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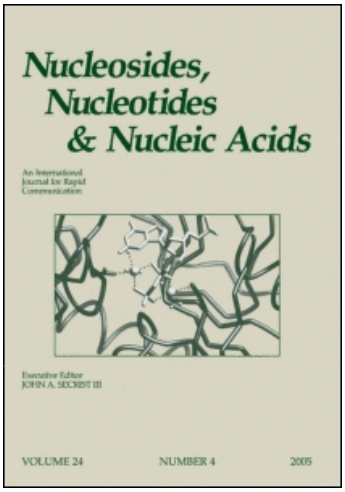

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
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Bis-Ketol Nucleoside Triesters as Prodrugs of the Antiviral Nucleoside Triphosphate Analogues of 3'-Deoxythymidine and 3'-Deoxy-2',3'-didehydrothymidine

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Online publication date: 03 December 2004

To cite this Article Calvo, Kim C. , Wang, Xiaohong and Koser, Gerald F.(2004) 'Bis-Ketol Nucleoside Triesters as Prodrugs of the Antiviral Nucleoside Triphosphate Analogues of 3'-Deoxythymidine and 3'-Deoxy-2',3'-didehydrothymidine', Nucleosides, Nucleotides and Nucleic Acids, 23: 3, 637 — 646
To link to this Article: DOI: 10.1081/NCN-120030723
URL: http://dx.doi.org/10.1081/NCN-120030723

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, No. 3, pp. 637–646, 2004

Bis-Ketol Nucleoside Triesters as Prodrugs of the Antiviral Nucleoside Triphosphate Analogues of 3′-Deoxythymidine and 3′-Deoxy-2′,3′-didehydrothymidine

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ABSTRACT

Derivatives of 3′-deoxythymidine (ddT) and 3′-deoxy-2′,3′-didehydrothymidine (d4T) were prepared in which the 5′-hydroxyl group of the nucleoside was esterified to a bis-ketol phosphate. The resulting phosphate triesters are postulated to be prodrugs of the corresponding 5′-mononucleotides, which are formed intracellularly by the hydrolysis of the two ketol ester groups. The triesters were tested for anti-HIV activity with the result that those derived from ddT showed enhanced antiviral activity when compared to the parent nucleoside.

Key Words: Bis-ketol; Triesters; Prodrugs; Triphosphate; Didehydrothymidine.

INTRODUCTION

Analogs of deoxyribonucleosides and nucleotides find use as antiviral compounds.[1–4] They constitute one of the important components of the combination therapy currently used in the treatment of AIDS.[5] These nucleoside analogs are really prodrugs of the biologically active form, the 5′-triphosphate,[6] which inhibits viral replication by interaction with the viral reverse transcriptase. The nucleoside analogs

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DOI: 10.1081/NCN-120030723 1525-7770 (Print); 1532-2335 (Online)
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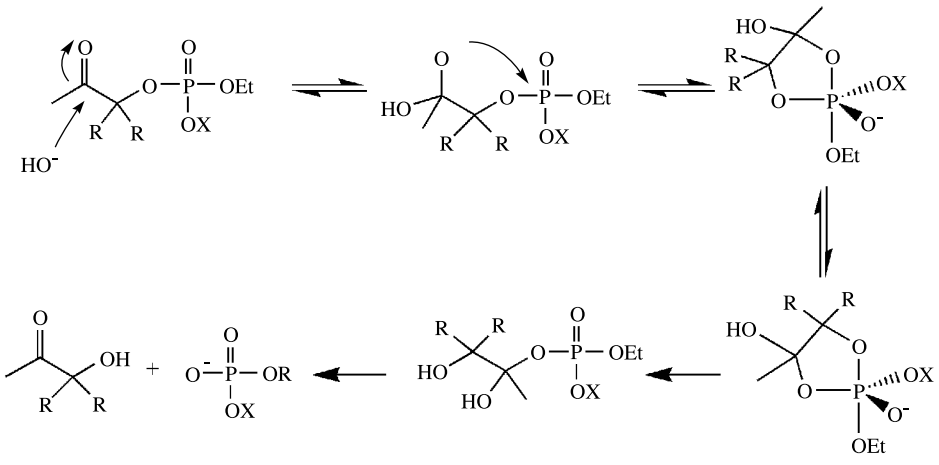
are converted to the active triphosphate form by three successive phosphorylation reactions catalyzed by various cellular kinases. Several nucleoside analogs, which are capable of potent inhibition of reverse transcriptase when present as the triphosphate form, do not show antiviral activity in screening assays because they are poor substrates for the cellular kinases.[7] The mono-, di- or triphosphate forms of the nucleosides are too polar to pass through the cell membrane and so cannot be used as drugs.

McGuigan has examined a strategy, named the kinase bypass, which attempts to circumvent the first phosphorylation reaction needed to activate all nucleoside analog prodrugs.[8–12] In this strategy, the double negative charge associated with a nucleoside analog monophosphate is masked by forming a nucleoside analog monophosphate triester. The triester is sufficiently lipophilic that it is able to penetrate the cell membrane. Related strategies include the use of a bis-S-acyl-2-thioethyl ester[13] and the use of the bis-pivaloyloxymethyl ester.[14] It appears that by judicious choice of the alcohols used in triester formation, it should be possible to direct intracellular hydrolysis to form the nucleoside analog monophosphate. The alcohols incorporated into the triester group should allow rapid triester hydrolysis to the diester monoanion, which should be retained within the cell since it is too polar to pass through the membrane, and subsequently to the monoester dianion. In addition, hydrolysis of the triester and diester should occur in a way that leads to formation of the nucleoside analog monophosphate, and not to the original nucleoside.

The ketol esters of phosphoric acid satisfy both of these criteria. The rate constant for the hydrolysis of a bis-ketol alkyl phosphate triester is about 6.2×10^{-4} s^{-1} at pH 7.4 and 37°C, giving a half life for the triester of about 19 min.[15] In addition, the mechanism for the hydrolysis as given in Scheme 1, demands that the ketol group must always be lost during hydrolysis to the diester.[16] Pseudorotation of the intermediate phosphorane places the alcohol oxygen of the ketol in the apical position. After the ketol ester group is expelled, the phosphorylated carbonyl hydrate eliminates the phosphate diester. Once the diester is formed, two hydrolysis reactions are possible.



Scheme 1. Mechanism of base hydrolysis of ketol phosphate triesters.

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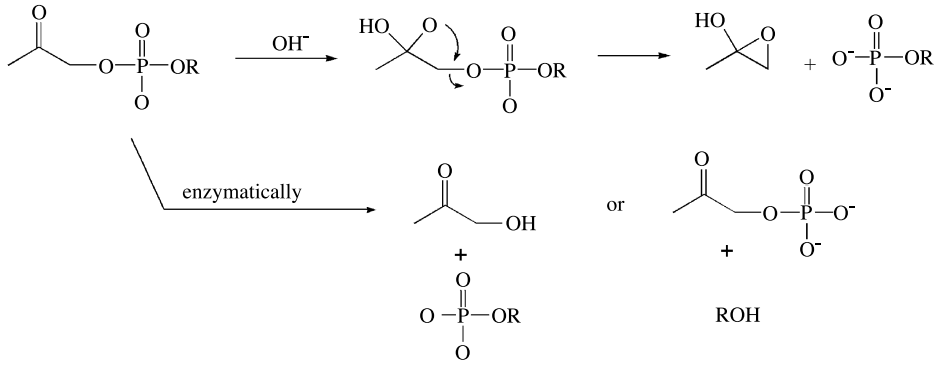

Scheme 2. Proposed routes for intracellular hydrolysis of mono-ketol phosphate diesters.

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The monoketol diester can either hydrolyze non-enzymatically and regioselectively via phosphate elimination from the ketol[17] to give the nucleoside monophosphate or enzymatically to produce either the nucleoside monophosphate and the ketol, or the ketol phosphate and the nucleoside analog (Scheme 2). Hydrolysis to give the ketol phosphate does not accomplish the kinase bypass.

In this paper we describe the synthesis of various bis-ketol ddT and d4T triesters and the results of antiviral screening of the compounds.

EXPERIMENTAL

General. NMR spectra were recorded on a Varian 300 MHZ NMR. The chemical shifts for ^{1}H spectra are referenced to the residual protio form of the deuterated NMR solvent. The chemical shifts for ^{13}C spectra are referenced to the solvent carbons, while the ^{31}P NMR shifts (proton-decoupled) are referenced to external 85% phosphoric acid capillary in the NMR solvent. Reagents were obtained from Aldrich, Lancaster or Acros. Solvents used in reactions were purified by standard methods by distillation from appropriate drying agents. The ddT was prepared by literature procedures.[18]

Tris-Ketol Phosphoric Acid Esters (1a–c). These compounds were prepared by treatment of crystalline phosphoric acid with 3 mole equivalents of $PhI(OAc)_2$ and 6 to 6.5 mole equivalents of the appropriate silyl enol ethers in *t*-butyl alcohol as reported in[19] for the analogous procedure with p-(difluoroiodo)toluene . Beginning with H_3PO_4 (10.0 mmol for **1a**, 15.3 mmol for **1b**, and 25.5 mmol for **1c**), the product yields were **1a** (22%, mp 107–9°C), **1b** (44%, mp 43–44°C), and **1c** (56%, mp 142–4°C). Selected NMR data (^{1}H, ^{13}C, and ^{31}P) for **1a** and **1c** are reported in.[19]

Lithium Bis-Ketol Phosphate Diesters (2a–c). These compounds were prepared as described in[19] Solutions of the tris-ketol phosphates [**1a** (5.89 mmol), **1b** (3.90 mmol), and **1c** (3.49 mmol)] and LiBr (1 to 1.3 equivalents) in dry acetone (120 or 130 mL) were stirred and heated under reflux (3 to 5 hr). During this time, the

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lithium bis-ketol phosphates precipitated and were isolated by filtration: yields; **2a** (89%, mp 221–2°C), **2b** (93%, mp 214–6°C), and **2c** (100 %, mp 189–93°C). Selected NMR data (^{1}H, ^{13}C, and ^{31}P) for 2**a** and 2**c** are reported in.[19] Combustion analysis of samples of **2a–2c**, previously prepared in our laboratory, indicate that these compounds may be hemi-hydrates.[19]

Bis-Ketol Hydrogen Phosphoric Acid Diesters (3a-c). Mixtures of the lithium bis-ketol phosphates [**2a** (2.57 mmol), **2b** (2.00 mmol), and **2c** (1.35 mmol)] and Dowex (50w × 8, H^+ form) ion exchange resin [14 g for **2a**, 7 g for **2b** and **2c**] in acetonitrile (50 mL) were stirred and heated under reflux for 3 h. The salts dissolved as they were converted to **3**. The resin was then removed by filtration and washed with methylene chloride (2 × 20 mL). Concentration of the filtrate and washings gave white solids, identified as **3a** (89%, mp 105–7°C), **3b** (90%, mp 72–4°C), and **3c** (92%).

4b-3′-Deoxythymidine-5′-bis(heptanoylmethyl)phosphate. Dicyclohexylcarbodiimide (DCC, 0.660 g, 3.20 mmol) was added at rt to a stirred solution of the bis-ketol hydrogen phosphate **3b** (0.561 g, 1.60 mmol) in dry CH_2Cl_2 (10 mL); a white solid (dicyclohexyl urea) separated immediately. After 4 h, the mixture was passed through a pipet packed with a glass wool plug. Triethylamine (25 drops) and ddT (91 mg, 0.40 mmol) were added to the filtrate, and the reaction mixture was allowed to stir for 20 h at rt. The solvent was then removed (rotary evaporator), and the residual yellow oil was subjected to flash column chromatography on silica gel (20 g) with $CHCl_3$ (300 mL), $CHCl_3/CH_3OH$ (200:1 (v/v), 100 mL), $CHCl_3/CH_3OH$ (100:1 (v/v), 100 mL), and $CHCl_3/CH_3OH$ (75:1 (v/v), 150 mL) to give **4b** as a pale yellow oil which gradually (1 week) solidified under mechanical vacuum; white solid; yield 202 mg (90%); mp 51.5–53°C. ^{1}H NMR ($CDCl_3$): 0.88 (t, J = 6.9 Hz, 6H), 1.28 (m, 12H), 1.61 (m, 4H), 1.93 (d, J = 1.1 Hz, 3H), 2.01–2.18 (m, 3H), 2.38–2.46 (m, 5H), 4.28–4.54 (m, 3H), 4.63–4.82 (m, 4H), 6.11–6.15 (m, 1H), 7.54 (d, J = 1.1 Hz, 1H), 8.50 (br s, 1H). ^{13}C NMR ($CDCl_3$): 12.60, 14.22, 22.65, 23.39, 25.56, 28.99, 31.69, 32.21, 38.60, 68.98 (d, J_{CP} = 5.4 Hz), 71.16 (t, J_{CP} = 5.1 Hz), 78.79 (d, J_{CP} = 8.5 Hz), 86.14, 110.96, 135.86, 150.42, 163.78, 204.05 (t, J_{CP} = 3.8 Hz). ^{31}P NMR ($CDCl_3$): 0.009 (s). IR (neat): 1694 cm^{-1} (C = O), 1270 cm^{-1} (P = O). Anal. Calcd. for $C_{26}H_{43}N_2O_9P$; C, 55.90; H, 7.76; N, 5.01. Found: C, 56.19; H, 7.99; N, 5.10.

4a-3′-Deoxythymidine-5′-bis(2,2-dimethylpropanoylmethyl)phosphate. Yield 221 mg (88%). ^{1}H NMR ($CDCl_3$): 1.19 (d, J = 2.1 Hz, 18H), 1.93 (d, J = 1.0 Hz, 3H), 2.02–2.15 (m, 3H), 2.35–2.44 (m, 1H), 4.29–4.59 (m, 3H), 4.89–5.11 (m, 4H), 6.13–6.16 (m, 1H), 7.58 (d, J = 1.1 Hz, 1H), 8.15 (br s, 1H). ^{13}C NMR ($CDCl_3$): 12.58, 25.53, 26.35, 32.26, 42.87, 68.03 (dd, J_{CP} = 5.3 Hz), 68.83 (d, J_{CP} = 5.5 Hz), 78.85 (d, J_{CP} = 7.3 Hz), 86.05, 110.92, 135.96, 150.50, 163.90, 208.16 (t, J_{CP} = 3 Hz). ^{31}P NMR ($CDCl_3$): 0.46 (s). mp: 114–115°C. IR (neat): 1695 cm^{-1} (C = O), 1270 cm^{-1} (P = O). Anal. Calcd. for $C_{22}H_{35}N_2O_9P$; C, 52.59; H, 7.02; N, 5.57. Found: C, 52.54; H, 7.04; N, 5.59.

4c-3′-Deoxythymidine-5′-bis(phenacyl)phosphate. Yield 153 mg (88%). ^{1}H NMR ($CDCl_3$): 1.90 (d, J = 0.9 Hz, 3H), 2.03–2.19 (m, 3H), 2.36–2.46 (m, 1H), 4.32–4.42 (m, 1H), 4.46–4.68 (m, 2H), 5.41–5.61 (m, 4H), 6.12–6.16 (m, 1H), 7.48–7.55 (m,

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4H), 7.58–7.68 (m, 2H), 7.87–7.93 (m, 4H), 8.65 (br s, 1H). ^{13}C NMR ($CDCl_3$): 12.57, 25.61, 32.18, 69.14 (d, J_{CP} = 6.0 Hz), 69.73 (dd, J_{CP} = 6.4 Hz), 78.79 (d, J_{CP} = 8.5 Hz), 86.06, 111.02, 127.90, 129.21, 133.93, 134.41, 135.89, 150.45, 163.77, 192.32 (t, J_{CP} = 3.7 Hz). ^{31}P NMR ($CDCl_3$): 0.52 (s). mp: 58–59°C. IR (neat): 1690 cm^{-1} (C = O), 1266 cm^{-1} (P = O). Anal. Calcd. for $C_{26}H_{27}N_2O_9P\cdot{}^1/_2H_2O$; C, 56.62; H, 5.12; N, 5.08. Found: C, 56.63; H, 5.12; N, 5.08.

5b-3′-Deoxy-2′,3′-didehydrothymidine-5′-bis(heptanoylmethyl)phosphate. DCC (1.41 g, 6.83 mmol) was added at rt to a stirred solution of **3b** (1.20 g, 3.42 mmol) in dry CH_2Cl_2 (15 mL); a white solid separated immediately. After 5h, the mixture was passed through a pipet packed with a glass wool plug. Triethylamine (25 drops) and d4T (0.192 g, 0.86 mmol) were added to the filtrate and the reaction mixture was allowed to stir for 2–3 days at rt. The solvent was then removed and the residual yellow oil was subjected to flash column chromatography on silica gel (26 g) with $CHCl_3$ (300 mL), $CHCl_3/CH_3OH$ (200:1 (v/v), 100 mL), $CHCl_3/CH_3OH$ (100:1 (v/v), 100 mL), and $CHCl_3/CH_3OH$ (75:1 (v/v), 150 mL) to give **5b** as a pale yellow oil which gradually solidified (1 week) under mechanical vacuum. yield 320 mg (67%). Further purification of this material by a second chromatography on silica gel (6 g) with $CHCl_3$ (150 mL) and $CHCl_3/CH_3OH$ (200:1 (v/v), 150 mL) returned 0.280 g of **5b**. mp: 90–92°C. ^{1}H NMR ($CDCl_3$): 0.88 (t, J = 7.0 Hz, 6H), 1.21–1.28 (m, 12H), 1.54–1.64 (m, 4H), 1.89 (d, J = 1.2 Hz, 3H), 2.38–2.42 (m, 4H), 4.35–4.52 (m, 2H), 4.60–4.83 (m, 4H), 4.99–5.06 (m, 1H), 5.86–5.92 (m, 1H), 6.39–6.42 (m, 1H), 7.01–7.04 (m, 1H), 7.36 (d, J = 1.2 Hz, 1H), 8.82 (br s, 1H). ^{13}C NMR ($CDCl_3$): 12.39, 14.19, 22.61, 23.37, 28.96, 31.66, 38.55, 68.37 (d, J_{CP} = 6.1 Hz), 71.07 (dd, J_{CP} = 6.1 Hz), 84.78 (d, J_{CP} = 8.6 Hz), 89.77, 111.46, 127.58, 133.40, 136.28, 151.03, 163.96, 203.92 (t, J_{CP} = 4.8 Hz). ^{31}P NMR ($CDCl_3$): − 0.15 (s). . IR (neat): 1699 cm^{-1} (C = O), 1265 cm^{-1} (P = O). Anal. Calcd. for $C_{26}H_{41}N_2O_9P$; C, 56.11; H, 7.42; N, 5.03. Found: C, 56.32; H, 7.08; N, 5.31.

5a-3′-Deoxy-2′,3′-didehydrothymidine-5′-*bis*(2,2-dimethylpropanoylmethyl)-phosphate. Yield 300 mg (66%). ^{1}H NMR ($CDCl_3$): 1.18 (d, J = 3.5 Hz, 18H), 1.90 (d, J = 1.2 Hz, 3H), 4.37–4.57 (m, 2H), 4.83–5.12 (m, 5H), 5.87–5.91 (m, 1H), 6.42–6.46 (m, 1H), 7.04–7.07 (m, 1H), 7.40 (d, J = 1.2 Hz, 1H), 8.55 (br s, 1H). ^{13}C NMR ($CDCl_3$): 12.36, 26.35, 42.87, 67.93 (dd, J_{CP} = 4.8 Hz), 68.26 (d, J_{CP} = 6.1 Hz), 84.90 (d, J_{CP} = 8.6 Hz), 89.74, 111.43, 127.40, 133.64, 136.47, 150.97, 163.88, 208.15 (t, J_{CP} = 3 Hz). ^{31}P NMR ($CDCl_3$): 0.22 (s). mp: 121–122°C. IR (neat): 1700 cm^{-1} (C = O), 1262 cm^{-1} (P = O). Anal. Calcd. for $C_{22}H_{33}N_2O_9P$; C, 52.80; H, 6.65; N, 5.60. Found: C, 53.06; H, 6.84; N, 5.85.

5c-3′-Deoxy-2′,3′-didehydrothymidine-5′-bis(phenacyl)phosphate. Yield 310 mg (87%). ^{1}H NMR ($CDCl_3$): 1.89 (d, J = 1.1 Hz, 3H), 4.48–4.68 (m, 2H), 5.05–5.15 (m, 1H), 5.37–5.61 (m, 4H), 5.89–5.92 (m, 1H), 6.47–6.51 (m, 1H), 7.04–7.08 (m, 1H), 7.44 (d, J = 1.3 Hz, 1H), 7.48–7.54 (m, 4H), 7.60–7.67 (m, 2H), 7.86–7.92 (m, 4H), 8.56 (br s, 1H). ^{13}C NMR ($CDCl_3$): 12.39, 68.48 (d, J_{CP} = 6.1 Hz), 69.64 (dd, J_{CP} = 5.3 Hz), 84.89 (d, J_{CP} = 8.5 Hz), 89.74, 111.48, 127.49, 127.86, 129.21, 133.58, 133.84, 134.42, 136.39, 151.00, 163.91, 192.24 (t, J_{CP} = 4.2 Hz). ^{31}P NMR ($CDCl_3$): 0.29 (s). mp: 72–74°C. IR (neat): 1703 cm^{-1} (C = O), 1102 cm^{-1} (P = O). Anal. Calcd. for $C_{26}H_{25}N_2O_9P\cdot{}^1/_2H_2O$; C, 56.83; H, 4.77; N, 5.10. Found: C, 56.94; H, 4.68; N, 5.15.

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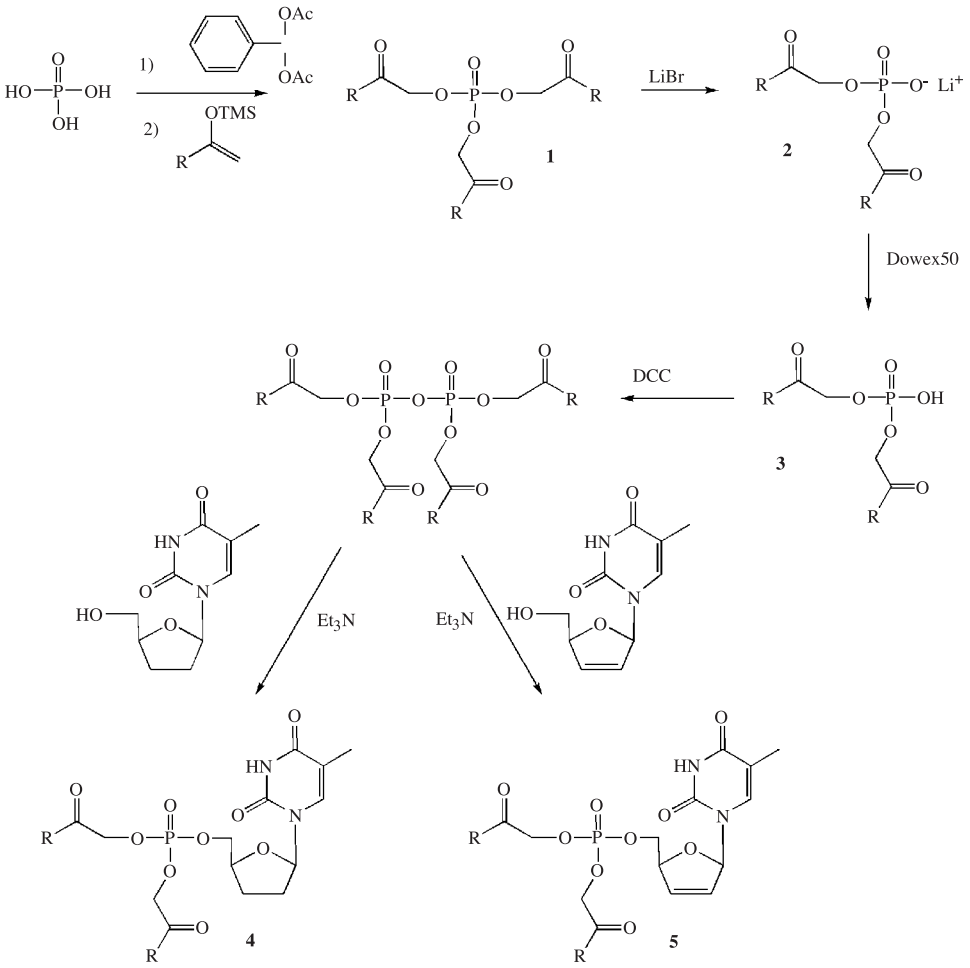
Hydrolysis rate constant for 5c: The hydrolysis of 5c was determined as indicated in reference 8. At 25°C, pH 7.68, the hydrolysis rate constant is 0.0260 min^{-1}. Using an activation energy of 18.40 kcal/mole,[15] we estimate that at pH 7.4 and 37°C, the half life of 5c is about 42 min.

Assay of anti-HIV activity: The bis-ketol ddT and d4T derivatives were tested for anti-HIV activity at the National Cancer Institute by their standard protocol.[20] In this protocol, the compounds are dissolved in DMSO and then serially diluted into the cell culture medium. CEM cells are used as the T4 lymphocytes. These cells are added to the culture medium followed by HIV-1. An uninfected control in the presence of the compound being tested is used as a toxicity control, and infected and uninfected cells without the compound serve as basic controls. Cell viability is assessed by the formazan color development following addition of the tetrazolium salt, XTT. The IC50 values represent the molar concentration of compound that results in 50% inhibition of



Scheme 3. Synthesis of bis-ketol phosphate derivatives of deoxy and dideoxythymidine.

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uninfected cell growth. The EC50 values represent the molar concentration of compound that results in survival of 50% of infected cells. The TI50 is the ratio of IC50/EC50.

RESULTS AND DISCUSSION

The synthesis of the bis-ketol ddT and d4T monophosphate triesters was accomplished by phosphorylation of the nucleoside with the tetra-kis ketol pyrophosphates generated in situ from bis-ketol hydrogen phosphates and DCC, Scheme 3. Formation of the bis-ketol hydrogen phosphates was made possible by using an ion exchange resin to exchange lithium for proton. The formation of the lithium bis-ketol phosphates proceeded almost quantitatively from the tris-ketol phosphates using LiBr in acetone. Overall, the synthesis proceeded in good yield from the nucleoside derivative to give the desired bis-ketol phosphate triesters.

Table 1 summarizes the in vitro anti-HIV test results for the bis-ketol phosphate triesters of ddT and d4T given in Scheme 4. In the assay conducted by the NCI, ddT is classified as inactive in inhibiting the growth of HIV in CEM T4 lymphocytes. This category is reserved for compounds showing an EC50 greater than about 200 μM. When the bis-ketol phosphate is added to the 5-hydroxyl of ddT, all of the derivatives become moderately active in inhibiting the growth of HIV. The EC50 values for the bis-ketol phosphate ddT triesters ranged from 19 to 25 μM which represents a decrease in EC50 of at least 10 fold (the NCI assay did not always determine the higher limit of activity) when compared to the parent ddT. This increase in anti-viral activity is attributed to the formation of increased levels of the monophosphate of ddT in the cells.

Table 1. Anti-HIV activity of compounds **4a–c** and **5a–c**.[a]

Compound	IC50[b] μM	EC50[c] μM	TI50[d]
ddT	> 200	INACTIVE[e]	
4a	> 200	23.	> 8.7
4b	> 200	19.	> 10
4c	> 200	25.	> 8
d4T	> 200	0.60	333
5a	> 200	1.3	> 154
5b	> 180	0.51	> 353
5c	110	0.89	124

[a]Compounds were tested against HIV-1 in CEM cells according to the NCI protocol.[20]
[b]Concentration that gives a cell growth of 50% relative to a culture not exposed to the compound or virus.
[c]Concentration that leads to 50% protection of virus infected cells.
[d]The ratio IC50/EC50.
[e]According to the National Cancer Institute, the EC50 of this compound is greater than 200 μM and the compound is labeled as inactive. Compounds 4a–c are labeled moderately active. Compounds 5a–c are all labeled as active by the NCI.

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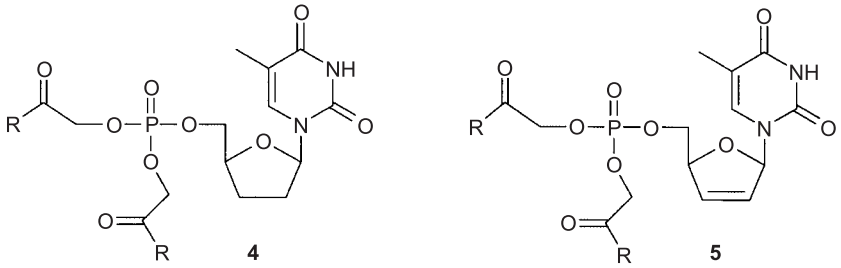

R = *t*-butyl (a), hexyl (b), phenyl (c)

Scheme 4. Structural formulas of nucleotides tested for anti-HIV activity.

Both ddT and d4T have been assayed for anti-HIV activity in the past. When ddT and d4T were examined in the same assay system and the data reported in the same paper,[21,22] ddT was found to have an EC50 about 20 times larger than d4T. When the triphosphates of ddT and d4T were analyzed as inhibitors of HIV reverse transcriptase using the same enzyme and the same polymeric substrate,[23] the inhibition constant for ddT was about 3 times that for d4T. It is clear that both ddT and d4T are active against HIV in an in vitro assay or reverse transcriptase assay. The fact that d4T is only three times better at inhibiting reverse transcriptase but 20 fold better at protecting cells from HIV indicates that the two compounds lead to significantly different levels of triphosphate inside the cells when administered as the nucleosides. The data on the EC50 values of the bis-ketol phosphate triesters of ddT and d4T in this study indicate that the bis-ketol derivatives of d4T inhibit HIV at concentrations about 25 fold lower than the bis-ketol derivatives of ddT. However, in the assay used to assess anti-HIV activity, the EC50 of d4T is about 300 fold lower than the EC50 of ddT. The addition of the bis-ketol phosphate group to ddT has decreased the EC50 value by at least 10 fold. Although the addition of a bis-ketol triester to d4T did not significantly improve the EC50, it did not render the nucleoside analog inactive. This is in contrast to a series of bis-ketol phosphate AZT derivatives that were tested previously[24] where the bis-ketol derivative was 1.5 to 77 times more active than the parent nucleoside analog. In the case of ddT, the addition of a bis-ketol triester significantly improved the EC50 value. The bis-ketol phosphate triesters of ddT behave as though they were pro-drugs of ddT monophosphate esters.

ACKNOWLEDGMENT

This research was supported in part by NIH grant AI043975 awarded to G.F. Koser and K.C. Calvo.

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Received July 1, 2003
Accepted December 31, 2003

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