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Mononucleoside phosphorodithiolates as mononucleotide prodrugs

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ABSTRACT

The synthesis and *in vitro* anti-HIV activity of a novel series of pronucleotides are reported. These prodrugs were characterized by a phosphorodithiolate structure, incorporating two *O*-pivaloyl-2-oxyethyl substituents as biolabile phosphate protections. The compounds were obtained following an original one-pot three-step procedure, involving the formation of a phosphorodithioite intermediate which is *in situ* oxidized. *In vitro*, comparative anti-HIV evaluations demonstrate that such original prodrugs are able to allow the efficient intracellular release of the corresponding 5'-mononucleotide. The pronucleotide of 2',3'-dideoxyadenosine (ddA) **3** exhibited a very potent antiretroviral effect with 50% effective concentration (EC₅₀) values in nanomolar concentration range in various cell lines. In primary monocytes/macrophages, this derivative was 500 times more potent in inhibiting HIV replication (EC₅₀ 0.23 pM) than ddA and the selectivity index of the prodrug is fifty times higher than the one of the parent nucleoside.

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1. Introduction

A large number of nucleoside and nucleotide analogues are currently used in the treatment of cancers and viral infections [1]. In the early 1990s, various strategies have been developed in order to improve the therapeutic potential of this family of drugs by using mononucleotide prodrugs (pronucleotides). Such a kind of prodrugs was developed to address not only bioavailability issues but also poor *in vivo* conversion of the parent nucleoside into its corresponding 5'-monophosphorylated form (5'-mononucleotide). Their rational design has been widely discussed in the past [2]. Briefly, their structure typically consists in a 5'-mononucleotide analogue where the negatively charged phosphate (or phosphonate) function is masked by substituents able to be hydrolyzed through a sequence involving enzymatic and/or chemical steps. We previously explored symmetrical phosphotriesters [3], phosphoramidate monoesters [4] and aryl phosphotriesters [5]. All these derivatives were shown to behave as pronucleotides and led to the

intracellular delivery of the 5'-mononucleotide, following esterase and then phosphoramidase or phosphodiesterase-mediated activation, respectively [2b,c].

Our work on this topic has started by the study of mononucleoside phosphotriesters where the thiol function of a thioethyl chain was engaged in a thioester link resulting in a biolabile phosphate protecting group called SATE (*S*-acyl-2-thioethyl) group (Fig. 1). Our first studies on bis(SATE) pronucleotides of anti-human immunodeficiency virus (anti-HIV) nucleoside analogues have shown *in vitro* and *in vivo* increased anti-HIV activities compared to the parent nucleosides [2b]. Thus, we decided to extend our investigations to the design of a new series of biolabile phosphate protections through the synthesis and the study of an isomeric form of these mononucleotide prodrugs, namely mononucleoside *S,S'*-bis(*O*-acyl-2-oxyethyl) phosphorodithiolates or bis(isoSATE) pronucleotides (Fig. 1).

2. Results and discussion

Here, we report the synthesis, antiviral evaluation and stability studies in various media of the mononucleoside phosphorodithiolate derivatives **1–3** (Fig. 1) of three well-known anti-HIV pyrimidine and purine containing nucleosides: 2',3'-dideoxy-2',3'-dideoxythymidine (d4T), 2',3'-dideoxythymidine (ddT), and

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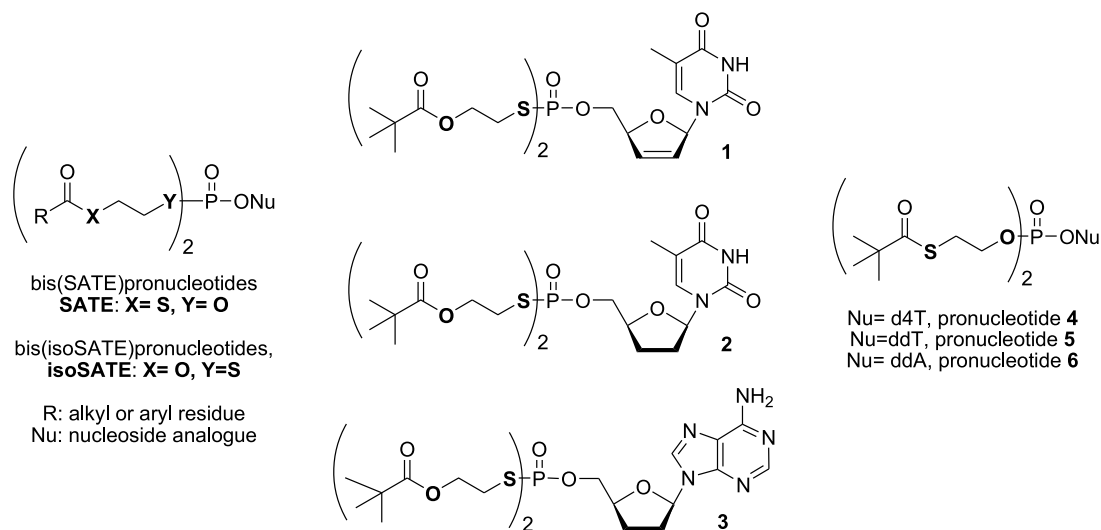


Fig. 1. Structures of the pronucleotides investigated in this study.

2',3'-dideoxyadenosine (ddA). As described for AZT (3'-azido-2',3'-dideoxythymidine) [6], these 2',3'-dideoxynucleoside analogues act as nucleoside reverse transcriptase inhibitors (NRTIs) after their metabolization into their corresponding 5'-triphosphate derivatives [7]. Thus, the studied bioconstructs are characterized by the presence of the *O*-pivaloyl-2-oxoethyl group (Fig. 1) as a first model of biolabile phosphate protection and their studies are performed in comparison to their corresponding mononucleoside phosphotriesters **4–6** bearing the *S*-pivaloyl-2-thioethyl (*t*BuSATE) group.

2.1. Chemistry

During the last decades, the replacement of oxygen by sulfur as modification of DNA's backbone has extensively been studied for mechanistic and structural investigations [8]. Thus, the variation, in which a sulfur atom replaces one of the non-bridging oxygen atoms in a phosphate group (phosphorothioation) that links DNA nucleotides together, is the first known modification of DNA's backbone [9] and confers increased resistance to nuclease degradation of the corresponding phosphorothioate oligonucleotides [10]. In addition, the *S*-phosphorothiolate analogues of oligonucleotides, in which 3'- or 5'-bridging oxygen atom in the phosphodiester linkage is replaced by a sulfur atom, have been developed for investigating involvement of metal ions in phosphoester transfer reactions [11]. In contrast, few works reported the study of phosphorodithiolates in nucleic acids chemistry. Mononucleoside phosphorodithiolates appeared as intermediates in the oligonucleotide synthesis using a phosphotriester (P^V) approach and are prepared by the reaction of a nucleoside with a phosphorodithiophosphate (as phosphorylating agent) in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride or mesitylenedisulphonyl chloride [12]. Such entities can also be obtained according to P^{III} strategies using the transient formation of a phosphorodithioite, resulting from a chlorophosphorodithioite [13] or a phosphorodichloridite [14]. In addition, some phosphorodithioates have been prepared following *H*-phosphonate approaches by the reaction of a hydroxylic component with the reactive species generated *in situ* from ammonium phosphinate [15] or phosphonic acid [12a]. Finally, the use of thiophosphoramidites for the synthesis of phosphorothioate DNA has also been reported [16]. The initial works on the synthesis of these modified nucleic acids have revealed that coupling reactions with a

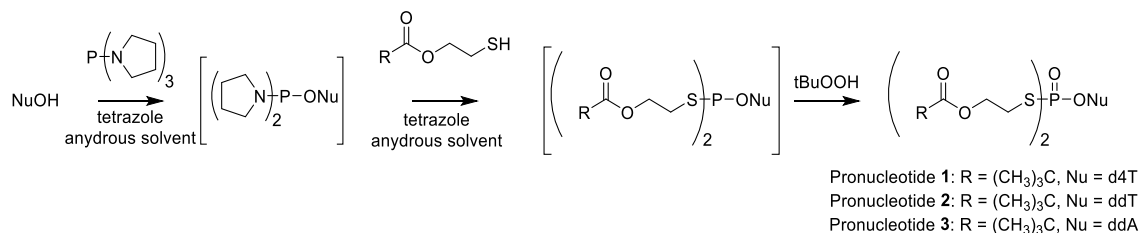
phosphorothioamidite gave low yields and are accompanied by the formation of side-products. Most of them are associated with the decreased reactivity of the thiophosphoramidite intermediate compared to the corresponding phosphoramidite under standard coupling conditions [17]. The slow or incomplete conversion of a thiophosphoramidite in presence of tetrazole led to prolonged reaction times and formation of side products via hydrolysis, oxidation/sulfurization and rearrangements.

Based on these literature data, the synthesis [18] of the targeted bis(isoSATE) pronucleotides **1–3** (Fig. 1) has been envisaged following a one-pot, three-step general procedure (Scheme 1). Compared to other alkylphosphines, the *tris*(pyrrolidino)phosphine [16c] was selected as phosphitylating agent due to its enhanced reactivity (associated to the reduced steric bulk surrounding phosphorus) with thiol derivatives [14a].

The phosphitylation of the nucleoside analogue (NuOH) was achieved with *tris*(pyrrolidino)phosphine (1.1 eq.) in presence of tetrazole (0.35 eq.) to afford a *bis*(pyrrolidino)phosphite derivative. Without isolation, the phosphite was converted to a phosphorodithioite product by adding tetrazole and then the thiol reagent (3 eq.). A large excess of tetrazole (5.2 eq.) was used to increase the reaction rate and to buffer the basicity of pyrrolidine (causing putative deacylation reactions) by the formation of the pyrrolidinium tetrazolide salt. Finally, *in situ* oxidation was carried out using *tert*-butyl hydroperoxide and led to the desired phosphorodithiolate structure.

Applied to 2-mercaptoethyl pivalate, previously obtained from mercaptoethanol [19], this procedure yielded the corresponding pronucleotides **1–3** (Fig. 1) in 27–33% overall yield. These compounds were characterized by high-field multinuclear NMR spectroscopy, UV, HPLC analysis, HRMS, microanalysis, and all resulting data were consistent with their structure and purity.

Using d4T as model nucleoside analogue, the reaction was monitored by ^{31}P NMR spectroscopy (Fig. 2). The fractional addition of the tetrazole (A) to a mixture of the nucleoside and the *tris*(pyrrolidino)phosphine ($\delta = 104$ ppm) led to a single signal at 136 ppm corresponding to the *bis*(pyrrolidino)phosphite. The successive addition of 1*H*-tetrazole and 2-mercaptoethyl pivalate (B) resulted into the transient formation of the phosphorodithioite intermediate ($\delta = 163$ ppm) and a minor side-product **7** ($\delta = 93$ and 94 ppm). In presence of *tert*-butyl hydroperoxide, the expected phosphorodithiolate derivative **1** was obtained ($\delta = 57$ ppm) along



Scheme 1. General procedure developed for the synthesis of bis(isoSATE) pronucleotides according to a phosphoramidite strategy.

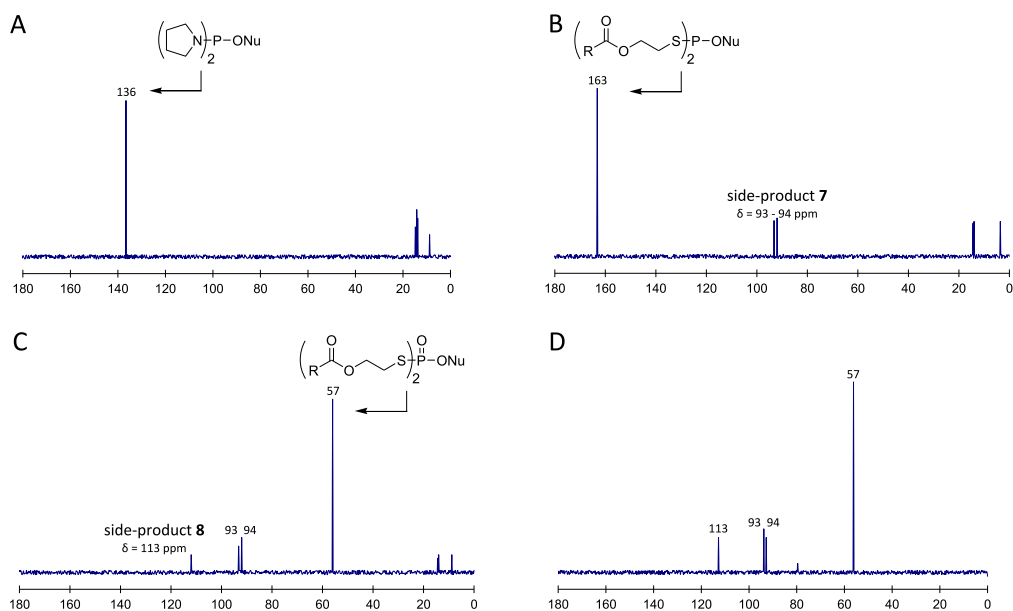


Fig. 2. ³¹P NMR monitoring of the synthesis of pronucleotide **1** following a phosphoramidite strategy. A: nucleoside (d4T), tris(pyrrolidino)phosphine (1.1 eq.), 1*H*-tetrazole (0.35 eq.), rt, 45 min; B: 1*H*-tetrazole (5.2 eq.), then 2-mercaptoethyl pivalate (3 eq.), rt, 45 min; C: *tert*-butyl hydroperoxide (2.8 eq.), -40 °C to rt, 45 min; D: organic layers after work-up (Na₂S₂O₃), extraction and decantation.

with by-product **8** ($\delta = 113$ ppm). Work-up of the reaction mixture (D) did not affect the NMR reaction profile.

Multiple chromatography purifications of the complex reaction mixture allowed separation and identification of the two side-products **7** and **8**. The formation of **7** was observed during the coupling step between the 2-mercaptoethyl pivalate and the bis(pyrrolidino)phosphite (Fig. 2B). This compound was found to be unreactive under the conditions of the last oxidative step carried out in presence of *tert*-butyl hydroperoxide. Comparison of the physicochemical properties of the side-product **7** with literature data suggested a thionophosphoramidothiolate structure (Fig. 3). Indeed, the ³¹P NMR ($\delta = 92.9$ and 94.3 ppm) chemical shifts of both

diastereoisomers were in agreement with those given for related structures [20,21], and the germinal coupling constant ($J_{P-C} = 2.5$ Hz) appears to be in the same range as that observed for some representative phosphorothiolates [22]. In addition, FAB-MS (in positive- and negative-ion modes) and HRMS (ESI-MS) analysis corroborated the chemical structure of **7**. No by-products corresponding to a thionophosphoramidate structure ($J_{P-C} = 90$ –100 Hz) [23] were identified, despite the fact that thionophosphoramidate derivatives may be subject to Arbuzov rearrangements [14a,14b,16b].

The side-product **8** was observed during the last oxidative step (Fig. 2C). Its ³¹P NMR chemical shifts appears to be consistent

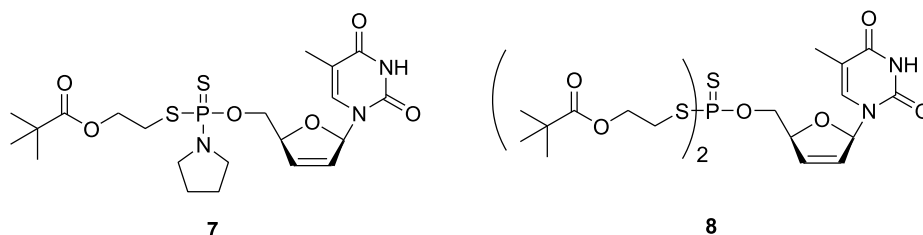


Fig. 3. Proposed structures of the side-products **7** and **8** isolated during the synthesis of pronucleotide **1** according to a phosphoramidite strategy.

[24,25] with a thionophosphorodithiolate structure (Fig. 3). This hypothesis was corroborated by FAB-MS (in positive- and negative-ion modes) and HRMS (ESI-MS) analysis, in agreement with the presumed structure.

The formation of the side-products **7** and **8** could be the result of a sulfurization processes involving a Schönberg reaction [26]. Thus, derivative **7** may come from the nucleophilic attack of the transient thiophosphoramidite on a symmetrical disulfide, generated *in situ* from the dimerization of 2-mercaptoethyl pivalate, while the formation of **8** could be explained by a similar mechanism involving the reaction of the phosphorodithioite intermediate with the same disulfide. Finally, the same reaction profile and related side-products were observed during the synthesis of pronucleotides **2** and **3** (see supporting information).

In addition, we synthesized the phosphorothiolate diester derivative of ddA **9** and its phosphodiester isomeric form **10** (Fig. 4) to be used as reference samples in the stability studies (*vide infra*).

After multiple and unsuccessful attempts involving phosphite and phosphoramidite intermediates, the phosphorothiolate diester **9** was obtained from the *H*-phosphonate derivative of ddA **11** (Scheme 2), previously obtained following a procedure reported by Stawinski et al. [27]. The *H*-phosphonate **11** was treated with a large excess (4 eq.) of chlorotrimethylsilane in presence of triethylamine, followed by the succinimide reagent **12** [15,28] to afford the desired phosphorothiolate diester derivative of ddA **9** in 78% yield. It is assumed that the reaction proceeds by the electrophilic attack of the succinimide derivative on a bis(trimethylsilyl) phosphite intermediate.

The *S*-pivaloyl-2-thioethyl phosphodiester derivative of ddA **10** (Fig. 4) was synthesized following a previously published procedure [29]. All compounds were characterized on the basis of their spectrometric properties (NMR, UV, MS, analysis) and data were consistent with their expected structure and purity.

2.2. Antiviral activity

The inhibitory effects on the replication of HIV-1 of the mononucleoside phosphorodithiolates **1–3** were evaluated in cell culture systems in comparison to their nucleoside parent (d4T, ddT and ddA) and the corresponding mononucleoside bis(*t*BuSATE)phosphotriester derivatives **4–6** (Fig. 1). The related cytotoxicity (CC₅₀) was also determined in uninfected cells. These results are summarized in Tables 1 and 2.

In human T4-lymphoblastoid cells, CEM-SS and MT-4, the pronucleotides **1** and **2** showed similar or slightly higher anti-HIV activity than their parent nucleosides d4T and ddT, respectively (Table 1). With 50% effective concentration (EC₅₀) values about micro- to nanomolar range in these two cell lines, no significant difference in antiretroviral activity was observed between the bis(isoSATE) pronucleotides **1** and **2** and their SATE isomeric forms **4** and **5**, respectively.

The CEM/TK⁻ cell line, highly deficient in thymidine kinase, should be considered as a predictive model to investigate the

efficiency of nucleotide prodrugs of pyrimidine nucleoside analogues [30]. In contrast to d4T or ddT, the corresponding bis(isoSATE) pronucleotides **1** and **2**, as their previously described SATE isomers, exhibited significant anti-HIV effects in CEM/TK⁻ cells, similar to that observed in infected CEM-SS cells. These results demonstrate the successful release of the corresponding 5'-mononucleotide inside cells.

The inhibitory effects on the replication of HIV-1 of the phosphorodithiolate derivative of 2',3'-dideoxyadenosine (ddA) **3** was evaluated in four cell culture systems in comparison to the corresponding bis(*t*BuSATE)phosphotriester **6** and the parent nucleoside ddA (Table 2). The bis(isoSATE) pronucleotide **3** exhibited a very potent antiretroviral effect in human lymphoblastoid cells (CEM-SS, MT-4), stimulated and unstimulated primary cultured human cell lines such as peripheral blood mononuclear (PBM) cells or monocyte-derived macrophages (MDM). The last two cell lines are primary cells which may be considered as most physiologically relevant models in the context of HIV-infection.

This effect was similar to that observed for its corresponding SATE derivative **6**, both types of isomeric prodrugs being more potent inhibitors than ddA by 2 or 3 orders of magnitude. The tested pronucleotides **3** and **6** exhibited 50% effective concentration (EC₅₀) values in the nanomolar concentration range in CEM-SS, MT-4 and PBM cells. In primary monocytes/macrophages, very low EC₅₀ values at 0.23 pM and 0.16 pM were measured for compounds **3** and **6** respectively. In this cell line, the bis(isoSATE) pronucleotide **3** was 500 times more potent in inhibiting HIV replication, than ddA, and the selectivity index (ratio CC₅₀/EC₅₀) of the prodrug is fifty times higher than the one of the nucleoside.

These results may be explained in relation to the well-established ddA/ddI cellular metabolic pathway (Scheme 3) [7b,31]. Briefly, ddA is not readily transformed to its corresponding 5'-monophosphate (ddAMP) by cellular kinases *in vivo* but metabolized to ddI (2',3'-dideoxyinosine) by the ubiquitous cellular enzyme adenosine deaminase. This nucleoside is phosphorylated to ddIMP by 5'-nucleotidase, a cellular enzyme whose activity remains relatively constant during all phases of the cell cycle. The resulting ddIMP is converted to ddAMP by adenylosuccinate synthase and lyase, and then subsequent phosphorylated to ddA 5'-triphosphate (ddATP), the active metabolite responsible for inhibiting viral reverse transcriptase. Additionally, ddA and ddI could be hydrolyzed by purine nucleoside phosphorylase, to release the sugar dideoxyribose and the corresponding nucleobases, which will be further degraded. In such metabolic pathway, the intracellular conversion of ddIMP constitutes the rate-limiting step for obtaining ddAMP.

As observed with anti-HIV pyrimidine nucleoside models (Table 1), these results (Table 2) strongly support the hypothesis that the bis(isoSATE) pronucleotide **3**, as previously demonstrated for isomeric SATE forms, exerts its biological effects via the intracellular delivery of the corresponding 5'-mononucleotide, circumventing the first limiting steps in the anabolism pathway of ddA (Scheme 3).

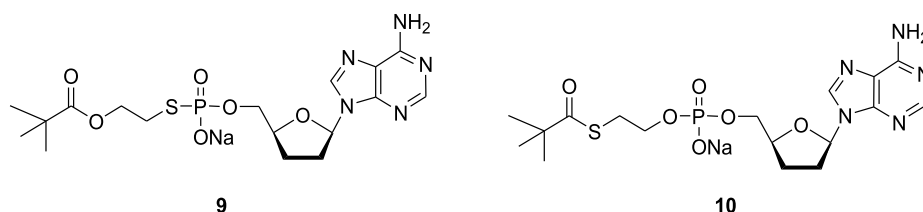
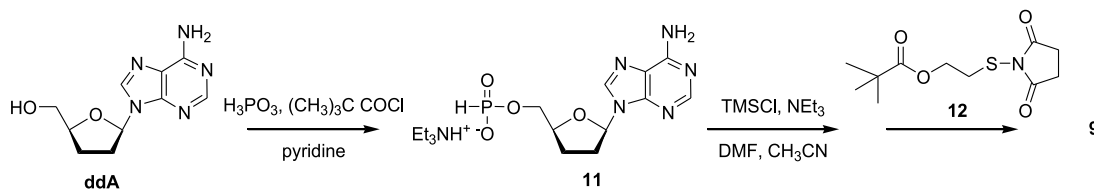


Fig. 4. Expected metabolites **9** and **10** resulting from the decomposition of the pronucleotides **3** and **6**, respectively.



Scheme 2. Synthesis of the phosphorothiolate diester derivative of ddA **9**.

Table 1

Anti-HIV activity (μM)^a in four cell culture systems of pronucleotides **1** and **2** compared to their corresponding mononucleoside bis(*t*BuSATE)phosphotriesters **4** and **5**, as well as their parent nucleosides (d4T and ddT).

Compound	CEM-SS		CEM/TK ⁻		MT-4	
	EC ₅₀ ^b	CC ₅₀ ^c	EC ₅₀ ^b	CC ₅₀ ^c	EC ₅₀ ^b	CC ₅₀ ^c
1 (isoSATE)	0.027	>10	0.005	8	0.099	>10
4 (SATE)	0.03	>10	0.021	4.6	0.26	>10
d4T	0.059	>100	>10	>100	0.28	>100
2 (isoSATE)	0.08	>10	0.05	>10	0.9	>10
5 (SATE)	0.22	>10	0.05	>10	2.5	>10
ddT	0.7	>100	>100	>100	16	>100

^a All data represents average values for at least three separate experiments. The variation of these results under standard operating procedures is below $\pm 10\%$.

^b EC₅₀: effective concentration or concentration required to inhibit the replication of HIV-1 by 50%.

^c CC₅₀: cytotoxic concentration or concentration required to reduce the viability of uninfected cells by 50%.

Table 2

Anti-HIV activity (μM)^a in four cell culture systems of pronucleotide **3** compared to its corresponding mononucleoside bis(*t*BuSATE)phosphotriester **6**, as well as its parent nucleoside ddA.

Compound	CEM-SS		MT-4		PBMC		MDM	
	EC ₅₀ ^b	CC ₅₀	EC ₅₀ ^b	CC ₅₀	EC ₅₀ ^b	CC ₅₀	EC ₅₀ ^b	CC ₅₀
3 (isoSATE)	0.025	>10	0.27	>1	0.02	>10	0.00023	8.6
6 (SATE)	0.018	5.9	0.32	9	0.047	1.7	0.00016	3.6
ddA	0.49	>100	7.8	>100	0.24	>100	0.12	100

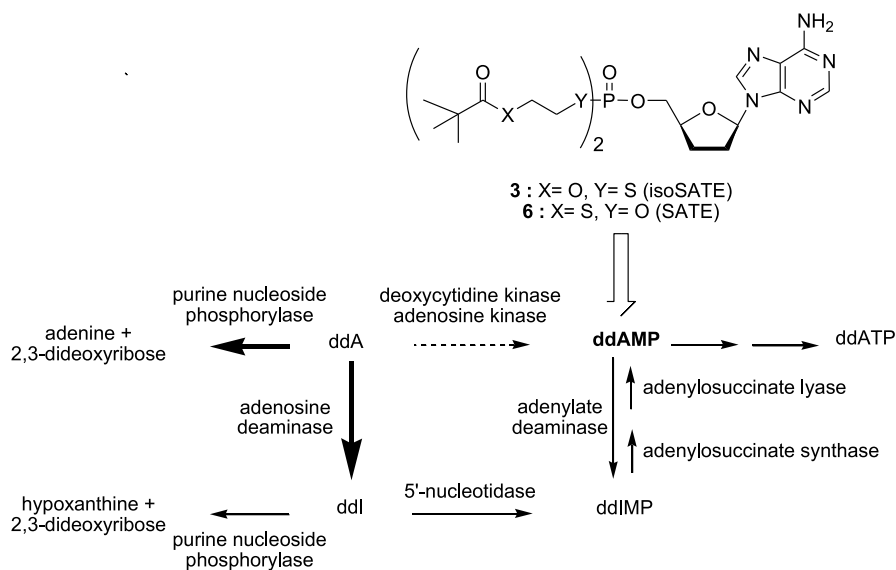
^{a, b, c} See Table 1.

The SATE and isoSATE biolabile phosphate protecting groups were designed to be hydrolyzed with similar decomposition pathways producing the same degradation products (*see below*). The potential toxicity of SATE groups and products originating from their decomposition was evaluated using a large number of experimental assays [2b]. All these studies demonstrated that the increase toxicity observed (in some cases) for bis(SATE) pronucleotides compared to the parent nucleosides reflect the intracellular accumulation of the phosphorylated forms, which may possibly interact with host cellular enzymes. This result does not hamper the potential interest of a pronucleotide approach, but raises the importance of the selectivity of the corresponding 5'-triphosphate derivatives for the viral polymerase [30].

2.3. Stability studies

The decomposition pathways and kinetic data of prodrugs **3** and **6** (initial concentration $5 \cdot 10^{-5}$ M) were studied at 37 °C in: (i) a phosphate buffer (pH 7.4), (ii) RPMI 1640, (iii) RPMI 1640 containing 10% heat-inactivated fetal calf serum (culture medium, CM), and finally (iv) total cell extract (TCE) from CEM-SS cells in order to mimic the behavior of the studied compounds inside the cells.

Studies in the phosphate buffer and RPMI were used to differentiate between chemical and enzyme-mediated hydrolysis of the two pronucleotide isomers. Crude aliquots of incubates were analyzed by HPLC using an on-line internal-surface reversed-phase (ISRP) cleaning method, previously described [3]. Authentic phosphorothiolate diester derivative **9** and its phosphodiester isomer **10**



Scheme 3. Metabolic pathways of ddl, ddA [7b] and expected metabolism of the bis(isoSATE) and bis(SATE) pronucleotides of ddA.

(Fig. 4) were used for calibration and identification of the decomposition products. The rate constants of the disappearance of the pronucleotides were calculated according to pseudo-first-order kinetic models, which are in accordance with the experimental data. In non-enzymatic media (*i.e.* phosphate buffer and RPMI), both prodrugs **3** and **6** were very weakly hydrolyzed to their corresponding metabolites **9** and **10**, with half-lives higher than several days (Table 3). As observed in solvolysis studies of some S-alkyl phosphorothioates [32], this decomposition process may result from direct attacks on the phosphorus atom by nucleophiles, more present in an enriched growth media used in cell culture such as RPMI than the phosphate buffer. As expected, the lower chemical stability of phosphorodithiolate **3** compared to its corresponding phosphotriester isomer **6** is the consequence of facilitated attack at phosphorus, the bond energy of the P–S bond being considerably less than P–O bond energy (by about 120 kJ/mol) due to less efficient $p_{\pi}-d_{\pi}$ bonding [33]. In both medium, the metabolites **9** and **10** were found stable to further chemical hydrolysis due to the presence of the negative charge of the phosphate [34].

Then, the stability of pronucleotide **3** and its isomeric form **6** were evaluated in CM and CE (Table 3). Both prodrugs were converted to their corresponding metabolite diesters **9** and **10** (Fig. 5 and 6a) in these media and the transformation was faster in cell extracts (Fig. 6a). This is a prerequisite for prodrugs designed to release the parent mononucleotides inside cells.

In addition to the chemical process observed in RPMI, the existence of enzymatic activities in CM (provided by the heat-inactivated serum) contribute to the slight increase of the decomposition kinetics of studied pronucleotides. This effect is more pronounced in the case of the SATE group (ratio of $t_{1/2}$ CM/RPMI: 1.8 and 1.3 respectively for **6** and **3**), as the phosphotriester structure ($t_{1/2}$: 6.9 days for **6**) appeared four times more stable in this medium than that its phosphorodithiolate isomer ($t_{1/2}$: 1.6 days for **3**). In CE, the observed half-lives of such bioconstructs essentially depend on enzymatic processes. As previously demonstrate through the stability studies of various enzymolabile phosphate protecting groups characterized by the same phosphorus environment [35], a thioester link is more rapidly hydrolyzed in this medium than an ester function. Thus, in contrast to CM, the SATE pronucleotide **6** exhibited a lower half-life in CE compared to that of its isoSATE isomer **3**. Once formed, the metabolites **9** and **10** were essentially converted into the 5'-mononucleotide (ddAMP) but the mechanism of this transformation differed according to the medium. Finally, in both media, the 5'-mononucleotide was dephosphorylated to the parent nucleoside (ddA) due to a remaining phosphatase activity.

As illustrated by the incubation of the SATE phosphodiester derivative of ddA **10** (Fig. 6b) and previously published works in this topic (Scheme 4), its main decomposition in CE involved an esterase-mediated hydrolysis leading to a new deacylated phosphodiester (Fig. 6b and Scheme 4, metabolite X), which in turn was relatively slowly converted into the 5'-mononucleotide. In contrast, this intermediate was not observed in CM (Fig. 5) suggesting that

the 5'-mononucleotide delivery may result in this case from a phosphodiesterase activity.

The design of the bis(isoSATE) pronucleotides (Scheme 4) was rationalized in terms of the formation of a five-membered cyclic intermediate in which pseudorotation is permitted but subject to certain restrictions. Thus, the proposed decomposition pathway of such bioconstructs may involve an esterase-mediated activation leading to a new phosphorodithiolate (A) and the nucleophilic attack of the resulting hydroxyl function on the phosphorus atom giving the five-covalent intermediate (B). The constraint imposed by the five-membered ring is sufficient to undergo rapid rearrangement of ligand positions *via* Berry pseudo-rotation, leading the cyclic sulfur to preferentially adopt an apical position (as leaving group) in the trigonal bipyramidal intermediate. This transient conformation leads to a P–S bond cleavage and the driving force for this reaction is presumably ring strain, with a difference in energy of activation for the two hydrolysis (endo *versus* external to the ring) estimated at 20–25 kJ/mol [36]. A such $S_N2(P)$ pathway gave rise to the unstable 2-mercaptoethyl phosphorothiolate (C) which spontaneously decomposes according to the HSAB concept with an episulfide elimination (as observed with the SATE group) affording the corresponding phosphorothiolate diester (Scheme 4, metabolite **9** from **3**). This metabolite is the only one observed when pronucleotide **3** is incubated in CE (Fig. 6a), demonstrating that the reaction occurs rapidly and that the different steps of the proposed mechanism were not limiting factors in this hydrolysis process. In CE, the phosphorothiolate diester derivative of ddA **9** appeared ten times more stable (Table 2, $t_{1/2}$: 10.5 h) than that its phosphodiester isomer **10** ($t_{1/2}$: 1.1 h). Consequently, metabolites resulting from its decomposition (such as ddAMP) cannot accumulate in the medium, their relative concentrations being between 2 and 5% over the incubation period (Fig. 6b). Unfortunately, these stability studies do not allow to determine the relative part between an esterase-mediated process and a phosphodiesterase hydrolysis during the conversion of metabolite **9** into its corresponding 5'-mononucleotide. In the past, we have already been confronted to these limitations associated with biological models and due to the (partial) denaturation of the enzymatic content of the cell extract during its preparation [4,37]. As example, no enzymatic activity involved in the anabolism (*i.e.* phosphorylation steps) of nucleosides or nucleotide analogues was observed in CE, the decomposition of the mononucleotide into the parent nucleoside being only observed because of residual phosphatase activity. This highlights the importance to develop this type of prodrug approaches on the basis of nucleoside analogues for which biological activity is known to be limited by a rate-limiting step in the anabolic pathway such as ddA (Scheme 3). In this context, when a significant increase of the biological response is detected in cell cultures (which is the case for pronucleotide **3** in the all cell lines studied), one can assume that it is associated with the release of the 5'-mononucleotide inside the cells and does not result from a possible extracellular decomposition of the prodrug into its parent nucleoside.

3. Conclusion

The present study demonstrates that the studied mononucleoside phosphorodithiolate derivatives **1–3** constitute the first examples of a new series of pronucleotides, allowing the efficient intracellular delivery of the parent 5'-mononucleotide. The proposed decomposition mechanism may involve in a first step an esterase-mediated activation. Compared to their well-known SATE phosphotriester isomers, such bioconstructs shown a lower chemical stability probably due to facilitated nucleophilic attacks at phosphorus atom. These results encourage us to pursue the design

Table 3

Calculated half-lives of the pronucleotides **3** and **6** and their metabolites in phosphate buffer, RPMI 1640, culture medium (CM), and CEM-SS cell extracts (CE).

Media	Compound			
	3 (isoSATE)	6 (SATE)	9 (from 3)	10 (from 6)
Phosphate buffer (pH 7.4)	2.9 days	18 days	stable	stable
RPMI (pH 7.45)	2.2 days	12.6 days	stable	stable
CM	1.6 days	6.9 days	9.9 h	46 h
CE	5.3 h	1 h	10.5 h	1.1 h

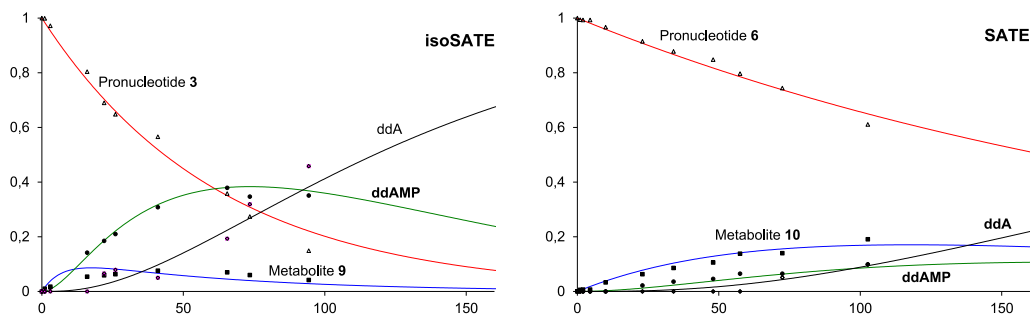


Fig. 5. Decomposition kinetics of the pronucleotides **3** and **6** (red) at 5×10^{-5} M initial concentration and their degradation products **9** and **10** (blue), ddAMP (green) and ddA (black) in cell culture medium (CM) at 37 °C. The abscissa shows the incubation time (h) and the ordinate the relative concentration of each species.

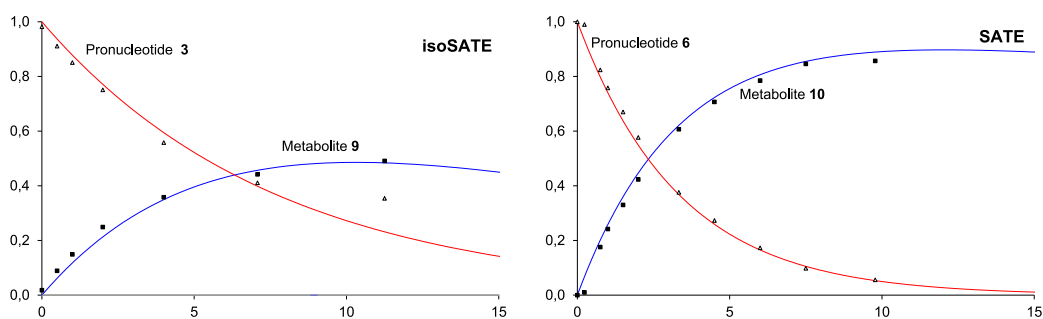


Fig. 6a. Decomposition kinetics of the pronucleotides **3** and **6** (red) at 5×10^{-5} M initial concentration and their degradation products **9** and **10** (blue) in CEM-SS cell extracts (CE) at 37 °C. The abscissa shows the incubation time (h) and the ordinate the relative concentration of each species.

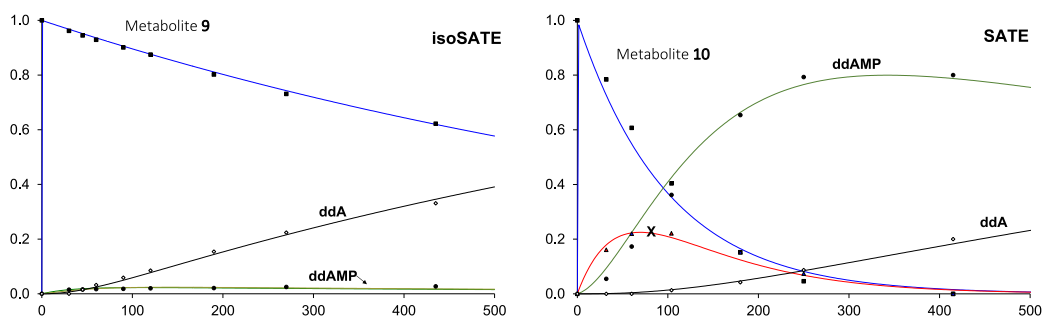


Fig. 6b. Decomposition kinetics of the metabolites **9** and **10** (blue) and their degradation products X (orange), ddAMP (green) and ddA (black) at 5×10^{-5} M initial concentration in CEM-SS cell extracts (CE) at 37 °C. The abscissa shows the incubation time (min) and the ordinate the relative concentration of each species.

of new pronucleotide series in which the terminal functions (thiol or hydroxyl) of the 2-thioethyl or 2-oxethyl linkers are engaged in other enzymolabile bonds and open the way to more specific enzymatic activation systems in order to target cells or tissues. Works in this topic are currently in progress in our group.

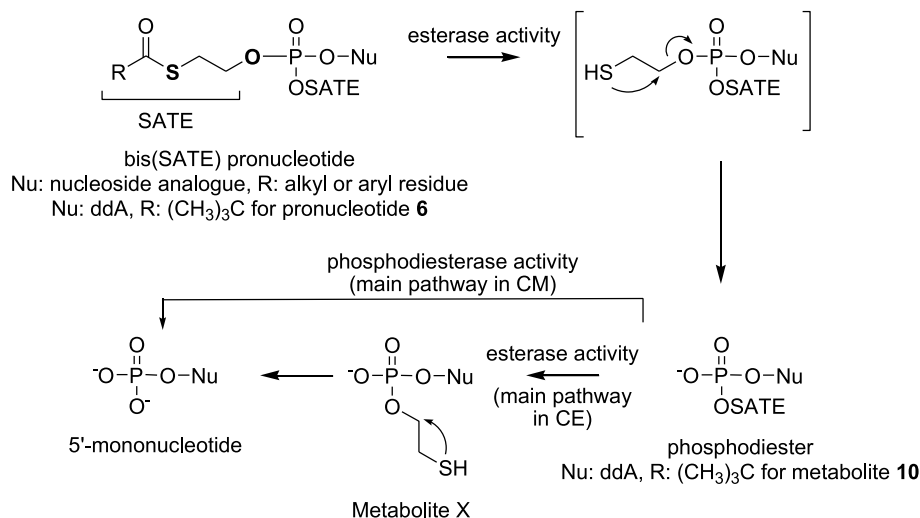
4. Experimental section

4.1. General information

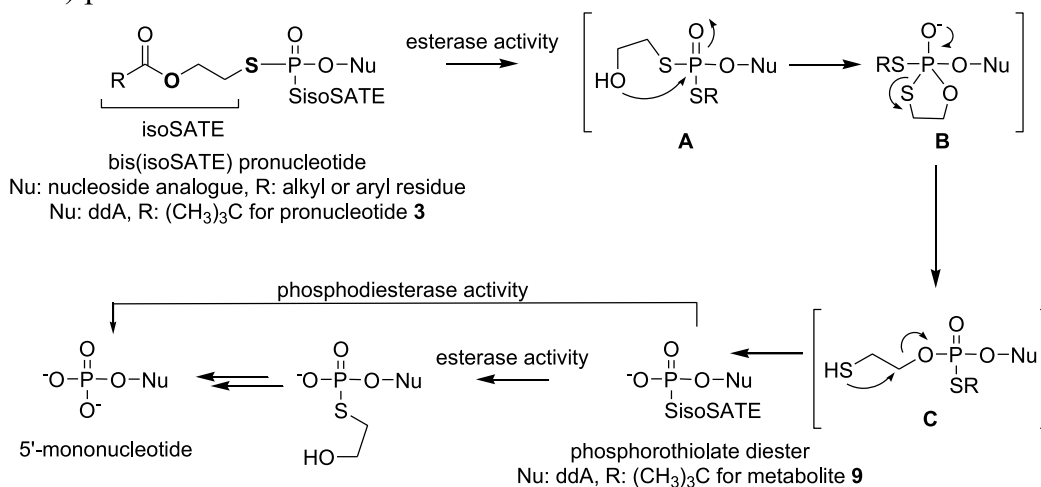
^1H NMR were recorded using a Bruker AC 250 or a Bruker AC 400 spectrometer at ambient temperature in CDCl_3 . Chemical shifts are given in δ -values referenced to the residual solvent peak (7.26 ppm). Deuterium exchange, decoupling and COSY experiments were performed in order to confirm proton assignments. ^{31}P NMR spectra were recorded at ambient temperature on a Bruker AC 250 spectrometer at 101.2 MHz with proton decoupling. Chemical

shifts are reported relative to external H_3PO_4 . ^{31}C NMR spectra were measured on a Bruker AC 400 spectrometer at 100.6 MHz with proton decoupling using CDCl_3 (77.00 ppm) as internal standard. Coupling constants, J , are reported in Hertz. FAB mass spectra were recorded in the positive-ion or negative-ion mode on a JEOL DX 300 mass spectrometer operating with a JMA-DA 5000 mass data system using thioglycerol/glycerol (1:1, v/v, G-T) as matrix. Xe atoms were used for the gun at 3 kV with a total discharge current of 20 mA. HRMS using electrospray ionization-mass spectrometry (ESI-MS) were performed using a SSQ 7000 single quadrupole mass spectrometer (Finnigan, San Jose, California, USA) in the negative-ion mode with a spray voltage at -4.5 kV. The capillary temperature was maintained at 250 °C. Nitrogen served both as sheath gas (operating pressure of 80 psi) and as auxiliary gas with a flow rate of 15 units. Under these conditions, full scan data acquisition was performed from m/z 200 to 800 in centroid mode and using a cycle time of 1.0 s. UV spectra were recorded on an Uvikon 810 (Kontron)

Bis(SATE) pronucleotides



Bis(isoSATE) pronucleotides



Scheme 4. Proposed decomposition pathways of the bis(SATE) and bis(isoSATE) pronucleotides.

spectrometer in ethanol (95%).

TLC was performed on precoated aluminum sheets of silica gel 60 F₂₅₄ (Merck), visualization of products being accomplished by UV absorbance followed by charring with 5% ethanolic sulfuric acid with heating; phosphorus-containing compounds were detected by spraying with Hanes molybdate reagent. Column chromatography was carried out on silica gel 60 (Merck).

High-performance liquid chromatography (HPLC) studies were carried out on a Waters Assoc. unit equipped with a model 616 pump system, a model 600S system controller, a model 996 photodiode array detector and a Millennium data workstation. The column was a reverse-phase analytical column (Macherey-Nagel, C₁₈, 150 × 4.6 mm, 5 μm) protected by a prefilter and a precolumn (Nucleosil, C₁₈, 5 μm). The compound to be analyzed was eluted using a linear gradient of 0%–80% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7) programmed over a 40 min period with a flow rate of 1 mL/min and detection at 260 nm.

All solvents used in reactions involving trivalent phosphorus compounds were degassed by an argon stream before use. All reactions were carried out under rigorous anhydrous conditions under an argon atmosphere. ddA was supplied by Sigma-Aldrich and was dried over P₂O₅ under reduced pressure at rt prior to

use. *Tris*(pyrrolidino)phosphine [16c], the 2-mercaptoethyl pivalate [19a] and the mononucleoside bis(*S*-pivaloyl-2-thioethyl)phosphotriester derivatives 4–6 were prepared as previously described [38]. The *S*-pivaloyl-2-thioethyl phosphodiester derivative of ddA 10 was synthesized following a published procedure [29], characterized on the basis of its spectrometric properties (NMR, UV, MS, analysis) and data were consistent with its structure and purity. The corresponding *H*-phosphonate derivative 11 was obtained by adapting a published procedure [27] and its physicochemical properties were in accordance with the literature data [39]. The *N*-(*O*-pivaloyl-2-oxyethylsulfanyl)succinimide 12 was synthesized following published procedures [15,28], characterized on the basis of its spectrometric properties (NMR, MS, analysis) and data were consistent with its structure and purity.

4.1.1. General procedure for the synthesis of mononucleoside *S,S'*-bis(*O*-pivaloyl-2-oxyethyl) phosphorodithiolates

The nucleoside analogue (0.5 mmol) was dissolved in dichloromethane (ddT, 6.0 mL), or a mixture DMF/dichloromethane (d4T, 6.0 mL, 1/3, v/v) or DMF (dda, 6.0 mL) by warming the solution at 50 °C. After cooling to room temperature, the solution was stirred for 2 h over 3A° molecular sieve (500 mg). *Tris*(pyrrolidino)

phosphine (120 mg, 0.55 mmol) was added, followed by 1*H*-tetrazole in seven aliquots (7 × 50 µL of 0.5 M tetrazole in acetonitrile, 0.175 mmol) at 3 min intervals. After stirring at room temperature for 15 min, 1*H*-tetrazole (5.2 mL 0.5 M tetrazole in acetonitrile, 2.60 mmol) was added, immediately followed by the addition of the 2-mercaptoethyl pivalate [19a] (243 mg, 1.5 mmol). The reaction mixture was stirred at room temperature for 45 min and then cooled to -40 °C. *Tert*-butyl hydroperoxide (360 µL, 3 M in toluene) was added and the reaction mixture allowed to warm to room temperature over 45 min. The reaction mixture was concentrated under reduced pressure to approximately 2 mL and diluted with dichloromethane (10 mL). The excess of oxidant was destroyed by the addition of Na₂S₂O₃ (10%, 5 mL). The organic phase was separated and the aqueous phase extracted twice with dichloromethane (2 × 10 mL). The combined organic layers were successively washed with brine (10 mL) and water (10 mL). The organic layer was dried with sodium sulfate, filtered and concentrated to dryness under reduced pressure.

4.1.1.1 *S,S'*-bis(*O*-pivaloyl-2-oxyethyl) *O*-2',3'-didehydro-2',3'-dideoxy-thymidin-5'-yl phosphorodithiolate (**1**). The crude was purified by silica gel column chromatography (ethyl acetate 30% in dichloromethane) to afford the title compound **1** (98 mg, 33%). *R*_f 0.28 (dichloromethane/ethyl acetate, 4/6, v/v); ¹H NMR (CDCl₃) δ 1.19, 1.20, (2s, 18H, *t*Bu), 1.94 (d, 3H, *J* = 1.1, CH₃), 3.17 (m, 4H, SCH₂), 4.30 (m, 5H, 5'-H, CH₂O), 4.42 (m, 1H, 5''-H), 5.04 (m, 1H, 4'-H), 5.94 (m, 1H, 2'-H), 6.36 (m, 1H, 3'-H), 7.01 (m, 1H, 1'-H), 7.21 (d, 1H, *J* = 1.2, 6-H), 8.0 (bs, 1H, NH); ¹³C NMR (CDCl₃) δ 12.8 (CH₃), 27.3 (C(CH₃)₃), 30.9 and 30.5 (2d, *J* = 2.7, *J* = 2.7, SCH₂), 38.9 (C(CH₃)₃), 62.7 and 62.8 (2d, *J* = 4.5, OCH₂), 67.3 (d, *J* = 7.8, 5'-C), 84.3 (d, *J* = 8.5, 4'-C), 89.7 (1'-C), 111.6 (5-C), 127.9 (2'-C), 133.1 (3'-C), 135.7 (6-C), 150.6 (2-C), 163.4 (4-C), 178.2 (C=O); ³¹P NMR (CDCl₃) δ 56.8; UV (ethanol 95) λ_{max} 264 nm (ε 7300); FAB MS (>0, G-T) *m/e* 593 [M+H]⁺; FAB MS (<0, G-T) *m/e* 1183 [2M - H]⁻, 591 [M - H]⁻; HRMS calculated for C₂₄H₃₉N₂O₉PS₂ (M + H)⁺: 593.1751. Found: 593.1747; HPLC *t*_R 32.3 min; Anal. Calculated for C₂₄H₃₇N₂O₉PS₂ (MW: 592.66): C, 48.64; H, 6.29; N, 4.73, S, 10.82. Found: C, 48.65; H, 6.38; N, 4.73, S, 10.97.

4.1.1.2 *S,S'*-bis(*O*-pivaloyl-2-oxyethyl) *O*-2',3'-dideoxythymidin-5'-yl phosphorodithiolate (**2**). The crude was purified by silica gel column chromatography (gradient ethyl acetate 20–100% in dichloromethane) to afford the title compound **2** (80 mg, 27%). *R*_f 0.20 (dichloromethane/ethyl acetate, 4/6, v/v); ¹H NMR (CDCl₃) δ 1.20, 1.21, (2s, 18H, *t*Bu), 1.97 (d, 3H, *J* = 1.1, CH₃), 2.09 (m, 3H, 2'-H, 3'-H, 3''-H), 2.42 (m, 1H, 2''-H), 3.20 (m, 4H, SCH₂), 4.3–4.5 (m, 7H, 4'-H, 5'-H, 5''-H, CH₂O), 6.12 (m, 1H, 1'-H), 7.41 (d, 1H, *J* = 1.2, 6-H), 8.2 (bs, 1h, NH); ¹³C NMR (CDCl₃) δ 12.6 (CH₃), 25.7 (3'-C), 27.1 (C(CH₃)₃), 30.6 and 30.5 (2d, *J* = 3.4, *J* = 3.4, SCH₂), 31.8 (2'-C), 38.8 (C(CH₃)₃), 62.5 and 62.6 (2d, *J* = 5.1, *J* = 4.8, OCH₂), 67.9 (d, *J* = 8.0, 5'-C), 78.1 (d, *J* = 7.9, 4'-C), 85.9 (1'-C), 110.9 (5-C), 135.2 (6-C), 150.3 (2-C), 163.6 (4-C), 178.0 (C=O); ³¹P NMR (CDCl₃) δ 57.1; UV (ethanol 95) λ_{max} 267 nm (ε 8700); FAB MS (>0, G-T) *m/e* 595 [M+H]⁺, 127 [BH₂]⁺; FAB MS (<0, G-T) *m/e* 1187 [2M - H]⁻, 593 [M - H]⁻; HRMS calculated for C₂₄H₄₀N₂O₉PS₂ (M + H)⁺: 595.1913. Found: 595.1877; HPLC *t*_R 34.0 min.

4.1.1.3 *S,S'*-bis(*O*-pivaloyl-2-oxyethyl) *O*-2',3'-dideoxyadenosin-5'-yl phosphorodithiolate (**3**). The crude was purified by silica gel column chromatography (gradient methanol 3–5% in dichloromethane) to afford the title compound **3** (85 mg, 28%). *R*_f 0.30 (methanol/dichloromethane, 1/9, v/v); ¹H NMR (CDCl₃) δ 1.20 (s, 18H, *t*Bu), 2.23 (m, 2H, 3'-H, 3''-H), 2.58 (m, 2H, 2'-H, 2''-H), 3.13 (m, 4H, SCH₂), 4.29 (t, 4H, *J* = 6.4, CH₂O), 4.39 (m, 3H, 4'-H, 5'-H, 5''-H), 5.3 (bs, 2H, NH₂), 6.31 (q, 1H, *J* = 3.9, *J* = 6.5, 1'-H), 8.07 (s, 1H, 8-H), 8.35 (s, 1H,

2-H); ¹³C NMR (CDCl₃) δ 26.0 (3'-C), 27.0 (C(CH₃)₃), 30.4 and 30.5 (2d, *J* = 3.1, *J* = 3.4, SCH₂), 32.2 (2'-C), 38.7 (C(CH₃)₃), 62.5 and 62.6 (2d, *J* = 4.6, *J* = 5.0, OCH₂), 67.9 (d, *J* = 8.0, 5'-C), 79.2 (d, *J* = 8.1, 4'-C), 85.5 (1'-C), 120.0 (5-C), 138.7 (8-C), 149.2 (4-C), 152.8 (2-C), 155.5 (6-C), 177.9 (C=O); ³¹P NMR (CDCl₃) δ 57.1; UV (ethanol 95) λ_{max} 259 nm (ε 14 900); FAB MS (>0, G-T) *m/e* 604 [M+H]⁺; FAB MS (<0, G-T) *m/e* 602 [M - H]⁻; HRMS calculated for C₂₄H₃₉N₅O₇PS₂ (M + H)⁺: 604.2020; HPLC *t*_R 26.0 min; Anal. Calculated for C₂₄H₃₈N₅O₇PS₂ (MW: 362.47): C, 47.75; H, 6.34; N, 11.60. Found: C, 48.21; H, 6.67; N, 11.79.

4.1.2 *S*-(*O*-pivaloyl-2-oxyethyl) *N,N*-tetramethylene *O*-2',3'-didehydro-2',3'-dideoxythymidin-5'-yl thionophosphoramidothiolate (**7**)

The crude resulting from the reaction of d4T and 2-mercaptoethyl pivalate was purified by silica gel column chromatography (gradient ethyl acetate 20–30% in dichloromethane). The fractions enriched in this side-product were purified several times in the same chromatographic conditions to obtain a pure sample of the title compound **7**. *R*_f 0.47 (dichloromethane/ethyl acetate, 4/6, v/v); ¹H NMR (CDCl₃) δ 1.19, 1.20, (2s, 9H, *t*Bu), 1.89 (m, 4H, CH₂CH₂N), 1.96 (d, 3H, *J* = 1.1, CH₃), 3.19 (m, 6H, SCH₂, CH₂CH₂N), 4.24 (m, 4H, 5'-H, 5''-H, CH₂O), 5.03 (m, 1H, 4'-H), 5.90 (m, 1H, 2'-H), 6.34 (m, 1H, 3'-H), 7.00 (m, 1H, 1'-H), 7.23 (d, 1H, *J* = 1.2, 6-H), 8.5 (bs, 1H, NH); ¹³C NMR (CDCl₃) δ 12.7 (CH₃), 26.5 (CH₂CH₂N), 27.3 (C(CH₃)₃), 32.0 (CH₂CH₂N), 38.9 (C(CH₃)₃), 48.2 (SCH₂), 63.2 and 63.4 (2d, *J* = 4.1, *J* = 4.3, OCH₂), 65.9 and 66.5 (2d, *J* = 5.9, *J* = 5.9, 5'-C), 84.9 (4'-C), 89.8 and 90.1 (2s, 1'-C), 111.4 (5-C), 127.3 and 127.4 (2s, 2'-C), 133.4 and 133.7 (2s, 3'-C), 135.7 and 135.9 (2s, 6-C), 150.8 (2-C), 163.7 (4-C), 178.3 (C=O); ³¹P NMR (CDCl₃) δ 92.9 and 94.3; UV (ethanol 95) λ_{max} 264 nm (ε 7400); FAB MS (>0, G-T) *m/e* 518 [M+H]⁺; FAB MS (<0, G-T) *m/e* 516 [M - H]⁻; HRMS calculated for C₂₁H₃₃N₃O₆PS₂ (M + H)⁺: 518.1543. Found: 518.1547; HPLC *t*_R 33.7 min.

4.1.3 *S,S'*-bis(*O*-pivaloyl-2-oxyethyl) *O*-2',3'-didehydro-2',3'-dideoxythymidin-5'-yl thionophosphorodithiolate (**8**)

The crude resulting from the reaction of d4T and 2-mercaptoethyl pivalate was purified by silica gel column chromatography (gradient ethyl acetate 20–30% in dichloromethane). The fractions enriched in this side-product were purified several times in the same chromatographic conditions to obtain a pure sample of the title compound **8**. *R*_f 0.47 (dichloromethane/ethyl acetate, 4/6, v/v); ¹H NMR (CDCl₃) δ 1.20, (s, 18H, *t*Bu), 1.97 (d, 3H, *J* = 1.1, CH₃), 3.20 (m, 4H, SCH₂), 4.27 (m, 5H, 5'-H, CH₂O), 4.39 (m, 1H, 5''-H), 5.04 (m, 1H, 4'-H), 5.92 (m, 1H, 2'-H), 6.34 (m, 1H, 3'-H), 7.01 (m, 1H, 1'-H), 7.21 (d, 1H, *J* = 1.2, 6-H), 8.5 (bs, 1H, NH); ¹³C NMR (CDCl₃) δ 12.8 (CH₃), 27.3 (C(CH₃)₃), 33.4 and 33.8 (2d, *J* = 2.9, *J* = 3.3, SCH₂), 38.9 (C(CH₃)₃), 62.4 and 62.6 (2d, *J* = 4.7, OCH₂), 67.2 (d, *J* = 8.6, 5'-C), 84.3 (d, *J* = 9.7, 4'-C), 89.8 (1'-C), 111.6 (5-C), 127.8 (2'-C), 133.2 (3'-C), 135.7 (6-C), 150.8 (2-C), 163.6 (4-C), 178.2 (C=O); ³¹P NMR (CDCl₃) δ 113.3; UV (ethanol 95) λ_{max} 264 nm (ε 7200); FAB MS (>0, G-T) *m/e* 609 [M+H]⁺; FAB MS (<0, G-T) *m/e* 607 [M - H]⁻; HRMS calculated for C₂₄H₃₈N₂O₈PS₃ (M + H)⁺: 609.1522. Found: 609.1505; HPLC *t*_R 37.2 min.

4.1.4 *S*-(*O*-pivaloyl-2-oxyethyl) *O*-2',3'-dideoxyadenosin-5'-yl phosphorothiolate diester, sodium salt (**9**). The *H*-phosphonate **11** [39] (59 mg, 0.147 mmol) was first co-evaporated with anhydrous pyridine then anhydrous DMF before to be dissolved in this last solvent (3 mL). To the resulting solution was added successively chlorotrimethylsilane (75 µL, 0.593 mmol), triethylamine (123 µL, 0.882 mmol) and a solution of the thiosuccinimide **12** (49 mg, 0.189 mmol) in anhydrous acetonitrile (1 mL). After 3 h stirring at room temperature under argon atmosphere, the reaction mixture

was added triethylammonium carbonate buffer (TEAB, 1 M, pH = 7) and concentrated under reduced pressure. The purification of the crude was performed by column chromatography, first on silica gel (gradient methanol 5–30% in dichloromethane), then on reverse phase (gradient methanol 0–50% in water). The appropriate fractions were collected, concentrated under reduced pressure and percolated on Dowex 50WX2 (sodium form). After freeze-drying in water, the title compound **9** was obtained as a white powder (55 mg, 78%). R_f 0.56 (iPrOH/NH₄OH/H₂O, 7/2/1, v/v/v) and 0.32 (MeOH/CH₂Cl₂, 35/65, v/v); ¹H NMR (DMSO-*d*₆) δ 1.05 (s, 9H, C(CH₃)₃), 2.06 (m, 2H, 3'-H, 3''-H), 2.41 (m, 2H, 2'-H, 2''-H), 2.66 (m, 2H, SCH₂), 3.73 (m, 2H, 5'-H, 5''-H), 4.07 (t, 2H, *J* = 7.2 Hz, OCH₂), 4.21 (m, 1H, 4'-H), 6.21 (m, 1H, 1'-H), 7.26 (s, 2H, NH₂), 8.11 (s, 1H, 8-H), 8.38 (s, 1H, 2-H); ¹³C NMR (DMSO-*d*₆) δ 26.3 (3'-C), 26.9 (C(CH₃)₃), 28.6 (d, *J* = 3.3 Hz, SCH₂), 31.7 (2'-C), 38.1 (C(CH₃)₃), 64.1 (d, *J* = 5 Hz, OCH₂), 66.1 (d, *J* = 5.9, 5'-C), 80.0 (d, *J* = 8.5, 4'-C), 84.1 (1'-C), 118.9 (5-C), 138.9 (8-C), 148.9 (4-C), 152.5 (2-C), 156.0 (6-C), 177.2 (C=O); ³¹P NMR (DMSO-*d*₆) δ 13.6; UV (ethanol 95) λ_{max} 260 nm (ϵ 12 800); FAB MS (>0, G-T) *m/e* 482 [M+H]⁺, 460 [M-Na+H]⁺, 136 [BH₂]⁺; FAB MS (<0, G-T) *m/e* 458 [M-Na]⁻, 134 [B]⁻; Anal. Calculated for C₁₇H₂₅N₅NaO₆PS (MW: 481.44) + 1.5H₂O: C, 40.16; H, 5.55; N, 13.77. Found: C, 40.20; H, 5.50; N, 13.75.

4.2. Biological methods

The origin of the viruses and the techniques used for measuring inhibition of virus multiplication were as previously described [3,40].

4.3. Stability studies

The studies were performed using already published methods [3,29,41]. Co-injection with authentic samples (ddA, ddAMP, metabolites **9** and **10**) were used for calibration and identification of the decomposition products. The rate constants of disappearance of the studied compounds were calculated according to pseudo-first-order kinetic models, which were in accordance with the experimental data.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.2021.113914>.

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