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Design of Substrate-Site-Directed Inhibitors of Adenylate Kinase and Hexokinase. Effect of Substrate Substituents on Affinity for the Adenine Nucleotide Sites

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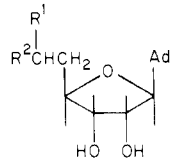
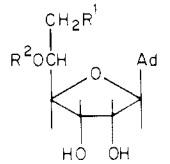
Nineteen derivatives of adenosine 5′-phosphate (AMP) bearing acylaminomethyl, acetoxy, or alkylaminomethyl substituents on the phosphate–ribose bridge (5′ and O-5′ positions) of AMP together with 2′,3′-*O*-ethylidene, 2′,3′-*O*-isopropylidene, and 2′,3′-di-*O*-acetyl derivatives of AMP have been synthesized. Their substrate and/or competitive inhibitor properties with pig and rabbit muscle AMP kinases indicate that all the substituents except 2′,3′-*O*-ethylidene with the pig enzyme permitted binding of AMP at its enzymic site. Determination of enzyme–inhibitor dissociation constants showed that several compounds with substituents on the ribose–phosphate bridge bind as well or better than AMP. The affinity is ascribed in part to interaction between substituents and a lipophilic region of the enzymes adjacent to the ribose–phosphate bridge in the enzyme–AMP complexes. The enzyme–inhibitor dissociation constants reveal a structural dissimilarity between the pig and rabbit enzymes in the vicinity of the lipophilic region. The substrate and inhibitor properties of eight ATP derivatives gave evidence that affinity of ATP for its substrate site on the AMP kinases is compatible with acetyl- or chloroacetylaminomethyl groups at the phosphate–ribose bridge or with 2′,3′-*O*-ethylidene or isopropylidene residues. The yeast hexokinase–ATP complex tolerated an acetylaminomethyl group at C-5′ or a benzoylaminomethyl group adjacent to O-5′. The present findings regarding substituent tolerance could be used in the design of adenine nucleotide site-directed irreversible inhibitors.

Adenylate kinase mediates reversible phosphate transfer from ATP to AMP to produce ADP and plays an important role in the energy economy of living systems.[1] To investigate the possibility of designing substrate-site-directed exo-site reagents with their potential to act as species-selective irreversible enzyme inhibitors,[2] we have introduced substituents at various positions of AMP and ATP and examined the effect on formation of enzyme–substrate complexes as indicated by substrate and inhibitor properties of the AMP and ATP derivatives. Studies with small substituents showed that binding to the AMP site of the kinase still occurs when a hydroxyl or cyano group[3,4] is inserted α to phosphorus (equivalent to O–5′ of AMP) in the phosphonate isostere of AMP (**1**, R^1 = H; R^2 = PO_3H_2) or when a methyl group is attached to C-5′ of AMP.[5] Substitution on the exocyclic N^6 of the adenine ring appears to prevent binding of AMP to its site because 1,N^6-etheno-AMP is not a substrate[6] and N^6-benzoyl-AMP is not a substrate and is a noncompetitive inhibitor with respect to AMP.[7] Formation of the ATP–enzyme complex was not prevented by introduction of a cyano group near O-5′.[4] This paper describes the substrate and inhibitor characteristics of derivatives of AMP and ATP with substituents on O-2′ and O-3′ (structure **4**) or on the phosphate–ribose bridge (5′ and O-5′ positions) (structures **1–3**).

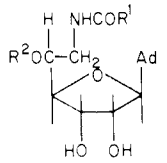
Chemical Syntheses. The syntheses of 5′-deoxy-5′-(*C*-acetoxyphosphonomethyl)adenosine (**1a**) and of the 5′-deoxy-5′-[*C*-(acylaminomethyl)phosphonomethyl]-adenosines **1c,d,f,g,h** were reported previously.[8] The benzamidomethyl-substituted phosphonate analogue of AMP, **1e**, was obtained in 40% yield as its purified disodium salt by treatment of 5′-deoxy-5′-[*C*-(aminomethyl)phosphonomethyl]adenosine (**1b**)[8] with 2 equiv each of benzoic acid and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.[9] The AMP analogues **1c** and **1e** were converted to the corresponding ATP analogues **1i** and **1j** by the general procedure of Michelson[10] for the conversion



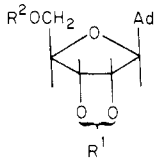
	R^1	R^2
1a	$OCOCH_3$	PO_3H_2
b	CH_2NH_2	PO_3H_2
c	$CH_2NHCOCH_3$	PO_3H_2
d	$CH_2NHCOC_2H_5$	PO_3H_2
e	$CH_2NHCOPh$	PO_3H_2
f	$CH_2NHCOOCH_3$	PO_3H_2
g	$CH_2NHCOOC_2H_5$	PO_3H_2
h	$CH_2NHCOOC_3H_7$	PO_3H_2
i	$CH_2NHCOCH_3$	$P_3O_9H_4$
j	$CH_2NHCOPh$	$P_3O_9H_4$



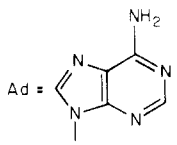
	R^1	R^2
2a	$NHCOCH_3$	PO_3H_2
b	$NHCOC_2H_5$	PO_3H_2
c	$NHCOCH_2Cl$	PO_3H_2
d	$NHCOC_6H_5$	PO_3H_2
e	$NHCOC_6H_4$-*p*-F	PO_3H_2
f	$NHCOC_6H_4$-*o*-CH_3	PO_3H_2
g	$NHCOC_6H_4$-*m*-CH_3	PO_3H_2
h	$NHCOC_6H_4$-*p*-CH_3	PO_3H_2
i	$NHCH_2CH_2CN$	PO_3H_2
j	$N(COC_6H_5)CH_2CH_2CN$	PO_3H_2
k	$NHCOCH_3$	$P_3O_9H_4$
m	$NHCOCH_2Cl$	$P_3O_9H_4$
n	$NHCH_2CH_2CN$	$P_3O_9H_4$



	R^1	R^2
3a	CH_3	PO_3H_2
b	C_2H_5	PO_3H_2
c	CH_3	$P_3O_9H_4$



	R^1	R^2
4a	$COCH_3$, $COCH_3$	PO_3H_2
b	$CHCH_3$	H
c	$CHCH_3$	PO_3H_2
d	$C(CH_3)_2$	PO_3H_2
e	$CHCH_3$	$P_3O_9H_4$
f	$C(CH_3)_2$	$P_3O_9H_4$



of nucleoside 5′-phosphates to phosphoanhydride derivatives. Alkaline phosphatase liberated 2.0 mol of inorganic phosphate from each mole of **1i** or **1j**, in additional confirmation of their structure. Compounds **1i** and **1j** were much less effective than ATP as substrates of potato ATPase. Compound **1b**[8] had been obtained in high yield by reduction of a 1:1 mixture of two epimers of 5′-deoxy-5′-(*C*-cyanophosphonomethyl)adenosine,[4] differing in configuration of the carbon α to phosphorus, and all the compounds of structure **1** can hence be presumed to also be mixtures of such epimeric forms.

The β-D-allo (structure **2**) and α-L-talo (**3**) epimers of 5′-(*C*-acylaminomethyl) derivatives of AMP and ATP were either described previously[11] or were prepared for the present studies by minor modifications of the previous

Table I. Properties of Compounds with Structures 1 and 4

Compd	Yield, %	Uv max (H_2O), nm ($\epsilon \times 10^{-3}$)	Electrophoresis[b] pH 7.5	Electrophoresis[b] pH 3.5	R_f system A	R_f system B	R_f system C	R_f system D	R_f system E	Phosphate/base ratio[a]	Formula	Analyses
1e	40	257 (14.9), 230 (23.0)	0.78		0.48	0.64					$C_{19}H_{21}N_6O_7PNa_2 \cdot 3H_2O$	C, H, N, P
1i	32	258 (14.7)		1.91			0.48		0.27	2.01	$C_{14}H_{19}N_6O_{13}P_3Na_4 \cdot 3H_2O$	P
1j	42	257 (14.9), 230 (23.1)		1.80			0.62		0.36	1.98	$C_{19}H_{21}N_6O_{13}P_3Na_4 \cdot 2H_2O$	P
4b	46	260 (14.7) (ethanol)			0.77			0.74			$C_{12}H_{15}N_5O_4 \cdot 0.3C_2H_5OH$	C, H, N
4c	49	259 (14.8)	0.92		0.51		0.84	0.38		0.98	$C_{12}H_{14}N_5O_7PNa_2 \cdot 1H_2O$	P
4e	54	258 (15.0)		2.37			0.57		0.34	3.08	$C_{12}H_{14}N_5O_{13}P_3Na_4 \cdot 3H_2O$	P
4f	67	260 (14.7)		2.30			0.60		0.40	2.92	$C_{13}H_{16}N_5O_{13}P_3Na_4 \cdot 3H_2O$	P

[a] The phosphate measured was that liberated by alkaline phosphatase. [b] AMP = 1.0.

procedures as described in the Experimental Section. The synthesis of 2′,3′-di-*O*-acetyl-AMP (**4a**) and of 2′,3′-*O*-isopropylidene-AMP (**4d**) was previously reported.[8] Treatment of adenosine with excess of acetaldehyde in the presence of 10 equiv of *p*-toluenesulfonic acid gave a crystalline 2′,3′-*O*-ethylideneadenosine **4b** in 46% yield. The ^{1}H NMR spectrum indicated that **4b** consisted of only one of the two possible diastereoisomers epimeric about the chiral acetal function. Acid-catalyzed condensation of uridine with acetaldehyde has likewise been reported[12] to give rise to a single diastereoisomer of 2′,3′-*O*-ethylideneuridine. In the ^{1}H NMR spectrum of **4b** in methyl-d_6 sulfoxide the methyl protons occurred as a doublet centered at δ 1.27–1.37 (after correction of the observed value for the use of an external standard of Me_4Si), thus indicating, from shielding considerations, that the methyl group is probably on the same side of the 1,3-dioxolane ring as H-2′ and H-3′.[13,14] Phosphorylation of **4b** by the dicyclohexylcarbodiimide–β-cyanoethylphosphate method of Tener[15] gave 2′,3′-*O*-ethylideneadenosine 5′-phosphate (**4c**) in 50% yield as its homogeneous disodium salt. This was readily converted by the Michelson procedure[10] to 2′,3′-*O*-ethylideneadenosine 5′-triphosphate (**4e**) which was isolated in homogeneous form as its tetrasodium salt after purification by anion-exchange chromatography on diethylaminoethylcellulose. The 2′,3′-*O*-isopropylidene derivative of ATP (**4f**) was prepared from 2′,3′-*O*-isopropylidene-AMP (**4d**)[8] via its phosphoroimidazolidate according to the procedure of Hoard and Ott.[16] The properties of the new compounds prepared for this work are given in Tables I and II.

Studies with Hexokinase. Substrate and inhibitor properties of the ATP derivatives studied with this enzyme are given in Table III. The α-acetylaminomethyl and α-benzoylaminomethyl phosphonates **1i** and **1j** are substrates, showing that the hexokinase–ATP complex can accommodate bulky substituents in the vicinity of O-5′ of ATP. The region of bulk tolerance extends, at least in some measure, to C-5′ of ATP as revealed by the substrate activity of the allo epimer of 5′-acetylaminomethyl-ATP (**2k**). The talo epimer **3c**, in contrast to **2k**, exhibited no substrate properties in the presence of 100 times the normal assay level of hexokinase and showed no inhibitory action at a level 10 times higher than the enzyme–inhibitor dissociation constant of **2k**. The marked difference in the properties of epimers **3c** and **2k** suggests that during catalysis the tripolyphosphate portion of ATP as well as its nucleosidic portion may interact with the enzyme, thereby restricting rotation of the C-4′–C-5′ and O-5′–C-5′ bonds and causing epimeric 5′ substituents to orient differently in relation to enzymic groups. The 2′,3′-*O*-ethylidene and 2′,3′-*O*-isopropylidene derivatives of ATP (**4e** and **4f**, respectively) were not substrates and were weak noncompetitive inhibitors with respect to ATP, thus indicating that these substituents significantly impede formation of the enzyme–ATP complex. The V_{max} values of 2′- and 3′-deoxy-ATP are ca. 6% that of ATP[17] and it is possible that the 2′ and 3′ hydroxyls of ATP interact with hexokinase during catalysis and that there is difficulty in accommodating O-2′ and O-3′ substituents within the enzyme–ATP complex. In regard to the adenine ring of ATP, previous work has shown that introduction of acyl,[18] aroyl,[7] or bulky alkyl[18] groups on N^6 or of bromine or amino groups on C-8 allows formation of the yeast hexokinase–ATP complex as evidenced by high V_{max} values of those ATP derivatives.

Studies with the AMP Site of AMP Kinase. Substrate specificity data and magnetic resonance studies[1,19]

Table II. Properties of Compounds with Structure 2

Compd	Formula	Analyses	Yield, %	R_f system A	B	C	D	E	Electrophoretic mobility rel to AMP pH 7.5	pH 4.5	pH 3.8	Absorption[a] ($\epsilon \times 10^{-3}$) pH 1	pH 12	Phosphate/base ratio[b]
2c	$C_{13}H_{16}N_6O_8ClPNa_2 \cdot 2H_2O$	C, H, N, Cl, P	74	0.34	0.69				0.90		0.74	14.9	15.1	
2e	$C_{18}H_{18}N_6O_8FPNa_2 \cdot 2H_2O$	P	81	0.55	0.77		0.34	0.58	0.84	0.90		14.8	15.0	1.04
2f	$C_{19}H_{21}N_6O_8PNa_2 \cdot 2H_2O$	P	83	0.58			0.34		0.81	0.90		14.8	15.0	0.97
2g	$C_{19}H_{21}N_6O_8PNa_2 \cdot 2H_2O$	P	76	0.57			0.33		0.80	0.89		14.7	14.8	1.01
2h	$C_{19}H_{21}N_6O_8PNa_2 \cdot 2H_2O$	P	81	0.58			0.33		0.80	0.90		14.6	14.7	1.07
2j	$C_{21}H_{22}N_7O_8PNa_2 \cdot 2H_2O$	P	80	0.42			0.30		0.75	0.75		14.8	15.0	1.10
2m	$C_{13}H_{16}N_6O_{14}ClP_3Na_4 \cdot 1.0H_2O$	P	48	0.14		0.48					2.05	15.0	15.3	3.05
2n	$C_{14}H_{18}N_7O_{13}P_3Na_4 \cdot 3H_2O$	P	43	0.09		0.41	0.05	0.45			1.46	14.8	15.0	3.10
AMP				0.26	0.63	0.56	0.10	0.43	1.00	1.00	1.00			
ATP				0.09		0.42	0.03	0.44			2.34			

[a] All compounds had maxima at 257 nm at pH 1 and 259 nm at pH 12. [b] The phosphate measured was that liberated by alkaline phosphatase.

Table III. Substrate and Inhibition Constants of ATP Derivatives with Muscle AMP Kinases and Yeast Hexokinase

Enzyme source	Compd	Substrate constants[a]			Inhibition properties		
		Protein per assay, μg	K_m, μM	V_{max}, rel %	Type[b]	K_i[c]	Inhibitor concn, μM
Rabbit AMP kinase	ATP	0.052	330	100[d]		330[h]	
	1i	1.0	640	0.7			
	1j	1.2		0	NC, L	110	104, 208
	2k	1.14	1250	5.4	C, NL	520, 180[e]	261, 1042
	2m	1.14		0	C, L	325	84, 167, 335
	2n	1.14		0	C-NC, NL	4000, 950[f]	500, 1000
	3c	1.14	270	1.2	C-NC, L	1270	270, 540, 1000
	4e	0.2	330	57			
	4f	0.2	160	45			
	8-Br-ATP	0.2	1180	50			
Pig AMP kinase	ATP	0.052	180	100[g]		180[h]	
	1i	1.0	740	0.7			
	1j	1.2		0	NC, L	475	104, 208
	2k	1.14	260	4.8	C, NL	860, 180[f]	261, 1042
	2m	1.14	320	1.3	C, L	285	84, 167
	2n	1.14		0			
	3c	1.14		0	C-NC, L	1520	270, 540, 1000
	4e	0.2	390	42			
	4f	0.2	290	50			
Carp AMP kinase	ATP	0.28	800	100[i]			
	4e	0.1	495	68			
	4f	0.1	625	73			
Hexokinase	ATP	0.1	100	100[j]			
	1i	1.0	390	0.9			
	1j	5.0	305	0.9			
	2k	10.0	560	1.9	C, L	820	577, 1154
	3c	10.0		0	No inhibition		8400
	4e	300.0		0	NC, L	4200	860, 1720
	4f	300.0		0	NC, L	3900	1520, 3040

[a] See footnote *a*, Table IV. [b] See footnote *b*, Table IV; C and NC are with respect to ATP. [c] Enzyme-inhibitor dissociation constant; the ATP levels used for the K_i determinations ranged from 100 to 600 μM for the AMP kinases and 100 μM to 1 mM for hexokinase. [d] V_{max} was 122 μmol of ATP/min/mg of protein. [e] The two values were obtained by linear replots of the inhibitions produced by 261 and 1042 μM, respectively, of 2k. [f] Values calculated as in footnote *e*. [g] V_{max} was 100 μmol of ATP/min/mg of protein. [h] For the source of this value see the Experimental Section. [i] V_{max} was 144 μmol/min/mg of protein. [j] V_{max} was 106 μmol/min/mg of protein.

have shown that adenylate kinase has nonequivalent binding sites for AMP and ATP, respectively. Kinetic parameters of the present series of AMP derivatives with rabbit and pig AMP kinases are given in Table IV. The substrate properties with both enzymes of the phosphonates **1a** and **1c** show that introduction of an acetoxy or acetylaminomethyl group adjacent to O-5′ of AMP does not prevent formation of enzyme–substrate complexes. With longer aliphatic chains (**1d,f–h**) substrate activity was not detectable but inhibition by these compounds remained linearly competitive with respect to AMP suggesting that affinity for all or part of the AMP site is retained. With the rabbit enzyme the K_I of **1c** is similar to the values of AMP (500 μM) and the parent phosphonate isostere of AMP (1, R^1 = H; R^2 = PO_3H_2) (440 μM[4]), and introduction of more methylene groups into the substituent of **1c** to give **1d**, or into the substituent of **1f** to give **1g** and **1h**, promotes binding of compounds **1** to the enzyme and indicates that their substituents interact with a lipophilic region.

The 5′-C-substituted AMP derivatives of the allo configuration (structure **2**) behaved as linear competitive inhibitors of the rabbit enzyme (Table IV), indicating that they combine solely with enzyme uncomplexed with AMP; the K_i values are hence most probably a measure of affinity of the compounds for the AMP site. The affinity increased when the lipophilicity of the 5′ substituent was increased by replacing the methyl group of **2a** by ethyl, phenyl, or *o*-, *m*-, or *p*-tolyl groups; on the other hand, the affinity decreased when the polar character of the 5′ substituent was increased by replacing ethyl by chloromethyl or phenyl by *p*-fluorophenyl. These findings clearly indicate interaction of the 5′ substituents with a lipophilic region of the rabbit enzyme. The results with the pig AMP kinase were more complex. The K_i values of the acetyl-, propionyl-, and chloroacetylaminomethyl derivative **2a–c** suggested, as for the rabbit enzyme, that these 5′ substituents interact with a lipophilic area of the pig enzyme. However, the kinetic data indicate that 5′-benzamidomethyl-AMP (**2d**) binds to the AMP site of the pig enzyme one-third as strongly as 5′-acetamidomethyl-AMP (**2a**) whereas it binds to the rabbit enzyme approximately twice as strongly as **2a**. From this it would appear that the pig enzyme possesses a structural feature (possibly a polar region near the benzene ring) which is responsible for the lessened affinity of **2d** and that in the rabbit enzyme this feature is absent or exerts a less pronounced effect. Further evidence that the two enzymes are structurally different in the regions which interact with the phenyl group of **2d** is that introduction of a fluorine into **2d** enhances affinity for the pig enzyme and decreases affinity for the rabbit enzyme. Introduction of a methyl group ortho, meta, or para in **2d** enhanced affinity for both pig and rabbit enzymes, possibly because the tolyl residues bind to a nonpolar region common to both enzymes.

The three 2′,3′-O-substituted derivatives of AMP (**4a,c,d**), with the exception of **4c** with the pig enzyme, were competitive inhibitors of the pig and rabbit kinases. Further work will be required to determine if affinity for the AMP site is retained when larger substituents are introduced at these positions of the ribofuranose ring. The ethylidene derivative **4c** inhibited the pig enzyme noncompetitively and thus apparently binds to the enzyme–AMP complex more strongly than to the free enzyme; that

Table IV. Substrate and Inhibition Constants of AMP Derivatives with AMP Kinases

		Substrate constants[a]			Inhibition properties		
Enzyme source	Compd	Proteins per assay, μg	K_m, μM	V_{max}, rel %	Type[b]	K_i,[c] μM	Inhibitor concn, μM
Rabbit muscle	AMP	0.052	320	100[d]		500[e]	
	1a	10.0	270	0.27	C, L	1020	170, 340
	1c	40.0	100	0.12	C, L	545	154, 308
	1d	40.0		0	C, L	340	190, 380
	1e	250.0		0			
	1f	40.0		0	C, L	450	320, 640
	1g	40.0		0	C, L	310	145, 290
	1h	40.0		0	C, L	195	140, 280
	2a	200.0		0	C, L	1900	550, 1100
	2b	200.0		0	C, L	930	550, 1100
	2c				C, L	5300	1100, 2200
	2d	200.0		0	C, L	1120[f]	840, 1680
	2e	200.0		0	C, L	1660[g]	1520, 3040
	2f					620[h]	1095
	2g					850[h]	1095
	2h					560[h]	1100
	2i				C, L	3800	1000, 2000
	2j				C, L	5220	650, 1300
	3a	100.0	1000	0.02	C-NC,[i] L	1320	550, 1100
	3b	100.0	1250	0.01	C-NC,[i] L	760	550, 1100
	4a	200.0		*j*	C, L	1000	123, 493
	4c	200.0		0	C, NL	410, 285[k]	140, 280
	4d	200.0		0	C, L	870	103, 309
	8-Br-AMP	0.57		0			
Pig muscle	AMP	0.052	250	100[l]			
	1a	10.0	670	0.62	C, L	1050	193, 387
	1c	40.0	200	0.08			
	1e	250.0		0			
	2a	200.0		0	C, L	1380	550, 1100
	2b	200.0		0	C, L	560	550, 1100
	2c				C, L	4100	1200, 2400
	2d	200.0		0	C, L	3950	820, 1640
	2e	200.0		0	C	3100	3000
	2f					1150[h]	1095
	2g					1560[h]	1095
	2h					1020[h]	1100
	2i	200.0		0	C, L	3140	565, 1130
	3a	100.0	330	0.01	C-NC, L	820	550, 1100
	3b	100.0	250	0.004	C-NC, L	510	550, 1100
	4a	200.0		*j*	C, L	1450	246, 493
	4c	200.0		0	NC, L	1290	720, 1440
	4d	200.0		0	C, L	830	103, 206

[a] K_m (Michaelis constant) = concentration of substrate for half-maximal velocity; V_{max} = maximal velocity. [b] L = linear, NL = nonlinear, C = competitive with respect to AMP, NC = noncompetitive. [c] Enzyme-inhibitor dissociation constant. The AMP levels used for the K_i determinations ranged between 100 μM and 2 mM. [d] V_{max} was 135 μmol of AMP transformed/min/mg of protein. [e] Value from ref 24. [f] The K_i value determined from a single level (835 μM) of **2d** and a single level (84.5 μM) of AMP (see Experimental Section) was 1300 μM. [g] A K_i value of 2000 μM was calculated from 980 μM **2e** and 84.5 μM AMP. [h] The K_i values were calculated from the reaction rates produced by the inhibitor levels listed (last column) in the presence of 84.5 μM AMP. [i] Principally NC. [j] Low activity (rel V_{max} 0.01%) was detected and may have arisen from possible traces of AMP in 4a. [k] The values are calculated from the two levels of **4c** used (140 and 280 μM, respectively). [l] V_{max} for AMP was 115 μmol/min/mg of enzyme.

4c might bind to a non-AMP site as well as to the AMP site of the rabbit enzyme is suggested by the observed decrease in apparent K_i with increase in **4c** concentration (Table IV).

Studies with the ATP Site of AMP Kinases. The amounts of AMP kinases which could be employed in tests for substrate activity of the ATP derivatives were more limited than in the studies with AMP derivatives because the enzyme preparations contained small levels of protein-bound ADP and/or ATP which after a period of time initiated a progressively accelerating conversion of AMP to ATP in the coupled enzyme assay system. The kinetic data obtained are shown in Table III. The acetylaminomethyl substituent of the ATP phosphonate analogue **1i** permits binding to the ATP site to occur as evidenced by the substrate activity of **1i** for both pig and rabbit kinases, whereas **1j** with its relatively bulky benzoylaminomethyl substituent has little tendency to bind to the ATP site as indicated by lack of substrate and competitive inhibitor properties. The kinetic data similarly indicate that at the adjoining C-5′ region of ATP the acetylaminomethyl and chloroacetylaminomethyl groups of the allo epimers **2k** and **2m** also permit binding to the ATP sites of the rabbit and pig kinases. The substrate activity of **3c** with the rabbit kinase shows that a 5′-*C*-(acetylaminomethyl) group in the talo configuration is tolerated within the ATP site. The pronounced substrate activity of 8-Br-ATP with the rabbit kinase and of **4e** and **4f** with the rabbit, pig, and carp kinases (Table III) shows that 8-bromo and 2′,3′-*O*-ethylidene and 2′,3′-*O*-isopropylidene substituents do not prevent formation of the respective enzyme–ATP complexes.

The kinetic results obtained provide evidence that acylaminomethyl groups can be introduced at the ribose–phosphate bridge (5′ and O-5′ positions) of ATP and AMP without preventing formation of the enzyme–ATP

complexes of hexokinase and AMP kinase and of the kinase–AMP complex. The evidence also indicates (except for **4c** and the pig enzyme) that ethylidene or isopropylidene groups can be introduced at O-2′ and O-3′ of AMP and ATP without preventing formation of the kinase–AMP or kinase–ATP complexes. Some of the derivatives tested (e.g., **1d,g,h, 2h,m**) bind to the enzymes as well or better than the parent substrate and it may be possible to design potent substrate-site-directed irreversible inhibitors by introducing suitably modified substituents at the same substrate atoms.

Experimental Section

Chemical Syntheses. General. Tri-*n*-butylammonium pyrophosphate was prepared at room temperature.[20] Pyridine and *N,N*-dimethylformamide were distilled from calcium hydride. Paper chromatography was performed on Whatman No. 1 or 3 MM paper in (A) 1-butanol–acetic acid–water (5:2:3), (B) 2-propanol–0.25 M triethylammonium bicarbonate (1:1), (C) isobutyric acid–1 M NH_4OH (10:6), (D) 2-propanol–concentrated ammonia–water (7:1:2), (E) 1-propanol–concentrated ammonia–water (55:10:35). Electrophoresis was performed with Whatman No. 1 paper in 0.05 M triethylammonium bicarbonate (pH 7.5) and 0.035 M citric acid–0.015 M sodium citrate (pH 3.5). Spots on chromatograms were detected by their ultraviolet absorption. Ultraviolet spectra were determined with a Cary Model 15 spectrophotometer and infrared spectra with a Perkin-Elmer spectrophotometer Model 137. ^1H NMR spectra were obtained with Varian XL-100-15 and Jeolco MH 60 spectrometers and are recorded as parts per million downfield from an external standard (concentric capillary) of $SiMe_4$; use of the external standard caused a downfield shift of 0.4–0.5 ppm for all protons. Evaporations were carried out in vacuo at bath temperatures below 30 °C. Elemental analyses were performed by Atlantic Microlabs, Atlanta, Ga., and Midwest Microlab, Ltd., Indianapolis, Ind. Phosphate analyses of nucleoside triphosphates were performed by the method of Lowry and Lopez[21] after treatment of ca. 1 μmol of these compounds for 60 min at 22 °C in 1 ml of Tris buffer, pH 10.4, containing 0.02 mg of alkaline phosphatase of calf intestinal mucosa (Type VII, Sigma Chemical Co.).

5′-Deoxy-5′-[*C*-(benzoylaminomethyl)phosphonomethyl]adenosine (1e). To a solution of 1b (0.17 mmol) in ethanol–water (3:2, 7.5 ml) was added *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.34 mmol) and benzoic acid (0.34 mmol). The solution was stirred at 37 °C (bath temperature) for 3 h and concentrated to dryness. The residue was extracted with ether (3 × 50 ml) and dissolved in water and applied to paper chromatograms. After development in solvent B two bands of equal intensity (R_f 0.57 and 0.64) were obtained. The band at R_f 0.64 was eluted with water, the solution was freeze-dried, and 1e was precipitated as its white disodium salt (40% yield) by addition of NaI in acetone to a solution in MeOH of the triethylammonium salt.[16,22] The product was homogeneous in solvents A and B and on electrophoretograms run at pH 7.6: ^1H NMR (D_2O) δ 8.71 (br m, 1, H-8), 8.53 (br s, 1, H-2), 7.77 (br m, 5, phenyl), 6.38 (br m, 1, H-1′), 5.10 (overlap of HDO and H-2′ bands caused uncertainty in location of H-2′ signal), 4.78 (overlap with HDO signal caused uncertainty in location of H-3′ and H-4′ signals), 3.94 (m, 2, H-7′), 2.43 (br s, 2, H-5′), 1.72 (br m, 1, H-6′). For other properties see Table I.

5′-Deoxy-5′-[*C*-(acetylaminomethyl)-*O*-(pyrophosphoryl)phosphonomethyl]adenosine (1i). An aqueous solution of compound 1c (0.12 mmol; purified in solvent B) was percolated through a column of Dowex 50 ion-exchange resin (pyridinium form) and the combined eluate and aqueous washings were evaporated to dryness. The residue was dried by distillation from it of pyridine (3 × 2 ml) and was dissolved in pyridine (2 ml) containing tri-*n*-butylamine (0.36 mmol). The solution was evaporated to dryness and again after dissolution of the residue in anhydrous *N,N*-dimethylformamide (1 ml). To the residue was added dioxane (0.5 ml), *N,N*-dimethylformamide (0.1 ml), diphenylphosphorochloridate (36 μl), and tri-*n*-butylamine (40 μl) and the solution was stirred at room temperature for 3 h. Volatiles were removed and ether (20 ml) was added. The mixture was stored at 0 °C for 1 h. The ether was decanted, dioxane (0.2 ml) was added, and the solution was concentrated to a syrup. A solution of tri-*n*-butylammonium pyrophosphate (0.4 mmol) in pyridine (0.55 ml) was added and the mixture was stirred at room temperature for 0.75 h. Volatiles were removed and ether was added. The ether was decanted and the residue was dissolved in water (2 ml) and chromatographed on paper in solvent C. Compound 1i was eluted with water and isolated as the white solid tetrasodium salt by the method of Hoard and Ott.[16] The product was homogeneous in the systems of Table I.

5′-Deoxy-5′-[*C*-(benzoylaminomethyl)-*O*-(pyrophosphoryl)phosphonomethyl]adenosine (1j). Compound 1e (0.044 mmol; purified as above in solvent B) was converted to 1j by the same procedures used for the synthesis of 1i and the tetrasodium salt was obtained as a white powder homogeneous in the systems of Table I.

β-D-Allo and α-L-Talo Epimers of 5′-(*C*-Acylaminomethyl) Derivatives of AMP and ATP (Structures 2 and 3, Respectively). The preparation of compounds **2a,b,d,i,k** and **3a–c** from the respective epimers of 5′-(*C*-aminomethyl)-AMP was reported previously.[11] The monophosphates **2e–h** and **2j** were prepared by method D previously described;[11] for the preparation of **2c**, method D was modified in that the acylation was carried out at 45–50 °C for 24 h and employed a fourfold excess of chloroacetic acid and of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. Monophosphates **2f–h** and **2j** were purified by descending chromatography on Whatman 3 MM paper in 1-propanol–NH_4OH–H_2O (7:1:2) and **2c** and **2e** were purified similarly in solvent A. The triphosphates **2m** and **2n** were prepared and isolated by the method of Hoard and Ott.[16] All these compounds were homogeneous in the systems of Table II.

2′,3′-*O*-Ethylideneadenosine (4b). A suspension of adenosine (5 g, 18.7 mmol) in acetaldehyde (250 ml) was cooled in an ice bath and to this was added slowly *p*-toluenesulfonic acid (36 g, 187 mmol). After dissolution was complete, the solution was allowed to rise to room temperature over 1 h. It was added to 0.5 M $NaHCO_3$ (1 l.) and the mixture evaporated to dryness. The residue was extracted at 25 °C with chloroform (2 l.); the dried ($MgSO_4$) chloroform was evaporated and the residue crystallized from ethanol to yield 1.5 g of 4b, mp 224–226 °C. The supernatant was subjected to preparative thin-layer chromatography on silica gel in tetrahydrofuran–EtOAc (1:1) and material at R_f 0.46 was eluted with ethanol and crystallized from ethanol to yield an additional 1 g of 4b: mp 225–228 °C; $[\alpha]^{26.8}D$ −70° (*c* 1, EtOH); ^1H NMR (100 MHz, Me_2SO-d_6) δ 8.71 (s, 1, H-8), 8.53 (s, 1, H-2), 7.68 (s, 2, NH_2), 6.52 (d, 1, J = 3 Hz, H-1′), 5.62 (m, 3, H-2′, 5′-OH and $CHCH_3$), 5.26 (m, 1, H-3′), 4.64 (m, 1, H-4′), 3.92 (m, 2, H-5′), 1.77 (d, 3, J = 5 Hz, $CHCH_3$). The sharpness of the H-2 and H-8 signals confirmed that the product consists of a single isomer. The product was homogeneous in the systems of Table I.

2′,3′-*O*-Ethylideneadenosine 5′-Monophosphate (4c). Compound 4b (0.5 g, 1.7 mmol) was converted to the β-cyanoethyl ester of 4c by the method of Tener.[15] The crude ester was dissolved in methanol–concentrated NH_4OH (1:1, 150 ml) and the solution was stirred overnight at 50 °C, then filtered, concentrated, and applied to paper chromatograms for development in solvent D. The band at R_f 0.38 was eluted with water and the product converted to its sodium salt as described for 1e. Compound 4c was homogeneous in the systems of Table I. It was unreactive toward 5′-nucleotidase (Sigma Chemical Co., Grade II, 2 mg/ml) upon incubation at pH 10.4, 37 °C, for 6 h, but was quantitatively converted to 4b upon reaction with alkaline phosphatase.

2′,3′-*O*-Ethylideneadenosine 5′-Triphosphate (4e). Compound 4c (0.24 mmol) was converted to 4e by the same procedure used for the preparation of 1i and purified by paper chromatography in solvent E. The band at R_f 0.34 was eluted with water and the product was isolated as its tetrasodium salt which was found to be homogeneous in the systems of Table I.

2′,3′-*O*-Isopropylideneadenosine 5′-Triphosphate (4f). 2′,3′-*O*-Isopropylideneadenosine 5′-phosphate[8] (0.11 mmol) was converted to 4f by the procedure of Hoard and Ott[16] and isolated as its chromatographically homogeneous tetrasodium salt after purification by paper chromatography in solvent E. See Table I for properties.

Treatment of 1i and 1j with Adenosine Triphosphatase and Adenosine Diphosphatase. To separate solutions of 1i (0.5

mg) and 1j (0.5 mg) in 100 μl of phosphate buffer (pH 6.5) was added potato apyrase (Sigma Chemical Co., Grade 1, 10 mg). The reactions were monitored by paper electrophoresis at pH 3.5 and by descending paper chromatography in solvent C. After 10 min 1i and 1j were unaffected whereas ATP itself was completely converted to AMP within 1 min. An additional 90 mg of apyrase was added to each solution. After a further 2 min ca. 30% conversion of 1i and 1j to 1c and 1e, respectively, had occurred. After 15 min the conversions were complete.

Enzyme Kinetic Studies. Pig and rabbit muscle adenylate kinase, rabbit muscle pyruvate kinase, yeast glucose-6-phosphate dehydrogenase (Grade II), and yeast hexokinase were purchased from Boehringer and carp muscle myokinase was a gift from Dr. Mildred Cohn. Lactate dehydrogenase (Type 1, rabbit muscle) was from Sigma Chemical Co. All assays were carried out at 22 °C by measuring the rate of change of optical density at 340 nm in a Cary Model 15 spectrophotometer using 1-cm cells containing a final volume of 1 ml. Initial velocities were in every case linear and proportional to the concentration of primary enzyme and independent of the level of secondary enzymes in the assay systems. Each substrate study employed four or more concentrations of substrate and unless noted otherwise each study of inhibition employed four or more levels of normal substrate for each of two levels of inhibitor. Substrate constants were determined from Lineweaver–Burk plots, all of which were linear, and enzyme–inhibitor dissociation constants (K_i values) were obtained from replots of inhibitor concentration vs. slopes of the Lineweaver–Burk plots and were reproducible to within 7%. The K_i value of AMP with rabbit AMP kinase is 500 μM;[23] the K_i values of ATP with the rabbit and pig kinases (Table IV) were obtained as discussed previously.[7] For **2f**, **2g**, and **2h** the K_i values (accuracy within 15%) were obtained from the inhibition produced by a single level of inhibitor with a single level of normal substrate assuming that the inhibitions were competitive as they were in the case of **2d** and **2e**.

For studies of substrate and inhibitor kinetics each adenylate kinase was employed in 1 ml of 0.1 M Tris–HCl (pH 7.6) containing $MgSO_4$ (0.92 mM), KCl (0.11 M), phosphoenolpyruvate cyclohexylammonium salt (0.31 mM), NADH (0.38 mM), AMP (0.39 mM; included in the studies of ATP and ATP analogues), ATP (0.24 mM; included when studying AMP and AMP analogues), pyruvate kinase (10 μg), and lactate dehydrogenase (10 μg). When 100 μg or more of AMP kinase was employed per assay the enzyme was collected by centrifugation and dissolved in the assay buffer in order to prevent inhibition of the reaction by ammonium sulfate present in the enzyme preparation. In studies of the AMP derivatives as inhibitors a stock solution of ATP (5 mg), phosphoenolpyruvate (5 mg), NADH (10 mg), 0.1 M Tris–HCl (pH 7.6, 1.42 ml), 4 M KCl (1 ml), 32 mM $MgSO_4$ (1 ml), 35 μl of PK (10 mg/ml), 35 μl of LDH (10 mg/ml), and the adenylate kinase (10 μl, 1.82 μg) was made daily and stored at 22 °C for 1 h to permit stabilization of the absorbancy at 340 nm; 100 μl of this solution was added to 0.1 M Tris–HCl after which the AMP derivative and/or AMP were added to bring the volume to 1 ml. For studies of reversible inhibition by ATP derivatives, ATP was omitted from the above stock solution which then required no storage period prior to use. The required amount of ATP was added to the assay mixture 1 h prior to addition of the ATP derivative after which the reaction was initiated by the addition of AMP (3.9 mM, 100 μl).

Hexokinase was studied in 1 ml of 0.1 M Tris–HCl (pH 7.6) containing $MgCl_2$ (6 mM), α-D-glucose (0.2 M), NADPH (0.78 mM), and glucose-6-phosphate dehydrogenase (35 μg).

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