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Synthesis, Anti-HIV Activity, and Stability Studies of 5′-Phosphorofluoridate Derivatives of AZT[1]

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The synthesis, *in vitro* anti-HIV activity and stability studies of the 5′-fluorophosphate derivative of 3′-azido-3′-deoxythymidine (AZT) are reported. The results support the hypothesis that this phosphorylated entity exerts its biological effect via the delivery of the corresponding 5′-mononucleotide through an enzymatic process. However, the antiviral evaluation in thymidine kinase-deficient CEM cells as well as the stability studies in culture medium and cell extract showed that this bioconversion is not specific to the intracellular medium. Attempts to improve the biological activity of mononucleoside 5′-fluorophosphates by the use of the *S*-pivaloyl-2-thioethyl (*t*BuSATE) group as biolabile phosphate protection are reported.

Key Words: fluorophosphate; nucleotide; prodrug; antiviral; AZT; HIV.

A large number of 2′,3′-dideoxynucleoside analogues (ddNs) have emerged as effective drugs against human immunodeficiency virus (HIV) (*1*). In all cases, ddNs need to be phosphorylated to the corresponding 5′-triphosphates (ddNTPs) prior to exerting their biological effects. At the HIV reverse transcriptase level, ddNTPs may inhibit the viral proliferation as competitive inhibitors or as alternate substrates leading to termination of the growing DNA chain (*2*). In many cases, the first phosphorylation step could be considered as rate-limiting due to the substrate specificity of nucleoside kinases or 5′-nucleotidases (*3*). Moreover, this dependence on kinase-mediated phosphorylation may limit the efficiency of ddNs in a cellular environment where the activity of phosphorylating enzymes is low (*4*). In order to improve the therapeutic potential of ddNs, various monophosphorylated prodrugs (pronucleotides) have been

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developed. These pronucleotides should release the 5′-mononucleotides (ddNMPs) intracellularly, which could be further metabolized to their corresponding ddNTPs in a nucleoside kinase nondependent way. Briefly, two strategies have been envisaged requiring either structural modifications or introduction of transient groups in order to reduce or to mask the phosphate negative charges (*5–7*). As an original class of modified nucleotide analogues, nucleoside 5′-fluorophosphates emerged as potent antiviral compounds (*8–11*). For example, the 5′-fluorophosphate derivative of AZT **1** (Fig. 1) has been reported to exhibit in cell culture experiments an anti-HIV activity higher than that of its parent nucleoside (*10*). Several hypothesis on the metabolic conversion of nucleoside 5′-fluorophosphates have been suggested (*10*): (i) hydrolysis in extracellular media or inside the cells into the corresponding nucleosides; (ii) hydrolysis of the P-F bond leading to the intracellular formation of the 5′-mononucleotides ; (iii) further phosphorylation of the nucleoside 5′-fluorophosphates into the di- and triphosphate analogues.

In order to evaluate the capacity of such constructs to act as pronucleotides, the biological evaluation of the phosphorofluoridate **1** has been extended to various established human lymphoblastoid cell lines infected with HIV-1, including thymidine kinase-deficient (TK^-) CEM cells. Moreover, stability studies have been performed in several biological media to correlate the antiviral evaluation data with the pathways and kinetics of decomposition of the mononucleoside fluorophosphate **1**. Finally, attempts to improve the biological activity of this phosphorylated entity using the *S*-pivaloyl-2-thioethyl (*t*BuSATE) group as biolabile phosphate protection (*12,13*) are reported.

MATERIALS AND METHODS

Chemical synthesis. ^{1}H NMR spectra were recorded at 400 MHz and ^{13}C NMR spectra at 100 MHz with proton decoupling at ambient temperature. Chemical shifts are given in δ values referenced to the residual solvent peak chloroform ($CDCl_3$) at 7.26 ppm and 77.0 ppm, dimethylsulfoxide (DMSO-d_6) at 2.49 ppm and 39.5 ppm relative to tetramethylsilane (TMS). Deuterium exchange, decoupling and COSY experiments were performed in order to confirm proton assignments. Coupling constants, *J* are reported in Hertz. 2D ^{1}H-^{13}C heteronuclear COSY were recorded for the attribution of ^{13}C signals. ^{31}P NMR spectra were carried out at ambient temperature at 80 MHz with proton coupling and decoupling. Chemical shifts are reported relative to external H_3PO_4. ^{19}F NMR spectra were recorded at ambient temperature at 235 MHz. FAB mass spectra were recorded in the positive-ion or negative-ion mode using thioglycerol/glycerol (1:1, v/v, G-T) as matrix. Elemental analysis were carried out

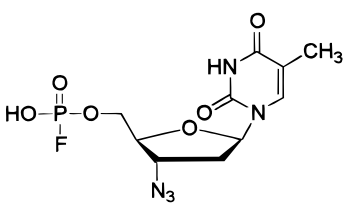

FIG. 1. Structure of the studied phosphorofluoridate derivative of AZT **1**.

by the Service de Microanalyses du CNRS, Division de Vernaison (France). Thin-layer chromatography (TLC) was performed on precoated aluminium sheets of silica gel $60F_{254}$ (Merck, Art. 9385), visualization of products was accomplished by UV absorbance followed by charred with ethanolic sulfuric acid 5% solution with heating; phosphorus-containing compounds were detected by spraying with Hanes molybdate reagent (*14*). Column chromatography was carried out on silica gel 60 (Merck) or on LiChroprep reverse-phase C_{18} (25–40 μM) from Merck. All moisture-sensitive reactions were carried out under anhydrous conditions and argon atmosphere using oven-dried glassware. Solvents were dried and distilled prior to use and solids were dried over P_2O_5 under reduced pressure at room temperature. AZT was purchased from Instel Marsing France. The 5′-fluorophosphate of AZT **1** (*10*), *S*-(2-hydroxyethyl) thiopivaloate **2** (*15*), and 5′-monophosphate of AZT (AZTMP) (*16*) were prepared as previously described.

*S-Pivaloyl-2-thioethyl N,N-bis(diisopropylamino)phosphine (**3**).* To a solution of *S*-(2-hydroxyethyl) thiopivaloate **2** (*15*) (6.08 g, 37.5 mmol) and triethylamine (5.75 ml, 41.3 mmol) in diethyl ether (65 ml) was added dropwise a solution of *N,N*-diisopropylphosphorodichloridite (*17*) (10 g, 37.5 mmol) in diethyl ether (65 ml) at 0°C. The reaction mixture was stirred for 2 h at room temperature and then filtered to remove triethylamine hydrochloride. The filtrate was concentrated *in vacuum* to about 10 ml and purified by flash column chromatography using ethyl acetate/cyclohexane (9/1, v/v) containing 1% of triethylamine as eluent. The appropriate fractions were collected and evaporated to give the title compound **3** (13 g, 88%) as colorless oil. ^{1}H NMR (DMSO-d_6) δ 3.55 (m, 2H, CH_2O), 3.47 (m, 2H, $CH(CH_3)_2$), 3.02 (t, 2H, J = 6.2, CH_2S), 1.16 (1s, 9H, $C(CH_3)_3$), 1.11, 1.09 (2d, 2 × 6H, J = 6.5, $CH(CH_3)_2$); ^{13}C NMR (DMSO-d_6) δ 205.4 (s, C=O), 62.3 (d, J_{PC} = 22.7, CH_2O), 45.9 (s, $C(CH_3)_3$), 43.8 (d, J_{PC} = 12.3, $CH(CH_3)_2$), 29.9 (d, J_{PC} = 9.5, CH_2S), 26.9 (s, $C(CH_3)_3$), 24.3, 23.5 (2d, J_{PC} = 7.9, J_{PC} = 5.7, $CH(CH_3)_2$); ^{31}P NMR (DMSO-d_6) δ 125.5; MS FAB>0 *m/z* 393 $(M + H)^+$; Anal Calcd for $C_{19}H_{41}N_2O_2PS$: C, 58.12; H, 10.53; N, 7.14. Found: C, 57.99; H, 10.31; N, 7.32.

*S-Pivaloyl-2-thioethyl hydrogenphosphonate, sodium salt (**4**).* To a solution of phosphine **3** (1.96 g, 5 mmol) in dry dioxane (25 ml) was added acetic acid (1.72 ml, 30 mmol). The reaction mixture was stirred for 8 h at room temperature and treated with excess pyridine-water. After evaporation of the solvent *in vacuum*, the residue was purified by column chromatography on silica gel using a stepwise gradient of methanol (0–10%) in dichloromethane containing 1% of triethylamine. Evaporation of the appropriate fractions, passage over Dowex 50 WX2 (Na^+ form) cation exchange resin and lyophilization afforded the title compound **4** (3.73 g, 90%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 6.53 (d, 1H, $^1J_{PH}$ = 573, PH), 3.62 (m, 2H, CH_2O), 2.96 (t, 2H, J = 6.6, CH_2S), 1.16 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (DMSO-d_6) δ 205.6 (s, C=O), 60.9 (d, J_{PC} = 4.6, CH_2O), 45.9 (s, $C(CH_3)_3$), 29.3 (d, J_{PC} = 6.5, CH_2S), 27.0 (s, $C(CH_3)_3$); ^{31}P NMR (DMSO-d_6) δ 2.30; MS FAB > 0 *m/z* 249 $(M + H)^+$, FAB < 0 *m/z* 225 $(M\text{-}Na)^-$; Anal Calcd for $C_7H_{14}O_4PSNa$: C, 33.87; H, 5.68; S, 12.92; Found: C, 33.79; H, 5.69; S, 12.90.

*3′-Azido-3′-deoxythymidin-5′-yl S-pivaloyl-2-thioethyl hydrogenphosphonate (**5**).* To a solution of *H*-phosphonate derivative **4** as triethylammonium form (1.84 g, 5.43 mmol) and 3′-azido-3′-deoxythymidine (1 g, 3.74 mmol) in pyridine (56 ml)

was added pivaloyl chloride (1.15 ml, 9.35 mmol). After 2 h, the reaction was quenched by addition of ammonium acetate buffer (0.5 M, pH 5.6, 10 ml) and dichloromethane (100 ml) was added. The organic layer was washed successively with ammonium acetate buffer (0.01 M, 100 ml) and water, dried over Na_2SO_4, filtered, and evaporated to dryness under reduced pressure. Column chromatography of the residue on silica gel using a stepwise gradient of methanol (0–2%) in dichloromethane containing 0.2% of acetic acid, afforded a diastereoisomeric mixture (1:1) of the title compound **5** (1.33 g, 75%) as a colorless foam. ^{1}H NMR (DMSO-d_6) δ 11.4 (s, 1H, NH), 6.93, 6.91 (2d, 1H, $^1J_{PH}$ = 715, PH), 7.48 (s, 1H, H-6), 6.12 (pt, 1H, H-1′), 4.48 (m, 1H, H-3′), 4.22 (m, 2H, H-5′, H-5″), 4.07 (m, CH_2O), 3.99 (m, 1H, H-4′), 3.11 (t, 2H, J = 6.3, CH_2S), 2.37 (m, 2H, H-2′, H-2″), 1.78 (s, 3H, CH_3), 1.16 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (DMSO-d_6) δ 205.1 (s, C=O), 163.7 (s, C-4), 150.4 (s, C-2), 136.0 (s, C-6), 110.0 (s, C-5), 83.7 (s, C-1′), 81.3, 81.2 (2d, J_{PC} = 7.1, C-4′), 64.5, 64.4 (2d, J_{PC} = 5.9, C-5′), 63.6, 63.5 (2d, J_{PC} = 5.6, CH_2O), 59.9,59.8 (2s, C-3′), 46.1 (s, $C(CH_3)_3$), 35.6, 35.5 (s, C-2′), 29.2 (d, J_{PC} = 6.9, CH_2S), 27.0 (s, $C(CH_3)_3$), 12.1 (s, CH_3); ^{31}P NMR (DMSO-d_6) δ 11.03, 10.58; MS FAB > 0 *m/z* 476 $(M + H)^+$, FAB < 0 *m/z* 474 $(M - H)^-$; Anal Calcd for $C_{17}H_{26}N_5O_7PS$: C, 42.94; H, 5.51; N, 14.63; Found: C, 43.21; H, 5.69; N, 14.52.

*3′-Azido-3′-deoxythymidin-5′-yl S-pivaloyl-2-thioethyl phosphorofluoridate (**6**).* To a solution of *H*-phosphonate diester **4** (150 mg, 0.32 mmol) in pyridine (6.5 ml) were added iodine (120 mg, 0.48 mmol) and triethylamine trihydrofluoride (5.2 ml, 0.32 mmol). The mixture was stirred at room temperature for 15 min and diluted with dichloromethane (50 ml). The organic layer was washed with 10% aqueous $Na_2S_2O_3$ solution, water, and dried over Na_2SO_4, filtered, and evaporated to dryness under reduced pressure. The residue was purified by a reverse-phase C_{18} column chromatography using acetonitrile/water (1:1, v/v) as eluent. Due to the limited stability of the desired phosphorofluoridate derivative in aqueous media, the appropriate fractions were rapidly extracted with dichloromethane, dried over Na_2SO_4, filtered, and concentrated *in vacuum* to afford a diastereoisomeric mixture (1:1) of **6** (54 mg, 33%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 8.7 (s, 1H, NH), 7.29, 7.26 (2d, 1H, J = 1.1, H-6), 6.19, 6.18 (2pt, 1H, H-1′), 4.43 (m, 3H, H-5′, H-5″, H-3′), 4.27 (m, 2H, CH_2O), 4.04 (m, 1H, H-4′), 3.17 (m, 2H, CH_2S), 2.42 (m, 2H, H-2′, H-2″), 1.94 (d, 3H, J = 1.1, CH_3), 1.24, 1.23 (2s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$) δ 205.5 (s, C=O), 163.3 (s, C-4), 150.0 (s, C-2), 135.1 (s, C-6), 111.7, 111.6 (s, C-5), 85.3, 85.1 (2s, C-1′), 82.0, 81.7 (2d, J_{PC} = 7.4, C-4′), 68.2, 68.1 (2d, J_{PC} = 5.8, C-5′), 67.8, 67.7 (2d, J_{PC} = 5.5, CH_2O), 59.8, 59.7 (2s, C-3′), 46.6 (s, $C(CH_3)_3$), 37.4 (s, C-2′), 28.2 (d, J_{PC} = 7.3, SCH_2), 27.2 (s, $C(CH_3)_3$), 12.5, 12.4 (2s, CH_3); ^{31}P NMR ($CDCl_3$) δ −8.27, −8.55, $^1J_{PF}$ = 989; ^{19}F NMR ($CDCl_3$) δ −79.96, −80.68, $^1J_{PF}$ = 989; MS FAB > 0 *m/z* 494 $(M + H)^+$, FAB < 0 *m/z* 492 $(M - H)^-$; HRMS Calcd for $C_{17}H_{26}FN_5O_7PS$ $(M + H)^+$ 494.1275, Found 494.1277.

*3′-Azido-3′-deoxythymidin-5′-yl methyl S-pivaloyl-2-thioethyl phosphate (**7**).* This compound (colorless oil) was isolated as by-product during the purification of **6** by silica gel column chromatography using a stepwise gradient of methanol (0–3%) in dichloromethane. ^{1}H NMR ($CDCl_3$) δ 8.84 (s, 1H, NH), 7.39 (d, 1H, J = 1.2, H-6), 6.23 (pt, 1H, J = 6.4, H-1′), 4.31 (m, 3H, H-5′, H-5″, H-3′), 4.13 (m, 2H, CH_2O), 4.02 (m, 1H, H-4′), 3.84, 3.81 (2d, 3H, $^3J_{PH}$ = 11.3, OCH_3), 3.14 (m, 2H, CH_2S),

2.48–2.30 (m, 2H, H-2′, H-2″), 1.94 (d, 3H, J = 1.2, CH_3), 1.23, 1.22 (2s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$) δ 205.6 (s, C=O), 163.5 (s, C-4), 150.1 (s, C-2), 135.1 (s, C-6), 111.6, 111.5 (2s, C-5), 84.8, 84.7 (2s, C-1′), 82.2 (d, J_{PC} = 7.7, C-4′), 66.5–66.3 (m, C-5′, CH_2O), 60.0, 59.9 (2s, C-3′), 54.8, 54.7 (2d, J_{PC} = 5.8, OCH_3), 46.6 (s, $C(CH_3)_3$), 37.5, 37.4 (2s, C-2′), 28.5 (d, J_{PC} = 7.2, CH_2S), 27.2 (1s, $C(CH_3)_3$), 12.4 (s, CH_3); ^{31}P NMR ($CDCl_3$) δ 1.25, 1.10; MS FAB $>$ 0 m/z 506 $(M + H)^+$, FAB $<$ 0 m/z 504 $(M - H)^-$.

Virology. The origin of viruses and techniques used for measuring inhibition of virus multiplication have been previously described (*18*). Briefly, in MT-4 cells, the determination of antiviral activity of the tested compounds was based on a reduction of HIV-1-IIIB-induced cytopathogenicity, the metabolic activity of the cells being measured by the property of mitochondrial dehydrogenases to reduce yellow 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to blue formazan. For CEM-SS or CEM/TK^- cells, the production of virus HIV-LAI was measured by quantification of the reverse-transcriptase activity associated with the virus particle released in the culture supernatant (*19*). Cells MT-4 and CEM were incubated with a $TCID_{50}$ of 50 or 100 of virus during 30 min; after virus adsorption, unbound particles were eliminated by two washes, and cells were cultured in the presence of different concentrations of test compounds for 5 days, or 6 days in the case of CEM/TK^- cells, before virus-production determination. The IC_{50} was derived from the computer-generated median effect plot of the dose/effect data (*20*). In parallel experiments, cytotoxicity of the tested compounds for uninfected cells was measured after an incubation of 5 or 6 days in their presence by means of the colorimetric MTT test. The CC_{50} is the concentration at which OD_{540} was reduced by one-half and was calculated by means of the program mentioned above.

Stability studies. RPMI 1640 was purchased from GibcoBRL. The CEM-SS cell extract was prepared according to a published procedure (*18*). Briefly, CEM cells logarithmic growth were separated from their culture medium by centrifugation (10^4 g, 4 min, 4°C). The residue (about 100 μl, 5 10^7 cells) was resuspended in 2 ml of buffer (10 mM Tris–HCl, 140 mM KCl, pH 7.4) and sonicated. The lysate was centrifuged (10^5 g, 1 h, 4°C) to remove membranes, organelles, and chromatin. The upper layer (0.48 mg/ml protein) was filtered (Millex GV, 0.22 μm, Millipore) and stored in a sterile container at −80°C. The stability studies were performed using a HPLC method previously described (*15*). HPLC analyses were carried out on a Waters unit equipped with a model 600S system controller, a model 717 autosampler injector, a model 996 photodiode array detector (detection at 265 nm), and a Millenium data workstation. The column was a reverse-phase analytical column (Hypersil, C_{18}, 100 × 4.6 mm, 3 μm) protected by a prefilter and a precolumn (Guard-Pak insert, Delta-Pak C_{18}, 100 Å) held in a Guard-Pak holder. The eluents used were A: 20 mM triethylammonium acetate buffer (pH 6.6), B: solution A containing 50% of acetonitrile. All solvents were filtered through 0.22 μm GV-type membrane (Millipore) before use. The crude sample (80 μl, initial concentration 5 10^{-5} M) was injected onto the precolumn and eluted during 5 min with A. Then a six port 7010 Rheodyne valve for connecting the precolumn to the column was activated and a linear gradient from A to B over 30 min was used with a flow rate of 1 ml/min. Co-injection with authentic samples (AZTMP and AZT) were used for calibration and identification of

the decomposition products. The retention times of AZTMP, compound **1** and AZT were 14.3, 17.5, and 18.3 min, respectively. The rate constants of disappearance of the fluorophosphate derivative **1** were calculated according to pseudo-first-order kinetic models (*15*), which were in accordance with the experimental data.

RESULTS AND DISCUSSION

Antiviral evaluation. The 5′-fluorophosphate derivative of AZT **1** was evaluated, in comparison to the parent nucleoside (AZT) and the corresponding 5′-mononucleotide (AZTMP), for its inhibitory effects on the replication of HIV-1 in T_4-human lymphoblastoid cells, CEM-SS and MT-4 (Table 1). As previously described in other cell lines (*10*), the phosphorofluoridate **1** proved to be markedly inhibitory to HIV-1 replication at submicromolar concentrations with 50% effective concentration (EC_{50}) values similar to those observed for AZT. Striking differences were found in the antiviral activities of the tested compounds in HIV-1-infected CEM/TK$^-$ cells. This cell line should be considered as an ideal *in vitro* system to investigate the efficiency of a pronucleotide for which the metabolism of parent nucleoside is known to be strictly dependent on cellular thymidine kinase-mediated phosphorylation. As expected, AZT proved to be completely inactive against HIV-1 replication in CEM/TK$^-$ cells at concentrations up to 100 μM. Under the assays conditions, the 5′-fluorophosphate derivative of AZT **1** showed no anti-HIV activity at concentrations up to 10 μM. This result seems to demonstrate that this phosphorylated derivative was not able to deliver selectively AZTMP inside the cells.

Stability studies. In order to explain the inefficiency of the 5′-fluorophosphate derivative of AZT **1** to act as a pronucleotide, its stability was studied (initial concentration 5 10^{-5} M) at 37°C in different biological media using a HPLC method previously published (*15*). The stability of compound **1** was evaluated in culture medium (CM, RPMI 1640 containing 10% heat-inactivated fetal calf serum), which is the extracellular medium used for antiviral evaluation in cell-culture systems, and in RPMI 1640,

TABLE 1

Anti-HIV-1 Activity of the 5′-Fluorophosphate Derivative of AZT **1** and Its *t*BuSATE Prodrug **6** Compared to the Nucleoside Parent AZT and the Corresponding 5′-Mononucleotide AZTMP in Three Cell Culture Systems[a]

	CEM-SS		MT-4		CEM/TK$^-$	
	EC_{50}[b]	CC_{50}[c]	EC_{50}	CC_{50}	EC_{50}	CC_{50}
1	0.005 ± 0.004	>100	0.022 ± 0.013	>100	46 ± 21	>100
6	0.003 ± 0.001	>100	0.008 ± 0.003	>100	18 ± 2	>100
AZT	0.006 ± 0.003	>100	0.017 ± 0.002	75 ± 5	>100	>100
AZTMP	0.007 ± 0.009	>100	0.022 ± 0.008	>100	39 ± 9	>100

[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] 50% effective concentration (μM) or concentration required to inhibit the replication of HIV-1 by 50%.

[c] 50% cytotoxic concentration (μM) or concentration required to reduce the viability of uninfected cells by 50%.