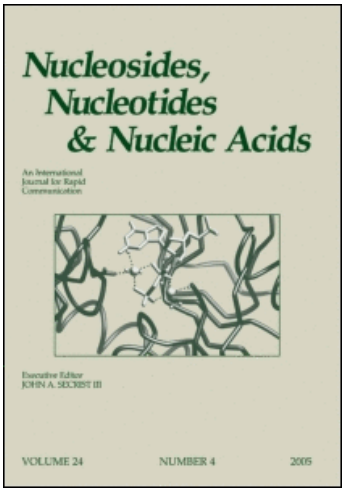

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Synthesis of Novel Alkyl Triphosphates and Their Substrate Properties Toward Terminal Deoxynucleotidyltransferase

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To cite this Article Jasko, Maxim , Khandazhinskaya, Anastasiya , Alexandrova, Ludmila , Shirokova, Elena , Ivanov, Alexander and Kukhanova, Marina(2007) 'Synthesis of Novel Alkyl Triphosphates and Their Substrate Properties Toward Terminal Deoxynucleotidyltransferase', Nucleosides, Nucleotides and Nucleic Acids, 26: 4, 323 — 334
To link to this Article: DOI: 10.1080/15257770701296713
URL: http://dx.doi.org/10.1080/15257770701296713

Nucleosides, Nucleotides, and Nucleic Acids, 26:323–334, 2007

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770701296713

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SYNTHESIS OF NOVEL ALKYL TRIPHOSPHATES AND THEIR SUBSTRATE PROPERTIES TOWARD TERMINAL DEOXYNUCLEOTIDYLTRANSFERASE

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□ *Novel triphosphate derivatives bearing bulky or small groups at α-position attached to the triphosphate residue through linkers of different structures and lengths were synthesized and studied as substrates toward terminal deoxynucleotidyltransferase. The substrate efficacy depends on the structure of substituents, linker length, and nature of metal activator. The replacement of hydrophobic groups by small substituents decreased the substrate efficacy by about 20 times in respect to hydrophobic residues. The dependence on metal activator is the following:* $Co^{2+} > Mn^{2+} >> Mg^{2+}$. *The model of interaction of alkyl triphosphates with linkers of different lengths bearing TdT active site is presented.*

Keywords Synthesis; terminal deoxynucleotidyltransferase; alkyl triphosphates; substrate specificity; kinetic parameters

INTRODUCTION

Recently we have shown that triphosphates bearing hydrophobic groups attached to triphosphate residue through linkers of different structures and lengths serve as unique substrates for both template-independent terminal deoxynucleotidyltransferase (TdT)[1,2] and mammalian template-

Received 13 September 2006; accepted 24 January 2007.
These studies were supported by Russian Foundation for Basic Research, project 06-04-48248, 05-04-49500, by grant of Presidium RAS on "Molecular and Cellular Biology."
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dependent DNA polymerases λ and β.[3,4] The obtained results demonstrate that neither the base nor the sugar moieties of the nucleosides play a crucial role for their incorporation into the primer 3′-ends by TdT or DNA polymerase λ and β, although the incorporation of alkyl residues by template-instructed DNA polymerases was limited.[3,4]

TdT belongs to the X polymerase family, which also includes DNA polymerases β, λ, μ, and some others. The members of this superfamily share a common signature in the active site and catalyze the same chemical reaction but have diverse biological roles.[5,6] TdT plays an important role in the diversification of immunoglobulins and T cell receptors.[7,8] DNA polymerases β and λ are involved in DNA reparation process.

Herein, we expanded the repertoire of alkyl triphosphates as potential substrates of TdT. We synthesized two new sets of triphosphates bearing bulky or small substitutes at α-position joined to triphosphate residue through linkers of different structure and length and evaluated them as substrates/inhibitors of TdT. A molecular model demonstrating the superposition of triphosphates bearing a fluorenylmethoxycarbonyl (Fmoc) fragment with the TdT active site is discussed to explain the dependence of substrate efficacy of the alkyl triphosphates on the linker length.

MATERIAL AND METHODS

Unlabeled dNTP and ddTTP were purchased from Boehringer Mannheim. [γ-^{32}P]ATP (6000 Ci/mmol) and [α-^{32}P]dTTP (3000 Ci/mmol) were from "Isotop" (Moscow, Russia); T4 polynucleotide kinase and TdT were obtained from Amersham Pharmacia Biothech (UK). The 19-mer oligonucleotide primer CCGTCAATTCCTGTAGTCT was obtained from "Litech" (Russia). The primer was labeled at the 5′-end with T4 polynucleotide kinase using [γ-^{32}P]ATP, purified on a Sephadex-G50 spin column and used for elongation reactions[9]. NMR spectra were registered on a Bruker AMX III-400 (USA) spectrometer at a working frequency of 400 MHz for ^{1}H NMR (using Me_4Si for $CDCI_3$ solution and sodium 3-(trimethylsilyl)-1-propanesulfonate for D_2O solutions as internal references), 162 MHz for ^{31}P NMR (with phosphorus-proton spin decoupling using 85% phosphoric acid as the external reference), and 100 MHz for ^{13}C NMR. Chemical shifts are given in ppm (δ scale) and spin coupling constants in Hz. UV spectra were registered on a Shimadzu UV-VIS-2401 P spectrophotometer (Japan). Mass spectra were registered on a Triple Quadrupole LC/MS Mass Spectrometer (Perkin-Elmer Sciex Instrument, Vernon Hills, IL, USA).

Synthesis of the Compounds

Compounds **1a–f** (Chart 1) were synthesized as described earlier.[10] A key stage of the synthesis of compounds **1 a–d**, **f** was phosphorylation of the

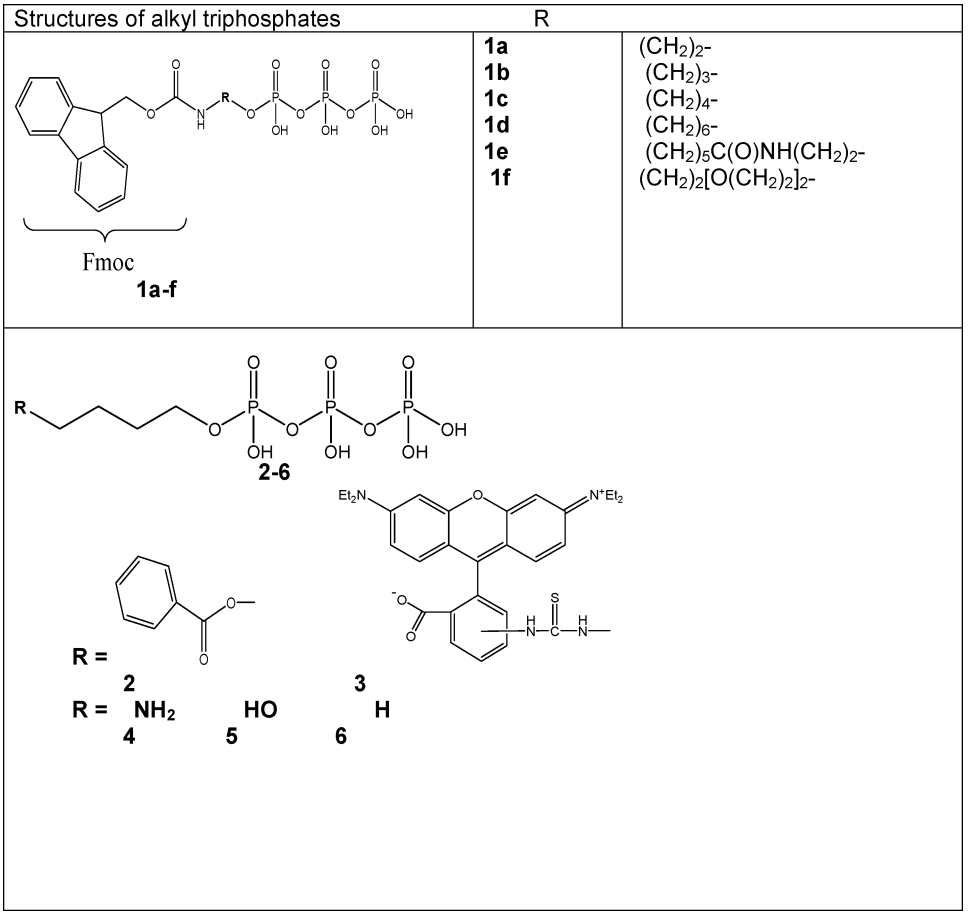

CHART 1

appropriate N-Fmoc-aminoalcohol according to the Ludwig procedure.[11] For the synthesis of **1e**, Fmoc-residue was introduced at the amino group of ε-aminocaproic acid to obtain the corresponding substituted caproyl chloride. The latter was coupled with the preliminarily silylated 2-aminoethyl phosphate. The resulting substituted phosphate was activated with 1,1′-carbonyldiimidazole and coupled with tributylammonium pyrophosphate to give the target **1e**.

N-(9-Fluorenylmethoxycarbonyl)-2-aminoethyl triphosphate 1a. Yield: 11%. ^{1}H-NMR (D_2O): 7.83, 7.69 (4H, 2d, *J* 7.5, H-1, 4, 5, 8 (Fmoc)), 7.42, 7.36 (4H, 2t, *J* 7.2, H-2, 3, 6, 7), 4.35 (2H, d, *J* 6.5, CH_2(Fmoc)), 4.24 (1H, t, H-9 (Fmoc)), 4.06 (2H, m, CH_2O), 3.44 (2H, m, CH_2N). ^{31}P-NMR (D_2O): −8.20 (1P, d, *J* 19.8, P_γ), −8.79 (1P, d, *J* 20.3, P_α), −20.73 (1P, dd, P_β). UV (H_2 O, pH 6): λ_{max} 265 nm ($\varepsilon = 17000$). Mass (m/e): 522.0 [M^+-1].

N-(9-Fluorenylmethoxycarbonyl)-4-aminopropyl triphosphate 1b. Yield: 7%. ^{1}H-NMR (D_2O): 7.83, 7.61 (4H, 2d, *J* 7.5, H-1, 4, 5, 8 (Fmoc)), 7.41, 7.33

(4H, 2t, J 7.2, H-2, 3, 6, 7), 4.69 (2H, d J 6.9, CH_2), 4.24 (1H, t, H-9), 3.82 (2H, m, CH_2O), 3.03 (2H, m, CH_2N), 1.65–1.53 (2H, m, (CH_2)). ^{31}P-NMR (D_2O): −9.74 (1P, d, J 20.3, P_γ), −10.48 (1P, d, J 19.3, P_α), −22.65 (1P, dd, Pβ). UV (H_2 O, pH 6): λ_{max} 265 nm ($\varepsilon = 17000$). Mass (m/e): 536.0 [M^+-1].

N-(9-Fluorenylmethoxycarbonyl)-4-aminobutyl triphosphate 1c. Yield: 12%. ^{1}H-NMR (D_2O): 7.89, 7.64 (4H, 2d, J 7.5, H-1, 4, 5, 8 (Fmoc)), 7.40, 7.48 (4H, 2t, J 7.5, H-2, 3, 6, 7), 4.64 (2H, d, J 6.9, CH_2), 4.20 (1H, t, H-9), 3.94 (2H, m, CH_2O), 3.02 (2H, m, CH_2N), 1.55–1.23 (4H, m, $(CH_2)_2$). ^{31}P-NMR (D_2O): −10.30 (1P, d, J 19.3, P_γ), −10.38 (1P, d, J 20.3, P_α), −22.79 (1P, dd, P_β). UV (H_2 O, pH 6): λ_{max} 265 nm (ε = 17000). Mass (m/e): 550.1 [M^+-1].

N-(9-Fluorenylmethoxycarbonyl)-6-aminohexyl triphosphate 1d. Yield: 8%. ^{1}H-NMR (D_2O): 7.94, 7.71 (4H, 2d, J 7.5, H-1, 4, 5, 8 (Fmoc)), 7.53, 7.45 (4H, 2t, J 7.2, H-2, 3, 6, 7), 4.69 (2H, d, J 6.8, CH_2), 4.34 (1H, t, H-9), 3.89 (2H, m, CH_2O), 3.03 (2H, m, CH_2N), 1.63–1.19 (8H, m, $(CH_2)_4$). ^{31}P-NMR (D_2O): −10.15 (1P, d, J 19.3, P_γ), −10.24 (1P, d, J 20.3, P_α), −22.67 (1P, dd, P_β). UV (D_2O, pH 6): λ_{max} 265 nm ($\varepsilon = 17000$). Mass (m/e): 578.1 [M^+−1].

N-[(9-Fluorenylmethoxycarbonyl)aminohexanoyl]-2-aminoethyl triphosphate 1e. Yield: 14%. ^{1}H-NMR (D_2O): 7.67, 7.47 (4H, 2d, J 7.5, H-1, 4, 5, 8 (Fmoc)), 7.53, 7.45 (4H, 2t, J 7.2, H-2, 3, 6, 7 (Fmoc)), 4.21 (2H, d, J 6.8, CH_2(Fmoc)), 4.04 (1H, t, H-9 (Fmoc)), 3.86 (2H, m, CH_2O), 3.25 (2H, m, CH_2N), 2.85 (2H, m, CH_2N), 2.19 (2H, m, CH_2 C(O)N), 1.53–1.10 (6H, m, $(CH_2)_3$). ^{31}P-NMR (D_2O): −10.24 (1P, d, J 20.3, P_γ), −10.64 (1P, d, J 19.3, P_α), −22.67 (1P, dd, P_β). UV (H_2 O, pH 6): λ_{max} 265 nm ($\varepsilon = 17000$). Mass (m/e): 635.1 [M^+-1].

N-(9-Fluorenylmethoxycarbonyl)-8-amino-3,6-dioxaoctyl triphosphate 1f. Yield: 11%. ^{1}H-NMR (D_2O): 7.80, 7.59 (4H, 2d, J 7.5, H-1, 4, 5, 8 (Fmoc)), 7.41, 7.33 (4H, 2t, J 7.5, H-2, 3, 6, 7 (Fmoc)), 4.47 (2H, m, CH_2(Fmoc)), 4.18 (1H, m, H-9 (Fmoc)), 4.02 (2H, m, CH_2OP), 3.64—3.38 (8H, m, $(CH_2)_4$), 3.11 (2H, m, CH_2N). ^{31}P-NMR (D_2O): −9.76 (1P, d, J 19.3, P_γ), −10.49 (1P, d, J 19.3, P_α), −22.49 (1P, t, P_β). UV (H_2 O, pH 6): λ_{max} 265 nm ($\varepsilon = 17000$). Mass (m/e): 610.1 [M^+-1].

4-Benzoyloxybutyl triphosphate 2 was obtained by phosphorylation of 4-benzoyloxybutanol according to[12]. Yield: 15%. ^{1}H-NMR (D_2O): 7.74 (2H, d, J 7.8, *o*-Bz), 7.42 (1H, t, J 7.1, *p*-Bz), 7.26 (2H, t, J 6.2, *m*-Bz), 4.11 (2H, m, CH_2O), 3.82 (2H, m, CH_2O), 1.63 (4H, m, $(CH_2)_2$), ^{31}P-NMR (D_2O): −5.71 (1P, d, J 18.2, P_γ), −10.06 (1P, d, J 19.1, P_α), −21.52 (1P, dd, P_β). UV (H_2 O, pH 6): λ_{max} 273 nm ($\varepsilon = 1100$). Mass (m/e): 432.0 [M^+-1].

N-(Rhodamineaminothiocarbonyl)-4-aminobutyl triphosphate 3 was obtained by the condensation of triphosphate **6** with rhodamine B isothiocyanate according to[13]. Yield: 75%. ^{1}H-NMR (D_2O): 7.75, 6.87, 6.54, 6.38, 6.25 (9H, 5m, aryl), 3.87 (2H, m, CH_2O), 3.21 (10H, m, $\underline{CH_2}NH$ and 4 $\underline{CH_2}CH_3$), 1.63 (4H, m, $(CH_2)_2$), 0.91 (12H, m, $4CH_2\underline{CH_3}$). ^{31}P-NMR

(D_2O): −10.07 (1P, d, J 18.3, P_γ), −10.34 (1P, d, J 19.3, P_α), −21.94 (1P, dd, P_β). UV (H_2O, pH 6): λ_{max} 556 ($\varepsilon = 59000$). Mass (m/e): 827.2 [M^+-1].

4-Aminobutyl triphosphate 4 was obtained by reaction of triphosphate **1c** with 25% aqueous NH_3 at +24°C for 18 h. Yield: 97%. ^{1}H-NMR (D_2O): 3.80 (2H, m, CH_2O), 2.89 (2H, m, CH_2N), 1.68–1.58 (4H, m, $(CH_2)_2$). ^{31}P-NMR (D_2O): −6.92 (1P, d, J 18.3, P_γ), −10.41 (1P, d, J 19.4, P_α), −22.07 (1P, dd, P_β). Mass (m/e): 328.0 [M^+-1].

4-Hydroxybutyl triphosphate 5 was obtained by reaction of triphosphate **2** with 25% aqueous NH_3 at +24°C for 18 h. Yield: 45%. ^{1}H-NMR (D_2O): 3.84 (2H, m, CH_2O), 3.48 (2H, t, J 5.9, $\underline{CH_2}OH$), 1.53 (4H, m, $2CH_2$). ^{31}P-NMR (D_2O): −9.52 (1P, d, J 18.2, P_γ), −10.35 (1P, d, J 19.1, P_α), −22.62 (1P, dd, P_β). Mass (m/e): 329.0 [M^+-1].

Butyl triphosphate 6 was obtained by phosphorylation of 1-butanol according to.[11] Yield: 6%. ^{1}H-NMR (D_2O): 3.61 (2H, m, CH_2O), 1.36 (2H, m, CH_2), 1.07 (2H, m, CH_2), 0.59 (3 H, t, J 7.5, CH_3). ^{31}P-NMR (D_2O): −7.92 (1P, d, J 18.5, P_γ), −10.14 (1P, d, J 17.1, P_α), −21.87 (1P, dd, P_β). Mass (m/e): 313.0 [M^+-1].

Inhibition assays. The reaction mixture (10 μl) contained 50 mM cacodylate buffer (pH 7.2), 2 mM Co^{2+}, if not stated otherwise, 1 mM DTT, 100 nM [5′-^{32}P]-19-mer primer, 2 μM [α-^{32}P]dTTP, and the tested compounds at varied concentrations (see Figures). The reactions were initiated by the addition of 0.25 U of TdT. After incubation for 10 min at 37°C, 8 μl of the reaction mixtures were spotted onto DE81 discs. The discs were washed with 0.5 M potassium phosphate buffer, pH 7.0, dried, and counted in a liquid scintillation counter.

Elongation assays. The reaction mixture (10 μl) contained 50 mM cacodylate buffer (pH 7.2), 100 nM [5′-^{32}P]-primer, 1 mM DTT, varied concentrations of Co^{2+}, Mn^{2+}, or Mg^{2+}, 0.25 U of TdT and the tested compounds. After incubation for 10 minutes at 37°C, the reactions were quenched by the addition of 5 μl of sequencing loading buffer containing 98% (v/v) deionized formamide/10 mM EDTA/1 mg/ml of each of bromophenol blue and xylene cyanol. The reaction mixtures were loaded onto a 20% denaturing PAAG and subjected to electrophoresis followed by the gel exposure with a Kodak film. The kinetic parameters of TdT catalyzed reactions were measured in the presence of 2 mM Co^{2+}, 0.25 U of enzyme, a saturated concentrations of the primer-template complex, varied concentrations of **1a**, **1b**, **1c** ranged from 0.05 to 5 μM or that of **1d** and **1e** ranged from 0.2 and 10 μM under the conditions described above. The reaction yields were measured in 5-minute time point for compounds **1a**, **1b**, **1c**, and ddTTP and 8 minutes for **1d** and **1f**. These time points were mid-points of the linear phase of the reactions, as was determined independently by the time-dependent incorporation experiments. The film after exposure was scanned with the aid of a computer densitometer (Molecular Dynamics,

Sunnyvale, CA, USA). The *Km* and relative k_{cat} values were calculated according to the Lineiweaver-Burk equation plot.

RESULTS

Novel alkyl triphosphates, lacking a nucleoside moiety but bearing bulky hydrophobic **1a–f**, **2**, **3**, or small **4–6** substituents attached to triphosphate residue through linkers of different structures and lengths were synthesized (Chart 1). The compounds **1a–f** had Fmoc-residues attached to the triphosphates through increasing linker lengths. The compounds **1c**, **2**, **3** have $(\text{-CH}_2\text{-})_4$- linker but substituents of different structures including the fluorescent residue of rodamine dye **3**. The compounds **4–6** bear small substituents at triphosphate residues.

The Inhibition of the TdT-Catalyzed Elongation Reactions with Triphosphate Derivatives

To understand the effects of the substituent nature and linker length on the ability to inhibit the elongation reaction, the incorporation of [α-^{32}P]dTMP into the primer 3′-ends was performed in the presence of triphosphates joined different substituent at α-position through linkers of different lengths and structures. Table 1 presents the concentrations of the compounds ensured a 50% inhibition of [^{32}P]dTMP incorporation into the 19-mer primer. The inhibition of the reaction with alkyl triphosphates **1a–d** bearing Fmoc-residues dropped almost by one order of magnitude while linker lengthened from two **1a** to six methylene groups **1d**. Further linker extension **1e–f** resulted in the compounds, whose inhibitory potential decreased by almost two orders of magnitude in respect to **1a**. Table 1 also shows that the compounds with the same linker but bearing bulky hydrophobic groups **1c**, **2** inhibited the reaction by 1–1.5 orders of magnitude stronger than the compounds with hydrophilic **4–5** or small **6** substituents.

TABLE 1 The concentrations of the compounds that inhibit the primer elongation with [α-^{32}P]dTTP by 50%

Inhibitor	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**	**2**	**4**	**5**	**6**
[μM]	1.0 ± 0.1	1.5 ± 0.1	3.0 ± 0.2	9 ± 1	20 ± 3	>80	4.0 ± 0.2	45 ± 5	50 ± 6	>60

The concentration of dTTP was 2 μM; TdT 0.25 U; reaction time 10 minutes. Values are mean of three independent experiments.

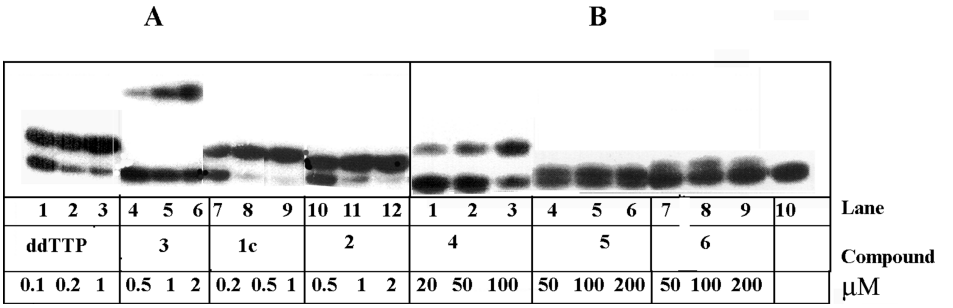

FIGURE 1 The radioautograph of denaturing PAAG after product separation of the [5′-^{32}P]-primer elongation reactions catalyzed by TdT in the presence of triphosphates bearing bulky hydrophobic (**A**) or small groups (**B**). TdT (0.25 U) was incubated in the presence of 100 nM [5′-^{32}P]-19-mer primer and varied concentrations of alkyl triphosphates for 10 minutes at 37°C. Concentrations of the compounds are shown in the Figure.

TdT Catalyzes the Incorporation of Alkyl Triphosphates into the Primer 3′-End

We investigated whether the alkyl triphosphate analogues could be incorporated into the primer 3′-end, or these compounds were non-substrate inhibitors of TdT. The substrate properties of alkyl triphosphates were evaluated by the ability of TdT to elongate the [5′-^{32}P]-19-mer primer in the presence of the compounds. The incorporation of the compounds into the primer 3′-ends was monitored using the [5′-^{32}P]-19-mer primer and different concentrations of the compounds. The reaction products were separated by denaturing PAGE followed by the exposure with a Kodak film. As one can see in Figure 1, the compounds **1c** (lanes 7–9) and **2** (lanes 10–12) were almost as good substrates as ddTTP (lanes 1–3) taken as a reference compound; the substrate efficacy of **3** (lanes 4–6) bearing a fluorescent residue of rodamine dye was about 5- to 10-fold lower in comparison with **1c**. The ability of TdT to incorporate triphosphates with small substituents **4–6** (Figure 1B) was 100–200-fold lower in respect to the triphosphates bearing bulky groups (Figure 1A). Electrophoretic mobilities of primers after incorporation of bulky hydrophobic substituents into the 3′-end differed from each other as well as the primers with small (H) and hydrophilic groups (NH_2, OH). The mobilities of the primers bearing the residues of compounds **5** and **6** (lanes 4–9) were very close to that of primer alone (lane 10).

Quantitation of the Primer Extension Reactions by Triphosphates Joined to Fmoc-Residues Through Linkers of Different Lengths

We evaluated the *Km* and relative k_{cat} values of the elongation reactions with **1a–e** under conditions described in Material and methods. The

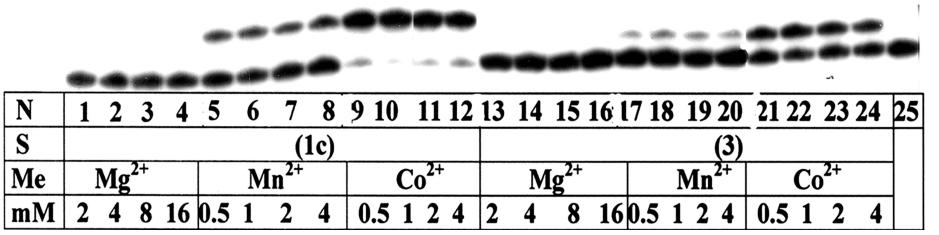

FIGURE 2 The effect of metal activators on the incorporation of alkyl triphosphates **1 c** (lanes 1–12) and **3** (lanes 13–24) into the primer 3′-ends. TdT (0.5 U) was incubated in the presence of 100 nM [5′-^{32}P]-19-mer primer and different concentrations of Mg^{2+} (lanes 1–4, 13–16), Mn^{2+} (lanes 5–8, 17–20), Co^{2+} (lanes 9–12, 21–24). Lane 25 shows the primer position. Reaction products were separated by a denaturing 20% PAAG electrophoresis followed by gel exposure with a film.

parameters for **1f** were not estimated because its incorporation was negligibly small. The band intensities within each lane were quantified with a computer densitometer. All the values were normalized to the total intensity of the corresponding band to exclude differences in the gel loading. The results are summarized in Table 2. The k_{cat} value for ddTTP was accepted as 1, and the k_{cat} values for other compounds were calculated relatively to this value. As is seen in Table 2, the *Km* and k_{cat} values of **1a–c** and ddTTP are close. The *Km* values for **1d** and **1e** were 7–10 times higher and relative k_{cat} were 4–5 times lower if compared with those for ddTTP. Thus, the efficacies of **1d** and **1e** as substrates for TdT were more than 1.5 orders of magnitude lower in respect to the potency of ddTTP. This fact implies that the linker length affects both the *Km* and k_{cat} of the reactions and has a major impact on properties of alkyl triphosphates as substrates for TdT.

Effect of Metal Ions on Incorporation of Alkyl Triphosphates into the Primer 3′-Ends

All the experiments discussed above were performed in the presence of Co^{2+} ions that were found to be an optimal metal activator for the TdT catalyzed polymerization of pyrimidine dNTP in vitro.[14] However, it is

TABLE 2 Kinetic parameters of incorporation reactions of compounds **1a–e** into the primer 3′-ends

Substrate	*Km*, μM	Relative k_{cat}	k_{cat}/Km
ddTTP	0.25 ± 0.05	1.0	4.0
1a	0.30 ± 0.08	0.85 ± 0.10	2.8
1b	0.35 ± 0.06	0.80 ± 0.11	2.3
1c	0.5 ± 0.08	0.95 ± 0.15	1.9
1d	1.85 ± 0.12	0.22 ± 0.06	0.12
1e	2.9 ± 0.3	0.18 ± 0.05	0.06

Values and experimental errors (± S.D.) are the means of three independent experiments. Experimental details are given in Material and Methods section.

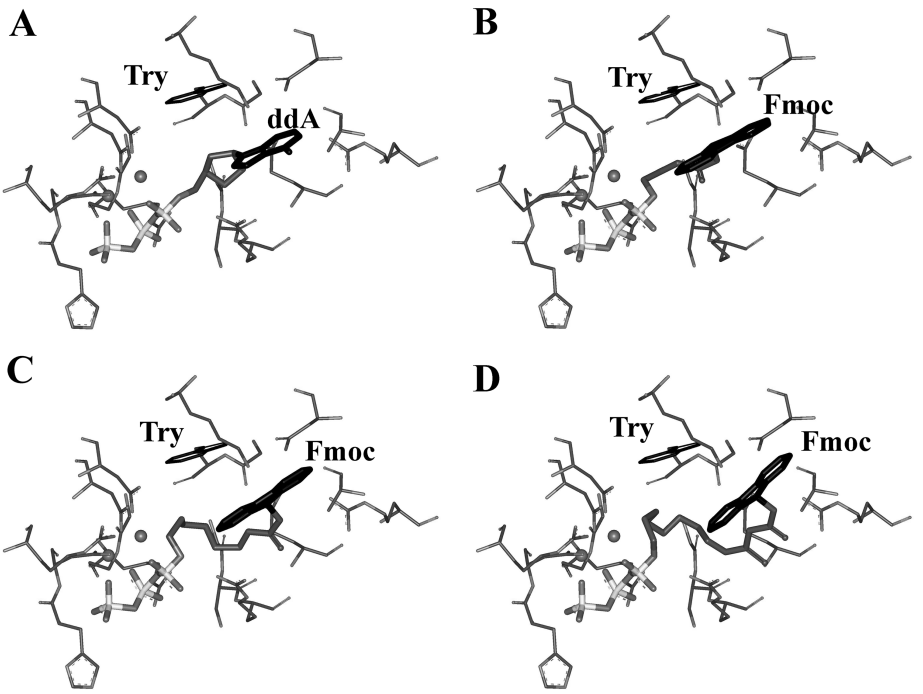

FIGURE 3 Superposition of ddATP (**A**) and alkyl triphosphates **1 a**, **1 d**, and **1f** on the TdT catalytic center using Software "WebLabViewerPro 3.7" (Accelrys, Inc., San Diego, CA, USA) Black (bold)–Fmoc-group in **1a** (B), **1d** (**C**), and **1f (D)**; gray (bold)–linkers in **1a**, **1d**, and **1f**, respectively. Grey-white–triphosphate residue. Triphosphate residues of the compounds were fixed in the same positions as that of ddATP in the crystal structure of [TdT + ddATP+Co^{2+}] complex (**A**) published earlier.[15]

known that some other metals such as Mg^{2+}, Mn^{2+}, Zn^{2+} also can support the reactions catalyzed by TdT. For elucidating the efficacy of the metal activation, we performed the elongation reactions with alkyl triphosphates 1c and 2 in the presence of Mg^{2+} (1–16 mM), Mn^{2+} and Co^{2+} (both at the concentrations of 0.5–4 mM). The pattern of elongation reactions with the compounds 1 c (lanes 1–12) and 2 (lanes 13–24) is presented in Figure 2. As is seen, Co^{2+} ions (lanes 9–12, 21–24) showed advantageous over Mn^{2+} (lanes 5–9, 17–21) while ions Mg^{2+} (lanes 1–4, 13–17) did not support the catalysis. This results are consistent with the previous data, when multiple incorporation of [^{32}P]dTMP occurred: Co^{2+} was also the superior and Mg^{2+} was the worst metal ion.[13]

A Model of the Superposition of 1 a, 1 d, and 1f on the TdT Active Site

Recently, 3-D crystal structures of the catalytic subunit of murine TdT and its two binary complexes, one with a primer and another with an incoming [ddATP+Co^{2+}] complex, were reported.[15,16] To explain the

differences in the substrate properties of **1a**, **1d**, and **1f** bearing terminal Fmoc-fragments linked to the triphosphate fragments through linkers of increasing length, the structures of these compounds were manually superimposed on the active site of [TdT+ddATP+Co^{2+}] complex using Software "WebLabViewerPro 3.7" (Figure 3). The positions of the triphosphate moieties were fixed in the same positions as ddATP in the [TdT + ddATP+Co^{2+}] complex.[16] Figure 3B shows that the position of the Fmoc-group of **1a** is similar to that of the adenine base in ddATP (Figure 3A). The aromatic ring of Trp450 in TdT is positioned in parallel to the hydrophobic Fmoc-group. When the linker becomes longer **1d**, it forms a loop which changes the position of the Fmoc-residue in respect to Trp450 (Figure 3C). Figure 3D represents the superposition of a very poor substrate of TdT **1f** on the active site of the enzyme. The loop formed by the linker is getting larger, and the position of the Fmoc-residue is no longer parallel to the Trp450 plane.

DISCUSSION

TdT is a template-independent DNA polymerase, which has been found only in lymphoid tissues and plays a role in somatic hypermutation of immunoglobulins.[7,8] Recently, 3-D structures were reported for [TdT + ddATP + Co^{2+}] and [TdT + oligonucleotide primer] complexes.[15,16] Based on these results and studies of the functional activity of mutant TdT,[17] the amino acid residues important for the dNTP binding and catalysis have been identified. Two Co^{2+} ions are coordinated by the two strictly conserved aspartate residues as well as by the phosphates of the incoming ddATP.[15] The aromatic ring of Trp450 is partially stacked with the adenine ring of the incoming nucleotide. Recently, it was shown that TdT can utilize not only dNTP analogues but also triphosphates bearing bulky alkyl groups joined to the triphosphate moiety through different linkers.[1,2,10]

Herein, we expended the repertoire of TdT substrates and synthesized novel triphosphates bearing different structures of substituents and linkers. We showed for the first time that triphosphates with small (H) and hydrophilic groups (NH_2 or OH) can be incorporated into the primer 3′-ends by TdT, although their efficacy was 1.5–2 orders of magnitude lower in comparison with triphosphates bearing hydrophobic substituents. According to these results, one can postulate that the triphosphate residue makes a major contribution to the substrate properties of the compounds toward TdT. The substrate efficacy of **3** bearing a fluorescent residue of rodamine dye only 4–5 times lower than that of **1 c**, which was one of the best substrate among the alkyl triphosphates with the same linker. This result implies that this system can be used for fluorescent labeling of the oligonucleotide 3′-ends.

The interaction of alkyl triphosphate derivatives with TdT depends on the linker length joining the substituents and triphosphate residue (Table 2). These results are consistent with the presented model of superposition of alkyl triphosphates on the TdT active site, from which it can be deduced that long linker forms a loop that obstructs the formation of the productive [TdT + alkyltriphosphate+Co^{2+}] complex due to steric hindrance. The nature of metal ions plays an important role for the catalytic activity of TdT.[14] Although the Co^{2+} -activated catalysis has been used most extensively for multiple elongation reactions in vitro, it is still unknown, which metal or metals function *in vivo*. Our data demonstrated that Co^{2+} and Mn^{2+} ions are able to support the incorporation of alkyl triphosphates into the primer 3′-ends, Co^{2+} being superior to Mn^{2+}. The Mg^{2+} ions failed to activate the reactions catalyzed by TdT with alkyl triphosphates as substrates.

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