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ATP ANALOGS WITH NON-TRANSFERABLE GROUPS IN THE γ POSITION AS INHIBITORS OF GLYCEROL KINASE

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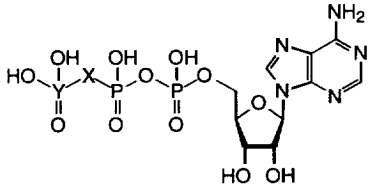
Abstract: β,γ-Difluoromethyleneadenosine-5'-triphosphate (AMP-PCF$_2$P, **3**) and γ-arsono-β,γ-methylene-adenosine-5'-diphosphate (AMP-PCAs, **4**) were synthesized and were found to be competitive inhibitors of glycerol kinase. Commercially available AMP-PCP and AMP-PNP also are competitive inhibitors. The structural similarities and differences of these ATP analogs and their effect on kinase inhibition are discussed.

Non-hydrolyzable analogs of ATP have been valuable tools for biochemical and structural studies of ATPases that involve the hydrolysis/transfer of the γ phosphate.[1,2] The general strategy for preparation of such stable analogs has been to replace the labile anhydride oxygen in the β,γ pyrophosphate moiety with a stable, non-hydrolyzable chemical group. In this paper we describe the synthesis of two non-hydrolyzable ATP analogs, β,γ-difluoromethyleneadenosine-5'-triphosphate (AMP-PCF$_2$P, **3**), a known but little studied ATP analog, and γ-Arsono-β,γ-methylene-adenosine-5'-diphosphate (AMP-PCAs, **4**), a new and previously unknown ATP analog. These novel ATP analogs are tested as inhibitors of glycerol kinase in comparison with commercially available and well established ATP analogs AMP-PCP (**1**) and AMP-PNP (**2**).



1 AMP-PCP: X = CH_2, Y = P
2 AMP-PNP: X = N, Y = P
3 AMP-PCF$_2$P: X = CF_2, Y = P
4 AMP-PCAs: X = CH_2, Y = As

Figure 1. ATP analogs with non-transferable groups in the γ position.

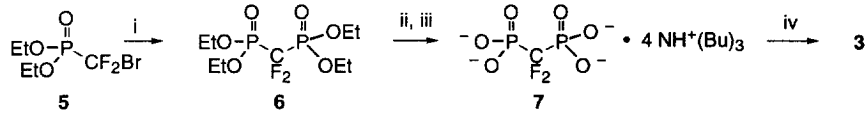
The ATP analog **1** has been known since 1963.[3] The degree of inhibition of ATPases by **1** is variable. This has been explained by the steric and electronic changes that result from the substitution of the methylene group for the anhydride oxygen. Attempts to improve the properties of non-hydrolyzable analogs led to the synthesis of **2** after the crystal structure of sodium imidobisphosphate was reported.[4,5] This analog has been widely accepted due to the perception that it may generally be a more reliable inhibitor since it appears to mimic the geometry and pK_a of pyrophosphate more closely than the methylene bisphosphonate. However, this analog suffers from slow hydrolysis under acidic conditions and also shows a range of inhibition efficiency with ATP dependent enzymes.

Blackburn suggested that the difluoromethylene phosphonates ($RCF_2PO_3H_2$) are the best non-hydrolyzable analogs of phosphates because the electronegativity of fluorine substituents should lower the pK_as of the phosphonate, making them closer to those of a phosphate. Determination of the pK_a, ^{31}P NMR, and computational analysis of mono and difluoromethylene phosphonates suggests that the steric and pK_a analogy between difluoromethylphosphonates and phosphates is very good.[6-8] The preparation of the difluoromethylene analog of ATP (**3**) was reported, without experimental details, in 1981.[7]

To extend the range of substitutions accessible for ATP analogs it was interesting to envision an ATP analog with arsenic replacing phosphorus at the γ phosphate position in tandem with a non-hydrolyzable β,γ methylene group. It is well known that arsenate is an excellent phosphate mimic and can often be utilized as a substrate in phosphate dependent enzymes.[9]

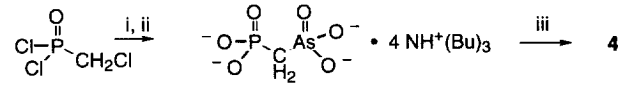
Syntheses of the ATP analogs **3** and **4** were accomplished as follows. Compound **6** was prepared from **5** by reaction with the sodium salt of diethylphosphite in benzene at reduced temperature. Ester hydrolysis was readily accomplished by treatment with trimethylsilyl iodide (TMSI) followed by aqueous hydrolysis and recrystallization of the cyclohexylamine salt. Cyclohexylamine was exchanged away and replaced by tributylamine to yield **7**. Coupling of the pyrophosphate analog **7** with AMP was accomplished via the technique described by Yount with minor modifications.[5] Compound **3** was purified via ion exchange chromatography and isolated as the sodium salt. Reverse phase HPLC indicated that the product was >95% pure.[10] Preparation of the arsono analog began with **9**, which was prepared from **8** (Scheme 2) as described by Webster with minor modifications.[11] In the coupling of **9** with AMP either the phosphate or the arsonate can form an anhydride with AMP. Because no suitable protection strategies for arsonates exist, two equivalents of AMP were coupled to each equivalent of arsonomethylphosphonic acid to produce both the P–O–P and As–O–P anhydrides (i.e. AMP–OP(O)OH–CH_2–As(O)OH–O–AMP). During workup and purification, the undesirable arsenate-phosphate anhydride underwent spontaneous hydrolysis, leaving behind **4** as the sole, stable ATP analog. Reverse phase HPLC indicated that the material was >94% pure.[10,12] The analogs **1-4** were evaluated as inhibitors of glycerol kinase by the method described by Pettigrew and all were demonstrated to be competitive inhibitors versus ATP as summarized in Table 1.[13] It is noteworthy that **3** is the most potent inhibitor of glycerol kinase, better than the commercially available inhibitors and 11 fold better than the standard and generally accepted ATP analog **2**.

Scheme 1



i: $((EtO)_2PO)Na$, benzene, 0 °C. ii: (1)TMSI; 2) H_2O, cyclohexylamine. iii: Dowex-50 (H^+), excess tributylamine.
iv: AMP-diphenylphosphatephosphoanhydride, DMF, pyridine (35% based on AMP).

Scheme 2



i: (1) NaOH (aq), As_2O_3; (2) Dowex-50 (H^+), cyclohexylamine. ii: Dowex-50 (H^+), excess tributylamine.
iii: AMP-diphenylphosphatephosphoanhydride, DMF, pyridine (60% based on **9**).

In the series of analogs examined, there are five variables that are significantly changed by the substitution of X and Y positions indicated in Figure 1, P-X-Y bond angle, X-Y bond length, P-Y distance, presence of hydrogen bonding potential at the X position, and changes in the pK_a of the group at the corresponding γ position. A comparison of these properties is given in Table 2.

The arsonate analog demonstrates the weakest binding and the most significant differences in geometry and pK_a. The P-As distance is substantially longer than the P-P distance in pyrophosphate. The As-O bond lengths are much greater than the corresponding P-O bond lengths with As-O bond lengths around 1.7 Å compared to 1.5 Å for P-O bond lengths. Together, these differences suggest that the arsonate oxygens will not be able to occupy the same positions in the enzyme active site which are accessible to phosphate oxygens. Additionally, the significantly increased pK_a of the arsonate group certainly allows protons to occupy arsonate oxygens that would normally be ionized at the γ phosphate position of ATP. However, it appears that this is not a major determinant of binding due to the fact that **1**, which also reflects a substantially increased pK_a, binds nearly as well as the true substrate. Taking this information into account, is appears that **3** primarily acts as a very sterically demanding probe of the active site of GK.

Table 1. Comparison of K_i Values for Glycerol Kinase with Parameters Varied by Substitution

ATP Analog	K_m[a] or K_i[b] (mM)[c]	P-X-Y angle (degrees)	X-Y length (Å)	P-Y distance (Å)	H-bond at X	pK_{a4}
ATP	0.2±0.5[a]	128.7[d]	1.63[d]	2.94[d]	Yes	7.1[d]
1	0.3 (0.2, 0.5)[b]	117.0[d]	1.79[d]	3.05[d]	No	8.4[d]
2	0.9 (0.8, 1.2)[b]	127.2[d]	1.68[d]	3.01[d]	No	7.7[d]
3[e]	0.08 (0.05, 0.11)[b]	117.5[e]	1.84[e]	3.26[e]	Yes	6.7[f]
4	2.9 (2.1, 4.0)[b]	115.0[g]	1.95[g]	3.28[g]	No	9 (estimated)[h]

[a]Error given as the standard deviation. [b]Error ranges in parentheses are given at 65% confidence limits. [c]K_m for ATP and K_i for **1**, **2**, **3** & **4**. [d]Structural and pK_a data for ATP, **1** & **2** are taken from reference 5. [e]Geometry calculated at the 3-21G* level. [f]Data for pK_a of **3** taken from reference 7. [g]Structural data given for **4** are X-ray structural data for PCAs, taken from reference 14, and are used here with the assumption that AMP-PCAs should have similar data for these bond lengths and angles. [h]The pKa value for **4** is estimated from data presented and discussed in reference 9.

AMP-PNP (**2**), had an unexpectedly high K_i despite the appearance of excellent geometric complementarity and pK_a agreement with the true substrate. This may be explained by the conformational rigidity in the P-N-P moiety, which is the result of lone pair delocalization from the nitrogen into the P-N and P–O bonds. Available evidence suggests that such delocalization of the nitrogen lone pair is significant. The crystal structure of sodium imidodiphosphate clearly shows a planar arrangement of atoms around the nitrogen and the P-N bond length is shortened by 0.09 Å compared to phosphoramidate.[4] Finally, titration of imidodiphosphate clearly indicates that protonation of the nitrogen is difficult. Based on the crystal structures of enzyme/nucleotide complexes, it is clear that phosphate oxygens are the major determinants for positioning the phosphate chain in enzyme active sites. Therefore, if the positioning of β and γ phosphate oxygens is constrained through partial double bond character of the P-N bond then this may impose an unfavorable energetic cost for binding to some ATPases. However, it is likely that other ATPases establish a phosphate oxygen binding scaffold that can readily accommodates P-N-P rigidity. In these cases, we would expect that **2** would act as an effective analog of ATP and would bind with a lower K_i. It is also important to consider that **2** may be unable to participate in strong hydrogen bonding interactions at the imido nitrogen due to delocalization. This is contrary to the suggestion that **2** is generally a

better inhibitor than **1** because it has hydrogen bonding potential at the β,γ bridging position, albeit as a donor rather than an acceptor compared to ATP.

The analogs **1** and **3** are moderate isosteres of ATP and are likely to be less conformationally restricted than **2**. This steric agreement and conformational freedom are likely to be significant factors in the lower K_i values observed. Table 2 indicates that pK_a shifts and the potential for hydrogen bonding interactions are the most significant differences between **1** and **3**. It is possible that the increased extent of ionization of the γ phosphate also plays a role in improving binding as has been suggested by Blackburn. However, it appears that this effect is minimal in the case of glycerol kinase since fluorine substitution lowers the pK_a by 1.7 units and the K_i by only 3.75 fold in comparison to **1**.

This study suggests that the binding of ATP analogs is significantly dependent on the active site that is being probed. Several avenues of investigation are underway to further examine the relationship between the binding of ATP analogs in the context of a number of enzymes. A survey of inhibition of several different ATPases with the analogs described in this study will expand the understanding of active site context on analog binding. X-ray crystallographic studies of these analogs in complex with glycerol kinase are underway to detail the differences in binding at high resolution.

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