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Title

An original pronucleotide strategy for the simultaneous delivery of two bioactive drugs

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Abstract

The synthesis and *in vitro* anti-HIV activity of a novel series of phosphoramidate pronucleotides including a *S*-pivaloyl-2-thioethyl (*t*BuSATE) group as biolabile phosphate protecting group are reported. Such constructs, obtained through different phosphorus chemistries, are characterized by the association of two different anti-HIV nucleoside analogues linked to the phosphorus atom respectively by the sugar residue and the exocyclic amino function of the nucleobase. *In vitro*, comparative anti-HIV evaluation demonstrates that such original prodrugs are able to allow the efficient intracellular combination release of a 5'-mononucleotide as well as another nucleoside analogue. In human T4-lymphoblastoid cells, the pronucleotide **1** shows remarkable antiviral activity with an EC₅₀ in the nanomolar range (0.6 nM) and without additional cytotoxicity. In addition, these two pronucleotide models exhibit higher selectivity index than the equimolar mixture of their constitutive nucleoside analogues opening the way to further studies with regard to the current use of drug combinations.

Keywords: mononucleotide, prodrug, antiviral, drug combination

1. Introduction

During the last decade, the most significant advances in the treatment of viral infections have probably been associated with the development of 5'-mononucleotide prodrugs (pronucleotides), notably phosphoramidate pronucleotides^{1,2}. Thus, Sofosbuvir³ and Tenofovir alafenamide⁴ (Fig. 1) were approved by the United States Food and Drug Administration (FDA) on December 2013 and November 2016 for the treatment of chronic hepatitis C (HCV) and B (HBV) infections, respectively. More recently, the potential of Remdesivir (Fig. 1), a phosphoramidate pronucleotide with broad-spectrum activities against RNA viruses, has been proposed for the treatment of coronavirus disease-2019⁵.

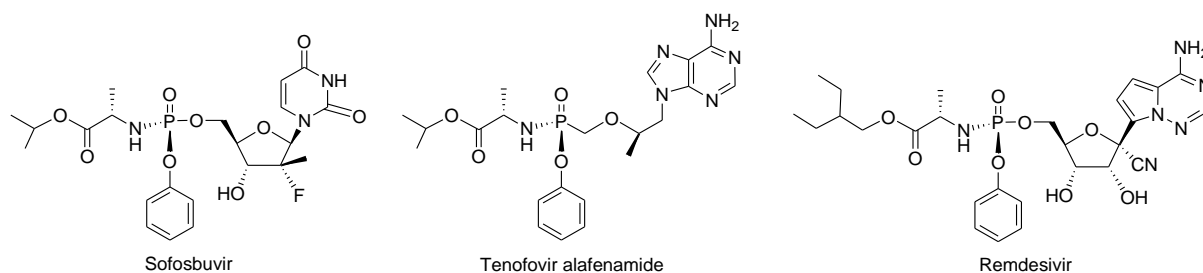
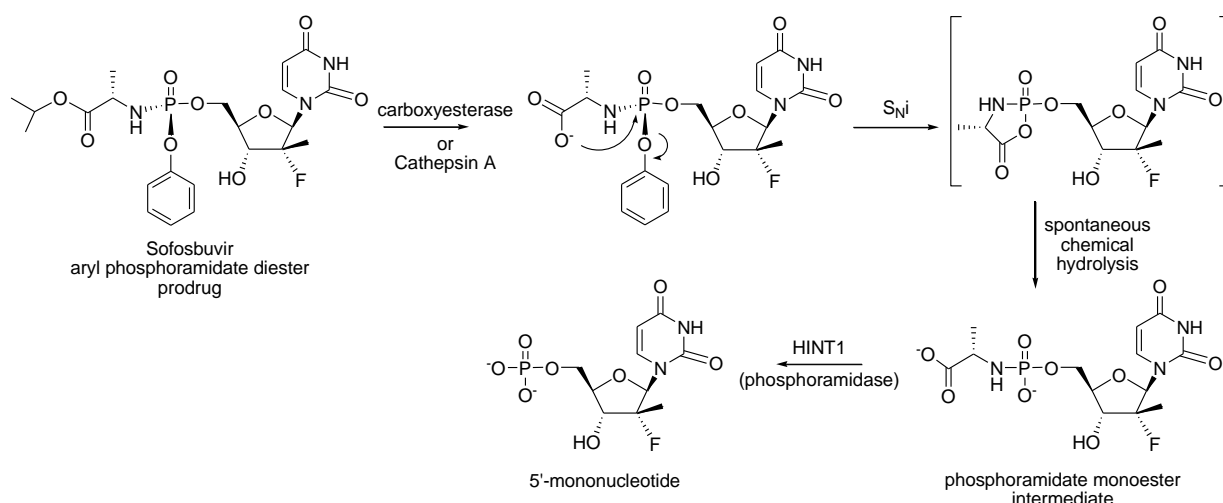


Fig. 1. Chemical structures of Sofosbuvir, Tenofovir alafenamide and Remdesivir.

First disclosed by McGuigan *et al.*⁶, such kind of pronucleotides (*i.e.* mononucleoside aryl phosphoramidate diesters) were 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 structure typically consists in a 5'-mononucleotide analogue where the phosphate (or phosphonate) group is masked by appending an aryloxy group (usually a phenol) and a α -amino acid ester (Fig. 1). This type of pronucleotides is commonly named McGuigan ProTides. As illustrated in the Scheme 1 for the Sofosbuvir decomposition pathway⁷, the phenyl phosphoramidate diester is hydrolyzed by a sequence involving enzymatic and chemical steps requiring either carboxyesterase or cathepsin A to cleave the terminal amino acid ester, then intramolecular displacement of the phenol substituent and the enzymatic cleavage of the amino acid moiety by a phosphoramidase or histidine triad nucleotide-binding protein 1 (HINT 1).



Scheme 1. Sofosbuvir decomposition pathway.

As part of our work in the field of pronucleotides, we have previously studied⁸ the potential of series of phosphoramidate diesters incorporating a *S*-acyl-2-thioethyl (SATE) phosphate protecting group as an alternative to the aryl substituent, and aliphatic (or aromatic) amino residues (Fig. 2). In cell culture experiments, such pronucleotides lead to the selective and efficient intracellular release of the parent 5'-mononucleotide, through a mechanism involving successively an esterase and a phosphoramidase activation step. Various amino residues could be introduced without affecting the biological activity of the corresponding prodrugs indicating a less restrictive intracellular decomposition process than previously reported in the literature.

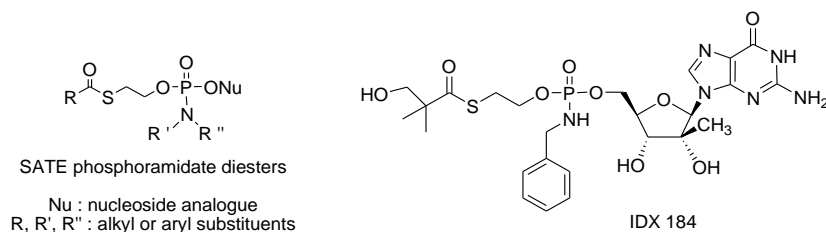
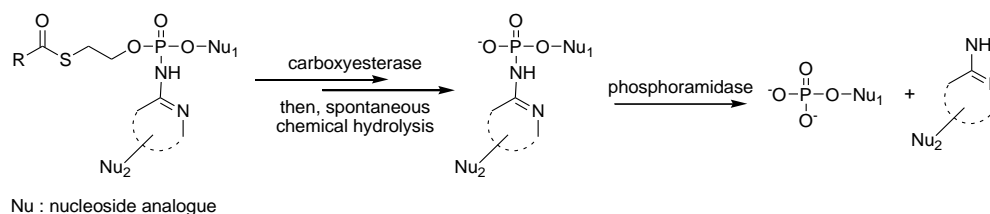


Fig. 2. Structures of SATE phosphoramidate diesters and IDX 184.

One of the most promising SATE phosphoramidate diester derivative was IDX 184 (Fig. 2), a 2'-methylguanosine pronucleotide, which demonstrated to be effective in patients with chronic HCV infections in phase II clinical trials⁹⁻¹⁰. Unfortunately, severe cardiotoxicity complications observed during phase III trials of INX 189, a McGuigan ProTide sharing the same nucleoside analogue parent, led to the withdrawal of IDX 184 from further clinical developments¹⁰.

The image of the Trojan horse has often been used to illustrate strategies aiming to the selective mononucleotide-delivery based on enzymatic and/or chemically induced cascade mechanisms. To pursue the comparison with Greek mythology, we report herein like Orthrus, the two-headed dog who guarded Geryon's cattle, an unprecedented phosphoramidate pronucleotide series designed to deliver two potent and different biologically active metabolites

into cells (Scheme 2). In such constructs, the amino function bound to the phosphorus atom is the one present on the nucleobase of the second nucleoside analogue. Their expected decomposition pathway could lead to the simultaneous release of a 5'-mononucleotide and of another nucleoside analogue. Thus, in addition to the benefit of the previously reported pronucleotide approach, developed to overcome the undesirable metabolism of a nucleoside analogue (Nu₁ Scheme 2), the concomitant delivery of another (properly selected) bioactive nucleoside (Nu₂) could increase the potency and the selectivity of the biological effect, as already observed in current combination antiretroviral therapy¹¹.



Scheme 2. Expected decomposition pathway of the studied pronucleotides.

Thus, we report the synthesis and biological evaluation of two SATE dinucleoside phosphoramidate diester models (Fig. 3, compounds **1** and **2**) incorporating a *S*-pivaloyl-2-thioethyl (*t*BuSATE) phosphate protecting group and characterized by the presence of the 2',3'-dideoxycytidine (ddC) linked to the phosphorus atom through its exocyclic amino group. In addition, pronucleotide **1** combines 3'-azido-2',3'-dideoxythymidine (AZT) while 2',3'-dideoxyadenosine (ddA) is used for derivative **2**.

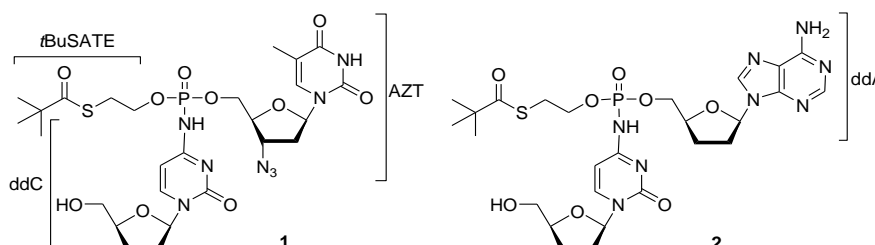


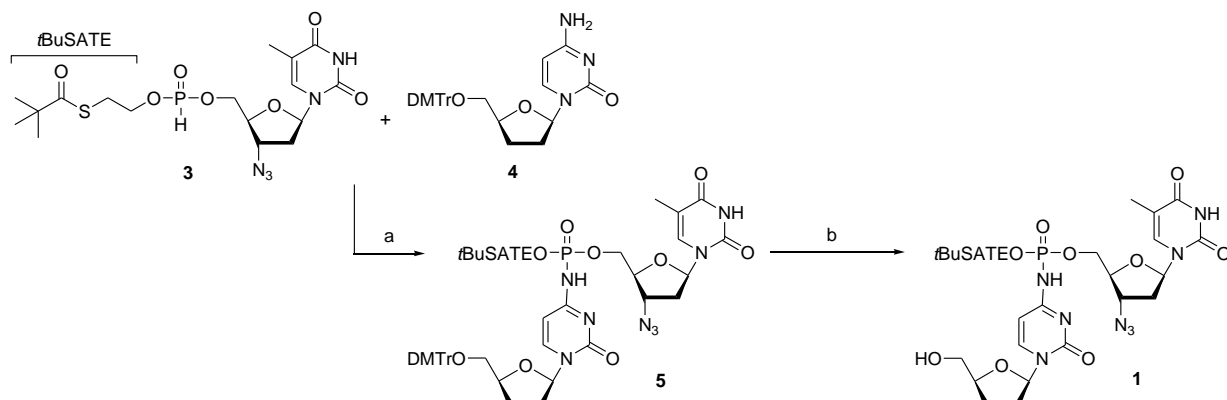
Fig. 3. Structures of the *t*BuSATE dinucleoside phosphoramidate diesters investigated in this study.

2. Results and discussion

2.1. Chemistry

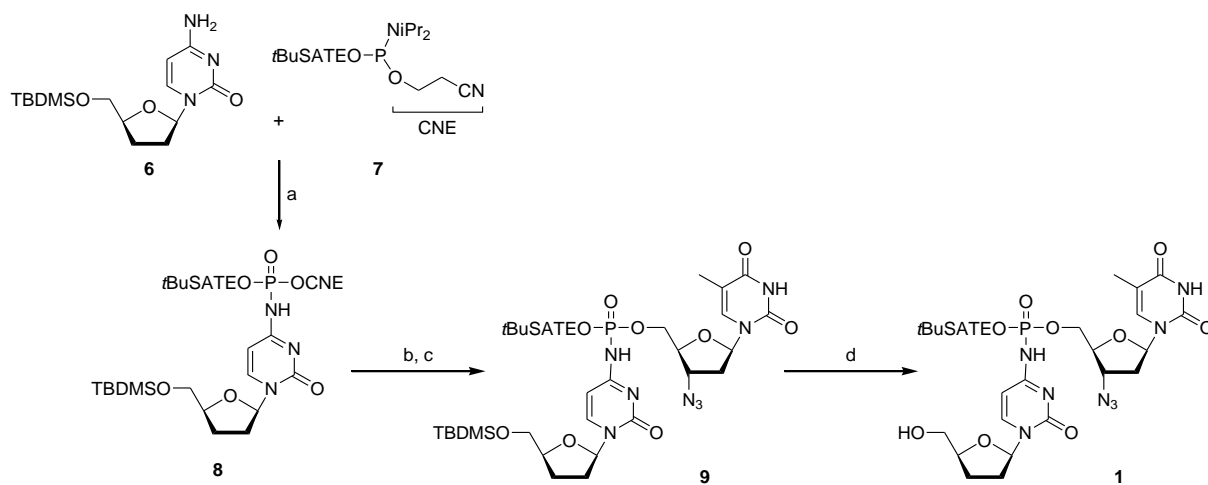
The pronucleotide **1** was first synthesized according to the hydrogenphosphonate approach (Scheme 3), previously reported for the preparation of various phosphoramidate diester derivatives of AZT⁸. Oxidative coupling of the *H*-phosphonate diester **3**¹² with the protected nucleoside **4**¹³, prepared beforehand according to a published procedure¹⁴, afforded the desired phosphorylated intermediate **5** in low yield (9%). Amidative oxidation reactions generally lead to satisfactory yields. Nevertheless, in the case of weakly basic amines (such as arylamines) the addition of a tertiary amine to the reaction medium is required¹⁵. In our case, the optimized reaction is using an excess of nucleoside **4** (4 eq.) in presence of 1,8-diazabicyclo-

[5.4.0]undec-7-ene (DBU, 3 eq.). Finally, acid treatment of **5** afforded the desired *t*BuSATE dinucleoside phosphoramidate **1** in 80% yield.



Scheme 3. Synthesis of the pronucleotide **1** following a hydrogenphosphonate strategy. Reagents and conditions: (a) CCl_4 , 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), pyridine, rt, 3h, 9%; (b) 3% $\text{CCl}_3\text{CO}_2\text{H}$ in CH_2Cl_2 , 15min, 80%.

The low yield observed during the amidative oxidation step led us to consider another synthetic strategy. After several attempts, the pronucleotide **1** was also obtained through a synthetic procedure involving the formation of the 4-*N*-phosphoramidate precursor (Scheme 4).



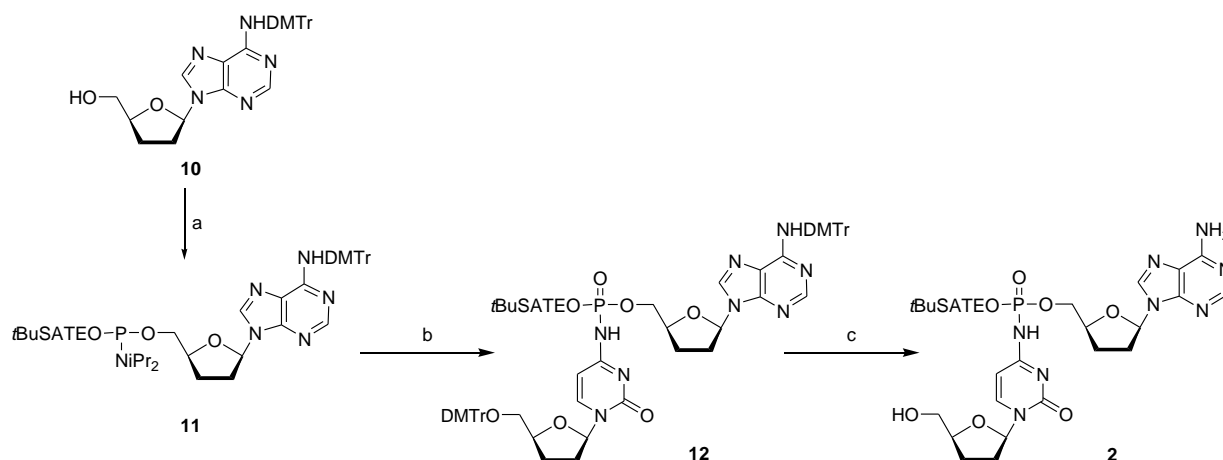
Scheme 4. Synthesis of the pronucleotide **1** according to the preliminary synthesis of 4-*N*-phosphoramidate precursor. Reagents and conditions: (a) 1*H*-tetrazole, CH_2Cl_2 , rt, 2h, then *t*BuOOH (3M in toluene), -20°C , 38%; (b) DBU, THF, rt, 18h, then (c) AZT, 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl), pyridine, rt, 24h, 83% over two steps; (d) HCl (1% in ethanol), rt, 20min, 80%.

The silylated nucleoside **6**¹⁶ was phosphitylated with phosphoramidite **7**, previously prepared from *S*-pivaloyl-2-thioethyl *N,N*-bis(diisopropylamino)phosphine¹², upon tetrazole activation and in presence of *N,N*-diisopropylamine. The resulting phosphoramidite was oxidized *in situ* with *tert*-butyl hydroperoxide to give rise to the 4-*N*-phosphoramidate **8** in 38% yield. The key step in this multi-step procedure results in the introduction of the second nucleoside analogue. The dinucleoside phosphoramidate **9** was obtained following a one-pot two steps procedure.

Thus, the β -cyanoethyl group of **8** was removed by using DBU to obtain a phosphoramidate monoester intermediate, which is directly condensed without purification with AZT in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) and affording the phosphorylated intermediate **9** in 83% yield over two steps. Subsequent acidic treatment gave rise to the desired pronucleotide **1** in 80% yield.

Then, we extended this dinucleoside pronucleotide approach to the study of a phosphoramidate derivative in which a purine nucleoside analogue replaces . AZT and we selected 2',3'-dideoxyadenosine (ddA). Indeed, we previously demonstrated that applied to this anti-human immunodeficiency virus (anti-HIV) drug, a pronucleotide approach led to an enhanced *in vitro* antiviral efficiency¹⁷. The synthesis of the resulting pronucleotide **2** (Fig. 3) was carried out using a strategy based on phosphoramidite intermediates (Scheme 5). The latter could not be used for the preparation of pronucleotide **1** because of the reactivity of phosphoramidites in presence of the azide group of AZT¹⁸.

Protected nucleoside **10**, synthesized according to a previously described procedure¹⁹, was reacted with *S*-pivaloyl-2-thioethyl *N,N*-bis(diisopropylamino)phosphine¹² to afford phosphoramidite **11** in 78% yield. Coupling this intermediate with nucleoside **4** in presence of 1*H*-tetrazole, followed by *in situ* oxidation with *tert*-butyl hydroperoxide, gave rise to the fully protected phosphoramidate **12** in 41% yield. Finally, a controlled acidic treatment (in order to avoid depurination) led to the desired pronucleotide **2** in 67% yield.



Scheme 5. Synthesis of the pronucleotide **2** using a phosphoramidite strategy. Reagents and conditions: (a) *S*-pivaloyl-2-thioethyl *N,N*-bis(diisopropylamino)phosphine, 1*H*-tetrazole, HN(iPr)₂, CH₂Cl₂/CH₃CN, rt, 1h, 78%; (b) **4**, 1*H*-tetrazole, CH₂Cl₂/CH₃CN, rt, 1h30, then *t*BuOOH (3M in toluene), -40°C, 41%; (c) AcOH/H₂O/dioxane (pH 2.5), rt, 24h, 67%.

Usually, *t*BuSATE dinucleoside phosphoramidate diesters displayed two closely spaced signals in ³¹P NMR associated with the presence of diastereoisomers on the phosphorus stereocenter. In some cases, distinct diastereoisomeric signals were also noted on the proton-decoupled ¹³C NMR spectra, from ¹H NMR spectroscopy and analytical HPLC studies.

Characteristic features of ¹H NMR spectra of the phosphorylated prodrugs were downfield shifts of the cytosine 5-H and 6-H protons that may be attributed to the electron-withdrawing effect of the 4-*N*-phosphorylation. The same effect has already been reported for 4-*N*-

phosphorylated ²⁰ and acylated ²¹ cytidine derivatives. No significant effect of *N*-substitution on the chemical shifts of the sugar moiety of ddC was observed.

Similarly, there were no significant effects of the *N*-phosphorylation on the chemical shifts of the sugar carbons of ddC. ¹³C NMR spectra of phosphoramidate derivatives showed an upfield effect for chemical shifts of the cytosine 2-C and 4-C carbons. Determination of P-C couplings is made difficult due to the complexity generated by the presence of diastereoisomers.

2.2. Antiviral activity

The inhibitory effects on the replication of HIV-1 of the *t*BuSATE dinucleoside phosphoramidate diesters **1** and **2** were evaluated, as diastereoisomeric mixtures, in three cell culture systems in comparison to AZT, ddC and ddA as well as an equimolar mixture of each constitutive nucleosides. The related cytotoxicity (CC₅₀) was also determined in parallel in uninfected cells. These results are summarized in Table 1.

With 50% effective concentration (EC₅₀) about nanomolar concentration range, pronucleotide **1** appeared to be most potent than AZT, ddC, as well as an equimolar mixture of the two nucleoside analogues in human T4-lymphoblastoid cells, CEM-SS and MT-4. In addition, the antiviral effect observed was not correlated with an increased cytotoxicity. Thus, no cytotoxicity was observed in MT-4 cells at the highest concentration tested (up to 500 μM), leading to a selectivity index (SI) more than five times higher than the one of the mixture of the parent nucleosides (AZT and ddC), whereas in CEM cells, the SI of pronucleotide **1** is two hundred times higher than the same mixture.

Table 1

Anti-HIV activity (μM)^a in three cell culture systems of pronucleotides **1** and **2** compared to the parent nucleosides as well as the equimolar mixture of each constitutive nucleoside analogues.

Compound	CEM-SS			MT-4			CEM/TK ⁻		
	EC ₅₀ ^b	CC ₅₀ ^c	SI ^d	EC ₅₀ ^b	CC ₅₀ ^c	SI ^d	EC ₅₀ ^b	CC ₅₀ ^c	SI ^d
1	0.0006	350	>580000	0.0065	>500	>76920	0.001	>500	>312500
AZT	0.024	>1000	>40000	0.017	140	8235	>100	>1000	-
ddC	0.067	16	239	2	180	90	0.015	7	467
AZT/ddC ^e	0.016	46	2875	0.083	130	15660	0.009	8.3	922
2	0.04	>160	>4000	0.79	>160	>203			
ddA	2	>110	>55	9	>110	>12			
ddA/ddC ^f	0.01	46	4600	1.2	>100	>83			

^a All data represent average values for at least three separate experiments. The variation of these results under standard operating procedures is below ± 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%. ^d SI: selectivity index, ratio CC₅₀/EC₅₀. ^e Equimolar mixture of AZT and ddC. ^f Equimolar mixture of AZT and ddA.

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 AZT ²². In contrast to

AZT, the SATE dinucleoside phosphoramidate **1** exhibited significant anti-HIV effects in CEM/TK⁻ cells, similar to that observed in infected CEM-SS cells. This result demonstrates the successful release of the corresponding 5'-mononucleotide (*i.e.* AZT 5'-monophosphate) inside cells. Using an already published method^{23, 24}, the stability of pronucleotide **1** was studied in culture medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum) and in total cell extracts from CEM-SS cells in order to mimic its behavior inside the cells (see supplementary data). Thus, pronucleotide **1** decomposed faster in cell extracts ($t_{1/2} = 2.3$ h) than in culture medium ($t_{1/2} = 165$ h). In both media, the formation of the corresponding phosphoramidate monoester was observed after a first step mediated by esterases (loss of the SATE group, Scheme 2). Then, this metabolite appeared to be stable in the culture medium and was very slowly hydrolyzed in cell extracts. These data are in agreement with the literature concerning the enzymatic stability of McGuigan ProTides and SATE phosphoramidate pronucleotides in these biological media used as models and may be associated with the partial denaturation of the enzymatic content during the preparation of the cell extracts^{8,25}.

We previously demonstrated the efficacy of the SATE pronucleotide approach applied to various anti-HIV nucleoside analogues that are hampered by the first phosphorylation step, through a dependence on kinase-mediated phosphorylation, or by a rate limiting-step in the anabolism pathway. Thus, the bis(SATE)phosphotriester derivatives of ddA emerged as very potent and selective inhibitors of HIV and simian immunodeficiency virus (SIV) replications *in vitro* in various human infected cells, stimulated and unstimulated primary cultured human cell lines such as peripheral blood mononuclear (PBM) cells or monocyte-derived macrophages¹⁷. The inhibitory effects of pronucleotide **2** on the replication of HIV-1 was evaluated in CEM-SS and MT-4 cells (Table 1). This derivative appeared to be most potent than its parent nucleoside ddA. As observed with analogue **1**, the SI of pronucleotide **2** is higher than that of the equimolar mixture of each constitutive nucleosides (ddA and ddC). As suggested by our previous works, the putative phosphoramidase activity that could be involved in the bioconversion of the dinucleoside phosphoramidate monoester intermediate into the corresponding 5'-mononucleotide (Scheme 2) seems to exhibit a broad substrate specificity, hydrolyzing phosphoramidates bearing arylamino substituents as well as pyrimidine aglycones.

3. Conclusion

The synthesis of novel SATE dinucleoside phosphoramidate derivatives has been performed using different phosphorus chemistries. Such constructs are characterized by the combination of two different anti-HIV nucleoside analogues linked to the phosphorus atom respectively by the sugar residue (5'-hydroxyl function) and the exocyclic amino function presents on the pyrimidine nucleobase. Comparative anti-HIV evaluation in three culture cell lines indicates that these original constructs are able to deliver efficiently a 5'-mononucleotide and another nucleoside analogue within infected cells. On the basis of the literature data, the proposed decomposition pathway may successively involve an esterase (hydrolysis of SATE phosphate protecting group), followed by another bioactivation step carried out by a putative phosphoramidase activity. These promising results encourage us to pursue the study of this new series of pronucleotides in order to evaluate their pharmacological potential with regard to the current use of drug combinations.

4. Experimental section

4.1. General Information

Unless otherwise stated, ^1H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra at 100 MHz with proton decoupling at 25 °C on a Bruker 300 Avance or DRX 400. Chemical shifts are given in δ values referenced to the residual solvent peak (CDCl_3 at 7.26 ppm and 77 ppm, $\text{DMSO-}d_6$ at 2.49 ppm and 39.5 ppm) relative to TMS. Deuterium exchange, decoupling and COSY experiments were performed in order to confirm proton assignments. Coupling constants, J , are reported in Hertz. 2D ^1H - ^{13}C heteronuclear COSY were recorded for the attribution of ^{13}C signals. ^{31}P NMR spectra were recorded at ambient temperature at 121 MHz with proton decoupling. Chemical shifts are reported relative to external H_3PO_4 . FAB mass spectra were recorded in the positive-ion or negative-ion mode on a JEOL JMS DX 300 using thioglycerol/glycerol (1:1, v/v, G-T) as matrix. 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 second. UV spectra were recorded on an Uvikon 931 (Kontron). Elemental analyses were carried out by the Service de Microanalyses du CNRS, Division de Vernaison (France). Analytical HPLC experiments were carried out on an Alliance 2690 system (Waters, Milford, Massachusetts, USA) equipped with 996-photodiode detector and a Millennium data workstation. A reverse-phase analytical column (Nucleosil, C_{18} , 150×4.6 mm, $5 \mu\text{m}$) equipped with a prefilter, and a precolumn (Nucleosil, C_{18} , $5 \mu\text{m}$) were used. Detection was monitored at 267 nm. Nucleotidic derivatives were eluted using a linear gradient of 0 to 80 % acetonitrile in 20 mM triethylammonium acetate buffer (pH 7) over 30 min at 1 mL/min flow rate. TLC was performed on precoated aluminum sheets of silica gel 60 F₂₅₄ (Merck, Art. 9385), visualization of products being accomplished by UV absorbance followed by charring with 5% ethanolic sulphuric acid with heating for nucleotides. Flash chromatography was carried out using 63 - 100 μm silica gel (Merck Art. N°115101) otherwise 40 - 63 μm silica gel (Merck Art. N°109385) was used. Solvents were reagent grade or purified by distillation prior to use, and solids were dried over P_2O_5 under reduced pressure at rt. Moisture sensitive reactions were performed under argon atmosphere using oven-dried glassware. All aqueous (aq.) solutions were saturated with the specified salt unless otherwise indicated.

AZT was from Brantford Chemicals Inc.; ddC was purchased from Carbosynth; *S*-pivaloyl-2-thioethyl *N,N*-bis(diisopropylamino)phosphine and *H*-phosphonate diester **3** were obtained following a previously published procedure¹².

4.1.1. 1-(2,3-Dideoxy-5-*O*-(4,4'-dimethoxytrityl)- β -*D*-ribofuranosyl) cytosine (**4**)

According to a published procedure¹⁴, 2',3'-dideoxycytidine (ddC, 1.05 g, 4.97 mmol) was co-evaporated (3x) with pyridine and suspended in the same solvent (6 mL). To this suspension were added first triethylamine (0.69 mL, 4.97 mmol) and then dichloroacetic acid (0.41 mL, 4.97 mmol). After 15 minutes stirring at room temperature, 4,4'-dimethoxytrityl chloride (2.02 g, 5.96 mmol) was added portion wise. After 2 hours at room temperature,

methanol (1 mL) was added. Then, the reaction mixture was diluted with ethyl acetate (50 mL), washed with saturated NaHCO₃ (30 mL) and finally with water (30 mL). The resulting organic layer was dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude was co-evaporated with toluene (3x) and purified by silica gel column chromatography (gradient methanol 0-5% in ethyl acetate/ 0.2% NEt₃) to afford **4** as white foam (2.14 g, 84%). R_f (CH₂Cl₂/CH₃OH/NEt₃, 89/10/1, v/v/v): 0.48; NMR ¹H (CDCl₃) data were in accordance with literature ¹³; NMR ¹³C (CDCl₃, 100 MHz) δ 165.5 (1s, C-4), 158.5 (1s, CH₃O*Ph* ipso), 155.9 (1s, C-2), 144.6 (1s, *Ph* ipso), 141.9 (1s, C-6), 135.7-135.6-130.1 (3s, CH₃O*Ph*), 128.2-127.9-126.9 (3s, *Ph*), 113.2 (1s, CH₃O*Ph*), 92.9 (1s, C-5), 87.2 (1s, C-1'), 86.5 (1s, C[(CH₃O*Ph*)₂Ph]), 81.2 (1s, C-4'), 63.7 (1s, C-5'), 55.2 (1s, CH₃O*Ph*), 33.6 (1s, C-2'), 24.9 (1s, C-3'); MS FAB>0 (GT) m/z 514 (M+H)⁺, 112 (BH₂)⁺; MS FAB<0 (GT) m/z 512 (M-H)⁻, 110 (B)⁻; UV (ethanol 95) λ_{max} 275 nm (ε 11 000), 233 (ε 25 000), λ_{min} 258 nm (ε 8 300); HPLC t_R 29.1 min; Anal. Calculated for C₃₀H₃₁N₃O₅ (MW: 513.58): C, 70.16; H, 6.08; N, 8.18. Found: C, 69.90; H, 6.10; N, 7.85.

4.1.2. O-(3'-Azido-2',3'-dideoxythymidin-5'-yl)-O'-(S-pivaloyl-2-thioethyl)-4-N-[2',3'-dideoxy-5'-O-(4,4'-dimethoxytrityl)cytidine] phosphoramidate (**5**)

The protected nucleoside **4** (2.24 g, 4.36 mmol) was dissolved in pyridine (6 mL) and 1,8-diazabicyclo[5.4.0]undec-7-en (DBU, 0.49 mL, 3.27 mmol) was added. After 30 minutes stirring at room temperature, the mixture was added to a solution of *H*-phosphonate diester **3** (0.52 g, 1.09 mmol), previously co-evaporated with pyridine (3x), in carbon tetrachloride (10 mL). After 3 hours stirring at room temperature, the mixture was diluted with chloroform (50 mL) and washed with saturated NaHCO₃ (25 mL) then with water (25 mL). The resulting organic layer was dried over Na₂SO₄, filtered and concentrated under reduced pressure. After co-evaporation with toluene, column chromatography on silica gel (gradient ethyl acetate 60-100% in hexane/ 0.1% NEt₃, then methanol 0-2% in ethyl acetate/ 0.1% NEt₃) gave a diastereoisomeric mixture of the desired phosphoramidate **5** (0.097 g, 9%). R_f (AcOEt/NEt₃, 99/1, v/v): 0.37; NMR ¹H (CDCl₃, 400 MHz) δ 10.4 (bs, 1H, Thy-NH), 8.6 (bs, 1H, P-NH), 7.98 (m, 1H, *H*-6_{ddC}), 7.48-7.46 (2d, 1H, *J* = 1.0 Hz, *H*-6_{AZT}), 7.34 (m, 2H, *Ph*), 7.21 (m, 7H, CH₃O*Ph* meta, *Ph*), 6.78 (d, 4H, *J* = 8.8 Hz, CH₃O*Ph*), 6.20 (m, 1H, *H*-1'_{AZT}), 5.97 (dd, 1H, *J* = 6.3 Hz, *J* = 1.5 Hz, *H*-1'_{ddC}), 5.50-5.48 (2d, 1H, *J* = 3.3 Hz, *H*-5_{ddC}), 4.37 (m, 1H, *H*-3'_{AZT}), 4.16 (m, 3H, *H*-5'_{AZT}, *H*-5''_{AZT}, *H*-4'_{AZT}), 3.98 (m, 3H, *H*-4'_{ddC}, OCH₂CH₂), 3.73 (s, 6H, CH₃O*Ph*), 3.47 (dd, 1H, *J* = 10.8 Hz, *J* = 2.4 Hz, *H*-5'_{ddC}), 3.26 (m, 1H, *H*-5''_{ddC}), 3.06-3.05 (2t, 2H, *J* = 6.8 Hz, OCH₂CH₂S), 2.32 (m, 3H, *H*-2'_{AZT}, *H*-2''_{AZT}, *H*-2'_{ddC}), 1.98 (m, 3H, *H*-2''_{ddC}, *H*-3'_{ddC}, *H*-3''_{ddC}), 1.87-1.85 (2d, 3H, *J* = 0.7 Hz, Thy-CH₃), 1.15-1.14 (2s, 9H, (CH₃)₃CSATE); NMR ³¹P (CDCl₃, 81 MHz) δ 8.07-7.90; MS FAB>0 (GT) m/z 987 (M+H)⁺, 961 (M-N₂+3H)⁺, 738 (M-AZT+2H)⁺. FAB<0 (GT) m/z 985 (M-H)⁻, 959 (M-N₂+H)⁻, 736 (M-AZT)⁻.

4.1.3. 1-(2,3-Dideoxy-5-O-*tert*-butyldimethylsilyl-β-D-ribofuranosyl) cytosine (**6**)

To a suspension of 2',3'-dideoxycytidine (ddC, 1.50 g, 7.10 mmol) in pyridine (30 mL) was added *tert*-butyldimethylsilyl chloride (1.61 g, 2.25 mmol). After stirring overnight at room temperature, the mixture was diluted in dichloromethane (70 mL) and washed with water. The resulting organic layer was dried over Na₂SO₄, filtered and concentrated under reduced

pressure. The purification of the crude was performed by column chromatography on silica gel (gradient methanol 3-10% in dichloromethane) affording the desired nucleoside **6** as a white powder (2.22 g, 96%). R_f (CH₂Cl₂/CH₃OH, 9/1, v/v): 0.30; NMR ¹H (CDCl₃) data were in accordance with literature ¹⁶; NMR ¹H (DMSO-*d*₆, 400 MHz) δ 7.88 (d, 1H, *J* = 7.4 Hz, *H*-6), 7.10 (bl, 2H, *J* = 14.1 Hz, Cyt-NH₂), 5.92 (m, 1H, *H*-1'), 5.64 (d, 1H, *J* = 7.4 Hz, *H*-5), 4.04 (m, 1H, *H*-4'), 3.91 (dd, 1H, *J* = 11.5 Hz, *J* = 2.8 Hz, *H*-5'), 3.70 (dd, 1H, *J* = 11.5 Hz, *J* = 3.1 Hz, *H*-5'), 2.27 (m, 1H, *H*-2'), 1.81 (m, 3H, *H*-2'', *H*-3', *H*-3''), 0.88 (s, 9H, (CH₃)₃CSi), 0.07-0.06 (2s, 6H, (CH₃)₂Si); NMR ¹³C (CDCl₃, 100 MHz) δ 165.6 (1s, *C*-4), 156.0 (1s, *C*-2), 141.8 (1s, *C*-6), 93.0 (1s, *C*-5), 87.0 (1s, *C*-1'), 82.1 (1s, *C*-4'), 63.5 (1s, *C*-5'), 33.7 (1s, *C*-2'), 25.9 (1s, (CH₃)₃CSi), 24.0 (1s, *C*-3'), 18.4 (1s, (CH₃)₃CSi), -5.4--5.5 (2s, (CH₃)₂Si); (DMSO-*d*₆, 100 MHz) δ 165.6 (1s, *C*-2), 155.0 (1s, *C*-4), 140.6 (1s, *C*-6), 93.0 (1s, *C*-5), 85.6 (1s, *C*-1'), 81.0 (1s, *C*-4'), 63.5 (1s, *C*-5'), 32.7 (1s, *C*-2'), 25.8 (1s, (CH₃)₃CSi), 24.2 (1s, *C*-3'), 18.0 (1s, (CH₃)₃CSi), -5.5 -5.6 (2s, (CH₃)₂Si); MS FAB>0 (GT) *m/z* 651 (2M+H)⁺, 326 (M+H)⁺, 112 (BH₂)⁺; FAB<0 (GT) *m/z* 324 (M-H)⁻, 110 (B)⁻; HRMS calculated for C₁₅H₂₈N₃O₃Si (M+H)⁺: 326.1900. Found: 326.1900; UV (ethanol 95) λ_{max} 275 nm (ε 10 700), λ_{min} 253 nm (ε 8 000); HPLC *t*_R 26.2 min.

4.1.4. O-(2-Cyanoethyl)-O'-(S-pivaloyl-2-thioethyl)-N,N-diisopropylaminophosphoramidite (**7**)

To a solution of 2-hydroxypropionitrile (0.38 μL, 5.61 mmol) in acetonitrile (55 mL) were added *S*-pivaloyl-2-thioethyl *N,N*-bis(diisopropylamino)phosphine (3.30 g, 8.41 mmol), *N,N*-diisopropylamine (1.57 mL, 11.22 mmol) and 1*H*-tetrazole (0.714 g, 11.22 mmol). The mixture was stirred at room temperature for 2 hours and then diluted in ethyl acetate (200 mL). This solution was washed with saturated aq. NaHCO₃. The resulting organic layer was dried over Na₂SO₄, filtered and concentrated under reduced pressure. After co-evaporation with cyclohexane/ 1% NEt₃, silica gel column flash-chromatography (gradient ethyl acetate 0-10% in cyclohexane/ 1% NEt₃) gave the desired phosphoramidite **7** as colorless oil (1.85 g, 84%). R_f (cyclohexane/AcOEt/NEt₃, 80/19/1, v/v/v): 0.58; NMR ¹H (DMSO-*d*₆, 400 MHz) δ 3.66 (m, 6H, OCH₂CH₂S, OCH₂CH₂CN, CH(CH₃)₂), 3.04 (t, 2H, *J* = 6.3 Hz, OCH₂CH₂S), 1.17 (1s, 9H, C(CH₃)₃), 1.13-1.11 (2d, 12H, *J* = 6.8 Hz, CH(CH₃)₂); NMR ¹³C (DMSO-*d*₆, 100 MHz) δ 205.4 (1s, CO), 118.9 (1s, CN), 61.6 (1d, *J*_{P-C} = 17.4 Hz, OCH₂CH₂S), 58.3 (1d, *J*_{P-C} = 18.2 Hz, OCH₂CH₂CN), 45.9 (1s, C(CH₃)₃), 42.5 (1d, *J*_{P-C} = 12.3 Hz, CH(CH₃)₂), 30.3 (1d, *J*_{P-C} = 7.1 Hz, OCH₂CH₂S), 26.9 (1s, C(CH₃)₃), 24.3 (1d, *J*_{P-C} = 7.3 Hz, CH(CH₃)₂), 19.8 (1d, *J*_{P-C} = 6.8 Hz, OCH₂CH₂CN); NMR ³¹P (DMSO-*d*₆, 81 MHz) δ 148.53; MS FAB>0 (GT) *m/z* 379 (M+O+H)⁺, 363 (M+H)⁺; Anal. Calculated for C₁₆H₃₁N₂O₃PS (MW: 362.47): C, 53.02; H, 8.62; N, 7.73; S, 8.85. Found: C, 52.77; H, 8.52; N, 7.61; S, 8.85.

4.1.5. O-(2-Cyanoethyl)-O'-(S-pivaloyl-2-thioethyl)-4-N-[2',3'-dideoxy-5'-O-tert-butylidimethylsilyl] cytidine phosphoramidate (**8**)

The silylated nucleoside **6** was co-evaporated with pyridine and acetonitrile (3x), and then solubilized in dichloromethane (32 mL). To this solution, kept under argon atmosphere, were added phosphoramidite **7** (1.771 g, 4.89 mmol) in dichloromethane (5 mL) and then 1*H*-tetrazole (0.571 g, 8.15 mmol). After 2 hours stirring, the mixture was cooled to -40°C and a

solution of *tert*-butyl hydroperoxide in toluene (3 M, 5.43 mL) was added. The reaction mixture was stirred for 1 hour and allowed to warm to 0°C. To reduce the excess of oxidant, sodium hydrogensulphite (10%) aqueous solution (3 mL) was added. This mixture was diluted with dichloromethane (70 mL) and washed with the previous reducing solution (30 mL) and then water (50 mL). After decantation, the organic layer was dried over Na₂SO₄, filtered and concentrated under reduced pressure. The purification of the crude was performed by column chromatography on silica gel (gradient methanol 0-2.5% in dichloromethane/ 0.2% acetic acid) to afford a diastereoisomeric mixture of the desired phosphoramidate **8** as colorless oil (0.747 g, 38%). *R_f* (CH₂Cl₂/CH₃OH, 95/5, v/v): 0.33; NMR ¹H (CDCl₃, 400 MHz) δ 8.14 (d, 1H, *J* = 7.4 Hz, *H*-6), 6.00 (d, 1H, *J* = 5.4 Hz, *H*-1'), 5.87 (m, 1H, *H*-5), 4.19 (m, 3H, OCH₂CH₂CN, *H*-4'), 4.06 (m, 3H, OCH₂CH₂S, *H*-5'), 3.70 (dd, 1H, *J* = 11.5 Hz, *J* = 1.5 Hz, *H*-5''), 3.12 (t, 2H, OCH₂CH₂S), 2.75 (m, 2H, OCH₂CH₂CN), 2.39 (m, 1H, *H*-2'), 2.05 (m, 1H, *H*-2''), 1.91 (m, 2H, *H*-3', *H*-3''), 1.20 (s, 9H, (CH₃)₃CSATE), 0.90 (s, 9H, (CH₃)₃CSi), 0.11-0.08 (2s, 6H, (CH₃)₂Si); NMR ¹³C (CDCl₃, 100 MHz) δ 205.7 (1s, CO_{SATE}), 160.0 (1s, *C*-4), 148.2 (1s, *C*-2), 140.9 (1s, *C*-6), 116.5 (1d, *J_{P-C}* = 1.7 Hz, CN), 101.3 (1d, *J_{P-C}* = 18.9 Hz, *C*-5), 86.6 (1s, *C*-1'), 82.3 (1s, *C*-4'), 65.3 (1d, *J_{P-C}* = 5.6 Hz, OCH₂CH₂S), 63.3 (1s, *C*-5'), 60.9 (m, OCH₂CH₂CN), 46.4 (1s, (CH₃)₃CSATE), 33.6 (1s, *C*-2'), 28.6 (1d, *J_{P-C}* = 7.5 Hz, OCH₂CH₂S), 27.2-27.1 (2s, (CH₃)₃CSATE), 25.8-25.7 (2s, (CH₃)₃CSi), 23.9 (1s, *C*-3'), 19.6 (1d, *J_{P-C}* = 6.9 Hz, OCH₂CH₂CN), 18.4 (1s, (CH₃)₃CSi), -5.4--5.5 (2s, (CH₃)₂Si); NMR ³¹P (CDCl₃, 81 MHz) δ 7.22-7.18; MS FAB>0 (GT) *m/z* 603 (M+H)⁺, 112 (BH₂)⁺; FAB<0 (GT) *m/z* 1203 (2M-H)⁻, 601 (M-H)⁻, 110 (B)⁻; UV (ethanol 95) λ_{max} 284 nm (ε 7 250), λ_{min} 254 nm (ε 2 800); HPLC *t_R* 31.4 min; Anal. Calculated for C₂₅H₄₃N₄O₇PSSi (MW: 602.76): C, 49.82; H, 7.19; N, 9.30. Found: C, 50.04; H, 7.34; N, 9.00.

4.1.6. *O*-(3'-Azido-2',3'-dideoxythymidin-5'-yl)-O'-(*S*-pivaloyl-2-thioethyl)-4-N-(2',3'-dideoxy-5'-O-*tert*-butyldimethylsilyl)cytidine phosphoramidate (**9**)

To a solution of phosphoramidate **8** (0.184 g, 0.305 mmol) in tetrahydrofuran (3 mL) was added 1,8-diazabicyclo[5.4.0]undec-7-en (DBU, 0.073 mL, 0.488 mmol). After stirring overnight at room temperature, the mixture was concentrated under reduced pressure, and co-evaporated with pyridine (3x). After addition of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCI, 2.713 g, 8.96 mmol), the resulting mixture was co-evaporated with pyridine (2x), then solubilized in the same solvent (5 mL). 3'-Azido-2',3'-dideoxythymidine (AZT, 0.40 g, 1.49 mmol) was added and the resulting solution was stirred under argon atmosphere at room temperature for 24 hours. The reaction was quenched by addition of aqueous NaHCO₃ (5%; 3 mL). After 15 minutes stirring, the mixture was diluted with chloroform (50 mL) and washed with aqueous NaHCO₃ (5%; 25mL), then water (25 mL). The organic layer was dried, filtered and concentrated under reduced pressure. The crude was co-evaporated with toluene and purified by column chromatography on silica gel (gradient methanol 0-3% in dichloromethane) to afford a diastereoisomeric mixture of the dinucleoside phosphoramidate diester **9** (0.66 g, 83%) as colorless oil. *R_f* (CH₂Cl₂/CH₃OH, 95/5, v/v) : 0.26; NMR ¹H (CDCl₃, 400 MHz) δ 10.6 (bs, 1H, Thy-NH), 8.6 (bs, 1H, P-NH), 8.18 (m, 1H, *H*-6_{ddc}), 7.56-7.55 (2d, 1H, *J* = 0.9 Hz, *H*-6_{AZT}), 6.27 (q, 1H, *H*-1'_{AZT}), 6.03 (m, 1H, *H*-1'_{ddc}), 5.83 (d, 1H, *J* = 7.9 Hz, *H*-5_{ddc}), 4.43 (m, 1H, *H*-3'_{AZT}), 4.26 (m, 3H, *H*-4'_{AZT}, *H*-5'_{AZT}, *H*-5''_{AZT}), 4.17 (m, 1H, *H*-4'_{ddc}), 4.09 (m, 3H, *H*-

5'*ddc*, OCH₂CH₂S), 3.71 (m, 1H, H-5''*ddc*), 3.12 (t, 2H, *J* = 6.8 Hz, OCH₂CH₂S), 2.44 (m, 1H, H-2'*ddc*), 2.34 (m, 2H, H-2'*AZT*, H-2''*AZT*), 1.98 (m, 6H, H-2''*ddc*, H-3'*ddc*, H-3''*ddc*, Thy-CH₃), 1.21-1.20 (2s, 9H, (CH₃)₃CSATE), 0.92 (s, 9H, (CH₃)₃CSi), 0.10--0.10 (2s, 6H, (CH₃)₂Si); NMR ¹³C (CDCl₃, 100 MHz) δ 205.8-205.7 (2s, CO_{SATE}), 163.5 (1s, C-4*AZT*), 160.3 (1d, *J*_{P-C} = 4.7 Hz, C-4*ddc*), 150.2-150.1 (2s, C-2*AZT*), 148.1 (1s, C-2*ddc*), 141.1 (1s, C-6*ddc*), 135.4 (m, C-6*AZT*), 111.6-111.4 (2s, C-5*AZT*), 101.5 (1d, *J* = 23.2 Hz, C-5*ddc*), 86.6 (1s, C-1'*ddc*), 84.6-84.5 (2s, C-1'*AZT*), 82.6-82.5 (2d, *J*_{P-C} = 7.9/7.8 Hz, C-4'*AZT*), 82.4 (1s, C-4'*ddc*), 65.3 (m, C-5'*AZT*, OCH₂CH₂S), 63.3 (1s, C-5'*ddc*), 60.3-60.2 (2s, C-3'*AZT*), 46.5 (1s, (CH₃)₃CSATE), 37.7-37.6 (2s, C-2'*AZT*), 33.7 (1s, C-2'*ddc*), 28.6 (1d, *J*_{P-C} = 7.5 Hz, OCH₂CH₂S), 27.3 (1s, (CH₃)₃CSATE), 25.9 (1s, (CH₃)₃CSi), 23.9 (1s, C-3'*ddc*), 18.4 (1s, (CH₃)₃CSi), 12.5-12.4 (2s, Thy-CH₃), -5.5--5.6 (2s, (CH₃)₂Si); NMR ³¹P (CDCl₃, 81 MHz) δ 8.22-8.01; MS FAB>0 (GT) *m/z* 799 (M+H)⁺, 773 (M-N₂+3H)⁺, . FAB<0 (GT) *m/z* 797 (M-H)⁻, 771 (M-N₂+H)⁻; HRMS calculated for C₃₂H₅₂N₈O₁₀PSSi (M+H)⁺: 799.3034. Found: 799.3173; UV (ethanol 95) λ_{max} 277 nm (ε 20 700), λ_{min} 245 nm (ε 11 150); HPLC *t*_R 32.6 min; Anal. Calculated for C₃₂H₅₁N₈O₁₀PSSi (MW: 798.92): C, 48.11; H, 6.43; N, 14.03. Found: C, 48.27; H, 6.64; N, 13.91.

4.1.7. O-(3'-Azido-2',3'-dideoxythymidin-5'-yl)-O'-(S-pivaloyl-2-thioethyl)-4-N-(2',3'-dideoxycytidine) phosphoramidate (**1**)

From the phosphoramidate 5. The tritylated phosphoramidate **5** (100 mg, 0.10 mmol) was treated by a solution of trichloroacetic acid (3%) in dichloromethane (2 mL). After 15 minutes stirring at room temperature, the mixture was neutralized by addition of triethylammonium carbonate buffer (TEAB, 1M, pH=7), then diluted in dichloromethane (50 mL). The organic layer was washed successively with aqueous NaHCO₃ (10%) and water, then filtered and finally concentrated under reduced pressure. Silica gel column chromatography of the residue (gradient 0-5% methanol in dichloromethane) gave rise to a diastereoisomeric mixture of the expected *t*BuSATE dinucleoside phosphoramidate diester **1** as colorless oil (0.056 g, 80%).

From the phosphoramidate 9. The silylated phosphoramidate **9** (339 mg, 0.50 mmol) was treated by a solution of hydrochloric acid (1%) in ethanol 95 (12.5 mL). After 20 minutes stirring at room temperature, the mixture was neutralized, at 0°C, by addition of TEAB (7 mL, 1M, pH=8.5), then diluted with dichloromethane (70 mL) and washed with water (30 mL). The resulting organic layer was dried over Na₂SO₄, filtered and concentrated under reduced pressure. Purification by column chromatography on silica gel (gradient methanol 2-5% in dichloromethane) gave rise to a diastereoisomeric mixture of the expected *t*BuSATE dinucleoside phosphoramidate diester **1** as colorless oil (0.274 g, 80%).

R_f(CH₂Cl₂/CH₃OH, 9/1, v/v): 0.26; NMR ¹H (CDCl₃, 400 MHz) δ 10.2 (bs, 1H, Thy-NH), 8.8 (bs, 1H, P-NH), 7.95 (m, 1H, H-6*ddc*), 7.51-7.49 (2d, 1H, *J* = 1.2/1.1 Hz, H-6*AZT*), 6.24-6.20 (2t, 1H, *J* = 6.5/6.4 Hz, H-1'*AZT*), 6.00 (m, 2H, H-1'*ddc*, H-5*ddc*), 4.45 (m, 1H, H-3'*AZT*), 4.31 (m, 3H, H-5'*AZT*, H-5''*AZT*, H-4'*ddc*), 4.06 (m, 4H, H-4'*AZT*, H-5'*ddc*, OCH₂CH₂), 3.72 (m, 1H, H-5''*ddc*), 3.13-3.12 (2t, 2H, *J* = 6.9/6.7 Hz, OCH₂CH₂), 2.40 (m, 3H, H-2'*AZT*, H-2''*AZT*, H-2'*ddc*), 2.13 (m, 1H, H-2''*ddc*), 1.97 (m, 5H, H-3'*ddc*, H-3''*ddc*, Thy-CH₃), 1.22-1.21 (2s, 9H, (CH₃)₃CSATE); NMR ¹³C (CDCl₃, 100 MHz) δ 205.9-205.8 (2s, CO_{SATE}), 164.1 (1s, C-4*AZT*), 160.5 (m, C-4*ddc*), 150.4-150.3 (2s, C-2*AZT*), 149.0 (m, C-2*ddc*), 141.2-141.1 (2s, C-6*ddc*), 136.0 (m, C-6*AZT*), 111.1-

111.0 (2s, C-5_{AZT}), 100.6 (m, C-5_{ddC}), 86.9 (1s, C-1'_{ddC}), 85.0-84.7 (2s, C-1'_{AZT}), 82.6 (m, C-4'_{AZT}), 82.3 (1s, C-4'_{ddC}), 65.3 (m, C-5'_{AZT}, OCH₂CH₂S), 62.6 (1s, C-5'_{ddC}), 60.2 (1s, C-3'_{AZT}), 46.4 (1s, (CH₃)₃CSATE), 37.6-37.3 (2s, C-2'_{AZT}), 33.1 (1s, C-2'_{ddC}), 28.5 (1d, J_{P-C} = 7.2 Hz, OCH₂CH₂S), 27.2 (1s, (CH₃)₃CSATE), 24.4 (1s, C-3'_{ddC}), 12.4 (1s, Thy-CH₃); NMR ³¹P (CDCl₃, 81 MHz) δ 7.28-6.97; MS FAB>0 (GT) m/z 685 (M+H)⁺, 659 (M-N₂+3H)⁺, 112 (BH₂ddC)⁺; FAB<0 (GT) m/z 1365 (2M-H)⁻, 683 (M-H)⁻, 125 (B_{AZT})⁻; HRMS calculated for C₂₆H₃₈N₈O₁₀PS (M+H)⁺: 685.2169. Found: 685.2198; UV (ethanol 95) λ_{max} 276 nm (ε 21 200), 212 (ε 24 950), λ_{min} 246 nm (ε 10 900); HPLC t_R 23.2 min; Anal. Calculated for C₂₆H₃₇N₈O₁₀PS (MW: 684.66): C, 45.61; H, 5.45; N, 16.37. Found: C, 45.34; H, 5.67; N, 16.16.

4.1.8. 1-(2',3'-Dideoxy-β-D-ribofuranosyl)-6-N-(4,4'-dimethoxytrityl) adenine (**10**)

To a solution of 2',3'-dideoxyadenosine (ddA, 1.25 g, 5.31 mmol) in pyridine (27 mL) was added at 0°C trimethylsilyl chloride (1.35 mL, 10.62 mmol). The reaction mixture was stirred for 1 hour at room temperature before the addition of 4,4'-dimethoxytrityl chloride (2.16 g, 6.37 mmol). The mixture was stirred overnight under argon atmosphere and then NH₄OH (28%, 5 mL) was added. After 20 minutes stirring, the mixture was diluted in dichloromethane (100 mL) and washed with water (50 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude was co-evaporated with toluene and purified by column chromatography on silica gel (gradient methanol 0-2% in dichloromethane/0.2% NEt₃) to afford the protected nucleoside **10** as a white powder (2.74 g, 96%). R_f (CH₂Cl₂/CH₃OH/NEt₃, 97/2/1, v/v/v): 0.27; NMR ¹H (CDCl₃, 400 MHz) δ 7.92 (s, 1H, H-2), 7.70 (s, 1H, H-8), 7.17 (m, 9H, CH₃O*Ph*, *Ph*), 6.88 (s, 1H, NH), 6.69 (d, 4H, J = 8.9 Hz, CH₃O*Ph*), 5.96 (dd, 1H, J = 7.6 Hz, J = 6.2 Hz, H-1'), 5.89 (d, 1H, J = 9.4 Hz, OH), 4.24 (m, 1H, H-4'), 3.89 (d, 1H, J = 12.6 Hz, H-5'), 3.68 (s, 6H, CH₃O*Ph*), 3.49 (m, 1H, H-5''), 2.69 (m, 1H, H-2'), 2.33 (m, 1H, H-3'), 2.23 (m, 2H, H-2''), 2.10 (m, 1H, H-3''); NMR ¹³C (CDCl₃, 100 MHz) δ 158.3 (1s, CH₃O*Ph* ipso), 154.5 (1s, C-6), 151.7 (1s, C-2), 147.7 (1s, C-4), 145.2 (1s, *Ph* ipso), 139.2 (1s, C-8), 137.3-137.2-130.0 (3s, CH₃O*Ph*), 128.3-127.9-126.8 (3s, *Ph*), 122.3 (1s, C-5), 113.1 (1s, CH₃O*Ph*), 88.3 (1s, C-1'), 81.6 (1s, C-4'), 70.7 (1s, C[(CH₃O*Ph*)₂Ph]), 65.1 (1s, C-5'), 55.2 (1s, CH₃O*Ph*), 32.2 (1s, C-2'), 26.2 (1s, C-3'); MS FAB>0 (GT) m/z 538 (M+H)⁺. FAB<0 (GT) m/z 1073 (2M-H)⁻, 536 (M-H)⁻, 436 (B)⁻; UV (ethanol 95) λ_{max} 276 nm (ε 22 300), λ_{min} 249 nm (ε 10 800); HPLC t_R 28.3 min; Anal. Calculated for C₃₁H₃₁N₅O₄ (MW: 537.61): C, 69.26; H, 5.81; N, 13.03. Found: C, 69.26; H, 5.92; N, 12.62.

4.1.9. O-[2',3'-Dideoxy-6-N-(4,4'-dimethoxytrityl)adenosin-5'-yl]-O'-(S-pivaloyl-2-thioethyl)-N,N-disopropylaminophosphoramidite (**11**)

To a solution of tritylated nucleoside **11** (2.40 g, 4.46 mmol) in dichloromethane (35 mL) and acetonitrile (9 mL) were added, under argon atmosphere, S-pivaloyl-2-thioethyl N,N-bis(diisopropylamino)phosphine (2.63 g, 6.69 mmol), N,N-diisopropylamine (1.25 mL, 8.92 mmol) and 1H-tetrazole (0.626 g, 8.92 mmol). After 1 hour stirring at room temperature, aqueous NaHCO₃ (10%, 5 mL) was added and the resulting mixture was diluted with ethyl acetate (100 mL) and washed with the same aqueous solution (30 mL). The organic layer was dried over Na₂SO₄, filtered, concentrated under reduced pressure and co-evaporated with cyclohexane/ 1% NEt₃. Purification of the crude was performed by silica gel column flash-

chromatography (gradient ethyl acetate 10-25% in cyclohexane/ 1% NEt₃) and afforded a diastereoisomeric mixture of the desired phosphoramidite **11** (2.88 g, 78%). R_f (AcOEt/hexane/NEt₃, 12/ 8/1, v/v/v): 0.39; NMR ¹H (CDCl₃, 400 MHz) δ 8.27-8.20-8.10 (3s, 2H, *H*-2, *H*-8), 7.40 (m, 2H, *Ph*), 7.30 (m, 7H, CH₃O*Ph* meta, *Ph*), 6.98 (s, 1H, *NH*), 6.84 (d, 4H, *J* = 8.8 Hz, CH₃O*Ph*), 6.33 (m, 1H, *H*-1'), 4.39 (m, 1H, *H*-4'), 3.83 (m, 4H, *H*-5', *H*-5'', OCH₂CH₂S), 3.83 (s, 6H, CH₃O*Ph*), 3.65 (m, 2H, (CH₃)₂CHN), 3.17 (m, 2H, OCH₂CH₂S), 2.54 (m, 2H, *H*-2', *H*-2''), 2.19 (m, 2H, *H*-3', *H*-3''), 1.25 (m, 21H, (CH₃)₂CHN, (CH₃)₃C_{SATE}); NMR ¹³C (CDCl₃, 100 MHz) δ 206.3-206.2 (2s, CO), 158.2 (1s, CH₃O*Ph* ipso), 154.0 (1s, C-6), 152.1-152.0 (2s, C-2), 148.1-148.0 (2s, C-4), 145.5 (1s, *Ph* ipso), 138.3-138.2 (2s, C-8), 137.5-130.1 (2s, CH₃O*Ph*), 128.8-127.8-126.7 (3s, *Ph*), 121.3-121.2 (2s, C-5), 113.0 (1s, CH₃O*Ph*), 88.5-88.4 (2s, C-1'), 80.9-80.7 (m, C-4'), 70.5 (1s, C[(CH₃O*Ph*)₂Ph]), 64.8-64.4 (2d, *J*_{P-C} = 16.1/15.5 Hz, C-5'), 62.3-62.2 (2d, *J*_{P-C} = 18.8/18.6 Hz, OCH₂CH₂S), 55.2 (2s, CH₃O*Ph*), 46.4 (1s, (CH₃)₃C_{SATE}), 42.9 (2d, *J*_{P-C} = 12.5 Hz, (CH₃)₂CHN), 33.0-32.8 (2s, C-2'), 30.0-29.9 (2d, *J*_{P-C} = 7.0/7.3 Hz, OCH₂CH₂S), 27.3-27.2 (2s, (CH₃)₃C_{SATE}), 26.3-26.1 (2s, C-3'), 24.7 (m, (CH₃)₂CHN); NMR ³¹P (CDCl₃, 81 MHz) δ 149.24-149.06; MS FAB>0 (GT) *m/z* 829 (M+H)⁺, 746 (M-N(*i*Pr)₂+2H)⁺; UV (ethanol 95) λ_{max} 274 nm (ε 21 700), λ_{min} 249 nm (ε 10 900); Anal. Calculated for C₄₄H₅₇N₆O₆PS (MW: 829.00): C, 63.75; H, 6.93; N, 10.14. Found: C, 63.39; H, 6.95; N, 9.80.

4.1.10. O-[2',3'-Dideoxy-6-N-(4,4'-dimethoxytrityl)adenosin-5'-yl]-O'-(S-pivaloyl-2-thioethyl)-4-N-[2',3'-dideoxy-5'-O-(4,4'-dimethoxytrityl)cytidine] phosphoramidate (**12**)

A mixture containing tritylated nucleoside **4** (0.77 g, 1.50 mmol) and phosphoramidite **11** (1.87 g, 2.25 mmol) was co-evaporated with anhydrous toluene (3x) and dissolved in dichloromethane (15 mL). To the resulting solution was added 1*H*-tetrazole (0.263 g, 3.75 mmol). After 1h30 stirring at room temperature under argon atmosphere, the reaction mixture was cooled at -40°C and a solution of *tert*-butyl hydroperoxide in toluene (3 M, 2.50 mL, 7.50 mmol) was added dropwise. The reaction mixture was stirred for 1 hour at room temperature, and then to reduce the excess of oxidant sodium hydrogen sulfite (10%) aqueous solution (5 mL) was added at 0°C. The mixture was diluted in dichloromethane (100 mL), washed with the previous aqueous solution (50 mL) and water (50 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated under reduced pressure. Column chromatography of the crude on silica gel (gradient acetone in ethyl acetate/ 0.2% NEt₃) gave a diastereoisomeric mixture of the titled phosphoramidate **12** (0.773 g, 41%) obtained as white foam. R_f (CH₂Cl₂/CH₃OH/NEt₃, 97/3/0.5, v/v/v) : 0.23; NMR ¹H (CDCl₃, 400 MHz) δ 8.22-8.21 (2s, 1H, *H*-8_{ddA}), 8.04 (1s, 1H, *H*-2_{ddA}), 7.95-7.94 (2d, 1H, *J* = 7.9 Hz, *H*-6_{ddC}), 7.71-7.52 (2m, 1H, P-*NH*), 7.40 (m, 4H, *Ph*), 7.25 (m, 14H, CH₃O*Ph* meta, *Ph*), 6.96 (s, 1H, *NH*_{ddA}), 6.84-6.83 (2d, 4H, *J* = 8.8/8.5 Hz, CH₃O*Ph*), 6.77 (d, 4H, *J* = 8.8 Hz, CH₃O*Ph*), 6.26 (m, 1H, *H*-1'_{ddA}), 6.00 (m, 1H, *H*-1'_{ddC}), 5.69-5.68 (2d, 1H, *J* = 7.8/7.9 Hz, *H*-5_{ddC}), 4.37 (m, 1H, *H*-4'_{ddA}), 4.23 (m, 3H, *H*-5'_{ddA}, *H*-5''_{ddA}, *H*-4'_{ddC}), 4.05 (m, 2H, CH₂CH₂O), 3.79-3.78 (2s, 6H, CH₃O*Ph*), 3.77-3.76 (2s, 6H, CH₃O*Ph*), 3.51 (dd, 1H, *J* = 10.8 Hz, *J* = 2.5 Hz, *H*-5'_{ddC}), 3.32 (m, 2H, *H*-5''_{ddC}), 3.11 (m, 2H, SCH₂CH₂), 2.54 (m, 2H, *H*-2'_{ddA}, *H*-2''_{ddA}), 2.33 (m, 2H, *H*-2'_{ddC}, *H*-2''_{ddC}), 2.12 (m, 2H, *H*-3'_{ddA}, *H*-3''_{ddA}), 1.98 (m, 2H, *H*-3'_{ddC}, *H*-3''_{ddC}), 1.20-1.19 (2s, 9H, C(CH₃)₃); NMR ¹³C

(CDCl₃, 100 MHz) δ 205.7 (1s, CO_{SATE}), 159.8 (1s, C-4_{ddC}), 158.6-158.2 (2s, CH₃O*Ph* ipso), 154.0 (1s, C-6_{ddA}), 152.0 (1s, C-2_{ddA}), 148.5 (1s, C-2_{ddC}), 148.1 (1s, C-4_{ddA}), 145.5-145.4 (2s, *Ph* ipso), 140.3 (1s, C-6_{ddC}), 138.6-138.5 (2s, C-8_{ddA}), 137.5-135.5-135.3-130.2-130.1 (5s, CH₃O*Ph*), 128.1-128.0-127.7-127.1-126.6 (5s, *Ph*), 121.2 (1s, C-5_{ddA}), 113.2-113.0 (2s, CH₃O*Ph*), 101.4 (1d, $J_{P-C} = 22.9$ Hz, C-5_{ddC}), 86.7 (1s, C[(CH₃O*Ph*)₂Ph]), 86.6 (1s, C-1'_{ddC}), 85.6-85.5 (2s, C-1'_{ddA}), 81.3 (1s, C-4'_{ddC}), 79.6 (1d, $J_{P-C} = 7.7$ Hz, C-4'_{ddA}), 70.5 (1s, C[(CH₃O*Ph*)₂Ph]), 67.2-67.1 (2d, $J_{P-C} = 5.6/5.7$ Hz, C-5'_{ddA}), 64.9-64.8 (2d, $J_{P-C} = 5.7/5.6$ Hz, OCH₂CH₂S), 63.6 (1s, C-5'_{ddC}), 55.2-55.1 (2s, CH₃O*Ph*), 46.4 (1s, (CH₃)₃C_{SATE}), 33.4 (1s, C-2'_{ddC}), 32.5 (1s, C-2'_{ddA}), 28.7 (1d, $J_{P-C} = 7.5$ Hz, OCH₂CH₂S), 27.3 (1s, (CH₃)₃C_{SATE}), 26.1-26.0 (2s, C-3'_{ddA}), 25.0 (1s, C-3'_{ddC}); NMR ³¹P (CDCl₃, 81 MHz) δ 7.36-7.30; MS FAB>0 (GT) m/z 1257 (M+H)⁺, 738 (M-B+2H)⁺; FAB<0 (GT) m/z 1255 (M-H)⁻, 736 (M-B)⁻. HPLC t_R 38.6 min (λ_{max} 275.6 nm).

4.1.11. O-(2',3'-Dideoxyadenosin-5'-yl)-O'-(S-pivaloyl-2-thioethyl)-4-N-(2',3'-dideoxycytidine) phosphoramidate (**2**)

Phosphoramidate **12** (0.754 g, 0.60 mmol) was dissolved in an acid mixture (pH 2.5) containing water, dioxane and acetic acid (120 mL, 25/25/17, v/v/v). After 24 hours stirring at room temperature, the reaction mixture was freeze-dried and purification of the crude was performed by column chromatography, first on silica gel (gradient methanol 0-10% in dichloromethane) then on reverse phase (gradient methanol 0-60% in water). After freeze-drying, a diastereoisomeric mixture of the desired *t*BuSATE dinucleoside phosphoramidate diester **2** was obtained as a white powder (255 mg, 67%). R_f (CH₂Cl₂/CH₃OH, 85/15, v/v) : 0.5; NMR ¹H (DMSO-*d*₆, 400 MHz) δ 11.2 (bs, 1H, P-NH), 8.27-8.26 (2s, 1H, H-8_{ddA}), 8.12 (1s, 1H, H-2_{ddA}), 8.02 (1d, 1H, $J = 7.0$ Hz, H-6_{ddC}), 7.21 (s, 2H, NH_{2,ddA}), 6.23 (m, 1H, H-1'_{ddA}), 6.20 (bs, 1H, H-5_{ddC}), 5.90 (dd, 1H, $J = 6.7$ Hz, $J = 3.0$ Hz, H-1'_{ddC}), 5.00 (bs, 1H, OH), 4.27 (m, 1H, H-4'_{ddA}), 4.07 (m, 1H, H-4'_{ddC}), 4.00 (m, 2H, H-5'_{ddA}, H-5''_{ddA}), 3.88 (m, 2H, OCH₂CH₂S), 3.68 (m, 1H, H-5'_{ddC}), 3.53 (m, 1H, H-5''_{ddC}), 3.01 (m, 2H, OCH₂CH₂S), 2.44 (m, 2H, H-2'_{ddA}, H-2''_{ddA}), 2.28 (m, 1H, H-2'_{ddC}), 2.12 (m, 2H, H-3'_{ddA}, H-3''_{ddA}), 1.95 (m, 1H, H-2''_{ddC}), 1.81 (m, 2H, H-3'_{ddC}, H-3''_{ddC}), 1.13-1.12 (2s, 9H, C(CH₃)₃); NMR ¹³C (DMSO-*d*₆, 100 MHz) δ 205.1 (1s, CO_{SATE}), 162.0 (1s, C-4_{ddC}), 156.0 (1s, C-6_{ddA}), 152.5 (1s, C-2_{ddA}), 148.9 (1s, C-4_{ddA}), 140.9 (m, C-6_{ddC}), 138.9-138.8 (2s, C-8_{ddA}), 119.0 (1s, C-5_{ddA}), 98.5 (m, C-5_{ddC}), 85.8 (1s, C-1'_{ddC}), 84.2 (1s, C-1'_{ddA}), 82.1 (1s, C-4'_{ddC}), 79.0 (1d, $J_{P-C} = 7.2$ Hz, C-4'_{ddA}), 67.3 (1s, C-5'_{ddA}), 64.1 (1s, OCH₂CH₂S), 61.8 (1s, C-5'_{ddC}), 45.9 (1s, (CH₃)₃C_{SATE}), 32.2 (1s, C-2'_{ddC}), 31.0 (1s, C-2'_{ddA}), 28.3 (1d, $J_{P-C} = 7.3$ Hz, OCH₂CH₂S), 26.9 (1s, (CH₃)₃C_{SATE}), 26.1-26.0 (2s, C-3'_{ddA}), 24.5 (1s, C-3'_{ddC}); NMR ³¹P (DMSO-*d*₆, 81 MHz) δ 5.82 (bs); MS FAB>0 (GT) m/z 653 (M+H)⁺; FAB<0 (GT) m/z 651 (M-H)⁻, 434 (M-ddA)⁻; HRMS C₂₆H₃₈N₈O₈PS (M+H)⁺: calculated: 653.2271. Found: 653.2294; UV (ethanol 95) λ_{max} 264 nm (ϵ 17 400), λ_{min} 240 nm (ϵ 10 500); HPLC t_R 20.6 min; Anal. Calculated for C₂₆H₃₇N₈O₈PS + 1.0 H₂O: C, 46.56; H, 5.86; N, 16.71. Found: C, 46.50; H, 5.77; N, 16.56.

4.2. Biological methods

The origin of the viruses and the techniques used for measuring inhibition of virus multiplication were as previously described²³.

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.

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Appendix A. Supplementary data

Supplementary data (NMR spectra for intermediates and final compounds) to this article can be found online at ...

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