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Laboratory note

Synthesis and antiviral activity of boranophosphonate isosteres of AZT and d4T monophosphates

Karine Barral [a], Stéphane Priet [a], Céline De Michelis [a], Joséphine Sire [b], Johan Neyts [c], Jan Balzarini [c], Bruno Canard [a], Karine Alvarez [a,*]

[a] Laboratoire d'Architecture et Fonction des Macromolécules Biologiques, UMR CNRS 6098, Equipe "Réplicases Virales: Structure, Mécanisme, et Drug-design", Universités Aix-Marseille I et II, Parc scientifique de Luminy, 163 av. de Luminy, Case 932, 13288 Marseille Cedex 9, France
[b] Unité des virus émergents, Faculté de Médecine, Marseille, France
[c] Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

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ABSTRACT

We report synthesis, in vitro antiviral activity, and stability studies in biological media of original boranophosphonate isosteres of AZT and d4T monophophates. A convenient route for the synthesis of 3′-Azido-3′-deoxythymidine-5′-boranophosphonate **8** and 2′,3′-Didehydro-3′-dideoxythymidine-5′-boranophosphonate **12** is described. H-phosphinates **7** and **11**, and α-boranophosphonates **8** and **12** exhibited no significant in vitro activity against HIV-infected cells, neither against a broad panel of viruses, up to 200 μM. The absence of activity of target compounds **8** and **12** can be partially explained by their short half-life in culture medium.

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1. Introduction

Nucleoside analogues like 3′-Azido-3′-deoxythymidine (AZT, zidovudine) and 2′,3′-Didehydro-3′-dideoxythymidine (d4T, stavudine) have been extensively used as antiviral drugs targeting HIV reverse transcriptase (RT) [1–3]. These nucleoside analogues need to be phosphorylated to its active triphosphate form in order to compete for DNA incorporation with the natural counterpart [4,5]. Nevertheless, under therapeutic pressure, the viral RT gene mutates and specifies enzymes bearing substitutions for the loss of efficacy of these nucleoside analogues [6,7].

One strategy to circumvent this problem is to develop nucleoside analogues that are still potent against mutant RTs. Nucleoside 5′-(α-P-borano)triphosphates are nucleotide analogues in which a borane (BH_3^-) group substitutes one nonbridging α-phosphate in nucleoside 5′-triphosphate. The borano modification acts as a general suppressor of resistance, therefore BH_3-nucleotide analogues such as α-BH_3-AZTTP and α-BH_3-d4TTP are HIV-1 RT inhibitors and have the capability to overcome resistance recovering sensitivity of the mutant RTs to these nucleoside analogue inhibitors [8–11]. However, the biologically active α-BH_3-AZTTP and α-BH_3-d4TTP cannot be administered orally, and a well-known problem in antiviral therapy is the poor bioavailability of nucleoside monophosphates (such as AZTMP and d4TMP) that are readily dephosphorylated in extracellular fluids [12,13]. Therefore, we expected a likely low stability of α-BH_3-AZTMP and α-BH_3-d4TMP (monophosphate form) in culture medium. Based on this knowledge, we decided to reinforce the bond stability between the 5′ nucleoside and the α-boranophosphate moiety by introducing a phosphonate modification, which shows advantages in terms of a long intracellular half-life. Indeed, there is considerable interest in phosphonates as biologically active mimics of natural phosphate. Phosphonate analogues [14] have been extensively studied as potent antiviral agents and present the advantage over their phosphate counterparts to be metabolically stable, as the phosphorus–carbon bond is not susceptible to phosphatase hydrolysis. Moreover, we recently demonstrated that α-BH_3-mediated suppression of HIV-1 RT resistance, formerly described with nucleoside 5′-(α-P-borano)triphosphate analogues, is also conserved at the phosphonate level, e.g. with nucleoside α-P-boranophosphonate diphosphate analogues [15].

Because of the advantages provided by the presence of an α-P-borane group into nucleoside triphosphates and by a 5′-phosphonate mimic modification, we have designed chimeric α-boranophosphonate

* Corresponding author. Tel.: +33 491 825 571; fax: +33 491 266 720.
E-mail address: Karine.Alvarez@afmb.univ-mrs.fr (K. Alvarez).

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nucleoside analogues, in order to obtain "synergy" between « α-P-boranо » derivative properties and « phosphonate » derivative properties. We explored the synthesis of 3′-azido-3′-deoxythymidine-5′-boranophosphonate (AZT analogue) 8 and 2′,3′-didehydro-3′-deoxythymidine-5′-boranophosphonate (d4T analogue) 12 in which one of the nonbridging oxygen atoms of the α-phosphonate group is replaced by a borane BH_3 group (Fig. 1). These compounds have not been described so far in the chemistry of nucleic acid analogs and they have not been evaluated in vitro against a panel of viruses.

2. Results and discussion

2.1. Chemistry

The methods initially described for the introduction of a BH_3 group on nucleoside monophosphates *via* phosphoramidite [16] or H-phosphonate [17] approaches are not suitable for the synthesis of α-boranophosphonate derivatives. An efficient procedure *via* the boronation of an activated H-phosphinate intermediate was described [18] and applied for the preparation of the target compounds **8** and **12**.

Treatment of β-thymidine **1** with methanesulfonyl chloride in anhydrous pyridine at 0 °C lead to 3′,5′-di-*O*-mesylthymidine [19] (Scheme 1). When this dimesyl derivative was heated at 55 °C with an 20% aqueous sodium hydroxide solution, the anhydronucleoside **2** [20] was isolated in 71% yield [21]. In our strategy the phosphonate group is introduced by a route originally published by Tanaka et al. [22,23] which involves the nucleophilic ring opening of the oxetane with an excess of diethylmethylphosphonate and the inversion of the 3′-center (*threo* furanoside instead of *erythro* furanoside in thymidine) [24], leading to phosphinate **3**, quantitatively.

The alcohol function of compound **3** was treated with methanesulfonyl chloride in anhydrous pyridine at 0 °C [19] to yield compound **4**, which is the starting point for divergent syntheses of α-boranophosphonate AZT and d4T analogues.

In order to obtain α-boranophosphonate AZT (Scheme 2), the simplest approach consisted in reduction of diethyl ester phosphonate by an effective method never applied to nucleotide analogues [25–27]. The reduction of the diethyl phosphonate **4** was obtained using lithium aluminium hydride ($LiAlH_4$) in THF mediated by the addition of a stoechiometric quantity of chlorotrimethylsilane (TMSCl) afforded phosphine **5** in 68% yield. This phosphine was oxidized with a 35% hydrogen peroxide in a mixture water/THF to result in the H-phosphinate **6** in quantitative yield [28,29]. Then, SN_2 displacement on C3′ of the mesyl group of **6** by azide, derived from sodium azide in dry dimethylformamide, afforded the AZT analogue **7** in 51% [19,30,31].

Because of the phosphorus atom in the H-phosphinate lacks a free electron pair and is not a suitable donor for the BH_3 group, the boronation procedure requires an intermediate activation of the H-phosphinate to a disilyl phosphonite and was readily achieved *in situ* with a silylating agent [32]. H-phosphinate **7** in anhydrous THF

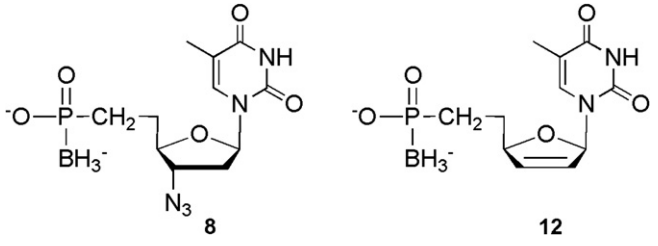


Fig. 1. Nucleoside α-boranophosphonate analogues of AZT **8** and d4T **12**.

and *N*,*O*-Bis(trimethylsilyl)acetamide (BSA) was activated in four hours into the corresponding disilyl phosphonite intermediate. To obtain compound **8**, we screened several borane complexes in different solvents to optimize boronation conditions. Quick and relative efficient boronation could be achieved with 10 equiv of diisopropylethylamine–borane (BH_3·DIPEA) complex. In these conditions, the disilyl phosphonite from **7** was converted in a di(trimethylsilyl)-α-boranophosphonate intermediate [33,34]. Without isolation, the *in situ* product obtained was treated with a mixture of water and methanol (v/v:1/1) to remove the trimethylsilyl group and gave the α-boranophosphonate **8**. The presence of the P–B bond was confirmed by ^{31}P NMR spectra [35], which showed a typical broad peak at 103 ppm [18]. The pure α-boranophosphonate **8** was then isolated by reverse phase chromatography in 20% yield.

Similarly, to obtain targeted compound **12** derived from d4T, we tried to eliminate the 3′-mesyl group of compound **5** in order to obtain the 2′,3′-didehydrohexofuranose phosphine form **10**. Unfortunately, traditional elimination conditions using potassium *tert*-butoxide in dry dimethylformamide [36] led to the degradation of compound **5** into several side-products. A similar degradation was observed when we applied these elimination conditions to compound **6**. Thus, compound **4** was used as a key synthon for the preparation of the target d4T analogue.

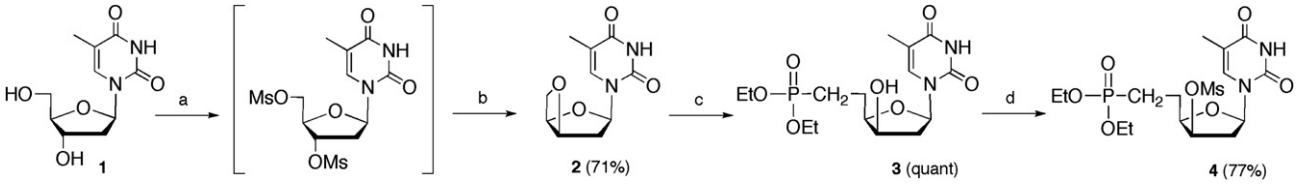
Elimination of the 3′-mesyl group of compound **4**, by treatment with potassium *tert*-butoxide in dry dimethylformamide [36], gave the d4T analogue **9** in good yield (Scheme 2). The same conditions of reduction with lithium aluminium hydride as described above were used to obtain compound **10**. The low yield (36%) of this reaction is due to the reduction of the sugar double bond. In an attempt to minimize the formation of this side-product, we reduced as much as possible (6 equivalents) the amount of $LiAlH_4$ and TMSCl to manage the best ratio between the consumption of the starting material and the appearance of the reduced product. Oxidation of the phosphine function of compound **10** with hydrogen peroxide provided H-phosphinate **11** in quantitative yield. The same boronation step as described for compound **8** was used in order to obtain the target α-boranophosphonate d4T **12** with 21% yield. The boronation of the compounds **7** and **11** is limiting because of the concomitant oxidation of the H-phosphinate function into the equivalent phosphonate function. No H-phosphinate starting material was detected after the reaction.

2.2. Antiviral activity assays

The target compounds **7**, **8**, **11** and **12** were screened for antiviral activity against HIV-1 in MAGI-CCR5 cells and HIV-1(III_B) and HIV-2(ROD) in CEM cell cultures. None of the compounds exhibited any activity and no toxicity at concentrations as high as 400 μM. Compounds **7**, **8**, **11** and **12** were also screened for antiviral activity against other viruses:hepatitis C virus (subgenomic replicon replication in Huh-5-2 cells); herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), vaccinia virus and vesicular stomatitis virus in E_6SM cells; coxsackie B4 virus and respiratory syncytial virus in Hela cells and para-influenza-3 virus, reovirus-1, sindbis virus and punta toro virus in Vero cells. No significant cytotoxicities were reported for any of the compounds up to 200 μM. None of these compounds exhibited any in vitro activity up to 200 μM.

2.3. Stability studies

To test the usefulness of 8 and 12, it is necessary to study their chemical and enzymatic stabilities. In order to measure the relative chemical and enzymatic stabilities of the compounds, kinetic data and decomposition pathways for compounds 7, 8, 11 and 12 were studied at 37 °C (a) in a pH buffer range of 1.2–11.5 (b) in culture

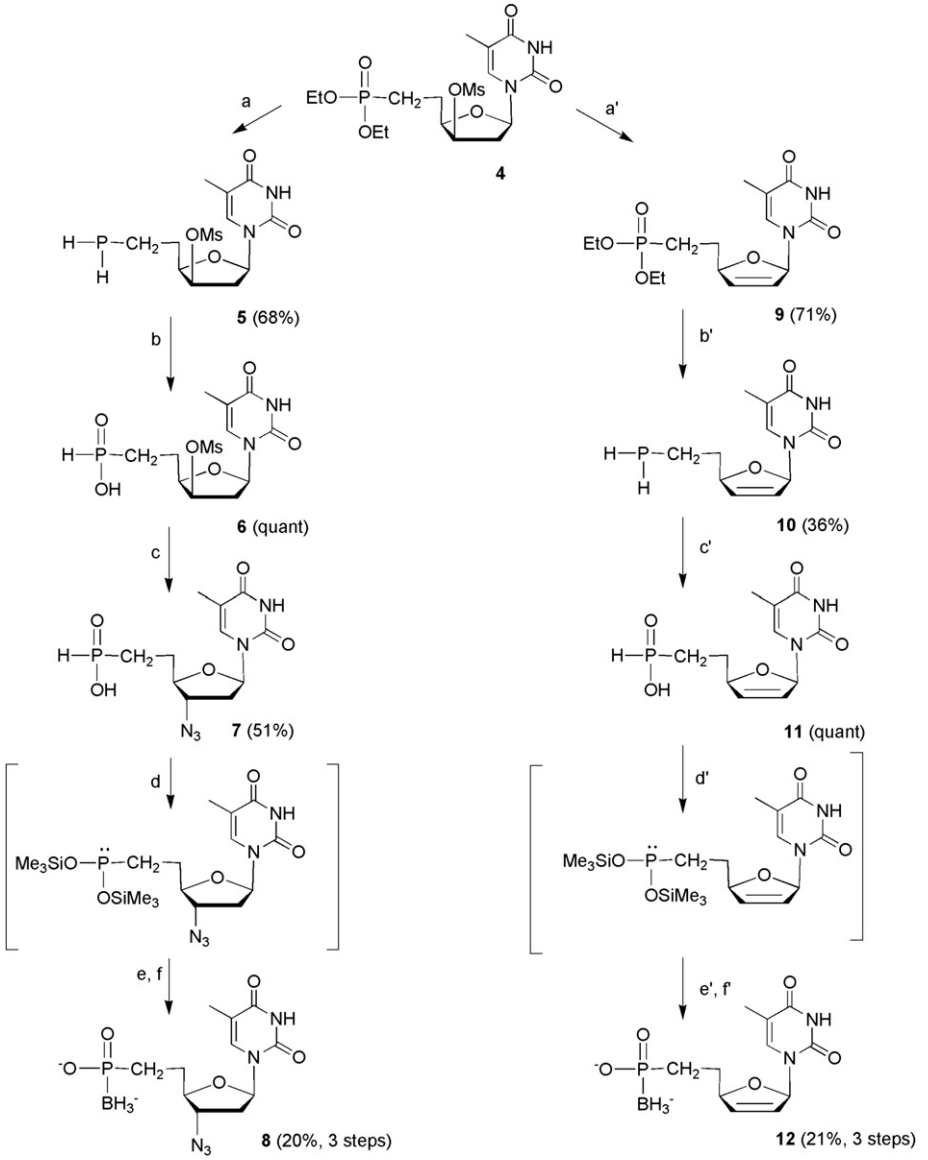


Scheme 1. Reagents and conditions: (a) $MeSO_2Cl$, pyridine, 0 °C then rt; (b) 20% NaOH aq., 55 °C; (c) $MePO(OEt)_2$, BuLi, $BF_3.Et_2O$, THF, −78 °C; (d) $MeSO_2Cl$, pyridine, 0 °C then rt.

medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum) (c) in total cell extracts (CEM-SS). These various test conditions were thought to be useful and relevant in vitro models for the different types of degradation that may affect the compounds *in vivo*. The culture medium is correlated with an enzymatic-nucleophile-enriched medium (extracellular medium mimic) and the conditions of total cell extracts (CEM-SS) are used to mimic the intracellular environment.

Crude aliquots of incubates were directly analyzed using an on-line HPLC cleaning method [37]. Half-lives were calculated and the results are summarized in Table 1.

In all tested media, compounds bearing the d4T scaffold **11** and **12** are less stable than compounds bearing the AZT scaffold **7** and **8**. Compound **7** is stable in all tested conditions for more than 3 days, while compound **8** was only stable in all three buffers. In total cell extract, α-boranophosphonate **8** is slowly hydrolyzed with a half-life value higher than 48 h. However, its half-life was approximately 20 times shorter in cell culture medium. Compound **8** decomposes into a single product, which is assigned to the H-phosphinate **7** by co-injection with an authentic sample. This bio-conversion is mediated by an ezymatic-nucleophile-enriched activity, responsible of the reduction of the P–B bond into a P–H bond. The



Scheme 2. Reagents and conditions: (a) $LiAlH_4$, TMSCl, −78 °C then rt; (b) H_2O_2, H_2O/THF, rt; (c) NaN_3, DMF, 65 °C; (d) BSA, THF, rt (e) BH_3.DIPEA, rt; (f) H_2O/MeOH (1/1). (a') *t*BuOK, DMF, rt; (b') $LiAlH_4$, TMSCl, −78 °C then rt; (c') H_2O_2, H_2O/THF, rt; (d') BSA, THF, rt (e') BH_3.DIPEA, rt; (f') H_2O/MeOH (1/1).

Table 1
Calculated half-lives of the derivatives 7, 8, 11 and 12 in several chemical and enzymatic media.

Compounds	$t_{1/2}$ buffer pH = 1.2	$t_{1/2}$ buffer pH = 7.3	$t_{1/2}$ buffer pH = 11.5	$t_{1/2}$ culture medium	$t_{1/2}$ CEM cell extracts
7	stable[a]	stable[a]	stable[a]	stable[a]	stable[a]
8	stable[a,b]	stable[a,b]	stable[a,b]	3.1 h[b]	>48h[b]
11	28 h[c]	>48 h[c]	>48 h[c]	11 h[c]	2 h[e]
12	16 h[c]	22 h[c]	>48 h[c]	1.6 h[d]	3 h[f]

$t_{1/2}$: half-life of the decomposition of nucleotides at a concentration of 0.1 mM and 37 °C in different media.
[a] Less than 20% degradation after 3 days.
[b] Single product of degradation = **7**.
[c] Single product of degradation = cleavage of the glycosidic bond and thymine release.
[d] Formation of product **11**, which, in turn, decomposes into thymine by glycosidic bond cleavage.
[e] Single product of degradation = cleavage of the glycosidic bond and thymine release.
[f] Formation of product **11**, which, in turn, decomposes into thymine.

difference of behaviour observed in culture medium ($t_{1/2} = 3.1$ h) and cell extracts ($t_{1/2} > 48$ h) might be most likely explained by the difference in enzyme content of both media (culture medium contains 10% foetal calf serum).

Compounds **11** and **12** slowly hydrolyse in the buffers. Their chemical stability appears to be pH dependent. Indeed, compounds **11** and **12** exhibit a increased stability in alkaline pH. Thus, compound **11** is about 3-fold more stable at pH 11.5 than at pH 1.2 (consistent with the acid lability of the d4T analogue [38]).

In cell culture medium, compound **11** is relatively stable ($t_{1/2} = 11$ h) while compound **12** quickly decomposes at a $t_{1/2}$ of 1.6 h. The decomposition pathways of compounds **11** and **12** are different and vary according to the medium used. Indeed, the sole decomposition pathway of compound **11**, in all media, results from the cleavage of the glycosidic bond which leads to thymine release, as identified by co-injection with an authentic sample. The decomposition pathway of compound **12** in enzymatic media goes through two steps: first, the quick reduction of the P–B bond into the P–H bond leading to the formation of compound **11**, which, then, decomposes more slowly into thymine by glycosidic bond cleavage. In contrast, in the buffers, compound **12** decomposes directly into thymine.

In all tested media, we didn't observe hydrolysis of the α-boranophosphonate group by nucleophilic substitution on the phosphorus atom, yielding a phosphonate formation.

3. Conclusion

To develop novel antiviral agents enable to circumvent HIV-1 RT-mediated drug resistance, we have synthesized AZT and d4T α-boranophosphonate analogues **8** and **12**, in which one of the non-bridging oxygen atoms of the 5′-phosphonate group is replaced by a borane BH_3 group. Target compounds were investigated for their antiviral activity in vitro against HIV and other DNA and RNA viruses. No significant cytotoxicity was observed for any of the compounds up to 200 μM. Compounds **7**, **8**, **11** and **12** did not exhibited any in vitro activity up to 200 μM against all tested viruses. The absence of activity of target compounds **8** and **12** might be due, in part, to their fast decomposition in cell extract and culture medium. It would be interesting to synthesis the diphosphate form of the derivatives **8** and **12** in order to evaluate their incorporation efficiency by HIV-1 wild-type and mutant RTs, and their capacity to circumvent drug resistance.

4. Experimental

4.1. Chemistry

All air-sensitive reactions were performed in oven-dried glassware under argon using extra-dry solvents purchased by Aldrich or Acros. All starting materials were purchased from Aldrich, Acros and Lancaster societies without further treatment. The ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were determined with a BRUKER AMX 250 MHz. Chemical shifts are expressed in ppm and coupling constants (J) are in hertz (s = singlet, d = doublet, dd = double doublet, t = triplet, dt = double triplet, td = triple doublet, bq = broad quadruplet, m = multiplet, dm = double multiplet). FAB Mass Spectra (MS) and High Resolution Mass Spectra (HRMS) were obtained on a JEOL SX 102 mass spectrometer (Laboratoire de Mesures Physiques RMN, USTL, Montpellier, France) using a cesium ion source and a glycerol/thioglycerol (GT) matrix. Electrospray ionization (ESI) High Resolution Mass Spectrometry was performed using a Micromass Q-TOF in the negative-ion mode with a spray voltage at −3 kV. The capillary temperature was maintained at 120 °C. Preparative flash column chromatographies were performed using silica gel (Merck) G60 230–240 mesh. Analytical thin layer chromatographies were performed on silica gel 60F 254 aluminium plates (Merck) of 0.2 mm thickness. The spots were examined with UV light and Cericdip Sray. HPLC was performed on a Waters 600E controller system equipped with a 996-photodiode array detector (detection 260 nm). Samples were eluted at a flow rate of 1 mL/min using a linear gradient 0–100% B in 60 min. Reverse phase (C18) chromatography was carried out on an X-Terra analytic column. Purification of H-phosphinate and α-boranophosphonate derivatives was achieved on an X-Terra semi-preparative column using a linear gradient 0–100% B. Eluant A: 0.05 M triethylammonium bicarbonate buffer (TEAB, pH 7.5); eluant B: solution A containing 50% of acetonitrile.

4.1.1. 1-(2′-Deoxy-3′,5′-epoxy-β-D-threo-pentofuranosyl)thymine (**2**)

To a stirred solution of β-thymidine **1** (4 g, 16.52 mmol) in dry pyridine (20 mL), methanesulfonyl chloride (2.8 mL, 36.32 mmol) was slowly added at 0 °C. The temperature was maintained at 0 °C during 2 h then brought to room temperature for one night. Pyridine was co-evaporated with toluene (20 mL). The residue was pre-purified by flash chromatography (CH_2Cl_2/MeOH: 93/7). The almost pure 3′,5′-dimesylthymidine was dissolved in a 20% aqueous sodium hydroxide solution (90 mL) and the solution was heated to 55 °C for 6 h. After completion of the reaction it was cooled to 0 °C and neutralized with a 32% hydrochloric acid solution. Water was evaporated under vacuo. Absolute ethanol (30 mL) was added and the solvent was evaporated under vacuo. The crude compound was purified by flash chromatography (CH_2Cl_2/MeOH 95:5) to afford 3′,5′-anhydrothymidine **2** (2.64 g, 71%) as a white powder. mp: 189–190 °C; litt [21] 188–190 °C.

^{1}H NMR (DMSO-d_6) δ: 11.35 (s, 1H), 8.03 (d, 1H, J = 1.2 Hz), 6.51 (t, 1H, J = 5.5 Hz), 5.49 (m, 1H), 4.90 (m, 1H), 4.70 (dd, 1H, J = 4.8, 8.2 Hz), 4.02 (dd, 1H, J = 1.5, 8.2 Hz), 2.50 (m, 2H), 1.79 (d, 3H, J = 1.2 Hz). ^{13}C NMR (DMSO-d_6) δ: 163.69, 151.14, 136.64, 109.61, 88.30, 86.89, 79.86, 75.17, 37.14, 12.40. HRMS (FAB) cald for $C_{10}H_{13}N_2O_4$ $(M+H)^+$ 225.2242, found 225.0875.

4.1.2. 1-[(3′R, 4′R)-2′,5′,6′-trideoxy-6′-(diethoxyphosphonyl)-β-hexofuranosyl]thymine (**3**)

Diethyl methylphosphonate (1.26 g, 8.26 mmol) was dissolved in freshly distilled THF (16.5 mL) and cooled to −78 °C. BuLi (2.5 M in hexanes, 3.3 mL, 8.26 mmol) was added dropwise over 10 min. The resulting solution was stirred at −78 °C for 90 min $BF_3 \cdot Et_2O$ (1 mL, 8.26 mmol) was added dropwise over 5 min. The solution was stirred for another 5 min. A solution of 3′,5′-anhydrothymidine **2**

(370 mg, 1.65 mmol) in dry THF (16.5 mL) was slowly added over 45 min. After 2 h at −78 °C, the reaction was quenched with saturated aqueous $NaHCO_3$ solution (1 mL) and solid $NaHCO_3$ (329 mg). The white suspension was allowed to warm to room temperature overnight. The solvent was evaporated, the resulting slimy residue resuspended in CH_2Cl_2 (60 mL), and the mixture filtered through Celite. The white residue was twice removed from the top of the Celite, washed with additional CH_2Cl_2 (40 mL), and refiltered. The combined organic layers were concentrated to dryness and purified by flash chromatography (CH_2Cl_2/MeOH 90:10) to afford compound **3** (662 mg, quant) as a colorless foam.

^{1}H NMR ($CDCl_3$) δ: 8.57 (s, 1H), 7.65 (s, 1H), 6.12 (dd, 1H, $J = 3.2$, 8.5 Hz), 4.31 (m, 1H), 4.05 (m, 4H), 3.71 (m, 1H), 2.55 (m, 1H), 2.05 (m, 1H), 2.00 (m, 2H), 1.83 (s, 3H), 1.70 (m, 2H), 1.27 (td, 6H, $J = 1.8$, 7.0 Hz). ^{13}C NMR ($CDCl_3$) δ: 164.15, 150.77, 137.95, 110.47, 84.76, 84.55, 69.38, 62.15 (d, $J = 6.5$ Hz), 40.85, 22.53 (d, $J_{CP} = 142.2$ Hz), 21.07 (d, $J = 4.5$ Hz), 16.45 (d, $J = 6.1$ Hz), 12.53. ^{31}P NMR ($CDCl_3$) δ: 32.74. HRMS (FAB) cald for $C_{15}H_{26}N_2O_7P$ $(M + H)^+$ 377.3544, found 377.1478.

4.1.3. 1-[(3′S,4′R)-3′-O-mesyl-2′,5′,6′-trideoxy-6′-(diethoxyphosphonyl)-β-hexofuranosyl]thymine (4)

To a stirred solution of compound **3** (438 mg, 1.17 mmol) in dry pyridine (6 mL), methanesulfonyl chloride (108 μL, 1.39 mmol) was slowly added at 0 °C. The temperature was maintained at 0 °C during 1 h. Then the reaction was allowed to warm to room temperature overnight. The solvent was co-evaporated with toluene (10 mL). The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 93:7) to give **4** (405 mg, 77%) as a white foam.

^{1}H NMR ($CDCl_3$) δ: 9.24 (s, 1H), 7.26 (d, 1H, $J = 1.2$ Hz), 6.16 (dd, 1H, $J = 3.2$, 8.5 Hz), 5.12 (dd, 1H, $J = 3.2$, 5.0 Hz), 4.05 (m, 5H), 3.02 (s, 3H), 2.77 (m, 1H), 2.35 (dd, 1H, $J = 3.2$, 16 Hz), 2.05 (m, 2H), 1.88 (d, 3H, $J = 1.2$ Hz), 1.85 (m, 2H), 1.27 (t, 6H, $J = 7.0$ Hz). ^{13}C NMR ($CDCl_3$) δ: 163.83, 150.52, 135.06, 111.25, 83.29, 82.15, 78.62, 61.94 (d, $J = 6.5$ Hz), 39.92, 38.72, 25.21 (d, $J_{CP} = 143.9$ Hz), 24.31 (d, $J = 5.0$ Hz), 16.51 (d, $J = 5.9$ Hz), 12.69. ^{31}P NMR ($CDCl_3$) δ: 30.46. HRMS (FAB) cald for $C_{16}H_{28}N_2O_9PS$ $(M + H)^+$ 455.4461, found 455.1253.

4.1.4. 1-[(3′S,4′R)-3′-O-mesyl-2′,5′,6′-trideoxy-6′-(phosphanyl)-β-hexofuranosyl]thymine (5)

Chlorotrimethylsilane (1.35 mL, 10.20 mmol) was added dropwise to a stirred solution of $LiAlH_4$ (443 mg, 10.20 mmol) in dry THF (8 mL) at −78 °C. The resulting mixture was allowed to warm to room temperature and stirred for 2 h. Compound **4** (800 mg, 1.70 mmol) in dry THF (8 mL) was added to the reducing mixture at −70 °C. The mixture was allowed to warm to room temperature and stirred for 1 h. The reaction was stopped by slow addition of H_2O (2 mL) at −70 °C. The mixture was allowed to warm to room temperature and filtered through Celite. The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. A purification by flash chromatography (CH_2Cl_2/MeOH 95:5) yielded **5** (402 mg, 68%) as a white foam.

^{1}H NMR ($CDCl_3$) δ: 9.34 (s, 1H), 7.26 (d, 1H, $J = 1.2$ Hz), 6.15 (dd, 1H, $J = 3.2$, 8.5 Hz), 5.09 (dd, 1H, $J = 3.2$, 5.0 Hz), 3.97 (m, 1H), 3.10 and 2.34 (dm, 2H, $J_{PH} = 190.0$ Hz), 2.99 (s, 3H), 2.77 (m, 1H), 2.28 (m, 1H), 2.02 (m, 1H), 1.89 (d, 3H, $J = 1.2$ Hz), 1.81 (m, 1H), 1.58 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 163.80, 150.56, 135.14, 111.19, 83.37, 82.69, 79.09, 39.95, 38.80, 31.86 (d, $J = 3.4$ Hz), 12.77, 10.36 (d, $J_{CP} = 9.2$ Hz). ^{31}P NMR ($CDCl_3$) δ: −136.52. HRMS (FAB) cald for $C_{12}H_{20}N_2O_6PS$ $(M + H)^+$ 351.3404, found 351.0780.

4.1.5. 1-[(3′S,4′R)-3′-O-mesyl-2′,5′,6′-trideoxy-6′-(hydroxyphosphinyl)-β-hexofuranosyl]thymine (6)

To a stirred solution of phosphine **5** (112 mg, 0.32 mmol) in water (1 mL) and THF (1 mL) was added dropwise 35% aqueous hydrogen peroxide (62 μL). The mixture was stirred at room temperature for 2 h then solvents were evaporated on vacuo to afford pure H-phosphinate **6** (120 mg, quant) as a white solid; HPLC purity >98%.

^{1}H NMR (D_2O) δ: 8.03 and 5.81 (dm, 2H, $J_{PH} = 555.0$ Hz), 7.45 (s, 1H), 6.05 (dd, 1H, $J = 2.9$, 8.0 Hz), 5.26 (dd, 1H, $J = 3.2$, 4.9 Hz), 4.17 (m, 1H), 3.17 (m, 1H), 3.12 (s, 3H), 2.80 (m, 1H), 2.41 (m, 1H), 2.10 (m, 1H), 2.03 (s, 3H), 1.79 (m, 2H). ^{13}C NMR (D_2O) δ: 165.73, 150.70, 136.27, 110.12, 83.90, 82.52, 79.40, 38.36, 36.98, 25.85 (d, $J_{CP} = 93.3$ Hz), 19.40 (d, $J = 2.0$ Hz), 10.90. ^{31}P NMR (D_2O) δ: 34.79. HRMS (FAB) cald for $C_{12}H_{20}N_2O_8PS$ $(M + H)^+$ 383.3392, found 383.0678.

4.1.6. 1-[(3′R,4′R)-3′-Azido-2′,3′,5′,6′-tetradeoxy-6′-(hydroxyphosphinyl)-β-hexofuranosyl]thymine (7)

Sodium azide (256 mg, 3.94 mmol) was added to a solution of the H-phosphinate **6** (188 mg, 0.49 mmol) in dry DMF (4 mL). The reaction was stirred for 24 h at 60 °C, then water (2 mL) was added and the solvent was evaporated under vacum. The crude residue was purified by HPLC with an X-Terra semi-preparative reversed-phase column (linear gradient 0–100% B). Product fractions were collected and evaporated to dryness. Excess triethylammonium bicarbonate was removed by repeated freeze-drying with deionized water to give **7** (83.2 mg, 51%) as a white powder. HPLC purity >97%

^{1}H NMR (D_2O) δ: 8.07and 5.92 (dm, 2H, $J_{PH} = 537.0$ Hz), 7.50 (s, 1H), 6.23 (t, 1H, $J = 6.7$ Hz), 4.31 (dd, 1H, $J = 5.6$, 12.0 Hz), 4.08 (dd, 1H, $J = 5.6$, 12.0 Hz), 2.56 (m, 2H), 1.98 (m, 2H), 1.93 (s, 3H), 1.72 (m, 2H). ^{13}C NMR (D_2O) δ: 167.50, 152.51, 137.17, 111.96, 84.73, 84.21, 83.94, 35.43, 26.80 (d, $J_{CP} = 92.9$ Hz), 25.49 (d, $J = 2.1$ Hz), 11.74. ^{31}P NMR (D_2O) δ: 29.49. HRMS (FAB) cald for $C_{11}H_{16}N_5O_5P$ $(M)^-$ 328.2447, found 328.0918.

4.1.7. 1-[(3′R,4′R)-3′-Azido-2′,3′,5′,6′-tetradeoxy-6′-(boranophosphono)-β-hexofuranosyl]thymine (8)

Compound **7** (50 mg, 0.14 mmol) was dried over P_2O_5 under vacuum for 4–5 h then dissolved in dry THF (15 mL). BSA (195 μL, 0.76 mmol) was added by syringe and the solution was stirred for about 1 h at room temperature. DIPEA·BH_3 (264 μL, 1.5 mmol) was added, and the solution stirred for 4 h, then a mixture H_2O–MeOH (1:1, 12 mL) was added. After the solvents were evaporated under reduce pressure, the residue was purified by reversed-phase column chromatography (linear gradient 0–100% B). Product fractions were collected and evaporated to dryness. Excess of triethylammonium bicarbonate was removed by repeated freeze-drying with deionized water to give compound **8** (10 mg, 20%) as a white powder. HPLC purity >98%.

^{1}H NMR (D_2O) δ: 7.32 (s, 1H), 6.04 (t, 1H, $J = 6.5$ Hz), 4.08 (dd, 1H, $J = 5.5$, 11.2 Hz), 3.83 (dd, 1H, $J = 5.6$, 11.0 Hz), 2.32 (t, 2H, $J = 6.5$ Hz), 1.75 (m, 5H), 1.42 (m, 2H), 0.5 (bq, 3H, $J = 82$ Hz). ^{31}P NMR (D_2O) δ: 103.54. HRMS (ESI) cald for $C_{11}H_{18}N_5O_5PB$ $(M)^-$ 342.1139, found 342.1123.

4.1.8. 1-[(4′R)-2′,3′-Didehydro-2′,3′,5′,6′-tetradeoxy-6′-(diethoxyphosphonyl)-β-hexofuranosyl]thymine (9)

Potassium *tert*-butoxide (185 mg, 1.65 mmol) was added to a solution of compound **4** (300 mg, 0.66 mmol) in dry DMF (4 mL). The reaction was stirred at room temperature for 2.5 h, then the solvent was evaporated on vacuo. The crude residue was purified by flash chromatography (CH_2Cl_2/MeOH 95:5) to afford **9** (167 mg, 71%) as a white foam.

^{1}H NMR ($CDCl_3$) δ: 9.00 (s, 1H), 6.90 (m, 2H), 6.28 (dt, 1H, $J = 1.5$, 5.8 Hz), 5.76 (dt, 1H, $J = 5.8$, 1.5 Hz), 4.77 (m, 1H), 4.03 (m, 4H), 2.02 (m, 2H), 1.84 (d 3H), 1.75 (m, 2H), 1.25 (td, 6H, $J = 1.5$, 6.7 Hz). ^{13}C NMR ($CDCl_3$) δ: 163.65, 150.82, 136.38, 135.06, 125.53, 111.42, 89.77, 85.91, 61.81 (d, $J = 6.5$ Hz), 28.87 (d, $J = 4.6$ Hz), 23.14 (d, $J_{CP} = 142.0$ Hz), 16.49 (d, $J = 6.0$ Hz), 12.53. ^{31}P NMR ($CDCl_3$) δ: 30.76.

HRMS (FAB) cald for $C_{15}H_{24}N_2OP$ $(M+H)^+$ 359.3391, found 359.1372.

4.1.9. 1-[(4′R)-2′,3′-Didehydro-2′,3′,5′,6′-tetradeoxy-6′-(phosphanyl)-β-hexofuranosyl]thymine (**10**)

Chlorotrimethylsilane (794 μL, 6.21 mmol) was added dropwise to a stirred solution of $LiAlH_4$ (260 mg, 6.21 mmol) in dry THF (5 mL) at −78 °C. The resulting mixture was allowed to warm to room temperature and stirred for 2 h. Compound **9** (370 mg, 1.04 mmol) in dry THF (6 mL) was added to the reducing mixture at −70 °C. The mixture was allowed to warm to room temperature and stirred for 1 h. The reaction was stopped by slow addition of MeOH (5 mL) at 0 °C. The mixture was allowed to warm to room temperature and filtered through Celite. The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. A purification by flash chromatography (CH_2Cl_2/MeOH 98:2) yielded **10** (94 mg, 36%) as a white foam.

^{1}H NMR ($CDCl_3$) δ: 9.29 (s, 1H), 6.92 (m, 2H), 6.26 (dt, 1H, $J = 1.5$, 6.0 Hz), 5.73 (dm 1H, $J = 6.0$ Hz), 4.78 (m, 1H), 3.07 and 2.29 (dm, 2H, $J_{PH} = 195.0$ Hz), 1.85 (d, 3H, $J = 1.2$ Hz), 1.79 (m, 2H,), 1.57 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 163.89, 150.96, 136.61, 135.20, 125.21, 111.34, 89.77, 86.25, 38.99 (d, $J = 3.3$ Hz), 12.64, 10.07 (d, $J_{CP} = 8.9$ Hz). ^{31}P NMR ($CDCl_3$) δ: −135.93. HRMS (FAB) cald for $C_{11}H_{16}N_2O_3P$ $(M+H)^+$ 255.2334, found 255.0899.

4.1.10. 1-[(4′R)-2′,3′-Didehydro-2′,3′,5′,6′-tetradeoxy-6′-(hydroxyphosphinyl)-β-hexofuranosyl]thymine (**11**)

To a stirred solution of phosphine **10** (84 mg, 0.33 mmol) in water (1 mL) and THF (1 mL) was added dropwise 35% aqueous hydrogen peroxide (64 μL). The mixture was stirred at room temperature for 2 h then solvents were evaporated under vacum to afford pure H-phosphinate **11** (94 mg, quant) as a white solid; HPLC purity >97%.

^{1}H NMR (D_2O) δ: 7.89 and 5.83 (dm, 2H, $J_{PH} = 515.0$ Hz), 7.26 (m, 2H), 6.74 (m, 1H), 6.39 (dt, 1H, $J = 1.5$, 6.2 Hz), 5.78 (m, 1H), 4.83 (m, 1H), 1.88 (m, 4H), 1.73 (s, 3H). ^{13}C NMR (D_2O) δ: 166.52, 152.19, 137.44, 136.84, 123.58, 111.41, 90.17, 86.40, 26.08 (d, $J_{CP} = 91.0$ Hz), 24.63 (d, $J = 2.6$ Hz), 11.28. ^{31}P NMR (D_2O) δ: 35.93. HRMS (FAB) cald for $C_{11}H_{15}N_2O_5P$ $(M)^-$ 285.2164, found 285.0727.

4.1.11. 1-[(4′R)-2′,3′-Didehydro-2′,3′,5′,6′-tetradeoxy-6′-(boranophosphono)-β-hexofuranosyl]thymine (**12**)

Compound 11 (25 mg, 0.09 mmol) was dried over P_2O_5 under vacuum for 4–5 h then dissolved in dry THF (5 mL). BSA (108 μL, 0.43 mmol) was added by syringe and the solution was stirred for about 1 h at room temperature. DIPEA·BH_3 (152 μL, 0.87 mmol) added, and the solution stirred for 5 h, then a mixture H_2O–MeOH (1:1, 12 mL) was added. After the solvents were evaporated under reduce pressure, the residue was purified by reversed-phase column chromatography (linear gradient 0–100% B). Product fractions were collected and evaporated to dryness. Excess triethylammonium bicarbonate was removed by repeated freeze-drying with deionized water to give compound **12** (5 mg, 21%) as a white powder. HPLC purity >97%.

^{1}H NMR (D_2O) δ: 7.26 (s, 1H), 6.73 (d, 1H, $J = 1.2$ Hz), 6.37 (dd, 1H, $J = 1.2$, 6.1 Hz), 5.71 (d, 1H, $J = 6.0$ Hz), 4.80 (m, 1H), 1.71 (s, 3H), 1.69 (m, 2H), 1.43 (m, 2H), 0.70 (bq, 3H, $J = 77$ Hz). ^{31}P NMR (D_2O) δ: 102.4. HRMS (ESI) cald for $C_8H_{16}N_5O_9P_3B$ $(M)^-$ 299.0974, found 299.0965.

4.2. Antiviral assays

4.2.1. Anti-HIV assays in MAGI-CCR5 cells

293T and MAGI-CCR5 cells were maintained in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal bovine serum. To obtain HIV-1 stocks, 293T cells were transiently transfected with an HIV-1NL4.3 molecular clone by the use of FuGENE 6 transfectant reagent (Roche) as recommended by the manufacturer. Two days post transfection, the CAp24 antigen was quantitated in cell-free culture supernatants by HIV-1 p24 antigen capture assay kit (Coulter). MAGI-CCR5 cells (10^4 cells), containing a *LacZ* reporter under control of an integrated HIV promoter, were seeded on 96-well microtiter culture plates and treated during 4 h with increasing amounts of AZT, d4T, 7, 8, 11 and 12 (0–400 μM) before being infected with 100 ng of HIV-1 CAp24 antigen by spinoculation as previously described [39]. Cells were then washed and grown in the presence of each compound dilution. Two days later, cells were stained for β-galactosidase activity and blue cells were counted. The 50% effective concentration (EC_{50}) corresponds to the compound concentration producing a 50% decrease of the number of blue cells in the virus-infected cell cultures. The 50% cytostatic concentration (CC_{50}) corresponds to the compound concentration required to inhibit cell proliferation by 50%. Standard compounds AZT and d4T inhibit HIV-1-infected cells at an IC_{50} value of 0.025 μM and 0.062 μM, respectively, in this assay.

4.2.2. Anti-HIV-1 and -2 assays in CEM cells

The compounds have also been evaluated against HIV-1(III_B) and HIV-2(ROD) in CEM cell cultures. Briefly, CEM cells (4.5×10^5 cells per ml) were suspended in fresh culture medium and infected with HIV-1 at 100 $CCID_{50}$ per ml of cell suspension. Then, 100 μl of the infected cell suspension were transferred to microplate wells, mixed with 100 μl of the appropriate dilutions of the test compounds, and further incubated at 37 °C. After 4–5 days, giant cell formation was recorded microscopically in the CEM cell cultures. The 50% effective concentration (EC_{50}) corresponds to the compound concentrations required to prevent syncytium formation by 50% in the virus-infected CEM cell cultures.

4.2.3. Antiviral assays other than HIV

Human embryonic lung (HEL) (ATCC-CCL 137), simian kidney (Vero) and human cervix carcinoma (HeLa) cells were propagated in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 0.075% bicarbonate. Herpes simplex virus type 1 (HSV-1) (KOS), HSV-2 (G), vaccinia virus and vesicular stomatitis virus were assayed in HEL cell cultures; Coxsackie virus B4 and respiratory syncytial virus in HeLa cell cultures, and para-influenza-3 virus, reovirus-1, Sindbis virus and Punta Toro virus in Vero cell cultures. Reference compounds, acyclovir and zidovudine were from Glaxo Smith Kline, ganciclovir from Hoffmann-La-Roche and cidofovir from Gilead. Cells were grown to confluency in 96-well microtiter plates and were inoculated with 100 times the 50% cell culture infective dose. Compounds were added after a 1–2 h virus adsorption period. The virus-induced cytopathic effect (CPE) was recorded microscopically at ≈3 days post infection and was expressed as percentage of the untreated controls. The 50% effective concentrations (EC_{50}) were derived from graphical plots. The minimal toxic concentration (MTC) was defined as the lowest concentration that resulted in a microscopically detectable alteration of cell morphology. The MTC 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.

Evaluation of antiviral activity and cytostatic activities of selected compounds in HCV genotype 1b subgenomic replicon carrying Huh-5-2 cells. Huh-5-2 cells [a cell line with a persistent HCV replicon I389luc-ubi-neo/NS3-3′/5.1; replicon with firefly luciferase-ubiquitin-neomycin phosphotransferase fusion protein and an EMCV-IRES driven NS3-5B HCV polyprotein] are cultured in RPMI medium

(GIBCO) supplemented with 10% fetal calf serum, 2 mM L-glutamine (Life Technologies), 1x non-essential amino acids (Life Technologies); 100 IU/ml penicillin and 100 μg/ml streptomycin and 250 μg/ml G418 (Geneticin, Life Technologies). Cells are seeded at a density of 7000 cells per well in 96-well View Plate™ (Packard) in medium containing the same components as described above, except for G418. Cells are allowed to adhere and proliferate for 24 h. At that time, culture medium is removed and 5 serial dilutions (5-fold dilutions starting at 100 μg/ml or 100 μM) of the test compounds are added in culture medium lacking G418. Interferon alfa 2a (500 IU) is included as a positive control in each experiment for internal validation. Plates are further incubated at 37 °C in a humidified 5% CO_2 atmosphere for 72 h. Replication of the HCV replicon in Huh-5-2 cells results in luciferase activity in the cells. Luciferase activity is measured by adding 50 μl of 1× Glo-lysis buffer (Promega) for 15 min followed by 50 μl of the Steady-Glo Luciferase assay reagent (Promega). Luciferase activity is measured with a luminometer and the signal in each individual well is expressed as a percentage of the untreated cultures. The 50% effective concentrations (EC_{50}) are calculated from these datasets. Parallel cultures of Huh-5-2 cells, seeded at a density of 7000 cells/well of classical 96-well cell culture plates (Becton–Dickinson) are treated in a similar fashion except that no Glo-lysis buffer or Steady-Glo Luciferase reagent is added. The effect of the compounds on the proliferation of the cells is measured 3 days after addition of the various compounds by means of The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS, Promega). In this assay 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) is bioreduced by cells into a formazan that is soluble in tissue. The number of cells correlates directly with the production of the formazan. The MTS stained cultures are quantified in a platereader.

4.3. Stability studies

4.3.1. Media and preparation of cell extracts

RPMI 1640 medium was purchased from GIBCO Life Technologies. Heat-inactivated fetal calf serum was purchased from PAN biotech. Culture medium was composed by RPMI 1640 containing 10% (v/v) heat-inactivated fetal calf serum and stored at −80 °C. CEM-SS cell extracts were prepared according to a published procedure [37]. Exponentially growing CEM-SS cells were recovered by centrifugation (500 g, 4 °C, 4 min), washed twice with PBS and resuspended in 10 mM Tris–HCl, 140 mM KCl (pH 7.4), 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, 4 °C, 20 min). The supernatant containing soluble proteins (3 mg/mL) was stored at −80 °C.

4.3.2. HPLC analysis

A Waters Model-600 gradient HPLC system equipped with two 600-pumps, a rheodine injector, a 996-photodiode array detector and an in-line degasser AF was used for reversed-phase chromatography. All solvents were of HPLC grade and filtered prior to use. A 1 M solution of triethylammonium bicarbonate buffer was prepared by adding dry ice to a 1 M triethylamine solution until the pH reached to 7.5. Triethylammonium bicarbonate solutions were made fresh by dissolving reagent-grade triethylammonium bicarbonate in HPLC-grade water prior to filtration.

The HPLC method has been described [37]. The cleaning precolumn is a guard-pak insert (Delta-Pak C18, 100 Å) in a guard-pak holder. The analytical column used is a Novapak C18, 3 μm, 100 Å, 4.6 × 150 mm. The elution system is prepared as follows: buffer A, 0.05 M TEAB; buffer B, 0.05 M TEAB (CH_3CN/H_2O, v/v, 50/50); flow rate, 1 mL/min. The crude sample (50 μl, initial concentration of **7**, **8**, **11**, **12**: 0.5 mM) is injected into the precolumn and eluted with buffer A during 3 min. Then, the switching valve for connecting the precolumn to the column is activated, and a linear gradient is applied from buffer A to buffer B, increasing buffer B from 0% at 0 min–20% at 40 min. The retention times are **7**, 29 min; **8**, 35 min; **11**, 23 min and **12**, 34 min. Kinetic data and decomposition pathways for compounds **7**, **8**, **11** and **12** were studied at 37 °C (a) in a pH buffers range 1.2–11.5 (3 conditions) (b) in culture medium RPMI 1640 containing 10% heat-inactivated fetal calf serum (c) in total cells extracts (CEM-SS). For each kinetic study, the compound solution is diluted with a freshly thawed aliquot of the considered medium to obtain an initial concentration of 0.1 mM. The mixture is incubated at 37 °C and for the required time, an aliquot (10% solution) is drawn and immediately frozen at −80 °C for further HPLC analysis. All compounds were analyzed under the same conditions. The amount of remaining parent compound at each time point was used to determine the half-life of the compound. The product of decomposition from parent derivative is determined by comparison with authentic samples and standard compounds.

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