

Effect of triphosphate modifications in 2'-deoxynucleoside 5'-triphosphates on their specificity towards various DNA polymerases

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Abstract Some natural and glycon-modified dNTPs with β,γ -pyrophosphate substitution at the triphosphate residue were synthesized and studied to evaluate the effect of these modifications on substrate properties of dNTPs in DNA synthesis catalyzed by human placental DNA polymerases α and β , avian myeloblastosis virus reverse transcriptase, and calf thymus terminal deoxynucleotidyl transferase. Reverse transcriptase proved to be the enzyme least specific to such modifications; the substrate activity of β,γ -methylenediphosphonate substituted dTTP and 3'-azido-3'-deoxy-dTTP decreased in the following order: $\text{CF}_2 = \text{CHF} > \text{CBr}_2 > \text{CFMe} \gg \text{CH}_2$. This order is individual for each DNA polymerase. It is interesting to mention that $\beta,\gamma\text{-CBr}_2$ substituted dTTP is neither a substrate nor an inhibitor of DNA polymerase β . This specificity distinguishes DNA polymerase β from other DNA polymerases studied.

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1. Introduction

dNTP are known to be rapidly dephosphorylated in inter- and intracellular media as well as during their diffusion into cells. This property limits their use in nucleic acids biosynthesis studies. Design of dNTP analogs which are stable enough in the presence of dephosphorylating enzymes may involve either replacement of the triphosphate residue by another group or protection of the triphosphate function by any chemical residue which is removed inside the cell. Recent studies have demonstrated that particular triphosphate modifications do not affect markedly the substrate properties of dNTP toward different DNA polymerases, but essentially increase the stability of the triphosphate in the presence of dephosphorylating enzymes [1,2]. However, triphosphate modifications for designing effective and selective substrates/inhibitors of DNA polymerases remain to be suggested. Besides, the dependence of substrate specificity of different DNA polymerases on the structure of modified triphosphate residue has received little study.

We synthesized several modified dNTP of Ia–c, IIa–c, and

IIIa,b types (Fig. 1). As a nucleoside component we used either natural thymidine (as in I), or the well known inhibitor of HIV replication, 3-azido-3'-deoxythymidine (as in II). As a structural basis for III, (Z)-1-[4-(phosphonomethoxy)but-2-enyl]thymine was used. Its β,γ -diphosphate, as well as diphosphates of the corresponding nucleotides with other nucleic bases, selectively terminated DNA synthesis at the catalysis by retroviral reverse transcriptases [3].

Substrate properties of I–III were evaluated in DNA synthesis reactions catalyzed by AMV reverse transcriptase, DNA polymerases α and β from human placenta, and calf thymus TDT. The above mentioned DNA polymerases revealed different selectivity towards dNTP with structurally similar substituents in the triphosphate residue.

2. Materials and methods

Thymidine 5'-monophosphate was from Sigma; AZT 5'-monophosphate was obtained according to Ref. [4]. (Z)-1-[4-(phosphonomethoxy)but-2-enyl]thymine was synthesized as described in Ref. [3]. Diphosphonic acids were prepared by dissolving the corresponding tetrakis(trimethylsilyl) diphosphonates [5] in aqueous pyridine followed by column chromatography of the obtained emulsion on Dowex 50W4 (H^+) and concentration of the eluted fractions in vacuo. Compounds I–III were synthesized as in Ref. [2] and analyzed by HPLC. ^{31}P NMR spectra with P-H decoupling were recorded at 162 MHz, 85% H_3PO_4 and D_2O were used as an external standard and solvent, respectively; ^{19}F NMR spectra were recorded on a Bruker AC 200F spectrometer at 188.3 MHz, CF_3COOH served as an external standard.

The yields and physicochemical parameters of the compounds prepared are presented in Table 1.

2.1. Enzymes and DNA

All the enzymes and template-primer complex were obtained as in Ref. [1]. All experiments were carried out as in Ref. [1].

3. Results

The compounds under study were examined using the template-tetradecadeoxynucleotide primer complex (Fig. 2) in DNA synthesis catalyzed by AMV reverse transcriptase (Fig. 3A), human DNA polymerase β (Fig. 3B), human DNA polymerase α (Fig. 3C), and calf thymus TDT (Fig. 3D), but in TDT assays no template was used. The primer was selected so that the next nucleotide residue in the growing DNA chain would be thymidylic acid or its analog.

It is evident from Fig. 3A that compounds of type I are substrates and of type II are terminating substrates for AMV reverse transcriptase. The most potent among the compounds of type I was Ia. It elongated primer with practically the same efficiency as dTTP (compare lanes 2 and 5 in Series E). The

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Abbreviations: dNTP, 2'-deoxynucleoside 5'-triphosphates; AMV, avian myeloblastosis virus; HIV, human immunodeficiency virus; TDT, terminal deoxynucleotidyl transferase; AZTTP, 3'-azido-3'-deoxythymidine 5'-triphosphate

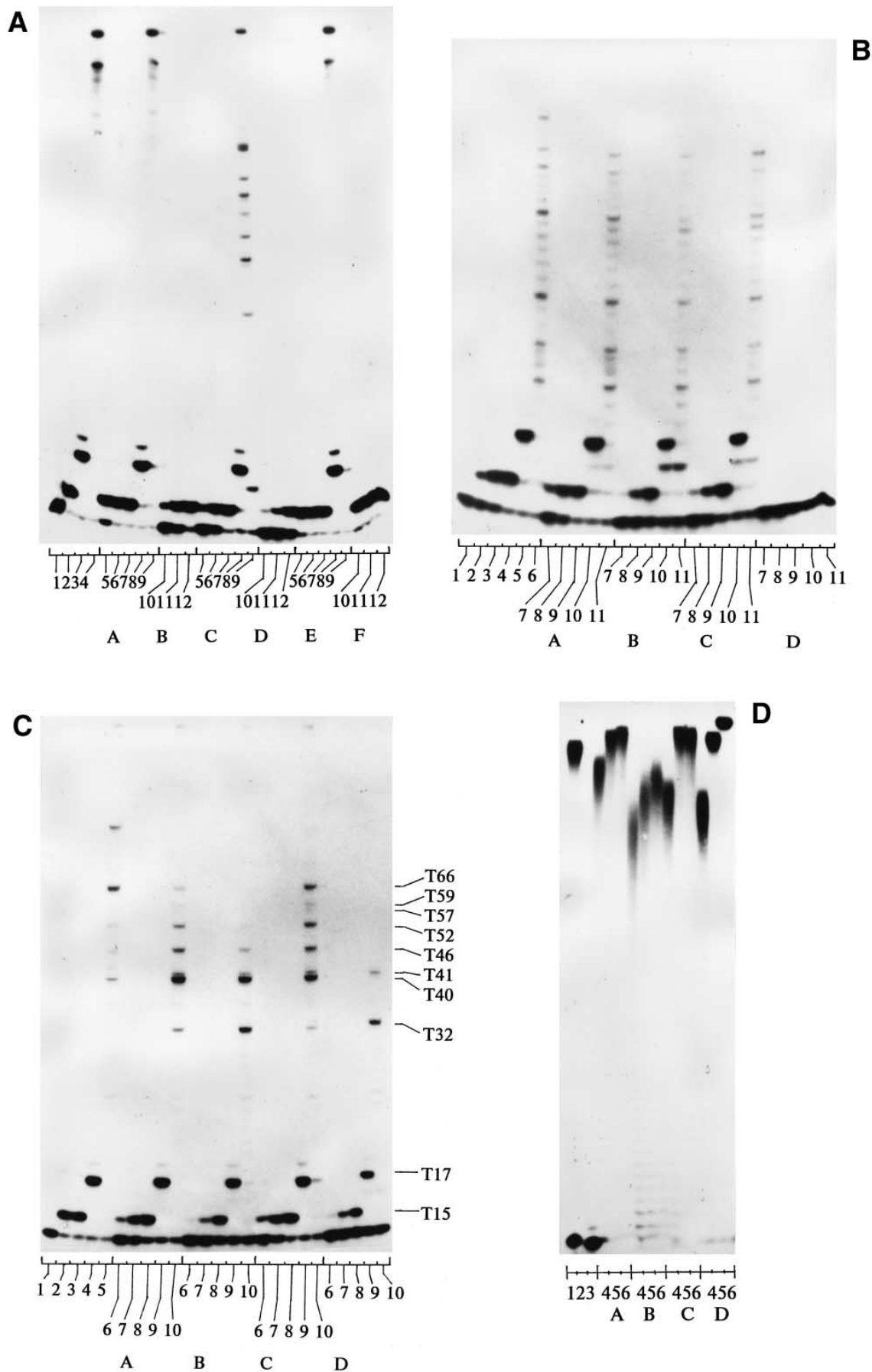


Table 2
Kinetic parameters for I and II in one-step elongation reaction catalyzed by AMV reverse transcriptase

Compound	K_m (μ M)	V_{max}/V_{max} dTTP
Ia	1.25±0.17	0.81
I, X = CH ₂	700 ^a	
Ib	1.30±0.12	0.98
Ic	11.38±2.27	1.15
Id	2.61±0.24	0.51
dTTP	1.02±0.01	1
IIa	0.98±0.09	0.52
IIb	10.34±1.68	0.82
IIc	12.31±1.34	1.42
AZTTP	0.76±0.02	0.68

^aAccording to Ref. [7].

Among the azido derivatives IIa–c, DNA polymerase β incorporated only IIb at a low efficiency (data not shown).

Triphosphates Ia–d were recognized by DNA polymerase α (Fig. 3C). The activity of the compounds Ia and Ib was approximately the same and was more than 50-fold lower than that for dTTP (compare lanes 6–8, Series C and A, with lanes 2–3); the activity of Ic (lanes 6–8, Series B) was even lower, and Id was incorporated in the DNA chain only at the concentration of 200 μ M (lanes 6–8, Series D). After incorporation of Ia–Id, the DNA chain was further extended if the incubation mixture contained dGTP (lanes 9, Series A–D, control lane 4), or dGTP+dATP+dCTP (lane 10, Series A–D, control lane 5). Pauses on lane 10 (Series A–D) correspond to the position of thymidylic acid residues (positions 32, 40, 41, 46, 52, 57, 59, and 66). This effect may be ascribed to the lower dissociation rate of halogenated methylenephosphonates after Ia–Id incorporation and, as a result, slower enzyme translocation along the template. This pattern is typical for DNA polymerase α due to its high polymerization activity (as compared with DNA polymerase β) and high fidelity (as compared with reverse transcriptase).

Fig. 3D illustrates tetradecanucleotide elongation by the synthesized compounds in the presence of TDT. It can be seen that all I were good substrates for this enzyme, Ic being slightly less efficient.

Compounds III were not substrates for HIV and AMV reverse transcriptases (data not shown).

4. Discussion

The results of our study demonstrate that replacement of the β,γ -pyrophosphate residue in dTTP and, to some extent in AZTTP, by substituted β,γ -methylenediphosphonate units maintains, as a rule, their substrate properties towards several DNA polymerases. Some dNTPs modified at the β,γ -pyrophosphate residue have earlier been studied. β,γ -Dibromomethylenediphosphonate and carboxyphosphonate analogs of dTTP were shown to be substrates for HIV, AMV, and RSV reverse transcriptases [6,7]; dTTP carboxyphosphonate analog was also a substrate for rat liver DNA polymerase β and calf thymus TDT, but was not utilized by calf thymus DNA polymerase α and *E. coli* DNA polymerase I [7]. Sugar-modified dTTP bearing a β,γ -methylenediphosphonate residue instead of the β,γ -pyrophosphate also revealed substrate properties towards HIV reverse transcriptase and TDT [8], though

its substrate activity was rather low. Guanine-related homolog of Ia was a substrate for *B. subtilis* DNA polymerase III, but was not recognized by *E. coli* DNA polymerase I [9].

Our data on the substrate properties of various modified dNTPs allowed us to formulate some principles concerning the specificity of different DNA polymerases.

All the compounds of type I were shown to be good substrates for AMV reverse transcriptase. Compounds IIa, IIb and IId were efficiently utilized by AMV reverse transcriptase, but IIc was a significantly less active substrate. TDT efficiently utilized Ia–Id as substrates, while DNA polymerase α recognized Ia and Ib better than Ic; triphosphate Id proved to be the least active substrate. For DNA polymerase β , Ib revealed the most potent substrate properties, Ia and Ic were slightly less active, and Id was not a substrate. Compounds III demonstrated no substrate properties towards any DNA polymerases.

Thus, substrate properties of I and II toward AMV reverse transcriptase decrease in the following order: CF₂ = CHF > CBr₂ > CFMe $\gg$ CH₂. For TDT, they may be arranged as follows: CF₂ = CBr₂ = CHF > CFMe. For DNA polymerase α , the relationship is different: CF₂ = CHF > CFMe $\gg$ CBr₂. For DNA polymerase β , substrate activity of Ia–Ic decreased in the following order: CHF > CF₂ > CFMe; Id (X = CBr₂) was neither a substrate nor an inhibitor of this enzyme. At the same time, DNA polymerase β incorporated IIb, although azido-dNTPs are not substrates for this enzyme.

The results obtained led us to the following conclusions.

1. Modification of the triphosphate residue in dNTP, particularly of the β,γ -pyrophosphate, allows one to modulate their substrate activity and selectivity, as it has been shown for glycon-modified dNTPs.
2. Possibilities for direct interpolation of the data on the activity of β,γ -modified dNTPs from one DNA polymerase to another one are limited, probably due to differences in the structure of triphosphate binding sites of DNA polymerase active centers.
3. The lack of specificity of DNA polymerase β towards Id was totally unpredictable, especially in view of the fact that this compound was a most potent substrate for AMV reverse transcriptase and TDT. It should be mentioned that DNA polymerase β did not utilize dTTP with bulky modifications at the γ -phosphate residue [1]. It is possible that bulky groups at the γ or β,γ -phosphate prevent correct dNTP binding to the DNA polymerase β active center because of spatial hindrances.

Thus, when designing dNTP analogs active and selective towards different DNA polymerases and stable in human blood, one should be aware of the fact that the substrate properties of these compounds differ for each enzyme and are defined by the particular combination of modifications in the dNTP molecule.

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