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Structure–Functional Analysis of Interactions of Terminal Deoxynucleotidyl Transferase with New Non-Nucleoside Substrates

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Abstract—New non-nucleoside esters of phosphoric acid containing various hydrophobic groups, namely (1) *N*-(2-tripticencarbonyl)-4-aminobutyl; (2) 5-phenylsubstituted *N*-(2,4-dinitrophenyl)-4-aminobutyl; (3) *N*-(4-phenylbenzoyl)- and *N*-(4-(*N*-benzylamino)benzoyl)-2-aminoethyl groups, as well as (4) diphenylmethyl and fluorenyl groups were synthesized and studied as substrates of terminal deoxynucleotidyl transferase. With the exception of the two latter derivatives, all the analogues displayed substrate properties and could incorporate into the deoxyoligonucleotide 3'-end. As it was shown in biochemical experiments and by computer modeling, a linker joining the triphosphate and hydrophobic fragments of the molecule was necessary for these compounds to display substrate properties.

Key words: non-nucleoside substrates, substrate properties, terminal deoxynucleotidyl transferase, triphosphate derivatives.

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INTRODUCTION

Template-independent terminal deoxynucleotidyl transferase (TdT, EC 2.7.7.31) is the only DNA polymerase capable of elongating single-stranded oligonucleotides in the presence of dNTP.[2] TdT belongs to the DNA polymerase X family, which also includes eukaryotic repair DNA polymerases β, λ, μ, and σ. Unlike other template-dependent DNA polymerases of the X family, TdT can utilize substrates of a wide spectrum [1–4]. The capacity of the enzyme to utilize both α-*D*- and α-*L*-dNTP provided a suggestion that the nucleoside fragment did not play an essential role in enzyme interaction [5]. It was shown earlier that triphosphates bearing hydrophobic groups joined with triphosphate residues via linkers were effective TdT substrates. The activity of these substrates was dependent on both the substituent structure and the linker structure and length [6–8].

In this work, we describe four types of new non-nucleoside TdT substrates from calf thymus. They contain hydrophobic groups joined with triphosphate residues via linkers of different lengths and structures. As hydrophobic groups, tripticen, 5-phenyl substituted *N*-(2,4-dinitrophenyl)-4-aminobutyl derivatives, *N*-(4-(*N*-benzylamino)benzoyl)-2-aminoethyl residues, and the compounds bearing a fluorenyl fragment or a diphenylethyl residue mimicking it were used.

RESULTS AND DISCUSSION

The structural formulas of the synthesized compounds are shown in the table.

Triphosphate (**I**) is one of the tripticen derivatives that have not been studied as TdT substrates. Compounds (**IIa**) and (**IIb**) continue the series of earlier-synthesized substituted *N*-(2,4-dinitrophenyl)-4-aminobutyl esters of triphosphoric acid: *N*-(2,4-dinitro-1-fluorophenyl)-4-aminobutyl, *N*-(2,4-dinitro-5-imidazolylphenyl)-4-aminobutyl, and *N*-(2,4-dinitrophenyl)-4-aminobutyl triphosphates [7], which demonstrated substrate properties towards TdT and inhibited mutant DNA polymerase λ Y505A [8]. New (**IIa**) and (**IIb**) derivatives contain even more bulky substituents, namely benzimidazole and benzotriazole residues.

Triphosphates (**IIIa**) and (**IIIb**) are analogues of the earlier synthesized 4-(4-phenylbenzoyl)oxybutyl triphosphate, an effective TdT termination substrate [8] which could also be incorporated into the 3'-end of the oligonucleotide primer in the presence of DNA polymerase λ wild and Y505A mutant types, as well as DNA polymerase β [8]. Incorporation was most effec-

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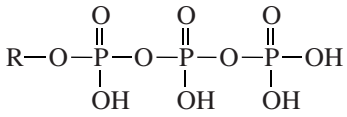
[2] Abbreviations: CDI, 1,1'-dicarbonyldiimidazole; TdT, terminal deoxynucleotidyl transferase.



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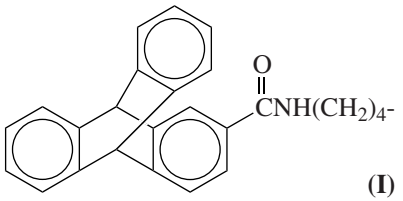
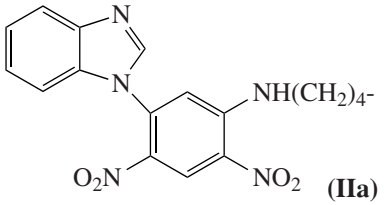
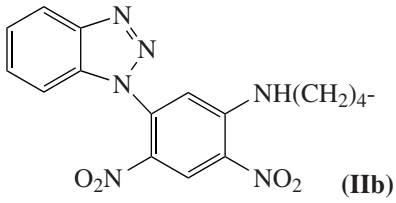


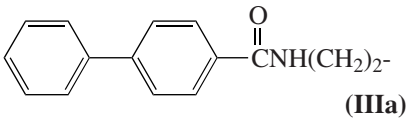
(I)



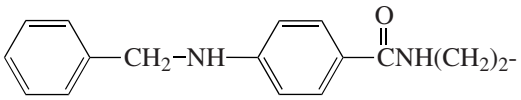
(IIa)



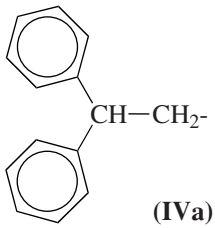
(IIb)



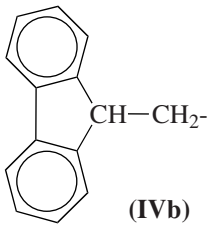
(IIIa)



(IIIb)



(IVa)



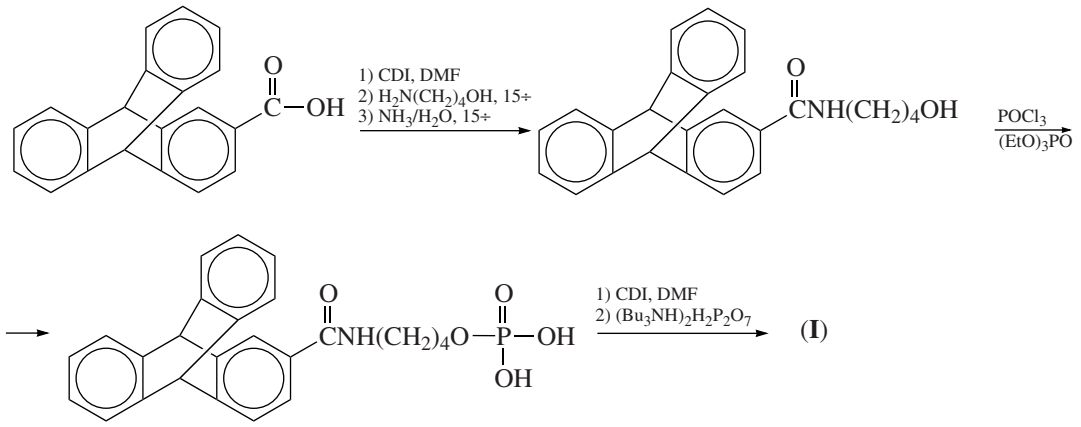
(IVb)

(I): R = *N*-(2-tripticencarbonyl)-4-aminobutyl,
(IIa): R = *N*-[(2,4-dinitro-5-benzimidazolylphenyl)]-4-aminobutyl,
(IIb): R = *N*-[(2,4-dinitro-5-benzotriazolylphenyl)]-4-aminobutyl,
(IIIa): R = *N*-(4-phenylbenzoyl)-2-aminoethyl,
(IIIb): R = *N*-(4-(*N*-benzylamino)benzoyl)-2-aminoethyl,
(IVa): R = 2,2,-diphenylethyl,
(IVb): R = 9-fluorenylmethyl.

tive against the template adenine in the presence of Mg^{2+} [8]. This is the first example of a substrate that lacked both a heterocyclic base and a carbohydrate fragment but could form a pair with the normal base.

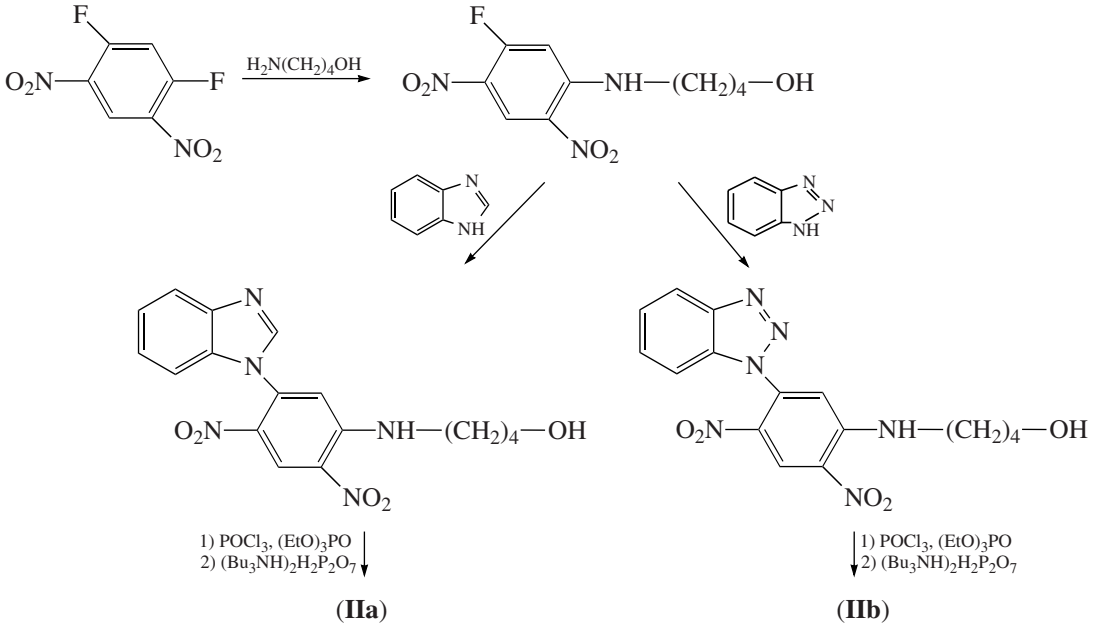
The fourth type of triphosphates, namely compounds (**IVa**) and (**IVb**), can be regarded as analogues of the earlier prepared *N*-(9-fluorenylmethoxycarbonyl)-ω-aminoalkyl, *N*-(9-fluorenylmethoxycarbonyl)-8-amino-3,6-dioxaoctyl-, and *N*-[(9-fluorenylmethoxycarbonyl)-6-aminohexanoyl]-2-aminoethyl esters [6]. All the earlier synthesized fluorenylmethoxycarbonyl-bearing triphosphates were substrates of calf thymus TdT and their substrate properties depended on the structure and length of the linker joining the 9-fluorenylmethoxycarbonyl residue with the triphosphate fragment, as well as of the nature of the metal activator [9]. Studies of new compounds (**IVa**) and (**IVb**) lacking an oxycarbonyl fragment and a linker between the aromatic and triphosphate residues enabled us to ascertain the impact of these fragments for manifestation of substrate properties towards TdT.

Triphosphate (**I**) was synthesized according to Scheme 1 using the method described in [10]. Coupling of 2-tripticencarboxylic acid activated with 1,1'-carbonyldiimidazole (CDI) with aminobutanol resulted in *N*-(2-tripticencarbonyl)-4-aminobutanol, which was then phosphorylated with $POCl_3$ in triethyl phosphate. After isolation on a DEAE cellulose column, the resulting monophosphate was transformed into the corresponding imidazolide in the presence of CDI followed by coupling with tributylammonium pyrophosphate. Triphosphate (**I**) was isolated by ion-exchange chromatography on a DEAE cellulose column in a gradient of NH_4HCO_3 and subsequent purification on a LiChroprep RP-18 column.



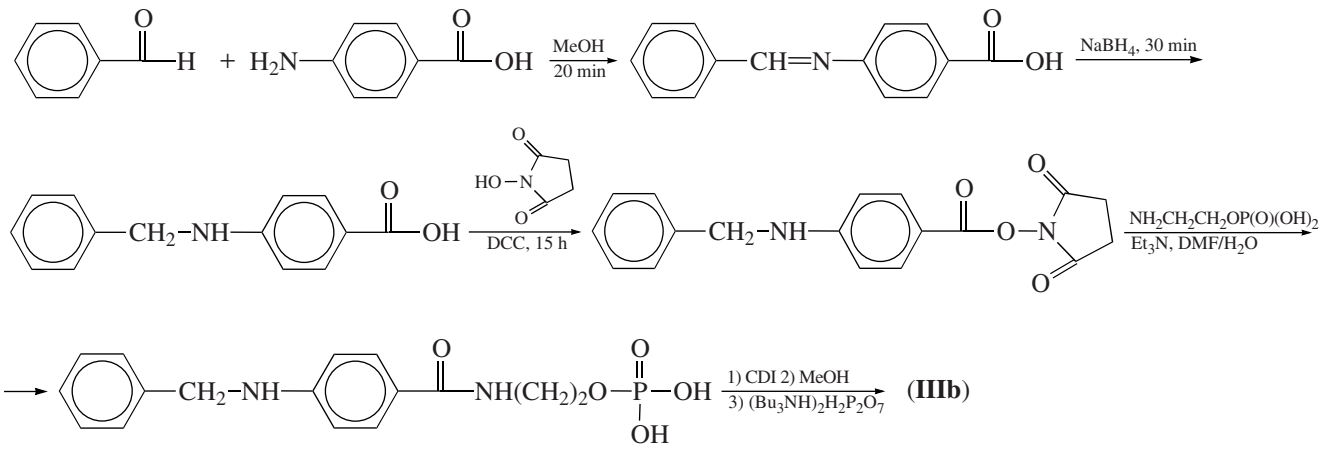
Scheme 1.

(2,4-Dinitrophenyl)-4-aminobutanol derivatives were synthesized by the reaction of aminobutanol with 1,5-difluoro-2,4-dinitrobenzene followed by treatment with a solution of the corresponding heterocycle in DMF (Scheme 2) [7]. Triphosphates (**IIa**) and (**IIb**) were obtained by phosphorylation by the Ludwig method [11] and isolated similarly to triphosphate (**I**) to give 25–30% of the product relative to the starting aminobutanol.



Scheme 2.

Triphosphates (**IIIa**) and (**IIIb**) were prepared from commercially available 4-phenylbenzoic acid for (**IIIa**) or 4-(*N*-benzylamino)benzoic acid for (**IIIb**). 4-(*N*-Benzylamino)benzoic acid was obtained by the coupling of benzaldehyde and 4-aminobenzoic acid followed by reduction with sodium borohydride (Scheme 3). The attempts to activate the arylcarboxylic acid with CDI (as in Scheme 1) failed. Therefore, we synthesized the corresponding succineimide esters which interacted with aminoethyl phosphate to give target monophosphates in high yields (75–80%). Monophosphates were activated with CDI and tributylammonium pyrophosphate was added. The resulting triphosphates (Scheme 3) were isolated and purified as described for the above compounds.



Sheme 3.

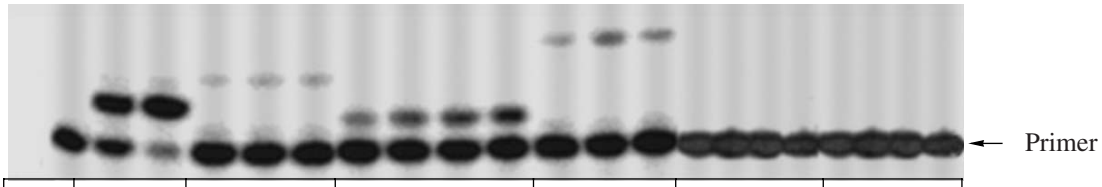
Compounds (**IVa**) and (**IVb**) were synthesized from the corresponding alcohols (diphenylmethanol for (**IVa**) and 9-fluorenylmethanol for (**IVb**)) by treatment with $POCl_3$ in triethyl phosphate followed by the addition of tributylammonium pyrophosphate similar to [6]. The target triphosphates were isolated and purified as described above.

Alkyl triphosphates (**I**)–(**IV**) were studied as TdT substrates (Fig. 1). 3'-Deoxythymidine triphosphate, an effective TdT substrate, served as a control. As is seen in the figure, the substrate efficacy of triphosphate (**IIa**) bearing a substituted 2,4-dinitrophenyl residue was lower than that of ddTTP: the 50% primer elongation indicating the reaction efficacy was not achieved at the concentration of triphosphate (**IIa**) of 10 μM, which exceeded the ddTTP concentration by more than two orders of magnitude. Similar data were obtained for triphosphate (**IIb**) (data not shown).

Compounds (**I**) and (**IIIb**) proved to be more effective TdT termination substrates, although less effective than reference ddTTP (Fig. 1). Compounds (**IVa**) and (**IVb**) did not display substrate properties towards TdT. The primer elongation was not observed even at a substrate concentration of 50 μM. We attempted to find out the reason for different substrate activities of compounds (**IIIb**), (**IVa**), and (**IVb**).

Recently, the crystal structure of the [TdT + ddATP + Co^{2+}] complex was published [12]. The main ddATP binding sites were triphosphate residue, whose position was coordinated with two Co^{2+} atoms, and the base, which interacted with the enzyme Trp450. Using the WebLabViewerPro 3.7 program (Accelrys Inc., San Diego, California, United States), we fixed the triphosphate residues of compounds (**IIIb**), (**IVa**), and (**IVb**) in the position which was occupied by this residue in ddATP within the [TdT + ddATP + Co^{2+}] complex (Fig. 2).

It is seen in Fig. 2a that the *N*-(4-(*N*-benzylamino)benzoyl)-2-aminoethyl residue of compound (**IIIb**) can also interact with Trp450, although this interaction must be weaker than that of the ddATP purine base, because only a six-membered purine fragment of (**IIIb**) could contact the Trp residue. At the same time, aromatic residues of compounds (**IVa**) and (**IVb**) (Fig. 2b, c) could not interact with the Trp450 residue (probably due to the absence of a linker between the triphosphate residue and the hydrophobic fragment of



K	(d_2T)		(**IIa**)			(**IIIb**)				(**I**)			(**IVa**)				(**IVb**)			
μM	0.1	1	1	2	10	0.4	1	2	10	1	2	10	1	2	10	50	1	2	10	50

Fig. 1. TdT-Catalyzed incorporation of triphosphate derivatives into the primer 3'-end. Compound concentrations are shown in the figure. Reaction products were analyzed as described in the Experimental section. The left lane indicates the primer position. The tracks above the primer show the primer elongation form the 3'-end after incorporation of the substrate monophosphate residue.

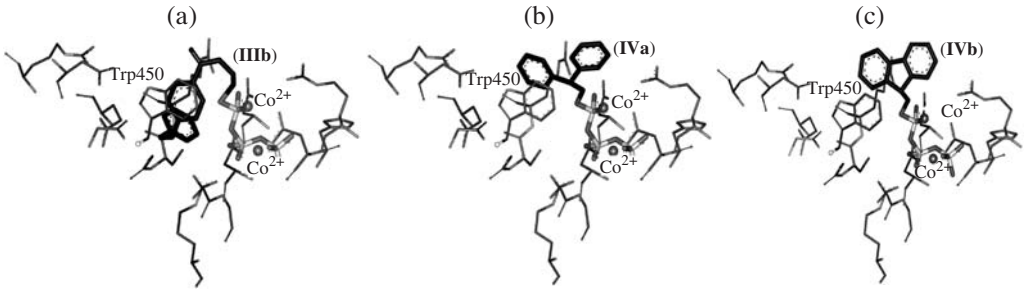


Fig. 2. Superposition of compounds (a) (**IIIa**), (b) (**IVa**), and (c) (**IVb**) in the TdT catalytic site. Substrate triphosphate residues were fixed in the position occupied by the triphosphate residue in ddATP molecule within the crystalline [TdT + ddATP + Co^{2+}] complex [12]. The compounds are in bold. The Trp450 and Co^{2+} ion positions are shown in the figure.

the molecule). This observation can explain the lack of substrate properties of these compounds.

To summarize, new nonnucleotide TdT substrates of four types, namely esters of triphosphoric acid, were synthesized. It was shown that the presence of a linker between the triphosphate residue and the aromatic part of the molecule was necessary for the manifestation of substrate properties.

EXPERIMENTAL

4-Aminobutanol, triethyl phosphate, 1,1'-carbonyldiimidazole, *N*-hydroxysuccineimide, dioxane, *N*-methylimidazole, benzaldehyde, *N*,*N*'-dicyclohexylcarbodiimide, sodium borohydride, and acetonitrile were from Fluka (Switzerland); 2-aminoethyl phosphate, phosphorus oxychloride, DMF, and 4-phenylbenzoic acid were from Aldrich (United States); tributylammonium pyrophosphate was from Sigma (United States). [γ-^{32}P]ATP (specific activity 6000 Ci/mM) was from Isotop (Russia). For enzymatic reactions, sodium cacodylate, 1,4-dithiothreitol, EDTA, acrylamide, formamide, and calf thymus TdT were from Amersham (Great Britain). The 18-mer deoxyoligonucleotide primer (5')CCGTCAATTCCTGTAGTC was obtained from Litex (Russia). Adsorption column chromatography was carried out on LiChroprep RP-18 (25–40 μm) and Kieselgel (63–100 μm) (Merck) columns. Ion-exchange column chromatography was performed on a Dowex-50 WX8 (Fluka, Switzerland) in the NH_4^+ form and a DEAE-Toyopearl (Toyosoda, Japan). TLC was performed on a Kieselgel 60 F_{254} (Merck, Germany); the eluting system was a 6 : 4 : 1 mixture of dioxane–water–25% NH_3. NMR spectra were registered on an AMX III-400 spectrometer (Bruker) with a working frequency of 400 MHz for ^{1}H NMR (chemical shifts are given in δ in ppm relative to the internal standard of sodium 3-trimethylsilyl-1-propanesulfonate (DSS); *J* are given in Hz) and 162 MHz for ^{31}P NMR (with hydrogen–phosphorus spin–spin decoupling; the external standard was 85% phosphoric acid; chemical shifts are given in δ in ppm). UV spectra were registered on a Shimadzu UV-2401PC spectrophotometer (Japan) (ε is the molar extinction coefficient).

N*-(2-Tripticencarbonyl)-4-aminobutyl triphosphate (I):** *N-(2Tripticencarbonyl)-4-aminobutanol.** Carbonyldiimidazole (123 mg, 0.75 mmol) was added to a solution of 2-tripticencarboxylic acid (149 mg, 0.5 mmol) (obtained as described in [13]) in DMF (3 ml), and the mixture was added in 10 min to a solution of 4-aminobutanol (270 mg, 3 mmol) in DMF (5 ml). The mixture was stirred for 20 h at room temperature, 24% aqueous ammonia (5 ml) was added, and the mixture was kept for 20 h. The solvents were evaporated in a vacuum, and a 1 : 1 mixture of 1 M HCl and chloroform (25 ml) was added. The aqueous phase was washed with chloroform (3 × 10 ml), and the chloroform fraction was dried with Na_2SO_4 and evaporated. The residue was dissolved in chloroform (2 ml), loaded on a silica gel column (2 ×18 cm), and eluted in a gradient of ethanol in chloroform (0 ⟶ 5%). The fractions containing the target compound were evaporated in a vacuum to give 147 mg (78%) of the product. UV (ethanol, λ_{max}, nm): 277 (ε 1570). ^{1}H NMR ($CDCl_3$): 7.38, 7.29, 6.99 (11 H, three m, aryl), 6.27 (1 H, br. s, NH), 5.45, 5.44 (2 H, two s, bridges), 3.69 (2 H, m, CH_2O), 3.43 (2 H, m, CH_2N), 1.53 (5 H, m $(CH_2)_2$ + OH).

N-(2-Tripticencarbonyl)-4-aminobutyl triphosphate (I) was obtained in a yield of 23% from *N*-(2-tripticencarbonyl)-4-aminobutanol as described in [10]. UV (H_2O, pH 6, λ_{max}, nm): 276 (ε 1450). ^{1}H NMR (D_2O): 7.62, 7.37, 6.96 (11 H, three m, aryl), 5.66, 5.64 (2 H, two s, bridges), 3.98 (2 H, m, CH_2O), 3.33 (2 H, m, CH_2N), 1.53 (4 H, m $(CH_2)_2$). ^{31}P NMR (D_2O): –10.07 (1 P, d, *J* 18.5, P^γ), –10.34 (1 P, d, *J* 20.3, P^α), –21.94 (1 P, dd, P^β).

***N*-[(2,4-Dinitro-5-benzimidazolylphenyl)]-4-aminobutyl triphosphate (IIa).** *N-[(2,4-Dinitro-5-benzimidazolylphenyl)]-4-aminobutanol.* Benzimidazole (73 mg, 0.62 mmol) and triethylamine (139 μl, 1 mmol) were added to a solution of *N*-[5-(2,4-dinitro-1-fluorophenyl)-4-aminobutanol (100 mg, 0.31 mmol) in acetonitrile (3 ml). The reaction mixture was refluxed for 2 h, evaporated, and the product was iso-

lated by chromatography on a silica gel column (2 × 20 cm) eluted with a 95 : 5 mixture of chloroforom–methanol in a yield of 70%.

N-[(2,4-Dinitro-5-benzimidazolylphenyl)]-4-aminobutyl triphosphate (IIa). Phosphorus oxychloride (12 μl, 0.13 mmol) was added to a solution of *N*-[5-(2,4-dinitro-1-benzimidazolylphenyl)-4-aminobutanol (35 mg, 0.1 mmol) in triethyl phosphate (2 ml). The reaction mixture was stirred for 3 h, and a solution of tributylammonium pyrophosphate (91 mg, 0.2 mmol) in DM (1 ml) was added. The mixture was kept for 1 h at room temperature, loaded on a Dowex-50 (NH_4^+) column (2 × 4 cm), and eluted with water. The eluate was evaporated and chromatographed on a LiChroprep RP-18 column (2 × 18 cm) eluted with water to give 30% of the product. UV (H_2O, pH 6, λ_{max}, nm): 242 (ε 6300), 365 (ε 7000). ^1H NMR (D_2O): 9.31 (1 H, s, H3), 8.48 (1 H, br. s, benzimidazole), 7.88, 7.46 and 7.42 (4 H, three m, benzimidazole), 7.31 (1 H, s, H6), 3.99 (2 H, m, CH_2O), 3.56 (2 H, t, *J* 6.8, CH_2N), 1.84–1.75 (4 H, m, $(CH_2)_2$). ^{31}P NMR (D_2O): –10.25 (1 P, d, *J* 19.3, P^γ), –10.37 (1 P, d, *J* 20.3, P^α), –22.68 (1 P, dd, P^β).

***N*-[(2,4-Dinitro-5-benzotriazolylphenyl)]-4-aminobutyl triphosphate (IIb)** was obtained in a yield of 25% similar to triphosphate (**IIa**) using benzotriazole. UV (H_2O, pH 6, λ_{max}, nm): 255 (ε 7000), 369 (ε 8000). ^1H NMR (D_2O): 9.31 (1 H, s, H3), 8.18, 7.72 and 7.61 (4 H, three m, *benzo*triazole), 7.41 (1 H, s, H6), 4.00 (2 H, m, CH_2O), 3.56 (2 H, t, *J* 6.8, CH_2N), 1.85–1.75 (4 H, m, $(CH_2)_2$). ^{31}P NMR (D_2O): –9.13 (1 P, d, *J* 19.3, P^γ), –10.12 (1 P, d, *J* 20.3, P^α), –21.77 (1P, dd, P^β).

***N*-(4-Phenylbenzoyl)-2-aminoethyl triphosphate (IIIa).** *N-Hydroxysuccineimide ester of p-phenylbenzoic acid.* Dicyclohexylcarbodiimide (227 mg, 1.1 mmol) was added to a solution of *p*-phenylbenzoic acid (198 mg, 1 mmol) and *N*-hydroxysuccineimide (127 mg, 1 mmol) in absolute dioxane (5 ml) and the mixture was stirred for 17 h at 20°C. The reaction mixture was filtered and the filtrate was evaporated. The residue was washed with petroleum, dissolved in a 2 : 1 ethyl acetate–hexane mixture, and loaded onto a silica gel column (2 × 18 cm). The mixture was eluted in a gradient of ethyl acetate in hexane (30 ⟶ 80%) and the target fractions were evaporated in a vacuum to give 215 mg (73%) of the product. ^1H MMR ($CDCl_3$): 7.95, 7.66, 7.42 (9 H, three m, aryl), 2.83 (4 H, m, $(CH_2)_2$).

N-(4-Phenylbenzoyl)-2-aminoethyl phosphate. A solution of *N*-hydroxysuccineimide ester of 4-phenylbenzoic acid (148 mg, 0.5 mmol) in DMF (6 ml), triethylamine (210 μl, 1.5 mmol), and methylimidazole (47 μl, 0.55 mmol) were added to a solution of aminoethyl phosphate (72 mg, 0.5 mmol) in water (2 ml), and the reaction mixture was stirred for 3 h at room temperature. Organic solvents were evaporated in a vacuum, the residue was dissolved in water (4 ml), and loaded onto a reverse-phase LiChroprep RP-18 column (2 × 18 cm) eluted in a linear gradient of ethanol in 0.01 M NH_4HCO_3 (0 ⟶ 20%). The target fractions were evaporated, dissolved in water (3 ml), and lyophilized to give 41 mg (23%) of the product. UV (H_2O, pH, 6, λ_{max}, nm): 274 (ε 24000). ^1H NMR (D_2O): 7.72, 7.53, 7.44 (9 H, three m, aryl), 4.02 (2 H, m, CH_2O), 3.68 ((2 H, m, CH_2N). ^{31}P NMR (D_2O): 1.73.

N-(4-Phenylbenzoyl)-2-aminoethyl triphosphate (IIIa) was obtained from the corresponding phosphate by the method described in [10] in a yield of 23% (8.5 mg). UV (H_2O, pH 6, λ_{max}, nm): 274 (ε 24000); ^1H NMR (D_2O): 7.73, 7.54 and 7.45 (9 H, three m, aryl), 4.12 (2 H, m, CH_2O), 3.78(2 H, m, CH_2N). ^{31}P NMR (D_2O): –7.77 (1 P, d, *J* 17.5, P^γ), –10.35 (1 P, d, *J* 19.3, P^α), –21.79 (1 P, dd, P^β).

***N*-(4-(*N*-Benzylamino)benzoyl)-2-aminoethyl triphosphate (IIIb) (Scheme 3).** *4-(N-benzylimino)benzoic acid.* Benzaldehyde (886 μl, 8 mmol) was added to a solution of 4-aminobenzoic acid (548 mg, 4 mmol) in ethanol (3 ml) and the mixture was kept for 20 h at +4°C. The residue was filtered and washed with cold methanol to give 540 mg (60%) of the product. UV (H_2O, pH 6, λ_{max}): 254 (ε 22700). ^1H NMR (DMSO-d_6): 12.22 (1 H, COOH), 7.98, 7.65, 7.33, 6.55 (9 H, four m, aryl), 5.81 (1 H, c, CH=N).

4-(*N*-Benzylamino)benzoic acid. Sodium borohydride (152 mg, 4 mmol) was added to a solution of 4-(*N*-benzylimino)benzoic acid (540 mg, 4 mmol) in ethanol (10 ml) under vigorous stirring. The reaction mixture was stirred for 1 h at room temperature and the solvents were evaporated in a vacuum. A 1 : 1 water–chloroform mixture was added to the residue, the aqueous phase was washed with chloroform (3 × 10 ml), acidified with 1 M HCl to pH 1.5, and the target acid was extracted with chloroform (3 × 10 ml). The extracts were dried with Na_2SO_4 and evaporated to give 452 mg (83%) of the product. UV (H_2O, pH 6, λ_{max}, nm): 286 (ε 12100). ^1H NMR (DMSO-d_6): 11.9 (1 H, COOH), 7.65, 7.30, 6.98, and 6.59 (9 H, four m, aryl), 4.34 (2 H, m, CH_2-N).

N-Hydroxysuccineimide ester of 4-(N-benzylamino)benzoic acid. *N*-Hydroxysuccineimide (253 mg, 2.2 mmol) was added to a solution of 4-benzylaminobenzoic acid (452 mg, 2 mmol) in dioxane (10 ml). After it was dissolved, DCC (474 mg, 2.3 mmol) was added and the reaction mixture was stirred for 20 h at room temperature. Ethyl acetate (5 ml) was added and the mixture was kept for 1 h at room temperature. The precipitate was filtered and the solvents were evaporated in a vacuum. The residue was dissolved in a 2 : 1 ethyl acetate–hexane mixture and loaded onto a silica gel column (2 × 18 cm). The product was eluted in a gradient of ethyl acetate concentration in hexane (30 ⟶ 80%). The target fractions were evaporated in a vacuum to give 505 mg (78%). UV (EtOH, pH 6, λ_{max}, nm): 289 (ε 12200). ^1H NMR ($CDCl_3$): 7.89, 7.35, 7.27, 6.59 (9H, four m, aryl), 4.39 (2 H, m, CH_2-N), 2.83 (4 H, m, $(CH_2)_2$).

***N*-(4-(*N*-Benzylamino)benzoyl)-2-aminoethyl phosphate.** A solution of *N*-hydroxysuccineimide ester of 4-(*N*-benzylamino)benzoic acid (162 mg, 0.5 mmol) in DMF (4 ml), triethylamine (210 μl, 1.5 mmol), and methylimidazole (47 μl, 0.55 mmol) were added to a solution of aminoethyl phosphate (212 mg, 1.5 mmol) in water (1 ml) and the reaction mixture was stirred for 3 h at room temperature. Organic solvents were evaporated in a vacuum, and the residue was dissolved in water (2 ml) and centrifuged. The solution was evaporated up to a volume of 2 ml, loaded on a reverse-phase LiChroprep RP-18 column (2 × 18 cm), and eluted with 0.01 M NH_4HCO_3. The target fractions were evaporated, and the residue was dissolved in water (3 ml) and lyophilized to give 45 mg (23%) of the product. UV (H_2O, pH 6, λ_{max}, nm): 290 (ε 11500). ^{1}H NMR (D_2O): 7.65, 7.43, 7.37 and 6.83 (9 H, four m, aryl), 4.43 (2 H, m, CH_2-aryl), 4.13 (2 H, m, CH_2O), 3.64 (2 H, m, CH_2N). ^{31}P NMR (D_2O): –1.3.

2,2-Diphenylethyl triphosphate (IVa) was obtained by treatment of diphenylmethanol with $POCl_3$ in triethyl phosphate followed by the addition of tributylammonium pyrophosphate similar to [6] to give 16% of the product. UV (H_2O, pH 6, λ_{max}, nm): 258 (ε 540).^{1}H NMR (D_2O): 7.39 (8H, m, Ph), 7.27 (2H, m, Ph), 4.55 (2 H, m, CH_2O), 3.56 (1 H, t, *J* 6.6, $CHCH_2$). ^{31}P NMR (D_2O): –9.25 (1 P, d, *J* 19.3, P^{γ}), –10.69 (1 P, d, *J* 20.3, P^{α}), –22.43 (1 P, dd, P^{β}).

9-Fluorenylmethyl phosphate (IVb) was obtained from 9-fluorenylmethanol using the procedure described in [6] to give 19% of the product. UV (H_2O, pH 6, λ_{max}, nm): 265 (ε 17000). ^{1}H NMR (D_2O): 7.82, 7.74 (4 H, two d, *J* 7.5, (Ar)), 7.34–7.43 (4 H, m, (Ar)), 4.26 (2 H, m, CH_2O), 4.20 (1 H, t, *J* 7.0, $C\underline{H}CH_2$). ^{31}P NMR (D_2O): –5.69 (1 p, d, *J* 19.1, P^{γ}), –10.45 (1 P, d, *J* 19.1, P^{α}), –21.41 (1 P, t, P^{β}).

Substrate properties. Primer **[5'-^{32}P]p**CCGTCAATTCCTGTAGTC was obtained by the procedure described in [5]. The reaction mixture contained a 0.02 μM primer, 0.2 U TdT, 100 mM sodium cacodylate (pH 7.2), 2 mM $CoCl_2$, 0.1 M dithiothreitol, and substrates at varied concentrations as shown in Fig. 1. The reaction mixture was incubated for 10 min at 37°C and terminated by the addition of formamide containing 0.5 mM EDTA and 0.1% phenol blue and xylenecyanol (50% vol of the reaction mixture volume). The products were separated by electrophoresis in 20% denatured PAG. The gels were exposed on a Kodak RX roentgen film (United States).

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