Brief Articles

Inhibition of Human Immunodeficiency Virus Reverse Transcriptase by Synadenol Triphosphate and Its *E*-Isomer

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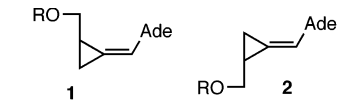
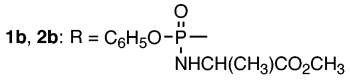
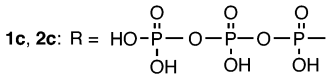
Triphosphate **1c** is a potent competitive inhibitor of wild-type HIV-1 reverse transcriptase with K_i close to ddATP. The *E*-isomer **2c** is about 30-times weaker. Triphosphates **1c** and **2c** interact with the same active site of reverse transcriptase as ddATP. The extent of inhibition of two mutant forms of reverse transcriptase (RT), RT_{M184V} and RT_{M184I}, with triphosphate **1c** was about 5 and 8 times lower than that of wild-type RT_{wt}.

Recently, we described a new series of nucleoside analogues with an unusual methylenecyclopropane unit replacing the furanose moiety. Purine derivatives are potent antiviral agents with broad selectivity.[1−3] This activity is particularly expressed in the *Z*-isomers, whereas the corresponding *E*-isomers are effective only exceptionally. From this group of analogues, synadenol (**1a**) exhibits the broadest range of antiviral selectivity, being effective against HIV-1, HCMV, EBV, HBV, and VZV.[1,3,4] The *E*-isomer **2a** is mostly inactive.



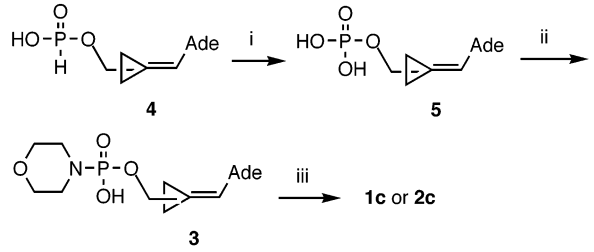
1a, **2a**: R = H





It is assumed but unproven that the mechanism of action of these agents follows the route of other nucleoside analogues, i.e., stepwise phosphorylation leading to the formation of triphosphates which then inhibit the relevant viral DNA polymerase or reverse transcriptase. Some indirect support for this scheme has already been obtained. Thus, phenyl phosphoralaninate **1b**, which is deemed capable of generating the free monophosphate inside the infected cells, was approximately 300−400 times more effective against HIV-1 than synadenol (**1a**).[3,5] It is also noteworthy, that the *E*-isomer **2b** exhibited a significant anti-HIV activity not observed in parent analogue **2a**. These results are in accord with an assumption that intracellular generation of phosphorylated species is essential for antiviral potency of these analogues.[6] In addition, pronucleotide **1b** is effective[3] against both HIV-1 and HIV-2, indicating that synadenol (**1a**) may fall into a category of nucleoside reverse transcriptase inhibitors (NRTIs) that must be activated by phosphorylation[7] (see reference[8] for an exception).

Scheme 1



3a, **4a**, **5a**: *Z*-isomers
3b, **4b**, **5b**: *E*-isomers

i. 1. TMSCl, I_2, pyridine. 2. H_2O
ii. DCC, morpholine, t-BuOH/H_2O, Δ.
iii. $[Bu_3NH]_2^{(2+)}$ $[H_2P_2O_7]^{(2-)}$, DMSO.

These results gave credence to a hypothesis that inhibition of reverse transcriptase (RT) via the respective triphosphates **1c** and **2c** is a possible mode of action of synadenol (**1a**) and prodrugs **1b**, **2b** against HIV-1. A final proof of this mechanism rests on inhibition of HIV-RT with triphosphates **1c** and **2c**. We have therefore synthesized both triphosphates and investigated their inhibition of HIV-1 RT.

Synthesis. Triphosphates of nucleoside analogues are usually prepared employing the reaction with $POCl_3$ in the first phosphorylation step.[9] Because synadenol **(1a)** gave a cyclization product under those conditions,[10] we proceeded as follows. The previously[11] obtained phosphite **4a** (Scheme 1) was oxidized with iodine to phosphate **5a** which, in turn, was converted to phosphoromorpholidate **3a** and, finally, triphosphate **1c** by an

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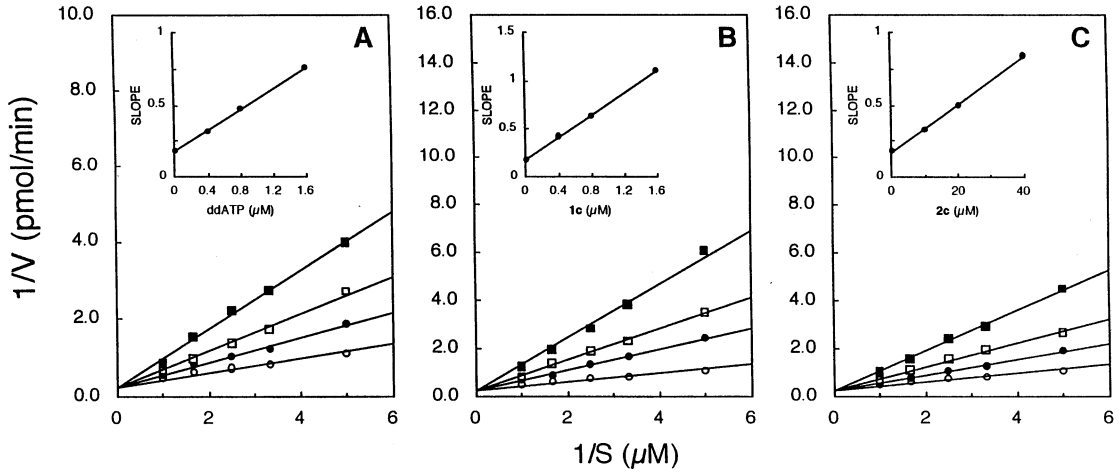

Figure 1. Lineweaver−Burk plots of inhibition of HIV-1 RT_{wt} with ddATP, synadenol triphosphate (**1c**) and the *E*-isomer **2c**. The reaction was of a distributive synthesis mode in which only [^{3}H]dATP was present as a nucleotide substrate in the reaction mixture as described in Experimental Procedures. Enzyme examined was HIV-1 RT_{wt}. Concentrations of ddATP and triphosphate **1c** were 0, 0.4, 0.8, and 1.6 μM (panel A and B), and those of triphosphate **2c** were 0, 10, 20, and 40 μM (panel C). The K_m values were determined from a plot in the absence of inhibitor and K_i values from the abscissa intercept of the replot (see figure insets). They are listed in Table 1.

Table 1. Kinetic Parameters[a] of Substrate Activity (dATP) and Inhibition (ddATP, triphosphates **1c** and **2c**) of HIV-1 RT_{wt}, RT_{M184V}, and RT_{M184I}

	dATP	ddATP		triphosphate **1c**		triphosphate **2c**	
enzyme	K_m	K_i	K_i/K_m	K_i	K_i/K_m	K_i	K_i/K_m
RT_{wt}	0.42 ± 0.005	0.51 ± 0.01	1.2	0.32 ± 0.01	0.8	10 ± 0.3	25
RT_{M184V}	0.38 ± 0.004	0.50 ± 0.01	1.3	1.7 ± 0.1	4.5	52 ± 0.8	137
RT_{M184I}	0.38 ± 0.007	1.2 ± 0.03	3.2	2.4 ± 0.1	6.3	71 ± 0.7	187

[a] All K_m and K_i values are in μM.

established procedure.[9] The *E*-isomer **2c** was obtained in a similar way by sequence **4b** → **5b** → **3b** → **2c**.

Inhibition of Reverse Transcriptase (RT). As expected, inhibition of the wild-type HIV-1 RT_{wt} by ddATP was competitive using dATP as a natural substrate (Figure 1). The Lineweaver−Burk plots indicate that inhibition with triphosphates **1c** and **2c** is also competitive. All slope vs inhibitor concentration plots (see Figure 1, insets) are linear. This is the first conclusive evidence that triphosphates **1c** and **2c** are the species ultimately responsible for anti-HIV activity of synadenol (**1a**) and prodrugs **1b**, **2b**. They act at the same site of RT as ddATP, the active species[12] of AIDS drug ddI (didanosine, Videx). The K_i values of ddATP and triphosphate **1c** were very close whereas the *E*-isomer **2c** is about 30 times weaker inhibitor (Table 1).

These results are in accord with the observed in vitro anti-HIV activity of synadenol (**1a**) and the respective phosphoralaninate pronucleotides **1b** and **2b**.[1,3,5] Our experiments also indicate that prodrugs **1b** and **2b** are first converted to free phosphates by a mechanism including possibly intracellular phosphoamidase action[13] and then to triphosphates **1c** or **2c**. It is likely that inhibition of replication of other viruses with synadenol (**1a**) and additional purine methylenecyclopropane nucleoside analogues follows a similar pattern where the respective triphosphates interact with appropriate viral DNA polymerases.

Our previous results have indicated that pronucleotide **1b** is 9−12 times less sensitive toward inhibition of mutant HIV-1 forms M184V and M184I.[14] Therefore, it was of interest to investigate inhibition of the corresponding RT mutants with triphosphate **1c** (Figure 2, Table 1). It is clear that the triphosphate **1c** is approximately 5 to 8 times weaker inhibitor of mutated forms RT_{M184V} and RT_{M184I} than that of wild-type RT_{wt}. These results parallel antiviral activities of pronucleotide **1b** against HIV-1 variants M184V and M184I. A similar trend in inhibition pattern of RT_{M184V} and RT_{M184I} vs RT_{wt} although at a higher concentration level was noted with the *E*-isomer **2c**. The inhibition with ddATP was only 2−3 times less sensitive to mutants of RT than triphosphate **1c**.

Experimental Section

General Methods. Bis(tributylammonium)pyrophosphate was prepared by passing the solution of the tetrasodium salt (4.46 g, 10 mmol) through Dowex 50 WX8/$H^{(+)}$/until the pH of the eluate increased to 5. Tributylamine (4.79 mL, 20 mmol) in ethanol (40 mL) was then added with an external ice-cooling, and the solution was evaporated in vacuo. Ethanol was evaporated twice from the residue followed by DMF, and the resultant pale yellow syrup was dissolved in DMSO (10 mL).

Synadenol Phosphoromorpholidate 3a. A suspension of phosphite[11] **4a** (triethylammonium salt, 300 mg, 0.79 mmol) in pyridine (30 mL) was sonicated for 30 min. Trimethylsilyl chloride (TMSCl, 0.32 mL, 2.52 mmol) was added at room temperature with stirring to give a clear solution followed, after 5 min, by a dropwise addition of iodine (300 mg, 1.18 mmol) in pyridine (3 mL). The stirring was continued for 30 min, pyridine was evaporated in vacuo, and the residue was partitioned between water and CH_2Cl_2. The aqueous phase (pH 7) was stirred with a mixture of charcoal and Celite (1:1, 10 g) which was then filtered. The filter bed was first washed with water (600 mL) and then with NH_4OH until the disappearance of the UV absorption. Evaporation of the latter eluate gave phosphate **5a** (130 mg, 53%) as an ammonium salt. To a

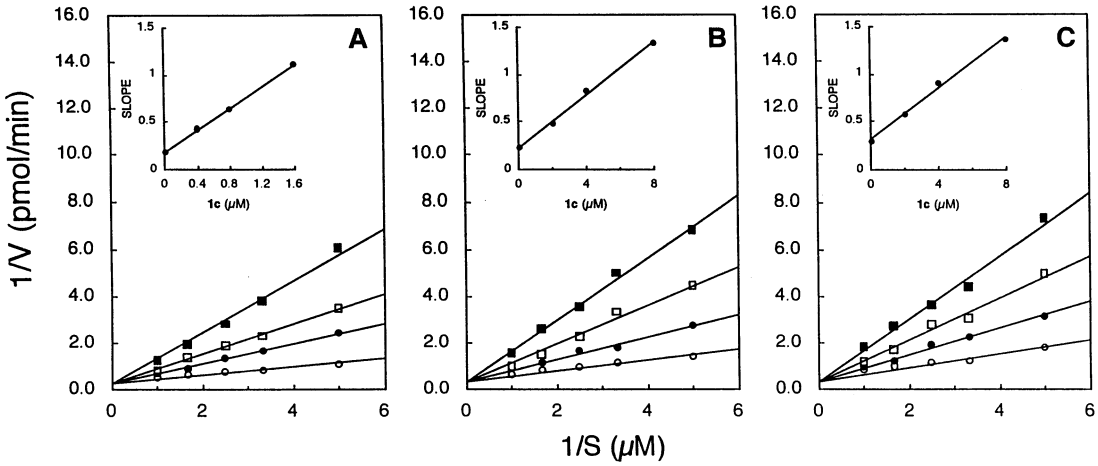

Figure 2. Lineweaver−Burk plots of inhibition of HIV-1 RT_{wt}, RT_{M184V}, and RT_{M184I} with synadenol triphosphate (**1c**). The reaction was perfomed as described in Figure 1. Enzymes examined were HIV-1 RT_{wt} (panel A), RT_{M184V} (panel B), and RT_{M184I} (panel C). Concentrations of triphosphate **1c** for RT_{wt} were 0, 0.4, 0.8, and 1.6 μM, and for RT_{M184V} and RT_{M184I} were 0, 2, 4, and 8 μM. The K_m and K_i values were determined as shown in Figure 1 (see Table 1).

refluxing solution of this product and morpholine (0.186 mL, 2.2 mmol) in tBuOH−water (1:1, 16 mL) was added *N*,*N*′-dicyclohexylcarbodiimide (DCC, 453 mg, 2.2 mmol) in tBuOH (11 mL) over a period of 30 min. The reflux was then continued for 2 h. After cooling, the volatile components were evaporated in vacuo, the residue was partitioned between CH_2Cl_2 (20 mL) and NH_4OH (20 mL), and the aqueous phase was extracted with CH_2Cl_2 (3 × 20 mL) whereupon it was evaporated to give a crude morpholidate **3a** as an ammonium salt. Chromatography on a silica gel column using CH_2Cl_2:MeOH:NH_4OH = 50:20:1 as an eluent gave a white solid (131 mg, 81%), mp. 94−96 °C. ^{1}H NMR (CD_3SOCD_3) δ 8.87 (s, 1H) and 8.16 (s, 1H, adenine H_8 and H_2), 7.47 (bs, 2, NH_2) and 7.39 (s, 1H, alkylidene, overlapped with $NH_4^{(+)}$, 4.01 (bm, 1H, CH_2OP, second half overlapped with CH_2OCH_2 of morpholine and H_2O at δ 3.37), 2.84 (bs, 4H, CH_2NCH_2 of morpholine), 2.21 (bm, 1H), 1.52 (t, 1H) and 1.25 (bt, 1H, cyclopropane); ^{31}P NMR 5.1.

***E*-Isomer 3b.** Following the procedure for morpholidate **3a**, phosphite[11] **4b** (190 mg, 0.5 mmol) was first transformed to phosphate **5b** (100 mg, 64%) and then to **3b** (110 mg, 89%), mp 115−118 °C. ^{1}H NMR (CD_3SOCD_3) δ 8.48 (s, 1H) and 8.16 (s, 1H, adenine H_8 and H_2), 7.55 (bs, 4, $NH_4^{(+)}$), 7.46 (d, 1H, alkylidene), 3.69 and 3.56 (2m, CH_2OP, partially overlapped with CH_2OCH_2 of morpholine and H_2O at δ 3.43), 2.88 (dd, 4H, CH_2NCH_2 of morpholine), 2.07 (m, 1H), 1.74 (dt, 1H) and 1.45 (m, 1H, cyclopropane); ^{31}P NMR 5.4.

Synadenol Triphosphate 1c. A mixture of phosphoromorpholidate **3a** (120 mg, 0.33 mmol) and bis(tri-*n*-butylammonium) pyrophosphate (4.95 mmol) in DMSO (20 mL) was stirred at room temperature for 3 days. The reaction mixture was applied to a column of DEAE-Sephadex A25 (10 g) and the column was eluted with water (2 L), followed by a linear gradient of water (1 L) to 0.4 M NH_4HCO_3 (1 L) and then with 0.4 M NH_4HCO_3 (1.5 L). The elution was continuously monitored at 254 nm. Appropriate fractions were combined and lyophilized to give triphosphate **1c** as a white solid (26 mg, 14%). Molecular weight was 569.17 corresponding to diammonium salt × 4.33H_2O as determined spectrophotometrically using ϵ_{260} 14 210 (pH 7) for synadenol **1a**. ^{1}H NMR (D_2O) δ 8.11 (s, 1H) and 7.73 (s, 1H, adenine H_8 and H_2), 6.83 (d, 1H, alkylidene), 3.80 (m, 1H) and 3.65 (m, 1H, CH_2OP), 2.06 (m, 1H), 1.40 (dd, 1H) and 1.14 (m, 1H, cyclopropane); ^{31}P NMR −5.23 (d, J = 21.9 Hz), −10.14 (d, J = 18.8 Hz), −20.68 (t, J = 19.8 Hz).

***E*-Isomer 2c.** Triphosphate **2c** was obtained from phosphoromorpholidate **3b** as described above for **1c**. Yield 19 mg (10%). Molecular weight was 561.84 corresponding to diammonium salt × 3.9 H_2O as determined spectrophotometrically using ϵ_{260} 14,280 (pH 7) for the *E*-isomer of synadenol **2a**. ^{1}H NMR (D_2O) δ 8.33 (s, 1H) and 8.15 (s, 1H, adenine H_8 and H_2), 7.21 (s, 1H, alkylidene), 3.98 (m, 2H, CH_2OP), 2.22 (m, 1H), 1.74 (t, 1H) and 1.53 (m, 1H, cyclopropane); ^{31}P NMR −5.23 (d, J = 20.7 Hz), −10.15 (d, J = 19.8 Hz), −20.64 (t, J = 19.8 Hz).

Enzyme Purification. RT_{wt} and two mutant RT-expression vectors were constructed as previously described.[15] Briefly, desired mutations were introduced into the *Xma*I-*Nhe*I region (759 base pairs) of pTZNX1, which encoded Gly-15 to Ala-267 of HIV-1 RT (strain BH10), by oligonucleotide-directed mutagenesis using T7 DNA polymerase (US Biochemical Co., Cleveland, OH). The presence of these intended mutations and the absence of unintended mutations were confirmed by determination of nucleotide sequence of the entire *Xma*I-*Nhe*I region. To construct each RT expression vector, the *Xma*I-*Nhe*I fragment carrying the intended mutation (s) was replaced with the *XmNh* linker region[15] of pKRT07. *Escherichia coli* JM109 (Promega), transformed with a RT_{wt}-expression vector or mutant RT-expression vectors, was cultured in ampicillin (100 μg/mL) containing 2xYT medium (1.6% tryptophan, 1% yeast extract, and 0.5% NaCl) at 37 °C and then exposed to 1 mM isopropyl 1-thio-β-D-galactoside (IPTG) and cultured for an additional 5 h. Cells were harvested, frozen at −20 °C for overnight, thawed on ice, and suspended in lysis buffer [50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 3 mM dithiothreitol (DTT), protease inhibitors (1 mM phenylmethylsulfonyl fluoride, PMSF, 3 μg/mL leupeptin, and 5 μg/mL aprotinin) and 10% glycerol]. To aid in solubilization of the obtained pellet, lysozyme (1 μg/mL) was added and mixed gently for 30 min at 4 °C. The pellet was disrupted by sonication 6 times for 30 s in an ice bath. After centrifugation (140 000 x *g* for 20 min at 4 °C), the supernatant was used as the RT crude extract. Thus obtained RT-containing fraction was applied to Q-column equilibrated with buffer Q (50 mM Tris-HCl, pH 8.0, 2 mM DTT, and 10% glycerol). Elution was carried out with a buffer with linearly increasing NaCl concentrations from 0 to 0.7 M (total 25 mL) at an elution flow rate of 1 mL/min. The RT containing fraction was eluted between 0.25 and 0.35 M NaCl. To partially purify RT, RT in the Q fraction was purified by SP-column chromatography (HPLC), previously equilibrated with buffer SP (50 mM Hepes-NaOH, pH 7.2, 2 mM DTT and 10% glycerol). RT was detected in the fractions eluted between 0.3 and 0.4 M NaCl. This RT preparation was concentrated using a Centricon-30 concentrator (Amicon, MA), dialyzed against 50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 2 mM DTT overnight at 4 °C, and stored at −20 °C until use. The purity of thus-prepared RT-derived polypeptides was >90% as assessed by a polyacrylamide gel analysis.

Enzyme Assays. A heteropolymeric template-primer (MS2/22A) was employed in enzymatic assays with RT. DNA primer 22A (5′ CGTTAGCCACTCCGAAGTGCGT-3′) was complementary to nucleotides 3326−3347 of phage MS2 genomic RNA.[15] The buffer used for steady-state kinetic analysis for the MS2/

22A contained 50 mM Tris-HCl, pH 7.8, 6 mM $MgCl_2$, 150 mM KCl, 0.01% Triton X-100, [^{3}H]dATP, and 10 μM each of the remaining dNTPs. After the reaction mixtures were equilibrated at 37 °C, the reaction was initiated by the addition of enzyme at 37 °C. Portions of the reaction mixture (20 μL) were removed periodically four or five times during the course of the assay and spotted on a DE81 filter, and the amount of [^{3}H]nucleotides incorporated into DNA and adsorbed on the filter was determined in liquid scintillation fluid using a scintillation counter. The K_m and K_i values were determined from initial linear steady-state velocities with Lineweaver−Burk plot analyses.

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References

(1) Qiu Y.-L.; Ksebati, M. B.; Ptak, R. G.; Fan, B. Y.; Breitenbach, J. M.; Lin, J.-S.; Cheng Y.-C.; Kern, E. R.; Drach J. C.; Zemlicka, J. (*Z*)- and (*E*)-((2-Hydroxymethyl)cyclopropylidene)methyladenine and guanine. New Nucleoside Analogues with a Broad Spectrum Antiviral Activity. *J. Med. Chem.* **1998**, *41*, 10−23.

(2) Qiu, Y.-L.; Ptak, R, G.; Breitenbach, J. M.; Lin, J.-S.; Cheng, Y.-C.; Kern, E. R.; Drach, J. C.; Zemlicka, J. (*Z*)- and (*E*)−(2-Hydroxymethyl)cyclopropylidenemethylpurines and Pyrimidines as Antiviral Agents. *Antiviral Chem. Chemother.* **1998**, *9*, 341−352.

(3) Uchida, H.; Kodama, E. N.; Yoshimura, K.; Maeda, Y.; Kosalaraksa, P.; Maroun, V.; Qiu Y.-L.; Zemlicka, J.; Mitsuya, H. In Vitro Anti-Human Immunodeficiency Virus Activities of *Z*- and *E*-Methylenecyclopropane Nucleoside Analogues and their Phosphoro-L-alaninate Diesters. *Antimicrob. Agents Chemother.* **1999**, *43*, 1487−1490.

(4) Cheng, C.; Shimo, T.; Somekawa, K.; Baba, M. 9-Hydroxymethylcyclopropylidene-methylenyladenine: The Design, Facile Synthesis, Isomer Separation and Anti-HIV-1 Activities. *Tetrahedron* **1998**, *54*, 2031−2040.

(5) Qiu, Y.-L.; Ptak, R. G.; Breitenbach, J. M.; Lin, J.-S.; Cheng, Y.-C.; Drach, J. C.; Kern, E. R.; Zemlicka, J.Synthesis and Antiviral Activity of Phosphoralaninate Derivatives of Methylenecyclopropane Analogues of Nucleosides. *Antiviral Res.* **1999**, *43*, 37−53.

(6) Zemlicka, J. Lipophilic phosphoramidates as antiviral pronucleotides. *Biochim. Biophys. Acta* (Molecular Basis of Disease) **2002**, *1587*, 276−286.

(7) Tanaka, H.; Baba, M.; Saito, S.; Miyasaka, T.; Takashima, H.; Sekiya, K.; Ubasawa, M.; Nitta, I.; Walker, R. T.; Nakashima, H.; De Clercq, E. Specific Anti-HIV-1 "Acyclonucleosides" which Cannot be Phosphorylated: Synthesis of Some Deoxy Analogues of 1-[(2-Hydroxy-ethoxy)methyl]-6-(phenylthio)thymine. *J. Med. Chem.* **1991**, *34*, 1508−1511.

(8) Buckheit, R. W., Jr.; Turpin, J. A.Mechanisms of anti-HIV Action of SJ-3366: Effects on Reverse Transcriptase and Cell Surface Events. *Antiviral Res.* **2001**, *51*, 2−3.

(9) Orr, D. C.; Figueiredo, H. T.; Mo, C.-L.; Penn, C. R.; Cameron, J. M. DNA Chain Termination Activity and Inhibition of Human Immunodeficiency Virus Reverse Transcriptase by Carbocyclic 2′,3′-Didehydro-2′,3′-dideoxyguanosine Triphosphate. *J. Biol. Chem.* **1992**, *267*, 4177−4182.

(10) Qiu, Y.-L.; J. Zemlicka, J. 3,5′-Anhydrosynadenol: a Polycyclic Anhydronucleoside Analogue. *Angew. Chem., Int. Ed.* **1998**, *37*, 1440−1441.

(11) Wang, R.; Corbett, T. H.; Cheng, Y.-C.; Drach, J. C.; Kern, E. R.; Mitsuya, H.; Zemlicka, J. Tryptophanyl Phosphoramidates as Prodrugs of Synadenol and its *E*-Isomer: Synthesis and Biological Activity. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2467−2470.

(12) Johnson, M. A.; Ahluwalia, G.; Connelly, M. C.; Cooney, D. A.; Broder, S.; Johns, D. G.; Fridland, A. Metabolic Pathways for the Activation of the Antiretroviral Agent 2′,3′-Dideoxyadenosine in Human Lymphoid Cells. *J. Biol. Chem.* **1988**, *263*, 15354−15357.

(13) Saboulard, D.; Naesens, L.; Cahard, D.; Salgado, A.; Pathirana, R.; Velasquez, S.; McGuigan, C.; De Clercq, E.; Balzarini, J. Characterization of the Activation Pathway of Phosphoramidate Triester Prodrugs of Stavudine and Zidovudine. *Mol. Pharmacol.* **1999**, *56*, 693−704.

(14) Yoshimura, K.; Feldman, R.; Kodama, E.; Kavlick, M. F.; Qiu, Y.-L.; Zemlicka, J.; Mitsuya, H. In Vitro Induction of Human Immunodeficiency Virus Type 1 Variants Resistant to Phosphoralaninate Prodrugs of *Z*-Methylenecyclopropane Nucleoside Analogues. *Antimicrob. Agents Chemother.* **1999**, *43*, 2479−2483.

(15) Ueno, T.; Shirasaka, T.; Mitsuya, H.Enzymatic Characterization of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Resistant to Multiple 2′,3′-Dideoxynucleoside 5′-Triphosphates. *J. Biol. Chem.* **1995**, *270*, 23605−23611.

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