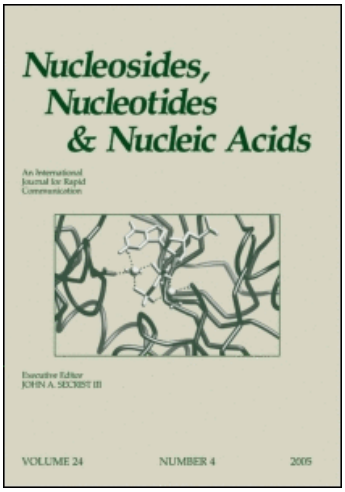

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SYNTHESIS OF NON-NUCLEOSIDE TRIPHOSPHATE ANALOGUES, A NEW TYPE OF SUBSTRATES FOR TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE

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ABSTRACT: A series of non-nucleoside triphosphate analogues were synthesized. In place of the nucleoside fragment, substituents bearing aromatic groups were introduced; the triphosphate component was replaced at α, β, or γ-positions by phosphonates. α-[2-N-(9-Fluorenylmethoxycarbonyl)aminoethylphosphonyl]-β,γ-difluoromethylenediphosphonate (**IIc**) revealed the best substrate properties toward terminal deoxynucleotidyl transferase.

INTRODUCTION

Terminal deoxynucleotidyl transferase (TDT)[1,2] occupies a specific place among DNA-polymerizing enzymes. Unlike other DNA polymerases, it is template-independent and randomly polymerizes dNTP onto initiator DNA.[3] It has been postulated that TDT may play a role in the development of immune system[4] as it is expressed maximally in pro-B and T-lymphocytes during immunological programming. It has been shown that the level of TDT in leukocytes of leukemia patients is very high.[5]

Several attempts were made to find cytotoxic agents for TDT-positive leukemia cells among 3'-deoxy- and 2',3'-dideoxynucleosides based on chain-terminating substrate properties of their triphosphates.[6,7] In 1994 triphosphate analogues with modified nucleoside residues (α- and β- anomers) belonging both to a D- and L-series were synthesized.[8] This compounds were shown to be TDT inhibitors in the cell-free system and their effect did not depend significantly

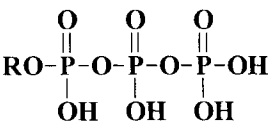
Dedicated to the memory of Professor A.A. Krayevsky

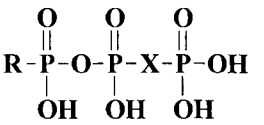
on their configuration. It was assumed that the interaction of TDT with the substrate was largely provided by dNTP triphosphate residues, whereas the nucleic base did not contribute to the binding of substrates to the TDT active site.[9] This implies that nucleoside-lacking triphosphates may serve as substrates of this enzyme. Here we describe the synthesis of non-nucleoside triphosphate analogues.

Abbreviations used: TDT - terminal deoxynucleotidyl transferase; 2-Npht - 2-naphtyl; Fmoc - 9-fluorenylmethoxycarbonyl; CDI - 1,1'-carbonyldiimidazole.

RESULTS AND DISCUSSION

To evaluate requirements to the substrates in the DNA chain elongation catalyzed by TDT, we have synthesized two groups of modified triphosphates bearing bulky substituents. The first series (**Ia-d**) included triphosphate monoesters, whereas the second one (**IIa-d**) contained α-phosphonate residues and, in some cases, an additional modification at β,γ-phosphorous atoms.





Ia, R = Ph
Ib, R = 4-$NO_2C_6H_4$
Ic, R = 2-Npht
Id, R = 4-$NO_2C_6H_4CH_2CH_2$

IIa, R = Ph, X = O
IIb, R = $FmocNHCH_2CH_2$, X = O
IIc, R = $FmocNHCH_2CH_2$, X = CF_2
IId, R = $FmocNHCH_2CH_2$, X = CBr_2

Compounds **Ia-c**, **IIa** were prepared by a coupling of aryl phosphates (phenylphosphonate for **IIa**) preactivated by CDI with pyrophosphate, according to the Hoard and Ott method.[10] The same approach was used earlier for the preparation of α-, β-, and γ-substituted triphosphates of natural and modified dNTPs.[11-13] Compound **Id** was prepared from 4-nitrophenethyl alcohol by treatment with $POCl_3$ followed by the addition of bis-(tri-*n*-butylammonium) pyrophosphate.[14]

To activate 2-N-(9-fluorenylmethoxycarbonyl)aminoethylphosphonic acid, 1,1'-carbonyldi(1,2,4-triazole) was used, which allowed us to obtain compounds (**IIb-d**) in moderate yields. The ambivalent character of the compounds **II** (they bear both highly hydrophilic

TABLE. Kinetic parameters of the TDT-catalyzed primer extension by ddTTP, **IIb**, and **IIc**

Compound	K_m, μM	$V_{max}/(V_{max}$ for ddTTP)
ddTTP	0.032 ± 0.001	1
IIb	0.033 ± 0.003	0.56
IIc	0.009 ± 0.001	0.56

phosphate groups and hydrophobic aromatic residues) impelled us to use successive chromatographic purification, first on DEAE-Toyopearl and then on reverse-phase silica gel LiChroprep RP-8 or RP-18. The structure of the compounds synthesized was confirmed by UV, ^{1}H-, and ^{31}P-NMR spectra.

The triphosphate analogues **I** and **II** were tested in extension reactions catalyzed by calf thymus TDT, human α- and β-polymerases and AMV reverse transcriptase.[15,16] Only TDT recognized the compounds of this type. The affinity of compounds **IIb** and **IIc** to TDT was close to that of ddTTP (Table). Other substances revealed weak substrate properties. The effectiveness of elongation with the compounds tested is decreasing in the following range: **IIc** > **IIb** > **IId** > **Id** > **Ic** > **Ib** > **Ia**. Compound **IIa** was inactive.

To conclude, non-nucleoside triphosphate analogues bearing aromatic groups are a new type of substrates of template-independent TDT and can serve as useful tools for the introduction of affinity labels and dyes at the 3'-end of oligonucleotides.

EXPERIMENTAL

Methylphosphonic acid, phenylphosphonic acid, phenylphosphate disodium salt, 4-nitrophenylphosphate disodium salt, 2-naphtyl phosphate disodium salt, 2-aminoethylphosphonic acid, and N-(9-fluorenylmethoxycarbonyloxy)succinimide were from Aldrich; 4-nitrophenethyl alcohol, CDI, 1,1'-carbonyldi(1,2,4-triazole), and Dowex 50 Wx8 were from Fluka; DEAE-Toyopearl 650M were from Toyo Soda; LiChroprep RP-18 (25-40 μm) and LiChroprep RP-8 (40-63 μm) were from Merck. Difluoromethylene- and dibromomethylenediphosphonic acids were prepared according to the reported procedures.[17,18]

UV spectra were measured on a Karl-Zeiss Specord UV-VIS M40 spectrophotometer in water at pH 7.0. ^{1}H-NMR spectra were recorded on a Bruker WP-200 SY spectrometer (USA) at 200.13 MHz, ^{31}P-NMR spectra were recorded at 81 MHz with ^{1}H-decoupling and 85%

H_3PO_4 as an external standard, in D_2O if not stated otherwise. Chemical shifts (δ) are in p.p.m. and coupling constants (*J*) in Hz.

For kinetic assays, the reaction mixture (6 μl) contained 0.02 μM [5'-^{32}P]primer, two activity units of TDT (USB Amersham Life Science, lot A1601-2), ddTTP or compounds **IIb,c** in the 100 mM sodium cacodylate buffer (pH 7.2), 2 mM $CoCl_2$ and 0.05 mM dithiothreitol. The reaction was carried out for 2 min at 37°C and stopped by adding 3 μl of formamide containing 0.5 M EDTA, 0.1% of bromophenol blue and xylene cyanol. The products were separated by electrophoresis in a 20% polyacrylamide/7 M urea sequencing gel. The autoradiographs were scanned on a Molecular Dynamics 300A Computing densitometer. The apparent K_m and V_{max} were determined from the rate of the product formation as a function of the substrate concentration.

Phenyl, 4-nitrophenyl, and 2-naphtyl phosphates, tri-*n*-butylammonium salts. A solution of the corresponding phosphate ester disodium salt (1 mmol) in water (2 ml) was filtered through the column (5 x 1 cm) with Dowex 50 (PyH^+). After washing with water, the UV-absorbing fractions (about 10 ml) were diluted with an equal volume of DMF. Tri-*n*-butylamine (240 ml, 1 mmol) was added and the mixture was stirred until a complete solution. The solvents were evaporated, the residue was dissolved in dry DMF and coevaporated with DMF (3 x 2 ml) *in vacuo*. The residue was dissolved in dry DMF (2 ml) to give 0.5 M stock solutions.

Phenyl (Ia), 4-nitrophenyl (Ib), and 2-naphtyl (Ic) triphosphates. CDI (243 mg, 1.5 mmol) in dry DMF (2 ml) was added to 0.5 M stock solutions of the respective phosphate ester tri-*n*-butylammonium salt (1 ml, 0.5 mmol), and the reaction mixture was stirred for 1 h at 20°C. Anhydrous MeOH (0.82 ml, 20 mmol) was added, and after 40-min stirring the solvents were evaporated and the residues were dissolved in dry DMF (1 ml). Then 0.5 M solution of bis-(tri-*n*-butylammonium) pyrophosphate (4 ml, 2 mmol) was added, and the mixture was stirred at 37°C for 2 h in the case of 4-nitrophenyl triphosphate **(Ib)** or overnight for phenyl (**Ia**) and 2-naphtyl (**Ic**) triphosphates. After dilution with water (200 ml), the mixture was loaded on a Toyopearl DEAE (HCO_3^-) column (2.5 x 20 cm). The column was washed with water (300 ml), and the products were eluted in a linear gradient of NH_4HCO_3 (0→0.4 M) in 1,4-dioxane (10→20%) (pH 7.5; total volume 1 l). The target fractions were collected, evaporated, coevaporated with water, and the residues were quenched with water (1 ml), and purified on a LiChroprep RP-18 column (1.5 x 20 cm). The products were eluted with water under the UV

control, and eluates were freeze-dried to give **Ia** 28 mg (15%), **Ib** 34 mg (16%), **Ic** and 21 mg (10%) as ammonium salts. **Ia**, UV: λ_{max} 212 nm (ε 2900). ^{1}H-NMR: 7.14dd (2H, *J* 7.9, 7.5, *m*-Ph), 7.01d (2H, *J* 7.9, *o*-Ph), 6.95t (1H, *p*-Ph). ^{31}P-NMR: -10.2d (1P, $J_{P\gamma,P\beta}$ 17, P_γ), -15.3d (1P, $J_{P\alpha,P\beta}$ 18, P_α), -22.5dd (1P, P_β). **Ib**, UV: λ_{max} 308 nm (ε 9400). ^{1}H-NMR: 7.23d (2H, *m*-Ph), 7.09d (2H, *J* 9, *o*-Ph). ^{31}P-NMR: -9.9d (1P, $J_{P\gamma,P\beta}$ 16, P_γ), -16.4d (1P, $J_{P\alpha,P\beta}$ 17.5, P_α), -22.7dd (1P, P_β). **Ic**, UV: λ_{max} 221.5 nm (ε 73000). ^{1}H-NMR: 7.75m (3H, Ar), 7.58s (1H, Ar), 7.34m (3H, Ar). ^{31}P-NMR: -9.1d (1P, $J_{P\gamma,P\beta}$ 19, P_γ), -14.6d (1P, $J_{P\alpha,P\beta}$ 20, P_α), -22.0dd (1P, P_β).

4-Nitrophenethyl triphosphate (Id). $POCl_3$ (93 ml, 1 mmol) was dropped to the precooled (0°C) solution of 4-nitrophenethyl alcohol (83 mg, 0.5 mmol) in triethyl phosphate (1 ml), the reaction was kept at 0°C for 20 h, then 0.5 M solution of bis-(tri-*n*-butylammonium) pyrophosphate (4 ml, 2 mmol) in DMF and tri-*n*-butylamine (715 ml, 3 mmol) were added. The reaction mixture was stirred for 2 h at 20°C and diluted with water (50 ml). The target product was purified as described above to give 31 mg (15%) of **Id** as ammonium salt. UV: λ_{max} 274 nm (ε 8800). ^{1}H-NMR: 8.03d (2H, *J* 8.3, Ph), 7.45d (2H, Ph), 4.15m (2H, CH_2OP), 3.02t (2H, *J* 6.2, $\underline{CH_2}Ph$). ^{31}P-NMR: -9.3d (1P, $J_{P\gamma,P\beta}$ 18.5, P_γ), -11.0d (1P, $J_{P\alpha,P\beta}$ 19.5, P_α), -22.6dd (1P, P_β).

α-Phenylphosphonyl-β,γ-diphosphate (IIa). To a solution of phenylphosphonic acid (79 mg, 0.5 mmol) and tri-*n*-butylamine (119 ml, 0.5 mmol) in DMF (1 ml) was added CDI (810 mg, 5 mmol) and the mixture was kept overnight at 20°C. After 40-min strring with anhydrous MeOH (0.81 ml, 20 mmol), the solution was evaporated, the residue was dissolved in DMF (1 ml), and added to the 0.5 M solution of bis-(tri-*n*-butylammonium) pyrophosphate (4 ml, 2 mmol). The reaction mixture was stirred overnight at 20°C, then diluted with water (100 ml), and separated as described above to give 32 mg (20%) of **IIa** as ammonium salt. UV: λ_{max} 212 nm (ε 2800). ^{1}H-NMR: 7.65dd (2H, *J* 13.5, 6.7, *o*-Ph), 7.30m (3H, *m*-Ph, *p*-Ph). ^{31}P-NMR: 6.5d (1P, $J_{P\alpha,P\beta}$ 30, P_α), -9.6d (1P, $J_{P\gamma,P\beta}$ 25, P_γ), -22.7m (1P, P_β).

2-N-(9-Fluorenylmethoxycarbonyl)aminoethylphosphonic acid. 2-Aminoethylphosphonic acid (50 mg, 0.4 mmol) and triethylamine (112 ml, 0.8 mmol) were dissolved in water (2 ml) and the solution of N-(9-fluorenylmethoxycarbonyloxy)succinimide (270 mg, 0.8

mmol) in THF (2 ml) was added. The reaction mixture was stirred for 5 h at 20°C, diluted with water (50 ml) and applied onto a DEAE-Toyopearl column (3 x 10 cm). The column was washed with 5% aqueous MeOH (100 ml) and eluted in a linear gradient of NH_4HCO_3 in 5% aqueous MeOH (0→0.25 M, 1 l). The target fractions were evaporated, coevaporated with water (5 x 10 ml), and the residue was purified on a LiChroprep RP-8 column (1 x 15 cm) eluting with water. The resulting solution was freeze-dried to yield 40 mg (27%) as ammonium salt. UV: λ_{max} 265 nm (ε 17500). ^{1}H-NMR (CD_3OD): 7.79d and 7.64d (4H, *J* 7, H-1, 4, 5, 8, Fmoc), 7.35m (4H, H-2, 3, 6, 7, Fmoc), 4.32m (2H, CH_2O), 4.20m (1H, H-9, Fmoc), 3.37m (2H, CH_2N), 1.81m (2H, CH_2P). ^{31}P-NMR: 21.6s.

α–[2-N-(9-Fluorenylmethoxycarbonyl)aminoethylphosphonyl]-β,γ–diphosphate (IIb), α–[2-N-(9-fluorenylmethoxycarbonyl)aminoethylphosphonyl]-β,γ–difluoromethylenediphosphonate (IIc) and α–[2-N-(9-fluorenylmethoxycarbonyl)aminoethylphosphonyl]-β,γ–dibromomethylenediphosphonate (IId). 2-N-(9-Fluorenylmethoxycarbonyl)aminoethylphosphonic acid (25 mg, 0.07 mmol) and 1,1'-carbonyldi(1,2,4-triazole) (32 mg, 0.2 mmol) were dissolved in DMF (2 ml). The solution was kept for 5 h at 5°C and MeOH (0.41 ml, 10 mmol) was added. After 30 min, a solution of 0.5 M bis-(tri-*n*-butylammonium) pyrophosphate or bis-(tri-*n*-butylammonium) difluoromethylenedi-phosphonate or bis-(tri-*n*-butylammonium) dibromomethylenediphosphonate (0.52 ml, 0.26 mmol) in DMF was added under stirring. After 4 h at 20°C, water (40 ml) was added and the solution was applied onto a DEAE-Toyopearl column (3 x 10 cm). The products were eluted in a linear gradient of NH_4HCO_3 (0→0.4 M, 1 l) in 20% aqueous CH_3CN. The solutions were evaporated, coevaporated with 50% aqueous ethanol, dissolved in water, and freeze-dried to yield **IIb** (10 mg, 28%), **IIc** (6 mg, 15%), and **IId** (8 mg, 16%) as ammonium salts. **IIb**, UV: λ_{max} 265 nm (ε 17000). ^{1}H-NMR: 7.76d and 7.55d [4H, *J* 7.5, H-1, 4, 5, 8 (Fmoc)], 7.31m [4H, H-2, 3, 6, 7 (Fmoc)], 4.37d (2H, *J* 5.5, CH_2O), 4.17t [1H, H-9 (Fmoc)], 3.14m (2H, CH_2N), 1.75m (2H, CH_2P). ^{31}P-NMR: 15.7d (1P, $J_{P\alpha,P\beta}$ 24, P_α), -10.6d (1P, $J_{P\gamma,P\beta}$ 21.5, P_γ), -23.2dd (1P, P_β). **IIc**, UV: λ_{max} 265 nm (ε 16500). ^{1}H-NMR: 7.72d and 7.52d [4H, *J* 7.5, H-1, 4, 5, 8 (Fmoc)], 7.31t and 7.24t [4H, *J* 7.5, H-2, 3, 6, 7 (Fmoc)], 4.33d (2H, *J* 5.5, CH_2O), 4.12t [1H, H-9 (Fmoc)], 3.11m (2H, CH_2N), 1.74dt (2H, $J_{H,P}$ 18, $J_{H,H}$ 8, CH_2P). ^{31}P-NMR: 16.6d (1P, $J_{P\alpha,P\beta}$ 33, P_α), 3.4dt (1P, $J_{P\gamma,P\beta}$ 59, $J_{P\gamma,F}$ 82, P_γ), -5.3ddt (1P, $J_{P\beta,F}$ 89, P_β). **IId**, UV: λ_{max} 264 nm

(ε 18000). ^{1}H-NMR: 7.74d and 7.53d [4H, *J* 7, H-1, 4, 5, 8 (Fmoc)], 7.25-7.30m [4H, H-2, 3, 6, 7 (Fmoc)], 4.35d (2H, *J* 5.5, CH_2O), 4.14t [1H, H-9 (Fmoc)], 3.12m (2H, CH_2N), 1.74m (2H, CH_2P). ^{31}P-NMR: 15.9d (1P, $J_{P\alpha,P\beta}$ 33, P_α), 8.2d (1P, $J_{P\gamma,P\beta}$ 15, P_γ), 0.3dd (1P, P_β).

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