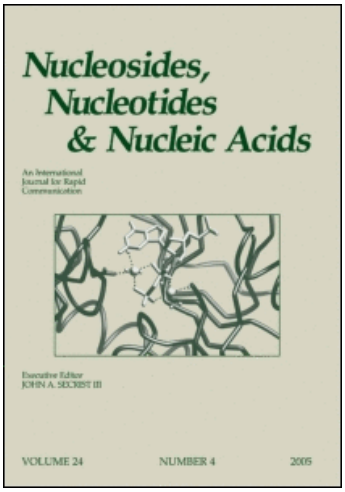

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ACYCLIC 2',3'-DIDEOXY-2',3'-DIDEHYDRONUCLEOSIDE 5'-TRIPHOSPHATES AS TERMINATION SUBSTRATES OF BROAD SET OF DNA POLYMERASES

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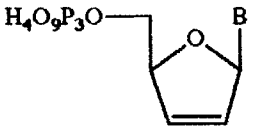
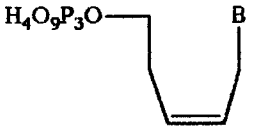
Abstract: O4'-Nor-2',3'-dideoxy-2',3'-didehydronucleoside 5'-triphosphates (acyclo-d_4NTP) have been shown to have the properties of effective termination substrates for DNA biosynthesis, catalyzed by several different DNA polymerases.

Among several kinds of nucleoside 5'-triphosphates with modified sugar residues highly active for several DNA polymerases were 2',3'-dideoxy-2',3'-didehydronucleoside 5'-triphosphates (d_4NTP)[1,2]. d_4NTP were incorporated into the 3'-end of growing DNA chains during synthesis catalyzed by mammalian and human DNA polymerases ß and γ, retroviral reverse transcriptases (RT), terminal deoxynucleotidyl transferase (TdT), *Echerichia coli* DNA polymerase I but not by DNA polymerases α. Here we describe



d_4NTP



acyclo-d_4NTP

Ia B=Ade
Ib B=Cyt
Ic B=Thy

properties of acyclic d_4NTP analogs (acyclo-d_4NTP). These compounds have turned out to be highly effective termination substrates for DNA polymerases α and ϵ from human placenta, ß from rat liver, TdT from calf thymus, human immunodeficiency virus (HIV) and avian myeloblastosis virus (AMV), but they did not terminate synthesis catalyzed by the *E.coli* DNA polymerases I, Klenow fragment.

EXPERIMENTAL

Synthesis of acyclo-d_4NTP was described briefly in the work[3] and manuscript is now in preparation.

The phage M13mp10 single-stranded DNA was isolated from the cultural liquid of the recipient strain *E.coli* K12XLI according to[4]. The primer tetradecanucleotide (Scheme) was labeled at 5'-position with [γ-^{32}P]ATP (specific activity 1500 Ci/mmol, Radioisotop, Russia), using T4 polynucleotide kinase (Amersham, England). After phenol extraction and reprecipitation with ethanol the [^{32}P]oligonucleotide was dissolved in water until the final concentration 1 nmol/ml and used either directly in the reactions of substrate binding with the TdT (Amersham) participation according to[5], or - after hybridization with the phage DNA and the primer- template purification on Biogel A-1.5M in reactions catalyzed by DNA polymerases. DNA polymerase I Klenow fragment (Amersham), rat liver DNA polymerase ß[6] (a kind gift of Dr A.M.Atrazhev), DNA polymerase α and ϵ from human placenta[7], reverse transcriptases of AMV (Omutninsk, Russia) and HIV-1 from the superproducer *E.coli* RPI (a king gift of Dr.S.Wilson, National Cancer Institute, Bethesda, USA) have been throughout this work. The paper on the isolation of HIV-1 reverse transcriptase is in preparation. [α-^{32}P]dATP of specific activity 2000 Ci/mmol (Radioisotop) was used.

DNA synthesis catalyzed by all DNA polymerases but not by DNA polymerase α, has been described in[9]. The substrate and inhibitor concentrations are induced in the legends to Figures. The method for DNA polymerase α has been modified and is described in the legend to Figure 2. Inhibition of the DNA synthesis reaction by acyclo-d_4NTP has been carried out using the unlabelled primer-template complex in the presence of [α-^{32}P]dATP as described in[9].

RESULTS

Investigation of the acyclo-d_4NTP properties in the DNA containing cell free systems has been carried out in two variants. In the first one (a monosubstrate system) the DNA of phage M13 as a template and 5'-[^{32}P]tetradecanucleotide as a primer has been used. The Scheme shows the structure of the primer and the working part of the template. Thymidine 5'-triphosphate (dTTP) and a known termination substrate d_4TTP (controls) or acyclo-d_4TTP were added to incubation mixture. The reaction products have been analyzed by electrophoresis in 20% denaturating polyacrylamide gel (PAAG).

Scheme

```
                10        20        30        40        50
          GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGAACCCGACGTC
5'-[32P]-CCCAGTCACGACGT
                60        70
          CAGCTGAGATCTCCTAGGGG-5'
```

In the second variant, the [^{32}P]primer-template complex, dNTP and an acyclo-d_4NTP in the appropriate concentrations were added to the reaction mixtures. All the components of incubation mixture are indicated in legends to Figures. The reaction was performed under the optimal conditions for each enzyme and then the reaction products were separated in 8% denaturating PAAG.

Fig.1 shows the incorporation of Ic nucleotide residue into 5'-[^{32}P]primer in the monosubstrate reaction. dTTP (track 3) and d_4TTP (track 4) served as controls. As is seen from Fig.1, the AMV reverse transcriptase (A series) as well as DNA polymerase ß (B) and α (C) elongate the primer by one nucleoside residue both in two controls and in the presence of Ic. The results of an experiment with TdT are shown in the tracks 7-10. In controls (tracks 8D and 8E) one can see multiple elongation of 5'-[^{32}P]-primer, whereas in the cases with d_4TTP (tracks 9) and Ic (tracks 10) it is elongated by only one nucleotide residue and the formed pentadecanucleotide is not elongated anymore (tracks 9 and 10E). Formation of more elongated forms after additional incubation of the samples with dTTP, seen in the track 9D, is due to the elongation of admixtured starting tetradecanucleotide and represented by an appropriate band in the track 9D. In the case of equal concentrations of the termination substrates d_4TTP or Ic, the first one elongates tetradecanucleotide with the significantly lower effectivity than Ic, as is seen from the tracks 9 and 10.

Fig.2 shows the pattern of DNA synthesis termination with acyclo-d_4NTP in the complete system with the DNA polymerases α. For control comparison there is the primary structure of DNA (-)strand, obtained in the presence of ddNTP and HIV reverse transcriptase. On the tracks with acyclo-d_4NTP termination bands are seen, allowing to read the nucleotide sequence in the synthesized DNA up to 90 residues. Finally, it can be seen from analysis of tracks 5-8 (A,B,C), that the I/dNTP ratio increasing (where dNTP - a

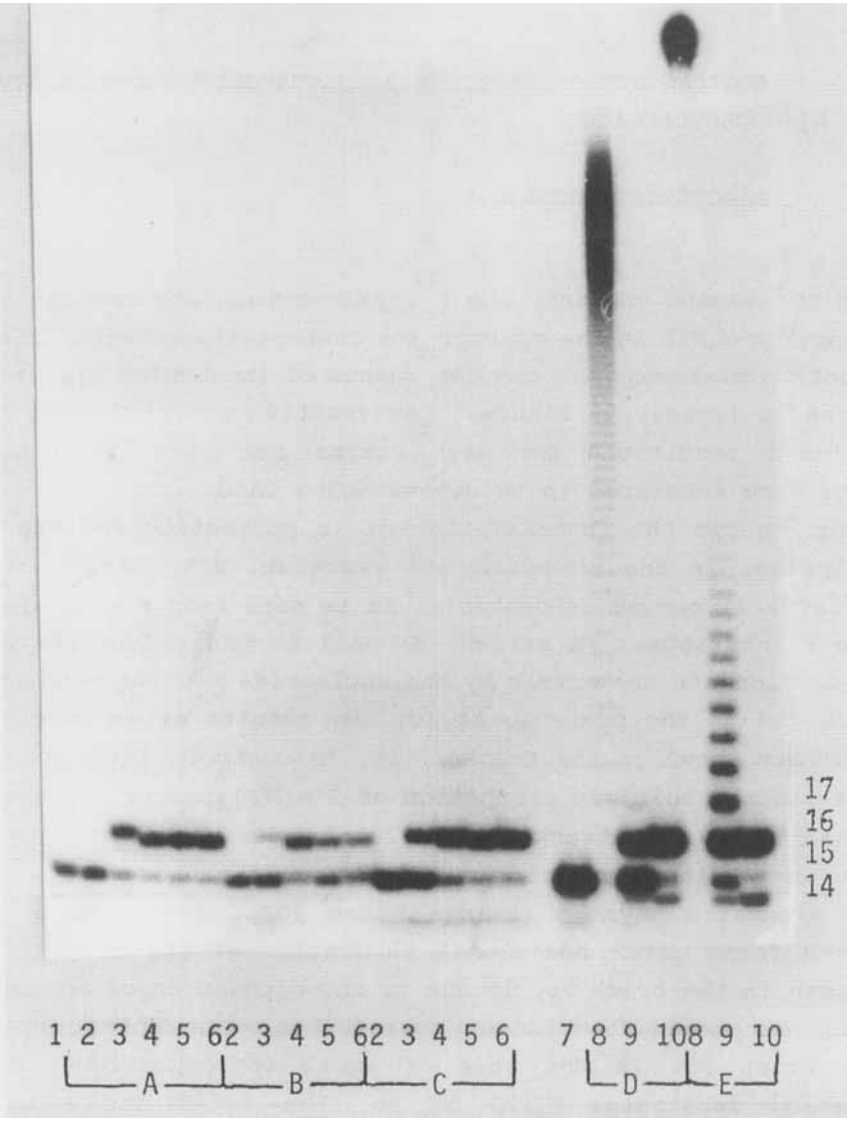


Figure 1. The autoradiogram of separation in 20% PAAG of products of [5'-^{32}P]primer elongation, catalyzed by the AMV reverse transcriptase (A series, 3 activity units, 10 min, 37°C), DNA polymerase ß (B series, 2 units, 20 min, 37°C), α (C series, 1 unit, 20 min, 37°C) and TdT (D series, 3 units, 40 min, 37°C; E series - the same as in D series + additional incubation after adding 300 μM dTTP): track 1 - primer-template; 2 - primer-template +appropriate enzyme; 3 - the same + 4 μM dTTP; 4 - as 2 + 100 μM d_4TTP; 5 and 6 - as 2 + 100 μM and 1 μM Ic, respectively; 7 - primer; 8 - the same+ 12μM dTTP; 9 - as 7 + 120 μM d_4TTP; 10 - as 7 + 120 μM Ic.

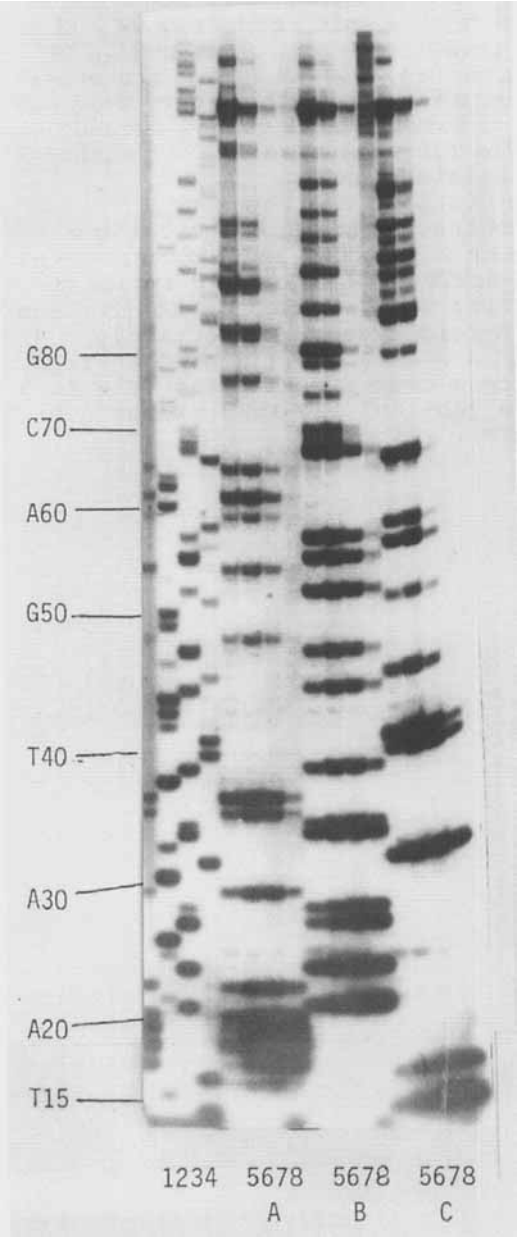


Figure 2. The autoradiogram of separation of [^{32}P]oligonucleotides.Reaction was catalyzed by DNA polymerase α in the presence of compounds Ia (series A), Ib (B) and Ic (C), taken in 2-, 10-, 50- and 200-fold molar excess relative the corresponding dNTP (tracks 5,6,7 and 8, respectively). The 6 μM samples, containing 0.05 pmols (~0.07 μCi) of [^{32}P]primer-template complex, 2 activity units of enzyme, 10 mM Tris.HCl, pH 7.4, 8 mM $MgCl_2$, 20 μg/ml BSA, dNTP of the same as corresponding I base nature - 6 μM, other three dNTP - 60 μM, were incubated for 1 hour at 37°C and then 30 min more after adding of 400 μM four dNTP. The reaction was stopped with 3 μl of 95% formamide, 20 mM EDTA, pH 8.0, containing 0.01% each xylene-cyanol and bromophenol blue. Track 1-4 (controls) were obtained as a result of reaction catalyzed by HIV reverse transcripase (3 activity units, 20 min, 37°C) in the presence of 0.5 μM ddATP (1), 1 μM ddGTP (2), 1 μM ddCTP (3) and 2 μM ddTTP (4), respectively.

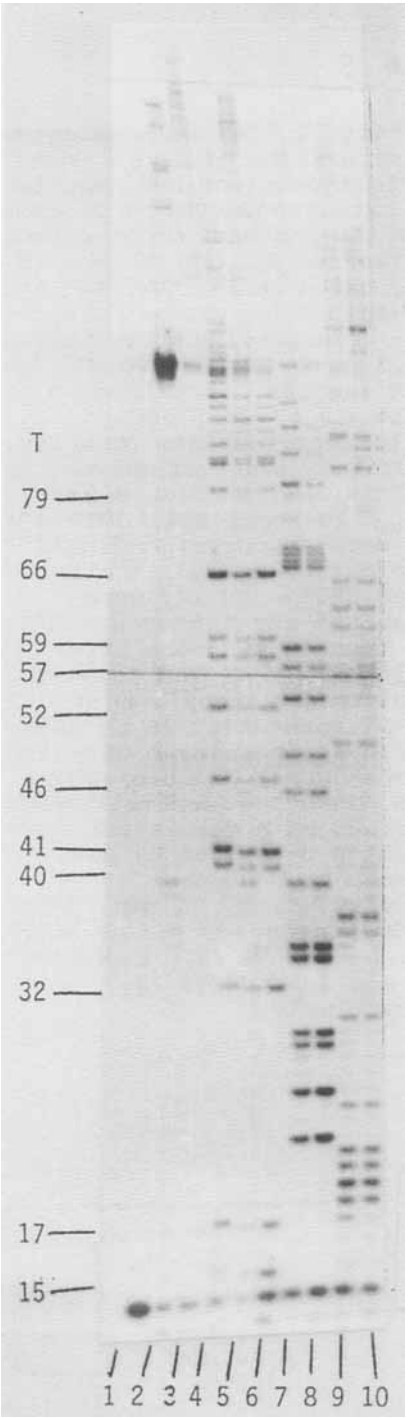


Figure 3. The autoradiogram of separation of [^{32}P]oligonucleotides. The reaction was catalyzed by DNA polymerase ϵ. Samples (5 μl), containing 0.05 pmole (~0.07 μCi) of the [^{32}P]primer-template complex, 1 activity unit of the enzyme, 15 mM Tris.HCl, pH 7.4, 8 mM $MgCl_2$, 20 μg/ml BSA, 0.3 mM EDTA, all other conditions as in the legend to Fig.2 with exception of second incubation time (10 min). Track 1 - primer-template + enzyme without incubation; 2 - the same + substrates and first incubation; 3 - as in 2 + second incubation; 4 - as 3+ ddTTP(3'NH_2) (20-fold excess over dTTP); 5 and 6 - as 3 + Ib (20- and 120-fold excess, respectively); 7 and 8 - as 3 +Ic (12- and 40-fold excess over dCTP); 9 and 10 - as 3 +Ia (40- and 120-fold excess over dATP).

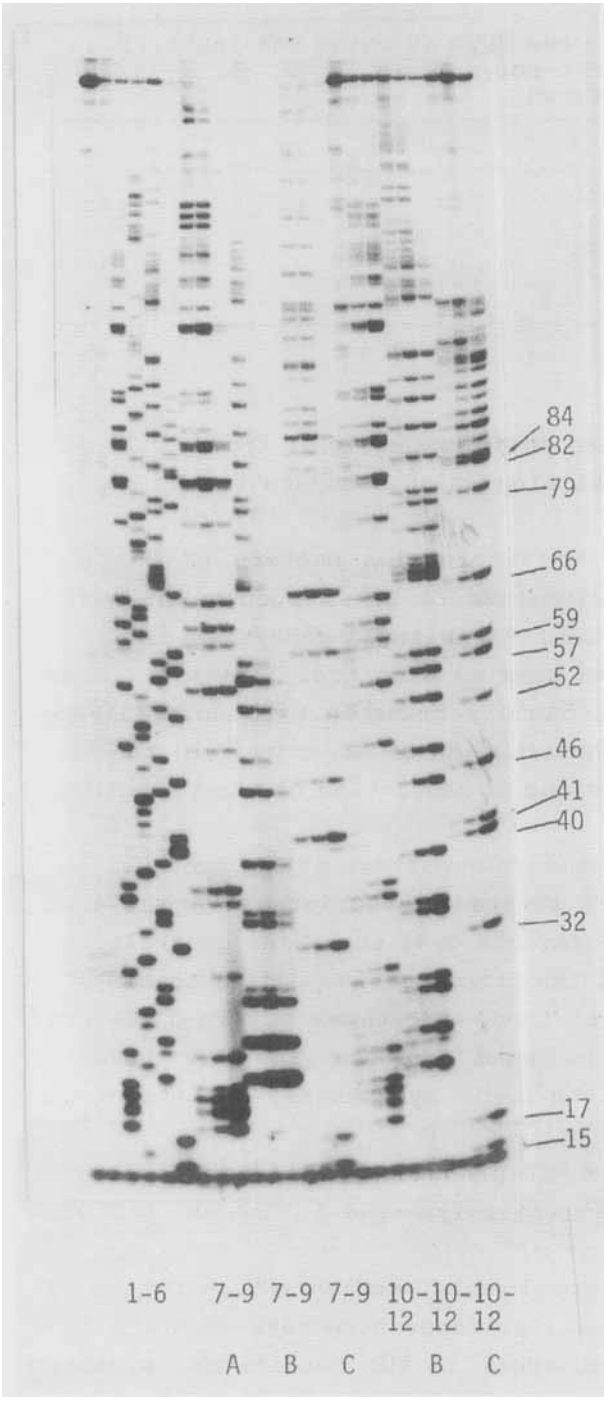


Figure 4. The autoradiogram of separation of [^{32}P] oligonucleotides. Reaction was catalyzed by the HIV (tracks 1-9 in the series A,B and C) and AMV (tracks 10-12, series A,B and C) reverse transcriptases in the presence of compounds Ia (A), Ib (B) and Ic (C), taken in 0.5, 2, and 10 fold molar excess over the corresponding dNTP (tracks 7 and 10, 8 and 11, 9 and 12, respectively). Samples (6 μl), containing 0.05 pmole (~0.07 μCi) [^{32}P]primer-template, 3 activity units of each enzyme, 10 mM Tris.HCl, pH 8.2, 5 mM $MgCl_2$, 40 mM KCl and 1 mM dithiotreitol (for HIV enzyme 2% glycerol, 0.05% Triton X-100, 0.05 μM EDTA were also added), a substrate of the same base nature as terminator 1 μM, the another three - 20 μM each. Probes were incubated for 20 min at 37°C and then for 20 min more after adding excess of dNTP. The reaction was stopped with 3 μl of the dyes containing formamide. Track 1 - a complete system without terminators; 2 - the same with additional incubation in the presence of four dNTP (200 μM); 3,4,5 and 6 - as 2 + 0.5 μM ddATP, 1 μM ddGTP, 1 μM ddCTP and 2 μM ddTTP, respectively.

Table

Molar ratios of acyclo-d_4NTP to the dNTP at which 50% inhibition of DNA synthesis catalyzed by DNA polymerases I, α, ß, ϵ and HIV reverse transcriptase are observed

Enzyme	Ia	Ib	Ic
DNA polymerase I	700	-	700
α	10	16	14
ß	14	20	-
ϵ	20	20	-
HIV reverse transcriptase	1.1	0.9	1.2

nucleotide with the similar base structure as in I) from 2 till 200 (mol/mol) sharply decreases DNA elongation: compare tracks 5-8 in each series.

Fig.3 points out a similar termination pattern of the DNA synthesis catalyzed by DNA polymerase ϵ. The structure of newly synthesized DNA is clearly seen. 3'-Amino-3'-deoxythymidine 5'-triphosphate [ddTTP(3'NH_2)] was used as a control (track 4). Some difference in mobility of the bands terminated with ddTTP(3'NH_2) and Ic is due to the charge contribution of NH_2-group (in the NH_3^+ form) of the aminothymidine residue at the 3'-end of the terminated DNA fragment.

Fig.4 demonstrates the termination pattern of DNA synthesis in the systems with the HIV and AMV reverse transcriptases in the case of Ia, Ib and Ic action. ddNTP (tracks 3-6) served as controls. As is seen from the comparison of the tracks corresponding to HIV (7-9) and AMV (10-12), almost all bands for three acyclo-d_4NTP are present in the gel (only the band T57 for the HIV reverse transcriptase is absent) and the newly synthesized DNA structure can be interpreted.

At the same time acyclo-d_4NTP practically did not terminate the DNA synthesis, catalyzed by DNA polymerase I (the gel pattern is not shown).

The Table points out the acyclo-d_4NTP concentration ratios to those of substrates with the similar bases structure when the 50% DNA synthesis inhibition is observed in the conditions, similar with the described in the methods. As is seen from the Table, the synthesis catalyzed by the DNA polymerases α, ß and ϵ is effectively inhibited by acyclo-d_4NTP, and in case of HIV reverse

transcriptase the effect was even by 15-20 times stronger. Approximately the same inhibition levels can be seen from the termination patterns. Optimum ratios of the termination substrate concentrations to those of dNTP with the nucleic base of the same structure, necessary for obtaining rather long DNA fragments, were 10-50 for DNA polymerases α and ε and 2-10 for reverse transcriptase.

DISCUSSION

The obtained results clearly show that acyclo-d_4NTP reveal the termination substrate properties relative to a broad set of DNA polymerases - α and ε from human placenta, ß from rat liver, TdT from calf thymus and HIV and AMV reverse transcriptases. The only exception is DNA polymerase I from *E.coli* which neither incorporate the acyclo-d_4NTP nucleotide residues into the DNA strand 3'-end, nor inhibit the DNA synthesis.

Such data are rather unusual, especially concerning the α type of DNA polymerases. Up to present moment only three groups of effective termination substrates for these very specific enzymes have been known. These are ddNTP(3'NH_3)[9], 3'-amino-3'-deoxyarabinonucleoside 5'-triphosphates[10] and arabinonucleoside 5'-triphosphates[11] (the last group of compounds able not only to be incorporated into 3'-end of DNA strand, but to be elongated further, although at a much lower rate). For this reason it was suggested that DNA polymerases α recognize substrate analogs only if they contain at their 3'-position a group able to be proton donor (HO, NH_2)[12]. As is seen from the above results the situation with DNA polymerases α is more complicated, and although the termination activity of acyclo-d_4NTP does not exclude recognition of a 3'-substituent in the substrate analog by the enzyme, it becomes evident that some other parameters of substrate analogs are also rather important. We think that this is conformation of the sugar residue or its substitute.

It seems interesting to find out which conformation elements of acyclo-d_4NTP as well as of other compounds with conformational restrictions of their carbohydrate residues, are essential for activity as termination substrates. We have suggested earlier that such contribution is due to the carbohydrate residue conformation flattening[1], especially because natural dNTP complexed with *E.coli* DNA

polymerase I also undergo conformational rearrangement, resulting in significant flattening the deoxyribose residue[13,14]. In fact, the X-ray analysis has confirmed flattening of the furanose ring in 2',3'-dideoxy-2',3'-didehydronucleosides[15-17] and some other termination substrates of some DNA polymerases, such as 2',3'-riboanhydroadenosine[18] and 2',3'-lyxoanhydrothymidine[19]. Their termination properties are described in[20,21]. However, all these cyclic nucleosides represents quite rigid molecules because of their furanoside structure, and this, possibly, makes more difficult their conformational transformations, necessary for the complete enzymatic cycle of their incorporation into 3'-end of DNA strand, namely formation of the reaction-competent complex and catalytic action. We can think that during such single DNA elongation step substrate undergoes a series of conformational rearrangements, for which both are essential, the initial substrate or its analog conformation and its definite conformational flexibility. It is possible that the combination of pentene residue planar conformation in acyclo-d_4NTP (depending on the *cys*-double bond properties) with a definite conformational flexibility due to the absence of cyclization explaines high effectivity of acyclo-d_4NTP as termination substrates.

We would like to stress that detecting the termination substrate properties in acyclo-d_4NTP disclose the investigation of a new family with potential antiviral and anticancer feature.

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