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Selective inhibition of Human Immunodeficiency Virus type 1 (HIV-1) by a novel family of tricyclic nucleosides

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ABSTRACT

Nucleoside 1, with an unusual tricyclic carbohydrate moiety, specifically inhibits HIV-1 replication while being inactive against HIV-2 or other (retro) viruses. In an attempt to increase the inhibitory efficacy against HIV-1, and to further explore the structural features required for anti-HIV-1 activity, different types of modifications have been carried out on this prototype compound. These include substitution of the ethoxy group at the C-4″ position by alkoxy groups of different length, branching, conformational freedom or functionalization. In addition, the 4″-ethoxy group has been removed or substituted by other functional groups. The role of the *tert*-butyldimethylsilyl (TBDMS) group at the 2′ position has also been studied by preparing the corresponding 2′-deprotected derivative or by replacing it by other silyl (*tert*-hexyldimethylsilyl) or acyl (acetyl) moieties. Finally, the thymine of the prototype compound has been replaced by *N*-3-methylthymine, uracil or thiophenyl. Some of these compounds were endowed with a 6- to 7-fold higher selectivity than the prototype 1. The tricyclic nucleosides here described represent a novel type of selective anti HIV-1 inhibitors, targeted at the HIV-1-encoded reverse transcriptase.

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1. Introduction

In the last few years the design and synthesis of nucleosides with bicyclic carbohydrate moieties have attracted considerable attention to restrict the conformational flexibility of the nucleoside into determined conformations which were ideal for nucleic acid recognition (Mathé and Périgaud, 2008; Kaur et al., 2007; Jepsen et al., 2004; Kumar et al., 2006; Gagneron et al., 2005). On the other hand, a number of structurally diverse bicyclic nucleosides have been synthesized in order to identify conformational preferences of some receptors and enzymes involved in the metabolism or polymerization of nucleosides and to study the interaction of such compounds with their enzymatic targets (Marquez et al., 2006; Comin et al., 2003). Some bicyclic nucleosides have shown to moderately inhibit HIV replication (Chuanzheng and Chattopadhyaya, 2009; Díaz-Rodríguez et al., 2009; Tronchet et al., 1994, 1995) although none of them are specific for HIV-1.

In order to further restrict the conformation of the bicyclic nucleosides, the introduction of additional bridges generating tricyclic nucleosides was also undertaken (Neogi et al., 2006; Ravn et al., 2001). However, no biological evaluation against HIV of these compounds have been reported so far.

As a part of an ongoing work in our laboratories directed to the synthesis and biological evaluation of bi- and tricyclic nucleosides, we found that the tricyclic nucleoside **1** (Bonache et al., 2004) (Fig. 1) inhibited HIV-1 replication while being inactive against HIV-2 or other (retro)viruses. The selectivity displayed by this compound prompted us to perform systematic modifications on this prototype with the aim of determining the minimal structural features essential for HIV-1 inhibition. This structure–activity study constitutes the subject matter of this paper.

First, the ethoxy group at the C-4″ position has been replaced by alkoxy groups of different length, branching, conformational freedom or functionalization. In addition, the ethoxy group has been removed or substituted by other functional groups. Next, the role of the *tert*-butyldimethylsilyl (TBDMS) group at the 2′ position has been studied by preparing the corresponding 2′-deprotected derivative or by replacing it by other silyl (*tert*-hexyldimethylsilyl) or lipophilic (acetyl) moieties.

Modifications on the nucleobase have been also examined. Thus, the *N*-3 methyl analog of **1** has been prepared and the thymine of the prototype compound has been replaced by uracil or by an aromatic moiety such as thiophenyl.

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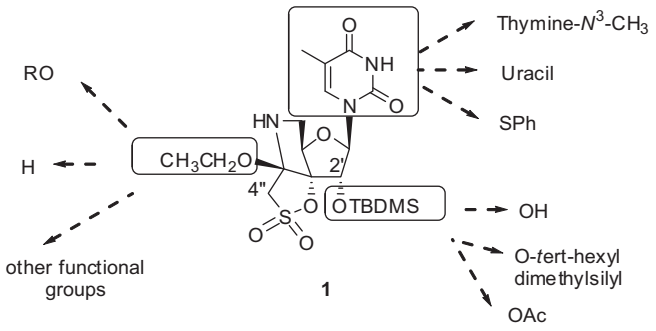


Fig. 1. Modifications carried out on the tricyclic nucleoside **1.**

2. Materials and methods: chemistry

2.1. General methods

Commercial reagents and solvents were used as received from the suppliers without further purification unless otherwise stated. Dichloromethane and acetonitrile were dried prior to use by distillation from CaH_2 and stored over Linde type activated 4 Å molecular sieves as described.

Analytical thin-layer chromatography (TLC) was performed on aluminum plates pre-coated with silica gel 60 (F_{254}, 0.25 mm). Products were visualized from the TLC by exposure to ultraviolet light (254 nm) or by heating on a hot plate (aprox. 200 °C), directly or after treatment with a 5% solution of phosphomolybdic acid or vanillin in ethanol. Separations were performed by preparative flash column chromatography on silica gel 60 (PF_{254}, 230–400 mesh) or by preparative Centrifugal Circular Thin-Layer Chromatography (CCTLC) (Kiesegel 60 PF_{254} gipshaltig, layer thickness of 1 mm, flow rate 4 mL min^{-1}). Mass spectra were registered in a quadrupole mass spectrometer 1100 equipped with an electrospray source.

2.1.1. NMR procedures

Monodimensional ^1H and ^{13}C NMR spectra were obtained using standard conditions and were registered in $CDCl_3$ or acetone-d_6 as solvents with 300, 400 or 500 NMR spectrometers. Solvents were used with higher deuteration degree than 99.5% and were filtered through a pad of neutral alumina in order to eliminate traces of water and acid prior to use. Chemical shifts for protons are reported in parts per million (ppm) downfield from tetramethylsilane referred to protonated residual peaks or specific signals due to deuterated solvents as internal references. Chemical shifts for proton-decoupled carbons are reported in parts per million (ppm) referenced to the deuterated solvents as internal standards.

Carbon and proton assignments were based on DEPT, HSQC, HMBC and NOE-differential experiments.

2.2. Biological methods

2.2.1. Activity assay of test compounds against HIV-1 and HIV-2 in cell cultures

The antiviral (i.e. HIV-1 and HIV-2) activity of the compounds was independently investigated in parallel in two different (CEM and MT-4) T-lymphocyte cell lines. The MT-4 cells are transformed by HTLV-I and exquisitely sensitive to HIV-1 infection whereas the CEM cells were not transformed by HTLV-I. The virus input for productive infection of MT-4 cell cultures is much lower (>100-fold) than required for CEM cell cultures. Therefore, it is of interest to reveal the antiviral activity of the test compounds in both cell lines. A total number of 4×10^5 CEM or 3×10^5 MT-4 cells per milliliter were infected with HIV-1(IIIB) or HIV-2 (ROD) at ~100 $CCID_{50}$ (50% cell culture infective dose) per milliliter of cell suspension. Then an amount of 100 μl of the infected cell suspension was transferred to microtiter plate wells and mixed with 100 μl of the appropriate dilutions of the test compounds. Giant cell formation (CEM) and HIV-induced cytopathicity (MT-4) was recorded microscopically (CEM) and by trypan blue dye exclusion (MT-4) in the HIV-infected cell cultures after 4 days (CEM) or 5 days (MT-4). The 50% effective concentration (EC_{50}) of the test compounds was defined as the compound concentration required to inhibit virus-induced cytopathicity (CEM) or to reduce cell viability (MT-4) by 50%. The 50% cytostatic concentration (CC_{50}) was defined as the compound concentration required to inhibit CEM cell proliferation by 50%.

2.2.2. Reverse transcriptase assay

Recombinant wild type p66/p51 HIV-1 RT was expressed and purified as described (Auwerx et al., 2002). The RT assay is performed with the EnzCheck reverse transcriptase assay kit (Molecular Probes, Invitrogen), as described by the manufacturer. The assay is based on the dsDNA quantitation reagent PicoGreen. This reagent shows a pronounced increase in fluorescence signal upon binding to dsDNA or RNA-DNA heteroduplexes. Single-stranded nucleic acids generate only minor fluorescence signal enhancement when a sufficiently high dye:basepair ratio is applied (Singer et al., 1997). This condition is met in the assay.

A poly(rA) template of approximately 350 bases long, and an oligo(dT)16 primer, are annealed in a molar ratio of 1:1.2 (60 min. at room temperature). Fifty-two ng of the RNA/DNA is brought into each well of a 96-well plate in a volume of 20 μl polymerization buffer (60 mM Tris–HCl, 60 mM KCl, 8 mM $MgCl_2$, 13 mM DTT, 100 μM dTTP, pH 8.1). 5 μl of RT enzyme solution, diluted to a suitable concentration in enzyme dilution buffer (50 mM Tris–HCl, 20% glycerol, 2 mM DTT, pH 7.6), is added. The reactions are incubated at 25 °C for 40 min and then stopped by the addition of EDTA (15 mM fc). Heteroduplexes are then detected by addition of PicoGreen. Signals are read using an excitation wavelength of 490 nm and emission detection at 523 nm using a spectrofluorometer (Safire2, Tecan). To test the activity of compounds against RT, 1 μl of compound in DMSO is added to each well before the addition of RT enzyme solution. Control wells without compound contain the same amount of DMSO. Results are expressed as relative fluorescence i.e. the fluorescence signal of the reaction mix with compound divided by the signal of the same reaction mix without compound.

2.2.3. RNase H assay

The RNase H assay was developed also employing the dsDNA quantitation reagent PicoGreen (Parniak et al., 2003). A RNA/DNA heteroduplex is formed by annealing a 40 bases long RNA oligonucleotide (5′-CCAGCAGGAAACAGCUAUGACGAUCUGAGCCUGGGAGCU-3′) and 120 bases long DNA oligonucleotide (5′-AGCTC CCAGGCTCAGATCGTCATAGCTGTTTCCTGCTGGCAGCTCCCAGGCTCAGATCGTCATAGCTGTTTCCTGCTGGCAGCTCCCAGGCTCAGATCGTCATAGCTGTTTCCTGCTGGC-3′) in a 4:1 M ratio.

An amount of 76 ng of the annealed complex is brought into each well of a 96-well plate in a volume of 20 μl RNase H buffer (60 mM Tris–HCl, 60 mM KCl, 8 mM $MgCl_2$, 13 mM DTT, pH 8.1) and 5 μl of suitably diluted RT solution is added. Reactions are stopped after 60 min at 25 °C by the addition of EDTA (15 mM fc). PicoGreen is then added to measure the amount of heteroduplexes and thus the decrease in signal upon RNA hydrolysis in the presence of enzyme activity and signals are read as described for the reverse transcriptase assay. The results are expressed relative to the amount of heteroduplexes measured in a negative

control sample without RT enzyme, after subtraction of the background signal obtained with only ssDNA.

To test compounds for activity against RNase H, 1 μl of compound in DMSO is added to each well before the addition of RT enzyme solution. Control wells without compound contain the same amount of DMSO. Positive compounds were tested for autofluorescence in a separate test.

2.2.4. Drug resistance studies

Compound **24** was exposed to HIV-1(III_B)-infected CEM cell cultures in a dose-escalating manner (starting at 40 μM). The virus-infected cultures were subcultured every 5 days by transferring 20 μl virus-infected cell suspension to fresh 180 μl CEM cell cultures containing new test compound. As soon as a full cytopathic effect was recorded the compound dose was increased for the next subcultivation (i.e. 100, 200, 400 μM). After the 6th passage, the cell cultures were split in two parallel subcultivations, transferring either 20 μl culture medium supernatant or 20 μl cell suspension to the next fresh cell cultures. At the 10th subcultivation, when in both cultures 400 μM test compound was reached, genotypic (RT gene) and phenotypic (drug sensitivity) characterization of the virus was performed according to previously reported procedures (Balzarini et al., 1993b).

3. Results and discussion

3.1. Chemical results

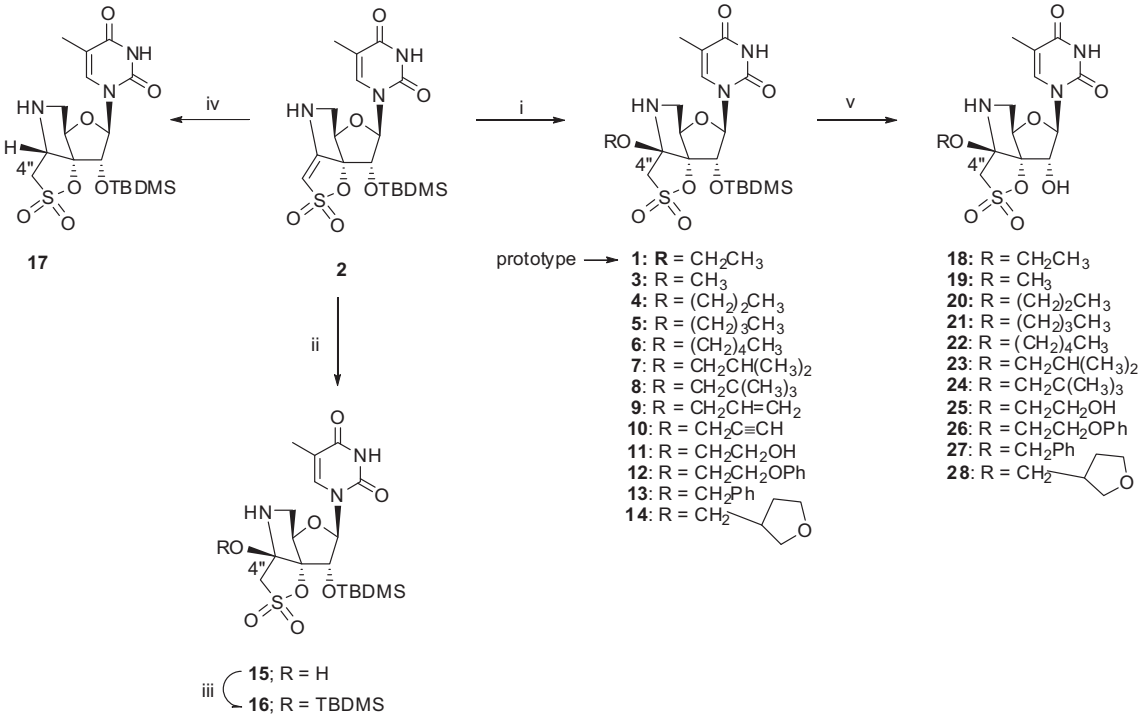
We studied first the effect of the substitution of the ethoxy moiety of the prototype compound **1** by flexible alkoxy moieties of different length and branching. These compounds were readily prepared in one step by reaction of **2** (Bonache et al., 2004) with the corresponding alcohol in refluxing acetonitrile (Scheme 1). Thus, reaction of different primary alcohols such as methanol, ethanol, propanol, butanol, pentanol, isobutyl alcohol and 2,2-dimethyl-1-propanol afforded the corresponding tricyclic derivatives: **3** (58%), the prototype **1** (70%), **4** (60%) (Bonache et al., 2004), **5** (78%), **6** (79%) (Bonache et al., 2004), **7** (72%) (Bonache et al., 2004) and **8** (83%) (Bonache et al., 2004) in satisfactory yields (58–83%).

Also, compounds **9** (80%) and **10** (76%) with a double or triple bond, respectively were prepared (Scheme 1). On the other hand, the functionalization of the alkoxy moiety was studied by preparing compounds **11** (74%) and **12** (67%), with an hydroxy or phenoxy moiety at the terminal position of the alkoxy moiety, or compounds **13** (80%) or **14** (67%) with benzyloxy or tetrahydrofuranylmethoxy groups at the C-4″ position (Scheme 1). These compounds were prepared following a similar procedure to that described for the synthesis of **1**. Thus, the reaction of **2** with 2-propen-1-ol, 2-propyn-1-ol, 1,2-ethanediol, 2-phenoxy-1-ethanol, benzyl alcohol and tetrahydro-2-furanmethanol in refluxing acetonitrile afforded the corresponding tricyclic nucleosides **9–14** in high yields (67–80%).

In addition, the 4″-*O*-*tert*-butyldimethylsilyl derivative **16** was prepared in 73% yield by treatment of the 4″-hydroxy derivative **15** (Bonache et al., 2009) with *tert*-butyldimethylsilyl chloride in pyridine at room temperature (Scheme 1).

As it was previously determined for the prototype **1**, these transformations proceeded with total regio- and stereoselectivity. The geometry of the new stereocentre created on C-4″ in this series of compounds was unequivocally determined as *S* on the basis of NOE difference experiments.

Compound **17** in which the ethoxy moiety at the C-4″ position has been removed was also prepared (Scheme 1). The synthesis of this compound was attempted first by hydrogenation of **2** (Bonache et al., 2004) using 10% palladium on charcoal as catalyst. However, under these conditions, the starting compound **2** remained unchanged, even upon prolonged reaction times. Finally, when platinum oxide was used as catalyst, compound **17** was obtained in 30% yield.



Scheme 1. Synthesis of tricyclic nucleosides **1** and **3–28**. *Reagents and conditions:* (i) ROH, CH_3CN, 80 °C (ii) H_2O, CH_3CN/AcOH (pH 5–6), 80 °C (iii) $Si(Me)_2tButCl$, pyridine, rt (iv) H_2/PtO_2, AcOEt (v) NH_4F, MeOH, rt.

A NOE difference experiment carried out on **17**, showed that the signal at δ 4.54 ppm, corresponding to the H-4″ proton, correlates with the signal of the H-2′ proton of the sugar, at δ 4.90 ppm, indicating that the H-4″ proton is on the upper side of the pyrrolidine ring (Fig. 2).

Next, modifications at the 2′ position were investigated (Schemes 1 and 2). First, the 2′ deprotected derivatives **18–28** were obtained in 65–83% yield by reaction of the corresponding 2′-TBDMS derivatives with ammonium fluoride in methanol (Scheme 1). In addition, the 2′ acetyl derivative **29** was obtained in 86% yield by acetylation of the 2′ deprotected derivative **18** with acetic anhydride/pyridine. Reaction of **18** with *tert*-hexyl dimethylsilyl chloride in acetonitrile at room temperature afforded the corresponding 2′-*O*-silylated nucleoside **30** in 75% yield (Scheme 2).

With respect to the modifications on the nucleobase, the introduction of a methyl group at the *N*-3 position of thymine was first carried out (Scheme 3). Thus, compound **1** was transformed into the corresponding 3-*N*-methyl nucleoside **31** (75%) by selective *N*-3-alkylation using methyl iodide in the presence of potassium carbonate.

Next, compounds **41** and **46** in which the thymine of the prototype has been replaced by uracil or thiophenyl moieties, respectively, were prepared using a convergent strategy, in which the 5-*O*-tosyl-3-cyano mesyl ribofuranose **33** was employed as a common sugar precursor (Scheme 4).

In turn, compound **33** was prepared from the previously described 5-*O*-tosyl derivative **32** (Cordeiro et al., 2006). Thus, hydrolysis of the 1,2-*O*-isopropylidene moiety of **32**, with aqueous trifluoroacetic acid, followed by reaction with acetic anhydride/pyridine afforded a 1:1.5 mixture of the two anomeric forms (α and β) of the diacetate derivative **33** in 90% yield (Scheme 4). Condensation of **33** with silylated uracil under modified Vorbrüggen (Vorbrüggen and Höfle, 1981) conditions afforded the 3′-cyano mesyl β-nucleoside **34** in 58% yield. The coupling constant value $J_{1',2'}$ = 7 Hz observed for this compound is in good agreement with the data for other β-cyanomesyl nucleosides previously described by our group (Camarasa et al., 1992).

On the other hand, reaction of **33** with thiophenol in the presence of boron trifluoride diethyl etherate afforded the β aryl thioglycoside **36** in 62% yield together with the α aryl thioglycoside **35** as a very minor compound (1%) (Scheme 4). In these sugar derivatives the similar values of the coupling constant $J_{1,2}$ observed for the α ($J_{1,2}$ = 5.9 Hz) and β anomers ($J_{1,2}$ = 5.8 Hz) precluded the unambiguously assignment of their anomeric configuration. These, were unequivocally determined by a NOE experiment (Fig. 2).

Thus, for the major isomer **36**, irradiation of H-1 caused enhancement of the signals for H-4 indicating that both protons, H-1 and H-4, are at the lower face (α face) of the furanose ring, and therefore the anomeric configuration of this compound is β. For the minor isomer **35**, irradiation of H-1 causes enhancement of the signals H-2 and H-5 indicating that all of these protons are at the upper face (β face) of the furanose ring, and therefore the anomeric configuration of this compound is α.

Once formed, nucleoside **34** was transformed into the desired uracil nucleoside **41** as shown in Scheme 5.

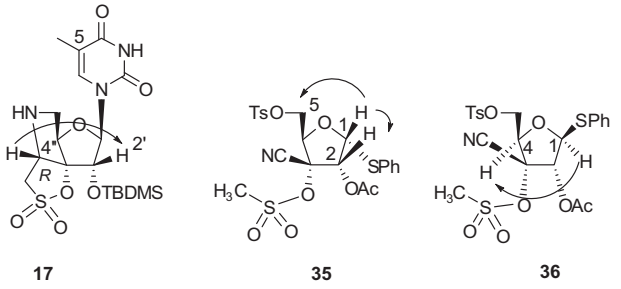
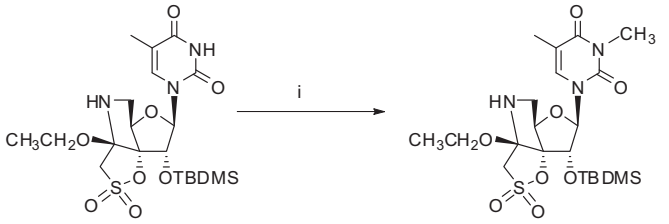
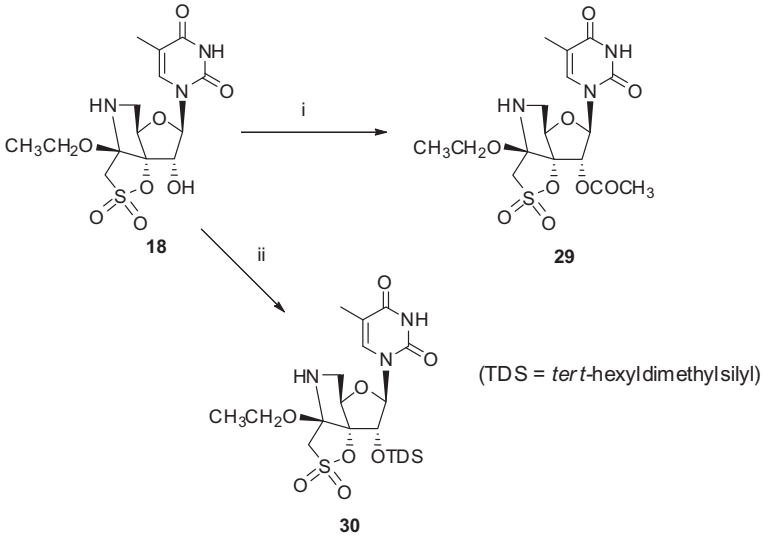


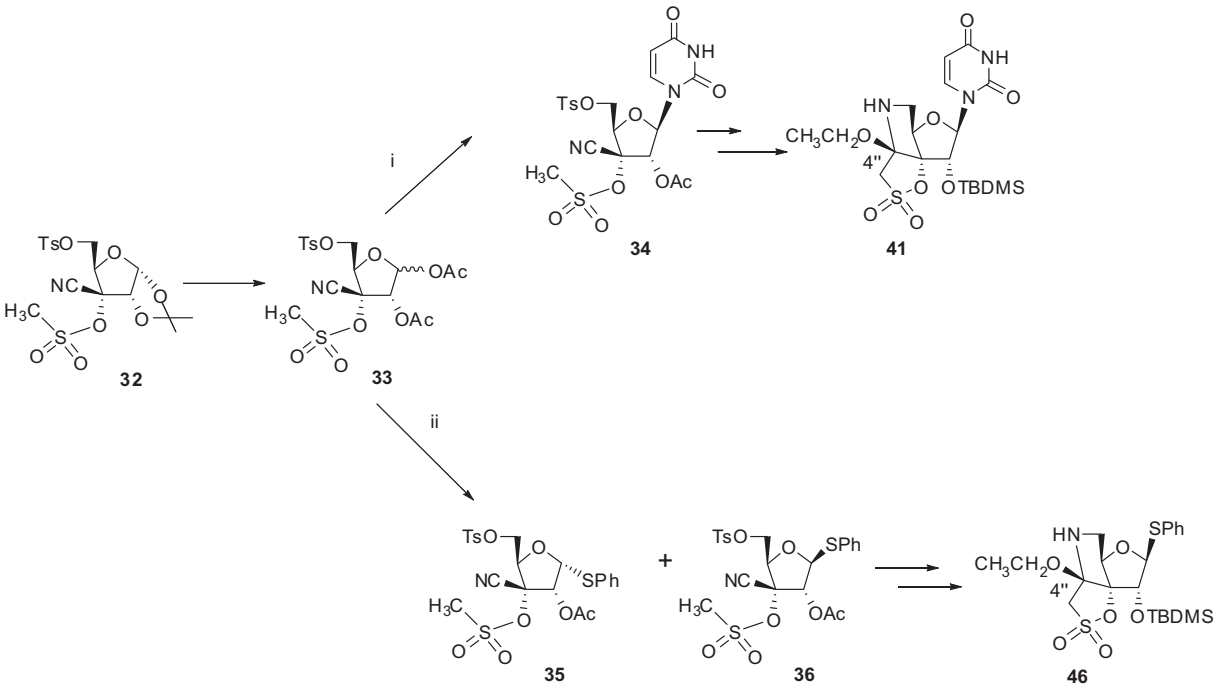
Fig. 2. Observed NOE's for compounds **17**, **35** and **36**.



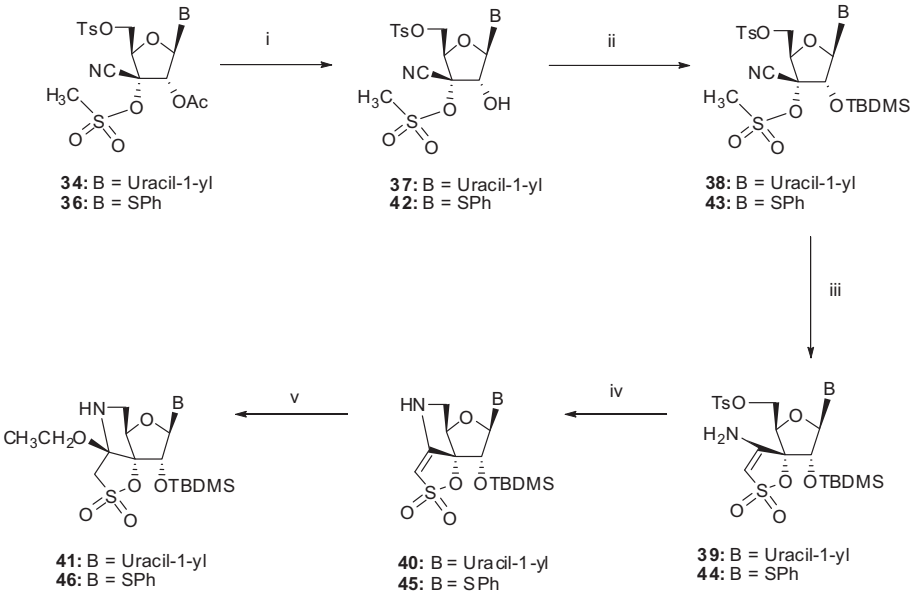
Scheme 3. Synthesis of **31**. *Reagents and conditions:* (i) MeI, K_2CO_3, dry acetone, 80 °C.



Scheme 2. Synthesis of **29** and **30**. *Reagents and conditions:* (i) $(CH_3CO)_2O$, pyridine, rt (ii) $Si(Me)_2tHexCl$, DMAP, dry acetonitrile, rt.

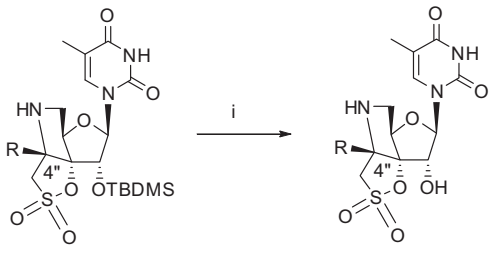


Scheme 4. Synthesis of carbohydrates **33–36**. *Reagents and conditions:* (i) Uracil, TMSTriflate, dry acetonitrile (ii) PhSH, $BF_3.OEt_2$, dry dichloromethane.



Scheme 5. Synthesis of **37–46**. *Reagents and conditions:* (i) NH_3, MeOH, 0 °C, 10 min (ii) TBDMSCl, DMAP, 80 °C, 1 h (iii) DBU, dry acetonitrile (iv) K_2CO_3, dry acetonitrile, 80 °C (v) CH_3CH_2OH, 80 °C.

Thus, 2′ deprotection of nucleoside **34** with saturated methanolic ammonia gave the deprotected nucleoside **37** (90%). Reaction of **37** with *tert*-butyldimethylsilyl chloride at 80 °C for 1 h afforded the 2′-*O*-silylated nucleoside **38** (73%). It should be mentioned that the substitution of the 5′-tosyl group by chloride was observed upon prolonged reaction times as a secondary reaction. Replacement of the tosyl moiety at 5′ position by chloride was determined in the ^{1}H NMR spectrum by the disappearance of the signals corresponding to the tosyl group and the upfield shift ($\sim\delta$ 0.4 ppm) of the signals corresponding to the H-5′ protons with respect to the same signals in the tosyl derivative (δ 4.40 and 4.50 ppm). However, the 5′-chloro derivative obtained behaves exactly as the corresponding 5′-*O*-tosyl derivative **38** and for this reason when the substitution takes place the resulting 5′-*O*-tosyl and 5′-Cl- mixture was used directly without separation. Nucleoside **38** was treated with DBU to give the spiro derivative **39** (74%). Reaction of **39** with potassium carbonate at 80 °C for 5 h afforded the cyclic enamine sulfonate **40** in 73% yield that was treated with ethanol to afford **41** in 70% yield. A similar synthetic sequence was followed with the thiophenyl



Scheme 6. Synthesis of **50–52**. *Reagents and conditions:* (i) NH_4F, MeOH, rt.

derivative **36** to afford the deprotected derivative **42** (65%) that was successively transformed into **43** (65%), **44** (77%), **45** (73%) and **46** (79%) as described above (Scheme 5).

Finally, for comparative purposes the 2′-deprotected tricyclic nucleosides **50–52** were prepared in 60–78% yield respectively by reaction of the corresponding 2′-TBDMS derivatives **47** (Bonache et al., 2004), **48** (Bonache et al., 2004) and **49** (Bonache et al., 2004) with ammonium fluoride in methanol (Scheme 6).

3.2. Biological results

The tricyclic nucleoside analogs **3–31**, **41** and **46** were tested for their *in vitro* inhibitory effects on HIV-1 and HIV-2 replication. In addition, for comparative purposes we tested the tricyclic nucleosides **47**, **48** and **49** with ethylthio, cyano and carboxamide moieties at the C-4″ position that were previously synthesized in our laboratory (Bonache et al., 2004) and their corresponding 2′-deprotected derivatives **50–52.**

Table 1 summarizes the results of the biological evaluation of the test compounds. The inhibitory activity of the compounds was expressed as EC_{50} values or compound concentrations required to inhibit virus-induced giant cell formation (CEM) or cytopathicity (cell destruction) (MT-4) by 50%. We decided to evaluate the compounds against both virus infection models because both assays used a different read-out, but should correspond to each other in terms of antiviral activity of test compounds. The antiviral data on the prototype compound (**1**) is also reported as reference.

Whereas several compounds inhibited HIV-1 replication in the lower micromolar concentration range, none of the compounds proved active against HIV-2 at subtoxic concentrations (Table 1). Therefore, the active compounds should be considered as specific inhibitors of HIV-1 replication. Their anti-HIV activity in CEM cells were very similar to those observed in HIV-1 infected MT-4 cell cultures (Table 1).

A structure–activity relationship (SAR) could be recognized for the 2′-TBDMS protected test compounds with regard of their cellular cytostatic profile. Small alkyl entities were clearly preferable over more bulky, either linear or branched, groups [order of lesser toxicity to highest toxicity: R = methyl (**3**) < ethyl (**1**) ∼ propyl (**4**) ∼ butyl (**5**) < isopropyl (**7**), isobutyl (**8**) < pentyl (**6**)]. The unsaturated propenyl (**9**) and propynyl (**10**) derivatives showed a 2-fold lesser cytostatic activity than the propyl derivative (**4**). Hydroxyethyl (**11**) resulted in far less cytostatic activity than phenyloxyethyl (**12**), and the benzyl (**13**) derivative was 2-fold less cytostatic than the phenyloxyethyl (**12**) derivative. The highly bulky OTBDMS (**16**) resulted in a most cytostatic compound (IC_{50}: 7 μM), whereas the OH-substituted compound (**15**) was not cytostatic at all (IC_{50}: >250 μM).

Given the wide varying range of the cytostatic effect of the individual derivatives, an exact comparison of the antiviral potential of the individual compounds to delineate an antiviral SAR could not be easily made, since several compounds could not be evaluated at concentrations higher than 5 or 10 μM.

Nucleosides **3** and **4**, with one more or one less carbon atom in the alkoxy chain than the prototype (**1**) showed an antiviral activity that was superior to the antiviral potential of **1**. Moreover, **3** was 4-to 5-fold less cytostatic than **1**. Consequently, the selectivity index (ratio CC_{50} (CEM)/EC_{50} (CEM)) increased from 3.3, for the prototype compound **1**, to 20–22 for compounds **3** and **4** due to both a higher antiviral potency and lower cytostatic effect. However, compounds with longer alkoxy chains (**5** or **6**) were devoid of antiviral activity at sub-cytostatic concentrations. Thus, the propyloxy derivative **4** was the most inhibitory to HIV-1 replication in CEM cell cultures of this series.

The isobutyloxy derivative **7** showed an antiviral activity comparable to that of the unbranched derivative **4** in MT-4 cell cultures. It was less potent than **4** in CEM cell cultures.

Replacement of the ethoxy moiety in the prototype by OH or OTBDMS resulted in compounds (i.e. **15** and **16**) that lack anti-HIV-1 activity. The presence of other functional groups (CN and $CONH_2$) also gave inactive compounds (**48** and **49**). In contrast, nucleoside **17**, lacking the ethoxy moiety, or **47** with an ethyl chain

Table 1
Inhibitory effects of test compounds on HIV-1 and HIV-2 replication in MT-4 and CEM cell culture.

CAM	EC_{50} (μM) [a]				CC_{50} (μM) [b]	
	MT-4		CEM		CEM	MT-4
	HIV-1	HIV-2	HIV-1	HIV-2		
3	8.4 ± 3.9	>50	5.5 ± 0.7	>50	111 ± 14.8	63 ± 24
4	5.1 ± 0.4	>10	1.4 ± 0.8	>10	28.5 ± 2.0	21.6 ± 1.6
5	>10	>10	>10	>10	34.8 ± 13.7	18.0 ± 0.64
6	⩾5	>5	>5	>5	10.1 ± 0.1	9.0 ± 1.4
7	5.2 ± 0.6	>10	8.0 ± 2.8	>10	21.1 ± 3.6	16 ± 2.1
8	>10	>10	>10	>10	21.5 ± 1.6	12 ± 0.42
9	>10	>10	15.0 ± 5.0	>50	57.8 ± 9.3	24 ± 0.57
10	>25	>25	>25	>25	66.2 ± 4.9	49 ± 2.1
11	9.5 ± 0.2	>25	11 ± 4.0	>25	73 ± 10	59 ± 11
12	>5	>5	>5	>5	10.4 ± 0.7	4.8 ± 1.4
13	>10	>10	⩾10	>10	20.4 ± 1.1	6.4 ± 0.98
14	>10	>10	>50	>50	72.0 ± 1.7	29 ± 1.8
15	>250	>250	>250	>250	>250	>250
16	>2	>2	>2	>2	7.04 ± 1.8	9.1
17	30.4 ± 23.5	>125	30.0 ± 7.1	>125	>125	⩾125
18	–	–	>250	>250	⩾250	>250
19	98.9 ± 8.7	>250	35.0 ± 21.2	>250	>250	>250
20	>250	>250	>250	>250	>250	>250
21	>125	>125	⩾125	>125	>125	>125
22	143 ± 0.0	>250	160 ± 79.4	>250	>250	⩾250
23	>250	>250	>250	>250	>250	>250
24	24.2 ± 1.9	>250	22.5 ± 3.5	>250	>250	237 ± 18
25	>250	>250	>250	>250	>250	>250
26	>250	>250	>250	>250	⩾250	⩾250
27	>250	>250	⩾100	>250	>250	>250
28	23.9 ± 1.13	>250	20.0 ± 0.0	>250	⩾250	⩾250
29	>250	>250	>250	>250	>250	>250
30	–	–	>10	>10	27.0 ± 3.6	–
31	3.6 ± 2.3	⩾50	3.0 ± 1.4	⩾50	32.0 ± 2.0	49 ± 2.3
41	>50	>50	>50	>50	111.0 ± 1.4	119 ± 16
46	–	–	>10	>50	131.0 ± 12.7	–
47	31.1 ± 16.5	>50	32.5 ± 3.5	>50	80.6 ± 8.6	42 ± 14
48	>50	>50	>50	>50	103.0 ± 7.9	101 ± 8.1
49	>125	>125	>125	>125	⩾125	108 ± 21
50	111 ± 3.5	>250	30.0 ± 0.0	>250	⩾250	208 ± 73
51	>250	>250	>250	>250	⩾250	⩾250
52	>250	>250	>250	>250	>250	⩾250
1	13.8 ± 5.4	>10	7.7 ± 4.0	>50	25.0 ± 1.2	24.8 ± 5.94

Data are the mean ± S.D. of at least 2–3 independent experiments.
[a] 50% effective concentration, or the compound concentration required to inhibit HIV-induced cytopathicity by 50%.
[b] 50% cytostatic concentration, or the compound concentration required to inhibit cell proliferation (CEM) or to reduce cell viability (MT-4) by 50%.

containing a sulfur atom at C-4″ were endowed with anti-HIV-1 activity, although being approximately 2-fold less active than the prototype compound.

Substitution of the TBDMS group at the 2′ position of the ribofuranose by other lipophilic moieties, such as acetyl (**29**) or *tert*-hexyl (**30**) gave inactive compounds. The 2′-deprotected derivative (**18**) was also inactive.

The 3-*N*-methylthymine derivative **31** was 2- to 4-fold more active than its unsubstituted counterpart (**1**). Substitution of the thymine moiety by uracil or thiophenyl resulted in inactive compounds (**41** and **46**), respectively.

The 2′-deprotected derivatives (**18–28** and **50–52**) were, as a rule, not cytostatic at concentrations as high as 250 μM (Table 1). Also, they showed marginal (i.e. **19**, **22**, **50**), if any, anti-HIV activity (EC_{50}: ⩾50 μM), except for compounds **24** and **28** that proved moderately inhibitory to HIV-1 replication in MT-4 and CEM cell cultures (EC_{50}: 20–24 μM).

Our biological results indicates that shorter substituents in the 4″ position improve potency when the TBDMS group is present, while in its absence, branched and bigger alkoxy groups are needed. Taking in consideration that the bulky 2′-OTBDMS and the OR moiety on the C-4″ carbon are respectively at the lower and the upper side of the furanose ring (Bonache et al., 2004) this biological result indicates that these two groups might define the length of the cavity that accommodates the tricyclic nucleosides. The distance between the "roof" and "floor" of the cavity might not be larger enough to accommodate simultaneously two bulky groups at the top and bottom faces of the furanose ring.

The most active compounds **3** and **4** were evaluated against recombinant HIV-1 reverse transcriptase using polyA-oligodT as the template/primer. The activity measurements were based on a fluorescence (PicoGreen) directed assay. Compounds **3** and **4** dose-dependently inhibited the enzyme reaction. The IC_{50} (50% inhibitory concentration) was around 100 μM. Nevirapine (used as a control) also inhibited 50% of the enzyme reaction at ~0.7 μM (Fig. 3). It should be noticed that when using a poly C-oligo dG template, the RT reaction was also inhibited by nevirapine (data not shown), but not by **3** and **4** at 100 μM, pointing to a certain degree of template preference of the test compounds to inhibit the RT reaction. The compounds have also been tested for their inhibitory activity against RNase H-associated RT but were found inactive at 100 μM.

Compounds **3** and **4** have also been evaluated for their inhibitory activity against mutant K103N RT, E138K RT and Y181C RT HIV-1 replication in CEM cell cultures. Similarly as observed for TSAO derivatives both compounds markedly lost inhibitory potential against the mutant E138K RT and Y181C RT HIV-1 strains (EC_{50} > 20 μM). The EC_{50}'s of **3** and **4** for the mutant K103N RT HIV-1 strains were 13 and >20 μM, respectively. Thus, the new compound derivatives act somewhat similar to TSAO derivatives in terms of (mutant) antiviral spectrum.

Since compound **24** was devoid of any cytostatic effect at 250 μM, this compound was chosen to select for drug-resistant HIV-1 strains. Virus-exposed cell cultures were cultured in the presence of escalating concentrations of the test compounds. During the first six subcultivations, a cell suspension was transferred to fresh culture medium containing the compounds. At the sixth subcultivation, both cell suspension or supernatant was, in parallel, transferred to fresh culture medium for another 4 subcultivations. After the 10th subcultivation, the virus in both cell cultures was isolated and further phenotypically and genotypically analysed. The two virus strains isolated under dose-escalating cultivation conditions were found to be insensitive to the inhibitory activity of compound **24** at 250 μM. Interestingly, amino acid mutations were observed in the reverse transcriptase of these virus strains: i.e. K101K/E, K102K/E, V108V/I and E138E/K) for the virus isolate for which cell suspension was transferred during the last four subcultivations (HIV-1/**24**$^{res\#1}$) and K101K/E + V108 V/I + E138E/K + Y181Y/C for the virus isolate for which supernatant was transferred during the last four subcultivations (HIV-1/**24**$^{res\#2}$). The emergence of mutations lining the NNRTI pocket of RT occurred at a similar speed as observed for first-generation NNRTIs such as nevirapine and TSAO-m^3T (Balzarini et al., 1993b). In contrast to TSAO derivatives, that consistently select for a E138K mutation in the HIV-1 RT, compound **24** seems to select for additional mutations in the 98–108 amino acid stretch of HIV-1 RT (Table 2). It should be noticed that mutations at amino acid position K102 of the HIV-1 RT occur rather rarely (Ceccherini-Silberstein et al., 2007) whereas mutations at amino acid positions 101, 108, 138 and 181 are more common. Our findings (lack of anti-HIV-2 activity and the appearance of NNRTI-characteristic RT mutations) strongly suggest that the compounds behave as NNRTIs and likely bind in the NNRTI-binding pocket (resulting in mutations at amino acid positions 101, 102, 108, 138 and 181) to escape drug pressure). The amino acid mutations at positions 102 and 181 seem to have appeared most likely after the amino acid mutations at positions 101, 108 and 138. It is interesting to observe the appearance of the E138 K mutation in the mutant virus strains. An E138 K RT mutation is very characteristic for the TSAO derivatives that contain, beside the oxathioledioxide spiro moiety, also bulky *tert*-butyldimethylsilyl groups at C-2′ and C-5′. The latter groups are not present in the tricyclic nucleoside **24**, pointing to other parts of the molecule (i.e. the oxathioledioxide ring or the pyrrolidine

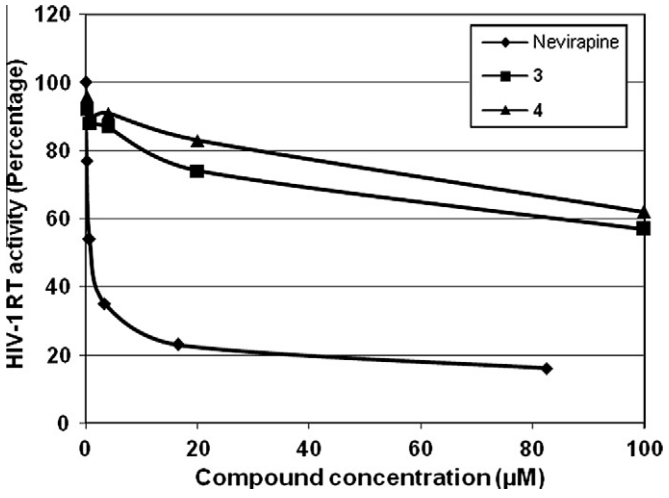


Fig. 3. Anti-HIV-1 RT activity of compounds **3**, **4** and nevirapine.

Table 2
Inhibitory activity of **24**, NNRTIs and the NRTI tenofovir against mutant HIV-1 strains in CEM cell cultures.

Compound	EC_{50} [a] (μM)		
	HIV-1(III_B) (WT)	HIV-1/**24**$^{res\#1b}$	HIV-1/**24**$^{res\#2c}$
24	32 ± 8	>250	>250
TSAO-m^3T	0.01	0.45 ± 0.07	>20
Nevirapine	0.013 ± 0.004	0.034 ± 0.03	0.055 ± 0.007
UC-781	0.001 ± 0.0007	0.008 ± 0.003	0.012 ± 0.003
Tenofovir	4.2 ± 1.8	16 ± 5.7	11 ± 1.4

Data are the mean ± S.D. of at least 2–3 independent experiments.

[a] 50% effective concentration or compound concentration required to inhibit virus-induced giant cell formation in CEM cell cultures by 50%.

[b] Following amino acid mutations were observed: K101K/E + K102K/E + V108 V/I + E138E/K.

[c] Following amino acid mutations were observed: K101K/E + V108V/I + E138E/K + Y181Y/C.

ring) that determines the resistance to the E138K RT mutation. It has recently been suggested that E138 and surrounding residues are involved in the entry of TSAO into the NNRTI binding pocket. This suggestion was based on the coordinates of a crystallographic HIV-1 RT/TSAO-m^{3}T complex (Das et al., 2011). It would be interesting to reveal whether this would also be the case for the novel tricyclic nucleoside analog **24**. When the phenotypic sensitivity of both virus isolates was determined, full resistance was observed for **24** (EC_{50} > 250 μM). Also, virus isolates # 1 and # 2 were 45- and >2000-fold resistant to TSAO-m^{3}T, presumably due to the presence of the TSAO-characteristic E138K mutation in the RT and the combination of E138K with Y181C. Instead, the antiviral potential of the NNRTIs nevirapine and UC-781 and the NRTI tenofovir were only marginally affected for both mutant virus strains.

4. Conclusions

In summary, we report on novel derivatives of the prototype tricyclic nucleoside **1** by modifying the ethoxy moiety at the C-4″ position, the nucleobase and the substituent at the 2′ position. Several members of this class of compounds show specific anti-HIV-1 activity comparable or superior to those of the prototype tricyclic nucleoside. Our studies demonstrate that both, the presence of thymine and a *tert*-butyldimethylsilyl group at the 2′-position of the sugar are important structural components since deletion of either of them is detrimental to the anti-HIV-1 activity. Modifications at the alkoxy moiety at C-4″ position were less stringent to keep anti-HIV-1 activity. Thus, the ethoxy moiety can be replaced by methoxy, propyloxy, hydrogen and ethylthio moieties. The methoxy (**3**) and propyloxy (**4**) derivatives were endowed with a 6- to 7-fold higher selectivity than the prototype **1**. Introduction of a methyl group at the position *N*-3 of the thymine enhanced the antiviral activity by 2- to 4-fold. Also several 2′-deprotected analogs (**24** and **28**) were endowed with selective anti-HIV-1 activity. The tricyclic nucleosides here described represent novel type of selective anti HIV-1 inhibitors, targeting HIV-1 reverse transcriptase by likely binding to the NNRTI-binding pocket.

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Further reading

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