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# PMPA and PMEA Prodrugs for the Treatment of HIV Infections and Human Papillomavirus (HPV) Associated Neoplasia and Cancer.

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**Abstract**

The synthesis and *in vitro* biological evaluation of novel phosphonamidate and phosphonodiamidate prodrugs of adefovir and tenofovir are reported. The selected synthetic approach from free phosphonic acid *via* bis-trimethylsilyl ester intermediates affords (*L*)-alanine ester derivatives in 10-70% yields. When assessed for their anti-HIV activity, all the prodrugs showed submicromolar activity. Noteworthy, the most potent derivative in the adefovir series contained a 5,6,7,8-tetrahydronaphtyl group, herein reported for the first time as an aryl moiety in a ProTide. A pronounced cytostatic activity of the above prodrugs is also reported. Selected compounds were tested for their antiproliferative activity against HPV-transformed cells and they were found significantly more active in comparison to their parent compounds. In this study a slightly improved activity of the adefovir derivatives over those of tenofovir was also noticed. However, no specificity for naturally HPV-transformed cell lines was observed.

## 1. Introduction

Acyclic nucleoside phosphonates (ANPs) are broad spectrum antiviral agents active against DNA viruses and retroviruses. The common structural attribute of ANPs is a nucleobase attached to an aliphatic side chain containing a phosphonomethyl residue [1-5]. The methylene bridge between the phosphonate moiety and the rest of the molecule excludes the possibility of enzymatic dephosphorylation. Three ANP compounds, namely cidofovir (HPMPC), adefovir (PMEA) **1** and

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tenofovir ((*R*)-PMPA) **2** are in current clinical use. Cidofovir is approved for the treatment of human cytomegalovirus (HCMV) retinitis in AIDS patients. Adefovir in its oral prodrug form, adefovir dipivoxil (Hepsera) **3,** is approved for the treatment of Hepatitis B Virus (HBV) infections, and tenofovir disoproxil fumarate (TDF, Viread) **4** has been approved for the treatment of HIV and HBV infections [6].

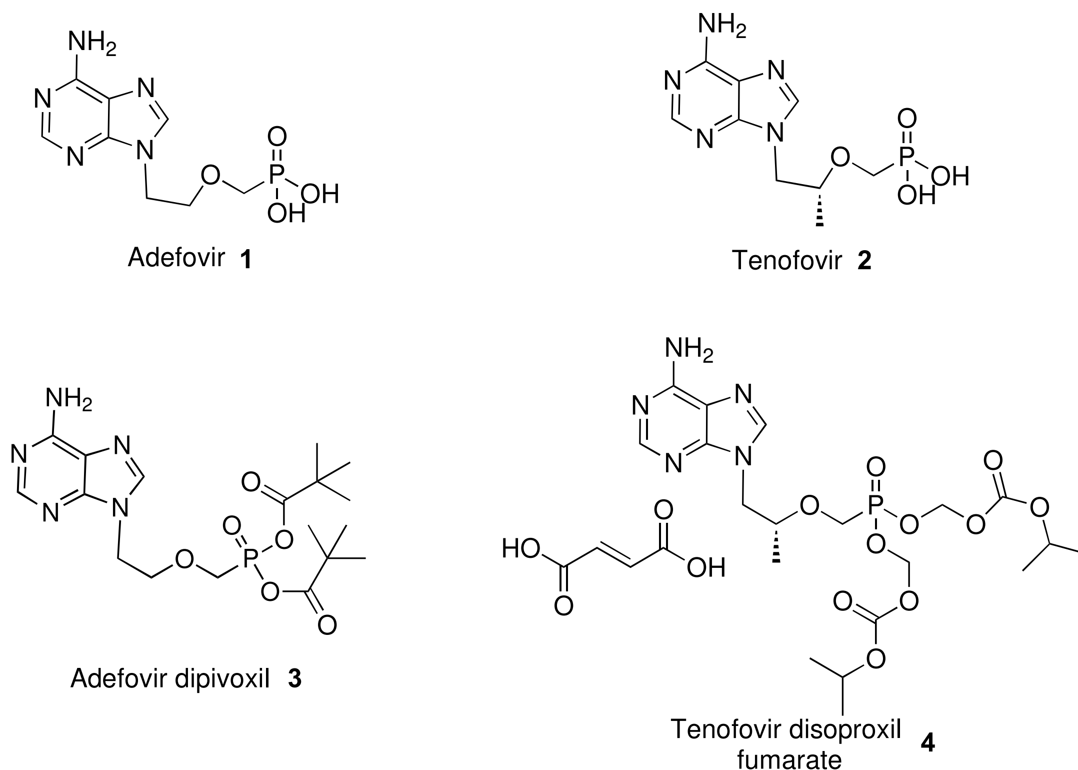



**Figure 1.** Structure of Adefovir **1**, Tenofovir **2** and their prodrugs **3** and **4**.

Since the discovery of these compounds, intensive drug discovery programs have led to the development of other ANPs with diverse nucleobases, aglyco modifications and other structural variations. The major limitation of ANPs relates to their poor oral bioavailability, most likely due to the presence of a negatively charged free phosphonate. Therefore, several prodrug approaches are needed to improve the bioavailability of ANPs. The importance of ANP prodrugs has been recently reviewed [7, 8].

Our ProTide technology was successfully applied to PMEA and (*R*)-PMPA by Ballatore *et al.[9]*. Mirroring this work, Gilead Sciences reported an extensive study on the application of the ProTide approach to (*R*)-PMPA. In these studies, the phenyloxy isopropyl-(*L*)-alaninyl phosphonamidate

prodrug of (*R*)-PMPA (GS-7340) emerged as a lead compound and is now in a phase III clinical trial for the treatment of HIV (Figure 2). Similarly, several ANP phosphonodiamidates containing (*L*)-alanine esters were reported to exhibit potent antiviral activities, notably bis(isobutyl-(*L*)-phenylalaninyl)phosphonodiamidate-PME-N6-(cyclopropyl)DAP (GS-9191) the most active of the series against human papillomavirus (HPV). This compound has currently completed Phase I clinical trials as a topical prodrug for the treatment of HPV lesions [10].

Some ANPs have also demonstrated an interesting cytostatic potential, a little explored therapeutic opportunity in the case of ANPs. In particular, PMEA has been shown to be a potent inhibitor of highly aggressive choriocarcinoma tumors which may be of particular interest [11, 12], given the occurrence of several types of cancer, including Kaposi sarcoma, in patients with AIDS.

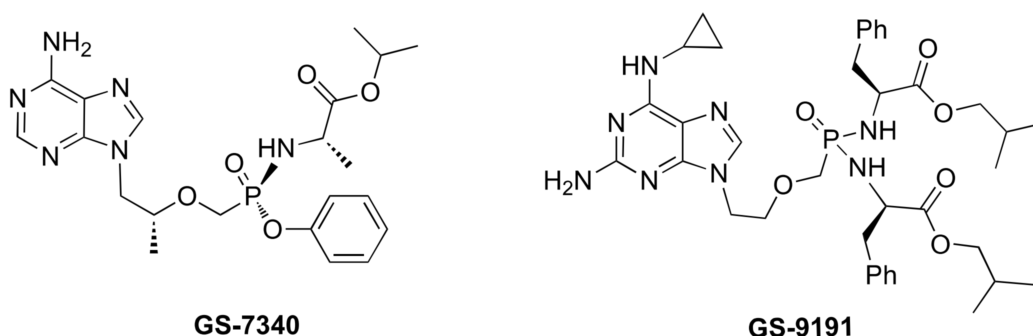



**Figure 2.** Structures of GS-7340 and GS-9191.

Noteworthy, cidofovir also displays antineoplastic activity, increasingly showing positive efficacy in HPV associated lesions [13]. HPV is the primary causative agent of cervical cancer, and also gynaecological and anogenital epithelial lesions such as Vulval Intraepithelial Neoplasia (VIN). VIN is an uncommon chronic skin disorder, which has a high risk of recurrence post treatment and a significant risk of progression to invasive vulval carcinoma. The current treatment options including interferon, 5-FU and several forms of surgery, all have considerable disadvantages [14]. One pilot study has shown promise for the treatment of VIN with topical cidofovir [15]. With this in mind, there is a considerable need for a treatment option that specifically targets the cause of the lesion with minimal side effects.

Due to the high success of the ProTide approach applied to ANPs and other nucleosides, with the aim to expand the SAR study of PMEA and (*R*)-PMPA derivatives as anti-HIV agents, we present

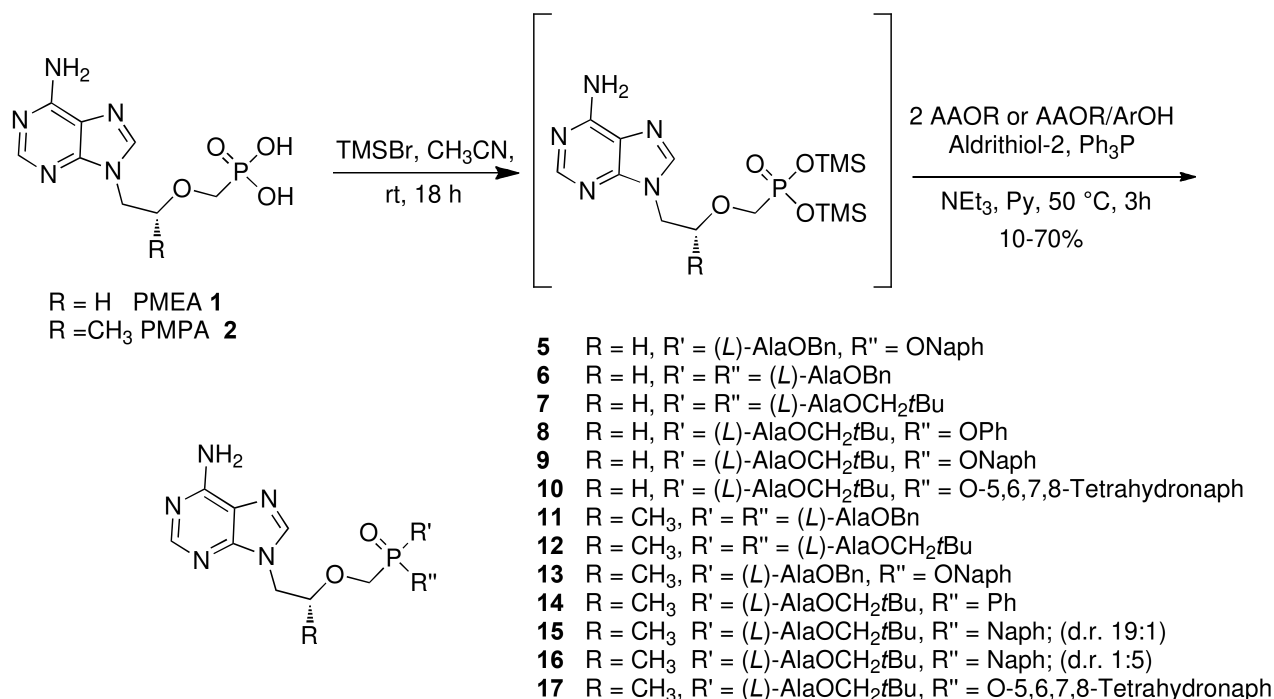
here a series of novel phosphonamidate and phosphonodiamidate prodrugs of both adefovir and tenofovir. Considering the therapeutic potential of PMEA for the treatment of neoplastic diseases and the positive effect of cidofovir in the treatment of the HPV-associated VIN, we assessed the antiproliferative activity of these derivatives in the treatment of HPV and related agents.

## 2. *Results and discussion.*

### *2.1. Chemistry.*

In 2001, we reported the synthesis of a number of adefovir and tenofovir amidate prodrugs. However, the yields achieved with established chemical methodologies were very modest [9]. More recently, an efficient one-pot procedure for the synthesis of ANP diamidate prodrugs from free phosphonic acid has been reported [16].

We thus endeavored to apply this novel methodology. First, we prepared new diamidate derivatives according to the approach highlighted in Scheme 1. (*L*)-Alanine neopentyl and benzyl ester amino acids were selected as designated pro-moieties on the basis of the excellent results obtained during our anti-HCV synthesis program [17]. Thus, overnight reaction of free ANPs with trimethylsilyl bromide in acetonitrile afforded the intermediate bis(trimethylsilyl) phosphonate esters, which without purification were reacted with an excess of the desired (*L*)-alanine amino acid ester in pyridine in the presence of triethylamine, aldrithiol-2 and triphenylphosphine. This two-step reaction gave the corresponding (*L*)-Alanine diamidate prodrugs **6**, **7**, **11** and **12** in 22-70% overall yields, after purification by column chromatography. The reaction can be monitored by $^{31}$P NMR spectroscopy analysis to ensure the completion of each step.




**Scheme 1**: General procedure for the synthesis of amidate ANP prodrugs **5-17**.

When we slightly modified this procedure, adding in the second step a 1:1 mixture of the amino acid ester and the aryl alcohol instead of solely the amino acid ester, the desired phosphonoamidate **5**, **8-10** and **13-17** were obtained, in 10-30% yields after column chromatography. All products were isolated as a roughly 1:1 mixture of two diastereoisomers. It is worthy to note that in the case of the neopentyloxy-(*L*)-alanyl naphtyloxyphosphonoamidate derivatives of tenofovir we succeeded, after column chromatography, to partially separate the two diastereoisomers obtaining two fractions enriched respectively with one of the two diastereoisomers (**15** d.r. 19:1 and **16** d.r. 1:5).

*3. Antiviral activity.*

*3.1. Anti-HIV activity.*

All compounds were tested against HIV-1 and HIV-2 to determine their antiviral activity *in vitro*. All prodrugs showed marked anti-HIV activity when compared to the parent ANPs, likely due to improved cellular permeability (Table 1).

**Table 1:** Anti-HIV activity ($EC_{50}$) and cytotoxicity ($CC_{50}$) data of the test compounds.

| Compounds | $EC_{50}$ (μM) [a] | | $CC_{50}$ (μM) [a] |
|---|---|---|---|
| | HIV-1 ($III_B$) | HIV-2 (ROD) | (CEM) |
| **1** | 8.4 ± 5.4 | 8.2 ± 4.0 | ≥50 |
| **5** | 0.030 ± 0.009 | 0.050 ± 0.005 | 0.12 ± 0.04 |
| **6** | 0.040 ± 0.003 | 0.025 ± 0.012 | 0.14 ± 0.09 |
| **7** | 0.024 ± 0.003 | 0.019 ± 0.004 | 0.20 ± 0.04 |
| **8** | 0.056 ± 0.004 | 0.044 ± 0.003 | 0.40 ± 0.04 |
| **9** | 0.032 ± 0.002 | 0.019 ± 0.004 | 0.045 ± 0.045 |
| **10** | 0.017 ± 0.011 | 0.007 ± 0.0002 | 0.019 ± 0.009 |
| **2** | 3.9 ± 0.98 | 3.7 ± 1.2 | >500 |
| **11** | 0.030 ± 0.001 | 0.014 ± 0.004 | 6.9 ± 6.4 |
| **12** | 0.004 ± 0.003 | 0.002 ± 0.0006 | 1.2 ± 0.1 |
| **13** | 0.019 ± 0.013 | 0.011 ± 0.001 | 4.4 ± 0.6 |
| **14** | 0.018 ± 0.009 | 0.012 ± 0.006 | 2.3 ± 1.1 |
| **15** (ratio 19:1) | 0.009 ± 0.002 | 0.007 ± 0.005 | 6.9 ± 1.7 |
| **16** (ratio 1:5) | 0.003 ± 0.0006 | 0.004 ± 0.002 | 3.9 ± 1.7 |
| **17** | 0.011 ± 0.0 | 0.009 ± 0.002 | 2.0 ± 0.7 |
| GS 7340 | 0.005[b] | - | 40[b] |

[a] in CEM cells. [b] in MT-2 cells[18]

From the data collected in Table 1, it can be appreciated that adefovir and tenofovir derivatives display low micromolar activity against HIV-1 and HIV-2. The $EC_{50}$ of tenofovir value is slightly (~ 3-fold) lower compared to that of adefovir. However, their prodrugs consistently showed nanomolar anti-HIV activities, up to 600- to 1,000-fold stronger for the adefovir prodrugs or even up to 1,000- to 2,000-fold stronger for the tenofovir prodrugs depending the nature of the $R_1$ and

$R_2$ groups on the phosphonate moiety. Interestingly, in the adefovir series, the derivative bearing the novel 5,6,7,8-tetrahydro-1-naphthol entry as a hydrolysable aryl moiety (**10**) showed the most increased antiviral activity compared to all other adefovir protides. This is the first time this aryl unit has been reported in ProTide. When the amino acid ester is taken into account, in general, neopentyl diamidates were more active than the respective benzyl derivatives (**6** vs **7** and **11** vs **12**, Table 1). These results may suggest that steric factors in the amino acid moiety may have a defined influence on the antiviral activity. The increased antiviral potency was more pronounced in the tenofovir than the adefovir prodrug series. It is also evident that an increased anti-HIV activity is observed, especially in the neopenthyl ester Protide for both adefovir and tenofovir series going from phenol towards the presumably more lipophilic and sterically encumbered tetrahydro-1-naphthol.

In case of the tenofovir L-Ala-Oneopentyl-naphthyl derivative, we were able to obtain two fractions **15** and **16** each of one enriched in one of the diastereoisomers. They showed a difference in activity between the two fractions. This suggests that prodrug stereochemistry may be important for the eventual metabolic conversion/activation of these prodrugs.

The compounds have also been evaluated against DNA virus replication in HEL cell cultures such as herpes simplex virus type 1 (HSV-1), HSV-2, thymidine kinase-deficient HSV-1 and vaccinia virus. The prodrugs of PMEA (**1**) but also of PMPA (**2**) were invariably more antivirally active than the parent compounds (Table 2).

**Table 2:** Anti-DNA virus activity of the test compounds

| Compounds | $EC_{50}$[a] (µM) | | | | MIC[b] (µM) |
|---|---|---|---|---|---|
| | HSV-1 (KOS) | HSV-2 (G) | HSV-1 TK$^-$ (ACV$^R$ KOS) | VV | |
| **1** | 4.7 | 6.3 | 7.0 | - | >100 |
| **5** | 0.02 ± 0.01 | 0.008 ± 0.003 | 0.7 ± 0.2 | 9.5 ± 3.5 | >100 |
| **6** | 0.7 ± 0.2 | 0.25 ± 0.07 | 0.6 ± 0.3 | 5.0 ± 1.7 | >100 |
| **7** | 0.3 ± 0.07 | 0.2 ± 0.1 | 0.3 ± 0.1 | 2.7 ± 1.5 | >100 |
| **8** | 0.6 ± 0.3 | 0.15 ± 0.07 | 0.55 ± 0.4 | 3.7 ± 0.6 | >100 |
| **9** | 0.25 ± 0.07 | 0.1 ± 0.1 | 0.2 ± 0 | 1.6 ± 0.7 | >100 |

| | | | | | |
|---|---|---|---|---|---|
| **10** | 0.13 ± 0.09 | 0.05 ± 0.03 | 0.12 ± 0.1 | 1.3 ± 0.6 | >100 |
| **2** | 105 | 122 | 151 | >100 | >100 |
| **11** | 10 ± 3.5 | 3 ± 1.4 | 7 ± 4.2 | 20 ± 0 | >100 |
| **13** | 1.3 ± 0.7 | 0.7 ± 0.1 | 0.8 ± 0 | 5.5 ± 2.1 | >100 |
| **12** | 1.5 ± 0.7 | 0.7 ± 0.1 | 2 ± 1.4 | 6 ± 2.1 | >100 |
| **14** | 1.3 ± 0.7 | 0.7 ± 0.1 | 1 ± 0 | 8 ± 1.4 | >100 |
| **15** | 1.5 ± 0.7 | 0.7 ± 0.1 | 2 ± 1.4 | 4 ± 0 | >100 |
| **16** | 2 ± 1.4 | 0.8 ± 0 | 2 ± 0 | 4 ± 0 | >100 |
| **17** | 1.4 ± 0 | 1.4 ± 0 | 5.5 ± 2.1 | 10.5 ± 2.1 | >100 |

[a]50% Effective concentration or compound concentration required to inhibit virus-induced cytopathicity by 50%. [b] Minimal inhibitory concentration or compound concentration required to afford a microscopically visible alteration of HEL cell morphology.

The PMEA prodrugs showed more antiviral efficacy than those of PMPA but given the much poorer antiherpetic activity of PMPA *versus* PMEA, the antiherpetic improvement for the PMPA prodrugs was much more pronounced than observed for the PMEA prodrugs. In fact, PMPA as such proved poorly active ($EC_{50}$: 105-151 µM), but its prodrugs (except for **13**) were inhibitory in the higher nanomolar/lower micromolar range for the herpesviruses (0.7-2 µM), and in the lower micromolar range for VV (4-5.5 µM). In the PMEA prodrug series, the $EC_{50}$ values for antiherpes activity ranged between 0.12 µM and 0.7 µM and for VV between 1.3 and 5 µM. In none of the cases, cellular toxicity against the HEL cell monolayers was noticed at 100 µM.

*3.2. Cytostatic activity.*

The *in vitro* inhibitory effect of all compounds on the proliferation of murine leukemia cells (L1210), human $CD4^+$ T-lymphocyte cells (CEM) and human cervix carcinoma cells (HeLa) was also evaluated. In the tenofovir prodrug series, low micromolar $IC_{50}$ values (between 1.2 and 17 µM, Table 3) were observed. On the contrary, adefovir prodrugs showed submicromolar cytostatic activity ($IC_{50}$ between 0.019 and 1.6 µM) (Table 3). An increased cytostatic activity in the adefovir and tenofovir series is observed on replacing the phenyl by either the naphthyl or

tetrahydronaphthyl groups, with the latter showing a >100-fold increase in antiproliferative activity when compared to the parent nucleoside phosphonate.

**Table 3:** Antiproliferative activity ($IC_{50}$) of adefovir derivatives in different cell lines.

| Compounds | $IC_{50}$[a] (μM) | | |
|---|---|---|---|
| | L1210 | CEM | HeLa |
| **1** | 3.0 ± 1.0 | 24 ± 11 | 181 ± 166 |
| **2** | 11 ± 12 | 6.9 ± 6.4 | 17 ± 10 |
| **5** | 0.041 ± 0.018 | 0.12 ± 0.04 | 1.6 ± 0.7 |
| **6** | 0.21 ± 0.08 | 0.14 ± 0.09 | 0.67 ± 0.26 |
| **7** | 0.24 ± 0.01 | 0.20 ± 0.04 | 1.6 ± 0.8 |
| **8** | 0.15 ± 0.07 | 0.40 ± 0.04 | 2.2 ± 1.5 |
| **9** | 0.068 ± 0.016 | 0.045 ± 0.045 | 0.92 ± 0.22 |
| **10** | 0.026 ± 0.009 | 0.019 ± 0.009 | 0.45 ± 0.17 |
| **11** | 120 ± 28 | >500 | >500 |
| **12** | 1.5 ± 0.9 | 1.2 ± 0.1 | 4.0 ± 0.1 |
| **13** | 3.7 ± 1.8 | 4.4 ± 0.6 | 11 ± 1.0 |
| **14** | 4.0 ± 0.2 | 2.3 ± 1.1 | 8.0 ± 3.0 |
| **15** | 5.6 ± 2.3 | 6.9 ± 1.7 | 17 ± 1.0 |
| **16** | 2.2 ± 1.3 | 3.9 ± 1.7 | 12 ± 1.0 |
| **17** | 1.3 ± 0.5 | 2.0 ± 0.7 | 5.3 ± 3.9 |

[a]50% Inhibitory concentration.

*3.3. Antiproliferative activity against naturally HPV-transformed cell lines.*

To assess the antiproliferative activity of the phosphonodiamidates and protides of adefovir and tenofovir for the potential treatment of HPV-induced lesions, the prodrugs **12-15**, **16**, **9** and **7** and the parent ANPs were initially examined in the SiHa (HPV 16+), HeLa (HPV 18+) and C33A (HPV-) cell cultures (Table 4).

**Table 4**: $IC_{50}$ values of the compounds against HPV+ and HPV- cells

| **Entry** | **Compound** | $IC_{50}$ (μM) | | |
|---|---|---|---|---|
| | | SiHa | HeLa | C33A |
| **1** | **2** | - | - | - |
| **2** | **12** | 0.73 | 0.54 | 0.7 |
| **3** | **15** (ratio 19:1) | - | 0.53 | 7.6 |
| **4** | **16** (ratio 1:5) | 7.8 | 6.2 | 6 |
| **5** | **1** | - | - | 7.8 |
| **6** | **9** | 0.6 | 0.5 | 0.54 |
| **7** | **7** | 0.66 | 0.6 | 0.84 |

From the data in Table 4, it can be seen that the adefovir protide compounds are significantly more cytostatic in comparison to the parent compounds (**1** and **2**). Compounds **7** and **9** and the tenofovir protide compounds **12**, **15** and **16** were further examined in cells derived from normal, neoplastic, and tumour tissue: SiHa cells, M08 vulval keratinocytes and human epidermal keratinocytes (Table 5). Unfortunately, no specificity to naturally HPV-transformed cell lines was observed in this study.

**Table 5:** $IC_{50}$ values of **9** and **7** against normal, neoplastic and tumor cell lines

| Cell Line (HPV Status) | Cell Origin | $IC_{50}$ (μM) | |
|---|---|---|---|
| | | **9** | **7** |
| SiHa (HPV16) | Malignant | 0.09 | 0.83 |
| M08 (HPV16) | Neoplastic | 0.048 | 0.82 |
| HEK (HPV negative) | Normal | 0.076 | 0.3 |

*3.4. Mechanism of activation of the phosphonamidate derivatives.*

The metabolic activation of the phosphonamidates is generally assumed to follow the same two enzymatic steps involved in the activation of the phosphoramidates [19]. In order to prove that the tetrahydronaphthyl derivative **10** is activated in a similar manner, a $^{31}$P NMR study has been performed to investigate the interaction of this compound with a carboxypeptidase-type enzyme. Compound **10** was therefore incubated in an NMR tube with carboxypeptidase Y in acetone-$d_6$ buffered at pH = 7.6 with Trizma. The reaction was monitored by $^{31}$P NMR. The spectra in Figure 3 show conversion of the starting material ($\delta_P$ = 23.2, 23.1 ppm) to the corresponding aminoacyl phosphonamidate ester ($\delta_P$ = 15.0 ppm). *In vivo*, the ester is then believed to undergo P-N cleavage, mediated by a phosphonamidase or resulting from simple hydrolysis in a more acidic subcellular compartment, to eventually release the parent drug.

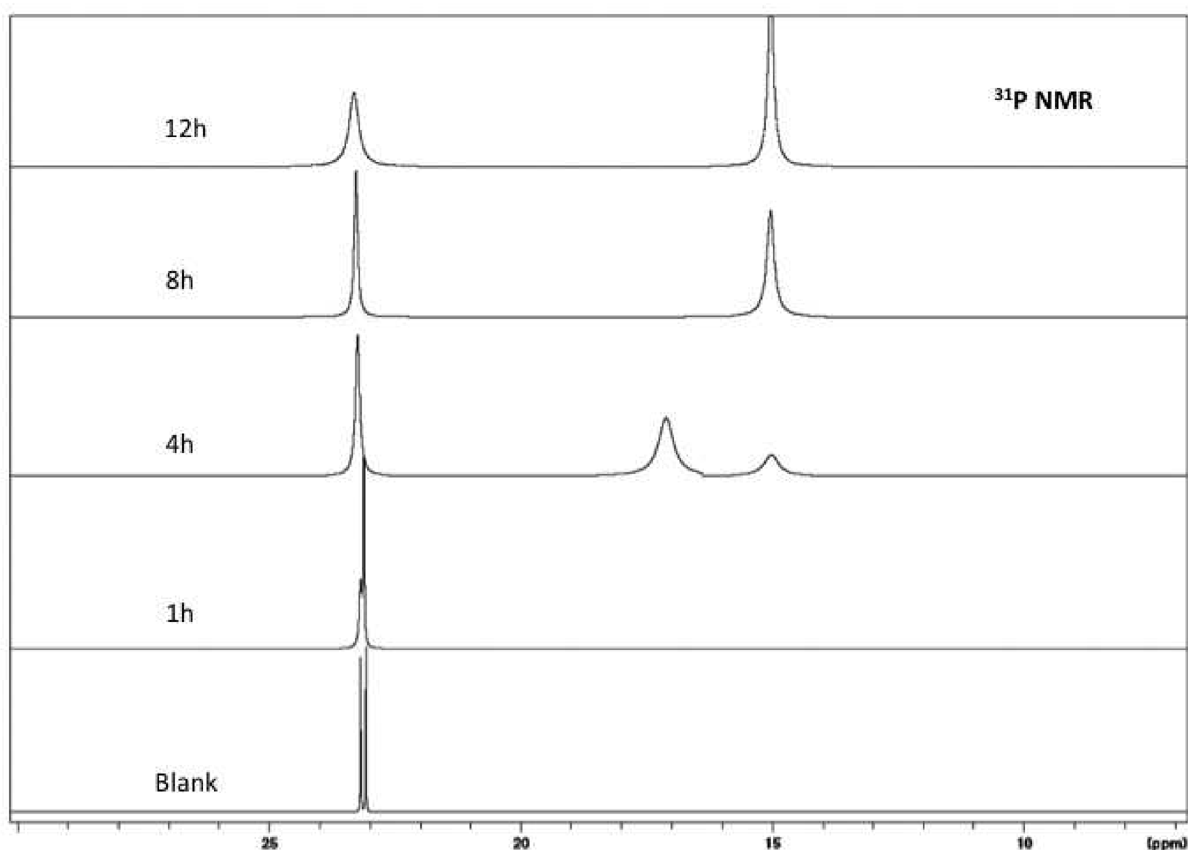



**Figure 3.** Carboxypeptidase Y assay applied on ProTide **10** and monitored by $^{31}$P NMR, 25 °C**.**

## 4. *Conclusion.*

In conclusion, we have successfully prepared a family of adefovir and tenofovir phosphonodiamidates and protides with improved *in vitro* anti-HIV activity compared to previously reported data for the parent ANP. For the first time, a 5,6,7,8-tetrahydro-1-naphthol unit was introduced as hydrolysable aryl unit in the proTide motif of ANPs. Phosphonoamidates bearing this structurally innovative modification displayed an improved antiviral activity compared with the "common" naphthyl and phenol protide units. This modification appears to be an excellent candidate for future improvement of the ProTide motif. The prodrugs obtained were evaluated *in vitro* for antiviral (HIV type 1 and 2) and cytostatic (murine leukemia cells and human T-lymphocyte, human cervix carcinoma) activity. These data showed that the 5,6,7,8-tetrahydro-1-naphthol derivative has improved inhibitory activity compared to the phenol and naphthyl moieties in both a cytostatic and an anti-HIV context. An enzyme study shows processing by a parallel mechanism to known aryl entities.

Prodrug compounds **7** and **9** (adefovir) and compounds **15**, **16** and **12** (tenofovir) were also tested for their antiproliferative activity against HPV-infected neoplastic cells and were found to be significantly more active in comparison to the parent compounds from which they were derived. A slightly better activity of the adefovir derivative was also noticed. However, no preferential specificity to HPV+ cell lines or transformed cell lines was observed in this study.

The compounds of the present study warrant further investigation in antiviral and cytotoxic settings.

## 5. *Experimental section.*

### 5.1. *Chemistry.*

Tenofovir was obtained from Ningbo Haishu Hobid Imp & Exp Co., Ltd, Ningbo, China and Adefovir was obtained from Hubei Maxsource chemical Co., Ltd, Wuhan, China.

The numbering of compounds follows the recommended IUPAC nomenclature guidelines. The naming of the compounds follows IUPAC nomenclature and/or standard accepted nomenclature for nucleoside chemistry.

All solvents used were anhydrous and used as supplied by Aldrich. All nucleosides and solid reagents were dried for several hours under high vacuum over potassium hydroxide. All glassware

was oven-dried at 130 °C for several hours or overnight and allowed to cool in a desiccator or under a stream of dry nitrogen. For thin-layer chromatography (TLC), precoated aluminium-backed plates (60 F-54, 0.2 mm thickness; supplied by E. Merck AG, Darmstadt, Germany) were used and developed by an ascending elution method. After solvent evaporation, compounds were detected by quenching of the fluorescence, at 254 or 336 nm depending on the compound, upon irradiation with a UV lamp. For column chromatography: Glass columns were slurry-packed in the appropriate eluent or pre-adsorbed onto silica gel. Fractions containing the product were identified by TLC and pooled, and the solvent was removed *in vacuo*.

$^{1}$H, $^{31}$P and $^{13}$C NMR spectra were recorded in a Bruker Avance 500 spectrometer at 500 MHz, 202 MHz and 125 MHz, respectively and auto-calibrated to the deuterated solvent reference peak in case of $^{1}$H and $^{13}$C-NMR and 85% $H_3PO_4$ for $^{31}$P-NMR experiments. All $^{31}$P and $^{13}$C NMR spectra were proton-decoupled. Coupling constants (*J*) are measured in Hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), ddd (doublet of doublet of doublet), dt (doublet of triplet).

Low resolution mass analysis were performed by the service at the Department of Chemistry, University of Wales, Cardiff, UK, using electrospray ionization technique (ES).

All analytical high-performance liquid chromatography (HPLC) experiments were done on a Thermo Fisher Scientific Spectra System SCM1000 provided with a System Controller SN4000, a pump Spectra System P4000 and a Spectra UV2000 detector set and using a 5 µM Hypersil GOLD (150 × 4.6 mm) reverse phase column, eluting with the indicated mobile phase. The software used was ChromQuest 5.0. All final compounds were isolated with purity ≥ 95%.

#### *5.1.1. Procedure A: Synthesis of phosphonodiamidates. [16]*

To a solution of the acyclic nucleoside in dry ACN (10 ml/mmol), TMSBr (5 eq) was added under argon and the reaction was stirred at RT overnight. The solvents were removed under reduced pressure without any contact with air. The residue was dissolved in anhydrous $Et_3N$ (2 ml/mmol) and pyridine (8 ml/mmol) and some amino acid ester (4 eq) was added. In a separated flask, Aldrithiol-2 (6 eq) and $Ph_3P$ (6 eq) were dissolved in anhydrous pyridine (10 ml/mmol) and this solution was immediately added to the reaction. The resulting mixture (green solution) was stirred at 50 °C for 3-5 h, then cooled to room temperature and the solvent completely evaporated on a

rotary evaporator (temp. water bath 40 ˚C). The residue was taken up in a mixture of methanol, water, toluene and hexane (15 mL each) and then transferred into a separating funnel. The upper phase (toluene and hexane) was removed and the lower phase (water/methanol) was extracted again with a 1:1 mixture of toluene/hexane (3 x 10 mL). The product was then extracted from the water /methanol phase with 3 dichloromethane washing (30 mL each). The dichloromethane washing were combined and dried over $MgSO_4$, filtered and evaporated to dryness.

The residue was purified by flash chromatography on silica gel eluted with DCM/MeOH 95:5 to obtain the desired products.

### *5.1.2. Procedure B: synthesis of phosphonoamidates [16].*

To a solution of the acyclic nucleoside in dry ACN (10 ml/mmol), TMSBr (5 eq) was added under argon and the reaction was stirred at RT overnight. Then the solvents were removed under reduced pressure without any contact with air. The residue was dissolved in anhydrous $Et_3N$ (2 ml/mmol) and pyridine (8 ml/mmol) and some amino acid ester (1.5 eq) and aryl alcohol (1.5 eq) were added. In a separated flask, Aldrithiol-2 (6 eq) and $Ph_3P$ (6 eq) were dissolved in anhydrous pyridine (10 ml/mmol) and this solution was immediately added to the reaction. The resulting mixture (green solution) was stirred at 50 °C for 3 h.

The residue was taken up in a mixture of methanol, water, toluene and hexane (15 mL each) and then transferred into a separating funnel. The upper phase (toluene and hexane) was removed and the lower phase (water/methanol) was extracted again with a 1:1 mixture of toluene/hexane (3 x 10 mL). The product was then extracted from the water /methanol phase with 3 dichloromethane washing (30 mL each). The dichloromethane washing were combined and dried over $MgSO_4$, filtered and evaporated to dryness. The residue was purified by flash chromatography on silica gel eluted with DCM/MeOH 95:5 to obtain the desired products.

### *5.1.3. 9-[2-(Naphthyloxy-(benzyloxy-L-alaninyl)-Phosphonomethoxy)ethyl]adenine. (**5**)*

Following procedure B with adefovir (400 mg, 1.46 mmol), 2,2-dimethylpropoxy-L-alaninyl ester *p*-TSA salt (790.42 mg, 2.34 mmol) and phenol (337.75 mg, 2.34 mmol)), the title compound was obtained as an off-white solid (55%, 450 mg). $^1$H NMR (500 MHz, MeOH-$d_4$): δ 8.18 (s, 1H, H-2), 8.16 (s, 1H, H-2), 8.10 (s, 1H, H-8), 8.07 (s, 1H, H-8), 8.01 (d, $J$ = 10.0 Hz, 1H, Naph), 7.98 (d, $J$ = 10.0 Hz, 1H, Naph), 7.87 (d $J$ = 10.0 Hz, 2H, Naph), 7.69 (d, $J$ = 5.0 Hz, 1H, Naph), 7.68 (d, $J$

= 5.0 Hz, 2H, Naph), 7.53-7.50 (m, 2H, Naph), 7.47-7.45 (m, 2H, Naph), 7.39-7.31 (m, 4H, Naph), 7.30-7.27 (m, 10H, C$H_2$Ph), 5.08, 5.02 (ABq, 2H, $J_{AB}$= 12.2 Hz, C$H_2$Ph), 5.03 (s, 2H, C$H_2$Ph), 4.40-4.35 (m, 4H, N-$CH_2$), 4.10-3.97 (m, 6H, $CH_2$-P, N-CH), 3.92-3.88 (m, 4H, O$CH_2$$CH_2$N), 1.23 (d, $J$ = 7.0 Hz, 3H, $CH_3$-aa), 1.19 (d, $J$ = 7.0 Hz, 3H, $CH_3$-aa) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 175.31 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, C=O), 174.97 (d, $^3J_{C\text{-}P}$ = 4.2 Hz, C=O), 157.27, 157.25 (C-6), 153.73, 153.69 (CH-2), 150.65 (C-4), 147.45 (d, $^2J_{C\text{-}P}$ = 8.7 Hz, C-*ipso* ONaph), 147.40 (d, $^2J_{C\text{-}P}$ = 8.7 Hz, C-*ipso* ONaph), 143.19 (CH-8), 137.57 (C-*ipso* $CH_2$*Ph*), 137.33 (C-*ipso* $CH_2$*Ph*), 136.36 (C-Naph), 129.56, 129.33, 129.31 (CH-Ph), 128.83, 128.77 (CH-Naph), 128.08 (d, $^3J_{C\text{-}P}$ = 4.2 Hz, C-Naph), 127.98 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, C-Naph), 127.47, 127.39, 126.45, 126.42, 125.98, 125.92, (CH-Naph), 122.84, 122.57 (CH-Naph), 119.98, 119.92 (C-5), 116.82 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, CH-Naph), 116.68 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, CH-Naph), 72.46 (d, $^3J_{C\text{-}P}$ = 13.7 Hz, O$CH_2$$CH_2$N), 72.35 (d, $^3J_{C\text{-}P}$ =13.7 Hz, O$CH_2$$CH_2$N), 67.97, 67.87 (O$CH_2$Ph), 67.78 (d, $^1J_{C\text{-}P}$ = 155.0 Hz, $CH_2$-P), 68.29 (d, $^1J_{C\text{-}P}$ = 153.7 Hz, $CH_2$-P), 50.98, 50.92 (N-CH), 44.64, 44.58 (N-$CH_2$), 20.81 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, $CH_3$-aa), 20.32 (d, $^3J_{C\text{-}P}$ = 6.2 Hz, $CH_3$-aa) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 24.26, 23.13 ppm. MS (ES+) *m/z*: 561 [M+H$^+$]. Reverse-phase HPLC, eluting with $H_2O$/MeOH from 90/100 to 0/100 in 30 min, Flow = 1 mL/min, λ = 263 nm, $t_R$ = 15.14 min.

5.1.4. 9-[2-(bis-(benzyloxy-L-alaninyl)-Phosphonomethoxy)ethyl]adenine. (**6**)

Following procedure A with adefovir (210 mg, 0.77 mmol) and benzyloxy-L-alaninyl ester p-TSA salt (1.04 g, 3.07 mmol), the desired product (FP137=Cf3557) was obtained as an off-white solid (22%, 82 mg). 1H NMR (500 MHz, MeOH-d4): □ 8.24 (s, 1H, H-2), 8.19 (s, 1H, H-8), 7.38-7.30 (m, 10H, Ph),5.16, 5.13 (ABq, 2H, JAB=12.2 Hz, CH2Ph), 5.13, 5.08 (ABq, 2H, JAB= 2.2 Hz, CH2Ph), 4.37 (t, J = 4.8 Hz, 2H, N-CH2), 3.97 (m, 2H, 2 x N-CH), 3.82 (t, J = 5.0 Hz, 2H, OCH2CH2N), 3.72 (dd, JHP = 8.2 Hz, J = 10.2 Hz, 1H, CH2-P), 3.67 (dd, JHP = 8.9, J = 13.2 Hz, 1H, CH2-P), 1.34 (d, J =7.1 Hz, 3H, CH3-aa), 1,28 (d, J = 7.1 Hz, 3H, CH3-aa); 13C-NMR (125 MHz, MeOH-d4): □ 175.41 (d, 3JC-P = 3.9 Hz, C=O), 175.37 (d, 3JC-P = 2.6 Hz, C=O), 156.82 (C-6), 153.02 (CH-2), 150.71 (C-4), 143.71 (CH-8), 137.35 (C-ipso Ph), 137.33 (C-ipso Ph), 129.64, 129.61, 129.42, 129.40, 129.36 (Ph), 119.92 (C-5), 72.22 (d, 2JC-P = 13.2 Hz, OCH2CH2N), 68.55 (d, 1JC-P = 135.1 Hz, P-CH2), 67.99, 67.93 (OCH2Ph), 50.21, 49.88 (N-CH), 44.65 (N-CH2), 21.14 (d, 3JC-P = 4.9 Hz, CH3-aa), 20.95 (d, 3JC-P = 6.2 Hz, CH3-aa) ppm;

31P NMR (202 MHz, MeOH-d4):  23.10 ppm. MS (ES+) m/z: 596 [M+H]. Reverse-phase HPLC, eluting with H2O/MeOH from 90/10 to 0/100 in 30 min, Flow = 1 mL/min,  = 263 nm, tR = 19.44 min.

*5.1.5.* 9-[2-(bis-(2,2-dimethylpropoxy-L-alaninyl)-Phosphonomethoxy)ethyl]adenine. (**7**) Following procedure A with adefovir (200 mg, 0.73 mmol) and 2,2-dimethylpropoxy-L-alaninyl ester *p*-TSA salt (970 mg, 2.93 mmol), the desired product was obtained as an off-white solid (64%, 273 mg). $^{1}$H NMR (500 MHz, MeOH-$d_4$): δ 8.21 (s, 2H, H-2, H-8), 4.44 (m, 2H, N-$CH_2$), 3.97 (m, 2H, 2 x N-CH), 3.94 (m, 2H, O*$CH_2$*$CH_2$N), 3.90, 3.79 (AB, 2H, $J_{AB}$= 11.0 Hz, O*$CH_2$*C$(CH_3)_3$), 3.86, 3.73 (AB, 2H, $J_{AB}$ = 11.0 Hz, O*$CH_2$*C$(CH_3)_3$), 3.78 (dd, $J_{HP}$ = 8.2 Hz, $J$ = 13.7 Hz, 1H, $CH_2$-P), 3.74 (dd, $J_{HP}$= 9.3 Hz, $J$ = 13.9 Hz, 1H, $CH_2$-P), 1.36 (d, $J$ = 7.2 Hz, 3H, $CH_3$-aa), 1.34 (d, $J$ = 7.3 Hz, 3H, $CH_3$-aa), 0.95 (s, 9H, C$(CH_3)_3$), 0.94 (s, 9H, C$(CH_3)_3$) ppm; $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 175.69 (d, $^{3}J_{C-P}$ = 5.2 Hz, C=O), 175.62 (d, $^{3}J_{C-P}$ = 3.9 Hz, C=O), 157.26 (C-6), 153.70 (CH-2), 150.73 (C-4), 143.47 (CH-8), 119.91 (C-5), 75.42, 75.34 (O*C*$H_2$C$(CH_3)_3$), 72.26 (d, $^{3}J$= 13.2 Hz, O*C*$H_2$$CH_2$N), 68.51 (d, $^{1}J_{C-P}$ = 136.3 Hz, $CH_2$-P), 50.20, 49.78 (N-CH), 44.62 (N-$CH_2$), 32.31, 32.29 (*C*$(CH_3)_3$), 26.76, 26.74 (C(*C*$H_3)_3$), 21.39 (d, $^{3}J_{C-P}$ = 4.8 Hz, $CH_3$-aa), 21.26 (d, $^{3}J_{C-P}$ = 5.8 Hz, $CH_3$-aa) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 23.19 ppm. MS ($ES^+$) m/z: 578.28 [M+Na]. Reverse-phase HPLC, eluting with $H_2O$/MeOH from 90/10 to 0/100 in 30 min, Flow = 1 mL/min, λ = 263 nm, $t_R$ = 15.44 min.

5.1.6. *9-[2-(Phenyloxy-(2,2-dimethylpropoxy-L-alaninyl)-Phosphonomethoxy)ethyl]adenine.* (**8**)

Following procedure B with adefovir (218 mg, 0.80 mmol), 2,2-dimethylpropoxy-L-alaninyl ester *p*-TSA salt (423.16 mg, 1.28 mmol) and phenol (120.16 mg, 1.28 mmol), the title compound was obtained as an off-white solid (25%, 100 mg). $^{1}$H NMR (500 MHz, MeOH-$d_4$): δ 8.21 (s, 1H, H-2), 8.20 (s, 1H, H-2), 8.18 (s, 1H, H-8), 8.16 (s, 1H, H-8), 7.30-7.22 (m, 4H, Ph), 7.14 (t, $J$ = 8.2 Hz, 2H, Ph), 7.12 (t, $J$ = 8.1 Hz, 2H, Ph), 7.06 (d, $J$ = 8.1 Hz, 2H, Ph), 4.43 (m, 4H, N-$CH_2$), 4.07 – 3.85 (m, 10H, $CH_2$-P, N-CH, O*$CH_2$*$CH_2$N), 3.81, 3.71 (AB, $J_{AB}$ = 10.0 Hz, 2H, O*$CH_2$*C$(CH_3)_3$), 3.80, 3.70 (AB, $J_{AB}$ = 10.0 Hz, 2H, O*$CH_2$*C$(CH_3)_3$), 1.29 (d, $J$ = 7.3 Hz, 3H, $CH_3$-aa), 1.28 (d, $J$ = 7.3 Hz, 3H, $CH_3$-aa), 0.95 (s, 9H, C$(CH_3)_3$), 0.94 (s, 9H, C$(CH_3)_3$) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 175.33 (d, $^{3}J_{C-P}$ = 4.0 Hz, C=O), 175.09 (d, $^{3}J_{C-P}$ = 4.0 Hz, C=O), 157.32,157.30 (C-

6), 153.76, 153.72 (CH-2), 151.50 (d, $^{2}J_{C\text{-}P}$ = 9.0 Hz, C-*ipso* OPh), 151.41 (d, $^{2}J_{C\text{-}P}$ = 9.0 Hz, C-*ipso* OPh), 150.75, 150.73 (C-4), 143.41, 143.38 (CH-8), 130.76, 130.74, 126.17, 126.11 (Ph), 121.94 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, Ph), 121.76 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, Ph), 119.99, 119.95 (C-5), 75.40, 75.37 (O*C*$H_2C(CH_3)_3$), 72.37 (d, $^{3}J_{C\text{-}P}$ = 11.9 Hz, O$CH_2$$CH_2$N), 72.26 (d, $^{3}J_{C\text{-}P}$ =12.9 Hz, O$CH_2$$CH_2$N), 67.51 (d, $^{1}J_{C\text{-}P}$ = 155.3 Hz, $CH_2$-P), 67.48 (d, $^{1}J_{C\text{-}P}$ = 155.3 Hz, $CH_2$-P), 51.02, 50.92 (N-CH), 44.69, 44.65 (N-$CH_2$), 32.34 (*C*$(CH_3)_3$), 26.77, 26.74, (C(*C*$H_3)_3$), 21.26 (d, $^{3}J_{C\text{-}P}$ = 5.3 Hz, $CH_3$-aa), 20.74 (d, $^{3}J_{C\text{-}P}$ = 5.3 Hz, $CH_3$-aa) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 23.71, 22.68 ppm. MS (ES+) *m/z*: 513.49 [M+$Na^+$]. Reverse-phase HPLC, eluting with $H_2O$/MeOH from 90/100 to 0/100 in 30 min, Flow = 1 mL/min, λ = 263 nm, $t_R$ = 15.74 min.

### 5.1.7. 9-[2-(□-Napthyloxy-(2,2-dimethylpropoxy-L-alaninyl)-Phosphonomethoxy)ethyl]adenine. (**9**)

Following procedure B with adefovir (210 mg, 0.76 mmol), 2,2-dimethylpropoxy-L-alaninyl ester *p*-TSA salt (384 mg, 1.16 mmol), and naphthol (167 mg, 1.16 mmol), the desired product (**9)** was obtained as an off-white solid (45%, 189 mg). $^{1}$H NMR (500 MHz, MeOH-$d_4$): δ 8.19 (s, 1H, H-2), 8.16 (s, 1H, H-8), 8.13 (s, 1H, H-8), 8.11 (s, 1H, H-8), 8.03 (d, *J* = 10.0 Hz, 1H, Naph), 8.00 (d, *J* = 10.0 Hz, 1H, Naph), 7.87 (d, *J* = 10 Hz, 2H, Naph), 7.69 (d, *J* = 5.0 Hz, 1H, Naph), 7.68 (d, *J* = 5.0 Hz, 1H, Naph), 7.54-7.51 (m, 2H, Naph), 7.48-7.35 (m, 2H, Naph) 7.32-7.34 (m, 4H, Naph), 4.43 (m, 4H, N-$CH_2$), 4.16 – 4.02 (m, 10H, $CH_2$-P, N-CH, O$CH_2$$CH_2$N), 3.80, 3.66 (AB, $J_{AB}$ = 10.5 Hz, 2H, O$CH_2$$C(CH_3)_3$), 3.76, 3.68 (AB, $J_{AB}$ = 10.5 Hz, 2H, O$CH_2$$C(CH_3)_3$), 1.25 (d, *J* = 6.9 Hz, 3H, $CH_3$-aa), 1.23 (d, *J* = 7.1 Hz, 3H, $CH_3$-aa), 0.90 (s, 9H, $C(CH_3)_3$), 0.88 (s, 9H, $C(CH_3)_3$) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 175.31 (d, $^{3}J_{C\text{-}P}$ = 3.7 Hz, C=O), 174.97 (d, $^{3}J_{C\text{-}P}$ = 4.2 Hz, C=O), 157.27, 157.25 (C-6), 153.74, 153.68 (CH-2), 150.74, 150.71 (C-4), 147.45, 147.40 (d, $^{2}J_{C\text{-}P}$ = 8.7 Hz, C-*ipso* ONaph), 143.23, 143.20 (CH-8), 136.29 (C-Naph), 128.84, 128.80 (CH-Naph), 128.08 (d, $^{3}J_{C\text{-}P}$ = 4.2 Hz, C-Naph), 127.98 (d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, C-Naph), 127.76, 127.45, 127.41, 126.48, 126.45, 125.99, 125.94, (CH-Naph), 122.82, 122.62 (CH-Naph), 119.96, 119.94 (C-5), 116.84 (d, $^{3}J_{C\text{-}P}$ = 3.9 Hz, CH-Naph), 116.70 (d, $^{3}J_{C\text{-}P}$ = 3.7 Hz, CH-Naph), 75.39, 75.35 (O*C*$H_2C(CH_3)_3$)), 72.22 (d, $^{3}J_{C\text{-}P}$ = 12.5 Hz, O*C*$H_2CH_2$N), 72.20 (d, $^{3}J_{C\text{-}P}$ = 12.5 Hz, O*C*$H_2CH_2$N), 67.78 (d, $^{1}J_{C\text{-}P}$ = 155.3 Hz, $CH_2$-P), 67.72 (d, $^{1}J_{C\text{-}P}$ = 154.5 Hz, $CH_2$-P), 50.95, 49.90 (N-CH), 44.69, 44.61 (N-$CH_2$), 32.37, 32.36 (*C*$(CH_3)_3$), 26.81 (C(*C*$H_3)_3$), 21.18 (d, $^{3}J_{C\text{-}P}$ = 5.2 Hz, $CH_3$-

aa), 20.66 (d, $^3J_{C-P}$ = 6.2 Hz, $CH_3$-aa), ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 24.29, 23.34 ppm. MS (ES+) *m/z*: 541 [M+H$^+$]. Reverse-phase HPLC, eluting with $H_2O$/MeOH from 90/100 to 0/100 in 30 min, Flow = 1 mL/min, λ = 263 nm, $t_R$ = 15.8 min.

5.1.8. 9-[2-(5,6,7,8-Tetrahydro-1-naphthyloxy-(2,2-dimethylpropoxy-L-alaninyl)-Phosphonomethoxy)ethyl]adenine. (**10**)

Following procedure B with adefovir (230 mg, 0.84 mmol), 2,2-dimethylpropoxy-L-alaninyl ester *p*-TSA salt (421 mg, 1.27 mmol), and tetrahydronaphthol (188 mg, 1.27 mmol), the desired product (FP137= Cf3476) was obtained as an off-white solid (8 %, 35 mg). $^1$H NMR (500 MHz, MeOH-$d_4$): δ 8.22 (s, 1H, H-2), 8.20 (s, 1H, H-8), 8.17 (s, 1H, H-2), 8.15 (s, 1H, H-8), 7.06-6.94 (m, 4H, C*H*-tetrahydronaph), 6.86 (t, *J* = 6.4 Hz, 2H, C*H*-tetrahydronaph), 4.45 (m, 4H, N-$CH_2$), 4.09 – 3.92 (m, 10H, $CH_2$-P, N-CH, O$CH_2$$CH_2$N), 3.83, 3.73 (AB, $J_{AB}$ = 11.0 Hz, 2H, O$CH_2$C$(CH_3)_3$), 3.81, 3.71 (AB, $J_{AB}$ = 9.0 Hz, 2H, O$CH_2$C$(CH_3)_3$), 2.76-2.71 (m, 4H, $CH_2$-tetrahydronaph), 2.57-2.52 (m, 4H, $CH_2$-tetrahydronaph), 1.76-1.75 (m, 8H, $CH_2$-tetrahydronaph), 1.31 (d, *J* = 6.9 Hz, 3H, $CH_3$-aa), 1.30 (d, *J* = 7.1 Hz, 3H, $CH_3$-aa), 0.93 (s, 9H, C$(CH_3)_3$), 0.92 (s, 9H, C$(CH_3)_3$) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 175.40 (d, $^3J_{C-P}$ = 3.8 Hz, C=O), 175.05 (d, $^3J_{C-P}$ = 4.2 Hz, C=O), 152.44 (C-6), 150.5 (C-4), 149.80 (d, $^2J_{C-P}$ = 6.2 Hz, C-*ipso* OTetrahydronaph), 149.70 (d, $^2J_{C-P}$ = 6.2 Hz, C-*ipso* OTetrahydronaph), 146.43, 146.41 (CH-2), 145.71, 145.67 (CH-8), 140.70 (C-Tetrahydronaph), 129.90 (d, $^3J_{C-P}$ = 6.2 Hz, *C*-Tetrahydronaph), 129.76 (d, $^3J_{C-P}$ = 6.2 Hz, *C*-Tetrahydronaph), 126.86, 126.82, 126.79, 126.76 (CH-Tetrahydronaph), 119.68, 119.66 (C-5), 118.26 (d, $^3J_{C-P}$ = 3.4 Hz, CH-Tetrahydronaph), 75.42, 75.40 (O$CH_2$C$(CH_3)_3$)), 72.22 (d, $^3J_{C-P}$ = 12.5 Hz, O$CH_2$$CH_2$N), 72.20 (d, $^3J_{C-P}$ = 12.5 Hz, O$CH_2$$CH_2$N), 67.70 (d, $^1J_{C-P}$ = 153.7 Hz, $CH_2$-P), 50.95, 50.93 (N-CH), 45.15, 45.16 (N-$CH_2$), 32.37, 32.36 (*C*$(CH_3)_3$), 30.52 ($CH_2$-Tetrahydronaph), 26.81 (C$(CH_3)_3$), 24.60, 24.58, 23.88, 23.85, 23.76 ($CH_2$-Tetrahdronaph), 21.40 (d, $^3J_{C-P}$ = 5.0 Hz, $CH_3$-aa), 20.81 (d, $^3J_{C-P}$ = 6.2 Hz, $CH_3$-aa) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 23.41, 22.47 ppm. MS (ES+) *m/z*: 545.2 [M+H$^+$]. Reverse-phase HPLC, eluting with $H_2O$/MeCN from 90/10 to 40/60 in 15 min, then 40/60 isocratic for 15 min. then 40/60 to 0/100 in 10 min., Flow = 1 mL/min, λ = 263 nm, $t_R$ = 26.7 min.; 27.6 min.

5.1.9. 9-[(R)-2-(bis-(benzyloxy-L-alaninyl)-phosphonomethoxy)propyl]adenine (**11**). Following procedure A with tenofovir (200 mg, 0.69 mmol) and benzyloxy-L-alaninyl ester *p*-TSA salt (970 mg, 2.76 mmol), the desired product **11** was obtained as an off-white solid (75%, 0.320 g). $^{1}$H NMR (500 MHz, MeOH-$d_4$): δ 8.22 (s, 1H, H-2), 8.17 (s, 1H, H-8), 7.37-7.29 (m, 10H, 2 x O-$CH_2$*Ph*), 5.19, 5.15 (AB, $J_{AB}$ = 12.0 Hz, 1H, O*$CH_2$Ph*), 5.08, 5.02 (AB, $J_{AB}$ = 12.5 Hz, 1H, O*$CH_2$Ph*), 4.32 (dd, 1H, *J* = 14.5, 3.1 Hz, 1H, N-$CH_2$), 4.14 (dd, 1H, *J* = 14.5, 7.1 Hz, N-$CH_2$), 4.02-3.98 (m, 1H, N-CH), 3.97-3.92 (m, 1H, N-CH), 3.86-3.80 (m, 1H, O-CH), 3.77 (dd, *J* = 13.0 Hz, $J_{HP}$ = 7.7 Hz, 1H, P-$CH_2$), 3.48 (dd, *J* = 13.0 Hz, $J_{HP}$ = 10.9 Hz, 1H, P-$CH_2$), 1.37 (d, *J* = 7.2 Hz, 3H, $CH_3$-aa), 1.25 (d, *J* = 7.1 Hz, 3H, $CH_3$-aa), 1.12 (d, *J* = 6.2 Hz, 3H, $CH_3$-tenofovir) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 175.42 (d, $^{3}J_{C\text{-}P}$ = 5.3 Hz, C=O), 175.35 (d, $^{3}J_{C\text{-}P}$ = 2.9 Hz, C=O), 157.25 (C-6), 153.70 (C-2), 150.99 (C-4), 143.92 (C-8), 137.33 (C-*ipso* Ph), 137.24 (C-*ipso* Ph), 129.54, 129.36, 129.32, 129.30 (Ph), 119.79 (C-5), 77.52 (d, $^{3}J_{C\text{-}P}$ = 13.0 Hz, O-CH), 67.97, 67.89 (2 x O-*C*$H_2$Ph), 66.07 (d, $^{1}J_{C\text{-}P}$ = 137.1 Hz, P-$CH_2$), 50.18, 49.71 (2 x N-CH), 49.19 (N-$CH_2$), 21.08 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz, $CH_3$-aa), 20.81 (d, $^{3}J_{C\text{-}P}$ = 6.3 Hz, $CH_3$-aa), 16.61 ($CH_3$-tenofovir) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 23.75 ppm. MS (ES+) *m/z*: 632 (M+$Na^+$, 100%), 610 (M+$H^+$, 46%). Reverse-phase HPLC, eluting with $H_2O$/ACN from 90/100 to 0/100 in 25 min, Flow = 1 mL/min, λ = 254 nm, $t_R$ = 13.37 min.

5.1.10. 9-[(R)-2-(bis-(2,2-dimethylpropoxy-L-alaninyl)-phosphonomethoxy)propyl]adenine (**12**).

Following procedure A with tenofovir (200 mg, 0.69 mmol) and neopentyloxy-L-alaninyl ester *p*-TSA salt (928 mg, 2.76 mmol). The desired product **12** was obtained as an off-white solid (76%, 0.298 g). $^{1}$H NMR (500 MHz, MeOH-$d_4$): δ 8.27 (s, 1H, H-2), 8.24 (s, 1H, H-8), 4.43 (dd, *J* = 14.5, 3.1 Hz, 1H, N-$CH_2$), 4.25 (dd, *J* = 14.6, 7.3 Hz, 1H, N-$CH_2$), 4.05-3.93 (m, 3H, 2 x N-CH, O-CH), 3.94, 3.82 (AB, $J_{AB}$ = 10.0 Hz, 2H, O*$CH_2$*C$(CH_3)_3$), 3.86 (dd, *J* = 12.5 Hz, $J_{HP}$ = 7.5 Hz, 2H, P-$CH_2$), 3.78, 3.67 (AB, $J_{AB}$ = 10.0 Hz, 2H, O*$CH_2$*C$(CH_3)_3$), 3.58 (dd, *J* = 12.8 Hz, $J_{HP}$ = 11.0 Hz, 2H, P-$CH_2$), 1.43 (d, *J* = 7.2 Hz, 3H, $CH_3$-aa), 1.36 (d, *J* = 7.2 Hz, 3H, $CH_3$-aa), 1.23 (d, *J* = 6.3 Hz, 3H, $CH_3$-tenofovir), 0.99 (s, 9H, C$(CH_3)_3$), 0.94 (s, 9H, C$(CH_3)_3$) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 175.77 (d, $^{3}J_{C\text{-}P}$ = 4.6 Hz, C=O), 175.35 (d, $^{3}J_{C\text{-}P}$ = 2.7 Hz, C=O), 157.28 (C-6), 153.70 (C-2), 151.07 (C-4), 144.04 (C-8), 119.82 (C-5), 77.69 (d, $^{3}J_{C\text{-}P}$ = 13.3 Hz, O-CH),

75.50, 75.36 (2 x O-$CH_2$), 66.12 (d, $^{1}J_{C\text{-}P}$ = 136.2 Hz, P-$CH_2$), 50.26, 49.69 (2 x N-CH), 48.53 (N-$CH_2$), 32.37, 32.27 ($C(CH_3)_3$)), 26.79, 26.76 ($C(CH_3)_3$)), 21.37 (d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, $CH_3$-aa), 21.15 (d, $^{3}J_{C\text{-}P}$ = 6.2 Hz, $CH_3$-aa), 16.70 ($CH_3$-tenofovir) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 23.91 ppm. MS (ES+) *m/z*: 570 (M+$Na^+$). Reverse-phase HPLC, eluting with $H_2O$/MeOH from 90/100 to 0/100 in 30 min, Flow = 1 mL/min, λ = 254 nm, $t_R$ = 26.04 min.

### 5.1.11. 9-[(R)-2-(naphtyloxy-(benzyloxy-L-alaninyl))-phosphonomethoxy)propyl]adenine (**13**).

Following procedure B with tenofovir (200 mg, 0.69 mmol), benzyloxy-L-alaninyl ester *p*-TSA salt (364 mg, 1.03 mmol) and 1-naphtol (149 mg, 1.03 mmol), the title compound **13** was obtained as an off-white solid (120 mg, ratio: 1:1, yield: 30%). $^{1}$H NMR (500 MHz, MeOH-$d_4$): δ 8.17 (s, 1H, H-2), 8.13 (s, 1H, H-2), 8.10 (s, 1H, H-8), 8.06 (s, 1H, H-8), 8.03 (d, 1H, *J* = 8.0 Hz, Naph), 7.99 (d, 1H, *J* = 8.0 Hz, Naph), 7.88 (d, 2H, *J* = 8.0 Hz, Naph), 7.71-7.68 (m, 2H, Naph), 7.55-7.51 (m, 2H, Naph), 7.50-7.40 (m, 5H, Naph), 7.33-7.29 (m, 11H, Naph, Ph), 5.07, 5.00 (AB, $J_{AB}$ = 12.0 Hz, 2H, O$CH_2$Ph), 5.04 (s, 2H, O$CH_2$Ph), 4.34 (dd, 1H, *J* = 9.5 Hz, 3.0 Hz, N-$CH_2$), 4.31 (dd, 1H, *J* = 10 Hz, 3.0 Hz, N-$CH_2$), 4.19 (dd, 1H, *J* = 9.0 Hz, 7.0 Hz, N-$CH_2$), 4.16 (dd, 1H, *J* = 7.5 Hz, 5.5 Hz, N-$CH_2$), 4.12-4.06 (m, 4H, P-$CH_2$, O-CH), 4.02-3.99 (m, 1H, N-CH), 3.95 (dd, 1H, *J* = 13.0 Hz, $J_{HP}$ = 9.5 Hz, P-$CH_2$), 3.94-3.91 (m, 1H, N-CH), 3.85 (dd, 1H, *J* = 13.5 Hz, $J_{HP}$ = 9.5 Hz, P-$CH_2$), 1.25 (d, 3H, *J* = 7.0 Hz, $CH_3$-aa), 1.23 (d, 3H, *J* = 7.0 Hz, $CH_3$-aa), 1.18 (d, 3H, *J* = 6.5 Hz, $CH_3$-tenofovir), 1.08 (d, 3H, *J* = 6.5 Hz, $CH_3$-tenofovir) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 174.99 (d, $^{3}J_{C\text{-}P}$ = 3.6 Hz, C=O), 174.70 (d, $^{3}J_{C\text{-}P}$ = 3.6 Hz, C=O), 157.19 (C-6), 153.70, 153.63 (C-8), 150.95 (C-4), 147.50 (d, $^{2}J_{C\text{-}P}$ = 3.6 Hz, C-*ipso* ONaph), 147.43 (d, $^{2}J_{C\text{-}P}$ = 3.6 Hz, C-*ipso* ONaph), 143.67, 143.47 (C-2), 137.19, 137.18 (C-*ipso* $CH_2$*Ph*), 136.27, 129.55, 129.33, 129.32, 128.78, 128.05, 127.73, 127.41, 127.28, 126.43, 126.42, 125.94, 125.88, 122.95, 122.67 (Naph, Ph), 119.73 (C-5), 116.74 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, C-2 Naph), 116.66 (d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, C-2 Naph), 77.88 (d, $^{3}J_{C\text{-}P}$= 13.7 Hz, O-CH), 77.63 (d,$^{3}J_{C\text{-}P}$= 13.1 Hz, O-CH), 68.00, 67.89 (O-*C*$H_2$Ph), 65.51 (d, $^{1}J_{C\text{-}P}$ = 157.2 Hz, P-$CH_2$), 65.35 (d, $^{1}J_{C\text{-}P}$ = 156.7 Hz, P-$CH_2$), 50.97 (d, *J* = 4.5 Hz, N-CH), 49.18 (N-$CH_2$), 20.88 (d, $^{3}J_{C\text{-}P}$ = 5.3 Hz, $CH_3$-aa), 20.22 (d, $^{3}J_{C\text{-}P}$ = 6.2 Hz, $CH_3$-aa), 16.79, 16.60 ($CH_3$-tenofovir) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 24.89, 23.60 ppm. MS

(ES+) *m/z*: 575 (M+H$^+$). Reverse-phase HPLC, eluting with $H_2O$/MeOH from 90/100 to 0/100 in 30 min, Flow = 1 mL/min, λ = 254 nm, $t_R$ = 16.29 min.

5.1.12. 9-[(R)-2-(phenyloxy-(2,2-dimethylpropoxy-L-alaninyl))-phosphonomethoxy)propyl]adenine (**14**).

Following procedure B with tenofovir (200 mg, 0.69 mmol), neopentyloxy-L-alaninyl ester *p*-TSA salt (343 mg, 1.03 mmol) and phenol (97 mg, 1.03 mmol), the title compound **14** was obtained as an off-white solid (37 mg, ratio: 2:1, yield: 10%). $^1$H NMR (500 MHz, MeOH-$d_4$): δ 8.23 (s, 1H, H-2), 8.22 (s, 1H, H-8), 8.20 (s, 1H, H-2), 8.19 (m, 1H, H-8), 7.35-7.32 (m, 2H, Ph), 7.29-7.25 (m, 2H, Ph), 7.20-7.12 (m, 4H, Ph), 7.00 (d, 2H, *J* =8.5 Hz, Ph), 4.42 (dd, 1H, *J* = 14.5, 3.5 Hz, 1H, N-$CH_2$), 4.38 (dd, 1H, *J* = 15.0, 3.5 Hz, 1H, N-$CH_2$), 4.27 (dd, 1H, *J* = 14.5, 7.0 Hz, N-$CH_2$), 4.23 (dd, 1H, *J* = 14.5, 8.0 Hz, N-$CH_2$), 4.06-3.97 (m, 4H, O-CH, O-$CH_2$), 3.87-3.71 (m, 4H, P-$CH_2$, O-$CH_2$), 1.31 (d, 3H, *J* = 7.5 Hz, $CH_3$-aa), 1.27 (d, 3H, *J* = 7.0 Hz, $CH_3$-aa), 1.24 (d, 3H, *J* = 5.5 Hz, $CH_3$-tenofovir), 1.21 (d, 3H, *J* = 6.0 Hz, $CH_3$-tenofovir), 0.94 (s, 9H, $C(CH_3)_3$), 0.93 (s, 9H, $C(CH_3)_3$) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 175.27 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, C=O), 175.07 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, C=O), 157.28, 157.22 (C-6), 153.71, 153.61 (C-8), 151.53 (d, *J* = 7.1 Hz, *C-ipso* OPh), 151.50, 151.43 (C-4), 143.83, 143.63 (C-2), 130.68, 126.12, 126.02, 121.94, 121.91, 121.68 (d, $J_{CP}$ = 7 Hz, *Ph*), 119.95 (C-5), 77.76 (d, $^3J_{C\text{-}P}$= 12.7 Hz, O-CH), 77.63 (d, $^3J_{C\text{-}P}$= 13.7 Hz, O-CH), 75.38 (O-$CH_2$), 65.27 (d, $^1J_{C\text{-}P}$ = 156.1 Hz, P-$CH_2$), 65.17 (d, $^1J_{C\text{-}P}$ = 156.0 Hz, P-$CH_2$), 51.02, 50.85 (N-CH), 49.20 (N-$CH_2$), 32.33($\mathit{C}(CH_3)_3$), 26.74, 26.70 ($C(\mathit{CH}_3)_3$), 21.26 (d, $^3J_{C\text{-}P}$ = 5.25 Hz, $CH_3$-aa), 20.58 (d, $^3J_{C\text{-}P}$ = 6.0 Hz, $CH_3$-aa), 16.77, 16.72 ($CH_3$-tenofovir) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 24.34, 22.99 ppm. MS (ES+) *m/z*: 505 (M+H$^+$). Reverse-phase HPLC, eluting with $H_2O$/ACN from 90/100 to 0/100 in 30 min, Flow = 1 mL/min, λ = 254 nm, $t_R$ = 15.15 min.

5.1.13. 9-[(R)-2-(naphtyloxy-(2,2-dimethylpropoxy-L-alaninyl))-phosphonomethoxy)propyl]adenine (**15**).

Reaction was carried out following procedure B with tenofovir (200 mg, 0.69 mmol), neopentyloxy-L-alaninyl ester *p*-TSA salt (228 mg, 0.69 mmol) and 1-naphtol (100 mg, 0.69 mmol). Purification by column chromatography allows isolation of compound **15** (27 mg, ratio $S_P$ : $R_p$ : 19:1, 7%) as off-white solid: $^1$H NMR (500 MHz, MeOH-$d_4$, *only the major diastereoisomer*

*is described*): δ 8.16 (s, 1H, H-2), 8.11 (s, 1H, H-8), 8.05 (d, 1H, $J$ = 8.5 Hz, Naph), 7.87 (d, 1H, $J$ = 8.0 Hz, Naph) 7.70-7.69 (m, 1H, Naph), 7.54-7.46 (m, 2H, Naph), 7.42-7.41 (m, 2H, Naph), 4.39 (dd, $J$ = 14.6, 3.2 Hz, 1H, N-$CH_2$), 4.23 (dd, $J$ = 14.6, 6.9 Hz, 1H, N-$CH_2$), 4.14 (dd, 1H, $J$ = 13.0 Hz, $J_{HP}$ = 9.0 Hz, P-$CH_2$), 4.10, 4.07 (m, 1H, O-CH), 4.05-4.01 (m, 1H, N-CH), 3.94 (dd, 1H, $J$ = 13.0 Hz, $J_{HP}$ = 9.0 Hz, P-$CH_2$), 3.79, 3.67 (AB, 2H, $J_{AB}$ = 10.5 Hz, O-$CH_2$), 1.24 (d, $J$ = 7.2 Hz, 3H, $CH_3$-aa), 1.12 (d, $J$ = 6.3 Hz, 3H, $CH_3$-tenofovir), 0.90 (s, 9H, -$C(CH_3)_3$) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$), *only the major diastereoisomer is described*): δ 175.34 (d, $^3J_{C\text{-}P}$ = 3.5 Hz, C=O), 157.20 (C-6), 153.65 (C-8), 150.98 (C-4), 147.48 (d, $^2J_{C\text{-}P}$ = 5.5 Hz, C-*ipso* Naph), 143.69 (C-2), 136.30 (C-Naph), 128.80 (C-H Naph), 128.07 (d, $J$ = 5.4 Hz, C-Naph), 127.78, 127.33, 126.47, 125.98, 122.98 (C-H Naph), 119.77 (C-5), 116.80 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, CH-Naph), 78.98 (d, $^3J_{C\text{-}P}$ = 13.3 Hz, O-CH), 75.43 (O-$CH_2$), 65.44 (d, $^1J_{C\text{-}P}$ = 156.3 Hz, P-$CH_2$), 50.96 (N-CH), 48.89 (N-$CH_2$), 32.30 (*C*$(CH_3)_3$), 26.75 (C$(\mathit{CH_3})_3$), 20.57 (d, $^3J_{C\text{-}P}$ = 6.3 Hz, $CH_3$-aa), 16.74 ($CH_3$-tenofovir) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$, *only the major diastereoisomer is described*): δ 24.90 ppm. MS (ES+) *m/z*: 577 (M+$Na^+$). Reverse-phase HPLC, eluting with $H_2O$/MeOH from 90/100 to 0/100 in 30 min, Flow = 1 mL/min, λ = 254 nm, $t_R$ = 22.45 min.

Further elution afforded **16** (16 mg, ratio $S_P$ : $R_p$ 1:5, 4%) as an off-white solid: $^1$H NMR (500 MHz, MeOH-$d_4$, *only the major diastereoisomer is described*): δ 8.17 (s, 1H, H-2), 8.12 (s, 1H, H-8), 7.96 (d, $J$ = 8.1 Hz, 1H, Naph), 7.86 (d, $J$ = 8.0 Hz, 1H, Naph), 7.68-7.66 (m, 1H, Naph), 7.53-7.44 (m, 2H, Naph), 7.36-7.34 (m, 2H, Naph), 4.36 (dd, $J$ = 14.6, 3.2 Hz, 1H, N-$CH_2$), 4.22 (dd, $J$ = 14.6, 7.2 Hz, 1H, N-$CH_2$), 4.13-4.03 (m, 3H, P-$CH_2$, N-CH, O-CH), 4.06 (dd, $J_{HP}$ = 10.0 Hz, $J$ = 12.5 Hz, 1H, P-$CH_2$) 3.76, 3.68 (AB, $J_{AB}$ = 10.5 Hz, 2H, O-$CH_2$), 1.26 (d, $J$ = 7.2 Hz, 3H, $CH_3$-aa), 1.22 (d, J = 6.2 Hz, 3H, $CH_3$-tenofovir), 0.88 (s, 9H, $C(CH_3)_3$) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$, *only the major diastereoisomer is described*): δ 174.95 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, C=O), 155.34 (C-6), 150.94 (C-8), 150.79 (C-4), 147.39 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, C-*ipso* Naph), 144.35 (C-2), 136.27 (C-Naph), 128.82 (CH-Naph), 128.00 (d, $J_{CP}$ = 6.2 Hz, C-Naph), 127.74, 127.38, 126.46, 125.87, 122.62 (CH-Naph), 119.59 (C-5), 116.57 (d, $^3J_{C\text{-}P}$ = 3.6 Hz, CH-Naph), 77.60 (d, $^3J_{C\text{-}P}$= 13.4 Hz, O-CH), 75.35 (O-$CH_2$), 65.51 (d, $^1J_{C\text{-}P}$ = 157.1 Hz, P-$CH_2$), 50.90 (N-CH), 49.00 (N-$CH_2$), 32.30 (*C*$(CH_3)_3$), 26.67 (C$(\mathit{CH_3})_3$), 21.18 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, $CH_3$-aa), 16.86 ($CH_3$-tenofovir) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$, *only the major diastereoisomer is described*): δ 23.64 ppm. MS (ES+)

*m/z*: 577 ($M+Na^+$). Reverse-phase HPLC, eluting with $H_2O$/MeOH from 90/100 to 0/100 in 30 min, Flow = 1 mL/min, $\lambda$ = 254 nm, $t_R$ = 14.63 min.

5.1.14. 9-[2-(5,6,7,8-Tetrahydro-1-naphthyloxy-(2,2-dimethylpropoxy-L-alaninyl)-Phosphonomethoxy)propyl]adenine (**17**).

Reaction was carried out following procedure B with tenofovir (500 mg, 1.74 mmol), neopentyloxy-L-alaninyl ester *p*-TSA salt (881 mg, 2.61 mmol) and 5,6,7,8-tetrahydro-1-naphtol (387 mg, 2.61 mmol). The desired product (**17**) was obtained as an off-white solid (8 %, 35 mg).$^1$H NMR (500 MHz, MeOH-$d_4$): δ 8.20 (s, 1H, H-2), 8.18 (s, 2H, H-2, H-8), 8.10 (s, 1H, H-8), 7.07-7.01 (m, 2H, Naph), 6.98-6.95 (m, 2H, Naph), 6.89-6.86 (m, 2H, Naph), 4.43 (dd, $J$ = 14.6, 3.2 Hz, 1H, N-$CH_2$), 4.37 (dd, $J$ = 14.6, 3.1 Hz, 1H, N-$CH_2$), 4.26 (dd, $J$ = 14.7, 6.8 Hz, 1H, N-$CH_2$), 4.23 (dd, $J$ = 15.7, 7.5 Hz, 1H, N-$CH_2$), 4.08-3.97 (m, 6H, P-$CH_2$, N-CH, O-CH), 3.86-3.80 (m, 4H, P-$CH_2$, O-$CH_2$), 3.75-3.72 (m, 2H, O-$CH_2$), 2.75-2.73 (m, 4H, $CH_2$-Tetrahydronaph), 2.57-2.55 (m, 4H, $CH_2$-Tetrahydronaph), 1.74-1.72 (m, 8H, $CH_2$-Tetrahydronaph), 1.35 (d $J$ = 7.15 Hz, 3H, CH3-aa), 1.30 (d $J$ = 6.9 Hz, 3H, CH3-aa), 1.24 (d, $J$ = 7.2 Hz, $CH_3$-tenofovir), 1.18 (d, $J$ = 6.26 Hz, 3H, $CH_3$-tenofovir), 0.94 ($C(CH_3)_3$), 0.95 (9H,s, $C(CH_3)_3$) ppm. $^{13}$C-NMR (125 MHz, MeOH-$d_4$): δ 175.44 (d, $^3J_{C-P}$ = 2.5 Hz, C=O), 175.12 (d, $^3J_{C-P}$ = 3.7 Hz, C=O), 152.20, (C-6), 157.25 (C-6), 153.68, 153.63 (CH-2), 151.03, 150.97(C-4), 149.88 (d, $^2J_{C-P}$ = 10.0 Hz, C-*ipso* OTetrahydronaph), 149.80 (d, $^2J_{C-P}$ = 10.0 Hz, C-*ipso* OTetrahydronaph), 143.76, 143.65 (CH-8), 140.64 (C-Tetrahydronaph), 129.90 (d, $^3J_{C-P}$ = 5.0 Hz, *C*-Tetrahydronaph), 126.80, 126.76, 126.73, 126.69 (CH-Tetrahydronaph), 119.77 (C-5), 118.19 (d, $^3J_{C-P}$ = 2.5 Hz, CH-Tetrahydronaph), 118.17 (d, $^3J_{C-P}$ = 3.7 Hz, CH-Tetrahydronaph), 77.9 (d, $^3J_{C-P}$= 13.75 Hz, O-CH), 77.7 (d, $^3J_{C-P}$= 12.5 Hz, O-CH), 75.43, 75.41 (O-$CH_2$), 65.58 (d, $^1J_{C-P}$ = 156.3 Hz, P-$CH_2$), 65.44 (d, $^1J_{C-P}$ = 156.3 Hz, P-$CH_2$), 50.94, 50.96 (N-CH), 49.88, 49.65 (N-$CH_2$), 32.34, 32.31 ($\mathit{C}(CH_3)_3$), 30.76, 30.49 ($CH_2$-Tetrahydronaph), 26.75, 26.75 ($C(\mathit{C}H_3)_3$), 24.56, 24.51, 23.84, 23.81, 23.71 ($CH_2$-Tetrahydronaph), 21.38 (d, $^3J_{C-P}$ = 5.0 Hz, $CH_3$-aa), 20.62 (d, $^3J_{C-P}$ = 6.3 Hz, $CH_3$-aa), 16.92, 16.79 ($CH_3$-tenofovir) ppm. $^{31}$P NMR (202 MHz, MeOH-$d_4$): δ 23.96, 22.75 ppm. MS (ES+) *m/z*: 559 ($M+H^+$). Reverse-phase HPLC, eluting with $H_2O$/MeOH from 90/100 to 0/100 in 30 min, Flow = 1 mL/min, $\lambda$ = 254 nm, $t_R$ = 17.67 min.

*5.2. Carboxypeptidase Y (EC 3.4.16.1) Assay.*

The experiment was carried out by dissolving compound **10** (3.0 mg) in acetone-*d6* (0.15 mL) and by adding 0.30 mL of Trizma buffer (pH 7.6). After the $^{31}$P NMR data were recorded at 25 ˚C as a control, a previously defrosted carboxypeptidase Y (0.1 mg dissolved in 0.15 mL of Trizma) was added to the sample. Next, the sample was submitted to $^{31}$P NMR experiments (at 25 ˚C) and the spectra were recorded every 7 min over 12 h. $^{31}$P NMR recorded data were processed and analyzed with the Bruker Topspin 2.1 program.

*5.3. HPV Assay.*

*5.3.1. Methods*

Parent and daughter compounds were formulated in ~1% Dimethyl sulfoxide (Fisher Scientific, Loughborough, UK) and pH 7.4 phosphate buffered saline (Sigma-Aldrich, Dorset, UK) for in vitro assays.

*5.3.2. Cell Culture:*

SiHa (HPV16+), HeLa (HPV18+) and C33A (HPV-) human epithelial cervical carcinoma cell lines (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's Medium (Sigma-Aldrich, Dorset, UK) supplemented with 10% foetal bovine serum (Autogen Bioclear, Wiltshire, UK) and 100units/mL penicillin and 100µg/mL streptomycin (Sigma-Aldrich, Dorset, UK). Human epidermal keratinocytes (GIBCO/Invitrogen, Paisley, UK) were grown in EpiLife culture medium with supplemented calcium with Human Keratinocyte Growth Supplement (GIBCO/Invitrogen, Paisley, UK). The M08 vulval keratinocytes were a HPV16 positive short term cell line derived from a biopsy of VIN3, and were grown in Glasgow Modified Eagles Medium (Sigma-Aldrich, Dorset, UK) supplemented with 10% foetal bovine serum (Autogen Bioclear, Wiltshire, UK), 0.01µg/mL EGF (Sigma-Aldrich, Dorset, UK), 0.5 µg/mL Hydrocortisone (Sigma-Aldrich, Dorset, UK), 0.1nM Cholera Toxin (Sigma-Aldrich, Dorset, UK), 4mM Glutamine (Sigma-Aldrich, Dorset, UK) and 100units/mL penicillin and 100µg/mL streptomycin (Sigma-Aldrich, Dorset, UK). All cell types were cultured at 37°C in 5% $CO_2$.

*5.4. Assessment of the IC50 values.*

*5.4.1. Initial prodrug screen:*

Prior to treatment SiHa, HeLa and C33A cells were seeded at 4 x $10^3$ cells per well in 96 well plates and allowed to adhere for 24 hours. After this period cells were treated with 1µM, 10µM and 100µM parent and protide compounds in triplicate. After 72 hours cells were detatched with trypsin/EDTA (Sigma-Aldrich, Dorset, UK) and re-suspended in a 1µg/mL 7-Aminoactinomycin D (Sigma-Aldrich, Dorset, UK)/FACS Flow (BD Biosciences, Oxford, UK) solution and analysed for cell viability on an Accuri C6 flow cytometer (BD Bioscience, Oxford, UK).

*5.4.2. Further Examination of compounds **7** and **9** in models representing cells at different stages of neoplasia:*

Prior to treatment SiHa cells and M08 vulval keratinocytes were seeded at 4 x $10^3$ cells per well in 96 well plates and human epidermal keratinocytes were seeded at 3.5 x $10^4$ cells per well in 24 well plates. Cells were allowed to adhere for 24 hours. After this period cells were treated with 0.1µM and 1µM of compounds **9** and **7** in replicate. After 96 hours cells were detatched with trypsin/EDTA (Sigma-Aldrich, Dorset, UK) and re-suspended in pH 7.4 Phosphate Buffered Saline (Sigma-Aldrich, Dorset, UK) and manually counted in 0.4% Trypan Blue (Sigma-Aldrich, Dorset, UK) solution using a Neubauer haemocytometer.

*5.5. Antiviral and cytostatic assays.*

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 (ACVr), herpes simplex virus type 2 (HSV-2) strain G, and vaccinia virus Lederle strain. 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) 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 $EC_{50}$ or compound concentration required to reduce virus-induced cytopathogenicity.

Inhibition of HIV-1 ($III_B$)- and HIV-2(ROD)-induced cytopathicity in CEM cell cultures was measured in microtiter 96-well plates containing ~ 3 × $10^5$ CEM cells/mL infected with 100 $CCID_{50}$ of HIV per milliliter and containing appropriate dilutions of the test compounds. After 4−5 days of incubation at 37 °C in a $CO_2$-controlled humidified atmosphere, CEM giant (syncytium)

cell formation was examined microscopically. The $EC_{50}$ (50% effective concentration) was defined as the compound concentration required to inhibit HIV-induced giant cell formation by 50%.

For the cytostatic experiments, all assays were performed in 96-well microtiter plates. To each well were added (5−7.5) × $10^4$ tumor cells and a given amount of the test compound. The cells were allowed to proliferate for 48 h (murine leukemia L1210 cells) or 72 h (human lymphocytic CEM and human cervix carcinoma HeLa cells) 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%.

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## LIST OF CAPTIONS:

**Figure 1.** Structure of Adefovir **1**, Tenofovir **2** and their prodrugs **3** and **4**.

**Figure 2.** Structures of GS-7340 and GS-9191.

**Figure 3.** Carboxypeptidase Y assay applied on ProTide **10** and monitored by $^{31}$P NMR, 25 ˚C**.**

**Scheme 1**: General procedure for the synthesis of amidate ANP prodrugs **5-17**.

**Table 1:** Anti-HIV activity ($EC_{50}$) and cytotoxicity ($CC_{50}$) data of the test compounds.

**Table 2:** Anti-DNA virus activity of the test compounds

**Table 3:** Antiproliferative activity ($IC_{50}$) of adefovir derivatives in different cell lines.

**Table 4**: $IC_{50}$ values of the compounds against HPV+ and HPV- cells

**Table 5:** $IC_{50}$ values of **9** and **7** against normal, neoplastic and tumor cell lines