Truncated phosphonated C-1′-branched N,O-nucleosides: A new class of antiviral agents

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ABSTRACT

Truncated phosphonated C-1′-branched N,O-nucleosides have been synthesized in good yields by 1,3-dipolar cycloaddition methodology, starting from N-methyl-C-(diethoxyphosphoryl)nitrone 7. Preliminary biological assays show that β-anomers are able to inhibit HIV in vitro infection at concentrations in the micromolar range. Higher SI values with respect to AZT indicated that the compounds were endowed with low cytotoxicity.

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1. Introduction

Natural psicofuranosyl nucleosides, bearing a hydroxymethyl group at the anomeric carbon atom, have been reported to possess different and relevant biological activities.[1] Typical examples are represented by angustmycin A **1** and C **2**, which show interesting antimicrobial and antiviral properties,[2] and by hydantocidin **3**, a spironucleoside, which exhibits herbicidal activity, able to regulate plant growth[3] (Fig. 1). Besides their potential biological activity as antiviral agents, the C1′-branched nucleosides show further great interest, linked to the availability of model nucleosides which may allow the study of the formation and evolution of radical species generated during DNA/RNA damage.[4]

N,O-psiconucleosides **4** constitute a particular class of modified psiconucleosides where an isoxazolidine system mimics the ribose ring of natural nucleosides and a hydroxymethyl group is linked at the anomeric carbon atom.[5] These derivatives show synthetic interest for their potential antiviral or anticancer activity which have been also discovered in other N,O-nucleosides.[6–11]

Our research group has reported a versatile route towards the synthesis of N,O-psiconucleosides both in racemic and in enantio-pure form.[5,12–15]

More recently, the use of a chiral auxiliary has promoted the enantioselective synthesis of a series of psiconucleosides.[16] However, none of the reported compounds has shown a remarkable biological activity, probably due to the lack of efficient phosphorylation towards the triphosphate derivatives, the active form of nucleoside RT inhibitors.

In the field of nucleoside analogs, N,O-modified nucleosides have been proved to efficiently block the in vitro and in vivo virus infections caused by HIV, HBV, and HTLV-1.[17–22] Following intracellular phosphorylation to their 5′-triphosphate forms, they are able to serve as chain terminators, thus acting as inhibitors in the viral reverse transcription reaction.[18,19] Several strategies to overcome the initial selective phosphorylation step have been designed;[23] in particular, phosphonate analogues,[20] by miming the nucleoside monophosphates, overcome the instability of nucleotides towards phosphodiesterase and enhance the cellular uptake by bypassing the initial phosphorylation step.

In this context, we have recently reported the synthesis of phosphonated carbocyclic 2′-oxa-3′-azanucleosides (PCOANS) **5**, which have shown to be potent inhibitors of RT of different retroviruses[21] (Fig. 2). Also truncated phosphonated azanucleosides (TPCOANS) **6**, where the phosphonate group is directly linked to the C-4′ position of the pseudosugar moiety, are able to inhibit the HIV and HTLV-1 viruses at concentration in the nanomolar range, with a potency comparable with that of Tenofovir.[22]
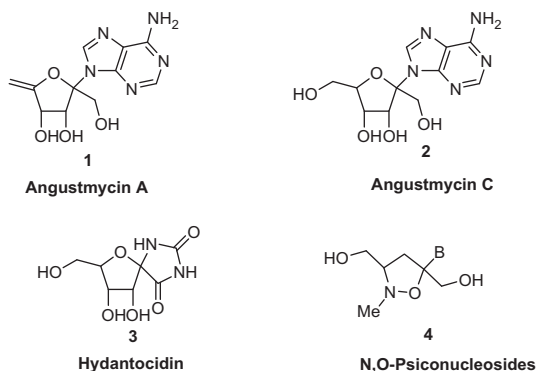
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Figure 1. Modified nucleosides, B = nucleobase.

Scheme 1. Retrosynthetic analysis of truncated phosphonated N,O-psiconucleosides.

Figure 2. Tenofovir, truncated nucleosides and phosphonated N,O-nucleosides.

Scheme 2. Synthesis of phosphonated nitrone **7**. Reagents and conditions route a: (a) Swern oxidation; (b) MeNHOH·HCl, Et$_3$N, −78 °C; route b: (a) MeSO$_2$Cl, CH$_2$Cl$_2$, Et$_3$N, rt; (b) Et$_3$N, MeNHOH, reflux, 6 h; (c) MnO$_2$, CH$_2$Cl$_2$.

In connection with our work addressed to the search of new and potent antiviral agents, we have extended our interest to the synthesis of the new generation of truncated phosphonated C-1′-branched N,O-nucleosides. The rational of our choice is based on the consideration that the presence of the phosphonic unit could bypass the limiting monophosphorylation step, thus promoting the cellular uptake and leading to biologically active compounds. We report in this paper the synthetic approach towards these derivatives and their preliminary biological evaluation. To the best of our knowledge, no example of this kind of compounds has been reported in the literature until now.
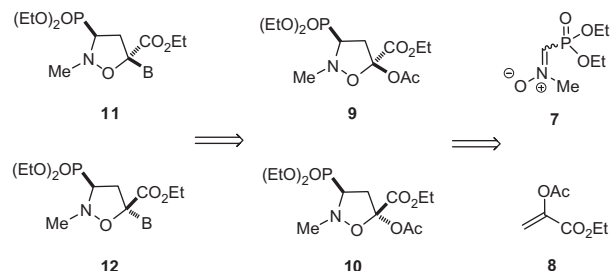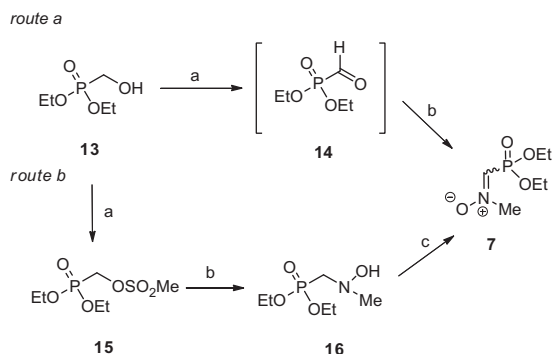
2. Results and discussion

2.1. Chemistry

According to the retrosynthetic analysis shown in Scheme 1, the key step of the synthetic route involves the 1,3-dipolar cycloaddition of the N-methyl-C-(diethoxyphosphoryl)nitrone **7** with the acrylate **8**.

The nitrone **7** can be prepared from the commercially available diethyl hydroxymethyl phosphonate **13** (route a), as previously described.[24] We have designed an alternative methodology towards **7** (route b), which is based on the conversion of the phosphonated alcohol **13** into the corresponding mesylate **15**; the subsequent reaction with N-methyl hydroxylamine afforded the derivative **16** which, by oxidation with MnO$_2$, gave the target nitrone **7** in a 60% yield (Scheme 2).

In comparison to the route A, this second approach is performed in milder conditions, at room temperature, thus allowing to avoid the severe experimental conditions required by the Swern oxidation (−78 °C) and the possible decomposition of intermediate **14** to carbon monoxide and dialkyl phosphate, with the risk of explosion, during the work up.

Dipolarophile **8** has been prepared starting from ethyl pyruvate, by heating with acetic anhydride.[5,25] The cycloaddition of **7** with ethyl 2-acetyloxyacrylate **8**, in THF at reflux for 24 h, afforded a mixture of cis/trans isoxazolidines **9** and **10** in an isomeric ratio of 1:4.5 and a combined yield of 80% (Scheme 3).

The crude reaction mixture was purified by flash chromatography (CH$_2$Cl$_2$/Me$_2$CHOH 98:2 as eluent) and two cycloadducts **9** and **10** were obtained in pure form. The cis/trans stereochemistry of both adducts was deduced on the basis of ^{1}H NMR spectroscopy and by means of NOE experiments. Thus, in the major trans compound **10**, the resonance of H$_{4a}$ appears as ddd at 2.85 ppm, while H$_{4b}$ resonates at 2.96 ppm (ddd). Moreover, H$_3$ resonates as ddd at 3.27 ppm. For the cis compound **9**, the resonance of H$_{4a}$ and H$_{4b}$ appears as ddd at 2.81 and 2.93 ppm respectively; H$_3$ resonates as ddd at 3.23 ppm. NOE difference experiments were conclusive in the stereochemical assignment: (a) on irradiation at 2.96 ppm (H$_{4b}$ of trans compound **10**), a diagnostic positive NOE was observed for H$_3$ (δ = 3.27 ppm) and the methyl of the OAc group, thus confirming their cis relationship, and (b) on irradiation of H$_{4b}$ of cis derivative **9**, at δ = 2.93 ppm, a positive NOE was observed for H$_3$ (δ = 3.23 ppm) but not for the methyl protons of OAc group.
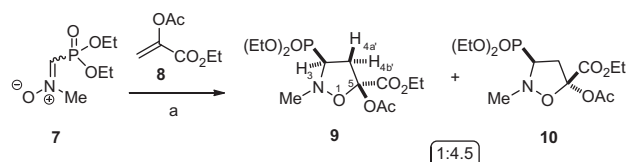
Scheme 3. Cycloaddition reaction. Reagents and conditions: nitrone **7**, ethyl 2-acetyloxyacrylate **8**, THF, reflux, 24 h, overall yield 80%.
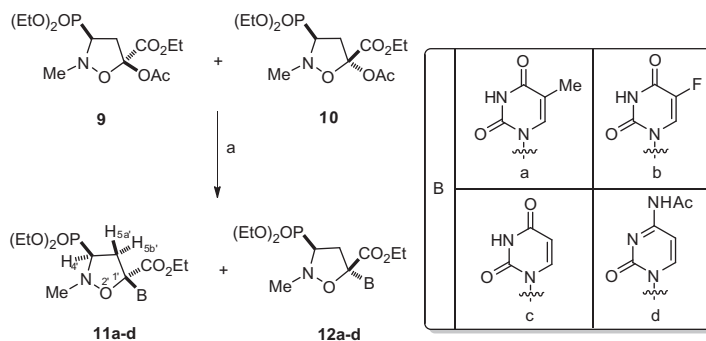
Scheme 4. Nucleosidation reaction. Reagents and conditions: isoxazolidines **9** and **10**, MeCN, TMSOTf, silylated Thy (overall yield 72%), 5-Fu (overall yield 80%), U (overall yield 71%), or Ac-Cy (overall yield 61%).
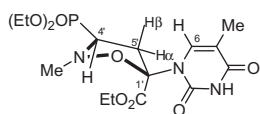

Figure 3. Preferred conformation of **11a**.

The crude mixture of isoxazolidines **9** and **10** was then transformed into the pyrimidine nucleoside analogues **11** and **12**, as depicted in Scheme 4.

The condensation with silylated thymine, uracil or acetylcytosine, using the glycosylation methodology developed by Vorbrüggen, resulted in nucleoside products consisting of β-**11a,c,d** and α-**12a,c,d** anomers in a 3:2 ratio and in 61–80% combined yield. The nucleosidation reaction performed with 5-fluoruracil showed a better diastereoselectivity in favor of the β-anomer **11b** (β/α ratio 4:1; combined yield: 85%).

The ratio between α and β nucleosides did not change if the nucleosidation was performed starting from the separated cis/trans isoxazolidines **9** and **10**. As previously reported for similar compounds,[13] these results indicate that the coupling reaction occurs without selectivity with respect to the anomeric center. The anomers have been separated by flash chromatography, the cis isomer (β) showing the lower rf. The anomeric configuration of obtained modified nucleosides was assigned on the basis of ^{1}H NMR and NOE experiments.

The ^{1}H NMR spectrum of the major isomer **11a**, chosen as reference, showed a different set of signals in comparison with **12a**. In particular, the H4′ proton resonated at 3.25 ppm (ddd), H5′β at 2.93 ppm (ddd) and H5′α at 3.28 ppm (ddd). Positive NOE effects were observed within H5′β–H6 and H4′–H5′α pairs of protons and fully supported the cis configuration of the substituents at C1′ (thymine) and C4′ (diethoxyphosphoryl) in the β isomer **11a**. Analogous NOE effects have been detected for the fluorouracil, uracil and cytosine derivatives **11b–d**.

Since values of all vicinal coupling constants were successfully extracted from the ^{1}H and ^{13}C NMR spectra of **11a**, detailed conformational analysis was accomplished. On the basis of vicinal couplings [J (H-C4′–C5′Hβ) = 10.5 Hz, J (H-C4′–C5′Hα) = 8.5 Hz, J (P-C4′–C5′β) = 16.5 Hz, J (P-C4′–C5′α) = 4.0 Hz, J (C1′-C–C–P) = 11.5 Hz] it was concluded that the isoxazolidine ring exists in an $E_{1'}$ conformation in which the thymine residue occupies the equatorial position, while the ethoxyphosphoryl group is located pseudoequatorially (Fig. 3).

2.2. Biological results

For testing the potential activity of the new truncated phosphonated C-1′-branched N,O-nucleosides against human retroviruses, we assessed their ability to inhibit both HIV and HTLV-1 in vitro

Table 1
Inhibitory activity of the compounds **11a–d** on HIV infection

Compound	HIV infection IC$_{50}$[a] (μM)	SI[b]	CC$_{50}$ Molt-3[c] (μM)
11a	>1000	n.d	>1000
11b	220	4.5	>1000
11c	132	7.57	>1000
11d	>1000	n.d.	>1000
11e	180	7.02	>1000
AZT	7.7	1.57	12.1

[a] IC$_{50}$: inhibitory concentration required to inhibit 50% HIV infection.
[b] SI: selectivity index based on the ratio CC$_{50}$/IC$_{50}$.
[c] CC$_{50}$ is the cytotoxic concentration 50% required to inhibit 50% metabolic activity evaluated in cell line MOLT-3 by MTS.

infections. Zidovudine (AZT) was used as an internal positive control in the assay, since it is the prototype of nucleoside inhibitors of HIV reverse transcriptase, acting as chain terminator, equally active in comparison with other nucleoside and nucleotide analogues, towards HIV in vitro infection[22] The results reported in Table 1 showed that compounds **11a** and **11d** were not active, while **11b** and **11c** inhibited HIV infection at an inhibitory concentration 50 (IC$_{50}$) of 220 and 132 μM respectively. Conversely AZT showed an IC$_{50}$ 28 times lower in comparison with that of the tested compounds. On the other hand, the compounds were unable to inhibit HTLV-1 infection (data not shown). Moreover, cytotoxicity indicated that **11b** and **11c** (CC$_{50}$ >1000 μM) were at least eighty three times less cytotoxic than AZT (CC$_{50}$ 12.1 μM), as demonstrated by the values of cytotoxic concentration 50 (CC$_{50}$) shown in Table 1. Actually this is summarized by the values of the selectivity index (SI) calculated on the basis of the ratio between the CC$_{50}$ and IC$_{50}$ values. Table 1 shows the comparison between the anti HIV activity of **11b** and **11c** versus AZT, by reporting the SI for each compound. The SI of **11c**, (7.6) was higher than that of **11b** (4.5) and than that of AZT (1.57). A high SI value indicated that the compounds were endowed with low cytotoxicity.

Thus, the low cytotoxic effect of the compounds could balance their higher IC$_{50}$ with respect to the positive control. In addition, the activity of **11b** and **11c** seems to be rather specific toward HIV infection, since they were unable to inhibit HTLV-1 infection (data not shown). Moreover, it is interesting to put in evidence that compounds **12a–d** did not show any activity against HIV and HTLV-1 viruses, in agreement with the α-nature of these derivatives.

3. Conclusion

Truncated phosphonated C-1′-branched N,O-nucleosides have been synthesized in good yields by the 1,3-dipolar cycloaddition methodology, starting from the nitrone **7**. Preliminary biological

assays show that the β-anomers are able to inhibit infection of HIV at concentrations in the micromolar range. Although twenty eight times less active than AZT, they are certainly less cytotoxic than AZT, as deduced from the calculated SI values. In addition, they seem to be rather specific in inhibiting HIV infection, while they were unable to exert the same effect on HTLV-1 infection.

Truncated phosphonated represent a new template of cyclic nucleoside analogs which deserve further investigations as lead compounds for extending the current spectrum of antiviral activity of modified nucleosides, avoiding some unwanted side effects.

4. Experimental section

Melting points were recorded on a capillary melting point apparatus and are uncorrected. Elemental analyses were recorded on a Perkin-Elmer elemental analyzer. The elemental analyses of all final compounds were within ±0.4% of the expected values. NMR spectra were performed on a Varian instrument at 500 MHz (1H) and at 125 MHz (13C) using deuterochloroform; chemical shifts are given in ppm from TMS. The NOE difference spectra were obtained by subtracting alternatively right off-resonance free induction decays (FIDS) from right-on-resonance-induced FIDS. Thin-layer chromatographic separations were performed on Merck silica gel 60-F254 precoated aluminum plates. Preparative separations were carried out by flash chromatography using Merck silica gel 0.035–0.070 mm. All reagents were purchased from Aldrich Chemicals Ltd.

4.1. Synthesis of *N*-methyl-*C*-(diethoxyphosphoryl) nitrone 7

To a solution of diethyl hydroxymethyl phosphonate (500 mg, 2.97 mmol) in dry dichloromethane (20 mL), triethylamine (2.4 mL, 17 mmol) and mesyl chloride (1.45 g, 12 mmol) were added at −10 °C. The mixture was stirred at room temperature for 2 h and, then, washed with a saturated solution of $NaHCO_3$ and concentrated in vacuo. The residue was purified by flash chromatography (dichloromethane/methanol 99:1 as eluent) to afford the mesylate **15**[26] in 90% yield. A solution of **15** in 25 mL of triethylamine was then reacted with methyl hydroxylamine (668 mg, 7.9 mmol) and left under reflux for 5 h. The reaction mixture was evaporated and the residue was extracted with ether to afford compound **16** as an yellow oil (50% yield). ^{1}H NMR ($CDCl_3$): δ = 5.80 (bs s, OH), 4.30 (m, 4H, CH_2OP), 3.32 (d, J = 14.9 Hz, CH_2P, 2H), 2.95 (s, NCH_3), 1.45 (t, J = 7.0 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ = 62.02 (d, $^2J_{POC}$ = 6.2 Hz), 57.99 (d, $^1J_{PC}$ = 162.0 Hz), 49.87 (d, $^3J_{PCNC}$ = 17.2 Hz), 16.22 (d, $^2J_{POCC}$ = 6.2 Hz). Anal. Calcd for $C_6H_{16}NO_4P$: C, 36.55; H, 8.18; N, 7.10. Found: C, 36.69; H, 8.17; N, 7.04.

Phosphonated hydroxylamine **16** (330 mg, 1.6 mmol) in 15 mL of dichloromethane was oxidized with activated MnO_2 (145 mg, 1.7 mmol) at room temperature overnight to afford, after filtration on celite, a residue which was purified by medium pressure chromatography to give nitrone **7**. Spectral data correspond perfectly with earlier reported data.[23]

4.2. Synthesis of isoxazolidines 9 and 10

A solution of *C*-diethoxyethylphosphoryl-*N*-methyl nitrone (**7**; 3.0 g, 22.9 mmol) and ethyl 2-acetyloxyacrylate (**8**; 3.7 g, 23 mmol) in THF (100 mL) was stirred at reflux for 24 h. The solvent was evaporated and the residue was purified by flash chromatography (dichloromethane/isopropanol 98:2). The product eluted first was the ethyl (3*SR*,5*RS*)-5-(acetyloxy)-3-(diethoxyphosphoryl)-2-methyl-isoxazolidine-5-carboxylate **9**; 18% yield; yellow oil. ^{1}H

NMR ($CDCl_3$): δ = 4.28–4.12 (m, 6H, 2× CH_2–O–P + CH_2–O–C), 3.23 (ddd, J = 9.1, 6.9 and 6.5 Hz, H-C3), 2.93 (ddd, J = 12.5, 12.6 and 9.1 Hz, Hβ–C4), 2.84 (s, 3 H, CH_3–N), 2.81 (ddd, J = 18.8, 12.5 and 6.9 Hz, Hα-C4), 2.07 (s, 3H, CH_3–CO), 1.31 (t, 6H, J = 6.5 Hz), 1.24 (t, 3H, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$): δ = 170.16 (s, C=O), 165.50 (s, C=O), 102.19 (d, $^3J_{PCCC}$ = 9.5 Hz, C5), 64.57 (d, $^1J_{PC}$ = 163.1, C3), 63.61 (d, $^2J_{POC}$ = 6.0 Hz), 62.71 (d, $^2J_{POC}$ = 7.8 Hz), 62.61 (s, CH_2–O), 46.08 (s, CH_3–N), 44.50 (d, $^2J_{PCC}$ = 1.5 Hz, C4), 20.84 (s, CH_3–C=O), 16.38 (d, $^3J_{POCC}$ = 6.0 Hz), 13.86 (s, CH_3); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ 22.77. Anal. Calcd for $C_{13}H_{24}NO_8P$: C, 44.19; H, 6.85; N, 3.96. Found: C, 44.26; H, 6.88; N, 3.94.

The fraction eluted second was the ethyl (3*SR*,5*SR*)-5-(acetyloxy)-3-(diethoxyphosphoryl)-2-methylisoxazolidine-5-carboxylate **10**; 62% yield; yellow oil. ^{1}H NMR ($CDCl_3$): δ = 4.27-4.09 (m, 6H, 2× CH_2–O–P + CH_2–O–C), 3.27 (ddd, J = 12.0, 6.1 and 1.5 Hz, H-C3), 2.96 (ddd, J = 16.5, 13.0 and 12.0 Hz, Hβ-C4), 2.94 (d, J = 0.9 Hz, 3H, CH_3–N), 2.85 (ddd, J = 13.0, 6.1, 4.3 Hz, Hα-C4), 2.05 (s, 3H, CH_3–CO), 1.32 (t, 6H, J = 6.5 Hz), 1.23 (t, 3H, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$) δ = 169.48 (s, C=O), 164.84 (s, C=O), 102.24 (d, $^3J_{PCCC}$ = 13.5 Hz, C5), 64.00 (d, J = 6.0 Hz, C–O–P), 62.78 (d, $^1J_{PC}$ = 171.7, C3), 62.70 (d, J = 8.2 Hz, C–O–P), 62.63 (s, CH_2–O), 49.24 (d, J = 4.5 Hz, C–N–C–P), 43.01 (d, $^2J_{PCC}$ = 1.5 Hz, C4), 21.07 (s, CH_3–C=O), 16.38 (d, J = 6.0 Hz), 13.86 (s, CH_3); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ 21.66. Anal. Calcd for $C_{13}H_{24}NO_8P$: C, 44.19; H, 6.85; N, 3.96. Found: C, 44.22; H, 6.87; N, 3.99.

4.3. General procedure for the preparation of truncated phosphonated *N,O*-psiconucleosides 11 and 12

A suspension of nucleobases (2 mmol) in dry acetonitrile (30 mL) was treated with bis(trimethylsilyl)acetamide (1.5 mL, 6 mmol) and left under stirring until the solution was clear. A solution of a mixture of isozaxolidines **9** and **10** (282 mg, 1 mmol) in dry acetonitrile (10 mL) and trimethylsilyl triflate (72 μL, 0.4 mmol) was then added, and the reaction mixture was heated at 70 °C for 5 h. After being cooled at 0 °C, the solution was carefully neutralized by addition of aqueous 5% sodium bicarbonate and then concentrated in vacuo. After addition of dichloromethane (20 mL), the organic phase was separated, washed with water (2 × 10 mL), dried over sodium sulfate, filtered, and evaporated to dryness. The ^{1}H NMR spectrum of the crude reaction mixture shows the presence of β-anomers as major adducts, while the α-anomers are present only in low amount. The residue was purified by MPLC on a silica gel column using as eluent a mixture of CH_2Cl_2/Me_2CHOH 98:2 to afford β-nucleosides **11a–d** and α-nucleosides **12a–d**.

4.3.1. Ethyl (3*SR*,5*RS*)-3-(diethoxyphosphoryl)-2-methyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-pyrimidin-1(2*H*)-yl)isoxazolidine-5-carboxylate 11a

Yield: 43.2%; white solid, mp 193–195 °C. ^{1}H NMR ($CDCl_3$): δ = 8.71 (s, NH, 1H), 7.52 (br q, J = 1.0 Hz, CH=, 1H), 4.26 (q, J = 7.0 Hz, 2H), 4.30–4.08 (m, 4H), 3.84 (ddd, J = 14.5, 8.5 and 4.0 Hz, Hα–C5′, 1H), 3.25 (ddd, J = 10.5, 8.5 and 3.0 Hz, H–C4′, 1H), 3.06 (s, 3H), 2.88 (ddd, J = 16.5, 14.5 and 10.5 Hz, Hβ–C5′, 1H), 1.97 (d, J = 1.0 Hz, 3H), 1.34 (t, J = 7.0 Hz, 3H), 1.28 (t, J = 7.0 Hz, 3H), 1.25 (t, J = 7.0 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ = 164.7, 164.2 (C4), 150.3 (C2), 134.7 (C6), 109.3 (C5), 92.5 (d, $^3J_{PCCC}$ = 11.5 Hz, C1′), 65.2 (d, $^1J_{PC}$ = 155.2 Hz, C4′), 63.5 (d, $^2J_{POC}$ = 6.0 Hz), 63.3 (CH_2–O), 62.5 (d, $^2J_{POC}$ = 6.8 Hz), 46.2 (CH_3N), 45.4 (D, $^2J_{PCC}$ = 4 Hz, C5′), 16.4 (d, $^3J_{POCC}$ = 2.2 Hz), 16.3 (d, $^3J_{POCC}$ = 2.2 Hz), 13.8 (CH_3–CH=), 12.7. ^{31}P NMR (121.5 MHz, $CDCl_3$): δ 21.52. Anal. Calcd for $C_{16}H_{26}N_3O_8P$: C, 45.82; H, 6.25; N, 10.02. Found: C, 45.86; H, 6.23; N, 10.01.

4.3.2. Ethyl (3*SR*,5*RS*)-3-(diethoxyphosphoryl)-5-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-2-methylisoxazolidine-5-carboxylate 11b

Yield: 68%; white solid, mp 200–201 °C. ^{1}H NMR (CDCl$_3$): δ = 8.91 (s, 1H), 7.77 (d, *J* = 6.5 Hz, 1H), 4.30–4.10 (m, 6H), 3.83 (ddd, *J* = 14.0, 8.5 and 4.0 Hz, 1H), 3.25 (ddd, *J* = 10.0, 8.5 and 3.0 Hz, 1H), 2.95 (s, 3H), 2.93 (ddd, *J* = 16.5, 14.0 and 10.0 Hz, 1H), 1.35 (t, *J* = 7.5 Hz, 3H), 1.28 (m, 6H). ^{13}C NMR (125 MHz, CDCl$_3$): δ = 164.12, 156.93 (d, *J* = 26.7 Hz), 148.74, 139.90 (d, *J* = 235.1 Hz), 123.73 (d, *J* = 35.2 Hz), 92.63 (d, *J* = 10.6 Hz), 65.13 (d, *J* = 163.7 Hz), 63.43 (d, *J* = 7.3 Hz), 63.35, 62.61 (d, *J* = 6.8 Hz), 46.20, 45.17, 16.41, 16.21, 13.84. Anal. Calcd for C$_{15}$H$_{23}$FN$_3$O$_8$P: C, 42.56; H, 5.48; N, 9.93. Found: C, 42.59; H, 5.50; N, 9.91.

4.3.3. Ethyl (3*SR*,5*RS*)-3-(diethoxyphosphoryl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-2-methylisoxazolidine-5-carboxylate 11c

Yield: 47.61%; white solid, mp 171–174 °C. ^{1}H NMR (CDCl$_3$): δ = 8.61 (bs s, 1H), 7.72 (d, *J* = 8.2 Hz, 1H), 5.77 (d, *J* = 8.2 Hz, 1H), 4.37–4.02 (m, 6H), 3.92 (ddd, *J* = 14.2, 8.5 and 4.1 Hz, Hα–C5′, 1H), 3.31 (ddd, *J* = 10.1, 8.5 and 2.7 Hz, H–C4′, 1H), 3.00 (ddd, *J* = 16.3, 14.2 and 10.1 Hz, Hβ–C5′, 1H),. 2.98 (s, 3H),), 1.34 (t, *J* = 7.1 Hz, 3H), 1.32 (m, 6H). ^{13}C NMR (125 MHz, CDCl$_3$): δ = 164.42, 163.31, 150.28, 139.01, 100.96, 92.84 (d, *J* = 12.0 Hz), 65.04 (d, *J* = 164.9 Hz), 63.69 (d, *J* = 6.0 Hz), 63.44, 62.49 (d, *J* = 7.2 Hz), 46.11, 45.28, 16.50, 16.41, 16.31, 13.84. Anal. Calcd for C$_{15}$H$_{24}$N$_3$O$_8$P: C, 44.45; H, 5,97; N, 10.37. Found: C, 44.41; H, 5.96 N, 10.40.

4.3.4. Ethyl (3*SR*,5*RS*)-5-[4-(acetylamino)-2-oxopyrimidin-1(2*H*)-yl]-3-(diethoxyphosphoryl)-2-methylisoxazolidine-5-carboxylate 11d

Yield: 41.27%; yellow sticky oil. ^{1}H NMR (CDCl$_3$): δ = 8.91 (bs s, 1H), 8.10 (d, *J* = 7.6 Hz, 1H), 7.51 (d, *J* = 7.6 Hz, 1H), 4.22–4.08 (m, 6H), 3.95 (ddd, *J* = 13.5, 8.4 and 3.4 Hz, Hα–C5′, 1H), 3.28 (ddd, *J* = 11.2, 8.4 and 2.9 Hz, H–C4′, 1H), 3.07 (s, 3H), 2.92 (ddd, *J* = 16.2, 13.5 and 10.3 Hz, Hβ–C5′, 1H), 2.26 (s, 3H), 1.35 (t, *J* = 7.1 Hz, 3H), 1.33 (m, 6H). ^{13}C NMR (125 MHz, CDCl$_3$): δ = 170.96, 164.32, 163.51, 155.18, 143.69, 95.92, 92.84 (d, *J* = 10.6 Hz), 65.23 (d, *J* = 162.6 Hz), 63.61 (d, *J* = 69 Hz), 63.15, 62.41 (d, *J* = 6.6 Hz), 46.12, 44.86, 24.76, 16.41 (d, *J* = 4.4 Hz), 16.30 (d, *J* = 5.4 Hz), 13.77. Anal. Calcd for C$_{17}$H$_{27}$N$_4$O$_8$P: C, 45.74; H, 6.10; N, 12.55. Found: C, 45.78; H, 6,16 N, 12.53.

4.3.5. Ethyl (3*SR*,5*RS*)-5-[4-amino-2-oxopyrimidin-1(2*H*)-yl]-3-(diethoxyphosphoryl)-2-methylisoxazolidine-5-carboxylate 11e

Yield: 41.42%; yellow sticky oil. ^{1}H NMR (CDCl$_3$): δ = 8.15 (d, *J* = 7.5 Hz, 1H), 7.50 (d, *J* = 7.5 Hz, 1H), 6.70 (bs s, 1H), 4.20–4.08 (m, 6H), 4.01 (ddd, *J* = 13.4, 8.2 and 3.4 Hz, Hα–C5′, 1H), 3.30 (ddd, *J* = 11.2, 8.2 and 3.0 Hz, H–C4′, 1H), 3.02(s, 3H), 2.80 (ddd, *J* = 16.2, 13.4 and 10.3 Hz, Hβ–C5′, 1H), 1.35 (t, *J* = 7.1 Hz, 3H), 1.29 (m, 6H). ^{13}C NMR (125 MHz, CDCl$_3$): δ =165.06, 163.50, 154.89, 142.12, 96.28, 94.544 (d, *J* = 10.4 Hz), 66.02 (d, *J* = 162.4 Hz), 64.05 (d, *J* = 67 Hz), 63.14, 62.38 (d, *J* = 6.4 Hz), 48.89, 46.85, 15.89 (d, *J* = 4.4 Hz), 16.22 (d, *J* = 5.5 Hz), 12.70. Anal.Calcd for C$_{15}$H$_{25}$N$_4$O$_7$P: C, 44.56; H, 6.20, N, 13.88. Found: C, 44.59; H, 6.29 N, 13.84.

4.3.6. Ethyl (3*SR*,5*SR*)-3-(diethoxyphosphoryl)-2-methyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-pyrimidin-1(2*H*)-yl)isoxazolidine-5-carboxylate 12a

Yield: 28.8%; white solid, mp 198–200 °C. ^{1}H NMR (CDCl$_3$): δ = 8.23 (br s, NH, 1H), 7.61 (br q, *J* = 0.9 Hz, C*H*═, 1H), 4.30–4.11 (m, 6H), 3.84 (ddd, *J* = 15.0, 10.2 and 2.8 Hz, Hα–C5′, 1H), 3.15–3.08 (m, H–C4′ and Hβ–C5′, 2H), 3.13 (s, 3H), 1.97 (d, *J* = 0.9 Hz, 3H), 1.34 (t, *J* = 7.0 Hz, 3H), 1.26 (t, *J* = 7.0 Hz, 3H), 1.24 (t,

J = 7.0 Hz, 3H). ^{13}C NMR (CDCl$_3$): δ = 165.19, 163.46 (C4), 150.13 (C2), 134.61 (C6), 109.92 (C5), 92.69 (d, *J* = 14.2 Hz, C1′), 63.33 (CH$_2$-O), 63.24 (d, *J* = 6.9 Hz), 62.15 (d, *J* = 163.2 Hz, C4′), 62.70 (d, *J* = 6.9 Hz), 45.43 (CH$_3$N), 43.95 (C5′), 16.43 (d, *J* = 4.5 Hz), 13.95 (d, *J* = 7.5 Hz), 13.80 (CH$_3$–CH═), 12.86. ^{31}P NMR (121.5 MHz, CDCl$_3$): δ 20.79. Anal. Calcd for C$_{16}$H$_{26}$N$_3$O$_8$P: C, 45.82; H, 6.25; N, 10.02. Found: C, 45.87; H, 6.27; N, 9.98.

4.3.7. Ethyl (3*SR*,5*SR*)-3-(diethoxyphosphoryl)-5-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-2-methylisoxazolidine-5-carboxylate 12b

Yield: 12%; white solid, mp 193–195 °C. ^{1}H NMR (CDCl$_3$): δ = 8.65 (bs s, 1H), 7.86 (d, *J* = 6.6 Hz, 1H), 4.29–4.14 (m, 6H), 3.81 (ddd, *J* = 15.0, 14.6 and 1.3 Hz, H-C5′, 1H), 3.18–3.02 (bm, H-C4′ and H-C5′, 2H), 3.13 (s, N–CH$_3$, 3H), 1.38 (t, *J* = 8.5 Hz, 3H), 1.35 (t, *J* = 8.2 Hz, 3H), 1.25 (t, *J* = 8.2 Hz, 3H). ^{13}C NMR (CDCl$_3$): δ = 164.66, 159.82 (d, *J* = 30.4 Hz), 156.20, 139.95 (d, *J* = 237.0 Hz), 123.60 (d, *J* = 35.8 Hz), 92.55 (d, *J* = 12.5 Hz), 64.07 (d, *J* = 6.5 Hz), 63.88 (d, *J* = 175.0 Hz), 63.80, 62.90 (d, *J* = 6.5 Hz), 53.43, 42.62, 16.48, 16.37, 13.87. Anal. Calcd for C$_{15}$H$_{23}$FN$_3$O$_8$P: C, 42.56; H, 5.48; N, 9.93. Found: C, 42.53; H, 5.51; N, 9.94.

4.3.8. Ethyl (3*SR*,5*SR*)-3-(diethoxyphosphoryl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-2-methylisoxazolidine-5-carboxylate 12c

Yield: 23.8%; white solid, mp 178–180 °C. ^{1}H NMR (CDCl$_3$): δ = 8.49 (bs s, 1H), 7.81 (d, *J* = 8.3 Hz, 1H), 5.79 (d, *J* = 8.3 Hz, 1H), 4.37–4.10 (m, 6H), 4.02 (ddd, *J* = 14.3, 8.2 and 1.3 Hz, Hα–C5′, 1H), 3.16–3.05 (m, H–C4′, and H–C5′, 2H), 3.13 (s, 3H), 1.35 (t, *J* = 7.2 Hz, 3H), 1.26 (t, *J* = 7.0 Hz, 3H), 1.24 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, CDCl$_3$): δ = 169.33, 166.44, 150.16, 138.88, 101.53, 90.77 (d, *J* = 12.3 Hz), 64.07 (d, *J* = 6.0 Hz), 63.14 (d, *J* = 176.8 Hz), 63.31, 62.81 (d, *J* = 7.1 Hz), 47.27, 42.67, 16.41 (d, *J* = 4.5 Hz), 14.13, 13.86. Anal. Calcd for C$_{15}$H$_{24}$N$_3$O$_8$P: C, 44.45; H, 5,97; N, 10.37. Found: C, 44.47; H, 5.94 N, 10.41.

4.3.9. Ethyl (3*SR*,5*SR*)-5-[4-(acetylamino)-2-oxopyrimidin-1(2*H*)-yl]-3-(diethoxyphosphoryl)-2-methylisoxazolidine-5-carboxylate 12d

Yield: 19.84%; yellow sticky oil. ^{1}H NMR (CDCl$_3$): δ = 8.89 (bs s, 1H), 7.75 (d, *J* = 7.3 Hz, 1H), 7.52 (d, *J* = 7.3 Hz, 1H), 4.27–4.03 (m, 6H), 4.02 (ddd, *J* = 13.9, 7.8 and 1.7 Hz, Hα–C5′, 1H), 3.33–3.22 (m, H–C4′, and H–C5′, 2H), 3.08 (s, 3H), 2.24 (s, 3H), 2.24 (s, 3H), 1.29 (t, *J* = 7.2 Hz, 3H), 1.27 (t, *J* = 7.2 Hz, 3H), 1.25 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (125 MHz, CDCl$_3$): δ = 170.16, 164.30, 162. 22, 155.10, 143.87, 113.88, 93..82 (d, *J* = 11.9 Hz), 65.85 (d, *J* = 105.0 Hz), 63.53 (d, *J* = 6.0 Hz), 63.21, 62.42 (d, *J* = 6.5 Hz), 46.17, 44.87, 24.95, 16.39 (d, *J* = 6.3 Hz), 16.41, 13.96 (d, *J* = 16.0 Hz). Anal. Calcd for C$_{17}$H$_{27}$N$_4$O$_8$P: C, 45.74; H, 6.10; N, 12.55. Found: C, 45.70; H, 6.14; N, 12.58.

4.4. Biological assay

The compounds were tested for their inhibitory activity on HTLV-1 and HIV infection. HTLV-1 infection was carried out as previously shown.[26] Peripheral blood mononuclear cells were co-cultivated with a HTLV-1 chronically infected cell line and infection was evaluated as production of the viral core protein p19. HIV infection was carried on by using a stable T cell line (CEM) containing a plasmid encoding a green fluorescence protein (GFP) driven by the HIV-1 long terminal repeat.[27] Infection was carried on as previouslyb shown with some modification.[28] Briefly, 5×10^5 CEM-GFP were infected with a volume of supernatant from HIV chronically infected H9 cells equivalent to 20 ng/mL of HIV p24, for 2 h in 100 μl CM in presence of 1000, 100, 10 and 1 μM concentration of compounds. Then medium was added and the

cultures were incubaterd for 72 h. The inhibition was assessed on the basis of GFP expression in the different culture conditions. Cito-toxicity assays were performed by MTS assay kit (Promega Corporation, Madison, Winsconsin).BrieflyInhibition of cell metabolic activity revealed by reduction of the oxidative burst was detected through formazan product formation, using a commercial colorimetric kit (MTS [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt]) (Cell Titer 96 Aqueous One Solution; Promega). The assay was performed by seeding 1×10^4 MOLT-3 cells in the presence or absence of the different compounds at concentrations ranging from 1000 to 1 μM.

Acknowledgments

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.03.047. These data include MOL files and InChiKeys of the most important compounds described in this article.

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