

Phosphoramidate derivatives of *N,O*-nucleosides as inhibitors of reverse transcriptase

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Dedicated to Prof Nicolò Vivona on his 70th birthday

Abstract

Phosphoramidate derivatives of adenine and 5-fluorouracil *N,O*-nucleoside analogues have been synthesized as potential antiviral prodrugs. In particular, dimethoxyphenyl phosphates, linked via nitrogen to L-leucine methyl ester were studied. The synthesized compounds were also subjected to *in vitro* evaluation for their RT inhibition. Results show that phosphoramidate derivatives, in comparison with their corresponding *N,O*-nucleosides, present a promising antiviral activity, though of micro-molar order.

Keywords: Phosphoramidates, *N,O*-nucleosides, 1,3-dipolar cycloadditions, nitrones, RT inhibitors

Introduction

The nucleoside structure has proven to be an effective template for the development of therapeutically useful agents with antiviral and antitumor activity. In particular nucleoside analogues were approved for the treatment of pathologies induced by infections from HCMV, HSV, HIV, and HBV.¹ Dideoxynucleoside analogues exert their antiviral activity by the competitive reversible inhibition of Reverse Transcriptase (RT) and/or viral DNA chain termination. These antiviral agents, acting *via* a nucleoside analogue mode, need to be phosphorylated into their corresponding 5'-triphosphates by cellular and/or viral enzymes.²

As a part of our continuing efforts to the search of new templates for the development of new antiviral agents, in the last years, our research group has been interested in the synthesis and

evaluation of the antiviral activity of nucleoside analogues, where the ribose moiety is replaced by an isoxazolidine ring.³

Some *N,O*-nucleosides have shown relevant biological properties as antiviral agents or antimetabolites.⁴ However, the analysis of the biological profile for some other derivatives revealed a moderate or no antiviral activity. The conversion of these latter nucleosides into the corresponding phosphonated structures⁵ have furnished often a higher potency in activity, suggesting that the inefficient conversion of *N,O*-nucleosides into the 5'-monophosphate forms by the kinase, is responsible of the lack of biological activity.

Several strategies directed to overcome the problem of the initial selective and regulated phosphorylation step could be foreseen. The use of the nucleotide monophosphate might bypass the first phosphorylation step. However, as unmodified agents, nucleoside monophosphates are unstable in biological media and they also show poor membrane permeation owing to the associated negative charges at physiological pH.

On the basis of these considerations, we decided to improve the pharmacological characteristics of these *N,O*-nucleosides designing the corresponding phosphoramidates as derivatives which can mask the negative charge of phosphate group conferring better lipophilic features. These nucleotide prodrugs, after their cellular uptake, may release the monophosphorylated nucleoside analogue which will be converted into di- and triphosphates by unspecific kinases. This technology greatly increases the lipophilicity of the nucleoside monophosphate analogue with a consequent increase of membrane permeation and intracellular availability.

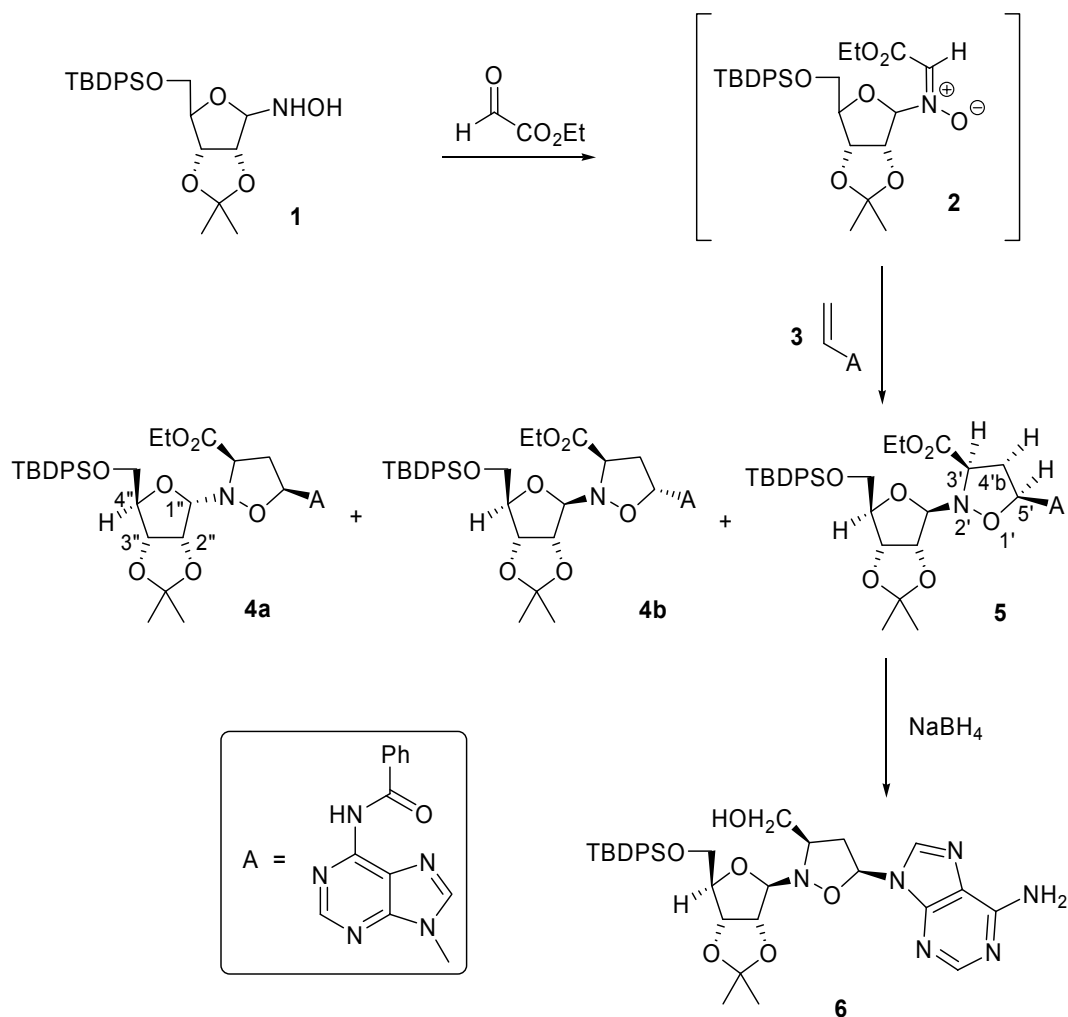
Herein, we describe the preparation of phosphoramidates of adenine and 5-fluorouracil *N,O*-nucleosides, **15** and **17** respectively, and their antiviral evaluation against two commercial RT [avian myeloblastosis virus RT (AMV-RT), and moloney murine leukemia virus RT (MLV-RT)] in comparison with the corresponding *N,O*-nucleosides. In particular, phosphoramidate derivatives with dimethoxyphenyl phosphates linked *via* nitrogen to methyl-esterified L-leucine were examined. Besides the strong effect activating of dimethoxyphenyl in enhancing the lipophilicity of molecules⁶ it would appear that a more potent antiviral action of the phosphoramidate derivatives requires an aminoacid which has a methylene unit adjacent to its chiral center, such as L-leucine in spite of its steric encumbrance.⁷

Results and Discussion

The synthesis of phosphoramidate **15**, containing the adenine as nucleobase, was accomplished through two simple steps: i) the preparation of isoxazolidinyladenine **6**, and ii) the phosphoramidation of this modified nucleoside by means of methyl *N*-[chloro-(2,6-dimethoxyphenoxy)phosphoryl]-L-leucinate **14**.

Cis- β -nucleoside **6** was prepared by two different synthetic approaches of which the first is based on the nitrene 1,3-dipolar cycloaddition, and the other one on the Hilbert Jones nucleosidation performed on 5-*O*-acetylated isoxazolidine **8**.^{3a}

The cycloaddition reaction of *N*-glycosyl nitrene **2**,⁸ easily prepared *in situ* as previously described by us^{3c} and vinyl-*N*⁶-benzoyladenine **3**⁹ in chloroform solution were allowed to react in a sealed tube for 18 h. After evaporation and purification, three adducts **4a,b** and **5** (Scheme 1), in a 1:6:6 ratio, and 65% global yield, were obtained and separated. The diastereomeric ratio of the products was determined by ¹H-NMR spectroscopy on crude mixture, while the relative configuration was assigned by NOESY and T-Roesy spectra.



Scheme 1

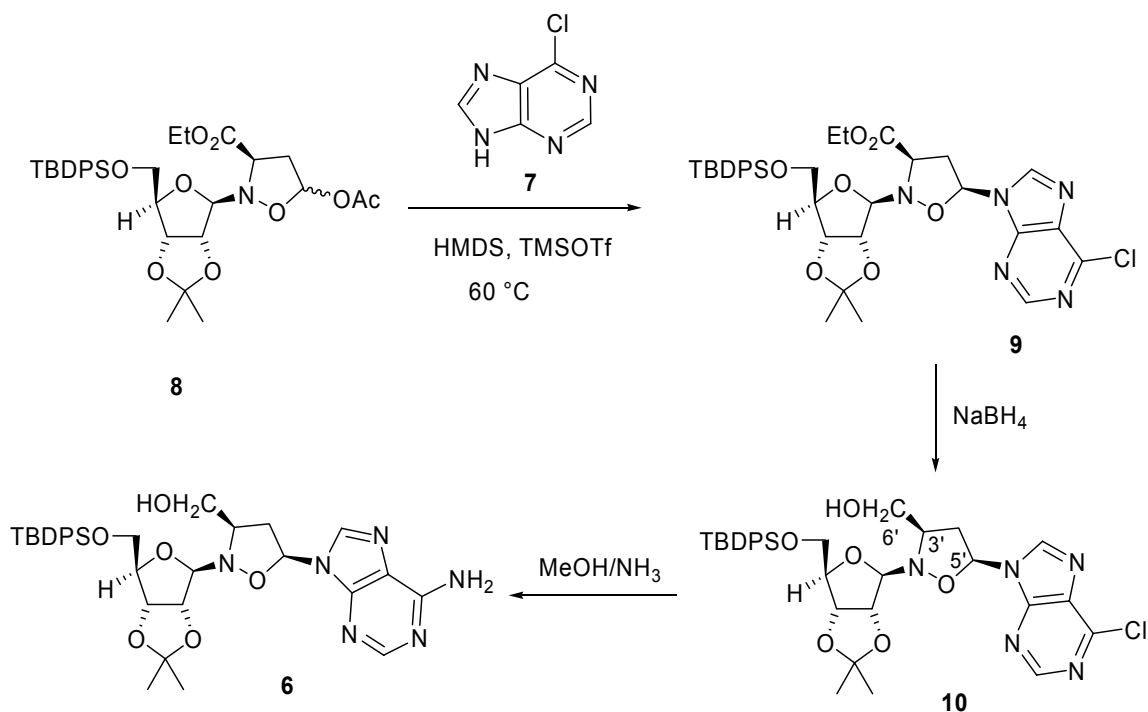
In particular, the irradiation of the H_{5'} proton in compound **5**, chosen as a reference compound, induced a positive NOE effect on the H_{4'b} proton (3.11 ppm); in turn, the irradiation of this latter H_{4'b} proton gave rise to an enhancement of the H_{3'} resonance. Moreover, the irradiation of the H_{1''} proton produced a strong positive effect on the H_{4''} proton, thus indicating

that all these protons are in a topological *cis* arrangement. As a confirmation, T-Roesy experiments reveal a NOE effect between H_{5'}–H_{3''} protons, and H_{1''}–H_{4'} protons.

Besides a complete diastereofacial selectivity, the cycloaddition reaction, therefore, shows a low *cis/trans* diastereoselectivity, which can be rationalized by assuming that the *E-endo* attack of the dipolarophile on the *re*-face of the nitrene is the preferred reaction pathway, because of secondary orbital interactions exerted by the purine ring of nucleobases. PM3 semi-empirical calculations confirm this result insofar as, an energy difference of 2.3 kcal/mol is observed in favour of the *re*-face attack with respect to the *si*-face one. This behaviour is also in agreement with DFT calculations on *C*-methoxycarbonyl-*N*-methyl nitrene with electron-rich dipolarophiles.¹⁰ On these basis, the absolute configuration of the cycloadducts **4b** and **4a,5** can be tentatively assigned as (3*R*,5*R*) and (3*R*,5*S*) respectively.

Compound **5** was then reduced and debenzoylated with NaBH₄ in dioxane/methanol 1:1 to afford 3'-hydroxymethyl derivative **6** in 75% yield (Scheme 1).

A valuable improvement of the synthetic strategy leading to **6** has been exploited through the reaction of 6-chloropurine **7** with an epimeric mixture of 5-*O*-acetyl-*N,O*-isoxazolidinyl derivatives **8** (*cis/trans*),^{3a} via the intermediate **9**, followed by NaBH₄ reduction to give **10** and finally by methanol/ammonia treatment (Scheme 2).



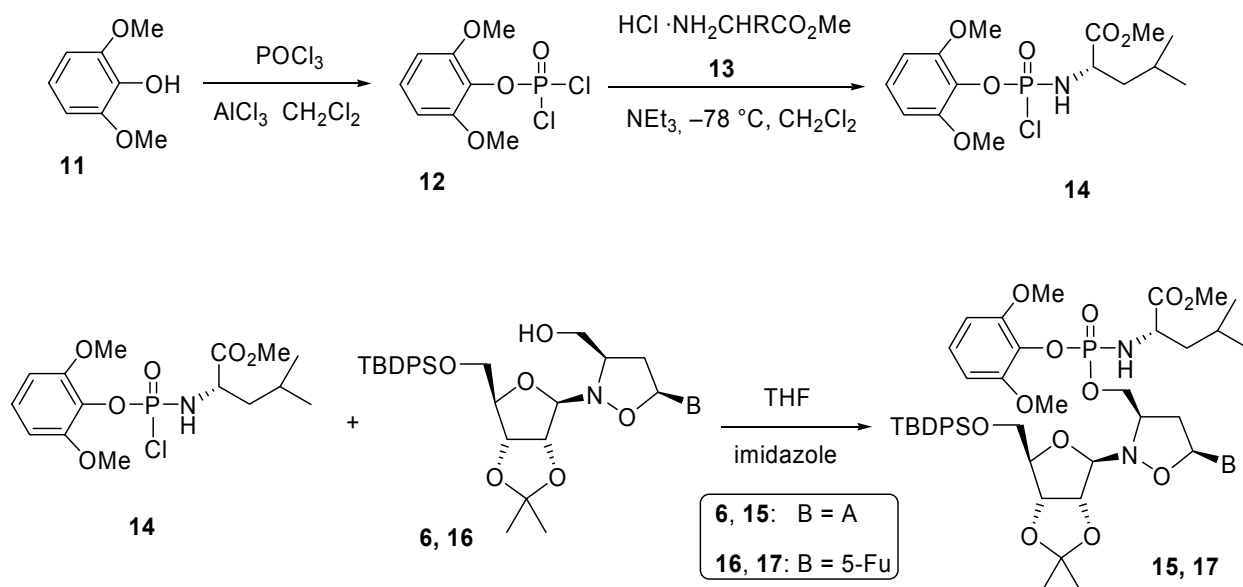
Scheme 2

In particular, 6-chloropurine **7** was refluxed with HMDS for 2 h; after removal of HMDS, the residue was treated with a dichloroethane solution of epimeric mixture of isoxazolidines **8** and TMSOTf in the presence of molecular sieves at 60 °C for 2 h to afford **9** in 75% yield.

According to similar nucleosidations performed at high temperature,¹¹ nucleoside **9** show to be the β -anomer as a result of its major thermodynamic stability in comparison with α -anomer.

Compound **9** was then reduced with NaBH₄ in dioxane/methanol (1:1) into **10** (83%) which was finally treated with a methanol solution of ammonia in a sealed tube at 80 °C overnight to give adenine nucleoside **6** (95%) (Scheme 2).

The synthetic approach to phosphoramidate **15**, was accomplished by means of the three simple steps illustrated in Scheme 3.



Scheme 3

2,6-Dimethoxyphenyl phosphorodichloridate **12** was prepared according to the procedure of Orloff *et al.*¹² by the reaction of **11** with POCl₃ in the presence of AlCl₃, and immediately used to prepare a diastereomeric mixture of **14** in 80 % yield by reaction with L-leucine methyl ester hydrochloride **13**. Diastereomeric mixture of **14** was then converted into **15**, as single diastereomer, in 57% yield by reaction with **6** in THF solution in the presence of imidazole at room temperature. Structures of phosphorochloridates **14** and phosphoroamidate **15** were assigned on the basis of ¹H-NMR data. In particular, as expected, the ¹H-NMR spectrum of **14** reveals the presence of duplicate peaks due to the presence of two diastereoisomers, in a 1:1 ratio, produced by nucleophilic reaction displacement of chlorine in **12** by **13**. On the contrary, the ¹H-NMR spectrum of **15** shows the presence of a single stereoisomer, whose formation is, probably, attributable to the bulky effect promoted by nucleoside moiety during the reaction of **14** with **6** at the phosphorus centre.

In order to valuate the antiviral activity of one pyridine and one purine phosphoroamidate derivatives, compound **17** as single stereoisomer was similarly prepared, in 60% yield, by reaction of **14** with **16**.^{3a}

Biological evaluations

The two *N,O*-nucleosides **6** and **16** and the corresponding phosphoramidate derivatives **15** and **17** were evaluated for their antiviral activity by measuring their ability to inhibit the reverse transcriptase of avian myeloblastosis virus and moloney murine leukaemia virus on a cell free assay along with respect to a reference antiviral compound such as AZT.^{4b} Moreover, the same compounds were tested in vitro for their cytotoxicity on Molt-3 cells. Compounds **15** and **17** completely inhibited the RT activity at 10 and 1 μM concentration, respectively, while compounds **6** and **16** are completely inactive at the higher tested concentration. Although compounds **15** and **17** inhibit the RT at concentrations 1000- and 100-fold, respectively, higher than that showed by AZT, these data show that the conversion of *N,O*-nucleosides into the corresponding phosphoramidates determines the switching from not active to active compounds (Table 1). No significant toxicity was observed at the higher tested concentration

Table 1. Inhibitory activity and Cytotoxicity for Compounds **6**, **15-17**

Comp.	AMV-RT ^a MRTCI, μM	MLV-RT ^a MRTCI, μM	CC ₅₀ ^b μM , Molt-3
AZT	$1 \cdot 10^{-2}$	$1 \cdot 10^{-2}$	12,14
6	>1000	>1000	>1000
15	10	10	>1000
16	>1000	>1000	>1000
17	1	1	>1000

^a MRTCI towards Reverse Transcriptase, determined as the minimal concentration required to completely inhibit reverse transcription evaluated in a cell free assay through an amplification program cycle. ^bConcentration of compound required to cause a 50% toxicity detected by a commercial viability assay (CellTiter 96[®] AQueous One Solution assay, Promega Co., Madison WI).

Conclusions

Two *N,O*-nucleosides containing 5-fluorouracil or adenine moiety and their corresponding derivatives with a phosphoramidate group at the C-5' position were synthesized and evaluated for their antiviral activity. The obtained results indicate that the introduction of the dimethoxyphenyl phosphate group, linked via nitrogen to L-leucine methyl ester, determine a switching in the activity of compounds allowing the possible speculation that the weak antiviral activity, sometimes showed by *N,O*-nucleosides, is linked to their inefficient conversion into the 5'-monophosphate forms, rather than to the lower affinity to viral polymerases. Therefore, the development of the phosphoramidate chemistry applied to *N,O*-nucleosides could improve the intracellular delivery with a concomitant overcome of the rate limiting first phosphorylation.

Experimental Section

General Procedures. Melting points were determined with a Kofler apparatus and are uncorrected. Elemental analyses were performed with a Perkin-Elmer elemental analyzer. NMR spectra were recorded at 200 or 500 MHz (^1H), at 50 or 125 MHz (^{13}C) and at 205 MHz (^{31}P) using CDCl_3 as solvent; chemical shifts are given in ppm from TMS as internal standard. All ^{31}P chemical shifts are quoted in ppm using H_3PO_4 as external reference.

Thin-layer chromatography was done on Merck silica gel 60-F₂₅₄ precoated aluminium plates. Preparative separations were made by flash column chromatography using Merck silica gel 0.063–0.200 mm and 0.035–0.070 mm. Chemical reagents were purchased from Aldrich except where noted and used without further purification. Solvent for chromatography were distilled at atmospheric pressure prior to use and dried using standard procedures. Isoxazolidines **4a,b** and **5**^{3a} and *N*⁶-benzoyl vinyladenine **13**⁹ were prepared according to literature methods.

Preparation of 6-amino-9-[(3*R*,5*S*)-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)- β -D-ribofuranosyl]-3-(hydroxymethyl)isoxazolidin-5-yl]-9*H*-purine (**6**).

Method A. A suspension containing vinyl-*N*⁶-benzoyladenine **3** (354 mg, 1.3 mmol), ribosyl hydroxylamine **1** (599 mg, 1.4 mmol) and ethyl glyoxalate (321 μL , 1.2 mmol; 50% in toluene) in chloroform (20 mL) was heated in a sealed vessel at 80 °C under stirring, until the ribosyl hydroxylamine was consumed (8 h). Removal of the solvent in vacuo affords a crude material, which was purified by flash chromatography to give a mixture of homochiral isoxazolidines **4a,b** and **5** (1:6:6, 65% global yield), which were purified by flash chromatography (cyclohexane/ethyl acetate 3:2) and then by HPLC with a linear gradient of 2-propanol (6–10%, 0–10 min, flow 3.5 mL/min) in *n*-hexane.

The first eluted product was ethyl (3*R*,5*S*)-5-[6-(benzoylamino)-9*H*-purin-9-yl]-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)- α -D-ribofuranosyl]-isoxazolidine-3-carboxylate **4a**.

Ethyl (3*R*,5*S*)-5-[6-(benzoylamino)-9*H*-purin-9-yl]-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)- α -D-ribofuranosyl]-isoxazolidine-3-carboxylate (4a**).** Yield 5 %; light yellow solid; mp 44–46 °C; $[\alpha]_{\text{D}}^{25} = -31.25$ (*c* 10.6, CHCl_3). ^1H NMR (CDCl_3 , 500 MHz) δ 1.08 (s, 9H), 1.25 (s, 3H), 1.27 (t, 3H, $J = 7.0$ Hz), 1.53 (s, 3H), 2.87 (ddd, 1H, $J = 2.5, 5.5, 14.0$ Hz, $\text{H}_{4\text{'a}}$), 3.01 (ddd, 1H, $J = 8.0, 10.0, 14.0$ Hz, $\text{H}_{4\text{'b}}$), 3.83 (d, 2H, $J = 6.0$ Hz, $\text{H}_{5\text{'a,b}}$), 4.23 (m, 4H), 4.49 (dd, 1H, $J = 3.5, 6.5$ Hz, $\text{H}_{3\text{'c}}$), 4.72 (dd, 1H, $J = 2.0, 6.5$ Hz, $\text{H}_{2\text{'c}}$), 5.10 (d, 1H, $J = 2.0$ Hz, $\text{H}_{1\text{'c}}$), 6.31 (dd, 1H, $J = 2.5, 8.0$ Hz, $\text{H}_{5\text{'d}}$), 7.35–7.74 (m, 13H), 8.03 (d, 2H, $J = 7.5$ Hz), 8.51 (s, 1H, H_8), 8.74 (s, 1H, H_2), 9.16 (bs, 1H, NH). ^{13}C NMR (CDCl_3 , 125 MHz) δ 60.3, 60.6, 62.1, 63.7, 64.3, 80.7, 81.7, 82.8, 86.6, 97.4, 113.2, 127.7, 127.7, 127.8, 128.7, 129.8, 129.8, 130.0, 132.7, 133.2, 133.3, 133.6, 135.5, 135.6. *Anal.* Calcd. for $\text{C}_{42}\text{H}_{48}\text{N}_6\text{O}_8\text{Si}$: C, 63.62; H, 6.10; N, 10.60. Found: C, 63.64; H, 6.07; N, 10.64.

The second eluted fraction was (3*R*,5*S*)-5-[6-(benzoylamino)-9*H*-purin-9-yl]-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)-β-*D*-ribofuranosyl]-isoxazolidine-3-carboxylate **5**.

(3*R*,5*S*)-5-[6-(Benzoylamino)-9*H*-purin-9-yl]-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)-β-*D*-ribofuranosyl]-isoxazolidine-3-carboxylate (5**)**. Yield 30%; white solid; mp 47–49 °C; $[\alpha]_D^{25} = +15.31$ (*c* 9.6, CHCl₃). ¹H NMR (CDCl₃, 500 MHz) δ 1.08 (s, 9H), 1.09 (t, 3H, *J* = 7.0 Hz), 1.46 (s, 3H), 1.53 (s, 3H), 2.96 (ddd, 1H, *J* = 3.0, 7.5, 14.0 Hz H_{4'a}), 3.11 (m, 1H, H_{4'b}), 3.75 (dd, 1H, *J* = 4.5, 11.5 Hz, H_{5''a}), 3.84 (dd, 1H, *J* = 6.5, 11.5 Hz, H_{5''b}), 4.00 (m, 2H), 4.20 (dd, 1H, *J* = 3.5, 9.5 Hz, H_{3'}), 4.26 (m, 1H, H_{4''}), 4.72 (dd, 1H, *J* = 2.5, 6.5 Hz, H_{2''}), 4.76 (dd, 1H, *J* = 1.5, 6.5 Hz, H_{3''}), 4.83 (d, 1H, *J* = 2.5 Hz, H_{1''}), 6.70 (dd, 1H, *J* = 3.0, 8.0 Hz, H_{5'}), 7.37-7.67 (m, 13H), 8.09 (d, 2H, *J* = 7.5 Hz), 8.60 (s, 1H, H₈), 8.79 (s, 1H, H₂), 9.29 (bs, 1H, NH). ¹³C NMR (CDCl₃, 125 MHz) δ 13.8, 19.1, 25.1, 26.7, 26.8, 38.6, 61.3, 61.8, 63.7, 80.0, 82.7, 83.1, 85.8, 100.3, 113.3, 122.6, 127.6, 127.7, 127.8, 128.6, 128.9, 129.8, 132.5, 132.7, 132.8, 133.5, 135.3, 135.4, 149.4, 151.5, 164.7, 170.6. *Anal.* Calcd. for C₄₂H₄₈N₆O₈Si: C, 63.62; H, 6.10; N, 10.60. Found: C, 63.65; H, 6.07; N, 10.62.

The third eluted product was ethyl (3*R*,5*R*)-5-[6-(benzoylamino)-9*H*-purin-9-yl]-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)-β-*D*-ribofuranos-yl]isoxazolidine-3-carboxylate **4b**.

Ethyl (3*R*,5*R*)-5-[6-(benzoylamino)-9*H*-purin-9-yl]-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)-β-*D*-ribofuranos-yl]isoxazolidine-3-carboxylate (4b**)**. Yield 30%; yellow oil; $[\alpha]_D^{25} = -27.04$ (*c* 8.6, CHCl₃). ¹H NMR (CDCl₃, 500 MHz) δ 1.06 (s, 9H), 1.14 (t, 3H, *J* = 7.0 Hz), 1.28 (s, 3H), 1.46 (s, 3H), 3.14 (ddd, 1H, *J* = 3.0, 7.0, 13.5 Hz, H_{4'a}), 3.27 (m, 1H, H_{4'b}), 3.75 (dd, 1H, *J* = 4.5, 11.0 Hz, H_{5''a}), 3.80 (dd, 1H, *J* = 7.0, 11.0, H_{5''b}), 4.02 (dq, 1H, *J* = 7.0, 11.0 Hz), 4.11 (dq, 1H, *J* = 7.0, 11.0 Hz), 4.24 (m, 1H, H_{4''}), 4.48 (dd, 1H, *J* = 3.0, 7.0 Hz, H_{3'}), 4.69 (dd, 1H, *J* = 2.5, 6.5 Hz, H_{2''}), 4.72 (dd, 1H, *J* = 2.5, 6.5 Hz, H_{3''}), 5.10 (d, 1H, *J* = 2.5 Hz, H_{1''}), 6.51 (t, 1H, *J* = 7.0 Hz, H_{5'}), 7.37-7.67 (m, 13H), 8.04 (d, 2H, *J* = 7.5 Hz), 8.25 (s, 1H, H₈), 8.77 (s, 1H, H₂), 9.25 (bs, 1H, NH). ¹³C NMR (CDCl₃, 125 MHz) δ 13.5, 13.8, 19.1, 19.5, 23.8, 25.1, 26.7, 36.3, 61.7, 62.1, 63.7, 81.2, 83.2, 85.4, 86.0, 99.1, 113.0, 123.4, 127.7, 127.8, 128.6, 128.8, 129.8, 132.6, 132.8, 132.9, 133.4, 135.3, 135.4, 149.5, 151.4, 164.7, 169.9. *Anal.* Calcd. for C₄₂H₄₈N₆O₈Si: C, 63.62; H, 6.10; N, 10.60. Found: C, 63.60; H, 6.12; N, 10.58.

A stirred solution of **5** (203 mg, 0.4 mmol) in a 1:1 methanol/dioxane mixture (50 mL), was added at 0 °C NaBH₄ (240 mg, 6 mmol) and the obtained mixture was stirred for 5 hours. At the end of this time the solvent was evaporated under reduced pressure, and the residue was then purified by flash chromatography (chloroform/methanol 85:15) to give **6**.

6-Amino-9-[(3*R*,5*S*)-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)-β-*D*-ribofuranosyl]-3-(hydroxymethyl)isoxazolidin-5-yl]-9*H*-purine (6**)**. Yield 75%; foam solid; $[\alpha]_D^{25} = -2.44$ (*c* 3.0, CHCl₃). ¹H NMR (CDCl₃, 200 MHz) δ 1.08 (s, 9H), 1.29 (s, 3H), 1.51 (s, 3H), 2.10 (bs, 1H, OH), 2.75 (m, 1H, H_{4'a}), 2.97 (m, 1H, H_{4'b}), 3.61 (m, 1H, H_{3'}), 3.68 (m, 1H, H_{6'a}), 3.80 (m, 3H, H_{5''a}, H_{5''b}, H_{6'b}), 4.25 (m, 1H, H_{4''}), 4.56 (m, 1H, H_{3''}), 4.68 (m, 1H, H_{2''}), 4.86 (s, 1H, H_{1''}), 5.92 (d, 2H, NH₂), 6.47 (m, 1H, H_{5'}), 7.40–7.67 (m, 10H), 8.16 (s, 1H, H₈),

8.33 (s, 1H, H₂). ¹³C NMR (CDCl₃, 50 MHz) δ 19.2, 25.1, 26.9, 29.7, 37.3, 62.8, 63.4, 64.4, 80.7, 82.9, 83.3, 85.8, 101.2, 113.4, 127.9, 130.0, 132.7, 132.9, 135.6, 149.6, 151.4, 152.0, 155.5.

Anal. Calcd. for C₃₃H₄₂N₆O₆Si: C, 61.28; H, 6.54; N, 12.99. Found: C, 60.99; H, 6.52; N, 12.77.

Method B: A suspension of 6-chloropurine **7** (750 mg, 4.8 mmol) in HMDS (4.6 g, 28.9 mmol) was refluxed to 110 °C for 2 hours under stirring. After this time the homogeneous mixture was concentrated and the solid residue was reconcentrated from toluene (20 mL) and placed under vacuum for 1 h. The resulting solid was combined with a mixture of isoxazolidines **8** (1.0 g, 1.6 mmol) and dissolved in anhydrous 1,2-dichloroethane (13 mL). Powdered 4Å the molecular sieves (0.55 g) were added and the mixture stirred for 15 minutes. TMSOTf (0.58 mL, 3.2 mmol) was added and the reaction was then heated to 60 °C for 2 hours, cooled and quenched by the careful addition of saturated NaHCO₃ solution. Ethyl acetate (3×10 mL), was added and the mixture was filtered through a sintered glass funnel. The filtrate was extracted with ethyl acetate (3×10 mL) and the combined organic layers were washed with brine, dried (Na₂SO₄), filtered and concentrated. The residue was purified by flash chromatography (chloroform/methanol 85:15) to afford ethyl (3*R*,5*S*)-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)-β-D-ribofuranosyl]-5-(6-chloro-9*H*-purin-9-yl)isoxazolidine-3-carboxylate **9**.

Ethyl (3*R*,5*S*)-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)-β-D-ribofuranosyl]-5-(6-chloro-9*H*-purin-9-yl)isoxazolidine-3-carboxylate (9**).** Yield 75 %; yellow oil; [α]_D²⁵ = +17.0 (*c* = 0.56, CH₂Cl₂). ¹H NMR (CDCl₃, 500 MHz) δ 0.91 (t, 3H, *J* = 7.2, Hz), 1.11 (s, 9H), 1.34 (s, 3H), 1.53 (s, 3H), 2.95 (ddd, 1H, *J* = 3.2, 3.3, 13.9 Hz, H_{4'a}), 3.10 (ddd, 1H, *J* = 7.8, 9.5, 13.9 Hz, H_{4'b}), 3.73 (dd, 1H, *J* = 4.3, 11.0 Hz, H_{5'a}), 3.82 (dd, 1H, *J* = 6.8, 11.0 Hz, H_{5'b}), 3.97 (d, 2H, *J* = 7.2 Hz), 4.18 (dd, 1H, *J* = 3.3, 9.5 Hz, H_{3'}), 4.27 (ddd, 1H, *J* = 2.0, 4.3, 6.8 Hz, H_{4''}), 4.70 (dd, 1H, *J* = 2.6, 6.3 Hz, H_{2''}), 4.76 (dd, 1H, *J* = 2.0, 6.3 Hz, H_{3''}), 4.81 (d, 1H, *J* = 2.6 Hz, H_{1''}), 6.67 (dd, 1H, *J* = 3.2, 7.8 Hz, H_{5'}), 7.38–7.67 (m, 10H), 8.69 (s, H₈), 8.74 (s, H₂). ¹³C NMR (CDCl₃, 125 MHz) δ 13.9, 19.2, 25.2, 26.8, 26.9, 38.9, 61.5, 62.0, 63.8, 81.2, 82.9, 83.4, 86.0, 100.5, 113.5, 127.8, 127.9, 128.0, 130.0, 131.7, 132.8, 133.0, 135.4, 135.6, 140.3, 151.1, 152.0, 170.5. *Anal.* Calcd. for C₃₅H₄₂ClN₅O₇Si: C, 59.35; H, 5.98; N, 9.89. Found: C, 59.37; H, 5.99; N, 9.91.

A stirred solution of **9** (450 mg, 0.65 mmol) in a 1:1 methanol/dioxane mixture (36 mL), was added at 0 °C NaBH₄ (120 mg, 3 mmol) and the obtained mixture was stirred for 5 hours. At the end of this time the solvent was evaporated under reduced pressure, and the residue was then purified by flash chromatography (cyclohexane/ethyl acetate 1:1) to give 9-[(3*R*,5*S*)-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)-β-D-ribofuranosyl]-3-(hydroxymethyl)isoxazolidin-5-yl]-6-chloro-9*H*-purine **10**.

9-[(3*R*,5*S*)-2-[5-*O*-[*tert*-Butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)-β-D-ribofuranosyl]-3-(hydroxymethyl)isoxazolidin-5-yl]-6-chloro-9*H*-purine (10**).** Yield 83%; amorphous solid; [α]_D²⁵ = +17.7 (*c* = 0.25, CH₂Cl₂). ¹H NMR (CDCl₃, 500 MHz) δ 1.12 (s, 9H), 1.32 (s, 3H), 1.55 (s, 3H), 2.73 (ddd, 1H, *J* = 2.5, 6.5, 13.7 Hz, H_{4'a}), 3.08 (ddd, 1H, *J* = 5.3, 8.6, 13.7 Hz, H_{4'b}), 3.17 (bt, 1H, OH), 3.47 (m, 1H, H_{3'}), 3.71 (m, 1H, H_{6'a}), 3.75 (dd, 1H, *J* = 4.5, 11.5 Hz, H_{5'a}), 3.81 (dd, 1H, *J* = 6.8, 11.5 Hz, H_{5'b}), 3.96 (m, 1H, H_{6'b}), 4.26 (ddd, 1H, *J* = 1.7, 4.5, 6.8 Hz,

H₄'), 4.47 (dd, 1H, $J = 1.7, 6.2$ Hz, H₃'), 4.64 (dd, 1H, $J = 2.5, 6.2$ Hz, H₂'), 4.82 (d, 1H, $J = 2.5$ Hz, H₁'), 6.56 (dd, 1H, $J = 2.5, 8.6$ Hz, H₅'), 7.40–7.68 (m, 10H), 8.64 (s, 1H, H₈), 8.74 (s, 1H, H₂). ¹³C NMR (CDCl₃, 125 MHz) δ 19.2, 25.0, 26.8, 29.7, 38.4, 62.1, 64.7, 65.0, 80.7, 82.2, 83.2, 86.3, 102.0, 113.4, 128.0, 130.2, 131.7, 132.8, 135.6, 151.2, 151.4, 152.0, 152.0. *Anal.* Calc for C₃₃H₄₀ClN₅O₆Si: C, 59.49; H, 6.05; N, 10.51. Found: C, 59.45; H, 6.03; N, 10.53.

A solution of **10** (390 mg, 0.59 mmol) in methanol (3 mL) was saturated with ammonia (ca 7% w/v). The resulting solution was stirred at 80 °C for 16 h, then concentrated under reduce pressure and purified by flash chromatography (MeOH/CH₂Cl₂ 2–5%) to give **6** in 95% yield.

General procedure for preparation of phosphoramidates **15** and **17**

A mixture of aluminum chloride (0.05 g 0.36 mmol) and phosphoryl chloride (5.6 mL 60.4 mmol) was added 2,6-dimethoxyphenol **11** (1.5 g, 9.73 mmol) dropwise and refluxed for 6 hours under stirring. At end of this time the reaction mixture was evaporated to dryness to afford 2,6-dimethoxyphenyl phosphorodichloridate (**12**) as a yellow oil pure enough for the next transformation.

2,6-Dimethoxyphenyl phosphorodichloridate (12). ¹H NMR (CDCl₃, 200 MHz) δ 3.86 (s, 6H), 6.62 (m, 2H), 7.16 (m, 1H). ¹³C NMR (CDCl₃, 50 MHz) δ 55.9, 104.7 (d, $J = 2.5$ Hz), 126.9 (d, $J = 3.1$ Hz), 140.2, 151.5 (d, $J = 5.0$ Hz).

A solution of triethylamine (0.1 mL, 0.55 mmol) in anhydrous dichloromethane (2mL) was added dropwise with vigorous stirring to a solution of L-leucine methyl ester hydrochloride (**13**) (50.0 mg, 0.28 mmol) and **12** (0.3 mmol) in anhydrous dichloromethane (2mL) at –78 °C. The reaction mixture was slowly warmed to ambient temperature with stirring for 6 h and the solvent was then removed in vacuum. The residue was treated with ethyl acetate (2mL), the mixture filtered, and the filtrate evaporated in vacuum to yield methyl *N*-[chloro(2,6-dimethoxyphenoxy)phosphoryl]-L-leucinate **14**.

Methyl *N*-[chloro(2,6-dimethoxyphenoxy)phosphoryl]-L-leucinate (14). Yield 98%; yellow oil. ³¹P NMR (CDCl₃, 205 MHz) δ 5.34, 5.75. ¹H NMR (CDCl₃, 200 MHz) δ 0.93–0.95 (m, 6H), 1.61–1.87 (m, 3H), 3.71 (s, 3H), 3.79 (s, 6H), 4.01–4.55 (m, 2H), 6.52–6.95 (m, 2H), 7.06–7.14 (m, 1H). ¹³C NMR (CDCl₃, 50 MHz) δ 22.5, 24.0, 39.3, 44.1, 53.0, 56.0, 104.9 (d, $J = 2.0$ Hz), 124.8, 129.8, 152.0 (d, $J = 3.4$ Hz), 169.8. *Anal.* Calcd. for C₁₅H₂₃ClNO₆P: C, 47.44; H, 6.10; N, 3.69. Found: C, 47.45; H, 6.09; N, 3.68.

A THF (6 mL) solution of **6**, or **16** (0.11 mmol), **14** (150 mg, 0.38 mmol) and imidazole (460 mg, 0.67 mmol) was stirred for 12 h, at room temperature. The solvent was removed under vacuum, and the residue was dissolved in dichloromethane (5 mL), and extracted with saturated sodium bicarbonate solution, and water. The organic phase was dried over sodium sulphate, filtered, and evaporated to dryness. The residue was purified by radial chromatography CH₂Cl₂/MeOH 1:10 ($R_f = 0.2$) to give β - phosphoramidates **15**, **17**.

Methyl *N*-[({(3*R*,5*S*)-5-(6-amino-9*H*-purin-9-yl)-2-[5-*O*-[*tert*-butyl(diphenyl)silyl]-2,3-*O*-(1-methylethylidene)- β -D-ribofuranosyl]isoxazolidin-3-yl}methoxy)(2,6-dimethoxyphenoxy)phosphoryl]-L-leucinate (15). Yield 57%; amorphous solid. ³¹P NMR

(CDCl₃, 205 MHz) δ 4.35. ¹H NMR (CDCl₃, 200 MHz) δ 0.89 (6H), 1.09 (s, 9H), 1.29 (s, 3H), 1.53 (s, 3H), 1.55 (m, 2H), 1.77 (m, 1H), 2.74 (m, 1H, H_{4'a}), 2.97 (m, 1H, H_{4'b}), 3.57–3.88 (m, 14H), 4.14 (m, 1H), 4.25 (m, 1H), 4.55 (d, 1H, J = 6.0 Hz, H_{3''}), 4.77 (dd, 1H, J = 2.5, 6.0 Hz, H_{2''}), 4.84 (d, 1H, J = 2.5 Hz, H_{1''}), 5.96 (bs, NH₂), 6.48 (m, 3H, H_{5'}, H_{3'''}, H_{5'''}), 6.89 (t, 1H, J = 8.5 Hz, H_{4'''}), 7.38–7.46 (m, 6H), 7.66–7.67 (m, 4H), 8.16 (s, 1H, H₂), 8.31 (s, 1H, H₈). ¹³C NMR (CDCl₃, 50 MHz) δ 19.2, 22.4, 22.7, 24.5, 25.2, 26.9, 27.0, 29.7, 38.5, 44.6, 51.6, 53.7, 55.9, 62.8, 64.9, 66.2, 80.8, 83.0, 83.2, 85.9, 101.2, 105.4, 113.5, 118.8, 119.7, 123.3, 127.8 (d, J = 7.5 Hz), 130.0 (d, J = 4.6 Hz), 132.9, 135.6 (d, J = 4.5 Hz), 139.5, 149.6, 152.8, 155.5, 175.7. *Anal.* Calcd. for C₄₈H₆₄N₇O₁₂PSi: C, 58.23; H, 6.52; N, 9.90. Found: C, 58.29; H, 6.50; N, 9.87.

Methyl N-[[[(3RS,5SR)-2-(5-O-tert-butyldiphenilsilyl)-2,3-O-isopropyliden- β -D-ribofuranosyl)-5-[5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl]isoxazolidin-3-yl]methoxy}(2,6-dimethoxyphenoxy)phosphoryl]-L-leucinate (17). Yield 60%; white solid; p.f. 65–66 °C, [α]_D²⁵ +11.1 (c = 0.90, CH₂Cl₂). ³¹P NMR (CDCl₃, 205 MHz) δ 4.87. ¹H NMR (CDCl₃, 500 MHz) δ 0.88 (d, 6H, J = 6.5 Hz), 1.06 (s, 9H), 1.30 (s, 3H), 1.50 (s, 3H), 1.54 (m, 2H), 1.76 (m, 1H), 2.26 (ddd, 1H, J = 4.0, 7.0, 14.0 Hz, H_{4'a}), 2.89 (ddd, 1H, J = 5.0, 8.5, 14.0 Hz, H_{4'b}), 3.35 (dddd, 1H, J = 3.0, 4.0, 4.5, 8.5 Hz, H_{3'}), 3.60 (m, 10H), 3.83 (m, 3H), 4.14 (m, 1H), 4.25 (m, 1H), 4.56 (dd, 1H, J = 3.0, 5.5 Hz, H_{3''}), 4.74 (m, 2H), 6.26 (dd, 1H, J = 5.0, 7.0 Hz, H_{5'}), 6.43 (d, 2H, J = 8.5 Hz, H_{3'''}, H_{5'''}), 6.85 (t, 1H, J = 8.5 Hz, H_{4'''}), 7.38–7.44 (m, 6H), 7.64–7.65 (m, 4H), 7.94 (d, 1H, J = 6.0 Hz). ¹³C NMR (CDCl₃, 50 MHz) δ 19.1, 22.4, 22.6, 24.5, 25.0, 25.1, 26.8, 38.5, 44.4, 51.6, 53.8, 55.7, 64.4, 62.2, 64.5, 80.9, 82.8, 83.4, 86.2, 101.6, 105.2, 113.4, 126.2, 127.9, 132.2, 132.7, 135.5, 140.3, 149.1, 152.7, 157.1 (d, J = 1.2 Hz), 175.8. *Anal.* Calcd. for C₄₃H₆₀N₂O₁₂PSi: C, 60.33; H, 7.06; N, 3.27. Found: C, 59.32; H, 7.05; N, 3.26.

Biological assays

Evaluation of toxicity. Toxicity was evaluated by a commercial viability assay (CellTiter 96^R Aqueous One Solution Assay, Promega Co., Madison WI), according to manufacturer's instructions. This assay is based on the principle that cells, at death, rapidly lose the ability to reduce MTS tetrazolium. Briefly, Molt-3 and Vero cells were cultured in optimal culture conditions for 20 h in 96-well plates, in the absence of the compounds or in their presence, at concentrations ranging from 1 to 1000 μ M. At the end of the incubation time, the MTS-tetrazolium-based reagent was added to each well. After a further incubation of one hour at 37 °C in a humidified, 5% CO₂ atmosphere, the absorbance of the samples was recorded at 490 nm using a 96-well spectrophotometer. The assays were performed in triplicate. The cytotoxic concentrations (CC₅₀) were calculated as the concentrations of the compounds required to cause 50% reduction of absorbance values.

Reverse-transcriptase inhibition assay. The capacity of the described compounds to inhibit avian myeloblastosis virus RT (Promega Co.) activity was investigated by evaluating their activity towards cDNA. Generation from an RNA template using a cell-free RT reaction assay, was recently described by us.^{4b} This assay is based on routinely adopted RT-PCR procedures.

The reactions were performed in the presence or in the absence of the activated compounds, at the concentrations ranging from 10^{-2} to 10^3 μM for 1 h at 37 °C. The newly synthesized compounds and AZT (Sigma–Aldrich Co.) were activated in vitro through incubation with a crude extract from 1×10^6 PBMCs, that served as enzyme supplier for phosphorylation processes. The effective concentrations (EC_{100}) were determined as the concentrations of the compounds required to cause complete (100%) inhibition of RTPCR

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