Adenosine-5'-carboxaldehyde: A Potent Inhibitor of S-Adenosyl-L-homocysteine Hydrolase

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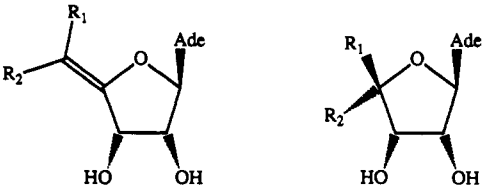
Adenosine-5'-carboxaldehyde (3) and its 4'-epimer (4) were synthesized and shown to be potent type I mechanism-based inhibitors of recombinant rat liver AdoHcy hydrolase with k_2/K_I values of 16.7×10^{-3} and 5.5×10^{-3} nM^{-1} min^{-1}, respectively. The observation that 3 and 4 are potent inhibitors of AdoHcy hydrolase supports the hypothesis that they function as key intermediates in the mechanism by which the (Z)- and (E)-4',5'-didehydro-5'-deoxy-5'-fluoroadenosines 1 and 2 inactivate this enzyme.

Introduction

The cellular enzyme S-adenosyl-L-homocysteine (AdoHcy) hydrolase (EC 3.3.1.1) has emerged as an attractive target for the design of antiviral agents.[1,2] This enzyme catalyzes the reversible hydrolysis of AdoHcy to adenosine (Ado) and homocysteine (Hcy). Inhibition of AdoHcy hydrolase results in cellular accumulation of AdoHcy, which is a product inhibitor of S-adenosylmethionine (AdoMet)-dependent methyltransferases. Enzymes sensitive to elevated cellular levels of AdoHcy include viral mRNA methyltransferases which are essential for viral replication.[3-5] For example, Ransohoff et al.[5] have shown that undermethylation of the viral mRNA-capped structure induced by the inhibition of AdoHcy hydrolase can be correlated with the inhibition of influenza viral replication. In addition, Cools and De Clercq[6] have shown that a close correlation exists between the antiviral potency of Ado analogs and their inhibitory effects on AdoHcy hydrolase. Furthermore, Hasobe et al.[7] and Cools and De Clercq[8] have shown that a close correlation exists between the antiviral potency of the nucleosides and their ability to elevate the cellular level of AdoHcy.

Various adenosine analogs and adenine carbocyclic nucleosides have been shown to be potent inhibitors of AdoHcy hydrolase.[1-4] Many of these compounds are type I mechanism-based inhibitors of AdoHcy hydrolase,[2] which inactivate the enzyme by reducing the enzyme-bound NAD^+ to NADH. In the process of inactivation, the inhibitor is oxidized stoichiometrically to the corresponding 3'-keto nucleoside.[2] Recently, McCarthy et al.[9] and Mehdi et al.[10] reported the synthesis and potent AdoHcy hydrolase inhibitory effects of the 4',5'-didehydro-5'-deoxy-5'-fluoro analogs 1 and 2 of Ado (Figure 1). The vinyl fluorides 1 and 2 are particularly interesting because they were reported to inactivate the enzyme by reducing the enzyme-bound NAD^+ to NADH and quantitatively releasing fluoride ion. These results suggest that the vinyl fluorides might be the first examples of the so-called type II mechanism-based inhibitors of AdoHcy hydrolase that function by generating a reactive intermediate (e.g., 3'-keto derivative 7, Scheme I) at the enzyme active site, which then could react with a protein nucleophile (pathway b', Scheme I) to form a covalent adduct (e.g., 8, Scheme I). However, evidence against this type II mechanism includes the observation that [^{3}H]-1 did not become covalently bound to AdoHcy hydrolase upon enzyme inactivation.[10,11] Therefore, it was suggested[2,10] that the 3'-keto derivative 7 generated at the active site of AdoHcy hydrolase by oxidation of vinyl fluoride 1 might react with enzyme-sequestered water, releasing fluoride ion and generating the 3'-keto 5'-carboxaldehydes 5 and 6 (pathway b, Scheme I). In an effort to clarify this mechanism, we report here the syntheses of the 5'-carboxaldehydes 3 and 4 (Figure 1) and their inhibitory effects on AdoHcy hydrolase.



1 R_1 = F, R_2 = H, (Z) 3 R_1 = CHO, R_2 = H
2 R_1 = H, R_2 = F, (E) 4 R_1 = H, R_2 = CHO

Figure 1. Structures of inhibitors of AdoHcy hydrolase.

Results and Discussion

Chemistry. The synthesis of adenosine-5'-carboxaldehyde by irradiation of Ado with near-UV light in the presence of $FeCl_3$[12] and by Moffatt's oxidation of 2',3'-O-anisylideneadenosine and then deprotection with 80% acetic acid[13] has been reported. However, possibly because of its instability, purification and full characterization of this aldehyde were not reported. In this paper, we report two pathways for the syntheses of aldehydes 3 and 4 (Scheme II). One pathway employed N^6-benzoyl-5'-deoxy-2',3'-O-isopropylidene-5',5'-(N,N'-diphenylethylenediamino)adenosine (9) which was prepared by published procedures.[14] Compound 9 was deprotected with NH_4OH(aq) in DMF to yield 5'-deoxy-2',3'-O-isopropylidene-5',5'-(N,N'-diphenylethylenediamino)adenosine (10) in 90% yield. Hydrolysis of 10 in 90% trifluoroacetic acid solution at 0 °C gave a white solid which was a mixture of the 5'-carboxaldehydes 3 and 4. HPLC analysis of this mixture showed that the 5'-carboxaldehydes 3 and 4 existed in a ratio of ca. 5:1 (Figure 2, panel A). Using HPLC, the individual isomers 3 and 4 were separated and obtained in pure form (Figure 2, panel B). Characterization of 3

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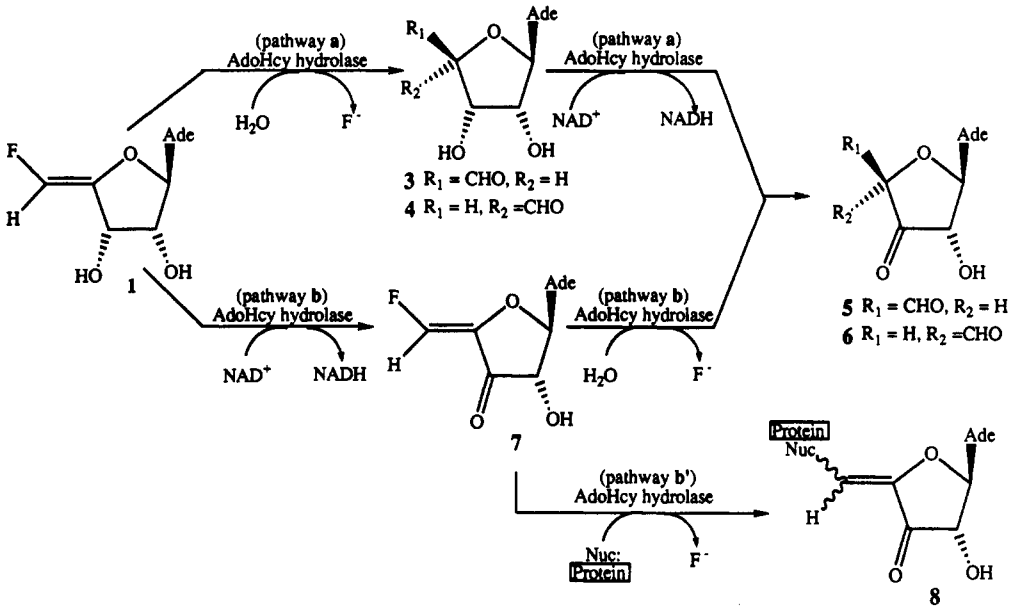
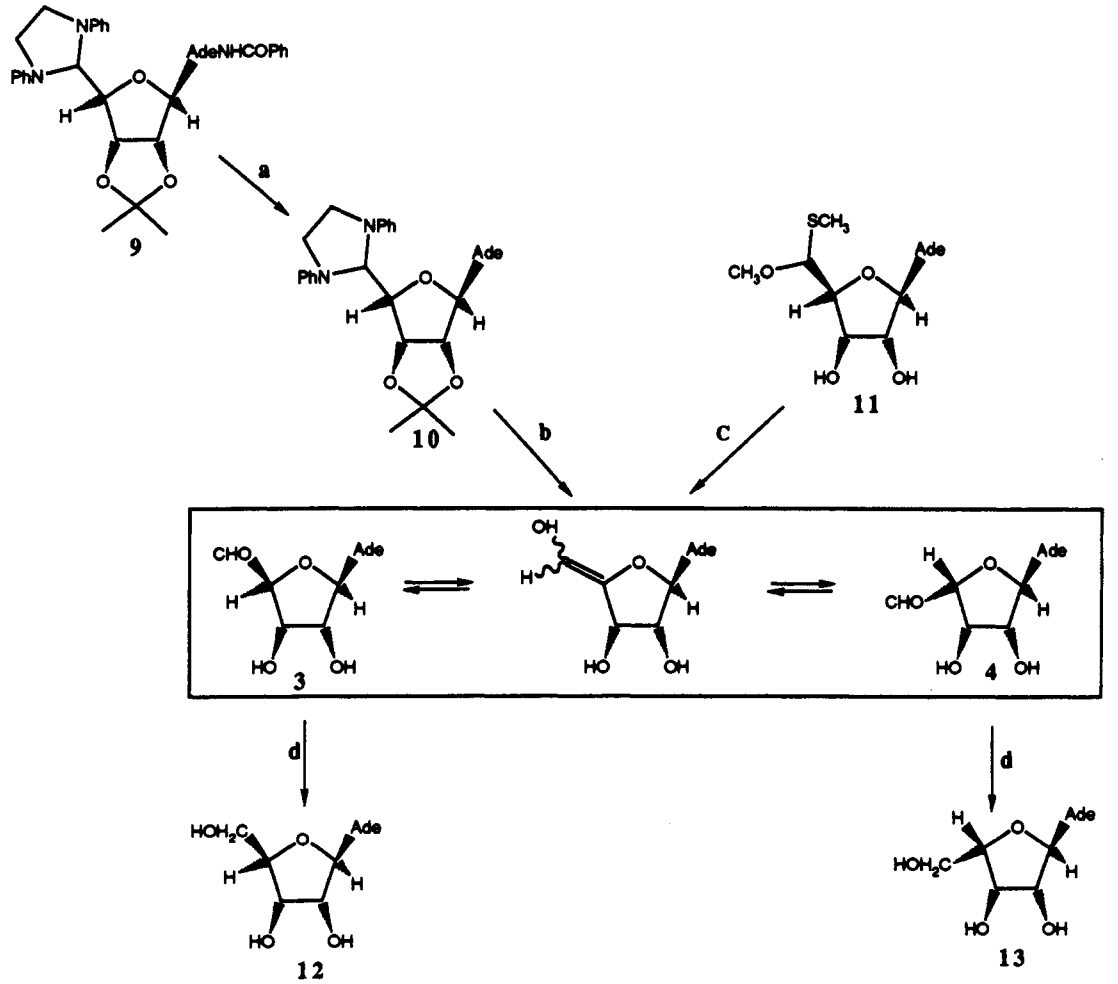
Scheme I. Possible Mechanisms by Which Vinyl Fluoride 1 Inactivates AdoHcy Hydrolase



Scheme II. Synthetic Pathways to Ado 5′-Carboxaldehydes 3 and 4[a]



[a] (a) 9 N NH_4OH(aq):DMF (5:2), 14 h, rt; (b) $CF_3COOH:H_2O$ (9:1), 0 °C, 20 min; (c) $CF_3COOH:H_2O$ (3:7), 5 °C, 14 h; (d) $NaBH_4$, H_2O, rt, 24 h.

and 4 was accomplished by ^1H and ^{13}C NMR spectrometry, mass spectrometry, and chemical transformation. ^1H NMR spectra of 3 and 4 showed that they existed mainly in the hydrate form in aqueous solution. As shown in Figure 2 (panel C), $NaBH_4$ reduction of 3 (retention time = 11.9 min) and 4 (retention time = 8.8 min) afforded products which coeluted on HPLC with adenosine (12, retention time = 30.7 min) and 9-(α-D-lyxofuranosyl)-adenine, an enantiomer of 13 (retention time = 16.1 min).

As an independent method of synthesis, the 5′-carboxaldehydes 3 and 4 were prepared by hydrolysis of 5′-*O*-methyl-5′-(methylthio)adenosine (11)[15] in 30% CF_3COOH.

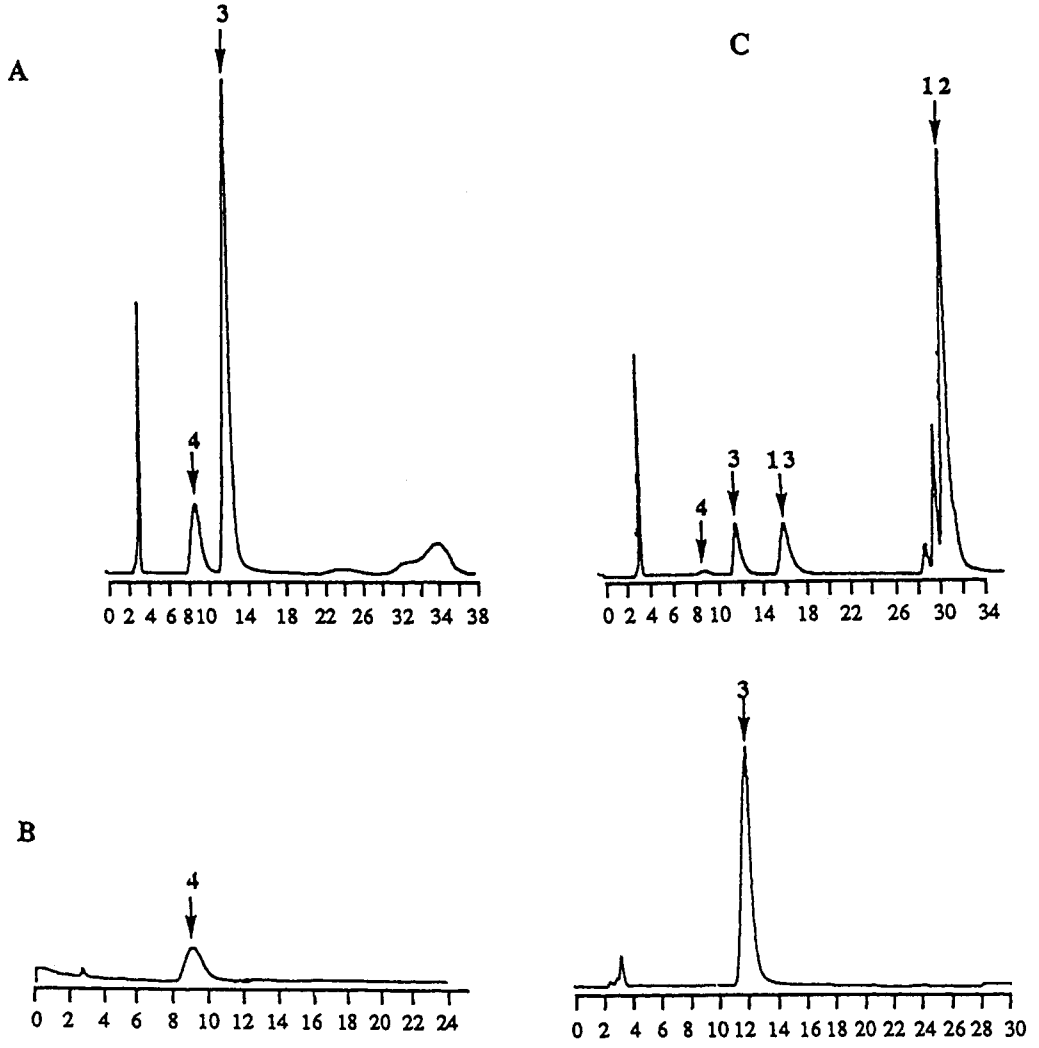


Figure 2. HPLC chromatograms of adenosine-5'-carboxaldehydes 3 and 4 and their $NaBH_4$ reduction products. HPLC was performed on a Shimadzu SLC-6A chromatography system using an Econosil C-18 reversed-phase column and eluting with CH_3CN (A) in H_2O (B) (program, 2% A in B from 0 to 15 min, a gradient of 2–6% A in B from 15 to 20 min, 6% A in B from 20 to 30 min, and then 6–100% A in B from 30 to 35 min; flow rate, 1.5 mL/min). Absorbance was monitored at 258 nm. Panel A, chromatograms of a mixture of 5'-carboxaldehydes 3 and 4 generated from hydrolysis of 10. Panel B, chromatograms of purified 5'-carboxaldehydes 3 and 4. Panel C, products derived from $NaBH_4$ reduction of 3 and 4. The peaks labeled 12 and 13 were identified by co-injection with authentic samples.

Purification by HPLC on a Dynamex C-18 reversed-phase preparative column afforded 3 in 58% yield. The products obtained by hydrolysis of 10 and 11 were shown by HPLC and ^1H and ^{13}C NMR spectrometry to be identical.

Biological. The enzyme inhibitory activities of the vinyl fluorides 1 and 2 and the 5'-carboxaldehydes 3 and 4 were determined using recombinant rat liver AdoHcy hydrolase.[16] As previously reported,[9,10] the vinyl fluorides 1 and 2 are time-dependent inactivators of AdoHcy hydrolase having similar affinities (K_I values) for the enzyme but slightly different rate constants for enzyme inactivation (k_2). Interestingly, the 5'-carboxaldehydes 3 and 4 are very potent inhibitors of AdoHcy hydrolase, having nearly identical K_I values but slightly different k_2 values (Table I). The 5'-carboxaldehyde 3 with the D-configuration (4'*S*) has a rate constant for enzyme inactivation approximately 3-fold greater than that for isomer 4 with the L-configuration (4'*R*). This observation is consistent with the fact that Ado, the natural substrate for this enzyme, has a D-configuration at the 4'-position.

Recently, our laboratory[11] showed that the vinyl fluorides 1 and 2 rapidly react with the NAD^+ form, the NADH form, or the apo (NAD^+ depleted) form of AdoHcy hydrolase to release fluoride ion. With the NADH and the apo forms of the enzyme, the vinyl fluorides 1 and 2 were shown to be transformed to a mixture of 3 and 4.[11] With the NAD^+ form of AdoHcy hydrolase, the vinyl fluorides 1 and 2 were shown to be transformed into a mixture of the 3'-keto 5'-carboxaldehydes 5 and 6. The 3'-keto 5'-carboxaldehydes 5 and 6 were also generated when the NAD^+ form of AdoHcy hydrolase was inactivated by 5'-carboxaldehydes 3 and 4. These results suggest that, in addition to pathway b (Scheme I) involving initial oxidation of the vinyl fluorides (e.g., 1) to the 3'-keto derivatives (e.g., 7) followed by reaction with water to yield

Table I. Inhibitory Activity of Vinyl Fluorides 1 and 2 and 5'-Carboxaldehydes 3 and 4 on Recombinant Rat Liver AdoHcy Hydrolase[a]

compd	k_2 (min^{-1})	K_I (nM)	k_2/K_I (nM^{-1} min^{-1})
1	0.082	39	2.1×10^{-3}
2	0.050	40	1.2×10^{-3}
3	0.65	39	16.7×10^{-3}
4	0.22	43	5.1×10^{-3}

[a] See Experimental Section for details concerning the procedures for conducting enzyme inactivation studies and data analysis for determination of K_I and k_2 values.

the 3′-keto 5′-carboxaldehydes 5 and 6, these vinyl fluorides upon binding to the NAD^+ form of the enzyme active site could be initially hydrolyzed to yield the 5′-carboxaldehydes 3 and 4 (pathway a, Scheme I). The 5′-carboxaldehydes 3 and 4, while bound to the enzyme active site, could then be oxidized to the 3′-keto 5′-carboxaldehydes 5 and 6, resulting in enzyme inactivation by a type I mechanism.[2]

Definitive evidence to support the mechanism depicted in pathway a (Scheme I) was obtained when the rate of inactivation of the NAD^+ form of AdoHcy hydrolase by the vinyl fluoride 1 was compared to the rate of NAD^+ to NADH conversion and the rate of fluoride ion release.[11] The results of these experiments revealed that the pseudo-first-order rate constants for loss of enzyme activity (k_{obs} = 0.06 min^{-1}) and NAD^+ depletion (k_{obs} = 0.057 min^{-1}) were almost identical. In contrast, the rate of fluoride ion release (K_{obs} = 1.17 min^{-1}) was approximately 20 times faster than enzyme inactivation or NAD^+ depletion.

It is interesting to note that the k_2 values for the 5′-carboxaldehydes are approximately 3–10-fold greater than the rate constants for inactivation of the enzyme by the vinyl fluorides (Table I). These differences may arise because in the case of 5′-carboxaldehydes 3 and 4, the species which bind and are being oxidized are probably the hydrated forms of the aldehydes. In contrast, the vinyl fluorides 1 and 2 react rapidly with sequestered water at the active site of AdoHcy hydrolase, probably generating 5′-carboxaldehydes 3 and 4, not the hydrated species. Therefore the differences in the k_2 values observed between the 5′-carboxaldehydes 3 and 4 and the vinyl fluorides 1 and 2 (Table I) may reflect the differences in the rates of oxidation of the hydrated forms of the carboxaldehydes versus the carboxaldehyde themselves. Different rates of oxidation of the aldehydes versus the hydrate forms of the aldehydes would not be unexpected.

Conclusion

In conclusion, the data presented in this paper clearly show that the 5′-carboxaldehydes 3 and 4 are potent type I mechanism-based inhibitors of AdoHcy hydrolase. When this is considered in light of the observation that the NADH and the apo forms of the enzyme can rapidly hydrolyze the vinyl fluorides 1 and 2 to the 5′-carboxaldehydes 3 and 4, it would appear that the vinyl fluorides 1 and 2 are simply "prodrugs" for the 5′-carboxaldehydes 3 and 4. The unique aspect of this mechanism is that conversion of the prodrug (vinyl fluorides 1 and 2) to the drug (5′-carboxaldehydes 3 and 4) actually occurs at the enzyme active site (pathway a, Scheme I). Alternatively, this mechanism could be viewed as a variation of a k_{cat} mechanism which does not involve formation of a covalent adduct but, rather, depletion of a cofactor (NAD^+ to NADH, type I mechanism-based inactivation[2]).

Experimental Section

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. NMR spectra were obtained on either a Varian XL-300 or Bruker AM-500 spectrophotometer. All ^1H chemical shifts are reported in ppm relative to the internal standard tetramethylsilane (TMS, δ 0.00) or H_2O in D_2O (δ 4.70). ^{13}C chemical shifts are reported in ppm relative to $CDCl_3$ (center of triplet, δ 77.0). Mass spectra were recorded on a Ribermag R10-10 quadrupole spectrometer. Elemental analyses were conducted at The University of Kansas. The purity of compounds 3 and 4 was established by HPLC using an Econosil C-18 reversed-phase column and elution with 2% A in B from 0 to 15 min, a gradient of 2–6% A in B from 15 to 20 min, 6% A in B from 20 to 30 min, and then 6–100% A in B from 30 to 35 min at a flow rate of 1.5 mL/min, where A is acetonitrile and B is water. Detection was at 258 nm. Column chromatography was accomplished with 70–230-mesh silica gel (Aldrich Chemical Co.).

5′-Deoxy-2′,3′-*O*-isopropylidene-5′,5′-(*N*,*N*′-diphenylethylenediamino)adenosine (10). N^6-Benzoyl-5′-deoxy-2′,3′-*O*-isopropylidene-5′,5′-(*N*,*N*′-diphenylethylenediamino)adenosine (9)[14] (2.16 g, 3.58 mmol) was suspended in a solution of 9 N NH_4OH(aq) (50 mL) and DMF (20 mL) and then stirred at room temperature for 14 h. After evaporation of the solvents, the residue was purified by column chromatography (3% EtOH in $CHCl_3$), and 1.60 g (90% yield) of 10 was obtained: mp 255–258 °C dec; ^1H NMR (300 MHz, $CDCl_3$) δ 8.31 (1 H, s), 7.60 (1 H, s), 7.20 (4 H, m), 6.80 (4 H, m), 6.65 (2 H, d, J = 8.6 Hz), 6.1 (1 H, d, J = 2.1 Hz), 5.77 (1 H, d, J = 3.2 Hz), 5.72 (2 H, s), 5.15 (2 H, m), 4.59 (1 H, m), 3.60 (4 H, m), 1.50 (3 H, s), 1.32 (3 H, s); ^{13}C NMR (300 Hz, $CDCl_3$) δ 155.5, 153.2, 149.5, 146.7, 146.5, 136.6, 129.3, 129.2, 119.7, 118.3, 118.2, 114.8, 113.7, 113.5, 88.6, 87.1, 83.9, 80.3, 73.6, 47.7, 46.7, 27.3, 25.6; MS (CI) *m/e* 500 (MH^+), 365, 239, 225, 213, 136. Anal. ($C_{27}H_{29}N_7O_3$) C, H, N.

Adenosine-5′-carboxaldehydes (3 and 4). Procedure A. 5′-Deoxy-2′,3′-*O*-isopropylidene-5′,5′-(*N*,*N*′-diphenylethylenediamino)adenosine (10) (1.0 g, 2 mmol) was mixed with a solution of CF_3CO_2H and H_2O (9:1, 1 mL) and stirred at 0 °C for 20 min until the solution became clear. Then saturated Na_2CO_3 solution was added to bring the pH of the solution of 10. The solution was washed with $CHCl_3$ (0.5 mL × 3). The aqueous layer as well as the precipitate formed was cooled to 0 °C. After it was filtered, the solid was washed with cold H_2O and then dried. A mixture of 3 and 4 (107 mg, 20% yield) was obtained. Separation of 3 and 4 was accomplished by HPLC using an Econosil C-18 reversed-phase column and eluting with 2% A in B for 15 min. Compound 3 eluted at 11.97 min and compound 4 eluted at 9.1 min: ^1H NMR (3, D_2O) δ 8.28 (1 H, s), 8.13 (1 H, s), 6.04 (1 H, dd, J_1 = 6.4 Hz, J_2 = 1.8 Hz), 5.26 (1 H, d, J = 3.8 Hz), 4.70–4.22 (3 H, m); ^{13}C NMR (D_2O) δ 158.34, 155.21, 151.14, 143.64, 121.85, 91.89, 90.98, 90.4, 76.14, 73.19; MS (FAB^+) *m/e* 266 (MH^+), 237, 221, 136; ^1H NMR (4, D_2O) δ 8.26 (1 H, s), 8.15 (1 H, s), 6.0 (1 H, d, J_1 = 7.7 Hz), 5.14 (1 H, m), 4.97 (1 H, m), 4.42 (1 H, t), 4.35 (1 H, m).

Procedure B. A solution of 5′-*O*-methyl-5′-(methylthio)-adenosine (11, 82 mg, 0.25 mmol)[15] in H_2O (7 mL) was cooled to ~5 °C (ice bath), and trifluoroacetic acid (3 mL) was added slowly. The mixture was stirred at ~5 °C for 14 h and flash evaporated in vacuo, and the residue was coevaporated twice with EtOH. The amorphous product consisting of a mixture of 3 and 4 was dissolved (16% A in B, 1.5 mL), filtered, and subjected to HPLC on a Dynamex C-18 reversed-phase preparative column (program: 16% A in B for 50 min followed by a gradient of 16–25% A in B for 50 min at a flow rate of 2.3 mL/min). Evaporation of appropriate fractions and drying in vacuo at ambient temperature gave the 5′-carboxaldehyde 3 (41 mg, 58%).

Reduction of 3 and 4. To an aqueous solution of 3 and 4 (4.6 mM, 1 mL) was added an aqueous solution of $NaBH_4$ (4.7 mM, 1 mL), which was then stirred at room temperature for 24 h. The solution was analyzed using HPLC on an Econosil C-18 reversed-phase column and eluted with 2% A in B from 0 to 15 min, a gradient of 2–6% A in B from 15 to 20 min, 6% A in B from 20 to 30 min, and then 6–100% A in B from 30 to 35 min at a flow rate of 1.5 mL/min. Two new peaks which appeared (retention time, 16.1 and 30.7 min) were characterized by co-injection with standard solutions of adenosine (12) and 9-(α-D-lyxofuranosyl)-adenine (enantiomer of 13). The peak with a retention time of 30.7 min coeluted with adenosine (12), and the peak with a retention time of 16.1 min coeluted with 9-(α-D-lyxofuranosyl)-adenine.

Determination of AdoHcy Hydrolase Inhibition Constants. Enzyme inhibition studies were conducted using a purified recombinant rat liver AdoHcy hydrolase.[16] Enzyme activity was determined in the synthetic direction using Ado (0.2 mM) and Hcy (5 mM) in 50 mM phosphate buffer (pH 7.2) containing 1 mM EDTA at 37 °C and incubating for 5 min. The reaction product AdoHcy was assayed[17] by HPLC after the reaction was stopped by addition of $HClO_4$. An aliquot (100 μL)

of the reaction mixture was analyzed by HPLC (Econosphere Alltech 25 cm × 4.6 mm C-18 reversed-phase column) at a flow rate of 1 mL/min. The elution gradient consisted of two sequential linear gradients: 6–15% A in C over 0–9 min and 15–50% A in C over 9–15 min, where mobile phase A was acetonitrile and C was 50 mM sodium phosphate buffer (pH 3.2) containing 10 mM heptanesulfonic acid. The peak area of AdoHcy was monitored by a UV detector at 258 nm to quantitate the AdoHcy. For the determination of inhibition constants, AdoHcy hydrolase was preincubated with various concentrations of inhibitors for varying amounts of time, and the remaining enzyme activity was measured. Experiments were run in duplicate. The pseudo-first-order rate constants (k_{obs}) were determined from plots of ln (% activity remaining) versus preincubation time. K_I and k_2 values were obtained from a plot of $1/k_{obs}$ versus 1/[inhibitor] using eq 1.

$$1/k_{obs} = (K_I/k_2)(1/[I]) + 1/k_2 \quad (1)$$

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Supplementary Material Available: Kitz and Wilson plots of compounds 3 and 4 (2 pages). Ordering information is given on any current masthead page.

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