Isozyme-Specific Enzyme Inhibitors. 14.[1] 5′(*R*)-*C*-[(L-Homocystein-*S*-yl)methyl]adenosine 5′-(β,γ-Imidotriphosphate), a Potent Inhibitor of Rat Methionine Adenosyltransferases

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The title compound is a covalent adduct of L-methionine (Met) and β,γ-imido-ATP. In its synthesis the *N*-Boc derivative of 5′(*R*)-*C*-(aminomethyl)-N^6-benzoyl-5′-*O*-tosyl-2′,3′-*O*-isopropylideneadenosine was converted by the successive actions of CF_3CO_2H and HNO_2 into the corresponding 5′(*R*)-*C*-hydroxymethyl derivative. Treatment of this with disodium L-homocysteinate led to attack of sulfur at C6′, apparently via a 5′,6′-epoxide, and to total stereoselective inversion at C5′ to furnish, after debenzoylation, 5′(*R*)-*C*-(L-homocystein-*S*-ylmethyl)-2′,3′-*O*-isopropylideneadenosine. The 5′ configuration was established by conversion of this into the known 5′(*S*)-*C*-methyl-2′,3′-*O*-isopropylidene adenosine with Raney nickel. The α-amino acid residue was protected as an *N*-Boc methyl ester, after which the 5′-hydroxyl was phosphorylated with benzyl phosphate and dicyclohexylcarbodiimide. The phosphoanhydride bond with inorganic imidodiphosphate was then created by established methods. Finally, blocking groups were removed under conditions that gave the desired adduct with no racemization of its L-methionine residue. It was a potent inhibitor [K_M(ATP)/K_i = 1080; K_M(Met)/K_i = 7.7] of the M-2 (normal tissue) form of rat methionine adenosyltransferase and of the M-T (hepatoma tissue) form [K_M(ATP)/K_i = 670; K_M(Met)/K_i = 22]. Inhibitions were competitive with respect to ATP or to L-methionine, indicating a dual substrate site mode of binding to the enzyme forms.

As discussed previously,[2] differences in isozyme compositions of normal tissues and those of poorly differentiated malignant tissues are well documented, and suggest that isozyme-selective inhibitors are of interest as agents that could exhibit selective toxicity toward malignant tissue. Currently, evidence indicates that targets potentially suitable in this approach to cancer chemotherapy include certain isozymic variants of the following enzymes:[3] adenosine aminohydrolase,[4,5] adenylosuccinate synthetase,[6] alcohol dehydrogenase,[7,8] aldehyde dehydrogenase,[9] microsomal aminopeptidase,[10] diaphorase P,[11] acetylesterases A_4 and A_5,[12] galactosyltransferase,[13] γ-glutamyltranspeptidase,[14,15] β-hexosaminidase,[16-18] methionine adenosyltransferase,[19] 5′-nucleotide phosphodiesterase,[20] alkaline phosphatase,[21] phosphofructokinase,[22] certain protein kinases,[23-25] tRNA (guanine-N^2-)methyltransferase,[26] and thymidine kinase.[27]

Previous studies in the present series have examined approaches that might facilitate the systematic design of potent isozyme-selective inhibitors. For this purpose, model target isozymes were selected from the most common category, namely isozymes of unknown tertiary structure that catalyze reactions involving two substrates. We have reported that it is sometimes possible to generate potent isozyme-selective inhibitors relatively readily by means of a three-stage strategy.[28,29] The scope and limitations of this strategy, particularly with respect to its second and third stages, remain to be determined. The second stage comprises attempted derivation of a potent dual substrate site ("multisubstrate") inhibitor by covalent attachment of the two substrates, either directly or through bridging groups, via atoms involved in the enzyme-catalyzed reaction. This approach is well established as ef-

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(3) Listings are tentative and provisional to the extent that they are based on isozyme compositional analyses that, for most of the cited enzymes, have not yet been made available for a comprehensive variety of normal and malignant tissues. Major factors in target selection that have not been taken into account in this compilation are the importance of the catalyzed reactions for tumor cell multiplication or survival in vivo and the question of whether, in malignant tissue, some candidate targets may be present in chemotherapeutically disadvantageous excess over metabolic requirements. References are to key articles and/or ones that afford rapid access to the literature.
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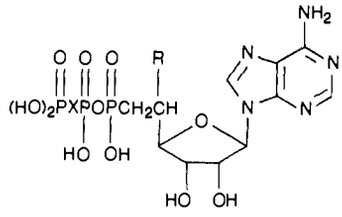
fective in producing potent inhibitors that tend to be selective for specific enzyme-catalyzed reactions.[30-35] In the first stage, systematic studies are carried out to identify short substrate substituents that produce isozyme-selective inhibitors. In the third stage, such substituents are attached at the corresponding atoms of adducts generated in stage 2, the objective being to impart isozyme selectivity while retaining the potency of inhibition. The present paper describes further studies of stage 2 in this overall design process.

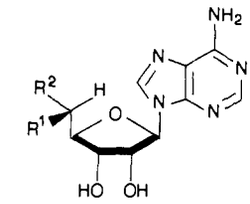
Earlier studies in the present series revealed that two-substrate adducts of the above type did not strongly inhibit rat hexokinase isozymes[36] but were potent inhibitors of rat thymidine kinase[36] and adenylate kinase[28] isozymes. More recently, **1b**, the more active of two 5′ epimers of an



1a: X = NH, R = L-$SCH_2CH_2CH(NH_2)CO_2H$
b: X = O, R = L-$SCH_2CH_2CH(NH_2)CO_2H$

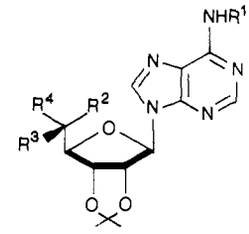
S–C5′ L-homocysteine–ATP adduct was shown to be a moderately effective inhibitor of the M-T variant (predominant in rat ascitic hepatoma cells) and the M-2 variant (predominant in most rat normal tissues) of methionine adenosyltransferase,[37] an enzyme that catalyzes attack of L-methionine sulfur on C5′ of ATP with expulsion of inorganic tripolyphosphate. On grounds outlined previously,[38] the M-T variant is of interest as a model mammalian target in the design of an isozyme-selective antineoplastic agent. Insertion of an amino nitrogen between C6′ and P^{α} of **1b**, to give **2**, has been found to enhance inhibitory activity against M-T 118-fold.[1] The present study continues a search for structural features in L-methionine–ATP adducts that could contribute to the effectiveness with which these adducts inhibit M-T. Thus **1a**, the β,γ-imido analogue of **1b**, inhibits M-T ca. 2.5-fold more effectively than **1b**[37] and, moreover, is potentially more stable to enzymatic attack in vivo in view of the phosphatase inertness of β,γ-imido-ATP.[39] Described here are the synthesis and some inhibitor properties of **3**, a novel adduct of β,γ-imido-ATP and L-methionine that differs most markedly from **1a** by the interposition of a methylene group between C5′ and L-homocysteine sulfur and that possesses the 5′*R* configuration that in **2** is essential for powerful inhibition of M-T and M-2.[1]

Chemical Synthesis. The previously described *N*-Boc derivative of the 5′*R* epimer, **4**, of 5′-*C*-(aminomethyl)-N^6-benzoyl-5′-*O*-tosyl-2′,3′-*O*-isopropylideneadenosine[1] was treated for 5 min at 4 °C with anhydrous trifluoroacetic



ATP: R^1 = H, R^2 = $OP(OH)(O)OP(OH)(O)OPO_3H_2$
2: R^1 = L-$S(CH_2)_2CH(NH_2)CO_2H$, R^2 = $CH_2NHP(OH)(O)OP(OH)(O)OPO_3H_2$
3: R^1 = CH_2-(L)-$S(CH_2)_2CH(NH_2)CO_2H$, R^2 = $OP(OH)(O)OP(OH)(O)NHPO_3H_2$

acid, bringing about selective removal of the *N*-Boc protecting group. The resulting amine was treated with sodium nitrite in tetrahydrofuran–acetic acid to give the 5′(*R*)-*C*-(hydroxymethyl)adenosine derivative **5** in homogeneous form in 42% yield overall. Treatment of **5** in ethanol solution with 3 equiv of disodium L-homocysteinate followed by debenzoylation at N^6 promoted by addition of methanol gave the 5′(*R*)-*C*-[(alkylthio)-methyl]adenosine derivative, **6**, in crystalline, homogeneous form in 73% yield. The C5′ configuration of **6** was established by treatment of it with Raney nickel catalyst in ethanol, which gave the known 5′(*S*)-*C*-methyl-2′,3′-*O*-isopropylideneadenosine,[40] **7**, as the sole product. When the foregoing reaction sequence was performed starting with the alternate 5′*S* epimer of **4**, the sole product was, correspondingly, 5′(*R*)-*C*-methyl-2′,3′-*O*-isopropylideneadenosine.[40] In both instances the reaction with Raney nickel produced no traces of 9-(5-deoxy-2,3-*O*-isopropylidene-β-D-allofuranosyl)adenine[41] (**8**), indicating that direct displacement of the 5′ tosylate of **5** by the thiolate anion of disodium homocysteinate did not occur under the conditions used. The total stereoselectivity observed in the conversion of **5** to **6** indicates an intermediate 5′,6′-epoxide that undergoes attack at C6′ by thiolate anion with resulting net inversion of configuration at C5′. In accord with this view, it was found that **5** was converted by triethylamine in methanol to a less polar product (presumably the 5′,6′-epoxide) and that this reacted rapidly with L-homocysteine to give **6**. By procedures found to be suitable in the synthesis of **1a**,**b**,[37] the α-amino and carboxyl groups of **6** were protected as *N*-Boc and methyl ester derivatives, respectively, by means of successive treatments with di-*tert*-butyl pyrocarbonate and diazomethane. This afforded homogeneous **9** in 82% yield from **6**.



no.	R^1	R^2	R^3	R^4
4	Bz	OTs	CH_2NH-Boc	H
5	Bz	OTs	CH_2OH	H
6	H	H	CH_2-L-$S(CH_2)_2CH(NH_2)CO_2H$	OH
7	H	H	CH_3	OH
8	H	H	H	CH_2OH
9	H	H	CH_2-(L)-$S(CH_2)_2CH(NH$-Boc$)CO_2Me$	OH
10	H	H	CH_2-(L)-$S(CH_2)_2CH(NH$-Boc$)CO_2Me$	OPO_3H_2
11	H	H	CH_2-(L)-$S(CH_2)_2CH(NH$-Boc$)CO_2Me$	$OP(OH)(O)OP(OH)(O)$-$NHPO_3H_2$

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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]

	M-2: K_i, μM (type of inhibn)[b]		M-T: K_i, μM (type of inhibn)	
compd	ATP varied	Met varied	ATP varied	Met varied
1a[c]	16 (C)	26 (C)	11 (C)	38 (NC)
2[d]	0.36 (C)	3.0 (NC)	0.27 (C)	2.3 (NC)
3	0.13 (C)	0.65 (C)	0.21 (C)	0.67[e] (M)

[a] When methionine (Met) was the variable substrate, [MgATP] was 2 mM with both MAT forms; with variable MgATP, [Met] was 60 μM with MAT-2 and 120 μM with MAT-T. For other conditions, see the Experimental Section. The Michaelis constant (K_M) of MgATP was 0.14 mM with both M-2 and M-T. The K_M of L-methionine was 5 μM with M-2 and 14.5 μM with M-T 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. [c] Data from ref 37. [d] Data from ref 1. [e] Calculated from double-reciprocal plots (see Experimental Section) as K_i (slope); K_i calculated from intercepts on the $1/V$ axis was 1.75 μM.

Phosphorylation of **9** by the Gilham–Tener benzyl phosphate–dicyclohexyl carbodiimide–pyridine method[42] gave the monobenzyl ester of the 5′-phosphate **10**. The benzyl group was removed by hydrogenolysis in acetic acid solution with a 2:1 ratio of palladium black to **9** to compensate for catalyst inhibition caused by the thioether group. The nucleoside 5′-phosphate derivative **10** was isolated in 51% yield after separation from inorganic phosphate by reversed-phase chromatography. Next, by known procedures modified previously in the synthesis of **1a**,[37] **10** was converted to its phosphoroimidazolidate and this was condensed with β,γ-imidodiphosphate to give the β,γ-imido-ATP derivative **11**. Blocking groups in **11** were removed by successive acidic and mild basic treatments under conditions that, as discussed previously,[37] permit retention of configuration of the L-homocysteine residue. This afforded the homogeneous tetrasodium salt of **3** (32% yield from **10**) with the expected elemental analyses and UV, ^{1}H NMR, and ^{31}P NMR spectral properties.

Enzyme Studies. Table I lists inhibition constants of **3** with M-2 and M-T determined under conditions that were the same for both MAT variants and the same as used in kinetic studies with **1a**[37] and **2**.[1] Adduct **3** was significantly more effective than the potent inhibitor **2** and gave competitive kinetics vs. MgATP [K_M(ATP)/K_i = 1080 and 670 respectively for M-2 and M-T] and vs. L-methionine [K_M(Met)/K_i = 7.7 and 22.4, respectively]. In the case of variable L-methionine with M-T, the inhibition showed also a component that was noncompetive.

Adduct **3** was 120-fold more inhibitory toward M-2 and 50-fold more inhibitory to M-T than was **1a** as indicated by the inhibition constants obtained with variable MgATP (Table I). Adduct **3** differs structurally from **1a** in two respects: it possesses an oxygen atom rather than a methylene between P(α) and C5′, and it has a methylene group interposed between C5′ and the sulfur of the L-homocysteine residue. Substrate and inhibitor properties of certain ATP derivatives with M-2 and M-T indicate that replacement of O5′ in ATP by CH_2 has little effect on affinity for the ATP site of M-2 or M-T.[43] The enhanced affinity of **3** thus appears to be ascribable largely to the CH_2 that bridges C5′ and S. Adduct **1a** inhibits M-T noncompetitively with respect to L-methionine, whereas **3** inhibits M-T in a mixed competitive-noncompetitive manner, suggesting that the added methylene enables the L-homocysteine residue of **3** to bind more avidly and with a larger segment of the L-methionine binding site of M-T.

That **3** is a powerful inhibitor of M-2 and M-T that is competitive or partly competitive with respect to both substrates indicates that it could bind simultaneously to the ATP and L-methionine sites. The K_i value with variable methionine was 3–5 times higher than with variable ATP. Although **3** presumably has more affinity for uncomplexed enzyme than for the enzyme–ATP or enzyme–methionine complexes, this finding suggests that under the test conditions **3** may be competing with ATP for both free enzyme and the enzyme–methionine complex, and competing with methionine for both free enzyme and the enzyme–ATP complex, and that **3** may have more affinity for enzyme–methionine than for enzyme–ATP and/or that enzyme–ATP may be formed more frequently than enzyme–methionine in the catalytic cycle. In this latter event it is possible that **3**, correspondingly, may undergo initial binding to M-T and M-2 more frequently via its ATP moiety than via its methionine moiety. That **3** inhibits M-T in a partially noncompetitive manner with respect to methionine implies reversible binding of **3** to an enzyme–methionine complex and/or reversible binding of methionine to an enzyme–**3** complex to furnish a ternary enzyme–methionine–**3** complex.

The present and a previous study[1] have shown that the adduct, **3**, of L-methionine and β,γ-imido-ATP is synthetically relatively accessible, and that it is a potent inhibitor of the M-2 form of methionine adenosyltransferase predominant in most normal rat tissues and of the M-T form predominant in certain rat hepatoma cells. A dual substrate site mode of inhibition is indicated from the present kinetic data. Rat M-T is potentially useful as a model mammalian target in the design of cancer chemotherapeutic agents. Adduct **3** inhibits M-2 slightly more effectively than M-T. Earlier work has shown, however, that a dual-site enzyme inhibitor can sometimes be rendered selective for a given isozymic form by attachment to the inhibitor of substrate substituents known to produce isozyme-selective inhibition.[28,29] Substrate and inhibitor properties of derivatives of ATP[38] and of methionine[44] with M-2 and M-T indicate that this approach may prove useful in molecular modifications of **3** aimed at imparting selectivity for M-T with respect to M-2. Further studies of potent and/or selective M-T inhibitors are in progress.

Experimental Section

Chemical Synthesis. General Procedures. *N,N*-Dimethylformamide (DMF) was distilled from CaH_2 and stored over molecular sieves. Thin-layer chromatograms were obtained with 0.25-mm layers of silica gel on glass from EM Laboratories in (A) $CHCl_3$–MeOH (19:1), (B) $CHCl_3$–MeOH–4% aqueous HOAc (3:2:1, lower layer), and (C) $CHCl_3$–MeOH (9:1). Reversed-phase chromatography employed 0.25-mm layers of C_{18} silica gel on glass from EM Laboratories in (D) MeOH–H_2O (1:1). Column chromatography employed Merck silica gel 60 (230–400 mesh). Paper chromatography was by the ascending technique on Whatman No. 1 paper in (E) 1-propanol–NH_4OH–H_2O (55:10:35) and (F) isobutyric acid–1 M NH_4OH (6:4). Electrophoresis was carried out on Whatman No. 1 paper at pH 6.8 (0.05 M citrate) and pH 7.5 (0.05 M $Et_3NH{\cdot}HCO_3$). UV spectra were obtained on a Varian Model 635 spectrophotometer. ^{1}H and ^{31}P NMR were obtained on a Nicolet NT 300 WB spectrometer. Chemical shifts are given as parts per million downfield from $SiMe_4$ or 85% H_3PO_4. The sign of ^{31}P shifts are in accord with the 1976 IUPAC convention.[45] Analytical HPLC was performed on a Waters Model 204 chro-

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matograph equipped with a dual solvent-delivery system (Model M-6000A) and a Model 660 programmer. Compounds were analyzed with a Waters RCM-100 unit using a 4-μm particle size Nova-Pak C_{18} cartridge (8 mm × 10 cm) and a 10-min linear gradient of 40–100% MeOH in H_2O at 2 mL/min flow rate. Elemental analyses were by Galbraith Laboratories Inc., Knoxville, TN, and were within ±0.4% of the theoretical values. All compounds were dried in vacuo over P_2O_5 at 22 °C.

N^6-Benzoyl-5′(*R*)-*C*-(hydroxymethyl)-2′,3′-*O*-isopropylidene-5′-*O*-(*p*-tolylsulfonyl)adenosine (5). A solution of **4** (0.9 g, 1.3 mmol) in cold CF_3CO_2H was kept at 4 °C for 5 min. Longer reaction times gave the desired amine, R_f 0.45 (solvent B), in admixture with a byproduct, R_f 0.32. The solution was evaporated immediately under reduced pressure and EtOH–toluene (2:3, 3×10 mL) was evaporated from the residue. To a solution of the residual oil in a mixture of acetic acid (6 mL), THF (12 mL), and H_2O (18 mL) was added $NaNO_2$ (828 mg, 12 mmol). The solution was kept at 22 °C for 0.5 h and then concentrated in vacuo to remove the THF. The aqueous solution was extracted with ethyl acetate (3 × 25 mL). The ethyl acetate extracts were combined, washed with H_2O (25 mL), dried (Na_2SO_4), and evaporated. The residue was purified by silica gel chromatography (4 × 15 cm column) with $CHCl_3$–MeOH (49:1). Fractions containing **5** were evaporated, giving a white foam (0.325 g, 42%), which was homogeneous on TLC, R_f 0.32 (A), and HPLC, t_R 7.2 min. ^1H NMR (300 MHz, $CDCl_3$): δ 1.32 and 1.57 (s, 3 each, CMe_2), 2.41 (s, 3 H, CH_3Ar), 3.56 (dd, 1 H, $J_{5',6'}$ = 4.8 Hz, $J_{6',6''}$ = 15.4 Hz, H-6′), 3.76 (dd, 1 H, $J_{5',6''}$ = 6.2 Hz, J = 15.4 Hz, H-6″), 4.45 (t, 1 H, $J_{3',4'}$ = 4 Hz, H-4′), 5.01 (m, 1 H, H-5′), 5.18 (dd, 1 H, $J_{2',3'}$ = 6.6 Hz, J = 4 Hz, H-3′), 5.30 (dd, 1 H, $J_{1',2'}$ = 3.5 Hz, J = 6.6 Hz, H-2′), 5.98 (d, 1 H, J = 3.5 Hz, H-1′), 7.3–8.05 (m, 5 H, aromatic), 8.09 and 8.74 (s, 1 each, H-2 and H-8). Anal. ($C_{28}H_{29}N_5O_8S\cdot CH_3OH$) C, H, N.

5′(*R*)-*C*-(L-Homocystein-*S*-ylmethyl)-2′,3′-*O*-isopropylideneadenosine (6). To an ethanolic solution of freshly prepared disodium salt of L-homocysteine[46] (1.5 mmol in 10 mL) was added **5** (310 mg, 0.5 mmol) in ethanol (5 mL). After 1 h, TLC (B) indicated complete conversion of **5** (R_f 0.76) to a product of R_f 0.29. MeOH (15 mL) was added and the solution was left at 22 °C for 18 h when UV analysis showed λ_{max} to be 260 nm. Evaporation of solvent gave a white foam that was partitioned between water (50 mL) and ethyl acetate (50 mL). The aqueous phase was adjusted to pH 5 and applied to a column of C_{18} silica gel (2.5 × 15 cm). The column was washed with water (100 mL) to remove excess amino acid; MeOH–H_2O (3:7, 200 mL) eluted **6**. Upon concentration, **6** separated as colorless needles (165 mg, 73%), mp 188–191 °C dec. The product was homogeneous by TLC [R_f 0.17 (B), 0.30 (C)] and HPLC (t_R 3.8 min). ^1H NMR (300 MHz, D_2O): δ 1.34 and 1.58 (s, 3 each, CMe_2), 2.14 (m, 2 H, H-8′,8″), 2.64 (m, 4 H, H-6′,6″,7′,7″), 3.62 (m, 2 H, H-5′,9′), 4.50 (t, 1 H, $J_{3',4'}$ = 1.7 Hz, H-4′), 4.99 (dd, 1 H, $J_{2',3'}$ = 5.7 Hz, J = 1.7 Hz, H-3′), 5.11 (dd, 1 H, $J_{1',2'}$ = 4.3 Hz, J = 5.7 Hz, H-2′), 6.05 (d, 1 H, J = 4.3 Hz, H-1′), 8.16 and 8.20 (s, 1 each, H-2 and H-8). Anal. ($C_{18}H_{26}N_6O_6S\cdot 2CH_3OH$) C, H, N.

5′(*R*)-*C*-[[L-Methyl *N*-(*tert*-butyloxycarbonyl)homocysteinat-*S*-yl]methyl]-2′,3′-*O*-isopropylideneadenosine (9). To a solution of **6** (135 mg, 0.3 mmol) in dry DMF (5 mL) were added NEt_3 (46 μL, 0.33 mmol) and di-*tert*-butyl pyrocarbonate (80 mg, 0.37 mmol).[47] After 1 h at 22 °C, TLC (B) showed a single ninhydrin-negative spot, R_f 0.50. The DMF was evaporated and glacial acetic acid (5 mL) was evaporated from the residue. Addition and evaporation of acetic acid was repeated four times. Toluene was added and evaporated, and the residue was dissolved in MeOH (5 mL) and treated with an excess of ethereal diazomethane. Volatiles were evaporated in vacuo, and the residue was eluted from a column (4 × 15 cm) of silica gel with chloroform–MeOH (97:3). Fractions containing **9** were pooled and evaporated to give a white foam (140 mg, 82%) homogeneous by TLC, R_f 0.40 (C), and HPLC, t_R 9.7 min. ^1H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 3 H, CMe_2), 1.39 (s, 9 H, CMe_3), 1.61 (s, 3 H, CMe_2), 1.85 (m, 1 H, H-8′), 2.07 (m, 1 H, H-8″), 2.53 (m, 2 H, H-7′,7″), 2.59 (dd, 1 H, $J_{5',6'}$ = 8.6 Hz, $J_{6',6''}$ = 13.1 Hz, H-6′), 2.70 (dd, 1 H, $J_{5',6''}$ = 6.2 Hz, J = 13.1 Hz, H-6″), 3.68 (s, 3 H, OMe), 3.85 (t, 1 H, $J_{8',9'}$ = 5.8 Hz, H-9′), 4.36 (m, 1 H, H-5′), 4.70 (s, 1 H, H-4′), 5.04 (d, 1 H, $J_{2',3'}$ = 5.9 Hz, H-3′), 5.13 (t, 1 H, J = 5.3 Hz, H-2′), 5.24 (d, 1 H, J = 8.7, OH), 5.86 (d, 1 H, $J_{1',2'}$ = 4.7 Hz, H-1′), 6.26 (s, 2 H, NH_2), 6.87 (br s, 1 H, NHBoc), 7.86 and 8.26 (s, 1 each, H-2 and H-8). Anal. ($C_{24}H_{36}N_6O_8S\cdot CH_3OH$) C, H, N.

5′(*R*)-*C*-(L-Homocystein-*S*-ylmethyl)adenosine 5′-(β,γ-imidotriphosphate) (3). A solution of **9** (130 mg, 0.23 mmol) and benzyl phosphate[48] (189 mg, 1 mmol) in pyridine (5 mL) was treated with dicyclohexylcarbodiimide (0.5 g, 2.5 mmol). The mixture was kept at 22 °C for 48 h. Water (5 mL) was added, and after 1 h the mixture was filtered, and the filtrate was evaporated. A solution of the residue in acetic acid (10 mL) contained palladium black (250 mg) was shaken with H_2 at 40 psi for 18 h, filtered, and evaporated. To a solution of the residue in H_2O–pyridine (5 mL) was added Et_3N (420 μL, 3 mmol). The solution was kept at 22 °C for 10 min and then evaporated. A solution of the residue in H_2O–MeOH (9:1, 25 mL) was applied to a column (2.5 × 15 cm) of C_{18} silica gel equilibrated with H_2O–MeOH (9:1). Elution with H_2O–MeOH (9:1, 100 mL) removed inorganic phosphate. H_2O–MeOH (3:7, 200 mL) eluted **10** (1750 OD_{260} units; 51%), which was homogeneous on HVE (pH 7.5) (mobility 0.67 relative to AMP) and HPLC (t_R 6.3 min). After removal of volatiles in vacuo, **10** (1700 OD_{260} units, 0.11 mmol) was converted to its phosphoroimidazolidate and this in turn to **3** via **11** by procedures identical with those detailed for the synthesis of **1a**.[37] After column chromatography over DEAE-cellulose as described for **1a**,[37] fractions containing **3** (550 OD_{260} units, 32%) were combined and evaporated. The residue, homogeneous on paper chromatography in E and F (R_f 0.38 in each), was dissolved in MeOH (0.5 mL) and 1 M NaI in MeOH (180 μL, 5 equiv) was added. Acetone (30 mL) was added. The precipitate was washed with acetone and dried in vacuo over P_2O_5 to give the sodium salt of **3** (21 mg) as a white powder. UV_{max} pH 2, 257 nm (ε 14 900); pH 11, 259 nm (ε 15 300). ^1H NMR (300 MHz, D_2O): δ 2.04 (m, 2 H, H-8′,8″), 2.64 (m, 4 H, H-6′,6″,7′,7″), 3.67 (t, 1 H, J = 6.5 Hz, H-9′), 4.45 (d, 1 H, J = 4 Hz, H-4′), 5.96 (d, 1 H, J = 6.3 Hz, H-1′), 8.07 and 8.37 (s, 1 each, H-2, H-8). ^{31}P NMR (121.46 MHz, D_2O): δ −13.10 (P^α), −11.92 (P^β), −3.17 (P^γ). Found for adenosine 5′-(β,γ-imidotriphosphate) tetralithium salt (Sigma Chemical Co.) in D_2O: δ −11.75, −8.4, −1.84 for P^α, ep^β, and P^γ, respectively. Anal. (**3**) ($C_{15}H_{22}N_7O_{14}P_3SNa_4\cdot 2H_2O\cdot 2CH_3OH$) C, H, N, P, S.

Establishment of the C5′ Configuration of 6. A solution of **6** (10 mg) in EtOH (5 mL) was treated with 150 mg (wet weight) of Raney nickel (Sigma Chemical Co.) and refluxed for 0.5 h. HPLC analysis (40% MeOH in H_2O, isocratic) showed the presence of a single compound with the same retention time (9.6 min) as 5′(*S*)-*C*-methyl-2′,3′-*O*-isopropylideneadenosine (**7**).[40] 5′(*R*)-*C*-Methyl-2′,3′-*O*-isopropylideneadenosine[40] had t_R 11.3 min and 2′,3′-*O*-isopropylidenehomoadenosine (**8**)[41] had t_R 5.8 min.

Enzyme Studies. Preparations of M-2 and M-T were obtained as described previously.[1] Enzyme assays were conducted for 10 min at 37 °C in a final volume of 0.1 mL containing 150 mM KCl–15 mM $MgCl_2$–5 mM dithiothreitol–50 mM Tris·HCl, pH 8.2.[49] Each mixture was made up in duplicate. L-[*methyl*-^{14}C]methionine (New England Nuclear Co., 54 Ci/mol) and MgATP were included at the levels specified below and in Table I, footnote *a*. A working enzyme solution was prepared freshly each day by 10-fold dilution of a stock solution with the buffer mixture agaisnt which it had been dialyzed and concentrated.[38] Reactions were started by addition of 10 μL of working enzyme solution [(9.5–10.5) × 10^{-6} units of activity; 1 unit gives a V_{max} with 2 mM ATP of 1 μmol of product/min] and terminated by addition of 10 μL of 4 N $HClO_4$–10 mM L-methionine after immersion of the solution in an ice bath. Each suspension was centrifuged and 50 μL of supernatant was applied to a 2.3-cm disk of phosphocellulose paper. Disks were washed as described,[50] then immersed in a toluene solution of phosphors and counted in a Packard liquid scintillation spectrometer (Model 2425). Controls

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were provided by incubations carried out in the absence of ATP. Reaction velocities were linear for at least 30 min and were proportional to the amount of enzyme added at the levels of enzyme activity employed.

Inhibition studies were made with six to eight levels of MgATP or L-methionine in the range (0.5–4.0) × K_M for each of two inhibitor levels that were in the range (1–10) × K_i and for control mixtures lacking inhibitor. Inhibitors were dissolved in the above pH 8.2 buffer solution prior to testing. Inhibition constants (K_i values) were obtained to within ±15% from replots of inhibitor concentrations vs. slopes or intercepts on the vertical ($1/V$) axis of double-reciprocal plots of velocity vs. substrate level. All of the latter plots were linear, as were the replots.

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Registry No. 3, 109214-84-8; 3·4Na, 109087-20-9; 4, 107961-45-5; 4 (Boc deblocked), 109087-25-4; 5, 109087-21-0; 6, 109087-22-1; 7, 35847-70-2; 9, 109087-23-2; 10, 109087-24-3; L-homocysteine disodium salt, 50615-55-9; benzyl phosphate, 1623-07-0; methionine adenosyltransferase, 9012-52-6.

Phosphorus Amino Acid Analogues as Inhibitors of Leucine Aminopeptidase

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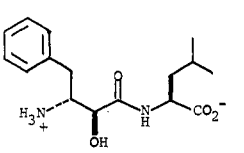
A variety of phosphorus amino acid and dipeptide analogues have been synthesized and evaluated as inhibitors of the metalloenzyme leucine aminopeptidase from porcine kidney. Two phosphonate dipeptides were found to be modest inhibitors of the enzyme (**8e**, K_i = 58 μM; **8h**, K_i = 340 μM). The phosphinic acid (17-OH) and phosphinamide (17-NH_2) analogues related to bestatin were prepared by condensation of the phosphinate amino acid derivative **11**, via a trivalent phosphonite ester **12**, with leucine isocyanate derivatives **13**. These compounds also proved to be unexceptional in their inhibition of LAP (17-O^-, K_i = 56 μM; 17-NH_2, K_i = 40 μM). A series of simple (α-aminoalkyl)phosphonic acid and -phosphinic acids were also evaluated, and the most potent inhibitors were found to be the phosphonic acid analogues of L-Leu and L-Phe ((*R*)-**3e**, K_i = 0.23 μM; (*R*)-**3h**, K_i = 0.42 μM). Slow-binding behavior was observed for (*R*)-**3e** (k_{on} = 400 ± 55 M^{-1} s^{-1}) and (*R*)-**3h** (k_{on} = 445 ± 50 M^{-1} s^{-1}). The phosphinic acid analogues of Leu and Phe are 100-fold less potent than the phosphonate derivatives. The fact that tetrahedral phosphorus analogues are less potent inhibitors of LAP than they are of other zinc peptidases suggests that the mechanism of LAP may be fundamentally different than that of the latter enzymes.

Aminopeptidases are a group of zinc-containing exopeptidases with specificity for cleavage at the amino terminus of a polypeptide chain. Enzymes with similar properties have been found in bacteria and many mammalian tissues.[1] They are of biochemical as well as medicinal importance because of their putative involvement in degradation of biologically active peptides such as the enkephalins and in certain pathological conditions such as human eye cataracts.[2] Although their detailed catalytic mechanism has yet to be elucidated,[3-6] for some aminopeptidases there is evidence for a catalytic role of the zinc ion and a nucleophilic group associated with it, either a water molecule or a side-chain residue.[7-10] Effective inhibitors reported for aminopeptidases include chelating agents, such as amino acid hydroxamates[7,11,12] and amino thiols,[13] and amino acid analogues capable of forming relatively stable tetrahedral adducts, such as boronic acid derivatives,[7,10] chloromethyl ketones,[14,15] and aminoaldehydes.[9,16] The latter compounds are thought to act as transition-state analogues because of the resemblance between their adducts and the tetrahedral intermediates involved in substrate hydrolysis. The most potent aminopeptidase inhibitors are bacterially derived peptide analogues,[17-20] of which bestatin (**1**) is the most



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