Toward the Synthesis of Isozyme-Specific Enzyme Inhibitors. Potent Inhibitors of Rat Methionine Adenosyltransferases. Effect of One-Atom Elongation of the Ribose-P^{α} Bridge in Two Covalent Adducts of L-Methionine and β,γ-Imido-ATP[1]

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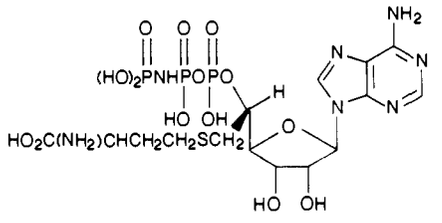
With 2',3'-*O*-isopropylideneadenosine or its N^6-benzoyl derivative as starting material, synthetic routes to two novel adducts of L-methionine and β,γ-imido-ATP have been devised. One adduct, **14** (2:3 mixture of 6' epimers), had a $P^{\alpha}OCH(R^1)CH_2$ system [R^1 = CH_2-L-$SCH_2CH_2CH(NH_2)CO_2H$] in place of the $P^{\alpha}OC(5')H_2$ system of ATP, while the other, **16** (2:3 mixture of 5' epimers), had a $P^{\alpha}OCH_2CH_2CH(R^2)$ system [R^2 = L-$SCH_2CH_2CH(NH_2)CO_2H$]. The ribose-P^{α} bridge in **14** and **16** contained one more methylene group than in two homologous methionine-ATP adducts studied previously. Adduct **14** was a potent inhibitor of the rat M-2 (normal tissue) and M-T (Novikoff ascitic hepatoma) variants of methionine adenosyltransferase and gave competitive kinetics vs MgATP (K_i = 0.39 and 0.63 μM, respectively) or vs L-methionine (K_i = 2.2 and 2.7 μM). Adduct **16** was likewise a potent inhibitor competitive vs MgATP (K_i = 0.44 and 0.81 μM, respectively) or L-methionine (K_i = 2.1 and 1.5 μM). The kinetic data indicate that **14** and **16** inhibit by binding simultaneously to the MgATP and L-methionine substrate sites and that the extra methylene group facilitates the interaction of their methionine residues with these methionine sites.

On grounds outlined previously,[1,2] enzyme inhibitors selective for certain isozymic variants predominant in isozyme profiles of tumor tissues have the potential to be more toxic toward poorly differentiated malignant tissue than toward many normal tissues of the host species and are thus of interest as possible progenitors of new antineoplastic drugs. The present series of studies is examining approaches that might be useful in the systematic design of potent, isozyme-selective inhibitors. Selected as model targets are mammalian isozymes that catalyze reactions involving two substrates. The work has revealed a three-stage strategy that can generate potent and isozyme-selective inhibitors relatively readily.[3,4] The scope and limitations of this strategy are as yet unclear. The second stage comprises attempted derivation of a potent dual substrate-site ("multisubstrate") inhibitor by the known procedure[5] of covalently coupling two substrates via atoms involved in the enzyme-catalyzed reaction. In the third stage, one or more substrate substituents that, from studies in the first stage, are known to produce isozyme-selective inhibitors are attached in the corresponding positions to adducts generated in the second stage.[3,4]

The present paper describes further studies of the second of the above stages. Earlier studies found that two-substrate adducts of the above type could act as potent inhibitors of rat isozymes of thymidine kinase[6] and adenylate kinase,[3] though not of hexokinase.[6] More recent papers reported that the 5'*R* epimers of adducts of L-methionine with β,γ-imido-ATP[1] or with a 5'-(aminomethyl)-5'-deoxy-ATP analogue[7] (**13** and **15**, respectively) are potent inhibitors 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. This enzyme catalyzes attack of methionine sulfur on C5' of ATP with expulsion of tripolyphosphate. As discussed earlier,[8] the M-T variant is of interest as a model mammalian target in the attempted design of an isozyme-selective type of antineoplastic agent. The present study explores the effect on inhibitory potency of ribose-P^{α} bridge elongation brought about by insertion of an additional CH_2 between C4' and C5' of **13**, giving **14**, and between C5' and C6' of **15**, giving **16**. Adduct **16** also differs from **15** in possessing a $POCH_2$ system, which is hydrolytically more stable at physiological pH than the $PNHCH_2$ system of **15** and in possessing a β,γ-imido group which, in β,γ-imido-ATP, imparts phosphatase inertness[9] and hence may render **16** more stable against such attack in vivo. Moreover, in structurally related methionine-ATP adducts a β,γ-imido group was found to increase inhibitory potency ca. 2.5-fold.[10] Adduct **14** was synthesized and evaluated here as a mixture, in known proportion, of its 6' epimers, and adduct **16** was synthesized and evaluated



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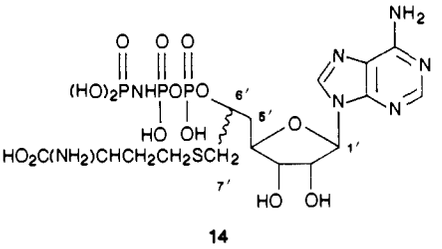

14

(1) This is part 15 in a series on the design of isozyme-specific enzyme inhibitors. For part 14, see: Kappler, F.; Vrudhula, V. M.; Hampton, A. *J. Med. Chem.* **1987**, *30*, 1599.
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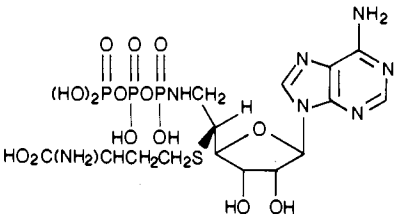
as a mixture of its 5′ epimers.



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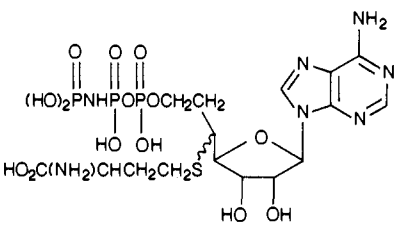
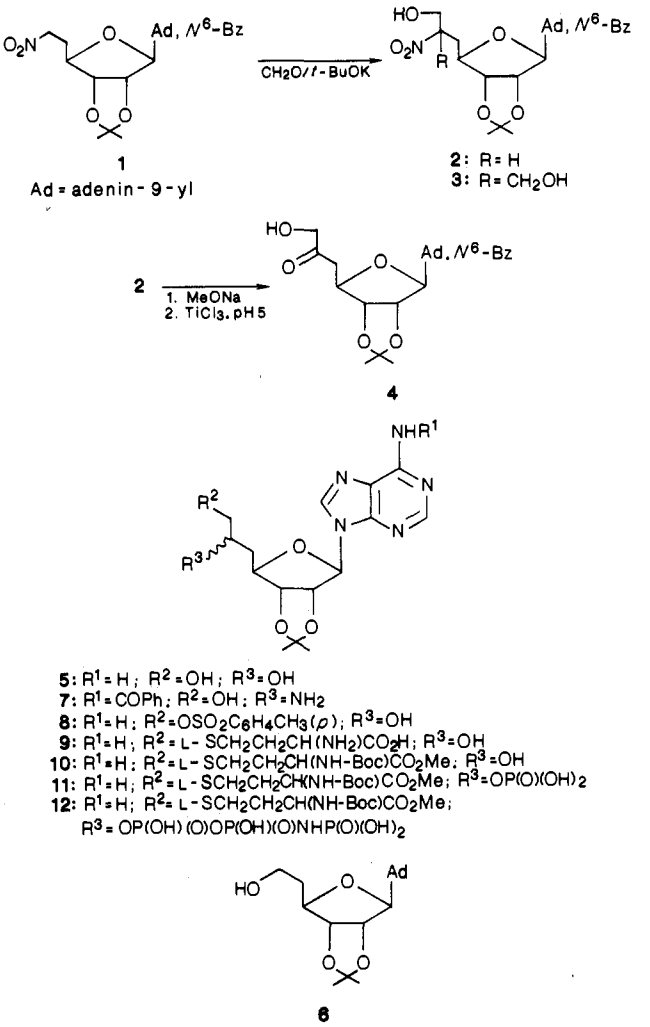


16

Synthesis of 14. The readily accessible N^6-benzoyl-5′-deoxy-5′-(nitromethyl)-2′,3′-*O*-isopropylideneadenosine (1; Scheme I),[11] when treated in *t*-BuOH with 1.2 equiv of CH_2O in the presence of 1 equiv of *t*-BuOK, gave a mixture of products from which the desired 6′-hydroxymethyl derivative **2** could be isolated in homogeneous form in 48% yield. HPLC and ^1H NMR analyses indicated that the two 6′ epimers of **2** were present in a 1:1 ratio. Also isolated from the reaction mixture were a more polar UV-absorbing product (30%), presumably the 6′,6′-bis(hydroxymethyl) derivative **3**, and unreacted **1** (10%). Platinum-catalyzed hydrogenation of **2** gave only low yields of a product that appeared to be the amine **7**. In a successful alternate approach to the desired diol **5**, **2** was converted in MeOH to a sodium nitronate, and this was reduced with $TiCl_3$ at pH 5 by the McMurry–Melton procedure[12] to give, via hydrolysis of the corresponding imine, the 6′-keto nucleoside **4** in about 60% yield. Also formed were N^6-benzoyladenine (10% yield) and **1** (10%). Treatment of crude **4** with $NaBH_4$ in MeOH brought about reduction of the keto group as well as debenzoylation at N^6 and furnished homogeneous **5** in 27% yield overall from **2**. A major byproduct was adenine. Elimination of adenine in this reaction and of N^6-benzoyladenine in the reduction of **2** presumably proceeds through a 6′ carbanion and is accompanied by formation of a 4′,5′ double bond. The same mechanism has been proposed for base-catalyzed elimination of adenine from 5′-cyano-5′-deoxyadenosine.[13]

In confirmation of their structure, a mixture of the two epimeric diols **5**, upon treatment with $NaIO_4$, yielded a single aldehyde, which, after reduction with $NaBH_4$, was converted to an alcohol that could not be distinguished from authentic 9-(5-deoxy-2,3-*O*-isopropylidene-β-D-allofuranosyl)adenine[14] (**6**) by reversed-phase HPLC. The diol **5** was converted quantitatively to a mono-*O*-tosylate that, presumably, was predominantly or exclusively the primary tosylate **8**. Without purification, this was treated in EtOH at 22 °C with disodium L-homocysteinate. The 6′-hydroxy 7′-thioether nucleoside derivative **9** formed as a 1:1 mixture of the two 6′ epimers and after reversed-phase chromatography was isolated in homogeneous form in 77% yield overall from **5** as a 2:3 mixture of 6′ epimers.

Scheme I



The α-amino acid functionality in **9** was protected by conversion to an *N*-Boc derivative of a methyl ester (**10**) by means of successive treatments with di-*tert*-butyl pyrocarbonate and CH_2N_2. This furnished homogeneous **10** in 62% yield from **9** with an unaltered ratio of 6′ epimers. By use of procedures described previously in the synthesis of **13**,[1] **10** was converted into the nucleoside 6′-phosphate derivative **11** (64% yield), and this in turn was converted to its imidopyrophosphoroxy derivative **12** (58% yield), after which removal of blocking groups afforded the homogenous adduct **14** (29% yield from **10**) as a 2:3 mixture of the two 6′ stereoisomers as indicated by the observation, from HPLC analysis of unreacted **10**, that the two 6′ stereoisomers of **10** had undergone phosphorylation at the same rate. Adduct **14** was isolated as a tetrasodium salt that possessed the expected elemental analyses and UV, ^1H NMR, and ^{31}P NMR spectral properties.

Synthesis of 16. N^6-Benzoyl-2′,3′-*O*-isopropylideneadenosine-5′-aldehyde, conveniently obtained through oxidation of the corresponding alcohol,[15] was condensed, without isolation, with (carbethoxymethylene)triphenylphosphorane to give, after preparative HPLC, the trans isomer of the 5′-deoxy-5′-(carbethoxyvinyl)adenosine derivative **17a** (Scheme II) in 73% yield. Treatment of **17a** in aqueous THF at pH 9 with a two-fold excess of sodium L-homocysteinate gave a 4:5 mixture of the 5′ epimers of the 5′-(alkylthio)-5′-deoxyadenosine derivative **18a**, isolated

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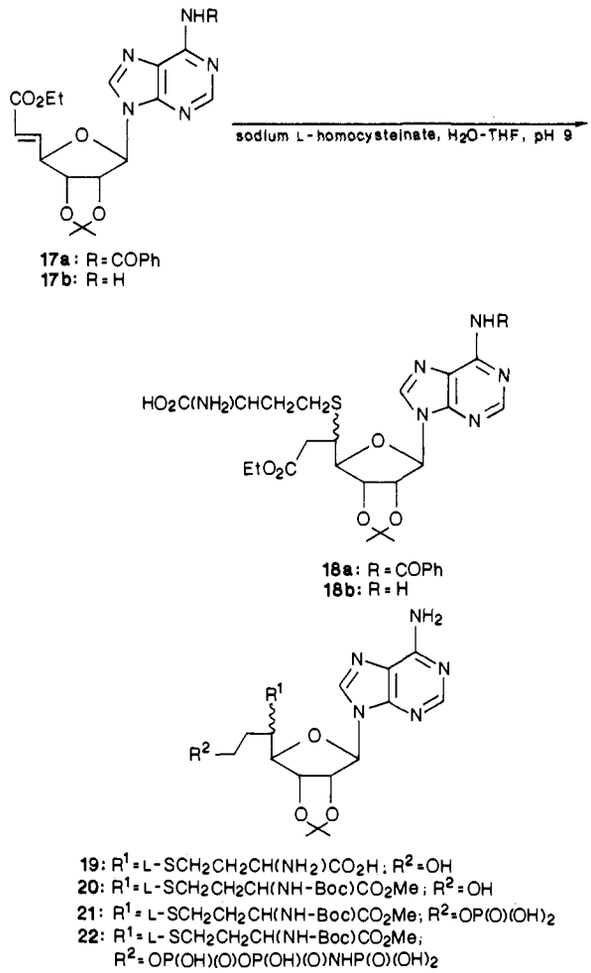
Scheme II



in 82% yield after reversed-phase chromatography. Selective reduction of the carbethoxy group to the corresponding alcohol was attempted with $LiBH_4$ in THF[16] or with $NaBH_4$ in MeOH[17] or EtOH.[6] These reactions, however, produced a variety of products that appeared to arise, at least partly, from loss of the benzoyl group at N^6 and/or elimination of the homocysteine residue. Reaction of the known olefin **17b**[18] with sodium L-homocysteinate gave the homogeneous mixed 5′ epimers of the 5′-(alkylthio)-5′-deoxy nucleoside **18b** in 80% yield and 4.5:5 ratio. Treatment of **18b** with $NaBH_4$ in EtOH yielded mixtures of the desired primary alcohol **19** with unchanged **18b**, free homocysteine, and unidentified UV-absorbing material that was less polar than **18b** or **19**. A study of the effect of temperature and the number of equivalents of $NaBH_4$ revealed that a temperature of 60 °C and 4–6 equiv of the $NaBH_4$ were roughly optimal for **19** formation and furnished homogeneous **19** in 38% yield. The mixed 5′ epimers (in 2:3 ratio) of the methyl ester **20** of the *N*-Boc derivative of **19** were then prepared in 80% yield from **19** by the methods used to obtain **10**. By the procedures used in the above synthesis of **14**, **20** was converted in 60% yield into the corresponding nucleoside 7′-monophosphate derivative **21**, and **21**, via its imidopyrophosphoroxy derivative **22**, was converted into **16** (37% yield of homogeneous material from **21**). Adduct **16** was isolated as a tetrasodium salt with the expected UV and ^{31}P NMR spectral properties. Anion-exchange HPLC analysis on a reversed-phase column showed the ratio of 5′ epimers in **16** to be the same (2:3) as in its blocked nucleoside precursor **20**.

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
13[c]	0.13 (C)	0.65 (C)	0.21 (C)	0.67[d] (M)
14	0.39 (C)	2.2 (C)	0.63 (C)	2.7 (C)
15[e]	0.36 (C)	3.0 (NC)	0.27 (C)	2.3 (NC)
16	0.44 (C)	2.1 (C)	0.81 (C)	1.5 (C)

[a] When methionine (Met) was the variable substrate, [MgATP] was 2 mM with both MAT forms; with variable MgATP, [Met] was 60 μM with M-2 and 120 μM with M-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 4.4 μM with M-2 and 12 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 1. [d] Calculated from double-reciprocal plots (see the Experimental Section) as K_i (slope); K_i calculated from intercepts on the $1/V$ axis was 1.75 μM. [e] Data from ref 7.

Enzyme Studies. Listed in Table I are inhibition constants of **13–16** with M-2 and M-T determined under the same conditions for each of these methionine adenosyltransferase variants. Adduct **14** was a potent inhibitor of both variants and produced competitive kinetics vs MgATP [K_M(ATP)/K_i = 360 and 220 for M-2 and M-T, respectively] and vs L-methionine [K_M(Met)/K_i = 2.0 and 4.5, respectively], suggesting that it may bind simultaneously to the MgATP and methionine enzymic sites in exerting these inhibitions. Inasmuch as one 5′ epimer of **15** inhibits M-T 74-fold more effectively than the other 5′ epimer,[7] it can be expected that the observed inhibitions by **14** are brought about mainly by one of its two 6′ epimers (present in a 2:3 ratio). Table I shows that if inhibition arose from the minor epimeric component, the potency of **14** for both enzyme forms would be almost equal to that of **13**; if arising from the major epimer, the potency would be 33% less. Adduct **14** inhibited M-T competitively with respect to methionine, whereas **13** gave a mixed competitive–noncompetitive type of inhibition, suggesting that the additional methylene group may permit the methionine residue in **14** to interact with the methionine binding site in a way that diminishes the ability of methionine to bind reversibly to an enzyme–adduct complex to form a ternary methionine–enzyme–adduct complex.

Adduct **16**, tested as the 2:3 mixture of its 5′ epimers, is seen from Table I to inhibit M-2 significantly more effectively than the structurally related adduct **15** irrespective of which 5′ epimer of **16** is the more inhibitory. Adduct **16** also inhibited M-T more effectively than did **15** as indicated by its inhibition constant with variable methionine and appeared from its inhibition constant with variable MgATP to be either as effective or almost as effective against M-T as **15**, depending upon whether the minor or the major 5′ epimer of **16** is the more active species. That adduct **16** inhibited both variants competitively with respect to either methionine or to MgATP suggests that it could be acting as a dual substrate site inhibitor. A β,γ-imidophosphate system in a homocysteine–ATP adduct was earlier reported to enhance inhibitory potency toward M-2 and M-T by a factor of ca. 2.5-fold,[10] and hence this system might enhance the potency of **16** also. Notably, with both enzyme variants, **16**

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inhibits in competitive fashion with respect to methionine, whereas **15** inhibits noncompetitively (Table I). Possibly, the longer P^{α}–C5′ bridge of **16** allows its methionine residue to interact with a larger segment of the methionine binding site, thereby hindering binding of methionine to the enzyme–**16** complex and suppressing noncompetitive inhibition. The ATP moiety of enzyme-bound **16** could, in the manner of ATP itself,[19] be bound mainly via its tripolyphosphate and 2′-OH loci as a result of rotations that can occur about the C2′–O2′ bond and about bonds in the P^{α}–C4′ system.

In summary, previous studies[1,7] have described two types of methionine–ATP adducts (**13** and **15**) that were potent inhibitors of the M-2 and M-T variants and that appeared to possess a dual substrate site mode of binding. The present study provides evidence that introduction of a single methylene group into the P^{α}–C5′ bridge of **13** or **15** (concomitantly with two isosteric atom replacements in the case of **15**) is compatible with maintenance of powerful dual-site inhibition and appears to permit the methionine moieties to interact more effectively with the enzymic methionine sites. The four potent inhibitors **13**–**16** exhibit no more than minor levels of isozyme selectivity. It is possible, however, that selectivity for the chemotherapeutic target M-T may be inducible by structural modification, for example by the introduction of substituents indicated to be promising from previous studies with derivatives of ATP and of methionine.[8,20] Studies of additional potent and/or selective M-T inhibitors are continuing.

Experimental Studies

Chemical Synthesis. General Procedures. *p*-Toluenesulfonyl chloride was recrystallized from hexane. *S*-Benzyl-L-homocysteine was prepared and purified as described[21] and converted to disodium L-homocysteinate as described.[22] H_2O used in reactions was degassed. Thin-layer chromatography (TLC) was run on Merck silica gel 60 F-254 plates in (A) CH_3COC-H_3–CH_2Cl_2 (3:7), (B) MeOH–$CHCl_3$ (1:19), (C) MeOH–$CHCl_3$ (1:9) (D) $CHCl_3$–MeOH–4% aqueous CH_3COOH (3:2:1, lower layer), (E) MeOH–$CHCl_3$ (3:7), and (EE) acetone–CH_2Cl_2 (1:4). Reversed-phase TLC was carried out on glass plates of silica gel RP-18 from EM Laboratories in (R1) MeOH–H_2O (1:1) and (R2) MeOH–H_2O (7:3). Paper chromatography was carried out by the ascending technique on Whatman No. 1 in (F) 1-propanol–NH_4OH–H_2O (55:10:35) and (G) isobutyric acid–1 M NH_4OH (6:4). Electrophoresis was carried out on Whatman No. 1 paper at pH 3.5 (0.05 M citrate) and pH 7.5 (0.05 M $Et_3NH{\cdot}HCO_3$). HPLC was performed on a Waters Model 204 chromatograph equipped with a Waters 740 data module, a dual solvent-delivery system (Model M-6000A), a Model 660 programmer, and a Waters RCM-100 radial compression unit containing a 4-μm particle size Nova-Pak C_{18} cartridge (8 mm × 10 cm). Gradients were linear and were run for 10 min at 2 mL/min flow rate in (H) 40–100% MeOH in H_2O, (J) 20–100% MeOH in H_2O, and (K) 0–30% MeOH in 0.1 M potassium phosphate–0.025 M $(n\text{-}Bu)_4NHSO_4$ (pH 7.6). Flash chromatography[23] was performed with Merck silica gel 60 (230–400 mesh). Baker bonded-phase octadecyl silica gel (flash chromatography grade) was used for column chromatography. Preparative HPLC was performed at 250 mL/min on a Waters Prep LC/System 500A equipped with one silica gel cartridge. UV spectra were obtained with a Varian Model 635 spectrophotometer, and ^1H and ^{31}P NMR spectra were obtained with a Nicolet NT-300 WB spectrometer. Chemical shifts are given as ppm (δ) downfield from $SiMe_4$ or 85% H_3PO_4. The sign of ^{31}P shifts are in accord with the 1976 IUPAC convention.[24]

Elemental analyses were by Galbraith Laboratories, Inc., Knoxville, TN, and were within ±0.4% of theoretical values unless stated otherwise. Compounds were dried in vacuo at 22 °C over P_2O_5 except for **2**, **5**, and **9** which were dried at 78 °C.

***N*^{6}-Benzoyl-5′-deoxy-5′-(2-hydroxy-1(*RS*)-nitroethyl)-2′,3′-*O*-isopropylideneadenosine (2).** To a stirred solution of 1 (2.33 g, 5.1 mmol) in *t*-BuOH (25 mL) under argon was added 0.2 M *t*-BuOK in *t*-BuOH (25.5 mL, 1 equiv). The suspension was stirred for 15 min. Gaseous HCHO generated from paraformaldehyde (185 mg, 1.2 equiv) was added, and the suspension was stirred for an additional 0.5 h. Solid NH_4Cl (1 g, 18.7 mmol) was added, *t*-BuOH was removed in vacuo, and the residue was partitioned between EtOAc (2 × 50 mL) and saturated aqueous NH_4Cl. The EtOAc was dried (Na_2SO_4) and evaporated in vacuo. The residue was subjected to flash chromatography on silica gel with acetone–CH_2Cl_2 (3:7) to give **2** (1:1 mixture of 6′ epimers by NMR H-1′ integration; 1.21 g, 48% from 1), presumed **3** (0.78 g, 30%), and 1 (0.23 g) as white foams homogeneous by silica gel TLC [R_f values, solvent A, 0.55 (1), 0.30 (**2**), 0.13 (**3**); solvent B, 0.42 (1), 0.26 (**2**), 0.19 (**3**)] and HPLC [solvent H; t_R 7.8 min, 8.1 min (**2**); 8.5 min (1); 7.8 min (**3**)]. **2**: UV max (EtOH) 280 nm (ε 18600); ^1H NMR ($CDCl_3$) δ 9.10 (br s, 1, N*H*CO), 8.78, 8.75 (2 s, 1, H-2 or H-8), 8.01 (m, 3, H-2 or H-8 and 2 H of C_6H_5), 7.53 (m, 3, C_6H_5), 6.07, 6.06 (2 d, 1, H-1′, $J_{1'2'}$ = 1.9, 1.2 Hz), 5.52, 5.50 (m, H-2′), 5.00, 4.97 (2 dd, 1, H-3′, $J_{2',3'}$ = 6.3, 6.3 Hz, $J_{3',4'}$ = 3.6, 3.5 Hz), 4.61, 4.58 (m, 1, H-4′), 4.29 (m, 1, H-6′), 3.85 (m, 2, H-7′), 2.41 (m, 2, H-5′), 1.58, 1.37, 1.36 (3 s, 6, $C(CH_3)_2$). Anal. ($C_{22}H_{24}N_6O_7{\cdot}0.75H_2O$) C, H, N.

5′-Deoxy-5′-(1(*RS*),2-dihydroxyethyl)-2′,3′-*O*-isopropylideneadenosine (5). To a stirred solution of **2** (0.97 g, 2 mmol) in MeOH (16.4 mL) at 20 °C under argon was added 1.5 M NaOMe in MeOH (2.7 mL, 2 equiv). After 2 min, a solution (16.4 mL) prepared from $TiCl_3$ (1.54 g), H_2O (7.7 mL), and 4 M aqueous NH_4OAc (15 mL) under argon was added, and the mixture was stirred for 20 min. MeOH was removed in vacuo, and to the white suspension was added saturated aqueous NH_4Cl (30 mL) and EtOAc (50 mL). The mixture was vigorously stirred for about 3 min and then filtered through a bed of Celite (1 × 5 cm) to remove titanium salts. The Celite was washed with EtOAc (3 × 50 mL). The aqueous phase in the combined filtrate and washings was extracted with EtOAc (50 mL). The combined EtOAc solutions were dried (Na_2SO_4) and evaporated in vacuo. Toluene (2 × 25 mL) was evaporated from the residual crude 4, and this was then dissolved in MeOH (10 mL) and treated with $NaBH_4$ (115 mg, 3 mmol). When effervescence ceased, the solution was refluxed for 0.5 h. MeOH was evaporated in vacuo, and the residue was partitioned between CH_2Cl_2 (30 mL) and water (3 × 10 mL). The combined aqueous solutions were concentrated to 5 mL and applied to a column (3 × 3 cm) of C_{18}-bonded silica gel, and this was eluted with water (100 mL) and then with MeOH–H_2O (3:7, 200 mL). The MeOH–H_2O eluate was evaporated in vacuo, and the pale yellow gum was triturated with CH_2Cl_2 (2 × 5 mL). The gum obtained after decantation of the CH_2Cl_2 was dried in vacuo to give **5** (8130 OD_{260} units, 0.54 mmol, 27% yield from **2**) as a white foam that was homogeneous by TLC (solvent C, R_f of the two 6′ epimers 0.15, 0.13) and HPLC (solvent J, t_R 7.0 min): ^1H NMR (CD_3CN) δ 8.19, 7.99, 7.97 (3 s, 2, H-2, H-8), 6.03, 6.02 (2 d, 1, H-1′, $J_{1'2'}$ = 3.2 and 2.7 Hz), 5.39 (m, 1, H-2′), 4.94, 4.86 (2 dd, 1, H-3′, $J_{2',3'}$ = 7.5, 6.3 Hz and $J_{3',4'}$ = 3.5 and 3.4 Hz), 4.32 (m, 1, H-4′), 3.60 (m, 1, H-6′), 3.29 (m, 2, H-7′), 1.69 (m, 2, H-5′), 1.53, 1.31 (2 s, 6, $C(CH_3)_2$). Integration of H-1′ revealed the 6′ epimers to be in a 1:1 ratio. Anal. ($C_{15}H_{21}N_5O_5{\cdot}CH_3OH{\cdot}0.5H_2O$) C, H, N.

5′-Deoxy-5′-(2-L-homocystein-*S*-yl-1(*RS*)-hydroxyethyl)-2′,3′-*O*-isopropylideneadenosine (9). A solution of **5** (7800 OD_{260} units, 520 μmol) and *p*-$CH_3C_6H_4SO_2Cl$ (158 mg, 1.6 equiv) in anhydrous pyridine (0.9 mL) was kept at −15 °C for 18 h, at which time silica gel TLC revealed quantitative conversion to **8** (R_f in solvent C, 0.50). The pyridine was evaporated in vacuo. A solution of the residue in EtOAc (20 mL) was washed with saturated aqueous $NaHCO_3$ (2 × 5 mL), dried (Na_2SO_4), and evaporated in vacuo. EtOH (10 mL) was evaporated in vacuo from the residue. A solution of the residue in EtOH (1 mL) was

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treated with 0.52 M disodium L-homocysteinate[22] in EtOH (1.5 mL, 1.5 equiv), and the orange solution was kept at ambient temperature for 2 h. EtOH was evaporated in vacuo, water (3 mL) was added to the residue, and the resulting turbid solution was adjusted to pH 6 and applied to a C_{18} silica gel column (3 × 3 cm), which was eluted in succession with H_2O (100 mL), MeOH–H_2O (1:9, 50 mL), MeOH–H_2O (3:7, 100 mL), and MeOH–H_2O (1:1, 50 mL). The aqueous 30% MeOH eluate was evaporated to dryness in vacuo, and the residue, after trituration with acetone (15 mL), was dried to give **9** (6000 OD_{260} units, 0.4 mmol, 77% from **5**) as a white foam homogeneous by TLC (R_f 0.10 in D; **8** had R_f 0.80) and HPLC (t_R 8.3, 8.6 min in J; **8** had t_R 9.8, 10.0 min): ^{1}H NMR (D_2O) δ 8.12, 8.10, 8.06, 8.05 (4 s, 2, H-2, H-8), 6.08, 6.04 (2 d, 1, H-1′, $J_{1',2'}$ = 1.2, 2.5 Hz), 5.63, 5.43 (2 dd, 1, H-2′, $J_{2',3'}$ = 6.1, 6.3 Hz), 4.91 (m, 1 H-3′), 4.33 (m, 1, H-4′), 3.60, 3.25 (m, 2, H-6′ and H-2″), 2.46, 2.42 (2 t, 2, H-4″, $J_{3'',4''}$ = 7.3, 7.6 Hz), 2.20 (m, 2, H-7′), 1.75 (m, 4, H-5′, H-3″), 1.46, 1.28, 1.26 (3 s, 6, $C(CH_3)_2$). Integration of H-1′ revealed the 6′ epimers to be in a 2:3 ratio. Anal. ($C_{19}H_{28}N_6O_6S$·0.75CH_3OH·H_2O) C, H, S; N: calcd, 16.47; found, 15.90.

The *N*-*tert*-Butyloxycarbonyl Derivative of 9, Methyl Ester 10. Compound **9** (94 mg, 0.20 mmol) was dissolved in dry DMF (2 mL) containing Et_3N (32 μL, 230 μmol), and di-*tert*-butyl pyrocarbonate (52 mg, 238 μmol) was added.[25] After 0.5 h at 22 °C, TLC analysis (E) showed that conversion to a single ninhydrin-negative spot, R_f 0.40, was complete. The DMF was evaporated. The residue was dissolved in MeOH (5 mL) and treated with excess of ethereal CH_2N_2. Volatiles were removed in vacuo, and the residue was eluted from a column (2 × 25 cm) of silica gel with $CHCl_3$–MeOH (97:3). Evaporation of eluant gave **10** as a white foam (86 mg, 74%) homogeneous by TLC, R_f 0.40 in C, and by HPLC, t_R 9.0 and 9.3 min in H (2:3 ratio of 6′ stereoisomers): NMR ($CDCl_3$) δ 1.35, 1.38, 1.59 (6 H, CMe_2), 1.40, 1.43 (9 H, CMe_3), 1.95 (4 H, H-5′, H-2″), 2.40 (2 H, H-4″), 2.58, (2 H, H-3″), 3.58 (1 H, H-6′), 3.72 (3 H, OMe), 3.96 (1 H, H-1″), 4.52 (1 H, H-4′), 4.93, 5.18 (1 H, H-3′), 5.40, 5.45 (1 H, H-2′), 5.80, 5.89 (1 H, H-1′), 7.91, 7.94, and 8.32 (2 H, H-2, H-8). Anal. ($C_{25}H_{38}N_6O_8S$·CH_3OH) C, H, N, S.

5′-Deoxy-5′-[[1(*RS*)-[(β,γ-imido)triphosphoroxy]-2-L-homocystein-*S*-yl]ethyl]adenosine (Mixture of Diastereomers) (14). Compound **10** (120 μmol) was converted to the homogeneous, purified triethylammonium salt of **14** in 29% yield overall by the procedures used in the synthesis of **13**.[1] To a solution of this in MeOH (1 mL) was added methanolic NaI (180 μL of 1 M) followed by acetone (75 mL). The resulting precipitate was washed with acetone and dried in vacuo to give 26 mg of tetrasodium **14** as a white powder, which was homogeneous by paper chromatography in systems (F) and (G), R_f 0.34 and 0.40, respectively: UV max pH 2, 260 nm (ε 14 700), pH 12, 260 nm (ε 14 800); ^{31}P NMR (121.46 MHz, D_2O) δ −11.58 (P^α), −6.50 (P^β), −1.47 (P^γ). The ^{1}H NMR spectrum in D_2O was consistent with the structure, but signal assignments could not be made due to complex overlapping resonances in the two 6′ stereoisomers. Anal. ($C_{16}H_{24}N_7O_{14}P_3S$ Na_4·3.5H_2O) C, H, S, P; N: calcd, 11.98; found, 12.45.

***N*⁶-Benzoyl-5′-deoxy-5′-(*trans*-carbethoxyvinyl)-2′,3′-*O*-isopropylideneadenosine (17a).** To a solution of N^6-benzoyl-2′,3′-*O*-isopropylideneadenosine (4.1 g, 10 mmol) in Me_2SO (50 mL) were added *N*,*N*′-dicyclohexylcarbodiimide (8.2 g, 40 mmol) and dichloroacetic acid (0.7 mL, 8.4 mmol). The mixture was stirred for 18 h. The precipitate of *N*,*N*′-dicyclohexylurea was removed by filtration and washed with small portions of Me_2SO (20 mL total). The filtrate was extracted with cyclohexane (3 × 50 mL). To the Me_2SO solution were added pyridine (0.7 mL) and carbethoxymethylene triphenylphosphorane (Alfa Products, Denvers, MA) (8 g, 22.4 mmol). Analysis of the solution after 1 h showed a single component by TLC (system EE), R_f 0.40 (R_f 0.06 for the 5′ aldehyde), and t_R 10.1 min by HPLC in system H. The solution was partitioned between EtOAc (200 mL) and water (100 mL). The aqueous phase was extracted with EtOAc (2 × 100 mL). The EtOAc extracts were combined, washed with H_2O (100 mL), dried (Na_2SO_4), and evaporated in vacuo. The residue was dissolved in CH_2Cl_2–Me_2CO (4:1, 100 mL) and filtered through a column of silica gel (5 × 10 cm) to remove excess of Wittig reagent. The column was washed with additional solvent to remove **17a**. Volatiles were removed in vacuo to give a pale yellow foam, which was purified by preparative HPLC (CH_2Cl_2–acetone, 88:12, one SiO_2 cartridge) to give 3.5 g (73.1%) of **17a** as a white foam: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (t, 3 H, J = 7.2 Hz, CH_2CH_3), 1.39 and 1.62 (s, 3 each, CMe_2), 4.10 (q, 2 H, J = 7.2 Hz, CH_2CH_3), 4.82 (m, 1 H, H-4′), 5.13 (dd, 1 H, $J_{3',4'}$ = 3.7 Hz, $J_{3'2'}$ = 6.2 Hz, H-3′), 5.54 (dd, 1 H, $J_{2'3'}$ = 6.2 Hz, $J_{1'2'}$ = 1.9 Hz, H-2′), 5.82 (dd, 1 H, $J_{6',5'}$ = 15.7 Hz, $J_{6',4'}$ = 1.6 Hz, H-6′), 6.18 (d, 1 H, $J_{1'2'}$ = 1.9 Hz, H-1′), 6.94 (dd, 1 H, $J_{5',6'}$ = 15.7 Hz, $J_{5',4'}$ = 5.5 Hz, H-5′), 8.02 (m, 5 H, aromatic). Anal. ($C_{24}H_{25}N_5O_6$) C, H, N.

***N*⁶-Benzoyl-5′-deoxy-5′(*RS*)-L-homocystein-*S*-yl-5′-(carbethoxymethyl)-2′,3′-*O*-isopropylideneadenosine (18a).** To an aqueous solution (10 mL) of freshly prepared disodium L-homocysteine (4 mmol),[22] adjusted to pH 9 with 50% HI, was added **17a** (0.96 g, 2 mmol) in THF (10 mL). HPLC showed the reaction to be complete in 0.5 h. The solution was evaporated. The residue was suspended in EtOH (20 mL) and centrifuged. The precipitate was washed with EtOH (2 × 10 mL). The EtOH solutions were combined and evaporated. The residue was dissolved in MeOH–H_2O (4:6, 30 mL) and applied to a column of C-18 silica gel (2.5 × 15 cm). The column was washed with MeOH–H_2O (4:6, 100 mL) to remove traces of amino acid, and **18a** was then eluted with MeOH–H_2O (4:1, 100 mL). Evaporation of volatiles in vacuo gave **18a** (1.05 g, 82%) as a white powder homogeneous by HPLC in system H (t_R 8.4 and 8.7 min; 5:4 mixture of diastereomers) and TLC (system D) (R_F 0.50): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.01 and 1.15 (t, 3 each, J = 7.2 Hz CH_2CH_3), 1.31, 1.34, 1.52, 1.54 (s, 3 each, CMe_2), 3.82 and 4.04 (q, 2 each, CH_2CH_3), 6.15 (d, 1 H, J = 3.8 Hz, H-1′), 6.22 (s, 1 H, H-1′), 8.35, 8.48, 8.65, 8.80 (s, 1 each, H-2, H-8). Anal. ($C_{28}H_{34}N_6O_8S$·1.5H_2O) C, H, N, S.

5′-Deoxy-5′-(*RS*)-L-homocystein-*S*-yl-5′-(carbethoxymethyl)-2′,3′-*O*-isopropylideneadenosine (18b). Disodium L-homocysteinate (3 mmol) was added to **17b** (1.5 mmol)[18] as described above for the preparation of **18a**. After 0.5 h, THF was removed in vacuo, and the aqueous solution was adjusted to pH 5. The resulting precipitate was removed by centrifugation, and the clear supernate was applied to a column of C-18 silica gel (2.5 × 15 cm). The column was washed well with water to remove the amino acid, and then **18b** was eluted with methanol–water (1:1, 100 mL). Removal of volatiles in vacuo gave **18b** as a white foam (0.61 g, 79.7%) homogeneous by TLC in (D), R_f 0.12, and (R2), R_f 0.48, and HPLC, t_R 6.0 and 6.6 min (5:4.5 mixture of diastereomers in system H): ^{1}H NMR (300 MHz, CD_3OD) δ 1.11 and 1.22 (t, 3 each, J = 7.1 Hz, CH_2CH_3), 1.38, 1.39, 1.58, 1.60 (s, 3 each, CMe_2), 6.13 (d, 1 H, J = 3.7 Hz, H-1′), 6.25 (d, 1 H, J = 2.0 Hz, H-1′), 8.21, 8.26, 8.27, and 8.32 (s, 1 each, H-2 and H-8). Anal. ($C_{21}H_{30}N_6O_7S$·H_2O) C, H, N, S.

5′-Deoxy-5′(*RS*)-L-homocystein-*S*-yl-5′-(2-hydroxyethyl)-2′,3′-*O*-isopropylideneadenosine (19). Compound **18b** (382 mg, 0.75 mmol) was dissolved in EtOH (50 mL), and a solution of $NaBH_4$ in EtOH (1 M, 3 mL) was added. The solution was kept at 60 °C for 2 h and then evaporated in vacuo. A solution of the residue in water 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 amino acid. Water–methanol (65:35; 100 mL) eluted **19**, which was obtained as a white foam (132 mg, 38%) after evaporation of volatiles in vacuo. It was homogeneous on TLC (D), R_f 0.06, and on RPTLC (R1), R_f 0.33. Anal. ($C_{19}H_{28}N_6O_6S$·H_2O) C, H, N, S.

The *N*-*tert*-Butyloxycarbonyl Derivative of 19, Methyl Ester 20. Compound **19** (117 mg, 0.25 mmol) was dissolved in DMF and triethylamine (40 μL, 288 μmol) and di-*tert*-butyl pyrocarbonate (65 mg, 298 μmol) were added. After 0.5 h at 22 °C, TLC analysis showed the reaction to be complete, R_f (D) 0.33 (ninhydrin negative) and RPTLC (R1) R_f 0.14. Volatiles were evaporated in vacuo. The residue was dissolved in MeOH (5 mL), and an excess of fresh ethereal CH_2N_2 was added. After removal of solvent in vacuo, the residue was chromatographed on a silica gel column (2.5 × 12 cm) in $CHCl_3$–MeOH (97.5:2.5). Fractions containing the diastereomers of **20** were evaporated to a white foam (116 mg, 79.7%), which was homogeneous on TLC (B) R_f

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0.14 and 0.17 (diastereomers) and (C) R_f 0.44 and 0.48 and HPLC (H) t_R 9.2 and 9.5 min (2:3 ratio of diastereomers). Anal. ($C_{25}H_{38}N_6O_8S \cdot CH_3OH$) C, H, N; S: calcd, 5.21; found, 4.73.

5′-Deoxy-5′-L-homocystein-*S*-yl-5′-[2-[(β,γ-imido)triphosphoroxy]ethyl]adenosine (Mixture of 5′ Epimers, 16). A solution of **20** (100 mg, 0.17 mmol) and benzyl phosphate (130 mg, 0.69 mmol) in pyridine (5 mL) was evaporated. This was repeated twice. The residue was dissolved in pyridine and treated with dicyclohexylcarbodiimide (345 mg, 1.72 mmol). The mixture was kept at 22 °C for 48 h. Water (5 mL) was added, and after 1 h the solution was filtered and evaporated. A solution of the residue in AcOH (10 mL) containing Pd black (200 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 (1:1, 5 mL) was added NEt_3 (290 μL, 2.1 mmol). The solution was kept at 22 °C for 10 min and evaporated. A solution of the residue in MeOH–H_2O (1:9, 20 mL) was applied to a column of C-18 silica gel (2.5 × 15 cm, equilibrated with MeOH–H_2O, 1:9). The column was washed with MeOH–H_2O (1:9, 100 mL) to remove inorganic phosphate. MeOH–H_2O (1:1, 100 mL) eluted **21** (1600 OD_{260} units, 62.7%), which was homogeneous on HVE (pH 7.5), EM_{AMP} 0.6. After removal of volatiles in vacuo, **21** (1550 OD_{260} units, 0.10 mmol) was dissolved in DMF (5 mL), and the solution was evaporated in vacuo. This was repeated twice. The resulting gum was dissolved in DMF (5 mL), and 1,1′-carbonyldiimidazole (80 mg, 0.5 mmol) was added. After 1 h at 22 °C, MeOH (34 μL) was added. The DMF was evaporated in vacuo after 0.5 h, and the residue was extracted several times with dry ether to remove most of the imidazole. The residual oil was dissolved in DMF (2 mL) and added to a solution of the tri-*n*-butylammonium salt of imidodiphosphate (0.5 mmol) in DMF (2 mL). The mixture was kept at 22 °C for 18 h, when paper electrophoresis showed the reaction to be complete. A solution of imidazole in DMF (1 mL, 1 M) was added. The precipitate was removed by centrifugation and washed with a small volume of DMF. The DMF solutions were combined and evaporated in vacuo. The residue was dissolved in H_2O and applied to a column of C-18 silica gel (1 × 15 cm). This was washed with water (100 mL) to remove imidodiphosphate. Water–MeOH (3:1, 100 mL) eluted **22** (880 OD_{260} units, 56.8%); removal of volatiles in vacuo gave **22** as a gum homogeneous by HVE at pH 3.5 and pH 7.5. The gum was dissolved in 90% trifluoroacetic acid (TFA) (2 mL), and after 10 min at 22 °C, the volatiles were immediately removed in vacuo. Toluene was evaporated twice from the residue to remove residual TFA. The residue was dissolved in buffer (1 mL) derived from 0.038 M $NaHCO_3$ and 0.025 M NaOH and adjusted to pH 10.4 with dilute NaOH. After 6 h at 22 °C, paper electrophoresis (pH 3.5) and PEI cellulose TLC (1 M LiCl) showed that hydrolysis was complete. The solution was chromatographed on a DEAE (HCO_3^-) cellulose column (2 × 15 cm) with a linear gradient of $Et_3NH \cdot HCO_3$ (0–0.4 M, 2 L). Fractions containing **16** (580 OD_{260} units, 37.4%) were combined and evaporated. The residue was homogeneous on paper chromatography in systems (F) and (G), R_f 0.33 and 0.40, respectively. The residue was dissolved in MeOH (500 μL), and 1 M NaI in MeOH (200 μL, 5 equiv) was added. Acetone (20 mL) was added. The precipitate was washed with acetone and dried in vacuo over P_2O_5 to give the tetrasodium salt (23 mg) of **16** as a white powder: HPLC (system K), t_R 10.8 and 12.3 min (2:3 ratio of 5′ epimers); UV max pH 2, 259 nm (ε 15 100), pH 11, 260 nm (ε 15 200); ^1P NMR (121.46 MHz, D_2O) δ −11.79 (P^α), −8.48 (P^β), −1.86 (P^γ). Anal. ($C_{16}H_{24}N_7O_{14}P_3SNa_4 \cdot 3.5H_2O$) C, H, N, P, S.

Enzyme Studies. Preparations of M-2 and M-T were obtained as described previously.[7] 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.[25] 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 against which it had been dialyzed and concentrated.[8] 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 per min) and terminated by addition of 10 μL of 4 N $HClO_4$–10 mM L-methionine after immersing the solution in an ice bath. Each suspension was centrifuged and 50 μL of supernatant was applied to a 2.3-cm disc of phosphocellulose paper. Discs were washed as described,[26] then immersed in a toluene solution of phosphors and counted in a Packard liquid scintillation spectrometer (Model 2425). Controls 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. Typical double-reciprocal plots have been illustrated in a previous publication.[7]

Acknowledgment. This work was supported by Public Health Service Research Grant CA-11196 from the National Cancer Institute and by grants to the Institute for Cancer Research (USPHS Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania). We thank Dr. Sidney Weinhouse, Temple University, for kindly providing the rat Novikoff ascitic hepatoma cells from which the M-T preparations were obtained.

Registry No. 1, 84757-71-1; **2** (*R*-epimer), 111268-99-6; **2** (*S*-epimer), 111269-14-8; **3**, 111269-00-2; **4**, 111269-01-3; **5** (*R*-epimer), 111269-02-4; **5** (*S*-epimer), 111269-15-9; **8** (*R*-epimer), 111323-50-3; **8** (*S*-epimer), 111269-16-0; **9** (*R*-epimer), 111269-03-5; **9** (*S*-epimer), 111269-17-1; **10** (*R*-epimer), 111269-04-6; **10** (*S*-epimer), 111269-18-2; **11** (*R*-epimer), 111269-05-7; **11** (*S*-epimer), 111269-19-3; **12** (*R*-epimer), 111269-06-8; **12** (*S*-epimer), 111269-20-6; **14** (*R*-epimer), 111291-44-2; **14** (*S*-epimer), 111269-30-8; **14**·4Na (*R*-epimer), 111269-07-9; **14**·4Na (*S*-epimer), 111269-21-7; **14**·Et_3N (*R*-epimer), 111291-45-3; **14**·Et_3N (*S*-epimer), 111269-31-9; **16** (*R*-epimer), 111269-32-0; **16** (*S*-epimer), 111269-34-2; **16**·4Na (*R*-epimer), 111269-08-0; **16**·4Na (*S*-epimer), 111269-22-8; **16**·Et_3N (*R*-epimer), 111269-33-1; **16**·Et_3N (*S*-epimer), 111269-35-3; **17a**, 111322-02-2; **17b**, 102044-62-2; **18a** (*R*-epimer), 111269-09-1; **18a** (*S*-epimer), 111269-23-9; **18b** (*R*-epimer), 111269-28-4; **18b** (*S*-epimer), 111269-29-5; **19** (*R*-epimer), 111269-10-4; **19** (*S*-epimer), 111269-24-0; **20** (*R*-epimer), 111269-11-5; **20** (*S*-epimer), 111269-25-1; **21** (*R*-epimer), 111269-12-6; **21** (*S*-epimer), 111269-26-2; **22** (*R*-epimer), 111269-13-7; **22** (*S*-epimer), 111269-27-3; H-Hcy-OH·2Na, 50615-55-9; $Cl_2CHCOOH$, 79-43-6; N^6-benzoyl-2′,3′-*O*-isopropylideneadenosine, 39947-04-1; methionine adenosyltransferase, 9012-52-6.

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