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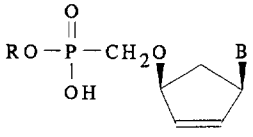
MODIFIED TRIPHOSPHATES OF CARBOCYCLIC NUCLEOSIDE ANALOGUES: SYNTHESIS, STABILITY TOWARDS ALKALINE PHOSPHATASE AND SUBSTRATE PROPERTIES FOR SOME DNA POLYMERASES

Natalya Dyatkina, Elena Shirokova*, Fritz Theil[a], Stanley M. Roberts[b], Alexander Krayevsky

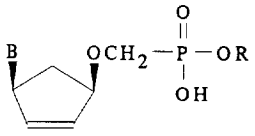
Engelhardt Institute of Molecular Biology Russian Academy of Sciences, Vavilov 32, Moscow 117984, Russia;
[a]Centre of Selective Organic Synthesis, Rudower Chaussee 5, D-12484 Berlin-Adlershof, Germany;
[b]Department of Chemistry, Liverpool University, P.O. Box 147, Liverpool L69 3BX, UK

Abstract New triphosphate derivatives of carbocyclic nucleoside analogues have been synthesized and shown to be potent substrates for terminal deoxynucleotidyltransferase and/or HIV reverse transcriptase; the compounds are stable to dephosphorylation with human placental alkaline phosphatase.

Today it is obvious that the active form of the approved anti-AIDS drugs of nucleoside origin are the corresponding triphosphates. The latter are efficiently utilized by human immunodeficiency virus reverse transcriptase (HIV RT) and terminate further elongation of the proviral DNA chain. Nucleoside triphosphorylation is performed by cellular nucleoside kinases and nucleotide kinases or nucleotidases. It is the high specificity of these enzymes that can explain the fact that most nucleoside analogs are not active in cells while their triphosphates are powerful inhibitors of DNA biosynthesis catalyzed by HIV RT in cell-free experiments. Unfortunately nucleoside 5'-triphosphates cannot be used as drugs, mainly because of their rapid dephosphorylation. The aim of our investigation is the synthesis of carbocyclic derivatives of nucleoside 5'-triphosphates bearing the only one hydrolysable bond, i.e. between the P_α and P_β - atoms.



D1a-D3c



L1a-L3c

a B=Gua, b B=Ade; 1, R=H; 2, R=P(O)(OH)OP(O)(OH)$_2$; 3, R=P(O)(OH)CBr$_2$P(O)(OH)$_2$
D and **L** correspond to the compounds mimicking D- and L-nucleotides, respectively

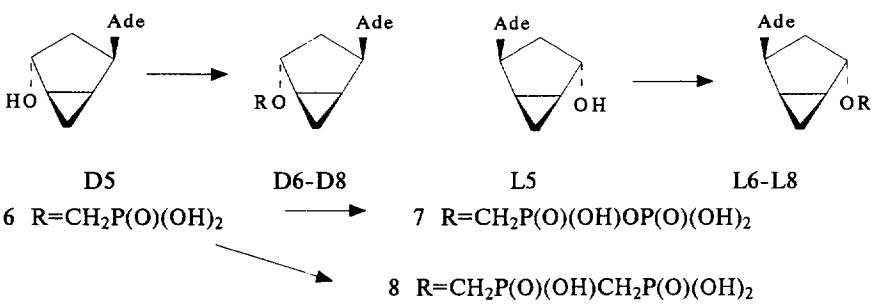
fax 7 095 135 4 14 05 E.mail aak@imb.imb.ac.ru

One of the approaches for obtaining enzymatically stable analogs of nucleoside triphosphates is the replacement of a P-O-P - bond by a P-C-P unit[1,2]. Carbocyclic phosphonates **1a,b** taken as starting materials for our synthesis were ineffective as antiviral agents in cell culture experiments[3]. This could be due to their failure to be phosphorylated by cellular enzymes and by adenylate kinase in particular[4]. The corresponding phosphonatediphosphates **L2a,b** as well as their enantiomers **D2a,b** have been evaluated previously, and shown to be potent terminating substrates of HIV RT[3,5]. We hoped that the compounds **L3a,b** with the β ,γ - dibromomethylenepyrophosphonate residue would combine enzymatic stability with good substrate properties towards HIV RT. The efficiency of such modification in the polyphosphate chain of 3'-azido-3'-deoxythymidine 5'-triphosphate (AZTTP) has been demonstrated earlier[6]. We present here the data for the compounds **L3a,b**[7], prepared by coupling of phosphonates **L1a,b** with dibromomethylene pyrophosphonate according to the procedure described previously in[8].

D4 L4

The compounds with the above -described properties are of great interest not only as potential antivirals but as tools for the investigation of different DNA polymerases in cell culture, and of terminal deoxynucleotidyltransferase (TDT) in particular. Little is known about the role of the latter enzyme in the cell[9]. We have described earlier that triphosphates **D4** and **L4** imitating α-D and α-L nucleotides, respectively, are specific terminating substrates for this enzyme[5]. With the goal of evaluation of new selective substrates that are stable to metabolic degradation we also investigated the triphosphonates **D7** and **D8** and their enantiomers **L7** and **L8**. Phosphonates **D6** and **L6** as well as triphosphosphonates **D7**, **L7**, **D8** and **L8**[11] were prepared as described earlier[8] from the corresponding α-carbonucleosides **D5** and **L5**[10] (scheme 1).

Scheme 1



The newly synthesized triphosphate analogs **L2**, **L3**, **L7**,**L8**, as well as their enantiomers **D2**, **D7**, and **D8** were evaluated as HIV RT and TDT substrates in a model cell-free system. The assay mixture contained ^{31}P-labelled template-primer, the compound under study, modified dNTP, the other three dNTPs, the enzyme and the appropriate buffer. The replacement of the bridging oxygen in **L2a,b** with the CBr_2-group resulted in a decrease

of affinity of **L3a,b** towards HIV RT by a factor of 5-10. This value agrees with previously obtained data for β,γ-methylenepyrophosphonates in the AZT series[12]. The affinity of **L3a,b** towards TDT falls by 2-5-fold.

Triphosphonate carboanalogs **D7, L7, D8, L8** revealed selective substrate properties towards TDT. They were less effective terminating substrates than triphosphates **D4** and **L4** by a factor of 4-5. It can be noted that replacement of inner oxygen by the CH_2-group in the β,γ-pyrophosphate residue does not affect the substrate properties of **D8** and **L8** when compared to **D7** and **L7**.

Enzymatic stability of thymidine 5'-triphosphate analogs has been previously studied in human blood serum[13]. We found that purine derivatives **2a,b, 3a,b, 7, 8** together with dATP and dGTP are not degraded over several days under these conditions whereas AZTTP has a half life time less than 5 min.

Alkaline phosphatases are known to be very active dephosphorylating enzymes. The carbocyclic analog of dGTP was shown to be a substrate for this enzyme[14]. We used human placental alkaline phosphatase[15] containing 7.5 mg protein/mL (20 units act./mL). The reaction was carried out at 37°C in 125 mM Tris HCl buffer containing 15 mM $MgCl_2$. The reaction mixture was analyzed by HPLC on a Alltech Hypersil APS (NH_2) column (250 x 4.6 mm). Elution was effected using a linear gradient of A-->B for 25 min., where A: 0.2 M KH_2PO_4 + 15v/v % MeOH; B: 1M KH_2PO_4 (pH 6.5). The extent of hydrolysis was assessed by measuring the amount of the starting triphosphate remaining. The half life time of the tested compounds is given in Table 1.

Table 1. Stability of the tested compounds to dephosphorylation

Compound	Half life time
AZTTP	<1 min
dATP	50 min
dGTP	1 hour
L2a	4 hours
L2b	3 hours
D2b	2 hours
D7, L7	4.5 hours
L3a, L3b, D8, L8	>7 days

*The triphosphate content in the reaction mixture after incubation with the enzyme was 90% relative to its concentration in the control assay (pH 9.8, 7 days, 37°C)

It can be seen that purine triphosphates are 50 times more stable towards alkaline phosphatase action that TTP. Replacement of the 2'-deoxyribosyl-5'-phosphate residue by the cyclopentenyloxyphosphonate one further increases their stability to dephosphorylation by 2-4-fold, the difference between the D-and L-series being insignificant. Introduction of the CBr_2-fragment instead of the bridging oxygen in the β,γ-pyrophosphate dramatically enhances the stability of the modified triphosphates towards enzymatic hydrolysis.

The results obtained testify that the 5'-triphosphate chain should not be a conserved feature in the structure of any nucleotide for potent inhibition of HIV RT and other DNA polymerases. Moreover, the proper modification of the triphosphate residue opens up new fields for the preparation of hydrolytically stable nucleotides as potential antiviral agents.

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7. **L3a,b**: Yield 25% related to **L2a,b**; ^{31}P NMR (162 MHz, D_2O): 0.15dd (33.1, 15.2, P_β), 8.16d (15.2, P_γ), 10.6d (33.1, P_α); FAB-mass, m/e: 659, 661, 663 [M^++1+NH_3] (**L3a**), 643, 645, 647 [M^++1+NH_3] (**L3b**).
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11. **D7**, **L7**:Yield 25% related to **D6, L6**; ^{31}P NMR (162 MHz, D_2O): -20.5bs (P_β), -8.1d (20.5, Pγ), 11.6d (26.3, P_α); FAB-mass, m/e: 503 [M^++1+NH_3];
D8, **L8**:Yield 20% related to **D6, L6**; ^{31}P NMR (162 MHz, D_2O): +15.4bs (P_γ), 11.2-10.0m (P_α+P_β); FAB-mass, m/e: 501 [M^++1+NH_3].
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