Synthesis, in vitro enzymatic and antiviral evaluation of d4T polyphosphate derivatives as chain terminators.

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Abstract. - A series of d4T di- or tri-phosphate derivatives have been synthesized and evaluated as effective substrates for HIV-1 RT, and also tested for their *in vitro* anti-HIV activity. The steady-state kinetic study of compounds **1-4** in an enzymatic incorporation assay by HIV-1 RT follows Michaelis-Menten profile. In addition, compounds **2-4** are able to inhibit HIV-1 replication to the same extent as d4T and d4TMP in MT-4 cells as well as in CEM/0 cells and CEM/TK⁻ cells. The data suggests that these d4T polyphosphate derivatives are hydrolysed to d4T and rephosphorylated to d4TTP before exerting their antiviral activity.

Introduction. - 2',3'-Dideoxynucleosides (ddN) and their derivatives are currently used as antiretroviral drugs. These Nucleoside RT inhibitors which lack both the 2'- and 3'-OH groups on their sugar moiety, such as AZT (3'-azido-2',3'-dideoxythymidine), d4T (2',3'-didehydro-2',3'-dideoxythymidine), DDI (2',3'-dideoxyinosine) and 3TC (2',3'-dideoxy-3'-thiacytidine), are transformed to the corresponding dideoxynucleoside triphosphates (ddNTPs) by the action of host kinases [1]. The intracellular generated ddNTPs inhibit retroviral transcriptase by competing with the natural nucleoside triphosphates (dNTPs) for binding and/or causing chain termination of viral DNA synthesis [1, 2]. Nowadays, NRTIs serve as critical components of highly active antiretroviral therapy (HAART), in combination with either protease inhibitors or nonnucleoside RT inhibitors (NNRTIs).

However, the benefits of HAART have been decreased by the high mutation rate of HIV and consequent drug resistance [3]. Besides HIV reverse transcriptase (RT), ddN di- and tri-phosphates are also inhibitors of eukaryotic DNA polymerases α , β and γ [4-6].

SNuPE (single-nucleotide primer extension) has become a valuable tool for the rapid and highly specific detection of point mutations and single-nucleotide polymorphisms in the field of human genetics [7]. In the primer extension reaction, a DNA polymerase is used to label a specific primer hybridized to the target sequence by incorporating a single labeled ddNTP. Sanger dideoxy sequencing using fluorescently labeled terminators is a useful method for sequencing DNA [8]. Therefore, the development of analogues of chain terminating nucleoside

triphosphates stays valuable research.

We have tested several d4T polyphosphate derivatives. [d4TP_n and d4TP_nd4T, n is the number of P (phosphate)], in an HIV-1 RT catalyzed incorporation assay as chain-terminating nucleotides at the 3' end of a growing DNA chain. As little is known about the precise boundaries of pyrophosphate and triphosphate linked dinucleotide substrates, we synthesized and evaluated four d4T di- or tri-phosphate analogues (**1-4**, Figure 1) as potential substrates of HIV-RT and further evaluated the antiviral activities in cells. Nucleotide dimers have been previously described as prodrugs of antiviral nucleosides, but they have not been studied as substrate for viral polymerases [9].

➔ Insert Figure 1 here

Results and discussion. - *Synthesis of d4T diphosphate derivatives.* The synthesis of the diphosphate derivatives of d4T (**1** and **2**) was carried out according to the method shown in scheme 1 [10, 11]. D4T monophosphate (d4TMP) and d4T diphosphate (**1**) were obtained from d4T by treatment with POCl₃. The direct phosphorylation (scheme 1a) yielded d4TMP and **1** in 24.5 % and 37.4 % after regular column chromatography. Additionally, compound **2** was formed as a side product when the reaction mixture was quenched with a mixture of ethanol and 1M TEAB (scheme 1b) before evaporation. Column chromatographic purification followed by HPLC was used to obtain compound **2**.

-> insert Scheme 1 here

Synthesis of the dinucleotide di- or tri-phosphate derivatives of d4T. The synthesis of dinucleotide di- or tri-phosphate derivatives of d4T (**3** and **4**) is described in scheme 2, which is similar to the general synthetic scheme described before for dinucleotide polyphosphates using a monophosphate as starting nucleotide [12]. Firstly, the phosphate group of d4TMP was activated as 5'-phosphorimidazolid. The activated compound was further reacted with the ammonium salt of d4TMP [or with d4TDP **1**] in a ZnCl₂-mediated reaction to give compounds **3** and **4**, respectively. After the reaction, 0.25 M EDTA disodium quench buffer was used to break down the nucleotide-metal complex. For compounds **3** and **4**, the products were obtained in 41.2 % and 24.4 % yields after column chromatographic purification.

➔ Insert Scheme 2 here

Single primer extension by HIV-1 RT with the polyphosphate derivatives of d4T (1-4) as substrates. The ability of the nucleotide polyphosphate analogues of d4T (**1-4**) to function as substrates for HIV-1 RT was investigated in a single nucleotide extension experiment, in which the incorporation of the chain-terminating nucleotide (d4TMP), is evaluated (Figure 2). The corresponding didehydrothymidine triphosphate (d4TTP) was used as reference.

Under identical conditions, compounds **1**, **2** and **4** proved to be good substrates for

HIV-RT, showing 85-95% conversion to a P + 1 strand at 50 μ M, 100 μ M and 20 μ M after 30 min polymerase reaction by HIV-RT, respectively. The use of compound **4** as substrate resulted in 70% conversion to a P + 1 strand in 30 min at 10 μ M. The triphosphate group of dinucleotide (**4**) is structurally more similar to the standard triphosphate substrate than compounds **1-3**, which may have a beneficial effect on catalysis. In contrast, dinucleotide diphosphate (**3**) is a suboptimal substrate for HIV-1 RT in the polymerase reaction and higher concentration (200 μ M) was needed to obtain 95 % conversion to a P + 1 strand in 60 min. Compound **3** is 4-fold less efficient than compound **1** in the single primer-extension assay. This suggests that the dinucleotide diphosphate (**3**) may bind less well in the active site of HIV RT or that the incorporation reaction itself is more difficult to achieve, leading to a less productive complex than the nucleotide diphosphate (**1**). As expected, a single incorporated d4TMP functions as a chain terminator of HIV-1 RT when compounds **1-4** were used as substrates in the single primer-extension assay by HIV-1 RT.

➔ Insert Figure 2 here

The efficiency of compounds **1-4** was further investigated by determining the kinetic parameters with steady-state kinetic analysis [13, 14]. The kinetic parameters K_m , K_{cat} and the derived value K_{cat}/K_m are given in Table 1. It was shown that the use of compounds **1-4** as substrates by HIV-1 RT results in 9.9, 12.2, 6.8 and 11.6-fold increase in the K_m value compared to that for d4TTP, respectively. Additionally, the

K_{cat} values (turnover number) of compounds **1-4** are 2.3, 1.1, 2.1 and 1.1-fold lower than that for d4TTP. Although the overall catalytic efficiency (K_{cat}/K_m) for compounds **1-4** is decreased 13 to 23-fold, the measured K_{cat} value indicates the catalytic efficiency of HIV-1 RT for these four compounds is only a little lower than for d4TTP. These data indicate efficient nucleophilic displacement of dideoxynucleotide polyphosphate derivatives when the substrate is bound in the active site.

➔ Insert Table 1 here

Antiviral evaluation of the polyphosphate derivatives of d4T (2-4) against HIV-1 and HIV-2. The antiviral activity of compounds **2-4** against HIV-1 and HIV-2 were evaluated in MT-4 cells, CEM/0 and CEM/TK⁻ cells (Table 2). In all of these experiments, d4T and d4TMP were used as references. Compounds **2-4** are as active as d4T and d4TMP in MT-4 cells and CEM/0 cells. Compounds **2-4** are more efficient in MT-4 cells than them in CEM/0 cells. Compound **3** shows antiviral activity against HIV-1 and HIV-2 at concentrations of 0.20 μ M and 0.15 μ M, respectively, in MT-4 cells. Additionally, compound **3** has similar activity as d4T in CEM/0 cells. However, all of these d4T polyphosphate derivatives are much less active in CEM/TK⁻. These results indicate that the d4T polyphosphate analogues and d4TMP itself were probably hydrolyzed to d4T in culture medium before taken up by the CEM/TK⁻ cells. In general, compounds **2-4** apparently serve as prodrugs of

d4T. None of these polyphosphate analogues possess marked cytotoxicity.

➔ Insert Table 2 here

Conclusion. - We demonstrated that the four d4T polyphosphate analogues (1-4) served as effective substrates of HIV-RT in a DNA chain termination reaction. Compounds **1**, **2** and **4** are good substrates for HIV-RT, resulting in 85-95% conversion to a P + 1 strand at 50 μ M, 100 μ M and 20 μ M after 30 min polymerase reaction time, respectively. Compound **3** is a suboptimal substrate of HIV-1 RT and higher concentrations (200 μ M) are needed to reach 95% incorporation after 60 min reaction time. The potency of the four compounds for enzymatic DNA chain termination increases in the order: compound **3** < compound **2** < compound **1** < compound **4**, when catalyzed by HIV-1 RT. Although the overall catalytic efficiency (K_{cat}/K_m) to HIV-1 RT for compounds **1-4** is decreased 13 to 23-fold, the measured K_{cat} values are only 2.3, 1.1, 2.1 and 1.1-fold lower than that for d4TTP. The steady-state kinetic study of compounds **1-4** in enzymatic incorporation by HIV-1 RT follows Michaelis-Menten profile. In addition, compounds **2-4** exhibit similar anti-HIV activity as d4T and d4TMP in MT-4 cells and CEM/0 cells, respectively. Compounds **2-4** are more efficient in MT-4 cells than them in CEM/0 cells. However, these compounds are less potent in CEM/TK⁻ cells, as also observed for d4T and d4TMP. These biological data suggest that compounds **2-4** are degraded to d4T before they are taken up by the cells.

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Experimental Part

General for chemical synthesis. For all reactions, analytical grade solvents were used. All moisture sensitive reactions were carried out in oven-dried glassware (135 °C). A Bruker Avance II 600 MHz or 500 MHz spectrometer and a Bruker Avance 300 MHz apparatus were used for ¹H NMR, ¹³C NMR and ³¹P NMR. All chemical shifts are listed in ppm. ³¹P NMR chemical shifts are referenced to an external 85 % H₃PO₄ standard (δ = 0.000 ppm). UV spectra were recorded on a Varian Cary-300-Bio UV/Vis Spectrophotometer. Mass spectrometry was performed on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard electrospray probe (Z-spray, Micromass, Manchester, UK). Samples were dissolved in acetonitrile/water (1:1 v/v) and infused with a flow rate of 5 μ L/min. Electrospray capillary voltage was set to 3000 V and cone voltage to 30 V. Nitrogen gas was used for nebulization and

desolvation. Accurate masses were determined by co-infusion of the samples with leucine enkephalin (YGGFL) and recalibration of the spectrum using the peak at m/z 556.2771 as lock mass. Precoated aluminum sheets (MN ALUGRAM SIL G/UV₂₅₄ 20 × 20 cm) were used for TLC; the spots were examined with UV light. Column chromatography was performed on ICN silica gel 63-200, 60 Å. An analytical sample was obtained by purification with preparative HPLC (waters 1525-2487 system) with a gradient of CH₃CN in 50 mM TEAB buffer (pH = 7.4) on using Prep C18 5 µm column 19 × 150 mm at the flow rate of 8 mL/min. Structures of all compounds synthesized were confirmed using ¹H, ¹³C, ³¹P NMR spectroscopy and MS.

Synthesis of d4T diphosphate derivatives (1 and 2). To a solution of 3'-deoxy-2',3'-didehydrothymidine (400 mg, 1.79 mmol) in trimethylphosphate (3.6 mL) was added phosphoryl chloride (0.48 mL, 5.15 mmol) slowly (20 min) at 0 °C. The reaction mixture was stirred at room temperature for 3 h and quenched by adding cold 1 M TEAB (pH 7.4) 30 mL. After the solvents were evaporated in vacuo, the residue was purified by silica column chromatography eluting with CHCl₃, CHCl₃/MeOH 5:1 to *i*-PrOH/NH₃/H₂O 7:1:1 and 6:1:1 to yield the desired d4T monophosphate ammonium salt (150 mg, 24.5 %) and d4T diphosphate ammonium salt (290 mg, 37.4 %) as white solid. In addition, if ethanol (25 mL × 3) was added to the reaction mixture before coevaporation and after quenching by adding 1 M TEAB, ethyl phosphodiester of d4T (**2**) was formed as side product and the purified sample (15 mg) was obtained by preparative HPLC with a gradient of CH₃CN in 50

mM TEAB buffer (pH = 7.4).

3'-Deoxy-2',3'-didehydrothymidine-5'-P^I, P²-diphosphate (1). TLC (*i*-PrOH/NH₃/H₂O 7:1:2, v/v): R_f 0.25 ; UV: (H₂O) λ_{max} = 266 nm (log ε = 3.97); ¹H NMR (500 MHz, D₂O, 5 °C): δ 7.59 (s, 1H, H-6), 6.94 (m, 1H, H-1'), 6.48 (d, J = 6.0 Hz, 1H, H-2'), 5.89 (d, J = 6.0 Hz, 1H, H-3'), 5.07 (m, 1H, H-4'), 4.10 (m, 2H, H-5' and H-5''), 1.85 (s, 3H, -CH₃); ¹³C NMR (125 MHz, D₂O, 5 °C): δ 166.26 (C-4), 151.70 (C-2), 137.59 (C-6), 133.70 (C-3'), 124.40 (C-2'), 110.90 (C-5), 89.26 (C-1'), 85.38 (d, ³J(C, P) = 7.5 Hz, C-4'), 65.49 (C-5'), 10.84 (CH₃); ³¹P NMR (202 MHz, D₂O): δ -9.73 (br), -11.62 (d, J = 21.0 Hz); HRMS for C₁₀H₁₄N₂O₁₀P₂ (M-H)⁻ calcd: 383.0051 , found: 383.0147.

3'-Deoxy-2',3'-didehydrothymidine-5'-P^I, (P²-ethyl)-phosphodiester (2). TLC (*i*-PrOH/NH₃/H₂O 7:1:2, v/v): R_f 0.39; UV: (H₂O) λ_{max} = 266 nm (log ε = 3.97); ¹H NMR (500 MHz, D₂O, 5 °C): δ 7.62 (s, 1H, H-6), 6.96 (m, 1H, H-1'), 6.49 (d, J = 6.0 Hz, 1H, H-2'), 5.92 (d, J = 6.0 Hz, 1H, H-3'), 5.09 (m, 1H, H-4'), 4.13 (m, 2H, H-5' and H-5''), 3.94 (m, 1H, -CH₂), 1.88 (q, 3H, -CH₃), 1.21 (t, J = 7.0 Hz, 3H, -CH₂CH₃); ¹³C NMR (125 MHz, D₂O, 5 °C): δ 166.58 (C-4), 151.94 (C-2), 137.78 (C-6), 133.66 (C-3'), 124.65 (C-2'), 110.90 (C-5), 89.35 (C-1'), 85.40 (d, ³J(C, P) = 8.8 Hz, C-4'), 65.77 (d, ²J(C, P) = 5.5 Hz, C-5'), 62.13 (d, ²J(C, P) = 5.7 Hz, -CH₂CH₃), 14.97 (d, ³J(C, P) = 7.5 Hz, -CH₂CH₃), 10.95 (CH₃); ³¹P NMR (202 MHz, D₂O): δ -11.03 (d, J₁ = 21.4 Hz), -11.70 (d, J₂ = 21.3 Hz); HRMS for C₁₂H₁₈N₂O₁₀P₂ (M-H)⁻ calcd: 411.0364, found: 411.0362.

Synthesis of dinucleotide di- or tri-phosphate derivatives of d4T (3 and 4). The

synthesis of d4T 5'-phosphorimidazolidine was carried out according to the standard method described for adenosine 5'-phosphorimidazolidine [15]. To a stirred solution of d4T 5'-phosphorimidazolidine (120 mg, 0.34 mmol) in 3 mL 0.2 M *N*-ethyl morpholine buffer (pH 7.5), d4TMP (103 mg, 0.34 mmol) or d4T diphosphate (130 mg, 0.34 mmol) ammonium salt was added under Argon followed by ZnCl₂ (46 mg, 0.34 mmol) and the reaction mixture was stirred at room temperature for 60 h. The reaction was quenched by adding 1 mL 0.25 M EDTA (pH 3.5-4), then lyophilized to dryness. The residue was purified by silica column chromatography eluting with *i*-PrOH/NH₃/H₂O (20:1:1, 9:1:1, 7:1:1 to 6:1:1) and yielding crude white solid compound **3** (88 mg, 41.2 %) and compound **4** (60 mg, 24.4 %) as ammonium salt. The analytical sample was further purified by preparative HPLC with a gradient of CH₃CN in 50 mM TEAB buffer (pH = 7.4).

Di-3'-deoxy-2',3'-didehydrothymidine 5',5''-P^I, P²-diphosphate (3). TLC (*i*-PrOH/NH₃/H₂O 7:1:2, v/v): R_f 0.42 ; UV: (H₂O) λ_{max} = 266 nm (log ε = 3.97); ¹H NMR (500 MHz, D₂O, 5 °C): δ 7.60 (d, J = 1.1 Hz, 2H, H-6), 6.92 (m, 2H, H-1'), 6.43 (m, 2H, H-2'), 5.89 (m, 2H, H-3'), 5.03 (m, 2H, H-4'), 4.10 (m, 4H, H-5' and H-5''), 1.85 (q, 6H, -CH₃); ¹³C NMR (125 MHz, D₂O, 5 °C): δ 166.58 (C-4), 151.94 (C-2), 137.72 (C-6), 133.41 (C-3'), 125.01 (C-2'), 110.81 (C-5), 89.26 (C-1'), 85.25 (C-4'), 65.87 (C-5'), 11.04 (CH₃); ³¹P NMR (202 MHz, D₂O): δ -11.62; HRMS for C₂₀H₂₄N₄O₁₃P₂ (M-H)⁻ calcd: 589.0742, found: 589.0750.

Di-3'-deoxy-2',3'-didehydrothymidine 5',5''-P^I, P³-triphosphate (4). TLC (*i*-PrOH/NH₃/H₂O 7:1:2, v/v): R_f 0.44 ; UV: (H₂O) λ_{max} = 266 nm (log ε = 3.96); ¹H

NMR (500 MHz, D₂O, 5 °C): δ 7.59 (d, J = 1.5 Hz, 2H, H-6), 6.90 (m, 2H, H-1'), 6.46 (m, 2H, H-2'), 5.86 (m, 2H, H-3'), 5.03 (m, 2H, H-4'), 4.13 (m, 4H, H-5' and H-5''), 1.84 (q, 6H, -CH₃); ¹³C NMR (150 MHz, D₂O, 5 °C): δ 166.21 (C-4), 151.64 (C-2), 137.74 (C-6), 133.72 (C-3'), 124.61 (C-2'), 110.90 (C-5), 89.29 (C-1'), 85.36 (d, ³J(C, P) = 9.0 Hz, C-4'), 65.83 (C-5'), 10.92 (CH₃); ³¹P NMR (202 MHz, D₂O): δ -11.91 (d, J_1 = 19.1 Hz), -23.68 (t, J_2 = 19.1 Hz); HRMS for C₂₀H₂₅N₄O₁₆P₃ (M-H)⁻ calcd: 669.0406 , found: 669.0413 .

Enzymatic synthesis. – *Oligodeoxyribonucleotides:* DNA oligonucleotides P₁ and T₁ were purchased from Sigma Genosys and Eurogentec. The concentrations were determined with a Varian Cary-300-Bio UV Spectrophotometer. The lyophilized oligonucleotides were dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at -20 °C. The primer oligonucleotides were 5'-labeled with [γ -³³P] ATP (Perkin Elmer) using T4 polynucleotide kinase (New England Biolabs) according to the standard procedures. Labeled oligonucleotides were further purified with Illustra™ Microspin™ G-25 columns (GE Healthcare).

DNA polymerase reactions: 5'-End-labeled primer was annealed to its template by combining primer and template at a molar ratio of 1:2 and heating the mixture to 70 °C for 10 min, followed by slow cooling to room temperature over a period of 2 h. The complex of P₁T₁ was used for the single nucleotide incorporation study of compounds **1-4**. A series of 20- μ L-batch reactions of the enzyme HIV-1 RT (Ambion, Inc.; 10 U/ μ L stock solutions) were prepared. The final mixture contained 125 nM primer-template complex, RT buffer (50 mM Tris-HCl, 50 mM KCl, 10 mM

MgCl₂, 0.5 mM spermidine, 10 mM DTT; pH 8.3), 0.025 U/μL HIV-1 RT, and different concentrations of phosphodiester substrates. Mixtures were incubated at 37 °C, and aliquots (2.5 μL) were removed and quenched after 10, 20, 30, 60, 120 min. In the control reaction with the natural nucleotides, 10 μM d4TTP was used.

Polyacrylamide electrophoresis: All polymerase reaction (2.5 μL) were quenched by the addition of 10 μL of loading buffer (90 % formamide, 0.05 % bromophenol blue, 0.05 % xylene cyanol, and 50 mM ethylenediaminetetraacetic acid (EDTA)). Samples were heated at 75 °C for 5 min prior to analysis by electrophoresis for 2.5 h at 2000 V on a 0.4-mm 20 % denaturing gel in the presence of a 100 mM Tris-borate, 2.5 mM EDTA buffer, pH 8.3. Products were visualized by phosphor imaging. The amount of radioactivity in the bands corresponding to product formation was determined with the imaging device Cyclone and the associated Optiquant image analysis software (Perkin-Elmer).

Steady-state kinetics of single nucleotide incorporation: To determine the kinetic parameters for the incorporation of compounds **1-4** and the reference d4TTP, a steady-state kinetics assay was carried out. The reaction was started by adding HIV-1 RT to P₁T₁ complex, RT buffer, compounds **1-4** and d4TTP. The final mixture (20 μL) contained 125 nM primer-template complex, and various concentrations of compounds **1-4** or d4TTP, buffer, 0.025 U/μL HIV-1 RT for compounds **1-4** and 0.0125 U/μL HIV-1 RT for d4TTP, respectively. The range of concentrations for compounds **1-4** was optimized according to the K_m value for the incorporation of an individual nucleotide. Enzymatic reactions were carried out to

attain 5-25 % incorporation with appropriate substrate concentrations (5-150 μM used for compounds **1-4** and 1-20 μM used for d4TTP) incubated at 37 °C and run for 8-10 different time intervals (1-7 min). The amount of primer template (125 nM) was 3.5-fold excess of HIV-1 RT (36.2 nM). Products were separated on a 20 % polyacrylamide gel and quantitated by phosphor imaging. The incorporation velocities were calculated based on the percentage of single-nucleotide extension product (P + 1 band). The kinetic parameters (V_{max} and K_m) were determined by plotting V (nM/min) versus substrate concentration (μM) and fitting the data point to a nonlinear Michaelis-Menten regression using GraphPad Prism software. The K_{cat} was calculated according to the used enzyme concentration.

Biological evaluation of the polyphosphate derivatives of dideoxynucleotides against HIV-1 and HIV-2. - The biological evaluation of the polyphosphate derivatives of d4T (**2-4**) against HIV-1 and HIV-2 was carried out including d4T and its 5'-O-monophosphate as references. The methodology of the anti-HIV assays was as follows. The anti-HIV activity and cytotoxicity of the compounds were evaluated against wild-type HIV-1 strain III_B and HIV-2 strain ROD in MT-4 cell cultures using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [16].

Briefly, virus stocks were titrated in MT-4 cells and expressed as the 50% cell culture infective dose (CCID₅₀). MT-4 cells were suspended in culture medium at 1×10^5 cells/mL and infected with HIV at a multiplicity of infection of 0.02. Immediately after viral infection, 100 μL of the cell suspension was placed in each

well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. The test compounds were dissolved in DMSO at 50 mM or higher. After 4 days of incubation at 37 °C, the number of viable cells was determined using the MTT method. Compounds were tested in parallel for cytotoxic effects in uninfected MT-4 cells.

CEM cells were infected as previously described. Briefly, 4×10^5 cells/mL were infected with HIV-2 at ≈ 100 CCID₅₀ (50 % cell culture infective dose) per mL of cell suspension. The thymidine kinase-deficient CEM (CEM/TK⁻) cell cultures were also infected with HIV-2. Then, 100 μ L of the infected cell suspensions was transferred into 96-well microtiter plate wells and mixed with 100 μ L of the appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, the giant cell formation was recorded microscopically in the HIV-infected cell cultures.

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Table 1. Steady-state kinetics of single nucleotide incorporation into P₁T₁ by HIV-1 RT

Compound	K _{cat} (min ⁻¹)	K _m (μM)	K _{cat} /K _m
			(×10 ⁻³ min ⁻¹ μM ⁻¹)
1	0.19 ± 0.01	35.33 ± 6.28	5.38
2	0.39 ± 0.01	43.64 ± 3.87	8.94
3	0.21 ± 0.01	24.19 ± 4.50	8.68
4	0.40 ± 0.03	41.54 ± 8.91	9.63
d4TTP	0.44 ± 0.02	3.57 ± 0.53	123.2

Table 2. Antiviral activity test of compounds **2-4** against HIV-1 and HIV-2

Compound	MT-4 cells ^a				CEM/0 ^b		CEM/TK- ^c
	IC ₅₀ (μM) [#]	CC ₅₀ (μM) [†]	IC ₅₀ (μM) [#]	CC ₅₀ (μM) [†]	IC ₅₀ (μM)	CC ₅₀ (μM)	IC ₅₀ (μM)
	HIV-1	HIV-1	HIV-2	HIV-2	HIV-2	HIV-2	HIV-2
2	0.5	168.6	0.3	140.2	2.2	303.2	179.8
3	0.2	72.8	0.2	97.1	0.8	211.7	84.0
4	0.2	97.6	0.2	95.9	1.2	186.5	84.8
d4T	0.3	149.0	0.2	142.4	3.0	267.6	217.4
d4TMP	0.4	185.4	0.4	188.7	1.6	410.9	170.1

^a HIV-1 (IIIB), HIV-2 (ROD); ^b CEM/0 cells (ROD); ^c CEM thymidine kinase deficient cells (ROD)

[#]IC₅₀ is the concentration required to inhibit HIV multiplication by 50%.

[†]CC₅₀ is the concentration drug which causes 50% cytotoxicity to uninfected cells.

[‡]SI corresponds to the ratio CC₅₀/IC₅₀.

LEGENDS

Figure 1. Structure of polyphosphate derivatives of d4T

Figure 2. Single incorporation of primer of P₁T₁ (125 nM) by HIV-1 RT with substrate concentrations and time intervals (min) as indicated, [HIV-1 RT] = 0.025 U/ μ L. Panel A: incorporation of compound **1**; panel B: incorporation of compound **2**; panel C: incorporation of compound **3**; panel D and E: incorporation of compound **4**; d4TTP (10 μ M) used as reference.

Scheme 1 Reagents and conditions: (a) POCl₃, PO(OMe)₃, 0°C ~ r. t., 3 h; (b) TEAB pH 7.4; (c) TEAB, EtOH, pH 7.4

Scheme 2 Reagents and conditions: (a) ZnCl₂, *N*-ethylmorpholine, d4TMP, r. t., 60 h; (b) ZnCl₂, *N*-ethylmorpholine, **1**, r. t., 60 h.

Figure 1

1, R = OH

2, R = OEt

Figure 2

Scheme 1

Scheme 2