Approaches to Isozyme-Specific Inhibitors. 17.[1] Attachment of a Selectivity-Inducing Substituent to a Multisubstrate Adduct. Implications for Facilitated Design of Potent, Isozyme-Selective Inhibitors

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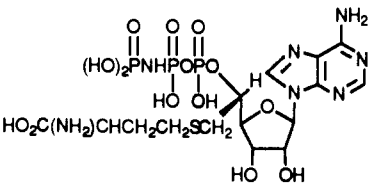
The synthesis is described of a methyl-C5′ adduct of L-methionine and β,γ-imido-ATP bearing a 6-S-*n*-Bu group in place of the 6-NH_2 group of the parent adduct. The latter is a potent multisubstrate inhibitor in a model system consisting of the M-2 and M-T isozymes of rat methionine adenosyltransferase. When attached to ATP, the 6-S-*n*-Bu group induces selectivity for M-T inhibition by elevating affinity for the ATP site of M-T but not of M-2. In the above adduct it exerted a similar effect, expressed by selectivity and increased inhibitory potency toward M-T. This affords a second illustration of the ability of this approach to generate, relatively readily, a potent inhibitor with moderate isozyme selectivity. An overview is given of extensive evidence from the present series of studies that moderate (ca. 10-fold) isozyme selectivity is often exhibited by substrate derivatives bearing a single short substituent at a variety of atoms. This, together with features of another feasible approach to isozyme-selective inhibitor design, suggests an approach that has potential to facilitate the design of potent inhibitors that are both isozyme-selective and selective for a given metabolic conversion. It comprises (1) evaluation of the above type of substrate derivatives as inhibitors of a chemotherapeutically significant set of isozymes (target and nontarget), (2) attempted derivation of a potent multisubstrate adduct inhibitor of the isozymes, (3) attachment to such an adduct of one or more selectivity-inducing substituents revealed in the first step, and, if desired, (4) systematic modification of substituents with a view to obtaining enhanced potency and/or isozyme-selectivity.

The present series of studies has aimed to devise approaches that could facilitate the design of potent enzyme inhibitors that exhibit species and/or tissue selectivity (referred to here as isozyme selectivity).[2] The studies have utilized enzymes of the type that catalyze reactions between two substrates, and have been directed to approaches that depend solely upon correlations between inhibitory potency and inhibitor structure and take no account of inhibitor–isozyme adduct structure. Approaches arising from these studies are hence potentially applicable to the majority of prospective target isozymes, in particular to isozymes that currently are of undetermined tertiary structure.[3]

In a survey that included groups of isozymes catalyzing four different reactions, we found that monosubstituted substrate derivatives possessing short (up to five-atom) substituents at a variety of substrate atoms exhibited inhibitions that were moderately (often ca. 10-fold) isozyme-selective in a high proportion of cases.[4-12] These derivatives usually did not produce inhibition that was potent. However, with a single model system of isozymes that was tested, it was found possible to retain the level of selectivity, yet to increase the potency 10^3-fold, by introducing the selectivity-inducing substituent at the corresponding atom of a potent, though nonselective multisubstrate adduct inhibitor.[6] Overall, the approach had generated a potent, moderately (45-fold) isozyme-selective inhibitor in a relatively direct fashion. In light of this, the present studies were carried out to explore the approach further, using for that purpose a second model system in which the isozymes catalyzed a different type of reaction. We had found that **1**, a CH_3-C5′ adduct of L-methionine



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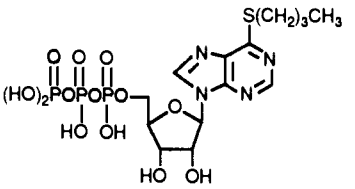

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and β,γ-imido-ATP, is a potent multisubstrate inhibitor of two isozymes of rat methionine adenosyltransferase.[13] In addition, we had found that replacement of the 6-amino group of the substrate ATP by a 6-S-*n*-Bu group, giving **2**, produces 3-fold selectivity for inhibition of the target isozyme of the model system.[11] The present paper describes a satisfactory route that we have developed for synthesis of an adduct **17** in which the selectivity-inducing 6-S-*n*-Bu group of **2** is substituted for the 6-NH_2 group of **1**. Inhibition constants for the interaction of **17** with the above isozymes of methionine adenosyltransferase have been determined, and the values are compared here with values given by **1** and **2**. In addition, we review certain

(1) Part 16: Vrudhula, V. M.; Kappler, F.; Afshar, C.; Ginell, S. L.; Lessinger, L.; Hampton, A. *J. Med. Chem.* **1989**, *32*, 885.
(2) The term isozymes is used herein to denote enzymes from different biological sources that catalyze the same metabolic conversion and possess different structures.
(3) For isozymes that can be obtained in homogeneous form or in suitable crystal forms, alternative approaches to the design of selective inhibitors are available, e.g., correlations between inhibitory potency and structural features of isozyme–inhibitor complexes revealed from solution NMR analyses or X-ray crystallographic analyses.
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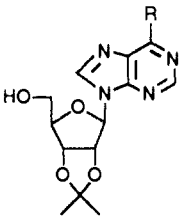
findings reported in the present series of papers, together with related findings reported by other laboratories. From this, we outline an approach that has potential to facilitate the design of potent inhibitors that possess both isozyme-selectivity and selectivity for a given metabolic conversion.

Synthesis of 17

The route that furnished **17** was a general one designed to permit introduction of a wide variety of thioether groups in place of the amino group at the 6-position of **1**. To that end, the last two steps of the sequence comprise a chemoselective S-alkylation of the methyl carboxylate ester of the 6-thiol analogue of **1** (**15**), followed by deesterification under conditions that preserve the L configuration of the homocysteine residue. The objective in the initial steps in both the present route to **17** and in the previously described route to its 6-amino counterpart **1**,[13] is to attach an L-methionine residue, via its methyl group, to C5′ of a suitably protected purine ribonucleoside. The present route accomplishes this in three steps (**4** → **10a,b**), and is markedly more efficient than the earlier route, which involved seven steps.

In the first step of the sequence, the readily accessible 6-chloro-9-β-D-ribofuranosylpurine[14] was converted into its 2′,3′-*O*-isopropylidene derivative, **3**. Compound **3** had

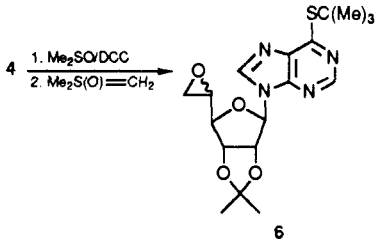


3: R = Cl
4: R = $SC(Me)_3$
5: R = SH

been obtained previously by reaction of 6-chloro-9-β-D-ribofuranosylpurine with acetone in the presence of 10 equiv of *p*-toluenesulfonic acid.[15] We examined the effect on this reaction of addition of 2,2-dimethoxypropane, because this ketal promotes acid-catalyzed reactions of acetone with a variety of ribonucleosides by creating more anhydrous conditions;[16] in addition, it can convert ribonucleosides to 2′,3′-*O*-isopropylidene derivatives under acidic conditions.[17] When a 5:1 ratio of 2,2-dimethoxypropane to nucleoside was employed, the amount of *p*-toluenesulfonic acid required to form **3** at roughly the same velocity was reduced 10-fold; this allowed use of a simplified isolation procedure that was particularly advantageous in larger scale (0.1 mol) preparations and reproducibly furnished **3** in 85% yield. Larger amounts of 2,2-dimethoxypropane in the reaction mixture produced increasing proportions of a second product that was less polar than **3** and that appeared, from its ^1H NMR spectrum, to be a mixed (monomethyl mononucleosidic) ketal resulting from reaction of 2,2-dimethoxypropane with the 5′- hydroxyl of **3**.

Because *tert*-butyl groups can be removed cleanly from *S-tert*-butyl thioethers by the action, at room temperature, of $Hg(OAc)_2$ at pH 4,[18] and because adenosine 5′-triphosphate is completely stable under these conditions,[19]

Scheme I



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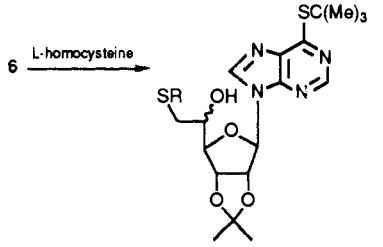
Scheme II



10a,b: R = $CH_2CH_2CH(NH_2)CO_2H$
11a,b: R = $CH_2CH_2CH(NH\text{-}Boc)CO_2Me$

we decided to evaluate the 6-*tert*-butylthio group as a precursor of the 6-mercapto group in the desired nucleoside triphosphate analogue **15**. 6-(*tert*-Butylthio)-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)purine, **4**, could be obtained in homogeneous form in 82% yield by treatment of **3** with sodium 2-methyl-2-propanethiolate in DMF–EtOH. Treatment of **4** for 1 h with $Hg(OAc)_2$ at pH 4 converted it quantitatively to a compound indistinguishable in TLC, HPLC, and UV spectral properties from 9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-6-mercaptopurine (**5**),[15] thereby demonstrating the potential suitability of the 6-*tert*-butylthio group for the proposed route to **17**. Quantitative conversion of **4** to **5** was mediated also by aqueous 90% trifluoroacetic acid (TFA) under the same conditions that had affected simultaneous and quantitative removal of *N*-Boc and 2′,3′-*O*-isopropylidene groups at the pentultimate step in earlier syntheses of **1** and other ATP–homocysteine adducts.[1] In the synthetic route to **17** that is described here, aqueous 90% TFA serves as a convenient and efficient reagent for removal of the *tert*-butyl group simultaneously with *N*-Boc and isopropylidene groups.

Compound **4** could be oxidized smoothly to the corresponding 5′-aldehyde by the Pfitzner–Moffatt Me_2SO–DCC procedure (Scheme I).[20] Treatment of the unisolated 5′-aldehyde in Me_2SO with dimethyloxosulfonium methylide[21] gave the diastereomeric 5′,6′-epoxides **6** in 25% yield after silica gel flash chromatography. When treated in MeOH with disodium L-homocysteinate, **6** gave the homogeneous 6′-thioethers **10a,b** (Scheme II) in 73% yield after reversed-phase chromatography.

The low overall yield (18%) of **10a,b** from **4** prompted us to investigate an alternative route (Scheme III) in which **4** would provide, via a Wittig reaction involving its 5′-aldehyde, the 5′,6′-alkene **7** from which the stereoisomeric 5′,6′ diols **8** could be obtained by OsO_4 treatment[22] and converted to their 6′-tosyloxy derivatives **9**, which, with disodium L-homocysteine, could then furnish **10a,b**.

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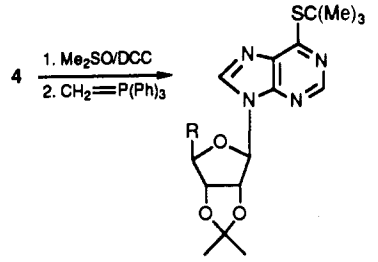
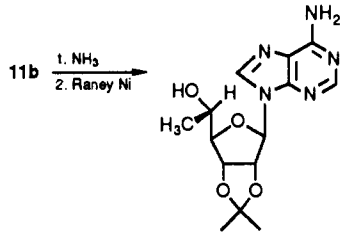
Scheme III



7: R = CH=CH$_2$
8: R = C(*R,S*)H(OH)CH$_2$OH
9: R = C(*R,S*)H(OH)CH$_2$OTs

Scheme IV



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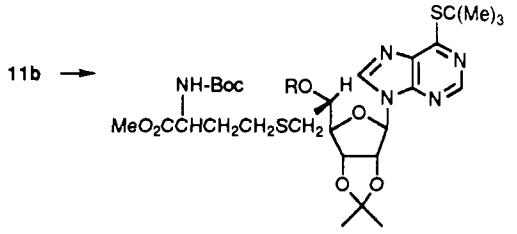
Support for this route was provided by earlier findings in this laboratory that N^6-benzoyl-2′,3′-*O*-isopropylideneadenosine could be thereby converted, in 70% yield, to the corresponding 5′,6′-alkene, and that this yielded, virtually quantitatively, a 6′-tosyloxy nucleoside analogous to **9** via the above two steps.[23] In addition, we had found earlier that a 7′-tosyloxy-6′-hydroxy nucleoside, when treated with disodium L-homocysteinate, gave the corresponding 7′-thioether in 77% yield.[24] Finally, we established that **4** (and hence presumably **7**) was stable under the above diol-forming conditions.[22] Reaction of the unisolated aldehyde of **4** (Scheme III) with methylenetriphenylphosphorane in THF or Me_2SO gave a complex mixture from which homogeneous **7** was isolatable in 20% yield by preparative silica gel TLC. The structure of **7** followed from its UV and ^{1}H NMR spectra, in particular from the chemical shifts of the 5′ and 6′ protons and the cis and trans coupling constants of the 6′ protons, which were identical with those reported for the 6′-amino analogue of **7**.[26] An alternative approach to **7**, which in certain cases has given better yields of olefin than the Wittig reaction, was to react the aldehyde from **4** with (trimethylsilylmethyl)magnesium chloride in ether–THF followed by elimination of trimethylsilanolate from the resulting secondary alcohol.[27] This gave only 25% yield of isolated **7**, however. The low yields of **6** and **7** might be related to the known base lability of nucleoside 5′ aldehydes[28] and to the basic character of the above two ylides and Grignard reagent because HPLC analysis showed that **6** or **7**, once formed, were stable in the reaction mixture.

The α-amino acid moiety in **10a,b** was converted into the *N*-Boc derivative of its methyl ester, **11a,b** (Scheme II), by modifications of procedures used previously with

Scheme V



13: R = PO$_3$H$_2$
14: R = P(OH)(O)OP(OH)(O)NHP(O)(OH)$_2$

homocysteine derivatives of similar structure to **10a,b**.[19] Reversed-phase semipreparative HPLC separated the more polar 5′ epimer, **11a**, (32% yield), from the other epimer, **11b** (55% yield). Reversed-phase HPLC separation of **10a,b** or their *N*-Boc derivatives was more difficult. To establish the C5′ configuration of **11b**, it was aminated at the 6-position under conditions similar to those that smoothly convert 6-(methylthio)-9-β-D-ribofuranosylpurine to adenosine[29] (Scheme IV). The product so obtained was treated with Raney nickel catalyst in EtOH. HPLC analysis of the sole product showed it to be identical with the known 5′(*S*)-*C*-methyl-2′,3′-*O*-isopropylideneadenosine (**12**) and different from its 5′*R* stereoisomer,[30] thereby showing **11b** to possess the 5′*R* configuration, which is present in **1** also.

Phosphorylation of **11b** with β-cyanoethyl phosphate–dicyclohexylcarbodiimide was attempted under conditions (3 days at room temperature) that normally are satisfactory with nucleoside secondary hydroxyls,[31] but less than 10% conversion to the desired cyanoethyl ester of the nucleoside 5′-phosphate **13** (Scheme V) took place. However, at 60–65 °C the reaction proceeded smoothly and was complete in about 3 h. The 5′*S* epimer **11a** underwent phosphorylation 6 times more slowly than **11b** over the first 0.5 h, consistent with a steric mechanism for the lowered reactivity of **11a** and **11b**. The reagent does not show the above effects with the 5′*R,S* forms of a structurally related adduct between L-ethionine and 2′,3′-*O*-isopropylideneadenosine,[1] suggesting a possible role for the 6-*tert*-butylthio group of **11a,b**.

The above phosphodiester derived from **11b** was readily converted in situ to the nucleoside 5′-phosphate **13** (Scheme V) by elimination of the cyanoethyl group through addition of *t*-BuOK, a procedure that, as described before,[1] leaves intact the carbomethoxy group that is needed for blocking purposes in the next operations. Nucleotide **13** was isolated in 50% yield and converted into its imidopyrophosphoroxy derivative **14** by procedures worked out previously for synthesis of a related methionine–ATP adduct.[19] Treatment of **14** with aqueous 90% TFA removed the isopropylidene, the Boc, and the *tert*-butyl groups simultaneously, giving **15**. The 6-mercapto group in **15** could be realkylated readily and completely at room temperature in DMF with 1.1 equiv of *n*-butyl iodide and 5 equiv of triethylamine. UV spectroscopy served as a sensitive indicator of the conversion of **15** into **16**. Finally, the carbomethoxy group of **16** was hydrolyzed with 1.1 equiv of NaOH to give **17**. The conditions used have been shown to cause <0.1% racemization of methyl esters of several dipeptides,[32] and hence are unlikely to

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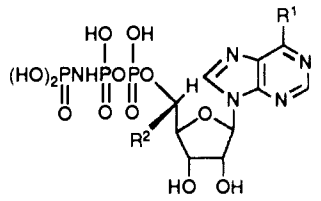

15: R^1 = SH; R^2 = $CH_2SCH_2CH_2CH(NH_2)CO_2Me$
16: R^1 = S-*n*-Bu; R^2 = $CH_2SCH_2CH_2CH(NH_2)CO_2Me$
17: R^1 = S-*n*-Bu; R^2 = $CH_2SCH_2CH_2CH(NH_2)CO_2H$

significantly racemize the L-homocysteine residue of **17**. Anion-exchange chromatography gave **17** in homogeneous form in 16% yield overall from **11b**. DEAE Trisacryl M (HCO_3^- form) was the preferred medium in the ion-exchange purification because it provided recoveries that were 20–30% higher than from DEAE cellulose (HCO_3^-), together with resolution that was equally good.

Studies of Isozyme Inhibition

Table I presents inhibition constants of **1**, **2**, and **17** with M-2 and M-T variants of rat methionine adenosyltransferase. The 6-desamino-6-*n*-butylthio adduct **17**, like its parent adduct **1**, was a potent inhibitor and exhibited competitive kinetics vs MgATP or vs L-methionine with both variants, except in the case of M-T and variable [Met], when it gave a mixed type of inhibition that was predominantly competitive. As concluded in the case of **1**,[13] these properties suggest concerted binding of **17** to the ATP and Met substrate sites of M-2 and M-T.[33]

The relative inhibitory potency values included in Table I show that **1** and **17** inhibit M-T more effectively than M-2, the effect being small with **1** and more pronounced with **17**. The observed K_i values indicate that **17** has a 1.8-fold higher affinity than **1** for the ATP site of M-T, and that **17** has an affinity equal to that of **1** for the Met site of M-T. With M-2, on the other hand, **17** and **1** have equal affinity for the ATP site, but **17** has 4.5-fold less affinity than **1** for the Met site.[35] On this basis, the selectivity of **17** toward M–T is higher than that of **1** by a factor of 8.

In earlier studies[11] it was possible to determine both a substrate and an inhibitor constant (K_M = 0.20 mM, K_i = 0.53 mM) in the particular instance of the interaction of 8-methylthio-ATP with M-T, and K_i/K_M was found to be ca. 2.5. If K_D/K_M for ATP (K_M = 0.14 mM) is also 2.5, then the K_D value for ATP with M-T would be ca. 0.35 mM. From this it would appear that the *n*-butylthio group in the ATP derivative **2** (K_i = 0.1 mM) interacts with M-T and enhances affinity for this isozyme by a factor of roughly 3.5. If ATP (K_M = 0.14 with M-2) is assumed to have a K_D value of 0.35 mM with M-2 also, then the K_i value (0.31 mM) of **2** indicates that its *n*-butylthio group has, in contrast, no detectable affect on affinity for the ATP site of M-2. In parallel with these properties of the 6-butylthio group of **2**, the 6-butylthio group of **17** enhances affinity for the ATP site of M-T by a factor of 1.8 but does not significantly influence affinity for the ATP site of M-2 (Table I). This suggests that those regions of enzyme-bound **2** and **17** that adjoin C6 are located in similar microenvironments, and that the microenvironment furnished by M-T differs from that furnished by M-2 in that portion of it has a net positive binding interaction with some or all of the 6-*n*-butylthio substituent. The present results, like those from an earlier parallel study with adenylate kinase isozymes,[6] demonstrate that it is feasible to transfer isozyme-selective inhibitory effects of a derivative of a single substrate to a weakly selective though potent multisubstrate adduct inhibitor, and to achieve thereby a large (here, 910-fold) evaluation of inhibitory potency. In the earlier example of this effect, elaboration of a monosubstituted substrate derivative into a multisubstrate adduct was found to increase potency 1000-fold while maintaining a 45-fold level of selective affinity for a targeted isozyme of adenylate kinase.[6] For reasons presented below, this procedure of attaching a small selectivity-inducing substituent at an appropriate atom of a multisubstrate adduct inhibitor is viewed as an integral phase of a potentially useful approach to the design of isozyme-selective inhibitors.

A Role for Monosubstituted Derivatives in Approaches to the Design of Potent, Isozyme-Selective Inhibitors

It is possible to distinguish three interrelated approaches that have yielded isozyme-selective inhibitors and that have been based solely upon correlations between inhibitor structure and inhibitor potency. The first of these approaches utilizes substrate site directed enzyme reagents made by attaching to a substrate analogue a substituent bearing an electrophilic group.[36] A detailed evaluation with groups of phosphokinase isozymes has suggested that this approach may not in general be efficient in generating inhibitors that are both isozyme-selective and substrate site directed.[37] Further, the presence of electrophilic groups in drug precursor structures will tend to promote toxicity that is nonselective rather than selective.

The second of the above approaches to isozyme selectivity involves systematic modifications of a nonelectrophilic substituent attached to a substrate or substrate analogue. That large differential effects can arise therefrom has been reported from several laboratories. Thus, B. R. Baker's groups found that affinity for substrate sites could be enhanced by several orders of magnitude by attachment to substrates of suitable lipophilic groups.[38] They reported that such enhancements could be markedly species-selective, as evidenced in the case of thymidine phosphorylase by a 500–900 times stronger binding of six uracil derivatives to the *Escherichia coli* enzyme than to the rabbit liver enzyme.[39] Another illustration of this approach is provided by *erythro*-9-(2-hydroxy-3-nonyl)-adenine (EHNA), which exhibits up to 5000-fold selectivity in its inhibitions of three calf intestinal adenosine aminohydrolase (AAH) isozymes,[40] and does not significantly

(33) 1 is a potent inhibitor also of homogeneous methionine adenosyltransferase from *E. coli*. The solution EPR spectrum of an enzyme–VO^{2+}–(β,γ-imido-ATP) complex, upon addition of L-methionine (Met), changes to one (ref 34) that is different from that of an enzyme–VO^{2+}–Met complex but very similar to that of an enzyme–VO^{2+}–**1** complex, providing a second, nonkinetic line of evidence that **1** binds to the enzyme via both its β,γ-imido-ATP portion and its L-methionine portion (G. D. Markham, unpublished results).
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(35) Substrate and inhibitor properties of mono-*C*-methylmethionines provide evidence for structural differences between M-2 and M-T at or near their Met binding sites (ref 12). Conceivably, the *n*-butyl group of **17** may interact with M-2 at or near portion of the Met site to give rise to the decreased affinity of **17** for that site.
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Table I. Inhibition Constants of Adenine Nucleotide Derivatives with Kidney (M-2) and Novikoff Ascitic Hepatoma (M-T) Forms of Rat Methionine Adenosyltransferase[a]

	K_i, μM (type of inhibition)[b]				inhibitory potency					
	with ATP varied		with Met varied		K_M(ATP)/K_i, ATP varied		K_M(Met)/K_i, Met varied		rel inhibitory potency, M-T/M-2	
compd	M-2	M-T	M-2	M-T	M-2	M-T	M-2	M-T	ATP site	Met site
2[c]	310 (C)	100 (C)			0.45	1.40			3.1	
1[d]	0.13 (C)	0.21 (C)	0.65 (C)	0.67 (M)[e]	1080	670	7.7	22	0.62	2.9
17	0.12 (C)	0.11 (C)	2.90 (C)	0.74 (M)[f]	1170	1270	1.7	22	1.1	13.0

[a] When methione (Met) was the variable substrate, [MgATP] was 2 mM with both enzyme forms; with variable MgATP, [Met] was 60 μM with M-2 and 120 μM with M-T. Remaining conditions are given in ref 24. The Michaelis constant (K_M) of MgATP was 0.14 mM with both M-2 and M-T in all runs. The K_M of L-Met with M-2 was 5 μM in all runs; with M-T it was 14.5 μM with 1 (ref 13) and 16 μM with 17 under the conditions of the K_i determinations. [b] C = competitive, NC = simple noncompetitive (the inhibitor reduces V_{max} and does not change K_M); M = mixed C and NC. K_i values for M type inhibitions were derived from double-reciprocal plots (ref 24) as K_i (slope) and provide a measure of the C component; K_i values calculated from intercepts on the vertical ($1/V$) axis (a measure of the NC component) are listed in footnotes. [c] Data from ref 11. [d] Data from ref 13. [e] K_i (intercept), 1.75 μM. [f] K_i (intercept), 2.05 μM.

Table II. Isozyme-Selective Inhibitory Potency[a] Shown by Substrate Derivatives Bearing a Single Short Substituent[b]

			substrate derivatives			substrate atoms used as substituent sites		range of relative inhibitory potencies, isozyme 1/isozyme 2
enzyme	isozymes studied	substrate[c]	no. studied	no. giving K_M or K_i values[d]	no. showing isozyme selectivity[e]	no. studied[f]	no. showing isozyme selectivity	
thymidine kinase	hamster; *E. coli*[g]	TdR[h]	27	10	10[i]	6	6	0.17->20
	rat M-TK; rat C-TK	TdR[j]	19	17	11	5	5	0.0025-26
adenylate kinase	rat AK-II; rat AK-M	ATP[k]	12	11	10[m]	4	4	0.11->30
	rat AK-III; *E. coli*	ATP[n]	3[o]	3	3	2	2	>2.4->350
	rat AK-M; rat AK-II	AMP[p]	24	20	13	11	6	3.0-11
	rat AK-M; rat AK-III	AMP[p]	25	22	13	11	6	2.6-9.3
pyruvate kinase	rat M; rat L	ADP[q]	15	15	9	8	6	0.5-13
	rat K; rat L	ADP[q]	13	13	10	7	5	0.45-7.6
methionine adenosyltransferase	rat M-1; rat M-2	ATP[r]	3	3	3	3	3	0.16-29
	rat M-T; rat M-2	ATP[r]	11	11	9	8	6	0.5-5.5
	rat M-T; rat M-2	Met[s]	12	3	3	3	3	2.0-3.1
		totals	164	128	94	68	52	

[a] Inhibitory potency = K_M of normal substrate/K_M or K_i of substrate derivative. K_i values represent inhibition that is competitive vs the normal substrate. [b] Substituents were of one to five spacer atoms, excluding H; all but three were unbranched. [c] TdR, thymidine; Met, L-methionine. [d] The listed derivatives gave K_M or K_i values with both isozymes or gave data that permitted an assignment of minimum possible K_M or K_i values. [e] Measured as relative inhibitory potency (footnote *a*) toward the two isozymes listed. [f] Chiral centers bearing a substituent are treated as two discrete substrate atoms. [g] The cytoplasmic variant from cultured kidney cells was used. [h] Data from ref 4. [i] Included are two compounds that activated the *E. coli* enzyme and inhibited the hamster enzyme. [j] Data from ref 7. [k] Data from refs 5, 6, and 9. [m] In several cases the data derives from comparison of AK-M with AK-III, which gave inhibitory potency values similar to those with AK-II with all the ATP derivatives studied. [n] Data from ref 5. [o] One compound had more than five spacer atoms (9 atoms). [p] Data from ref 8. [q] Data from ref 10. [r] Data from ref 11. [s] Data from ref 12.

inhibit AAH from *E. Coli* or *Saccharomyces cerevisiae*.[41] EHNA had been developed by H. J. Schaeffer's group as a potent inhibitor of the major isozyme in commercial calf intestinal AAH; to this end, they had measured inhibitions effected by a series of 9-substituted adenines in which the 9-substituents were systematically made more complex.[42] The 5000-fold lower affinity of EHNA for a minor isozyme of AAH thus demonstrates that atom-by-atom elaboration of high-affinity substituents is a procedure capable of producing large isozyme-selective effects. In our own laboratory, such variation in isozyme selectivity with substituent structure has been noted frequently. Examples are a 6-fold selectivity in inhibition of rabbit and pig AMP kinases given by a C5′-substituted AMP derivative[43] and a 9-fold effect on differential inhibition of *E. coli* and hamster thymidine kinases produced by addition of a single methyl group to the C5′-substituent of a thymidine derivative.[4]

A third type of approach to isozyme-selective inhibitors stems from results obtained during the present series of investigations. In this we determined, with isozymic variants of four enzymes, substrate and/or inhibitor constants of derivatives of five substrates, all with single short (up to five-atom) substituents located at a variety of substrate atoms. As judged by their inhibitory potency [K_M of normal substrate/K_M or K_i (for competitive inhibition) of inhibitor], a high proportion of this type of substrate derivative exhibited isozyme selectivity. We provide in Table II a comprehensive overview of these studies in order to facilitate assessment of the depth, scope, and potential usefulness of the findings. In Table II the tendency to generate isozyme-selective inhibition has been expressed in two ways: of the 128 tested compounds for which inhibitor or substrate constants could be determined, 94 exhibited significant isozyme selectivity; and of the total number (68) of substrate atoms used as sites for attachment of substituents, 52 were associated with isozyme selectivity. Table II shows that selectivity factors of 10 or more were commonly observed. Because high inhibitory potency was not often encountered among these derivatives, we studied two model design systems aimed at potency enhancement, i.e. the isozyme system of the present paper, and, earlier, an adenylate kinase isozyme system.[6] As discussed in the preceding section, with both these systems it proved feasible to transfer the isozyme-selective effect of a substrate substituent to a potent multisubstrate adduct inhibitor by attaching the substituent to the corresponding atom in the inhibitor. In both cases, this was attributable to a selective enhancement in inhibitory potency toward the target isozyme.

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The above findings illustrate a three-step approach that can generate, relatively readily, inhibitors that are potent, that are moderately isozyme-selective, and that, by reason of their multisubstrate character, are selective for a given metabolic reaction. The steps are (1) monosubstituted substrate derivatives are tested as inhibitors of a chemotherapeutically appropriate set of isozymes that catalyze a targeted multisubstrate reaction; (2) attempts are made to derive a potent multisubstrate adduct inhibitor (a ratio of the higher substrate K_M to the inhibitor K_i of $\geq 10^3$ is frequently obtainable) by the common procedure of joining the catalysis-involved substrate atoms via one or more spacer atoms (steps 1 and 2 would be reversed in cases where step 2 was expected to involve the lesser effort); (3) a substituent that, in step 1, was found to confer the desired selectivity is attached to the corresponding atom in the multisubstrate adduct inhibitor. After completion of this step, additional potency and/or selectivity, if desired, could presumably be imparted in many instances by modifying the substituent along the lines of the second approach described above. Potentially fruitful modifications of the above overall strategy could comprise the attachment of more than one selectivity-inducing substituent to a multisubstrate adduct inhibitor,[44] or the bridging of the two substrates in the adduct via atoms at which substituents had been found in step 1 to produce the desired direction of selectivity. This latter procedure could enable potent and isozyme-selective inhibitors to be secured at step 2 rather than at step 3.

In summary, some of the findings to date in the present series of studies are summarized and reviewed. They furnish two illustrations of an approach that can facilitate the design of potent inhibitors that exhibit selectivity for a given metabolic conversion as well as moderate (ca. 10-fold) levels of isozyme-selectivity. Evidence is adduced to indicate a likelihood that additional potency and/or isozyme selectivity, if desired, could be conferred on such inhibitors by making systematic structural modifications at specified sites.

Experimental Section

Chemical Syntheses. General Procedures. Sodium imidodiphosphate, barium (cyanoethyl)phosphate, and *t*-BuOK were from Fluka. *S*-Benzyl-L-homocysteine[45] was crystallized from boiling water and converted, as described,[46] to disodium L-homocysteine. Dimethyl sulfoxide (Me_2SO), pyridine, and dimethylformamide (DMF) were distilled from CaH_2 and stored over molecular sieves. Thin-layer chromatograms (TLC) were obtained with 0.25-mm layers of silica gel on glass from EM Laboratories in (A) CH_2Cl_2–Me_2CO (94:1), (B) CH_2Cl_2–Me_2CO (19:1), (C) ether–hexane (3:1), (D) $CHCl_3$–MeOH–4% aqueous HOAc (3:2:1, lower layer), (E) CH_2Cl_2–2-propanol (49:1), (F) $CHCl_3$–MeOH (19:1). Column chromatography employed Merck silica gel 60 (230–400 mesh) and J. T. Baker flash grade C_{18} silica gel. Paper chromatography was by ascending technique on Whatman No. 1 paper in (G) 1-propanol–NH_4OH–H_2O (55:10:35) and (H) isobutyric acid–NH_4OH–H_2O (66:1:33). DEAE Trisacryl M was obtained from LKB Instruments, Inc. UV spectra were obtained on a Varian Model 635 spectrophotometer. ^1H NMR was obtained on a Nicolet NT 300 WB spectrometer. Chemical shifts are given as parts per million downfield from TMS. Analytical HPLC 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 with a Waters RCM-100 unit using a NovaPak C_{18} cartridge (8 mm × 10 cm) in (J) a 10-min linear gradient of 40–100% MeOH in H_2O at a 2 mL/min flow rate, (K) MeOH–H_2O (3:1) at 2 mL/min, (L) a 10-min linear gradient of 0.1 M K_2HPO_4–0.025 M $(n\text{-Bu})_4NHSO_4$ (pH 7.6) to 40% MeOH in 0.02 M K_2HPO_4–0.005 M $(n\text{-Bu})_4NHSO_4$ (pH 7.6) at 2 mL/min. Preparative HPLC was performed at 250 mL/min on a Waters Prep LC/System 500 A equipped with two Prep Pak-500/Silica cartridges. Semipreparative HPLC was performed at 10 mL/min on a Phenomenex Spherex 5 C_{18} silica gel column (25 mm × 20.5 cm). Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN, and were within ±0.4% of theoretical values unless stated otherwise. All compounds were dried at 22 °C over P_2O_5. Evaporations of volatiles were conducted under reduced pressure (bath temperature below 35 °C).

For analysis of phosphate content, a solution of the nucleotide (1.5–2 mM) in Tris buffer, pH 10.4 (1 mL), was treated with alkaline phosphatase (0.02 mg, calf intestine, type VII, Sigma Chemical Co.) at 22 °C overnight. The solution was analyzed for phosphate by the method of Lowry and Lopez.[47]

6-Chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)purine (3). To a stirred suspension of 6-chloro-9-β-D-ribofuranosylpurine[14] (28.0 g, 98 mmol) in anhydrous acetone (600 mL) and 2,2-dimethoxypropane (60 mL, 0.49 mol) was added *p*-toluenesulfonic acid monohydrate (18.6 g, 98 mmol). After 4 h the resulting solution was poured slowly into stirred aqueous 0.5 M $NaHCO_3$ (300 mL). The solution was concentrated in vacuo to ca. 250 mL and extracted with $CHCl_3$ (4 × 80 mL). The combined $CHCl_3$ extracts were washed with brine (50 mL), dried (Na_2SO_4), and evaporated in vacuo. The residual pale yellow solid was crystallized firstly from EtOH and then from benzene to give **3** (27.2 g, 85%) as white granular crystals, mp 159–160 °C (lit.[15] mp 158–159 °C). It was homogeneous in system A (R_f 0.30) and in system F (R_f 0.37) and on paper chromatograms run in *n*-BuOH–H_2O (86:14) (R_f 0.83), and was identical with the authentic compound by mixed mp determination.

6-(*tert*-Butylthio)-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)adenine (4). A solution of sodium 2-methyl-2-propanethiolate (3.7 g, 33 mmol; Fluka Chemical Corp.) in EtOH (15 mL) was added dropwise to a stirred solution of **3** (9.8 g, 30 mmol) in DMF (30 mL). TLC (system A) showed that conversion of **3** to **4** was complete after 0.5 h. NaCl was filtered off and washed with EtOH. The combined filtrate and washings were evaporated. The residue was partitioned between $CHCl_3$ (200 mL) and H_2O (100 mL). The aqueous phase was extracted once with $CHCl_3$ (100 mL). The $CHCl_3$ extracts were combined, dried (Na_2SO_4), concentrated to about 30 mL, and purified by preparative HPLC (EtOAc–hexane, 1:1) to give **4** as a white foam (9.3 g, 81.6%) homogeneous by TLC, R_f 0.60 (A), and HPLC, t_R 11.4 min (J): UV_{max} (MeOH) 291 nm (ε 19 400); ^1H NMR ($CDCl_3$) δ 1.35 and 1.62 (s, 3 each, CMe_2), 1.71 (s, 9 H, $SCMe_3$), 3.78 (d, 1 H, H-5′A, $J_{5'A,5'B}$ = 12.8 Hz), 3.96 (d, 1 H, H-5′B), 4.52 (s, 1 H, H-4′), 5.09 (d, 1 H, $J_{2',3'}$ = 5.9 Hz, H-3′), 5.17 (t, 1 H, J = 5.3 Hz, H-2′), 5.87 (d, 1 H, J = 4.8 Hz, H-1′), 7.98 and 8.64 (s, 1 each, H-2, H-8). Anal. ($C_{17}H_{24}N_4O_4S$) C, H, S; N: calcd, 14.74; found, 14.31.

6-(*tert*-Butylthio)-9-[2′,3′-*O*-isopropylidene-5′(*R*,*S*),*O*$^{5'}$-methylidene-β-D-ribofuranosyl]purine (6). To a solution of **4** (7 mmol) and dicyclohexylcarbodiimide (DCC) (4.33 g, 21 mmol) in Me_2SO (25 mL) was added dichloroacetic acid (280 μL). TLC in system A showed that conversion of **4** (R_f 0.60) to the corresponding 5′-aldehyde (R_f 0.46, rosaniline-positive) was complete after 0.5 h. Dicyclohexylurea was filtered off and washed with small portions of Me_2SO. The combined Me_2SO solutions were extracted with cyclohexane (2 × 50 mL) to remove DCC and then added to a Me_2SO solution of dimethyloxosulfonium methylide (15 mmol, 15 mL).[21] The red solution was stirred for 1 h and poured into Et_2O (200 mL) and ice water (100 mL). The aqueous phase was extracted with Et_2O (100 mL). The ether extracts were dried (Na_2SO_4) and evaporated to an orange solid which was purified by silica gel flash chromatography[48]

(44) Findings with disubstituted derivatives of a multisubstrate adduct inhibitor of adenylate kinase[9] have suggested that polysubstituted multisubstrate adducts could prove to be flexible tools in the design of inhibitors with wide ranges of potency and isozyme selectivity.

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(CH_2Cl_2–acetone, 97:3), yielding **6** (668 mg, 25.1%) as a white noncrystalline foam homogeneous on TLC [R_f 0.44 (B), 0.35 (C)] and HPLC [t_R 11.7 min (J)]. Anal. ($C_{18}H_{24}N_4O_4S \cdot 1.2CH_3COCH_3$) C, H, N, S.

6-(*tert*-Butylthio)-9-[5′(*R*,*S*)-*C*-(L-homocystein-*S*-ylmethyl)-2′,3′-isopropylidene-β-D-ribofuranosyl]purine (10a,b). A solution of **6** (510 mg, 1.3 mmol) in anhydrous MeOH (10 mL) was added to a solution of freshly prepared disodium L-homocysteinate[46] (1.95 mmol) in MeOH (10 mL). TLC (D) indicated that after 1 h, **6** was completely converted to ninhydrin-positive material of R_f 0.56. The MeOH was evaporated. An aqueous solution of the residue was adjusted to pH 6 and chromatographed on a column (2.5 × 15 cm) of C_{18} silica gel. This was washed with H_2O (200 mL) to remove homocysteine. Water–MeOH (1:3, 200 mL) eluted **10a,b** (18 000 OD_{290} units, 72.9% yield; OD_{290} units = OD_{290} × volume in mL). Removal of volatiles gave a white foam homogeneous by normal-phase TLC (D), C_{18} silica gel TLC in MeOH–H_2O (3:1) (R_f 0.31), and HPLC [t_R 10.7 min (J)]. Anal. ($C_{22}H_{33}N_5O_6S_2 \cdot CH_3OH \cdot H_2O$) C, N, S; H: calcd, 6.76; found, 6.24.

6-(*tert*-Butylthio)-9-[5′(*R* and *S*)-C-[[L-methyl-*N*-(*tert*-butyloxycarbonyl)homocysteinat-*S*-yl]methyl]-2′,3′-*O*-isopropylidene-β-D-ribofuranosyl]purine (11a and 11b). To **10a,b** (527 mg, 1 mmol) in CH_2Cl_2 (25 mL) were added Et_3N (160 μL, 1.2 mmol) and di-*tert*-butyl pyrocarbonate (263 mg, 1.2 mmol). TLC (D) showed that formation of the *N*-Boc derivative was complete after 40–50 min. The solvent was evaporated in vacuo. Under argon, MeOH (25 mL) was added, followed by a small excess of fresh diazomethane in peroxide-free Et_2O. The solution was concentrated to small volume and subjected to semipreparative HPLC (MeOH–H_2O, 3:1; 25 mg/injection) to give 0.203 g (31.7%) of **11a** (first compound eluted) and 0.35 g (54.6%) of **11b**, both as white solids. They were homogeneous by TLC (E) (R_f **11a** = **11b** = 0.29) and HPLC (K) (t_R **11a** 15.3 min, **11b** 17.9 min). **11b**: UV_{max} (MeOH) 291 nm (ε 20 200). Anal. ($C_{28}H_{43}N_5O_8S_2$) C, H, N, S.

Establishment of the C5′ Configuration of 11b. A solution of **11b** (5 μmol) in EtOH (0.5 mL) and liquid ammonia (10 mL) was heated in a stainless steel bomb at 140–145 °C for 48 h. TLC (system F) showed complete conversion to a single UV-absorbing compound, R_f 0.24, of UV_{max} 260 nm. A solution of this in EtOH (5 mL) was refluxed for 1 h with 50 mg (wet weight) of Raney nickel (Sigma Chemical Co.). HPLC analysis (40% MeOH in H_2O, isocratic) showed the presence of a single compound with the same t_R (9.6 min) as 5′(*S*)-*C*-methyl-2′,3′-*O*-isopropylideneadenosine (**12**).[30] 5′(*R*)-*C*-Methyl-2′,3′-*O*-isopropylideneadenosine[30] had t_R 11.3 min under these conditions.

6-(*n*-Butylthio)-9-[5′(*R*)-*C*-(L-homocystein-*S*-ylmethyl)-β-D-ribofuranosyl]purine 5′-(β,γ-Imidotriphosphate) (17). A mixture of **11b** (100 mg, 150 μmol) and pyridinium β-cyanoethyl phosphate[31] (300 μmol) was rendered anhydrous by evaporations of dry pyridine, after which DCC (150 mg, 750 μmol) and dry pyridine (2 mL) were added. The mixture was stirred and heated at 60–65 °C. After 3 h, HPLC (J) indicated conversion of **11b** (t_R 12.3 min) to the cyanoethyl ester of **13** (t_R 8.6 min) as the major product. The mixture was cooled to 0 °C and *t*-BuOK (68 mg, 600 μmol) was added. The mixture was stirred at 0 °C for 0.5 h and then at 22 °C for 0.5 h, when HPLC (J) showed complete disappearance of the phosphodiester. H_2O (2 mL) was added and after 1 h at 22 °C the weakly acidic (pH 4) mixture was filtered from the dicyclohexylurea that had precipitated. This was washed with H_2O–MeOH (1:1), and the filtrate and washings were evaporated. A solution of the residue in MeOH–H_2O (1:10) was applied to a column of C_{18} silica gel (2 × 15 cm). This was washed with H_2O–MeOH (9:1, 200 mL) to remove inorganic phosphate. Water–MeOH (3:2, 200 mL) eluted **13** (1500 OD_{290} units, 50% yield from **11b**) in homogeneous form by HPLC analysis. The ratio of phosphate content (see General Procedures) to nucleoside moiety (determined spectrophotometrically) was 1.03 (theoretical 1.00). The residue obtained after removal of volatiles was dried by several evaporations with DMF. A solution of **13** in anhydrous DMF (2 mL) was treated with $(n\text{-Bu})_3N$ (36 μL, 150 μmol) and 1,1′-carbonyldiimidazole (61 mg, 375 μmol). After 6 h, MeOH (26 μL) was added. After 0.5 h, the solution was added to tri-*n*-butylammonium imidodiphosphate (750 μmol) in DMF (1 mL) and the mixture was stirred for 18 h. The precipitate was removed by centrifugation and washed with a small volume of DMF. The DMF was evaporated and the residue was dissolved in H_2O and chromatographed on a column (2.5 × 10 cm) of DEAE Trisacryl M (HCO_3^-) using a linear gradient of aqueous $Et_3NH \cdot HCO_3$ (0–0.4 M, 2 L). Fractions containing **14** (750 OD_{290} units, 25%) appeared as a single peak. These fractions were combined and evaporated. The residue was dissolved in pyridine, and $(n\text{-Bu})_3N$ (26 μL, 110 μmol) was added. The solution was evaporated. The residue was dissolved in H_2O and applied to a column of C_{18} silica gel (1 × 15 cm). This was washed with H_2O (100 mL) to remove a trace of inorganic imidodiphosphate and then with MeOH to remove **14**. The MeOH was evaporated and the residue was dissolved in aqueous 90% trifluoroacetic acid (TFA) (2 mL), and after 10 min at 22 °C, volatiles were immediately evaporated in vacuo (oil pump). Toluene was evaporated twice from the residue to remove remaining TFA. The residue (**15**) was dissolved in H_2O–DMF (1:9, 1 mL) containing Et_3N (25 μL, 180 μmol). 1-Iodo-*n*-butane (Fluka Chemical Corp.) (3.5 μL, 30 μmol) was added. Conversion of **15** to **16** was monitored by following the UV spectral shift from 322 nm to 290 nm. After 1 h, additional 1-iodobutane (1 μL) was added. A smooth spectral curve, UV_{max} (MeOH) 290 nm, was obtained after 0.5 h. The solution was evaporated. A solution of the residue in H_2O was adjusted to pH 7 with dilute NaOH. The solution was concentrated to about 0.5 mL and 1 N NaOH (30 μL) was added to convert **16** to **17**. After 2 h, the solution was diluted with H_2O and chromatographed on a DEAE Trisacryl M (HCO_3^-) column (2.5 × 10 cm) with a linear gradient of $Et_3NH \cdot HCO_3$ (0–0.4 M, 2 L). Fractions containing **17** (480 OD_{290} units, 16% from **11b**) were combined and evaporated. The residue was dissolved in MeOH (0.5 mL) and 1 M NaI in MeOH (120 μL, 5 equiv) was added. Acetone (50 mL) was added. The precipitate was washed with acetone and dried to give tetrasodium **17** (17 mg) as a white powder homogeneous on paper chromatography in (G)R_F 0.68 (ATP R_f 0.48) and (H)R_f 0.61 (ATP R_f 0.33) and on HPLC (L)t_R 35.1 min (ATP, t_R 11.1 min; β,γ-imino-ATP, 10.3 min; **1**, 15.9 min; **2**, 27.2 min). UV_{max}, pH 7, 291 nm (ε 20 400). FAB mass spectrum 815 $(M + H)^+$, 793 $(-Na + H)^+$, 771 $(-2Na + H)^+$, 749 $(-3Na + H)^+$. Anal. ($C_{19}H_{29}N_6O_{14}P_3S_2Na_4 \cdot 2H_2O \cdot 2.5MeOH$) C, H, N; S: calcd, 6.88; found, 8.10.

Enzyme Studies. Assay of M-2 and M-T activity and determination of inhibition constants were carried out as detailed previously.[24] The type of double-reciprocal plots so obtained have been illustrated previously.[49]

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