Ester prodrugs of acyclic nucleoside thiophosphonates compared to phosphonates: Synthesis, antiviral activity and decomposition study

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · MARCH 2013

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2013.02.039 · Source: PubMed

9 AUTHORS, INCLUDING:

Stéphane Priet
French National Centre for Scientific Resea…
27 PUBLICATIONS **809** CITATIONS
SEE PROFILE

Karine Alvarez
Aix-Marseille Université
29 PUBLICATIONS **536** CITATIONS
SEE PROFILE

European Journal of Medicinal Chemistry 63 (2013) 869–881

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Ester prodrugs of acyclic nucleoside thiophosphonates compared to phosphonates: Synthesis, antiviral activity and decomposition study

Loïc Roux [a], Stéphane Priet [a], Nadine Payrot [a], Clément Weck [a], Maëlenn Fournier [b], Fabien Zoulim [b], Jan Balzarini [c], Bruno Canard [a], Karine Alvarez [a,*]

[a] Laboratoire d'Architecture et Fonction des Macromolécules Biologiques, UMR CNRS 7257, Equipe "Chimie Médicinale et Virologie Structurale", Université Aix-Marseille, Parc scientifique de Luminy, 163 av. de Luminy, 13288 Marseille Cedex 9, France
[b] INSERM, U871, Université de Lyon, Hospices Civils de Lyon, 69003 Lyon, France
[c] Laboratory of Virology and Chemotherapy, Rega Institute for Medical Research, K.U. Leuven, B-3000 Leuven, Belgium

ARTICLE INFO

Article history:
Received 6 December 2012
Received in revised form
30 January 2013
Accepted 26 February 2013
Available online 14 March 2013

Dedicated to the memory of Dr Antonin Holý.

Keywords:
Nucleotide analogue
Prodrug
Thiophosphonate
Antiviral activity
Metabolism

ABSTRACT

9-[2-(Thiophosphonomethoxy)ethyl]adenine [S-PMEA, **8**] and (*R*)-9-[2-(Thiophosphonomethoxy)propyl] adenine [S-PMPA, **9**] are acyclic nucleoside thiophosphonates we described recently that display the same antiviral spectrum (DNA viruses) as approved and potent phosphonates PMEA and (*R*)-PMPA. Here, we describe the synthesis, antiviral activities in infected cell cultures and decomposition study of bis(pivaloyloxymethoxy)-S-PMEA [Bis-POM-S-PMEA, **13**] and bis(isopropyloxymethylcarbonyl)-S-PMPA [Bis-POC-S-PMPA, **14**] as orally bioavailable prodrugs of the S-PMEA **8** and S-PMPA **9**, in comparison to the equivalent "non-thio" derivatives [Bis-POM-PMEA, **11**] and [Bis-POC-PMPA, **12**]. Compounds **11**, **12**, **13** and **14** were evaluated for their *in vitro* antiviral activity against HIV-1-, HIV-2-, HBV- and a broad panel of DNA viruses, and found to exhibit moderate to potent antiviral activity. In order to determine the decomposition pathway of the prodrugs **11**, **12**, **13** and **14** into parent compounds PMEA, PMPA**,** **8** and **9**, kinetic data and decomposition pathways in several media are presented. As expected, bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** behaved as prodrugs of S-PMEA **8** and S-PMPA **9**. However, thiophosphonates **8** and **9** were released very smoothly in cell extracts, in contrast to the release of PMEA and PMPA from "non-thio" prodrugs **11** and **12**.

1. Introduction

Acyclic nucleoside phosphonates (ANPs) [1–3] are outstanding molecules in the fight against DNA virus and retrovirus infections. Three of those compounds have been formally licenced for the treatment of HCMV infections [4] in AIDS patients (HPMPC, cidofovir, Vistide®), chronic HBV infections [1,5,6] (PMEA, Adefovir dipivoxil, Hepsera®, (*R*)-PMPA, Tenofovir disoproxil fumarate, Viread®) and HIV infections (Tenofovir disoproxil fumarate, Viread®). Tenofovir is also available in a combination formulation with emtricitabine (Truvada®), or emtricitabine and efavirenz (Atripla®) or emtricibatine and rilpivirine (Complera®/Eviplera®) for the treatment of AIDS. In addition to these indications, there are various other clinical conditions in which ANPs have proven to be active [1,7].

ANPs are nucleotide analogues, and thus a phosphonate group is attached to the nucleoside moiety. Hence ANPs: 1) should resist any attack by esterases, phosphatases or any catabolic enzymes, thus enhancing physiological stability and half-life in biological fluids and cells, and 2) require only two phosphorylation steps to be converted into their diphosphate active form [8], thus circumventing the often rate-limiting first phosphorylation step of nucleoside analogues.

With respect to their mode of action, these nucleoside phosphonate analogues are phosphorylated by cellular kinases into nucleoside phosphonate diphosphates after endocytosis-mediated penetration into the infected cells. While they are generally poor substrates for cellular polymerases, these nucleotide analogues are,

Abbreviations: HIV, human immunodeficiency virus; HBV, hepatitis B virus; RT, reverse transcriptase; dNTP, deoxynucleotide; ANP, acyclic nucleoside phosphonate; PMEA, 9-[2-phosphonomethoxyethyl]adenine; S-PMEA, 9-[2-thiophosphono methoxyethyl]adenine; (*R*)-PMPA, (*R*)-9-[2-phosphonomethoxypropyl]adenine; (*R*)-S-PMPA, (*R*)-9-[2-thiophosphonomethoxypropyl]adenine; bis-POC-PMPA, (*R*)-9-{2-[O,O′-bis(isopropoxycarbonyloxymethyl)phosphonomethoxy]propyl}adenine; bis-POC-S-PMPA, (*R*)-9-{2-[O,O′-bis(isopropoxycarbonyloxymethyl)thiophosphono methoxy]propyl}adenine; bis-POM-PMEA, 9-{2-[O,O′-bis(pivaloyloxymethyl)phosphonomethoxy]ethyl}adenine; bis-POM-S-PMEA, 9-{2-[O,O′-bis(pivaloyloxymethyl)thiophosphonomethoxy]ethyl}adenine.

* Corresponding author. Tel.: +33 491 825 570; fax: +33 491 266 720.
E-mail address: Karine.Alvarez@afmb.univ-mrs.fr (K. Alvarez).

0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.02.039

on the other hand, fairly well incorporated into the growing viral DNA chain by viral reverse transcriptase (RT) or polymerase. As these analogues do not possess a free 3′-OH group, their incorporation results in a termination of DNA synthesis that is eventually responsible for the antiviral effect [9–11].

Given the antiviral potency, activity spectrum and long-term safety of ANPs, could their performance still be improved? To address this question, various structural modifications have been attempted [1], including structural diversity at both the side chain [12–14], the heterocyclic moiety [15–20] and the phosphonate. Among phosphonate moiety modifications, two strategies have been explored. One consists of designing prodrug adducts [21–26], such as alkoxyalkyl ester or phosphoramidate or *cyclo*Saligenyl derivatives to enhance bioavailability, and the other consists of modifying the environment of the phosphorus atom of the phosphonate moiety itself [27–30]. The latter strategy has been explored to target the resistance problem specifically, and to circumvent the loss of nucleotide analogue efficacy. Indeed, the phosphonate moiety represents the site of the polymerization reaction during nucleotide incorporation into the growing DNA chain and its modification may cause disturbances (electronic, steric effects) which could be advantageous in binding affinity and/or catalytic efficiency.

We previously reported [28,29] the synthesis of the α-thiophosphonates S-PMEA **8** and S-PMPA **9,** bearing a sulphur atom at the α-position of acyclic nucleoside phosphonate (Fig. 1). Target compounds **8** and **9** exhibit robust antiretroviral activity in cells infected by HIV-1 (LAI strain, and viral isolates from subtypes A to G), HIV-2, HBV and a moderate activity against herpes simplex viruses and vaccinia virus. We showed that their diphosphate forms S-PMEApp and S-PMPApp are readily incorporated by wild-type (WT) HIV-1 RT into DNA and act as DNA chain terminators [28,29].

Because these novel acyclic nucleoside thiophosphonates display promising antiviral activities, we decided to improve their pharmacokinetic properties and related efficacy by the synthesis of prodrug forms to make them potential candidates for HIV and HBV therapy.

A prodrug is a pharmacologically inactive compound that is converted to the active form of the drug by endogenous enzymes or intracellular metabolism, to overcome problems associated with stability, toxicity, lack of specificity and/or limited bioavailability. To be clinically useful a prodrug should fulfil pharmacokinetic and pharmacodynamic profiles which are mainly dependant on the kinetics of its conversion to the parent drug (sensitivity of the linkage between the prodrug moiety and the parent drug to hydrolysis) and on its membrane permeation properties (lipophilicity).

Prodrugs of ANPs have been largely described [31,32] and in particular, alkoxyalkyl ester prodrugs, which are the forms currently employed in the licenced Adefovir dipivoxil (Hepsera®) and Tenofovir disoproxil fumarate (Viread®). Historically, Srinivas et al. [33] described the first anti-HIV evaluation of the 9-{2-[O,O′-bis(pivaloyloxymethyl)phosphonomethoxy]ethyl}adenine "bis-POM-PMEA" or Adefovir dipivoxil. Antiviral activity was increased 30-fold, but pivaloyl-containing compounds generated pivaloic acid during release of the parent drug, which constituted a potential toxicity concern [34]. To overcome this issue, "bis-POC" prodrugs were developed [11]. The anti-HIV activity of (*R*)-9-{2-[O,O′-bis(isopropoxycarbonyloxymethyl)phosphonomethoxy]propyl}adenine "bis-POC-PMPA" or Tenofovir disoproxil was 100-fold higher than that of PMPA.

We describe here an efficient strategy to synthesize the alkoxyalkyl ester prodrugs: bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** via a thionation reaction of the corresponding "non-thio" derivatives bis-POM-PMEA **11** and bis-POC-PMPA **12** (Fig. 1). This thionation approach was also applied for the synthesis of the parent thiophosphonates S-PMEA **8** and S-PMPA **9**. In this study, we also evaluate the antiviral activity against a broad panel of viruses and the decomposition pathway in several media of the "thio"-prodrugs **13** and **14** in comparison with the "non-thio"-prodrugs **11** and **12**.

2. Results and discussion

2.1. Chemistry

We previously published [27,28] a synthetic pathway to obtain the thiophosphonates S-PMEA 8 and S-PMPA 9. We developed an original method to synthesize a H-phosphinate intermediate [27] that can easily serve as a precursor to generate phosphorus-modified nucleotides. This key step was applied to synthesize α-boranophosphonates [27], α-selenophosphonates and α-thiophosphonates [28] derived from PMEA and PMPA. However, this exploratory method was not feasible for large-scale synthesis, limited by the overall low yield. So, we decided to develop a new synthetic strategy allowing us to obtain target compounds and prodrugs derived thereof using a common strategy, thereby limiting the number of steps (Scheme 1).

The first step of the synthesis uses a methodology largely described [27,28,35–37] and consists of substituting adenine in order to obtain 9-(2-hydroxyethyl)adenine 1 for the S-PMEA series and (*R*)-9-(2-hydroxypropyl)adenine 2 for the S-PMPA series.

The following step, which consists of alkylating alcohols 1 and 2 with diethyl[[(*p*-toluenesulfonyl)oxy]methyl]phosphonate 3 [38] in DMF in the presence of alkoxide base, is the limiting step characterized by its low yield and side-product formation which makes purification difficult. Indeed, two side-products were identified following the reaction by HPLC/MS, the N7-regioisomer and the

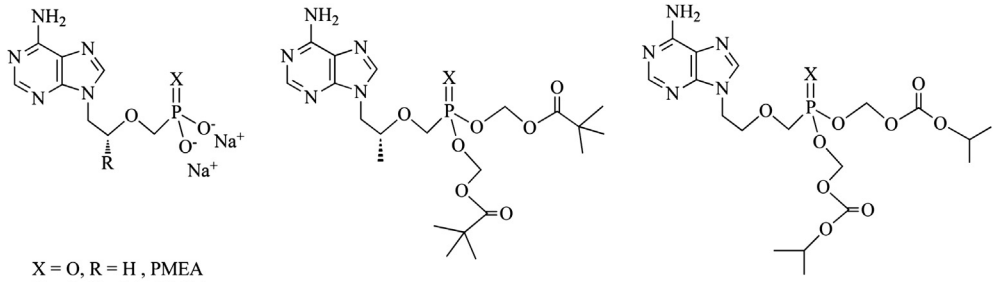
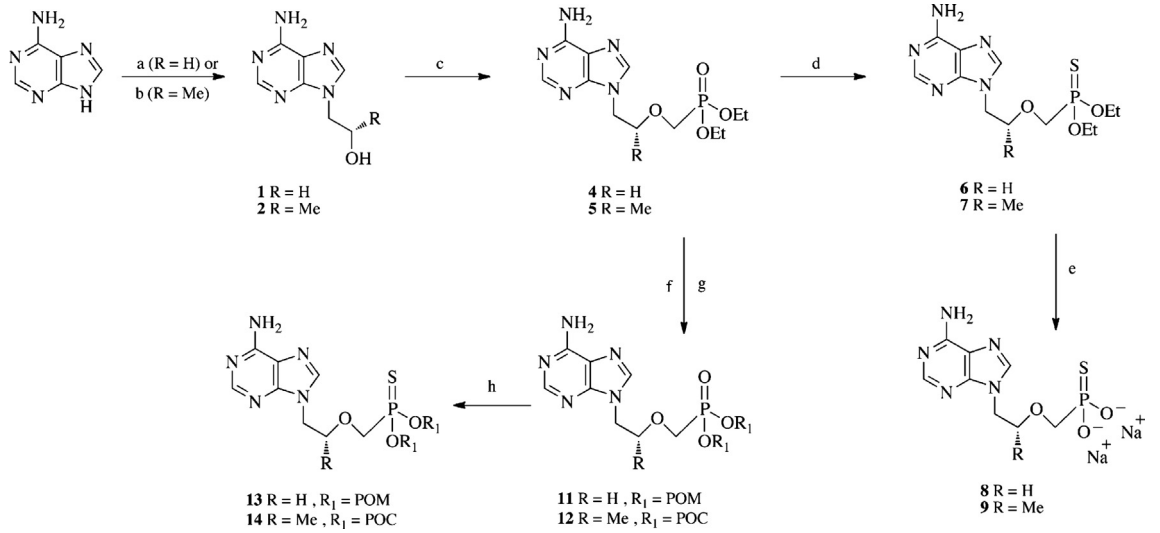


Fig. 1. Structure of PMEA, PMPA, S-PMEA **8** and S-PMPA **9**, bis-POM-PMEA **11**, bis-POM-S-PMEA **13**, bis-POC-PMPA **12** and bis-POC-S-PMPA **14**.



Scheme 1. Synthetic pathway for the synthesis of compounds **8, 9, 11, 12, 13** and **14**. Reagents and conditions : a) 2-bromoethylbenzoate, NaH, DMF, 60 °C, 16 h then NH_3/MeOH sat, 14 h; b) (R)-propylene carbonate, DMF, 140 °C, 16 h; c) $Mg(tBuO)_2$, **3**, DMF, 35 °C, 20 h; d) Davy Reagent, benzene, 80 °C, 2 h; e) TMSCl, NaBr, NMP, 90 °C, 12 h, then dowex Na^+ exchange after purification; f) TMSCl, NaBr, NMP, 60 °C, 12 h then POMCl, TEA, 60 °C, 6 h; g) TMSCl, NaBr, NMP, 60 °C, 12 h, then **10**, TEA, 60 °C, 6 h; h) Davy Reagent, benzene, 80 °C, 30 min.

elimination product. The formation of the N7-regioisomer has been reported by Hakimelahi, G. H [35]. and we also observed its formation using sodium *tert*-butoxide as alkoxide base. Indeed, The synthesis of compounds 4 and 5 with 1.1 eq of sodium *tert*-butoxide leads to a N-7/N-9 ratio 15/85 and 40/60 respectively. Regioisomers were not efficiently separated by purification on silica gel chromatography. We hypothesize that the elimination product results from a β-elimination reaction of the compound 5 which contains ether function. Under the action of strong bases such as organolithium or organosodium and to a lesser extent organomagnesium [39], hydrogen atom in beta position of the oxygen can be removed (Reformatsky reaction analogy). Elimination reaction leads to an alkene and an alcoholate.

In order to improve regioselectivity and efficiency, and to reduce side product formation, we tried several conditions of temperature (RT, 70 °C), solvent (DMF, NMP) and bases (tBuONa, tBuOK, NaH, LiH) [27,35,37,40]. The alkylation can be effected also with magnesium *tert*-butoxide [41–43] and demonstrated to be superior to the current manufacture production method. However, the strategy described is one-pot synthesis and diethylphosphonates 4 and 5 were not isolated. According to the recent literature [42,43], alkylation of compounds 1 and 2 was accomplished in dimethylformamide at 35 °C during 20 h in the presence of 1.5 equiv of magnesium *tert*-butoxide to lead to the corresponding diethylphosphonates 4 and 5 [17,27], with 52% and 53% yields, respectively.

The new approach we designed consists then to transfer a sulphur atom to diethylphosphonates **4** and **5** forming diethylthiophosphonates **6** and **7** using an efficacious thionation reagent (e.g., Belleau reagent, Lawesson reagent, Davy reagent) [44–47]. The Davy reagent in dry benzene at 90 °C lead to the corresponding diethylthiophosphonates **6** and **7** with 43% and 44% yields, respectively. Thionation of **4** and **5** proved to be fast and went to completion within 2 h. Inspection of the decoupling ^{31}P NMR spectra showed that the signals from **4** (δ: 21.56 ppm) and **5** (δ: 22.11 ppm) were replaced by new resonances at 88.02 ppm for **6** and 88.41 ppm for **7**.

Methyl and ethyl are current protecting groups of phosphonate diesters. However, while they can be removed by treatment with trimethylsilyl iodide or bromide, this method fails or gives low yields with thiophosphonate derivatives [48]. Deprotection of diethyl α,α-difluoromethylenephosphonothioic acid via a thiono–thiolo rearrangement followed by Pd-catalysed deallylation has been reported [49]. Our goal was to identify reaction conditions capable of generating thiophosphonic acids in a one step process. Since nucleophilic displacement is rate-determining for TMSBr-mediated hydrolysis, the use of TMSCl in conjugation with several exogenous nucleophiles (NaBr, NaI) was optimised for the hydrolysis of diethylthiophosphonates **6** and **7** [42]. Compounds **6** and **7** were treated with 40 equivalents of sodium bromide and trimethylsilyl chloride in *N*-methylpyrrolidone (NMP) at 90 °C, during 12 h, followed by hydrolysis with triethylammonium bicarbonate 0.05 M (TEAB). The deprotection reaction led to the formation of thiophosphonates **8** and **9** with 40% and 48% yields, respectively, after reverse phase chromatography purification.

In summary, a more efficient process has been developed to prepare thiophosphonates **8** and **9**. Even if the overall yield remains equivalent (10%), notable features include key alkylation using magnesium *tert*-butoxide, reduction of purification steps using reverse-phase chromatography, thionation of ANPs never described and efficient transsilylation of S-ANPs never reported.

To obtain the prodrug forms [29,30] bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** we used a similar synthesis pathway involving a thionation reagent to introduce the sulphur atom on the alkoxyalkyl ester phosphonate. The reaction involved the hydrolysis of the diethylphosphonates **4** and **5** followed by the conversion into the alkoxyalkyl phosphonate diesters **11** and **12** by base-promoted alkylation. After treatment of **4** and **5** with two equivalents of sodium bromide and four equivalents of trimethylsilyl chloride in NMP at 60 °C during 12 h, phosphonate diacids were obtained but not isolated. After ensuring the complete removal of excess of TMSCl by co-distillation with ethyl acetate, they were directly alkylated with triethylamine and the appropriate pivaloyloxymethyl chloride (POMCl) or chloromethylisopropyl carbonate **10** [50] (POCCl), in NMP at 60 °C during 6 h. Bis-POM-PMEA **11** and bis-POC-PMPA **12** were isolated by silica chromatography in 27% and 33% yield, respectively. The last step was the thionation reaction with the Davy Reagent in benzene at 80 °C during 30 min. The bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** were then obtained after silica gel chromatography with 29% and 32% yields, respectively.

2.2. Anti-HIV activity of thiophosphonate prodrugs **13** and **14** compared to phosphonate prodrugs **11** and **12** against wild-type (WT) HIV-1 (III_B) and HIV-2 (ROD) and cytotoxicity in CEM cell cultures

To evaluate the activity of the target compounds, a cell-based antiviral drug susceptibility assay was performed using WT HIV-1 (III_B) and HIV-2 (ROD) (Table 1). CEM cell cultures were treated with increasing concentrations of compounds **11**, **12**, **13** and **14**, and infected with 100 $TCID_{50}$ (50% tissue culture infectious doses) of HIV-1 (III_B) and HIV-2 (ROD). On day 3 post infection, reverse transcriptase (RT) activity in cell culture medium was measured to determine the antiviral properties of the compounds expressed as 50% reduction of viral replication (half maximal effective concentration or EC_{50}) and virus-induced cytopathicity was microscopically recorded (Table 1). Concomitantly, the antimetabolic effect (50% cytotoxic concentration, CC_{50}) of each compound was evaluated in the drug-exposed CEM cell cultures.

Under the assay conditions, all compounds significantly inhibited the replication of HIV-1 and HIV-2 in CEM cell cultures (Table 1). The alkoxyalkyl ester prodrugs bis-POM-PMEA **11**, bis-POC-PMPA **12**, bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** exhibited markedly increased antiviral activity against HIV-1 and HIV-2 compared to PMEA (60- and 110-fold, respectively [28]), PMPA (300- and 540-fold, respectively [28]), S-PMEA **8** and S-PMPA **9** (170- to 60-fold, respectively [28]). The prodrug strategy leads to an important decrease of EC_{50} compared to parent compounds [28]. Nevertheless the thiophosphonate prodrug **14** tends to display an increased EC_{50} (5- to 10-fold lower antiviral activity) as compared to the phosphonate prodrug bis-POC-PMPA **12**.

As described in the literature [11,33,51], cytotoxicity of the POM prodrugs also markedly increased. Indeed, as shown in Table 1, bis-POM-PMEA **11** and bis-POM-S-PMEA **13** inhibited the CEM cell growth at an CC_{50} of 1.6 and 2 μM, respectively. Consequently, the bis-POM prodrugs showed a substantially lower selectivity than the bis-POC prodrugs with selectivity ratios for the POM series of ≥10–35 and ≥270–1900 for the POC series. Both prodrug series are hydrolyzed to formaldehyde, carbon dioxide and the parent compound [11]. Bis-POM prodrugs also generate pivalic acid, whereas the bis-POC prodrugs release isopropyl alcohol. It is possible that the accumulation of pivalic acid contributes to the increased cytotoxicity.

The increased antiviral activity of alkoxyalkyl ester prodrugs of the thiophosphonates bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** and the phosphonate compounds bis-POM-PMEA **11** and bis-POC-PMPA **12**, can be attributed to increased cellular permeation of the prodrugs followed by effective intracellular conversion to the parent compounds, which undergo subsequent phosphorylation to their antivirally active diphosphate metabolite.

To conclude anti-HIV evaluation, thiophosphonates **13** and **14** are slightly less active (4–10 fold) than phosphonate compounds Adefovir Dipivoxil **11** and Tenofovir Disoproxil **12**.

Table 1
Antiviral activity and cytotoxicity of bis-POM-PMEA **11**, bis-POC-PMPA **12**, bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14**, in CEM cell cultures infected by 100 $TCID_{50}$ of HIV-1 (IIIB) or HIV-2 (ROD).

Compounds	EC_{50} (μM)[a] 100 $TCID_{50}$ HIV-1 (III_B)	EC_{50} (μM)[a] 100 $TCID_{50}$ HIV-2 (ROD)	CC_{50} (μM)[b] (CEM)
Bis-POM-S-PMEA **13**	≥0.2	≥0.2	2.0 ± 0.21
Bis-POM-PMEA **11**	0.045 ± 0.038	0.062 ± 0.021	1.6 ± 0.28
Bis-POC-S-PMPA **14**	0.092 ± 0.040	0.13 ± 0.0071	>25
Bis-POC-PMPA **12**	0.016 ± 0.011	0.013 ± 0.009	>25

[a] EC_{50} or 50% effective concentration : compound concentration required to inhibit syncytia formation by 50%.
[b] CC_{50} or 50% cytostatic concentration : compound concentration required to inhibit CEM cell proliferation by 50% in mock-infected cell cultures. CEM: human T-lymphoblastoid cells.

2.3. Anti-HBV activity of thiophosphonate prodrugs **13** and **14** compared to phosphonate prodrugs **11** and **12** and cytotoxicity in Huh7 cell cultures

Since prodrug forms of PMEA (Adefovir Dipivoxil) and PMPA (Tenofovir Disoproxil) are clinically approved to inhibit HBV replication in infected patients, the compounds were tested in a cell-based antiviral drug susceptibility assay. The inhibition of WT HBV replication was assessed in Huh7 cell cultures treated with increasing concentrations of compounds **11**, **12**, **13** and **14**. The amount of viral DNA produced in the cells was quantified to evaluate the antiviral properties of the compounds as the 50% reduction of viral DNA (EC_{50}) (Table 2). Concomitantly, the cytotoxic effect of each compound was evaluated as well.

Under the assay conditions, all compounds significantly inhibited the multiplication of HBV in huh-7 cell cultures (Table 2). None of the tested compounds exhibited any cytotoxicity at a concentration as high as 25 μM. Surprisingly, the alkoxyalkyl ester prodrugs bis-POM-PMEA **11**, bis-POC-PMPA **12,** bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** exhibited only a small increase of efficacy against HBV compared to PMEA (4-fold [28]), PMPA (5-fold [28]), S-PMEA **8** (3-fold [28]) and S-PMPA **9** (3-fold [28]). The prodrug strategy leads to a moderate decrease of EC_{50} compared to parent compounds [28].

To conclude anti-HBV evaluation, thiophosphonates **13** and **14** displayed the same activity (1.2–1.7 fold) than phosphonates **11** and **12**.

2.4. Evaluation of thiophosphonate prodrugs **13** and **14** compared to phosphonate prodrugs **11** and **12** against other DNA viruses in HEL cell culture

To further explore the antiviral selectivity of the compounds, the antiviral activity testing against a panel of DNA and RNA viruses was performed with herpes simplex virus type 1 and 2 (HSV-1 KOS, HSV-1 TK- KOS and HSV-2 G), and vaccinia virus. The inhibition of viral replication was assessed in HEL cell cultures treated with increasing concentrations of compounds **11, 12**, **13** and **14**. The virus-induced cytopathic effect (CPE) was recorded microscopically at 3 days post infection and the antiviral activity (EC_{50}) was expressed as the percentage of inhibition of the CPE compared with the untreated controls **(**Table 3**)**.

Under the assay conditions, except for bis-POC-S-PMPA **14** which is inactive up to 50 μM, all compounds inhibited the multiplication of HSV-1 and HSV-2 in HEL cell cultures (Table 3). None of the tested compounds exhibited any cytotoxicity at a concentration as high as 50 μM.

The alkoxyalkyl ester prodrugs bis-POM-S-PMEA **13**, bis-POM-PMEA **11** and bis-POC-PMPA **12** exhibited increased potency compared to S-PMEA **8 (**6- to 10-fold [28]**)**, PMEA **(**100- to 400-fold [28]**)** and PMPA (>20-fold [28]). However, the increase of antiviral

Table 2
Antiviral activity and cytotoxicity of bis-POM-PMEA **11**, bis-POC-PMPA **12**, bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14**, in Huh7 cell cultures transfected by HBV.

Compounds	EC_{50} (μM)[a]	CC_{50} (μM)[b] (Huh7)
Bis-POM-S-PMEA **13**	1.45 ± 0.53	>25
Bis-POM-PMEA **11**	0.83 ± 0.21	>25
Bis-POC-S-PMPA **14**	1.14 ± 0.92	>25
Bis-POC-PMPA **12**	0.88 ± 0.61	>25

[a] EC_{50} or 50% effective concentration : concentration of compound required to afford a 50% reduction of the level of HBV viral DNA in the Huh7 cell cultures. EC_{50} were determined from at least three independent experiments.
[b] CC_{50} or 50% cytotoxic concentration : concentration of compound required to afford a 50% reduction of the cell density.

Table 3
Antiviral activity and cytotoxicity of bis-POM-PMEA **11**, bis-POC-PMPA **12**, bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14**,, in HEL cell cultures infected by several DNA viruses.

Compounds	EC_{50} (μM)[a]				Minimum cytotoxic concentration (μM)[b]
	Herpes simplex virus-1 (KOS)	Herpes simplex virus-1 (TK- KOS)	Herpes simplex virus-2 (G)	Vaccinia virus	
Bis-POM-S-PMEA **13**	3.9 ± 1.9	3.9 ± 0.0	3.9 ± 0.0	29 ± 20	>50
Bis-POM-PMEA **11**	0.2 ± 0.0	0.2 ± 0.2	0.2 ± 0.2	4.0 ± 6.0	>50
Bis-POC-S-PMPA **14**	>50	>50	>50	>50	>50
Bis-POC-PMPA **12**	5.8 ± 3.8	9.6 ± 7.7	5.8 ± 0.0	>50	>50
Cidofovir	0.9 ± 1.1	0.9 ± 0.1	0.9 ± 1.1	22 ± 7.0	>250
Acyclovir	0.08 ± 0.12	29 ± 21	0.08 ± 0.12	>250	>250

[a] EC_{50} or 50% effective concentration : concentration of compound required to reduce virus-induced cytopathicity by 50%.
[b] Minimum compound concentration required to cause a microscopically detectable alteration of normal cell morphology.

efficacy was less pronounced for thiophosphonate prodrugs than phosphonates prodrugs. The alkoxyalkyl ester prodrugs **11** and **13** exhibited an efficacy equivalent to that of cidofovir and showed a better activity than acyclovir against thymidine kinase-deficient HSV-1 (TK- KOS).

As described previously [28], S-PMPA **9** displayed a moderate activity against HSV-1 and HSV-2 (EC_{50} 169 ± 13 μM and 48 ± 0.0 μM, respectively) in HEL cell cultures, while PMPA was inactive at 200 μM. Surprisingly, no antiviral activity up to 50 μM was observed against any of the DNA viruses tested with the alkoxyalkyl ester prodrug bis-POC-S-PMPA **14**, while the bis-POC-PMPA **12** displayed a good activity against HSV-1 and HSV-2 (EC_{50} 5.8 μM) in HEL cell cultures.

While S-PMEA **8** showed modest inhibitory properties against vaccinia virus in contrast to the inactivity of PMEA at 200 μM, surprisingly the alkoxyalkyl ester prodrug of PMEA **11** was 7 times more active than S-PMEA **13**. It is also intriguing that the prodrug derivative of PMEA was much more active than the corresponding prodrug of S-PMEA.

2.5. Decomposition studies and metabolism

It has been previously [52] demonstrated that the mechanism by which the bis-POM-PMEA **11** and bis-POC-PMPA **12** are converted to the corresponding PMEA and PMPA parent drugs involves, in a first step, a carboxyesterase-mediated decomposition process followed by a spontaneous C–O bond breakage and thus giving rise to the corresponding phosphonate monoesters "mono-POM-PMEA" and "mono-POC-PMPA", respectively, with the release of formaldehyde and pivalic acid or isopropyl alcohol. In a second step, the corresponding phosphonate monoesters are converted to the expected parent compounds PMEA and PMPA either following a decomposition process similar to that involved in the first step and/or by phosphodiesterase-mediated hydrolysis.

In order to determine whether bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** exert their biological effects via intracellular delivery of the corresponding S-PMEA **8** and S-PMPA **9**, we decided to establish the decomposition pathways of **13** and **14** in cell extracts and to determine their stability in various media.

The decomposition products, pathways and kinetics of thiophosphonates S-PMEA **8**, S-PMPA **9**, mono-POC-S-PMPA **16**, bis-POM-S-PMEA **13**, bis-POC-S-PMPA **14** and phosphonates PMEA, PMPA, mono-POC-PMPA **15**, bis-POM-PMEA **11** and bis-POC-PMPA **12** were determined upon incubation at 37 °C in culture medium (RPMI-1640 + 10% of heat-inactivated foetal calf serum; DMEM + 10% of heat-inactivated foetal calf serum), and in cell extracts (CEM; Huh7) in order to mimic their behaviour under the experimental conditions of the *in vitro* assays. All compounds were also incubated in water, in acidic buffer (pH 1.2), in RPMI-1640 and in DMEM medium to differentiate between chemical and enzyme-mediated hydrolysis. Additionally, compounds **8**, **9**, **11**, **12**, **13**, **14**, **15** and **16** were incubated in buffer with or without addition of purified pig liver carboxyesterases (PLE) and/or with snake venom phosphodiesterases (SVP). PLE is a widely accepted model of intracellular esterase activity [53–55].

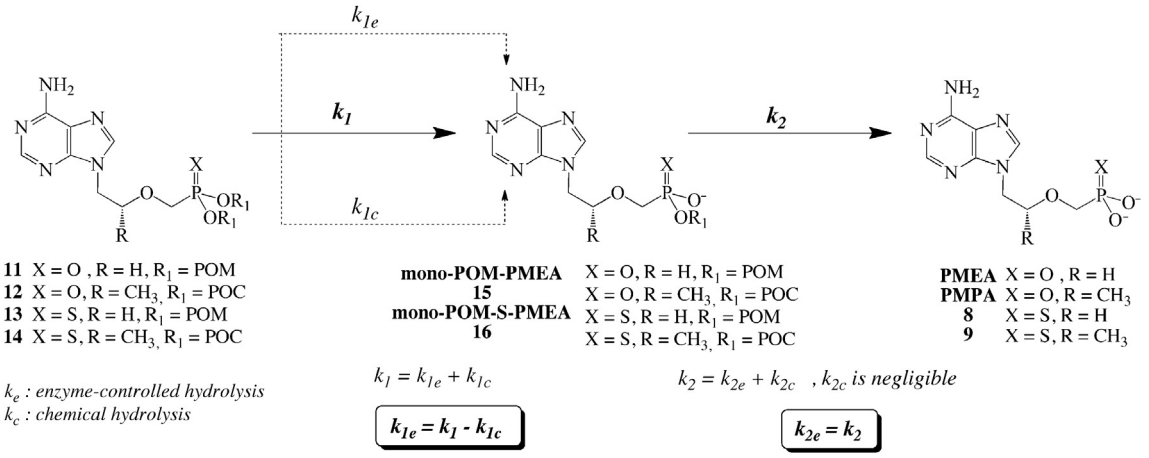
During incubation, samples were removed and analyzed using an "on-line cleaning" method [56]which allows the direct HPLC analysis of compounds and metabolites in biological media without any pre-treatment.

For each of the conditions studied, the whole sample was incubated at 37 °C and at each chosen time interval, 50 μL were sampled and directly injected onto an analytical HPLC column where the analytical conditions allowed visualising and properly separating the starting compound from the corresponding metabolites. All compounds could accurately be UV-detected (260 nm) and quantified. Identification of the metabolites was confirmed by comparative injection of authentic samples. For example, after incubation of compound **14** in the various investigated media, aliquots were HPLC-analyzed and several signals could be observed in resulting chromatograms: bis-POC-S-PMPA **14**, mono-POC-S-PMPA **16** (two diastereoisomers), S-PMPA **9** and PMPA. All signals were unambiguously assigned by co-injection with authentic samples (retention time, UV spectra).

Having assigned the various HPLC signal consecutive-competitives, the kinetic data could be treated according to a general " pseudo-first order" model. A proposed decomposition pattern is shown in Scheme 2, in which we established that the overall metabolic sequence implies two steps and the formation of an intermediate mono-POC or mono-POM during the conversion process. Each step of decomposition could be characterized by half-lives ($t_{½}$) and rate constants (k_1 and k_2). Moreover, the observed rate constant k is the sum of the rate constants of the chemical-mediated hydrolysis (k_{1c} and k_{2c}) and the enzyme-mediated hydrolysis (k_{1e} and k_{2e}) in the serum-containing media and cell extracts (Table 4).

In RPMI-1640 or DMEM, the rate constants obtained reflected the chemical hydrolysis kinetics. In both conditions, the rate constants k_1 for the first step of conversion of phosphonates diesters **11** and **12** were higher (5- to 9-fold) than the rate constants of the thiophosphonates diesters **13** and **14**, indicating that thiophosphonates were more resistant to chemical hydrolysis than the corresponding phosphonates ($t_{1/2} \approx 1$–2 h). For all tested compounds, the second conversion (k_2) was very slow and half-lives were higher than 72 or 48 h.

When tested in FCS-containing culture medium (RPMI-1640 + 10% FCS), the rate of decomposition (k_1) resulted predominantly from the chemical degradation but slightly decomposition of compounds **11**, **12**, **13** and **14** occurred, due to enzymatic degradation. k_{1e} was slow and equivalent for thiophosphonates (1.34 and 1.06 × 10^{-3} min^{-1}) and phosphonates derivatives (1.58 and 1.1 × 10^{-3} min^{-1}). In contrast, in DMEM + 10% FCS, the rate constant k_{1e} expressing the enzymatic degradation was higher especially for



Scheme 2. Proposed mechanism of decomposition of prodrugs **11, 12, 13** and **14**. The half-lives were deduced from experimental data and the calculated rate constants of each decomposition step in the different media are reported in Table 5.

phosphonate derivatives (6.93 and 6.41 $\times$ 10^{-3} min^{-1}). Such behaviour was correlated to the presence of heat-inactivated serum in the medium and demonstrated a higher remaining enzymatic activity in DMEM + 10% FCS than in RPMI-1640 + 10% FCS. For both series, the k_2 values were very low and half-lives were higher than 72 h or 48 h for compounds **13** and **14** and were 55 h/31 h and 33 h/>48 h for compounds **11** and **12**.

In cell extracts (CEM and Huh7), we observed a difference of behaviour between the POM and POC prodrug series, in addition to the impact of the thio modification. Indeed, bis-POM-S-PMEA **13** and bis-POM-PMEA **11** were rapidly deprotected in both cell extracts with equivalent k_{1e} values of 2.99 $\times$ 10^{-3} min^{-1} and 3.36 $\times$ 10^{-3} min^{-1} for compound **13** and 7.35 $\times$ 10^{-3} min^{-1} and 6.93 $\times$ 10^{-3} min^{-1} for compound **11**. As expected, thiophosphonate **13** was less efficiently deprotected than phosphonate **11**. However and unfortunately, the k_2 values for compound **13** were very low and half-lives were higher than 72 h or 48 h, meaning that the parent compound **8** was not efficiently released in cell extracts. In comparison, PMEA was released in cell extracts with a half-live of 11 h, being more compatible with the experimental conditions of the *in vitro* assays.

The combination of POC protection and thio modification was worse in terms of parent compound release. Indeed, the removal of the first POC protecting group in cell extracts (CEM and Huh7), was less efficient in comparison to the POM-protected (thio)phosphonates. Bis-POC-S-PMPA **14** was slowly hydrolysed in both cell extracts with k_{1e} values of 0.77 $\times$ 10^{-3} min^{-1} and 0.11 $\times$ 10^{-3} min^{-1}. Moreover, the k_2 values for compound **14** were very low and half-lives were higher than 72 h or 48 h, meaning that the parent compound **9** was not efficiently released in the cell extracts.

However, caution must be taken in extrapolating the kinetic findings to intact living cells: the concentration of protein in the cell lysate used in these experiments is certainly lower than that in intact cells; it is also possible that some of the enzymes responsible for metabolism in intact cells are bound to membranes or organelles and are removed during centrifugation or that enzyme activity is suboptimal when present in a crude extract compared to the (compartmentalized) intact cells.

Nevertheless, the foregoing studies demonstrated that bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** were prodrugs of S-PMEA **8** and S-PMPA **9,** but the kinetics of release was rather slow. The studies also revealed that mono-POM-S-PMEA and mono-POC-S-PMPA were intermediates in the conversion process and led to the establishment of a coherent kinetic scheme for the overall metabolic sequence.

We decided to investigate more carefully the metabolism of the thiophosphonate prodrugs to better understand the decomposition

Table 4
Pseudo-first-order rate constants ($\times 10^{-3}$ min^{-1}) related to the decomposition kinetic models of the compounds **8**–**16** in non-enzymatic media (RPMI-1640; DMEM), culture media (RPMI-1640 + 10% FCS; DMEM + 10% FCS) and cell extract (CEM-SS; Huh7).

Compound								
Rate constant ($\times 10^{-3}$ min^{-1})	Bis-POM-S-PMEA **13**		Bis-POM-PMEA **11**		Bis-POC-S-PMPA **14**		Bis-POC-PMPA **12**	
RPMI-1640/DMEM								
k_{1c}	0.86	0.71	4.2	4.62	0.72	0.94	6.6	5.14
k_{2c}	<0.16[a]	<0.24[b]	<0.16[a]	<0.24[b]	<0.16[a]	<0.24[b]	<0.16[a]	<0.24[b]
RPMI-1640 + 10% FCS/DMEM + 10% FCS								
k_1	2.2	3.3	5.78	11.55	1.78	2.1	7.7	11.55
k_{1e}	1.34	2.59	1.58	6.93	1.06	1.16	1.1	6.41
k_2	<0.16[a]	< 0.24[b]	0.21	0.37	<0.16[a]	<0.24[b]	0.34	<0.24[b]
CEM/Huh7								
k_1	3.85	4.07	11.55	11.55	1.49	1.05	11.55	6.6
k_{1e}	2.99	3.36	7.35	6.93	0.77	0.11	4.95	1.46
k_2	0.16[a]	<0.24[b]	1.05	0.26	<0.16[a]	<0.24[b]	0.33	<0.24[b]

[a] $t_{1/2}$ > 72 h.
[b] $t_{1/2}$ > 48 h. k_{1c}: rate constant of the first hydrolysis step chemically mediated. k_{2c}: rate constant of the second hydrolysis step chemically mediated. k_{1e}: rate constant of the first hydrolysis step enzymatically mediated. k_{2e}: rate constant of the second hydrolysis step enzymatically mediated. The observed rate constant k_1 is the sum of the rate constants of the chemical-mediated hydrolysis (k_{1c}) and the enzyme-mediated hydrolysis (k_{1e}). The observed rate constant k_2 is the sum of the rate constants of the chemical-mediated hydrolysis (k_{2c}) and the enzyme-mediated hydrolysis (k_{2e}).

pathway. Therefore, compounds **8**, **9**, **11**, **12**, **13**, **14**, **15** and **16** were incubated in buffer with or without addition of purified pig liver carboxyesterases (PLE) and/or snake venom phosphodiesterases (SVP).

As described previously, each step of decomposition could be characterized by half-lives ($t_{½}$) and rate constants (k_1 and k_2). Moreover, the observed rate constant k is the sum of the rate constants of the chemical-mediated hydrolysis (k_{1c} and k_{2c}) and the enzyme-mediated hydrolysis (k_{1e} and k_{2e}).

The half-lives were deduced from the experimental data and the calculated rate constants of each decomposition step in the different media are reported in Table 5.

Pig liver carboxyesterase (PLE) was used as a model for hydrolysis of the bis-POC and bis-POM prodrugs by intracellular esterase(s) [53–55]. At a concentration of 0.5 U/mL of carboxyesterase, the formation of the mono-POC or mono-POM intermediates was fast at 37 °C, with k_{1e} values from 60 to 130 × 10^{-3} min^{-1} but at this enzyme concentration, none of the parent compound formation was observed after several hours of incubation. The decomposition of thiophosphonate diesters **13** and **14** into monoesters was PLE-catalyzed, hence k_1 and k_{1e} values were equivalent meaning that the chemical hydrolysis was negligible. On the contrary, the decomposition of phosphonate diesters **11** and **12** into monoesters was predominantly chemical-mediated with a k_1 value (230 × 10^{-3} min^{-1}) markedly higher than the k_{1e} value (60 × 10^{-3} min^{-1}).

A relatively high concentration (5 U/mL) of enzyme was required to observe the release of parent thiophosphonates S-PMEA and S-PMPA, with k_2 values reaching 1.78 to 3.38 × 10^{-3} min^{-1}. Under these conditions, phosphonate monoesters were really poor substrates for carboxyesterase with a $t_{1/2}$ higher than 7 h (k_2 < 1.44 × 10^{-3} min^{-1}).

These model experiments have indicated that conversion of thiophosphonate diesters **13** and **14** into monoesters catalyzed by intracellular esterase(s) was efficient and thiophosphonate diesters **13** and **14** were better substrates for intracellular esterase(s) than the corresponding phosphonate diesters **11** and **12**. Esterase-catalyzed hydrolysis of thiophosphonate monoesters into parental thiophosphonates S-PMEA **8** and S-PMPA **9** was plausible but unfavourable. It has been shown that a carboxyesterase partially purified from rat liver was able to hydrolyse only one of the two carbethoxy groups of malathion and could attack only non-ionized substrates [57].

Snake venom phosphodiesterase (SVP) was used as a model for hydrolysis of the bis-POC and bis-POM prodrugs by intracellular phosphodiesterase(s). The incubation of thiophosphonate diesters **13** and **14** with a concentration of 0.5 U/mL SVP, led to formation of monoesters with k_1 values of 4.8 × 10^{-3} min^{-1} and 4.62 × 10^{-3} min^{-1}. In comparison, phosphonate diesters **11** and **12** were very rapidly hydrolyzed with k_1 values of 170 × 10^{-3} min^{-1} and 140 × 10^{-3} min^{-1}. However, this hydrolysis rate corresponded to a chemical degradation in the buffer employed and therefore, compounds **11**–**14** were found not to be significant substrates of SVP. On the contrary, the phosphonate monoesters were good substrates of SVP and were rapidly hydrolyzed into parent compounds PMEA and PMPA with k_2 values of 140 × 10^{-3} min^{-1} and 120 × 10^{-3} min^{-1}. Unfortunately, thiophosphonate monoesters were very poor substrates of SVP and k_2 values could not be accurately determined.

To confirm this difference of behaviour between phosphonate monoester and thiophosphonate monoester, mono-POC-PMPA **15** and mono-POC-S-PMPA **16** were incubated with 0.5 U/mL SVP. It should be noted that mono-POC-S-PMPA **16** was synthesized as a diastereoisomeric mixture. Diastereoisomers were separated by HPLC analysis, the more polar isomer was designated “fast” for fast-eluting (^{t}R 29.1 min in the S3 system analysis) and the more hydrophobic isomer was designated “Slow” for slow-eluting (^{t}R 29.3 min in the S3 system analysis).

Following the phosphodiesterase-catalyzed hydrolysis of thiophosphonate monoester **16** into parent compound S-PMPA **9** by HPLC, we observed that only the “Slow” diastereoisomer was smoothly hydrolyzed by SVP (k_2 value of 3.85 × 10^{-3} min^{-1}). Indeed, the observed signal area of the “Fast” isomer remained constant, meaning that this isomer was not substrate of SVP. In comparison, mono-POC-PMPA was a really good substrate of SVP (k_2 value of 230 × 10^{-3} min^{-1}). This result may indicate that thiophosphonate monoesters were still poor substrates of SVP and that the SVP showed clear stereopreference.

To propose a coherent decomposition scheme, compounds **11**–**14** were incubated with 0.5 U/mL of PLE and SVP as a mixture of enzymes. As we hypothesized, PLE should be involved in the first decomposition step and SVP should be involved in the second decomposition step.

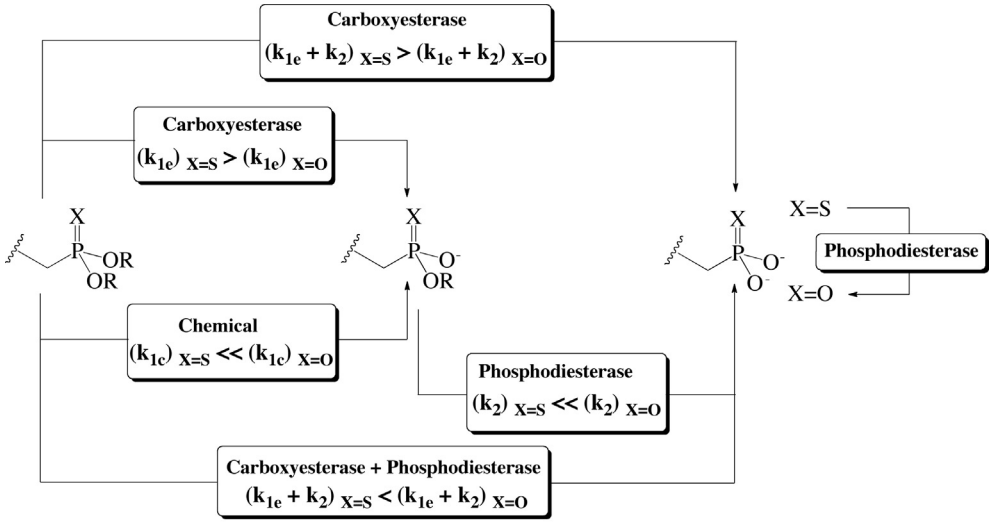
As observed previously, the decomposition of thiophosphonate diesters **13** and **14** into monoesters was PLE-catalyzed and the decomposition of phosphonate diesters **11** and **12** into monoesters was predominantly chemical-mediated (k_1 and k_{1e} values). The phosphonate monoesters were good substrates of SVP and were rapidly hydrolyzed into the parent compounds PMEA and PMPA with k_2 values of 140 × 10^{-3} min^{-1} and 60 × 10^{-3} min^{-1}. On the contrary, thiophosphonate monoesters were really poor substrates of SVP and k_2 values were low (k_2 values of 3.65 × 10^{-3} min^{-1} and 5.63 × 10^{-3} min^{-1}).

In summary, this study allowed to propose a reliable decomposition scheme for thiophosphonate prodrugs **12** and **14** in comparison to the phosphonate prodrugs **11** and **13**, as reported in Scheme 3.

Table 5
Pseudo-first-order rate constants (×10^{-3} min^{-1}) related to the enzymatic decomposition kinetic models of compounds **11**–**16** with purified enzymes: carboxyesterase (0.5 and 5 U/mL), phosphodiesterase (0.5 U/mL) and carboxyesterase + phosphodiesterase (0.5 U/mL).

	Carboxyesterase			Phosphodiesterase		Carboxyesterase + phosphodiesterase		
	0.5 U/mL		5 U/mL	0.5 U/mL		0.5 U/mL		
Rate constant (×10^{-3} min^{-1})	k_1	k_{1e}	k_2	k_1[a]	k_2	k_1	k_{1e}	k_2
13	140	130	1.78	4.80	NC[b]	120	110	3.65
11	230	60	<1.44	170	140	230	60	140
14	120	110	3.38	4.62	NC[b]	170	160	5.63
12	230	90	<1.44	140	120	150	10	60
16	ND[c]	ND[c]	ND[c]	ND[c]	3.85	ND[c]	ND[c]	ND[c]
15	ND[c]	ND[c]	ND[c]	ND[c]	230	ND[c]	ND[c]	ND[c]

[a] $k_1 = k_{1c}$ with $k_{1e} = 0$, compounds **11**–**14** were not substrate of phosphodiesterase.
[b] NC: k_2 could not be calculated.
[c] ND: not determined.



Scheme 3. Possible pathways of enzymatic decomposition of the compounds **11**–**16** and comparison of decomposition kinetic rates between thiophosphonate compounds **13**, **14** and **16** and phosphonate compounds **11**, **12** and **15**.

In cell extracts, bis-POM and bis-POC-thiophosphonates were readily transformed to mono-POM and mono-POC-thiopho sphonates. Very likely, this transformation occurred upon carboxyesterase activation of the thiophosphonate diester to the corresponding thiophosphonate monoester, which proved stable and was easily detected. The intermediate monoester was a poor substrate for cellular carboxyesterase(s) and for cellular phosphodiesterase(s). However, the parent compounds S-PMEA and S-PMPA were still efficiently released in cell extracts. In comparison, phosphonate diesters were hydrolyzed chemically predominantly into monoesters, which were then good substrates for intracellular phosphodiesterase(s).

In culture media, the same degradation pathway was observed, but the carboxyesterase-controlled step was significantly slower.

We observed also a minor degradation of parent compound S-PMEA and S-PMPA into PMEA and PMPA. This desulfuration may be mediated by phosphodiesterases [58]. It has been shown that phosphoramidases were also able to transform nucleoside 5′-O-phosphorothioates to nucleoside 5′-0-phosphates [59]. Additional studies concerning the elucidation of the mechanism of the enzyme-catalyzed desulfuration process should be performed.

3. Conclusion

In conclusion, we have investigated a novel practical synthetic procedure to efficiently obtain thiophosphonates S-PMEA **8** and S-PMPA **9** and their prodrug forms bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** using a common strategy, thereby limiting the number of synthetic steps. Compound **13** bis-POM-S-PMEA displayed interesting activity against HIV, HBV, HSV-1 and HSV-2 and compound **14** bis-POC-S-PMPA displayed potent activity against HIV-1, HIV-2 and HBV, but not HSV. Generally, the prodrug strategy led to an important increase of antiviral efficacy, especially against HIV for **13** and **14** and HSV for **13**. Sometimes (in case of PMEA) the increased antiviral activity was accompanied by an increase of the cytotoxicity. The thiophosphonate prodrugs **13** and **14** displayed the same antiviral specificity as the corresponding phosphonate prodrugs **11** and **12**.

Bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** behaved as prodrugs of S-PMEA and S-PMPA. Mono-POM-S-PMEA and mono-POC-S-PMPA **16** were intermediates in the conversion process and permitted the proposition of a coherent kinetic scheme for the overall metabolic conversion sequence. Thiophosphonate diesters were good substrates of carboxyesterase(s) leading to the formation of thiophosphonate monoesters, which were then rather poor substrates of phosphodiesterase(s). Consequently, the parent compounds S-PMEA **8** and S-PMPA **9** were released smoothly in the cell extracts. Furthermore, the release of S-PMEA or S-PMPA in the cell extracts may explain the unexpected antiviral activity of this kind of bis-POC/bis-POM prodrugs in infected cell cultures. Moreover, the metabolism in cells of prodrug forms to release parent compounds is sufficiently different between thiophosphonate and phosphonate series to induce important differences in antiviral efficacy. Because, thiosphosphonate prodrugs possess very long half-life in cell extracts, parent compounds are not effectively released and accumulated in cells and this is the limiting factor for antiviral efficacy. On the contrary, phosphonate prodrugs are quickly deprotected in cells, parent compounds are released efficiently, phosphorylated and could act effectively as chain terminators.

Finally, with this understanding of the conversion pathway and kinetic degradation of bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14** in various biological media, it should be possible to rationally design other prodrugs to identify compounds with better metabolic and pharmacological characteristics for clinical use. In particular, the slow removal of the two protecting groups represents a first target for improvement. Optimizing the delivery of these active nucleoside thiophosphonates remains our major goal for the successful treatment of viral infections with these type of agents.

4. Experimental

4.1. Chemistry

The ^{1}H NMR, ^{13}C NMR and ^{31}P NMR (Nuclear Magnetic Resonance) spectra were determined with a BRUKER AMX 250 MHz, a BRUKER Avance DPX 200 MHz and a BRUKER Avance III 600 MHz. Chemical shifts were expressed in ppm and coupling constants (*J*) are in hertz (s = singlet, d = doublet, dd = double doublet, ddd = double–double doublet, *t* = triplet, dt = double triplet,

m = multiplet). FAB High Resolution Mass Spectra (HRMS) were recorded in positive-ion mode on a JEOL SX 102 mass spectrometer using a caesium ion source and a glycerol/thioglycerol matrix; ESI High Resolution Mass Spectra were recorded in the negative-ion mode on a Micromass Q-TOF Waters (Laboratoire de Mesures Physiques RMN, USTL, Montpellier, France). Flash column chromatographies were performed on an MPLC SP4 system (Biotage®) with Biotage® SNAP cartridge HP-Sil columns. Analytical thin layer chromatographies (TLC) were performed on silica gel 60F 254 aluminium plates (Merck) of 0.2 mm thickness. The spots were examined with a UV light and Molybdenum blue spray. HPLC was performed on a Waters 600E controller system equipped with a 2998 photodiode array detector (detection 260 nm), auto-injector 717 and on-line degazer. HPLC-MS Coupling analyses were performed on a Thermofisher Accela 600 HPLC system (detection 260 nm) and a Finnigan survery MSQ (MS) with elecrospray ionization mode (ESI +/−). The UV-detector was connected to the mass spectrometer, and the whole eluting flow was both UV and mass analyzed. Analyses were performed on a column Hypersil Gold C18 (2.1 × 50 mm, 1.9 μm) and samples were eluted at a flow of 550 μL/min and detection at 260 nm. Eluent A: 0.1% of Formic acid (Sigma Aldrich) in water; eluent B: 0.1% of Formic acid in acetonitrile. The gradient starts at 98% eluent A with an increase to 10% eluent B after 5 min, to 50% eluent B after 9 min and continues at 50% eluent B until 10 min, to 100% eluent B after 11 min and continues at 100% eluent B until 12 min.

Buffer solutions: HPLC, MPLC and HPLC-MS buffers are prepared daily. TEAB buffer: A 1 M triethylamine solution in water was prepared mixing 140 mL of a triethylamine solution with 860 mL of deionized water. A stock solution of triethylammonium bicarbonate buffer TEAB (pH 7.5) 1 M was prepared by addition of dry-ice in a 1 M triethylamine solution in water to reach pH 7.5 and filtered with membrane 0.22 μM GV-type (Millipore).

Analytical reverse phase (RP) chromatography was carried out on a column X-Bridge (3.5 μm, 4.6 × 150 mm) + precolumn X-Bridge C18 (5 μM, 100 Å, 10 × 10 mm) and samples were eluted at a flow rate of 1 mL/min and detection at 260 nm. Eluent A: 0.05 M triethylammonium bicarbonate buffer (pH 7.5); eluent B: solution A containing 50% of acetonitrile. S1: the gradient starts at 100% eluent A with an increase to 10% eluent B after 10 min, to 20% eluent B after 25 min, to 100% eluent B after 35 min and continues at 100% eluent B until 50 min.

Preparative purifications were achieved on HPLC system on a preparative column X-Bridge (5 μm, 10 × 250 mm) + precolumn X-Bridge C18 (5 μM, 100 Å, 10 × 10 mm) and the samples were eluted at a flow rate of 4.5 mL/min and detection at 260 nm Eluent A: 0.05 M triethylammonium bicarbonate buffer (pH 7.5); eluent B: solution A containing 50% of acetonitrile. (S2: .the gradient starts after 5 min at 100% eluent A with an increase to 20% eluent B after 35 min, to 100% eluent B after 40 min continues at 100% eluent B until 50 min).

*4.1.1. General procedure for the synthesis of 9-[2-(diethylphosphonomethoxy)ethyl]adenine (**4**) and (R)-9-[2-(diethylphosphonomethoxy)propyl]adenine (**5**)*

A solution of **1** or **2** (1 equiv, 50 mmol) and magnesium di-*tert*-butoxide (1 equiv, 50 mmol) in anhydrous *N*,*N*-dimethylformamide (440 mL) was stirred at room temperature under argon for 1 h. A solution of diethyl *p*-toluenesulfonyloxymethanephosphonate **3** (1 equiv, 50 mmol) in anhydrous *N*,*N*-dimethylformamide (33 mL) was added dropwise. The mixture was stirred for 14 h at 35 °C. Analytical samples (1 μL) were collected, quenched with 250 μL of triethylammonium bicarbonate 0.05 M and analysed by HPLC (S1). Solution of **3** (0.5 equiv, 25 mmol) in anhydrous *N*,*N*-dimethylformamide (100 mL) and magnesium di-*tert*-butoxide (0.5 equiv, 25 mmol) were added under argon and stirred for another 6 h. The reaction was quenched then with acetic acid 80% (10 mL) and concentrated under reduced pressure and co-evaporated with toluene. The residue dissolved in brine (500 mL) was extracted twice with hot chloroform (500 mL). The organic extracts were dried over magnesium sulphate, filtered, and concentrated under reduced pressure. The residue was purified by silica gel flash chromatography [eluent, stepwise gradient of methanol (0–10%) in dichloromethane] to give pure compound as a white solid.

*4.1.1.1. 9-[2-(diethylphosphonomethoxy)ethyl]adenine (**4**).*

Chemical formula : $C_{12}H_{20}N_5O_4P$, MW = 329.29 g/mol. Yield = 52%. According to the literature [17,27]. R_f value 0.37 (DCM/MeOH, 9:1). HPLC ^tR = 36.4 min (S1), purity > 95%. ^1H NMR (CD_3OD) δ: 8.10 (s, 1H, *H*-2), 8.03 (s, 1H, *H*-8), 4.35 (*t*, *J* = 5.0 Hz, 2H, CH_2N), 3.93 (dq, *J* = 8.0 and *J* = 7.0 Hz, 4H, OCH_2CH$_3$), 3.86 (*t*, *J* = 5.0 Hz, 2H, CH_2O), 3.76 (d, J_{PH} = 8.5 Hz, 2H, CH_2P), 1.11 (*t*, *J* = 7.0, 6H, OCH$_2$CH_3). ^{13}C NMR (CD_3OD) δ: 157.29, 153.71, 150.67, 143.34, 119.34, 72.15 (d, *J* = 11.9 Hz), 66.63 (d, J_{CP} = 166 Hz), 63.99 (d, *J* = 6.6 Hz), 44.54, 16.62 (d, *J* = 5.8 Hz). ^{31}P NMR (CD_3OD) δ: 21.56. MS (GT, FAB$^+$): 136 (Adenine + 1H)$^+$, 330 (M + 1H)$^+$, 352 (M + Na)$^+$.

*4.1.1.2. (R)-9-[2-(diethylphosphonomethoxy)propyl]adenine (**5**).*

Chemical formula : $C_{13}H_{22}N_5O_4P$, MW = 343.32 g/mol. Yield = 53%. According to the literature [17,27]. R_f value 0.44 (DCM/MeOH, 9:1). HPLC ^tR = 37.2 min (S1), purity > 97%. ^1H NMR: (CD_3OD) δ: 8.23 (s, 1H, *H*-2), 8.15 (s, 1H, *H*-8), 4.41 (dd, *J* = 14.5 Hz and *J* = 3.2 Hz, 1H, CH_aN), 4.22 (dd, *J* = 14.5 Hz and *J* = 7.7 Hz, 1H, CH_bN), 4.10 (m, 1H, C*H*O), 4.03–3.69 (m, 6H, OCH_2CH$_3$ and CH_2P), 1.33 (*t*, *J* = 7.1 Hz, 6H, OCH$_2$CH_3), 1.27 (d, *J* = 6.2 Hz, 3H, CH_3). ^{13}C RMN (CD_3OD) δ: 157.30, 153.70, 150.89, 143.63, 119.70, 77.65 (d, *J* = 12.3 Hz), 64.32 (d, J_{CP} = 167 Hz), 64.08 (d, *J* = 6.6 Hz), 63.95 (d, *J* = 6.6 Hz), 49.18, 16.73 (d, *J* = 5.8 Hz), 16.71, 16.70 (d, *J* = 5.8 Hz). ^{31}P RMN (CD_3OD) δ: 21.56. MS (GT, FAB$^+$) : 136 (Adenine+1H)$^+$, 344 (M+1H)$^+$, 366 (M + Na)$^+$, 687 (2M+1H)$^+$.

*4.1.2. General procedure for the synthesis of 9-[2-(diethylthiophosphonomethoxy)ethyl]adenine (**6**) and (R)-9-[2-(diethylthiophosphonomethoxy)propyl]adenine (**7**)*

A solution of **4** or **5** (1 equiv, 10 mmol) and 2,4-Bis(methylthio)-1,3,2,4-dithiadiphosphetane-2,4-disulfide (1 equiv, 10 mmol) in anhydrous benzene (450 mL) was stirred at 80 °C for 2 h. The mixture was then stirred at 0 °C for 2 h. After a filtration through a celite pad, benzene was concentrated under reduced pressure and the residue was purified by silica gel flash chromatography [eluent, stepwise gradient of methanol (0–5%) in dichloromethane] to afford the desired product as a white powder.

*4.1.2.1. 9-[2-(diethylthiophosphonomethoxy)ethyl]adenine (**6**).*

Chemical formula : $C_{12}H_{20}N_5O_3PS$, MW = 345.36 g/mol. Yield = 43%. R_f value 0.42 (DCM/MeOH, 9:1). HPLC ^tR 40.9 min (S1), purity > 85%. ^1H NMR: (CD_3OD) δ: 8.11 (s, 1H, *H*-2), 8.06 (s, 1H, *H*-8), 4.32 (*t*, *J* = 4.7 Hz, 2H, CH_2N), 3.98–3.83 (m, 6H, OCH_2CH$_3$ and CH_2O), 3.76 (d, J_{PH} = 4.2 Hz, 2H, CH_2P), 1.09 (*t*, *J* = 7.1 Hz, 6H, OCH$_2$CH_3). ^{13}C NMR: (CD_3OD) δ: 157.27, 153.63, 150.69, 143.55, 119.88, 72.37 (d, J_{CP} = 132.9 Hz), 71.87 (d, *J* = 8.6 Hz), 63.74 (d, *J* = 6.9 Hz), 44.68, 16.49 (d, *J* = 6.5 Hz). ^{31}P NMR: (CD_3OD) δ: 88.02. MS (TOF, ESI$^+$) : 346 (M+1H)$^+$. HRMS (TOF, ESI$^+$) calcd for $C_{12}H_{21}N_5O_3PS$ (M + H)$^+$ 346.1103, found 346.1105.

*4.1.2.2. (R)-9-[2-(diethylthiophosphonomethoxy)propyl]adenine (**7**).*

Chemical formula : $C_{13}H_{22}N_5O_3PS$, MW = 359.38 g/mol. Yield = 44%. R_f value 0.49 (DCM/MeOH, 9:1). HPLC ^tR 41.7 min (S1), purity > 85%. ^1H NMR: (CD_3OD) δ 8.11 (s, 1H, *H*-2), 8.05 (s, 1H, *H*-8), 4.26 (dd, *J* = 14.3 Hz and *J* = 2.8 Hz, 1H, CH_aN), 4.08 (dd, *J* = 14.3 Hz

and J = 8.1 Hz, 1H, CH_bN), 4.00–3.73 (m, 6H, OCH_2CH$_3$ and CH_2P), 4.03-3.69 (m, 6H, CHO), 1.33 (*t*, J = 7.1 Hz, 6H, OCH$_2$CH_3), 1.27 (d, J = 6.2 Hz, 3H, CH_3). ^{13}C NMR (CD_3OD) δ 157.60, 153.60, 150.91, 143.89, 119.72, 77.35 (d, J = 9.1 Hz), 70.06 (d, J_{CP} = 134.6 Hz), 63.73 (d, J = 7.3 Hz), 63.62 (d, J = 7.3 Hz), 16.81, 16.55(d, J = 1.3 Hz), 16.45 (d, J = 1.1 Hz). ^{31}P NMR: (CD_3OD) δ: 88.407. MS (TOF, ESI$^+$) : 360 (M+1H)$^+$. HRMS (TOF, ESI$^+$) calcd for $C_{13}H_{23}N_5O_3PS$ (M + H)$^+$ 360.1260, found 360.1254.

4.1.3. General procedure for the synthesis of 9-[2-(thiophosphonomethoxy)ethyl]adenine (**8**) and (R)-9-[2-(thiophosphonomethoxy)propyl]adenine (**9**)

A solution of **6** or **7** (1 equiv, 3 mmol) and sodium bromide (40 equiv, 160 mmol) in anhydrous *N*-methylpyrrolidinone (20 mL) was stirred vigorously under argon. Trimethylsilyl chloride (40 equiv, 160 mmol) was added dropwise and the mixture was heated at 90 °C for 12 h. The reaction was quenched with 5 mL of triethylammonium bicarbonate 0.05 M and stirred for 1 h. After solvents were concentrated *in vacuo* and freezed-drying, the residue was purified by reversed-phase column chromatography (S2). Product fractions were collected, evaporated to dryness and lyophilized. Excess of triethylammonium bicarbonate was removed by repeated freezed-drying with deionized water. The residue was dissolved in water and eluted on a Dowex 50WX2 column (Na^+ exchange) to give the pure compound as disodium salt as a white powder after freeze-drying.

4.1.3.1. 9-[2-(thiophosphonomethoxy)ethyl]adenine ***(8)***. Chemical formula : $C_8H_{10}N_5Na_2O_3PS$, MW = 333.22 g/mol. Yield = 40%. HPLC ^{t}R 19.5 min (S1), purity > 98%. ^{1}H NMR : (D_2O) δ 8.13 (s, 1H, *H*-8), 8.01 (s, 1H, *H*-2), 4.28 (*t*, J = 4.8 Hz, 2H, CH_2N), 3.90 (*t*, J = 4.8 Hz, 2H, CH_2O), 3.62 (d, J = 5.2 Hz, 2H, CH_2P). ^{13}C NMR: (D_2O) δ 152.23, 149.12, 146.98, 141.98, 116.38, 73.01 (d, J_{CP} = 120 Hz), 68.57 (d, J_{CP} = 10.6 Hz), 42.27. ^{31}P NMR : (D_2O) δ 61.76 (*t*, J = 5.2 Hz). MS (TOF, ESI$^+$) : 290 (M+1H)$^+$. HRMS (TOF, ESI$^+$) calcd for $C_8H_{13}N_5O_3PS$ (M + H)$^+$ 290.0477, found 290.0576.

4.1.3.2. (R)-9-[2-(thiophosphonomethoxy)propyl]adenine ***(9)***. Chemical formula : $C_9H_{12}N_5Na_2O_3PS$, MW = 347.24 g/mol. Yield = 48%. HPLC ^{t}R 21.7 min (S1), purity > 99%. ^{1}H NMR (D_2O) δ 8.12 (s, 1H, *H*-8), 7.98 (s, 1H, *H*-2), 4.22 (dd, J = 3.1 Hz and J = 14.6 Hz, 1H, CH_aN), 4.05 (dd, J = 6.7 Hz and J = 14.6 Hz, 1H, CH_bN), 3.91 (m, 1H, CHO), 3.62 (dd, J = 5.6 Hz and J = 12.7 Hz, 1H, CH_aP), 3.46 (dd, J = 6.1 Hz and J = 12.8 Hz, 1H, CH_bP), 1.05 (d, J = 6.2 Hz, 3H, CH_3). ^{13}C NMR (D_2O) δ 152.65, 149.12, 147.25, 142.25, 116.26, 74.46 (d, J_{CP} = 10.7 Hz), 70.89 (d, J_{CP} = 121.1 Hz), 46.39, 14.73. ^{31}P NMR (D_2O) δ 61.11 (*t*, J = 5.6 Hz). MS (TOF, ESI$^+$) : 304 (M+1H)$^+$. HRMS (TOF, ESI$^+$) calcd for $C_9H_{15}N_5O_3PS$ (M + H)$^+$ 304.0633, found 304.0619 (M)$^-$.

4.1.4. General procedure for the synthesis of 9-{2-[O,O′-bis(pivaloyloxymethyl)phosphonomethoxy]ethyl}adenine (**11**) and (R)-9-{2-[O,O′-bis(isopropoxycarbonyloxymethyl)phosphonomethoxy]propyl}adenine (**12**)

A solution of **4** or **5** (1 equiv, 10 mmol) and sodium bromide (2.1 equiv, 21 mmol) in anhydrous *N*-methylpyrrolidinone (10 mL) was stirred vigorously under argon. Trimethylsilyl chloride (4 equiv, 40 mmol) was added dropwise and the mixture was heated at 60 °C for 12 h. The reaction was monitored by HPLC until complete dispartition of the starting material (S1). When completion was observed, the reaction mixture was diluted with anhydrous ethyl acetate (16 mL) and stirred for 5 min. The ethyl acetate and remaining trimethylsilyl chloride were then concentrated *in vacuo* at 60 °C. Anhydrous isopropyl alcohol (1.5 mL) was then added to the reaction mixture and stirred for 15 min. The mixture was diluted with anhydrous ethyl acetate (16 mL) and solvents were evaporated under reduced pressure at 60 °C. Chloromethyl pivalate (POMCl, 5 equiv, 50 mmol) for the synthesis of **11** or chloromethyl isopropylcarbonate **10** [50] (POCCl, 5 equiv, 50 mmol) for the synthesis of **12** and triethylamine (3 equiv, 30 mmol) were added to the mixture and the reaction mixture was stirred at 60 °C for 6 h. The reaction was diluted in anhydrous ethyl acetate (16 mL), stirred for 5 min, and the ethyl acetate and remaining triethylamine were concentrated *in vacuo* at 60 °C. The reaction mixture was diluted in ethyl acetate (160 mL) and extracted with water (60 mL), dried over magnesium sulphate, filtered, and concentrated to dryness under reduced pressure. The resulting oil was purified by silica gel flash chromatography [eluent, stepwise gradient of methanol (0–5%) in dichloromethane] to afford the desired product as a white powder.

4.1.4.1. 9-{2-[O,O′-Bis(pivaloyloxymethyl)phosphonomethoxy]ethyl} adenine ***(11)***. Chemical formula : $C_{20}H_{32}N_5O_8P$, MW = 501.47 g/mol. Yield = 27%. According to the literature [33]. R_f value 0.32 (DCM/MeOH, 9.5:0.5). HPLC ^{t}R 42.5 min (S1), purity > 90%. ^{1}H NMR ($CDCl_3$) δ 8.30 (s, 1H, *H*-8), 7.90 (s, 1H, *H*-2), 6.16 (s, 2H, NH_2), 5.69–5.54 (m, 4H, OCH2O), 4.37 (*t*, J = 4.9 Hz, 2H, CH_2N), 3.92 (*t*, J = 4.9 Hz, 2H, CH_2O), 3.83 (d, J = 7.6 Hz, 2H, CH_2P), 1.18 (s, 18H, CH_3 (*t*-Bu)). ^{13}C NMR ($CDCl_3$) δ 155.76, 153.00, 149.99, 141.43, 119.52, 81.76 (d, J = 6.3 Hz), 71.48, 65.63 (d, J = 166.1 Hz), 43.43, 38.79, 26.89. ^{31}P NMR: ($CDCl_3$) δ 20.56. MS (TOF, ESI$^+$): 502 (M+1H)$^+$. HRMS (TOF, ESI$^+$) calcd for $C_{20}H_{33}N_5O_8P$ (M + H)$^+$ 502.2067, found 502.2057.

4.1.4.2. (R)-9-{2-[O,O′-Bis(isopropoxycarbonyloxymethyl)phosphonomethoxy]propyl}adenine ***(12)***. Chemical formula : $C_{19}H_{30}N_5O_{10}P$, MW = 519.44 g/mol. Yield = 33%. According to the literature [51]. R_f value 0.32 (DCM/MeOH, 9.5:0.5). HPLC ^{t}R 42.6 min (S1), purity > 90%. ^{1}H NMR ($CDCl_3$) δ 8.31 (s, 1H, *H*-8), 8.02 (s, 1H, *H*-2), 6.49 (s, 2H, NH_2), 5.70–5.57 (m, 4H, OCH_2O), 4.96–4.87 (m, 2H, CH(*i*-Pr)), 4.37 (dd, J = 14.5 and 3.1 Hz, 1H, CHaN), 4.15 (dd, J = 14.5 and 7.3 Hz, 1H, CHbN), 3.94 (m, 2H, OCH_2P), 3.72 (dd, J = 13.9 and 9.0 Hz, 1H, CHO), 1.32–1.29 (m, 12H, CH_3(*i*-Pr)), 1.22 (d, J = 6.2 Hz, 3H, CH_3). ^{13}C NMR: ($CDCl_3$) δ 155.29, 153.24, 153.21, 142.29, 84.44, 77.37, 77.16, 76.94, 76.71, 73.59, 63.76, 62.64, 48.38, 32.07, 29.84, 22.83, 21.77, 16.53, 14.24, 1.15. ^{31}P NMR : ($CDCl_3$) δ 20.99. MS (TOF, ESI$^+$): 520 (M + 1H)$^+$. HRMS (TOF, ESI$^+$) calcd for $C_{19}H_{31}N_5O_{10}P$ (M)$^+$ 520.1809, found 520.1802.

4.1.5. General procedure for the synthesis of 9-{2-[O,O′-bis(pivaloyloxymethyl)thiophosphonomethoxy]ethyl}adenine (**13**) (R)-9-{2-[O,O′-bis(isopropoxycarbonyloxymethyl) thiophosphonomethoxy]propyl}adenine (**14**)

A solution of **11** or **12** (1 equiv, 2.5 mmol) and 2,4-Bis(methylthio)-1,3,2,4-dithiadiphosphetane-2,4-disulfide (1 equiv, 2.5 mmol) in anhydrous benzene (40 mL) was stirred at 80 °C for 30 min. The mixture was then stirred at 0 °C for 2 h. After filtration through a celite pad, benzene was concentrated *in vacuo* and the residue purified by silica gel flash chromatography [eluent, stepwise gradient of methanol (0–5%) in dichloromethane] to afford the desired product as a white powder.

4.1.5.1. 9-{2-[O,O′-Bis(pivaloyloxymethyl)thiophosphonomethoxy] ethyl}adenine ***(13)***. Chemical formula : $C_{20}H_{32}N_5O_7PS$, MW = 517.54 g/mol. Yield = 29%. R_f value 0.39 (DCM/MeOH, 9.5:0.5). HPLC ^{t}R 46.1 min (S1), purity > 97%. ^{1}H NMR : ($CDCl_3$) δ: 8.73 (s, 1H, *H*-8), 8.11 (s, 1H, *H*-2), 7.26 (s, 2H, NH_2), 5.69-5.60 (m, 4H, OCH_2O), 4.40 (*t*, J = 4.9 Hz, 2H, CH_2N), 3.84 (*t*, J = 4.9 Hz, 2H, CH_2O), 3.96 (d, J = 5 Hz, 2H, CH_2P), 1.21 (s, 18H, CH_3(*t*-Bu)). ^{13}C NMR ($CDCl_3$) δ 155.64, 152.68, 149.63, 143.84, 122.7, 81.83 (d, J = 6.2 Hz), 71.23, 65.17 (d, J = 166.1 Hz), 48.66, 38.86, 26.95. ^{31}P RMN ($CDCl_3$) δ 88.89.

MS (TOF, ESI$^+$) : 518 (M+1H)$^+$. HRMS (TOF, ESI$^+$) calcd for $C_{20}H_{33}N_5O_7PS$ (M + H)$^+$ 518.1838,found 518.1851.

4.1.5.2. (R)-9-{2-[O,O′-Bis(isopropoxycarbonyloxymethyl)thiophosphonomethoxy]propyl}adenine ***(14)***. Chemical formula : $C_{19}H_{30}N_5O_9PS$, MW = 535.51 g/mol. Yield = 32%. R_f value 0.38 (DCM/MeOH, 9.5:0.5). HPLC ^{t}R 46.3 min (S1), purity > 98%. ^{1}H NMR: ($CDCl_3$) δ 8.31 (s, 1H, *H*-8), 8.23 (s, 1H, *H*-2), 6.49 (s, 2H, NH_2), 5.72–5.54 (m, 4H, OCH_2O), 4.91 (tq, *J* = 12.3 and *J* = 6.2 Hz, 2H, C*H*(*i*-Pr)), 4.43 (d, *J* = 13.7, 1H, C*H*aN), 4.16 (dd, *J* = 14.3 and *J* = 7.6 Hz, 1H, C*H*bN), 4.09–3.95 (m, 2H, OCH_2P), 3.75 (dd, *J* = 13.6 and *J* = 4.3 Hz, 1H, C*H*O), 1.32–1.29 (m, 12H, CH_3 (*i*-Pr))1.31 (d, *J* = 6.1, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ 153.24, 153.21, 151.74, 144.66, 118.45, 76.30 (d, *J* = 8.5 Hz), 73.58, 69,27 (d, *J* = 137 Hz), 48.90, 46.02, 29.82, 16.56, 8.77. ^{31}P NMR ($CDCl_3$) δ 88.82. MS (TOF, ESI$^+$) : 536 (M + 1H)$^+$. HRMS: (TOF, ESI$^+$) calcd for $C_{19}H_{31}N_5O_9PS$ (M + H)$^+$536.1580, found 536.1572.

*4.1.6. General procedure for the synthesis of (R)-9-{2-[O-mono(isopropoxycarbonyloxymethyl)phosphonomethoxy]propyl} adenine (**15**) (R)-9-{2-[O-mono(isopropoxycarbonyloxymethyl) thiophosphonomethoxy]propyl}adenine (**16**)*

A solution of **12** or **14** (1 equiv, 0.05 mmol) in water (5 mL) was stirred vigorously at 60 °C. The reaction was maintained at 60 °C and monitored by HPLC-MS for complete conversion to **15** or **16**. Typical retention times: ^{t}R = 0.49 min, PMPA, ESI-MS [M + H] *m/z* = 288.0; ^{t}R = 0.48 min, **9**, ESI-MS [M + H] *m/z* = 304.10; ^{t}R = 4.78 min, **15**, ESI-MS [M + H] *m/z* = 404.09; ^{t}R = 6.36 and 6.50 min (2 diastereoisomers), **16**, ESI-MS [M + H] *m/z* = 420.09; ^{t}R = 8.04 min, **12**, ESI-MS [M + H] *m/z* = 520.19; ^{t}R = 8.92 min, **14**, ESI-MS [M + H] *m/z* = 536.10. When completion was observed, the reaction mixture was extracted with ethyl acetate (5 mL) and the aqueous layer concentrated *in vacuo* and lyophilized. The crude product was purified on reverse phase C18 (biotage® SNAP cartridge KP–C18-HS 33g, flow : 10 mL/min, UV-detector : 260 nm, Eluent A : TEAB 0.05 M, eluent B : solution A containing 50% of acetonitrile, gradient starts at 100% eluent A with an increase to 10% eluent B after 66 min, to 20% eluent B after 83 min and to 100% eluent B after 116 min). Each fraction was analysed by HPLC (S1), pure fractions were collected and eluted on a Dowex 50WX2 column (Na^+ exchange) to give the pure compound as sodium salt as a white powder after freeze-drying.

4.1.6.1. (R)-9-{2-[O-Mono(isopropoxycarbonyloxymethyl)phosphonomethoxy]propyl}adenine ***(15)***. Chemical formula : $C_{14}H_{21}N_5NaO_7P$, MW = 425.31 g/mol. Yield = 38%. HPLC ^{t}R 37.7 min (S1), purity > 98%. ^{1}H NMR: (D_2O) δ: 8.26 (s, 1H, *H*-8), 8.23 (s, 1H, *H*-2), 5.29 (m, 2H, OCH_2O), 4.70 (dd, *J* = 12.6 and *J* = 6.3 Hz, 1H, C*H*(*i*-Pr)), 4.40 (dd, *J* = 14.8 and *J* = 2.7 Hz, 1H, C*H*aN), 4.24 (dd, *J* = 14.8 and *J* = 7.7 Hz, 1H, C*H*bN), 3.78 (dd, *J* = 13.1 and *J* = 8.7 Hz, 2H, OCH_2P), 3.46 (dd, *J* = 13.2 and *J* = 10.5 Hz, 1H, C*H*O), 1.23 (d, *J* = 6.3 Hz, 3H, CH_3), 1.18 (d, *J* = 6.2 Hz, 6H, CH_3 (*i*-Pr)). ^{31}P RMN (D_2O) δ 17.10. MS (ESI$^+$): 404.09 (M + H)$^+$; 807.23 (2M + H)$^+$.

4.1.6.2. (R)-9-{2-[O-Mono(isopropoxycarbonyloxymethyl)thiophosphonomethoxy]propyl}adenine ***(16)***. Chemical formula: $C_{14}H_{21}N_5NaO_6PS$, MW = 441.38 g/mol. Yield = 30%. HPLC ^{t}R 38.7 (fast-eluting isomer) and 38.9 (slow-eluting isomer) min (S1), purity > 98%. ^{1}H NMR (peaks of both diastereoisomers A and B are described): (D_2O) δ 8.29 (s, 4H, *H*-8 of A and B and *H*-2 of A and B), 5.50 (ddd, *J* = 21.0, *J* = 14.9 and *J* = 5.6 Hz, 2H, OCH_2O of A), 5.39–5.34 (m, 2H, OCH_2O of B), 4.83–4.79 (m, 2H, C*H*(*i*-Pr) of A and B), 4.46–4.41 (m, 2H, C*H*aN of A and B), 4.30–4.22 (m, 2H, C*H*bN of A and B), 4.09–4.03 (m, 2H, CHO of A and B), 3.91 (dd, *J* = 13.2 and *J* = 5.1 Hz, 1H, OCH_aP of A), 3.83 (dd, *J* = 13.0 and *J* = 3.1 Hz, 1H, OCH_aP of B), 3.64 (dd, *J* = 13.0 and *J* = 8.0 Hz, 1H, OCH_bP of A), 3.57 (dd, *J* = 13.2 and *J* = 5.0 Hz, 1H, OCH_bP of B), 1.29–1.21 (m, 18H, CH_3(*i*-Pr) and CH_3 of A and B). ^{31}P NMR (D_2O) δ 72.91, 72.26 (peaks of both diastereoisomers are described). MS (ESI$^+$): 420.09 (M + H)$^+$; 839.18 (2M + H)$^+$.

4.2. Antiviral activity in cells

4.2.1. Cells

CEM cells were grown in RPMI-1640 medium supplemented with antibiotics, and 10% foetal calf serum (FCS). The human hepatoma cell line Huh7 was grown in DMEM culture medium supplemented with 10% (FCS), 100 IU/ml penicillin, 50 μg/ml streptomycin. Human embryonic lung HEL cells were propagated in minimal essential medium (MEM) supplemented with 10% FCS, 2 mM L-glutamine, and 0.075% bicarbonate.

4.2.2. Anti-HIV assay in cell cultures

The methodology of the anti-HIV assays was as follows: human CEM (~3×10^5 cells/cm^3) cells were infected with 100 $CCID_{50}$ of HIV(III_B) or HIV-2(ROD)/ml and seeded in 200 μL wells of a microtiter plate containing appropriate dilutions of the tested compounds **11**, **12**, **13** and **14**. After 4 days of incubation at 37 °C, HIV-induced CEM giant cell formation was examined microscopically. The 50% effective concentration (EC_{50}) was defined as the compound concentration required to inhibit syncytia formation by 50%. The 50% cytostatic concentration (CC_{50}) was defined as the compound concentration required to inhibit CEM cell proliferation by 50% in mock-infected cell cultures.

4.2.3. Anti-HBV assay in cells

For the Anti-HBV assay in the Huh7 cell cultures, the plasmid pTriEXMod-HBV, which contains 1.1 U of the HBV genome, enabled after cell transfection the production of pregenomic RNA under the control of the chicken beta actin promoter and therefore triggers HBV DNA synthesis. Huh7 cells (2×10^5) were seeded in 12-well plates and were transfected 1 day post plating with 1.5 μg/well of pTriEXMod-HBV plasmid containing wt HBV genome, using the TransIT®-2020 Transfection Reagent (Mirus) according to the manufacturer's instructions. Medium was changed daily, and compound administration of **11**, **12**, **13** and **14**, with increasing concentrations (0, 1.25, 2.5, 5, 10, 25 μM) was performed from day 3 to day 7 post transfection. Cells were then lysed for analysis of intracellular viral DNA. HBV DNA was purified from intracellular core particles as described earlier [60] and the quantity of HBV DNA was quantified by qPCR using Sybr Green. The qPCR was performed in a reaction volume of 20 μL using the *Light Cycler* 480 *SYBR Green* I Master mix with forward primer (5′-GCTGACGCAACCCCCACT-3′; final concentration: 500 nM) and reversed primer (5′-AGGAGTTCCGCAGTATGG-3′; final concentration: 500 nM). The reaction was analysed using a Light Cycler 480 apparatus (Roche). A plasmid containing the full-length insert of the HBV genome was used to prepare the standard curve. The amount of viral DNA produced in drug-treated cell cultures was expressed as a percentage of the untreated samples.

4.2.4. Antiviral assay against a panel of DNA viruses

Herpes simplex virus type 1 (HSV-1) (KOS and KOS ACV TK$^-$), HSV-2 (G), and vaccinia virus were assayed in HEL cell cultures. Cells were grown to confluency in 96-well microtiter trays and were inoculated with 100 times the 50% cell culture infective dose. Compounds **11**, **12**, **13** and **14**, were added to the virus-infected cell cultures. The virus-induced cytopathic effect (CPE) was recorded microscopically at 3 days post infection and the inhibition was expressed as a percentage of inhibition of the CPE in the untreated

controls. The 50% effective concentrations (EC_{50}) were derived from graphical plots. The minimal cytotoxic concentration (MCC) was defined as the minimal drug concentration that resulted in a microscopically detectable alteration of cell morphology. The MCC was determined in uninfected confluent cell cultures that were incubated, akin to the cultures used for the antiviral assays, with serial dilutions of the compounds for the same time period. Cultures were inspected microscopically for alteration of cell morphology.

4.3. Decomposition studies and metabolism

4.3.1. Media and preparation of cell extracts

Acidic buffer (pH 1.2) was prepared from 0.1 N HCl and the pH measured at 25 °C. RPMI-1640 medium and DMEM were purchased from Invitrogen. Heat-inactived foetal calf serum (FCS) was purchased from PAN biotech. Culture media were supplemented with 10% FCS, 100 IU/mL penicillin and 50 μg/mL streptomycin. CEM and Huh7 cell extracts were prepared as previously described [27,56]. Briefly, exponentially growing CEM or Huh7 cells were pelleted by centrifugation (500 *g*), washed with PBS, and resuspended in 10 mM Tris-HCl pH 7.4, 140 mM KCl, at the concentration of 30×10^6 cells/mL. Cells were lyzed by ultrasonic treatment and cellular debris were removed by centrifugation (10,000 *g*, 20 min). The supernatant containing soluble proteins (3 mg/mL) was stored at −80 °C. Kinetic data of metabolism of compounds **8**, **9**, **11**, **12**, **13** and **14** were studied at 37 °C: (a) in water, (b) in acidic buffer (pH 1.2) prepared from 0.1 N HCl, (c) in non-enzymatic medium (RPMI-1640), (d) in culture medium (RPMI-1640 containing 10% heat-inactivated foetal calf serum), (e) in total cell extract (CEM cell) to mimic anti-HIV assay conditions, (f) in non-enzymatic medium (DMEM), (g) in culture medium (DMEM containing 10% heat-inactivated foetal calf serum), (h) in total cell extract (Huh7) to mimic anti-HBV assay conditions.

4.3.2. HPLC analyses

We used a previously described on-line HPLC cleaning method [56]. Analyses were performed on a column Nova-pak C18 (4 mm, 3.9 × 150 mm) + precolumn Nova-pack C18 (100 Å) with an on-line filtration system. Samples were eluted using a linear gradient of 0.005 M PIC A buffer in 100% water (buffer A) to a 0.005 M PIC A buffer in 50% acetonitrile (buffer B), programmed over a 60 min period with a flow rate of 1 mL/min and detection at 260 nm. A 0.005 M solution of tetrabutylammonium hydrogen sulphate buffer (PIC A) was prepared by addition of a vial of PIC A Low UV Reagent (WATERS) in 1 L of deionized water and filtered with membrane 0.22 μ GV-type (Millipore).

For each kinetic study, the compound solution was diluted with a freshly thawed aliquot of the considered medium to obtain a final concentration of 0.1 mM and the mixture was incubated at 37 °C. At the desired time, samples were injected into an analytical HPLC column where the analytical conditions make it possible to visualise and properly separate the starting compound from the corresponding metabolites. The crude sample (50 μL) was injected into the precolumn and eluted with buffer A during 4 min. Then, the switching valve for connecting the precolumn to the column was activated and a gradient S3 was applied : the gradient started at 100% eluent A with an increase to 10% eluent B after 6 min, to 15% eluent B after 11 min, to 100% eluent B after 21 min and continued at 100% eluent B until 33 min. The retention times are listed in Table 6.

4.3.3. Constant rate determination

All compounds could be accurately UV-detected (260 nm) and quantified. The nature of the metabolites was confirmed by injection of authentic samples. Having assigned the various HPLC signals, the kinetic data were treated according to a general "consecutive-competitive pseudo-first order" model. The relative concentration of each component was calculated from the areas at 260 nm of the corresponding signals. The half-lives were deduced from experimental data following the disappearance of the peak area of the desired compound and the calculated rate constant of each decomposition step in different media were calculated with the equation (1)

$$K = \frac{\ln(2)}{t_{1/2}}(\text{competitive} - \text{consecutive pseudo} - \text{first order}) \quad (1)$$

The amount of remaining compound at each time point was used to determine the half-life of the compound.

Table 6
Retention times (gradient : S3) of PMEA, S-PMEA **8**, PMPA, S-PMPA **9**, bis-POM-PMEA **11**, bis-POC-PMPA **12**, bis-POM-S-PMEA **13** and bis-POC-S-PMPA **14**.

Compounds	Retention time ^tR (min)
S-PMEA **8**	23.0
PMEA	20.7
S-PMPA **9**	24.6
PMPA	22.2
Mono-POM-S-PMEA	29.4
Mono-POM-PMEA	27.5
Mono-POC-S-PMPA Fast-eluting isomer **16**	29.1
Mono-POC-S-PMPA Slow-eluting isomer **16**	29.3
Mono-POC-PMPA **15**	27.4
Bis-POM-S-PMEA **13**	36.8
Bis-POM-PMEA **11**	32.0
Bis-POC-S-PMPA **14**	34.7
Bis-POC-PMPA **12**	31.0

4.3.4. Purified enzyme incubation studies

Porcine liver esterase (E9019) and phosphodiesterase I from *Crotalus adamanteus* venom (P3134) were purchased from Sigma–Aldrich. Porcine liver esterase was solubilized in a water/glycerol solution (50:50) at a final concentration of 50 U/mL. Phosphodiesterase I from *C. adamanteus* venom was solubilized in a water/glycerol solution (50:50) at a final concentration of 20 U/mL.

Porcine liver esterase assay (0.5 U/mL enzyme/50 μM substrate) [61,62]. 8 μL of a solution of compound **11**, **12**, **13**, or **14** in MeOH (4 mM) in 792 μL of buffer containing 80 μL of Tris–HCl/KCl (pH 7.5) and 8 μL of Porcine liver esterase solution (50 U/mL) were incubated at 37 °C. At different time intervals (0, 15, 45, 90 min), 200 μL of the sample were withdrawn and analyzed.

Porcine liver esterase assay (5 U/mL enzyme/50 μM substrate) [61,62]. 4 μL of a solution of compound **11**, **12**, **13**, or **14** in MeOH (5 mM) in 676 μL of buffer containing 80 μL of tris–HCl/KCl (pH 7.5) and 40 μL of Porcine liver esterase solution (50 U/mL) were incubated at 37 °C. At different time intervals (0, 60, 120, 240 min), 200 μL of the sample were drawn and analyzed.

Phosphodiesterase I from *C. adamanteus* venom assay (0.5 U/mL enzyme/50 μM substrate) [61,62]. 8 μL of a solution of compound **11**, **12**, **13**, or **14** in MeOH (5 mM) or 20 μL of a solution of compound **15** or **16** in deionized water (2 mM) in 1768 or 1756 μL of buffer containing 200 μL of CHES/KCl (pH 9), 4 μL of $MgCl_2$ (1 M) and 20 μL of phosphodiesterase I from *C. adamanteus* venom solution (20 U/mL) were incubated at 37 °C. At different time intervals (0, 20, 30, 40, 60, 120, 240, 480 min) 200 μL of the sample were withdrawn and analyzed.

Phosphodiesterase I from *C. adamanteus* venom and Porcine liver esterase assay (0.5 U/mL enzyme/50 μM substrate) [61,62]. 8 μL of a solution of compound **11**, **12**, **13**, or **14** in MeOH (5 mM) in

1760 μL of buffer containing 200 μL of CHES/KCl (pH 9), 4 μL of $MgCl_2$ (1 M), 20 μL of phosphodiesterase I solution (20 U/mL) and 8 μL of Porcine liver esterase solution (50 U/mL) were incubated at 37 °C. At different time intervals (0, 20, 30, 40, 60, 120, 240, 480 min) 200 μL of the sample were drawn and analyzed. Each aliquot was centrifuged on Vivacon500® (Sartorius Stedim Biotech, 2000 MWCO) for 45 min at 14000 G at 4 °C to remove protein materials. 300 μL of filtrate were collected and diluted with 300 μL of TEAB 0.1 M.

HPLC analyses were performed on a column Nova-pak C18 (4 mm, 3.9 × 150 mm) + precolumn Nova-pack C18 (100 Å). Eluent A: 0.1 M triethylammonium bicarbonate buffer (pH 7.5); eluent B: solution A containing 50% of acetonitrile. Gradient S4 was applied : the gradient started at 100% eluent A with an increase to 10% eluent B after 20 min, to 20% eluent B after 25 min, to 100% eluent B after 35 min and continued at 100% eluent B until 45 min. All compounds were accurately UV-detected (260 nm) and quantified. The nature of the metabolites was confirmed by injection of authentic samples. Having assigned the various HPLC signals, the kinetic data were treated according to a general "consecutive-competitive pseudo-first order" model. The relative concentration of each component was calculated from the areas at 260 nm of the corresponding signals. The half-lives were deduced from experimental data following the disappearance of the peak area of the desired compound and the calculated rate constant of each decomposition step in different media were calculated with the equation (1).

Acknowledgements

This work was supported by grant from the French "Agence Nationale de Recherche Contre le Sida et les hépatites virales" (ANRS), by SIDACTION and KU Leuven (GOA no. 10/14). Loic Roux is particularly grateful to SIDACTION for a his PhD fellowship. We wish to thank, A. Lebrun and Dr G. Valette (Université Montpellier II, Montpellier, France) for their expertise in conducting NMR experiments and mass spectrometry analysis, and L. Ingels, L. Persoons and F. De Meyer for excellent technical assistance in the antiviral assays.

Appendix A. Supporting information

Supporting information related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.02.039.

References

[1] E. De Clercq, A. Holy, Nat. Rev. Drug Discovery 4 (2005) 928–940.
[2] E. De Clercq, Antiviral. Res. 75 (2007) 1–13.
[3] A. Holý, Curr. Pharm. Des. 9 (2003) 2567–2592.
[4] J. Lalezari, T. Schacker, J. Feinberg, J. Gathe, S. Lee, T. Cheung, F. Kramer, H. Kessler, L. Corey, W.L. Drew, J. Infect. Dis. 176 (1997) 892–898.
[5] A. Duarte-Rojo, E.J. Heathcote, Ther. Adv. Gastroenterol. 3 (2010) 107–119.
[6] A.M. Jenh, P.A. Pham, Expert Rev. Anti. Infect. Ther. 8 (2010) 1079–1092.
[7] E. De Clercq, Clin. Microbiol. Rev. 16 (2003) 569–596.
[8] P.L. Sharma, V. Nurpeisov, B. Hermandez-Santiago, T. Beltran, R.F. Schinazi, Curr. Top. Med. Chem. 4 (2004) 895–919.
[9] B.L. Robbins, M.C. Connelly, D.R. Marshall, R.V. Srinivas, A. Fridland, Mol. Pharmacol. 47 (1995) 391–397.
[10] B.L. Robbins, J. Greenhaw, M.C. Connelly, A. Fridland, Antimicrob. Agents Chemother. 39 (1995) 2304–2308.
[11] B.L. Robbins, R.V. Srinivas, C. Kim, N. Bischofberger, A. Fridland, Antimicrob. Agents Chemother. 42 (1998) 612–617.
[12] A. Holý, Collect. Symp. Ser. 5 (2002) 24–35.
[13] D. Hocková, A. Holý, M. Masojídková, G. Andrei, R. Snoeck, E. De Clercq, J. Balzarini, J. Med. Chem. 46 (2003) 5064–5073.
[14] P. Dolakova, M. Dracinsky, M. Masojidkova, V. Solinova, V. Kasicka, A. Holý, Eur. J. Med. Chem. 44 (2009) 2408–2424.
[15] J. Balzarini, P.C.E. De Clercq, S. Aquaro, C.F. Perno, H. Egberink, A. Holy, Antimicrob. Agents Chemother. 46 (2002) 2185–2193.
[16] A. Holý, I. Votruba, E. Tloustova, M. Masojidkova, Collec. Czech. Chem. Commun. 66 (2001) 1545–1592.
[17] A. Holý, J. Günter, H. Dvoráková, M. Masojídková, G. Andrei, R. Snoeck, J. Balzarini, E. De Clercq, J. Med. Chem. 42 (1999) 2064–2086.
[18] A. Holý, H. Dvorakova, J. Jindrich, M. Masojidkova, M. Budesinsky, J. Balzarini, G. Andrei, E. De Clercq, J. Med. Chem. 39 (1996) 4073–4088.
[19] C. Ying, A. Holý, D. Hocková, Z. Havlas, E. De Clercq, J. Neyts, Antimicrob. Agents Chemother. 49 (2005) 1177–1180.
[20] E. De Clercq, G. Andrei, J. Balzarini, P. Leyssen, L. Naesens, J. Neyts, C. Pannecouque, R. Snoeck, C. Ying, D. Hocková, A. Holý, Nucleosides, Nucleotides Nucleic Acids 24 (2005) 331–341.
[21] T. Tichy, G. Andrei, M. Dracinsky, A. Holy, J. Balzarini, R. Snoeck, M. Krecmerova, Bioorg. Med. Chem. 19 (2011) 3527–3539.
[22] J. Ruiz, J.R. Beadle, M. Buller, J. Schreiwer, M.N. Prichard, K.A. Keith, K.C. Lewis, K.Y. Hostetler, Bioorg. Med. Chem. 19 (2011) 2950–2958.
[23] C. Meier, U. Görbig, C. Müller, J. Balzarini, J. Med. Chem. 48 (2005) 8079–8086.
[24] S.J. Hecker, M.D. Erion, J. Med. Chem. 51 (2008) 2328–2345.
[25] M.D. Erion, K.R. Reddy, S.H. Boyer, M.C. Matelich, J. Gomez-Galeno, R.H. Lemus, B.G. Ugarkar, T.J. Colby, J. Schanzer, P.D. Van Poelje, J. Am. Chem. Soc. 126 (2004) 5154–5163.
[26] R.L. Mackman, L. Zhang, V. Prasad, C.G. Boojamra, J. Douglas, D. Grant, H. Hui, C.U. Kim, G. Laflamme, J. Parrish, A.D. Stoycheva, S. Swaminathan, K. Wang, T. Cihlar, Bioorg. Med. Chem. 15 (2007) 5519–5528.
[27] K. Barral, S. Priet, J. Sire, J. Neyts, J. Balzarini, B. Canard, K. Alvarez, J. Med. Chem. 49 (2006) 7799–7806.
[28] K. Barral, C. Weck, N. Payrot, L. Roux, C. Durafour, F. Zoulim, J. Neyts, J. Balzarini, B. Canard, S. Priet, K. Alvarez, Eur. J. Med. Chem. 46 (2011) 4281–4288.
[29] B. Canard, K. Alvarez, K. Barral, J.L. Romette, J. Neyts, J. Balzarini, PCT/IB2007/004233, 2008.
[30] X. Kui, W. Wei, L. Jingxing, 200910116015, 2009.
[31] T. Calogeropoulou, A. Detsi, E. Lekkas, M. Koufaki, Curr. Top. Med. Chem. 3 (2003) 1467–1495.
[32] A.S. Ray, K.Y. Hostetler, Antiviral Res. 92 (2011) 277–291.
[33] R.V. Srinivas, B.L. Robbins, M.C. Connelly, Y.F. Gong, N. Bischofberger, A. Fridland, Antimicrob. Agents Chemother. 37 (1993) 2247–2250.
[34] L. Nassens, J. Balzarini, E. De Clercq, Med. Virol. 4 (1994) 147–159.
[35] G.H. Hakimelahi, T.W. Ly, A.A. Moosavi-Movahedi, M.L. Jain, M. Zakerinia, H. Davari, H.-C. Mei, T. Sambaiah, A.A. Moshfegh, S. Hakimelahi, J. Med. Chem. 44 (2001) 3710–3720.
[36] P. Pospisil, B.D. Pilger, S. Marveggio, P. Schelling, C. Wurth, L. Scapozza, G. Folkers, Helv. Chim. Acta 85 (2002) 3237–3250.
[37] L.M. Schultze, H.H. Chapman, R.J.J. Dubree, K.M. Kent, T.T. Lee, M.S. Louie, M.J. Postich, E.J. Prisbe, J.C. Rohloff, R.H. Yu, Tetrahedron Lett. 39 (1998) 1853–1856.
[38] A. Holý, H. Dvorakova, M. Masojidkova, Collec. Czech. Chem. Commun. 60 (1995) 1390–1409.
[39] P.R. Jenkins, Organometallic Reagents in Synthesis, Oxford University Press, Oxford, 1994.
[40] A.L. Jeffery, J.-H. Kim, D.F. Wiemer, Tetrahedron 56 (2000) 5077–5083.
[41] M.W. Becker, H.H. Chapman, T. Cihlar, E.J. Eisenberg, G.-X. He, M.R. Kernan, W.A. Lee, E.J. Prisbe, J.C. Rohloff, M.L. Sparacino, WO/0208241A32002, 2002.
[42] S.R. Houghton, J. Melton, J. Fortunak, B.R.D. H, C.N. Boddy, Tetrahedron 66 (2010) 8137–8144.
[43] D.H.B. Ripin, D. Teager, J. Fortunak, K. Basha, N. Bivins, C.N. Boddy, S. Byrn, K. Catlin, S.R. Houghton, S.T. Jagadeesh, K.A. Kumar, J. Melton, K. Muneer, L.N. Rao, R.V. Rao, N.G. Reddy, R.M. Reddy, K.C. Shekar, T. Silverton, D.T. Smith, R. Stringham, F. Talley, A. Williams, Org. Process. Res. Dev. 14 (2010) 1194–1201.
[44] S.R. Piettre, Tetrahedron Lett. 37 (1996) 4707–4710.
[45] S.R. Piettre, P. Raboisson, Tetrahedron Lett. 37 (1996) 2229–2232.
[46] H. Lu, C.E. Berkman, Bioorg. Med. Chem. 9 (2001) 395–402.
[47] C. Lopin, G. Gouhier, S.R. Piettre, Tetrahedron Lett. 44 (2003) 8837–8840.
[48] B. Boerecka, J. Chojnowski, M. Cyprvk, J. Michalski, J. Zielinska, J. Organomet. Chem. 171 (1979) 17–34.
[49] T. Yokomatzu, H. Takechi, T. Murano, S. Shibuya, J. Org. Chem. 65 (2000) 5858–5861.
[50] J.D. Thomas, K.B. Sloan, Tetrahedron Lett. 48 (2007) 109–112.
[51] M.N. Arimilli, C.U. Kim, J. Dougherty, A.S. Mulato, R. Oliyai, J.P. Shaw, K.C. Cundy, N. Bischofberger, Antiviral Chem. Chemother. 8 (1997) 557–564.
[52] L.-C. Yuan, T.C. Dalh, R. Oliyai, Pharm. Res. 18 (2001) 234–237.
[53] J. Zemlicka, Biochim. Biophys. Acta 1587 (2002) 276–286.
[54] C. McGuigan, K.G. Devine, T.J. O'Connor, D. Kinchington, Antiviral. Res. 15 (1991) 255–263.
[55] D. Farquhar, R. Chen, S. Khan, J. Med. Chem. 38 (1995) 488–495.
[56] F. Puech, G. Gosselin, I. Lefebvre, A. Pompon, A.-M. Aubertin, A. Kirn, J.-L. Imbac, Antiviral. Res. 22 (1993) 155–174.
[57] R.S.H. Yang, E. Hodgson, W.C. Dauterman, J. Agric. Food Chem. 19 (1971) 10–13.
[58] C.B. Reese, H. Yan, Tetrahedron Lett. 44 (2003) 2501–2504.
[59] M. Ozga, R. Dolot, M. Janicka, R. Kaczmarek, A. Krakowiak, J. Biol. Chem. 285 (2010) 40809–40818.
[60] A.L. Horwich, K. Furtak, J. Pugh, J. Summers, J. Virol. 62 (1990) 642–650.
[61] I. Hynie, M. M, W.J. Poznanski, Clin. Chem. 21 (1975) 1383–1387.
[62] W.E. Razzell, H.G. Khorana, J. Biol. Chem. 234 (1959) 2105–2113.