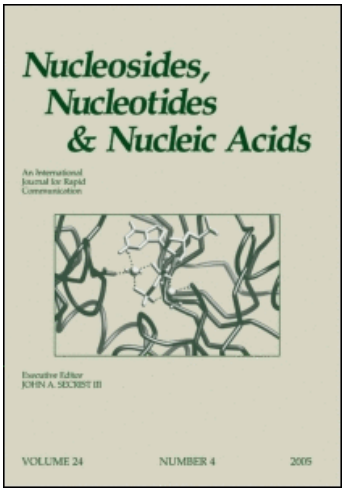

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dNTP Modified at Triphosphate Residues: Substrate Properties Towards DNA Polymerases and Stability in Human Serum

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REVIEW

dNTP MODIFIED AT TRIPHOSPHATE RESIDUES: SUBSTRATE PROPERTIES TOWARDS DNA POLYMERASES AND STABILITY IN HUMAN SERUM

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ABSTRACT: Substrate and terminating substrate properties of dNTP with phosphate groups replaced by phosphonates at α-, γ-, β,γ-, and α,β,γ-positions towards different human DNA polymerases and retroviral reverse transcriptases are reviewed. Substitution of the phosphate group by the phosphonate at any of the three phosphate positions of dNTP increased their stability towards dephosphorylating enzymes of human blood. In some cases hydrophobicity of these compounds was markedly enhanced.

It is well known that dNTP are characterized by a high elimination rate of phosphate residues in the presence of various cellular phosphatases, phosphoesterases and nucleotidases. It is dictated by the role of dNTP in cells and intercellular media. Another property of dNTP is increased hydrophilicity resulting from high acidity of dNTP phosphate residues in biological liquids.

Abbreviations: HIV, AMV and Mo-MLV - human immunodeficiency, avian myeloblastosis and Molony murine leukemia viruses, respectively; RT - reverse transcriptase; TDT - calf thymus terminal deoxynucleotidyl transferase; AZT - 3'-azido-2',3'-dideoxythymidine and AZTTP - its 5'-triphosphate. All DNA polymerases if not stated otherwise, are from human placenta.

This paper is dedicated to the memory of Professor Tsujiaki Hata

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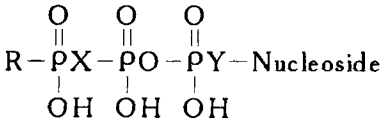
These two properties define that dNTP practically are not able to penetrate into cells. This prohibits their use in cell biology and biological systems other than model cell-free ones.

If one succeeded in designing stable and diffusing into cells modified dNTP it could made possible to transfer these compounds to intracellular targets. The dNTP bearing ligand and reporter groups and possessing the properties indicated above could be of use for studies dealing with labelling DNA fragments synthesized in the selective processes of replication, repairing, reverse transcription and recombination.

Application of stable dNTP for design of medical drugs (antivirals, anticancer drugs and others) also opens some new possibilities. It allows to prepare substrate inhibitors independent on properties and activity of intracellular phosphorylating enzymes. It might provide nonstimulated immune cells with antimetabolites of DNA synthesis, rather than their precursors, which could result in inhibition of virus replication both before and after penetration of HIV genetic material in intracellular media.

As a dNTP triphosphate chain modification we selected the replacement of phosphate residues by different types of phosphonate groups with the enzymatically nonhydrolyzed P-C bond. There were two main reasons for such a choice. i. The replacement of phosphate units by phosphonates leads to new chemical properties, high chemical stability towards dephosphorylating enzymes in particular. ii. Variability of chemical types of phosphonates of different electron structure and bond length allows to investigate their combinations and to select most suitable for DNA polymerases as well as for other types of enzymes. These modifications are also favourable for attaching ligand groups to the modified dNTP. It should be kept in mind that the anhydride bond in dNTP between P-α and P-β has to be preserved because it is the bond that provides the possibility of incorporation of modified dNMP into the growing DNA chain.

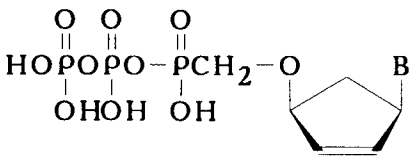
We present herein the data on evaluation of substrate properties of nucleoside 5'-triphosphate analogs of the general structure A:



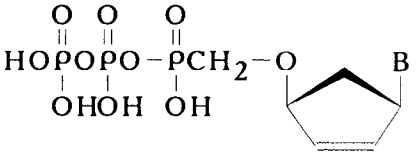
A

1. Modification of the α-phosphate

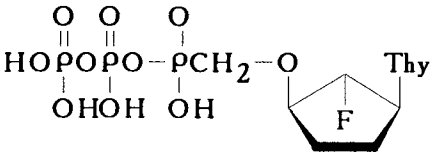
There are several types of modifications at the P-α atom together with the glycone modification known for nucleoside analogs.



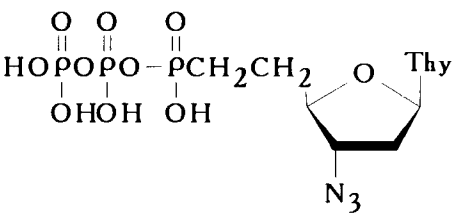
D-I



L-I



II

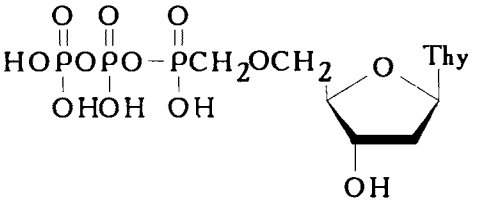


III

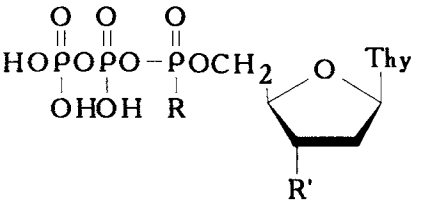
The cyclopentene analogs of dNTP both of D- (D-I) and L-series (L-I) (B =Ade, Gua) and II proved to be effective terminating substrates for HIV RT [1-3], whereas nucleotide III demonstrated significantly more poor substrate properties [4]. Affinity of these compounds towards other DNA polymerases was not evaluated. The theoretical basis for the similar activity of D- and L-compounds towards RTs was given in [5].

Recently it was demonstrated that both D-I and L-I (B=Ade) efficiently terminated the DNA chain elongation at the catalysis by HIV and AMV RTs [6,7]. At the same time compound IV, which is nonisosteric to natural dNTP, was selectively recognized by mammalian DNA polymerase α

and calf thymus DNA polymerase δ, but its incorporation into the growing DNA chain by the latter enzyme took place only in the presence of PCNA (proliferating cell nuclear antigen) [8,9].



IV



V

These data demonstrate that the substitution of the α-phosphate by the phosphonate unit in modified dNTP isosteric to natural ones preserves their substrate properties at least towards retroviral RTs.

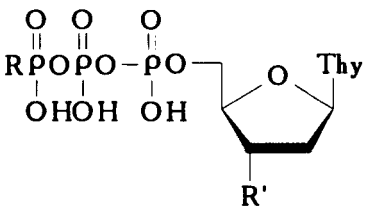
Substrate properties of compounds V (R'=OH) and terminating substrate ones of V (R'=N_3) with R=Me [10] and Ph [11] should also be mentioned. However, the affinity of V towards different DNA polymerases was significantly lower than that of natural dNTP.

Thus, the replacement of 5'-O- and 5'-CH_2- in dNTP does not significantly affect the affinity of such nucleoside 5'-phosphonate diphosphates in the reactions catalyzed by RTs.

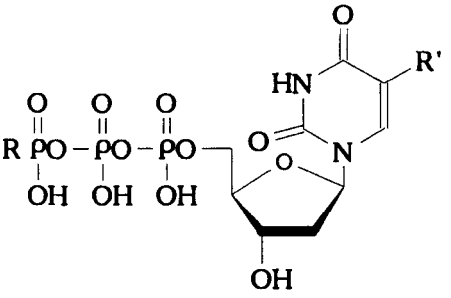
2. Substitution at the γ -phosphate

The effect of the γ-phosphate modification to dNTP substrate properties towards different DNA polymerases was studied only for γ-phosphate esters and amides. No characteristic properties were noted, probably due to the presence of esterase and amidase impurities which are usually not tested in DNA polymerases preparations (for references see [12]).

We have synthesized a series of thymidine γ-phosphonyl-α,β-diphosphates VIa,b (R'=OH) and their 3'-azido-2',3'-dideoxy counterparts VIc,d (R'=N_3) [12].



VIa, R=Ph, R'=OH
VIb, R=Me, R'=OH
VIc, R=Ph, R'=N_3
VId, R=Me, R'=N_3



VIIa, R'=CH_3, R=$N_3CH_2CH_2$-
VIIb, R'=CH_3, R=$NH_2CH_2CH_2$-
VIIc, R'=CH_3, R=DNP*-$NHCH_2CH_2$-
VIId, R'=CH_3, R=DNP*-$NH(CH_2)_5CONHCH_2CH_2$-
VIIe, R'=$CH_2O(CH_2)_6N_3$, R=C_6H_5
VIIf, R'=$CH_2O(CH_2)_6NH_2$, R= C_6H_5
VIIg, R'= $CH_2O(CH_2)_6N_3$, R=OH

where *DNP - 2,4-dinitrophenyl

Compounds VIa and VIb with R=Ph, Me showed substrate activity rather similar to that for natural dTTP. Phosphonate diphosphates VIc and VId also efficiently terminated DNA synthesis catalyzed by HIV and AMV RTs (Table 1). Moreover, selectivity of VIc and VId, especially for R=Ph, towards RTs is higher than that of AZTTP [12].

Modified triphosphates VIIa-VIId as well as VIIg proved to be good substrates for AMV RT whereas VIIe-VIIf affinity to DNA synthesizing complexes was decreased by 100-fold (paper in preparation).

The results obtained indicate that HIV and AMV RTs have no sterical obstracles for binding with modified dNTP bearing a bulky substituent at the γ-position.

It should be mentioned that γ-alkylphosphonates and γ-aryl or arylalkylphosphonates in particular, were markedly more hydrophobic than the corresponding dNTP. Their hydrophobicity is similar to that of corresponding mononucleotides according the TLC and HPLC data (see below, Table 5).

Nucleoside diphosphate γ-phosphonates possess another useful property. They reveal increased stability towards dephosphorylating enzymes. Enzymatic

TABLE 1. Kinetic parameters for VI in one-step elongation reaction catalyzed by AMV RT*

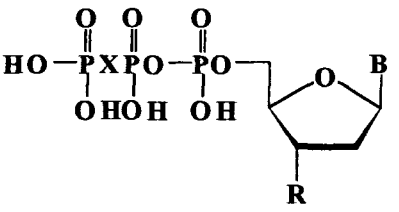
Compound	Km, μM	V_{max}/V_{max}dTTP	V_{max}/Km
VIa	3.0	0.78	0.26
VIb	4.4	0.74	0.17
VIc	20	0.68	0.03
VId	10	0.68	0.07
dTTP	1.3	1	0.77
AZTTP	0.96	0.92	0.96

*According to [6]

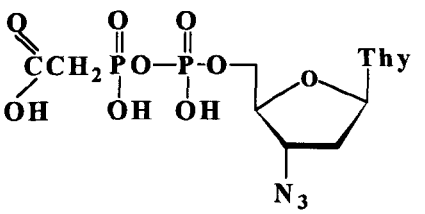
degradation of VIa and VIc in human serum proceeds by about 10 fold slower than that for dTTP [12].

3. Substitutuon at β,γ-diphosphates

The first data on terminating substrate activity of 3'-azido-2',3'-dideoxythymidine 5'-(β,γ-methylenediphosphonyl)-α-phosphate (VIIIa) in the reactions catalyzed by HIV and Mo-MLV RTs were published in 1988. It was shown to be 100-350-fold less active than AZTTP [13]. Substrate properties of the corresponding 2'-deoxyguanosine derivative (VIIIb) and 2'-deoxyguanosine 5'-(β,γ-difluoromethylenediphosphonyl)-α-phosphate (VIIIc) were close to those for dGTP towards DNA polymerase III from *Bacillus subtilis*, but significantly weaker than towards DNA polymerase from *E.coli* and α from calf thymus [14].



VIIIa, B=Thy, X=CH_2, R=N_3
VIIIb, B=Gua, X=CH_2, R=OH
VIIIc, B=Gua, X=CF_2, R=OH
VIIId, B=Thy, X=CBr_2, R=N_3



IX

VIIIe, B=Thy, X=CBr_2, R=OH
VIIIf, B=Thy, X=CF_2, R=OH
VIIIg, B=Thy, X=CHF, R=OH
VIIIh, B=Thy, X=CFMe, R=OH
VIIIi, B=Thy, X=CF_2, R=N_3
VIIIj, B=Thy, X=CHF, R=N_3
VIIIk, B=Thy, X=CFMe, R=N_3

Triphosphate analogs, 3'-azido-2',3'-dideoxythymidine 5'-(β,γ-dibromomethylenediphosphonyl)-α-phosphate (**VIIId**) and 3'-azido-2',3'-dideoxythymidine 5'-(β-carboxymethylenephosphonyl)-α-phosphate (**IX**), were shown to be terminating substrates for HIV RT and their activity can be arranged as follows: AZTTP : VIIId : IX as 1 : 3 : 17 [15]. Later similar results were obtained for HIV RT in another cell-free system [16].

More systematic study has been performed by us recently [17]. Significant effect of the X structure on substrate activity was demonstrated for 5'-(β,γ-methylenediphosphonyl)-α-phosphates of thymidine (**VIIIe,f,g,h**) and 3'-azido-2',3'-deoxythymidine (**VIIId,i,j,k**). Moreover, this activity varied for different DNA polymerases.

Compounds **VIIIe,f,g,h** were tested as substrates and **VIIId,i,j,k** - as terminating substrates. For AMV RT activity both for **VIIIe,f,g,h** and for **VIIId,i,j,k** was decreased in the order X: CF_2 = CHF > CBr_2 > CFMe (Table 2). For TDT, they may be arranged as follows: CF_2 = CBr_2 = CHF > CFMe. For DNA polymerase α, the relationship was different: CF_2 = CHF > CFMe >> CBr_2. For DNA polymerase β, substrate activity of **VIIIf,g,h** decreased in the following order: CHF > CF_2 > CFMe; **VIIIe** was neither a substrate nor an inhibitor of this enzyme.

The results obtained led to the following conclusions.

a. Modification of the β,γ-pyrophosphate in the triphosphate residue of dNTP allows one to modulate their substrate activity and selectivity towards different types of DNA polymerases.

b. Possibilities for direct interpolation of the data on the activity of β,γ-modified dNTPs from one DNA polymerase to another one are limited,

TABLE 2. Kinetic parameters for VIII in one-step elongation reaction catalyzed by AMV reverse transcriptase*

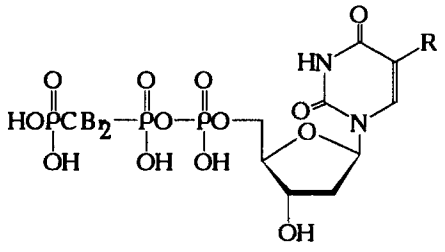
Compound	Km, μM	V_{max}/V_{max}dTTP	V_{max}/Km
VIIIe	2.61	0.51	0.19
VIIIf	1.25	0.81	0.65
VIIIg	1.30	0.98	0.75
VIIIh	11.38	1.15	0.10
VIIIi	0.98	0.52	0.53
VIIIj	10.34	0.82	0.08
VIIIk	12.31	1.42	0.11
dTTP	1.02	1	0.98
AZTTP	0.76	0.68	0.89

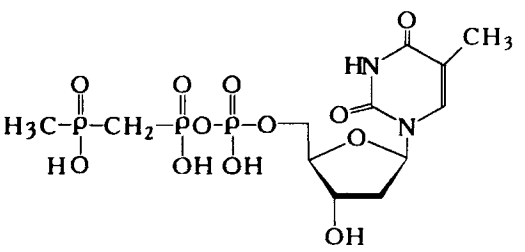
*According to Ref. [6]

probably due to differences in the structure of triphosphate binding sites of DNA polymerase active centers.

c. The lack of activity of DNA polymerase β did not utilize dTTP with bulky modifications at the γ-phosphate residue [12]. 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.

Another group of compounds modified both at the thymine and β,γ-diphosphate residues (Xa-e, XI) are presented below.





Xa, R=$CH_2O(CH_2)_6N_3$
Xb, R=$CH_2O(CH_2)_6NH_2$
Xc, R=$CH_2O(CH_2)_6NHCO(CH_2)_5$NH-Biotinyl
Xd, R=$CH_2O(CH_2)_6$NH-Flu*
Xe, R=$CH_2O(CH_2)_6$NH-TMR**
*Flu - fluoresceinyl; **TMR - tetramethylrhodaminyl

XI

Modified both at the nucleic base and γ-phosphate Xa was utilized by AMV RT, with the affiinity lower than that of VIIIe. After incorporation of Xa the growing DNA chain could be elongated only once. Replacement of the azido-group in Xa by the amino group results in total loss of of substrate activity by Xb. Compounds Xc-e were not recognized AMV RT either.

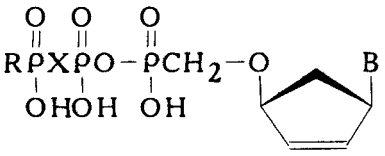
The β,γ-phosphonate analog of dTTP (XI) bearing the γ-phosphinate was utilized by AMV RT although 50-100-fold less efficiently than dTTP. Primer extention catalyzed by HIV RT in the presence of XI was also shown but its incorporation was one order of magnitude less effective than in the case of AMV RT.

At the same time X and XI were not recognized by mammalian DNA polymerases α and β, as well as TDT.

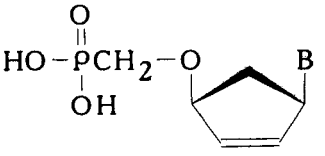
4. Substitution at α,β,γ-phosphates

The next group of compounds includes modified nucleotides in which all the three phosphate residues are substituted by phosphonates (XII). These compounds bear the only anhydride bond sensitive to enzymatic hydrolysis. This bond is nessessary for nucleotide residue incorporation into the DNA chain. Synthetic routes for compounds XII were reported in [18].

The experiments in model cell free systems with DNA polymerases demonstrated that triphosphonates XII were potent substrates for HIV and AMV RTs (Table 3). The affinity of XIIa-d towards DNA-synthesizing complexes was one order of magnitude lower than that of natural dNTP, D-I and L-I. DNA polymerase



XIIa B=Ade, X=CBr_2, R=OH
XIIb B=Ade, X=CF_2, R=OH
XIIc B=Gua, X=CBr_2, R=OH
XIId B=Gua, X=CF_2, R=OH
XIIe B=Ade, X=CH_2, R=Me



XIIIa B=Ade
XIIIb B=Gua

TABLE 3. Kinetic constants for XIIc,d in primer extention reaction catalyzed by AMV RT [6]

	Km, μM	Vmax/Vmax for ddGTP	Vmax/Km
XIIc	0.17	0.85	5.1
XIId	0.22	2.68	12.2
L-I, B=Gua	0.017	0.86	50.6
ddGTP	0.005	1	200

α and β did not recognize XIIa-e. In the case of TDT the replacement of the β,γ-diphosphate by dibromo- or difluorodiphosphonate units decreases terminating properties of the compounds by ten-fold. The triphosphonate XIIe is more than 100 times a weaker substrate than XIIa-d.

Thus triphosphonates of XIIa-d structure selectively inhibit DNA synthesis catalyzed by RTs and TDT and do not affect the process catalyzed by DNA polymerases α and β.

Decrease in activity of modified at the triphosphate dNTP may be a result of partial electronic and steric dissimilarity of the phosphonate groups in respect to the phosphate one [19].

5. The effect of the substitution of phosphates in dNTP by phosphonates on their stability and hydrophobicity

Table 4 demonstrates the data on enzymatic stability of compounds XII in human blood serum. Hydrolysis of XIIa and XIIb was shown to be 70-200 times as slow as that for the corresponding natural dNTP.

Significant increase of hydrophobicity of dNTP γ-phosphonates should be mentioned individually [12]. As can be seen in Table 4, HPLC retention time of VId and XIIa-d is more than that of AZT 5'-phosphate but less than for AZT. Values of Rf at TLC control for VIIa, VIIc-f, Xd and XI also evidence about increased hydrophobicity of the compounds under discussion (Table 5). It can be a result of the presence of hydrophobic residues in the γ-position of dNTP and decreased acidity of phosphonic groups if to compare with phosphate ones.

TABLE 4. Half life time of compounds XII in blood serum and retention time at HPLC*

Compound	Half-life time	Retention time, min*
XIIa	110 hours	17.7
XIIb	30 hours	17.5
XIIc	31 hours	16.4
XIId	45 hours	16.3
dATP	<30 min	12.6
dGTP	<30 min	11.6
dTTP	5 min	5.5
VIa	55 min**	18**
AZTTP	5 min**	7**
AZT 5'-phosphate		11**
AZT		24**

*reversed phase HPLC, Nucleosil 120 C18 column (4 x 150 mm, 5 μ), eluent -a linear gradient of methanol from 0 to 7.5% in 0.01 M buffer of potassium dihydrogenphosphate for 25 min. The flow rate - 0.7 ml/min. **According to [12]

TABLE 5. R_f values of the synthesized compounds, TLC data*

Compound	Dioxane-25% NH_4OH-water 4:1:2 (v/v)	i-PrOH-25% NH_4OH-water 7:1:2(v/v)
VIIa	0.41	0.27
VIIb	0.14	0.09
VIIc	0.43	0.23
VIId	0.47	0.21
VIIe	0.38	0.32
VIIf	0.29	0.11
dUTP, dTTP, AZTTP	0.04	0.00
VIIg	0.08	
dUTP($5CH_2O(CH_2)_6NHCO(CH_2)_5NHBio$	0.15	
dUMP($5CH_2O(CH_2)_6N_3$)	0.48	0.35
dTMP	0.40	0.21
AZTMP	0.56	0.35
Xa	0.08	
Xb	0.04	0.00
Xc	0.14	
Xd	0.22	
Xe	0.12	
XI	0.22	0.14

*TLC plates Kieselgel 60 F_{251} were from Merck. Visualization was made at 254 nm.

Preliminary data demonstrate that some of the triphosphonates XII inhibit HIV (data of J. Balzarini, E. De Clercq) and Mo-MSV replication (data of L. Goryunova and R. Beabealashvilli). Antiviral activity of XII in the case of Mo-MSV was 1-2 orders of magnitude higher than that of the corresponding monophosphonates XIII used as controls. It supports the hypothesis on the direct inhibitory effect of XII on virus replication. If XII were hydrolyzed to XIII in cell cultures with subsequent intracellular diphosphorylation to the compounds of L-I type the activity of the latter could not be higher than that of XIII.

Summarizing the data obtained one can conclude that modified nucleoside triphosphonates can represent potential anti-HIV drugs active both in virus infected cells and in early steps of virus replication before its penetration into cells.

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