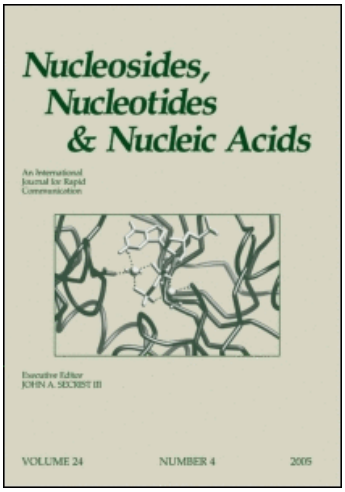

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Synthesis and Substrate Properties of Thymidine 5'-Triphosphate Analogs with Large Hydrophobic Substituent Groups At α-P Atom

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SYNTHESIS AND SUBSTRATE PROPERTIES OF THYMIDINE 5'-TRIPHOSPHATE ANALOGS WITH LARGE HYDROPHOBIC SUBSTITUENT GROUPS AT α-P ATOM

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Abstract: The new thymidine 5'-O-triphosphate analogs - thymidine 5'-O-α-phenylphosphonyl-β,γ-diphosphate **4**, 3'-fluoro-3'-deoxythymidine 5'-O-α-phenylphosphonyl-β,γ-diphosphate **5** and thymidine 5'-O-α-decylphosphonyl-β,γ-diphosphate **6** were synthesized. Their substrate properties towards avian myeloblastosis virus and human immunodeficience virus reverse transcriptases, DNA polymerase β from rat liver and terminal deoxynucleotidyltransferase from calf thymus were demonstrated. The mixture of Rp and Sp diastereomers of **4** was separated by reversed phase HPLC, diastereomeric selectivity of the reaction of DNA chain elongation was noted.

Modified nucleoside 5'-triphosphates are extensively used for studying the mechanism of DNA polymerases functioning. Usually the modification of the dNTP molecule involves a substitution of a native group by an isosteric or/and isoelectronic one to minimize the distortion of the natural dNTP structure. Another approach is the introduction of a bulky substituent into the molecule. Specific information about the structure of an enzyme active center may be obtained only in the case if this modified triphosphate binds to DNA polymerase. Such binding can result in a productive complex formation followed by single or multiple incorporation of the substrate analog into the growing DNA chain. Non-productive complex formation can lead to competitive inhibition of DNA biosynthesis.

Substrate properties of the dNTP analogs with large substituents in the carbohydrate and heterocyclic parts of the molecule are widely investigated[1] Here we describe the synthesis of dNTP analogs with large hydrophobic

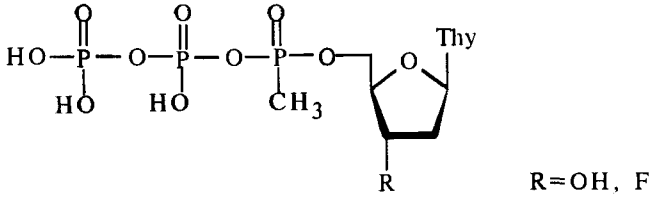

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Figure 1

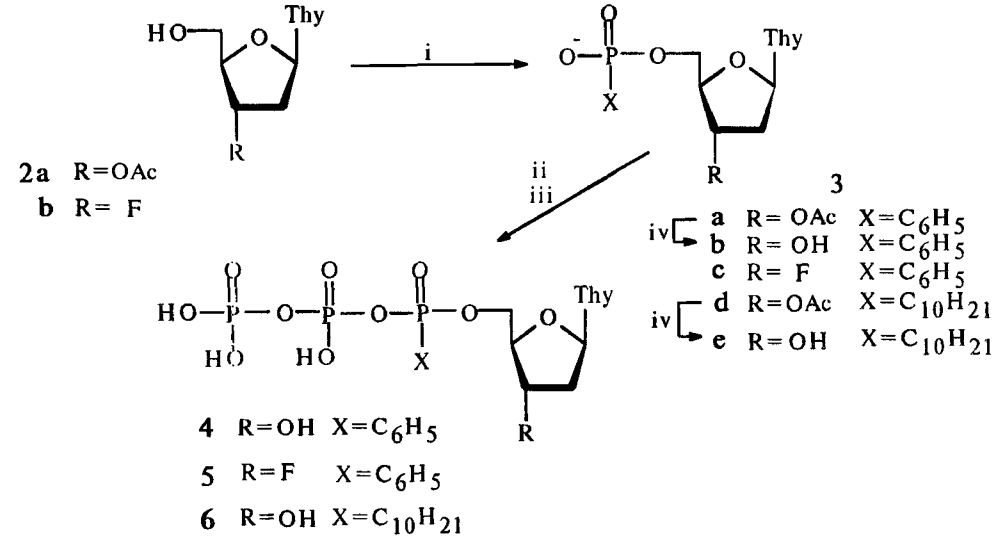
substituents at α-P atom and evaluation of their substrate properties towards DNA polymerases of different origin.

It has been shown earlier that α-P methylated TTP analogs (**1**) (Fig. 1) are substrates for terminal deoxynucleotidyl transferase (TdT)[2], human immunodeficiency virus (HIV) and avian myeloblastosis virus (AMV) reverse transcriptases as well as for DNA polymerase β from rat liver[3]. It seemed interesting to find out whether these enzymes could recognize the TTP analogs with large substituents at α-P atom. As a bulky substituent at α–P atom we have chosen a phenyl or decyl group. Besides, the introduction of a large substituent could enable the separation of a mixture of R- and S-diastereomers. We failed to separate such a mixture in the case of **1**[4].

Previously the stereoselectivity of DNA-polymerases has been shown when pure stereoisomeric nucleoside α-thiotriphosphates were used as substrates[5]. Higuchi and co-authors demonstrated the stereoselectivity of TdT towards **1**(R=OH). After the hydrolytic degradation of the methylated DNA chain by nuclease P1 and alkaline phosphatase only stereoisomerically pure di- and trinucleotides were isolated[2]. We aimed to investigate the stereoselectivity of different DNA polymerases in a direct way.

Enzymatic incorporation of large hydrophobic thymidilic residues into a DNA chain could permit to get pure stereoisomers of P-modified DNA fragments[6] unavailable by chemical synthesis and obtain DNA chains bearing reporter groups linked to the phosphorus atom.

The synthesis was carried out according to the Scheme 1 starting from 3'-acetylated thymidine (**2a**) or 3'-fluorothymidine (**2b**) as described for **1**[3]. The reaction of phenyl and decyl phosphonic acids with nucleosides **2a** and **2b** in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) resulted in



i) Phenyl or decyl phosphonic acid, TPSCl, Pyr; ii) CDI, DMF; iii) bis-tri-n-butylammonium salt of pyrophosphoric acid, DMF; iv) NH_4OH

Scheme 1

phosphonates **3a,c,d**. Ammonolysis of phosphonates **3a,d** led to phosphonates **3b,e**, respectively. The latter compounds as well as **3c** were activated with N,N-carbonyldiimidazole (CDI) and intermediate nucleotide imidazolides were condensed with pyrophosphate[2,3]. This resulted in the formation of triphosphates **4-6**. Partial degradation of triphosphate derivatives **4-6** took place in the process of ion exchange chromatography on DEAE-Toyopearl. To accelerate the elution we used a gradient of TEAB buffer in 10% methyl alcohol. Fractions containing **4-6** were frozen immediately and lyophylized. Final purification of **4-6** was carried out by the low pressure reversed phase chromatography on LiChroprep RP-8 in 5% methanol in water.

The structure of triphosphates **4-6**, obtained as a mixture of R- and S-diastereomers, was confirmed by ^1H and ^{31}P NMR spectra and FAB mass spectra. The obtained triphosphate solutions were concentrated by lyophylisation up to 10 mM and used as stock solutions in biochemical experiments. However, after storage for a week at -20°C we observed about 10% admixtures of corresponding phosphonates **3** in each stock solution (control by ion exchange and reversed phase HPLC). The main criteria of

substrate purity for DNA polymerase catalyzed reactions is the absence of triphosphate by-products. Therefore small amounts of monophosphate derivatives formed under the storage of the solutions could not significantly influence the DNA biosynthesis.

The obtained mixture of diastereomers **4** was separated by preparative RP HPLC in 0.05 M KH_2PO_4 buffer to prevent the degradation of triphosphates. In TEAB buffer fast pyrophosphate elimination with phosphonate **3b** formation was observed. The HPLC profiles before and after preparative HPLC separation are presented in Fig. 2. Appropriate fractions were lyophylized and desalted by low pressure RP chromatography in 5% methanol in water.

In ^1H NMR spectra of **4a** and **4b** the upfield shift of H-6, CH_3 and H-1' signals as compared to corresponding signals of phosphonate **3b** appeared. In the case of **4a** this difference was more pronounced. For example, the signal of CH_3 -group of **4a** was shifted at 0.19 ppm while for **4b** only at 0.03 ppm. The chirality of **4a** and **4b** was not assigned.

All the synthesized compounds **4-6** were evaluated in a cell-free system with several DNA polymerases (Table 1).

One can see that compounds **4-6** up to 1mM concentration are not substrates for DNA polymerases α, KF, CMV and HSV. While the isomer **4b** showed no substrate properties also towards the other enzymes studied, **4a**, **5**, **6** demonstrated such activities toward DNA polymerase β, TdT, and reverse transcriptases of HIV and AMV , all of which incorporated one residue of **5** or several residues in the case of **4a** and **6** into a growing DNA chain. Fig. 3 presents a pattern of primer elongation by **4a** catalyzed by AMV RT. In the system containing the template-primer complex and the enzyme **4a** elongates the primer by one unit (lanes 1-4, band 15T'), the efficiency of incorporation being proportional to the reaction duration. The mobility of the resulting pentadecanucleotide differs from that observed in the control assay (lane 7) due to the hydrophobic phenyl residue. The slight band at the level of control pentadecanucleotide (15T) is due to the presence of TTP trace amounts in the enzyme preparation. In control assays with primer-template complex and enzyme and without exogenous dNTP a faint band at the level of 15T oligonucleotide but not at the level of 15T' oligonucleotide was observed (data are not shown). After addition of dGTP to the system containing primer-template complex, **4a** and AMV RT one can see the elongation of 15T' band by one

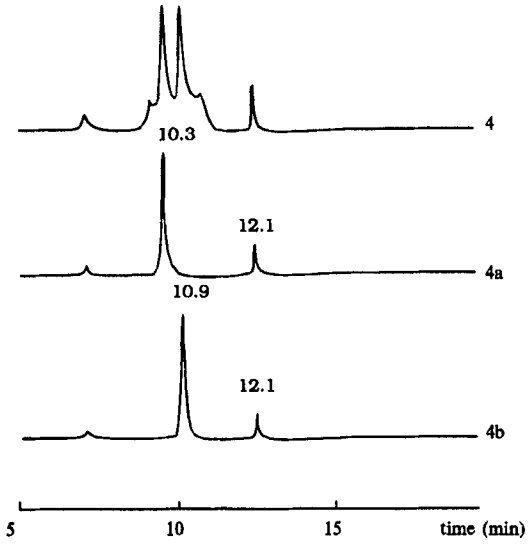


Figure 2. HPLC analysis of diastereomeric mixture of triphosphate **4** and pure isomers **4a** and **4b** after the preparative RP HPLC. 12.1 min - the retention time of phosphonate **3b**. The conditions as for preparative separation: SilaSorb RP-18 (10x250 mm, 12 μ) column, flow rate -5 ml/min, linear gradient of acetonitrile in 0.05 M KH_2PO_4 (0->35%, 25 min).

```
          10        20        30        40
3'-GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGA
5'-CCCAGTCACGACGT-->  the direction of elongation
```

Scheme 2. The primer-template complex

Table 1. Substrate properties of compounds **4-6** towards DNA polymerases.

Sub-strate	DNA polymerases*						Reverse transcriptases	
	α	β	CMV	HSV	TdT	KF	HIV	AMV
4a	-	+	-	-	+	-	+	+
4b	-	-	-	-	-	-	-	-
5	-	+	-	-	+	-	+	+
6	-	+	-	-	+	-	+	+

* α from human placenta; β from rat liver; TdT from calf thymus; human cytomegalovirus (CMV); herpessimplex virus type 1 (HSV); *E. coli* DNA polymerase I Klenow fragment (KF).

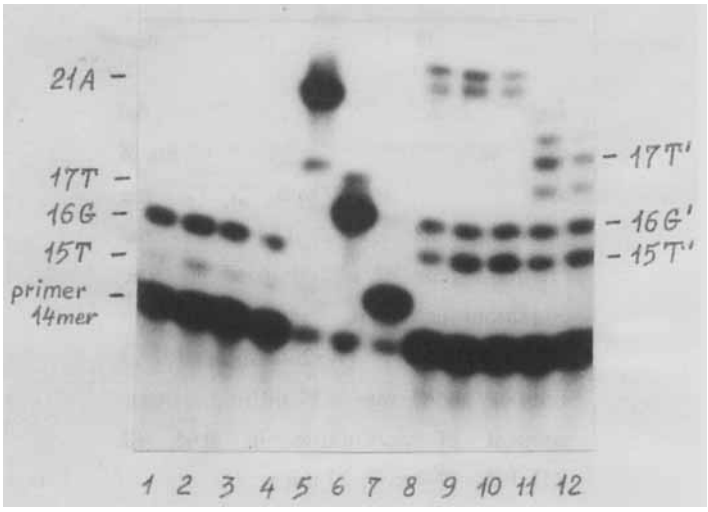


Figure 3. Electrophoregram in 20% PAAG of the reaction products of complex [template-[5'-^{32}P]-labeled primer+AMV reverse transcriptase] with **4a** (200 μM). Lanes 1-4 - incubation for 10 min (4), 20 min (3), 40 min (2) and 60 min (1);lane 5 - complex+TTP+dGTP+ dATP, 3 μM of each; lane 6 - complex+TTP+GTP, 3 μM of each ; lane 7 - complex+TTP, 3 μM, incubation time 10 min . Lanes 8-10 - complex+dGTP+dATP (3 μM of each) + **4a** (200 μM); incubation time 20 min (10), 40 min (9) and 60 min (8); lanes 11-12 - complex +3 μM dGTP+**4a** (200 μM), incubation time 20 min (11) and 40 min (12). The length of natural oligonucleotides are shown on the left, oligonucleotides containing **4a** are on the right and marked with "'".

nucleotide (16G' band) and following incorporation the next residue from **4a** (17T' band) according to the template context (lanes 11, 12). The position of 17T' band corresponds to the mobility of oligonucleotide with two hydrophobic residues. The faint band located between 16G' and 17T' can be explained probably by extension of 16G'-oligonucleotide by TTP residue. In the system containing template primer complex, enzyme, **4a**, dGTP and dATP one can see further elongation of 17T' oligonucleotide by dATP residues according to template context (lanes 8-10). The incorporation of two **4a** residues in 17T' oligonucleotide is also confirmed by time dependence of the reaction. Thus, the incorporation of **4a** residue is observed twice and the DNA chain is elongated after each incorporation. Control band is the 21-membered oligonucleotide (lane 5).

The properties of triphosphates **4a-6** in the cell-free system with TdT are demonstrated in Fig.4. The pattern A presents the results of the reaction of tetradecanucleotide with **4a-6** catalyzed by TdT. The pattern B - the same reaction with the subsequent chain elongation by dTTP. Triphosphate **4a** elongates the primer by one unit (lanes 2-5, A). The increase of the concentration of **4a** up to 1000 μM results in the inhibition of DNA synthesis (lanes 4-5, A). Such inhibition at high concentrations of **4a** is observed in the experiments with subsequent chain elongation (lanes 4-5, B). The compound **5** is incorporated into the primer once (lanes 6-9, A). No further synthesis takes place in the presence of dTTP (lanes 6-9, B). The properties of **6** are similar to those of **4a** (lanes 10-12, A and B). Its inhibition effect is stronger as compared to **4a** being total at 1000 μM (lanes 13, A and B).

Table 2 presents ratios of concentrations (moles) of compounds **4a-6** to those of the natural TTP whereby DNA synthesis is inhibited by 50%. The ratios have been obtained from the curves of their inhibitory effect on the DNA (-) chain synthesis catalyzed by different enzymes. It can be seen that **4a** has the lowest inhibitory activities, whereas those of **6** are the highest and its inhibitory effect towards HIV reverse transcriptase is the most powerful.

In conclusion we can state that:

- AMV and HIV reverse transcriptases, DNA polymerase β, and TdT are able to incorporate the thymidilic residue with large hydrophobic substituents at α-P atom into the 3'-end of the DNA chain and further elongation can be observed if residue has 3'-OH group;
- only one diastereoisomer of triphosphate **4** is a substrate of the enzymes mentioned above.

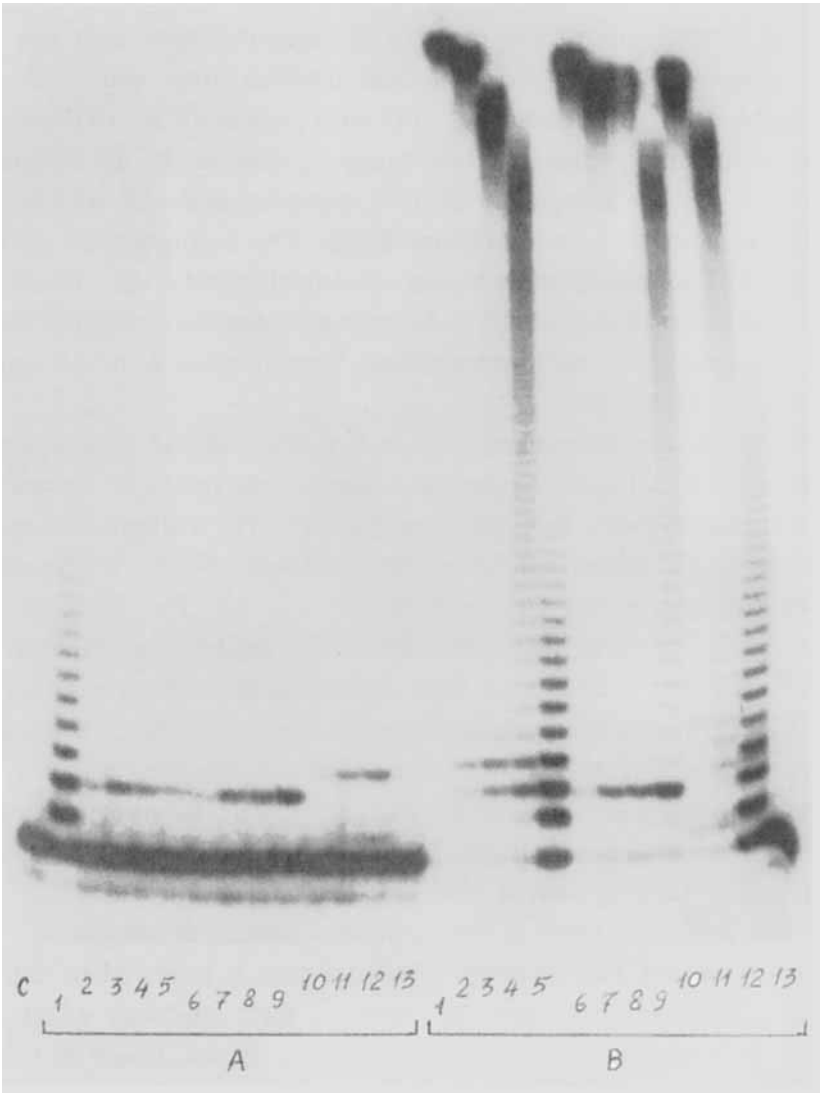


Figure 4. Electrophoregram of the extension of the [5'-^{32}P]-labeled primer by TdT. A - the reaction during 30 min at 37^{0}C, B - as in A, then 600 μM dTTP was added and the incubation was continued for 30 min. C - control (primer+enzyme); 1 - C+10 μM dTTP; 2-5 - C+**4a** at the concentrations 10 μM (2), 100 μM (3), 200 μM (4) 1000 μM (5); 6-9 - C+**5** at the concentrations 10 μM (6), 100 μM (7), 200 μM (8), 1000 μM (9); 10-13 - C+**6** at the concentrations 10 μM (10), 100 μM (11), 200 μM (12), 1000 μM (13).

Table 2. Inhibitor : substrate concentration ratios whereby DNA synthesis is inhibited by 50%

Compound	Reverse transcriptases		DNA polymerases	
	AMV	HIV	I (Klenow fragment)	α from human placenta
4a	215	43	280	8.3
5	15	10	60	8.3
6	25	2	14.5	4.1

Experimental Section

TLC was performed on Kieselgel 60 F_{254} (Merck) in 2-propanol: NH_4OH:water 7:1:2 (v/v) (A) or dioxane-NH_4OH-water 6:1:4 (v/v) (B). HPLC was carried out on the columns Spherisorb C-18, 8μk, 4x150 mm and Hypersil APS, 5μk, 4.6x250 mm at the flow rate 0.8 ml/min. Elution was done, respectively, with a linear gradient of CH_3CN in 0.1 M TEAB, pH 7.0 from 0 to 30% during 30 min (C), and with a linear gradient of KH_2PO_4 pH 5.5 from 0.05 to 1.0 M during 25 min and 1.0 M during 5 min (D).

UV spectra were registered on Specord M40 in water. For all compounds λ_{max} was 267 nm. For column chromatography 650 M DEAE Toyopearl from Toyosoda (Japan) and LiChroprep RP-8 (25-40μ) from Merck were used. ^{1}H-NMR spectra were registered on Bruker 250 spectrometer in D_2O with *t*-BuOH as an internal standard. ^{31}P-NMR spectra were recorded with ^{1}H-decoupling at 101.26 MHz on Bruker 250 spectrometer in D_2O with 85% H_3PO_4 as an external standard. FAB mass spectra were determined with Kratos MS 50TC mass spectrometer. The yields were estimated spectrophotometrically using molar extinction coefficients (ε) of the parent nucleosides.

M13mp 10 phage DNA was isolated from the culture fluid of a recipient K12XL1 *E.coli* strain[7] . The primer tetradecadeoxynucleotide (see Scheme 2) was labelled at the 5'-position by T4 polynucleotide kinase (Amersham, England)[7] using [γ -^{32}P]ATP with a specific activity of 1500 Ci/mmol (Radioisotop, Russia). After phenol extraction and reprecipitation with ethanol the [^{32}P]-tetradecanucleotide was dissolved in water up to the

concentration of 1 nmol/ml and used either directly in the reaction with TdT (Amersham)[8] or after primer-template complex purification on BioGel A-1.5M in reactions catalyzed by KF (Amersham), reverse transcriptases AMV (Omutninsk Chemical Plant, Russia), HIV-1 (a kind gift of Dr. T.Rosovskaya), DNA-polymerases α from human placenta[9], β from rat liver[10], HSV[11], CMV[11].

A general procedure for synthesis of 5'-phosphonates 3 b,c,e

The nucleosides **2a,b** (0.3 mmol) were dissolved in dry pyridine (5 ml) and the solution of tri-n-butylammonium salt of corresponding phenyl- or decylphosphonic acid (0.5 mmol) in DMF (1 ml) and then TPSCl (240 mg, 0.8 mmol) were added. The reaction mixture was allowed to stand at room temperature till the starting nucleoside disappeared (TLC control in $CHCl_3$:MeOH 9:1, v/v). Water was added (30 ml) and the mixture was stirred for 15 min and filtered. The filtrate was diluted with water (300 ml) and put onto a DEAE Toyopearl (HCO_3^-) column (2x20 cm). The column was washed with 300 ml of water and then with a linear gradient of ammonium bicarbonate buffer (0-->0.2 M, pH 7.5), total volume 600 ml. The product was eluated at ~0.15M NH_4HCO_3. The appropriate fractions were combined, evaporated to dryness and coevaporated with water (3x15 ml). In the case of phosphonates **3a,d** the residue was dissolved in NH_4OH (10 ml), stored overnight at room temperature and evaporated to give phosphonates **3b,e**. The phosphonates **3b,c,e** were applied onto a LiChroprep RP-18 column (2.5x15 cm). Elution with 12% methanol in water followed by freeze drying yielded nucleotides **3b,c,e** as NH_4^+ salts with average yield 65%.

Thymidine 5'-O-phenylphosphonate 3b: Rf 0.63(A), retention time 20 min (C) and 16 min (D). MS: m/z 382(M^+), 399(M^++NH_3). ^{1}H-NMR δ, ppm: 7.73-7.53m (5H, C_6H_5), 7.51d (1H, H-6), 6.27t (1H, H-1', $J_{1',2'a}=J_{1',2'b}=$ 6.2 Hz), 4.60-4.40m (2H, H-3', H-4'), 4.10-4.00m (2H, H-5'a,b), 2.50-2.39m (2H, H-2'a,b), 1.78d (3H, CH_3). ^{31}P-NMR δ, ppm: 15.59s.

3'-Fluoro-3'-deoxythymidine 5'-O-phenylphosphonate 3c: Rf 0.67 (A), retention time 27 min (C) and 5 min (D). MS: m/z 384 (M^+), 401 (M^++NH_3). ^{31}P-NMR δ, ppm: 15.41s.

Thymidine 5'-O-decylphosphonate 3e: Rf 0.72 (A), retention time 23 min (C) and 15 min (D). MS: m/z 446 (M^+), 463 (M^++NH_3). ^{31}P-NMR δ, ppm: 29.55s.

A general procedure for synthesis of triphosphate analogs 4,5,6

Phosphonate **3b**, **3c** or **3e** (0.2 mmol) was dissolved in 5 ml of dry pyridine and N,N'-carbonyldiimidazole (162 mg, 1 mmol) was added. The mixture was allowed to stay at room temperature for 3 h followed by addition of 2 ml of 1 M bis-(tri-n-butylammonium) pyrophosphate (2 mmol) in DMF (2 ml). The mixture was stirred for 1.5 h and then diluted with water to the total volume 150 ml. The solution was loaded onto the DEAE Toyopearl (HCO_3^-) column (2x20 cm). The column was washed with water up to the absence of optical density in the eluate, the elution was carried out with a linear gradient of NH_4HCO_3, pH 7.5 (0->0.4 M in 10% methanol, total volume 600 ml). Starting nucleotide **3b**, **3c** or **3e** was eluted with 0.05 M buffer while **4,5** or **6** with 0.3 M buffer. Corresponding fractions were freeze dried and repurified by reversed phase chromatography in 5% methanol in water. The average yield was 20%.

3'-Fluoro-3'-deoxythymidine 5'-O-α-phenylphosphonyl-β,γ-diphosphate ***5*** (R and S mixture): Rf 0.35(B), retention time 12.0 min and 12.5 min (C), 25 min (D). MS: m/z 544 (M^+), 561 (M^++NH_3). ^{31}P-NMR δ, ppm: 11.23d and 10.93d (α–P, $J_{\alpha,\beta}$=23.9 Hz, $J_{\alpha,\beta}$=23.5 Hz), -10.18bs, (γ-P), -23.65m (β–P).

Thymidine 5'-O-α-decylphosphonyl-β,γ-diphosphate ***6*** (R and S mixture): Rf 0.43(B), retention time 13.8 min and 15.1 min (C), 17.2 min (D). MS: m/z 606 (M^+), 623 (M^++NH_3). ^{31}P-NMR δ, ppm: 27.80d and 27.40d (α–P, $J_{\alpha,\beta}$=25.0 Hz, $J_{\alpha,\beta}$=25.5 Hz), -13.06m (γ-P), -26.03m (β–P).

Thymidine 5'-O-α-phenylphosphonyl-β,γ-diphosphate ***4a*** *and* ***4b.*** The mixture of R and S isomers was separated by preparative RP HPLC on the SilaSorb RP-18 column (10x250 mm, 12 μ) at flow rate 5 ml/min of linear gradient of acetonitrile in 0.05 M KH_2PO_4 (0->35%, 25 min). Triphosphates **4a** and **4b** were desalted by low pressure reversed phase chromatography as mentioned above. ***4a:*** Rf 0.32(B), retention time 12.0 min (C) and 19.0 min (D). MS: m/z 542(M^+), 559(M^++NH_3). ^{1}H-NMR δ, ppm: 7.90-7.52m (5H, C_6H_5), 7.35d (1H, H-6), 6.16t (1H, H-1', $J_{1',2'a}$=$J_{1',2'b}$=6.3 Hz), 4.60-3.95m (4H, H-3', H-4', H-5'a,b), 2.58-2.32m (2H, H-2'a,b), 1.59d (3H, CH_3). ^{31}P-NMR δ, ppm: 12.75d (α–P, $J_{\alpha,\beta}$=24.1 Hz), -9.5br s (γ-P), -22.5m (β–P).

4b: Rf 0.32(B), retention time 12.4 min (C) and 21.0 min (D). MS: m/z 542(M^+), 559(M^++NH_3). ^{1}H-NMR δ, ppm: 7.92-7.53m (5H, C_6H_5), 7.42d (1H, H-6), 6.22t (1H, H-1', $J_{1',2'a}$=$J_{1',2'b}$=6.5 Hz), 4.70-4.00m (4H, H-3', H-

4', H-5'a,b), 2.48-2.32m (2H, H-2'a,b), 1.75d (3H, CH_3). ^{31}P-NMR δ, ppm: 12.70d (α–P, $J_{\alpha,\beta}$=22.7 Hz), -8.5br s (γ-P), -22.2m (β–P).

Extention of the [5'-^{32}P] oligonucleotide catalyzed by TdT

A mixture (volume 5μl) containing 100 mM sodium cacodylate pH 7.2, 0.6 μM [5'-^{32}P]d(CCCAGTCACGACGT), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiotreitol, 3 activity units of the enzyme and dTTP, **4a**, 5 or **6** was incubated 40 min at 37° C. The reaction was stopped by the addition of 2 μl of deionized formamide containing 0.1% of bromophenol blue, 0.1% of xylenecyanol and 20 mM EDTA, pH 8.0. The products were analyzed by electrophoresis in denaturating 20% PAAG.

The template dependent elongation of the [5'-^{32}P]-oligonucleotide catalyzed by DNA polymerases

The M13mp10 DNA (0.5 μM) was hybridized with 0.75 μM [5'-^{32}P] primer in the following buffers: 10 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 1 mM dithiotreitol (*A*) - for KF, as *A* +40 mM KCl, pH 8.2 - for RT, 10 mM Tris-HCl pH 7.4, 6 mM $MgCl_2$, 0.4 mM dithiotreitol (*C*) - for DNA polymerase α; as *C*, pH 8.5 - for DNA polymerase β, 20 mM HEPES pH7.5, 60 mM $(NH_4)_2SO_4$, 6 mM $MgCl_2$, 1 mM dithiotreitol, BSA (*D*) - for HSV; as *D*, but 100 mM KCl instead of $(NH_4)_2SO_4$ - for CMV. The reaction of a substrate **4a**, **5** or **6** with 0.01 μM primer-template complex was carried out in a mixture (volume 6 μl) of the appropriate buffer solution, enzyme (0.5 activity unit of KF or β; 1 activity unit of α, HSV or CMV; 4 activity units of RT) and the corresponding dNTPs. After incubation for 20 min at 37°C (10 min at 20°C for KF, 10 min at 37°C for HSV and CMV) the reaction was stopped by adding of 3 μl of formamide containing dyes and EDTA. The products were separated by electrophoresis in the denaturating 20% PAAG.

Inhibition of DNA synthesis by 4a, 5, 6

Reaction mixture (6 μl) contained DNA polymerases in appropriated buffer, 0.02 μM primer-template complex, 20 μM dATP and dGTP, 10 μM dCTP (1-2 μCi [α–^{32}P]dCTP), 3 μM dTTP and **4a**, **5** or **6** in different concentrations. The reactions proceeded for 15 min at 20°C (0.5 activity unit of KF); 15 min at 40°C (4 activity units of reverse transcriptases) or 1 h at 37°C (1 activity unit of DNA polimerase α). Then EDTA was added up to 50 mM. The aliquots were placed on 0.5 x 1 cm strips of Whatman DE-81, the

strips were washed with 0.2 M NaCl containing 0.5 mM EDTA, pH 8, fixed with ethanol and the incorporation of the radiolabelled substrates was measured by liquid scintillation counting.

Acknowledgement

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