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CARBOCYCLIC PHOSPHONATE ANALOGS OF 2',3'-DIDEOXYADENOSINE-5'-MONOPHOSPHATE AS SUBSTRATES OF 5-PHOSPHORIBOSYL-1-PYROPHOSPHATE (PRPP) SYNTHETASE

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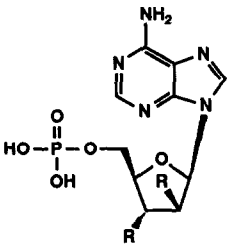
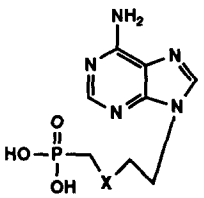
Abstract: Several carbocyclic phosphonate analogs of 2',3'-dideoxyadenosine-5'-monophosphate (ddAMP) were pyrophosphorylated by *E.coli* 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase in the presence of PRPP. Structure-activity relationships are discussed.

Since the discovery of human immunodeficiency virus (HIV) as the agent responsible for acquired immunodeficiency syndrome (AIDS), there has been intense effort to find compounds that selectively block the replication of HIV. Several dideoxynucleosides have proven to be potent and selective HIV inhibitors *in vitro*[1] as for example 2',3'-dideoxyadenosine (ddA) and 2',3'-dideoxyinosine (ddI). Recently, ddI was approved for clinical use by the FDA. ddA and ddI are believed to exert their antiviral effect after intracellular conversion into 2',3'-dideoxyadenosine triphosphate (ddATP) which acts as a potent inhibitor of HIV reverse transcriptase[2]. 2',3'-Dideoxyadenosine monophosphate (ddAMP, **1**, scheme 1) has been identified as a common key intermediate in the metabolic process required for antiviral activity of ddA and ddI[3]. This finding and the fact that phosphonates such as 9-(2-phosphonomethoxyethyl)adenine (PMEA, **3**, scheme 1) are effective antiviral agents *in vivo*[4] led us to design new stable synthetic analogs of ddAMP as potential anti-HIV agents by replacing the oxygen in the sugar ring by a methylene group and by replacing the labile phosphate monoester group by phosphonomethyl, phosphonodifluoromethyl and phosphonomethoxy groups (compounds **5, 7, 8**, scheme 1).

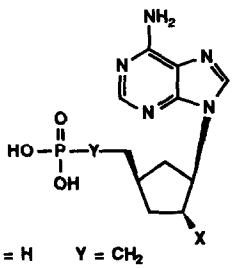
Scheme 1



1. ddAMP (R = H)
2. ara-AMP (R = OH)



3. PMEA (X = O)
4. X = $(CH_2)_2$



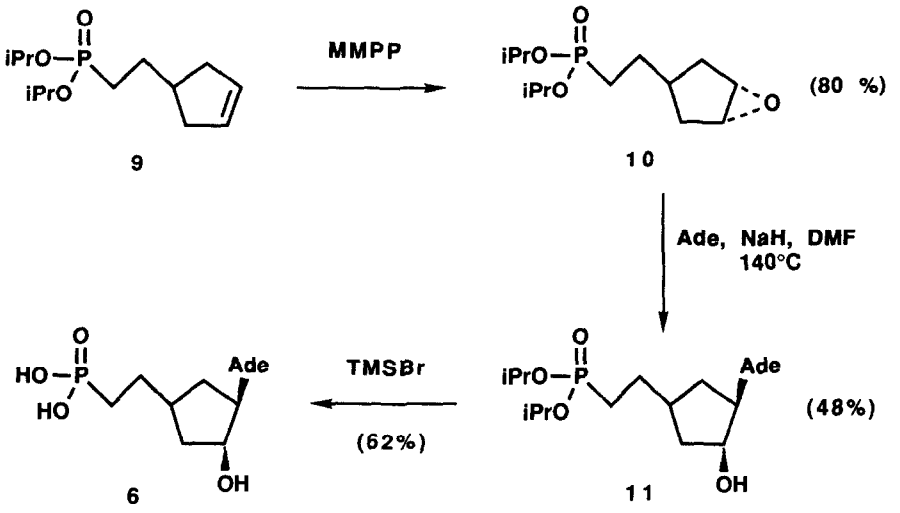
5. X = H Y = CH_2
6. X = OH Y = CH_2
7. X = H Y = CH_2O
8. X = H Y = CF_2

The potential antiviral activity of such compounds is based upon their capability to act as substrates of cellular kinases. The efficiency of conversion into diphosphate derivatives (nucleoside triphosphate analogs) is a critical factor for these mononucleotide analogs to be useful anti-HIV agents since it determines the level of their active form in cells. Ultimately, the antiviral activity is due to a selective inhibition of reverse transcriptase by the nucleoside triphosphate analogs[1].

A possible pathway for phosphorylation of the above mentioned mononucleotide analogs is that involved in the conversion of AMP (or dAMP) into ATP (or dATP). AMP is converted into ADP by adenylate kinase using ATP as the phosphate donor. ADP is subsequently phosphorylated by nucleoside diphosphate kinase, using again ATP as the phosphate donor. Recently, another possible pathway for phosphorylation of AMP analogs was proposed by Balzarini and De Clercq[5,6]. They found that 9-β-D-arabinofuranosyladenine-5'-monophosphate **2** (the 5'-monophosphate of the antiviral drug vidarabine) and the anti-retroviral agent PMEA **3** (scheme 1) are converted directly to their antivirally active form by 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase from *E.coli* through a direct pyrophosphate transfer from PRPP[5,6]. For a better understanding of the potential *in vivo* metabolism of the carbocyclic phosphonates **5, 6, 7, 8** and in an attempt to design more rationally new antiviral agents in the adenine series, we felt that it was important to study these carbocyclic phosphonates as potential substrates of adenylate kinase and of PRPP synthetase. We report here the results of these studies.

The synthesis of compounds **5, 7, 8** was performed as previously reported[7]. 9-(5-Phosphonopentyl)adenine **4** was prepared from adenine and 5-bromo-1-(diethylphosphono)pentane according to a published procedure[8]. The carbocyclic phosphonate derivative **6** was prepared from 2-(3-cyclopenten-1-yl)ethyl phosphonic acid, diisopropyl ester **9**, according to scheme 2.

Scheme 2



Oxidation of **9** with magnesium monoperoxyphtalate (MMPP) produces a 4/1 mixture of epoxydes in which **10** was identified as the major isomer. Condensation of **10** with the sodium salt of adenine gives the expected adduct **11** in 48% yield. The final product **6** was obtained in 62% yield by reacting **11** with excess TMSBr in acetonitrile, aqueous work-up and recrystallization.

The carbocyclic mononucleotide analogs **5, 6, 7, 8** and 9-(5-phosphonopentyl)adenine **4** were first evaluated as potential substrates of adenylate kinase[9] (myokinase). None of these compounds was found to be phosphorylated by adenylate kinase in the presence of ATP even when tested at concentrations at least 4 times higher than the K_m value of AMP (0.13 mM) and in the presence of an enzyme concentration 20 times higher[10] than that used for AMP.

Table I. Inhibition constants and kinetic parameters of AMP analogs for PRPP synthetase.

Compound	As Inhibitor	As Substrate[a]		
	IC_{50} (mM)	K_m (mM)	V_{max}[b] (%)	F_A[c]
AMP	-	0.29	100	1
2 (ara-AMP)	5.1	1.7	45	13
3 (PMEA)	3.2	N.D.	0.2*	-
4	N.D.	0.69	7.5	32
5	N.D.	0.55	3.0	63
6	N.D.	0.24	7.4	11
7	0.6	0.26	0.5	179
8	1.0	N.D.	0.07*	-

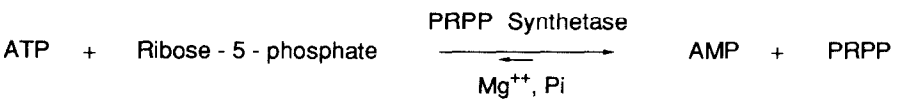
[a] For determination of kinetic parameters of AMP, 0.4 µg of enzyme was used per assay. For **2,3,4,5,6,7** and **8**, the enzyme concentration was respectively 3,10, 6, 6, 6, 8 and 40 times higher than that used for AMP. Incubation times varied from 2 to 10 min for good substrates up to 60 to 120 min for poor substrates. Data are means of two separate determinations and standard deviations on K_m and V_{max} did not exceed 22 and 18 %, respectively.

[b] V_{max} of AMP = 34.5 µmol. h^{-1}. (mg protein)$^{-1}$; (*) for compounds **3** and **8**, rates were measured at substrate concentrations 7.2 and 3.1 times higher than their IC_{50} / 2 value, respectively.

[c] $F_A = V_{max} / K_m$ of AMP divided by that of tested compound.

The carbocyclic mononucleotide analogs were then studied as potential substrates[11] of the reverse reaction catalyzed by *E. coli* PRPP synthetase i.e. in the presence of a constant excess of PRPP (scheme 3). For several AMP analogs, the binding constants to the AMP-binding site (IC_{50}) were also determined[12]. As shown in table I, all the compounds tested were pyrophosphorylated by PRPP synthetase albeit at very different rates. For all compounds with the exception of **8** (linearity observed only up to 30 min), the formation of products was linear with time. In all cases where K_m and V_{max} values were determined, the pyrophosphorylation reaction followed Michaelis-Menten kinetics. K_m and V_{max} measured for AMP were in excellent agreement with those published[5]. Considering the ratios of the V_{max}/K_m values (i.e. F_A), ara-AMP **2** was pyrophosphorylated 13 times less efficiently than AMP (table I). This agrees well with the value of 21 found by Balzarini and De Clercq[5]. Among the carbocyclic mononucleotide analogs, compound **6** emerged as the most efficient substrate (F_A = 11). Compound **5** (F_A = 63), the 2'-deoxy derivative of **6**, was 6 times less active than **6** indicating that the 2'-OH group plays a significant role in the activity of **6**. Unexpectedly, 9-(5-phosphonopentyl)adenine **4** which can be regarded as an acyclic derivative of the carbocyclic phosphonate **5** proved to be 2-fold more active than **5** and only 32-fold less active than AMP. The K_m values of **4** and **5** are similar and roughly 2-fold higher than the Km of AMP. This indicates that the ribofuranose ring of AMP does not play a critical role in the binding of AMP to the enzyme. Substitution of the phosphonomethyl group in the carbocyclic analog **5** (F_A = 63) by a phosphonomethoxy group (compound **7**, F_A = 179) lead to a 3-fold decrease in the efficiency of pyrophosphorylation. Comparison of the kinetic parameters shows that K_m is improved by 2-fold whereas V_{max} is decreased by 6-fold. Substitution of the phosphonomethyl group in **5** by a phosphonodifluoromethyl group (compound **8**) resulted in a dramatic (42-fold) decrease in the rate of pyrophosphorylation. The K_m value of **8** could not be determined. The IC_{50} of **8** (measured at a concentration of AMP equal to the K_m of AMP) was 1 mM. Provided that **8** occupies the same site as AMP, one would expect for **8** a K_m value around 0.5 mM i.e. similar to that of **5**. The large difference in reaction rates observed between **5** and **8** may be due, at least in part, to the strong electron-withdrawing effect of fluorine which can explain a slower nucleophilic attack of the difluorophosphonate anion on the α-phosphorus atom of the pyrophosphate group of PRPP[13].

Scheme 3



In summary, we have shown that carbocyclic phosphonate analogs of ddAMP are not substrates of AMP kinase (myokinase) but can be pyrophosphorylated by *E. coli* PRPP synthetase in the presence of PRPP. This last observation is not restricted to bacterial PRPP synthetase since we recently found that the recombinant isoform PRS I of rat PRPP synthetase[14] is also able to catalyze the pyrophosphorylation of the carbocyclic phosphonate analogs of ddAMP (to be reported elsewhere).

The PRPP synthetase reaction is easily reversible[15] and usually catalyzes the formation of PRPP and AMP from ribose-5-phosphate and ATP (forward reaction). Indeed, ara-ATP and PMEA-diphosphate have been found to serve as substrates for this reaction[5,6]. Therefore, one may expect that the diphosphate derivatives of the acyclic and carbocyclic phosphonates described in this study also act as substrates of the forward reaction. Hence, we extend and generalize to a large variety of adenine nucleotide analogs the assumption that PRPP synthetase may play a crucial role in the regulation of their triphosphate level.

References and notes

1. Mitsuya, H.; Yarchoan, R.; Broder, S. *Science* **1990**, 249, 1533.

2. (a) Cooney, D.A.; Ahluwalia, G.; Mitsuya, H.; Fridland, A.; Johnson, M.; Hao, Z.; Dalal, M.; Balzarini, J.; Broder, S.; Johns, D.G. *Biochem. Pharmacol.* **1987**, 36, 1765. (b) Hao, Z.; Dalal, M.; Cooney, D.A.; Balzarini, J.; Mitsuya, H.; Broder, S.; Johns, D.G. *Proc. Am. Ass. Cancer Res.* **1987**, 28, 323, abstract 1279. (c) Dalal, M.; Cooney, D.A.; Mitsuya, H.; Balzarini, J.; Hao, Z.; Nadkarni, M.; Broder, S.; Johns, D.G. *Proc. Am. Ass. Cancer Res.* **1987**, 28, 327, abstract 1297.

3. Johnson, M.A.; Fridland, A. *Mol . Pharmacol.* **1989**, 36, 291.

4. (a) Balzarini, J.; Naesens, L.; Herdewijn, P.; Rosenberg, I.; Holy, A.; Pauwels, R.; Baba, M.; Johns, D.G.; De Clercq , E. *Proc. Natl. Acad. Sci. USA*, **1989**, 86, 332. (b) Egberink, H.; Borst, M.; Niphuis, H.; Balzarini, J.; Neu, H.; Schellekens; H., De Clercq, E.; Horzinek, M.; Koolen, M. *Proc. Natl. Acad. Sci. USA* **1990**, 87, 3087.

5. Balzarini, J.; De Clercq, E. *Biochem. Biophys. Res. Commun.* **1990**, 173, 781.

6. Balzarini, J.; De Clercq, E. *J. Biol. Chem.* **1991**, 266, 8686.

7. Wolff-Kugel, D.; Halazy, S. *Tetrahedron Lett.* **1991**, 32, 6341.

8. Nakamura, C.E.; Chu, S.-H.; Stoeckler, J.D.; Parks, R.E., Jr. *Biochem. Pharmacol.* **1986**, 35, 133.

9. The enzymatic reaction was performed at 25°C in 0.1 M triethanolamine buffer, pH 7.6, containing 0.05 % BSA, 10 mM $MgCl_2$, 75 mM KCl, 5 mM dithiothreitol, 1.5 mM phosphoenol pyruvate, 0.15 mM NADH, 5 units/ml pyruvate kinase, 6.6 units/ml lactate dehydrogenase, 1 mM ATP, the substrate (e.g. AMP) and 0.01 µg of AMP kinase (from rabbit muscle; Sigma) in 1ml. NADH consumption was measured at 340 nm. The specific activity was 444 µmol AMP phosphorylated min^{-1}.(mg protein)$^{-1}$.

10. Substrates displaying reaction rates equal to 0.2% of the maximal velocity calculated for AMP under similar conditions could be detected under these conditions.

11. The enzymatic reaction was performed at 37°C in 50 mM triethanolamine buffer, pH 8.0, containing 0.1% BSA, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 2.5 mM PRPP, the substrate (AMP or AMP analogs) and PRPP synthetase (from E coli; Sigma) in 0.1 ml . Incubation was stopped by addition of 150 µl of ice-cold methanol and proteins were removed by centrifugation. After evaporation under nitrogen, the samples were resuspended in 150 µl HPLC buffer A containing 2% acetonitrile. The nucleoside triphosphate analogs were analysed by HPLC (Lichrospher column 100 RP-18E, 250-4, Merck at 40°C; buffer A: 0.1M KH_2PO_4, 5 mM tetrabutyl ammonium dihydrogen phosphate, pH 5.0; buffer B: buffer A at pH 5.5 containing 40% acetonitrile). Using a linear gradient (20 to 44%B, 24 min.), the retention times of the diphosphate derivatives of AMP and of **2**, **3**, **4**, **5**, **6** were 15.3, 16.5, 14.7, 17.6, 22.0, 15.1 min. Using a linear gradient (30 to 54%B, 24 min), the retention times of **7** and **8** were 14.0 and 18.0 min.

12. The assay mixture was as described in reference 11 but contained 0.5 µCi [^{3}H]AMP, 0.25 mM AMP and various concentrations of tested compound. After 12 min at 37°C, an aliquot (10 µl) was spotted on a PEI cellulose plastic sheet and eluted with a 0.3 M LiCl - 0.5 M formic acid solution. The band containing [^{3}H]AMP was extracted for 10 min in 1.5 ml of 2 M ammonium formate - 16 M formic acid solution and the radioactivity was counted.

13. For details on the mechanism of PRPP synthetase, see Walsh C., Enzymatic Reaction Mechanisms, W.H. Freeman and Cie, New York, 1979, pp 259-261.

14. The mammalian enzyme was kindly provided by Dr. M. Tatibana (Chiba University, Japan). For details on isolation of PRPP synthetase isoforms, see Ishijima, S.; Kita, K.; Ahmad, I.; Ishizuka, T.; Taira, M.; Tatibana, M. *J. Biol. Chem.* **1991**, 266, 15693.

15. (a) Switzer, R.L. *J. Biol. Chem.* **1969**, 244, 2854. (b) Sabina, R.L.; Holmes, E.W.; Becker, M.A. *Science* **1984**, 223, 1193.