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Isosteric Triphosphonate Analogues of dNTP: Synthesis and Substrate Properties toward Various DNA Polymerases

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Abstract—Isosteric triphosphonate derivatives of 2',3'-dideoxy-2',3'-didehydroadenosine and 3'-deoxy-2',3'-didehydrothymidine and their β,γ-substituted analogues were synthesized. Their substrate properties toward a number of reverse transcriptases of the human immunodeficiency and avian myeloblastosis viruses, human DNA polymerases α and β, and the Klenow fragment of *Escherichia coli* DNA polymerase I were studied.

Key words: isosteric analogues of dNTP, substrate properties; human DNA polymerases; HIV and avian myeloblastosis reverse transcriptases; Klenow fragment

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INTRODUCTION

The development of novel efficient antiretroviral agents is an important and urgent problem, which has been the subject of intensive investigations over the last 25 years.[2] Many research groups attempted to synthesize various modified nucleosides and study their antiviral properties. A rather great number of compounds of nucleoside nature that exhibit the anti-HIV activity are now known [1]. It was found that a modified nucleoside has to go through two phosphorylation stages inside the cell to form the corresponding nucleoside-5'-triphosphate to be incorporated into the growing DNA chain, [2]. Therefore, in the development of new antiviral agents, a great attention is given to the study of both the pathways of metabolism of modified nucleosides and the substrate specificity of the corresponding nucleoside-5'-triphosphates toward different DNA polymerases [3].

It is known that dNTPs hardly penetrate through cell membranes and are rapidly dephosphorylated in intercellular and intracellular media. Therefore, one of possible ways to create new antiviral preparations is the synthesis of compounds modified at the triphosphate residue that are resistant to enzymatic dephosphorylation and simultaneously are the selective substrates for retroviral reverse transcriptases [4].

It has earlier been shown that the isosteric phosphonate analogues of nucleotides (**Ia**) and (**Ib**) have an antiviral activity [5]. The synthesis and some properties of isosteric analogues of nucleoside triphosphates containing a cyclopentenyl residue instead of glycone and a triphosphonate residue instead of triphosphate was described [6]. It was found that these compounds are highly stable in human blood serum and some of them are selective terminating inhibitors of DNA synthesis catalyzed by HIV RT.

Here we describe the synthesis of isosteric analogues of nucleoside 5'-triphosphates (**IIa**)–(**IIf**) and their substrate properties in DNA elongation reactions catalyzed by HIV RT and BM RT, human DNA polymerases α and β, and the Klenow fragment of *Escherichia coli* DNA polymerase I.

RESULTS AND DISCUSSION

We carried out the synthesis of compounds (**IIa**)–(**IIf**) and (**IIIa**)–(**IIIc**) according to the scheme shown below. The oxidation of 2'-deoxynucleosides (**IVa**) and (**IVb**) to their 5'-carboxy derivatives (**Va**) and (**Vb**) is the key reaction in the subsequent multistage synthesis.

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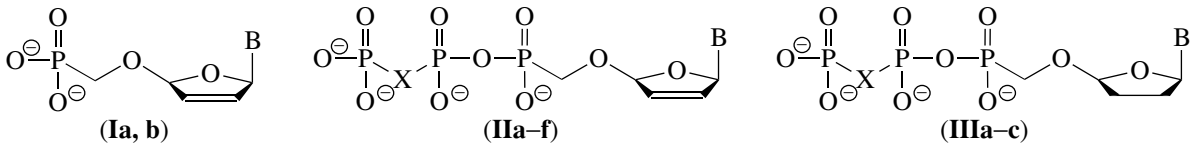
[2] Abbreviations: AMV RT and HIV RT, reverse transcription nucleases of human immunodeficiency virus and avian myeloblastosis virus; CDI, 1,1'-carbonyldiimidazole; DTT, dithiothreitol; ddNTP, 2',3'-dideoxynucleoside-5'-triphosphate; d_4NTP, 2',3-dideoxy-2',3'-didehydronucleoside-5'-triphosphate; KF, Klenow fragment; Piv, pivaloyl; and TCA, trichloroacetic acid.



(**Ia, b**) (**IIa–f**) (**IIIa–c**)

B = Ade, Thy; X = O, CF_2, CBr_2 B = Thy; X = O, CCl_2, NH

It is known that the oxidation of nucleosides proceeds in higher yields after the preliminary protection of 3'-hydroxy group [7, 8]. At the same time, a reproduction of these methods usually requires to oxidize the nucleoside in several stages, involving the protection of amino group (for 2'-deoxyadenosine), 5'-OH group, and 3'-OH group and removal of the protecting group from the 5'-OH group. As a result, the total yield does not exceed 30–40%. We tried four different methods for the oxidation of unprotected 2'-deoxynucleoside: by a solution of potassium permanganate in alkaline medium [9], by oxygen with a platinum catalyst in an aqueous suspension [10], by sodium periodate in the presence of ruthenium chloride [11], and by chromium trioxide in pyridine [12]. The method of oxidation by a potassium permanganate solution was chosen for obtaining (**Va**), because it provides a stable yield of 25–30%, and the isolation and purification of the product proceed much easier than by other schemes. The purity of the resulting (**Va**) was confirmed by UV and ^{1}H NMR. The UV spectrum of (**Va**) had a maximum at 260 nm, which corresponds to the λ_{max} of adenine, and the ^{1}H NMR spectrum showed a signal typical of the proton of carboxyl group and a simplified signal of proton at C4' (doublet) as compared with the signal of proton at C3' and contained no signal of 5-CH_2.

The decarboxylation of (**Va**) with the formation of derivative (**VIa**) proceeded in a high yield (more than 60%) only using freshly distilled *N,N*-dimethylformamide dineopentylacetal; in other cases, the yields were much lower.

Compound (**VII**) was synthesized by the method proposed in [13], with the only difference that the solvent was dichloroethane instead of pyridine, and the base was triethylamine. This enabled to achieve 85% yield of (**VII**), i.e., to increase it almost twofold.

In the synthesis of (**VIIIa**) and (**VIIIb**) we used diethyl(hydroxymethyl)phosphonate as a phosphonate component instead of dimethyl(hydroxymethyl)phosphonate used in [13]; however, this substitution did not substantially affect the final yield of the target compounds.

The elimination of HI from (**VIIIa**) and (**VIIIb**) by the action of a base in THF solution led to (**IXa**) and (**IXb**) (yields were about 95%). The ^{1}H NMR spectrum contains signals corresponding to the protons at the C3'–C4' double bond.

We did not reveal any changes in ^{31}P NMR spectra of (**IXa**) and (**IXb**) compared with the ^{31}P NMR spectra of compounds (**VIIIa**) and (**VIIIb**), respectively.

Compounds (**Ia**) and (**Ib**) were synthesized by the successive removal of protecting groups without isolation of intermediates.

Compound (**IVb**) was oxidized by pyridinium chromate to acid (**Vb**) in a high yield by the procedure described in [14]. In this case, the use of DMF as a solvent instead of pyridine reduced the reaction time from 22 to 3–5 h and increased its yield. For purification of acid (**Vb**), the excess of unreacted chromium oxide was reduced by β-mercaptoethanol. The subsequent treatment with disubstituted ammonium phosphate and routine chromatographic purification led to (**Vb**) in 35% yield.

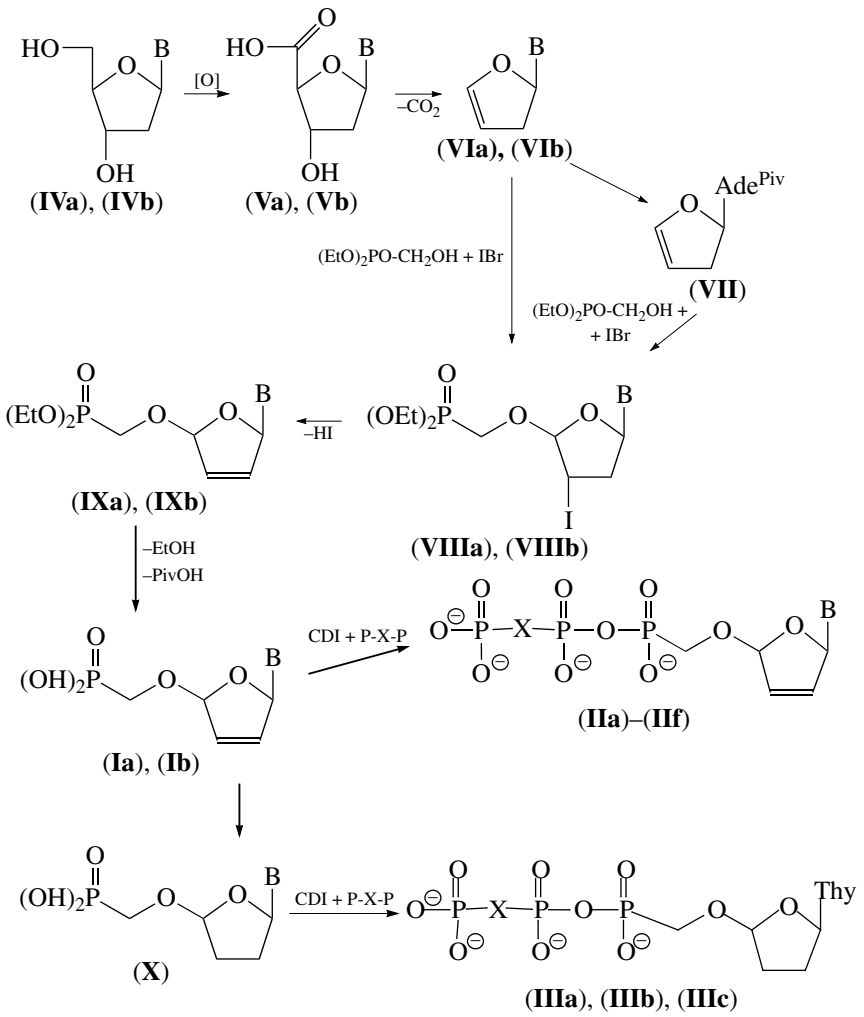
Compounds (**VIb**) and then (**VIIIb**) were obtained similarly to (**VIa**) and (**VIIIa**), but in higher yields. However, we had to change the scheme of synthesis of (**Ib**). The formation of (**IXb**) from (**VIIIb**) proceeded rather smoothly; however, the removal of the protecting ethyl groups from the 2',3'-dideoxy-2',3'-didehydro derivative (**IXb**) did not lead to (**Ib**). The major product of this reaction invariably was thymine, which accumulated rapidly as the phosphonate group was deblocked. Therefore, for obtaining (**Ib**), we first deblocked phosphonate and then, without isolating the intermediate, split off HI under conditions similar to those I the synthesis of (**IXa**). As a result of this modification, the yield of (**Ib**) was substantially higher than that of (**Ia**) (70%).

Derivatives (**IIa**)–(**IIf**) were obtained by the condensation of the tributylammonium salt of pyrophosphate (or its corresponding analogue) either with (**Ia**) or (**Ib**) activated by CDI as described in [15]. The yields of the final products (**IIa**)–(**IIf**) varied between 35 and 65%. Dideoxythymidine derivatives (**IIIa**)–(**IIIc**) were obtained by the method proposed in [16].

Substrate Properties

Complex DNA–14A:
3'-CATTTTGCTGCCGGTCACGGTTCGAACCCGACGTCCAGCTG…
5'[^{32}P]-GTAAAACGACGGCC

Complex DNA–14T:
3'-GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGA…
5'[^{32}P]-CCCAGTCACGACGT



Compound	B	X	Compound	B
(**Ia**)	Ade	–	(**IVa**)	Ade
(**Ib**)	Thy	–	(**IVb**)	Thy
(**IIa**)	Ade	O	(**Va**)	Ade
(**IIb**)	Ade	CF_2	(**Vb**)	Thy
(**IIc**)	Ade	CBr_2	(**VIa**)	Ade
(**IId**)	Thy	O	(**VIb**)	Thy
(**IIe**)	Thy	CCl_2	(**VII**)	^{Piv}Ade
(**IIf**)	Thy	NH	(**VIIIa**)	Ade
(**IIIa**)	Thy	O	(**VIIIb**)	Thy
(**IIIb**)	Thy	CCl_2	(**IXa**)	^{Piv}Ade
(**IIIc**)	Thy	NH	(**IXb**)	Thy
			(**X**)	Thy

Scheme of synthesis of compounds (**I**)–(**X**).

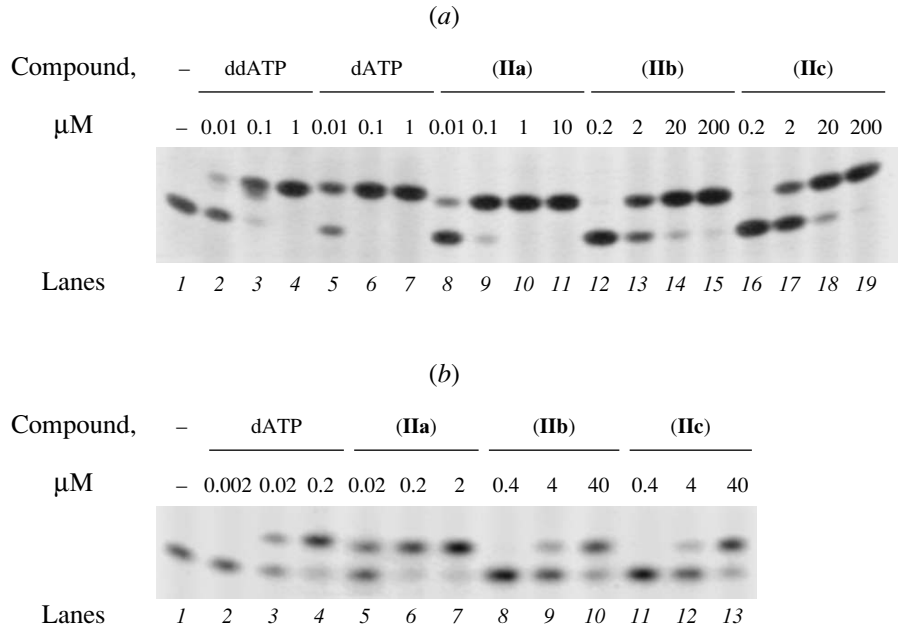


Fig. 1. Electrophoregrams of the reaction products of primer elongation in a DNA–14A complex by (**IIa**), (**IIb**), and (**IIc**): (*a*) catalysis by HIV RT and (*b*) catalysis by BMV RT. Control (lanes *1*) was primer–template complex + enzyme. For comparison, dATP and ddATP were used as substrates.

In the first series of experiments with the use of a DNA–14A primer–template complex, we studied the ability of the isosteric ddATP analogues (**IIa**)–(**IIc**) to elongate the primer by one nucleotide residue and thereby exhibit the properties of terminating substrates for both HIV RT and AMV RT and human DNA polymerases α and β. The results of testing the substrate properties of these compounds toward HIV RT and AMV RT are given in Figs. 1*a* and 1*b*, respectively. Control substrates were dATP (Fig. 1*a*, lanes *5–7*, and Fig. 1*b*, lanes *2–4*) and ddATP (Fig. 1*a*, lanes *2–4*). One can see that HIV RT and AMV RT catalyze the incorporation of (**IIa**) into DNA (Fig. 1*a*, lanes *8–11*; and Fig. 1*b*, lanes *5–7*) in the same efficiency as the incorporation of dATP and ddATP. Compounds (**IIb**) and (**IIc**) (Fig. 1*a*, lanes *12–15* and *16–19*; and Fig. 1*b*, lanes *8–10* and *11–13*, respectively) were incorporated into DNA much less efficiently. The HIV RT–catalyzed elongation of primer 14A by one nucleotide residue occurs with the same efficiency both in the presence of 0.1 μM dATP, ddATP and (**IIa**) and in the presence of 20 μM (**IIb**) and (**IIc**); the BMV RT–catalyzed elongation of primer occurs with an equal efficiency both in the presence of 0.2 μM dATP and (**IIa**) and in the presence of 40 μM (**IIb**) and (**IIc**), respectively.

A comparison of the kinetic parameters of a single-substrate elongation of the primer for compounds (**IIa**)–(**IIc**) shows that the modifications in the β,γ-pyrophosphate moiety of the molecule reduces the affinity of substrate to HIV RT 30–50 times (Table 1). At the same time, the rate of incorporation of the modified compounds (**IIa**)–(**IIc**) into DNA by this enzyme is rather high and close to the rate of ddATP incorporation.

The reparative human DNA polymerase β was also capable of utilizing the nucleoside triphosphate analogues (**IIa**)–(**IIc**) as terminating substrates for DNA synthesis, although with a very low efficiency (Fig. 2). Compound (**IIa**) was incorporated into the primer approximately 20 times less efficiently than dATP (lanes *6* and *2*, respectively). Compounds (**IIb**) and (**IIc**) were even less efficient substrates. The compounds tested in this series of experiments can be arranged in the order of decreasing substrate properties toward DNA polymerase β as follows: dATP (5 μM) > (**IIa**) (100) μM > (**IIb**) (1 mM) ⩾ (**IIc**) (1 mM) (in parentheses, the concentrations of compounds are given at which a 50% elongation under these conditions occurs). It should be noted that the carbocyclic isosteric analogues of d_4ATP (both without additional modifications of the phosphonate residue and with β,γ-CF_2- and β,γ-CBr_2-substituents) exhibited no substrate properties toward human DNA polymerase β [17].

We found that compounds (**IIa**)–(**IIe**) synthesized in this study exhibit no substrate properties in the reactions catalyzed by human DNA polymerase α; they are not incorporated into DNA (data not shown). It has previously been reported that d_4NTP s are not substrates for calf thymus DNA polymerase α [18]. Thus, the introduction of an isosteric modification (–C-5'–O–) and additional modifications of the β,γ-phosphate moiety of the molecule did not lead to the appearance in the compounds of substrate properties toward this enzyme.

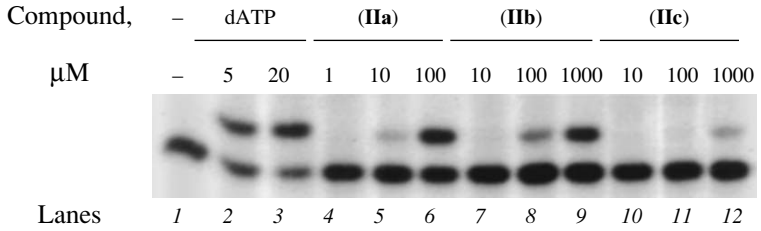


Fig. 2. Electrophoregram of the products of the DNA polymerase β–catalyzed primer elongation in a DNA–14A complex by (**IIa**), (**IIb**), and (**IIc**). Control (lane *1*) was primer–template complex + enzyme.

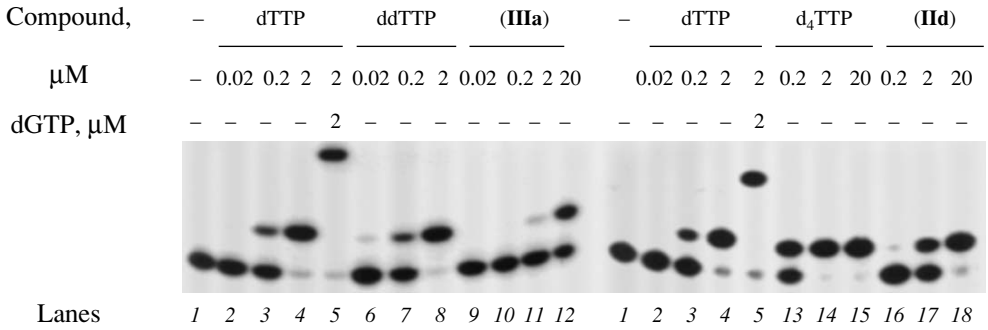


Fig. 3. Electrophoregram of the reaction products of HIV RT–catalyzed primer elongation in the DNA–14T complex by (**IIIa**) and (**IId**). Control (lanes *1*) was primer–template complex + enzyme. For comparison, dTTP, ddTTP, and d_4TTP were used as substrates.

We studied the substrate properties of thymidine derivatives (**IId**)–(**IIf**) and (**IIIa**)–(**IIIc**) toward HIV RT and KF of DNA polymerase I using a DNA–14T primer–template complex in the second series of experiments. The isosteric ddTTP analogue (**IIIa**) was nearly 20 times less efficient terminating substrate for HIV RT than ddTTP (Fig. 3, lanes *9–12* and *6–8*, respectively), and the isosteric d_4TTP analogue (**IId**) was by one order of magnitude less efficient than d_4TTP (Fig. 3, lanes *16–18* and *13–15*, respectively).

The modification of the β,γ-phosphate moiety of the isosteric d_4TTP analogues markedly reduced the ability of (**IIe**) and (**IIf**) to incorporate into the DNA chain of HIV RT (Fig. 4*a*). For example, (**IId**) (lanes 5–7) incorporated into the primer by one order of magnitude less efficiently than dTTP (lanes *2–4*) but was an about 400 times more efficient terminating substrate than (**IIf**) (lanes *8–11*). Compound (**IIe**) (lanes *12–15*) practically did not exhibit any substrate properties within the concentration range studied.

Somewhat different results for these compounds were obtained in single-substrate reactions catalyzed by the KF of DNA polymerase I (Fig. 4*b*). Compound (**IId**) (lanes *5–7*) was poorly recognized by the enzyme than dTTP (lanes *2–4*) but 20 and 200 times better than (**IIf**) (lanes *8–11*) and (**IIe**) (lanes *12–15*).

It was difficult to determine the K_m values for (**IIe**) and (**IIf**) because of their very low substrate activity. Therefore, for a quantitative determination of the properties of (**IId**)–(**IIf**) as terminating substrates for HIV RT, we compared their ability to inhibit the incorporation of [^{3}H]dTTP into poly(rA)-oligo(dT), catalyzed by this enzyme. The results presented in Table 2 enable one to compare the molar concentrations of compounds (**IId**)–(**IIf**) at which the incorporation of the labeled substrate into oligo(dT) is inhibited by 50%. It is seen that additional modifications in the pyrophosphate moiety of (**IId**) led to an almost complete loss of substrate properties by (**IIe**) and (**IIf**).

Reverse transcriptases of retroviruses are usually least sensitive to the modifications of sugar and phosphate residues of dNTP. In our case, for the ddTTP ana-

Table 1. Michaelis constants (K_m) and the rates (V_{max}/V_{max}^{ddATP}) of HIV RT-catalyzed elongation of primer 14A by one nucleotide residue for ddATP and isosteric analogues (**IIa**), (**IIb**), and (**IIc**)

Compound	K_m, μM	V_{max}/V_{max}^{ddATP}
ddATP (control)	0.095 ± 0.025	1
(**IIa**)	0.185 ± 0.045	6.2
(**IIb**)	6.37 ± 0.89	1.32
(**IIc**)	6.44 ± 1.45	1.41

logue (**IIIa**), substituting the isosteric phosphonate, a diphosphate residue, for the phosphate residue led to a more than tenfold decrease in its substrate activity compared with dTTP and ddTTP (see Fig. 3). Previously it has been shown that the isosteric carbocyclic analogues of d_4ATP and d_4GTP are effective terminating substrates for HIV RT, and their β,γ-CF_2-substituted derivatives under the same conditions are ~100 times worse recognized by the enzyme [19]. At the same time, β,γ-CBr_2-substituted analogues practically completely lost the substrate properties in reactions catalyzed by this enzyme [17, 19]. In our case, the β,γ-substituted analogues of d_4ATP were found to be substrates for both HIV RT and BMV RT. Compounds (**IIb**) (β,γ-CF_2) and (**IIc**) (β,γ-CBr_2) showed almost the same substrate properties toward HIV RT but were by two orders of magnitude less active than the unsubstituted compound (**IIa**) (see Fig. 1 and Table 1).

Thus, it can be concluded that the previously detected anti-HIV activity of (**Ia**) and (**Ib**) is due to the ability of HIV RT to selectively recognize their diphosphate derivatives as substrates. After the recognition stage, these derivatives terminate the synthesis of viral DNA and HIV RT, having only a negligible effect on other DNA polymerases. At the same time, additional modifications of compounds (**II**) at the β,γ-phosphate residues of molecules sharply reduce their substrate properties toward all DNA polymerases tested.

Table 2. Inhibition of the HIV RT-catalyzed synthesis of poly(dT) by modified analogues of thymidine triphosphates (**IId**), (**IIe**), and (**IIf**)

Compound	A*
d_4TTP (control)	0.15
(**IId**)	1.5
(**IIe**)	>1000
(**IIf**)	100

Note: * A, the ratio of molar concentrations [modified nucleotide]/[dTTP] at which the DNA synthesis is inhibited by 50%.

EXPERIMENTAL

Thin layer chromatography was carried out on Kieselgel-60 plates (Merck, Germany) using 9 : 1 chloroform–ethanol (system A) and on Silufol plates (Kavalier, Czech Republic) in 6 : 3 : 4 dioxane–ammonia–water (system A).

Column chromatography was carried out using Kieselgel 60 (60–100 μm) (Merck, Germany), Dowex 50-8 (Sigma, United States), Toyopearl 650M DEAE (Toyo Soda, Japan), and DEAE cellulose (Reanal, Hungary). For HPLC, a Gilson chromatograph (France) and a Silasorb-Sph C-18 column (5 μm, 4 × 150 mm) (Elsiko, Russia) were used. UV spectra were measured on a Specord M40 spectrophotometer (Carl Zeiss Jena, Ger-

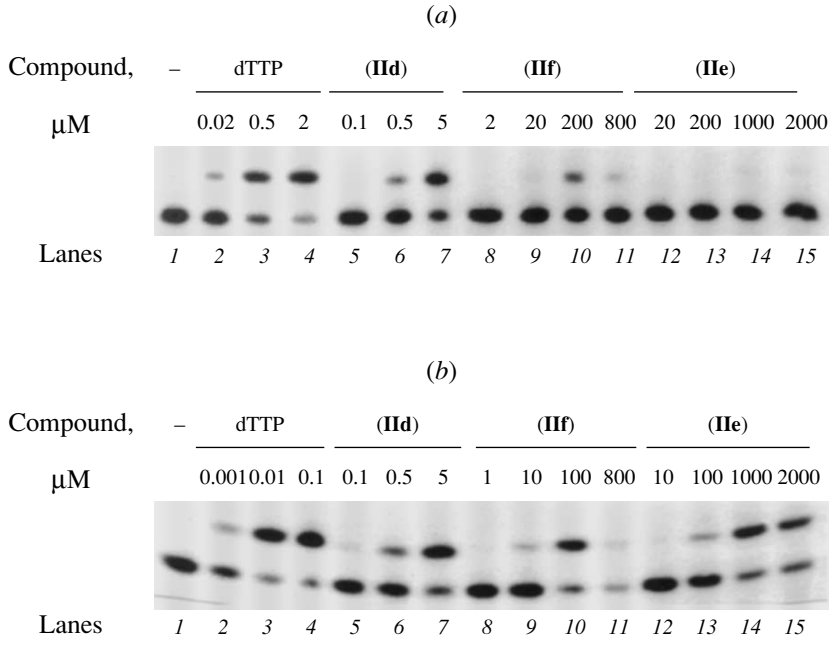


Fig. 4. Electrophoregrams of the reaction products of primer elongation in a DNA–14T complex by (**IId**), (**IIf**), and (**IIe**): catalysis by (*a*) HIV RT and (*b*) KF of *E. coli* DNA polymerase I. Control (lanes *1*) was primer–template complex + enzyme. For comparison, dTTP was used as a substrate.

many): in methanol for nucleosides and in water for nucleotides, unless otherwise indicated.

^{1}H- and ^{31}P NMR spectra were recorded on a Bruker 250 spectrometer (Bruker, Germany) at a working frequency of 101.3 MHz (δ, ppm, spin–spin coupling constants, Hz) (reference 85% H_3PO_4).

The following compounds were used: Bu_3N, CDI, phosphorus trichloride, 4-(*N*,*N* dimethylamino)pyridine (Fluka, Germany); 2'-deoxyadenosine and dineopentylacetal of *N*,*N*-dimethylformamide (Aldrich, United States); acrylamide and *N*,*N*'-methylene-bisacrylamide (Promega Corp., United States); and Tris-HCl (Sigma, United States). Prior to the use in biological experiments, dNTP, ddATP, and ddTTP (Boehringer Mannheim, Germany) were additionally purified by HPLC. d_4TTP was synthesized as described [20]; radioactively labeled compounds [^{3}H]dTTP with a specific activity of 46.6 Ci/mmol and [γ-^{32}P]ATP with a specific activity of 3000 Ci/mmol were from the Fosfor Center of Collective Use (Russian Academy of Sciences, Russia).

1-β-(Adenin-9-yl)-*D*-riburonic acid (Va). A solution of potassium permanganate (2.52 g, 16 mmol) in water (400 ml) and a solution of KOH (0.79 g, 12 mmol) in water (50 ml) were added at room temperature under stirring to a solution of 2'-deoxyadenosine (1.005 g, 4 mmol) in water (500 ml). After stirring for 2 days, excess permanganate was decomposed by a 30% H_2O_2 solution. The precipitated solid was filtered, the filtrate was evaporated to 30 ml, and pH of the solution was brought to 4.5 by concentrated HCl. The product precipitated as white crystals. The crystals were separated, dried, and analyzed by TLC in system B. Compound (**Va**) was obtained in yield of 0.3 g (28%); UV: λ_{max} 260 nm (H_2O); ^{1}H NMR (DMSO-d_6): 8.44 (1 H, s, H8), 8.17 (1 H, s, H2), 7.26 (2 H, s, NH_2), 6.53 (1 H, m, H1'), 4.65 (1 H, m, H3'), 4.35 (1 H, d, *J* 7, H4'), 2.18 (2 H, m, H2').

(2'*R*)-9-(2,3-Dihydrofuran-2-yl)adenine (VIa). 1-β-(Adenine-9-yl)-*D*-riburonic acid (**Va**) (0.26 g, 1 mmol) was heated with DMF dineopentylacetal (0.69 g, 3 mmol) in DMF (2 ml) at 80–85°C for 5 h, after which the mixture was evaporated to dryness. During evaporation, the product was crystallized, and its purification was carried out by recrystallization from ethanol with a drop of triethylamine. Compound (**VIa**) was obtained in a yield of 0.13 g (65%); TLC, system A; UV: λ_{max} 260 nm (95% ethanol); ^{1}H NMR (DMSO-d_6): 8.22 (2 H, s, H8), 6.78 (1 H, m, H5'), 6.35 (1 H, m, H2'), 6.27 (2 H, s, NH_2), 5.01 (1 H, m, H4'), 3.19 (2 H, m, H3').

(2'*R*)-9-(2,3-Dihydrofuran-2-yl)-*N*^{6}-pivaloyladenine (VII). Pivaloyl chloride (1.5 g, 12 mmol) and 4-(*N*,*N*dimethylamino)pyridine (170 mg) were added to a solution of (**VIa**) (2.0 g, 10 mmol) in 1,2-dichloroethane (10 ml) and triethylamine (1.7 ml, 12 mmol); the mixture was heated at 55–60°C for 6 h and evaporated in a vacuum. The residue (oil) was dissolved in dichloromethane (30 ml), washed successively with water, 20% H_3PO_4, and a saturated NaCl solution, dried with $MgSO_4$, and evaporated to dryness. The product was purified by column chromatography on silica gel, using dichloromethane–3% methanol as an eluent. Compound (**VII**) was obtained in yield 2.5 g (85%); TLC, system A; UV: λ_{max} 285 nm (95% ethanol); ^{1}H NMR ($CDCl_3$): 8.73 (1 H, s, H8), 8.51 (1 H, s, NH), 8.18 (1 H, s, H2), 6.48 (1 H, m, H5'), 6.41 (1 H, dd, *J* 3.5, 9.4, H2'), 5.24 (1 H, m, H4'), 2.29 (2 H, m, H3'), 1.24 (9 H, s, CH_3).

(2'*R*,3'*S*,5'*R*)-9-(2-Diethoxyphosphinoylmethoxy-3-iodotetrahydrofuran-5-yl)-*N*^{6}-pivaloyladenine (VIIIa). A solution of IBr (1.2 g, 6.0 mmol) in dichloromethane (10 ml) was added at –25°C for 5 min to a solution of (**VII**) (861 mg, 3.0 mmol) and diethyl(hydroxymethyl)phosphonate (1.6 g, 11 mmol) in dichloromethane (7 ml); mixture was stirred at –25°C for 45 min, extracted with dichloromethane with a $NaHCO_3$ solution; the organic phase was washed with an sodium bisulfite solution, dried with $MgSO_4$, and evaporated to dryness. The substance was chromatographed on a silica gel column eluted with dichloromethane–3% methanol. Compound (**VIIIa**) was obtained as a colorless oil; yield 1 g (65%); TLC, system A; UV: λ_{max} 285 nm (95% ethanol); ^{1}H NMR ($CDCl_3$): 8.69 (1 H, s, H8), 8.51 (1 H, s, NH), 8.27 (1 H, s, H2), 6.83 (1 H, t, *J* 6.5, H5'), 5.46 (1 H, s, H2'), 4.45 (1 H, d, *J* 5.9, H3'), 3.7–4.0 (2 H, m, PCH_2), 3.76 (4 H, m, CH_2 (Et)), 3.16 (1 H, ddd, *J* 5.9, 6.5, 14.9, H2'$_a$), 2.84 (2 H, dd, *J* 6.5, 14.9, H2'$_б$), 1.41 (6 H, m, CH_3 (Et)), 1.32 [9 H, s, CH_3 (Piv)]; ^{31}P NMR ($CDCl_3$): 20.34 (s).

(2'*R*,5'*R*)-9-(2-Diethoxyphosphinoylmethoxy-2,5-dihydrofuran-5-yl)-*N*^{6}-pivaloyladenine (IXa). 1,8-Diazabicyclo[5.4.0]undec-7-ene (610 mg, 4 mmol) was added to a solution of (**VIIIa**) (1.2 g, 2.1 mmol) in THF (20 ml), and the mixture was heated at 65°C for 50 min. The reaction mixture was evaporated, and the residue was dissolved in dichloromethane, washed with a 20% H_3PO_4 and a saturated NaCl solution, and evaporated to dryness. The product (**IXa**) was chromatographed on a silica gel column eluted with dichloromethane–5% methanol; yield 848 mg (90%); TLC, system A; UV: λ_{max} 285 nm (95% ethanol); ^{1}H NMR ($CDCl_3$): 8.66 (1 H, s, H8), 5.51 (1 H, s, NH), 8.04 (1 H, s, H2), 7.0 (1 H, d, *J* 1.5, H5'), 6.34 (1 H, dd, *J* 1.5, 6.0, H4'), 6.27 (1 H, d, *J* 6.0, H3'), 5.87 (1 H, s, H2'), 3.90 (2 H, m, PCH_{a2}), 3.64 (4 H, m, CH_2 (Et)), 1.40 (6 H, m, CH_3 (Et)), 1.31 [9 H, s, CH_3 (Piv)]; ^{31}P NMR ($CDCl_3$): 19.98 (s).

9-[(2*R*,5*R*)-2,5-Dihydro-5-phosphonomethoxyfuran-2-yl]adenine ammonium salt (Ia) A saturated solution of NH_3 (1 ml) in methanol was added to a solution of (**IXa**) (326 mg, 0.76 mmol) in methanol (1 ml). After stirring at 25°C for 12 h, the reaction mixture was evaporated to dryness, and the resulting white residue was dried over P_2O_5 in a vacuum. Without purification, the substance was dissolved in DMF (1 ml), and freshly

distilled trimethylsilyl bromide (2 ml) was added at 0°C to the solution. The solution was stirred for 1 h at 0°C and then for 2 h at 25°C. The mixture was evaporated, and a concentrated ammonia (3 ml) was added. The mixture was evaporated to dryness. The product obtained was purified by reversed-phase chromatography (silica gel C18), using water as an eluent. Compound (**Ia**) was obtained as a white amorphous powder; yield 114 mg (45%); TLC, system B; UV: λ_{max} 260 nm (H_2O); ^{1}H NMR (D_2O): 8.13 (1 H, s, H8), 7.87 (1 H, s, H2), 6.82 (1 H, s, H5'), 6.50 (1 H, d, *J* 6.0, H4'), 6.46 (1 H, d, *J* 6.0, H3'), 5.99 (1 H, s, H2'), 3.69 (1 H, dd, *J* 9.3, 13.2, PCH_a), 3.59 (1 H, dd, *J* 9.3, 13.2, PCH_b); ^{31}P NMR (D_2O): 15.9 (s).

Diphosphoryl-(2'*R*,5'*R*)-9-(5-phosphonomethoxy-2,5-dihydrofuran-2-yl)adenine (IIa). CDI (100 mg, 0.64 mmol) was added to a solution of ammonium salt of (**Ia**) (20 mg, 64 μmol) in DMF (1 ml). After 2 h, a 1 M (1.3 ml, 1.3 mmol) in DMF was added to the reaction mixture. After 4 h, the mixture was evaporated, and the product was applied onto a column of DEAE-Toyopearl and eluted with a gradient of $(NH_4)HCO_3$ concentration (from 0 to 0.5 M). The product was purified by HPLC on a silica gel C18 column of in an ion-pair regime (50 mM triethylammonium bicarbonate). The substance was eluted using a gradient of ethanol concentration (0 to 15%). The retention time of the substance was 12.8 min; TLC, system B. Compound (**IIa**) was obtained in yield 12 mg (40%); UV: λ_{max} 260 nm (H_2O); ^{1}H NMR (D_2O): 8.13 (1 H, s, H8), 7.87 (1 H, s, H2), 6.82 (1 H, s, H5'), 6.50 (1 H, d, *J* 6.0, H4'), 6.46 (1 H, d, *J* 6.0, H3'), 5.99 (1 H, s, H2'), 3.69 (1 H, dd, *J* 9.3, 13.2, PCH_a), 3.59 (1 H, dd, *J* 9.3, 13.2, PCH_b); ^{31}P NMR (D_2O): 8.9 (1 P, d, *J* 26, P^α), –10.0 (1 P, d, *J* 19, P^γ), –22.5 (1 P, dd, P^β).

(β,γ-Difluoromethylene)diphosphoryl-(2'*R*,5'*R*)-9-(5-phosphonylmethoxy-2,5-dihydrofuran-2-yl)adenine (IIb). The synthesis and purification of (**IIb**) were carried out as described for (**IIa**). Triethylammonium salt of (**Ia**) (20 mg, 64 μmol), CDI (100 mg, 0.64 mmol), and 1 M triethylammonium difluoromethylenediphosphonate (1.3 ml, 1.3 mmol) were used. The retention time of the product on the silica gel C18 column was 13.3 min. Compound (**IIb**) was obtained in yield 23 mg (73%); TLC, system B; UV: λ_{max} 260 nm (H_2O); ^{1}H NMR (D_2O): 8.13 (1 H, s, H8), 7.87 (1 H, s, H2), 6.82 (1 H, s, H5'), 6.50 (1 H, d, *J* 6.0, H4'), 6.46 (1 H, d, *J* 6.0, H3'), 5.99 (1 H, s, H2'), 3.69 (1 H, dd, *J* 9.3, 13.2, PCH_a), 3.59 (1 H, dd, *J* 9.3, 13.2, PCH_b); ^{31}P NMR (D_2O): 9.9 (1 P, m, P^α), 4.0 (1 P, m P^γ), –4.2 (1 P, m, P^β).

(β,γ-Dibromomethylene)diphosphoryl-(2'*R*,5'*R*)-9-(5-phosphonylmethoxy-2,5-dihydrofuran-2-yl)adenine (IIc) was synthesized, isolated, and purified as described for (**IIa**). Triethylammonium salt of (**Ia**) (20 mg, (64 μmol), CDI (100 mg, 0.64 mmol), and 1 M triethylammonium dibromomethylenediphosphonate (1.3 ml, 1.3 mmol) were used. The retention time of the product on the column was 14 min; TLC, system B. Compound (**IIc**) was obtained in yield 27 mg (69%); UV: λ_{max} 260 nm (H_2O); ^{1}H NMR (D_2O): 8.13 (1 H, s, H8), 7.87 (1 H, s, 1, H2), 6.82 (1 H, s, H5'), 6.50 (1 H, d, *J* 6.0, H4'), 6.46 (1 H, d, *J* 6.0, H3'), 5.99 (1 H, s, H2'), 3.69 (1 H, dd, *J* 9.3, 13.2, PCH_a), 3.59 (1 H, dd, *J* 9.3, 13.2, PCH_b); ^{31}P NMR (D_2O): 9.9 (1 P, m, P^α), 4.0 (1 P, m, P^γ), –4.2 (1 P, m, P^β).

1-β-(Thymin-1-yl)-*D*-riburonic acid (Vb). A solution of CrO_3 (12 g, 120 mmol) in pyridine (120 ml) was prepared under cooling (–20°C) and stirring after which the temperature of the solution was brought to room temperature under stirring. Pyridine was evaporated to dryness at a reduced pressure, DMF (100 ml) was added, and, after the complete dissolution of the complex, the mixture was evaporated to two thirds of the initial volume. Thymidine (3 g, 12.4 mmol) dissolved in DMF (10 ml) was added under stirring to the resulting Jones reagent in DMF and allowed to stand for 5 h at room temperature. The course of the reaction was monitored by TLC on Silufol plates in system B. Then water (500 ml) and a saturated solution of disubstituted ammonium phosphate (24 g, 180 mmol) in 1% ammonia were added. After a day, the precipitated solid was filtered, and inorganic salts were removed from the mother liquor by reversed-phase chromatography. The product was isolated by ion-exchange chromatography on DEAE cellulose using a gradient of ammonium bicarbonate concentration (0–0.3 M). The ammonium salt of substituted riburonic acid was converted into the acidic form by ion-exchange chromatography (Dowex-50, H^+ form). Compound (**Vb**) was obtained as a white crystalline substance; yield 1.1 g (34.7%); TLC, system B; UV: λ_{max} 267 nm; ^{1}H NMR (DMSO-d_6): 11.31 (1 H, s, COOH), 8.05 (1 H, s, 1, H6), 6.30 (1 H, m, H1'), 5.68 (1 H, s, 3'-OH), 4.44 (1 H, d, *J* 4.4, H4'), 4.29 (1 H, m, H3'), 2.12 (1 H, m, $H2'_a$), 1.95 (1 H, m, $H2'_b$), 1.76 (3 H, s, CH_3).

(2'*R*)-1-(2,3-Dihydrofuran-2-yl)thymine (VIb). A solution of (**Vb**) (0.90 g, 3.51 mmol) in DMF (37.5 ml) was heated to 100–110°C, *N*,*N*-dimethylformamide dineopentylacetal (2.0 g, 11.25 mmol) was added, and the mixture was stirred for 3–5 h. The reaction mixture was cooled to room temperature and evaporated to dryness in a vacuum. The residue was dissolved in water and extracted with chloroform (3 × 30 ml). The organic layer was separated and dried with magnesium sulfate. The product was chromatographed on a silica gel column (2.5 × 14 cm) eluted with 3% methanol in chloroform. After evaporation, 0.64 g (94%) of (**Vb**) as a white crystalline substance was obtained; TLC, system A; UV: λ_{max} 267 nm; ^{1}H NMR ($CDCl_3$): 9.08 (1 H, s, NH), 7.05 (1 H, s, H6), 6.77 (1 H, m, H5'), 6.49 (1 H, d, *J* 2.15, H2'), 5.16 (1 H, m, H4'), 2.56–3.25 (2 H, m, H3'), 1.94 (3 H, s, CH_3).

(2'*R*,3'*S*,5'*R*)-1-(2-Diethoxyphosphinoylmethoxy-3-iodotetrahydrofuran-5-yl)thymine (VIIIb). Diethyl (hydroxymethyl)phosphonate (0.87 g,

6.38 mmol) was added at –25°C to a solution of (**VIb**) (0.50 g, 2.60 mmol) in dichloromethane (4 ml), after which IBr (0.70 g, 3.48 mmol) dissolved in dichloromethane (5.8 ml) was added for 5 min dropwise under stirring. The mixture was stirred at –25°C for 50 min. Then chloroform (30 ml) and a saturated aqueous $NaHCO_3$ solution (50 ml) were added. The organic layer was separated, washed with $Na_2S_2O_3$ solution, and dried with magnesium sulfate. The product was chromatographed on a silica gel column (2.5 × 14 cm) eluted with 3% methanol in chloroform. After evaporation, 1.01 g (92%) of (**VIIIb**) as a colorless oil was obtained; TLC, system A; UV: λ_{max} 267 nm; ^{1}H NMR ($CDCl_3$): 8.45 (1 H, s, NH), 7.36 (1H, s, H6), 6.79 (1 H, t, *J* 7.16, H5'), 5.40 (1 H, s, H2'), 4.35 (1 H, m, H3'), 4.21 (4 H, m, CH_2 (Et)), 4.00 (1 H, m, PCH_a), 3.80 (1 H, m, PCH_b), 2.63 (2 H, dd, *J* 3.42, 7.16, H2'), 1.99 (3 H, s, 5-CH_3), 1.37–1.32 [6 H, m, CH_3 (Et)). ^{31}P NMR ($CDCl_3$): 17.75 (s).

(2'*R*,5'*R*)-1-(5-Phosphonylmethoxy-2,5-dihydrofuran-2-yl)thymine (Ib). Me_3SiBr (0.914 g, 6 mmol) was added at room temperature to a solution of (**VIIIb**) (0.50 g, 1.09 mmol) in DMF (1.9 ml) and stirred for 24 h. The reaction mixture was evaporated and dissolved in THF (4 ml) without isolation of the reaction product. 1,8-Diazabicyclo[5.4.0]undec-7-ene (0.254 g, 1.6 mmol) was then added under stirring at 50–60°C. The mixture was stirred for 3.5 h, evaporated, dissolved in chloroform, and extracted with water. The water layer was separated, and further purification was carried out by ion-exchange chromatography on a DEAE cellulose column. The product was eluted by a 0–0.2 M gradient of ammonium bicarbonate, evaporated, and purified from contaminations of inorganic salts by reversed-phase chromatography. Compound (**Ib**) was obtained as a white crystalline substance; yield: 0.229 g (70%); TLC, system A; UV: λ_{max} 267 nm; ^{1}H NMR (D_2O): 7.33 (1 H, s, H6), 6.78 (1 H, s, H5'), 6.37 (1 H, d, *J* 6.0, H4'), 6.16 (1 H, d, *J* 6.0, H3'), 5.83 (1 H, s, H2'), 3.81 (1 H, dd, *J* 9.34, 13.08, PCH_a), 3.67 (1 H, dd, *J* 9.35, 13.08, PCH_b), 1.79 (3 H, s, CH_3). ^{31}P NMR (D_2O): 15.39 (s).

(β,γ-Dichloromethylene)diphosphoryl-(2'*R*,5'*R*)-1-(5-phosphonylmethoxy-2,5-dihydrofuran-2-yl)thymine (IIe). Compound (**Ib**) (0.42 g, 0.14 mmol) was activated with CDI in DMF (2.5 ml) as described previously [15]. Then a solution of bis(tributylammonium) dichloromethylene diphosphate (0.19, 0.56 mmol) in DMF (1.5 ml) was added to the mixture, and the mixture was stirred for 24 h and then was dissolved in water. The product was isolated by ion-exchange chromatography on a DEAE-Toyopearl column. The product was eluted with a 0–0.5 M gradient of triethylammonium bicarbonate concentration. Compound (**IIe**) was obtained in yield of 0.016 g (36%); TLC, system B; UV: λ_{max} 267 nm; ^{1}H NMR (D_2O): 7.32 (1 H, s, H6), 6.76 (1 H, s, H5'), 6.39 (1 H, d, *J* 6.0, H4'), 6.12 (1 H, d, *J* 6.0, H3'), 5.90 (1 H, s, H2'), 3.99–3.87 (2 H, m, PCH_2), 1.79 (3 H, s, CH_3). ^{31}P NMR (D_2O): 8.71 (1 P, d, *J* 34.1, P^γ), 7.79 (1 P, d, *J* 18.3, P^α), –0.43 (1 P, dd, *J* 18.3, 34.1, P^β).

(β,γ-Imido)diphosphoryl-(2'*R*,5'*R*)-1-(5-phosphonylmethoxy-2,5-dihydrofuran-2-yl)thymine (IIf). CDI (0.086 g, 0.53 mmol) was added to a solution of triethylammonium salt of (**Ib**) (0.67 g, 0.13 mmol) in DMF (2.5 ml), and the mixture was stirred for 5 h. Methanol (0.1 ml) was then added, and the mixture was stirred for 25 min and evaporated in a vacuum to a minimum volume. After the addition of bis(tributylammonium) imidodiphosphate (0.285 g, 0.52 mmol) in DMF (3 ml), a white solid precipitated. The reaction mixture was stirred for 24 h, water (100 ml) was then added, and the filtered solution was applied onto a DEAE-Toyopearl column. The product was eluted with a gradient of triethylammonium bicarbonate concentration (0–0.5 M). Compound (**IIf**) was obtained in yield of 0.024 a (39%); TLC, system B; UV: λ_{max} 267 nm; ^{1}H NMR (D_2O): 7.31 (1 H, s, H6), 6.77 (1 H, s, H5'), 6.38 (1 H, d, *J* 6.0, H4'), 6.13 (1 H, d, *J* 6.0, H3'), 5.88 (1 H, s, H2'), 3.92–3.85 (2 H, m, PCH_2), 1.79 (3 H, s, CH_3). ^{31}P NMR (D_2O): 8.48 (1 P, d, *J* 27.0, P^α), 0.27 (1 P, s, P^γ), –12.41 (1 P, d, P^β).

(2'*R*,5'*R*)-1-(5-Phosphonylmethoxytetrahydrofuran-2-yl)thymine (X). Compound (**Ib**) (25 mg) was hydrogenated in 75% aqueous ethanol (3 ml) in the presence of 10% Pd/C (10 mg). After 7 h, the reaction mixture was passed through a column of Dowex 50 in NH_4 form of and evaporated to give 20 mg (80%) of (**X**); TLC, system B; UV: λ_{max} 267 nm; ^{1}H NMR (D_2O): 7.56 (1 H, s, H6), 6.3 (1 H, m, H5'), 5.21 (1 H, m, H2'), 3.63 (2 H, m, PCH_2), 2.03–2.33 (4 H, m, H3' + H4'), 1.83 (3 H, s, 5-CH_3). ^{31}P NMR (D_2O): 14.56 (s).

Enzymes and DNA. The following enzymes were used: T4 polynucleotide kinase (Amersham Pharmacia Biotech), DNA polymerase I from *E. coli*, the Klenow fragment [EC 2.7.7.7] (Boehringer Mannheim), HIV RT [EC 2.7.7.49] (Worthington), and AMV RT [EC 2.7.7.49] (Promega). DNA polymerases α and β were isolated from human placenta as described in [21, 22], respectively. Poly(rA)-$(dT)_{10}$ was from Calbiochem. Phage M13*mp*10 single-stranded DNA was isolated from a cultural liquid of the recipient strain *E. coli* K12XL1 as described in [23]. For single-substrate reactions catalyzed by the above-listed DNA polymerases, two types of primer–template complexes, DNA–14A and DNA–14T, were used, depending on which nucleic base (adenine or thymine) the dNTP analogue being tested contained. The tetradecanucleotide primers 14A and 14T of the Litekh company (Russia) were used without additional purification. The primers were labeled at the 5'-terminus by the method described in [24] using [γ-^{32}P]ATP and T4-polynucleotide kinase followed by the inactivation of the enzyme (10 min at 65°C). Primer–template complexes were obtained by incubating 5'-labeled primers with DNA in buffer containing 10 mM Tris-HCl, pH 8.0, and 5 mM $MgCl_2$ at 65°C for 10 min with subsequent cooling to 30°C for

1 h. The complexes were freed from free [γ-^{32}P]ATP by passing the solutions through a 1-ml column of Biogel A-1.5m in 10 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA. The fractions containing the primer–template complex were combined, stored at –20°C, and used in reactions without additional purification.

DNA synthesis. For the KF of DNA polymerase I, the reaction mixture for a single-substrate reaction contained in a volume of 6 μl: 10 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 1 mM DTT, 0.3 unit of the enzyme, a 10 nM primer–template complex, and test compounds at different concentrations; the reaction was carried out for 15 min at 25°C. In the case of HIV RT and MBV RT, the reaction mixture (6 μl) contained 10 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 1 unit of the enzyme, a 20 nM primer–template complex, and the corresponding substrates; the mixture was incubated for 30 min at 37°C. For DNA polymerase α, the reaction mixture (6 μl) contained 10 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 0.4 mM DTT, 1 unit of the enzyme, a 20 nM primer–template complex, and the corresponding substrates; the mixture was incubated for 20 min at 37°C. For DNA polymerase β, the reaction mixture (6 μl) contained 10 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 0.1 mM DTT, 1 unit of the enzyme, a 20 nM primer–template complex, and the corresponding substrates; the mixture was incubated for 20 min at 37°C. In all cases, the reactions were terminated by adding deionized formamide (3 μl to each sample) containing 20 mM EDTA and 0.1% dyes (Bromophenol Blue and Xylenecyanol). Samples were kept for 2 min at 100°C, and the reaction products were separated by electrophoresis in denaturing 14% or 20% PAG. Gels were exposed to a Retina RX X-ray film at –20°C.

Quantitative determination of reaction rates and the calculation of the kinetic constants were carried out as described in [25]. The Michaelis constants and V_{max} values in single-substrate reactions catalyzed by HIV RT and BMV RT were determined by the graphic method of Lineweaver–Burk. Optimal reaction conditions were the following: 3 nM DNA–14A complex, 0.2 unit of the enzyme, 5 min at 37°C in a volume of 6 μl. The average values of K_m for the examined compounds were obtained from the data of three independent experiments.

Inhibition of the incorporation of radioactive label into DNA by modified isosteric d_4TTP analogues. The ability of (**IId**), (**IIe**), and (**IIf**) to inhibit the HIV RT-catalyzed incorporation of [^{3}H]dTTP into poly(rA)–oligo(dT) was determined under the conditions described previously [26]. The reaction mixture (10 μl) contained 10 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, and 2 [^{3}H]dTTP, 150 ng/ml poly(rA)–oligo(dT), 1 unit of the enzyme, and the compounds tested. The mixture was incubated for 10 min at 37°C. The reaction was terminated by adding EDTA to a final concentration of 0.1 M. Samples were transferred onto GF/C filters preliminarily soaked in 5% TCA. Filters were washed five times with 5% TCA and one time with alcohol and dried, and their radioactivity was measured in a toluene scintillator using a liquid scintillation radioactivity counter (Intertechnique, France).

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