were dried (anhydrous sodium sulfate), and the residue obtained was subjected to thin-layer chromatography on 2000-μm silica gel G (Analtech) plates developed with 3:1 (v/v) $CHCl_3$/acetone containing 0.5% methanol. The green (red fluorescent) band was collected and further purified by high-pressure liquid chromatography on a Partisil 10-PAC column (9.4 × 250 mm) with a 20-min linear gradient of methanol into 1:1 (v/v) hexane/tetrahydrofuran. The demetallated porphyrin isomers obtained by treatment of the purified pigment with 5% H_2SO_4 in methanol[9,12] were then separated and further purified by high-pressure liquid chromatography on a Partisil 10-PAC column (9.4 × 500 mm) eluted with (v/v) 12:12:1 hexane/tetrahydrofuran/methanol. For NMR studies, zinc acetate in $CHCl_3$ was added to each of the isomers, and the resulting zinc complex of each isomer was finally purified by high-pressure liquid chromatography on a 4.6 × 250 mm Partisil 10-PAC column eluted with a 20-min linear gradient of methanol into 1:1 (v/v) hexane/tetrahydrofuran.

Cytochrome P-450 and heme assays were performed on an Aminco DW-2A spectrophotometer. Porphyrin electronic absorption spectra were recorded in CH_2Cl_2 on a Varian Cary 118 instrument. Field-desorption mass spectra were obtained as previously described[12] on a modified Kratos AEI MS-9 instrument. NMR studies were performed on a 360-MHz Nicolet NT-360 FT-NMR instrument. The $CHCl_3$ peak at 7.21 ppm was used as an internal reference. Conditions for nuclear Overhauser effect (NOE) and relaxation time measurements have been reported.[20,21] NMR samples were prewashed with NaCl solution to ensure the presence of chloride as the zinc counterion. The deuterated $CHCl_3$ used in the NMR work was stored over K_2CO_3 to eliminate acidic products.

Acknowledgment. The assistance of Dr. Bruce A. Mico with preliminary ethchlorvynol experiments and the acquisition of the field desorption mass spectrometric data by Dr. Kent L. Kunze are gratefully acknowledged. Access to the Biomedical and Environmental Mass Spectrometry Resource (supported by NIH Grant RR 00719) and to the University of California (Davis) 360-MHz NMR facility has been invaluable. This research was supported by NIH Grant GM 25515 and by an Alfred P. Sloan Research Fellowship (to P.R.O.M.).

Supplementary Material Available: NMR spectra of isomers II and III (Figure 5) and deuterium exchange and decoupling experiments with isomer I (Figure 6) (2 pages). Ordering information is given on any current masthead page.

Species- or Isozyme-Specific Enzyme Inhibitors. 8.[1] Synthesis of Disubstituted Two-Substrate Condensation Products as Inhibitors of Rat Adenylate Kinases

Francis Kappler, Ton T. Hai, Masanobu Abo, and Alexander Hampton*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received March 22, 1982*

Syntheses are described of 5′(*R*)- and 5′(*S*)-*C*-Me-ATP, 5′(*R*)- and 5′(*S*)-*C*-*n*-Pr-ATP, and the phosphonate isostere of ATP with a C(5′)-CH_2-P system. Determinations of K_M (ATP)/K_i for competitive inhibition showed that two of the five compounds inhibited rat muscle adenylate kinase (AK-M) 8–9.5 times more effectively than AK II (present in poorly differentiated rat hepatoma tissue) and that two other compounds inhibited AK II at least 2-fold more effectively than AK-M, further indicating that monosubstituted substrate derivatives are potentially useful probes in the design of isozyme-selective inhibitors. P^1-[8-(Ethylthio)adenosine-5′]-P^5-(adenosine-5′) pentaphosphate (8-SEt-Ap_5A) is a potent dual substrate site inhibitor of the rat AK isozymes with selectivity for AK II. Three derivatives of 8-SEt-Ap_5A were synthesized: P^1-[8-(ethylthio)adenosine-5′]-P^5-[5′(*R*)-*C*-methyladenosine-5′] pentaphosphate (I), its 5′(*R*)-*C*-*n*-Pr analogue (II), and di(8-SEt)-Ap_5A (III). Unsymmetrical pentaphosphates, such as I and II, are shown to be readily accessible via reaction of a derivative of ATP γ-piperidinate with an ADP derivative. Conversion of 8-SEt-ATP to 8-(ethylthio)adenosine 5′-trimetaphosphate, followed by reaction of the latter in situ with added piperidine, gave 8-SEt-ATP γ-piperidinate quantitatively. Except in the interaction of III with AK-M, I–III acted as two-site competitive inhibitors of AK-M and AK II with $K_i < K_M$ of AMP or ATP. Inhibitory potencies [K_M (ATP)/K_i] of I–III with the two isozymes varied over more than a 95-fold range, and inhibitory potencies for AK-M relative to those of AK II varied more than 61-fold. III was an effective inhibitor of AK II (K_M/K_i = 8 and 14 with AMP and ATP, respectively) and exhibited at least 4 times more selectivity for AK II [relative inhibitory potency, AK II/AK-M, >22] than 8-SEt-Ap_5A.

Evidence, summarized previously,[2] suggests that selective inhibitors of fetal-type isozymes of key metabolic enzymes could, if available, be potentially useful in the design of new types of antineoplastic agents. Studies of approaches that might have utility in the design of isozyme-selective inhibitors showed that such inhibitors were frequently produced when a single short (1 to 3 atoms) substituent was introduced at various atoms in turn of a substrate of the target enzyme. This result was obtained with each of three enzymic substrates studied.[1,3-6] The selective inhibitors so obtained were usually of weak or moderate potency, however. In the case of one target enzyme, adenylate kinase (AK), it proved possible to maintain the selectivity while enhancing potency 1000-fold by elaborating a monosubstituted substrate derivative into a monosubstituted two-substrate condensation product that appears to bind simultaneously to two adjoining substrate sites.[6] In the present study, two-substrate condensation products bearing a substituent at each substrate moiety were synthesized in order to further explore the potential of dual-site inhibitors for isozyme selectivity and with the hope of progressing further toward a potent and specific inhibitor of the isozyme AK II that predominates

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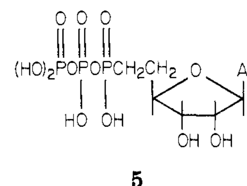
Table I. Physical Properties of Adenine Nucleotide Derivatives

compd	yield, %	UV λ_{max} (pH 6), nm ($\epsilon \times 10^{-3}$)	R_f[a] syst A	R_f[a] syst B	HPLC retn time,[a] min	formula	anal.	phosphate/ base ratio
ADP			0.36	0.23	3.8			
3a	80	260 (15.1)	0.40	0.32	3.9	$C_{11}H_{14}N_5O_{10}P_2Na_3\cdot H_2O\cdot 1.5(CH_3)_2CO$	C, H, N, P	
3b	75	260 (15.0)	0.53	0.46	3.9	$C_{13}H_{18}N_5O_{10}P_2Na_3\cdot H_2O\cdot MeOH$	C, H, N, P	
ATP			0.30	0.14	9.2			
5	75	260 (15.1)	0.30	0.18	9.2			2.06
5′(*R*)-Me-ATP	70	260 (15.2)	0.34	0.21	9.3	$C_{11}H_{14}N_5O_{13}P_3Na_4\cdot 2H_2O\cdot 0.5MeOH$	C, H, N, P	
5′(*R*)-Pr-ATP	60	260 (14.8)	0.46	0.39	10.8	$C_{13}H_{18}N_5P_3O_{13}Na_4\cdot 3.75H_2O$	C, H, N, P	
5′(*S*)-Me-ATP	75	260 (15.0)	0.42	0.23	9.3	$C_{11}H_{14}N_5O_{13}P_3Na_4\cdot H_2O\cdot 0.75MeOH$	C, H, N, P	
5′(*S*)-Pr-ATP	55	260 (14.9)	0.55	0.40	10.8	$C_{13}H_{18}N_5P_3O_{13}Na_4\cdot 3H_2O\cdot MeOH$	C, H, P; N[b]	
4a	44	265 (24.2)	0.46	0.33	17.8	$C_{23}H_{30}N_{10}P_5O_{22}SNa_5\cdot H_2O\cdot MeOH$	C, H, P, S; N[c]	2.38
4b	26	265 (24.0)[d]	0.59	0.48	19.5			2.43
di(8-SEt)Ap$_5$A		280 (32.5)[d]	0.56	0.32	22.8			2.44

[a] For conditions, see Experimental Section. [b] N: calcd, 9.68; found, 9.21. [c] N: calcd, 12.17; found, 11.76. [d] Calculated from the phosphate analysis.

in poorly differentiated rat hepatoma tissue.[7,8]

AK catalyzes the equilibrium ATP + AMP ⇌ 2ADP and possesses nonidentical binding sites for AMP and ATP. The previous studies[6] revealed that 8-SEt-Ap$_5$A acts as a potent two-site inhibitor and inhibits AK II (K_i = 0.07 μM) more effectively than the rat muscle isozyme AK-M (K_i = 3.25 μM). 8-SEt-ATP inhibits AK II more effectively than AK-M,[4] and it was concluded from this and other lines of evidence that the selectivity of 8-SEt-Ap$_5$A originates from interactions of its 8-(ethylthio)adenosine moiety at the ATP binding sites.[6] In the present work, single substituents have been attached to the unsubstituted adenosine moiety of 8-SEt-Ap$_5$A in attempts to increase the selectivity for inhibition of AK II by decreasing affinity for the AMP site of AK-M by a larger factor than affinity for the AMP site of AK II. In preceding studies,[1] a series of AMP derivatives monosubstituted at a variety of positions had been evaluated kinetically as inhibitors of the isozymes; for no compound could definitive evidence be obtained that inhibitory potency [K_M (AMP)/K_i for competitive inhibition] with AK II exceeded that with AK-M. The compound in the series most likely to possess that property was 5′(*R*)-*C*-*n*-Pr-AMP: this was a weak competitive inhibitor of AK II with K_M (AMP)/K_i = 0.025 and a weak noncompetitive inhibitor of AK-M with K_M (AMP)/K_i < 0.1, though of undetermined magnitude. 5′(*R*)-*C*-Me-AMP resembled its *n*-propyl homologue in its inhibitory properties toward the two isozymes; in addition, it was a substrate of AK II but, under the same conditions, was not a substrate of AK-M.[1] Hence, despite the incompletely defined relative inhibitory potencies of the 5′-(*R*)-*C*-alkyl-AMP derivatives toward the two isozymes, it was decided to determine the effect on isozyme-selective inhibition of introducing these substituents into 8-SEt-Ap$_5$A. The effect of introducing a second 8-SEt group into 8-SEt-Ap$_5$A was also investigated. This report describes the syntheses of P^1-[8-(ethylthio)adenosine-5′]-P^5-[5′-(*R*)-*C*-methyladenosine-5′] pentaphosphate (**4a**), its 5′-(*R*)-*C*-*n*-propyl homologue **4b**, and P^1,P^5-bis[8-(ethylthio)adenosine-5′] pentaphosphate [di(8-SEt)-Ap$_5$A], together with kinetic analyses of their inhibitor properties. In addition, in order to extend our studies of the tendency of ATP derivatives to exhibit isozyme-selective inhibitory effects with AK isozymes,[4] the corresponding 5′(*R*)-alkyl-ATP derivatives and their 5′(*S*) epimers and a phosphonate isostere of ATP (**5**) were synthesized, and their substrate and inhibitor properties with AK II and AK-M were evaluated.



5

Syntheses. 5′(*R*)-*C*-Me-ADP (**3a**), 5′(*R*)-*C*-*n*-Pr-ADP (**3b**), 5′(*R*)-*C*-Me-ATP, 5′(*S*)-*C*-Me-ATP, 5′(*R*)-*C*-*n*-Pr-ATP, and 5′(*S*)-*C*-*n*-Pr-ATP were synthesized by modifications of the method developed by Hoard and Ott[9] for the conversion of 2′-deoxynucleoside 5′-phosphates to 5′-triphosphates. This involved conversion of the tri-*n*-butylammonium salts of the appropriate 5′-*C*-alkyl derivatives of adenosine 5′-phosphate[1,10] to their 5′-phosphoroimidazolidates by the action of 1,1′-carbonyldiimidazole in DMF, followed by treatment of these derivatives with tri-*n*-butylammonium phosphate or tri-*n*-butylammonium pyrophosphate. The resulting 5′-*C*-alkyl derivatives of ADP or ATP were treated with aqueous ammonium hydroxide at room temperature to remove 2′,3′-cyclic carbonate residues,[11] after which they were purified by anion-exchange chromatography on a DEAE-cellulose (HCO_3^-) column and then isolated as their sodium salts in 55–80% yield. The 5′-*C*-alkyl-ADP and -ATP derivatives were homogeneous as judged by paper chromatography, UV extinction coefficient, anion-exchange HPLC, and elemental analysis (Table I). Application of

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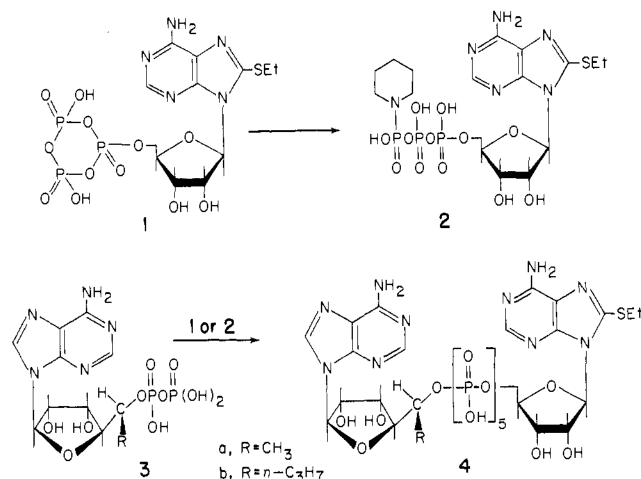
Scheme I



the same procedure to the phosphonate isostere of AMP[12] gave 5′-deoxy-5′-[[(hydroxypyrophosphoroxy)phosphinyl]methyl]adenosine (**5**) in 75% yield. Compound **5** has previously been prepared by reaction of AMP with inorganic phosphate in the presence of dicyclohexylcarbodiimide.[13]

As detailed previously,[6] Ap_5A can be conveniently synthesized in 55% yield by condensation of tributylammonium salts of adenosine 5′-trimetaphosphate (4 equiv) and ADP (1 equiv) in Me_2SO or DMF solution for 18 h at 35 °C. Substitution of ADP by 8-SEt-ADP in the procedure gave 8-SEt-Ap_5A in somewhat lesser yield (36%).[6] In the present work, reaction of 5′(*R*)-*C*-*n*-Pr-ADP (**3b**) with 8-(ethylthio)adenosine 5′-trimetaphosphate (**1**) in DMF under the above conditions and subsequent separation of the products by anion-exchange chromatography yielded 26% of pentasodium P^1-[8-(ethylthio)adenosine-5′]-P^5-[5′(*R*)-*C*-*n*-propyladenosine-5′] pentaphosphate (**4b**) together with 16% of P^1,P^5-bis[8-(ethylthio)adenosine-5′] pentaphosphate [di(8-SEt)-Ap_5A] (see Scheme I). The products were homogeneous by paper chromatography, anion-exchange HPLC, and phosphate/base ratio determined after enzymatic digestion with a mixture of venom phosphodiesterase and alkaline phosphatase. The enzymatic treatment of di(8-SEt)-Ap_5A was found by paper chromatography to yield 8-(ethylthio)adenosine as the only UV-absorbing product, and **4b** gave equimolar amounts of 8-(ethylthio)adenosine and 5′(*R*)-*C*-*n*-propyladenosine. Di(8-SEt)-Ap_5A presumably arises during synthesis of **4b** from decomposition of the 4-fold excess of **1**, because in DMF solution under the conditions used for the synthesis of **4b**, adenosine 5′-trimetaphosphate, in the absence of ADP, produced Ap_5A (3% yield) and Ap_6A (2%), together with 4% of several products with shorter HPLC retention times.

With the object of reducing the amounts of symmetrically substituted α,ω-di(adenosine-5′) polyphosphates formed as byproducts in the synthesis of unsymmetrically substituted Ap_5A derivatives, such as **4a** and **4b**, we studied the utility of ATP γ-piperidinate as a more stable, though less reactive, intermediate than adenosine 5′-trimetaphosphate. The phosphoropiperidinate group was selected because it has been shown to be a relatively reactive phosphoramidate in pyrophosphate bond synthesis.[14] Adenosine 5′-(tri-*n*-butylammonium trimetaphosphate) was found to be converted quantitatively to ATP γ-piperidinate when treated for 1 h, 22 °C, with 10 equiv of piperidine in Me_2SO solution. The product was used directly in the next step following its precipitation by the addition of diethyl ether. Reaction between the tri-*n*-butylammonium salts of ATP γ-piperidinate and ADP proceeded faster in DMF than in Me_2SO but was studied in a 1:1 mixture of the two solvents because of limited solubility of the ATP γ-piperidinate salt in DMF. At 35 °C, with ATP γ-piperidinate in 4-fold excess over ADP, anion-exchange HPLC analysis indicated that after 4 days ca. 50% conversion of ADP to Ap_5A had occurred and that the bulk of the starting materials remained undecomposed. Byproducts amounted to ca. 10% of the UV-absorbing components of the mixture and were principally of shorter retention times than ADP, except for ATP and/or Ap_4A (ca. 2%) and Ap_6A (ca. 1%). Omission of ADP from the reaction mixture showed that the Ap_6A and ATP and/or Ap_4A did not originate from the ATP γ-piperidinate. Prolongation of the reaction did not appear to increase the yield of Ap_5A but did increase the amounts of Ap_6A and ATP and/or Ap_4A, suggesting that these may arise from slow attack of ADP on Ap_5A[6] and subsequent reaction of ATP or AMP with ATP γ-piperidinate.

In view of the finding that Ap_5A could be synthesized in approximately the same yield from ATP γ-piperidinate as from adenosine 5′-trimetaphosphate but with formation of significantly less byproducts, the piperidinate route was employed for the synthesis of **4a**. 8-(Ethylthio)adenosine 5′-(tri-*n*-butylammonium triphosphate) was converted to the 5′-trimetaphosphate **1** by the action of excess of dicyclohexylcarbodiimide in Me_2SO. In situ treatment of **1** for 1 h, 22 °C, with 10 equiv of piperidine gave 8-(ethylthio)adenosine 5′-triphosphate γ-piperidinate **2** almost quantitatively after its precipitation from the mixture by addition of ether. Compound **2**, which appeared homogeneous at this stage as judged by paper chromatography and anion-exchange HPLC, was treated with 0.25 equiv of 5′(*R*)-*C*-methyladenosine 5′-(tri-*n*-butylammonium diphosphate) (**3a**) in DMF–Me_2SO at 35 °C. Anion-exchange HPLC analyses after 4 days showed that the mixture contained both the starting materials in significant amounts, **4a** (in ca. 50% yield), di(8-SEt)-Ap_5A (ca. 3% of **4a**), and trace amounts of six or more byproducts (total, 10% of **4a**) with retention times shorter than that of **4a**. Anion-exchange chromatography of the mixture gave **4a** in 44% yield as a pentasodium salt that was homogeneous as indicated by paper chromatography, anion-exchange HPLC, and elemental analysis. In addition, treatment of **4a** with a mixture of venom phosphodiesterase and alkaline phosphatase yielded 5 equiv of inorganic phosphate and 1 equiv each of 8-(ethylthio)adenosine and 5′(*R*)-*C*-methyladenosine.

The present studies on the synthesis of P^1,P^5-di(nucleoside-5′) pentaphosphates indicate that the phosphoropiperidinate route is the preferred one for synthesis of nonsymmetrically substituted pentaphosphates, such as **4a,b**, because concomitant formation of symmetrical pentaphosphates is not extensive due to the relative stability of ATP γ-piperidinate derivatives. On the other hand, the nucleoside 5′-trimetaphosphate route, because of its directness and rapidity, appears to be the preferred one for the synthesis of symmetrically substituted dinucleoside pentaphosphates, such as Ap_5A.

ATP Derivatives as Substrates and Inhibitors of Rat AK Isozymes. The four 5′-alkyl-ATP derivatives and

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the phosphonate isostere **5** of ATP did not show substrate activity with AK II or AK-M when the enzymatic assay for ADP employed in the kinetic studies was used. However, HPLC analysis showed that in the presence of higher levels of AK II or AK-M activity, all five compounds, except 5′(*R*)-*n*-Pr-ATP, were converted to the corresponding ADP derivatives with concomitant conversion of AMP to ADP. Inhibition constants of the five ATP derivatives are given in Table II. Since the K_M values of ATP with AK II and AK-M are not the same, the inhibitory potencies of the derivatives are expressed as K_M (ATP)/K_i for competitive inhibition. Except for 5′(*S*)-*n*-Pr-ATP, the present derivatives exhibited weak to moderate inhibitions with K_M (ATP)/K_i values ranging from 0.04 to 0.2 for AK II and <0.08 to 0.32 for AK-M. A similar tendency for monosubstituted substrate derivatives to act as weakly or moderately effective inhibitors has been noted in previous studies with ATP, AMP, or thymidine as substrates.[1,3-6] 5′(*R*)-Me- and 5′(*S*)-*n*-Pr-ATP inhibited AK-M more effectively than AK II by factors of 7.9 and 9.5, respectively, and 5′(*R*)-*n*-Pr-ATP and the phosphonate **5** inhibited AK II more effectively by a factor of more than 2. Possible differential inhibition by 5′(*S*)-Me-ATP could not be evaluated from its inhibitor properties. In addition to the present studies with substituents in the 5′(*S*) and 5′(*R*) configurations and at 0(5′) of ATP, previous studies were carried out with short substituents at N^6 or C(8) of ATP.[4,6] Selective inhibitions of rat AK isozymes are produced by all five types of ATP derivative. Likewise, a high proportion of AMP derivatives monosubstituted at various positions show selective inhibition of rat AK isozymes,[1] and seven of eight types of monosubstituted thymidine derivatives behave as isozyme-selective inhibitors of rat thymidine kinases,[5] indicating that monosubstituted substrate derivatives of this type are potentially useful in early stages of the design of isozyme-selective inhibitors.

Ap_5A Derivatives as Inhibitors of Rat AK Isozymes. Table II lists inhibition constants of the Ap_5A derivatives derived by kinetic analyses with variable AMP or variable ATP. Compound **4a** was a potent inhibitor of AK II with inhibition constants that were 29 and 60 times less than the K_M values of AMP and ATP, respectively. The fully or partly competitive nature of these inhibitions supports a two-site mode of binding of **4a** to AK II. The inhibition constants indicate that 8-SEt-ATP binds more strongly than 5′(*R*)-*C*-Me-ATP to the ATP site of AK II, while 5′(*R*)-*C*-Me-AMP binds more strongly than 8-SEt-AMP to the AMP site (Table II), suggesting that **4a** probably binds to AK II with its 8-(ethylthio)adenosine moiety positioned at the ATP site and its 5′-*C*-methyladenosine moiety at the AMP site. Consistent with this orientation of enzyme-bound **4a**, introduction of a 5′(*R*)-*C*-methyl group into either AMP or 8-SEt-Ap_5A increased the inhibition constants with variable AMP by similar orders of magnitude (123-fold and 47-fold, respectively). The inhibition constants indicated that the affinity of **4a** for the ATP site of AK II was 19 times less than that of 8-SEt-Ap_5A, and that with AK-M it was only 10 times less, and as a result **4a** exhibited approximately half as much selectivity for AK II as did 8-SEt-Ap_5A (Table II). Compound **4a** inhibited AK-M competitively with respect to both substrates. Since the inhibitory potency [K_M (substrate)/K_i] was ca. 20 for both substrates, **4a** appears to function as an effective two-site inhibitor of AK-M. The orientation of enzyme-bound **4a** at the active site of AK-M may be opposite to that with AK II. This could arise because 5′(*R*)-C-Me-ATP has more affinity than 8-SEt-ATP for the ATP site and because the corresponding AMP

Table II. Inhibition Constants of Adenine Nucleotide Derivatives with Rat Adenylate Kinase (AK) Isozymes

	AK II				AK-M				inhibitory potency [K_M (ATP)/K_i (ATP varied)]		rel inhibitory potency (ATP site), AK II/AK-M
	AMP varied		ATP varied		AMP varied		ATP varied				
compd	K_i, mM	type of inhibn[a]	K_i, mM	type of inhibn	K_i, mM	type of inhibn	K_i, mM	type of inhibn	AK II	AK-M	
AMP	0.08[b]				0.58[b]						
adenosine	92	NC	87	NC	19	NC	82	NC			
8-SEt-AMP	21[c]	NC			15[c]	NC					
5′(*R*)-Me-AMP	9.8[d]	C			16[d]	NC					
5′(*R*)-Pr-AMP	3.2[d]	C			4.5[d]	NC					
ATP			0.09[b]				0.57[b]				
8-SEt-ATP			0.08[e]	C			6.1	NC			
5′(*R*)-Me-ATP			2.2	C			1.8	C	0.04	0.32	0.13
5′(*R*)-*n*-Pr-ATP			0.45	C			4.5	NC	0.20	<0.084[f]	>2.4
5′(*S*)-Me-ATP			2.0	C			1.8	NC	0.045	<0.21[f]	
5′(*S*)-*n*-Pr-ATP			0.45	C			0.30	C	0.20	1.9	0.11
5			0.54	C			4.0	NC	0.17	<0.095[f]	>1.8
8-SEt-Ap_5A	0.00006[c]	C	0.00008[c]	C	0.0035[c]	C	0.003[c]	C	1125	190	5.9
di(8-SEt)Ap_5A	0.010	C-NC	0.0064	C	0.90	NC	0.60	NC	14	<0.63[f]	>22
4a	0.0028	C-NC	0.0015	C	0.031	C	0.031	C	60	18.4	3.3
4b	0.063	C-NC	0.052	C	0.16	C	0.12	C	1.7	4.8	0.36

[a] C = competitive; NC = noncompetitive; C-NC = mixed C and NC. [b] Value of the Michaelis constant. [c] Data from ref 6. [d] Data from ref 1. [e] Data from ref 4. [f] Calculated on the assumption that the K_i for competitive inhibition with respect to ATP would be at least 1.5 times the observed K_i value for noncompetitive inhibition.

derivatives have no detectable affinity for the AMP site (Table II).

The 5′(*R*)-*C*-propyl derivative **4b** of 8-SEt-Ap_5A also exhibited inhibitor properties with AK II and AK-M suggestive of a two-site mode of binding. The K_i values indicated that the affinities of **4b** for the AMP and ATP sites of AK II were ca. 20 and 35 times less, respectively, than those of **4a** even though the affinity of 5′(*R*)-*C*-Pr-AMP for the AMP site is 3 times higher than that of 5′(*R*)-*C*-Me-AMP. The inhibitor properties of 5′(*R*)-*C*-Pr-AMP and -ATP indicate that **4a** and **4b** are probably oriented in the same manner at the two substrate sites of AK II. In view of this, one implication of the relatively low affinity of **4b** is that the alkyl groups of the two enzyme-bound 5′(*R*)-*C*-alkyl-AMP derivatives may be in a different environment than the 5′(*R*)-alkyl groups of enzyme-bound **4a** and **4b**. The affinities of **4b** for the AMP and ATP sites of AK-M, as judged by the K_i values, were only 4–5 times less than those of **4a**, with the overall result that **4b** had an opposite selectivity than **4a**, and exhibited ca. a 3-fold more potent inhibition of AK-M than of AK II. This finding is difficult to interpret from the present data because 8-SEt-AMP, 8-SEt-ATP, 5′(*R*)-*C*-Pr-AMP, and 5′(*R*)-*C*-Pr-ATP all behaved as noncompetitive inhibitors of AK-M (Table II); hence, it is not possible to assign a probable orientation of **4b** on the AMP and ATP sites of that isozyme. A lack of substrate activity of 5′-(*R*)-*C*-Pr-ATP with AK-M, in contrast to a readily demonstrable substrate activity of its 5′-methyl homologue, is a further indication that **4a** and **4b** may not orient on AK-M in identical modes.

Di(8-SEt)-Ap_5A, despite the low affinity of 8-SEt-AMP for the AMP site of AK II or AK-M (Table II), behaved as an effective two-site inhibitor of AK II with K_M (AMP or ATP)/K_i values of 8 and 14 with AMP and ATP, respectively, as the varied substrate. The K_i values for varied ATP indicate that di(8-SEt)-Ap_5A inhibits AK II at least 22 times more powerfully than AK-M and possesses at least 4 times more selectivity for AK II than does 8-SEt-Ap_5A. With AK-M, di(8-SEt)-Ap_5A did not appear to act as a two-site inhibitor insofar as its K_i values were higher than the K_M values of AMP and ATP and the inhibitions were noncompetitive with respect to both substrates. This finding accords with the weak affinities of 8-SEt-AMP and 8-SEt-ATP for AK-M. Since adenosine is a weak noncompetitive inhibitor of AK II or AK-M when either AMP or ATP is varied (Table II) and since AMP and ATP induce conformational changes in pig muscle AK,[15,16] it is possible that competitive inhibition of AK II and AK-M may reflect, among other factors, an ability of the inhibitor to bind to forms of the enzymes that are different from the forms uncomplexed to AMP or ATP.

Few kinetic studies have been made of two-substrate condensation products as enzyme inhibitors.[6,17-19] The present findings show that both the potency and selectivity of inhibition can be varied over a wide range by introducing a substituent at each substrate moiety of a two-substrate condensation product that acts as a potent two-site inhibitor. In the present instance it proved difficult, for most of the isozyme–inhibitor complexes studied, to rationalize the effects of added substituents on inhibitory potency. These difficulties arose from unknown orientations of some inhibitors on the two substrate sites and from apparent variability in the environment of certain substituents, depending upon whether they were attached to an AK-bound substrate derivative or to an AK-bound two-substrate condensation product. The present and previous[6] investigations indicate that substituted two-substrate condensation products that can act as two-site inhibitors possess the potential to act as effective inhibitors with isozyme selectivity. Specifically, the present studies reveal that di(8-SEt)-Ap_5A is an effective inhibitor of AK II with significantly more selectivity for AK II than shown by compounds hitherto evaluated.

Experimental Section

Chemical Synthesis. General. *N,N*-Dimethylformamide and dimethyl sulfoxide were distilled from calcium hydride and stored over Linde 4A molecular sieves. Paper chromatography was carried out by descending technique on Whatman No. 1 paper in (A) 1-propanol–NH_4OH–water (55:10:35) or (B) isobutyric acid–1 M NH_4OH (6:4). Phosphorus-containing compounds were visualized on paper chromatograms with the molybdate spray of Hanes and Isherwood,[20] followed by ultraviolet irradiation.[21] Electrophoresis was carried out on Whatman No. 1 paper at pH 7.5 (0.05 M triethylammonium bicarbonate). Ultraviolet spectra were obtained on Cary Model 15 and Varian Model 635 spectrophotometers. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN; compounds were dried at 22 °C, except **4a** which was dried at 78 °C. Where analyses are indicated by the symbols of the elements, analytical results were within ±0.4% of the theoretical values. High-pressure liquid chromatography was performed on a Waters Model 204 chromatograph equipped with a dual solvent-delivery system (Model M-6000 A) and a Model 660 programmer. Compounds were analyzed on a μBondapak NH_2 column (30 cm × 4 mm) utilizing a 2 mL/min flow rate with a linear gradient of ammonium dihydrogen phosphate (pH 3, 0.05–0.5 M) over a 20-min period. The column eluent was monitored at 254 or 280 nm.

General Method for the Synthesis of 5′(*R*)- and 5′(*S*)-*C*-Alkyladenosine 5′-Di- and 5′-Triphosphates (Alkyl = Me or *n*-Pr) and 5′-Deoxy-5′-[[(hydroxypyrophosphoroxy)phosphinyl]methyl]adenosine (5). To an anhydrous DMF solution (5 mL) of the appropriate nucleoside 5′-(tri-*n*-butylammonium monophosphate) (0.5 mmol) was added *N,N*′-carbonyldiimidazole (0.4 g, 2.5 mmol). After 3 h, paper electrophoresis at pH 7.5 showed the reaction to be complete. Methanol (0.165 mL, 4 mmol) was added and, after 30 min, tri-*n*-butylammonium phosphate or bis(tri-*n*-butylammonium) pyrophosphate (2.5 mmol) was added. The mixture was stirred at room temperature for 18 h. The DMF was decanted, and the the residue was washed by centrifugation with DMF (10 mL). The residue obtained upon evaporation of the combined DMF solutions was dissolved in 1.5 N ammonium hydroxide (100 mL) and kept at room temperature for 2 h. The white solid obtained upon evaporation was dissolved in 100 mL of water and applied to a column (2.5 × 20 cm) of DEAE-cellulose bicarbonate. The column was washed with water and then eluted with a linear gradient of 0.0–0.3 M triethylammonium bicarbonate (2 L). The fractions corresponding to the di- or triphosphate were pooled and evaporated in vacuo. No 2′,3′-cyclic carbonates could be detected in chromatographic system B in which ATP has R_f 0.38 and ATP 2′,3′-cyclic carbonate has R_f 0.55. The residue was evaporated several times with ethanol to give the triethylammonium salt. This was converted to the sodium salt by dissolving the white solid in methanol (2 mL) and adding 1.0 M NaI in acetone (2 mL), followed by acetone (35 mL). The precipitate was washed with acetone (3 × 20 mL) and then dried in vacuo. The products were homogeneous in paper chromatographic systems A and B and on HPLC. Physical

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properties are given in Table I.

P^1-[8-(Ethylthio)adenosine-5′]-P^5-[5′(*R*)-*C*-*n*-propyladenosine-5′] Pentaphosphate (4b) and P^1,P^5-Bis[8-(ethylthio)adenosine-5′] Pentaphosphate. 8-(Ethylthio)adenosine 5′-(tri-*n*-butylammonium trimetaphosphate)[6] (0.4 mmol) and 5′(*R*)-*C*-*n*-propyladenosine 5′-[tris(tri-*n*-butylammonium) diphosphate] (0.1 mmol) were condensed in DMF under conditions described for the synthesis of 8-SEt-Ap_5A.[6] Purification was effected by chromatography on a column of DEAE-cellulose (HCO_3^-) (4 × 40 cm), which was washed with water (500 mL) and eluted with a linear gradient of 0–0.5 M triethylammonium bicarbonate (4 L). Compound **4b** eluted at 0.36–0.39 M salt (690 OD_{265} units, 29% yield) and di(8-SEt)-Ap_5A eluted at 0.40–0.44 M salt (580 OD_{280} units). Both **4b** and di(8-SEt)-Ap_5A contained trace contaminants, which were removed by paper chromatography on Whatman 3MM paper in solvent system A. The compounds were eluted from the chromatograms with water, and this solution was passed through a column (2.5 × 5 cm) of DEAE-cellulose (HCO_3^-). Elution with 0.5 M triethylammonium bicarbonate gave a methanol-soluble triethylammonium salt, which was converted to a sodium salt as above, yielding 29 mg of **4b** and 21 mg of di(8-SEt)-Ap_5A as white powders. See Table I for physical properties.

P^1-[8-(Ethylthio)adenosine-5′]-P^5-[5′(*R*)-*C*-methyladenosine-5′] Pentaphosphate (4a). An anhydrous solution of 8-(ethylthio)adenosine 5′-[tetrakis(tri-*n*-butylammonium) triphosphate] (1 mmol) and dicyclohexylcarbodiimide (605 mg, 3.3 mmol) in Me_2SO (5 mL) was stirred at room temperature for 1 h. Piperidine (1 mL, 10 mmol) was added, and stirring was continued for 1 h, when TLC in solvent A showed a single spot of R_f 0.75. The precipitate of *N,N*′-dicyclohexylurea was removed by filtration and the filtrate was added to anhydrous diethyl ether (100 mL). The gummy solid was triturated several times with ether and then dissolved in Me_2SO (4 mL). To the Me_2SO solution of **2** was added 5′-(*R*)-*C*-methyladenosine 5′-[tris(tri-*n*-butylammonium) diphosphate] (0.25 mmol) in DMF (4 mL) containing tri-*n*-butylamine (1.2 mL). The solution was maintained at 35 °C for 4 days, at which time HPLC analysis showed **4a**, starting materials, and seven trace impurities, one of which was di(8-SEt)-Ap_5A. The mixture was diluted with 0.15 M aqueous $Et_3NH{\cdot}HCO_3$ (100 mL) and applied to a column with DEAE-cellulose bicarbonate (4 × 35 cm) that had been equilibrated with 0.15 M $Et_3NH{\cdot}HCO_3$. The column was washed with 0.15 M $Et_3NH{\cdot}HCO_3$ (0.75 L) and then eluted with a linear gradient of 0.15–0.5 M $Et_3NH{\cdot}HCO_3$ (4 L). The product eluted at 0.38–0.43 M salt. Although the product appeared as a symmetrical peak in the elution profile, only the center one-third of the peak was homogeneous on analysis by HPLC. The leading and trailing edges contained a trace impurity, which was removed by chromatography on Whatman 3 MM paper in solvent A. Conversion of the three combined fractions to the sodium salt via the triethylammonium salt as described above gave 120 mg (44%) of **4a** as a white powder [dried at 78 °C (0.01 mm) over P_2O_5]. Properties of **4a** are given in Table I.

Enzymatic Conversion of P^1,P^5-Di(nucleoside-5′) Pentaphosphates and 5 to Nucleosides and Inorganic Phosphate. Enzymic digestion of ca. 1 μmol of **4a,b** or di(8-SEt)-Ap_5A was carried out for 2 h at 22 °C in 1 mL of Tris buffer, pH 10.4, containing 10 mg of phosphodiesterase from *Crotalus atrox* (Type IV, Sigma Chemical Co.) and 0.02 mg of alkaline phosphatase from calf intestine (Type VII, Sigma Chemical Co.). Paper chromatography in *n*-BuOH–H_2O (86:14) showed 8-(ethylthio)adenosine (R_f 0.67), 5′(*R*)-*C*-methyladenosine (R_f 0.40), or 5′(*R*)-*C*-*n*-propyladenosine (R_f 0.60) as the only UV-absorbing spots. Elution of the spots from digestion of **4a,b** showed a 1:1 ratio of nucleosides. Compound **5** was digested as above, except that the phosphodiesterase was omitted. Paper chromatography in *n*-BuOH–HOAc–H_2O (5:2:3) gave a single spot (R_f 0.30) identical with the authentic phosphonic acid isostere of AMP.[12] Phosphate analyses were performed by the method of Lowry and Lopez.[22]

Enzyme Kinetic Studies. Procedures for the determination of substrate and inhibitor constants with AK II and AK-M were the same as described previously.[6] All ATP derivatives and those Ap_5A derivatives with K_i values in excess of 0.10 mM were added in the form of 1:1 Mg complexes made by addition of $MgSO_4$ to the stock solutions.

Determination of substrate activity by HPLC analysis was accomplished by incubating the ATP analogue (0.5 mM), $MgSO_4$ (0.64 mM), and AMP (0.86 mM) in Tris buffer (0.1 M, pH 7.6) for periods up to 24 h at 22 °C with either AK II or AK-M (each at 10 × normal assay level). HPLC analysis was performed as described under "General" above but at pH 5 rather than at pH 3. This program separated ADP and ATP from the ADP and ATP derivatives, except for **5** and ATP which coeluted. However, the ADP analogue of **5** and ADP did separate as distinct peaks.

Acknowledgment. This work was supported by U.S. Public Health Service Research Grant CA-11196 from the National Cancer Institute and grants to the Institute for Cancer Research (USPHS Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania).

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Species- or Isozyme-Specific Enzyme Inhibitors. 9.[1] Selective Effects in Inhibitions of Rat Pyruvate Kinase Isozymes by Adenosine 5′-Diphosphate Derivatives

Ton T. Hai, Masanobu Abo, and Alexander Hampton*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received April 1, 1982*

Derivatives of adenosine 5′-diphosphate (ADP) with a substituent of 1–4 atoms at any of eight positions have been synthesized and evaluated as substrates and inhibitors of the liver (L), muscle (M), and kidney (K) isozymes of rat pyruvate kinase (PK). Inhibitory potencies of the compounds were expressed as K_M (ADP)/K_i or as K_M (ADP)/K_M when no K_i value was available. Nine of 14 ADP derivatives exhibited differential inhibitions. The M and K isozymes, which cross-react immunologically with each other but not with the L form, were inhibited differentially by 5 of the 14 derivatives. PK-K was preferentially inhibited by two derivatives, PK-L by three derivatives, and PK-M by two derivatives. Among the most selective and/or effective inhibitors were 3′-OMe-ADP [K_M (ADP)/K_i = 0.07 with PK-K; inhibitory potency, K/M/L, 7.6:6.0:1], N^6-Me,N^6-$(CH_2)_4$N(Me)COMe-ADP (prepared previously) [K_M (ADP)/K_M = 0.43 with PK-L; inhibitory potency, L/K/M, 3:2:1], and 8-NHEt-ADP [K_M (ADP)/K_i = 1.0 with PK-M; inhibitory potency, M/K/L, 7.1:1.2:1]. These and previous studies with two other enzymes indicate that monosubstituted substrate derivatives that bear short substituents (usually 1–4 atoms) at various positions are potentially useful probes in early stages of the attempted design of isozyme-selective inhibitors.

Evidence, summarized previously,[2] indicates that selective inhibitors for fetal-type isozymes are a class of compounds of potential utility in the design of new types of antineoplastic agents. It has been reported that in-

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