F = 18.7 Hz, H-1’), 5.31 (bs, 1H, NH), 4.90 (bs, 1H, NH), 4.82-4.72 (m, 1H, H-3’), 4.65-4.61 (m, 1H, H-5’), 4.56 (q, 1H, J = 6.6 Hz, OCH₂CH₃), 4.35-4.31 (m, 1H, H-1’), 4.19-4.18 (m, 1H, H-4’), 4.07-3.98 (m, 2H, 2x CH₃), 3.90, 3.88 (AB, 2H, J_AB = 10.4 Hz, OCH₂C(CH₃)₃), 3.71 (AB, 2H, J_AB = 10.4 Hz, OCH₂C(CH₃)₃), 1.27 (d, 3H, J_H-F = 22.7 Hz, 2’CH₃), 0.94 (s, 9H, OCH₂C(CH₃)₃), 0.92 (s, 9H, OCH₂C(CH₃)₃). ¹³C NMR (125 MHz, CDCl₃) δC 175.15 (d, J_C-P = 6.5 Hz, CO), 174.28 (d, J_C-P = 6.8 Hz, CO), 161.32 (C-6), 159.59 (C-2), 153.20 (C-4), 137.37 (C-8), 115.71 (C-5), 101.92, 100.48 (d, J_C-F = 180.0 Hz, C-2’), 89.23 (d, J_C-F = 40.3 Hz, C-1’), 79.97 (d, J_C-P = 7.6 Hz, C-4’), 74.82, 74.55 (OCH₂C(CH₃)₃), 71.93 (C-3’), 63.03 (OCH₂CH₃), 62.71, 62.57 (2d, J_C-P = 17.6 Hz, C-5’), 49.78, 49.43 (CHCH₃), 31.36, 31.23 (OCH₂C(CH₃)₃), 26.33, 26.25, 26.14 (OCH₂C(CH₃)₃), 21.37 (d, J_C-P = 6.1 Hz, CHCH₃), 21.02 (d, J_C-P = 8.1 Hz, CHCH₃), 16.59 (d, J_C-F = 25.2 Hz, 2’CH₃), 14.47 (OCH₂CH₃). ³¹P NMR (202 MHz, CDCl₃) δP 13.86. ¹⁹F NMR (470 MHz, CDCl₃) δF -162.43. MS (ES) m/z: 690.34 (M + H⁺, 100%). Reverse-phase HPLC, eluting with H₂O/CH₃CN from 90/10 to 10/90 in 30 min, flow= 1mL/min, λ = 275 nm, t_R = 20.24 min.

4.1.6 Synthesis of d4T-5’-O-bis(benzoxy-L-alaninyl)-phosphate (16).

Prepared according to standard procedure A, using (2) (0.50 g, 2.23 mmol) in anhydrous THF (5 mL), anhydrous Et₃N (0.31 mL, 2.23 mmol) POCl₃ (0.21 mL, 2.23 mmol).
Anhydrous CH$_2$Cl$_2$ (5 mL) and tosylate salt of benzoxy-L-alanine (2.35 g, 6.69 mmol) were added, followed by dropwise addition of anhydrous Et$_3$N (3.10 mL, 22.30 mmol). The reaction mixture was stirred at room temperature overnight. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (2% to 5%) in CH$_2$Cl$_2$ to yield product (16) as a white solid (11% 0.10 g).

$^1$H NMR (500 MHz, MeOD) δ$^H$ 7.40-7.30 (m, 11H, OCH$_2$Ph, H-5), 6.96-6.94 (m, 1H, H-1’), 6.34-6.32 (m, 1H, H-3’), 5.90-5.93 (m, 1H, H-2’), 5.17-5.10 (m, 4H, 2x OCH$_2$Ph), 4.91 (bs, 1H, H-1’), 4.11-4.09 (m, 2H, H-5’), 3.97-3.89 (m, 2H, 2x CHCH$_3$), 1.88 (s, 3H, CH$_3$), 1.35 (d, J = 7.0 Hz, 3H, CHCH$_3$), 1.33 (d, J = 7.0 Hz, 3H, CHCH$_3$).

$^{13}$C NMR (125 MHz, MeOD) δ$^C$ 175.37 (d, J$_{C-P}$ = 5.0 Hz, CO), 175.22 (d, J$_{C-P}$ = 5.0 Hz, CO), 166.42 (C-4), 152.82 (C-2), 137.96 (C-6), 137.35 (ipso OCH$_2$Ph), 137.33 (ipso OCH$_2$Ph), 134.99 (C-3’), 129.67, 129.41 (OCH$_2$Ph), 127.83 (C-2’), 112.17 (C-5), 91.20 (C-1’), 86.48 (d, J$_{C-P}$ = 8.7 Hz, C-4’), 67.97 (OCH$_2$Ph), 66.44 (d, J$_{C-P}$ = 8.7 Hz, C-5’), 51.17 (d, J$_{C-P}$ = 1.9 Hz, CHCH$_3$), 50.99 (d, J$_{C-P}$ = 1.9 Hz, CHCH$_3$), 20.84 (d, J$_{C-P}$ = 5.5 Hz, CHCH$_3$), 20.66 (d, J$_{C-P}$ = 5.5 Hz, CHCH$_3$), 12.73 (5-CH$_3$).

$^{31}$P NMR (202 MHz, MeOD) δ$_P$ 13.63. MS (ES+) m/z: 649.20 (M + Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 90/10 to 0/100 in 30 min; flow= 1 mL/min, λ= 280 nm, $t_R$= 18.61 min.

### 4.1.7 Synthesis of d4T-5’-O-bis-(cyclohexoxy-L-alaninyl)-phosphate (17).

Prepared according to standard procedure A, using (2) (0.53 g, 2.36 mmol) in anhydrous THF (5 mL), anhydrous Et$_3$N (0.33 mL, 2.36 mmol), and POCl$_3$ (0.22 mL, 2.36 mmol). Anhydrous CH$_2$Cl$_2$ (5 mL) and tosylate salt of cyclohexoxy-L-alanine (4.06 g, 11.82 mmol) were added, followed by dropwise addition of anhydrous Et$_3$N (3.30 mL, 23.63 mL...
mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (2% to 5%) in CH₂Cl₂ to afford the product (17) as a solid (46%, 0.66 g). ³¹H NMR (500 MHz, MeOD) δH 7.44 (s, 1H, H-6), 6.99 (m, 1H, H-1’), 6.45 (dd, J = 6.0, 1.2 Hz, 1H, H-3’), 5.99 (dd, J = 6.0, 1.2 Hz, 1H, H-2’), 5.02 (bs, 1H, H-4’), 4.78-4.74 (m, 2H, 2x OC₃H⁻cHx), 4.21-4.14 (m, 2H, H-5’), 3.90-3.84 (m, 2H, 2x C₃HCH₃), 1.92 (s, 3H, CH₃), 1.90-1.80 (m, 4H, cHx), 1.80-1.70 (m, 4H, cHx), 1.63-1.55 (m, 2H, cHx), 1.54-1.29 (m, 10H, cHx), 1.36 (d, J = 7.0 Hz, 3H, CH₃CCH₃), 1.32 (d, J = 7.0 Hz, 3H, CHCH₃), 1.30 (s, 3H, CH₃), 1.29 (s, 3H, CH₃), 1.28 (s, 3H, CH₃). ¹³C NMR (125 MHz, MeOD) δC 175.05 (d, JCP = 5.0 Hz, CO), 174.89 (d, JCP = 5.0 Hz, CO), 166.37 (C-4), 152.81 (C-2), 137.97 (C-6), 134.98 (C-3’), 127.95 (C-2’), 112.14 (C-5), 91.21 (C-1’), 86.57 (d, JC-P = 8.8 Hz, C-4’), 74.88, 74.86 (2x OCHcHx), 67.44 (d, JC-P = 5.0 Hz, C-5’), 51.22 (d, JC-P = 1.1 Hz, CHCH₃), 50.99 (d, JC-P = 1.1 Hz, CHCH₃), 32.57, 32.56, 32.50, 32.48 (CH₂-cHx), 26.48, 24.75, 24.72, 24.68 (CH₂-cHx), 21.15 (d, JC-P = 5.0 Hz, CHCH₃), 20.95 (d, JC-P = 5.0 Hz, CHCH₃), 12.73 (5-CH₃). ³¹P NMR (202 MHz, MeOD) δP 12.58. MS (ES+) m/z: 633.25 (M+Na⁺, 100%). Reverse-phase HPLC, eluting with H₂O/CH₃CN from 90/10 to 0/100 in 30 min, flow= 1 mL/min, λ= 254 nm, tᵣ = 18.48 min.

4.1.8 **Synthesis of d⁴T-5’-O-bis-(2,2-dimethylpropoxy-L-alaninyl)-phosphate (18).**

Prepared according to standard procedure A, using (2) (0.50 g, 2.23 mmol) in anhydrous THF (5 mL), anhydrous Et₃N (0.31 mL, 2.23 mmol), and POCl₃ (0.21 mL, 2.23 mmol). Anhydrous CH₂Cl₂ (5 mL) and tosylate salt of 2,2-dimethylpropoxy-L-alanine (2.22 g, 6.69 mmol) were added, followed by dropwise addition of anhydrous Et₃N (1.52 mL,
11.15 mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (2% to 5%) in CH₂Cl₂ to afford the product (18) as a white solid (12%, 0.15 g). ¹H NMR (500 MHz, MeOD) δ_H 7.44 (s, 1H, H-6), 7.01-6.90 (m, 1H, H-1’), 6.46-6.40 (m, 1H, H-3’), 6.00-5.90 (m, 1H, H-2’), 5.03 (bs, 1H, H-4’), 4.21-4.14 (m, 2H, H-5’), 3.98-3.92 (m, 2H, 2x CHCH₃), 3.91, 3.89, 3.78, 3.76 (2AB, J_AB = 10.0 Hz, 4H, 2x OCH₂C(CH₃)₃), 1.92 (s, 3H, CH₃), 1.40 (d, J = 7.0 Hz, 6H, 2x CHCCH₃), 0.97 (s, 9H, OCH₂C(CH₃)₃), 0.97 (s, 9H, OCH₂C(CH₃)₃). ¹³C NMR (125 MHz, MeOD) δ_C 175.64 (d, J_C-P = 5.0 Hz, CO), 175.47 (d, J_C-P = 5.0 Hz, CO), 166.41 (C-4), 152.85 (C-2), 137.97 (C-6), 134.98 (C-3’), 127.93 (C-2’), 112.14 (C-5), 91.22 (C-1’), 86.57 (d, J_C-P = 8.7 Hz, C-4’), 75.45 (OCH₂C(CH₃)₃), 67.54 (d, J_C-P = 5.0 Hz, C-5’), 51.19 (d, J_C-P = 1.1 Hz, CHCH₃), 50.82 (d, J_C-P = 1.1 Hz, CHCH₃), 32.36 (OCH₂C(CH₃)₃), 26.87, 26.60 (OCH₂C(CH₃)₃), 21.19 (d, J_C-P = 5.0 Hz, CHCH₃), 21.01 (d, J_C-P = 5.0 Hz, CHCH₃), 12.71 (5-CH₃). ³¹P NMR (202 MHz, MeOD) δ_P 13.73. MS (ES+) m/z: 609.27 (M+Na⁺, 100%). Reverse-phase HPLC, eluting with H₂O/CH₃CN from 90/10 to 0/100 in 30 min, flow = 1 mL/min, λ = 254 nm, t_R = 17.80 min.

4.1.9 Synthesis of ddA-5’-O-bis-(2,2-dimethylpropoxy-L-alaniny1)-phosphate (19).

Prepared according to standard procedure A, using (3) (0.10 g, 0.42 mmol) in anhydrous THF (2 mL), anhydrous Et₃N (0.06 mL, 0.42 mmol), and POCl₃ (0.39 mL, 0.42 mmol). Anhydrous CH₂Cl₂ (3 mL) and tosylate salt of 2,2-dimethylpropoxy (0.70 g, 2.10 mmol) were added, followed by dropwise addition of anhydrous Et₃N (0.29 mL, 2.10 mmol). The reaction mixture was stirred at room temperature for 18 h. After work-up, the crude
residue was purified by silica gel column chromatography eluting with a gradient of MeOH (2% to 5%) in CH$_2$Cl$_2$ to give the product (19) as a white solid (9%, 0.02 g). $^1$H NMR (500 MHz; MeOD) $\delta$H 8.55 (bs, 1H, H-8), 8.39 (bs, 1H, H-2), 6.38 (dd, $J = 6.5$, 3.3 Hz, 1H, H-1’), 4.44-4.39 (m, 1H, H-4’), 4.23-4.19 (m, 1H, H-5’), 4.14-4.10 (m, 1H, H-5’), 3.94-3.87 (m, 2H, 2x C$_2$H$_5$CH$_3$), 3.84, 3.72 (2AB, $J_{AB} = 10.4$ Hz, 4H, OCH$_2$C(CH$_3$)$_3$), 2.67-2.55 (m, 2H, H-2’), 2.21 (m, 2H, H-3’), 1.35 (d, $J = 7.1$ Hz, 6H, 2x CH$_2$H$_3$), 0.93 (s, 18H, 2x OCH$_2$C(CH$_3$)$_3$); $^{31}$P NMR (202 MHz; MeOD) $\delta$P 13.98. Reverse-phase HPLC, eluting with H$_2$O/MeOH from 90/10 to 0/100 in 30 min, flow= 1 mL/min, $\lambda$= 254 nm, $t_R$= 25.91 min.

4.1.10 Synthesis of 3’-azido-3’-deoxythymidine-5’-O-bis(benzoxy-L-alaninyl)-phosphate (20).

Prepared according to standard procedure A, using (4) (0.20 g, 0.75 mmol) in anhydrous THF (5 mL), anhydrous Et$_3$N (0.10 mL, 0.75 mmol), and POCl$_3$ (0.07 mL, 0.75 mmol). Anhydrous CH$_2$Cl$_2$ (10 mL) and tosylate salt of benzoxy-L-alaninyl (1.31 g, 3.74 mmol) were added, followed by dropwise addition of anhydrous Et$_3$N (1.04 mL, 7.48 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (0% to 3%) in CHCl$_3$ to give the product (20) as an off white solid (20%, 0.10 g). $^1$H NMR (500 MHz, MeOD) $\delta$H 7.51 (s, 1H, H-6), 6.16 (t, $J = 6.5$ Hz, 1H, H-1’), 5.17-5.10 (m, 4H, 2x OCH$_2$Ph), 4.38-4.35 (m, 1H, H-3’), 4.19-4.10 (m, 2H, H-5’), 4.02-3.95 (m, 3H, H-4’, 2x CHCH$_3$), 2.36 (m, 2H, H-2’), 1.89 (s, 3H, 5-CH$_3$), 1.39 (d, 3H, $J = 7.0$ Hz, CH$_2$H$_3$), 1.36 (d, 3H, $J = 7.0$ Hz, CH$_2$H$_3$). $^{13}$C (125 MHz, MeOD) $\delta$C 175.42 (d, $J_{C-P}$
= 3.8 Hz, CO), 175.35 (d, J_{C-P} = 6.3 Hz, CO), 166.25 (C-4), 152.22 (C-2), 137.69 (C-6),
137.29, 137.27 (ipso OCH_{2}Ph), 129.96, 129.68, 129.66, 129.44, 129.42, 129.38, 129.35,
129.12 (OCH_{2}Ph), 112.16 (C-5), 86.24 (C-1’), 83.85 (d, J_{C-P} = 8.8 Hz, C-4’), 68.03,
68.00 (OCH_{2}Ph), 66.04 (d, J_{C-P} = 5.0 Hz, C-5’), 61.85 (C-3’), 51.21, 51.16 (CHCH_{3}),
37.68 (C-2’), 20.85, 20.73 (2d, J_{C-P} = 6.3 Hz, CHCH_{3}), 12.71 (5-CH_{3}). ^{31}P NMR (202
MHz, MeOD) \delta_{P} 13.69. MS (ES+) m/z: 692.22 (M + Na^+, 100%). Reverse-phase HPLC,
eluting with H_{2}O/CH_{3}CN from 90/10 to 0/100 in 30 min, flow= 1mL/min, \lambda = 254 nm,
t_{R}= 20.83 min.

4.1.11 Synthesis of 3’-azido-3′-deoxythymidine-5′-O-bis(2,2-dimethylpropoxy)-phosphate
(21).

Prepared according to standard procedure A, using (4) (0.20 g, 0.75 mmol) in anhydrous
THF (5 mL), anhydrous Et_{3}N (0.10 mL, 0.75 mmol), and POCl_{3} (0.07 mL, 0.75 mmol).
Anhydrous CH_{2}Cl_{2} (10 mL) and tosylate salt of 2,2-dimethylpropoxy-L-alaninyl (1.24 g,
3.74 mmol) were added, followed by dropwise addition of anhydrous Et_{3}N (1.04 mL,
7.48 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-
up, the crude residue was purified by silica gel column chromatography eluting with a
gradient (0% to 3%) of MeOH in CHCl_{3} to give the product (21) as an off white solid
(25%, 0.10 g). ^{1}H NMR (500 MHz, MeOD) \delta_{H} 7.57 (s, 1H, H-6), 6.21 (t, J = 7.0 Hz, 1H,
H-1’), 4.48-4.45 (m, 1H, H-3’), 4.24-4.21 (m, 2H, H-5’), 4.08-4.06 (m, 1H, H-4’), 4.03-
3.96 (m, 2H, 2x CHCH_{3}), 3.91, 3.90, 3.79, 3.77 (2AB, 4H, J_{AB} = 10.5 Hz, 2x
OCH_{2}C(CH_{3})_{3}), 2.48-2.44 (m, 2H, H-2’), 1.94 (s, 3H, 5-CH_{3}), 1.45 (d, J = 7.0 Hz, 3H, 2x
CHCH_{3}), 1.44 (d, J = 7.0 Hz, 6H, 2x CHCH_{3}). ^{13}C NMR (125 MHz, MeOD) \delta_{C} 175.66
(d, $J_{C-P} = 3.8$ Hz, CO), 175.62 (d, $J_{C-P} = 6.3$ Hz, CO), 166.25 (C-4), 152.25 (C-2), 137.79 (C-6), 112.15 (C-5), 86.27 (C-1’), 83.90 (d, $J_{C-P} = 7.5$ Hz, C-4’), 75.47, 75.44 (OCH$_2$C(CH$_3$)$_3$), 66.09 (d, $J_{C-P} = 5.0$ Hz, C-5’), 61.90 (C-3’), 51.21, 51.12 (CHCH$_3$), 37.69 (C-2’), 30.78 (OCH$_2$C(CH$_3$)$_3$), 26.85, 26.70 (OCH$_2$C(CH$_3$)$_3$), 21.16 (d, $J_{C-P} = 5.0$ Hz, CHCH$_3$), 21.04 (d, $J_{C-P} = 6.3$ Hz, CHCH$_3$), 12.70 (5-C$_H$$_3$).

$^{31}$P NMR (202 MHz, MeOD) $\delta$P 13.79. MS (ES+) $m/z$: 652.59 (M + Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 90/10 to 0/100 in 30 min, flow= 1mL/min, $\lambda$= 254 nm, $t_R$= 19.93 min.

4.1.12 Synthesis of 3TC(-)’-O-bis(benzoxy-L-alaninyl)-N-bis(benzoxy-L-alaninyl)-diphosphate (22) and 3TC(-)-N-bis(benzoxy-L-alaninyl)-phosphate (23)

Prepared according to standard procedure A, using (5) (2.29 g, 10.00 mmol) in anhydrous THF (25 mL), anhydrous Et$_3$N (1.39 mL, 10.00 mmol), and POCl$_3$ (0.93 mL, 10.00 mmol). Anhydrous CH$_2$Cl$_2$ (25 mL) and tosylate salt of L-alanine benzyl ester (17.57 g, 50.00 mmol) were added, followed by dropwise addition of anhydrous Et$_3$N (14.0 mL, 100.00 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (1% to 10%) in CH$_2$Cl$_2$, followed by a second purification eluting with CH$_2$Cl$_2$/MeOH 95/5 to give compound (22) as an off-white solid (3%, 0.18 g). $^1$H NMR (500 MHz; MeOD) $\delta$H 8.13 (d $J$ = 7.6 Hz, 1H, H-6), 7.39-7.28 (m, 20H, 4 x OCH$_2$Ph), 6.29-6.22 (m, 2H, H-5, H-1’), 5.31-5.29 (m, 1H, H-4’), 5.19-5.11 (m, 6H, 3 x CH$_2$OPh), 5.08 (s, 2H, CH$_2$OPh), 4.28-4.22 (m, 2H, H-5’), 4.10-4.08 (m, 2H, 2x CHCH$_3$), 4.04-3.81 (m, 2H, 2x CHCH$_3$), 3.48 (dd, $J$ = 12.6, 4.4 Hz, 1H, H-2’), 3.01 (dd,
$J = 12.6, 4.4 \text{ Hz}, 1H, H-2'$), 1.38-1.41 (m, 12H, 4 x CHCH$_3$). $^{13}$C NMR (125 MHz; MeOD) $\delta$C 175.49, 175.12 (CO), 163.75 (C-4), 156.36 (C-2), 143.72 (C-6), 137.36, 137.34, 137.32, 137.30 (ipso OCH$_2$Ph), 129.66, 129.65, 129.62, 129.42, 129.38, 129.36, 129.26, 129.05 (OCH$_2$Ph), 98.06 (C-1'), 89.15 (C-5), 85.53 (d, $J_{C-P} = 8.7 \text{ Hz}$, C-5'), 68.01, 67.97, 67.96, 67.77 (OCH$_2$Ph), 66.94 (d, $J_{C-P} = 5.0 \text{ Hz}$, C-5'), 51.25, 51.13, 51.00, 50.94 (CHCH$_3$), 38.40 (C-2'), 20.83, 20.79, 20.76, 20.70 (CHCH$_3$). $^{31}$P NMR (202 MHz, MeOD) $\delta$P 13.56, 7.39. MS (ES+) $m/z$: 1056.31 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 90/10 to 0/100 in 30 min, flow= 1mL/min, $\lambda = 254 \text{ nm}$, $t_R = 16.34 \text{ min}$.

Further elution with CH$_2$Cl$_2$/MeOH 95/5 afforded (23) off-white solid (2%, 0.09 g).

$^1$H NMR (500 MHz; MeOD) $\delta$H 8.29 (d, $J = 7.3 \text{ Hz}$, 1H, H-6), 7.39-7.29 (m, 10H, 2x OCH$_2$Ph), 6.29 (dd, $J = 5.3, 3.1 \text{ Hz}$, 1H, H-1'), 6.20 (d, $J = 7.3 \text{ Hz}$, 1H, H-5), 5.32-5.30 (m, 1H, H-4'), 5.17 (s, 2H, CH$_2$OPh), 5.08 (s, 2H, CH$_2$OPh), 4.08 (m, 2H, 2x CH$_2$OPh), 4.05 (dd, $J = 12.2, 4.2 \text{ Hz}$, 1H, H-5'), 3.92 (dd, $J = 12.3, 4.2 \text{ Hz}$, 1H, H-5'), 3.59 (dd, $J = 12.6, 4.3 \text{ Hz}$, 1H, H-2'), 3.12 (dd, $J = 12.6, 4.3 \text{ Hz}$, 1H, H-2'), 1.42-1.38 (m, 6H, 2x CHCH$_3$). $^{13}$C NMR (125 MHz; MeOD) $\delta$C 175.23 (d, $J_{C-P} = 6.2 \text{ Hz}$, CO), 166.79 (C-4), 157.76 (C-2), 144.15 (C-6), 137.34 (ipso CH$_2$OPh), 129.60, 129.34, 129.32, 129.25, 129.07 (CH$_2$OPh), 97.26 (C-5), 89.15 (C-4'), 89.04 (C-1'), 67.96, 67.77 (CH$_2$OPh), 63.59 (C-5'), 51.01, 50.91 (CHCH$_3$), 39.08 (C-2'), 20.69 (d, $J_{C-P} = 6.2 \text{ Hz}$, CHCH$_3$). $^{31}$P NMR (202 MHz, MeOD) $\delta$P 7.40. MS (ES+) $m/z$: 654.18 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 90/10 to 0/100 in 30 min, flow= 1mL/min, $\lambda = 254 \text{ nm}$, $t_R = 14.34 \text{ min}$.  

34
4.1.13 Synthesis of N-acetyl-3TC-5′-O-bis(benzoxy-L-alaninyl)-phosphate (24).

Prepared according to standard procedure B, using (6) (0.50 g, 1.84 mmol) in trimethylphosphate (5 mL), and POCl₃ (0.18 mL, 1.84 mmol), the reaction mixture was stirred at 0 °C for 5 h. Anhydrous CH₂Cl₂ (5 mL) and tosylate salt of benzoxy-L-alanine (3.24 g, 9.22 mmol) were added, followed by dropwise addition of anhydrous DIPEA (3.21 mL, 18.43 mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (2% to 5%) in CH₂Cl₂ to give the product (24) as a white solid (1%, 0.01 g). ¹H NMR (500 MHz, MeOD) δH 8.26 (d, J = 7.5 Hz, 1H, H-6), 7.46 (d, J = 7.6 Hz, 1H, H-5), 7.35-7.33 (m, 10H, OCH₂Ph), 6.27 (dd, J = 5.3, 3.8 Hz, 1H, H-1’), 5.36-5.35 (m, 1H, H-4’), 5.17-5.11 (m, 4H, 2x CH₂OPh), 4.33-4.25 (m, 2H, H-5’), 4.03-3.98 (m, 2H, 2x CH₃), 3.59 (dd, J = 12.4, 4.6 Hz, 1H, H-2’), 3.17 (dd, J = 12.4, 4.6 Hz, 1H, H-2’), 2.19 (s, 3H, COCH₃), 1.41 (dd, J = 7.2, 0.8 Hz, 3H, CHCH₃), 1.38 (dd, J = 7.2, 0.6 Hz, 3H, CHCH₃). ³¹P NMR (202 MHz, MeOD) δP 13.75. MS (ES+) m/z: 674.20 (M+H⁺, 100%).


Prepared according to standard procedure B, using (5) (0.42 g, 1.84 mmol) in triethylphosphate (5 mL), and POCl₃ (0.18 mL, 1.84 mmol), the reaction mixture was stirred at 5 °C for 16 h. Anhydrous CH₂Cl₂ (5 mL) and tosylate salt of benzoxy-L-alanine (3.24 g, 9.20 mmol) were added, followed by dropwise addition of anhydrous DIPEA (3.21 mL, 18.43 mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography
eluting with a gradient of MeOH (2% to 7%) in CH₂Cl₂ to give the product (25) as an off white solid (6%, 0.07 g). ¹H NMR (500 MHz, MeOD) δH 7.72 (d, J = 7.6 Hz, 1H, H-6), 7.41-7.31 (m, 10H, OCH₂Ph), 6.41 (dd, J = 5.6, 2.9 Hz, 1H, H-1’), 5.85 (d, J = 6.3 Hz, 1H, H-5), 5.18-5.13 (m, 4H, 2x OCH₂Ph), 5.06-5.09 (m, 1H, H-4’), 4.09-3.92 (m, 3H, H-5’, CHCH₃), 2.72-2.60 (m, 2H, H-2’), 1.41-1.33 (m, 6H, 2x CHCH₃). ¹³C NMR (125 MHz, MeOD) δC 175.31 (d, Jc-p = 6.2 Hz, CO), 167.59 (C-4), 157.15 (C-2), 142.08 (C-6), 137.32 (ipso OCH₂Ph), 129.64, 129.54, 129.36, 129.30, 129.24 (OCH₂Ph), 96.52 (C-1’), 98.80 (d, Jc-p = 7.7 Hz, C-4’), 96.32 (C-5), 67.95, 67.88 (OCH₂Ph), 58.40 (d, Jc-p = 5.0 Hz, C-5’), 54.84 (CHCH₃), 30.13 (C-2’), 20.83 (d, Jc-p = 6.2 Hz, CHCH₃). ³¹P NMR (202 MHz, MeOD) δP 11.24. MS (ES+) m/z: 654.17 (M+Na⁺, 100%). Reverse-phase HPLC, eluting with H₂O/CH₃CN from 90/10 to 0/100 in 30 min, flow= 1 mL/min, λ= 254 nm, tᵣ= 14.64 min.

4.1.15 Synthesis of 4′-azidouridine-5′-O-bis(benzoxy-L-alaninyl)-phosphate (26).

Prepared according to standard procedure B, using (7) (0.25 g, 0.88 mmol) in triethylphosphate (1 mL), and POCl₃ (0.08 mL, 0.88 mmol), the reaction mixture was stirred at 5 °C for 16 h. Anhydrous CH₂Cl₂ (5 mL) and tosylate salt of benzoxy-L-alanine (1.54 g, 4.38 mmol) were added, followed by dropwise addition of anhydrous Et₃N (1.22 mL, 8.77 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (0% to 3%) in CHCl₃ to give the product (26) as an off white solid (20%, 0.10 g). ¹H NMR (500 MHz, MeOD) δH 7.51 (d, J = 8.0 Hz, 1H, H-5), 7.38-7.30 (m, 10 H, 2x OCH₂Ph), 6.14 (d, J = 4.0 Hz, 1H, H-1’), 5.80 (d, J = 8.0 Hz, 1H, H-
5), 5.18-5.09 (m, 4H, 2x OCH₂Ph), 4.44-4.38 (m, 2H, H-3’, H-2’), 4.10-4.02 (m, 2H, H-5’), 4.02-3.95 (m, 2H, 2x CHCH₃), 1.39 (d, 3H, J = 7.0 Hz, CHCH₃), 1.36 (d, 3H, J = 7.0 Hz, CHCH₃). ¹³C NMR (125 MHz, MeOD) δC 175.40 (d, J_{C-P} = 3.8 Hz, CO), 175.36 (d, J_{C-P} = 6.3 Hz, CO), 165.89 (C-4), 152.27 (C-2), 142.89 (C-6), 137.27, 137.25 (ipso OCH₂Ph), 129.67, 129.65, 129.40, 129.37, 129.33, 129.12 (OCH₂Ph), 103.83 (C-5), 98.94 (d, J_{C-P} = 8.8 Hz, C-4’), 92.58 (C-1’), 73.76, 73.58 (C-3’, C-2’), 68.07, 68.04 (OCH₂Ph), 67.60 (d, J_{C-P} = 3.8 Hz, C-5’), 51.16, 51.12 (CHCH₃), 20.85 (d, J_{C-P} = 6.3 Hz, CHCH₃), 20.65 (d, J_{C-P} = 6.3 Hz, CHCH₃). ³¹P NMR (202 MHz, MeOD) δP 13.57. MS (ES+) m/z: 710.18 (M+Na⁺, 100%). Reverse-phase HPLC, eluting with H₂O/CH₃CN from 90/10 to 0/100 in 30 min, flow= 1mL/min, λ= 254 nm, tᵣ= 15.97 min.

4.1.16 Synthesis of 4’-azidouridine-5’-O-bis(2,2-dimethylpropoxy-L-alaninyl)-phosphate (27).
Prepared according to standard procedure B, using (7) (0.25 g, 0.88 mmol) in triethylphosphate (1 mL), and POCl₃ (0.08 mL, 0.88 mmol), the reaction mixture was stirred at 5 ⁰C for 16 h. Anhydrous CH₂Cl₂ (5 mL) and tosylate salt of 2,2-dimethylpropoxy-L-alanine (1.45 g, 4.38 mmol) were added, followed by dropwise addition of anhydrous Et₃N (1.22 mL, 8.77 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (0% to 5%) in CHCl₃ to give the product (27) as an off white solid (17%, 0.10 g). ¹H NMR (500 MHz, MeOD) δH 7.55 (d, J = 8.0 Hz, 1H, H-5), 6.15 (d, J = 3.5 Hz, 1H, H-1’), 5.83 (d, J = 8.0 Hz, 1H, H-5), 4.45-4.41 (m, 2H, H-3’, H-2’), 4.13-4.06 (m, 2H, H-5’), 4.03-3.96 (m, 2H, 2x CHCH₃),
3.91, 3.79 (AB, 4H, J_{AB} = 10.5 Hz, 2x OCH_2C(CH_3)_3), 1.44, (d, J = 7.0 Hz, 3H, CHCH_3), 1.43 (d, J = 7.0 Hz, 3H, CHCH_3), 0.98 (s, 18H, 2x OCH_2C(CH_3)_3). 13C NMR (125 MHz, MeOD) δ_C 175.65 (d, J_{C-P} = 5.0 Hz, CO), 175.61 (d, J_{C-P} = 6.3 Hz, CO), 165.89 (C-4), 152.29 (C-2), 142.97 (C-6), 103.85 (C-5), 98.93 (d, J_{C-P} = 10.0 Hz, C-4’), 92.56 (C-1’), 75.53, 75.49 (OCH_2C(CH_3)_3), 73.78, 73.63 (C-3’, C-2’), 67.70 (d, J_{C-P} = 3.8 Hz, C-5’), 51.16, 51.09 (CHCH_3), 32.37, 32.36 (OCH_2C(CH_3)_3), 21.17 (d, J_{C-P} = 5.0 Hz, CHCH_3), 20.98 (d, J_{C-P} = 7.5 Hz, CHCH_3). 31P NMR (202 MHz, MeOD) δ_P 13.57. MS (ES+) m/z: 670.25 (M+Na^+, 100%). Reverse-phase HPLC, eluting with H_2O/CH_3CN from 90/10 to 0/100 in 30 min, flow= 1mL/min, λ= 254 nm, t_R= 17.55 min.

4.1.17 Synthesis of 4’-azidocytidine-5’-O-bis(benzyoxy-L-alaninyl)-phosphate (28).

Prepared according to standard procedure B, using (8) (0.25 g, 0.75 mmol) in trimethylphosphate (1 mL), and POCl_3 (0.07 mL, 0.75 mmol), the reaction mixture was stirred at 5 °C for 16 h. Anhydrous CH_2Cl_2 (5 mL) and tosylate salt of benzyoxy-L-alanine (1.32 g, 3.75 mmol) were added, followed by dropwise addition of anhydrous Et_3N (1.04 mL, 7.50 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (0% to 5%) in CHCl_3 to give the product (28) as an off white solid (16%, 0.08 g). 1H NMR (500 MHz, MeOD) δ_H 7.60 (d, J = 7.5 Hz, 1H, H-5), 7.27-7.19 (m, 10H, 2x OCH_2Ph), 6.00 (d, 1H, J = 4.5 Hz, H-1’), 5.85 (d, 1H, J = 7.5 Hz, H-5), 5.07-4.99 (m, 4H, 2x OCH_2Ph), 4.28 (d, J = 6.0 Hz, 1H, H-3’), 4.25 (dd, J = 6.0, 4.0 Hz, 1H, H-2’), 4.00-3.93 (m, 2H, H-5’), 3.90-3.84 (m, 2H, 2x CHCH_3), 1.26 (d, J = 7.0 Hz, 3H, CHCH_3). 13C NMR (125 MHz, MeOD) δ_C 175.83
(d, $J_{C-P} = 5.0$ Hz, CO), 175.37 (d, $J_{C-P} = 6.3$ Hz, CO), 167.71 (C-4), 158.22 (C-2), 143.38 (C-6), 137.24, 137.22 (ipso OCH$_2$Ph), 129.71, 129.68, 129.45, 129.40, 129.37, 129.12 (OCH$_2$Ph), 97.98 (d, $J_{C-P} = 9.0$ Hz, C-4’), 96.98 (C-5), 93.84 (C-1’), 74.52 (C-2’), 73.33 (C-3’), 67.61 (d, $J_{C-P} = 5.0$ Hz, C-5’), 51.16, 51.12 (CHCH$_3$), 20.84 (d, $J_{C-P} = 5.0$ Hz, CHCH$_3$), 20.73 (d, $J_{C-P} = 5.0$ Hz, CHCH$_3$). $^{31}$P NMR (202 MHz, MeOD) $\delta_P$ 13.53. MS (ES+) $m/z$: 709.20 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 90/10 to 0/100 in 30 min, flow= 1mL/min, $\lambda = 254$ nm, $t_R = 15.11$ min.

4.1.18 Synthesis of 4’-azidocytidine-5′-O-bis(2,2-dimethylpropoxy-L-alaninyl)-phosphate (29).

Prepared according to standard procedure B, using (8) (0.25 g, 0.75 mmol) in trimethylphosphate (1 mL), and POCl$_3$ (0.07 mL, 0.75 mmol), the reaction mixture was stirred at 5 °C for 16 h. Anhydrous CH$_2$Cl$_2$ (5 mL) and tosylate salt of 2,2-dimethylpropoxy-L-alanine (1.24 g, 3.75 mmol) were added, followed by dropwise addition of anhydrous Et$_3$N (1.04 mL, 7.50 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (0% to 5%) in CHCl$_3$ to give the product (29) as an off white solid (21%, 0.10 g). $^1$H NMR (500 MHz, MeOD): $\delta_H$ 7.76 (d, 1H, $J = 7.5$ Hz, H-5), 6.15 (d, 1H, $J = 4.0$ Hz, H-1’), 6.00 (d, 1H, $J = 7.5$ Hz, H-5), 4.41 (d, 1H, $J = 6.0$ Hz, H-3’), 4.36 (dd, 1H, $J = 6.0$, 4.5 Hz, H-2’), 4.16-4.08 (m, 2H, H-5’), 4.05-3.96 (m, 2H, 2x CHCH$_3$), 3.91, 3.90, 3.79, 3.78 (2AB, 4H, $J_{AB} = 10.5$ Hz, 2x OCH$_2$C(CH$_3$)$_3$), 1.44 (d, 6H, $J = 7.0$ Hz, 2x CHCH$_3$), 0.98 (s, 18H, 2x OCH$_2$C(CH$_3$)$_3$).

$^{13}$C NMR (125 MHz, MeOD) $\delta_C$ 176.74 (d, $J_{C-P} = 3.8$ Hz, CO), 175.63 (d, $J_{C-P} = 5.0$ Hz,
CO), 167.72 (C-4), 158.18 (C-2), 143.38 (C-6), 98.90 (d, $J_{C-P}$ = 9.0 Hz, C-4'), 96.98 (C-5), 93.96 (C-1'), 75.47, 75.46 (OCH$_2$C(CH$_3$)$_3$), 74.39 (C-2'), 73.32 (C-3'), 67.69 (d, $J_{C-P}$ = 5.0 Hz, C-5'), 51.16, 51.12 (CHCH$_3$), 32.34, 32.33 (OCH$_2$C(CH$_3$)$_3$), 26.79 (OCH$_2$C(CH$_3$)$_3$), 21.07 (d, $J_{C-P}$ = 5.0 Hz, CHCH$_3$), 20.94 (d, $J_{C-P}$ = 7.5 Hz, CHCH$_3$). $^{31}$P NMR (202 MHz, MeOD) $\delta_P$ 13.59. MS (ES+) m/z: 669.27 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 90/10 to 0/100 in 30 min, flow= 1mL/min, $\lambda$ = 254, $t_R$ = 16.59.

4.1.19 Synthesis of ribavirin-5'-O-bis(benzoxy-L-alaninyl)-phoshate (30).

Prepared according to standard procedure B, using (9) (0.30 g, 1.23 mmol) in trimethylphosphate (5 mL), and POCl$_3$ (0.11 mL, 1.23 mmol), the reaction mixture was stirred at -5 °C for 4 h. Anhydrous CH$_2$Cl$_2$ (5 mL), and a suspension of tosylate salt of benzoxy-L-alanine (2.16 g, 6.15 mmol) in anhydrous CH$_2$Cl$_2$ (5 mL) were added, followed by dropwise addition of anhydrous DIPEA (2.16 mL, 12.30 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (4% to 10%) in CH$_2$Cl$_2$ to give the product (30) as an off white solid (25%, 0.20 g). $^1$H NMR (500 MHz, MeOD) $\delta_H$ 8.69 (s, 1H, H-5), 7.36-7.31 (m, 10H, 2x OCH$_2$Ph), 5.95-5.94 (m, 1H, H-1'), 5.17-5.08 (m, 4H, 2x OCH$_2$Ph), 4.55-4.54 (m, 1H, H-2'), 4.45-4.43 (m, 1H, H-3'), 4.22-4.07 (m, 3H, H-4', H-5'), 3.98-3.91 (m, 2H, 2x CHCH$_3$), 1.34 (d, $J$ = 7.2 Hz, 3H, CHCH$_3$), 1.29 (d, $J$ = 7.0 Hz, 3H, CHCH$_3$). $^{13}$C NMR (125 MHz, MeOD) $\delta_C$ 175.40, 175.36 (CO), 163.24 (CONH$_2$), 158.71 (C-3), 146.88 (C-5), 137.33, 137.35 (ipso OCH$_2$Ph), 129.59, 129.35, 129.32, 129.31 (OCH$_2$Ph), 93.67 (C-1'), 84.93 (d, $J_{C-P}$ = 7.7 Hz, C-4'), 76.50 (C-2'), 71.83 (C-3'), 67.94, 67.91 (OCH$_2$Ph), 66.36 (d, $J_{C-}
$p = 5.1$ Hz, $\delta^p = 5.1$ Hz, $C-5'$), 51.09, 51.08 (CHCH$_3$), 20.74 (d, $J_{C-P} = 6.1$ Hz, CHCH$_3$), 20.59 (d, $J_{C-P} = 6.4$ Hz, CHCH$_3$). $^{31}$P NMR (202 MHz, MeOD) $\delta^p$ 13.75. MS (ES+) $m/z$: 669.20 (M+Na*, 100%). Reverse-phase HPLC, eluting with H$_2$O/MeOH from 90/100 to 0/100 in 30 min, flow= 1 mL/min, $\lambda$ = 254 nm, $t_R$ = 13.88 min.

4.1.20 Synthesis of ribavirin-5'-O-bis(2,2-dimethylpropoxy-L-alaninyl)-phoshate (31).

Prepared according to standard procedure B, using (9) (0.30 g, 1.23 mmol) in trimethylphosphate (5 mL), and POCl$_3$ (0.11 mL, 1.23 mmol), the reaction mixture was stirred at -5 $^\circ$C for 4 h. Anhydrous CH$_2$Cl$_2$ (5 mL) and a suspension of tosylate salt of 2,2-dimethylpropoxy-L-alanine (2.04 g, 6.15 mmol) in anhydrous CH$_2$Cl$_2$ (5 mL) were added, followed by dropwise addition of anhydrous DIPEA (2.16 mL, 12.30 mmol). The reaction mixture was stirred at room temperature for 20 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient (4% to 10%) of MeOH in CH$_2$Cl$_2$ to give the product (31) as an off white solid (21%, 0.16 g). $^1$H NMR (500 MHz, MeOD) $\delta^H$ 8.73 (s, 1H, H-5), 5.97 (m, 1H, H-1'), 4.56-4.55 (m, 1H, H-2'), 4.48-4.46 (m, 1H, H-3'), 4.28-4.13 (m, 3H, H-4', H-5'), 4.00-3.92 (m, 2H, 2x CHCH$_3$), 3.89-3.75 (m, 4H, 2x OC$_2$H$_2$C(CH$_3$)$_3$), 1.40-1.37 (6H, m, 2x CHC$_3$), 0.96 (s, 9H, 2x OCH$_2$C(CH$_3$)$_3$), 0.95 (s, 9H, 2x OCH$_2$C(CH$_3$)$_3$), 13C NMR (125 MHz, MeOD) $\delta^C$ 174.30 (d, $J_{C-P} = 4.1$ Hz, CO), 174.20 (d, $J_{C-P} = 3.2$ Hz, CO), 161.80 (CONH$_2$), 157.30 (C-3), 145.47 (C-5), 92.29 (C-1'), 83.53 (d, $J_{C-P} = 7.4$ Hz, C-4'), 74.81 (C-2'), 73.99, 73.96 (OCH$_2$C(CH$_3$)$_3$), 70.47 (C-3'), 65.11 (d, $J_{C-P} = 5.3$ Hz, C-5'), 49.68 (CHCH$_3$), 30.91, 30.90 (OCH$_2$C(CH$_3$)$_3$), 23.38 (OCH$_2$C(CH$_3$)$_3$), 19.66 (d, $J_{C-P} = 5.6$ Hz, CHCH$_3$), 19.54 (d, $J_{C-P} = 5.9$ Hz, CHCH$_3$). $^{31}$P NMR (202 MHz, MeOD) $\delta^p$ 13.84. MS (ES+) $m/z$:
607.28 (M+H\(^+\), 100%). Reverse-phase HPLC, eluting with H\(_2\)O/MeOH from 90/100 to 0/100 in 30 min, flow= 1 mL/min, \(\lambda= 254\) nm, \(t_R= 21.27\) min.

4.1.21 Synthesis of acyclovir-5'-O-bis[(benzoxyl-L-alaninyl)]-phosphate (32).

Prepared according to standard procedure B, using (\(\text{10}\)) (0.30 g, 1.33 mmol) in trimethylphosphate (5 mL), and POCl\(_3\) (0.12 mL, 1.33 mmol), the reaction mixture was stirred at -5 °C for 4 h. Anhydrous CH\(_2\)Cl\(_2\) (5 mL) and a suspension of tosylate salt of benzoxyl-L-alanine (2.34 g, 6.65 mmol) in anhydrous CH\(_2\)Cl\(_2\) (5 mL) were added, followed by dropwise addition of anhydrous DIPEA (2.32 mL, 13.30 mmol). The reaction mixture was stirred at room temperature for 45 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with gradient (2% to 10%) of MeOH in CH\(_2\)Cl\(_2\) to give a white solid, which was dissolved in CH\(_2\)Cl\(_2\) and washed with 0.05 N HCl (1 x 5 mL), brine (1 x 5 mL), 5% NaHCO\(_3\) (1 x 5 mL), brine (1 x 5 mL), dried over MgSO\(_4\), filtered and concentrated to give the product (32) as a white solid (26%, 0.22 g).

\(^1\)H NMR (500 MHz, MeOD) \(\delta_H\) 7.81 (s, 1H, H-8), 7.34-7.26 (m, 10H, 2x OCH\(_2\)Ph), 5.41 (s, 2H, H-1’), 5.12-5.10 (m, 4H, 2x OCH\(_2\)Ph), 4.02-3.93 (m, 4H, H-5’, 2x CHCH\(_3\)), 3.64 (t, \(J = 4.2\) Hz, 2H, H-4’), 1.35 (d, \(J = 7.1\) Hz, 3H, CHCH\(_3\)), 1.32 (d, \(J = 7.1\) Hz, 3H, CHCH\(_3\)). \(^{13}\)C NMR (125 MHz, MeOD) \(\delta_C\) 174.13 (d, \(J_{C-P} = 5.1\) Hz, CO), 158.14 (C-6), 154.34 (C-2), 151.90 (C-4), 138.35 (C-8), 135.87, 135.85 (ipso OCH\(_2\)Ph), 128.57, 128.24, 128.21, 127.99, 127.95, 127.92 (OCH\(_2\)Ph), 116.26 (C-5), 72.36 (C-1’), 68.16 (d, \(J_{C-P} = 7.3\) Hz, C-4’), 66.60, 66.57 (OCH\(_2\)Ph), 64.29 (d, \(J_{C-P} = 5.3\) Hz, C-5’), 49.76, 49.74 (CHCH\(_3\)), 19.50 (d, \(J_{C-P} = 6.2\) Hz, CHCH\(_3\)), 19.40 (d, \(J_{C-P} = 6.3\) Hz, CHCH\(_3\)). \(^{31}\)P NMR
(202 MHz, MeOD) $\delta_P$ 13.53. MS (ES+) $m/z$: 650.20 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 100/0 to 0/100 in 30 min, flow = 1 mL/min, $\lambda$ = 254 nm, $t_R$ = 16.65 min.

4.1.22 Synthesis of acyclovir-5′-O-bis[(2,2-dimethylpropoxy-L-alaninyl)]-phosphate (33).

Prepared according to standard procedure B, using (10) (0.30 g, 1.33 mmol) in trimethylphosphate (5 mL), and POCl$_3$ (0.12 mL, 1.33 mmol), the reaction mixture was stirred at -5 oC for 4 h. Anhydrous CH$_2$Cl$_2$ (5 mL) and a suspension of tosylate salt of 2,2-dimethylpropoxy-L-alanine (2.20 g, 6.65 mmol) in anhydrous CH$_2$Cl$_2$ (7 mL) were added, followed by dropwise addition of anhydrous DIPEA (2.32 mL, 13.30 mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient (3% to 10%) of MeOH in CH$_2$Cl$_2$. The product was dissolved in CH$_2$Cl$_2$ and washed with 0.05 N HCl (1 x 5 mL), brine (1 x 5 mL), 5% NaHCO$_3$ (1 x 5 mL), brine (1 x 5 mL), dried over MgSO$_4$, filtered and concentrated to give the product (33) as a white solid (22%, 0.17 g).

$^1$H NMR (500 MHz, MeOD) $\delta_H$ 7.92 (s, 1H, H-8), 5.52 (s, 2H, H-1′), 4.12-4.10 (m, 2H, H-5′), 3.98-3.95 (m, 2H, 2x CH$_3$), 3.89-3.86 (m, 2H, 2x C$_2$H$_5$C(CH$_3$)$_3$), 3.79-3.76 (m, 3H, H-4′, OCH$_2$C(CH$_3$)$_3$), 1.42-1.39 (m, 6H, 2x CHCH$_3$), 0.96 (s, 9H, OCH$_2$C(CH$_3$)$_3$), 0.95 (s, 9H, OCH$_2$C(CH$_3$)$_3$). $^{13}$C NMR (125 MHz, MeOD) $\delta_C$ 174.77 (d, $J_{C-P}$ = 5.9 Hz, CO), 174.72 (d, $J_{C-P}$ = 5.2 Hz, CO), 159.49 (C-6), 155.76 (C-2), 153.54 (C-4), 139.92 (C-8), 117.38 (C-5), 75.39 (d, $J_{C-P}$ = 5.7 Hz, C-4′), 73.81 (C-1′), 69.72 (d, $J_{C-P}$ = 7.0 Hz, OCH$_2$C(CH$_3$)$_3$), 65.76 (d, $J_{C-P}$ = 4.6 Hz, C-5′), 49.95 (CHCH$_3$), 32.37
(OCH₂C(CH₃)₃), 26.86 (OCH₂C(CH₃)₃), 21.14 (d, J_C-P = 6.0 Hz, CHCH₃), 21.06 (d, J_C-P = 6.1 Hz, CHCH₃). ³¹P NMR (202 MHz, MeOD) δP 13.69. MS (ES+) m/z: 610.26 (M+Na⁺, 100%). Reverse-phase HPLC, eluting with H₂O/CH₃CN from 100/0 to 0/100 in 30 min, flow= 1 mL/min, λ= 254 nm, tᵣ= 18.01 min.

Prepared according to standard procedure B, using (11) (0.20 g, 0.69 mmol) in trimethylphosphate (5 mL), and POCl₃ (0.06 mL, 0.69 mmol), the reaction mixture was stirred at -5 °C for 5 h. Anhydrous CH₂Cl₂ (5 mL) and a suspension of tosylate salt of benzoxy-L-alanine (1.23 g, 3.49 mmol) in CH₂Cl₂ (5 mL) were added, followed by dropwise addition of anhydrous DIPEA (1.22 mL, 6.98 mmol). The reaction mixture was stirred at room temperature for 15 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with CHCl₃/MeOH (95/5) to afford the product (34) as a white foam (47%, 0.23 g). ¹H NMR (500 MHz, MeOD) δH 7.67 (s, 1H, H-8), 7.32-7.26 (m, 10H, 2x OCH₂Ph), 6.08 (dt, J = 5.7, 2.1 Hz, 1H, H-2’), 5.90 (dt, J = 5.6, 2.2 Hz, 1H, H-3’), 5.49-5.46 (m, 1H, H-1’), 5.11-5.02 (m, 4H, 2x OCH₂Ph), 3.96-3.86 (m, 4H, H-5’), 3.03-3.00 (m, 1H, H-4’), 2.90-2.87 (m, 1H, CH-cPr), 2.71 (dt, J = 13.9, 8.5 Hz, 1H, H-6’), 1.59 (dt, J = 13.8, 6.2 Hz, 1H, H-6’), 1.35-1.31 (m, 6H, 2x CHCH₃), 0.84-0.80 (m, 2H, CH₂-cPr), 0.58-0.55 (m, 2H, CH₂-cPr). ¹³C NMR (125 MHz, MeOD): δC 175.50, 175.40 (CO), 161.90, 157.50 (C-6, C-2, C-4), 138.20 (C-2’), 137.37, 137.30 (ipso OCH₂Ph), 137.10 (C-8), 131.50 (C-3’), 129.62, 129.60, 129.38, 129.35, 129.32 (OCH₂Ph), 114.90 (C-5), 69.00 (d, J_C-P = 5.4 Hz, C-5’), 67.80 (OCH₂Ph), 60.50 (C-1’), 51.10 (CHCH₃), 47.10 (d, J_C-P = 8.3 Hz, C-4’), 35.90 (C-6’), 24.30 (CH-cPr),
20.70 (d, $J_{C,P} = 6.0$ Hz, CHCH$_3$), 20.60 (d, $J_{C,P} = 5.9$ Hz, CHCH$_3$), 7.67 (CH$_2$-cPr). $^{31}$P NMR (202 MHz, MeOD) $\delta_P$ 13.52. MS (ES+) $m/z$: 689.30 (M$^+$H$^+$, 100%). Reverse-phase HPLC eluting with H$_2$O/MeOH from 90/10 to 0/100 in 25 min, flow= 1 mL/min, $\lambda$= 254 nm $t_R= 22.15$ min.

4.1.24 Synthesis of abacavir-5'-O-bis-(2,2-dimethylpropoxy-L-alaninyl)-phosphate (35).

Prepared according to standard procedure B, using (11) (0.20 g, 0.69 mmol) in trimethylphosphate (5 mL), and POCl$_3$ (0.07 mL, 0.60 mmol), the reaction mixture was stirred at -5 °C for 5 h. Anhydrous CH$_2$Cl$_2$ (5 mL) and a suspension of tosylate salt of 2,2-dimethylpropoxy-L-alanine (1.16 g, 3.49 mmol) in CH$_2$Cl$_2$ (5 mL) were added, followed by dropwise addition of anhydrous DIPEA (1.22 mL, 6.98 mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with CHCl$_3$/MeOH (95:5) to afford the product (35) as a colorless oil (44%, 0.20 g). $^1$H NMR (500 MHz, MeOD) $\delta_H$ 7.70 (s, 1H, H-8), 6.16 (dt, $J = 5.5$, 2.0 Hz, 1H, H-2′), 5.95 (dt, $J = 5.5$, 2.0 Hz, 1H, H-3′), 5.55-5.51 (m, 1H, H-1′), 4.05-3.92 (m, 4H, H-5′, 2x CHCH$_3$), 3.85, 3.84, 3.74, 3.70 (2AB, $J_{AB} = 10.5$ Hz, 4H, 2x OCH$_2$C(CH$_3$)$_3$), 3.15-3.12 (m, 1H, H-4′), 2.93-2.91 (m, 1H, CH-cPr), 2.80 (dt, $J = 13.8$, 8.6 Hz, 1H, H-6′), 1.69 (dt, $J = 13.5$, 6.6 Hz, 1H, H-6′), 1.38-1.41 (m, 6H, 2x CHCH$_3$), 0.96 (s, 18H, 2x OCH$_2$C(CH$_3$)$_3$), 0.86-0.82 (m, 2H, CH$_2$-cPr), 0.62-0.59 (m, 2H, CH$_2$-cPr). $^{13}$C NMR (125 MHz, MeOD) $\delta_C$ 175.8 (d, $J_{C-P} = 5.0$ Hz, CO), 175.7 (d, $J_{C-P} = 4.8$ Hz, CO), 161.9, 157.5 (C-2, C-6, C-4), 138.1 (C-2′), 137.1 (C-8), 131.7 (C-3′), 114.9 (C-5), 75.38, 75.34 (OCH$_2$C(CH$_3$)$_3$), 69.1 (d, $J_{C,P} = 5.5$ Hz, C-5′), 60.5 (C-1′), 51.1 (CHCH$_3$), 47.2 (d, $J_{C-P} = 8.3$ Hz, C-4′), 36.0 (C-6′), 32.35, 32.33 (OCH$_2$C(CH$_3$)$_3$),
26.80, 26.79 (OCH₂C(CH₃)₃), 24.3 (CH-cPr), 21.06 (d, J_C-P = 6.1 Hz, CHCH₃), 21.02 (d, 
J_C-P = 5.8 Hz, CHCH₃), 7.67, 7.64 (CH₂-cPr). ³¹P NMR (202 MHz, MeOD) δP 13.65. MS 
(ES+) m/z: 649.36 (M+H⁺, 100%). Reverse-phase HPLC eluting with H₂O/MeOH 90/10 
to 0/100 in 25 min, flow= 1 mL/min, λ= 254 nm , t_R= 23.99 min.

4.1.25 Synthesis of 3-(2′-Deoxy-β-D-ribofuranosyl)-6-(4-n-pentylphenyl)-2,3-
dihydrofuro-[2,3-d]pyrimidin-2-one-5′-O-bis-(benzoxy-L-alaninyl)-phosphate (36).
Prepared according to standard procedure B, using (12) (0.26 g, 0.65 mmol) in 
trimethylphosphate (5 mL), and POCl₃ (0.06 mL, 0.65 mmol), the reaction mixture was 
stirred at -5 °C for 4 h. Anhydrous CH₂Cl₂ (5 mL) and a suspension of tosylate salt of 
benzoxy-L-alanine (1.14 g, 3.25 mmol) in anhydrous CH₂Cl₂ (5 mL) were added, 
followed by dropwise addition of anhydrous DIPEA (1.13 mL, 6.50 mmol). The reaction 
mixture was stirred at room temperature for 20 h. After work-up, the crude residue was 
 purified by silica gel column chromatography eluting with gradient of MeOH (2% to 
10%) in CH₂Cl₂ to give the product (36) as a yellow solid (19%, 0.10 g). ¹H NMR (500 
MHz, MeOD) δH 8.70 (s, 1H, H-4), 7.64 (d, J = 8.3 Hz, 2H, Ph), 7.36-7.21 (m, 12H, 
Ph, OCH₂Ph), 7.08 (s, 1H, H-5), 6.30 (t, J = 6.0 Hz, 1H, H-1’), 5.17-5.07 (m, 4H, 2x 
OCH₂Ph), 4.44-4.41 (m, 1H, H-3’), 4.31-4.28 (m, 1H, H-4’), 4.20-4.16 (m, 2H, 2x 
CHCH₃), 4.05-3.95 (m, 2H, H-5’), 2.68-2.63 (m, 1H, H-2’), 2.60 (t, J = 7.8 Hz, 2H, α-
CH₂), 2.24-2.19 (m, 1H, H-2’), 1.61 (qn, J = 7.5 Hz, 2H, β-CH₂), 1.41 (d, J = 7.2 Hz, 3H, 
CHCH₃), 1.37-1.28 (m, 7H, γ-CH₂, δ-CH₂, CHCH₃), 0.90 (t, J = 6.9 Hz, 3H, CH₂CH₃). 
¹³C NMR (125 MHz, MeOD) δC 175.45 (d, J_C-P = 5.6 Hz, CO), 175.39 (d, J_C-P = 4.5 Hz, 
CO), 172.91 (C-7a), 157.08 (C-2), 156.65 (C-6), 146.19 (para-C), 138.63 (C-4), 137.43,
137.21, 137.19, 130.16, 129.95, 129.63, 129.57, 129.37, 129.33, 129.30, 129.26, 129.20, 129.10, 128.28, 128.02, 127.33, 125.94 (OCH₂Ph, Ph), 110.17 (C-4a), 99.45 (C-5), 90.00 (C-1’), 87.60 (d, J_C-P = 8.2 Hz, C-4’), 71.30 (C-3’), 67.97, 67.92 (OCH₂Ph), 65.64 (d, J_C-P = 4.8 Hz, C-5’), 51.18 (CHCH₃), 42.63 (C-2’), 36.77, 32.62, 32.14, 23.61 ((CH₂)₄), 20.92 (d, J_C-P = 5.7 Hz, CHCH₃), 20.82 (d, J_C-P = 6.4 Hz, CHCH₃), 14.48 (CH₃). ³¹P NMR (202 MHz, MeOD) δ_P 14.03. MS (ES+) m/z: 823.29 (M+Na⁺, 100%). Reverse-phase HPLC, eluting with H₂O/CH₃CN from 100/0 to 0/100 in 30 min, flow= 1 mL/min, λ= 254 nm, t_R= 26.51 min.

4.1.26 Synthesis of 3-(2’-Deoxy-β-D-ribofuranosyl)-6-(4-n-pentylphenyl)-2,3-dihydrofuro-[2,3-d]pyrimidin-2-one-5’-O-bis-(2,2-dimethylpropoxy-L-alaninyl)-phosphate (37).

Prepared according to standard procedure B, using (12) (0.30 g, 0.75 mmol) in trimethylphosphate (4 mL), and POCl₃ (0.07 mL, 0.75 mmol), the reaction mixture was stirred at -5 °C for 4 h. Anhydrous CH₂Cl₂ (6 mL) and a suspension of tosylate salt of 2,2-dimethylpropoxy-L-alanine (1.25 g, 3.75 mmol) in anhydrous CH₂Cl₂ (6 mL) were added, followed by dropwise addition of anhydrous DIPEA (1.31 mL, 7.50 mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with gradient of MeOH (3% to 5%) in CH₂Cl₂ to give a pale yellow solid. The product was dissolved in CH₂Cl₂ and washed with 0.05 N HCl (2x 5 mL), brine (1 x 5 mL), 5% NaHCO₃ (2x 5 mL), brine (1 x 5 mL), dried over MgSO₄, filtered and concentrated to give the product (37) as a pale yellow solid (10%, 0.06 g). ¹H NMR (500 MHz, MeOD) δ_H 8.79 (s, 1H, H-4), 7.72 (d,
2H, $J = 8.3$ Hz, $Ph$), 7.28 (d, 2H, $J = 8.3$ Hz, $Ph$), 7.19 (s, 1H, $H$-5), 6.35 (t, $J = 6.0$ Hz, 1H, $H$-1”), 4.50-4.48 (m, 1H, $H$-3”), 4.38-4.34 (m, 1H, $H$-4”), 4.29-4.23 (m, 2H, $H$-5”), 4.05-3.94 (m, 2H, $H$-2”, $\alpha$-CH$_2$), 3.91-3.85 (m, 4H, 2x OCH$_2$C(CH$_3$)$_3$), 3.07-3.05 (m, 3H, H-2”), 3.89-3.84 (m, 3H, $\alpha$-CH$_3$), 2.70-2.64 (m, 3H, $\beta$-CH$_2$), 1.65 (qn, $J = 7.5$ Hz, 2H, $\beta$-CH$_2$), 1.46 (d, $J = 7.2$ Hz, 3H, CHC$_3$), 1.41 (d, $J = 7.2$ Hz, 3H, CHC$_3$), 1.39-1.31 (m, 4H, $\gamma$-CH$_2$, $\delta$-CH$_2$), 0.87 (t, $J = 6.9$ Hz, 3H, C$_3$H$_3$).

$^{13}$C NMR (125 MHz, MeOD): $\delta_C$ 175.72 (d, $J_{C-P} = 6.4$ Hz, CO), 175.58 (d, $J_{C-P} = 4.9$ Hz, CO), 173.02 (C-7a), 157.20 (C-2a), 156.73 (C-6), 146.29 (para-C), 138.72 (C-4), 130.17, 127.39, 125.96 (Ph), 110.28 (C-4a), 99.51 (C-3a), 89.94 (C-1’), 87.24 (d, $J_{C-P} = 8.2$ Hz, C-4”), 75.61, 75.40 (OCH$_2$C(CH$_3$)$_3$), 71.16 (C-3’), 65.67 (d, $J_{C-P} = 4.8$ Hz, C-5”), 51.16 (CHC$_3$), 51.08 (d, $J_{C-P} = 2.0$ Hz, CHC$_3$), 42.61 (C-2”), 36.77, 32.57, 32.34, 32.30, 32.15, 23.59 (OCH$_2$C(CH$_3$)$_3$, (CH$_2$)$_4$), 26.82, 26.77 (OCH$_2$C(CH$_3$)$_3$), 21.22 (d, $J_{C-P} = 5.5$ Hz, CHC$_3$), 21.13 (d, $J_{C-P} = 6.3$ Hz, CHC$_3$), 14.45 (CH$_3$). $^{31}$P NMR (202 MHz, MeOD) $\delta_P$ 14.18. MS (ES+) $m/z$: 783.35 (M+Na$^+$, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 100/0 to 0/100 in 30 min, flow= 1 mL/min, $\lambda$= 254 nm, $t_R$= 29.08 min.

### 4.1.27 Synthesis of acadesine-5'-O-bis(benzoxy-L-alaninyl)-phosphate (38).

Prepared according to standard procedure B, using (13) (0.30 g, 1.16 mmol) in trimethylphosphate (4 mL), and POCl$_3$ (0.11 mL, 1.16 mmol), the reaction mixture was stirred at -5°C for 5 h. Anhydrous CH$_2$Cl$_2$ (5 mL) and tosylate salt of benzoxy-L-alanine (2.04 g, 5.80 mmol) were added, followed by dropwise addition of DIPEA (2.00 mL, 11.61 mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a
gradient of MeOH (2% to 5%) in CH₂Cl₂ to afford the product (38) as a colorless oil (2%, 0.02 g). ¹H NMR (500 MHz, MeOD) δ_H 7.40-7.31 (m, 11H, OCH₂Ph and H-5), 5.56 (d, J = 6.0 Hz, 1H, H-1’), 5.18-5.10 (m, 4H, 2x OCH₂Ph), 4.41 (dd, J = 6.0, 6.1 Hz, 1H, H-2’), 4.23 (dd, J = 5.0, 3.5 Hz, 1H, H-3’), 4.16-4.09 (m, 3H, H-4’, H-5’), 3.97-3.89 (m, 2H, 2x CHCH₃), 1.40-1.31 (m, 6H, 2x CHC₂H₃). ¹³C NMR (125 MHz, MeOD) δ_C 175.46 (d, J_C-P = 5.0 Hz, CO), 175.40 (d, J_C-P = 5.1 Hz, CO), 169.25 (CONH₂), 145.44 (C-2), 130.21 (C-5), 137.29 (ipso OCH₂Ph), 129.63, 129.62, 129.39, 129.36, 129.32, 129.24 (OCH₂Ph), 113.82 (C-3), 89.54 (C-1’), 85.00 (d, J_C-P = 7.1 Hz, C-4’), 74.71 (C-2’), 71.42 (C-3’), 68.01 (OCH₂Ph), 66.27 (d, J_C-P = 5.0 Hz, C-5’), 51.17 (CHCH₃), 20.75 (d, J_C-P = 6.2 Hz, CHCH₃), 20.58 (d, J_C-P = 7.2 Hz, CHCH₃). ³¹P NMR (202 MHz, MeOD) δ_P 13.73. MS (ES+) m/z: 683.21 (M+Na⁺, 100%). Reverse-phase HPLC, eluting with H₂O/CH₃CN from 90/10 to 70/30 in 10 min; 70/30 to 40/60 in 20 min; 40/60 to 0/100 in 5 min; flow= 1 mL/min, λ= 265 nm, tᵣ= 18.72 min.

4.1.28 Synthesis of acadesine-5′-O-bis(methoxy-L-alaninyl)-phosphate (39).

Prepared according to standard procedure B, using (13) (0.20 g, 0.77 mmol) in trimethylphosphate (3.5 mL), and POCl₃ (0.18 mL, 1.77 mmol), the reaction mixture was stirred at -5 °C for 5 h. Anhydrous CH₂Cl₂ (5 mL) and chloridate salt of methoxy-L-alanine (0.54 g, 3.87 mmol) were added, followed by dropwise addition of anhydrous DIPEA (1.30 mL, 7.74 mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (2% to 20%) in CH₂Cl₂ to afford the product (39) as a colorless oil (3%, 0.01 g). ¹H NMR (500 MHz, MeOD) δ_H 7.41 (s, 1H, H-5), 5.56 (d, 1H,
$J = 6.0 \text{ Hz, } H-1\text{'}, 4.45 \text{ (dd, } J = 6.2, 6.0 \text{ Hz, } 1\text{H, } H-2\text{'}, 4.26 \text{ (dd, } 1\text{H, } J = 5.5, 3.0 \text{ Hz, } H-3\text{'}, 4.21-4.19 \text{ (m, } 3\text{H, } H-4\text{', } H-5\text{'}, 3.88-3.84 \text{ (m, } 2\text{H, } 2\times \text{CHCH}_3\text{), 3.73 \text{ (s, } 3\text{H, OCH}_3\text{), 3.72 \text{ (s, } 3\text{H, OCH}_3\text{), 1.39-1.34 \text{ (m, } 6\text{H, } 2\times \text{CHCH}_3\text{).}^{13}\text{C NMR (125 MHz, MeOD)} \delta_C 176.16 \text{ (d, } J_{C-P} = 4.6 \text{ Hz, CO), 176.09 \text{ (d, } J_{C-P} = 5.5 \text{ Hz, CO), 169.29 \text{ (CONH}_2\text{), 145.46 \text{ (C-2), 130.19 \text{ (C-5), 113.78 \text{ (C-3), 89.57 \text{ (C-1'), 85.07 \text{ (d, } J_{C-P} = 7.5 \text{ Hz, C-4'), 74.68 \text{ (C-2'), 71.53 \text{ (C-3'), 66.22 \text{ (d, } J_{C-P} = 4.6 \text{ Hz, C-5'}, 52.80 \text{ (OCH}_3\text{), 51.00 \text{ (d, } J_{C-P} = 1.7 \text{ Hz, CHCH}_3\text{), 51.99 \text{ (d, } J_{C-P} = 1.7 \text{ Hz, CHCH}_3\text{), 20.83 \text{ (d, } J_{C-P} = 6.4 \text{ Hz, CHCH}_3\text{), 20.65 \text{ (d, } J_{C-P} = 6.4 \text{ Hz, CHCH}_3\text{).}^{31}\text{P NMR (202 MHz, MeOD)} \delta_P 13.88. MS (ES+) } m/z: 531.15 (M+Na^+, 100%). Reverse-phase HPLC, eluting with H$_2$O/CH$_3$CN from 100/0 to 80/20 in 10 min; 80/20 to 70/30 in 10 min; 70/30 to 0/100 in 10 min; flow=1 mL/min, $\lambda = 265 \text{ nm, } t_R = 10.43 \text{ min.} 

4.1.29 Synthesis of acadesine-5'-O-bis(2,2-dimethylpentoxy-L-alaninyl)-phosphate (40).

Prepared according to standard procedure B, using (13) (0.30 g, 1.16 mmol) in trimethylphosphate (4 mL), and POCl$_3$ (0.11 mL, 1.16 mmol), the reaction mixture was stirred at -5 \text{ °C for 5 h. Anhydrous CH$_2$Cl$_2$ (5 mL) and tosylate salt of 2,2-dimethylpropoxy-L-alanine (1.92 g, 5.80 mmol) were added, followed by dropwise addition of anhydrous DIPEA (2.00 mL, 11.61 mmol). The reaction mixture was stirred at room temperature for 16 h. After work-up, the crude residue was purified by silica gel column chromatography eluting with a gradient of MeOH (2% to 10%) in CH$_2$Cl$_2$ to give the product (40) as a colorless oil (1%, 0.01 g). $^1$H NMR (500 MHz, MeOD) $\delta_H 7.41 \text{ (s, 1H, } H-5\text{), 5.56 \text{ (d, } J = 6.0 \text{ Hz, 1H, } H-1\text{'}, 4.42 \text{ (dd, } J = 5.6, 5.5 \text{ Hz, 1H, } H-2\text{'}, 4.25 \text{ (dd, } J = 5.5, 3.0 \text{ Hz, 1H, } H-3\text{'}, 4.22-4.17 \text{ (m, } 3\text{H, } H-4\text{', } H-5\text{'}, 4.00-3.94 \text{ (m, } 2\text{H, } 2\times \text{CHCH}_3\text{),}
3.90, 3.78 (2AB, J_AB = 11.0 Hz, 4H, 2x OCH2(CH3)3), 1.42 (d, J = 7.0 Hz, 3H, CHCH3),
1.40 (d, J = 7.0 Hz, 3H, CHCH3), 0.97 (s, 9H, OCH2C(CH3)3), 0.96 (s, 9H,
OCH2C(CH3)3). \(^{13}\text{C NMR (125 MHz, MeOD)} \delta_\text{C} 175.70 (d, J_{C-P} = 2.6 Hz, \text{CO}),
175.67 (d, J_{C-P} = 2.6 Hz, \text{CO}), 169.25 (CONH2), 145.44 (C-2), 130.21 (C-5),
113.82 (C-3), 89.58 (C-1'), 85.02 (d, J_{C-P} = 7.5 Hz, C-4'), 75.46 (OCH2(CH3)3),
75.42 (OCH2(CH3)3), 74.68 (C-2'), 71.48 (C-3'), 66.22 (d, J_{C-P} = 4.5 Hz, C-5'),
51.00 (d, J_{C-P} = 1.9 Hz, CHCH3),
32.35 (OCH2C(CH3)3), 26.79 (OCH2C(CH3)3), 21.07 (d, J_{C-P} = 6.4 Hz, CHCH3), 20.93
(d, J_{C-P} = 6.4 Hz, CHCH3). \(^{31}\text{P NMR (202 MHz, MeOD)} \delta_\text{P} 13.94. \text{MS (ES+)} m/z: 643.29
(M+Na\(^+\), 100%). Reverse-phase HPLC, eluting with H2O/CH3CN from 100/0 to 80/20 in
10 min; 80/20 to 50/50 in 20 min; 50/50 to 0/100 in 5 min; flow= 1 mL/min, \(\lambda\) = 265 nm,
t_R= 27.32 min.

4.2 Cytostatic activity assays.

The tumour cells were seeded in 96-well microtiter plates and exposed to different
concentrations of the test compounds. After 2 days (L1210, L1210/TK\(^-\)) and 3 days
(CEM, CEM/TK\(^-\), HeLa, HeLa TK\(^-\), Caco-2, Colo-320), cell number was determined
using a Particle counter (Coulter Z-1, Analis, Ghent, Belgium). The IC\(_{50}\) represents the
compound concentration required to inhibit tumour cell proliferation by 50%.

4.3 Antiviral assays.

The antiviral assays [except anti-human immunodeficiency virus (HIV) assays] were
based on inhibition of virus-induced cytopathicity in HEL [herpes simplex virus type 1
(HSV-1), HSV-2 (G), vaccinia virus and vesicular stomatitis virus], Vero (parainfluenza-
3, reovirus-1, Sindbis, Coxsackie B4, and Punta Toro virus), HeLa (vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus), CrFK (feline herpes virus), feline coronavirus (FIPV) and MDCK (influenza virus A (H1N1, H3N2) and B) cell cultures. Confluent cell cultures in microtiter 96-well plates were inoculated with 100 cell culture inhibitory dose-50 (CCID₅₀) of virus (1 CCID₅₀ being the virus dose to infect 50% of the cell cultures). After a 1 h virus adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations (200, 40, 8, … μM) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. The methodology of the anti-HIV assays was as follows: human CEM (~ 3 × 10⁵ cells/cm³) cells were infected with 100 CCID₅₀ of HIV-1(III₅) or HIV-2(ROD)/mL and seeded in 200 μL wells of a microtiter plate containing appropriate dilutions of the test compounds. After 4 days of incubation at 37°C, HIV-induced CEM giant cell formation was examined microscopically. MT-4 cells (~10⁴ cells per mL) were suspended in fresh culture medium and infected with 10 _L (0.7 ng of p24) of X4LAI.04 viral stock per mL of cell suspension. Infected cell suspensions were then transferred to microplate wells, mixed with 1 mL of medium containing the test compound at an appropriate dilution and further incubated at 37 °C. After 3 days, p24 production was measured in the MT-4 cell culture supernatants. The results are given as the mean ± standard error of the mean of the concentration required to suppress viral replication by 50% (median effective concentration [EC₅₀]). The value of EC₅₀ was calculated by fitting the data points to a sigmoidal dose-response curve, with Prism software, (version 4.0; GraphPad).
Viability assays. Viability assays were performed in the 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).

4.4 Enzymatic assays.

Compound 32 (5.0 mg) or 40 (4.8 mg) were dissolved in $d_6$-acetone (0.15 mL) and Trizma buffer (pH = 7.6) (0.30 mL) and a $^{31}$P-NMR was recorded (blank). Then a solution of carboxypeptidase Y (0.1 mg) in Trizma buffer (0.15 mL) was added and a $^{31}$P-NMR experiment was performed recording the experiment every 5 min, for 24 h at room temperature.

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