Doubly Homologated Dihalovinyl and Acetylene Analogues of Adenosine: Synthesis, Interaction with *S*-Adenosyl-L-homocysteine Hydrolase, and Antiviral and Cytostatic Effects

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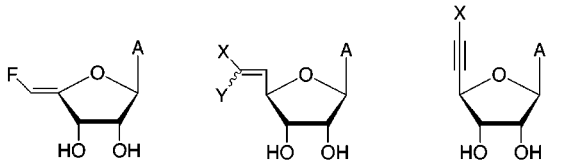
Treatment of the 6-aldehyde derived by Moffatt oxidation of 3-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-*ribo*-hexofuranose (**2c**) with the dibromo- or bromofluoromethylene Wittig reagents generated in situ with tetrabromomethane or tribromofluoromethane, triphenylphosphine, and zinc gave the dihalomethyleneheptofuranose analogues **3b** and **3d**, respectively. Acetolysis, coupling with adenine, and deprotection gave 9-(7,7-dibromo-5,6,7-trideoxy-β-D-*ribo*-hept-6-enofuranosyl)adenine (**5a**) or its bromofluoro analogue **5b**. Treatment of **5a** with excess butyllithium provided the acetylenic derivative 9-(5,6,7-trideoxy-β-D-*ribo*-hept-6-ynofuranosyl)-adenine (**6**). The doubly homologated vinyl halides **5a** and **5b** and acetylenic **6** adenine nucleosides were designed as putative substrates of the "hydrolytic activity" of *S*-adenosyl-L-homocysteine (AdoHcy) hydrolase. Incubation of AdoHcy hydrolase with **5a**, **5b,** and **6** resulted in time- and concentration-dependent inactivation of the enzyme (K_i: 8.5 ± 0.5, 17 ± 2, and 8.6 ± 0.5 μM, respectively), as well as partial reduction of enzyme-bound NAD^+ to E-NADH. However, no products of the "hydrolytic activity" were observed indicating these compounds are type I mechanism-based inhibitors. The compounds displayed minimal antiviral and cytostatic activity, except for **6,** against vaccinia virus and vesicular stomatitis virus (IC_{50}: 15 and 7 μM, respectively). These viruses typically fall within the activity spectrum of AdoHcy hydrolase inhibitors.

Introduction

The enzyme *S*-adenosyl-L-homocysteine (AdoHcy) hydrolase (EC 3.3.1.1) effects hydrolytic cleavage of AdoHcy to adenosine (Ado) and L-homocysteine (Hcy).[1] The cellular levels of AdoHcy and Hcy are critical because AdoHcy is a potent feedback inhibitor of crucial transmethylation enzymes,[1,2] and elevated plasma levels of Hcy in humans have been shown to be a risk factor in coronary artery disease.[3] A number of inhibitors which function as substrates for the "3′-oxidative activity" of AdoHcy hydrolase and convert the enzyme from its active form (NAD^+) to its inactive form (NADH, type I inhibition) have been prepared.[1] Inhibitors which function as substrates for the "5′/6′-hydrolytic activity" were also synthesized, which included vinyl fluoride[4] **A**, homovinyl halides[5] **B**, and oxime derivatives of adenosine 5′-aldehyde,[6] but not their 3′-deoxy analogues.[6a,7] Moreover, the propensity of homoadenosine 6′-aldehyde (formed during processing of **B** by the enzyme) to depurinate decreases the efficiency of enzyme inactivation by **B**.[5b]



A **B** X = I, Br, Cl, F; Y = H **C** X = Br, Y = Br or F **D** X = H **E** X = I, Br, Cl

Recently, geminal (dihalohomovinyl)adenosines **C** were designed as putative new substrates for the hydrolytic activity of AdoHcy hydrolase.[8] Addition of an enzyme-sequestered water molecule across the 5′,6′-double bond of bromo(fluoro)homovinyl analogue **C** (Y = F), followed by loss of bromide, was proposed to generate the homoAdo 6′-carboxyl fluoride at the active site of AdoHcy hydrolase.[8b] Nucleophilic attack by proximal amino acid functionalities caused type II (covalent binding) inhibition. The acetylenic analogue **D** derived from adenosine is a potent inhibitor of AdoHcy hydrolase[5a,9a] with antiviral[5a,9b] and cytostatic activity.[5a] Addition of water across the 5′,6′-triple bond of haloacetylenes[10] **E** followed by tautomerization of the hydroxyvinyl intermediates was postulated to generate similar acyl halides (C6′ hydroxyl attack) and/or α-halomethyl ketones (C5′ hydroxyl attack) at the enzyme active site. The X-ray structure determination of human AdoHcy hydrolase revealed an unusual dual role for a catalytic water molecule at the active site.[11]

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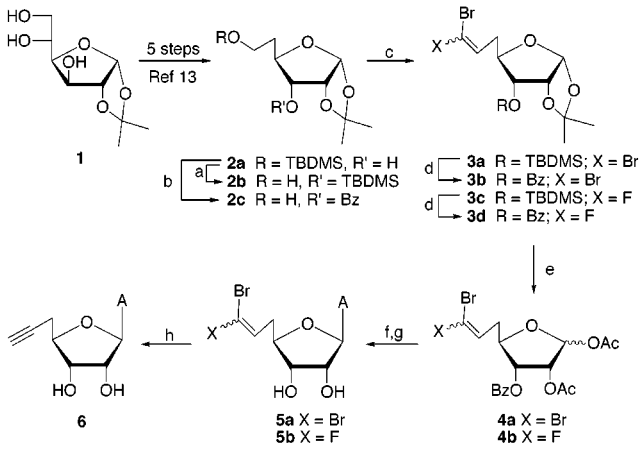
Scheme 1[a]



[a] (a) (i) TBDMSCl/imidazole/DMF, (ii) HCl/MeOH/H_2O; (b) (i) BzCl/pyridine, (ii) HCl/MeOH/H_2O; (c) (i) DCC/DMSO/Cl_2CHCO_2H, (ii) CBr_4 (or CBr_3F)/PPh_3/Zn/CH_2Cl_2; (d) (i) TBAF/THF, (ii) BzCl/pyridine; (e) (i) TFA/H_2O, (ii) Ac_2O/pyridine/DMAP; (f) adenine/$SnCl_4$/CH_3CN; (g) NH_3/MeOH; (h) BuLi/THF/−78 °C.

We now describe syntheses of the first "doubly homologated" vinyl halides **5** and acetylenic **6** adenine nucleosides, their interaction with AdoHcy hydrolase, and their antiviral activities. These derivatives (sp^3 hybridized C5′) should have greater conformational flexibility at C5′ relative to analogues that have vinylogous (sp^2; **B** or **C**) or acetylenic (sp; **D** or **E**) functions directly attached to the ribose ring. They also provide probes for evaluation of tolerated distances in the enzyme between the binding site for O3′ (presumably involved with oxidation at C3′) and the protein residues which are responsible for the "hydrolytic activity" (already shown to function at C5′and C6′).

Chemistry

Synthesis of the "doubly homologated" 6′,7′-unsaturated analogues **5** began with *ribo*-hexofuranose sugar precursors of type **2**, because homologation of adenosine to homoadenosine required multistep procedures which gave low overall yields.[12] Moreover, homoAdo 6′-aldehyde, which is the obvious intermediate for the synthesis of 7′-halovinyl nucleosides **5** via the Wittig approach, is known to be unstable.[5b] Commercial 1,2-*O*-isopropylidene-α-D-glucose (**1**) was converted to 6-*O*-TBDMS-1,2-*O*-isopropylidene-α-D-*ribo*-hexofuranose (**2a**) in five steps (∼55%).[13] Key steps involved regioselective oxidation [$(Bu_3Sn)_2O/Br_2$][14] of **1** to the 5-ketone, deoxygenation via its tosylhydrazone, and inversion of configuration at C3 via oxidation and reduction. Silylation (O3) of **2a** gave the 3,6-di-*O*-TBDMS derivative, and regioselective removal of the primary TBDMS group with dilute HCl/MeOH afforded the *ribo*-hexofuranose **2b**.[13c] Benzoylation (O3) of **2a** and desilylation gave **2c** (81%; Scheme 1).

Moffatt oxidation[15] of **2b**[13c] and treatment of the crude 6-aldehyde with (dibromomethylene)triphenylphosphorane ($CBr_4/Ph_3P/Zn$)[16] gave dibromovinylheptofuranose **3a** (89%). Compound **3a** was converted to its 3-*O*-benzoyl derivative **3b**, because attempted acetolysis of **3a** failed to give the 1-*O*-acetyl anomers [due to instability of the TBDMS group (O3) under acidic conditions]. Wittig-type olefination of **2c** with the dibromomethylene reagent also gave **3b** (75%). The isopropylidene group was removed from **3b** (TFA/H_2O) and the product was acetylated to give the anomeric acetates **4a** (85%; α/β, ∼1:4), which were partially separated by silica gel column chromatography. The β-anomer was obtained after crystallization. Coupling ($SnCl_4/CH_3CN$[17]) of the anomeric mixture of **4a** and adenine followed by deacylation gave the crystalline dibromovinyl compound **5a** (49%). Treatment of **5a** with excess BuLi[16,18] effected dehydrobromination to give the acetylenic derivative **6** (65%).

Analogously, oxidation of **2b**[13c] and treatment of the crude 6-aldehyde with the bromofluoromethylene Wittig reagent gave the bromofluorovinyl diastereomers **3c** [48%; *E*/*Z*, ∼45:55; $^3J_{F-H6'} = 13.2$ Hz (*Z*) and $^3J_{F-H6'} = 30.9$ Hz (*E*)]. Desilylation of **3c** followed by benzoylation gave **3d**, which was also prepared directly from **2c** (41%; *E*/*Z*, ∼45:55). The unreacted 6-aldehydes derived from **2b** [^{1}H NMR δ 9.71 (t, *J* = 2.4 Hz, 1, H6); ^{13}C NMR δ 200.28 (C6)] and **2c** were recovered in moderate yield (20−35%) from column chromatography of the Wittig olefination products. In contrast with the instability of homoAdo 6′-aldehyde,[5b] these homoribose 6-aldehydes were quite stable. Treatment of **3d** with TFA/H_2O and acetylation gave **4b** (80%; α/β, ∼1:4; *E*/*Z*, ∼40:60) which was coupled with adenine. Deacylation of this mixture and RP-HPLC purification gave 9-(7-bromo-5,6,7-trideoxy-7-fluoro-β-D-*ribo*-hept-6-enofuranosyl)adenine **(5b)** (31%; *E*/*Z*, ∼40:60).

Table 1. Inhibition of AdoHcy Hydrolase with **5a**, **5b**, and **6**

	% enzyme activity remaining[a]		
concn (μM)	**5a**	**5b**	**6**
0.1	87.5 ± 4.5	90.6 ± 5.0	98.3 ± 5.0
1.0	48.5 ± 2.4	87.4 ± 4.5	73.4 ± 3.8
10	17.1 ± 1.0	37.4 ± 2.0	38.9 ± 2.2
50	5.0 ± 0.5	9.7 ± 1.0	14.3 ± 1.1
100	4.0 ± 0.5	5.8 ± 1.0	8.0 ± 1.0

[a] AdoHcy hydrolase (42 nM) was incubated with **5a**, **5b**, and **6** in buffer A at 37 °C for 30 min, and the remaining activity was assayed as described in the Experimental Section. Data are the average of duplicate determinations.

Results and Discussion

Inactivation of Ado-Hcy Hydrolase. Recombinant human placental AdoHcy hydrolase was inactivated upon incubation with **5a**, **5b**, or **6** in a concentration-dependent (Table 1) and time-dependent (Figure 1) manner. However, these compounds did not produce complete inactivation of AdoHcy hydrolase even upon prolonged incubation. In the presence of 100 μM AdoHcy, AdoHcy hydrolase could be protected from inactivation by **5a**, **5b,** and **6** (data not shown). The K_i values of compounds **5a**, **5b,** and **6** were determined to be 8.5 ± 0.5, 17 ± 2, and 8.6 ± 0.5 μM, respectively.

Compounds **5a**, **5b**, and **6** were designed as putative substrates of the "hydrolytic activity" of AdoHcy hydrolase. Conceptually, addition of the enzyme's sequestered water[11] at the 6′- and/or 7′-positions of **5a** or **5b** could generate β,β-dihalo alcohols (halohydrins) and/or acyl halide products, respectively; and **6** would give a 6′-ketone and/or 7′-aldehyde. Based on our previous studies,[4b,5,8] most of the possible products should be released into solution. Some of them might produce covalent modification of the enzyme, but some should be detected by HPLC. However, incubation of AdoHcy

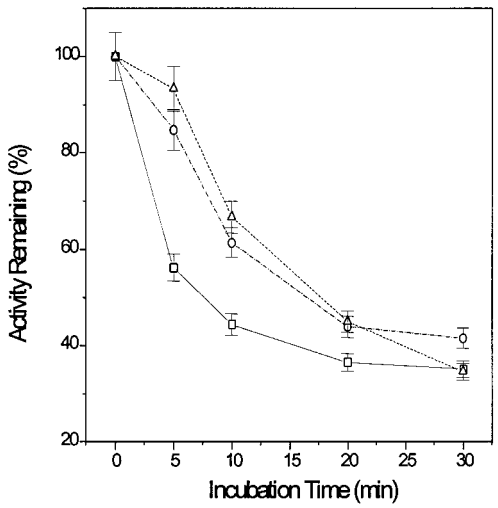


Figure 1. Time-dependent inactivation of AdoHcy hydrolase by **5a** (□), **5b** (△), and **6** (○). AdoHcy hydrolase (42 nM) was incubated with 10 μM **5a**, **5b**, and **6** in buffer A for various times. At the indicated times, remaining enzyme activity was determined in the synthetic direction as described in the Experimental Section. Data are the average of duplicate determinations.

Table 2. Effects of **5a**, **5b**, and **6** on NADH Content of AdoHcy Hydrolase[a]

	compd		
	5a	**5b**	**6**
NADH content (%)	30.2 ± 1.5	49.8 ± 2.5	38.1 ± 2.0

[a] AdoHcy hydrolase (21 μM, reconstituted NAD^+ form) was incubated with 100 μM **5a**, **5b**, and **6** in buffer A at 37 °C for 2 h, and the NAD^+/NADH content was assayed as described in the Experimental Section. Data are the average of duplicate determinations. The NADH content of the control enzyme is <1%.

hydrolase with 200 μM of each inhibitor for prolonged periods of time failed to generate any detected new products. These results indicate that compounds **5a**, **5b**, and **6** are not substrates for the "hydrolytic activity" of the enzyme.

The effects of **5a**, **5b**, and **6** on the NADH content of the enzyme were also determined. The data shown in Table 2 indicate that all three compounds cause partial conversion of the enzyme from its E-NAD^+ to E-NADH form. These results reveal that inactivation of AdoHcy hydrolase by **5a**, **5b**, and **6** involves a type I mechanism (cofactor depletion).[1] However, on the basis of the data presented in this study, we cannot totally rule out the possible involvement of a type II mechanism (covalent modification)[1] where the products arising from the enzyme's "hydrolytic activity" are tightly bound to the enzyme and are not released into the solution.

Antiviral and Cytostatic Activities. Compounds **5a**, **5b**, and **6** were examined for their antiviral, cytotoxic, and cytostatic activity in a broad-spectrum of antiviral tests [HSV-1 (KOS), HSV-2 (G), vaccinia, VSV, and TK^-HSV-1 (B2006) in E_6SM cells; polio-1, VSV, and RSV in HeLa cells; Reo-1, parainfluenza-3, Sindbis, coxsackie B4, and Punta Toro in Vero cells; CMV (AD-169) and CMV (Davis) in HEL cells; VZV (YS), VZV (Oka), TK^-VZV (07/1), and TK^-VZV (YS/R) in HEL cells; and HIV-1 and HIV-2 in CEM cells]. They showed little or no antiviral activity, except for compound **6,** against vaccinia virus and vesicular stomatitis virus (IC_{50}: 15 and 7 μM, respectively). Activity against these two viruses in E_6SM cells is consistent with what has been found with other AdoHcy hydrolase inhibitors.[19] These compounds were also evaluated for their cytostatic activity against a number of tumor cell lines [murine leukemia cells (L1210), murine mammary carcinoma cells (FM3A), and human T-lymphocyte cells (Molt4/C8, CEM)]; no marked inhibitory effects on tumor cell proliferation were observed.

Summary and Conclusions

We have synthesized the doubly homologated dibromovinyl **5a**, bromofluorovinyl **5b**, and acetylenic **6** nucleoside analogues that produced time- and concentration-dependent inactivation of AdoHcy hydrolase. Partial reduction of E-NAD^+ to E-NADH was also observed, indicating that inactivation of the enzyme by these inhibitors involves a type I mechanism. However, the vinyl or acetylenic compounds apparently were not substrates for the enzyme's "hydrolytic activity". This lack of "hydrolytic substrate activity" is in sharp contrast to analogues having vinyl or acetylenic bonds in the C5′−C6′ position.[5,8,10] These results suggest that the enzyme's sequestered water[11] and/or "activating functions" are not properly positioned to add water across vinyl or acetylenic bonds in the C6′−C7′ position. No significant antiviral or cytotoxic activity was observed with compounds **5a**, **5b**, and **6**.

Experimental Section

Uncorrected melting points were determined with a capillary apparatus. UV spectra were measured with solutions in MeOH. ^{1}H (400 MHz), ^{13}C (100 MHz) and ^{19}F [376.5 MHz ($CFCl_3$)] NMR spectra were determined with solutions in $CDCl_3$ unless otherwise specified. Mass spectra (MS and HRMS) were obtained with electron impact (20 eV), chemical ionization (CI, isobutane), or fast atom bombardment (FAB, thioglycerol matrix) techniques. Merck kieselgel 60-F_{254} sheets were used for TLC and products were detected with 254 nm light or by development of color with $Ce(SO_4)_2/(NH_4)_6Mo_7O_{24}\cdot 4H_2O/H_2SO_4/H_2O$. Merck kieselgel 60 (230−400 mesh) was used for column chromatography. Preparative reversed-phase (RP)-HPLC was performed with a Supelcosil LC-18S column with a Perkin-Elmer LC 200 binary pump system (gradient solvent systems are noted) or analytical C-18 reversed-phase column (Vydac 218TP54, 5 μm, 250 × 4.6 mm; Hesperia, CA) for enzymatic assays. Elemental analyses were determined by Galbraith Laboratories, Knoxville, TN. Reagent grade chemicals were used, and solvents were dried by reflux over and distillation from CaH_2 (except THF/potassium) under argon. Sonication was performed with a Branson 5200 ultrasonic bath.

3-*O*-Benzoyl-5-deoxy-1,2-*O*-isopropylidene-*β*-D-*ribo*-hexofuranose (2c). Procedure A. BzCl (3.0 mL, 3.6 g, 25.8 mmol) was added to dried 6-*O*-(*tert*-butyldimethylsilyl)-5-deoxy-1,2-*O*-isopropylidene-*β*-D-*ribo*-hexofuranose[13] (**2a**; 4.5 g, 14.2 mmol) in dried pyridine (35 mL) at 0 °C, and the mixture was stirred for 4 h at ambient temperature under N_2. H_2O (3 mL) was added, volatiles were evaporated in vacuo, and the residue was partitioned (EtOAc//$NaHCO_3/H_2O$). The organic layer was washed (HCl/H_2O, $NaHCO_3/H_2O$, brine), dried (Na_2SO_4), and evaporated to give 3-*O*-benzoyl-6-*O*-(*tert*-butyldimethylsilyl)-5-deoxy-1,2-*O*-isopropylidene-*β*-D-*ribo*-hexofuranose (5.41 g, 91%): ^{1}H NMR δ 0.01 (s, 6, 2 × Me), 0.85 (s, 9, *t*-Bu), 1.30 & 1.50 (2 × s, 2 × 3, 2 × Me), 1.72−1.95 (m, 2, H5,5′), 3.68−3.80 (m, 2, H6,6′), 4.36 (td, J = 8.7, 3.8 Hz, 1, H4), 4.63 (dd, J = 9.2, 4.8 Hz, 1, H3), 4.87 (t, J = 4.4 Hz, 1, H2), 5.80 (d, J = 3.9 Hz, 1, H1), 7.36−8.03 (m, 5, Arom); ^{13}C NMR δ −5.15, 18.26,

25.89, 26.44, 26.70, 35.38, 59.38, 74.03, 76.61, 77.36, 104.18, 112.78, 128.41, 129.46, 129.88, 133.31, 165.92; HRMS (CI) *m/z* 423.2209 (12, MH^+ [$C_{22}H_{35}O_6Si$] = 423.2203).

HCl/H_2O (0.1 M, 17.2 mL) was added to this product (4.14 g, 9.8 mmol) in MeOH (85 mL) and stirring was continued at ambient temperature for 30 min. Saturated $NaHCO_3/H_2O$ (6 mL) was added, and the mixture was stirred for 15 min and was then concentrated. The residue was partitioned (EtOAc//$NaHCO_3/H_2O$), and the organic layer was washed (brine), dried (Na_2SO_4), and evaporated. Chromatography of the residue (20→35% EtOAc/hexanes) gave **2c** (2.68 g, 89%) as an oil: ^{1}H NMR δ 1.34 & 1.54 (2 × s, 2 × 3, 2 × Me), 1.75−2.05 (m, 2, H5,5′), 2.40 (br s, 1, OH), 3.82 (t, J = 5.8 Hz, 2, H6,6′), 4.42 (td, J = 8.8, 3.6 Hz, 1, H4), 4.72 (dd, J = 9.1, 4.8 Hz, 1, H3), 4.92 (t, J = 4.4 Hz, 1, H2), 5.88 (d, J = 3.9 Hz, 1, H1), 7.40−8.08 (m, 5, Arom); ^{13}C NMR δ 26.46, 26.57, 34.59, 59.80, 75.74, 76.46, 77.15, 104.22, 112.99, 128.47, 129.27, 129.87, 133.44, 165.92; HRMS (CI) *m/z* = 309.1341 (100, MH^+ [$C_{16}H_{21}O_6$] = 309.1338).

7,7-Dibromo-3-*O*-(*tert*-butyldimethylsilyl)-5,6,7-trideoxy-1,2-*O*-isopropylidene-α-D-*ribo*-hept-6-enofuranose (3a). Procedure B. A solution of **2b**[13c] (3.18 g, 10 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC; 6.11 g, 30 mmol) in dried Me_2SO (25 mL) was cooled (∼5 °C) under argon, Cl_2CHCO_2H (0.41 mL, 645 mg, 5 mmol) was added, and stirring was continued for 90 min at ambient temperature. The brown solution was injected (syringe) into a mixture containing (dibromomethylene)triphenylphosphorane [generated in situ by stirring CBr_4 (6.63 g, 20 mmol), Ph_3P (5.25 g, 20 mmol), and activated Zn (dust; 1.30 g, 20 mmol) in dried CH_2Cl_2 (100 mL) for 5 h at ambient temperature under Ar; sonication was applied intermittently for a total of 30 min], stirring was continued for 10 h, and oxalic acid dihydrate (2.52 g, 20 mmol) in MeOH (20 mL) was added. After 20 min, the reaction mixture was concentrated (to ∼1/3 volume), dicyclohexylurea was filtered and washed with cold MeOH, and the combined filtrates were evaporated (in vacuo). The residue was partitioned ($NaHCO_3/H_2O$//$CHCl_3$), and the organic layer was washed (2 × H_2O, brine), dried ($MgSO_4$), and volatiles were evaporated. Column chromatography of the residue (15% EtOAc/hexane) gave **3a** (4.22 g, 89%) as a syrup: ^{1}H NMR δ 0.02 (s, 6, 2 × Me), 0.80 (s, 9, *t*-Bu), 1.20 & 1.41 (2 × s, 2 × 3, 2 × Me), 2.19 (dt, J = 15.7, 7.0 Hz, 1, H5′), 2.36 (ddd, J = 15.7, 6.6, 4.6 Hz, 1, H5), 3.47 (dd, J = 8.8, 4.6 Hz, 1, H3), 3.82 (ddd, J = 8.7, 6.9, 4.6 Hz, 1, H4), 4.28 (t, J = 4.2 Hz, 1, H2), 5.59 (d, J = 3.8 Hz, 1, H1), 6.40 (t, J = 6.9 Hz, 1 H6); ^{13}C NMR δ −4.81, −4.53, 18.13, 25.72, 26.55, 35.28 (C5), 76.24 (C3), 77.25 (C4), 78.97 (C2), 90.38 (C7), 103.83 (C1), 112.52, 134.17 (C6); HRMS (FAB) *m/z* 496.9940 (58, MNa^+ [$C_{16}H_{28}{}^{81}Br_2O_4SiNa$] = 496.9981), 494.9986 (100, MNa^+ [$^{81/79}Br_2$] = 495.0001), 493.0021 (45, MNa^+ [$^{79}Br_2$] = 493.0021).

3-*O*-Benzoyl-7,7-dibromo-5,6,7-trideoxy-1,2-*O*-isopropylidene-α-D-*ribo*-hept-6-enofuranose (3b). Method A. Procedure C. TBAF/THF (1 M, 10 mL) was added to a stirred solution of **3a** (4.00 g, 8.5 mmol) in dried THF (30 mL) at ∼0 °C under N_2. After 2 h, volatiles were evaporated, and the residue was partitioned ($NaHCO_3/H_2O$//EtOAc). The organic layer was washed (brine), dried ($MgSO_4$), and evaporated. Column chromatography of the residue (30% EtOAc/hexane) gave 7,7-dibromo-5,6,7-trideoxy-1,2-*O*-isopropylidene-α-D-*ribo*-hept-6-enofuranose (2.55 g, 84%) as a syrup: ^{1}H NMR δ 1.25 & 1.46 (2 × s, 2 × 3, 2 × Me), 2.33 (dt, J = 15.6, 7.2 Hz, 1, H5′), 2.49 (ddd, J = 15.6, 6.7, 4.4 Hz, 1, H5), 2.65 (d, J = 10.5 Hz, OH3), 3.54−3.61 (m, 1, H3), 3.73 (ddd, J = 8.8, 7.1, 4.5 Hz, 1, H4), 4.50 (t, J = 4.5 Hz, 1, H2), 5.72 (d, J = 3.9 Hz, 1, H1), 6.48 (t, J = 7.0 Hz, 1 H6); ^{13}C NMR δ 26.77, 26.88, 35.46 (C5), 75.40 (C3), 78.25 (C4), 78.74 (C2), 91.26 (C7), 104.09 (C1), 112.94, 134.30 (C6); HRMS (CI) *m/z* 360.9291 (50, MH^+ [$C_{10}H_{15}{}^{81}Br_2O_4$] = 360.9298), 358.9307 (100, MH^+ [$^{81/79}Br_2$] = 358.9317), 356.9339 (51, MH^+ [$^{79}Br_2$] = 356.9337).

Treatment of this product (1.38 g, 3.85 mmol) with BzCl (0.90 mL, 1.08 g, 7.7 mmol) by procedure A [6 h, DMAP (10 mg) added] and column chromatography (10% EtOAc/hexane) gave **3b** (1.73 g, 97%). Crystallization (MeOH) afforded **3b** (1.4 g, 79%) as white crystals: mp 73−75 °C; ^{1}H NMR δ 1.20 & 1.45 (2 × s, 2 × 3, 2 × Me), 2.40 (dt, J = 15.3, 6.8 Hz, 1, H5′), 2.48 (ddd, J = 15.7, 7.0, 5.0 Hz, 1, H5), 4.25 (dt, J = 8.6, 6.0 Hz, 1, H4), 4.57 (dd, J = 9.2, 4.8 Hz, 1, H3), 4.87 (t, J = 4.4 Hz, 1, H2), 5.77 (d, J = 3.8 Hz, 1, H1), 6.46 (t, J = 7.1 Hz, 1 H6), 7.31−7.98 (m, 5, Arom); ^{13}C NMR δ 26.90, 26.94, 35.58 (C5), 75.58 (C4), 76.23 (C3), 77.64 (C2), 92.11 (C7), 104.54 (C1), 113.48, 128.89, 129.56, 130.30, 133.56 (C6), 133.88, 166.20; HRMS (CI) *m/z* 464.9565 (44, MH^+ [$C_{17}H_{19}{}^{81}Br_2O_5$] = 464.9559), 462.9599 (100, MH^+ [$^{81/79}Br_2$] = 462.9579), 460.9594 (51, MH^+ [$^{79}Br_2$] = 460.9599). Anal. [$C_{17}H_{18}Br_2O_5$ (462.14)] C, H.

Method B. Treatment of **2c** (1.75 g, 5.68 mmol) by procedure B gave **3b** (1.98 g, 75%).

7-Bromo-3-*O*-(*tert*-butyldimethylsilyl)-5,6,7-trideoxy-7-fluoro-1,2-*O*-isopropylidene-α-D-*ribo*-hept-6-enofuranose (3c). Treatment of **2b**[13c] (1.59 g, 5 mmol) by procedure B (CBr_3F was used instead of CBr_4; column chromatography, 10→20% EtOAc/hexane) gave **3c** (986 mg, 48%; *E*/*Z*, ∼45:55): ^{1}H NMR δ 0.01 (s, 6, 2 × Me), 0.80 (s, 9, *t*-Bu), 1.20 & 1.40 (2 × s, 2 × 3, 2 × Me), 2.08−2.40 (m, 2, H5,5′), 3.58−3.64 (m, 1, H3), 3.87−3.95 (m, 1, H4), 4.39−4.42 (m, 1, H2), 5.15 (dt, J = 31.1, 7.5 Hz, 0.45, H6), 5.62 (dt, J = 12.9, 7.3 Hz, 0.55, H6), 5.70−5.72 (m, 1, H1); ^{13}C NMR δ −4.53, −4.43, −4.12, −4.04, 18.52, 26.09, 26.11, 26.95, 28.04 (J = 1.4 Hz) & 30.37 (J = 3.7 Hz, C5), 76.25 & 76.39 (C3), 78.07 & 78.22 (J = 1.7 Hz, C4), 79.42 (C2), 104.17 & 104.21 (C1), 106.44 (J = 17.5 Hz, C6*Z*), 108.39 (J = 12.4 Hz, C6*E*), 112.87, 112.89, 132.95 (J = 320.2 Hz, C7*E*), 136.56 (J = 314.3 Hz, C7*Z*); ^{19}F NMR δ −74.46 (d, J_{F-6} = 30.9 Hz, 0.45, F7*E*), −69.93 (d, J_{F-6} = 13.2 Hz, 0.55, F7*Z*); HRMS (CI) *m/z* 413.0981 (96, MH^+ [$C_{16}H_{29}{}^{81}BrFO_4Si$] = 413.0983), 411.1009 (100, MH^+ [^{79}Br] = 411.1003).

Further elution of the column (20→30% EtOAc/hexane) gave 6-aldehyde-**2b** (490 mg, 31%; 3-*O*-*tert*-butyldimethylsilyl-5-deoxy-1,2-*O*-isopropylidene-β-D-*ribo*-hexodialdofuranose): ^{1}H NMR δ 9.71 (t, J = 2.4 Hz, 1, H6); ^{13}C NMR δ 200.28 (C6).

3-*O*-Benzoyl-7-bromo-5,6,7-trideoxy-7-fluoro-1,2-*O*-isopropylidene-α-D-*ribo*-hept-6-enofuranose (3d). Method A. Treatment of **3c** (822 mg, 2 mmol) by procedure C gave 7-bromo-5,6,7-trideoxy-7-fluoro-1,2-*O*-isopropylidene-α-D-*ribo*-hept-6-enofuranose (481 mg, 81%; *E*/*Z*, ∼45:55): ^{1}H NMR δ 5.16 (dt, J = 30.9, 7.7 Hz, 0.45, H6), 5.62 (dt, J = 12.9, 7.5 Hz, 0.55, H6), 5.77 (d, J = 3.9 Hz, 1, H1); ^{19}F NMR δ −74.25 (d, J_{F-6} = 30.8 Hz, 0.45, F7*E*), −69.45 (d, J_{F-6} = 12.8 Hz, 0.55, F7*Z*); HRMS (CI) *m/z* 299.0117 (97, MH^+ [$C_{10}H_{15}{}^{81}BrFO_4$] = 299.0118), 297.0128 (100, MH^+ [^{79}Br] = 297.0138).

Benzoylation of this product (455 mg, 1.53 mmol) by procedure A gave **3d** (564 mg, 92%; *E*/*Z*, ∼45:55) as a syrup: ^{1}H NMR δ 1.30 & 1.49 (2 × s, 2 × 3, 2 × Me), 2.37−2.64 (m, 2, H5,5′), 4.28−4.38 (m, 1, H4), 4.69 (dd, J = 9.1, 4.7 Hz, 0.45, H3), 4.70 (dd, J = 9.1, 4.8 Hz, 0.55, H3), 4.96 (m, 1, H2), 5.20 (dt, J = 30.7, 7.7 Hz, 0.45, H6), 5.65 (dt, J = 12.7, 7.5 Hz, 0.55, H6), 5.88 (d, J = 3.8 Hz, 1, H1), 7.45−8.10 (m, 5, Arom); ^{13}C NMR δ 26.92, 26.97, 28.43 (J = 1.6 Hz) & 30.41 (J = 3.8 Hz, C5), 76.00 & 76.15 (C3), 76.13 & 76.17 (C4), 77.70 & 77.75 (C2), 104.52 & 104.57 (C1), 105.62 (J = 18.2 Hz, C6*Z*), 107.58 (J = 12.4 Hz, C6*E*), 113.44, 128.93, 129.59, 129.64, 130.27, 130.31, 133.90, 133.77 (J = 320.9 Hz, C7*E*), 137.49 (J = 316.1 Hz, C7*Z*), 166.21; ^{19}F NMR δ −73.75 (d, J_{F-6} = 30.9 Hz, 0.45, F7*E*), −68.55 (d, J_{F-6} = 12.8 Hz, 0.55, F7*Z*); HRMS (CI) *m/z* 403.0365 (96, MH^+ [$C_{17}H_{19}{}^{81}BrFO_5$] = 403.0379), 401.0399 (100, MH^+ [^{79}Br] = 401.0400). Anal. [$C_{17}H_{18}BrFO_5$ (401.23)] C, H.

Method B. Treatment of **2c** (924 mg, 3 mmol) by procedure B (CBr_3F was used instead of CBr_4) gave **3d** (493 mg, 41%; *E*/*Z*, ∼45:55). Further elution of the column (15→30% EtOAc/hexane) gave 6-aldehyde-**2c** (321 mg, 35%; 3-*O*-benzoyl-5-deoxy-1,2-*O*-isopropylidene-β-D-*ribo*-hexodialdofuranose): ^{1}H NMR δ 9.85 (t, J = 1.5 Hz, 1, H6); ^{13}C NMR δ 199.56 (C6).

1,2-Di-*O*-acetyl-3-*O*-benzoyl-7,7-dibromo-5,6,7-trideoxy-D-*ribo*-hept-6-enofuranose (4a). Procedure D. A solution of **3b** (462 mg, 1 mmol) in CF_3CO_2H/H_2O (9:1, 7 mL) was stirred at ∼0 °C (ice bath) for 1 h. Volatiles were evaporated under oil-pump vacuum (<10 °C), coevaporated with toluene

(3 ×), and kept under vacuum for 1 h. Pyridine (5 mL), DMAP (3 mg) and Ac_2O (0.47 mL, 510 mg, 5 mmol) were added, and the mixture was stirred at ∼5 °C overnight. Volatiles were evaporated, the residue was dissolved (EtOAc), and the solution was washed (dilute HCl/H_2O, saturated $NaHCO_3/H_2O$, brine), dried (Na_2SO_4), and evaporated. Chromatography (15→20% EtOAc/hexanes) of the residue resulted in partial separation of the anomers of **4a** (430 mg, 85%; β/α, ∼4:1). Crystallization (EtOAc/hexane, 1:4) of the less polar fractions gave β-anomer **4a** (195 mg, 39%): mp 109−110 °C; ^{1}H NMR δ 2.01 & 2.10 (2 × s, 2 × 3, 2 × Me), 2.46 (dt, J = 15.3, 6.9 Hz, 1, H5′), 2.57 (ddd, J = 15.3, 6.9, 5.3 Hz, 1, H5), 4.40 (q, J = 6.2 Hz, 1, H4), 5.36 (dd, J = 6.9, 5.0 Hz, 1, H3), 5.42 (d, J = 4.9 Hz, 1, H2), 6.14 (s, 1, H1), 6.46 (t, J = 7.2 Hz, 1, H6), 7.40−7.96 (m, 5, Arom); ^{13}C NMR δ 20.90, 21.54, 37.22 (C5), 73.59 (C3), 74.78 (C2), 80.12 (C4), 92.56 (C7), 98.55 (C1), 129.00, 129.20, 130.12, 133.24 (C6), 134.10, 165.76, 169.58, 169.76; HRMS (CI) m/z 530.9298 (48, MNa^+ $[C_{18}H_{18}{}^{81}Br_2O_7Na]$ = 530.9276), 528.9308 (100, MNa^+ $[^{81/79}Br_2]$ = 528.9296), 526.9315 (51, MNa^+ $[^{79}Br_2]$ = 526.9317). Anal. $[C_{18}H_{18}Br_2O_7$ (506.15)] C, H. More polar fractions yielded **4a** enriched in α-anomer: ^{1}H NMR δ 6.41 (d, J = 4.2 Hz, H1).

1,2-Di-*O*-acetyl-3-*O*-benzoyl-7-bromo-5,6,7-trideoxy-7-fluoro-D-*ribo*-hept-6-enofuranose (4b). Treatment of **3d** (401 mg, 1 mmol; *E*/*Z*, ∼45:55) by procedure D gave **4b** (356 mg, 80%; β/α, ∼4:1; *E*/*Z*, ∼40:60). Crystallization (EtOAc/hexane, 1:4) of the less polar fractions gave β-anomers **4b** (105 mg, 24%; *E*/*Z*, ∼40:60): mp 92−93 °C; ^{1}H NMR δ 2.06 & 2.14 (2 × s, 2 × 3, 2 × Me), 2.42−2.68 (m, 2, H5,5′), 4.38−4.47 (m, 1, H4), 5.20 (dtd, J = 30.6, 7.7, 2.6 Hz, 0.4, H6), 5.42−5.46 (m, 1, H3), 5.47−5.50 (m, 1, H2), 5.62 (dtd, J = 12.6, 7.6, 2.5 Hz, 0.6, H6), 6.20 (s, 1, H1), 7.41−8.01 (m, 5, Arom); ^{13}C NMR δ 20.48, 21.08, 29.78 & 31.86 (J = 3.6 Hz, C5), 76.10 & 76.17 (C3), 74.44 & 74.51 (C2), 80.34 & 80.37 (C4), 98.15 (C1), 104.87 (J = 18.5 Hz, C6*Z*), 106.78 (J = 12.5 Hz, C6*E*), 128.60, 128.81, 129.68, 133.68, 133.72 (J = 321.4 Hz, C7*E*), 137.42 (J = 316.1 Hz, C7*Z*), 165.35, 169.19, 169.37; ^{19}F NMR δ −73.15 (dd, J = 30.1, 1.9 Hz, 0.4, F7*E*), −68.02 (d, J_{F-6} = 12.8 Hz, 0.6, F7*Z*); HRMS (CI) m/z 469.0081 (94, MNa^+ $[C_{18}H_{18}{}^{81}BrFO_7Na]$ = 469.0097), 467.0105 (100, MNa^+ $[^{79}Br]$ = 467.0117). Anal. $[C_{18}H_{18}BrFO_7$ (445.24)] C, H. More polar fractions yielded **4b** enriched in α-anomers (β/α, ∼1:1): ^{1}H NMR δ 6.21 (s, 0.5, H1β), 6.488 (d, J = 4.4 Hz, 0.2, H1α,*E*), 6.492 (d, J = 4.3 Hz, 0.3, H1α,*Z*); ^{19}F NMR δ −73.15 (d, J_{F-6} = 29.8 Hz, 0.2, F7β,*E*), −73.35 (d, J_{F-6} = 30.1 Hz, 0.2, F7α,*E*), −68.02 (d, J_{F-6} = 12.8 Hz, 0.3, F7β,*Z*), −67.50 (d, J_{F-6} = 12.4 Hz, 0.3, F7α,*Z*).

9-(7,7-Dibromo-5,6,7-trideoxy-β-D-*ribo*-hept-6-enofuranosyl)adenine (5a). **Procedure E. (a) Coupling.** $SnCl_4$ (0.33 mL, 729 mg, 2.8 mmol) was added dropwise to a suspension of adenine (230 mg, 1.7 mmol) and anomeric **4a** (573 mg, 1.13 mmol) in dried CH_3CN (70 mL), and stirring was continued for 18 h at ambient temperature. Volatiles were evaporated, the residue was partitioned ($NaHCO_3/H_2O$//$CHCl_3$), and the organic layer was washed (brine) and dried ($MgSO_4$). Volatiles were evaporated and the residue was chromatographed (EtOAc→3% MeOH/EtOAc) to give 9-(2-*O*-acetyl-3-*O*-benzoyl-7,7-dibromo-5,6,7-trideoxy-β-D-*ribo*-hept-6-enofuranosyl)adenine (431 mg, 74%): HRMS (FAB) m/z 605.9622 (58, MNa^+ $[C_{21}H_{19}{}^{81}Br_2N_5O_5Na]$ = 605.9612), 603.9625 (100, MNa^+ $[^{81/79}Br_2]$ = 603.9631), 601.9641 (55, MNa^+ $[^{79}Br_2]$ = 601.9651).

(b) Deprotection. A solution of this product (400 mg, 0.69 mmol) in NH_3/MeOH (25 mL) was stirred for 5 h at ∼5 °C. Volatiles were evaporated, and the residue was flash chromatographed (EtOAc→8% MeOH/EtOAc) and crystallized (MeOH) to give **5a** (155 mg, 52%) as white crystals. RP-HPLC purification (preparative LC-18S column, gradient 15→60% CH_3CN/H_2O for 80 min at 3 mL/min; t_R = 59 min) of the mother liquor gave additional **5a** (44 mg, 14%; 66% total): mp 94−96 °C (soften); UV max 259 nm (ε 14800), min 228 nm (ε 2000); ^{1}H NMR (Me_2SO-d_6/D_2O) δ 2.42−2.62 (m, 2, H5′,5″), 4.01 (q, J = 5.5 Hz, 1, H4′), 4.11 (t, J = 4.8 Hz, 1, H3′), 4.73 (t, J = 5.2 Hz, 1, H2′), 5.91 (d, J = 5.2 Hz, 1, H1′), 6.72 (t, J = 7.1 Hz, 1, H6′), 8.17 (s, 1, H2), 8.33 (s, 1, H8); ^{13}C NMR (Me_2SO-d_6/D_2O) δ 36.90 (C5′), 72.80 (C2′/C3′), 82.14 (C4′), 88.84 (C1′), 90.26 (C7′), 119.59 (C5), 135.83 (C6′), 140.72 (C8), 149.67 (C4), 153.13 (C2), 156.32 (C6); HRMS (CI) m/z 437.9418 (52, MH^+ $[C_{12}H_{14}{}^{81}Br_2N_5O_3]$ = 437.9425), 435.9450 (100, MH^+ $[^{81/79}Br_2]$ = 435.9444), 433.9446 (56, MH^+ $[^{79}Br_2]$ = 433.9464). Anal. $[C_{12}H_{13}Br_2N_5O_3$ (435.08)] C, H, N.

9-(7-Bromo-5,6,7-trideoxy-7-fluoro-β-D-*ribo*-hept-6-enofuranosyl)adenine (5b). Treatment of anomeric **4b** (223 mg, 0.5 mmol) by procedure E gave **5b** [58 mg, 31%; *E*/*Z*, ∼40:60, overall yield after RP-HPLC purification and crystallization (MeOH)]: mp 79−83 °C (soften), 96−98 °C; UV max 259 nm (ε 14800), min 228 nm (ε 2000); ^{1}H NMR (CD_3OD) δ 2.44−2.61 (m, 2, H5′,5″), 4.01−4.10 (m, 1, H4′), 4.22−4.26 (m, 1, H3′), 4.76−7.81 (m, 1, H2′), 5.31 (dt, J = 31.6, 7.7 Hz, 0.4, H6′), 5.74 (dt, J = 13.2, 7.5 Hz, 0.6, H6′), 5.93 (d, J = 4.8 Hz, 0.4, H1′), 5.94 (d, J = 4.8 Hz, 0.6, H1′), 8.19 (s, 0.6, H2), 8.20 (s, 0.4, H2), 8.23 (s, 0.4, H8), 8.25 (s, 0.6, H8); ^{13}C NMR (CD_3-OD) δ 30.79 & 32.65 (J = 3.6 Hz, C5′), 74.13 & 74.21 (C2′), 74.68 (C3′), 84.10 (J = 1.7 Hz) & 84.23 (J = 2.3 Hz, C4′), 90.48 & 90.54 (C1′), 107.59 (J = 17.6 Hz) & 109.58 (J = 11.7 Hz, C6′), 120.67 (C5), 133.97 (J = 318.7 Hz) & 137.57 (J = 312.8 Hz, C7′), 141.65 (C8), 150.60 (C4), 153.88 (C2), 157.33 (C6); ^{19}F NMR (CD_3OD) δ −74.44 (dt, J = 31.9, 1.9 Hz, 0.4, F7′*E*), −69.93 (d, $J_{F-6'}$ = 13.2 Hz, 0.6, F7′*Z*); HRMS (CI) m/z 375.0181 (100, M^+ $[C_{12}H_{13}{}^{81}BrFN_5O_3]$ = 375.0165), 373.0193 (90, M^+ $[^{79}Br]$ = 373.0186). Anal. $[C_{12}H_{13}BrFN_5O_3 \cdot 0.5H_2O$ (383.17)] C, H, N.

9-(5,6,7-Trideoxy-β-D-*ribo*-hept-6-ynofuranosyl)adenine (6). BuLi/hexane (1.6 M; 3.3 mL, 5.3 mmol) was added dropwise to a solution of **5a** (152 mg, 0.35 mmoL) in dried THF (15 mL) at −78 °C and stirring was continued for 2.5 h with the temperature slowly increasing to ∼ −55 °C. The mixture was neutralized (AcOH, pH ∼6.5) and evaporated to dryness. The residue was flash chromatographed (EtOAc→6% MeOH/EtOAc) and fractions containing pure **6** [TLC ($MeOH/CHCl_3$, 1:9): R_f 0.45; **5a**: R_f 0.51] were evaporated. The residue was crystallized (MeOH) to give **6** (32 mg, 33%): mp 152−154 °C. The mother liquors and less pure fractions from flash chromatography were combined and repurified by RP-HPLC (preparative LC-18S column, gradient 12→35% CH_3CN/H_2O for 60 min at 2.5 mL/min; t_R = 42 min) to give additional **6** (30 mg, 31%; 64% total from **5a**): mp 154−155 °C (MeOH); UV max 259 nm (ε 14100), min 228 nm (ε 3100); ^{1}H NMR (Me_2SO-d_6) δ 2.55 (ddd, J = 17.1, 6.3, 2.5 Hz, 1, H5″), 2.72 (ddd, J = 16.9, 6.3, 2.6 Hz, 1, H5′), 2.90 (t, J = 2.5 Hz, 1, H7′) 3.96−4.01 (m, 1, H4′), 4.13 (q, J = 4.5 Hz, 1, H3′), 4.76 (q, J = 5.5 Hz, 1, H2′), 5.37 (d, J = 5.0 Hz, 1, OH3′), 5.55 (d, J = 6.1 Hz, 1 OH2′), 5.90 (d, J = 5.9 Hz, 1, H1′), 7.32 (br s, 2, NH_2), 8.12 (s, 1, H2), 8.31 (s, 1, H8); ^{13}C NMR (Me_2SO-d_6) δ 23.51, 73.31, 73.43, 73.65, 81.92, 83.04, 88.11, 119.91, 140.15, 150.34, 153.56, 156.90; HRMS (CI) m/z 276.1092 (100, MH^+ $[C_{12}H_{14}N_5O_3]$ = 276.1098). Anal. $[C_{12}H_{13}N_5O_3 \cdot 0.25H_2O$ (279.77)] C, H, N.

AdoHcy Hydrolase Activity. The assay of AdoHcy hydrolase activity in the synthetic direction was performed by measuring rates of formation of AdoHcy from Ado and Hcy. The enzyme was incubated with Ado (1 mM) and Hcy (5 mM) in potassium phosphate buffer [500 μL; 50 mM, pH 5.2, containing EDTA (1 mM)] (buffer A) at 37 °C for 5 min. After termination by addition of $HClO_4/H_2O$ (5 M, 25 μL), the reaction mixture was cooled in ice−water for 15 min, and the clear supernatant was collected and analyzed for AdoHcy by RP-HPLC as described.[4b]

The assay of AdoHcy hydrolase activity in the hydrolytic direction was performed spectroscopically by measuring the rate of the product (Hcy) formed by reaction with DTNB as described.[20] To the enzyme solution (800 μL) containing AdoHcy hydrolase (2 μg) in buffer A were added AdoHcy (500 μM, 200 μL) containing DTNB (250 μM) and Ado deaminase (4 units). The reaction was continuously monitored at 412 nm using an HP8452 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). The initial rate was obtained by fitting the data to zeroth-order kinetics.

Inactivation of AdoHcy Hydrolase. Incubation of AdoHcy hydrolase (42 nM) with various concentration of **5a**, **5b**, and

6 in buffer A at 37 °C for 30 min gave the data shown in Table 1 or with 10 μM of **5a**, **5b**, and **6** for different times (0−30 min) gave the data shown in Figure 1. The remaining activity was assayed in the synthetic direction as described above. When AdoHcy hydrolase was incubated with **5a**, **5b**, and **6** in the presence of 100 μM AdoHcy, the remaining activity was assayed in the hydrolytic direction as described above. The K_i values were calculated by fitting the data to the equation: $v_0/v = 1 + \{K_S/([S] + K_S)\}*[I]/K_i$, where the K_S value for AdoHcy was 7.5 μM.

Analysis of E-NAD$^+$ and E-NADH. The extent of conversion of the NAD^+ to the NADH form of the enzyme was analyzed by a fluorescence method as described.[4b] AdoHcy hydrolase (21 μM) was incubated with **5a**, **5b,** and **6** for 2 h at 37 °C. The cofactors were then released from the enzyme (0.2 mg) by addition of 3 volumes of 95% ethanol followed by centrifugation for 5 min at 4 °C. The precipitate was washed with 95% ethanol (0.2 mL) and centrifuged again. Pooled supernatants were lyophilized in the dark and the residue was then dissolved in H_2O (1.0 mL) and sodium pyrophosphate buffer [0.1 M, 1.0 mL; pH 8.8, containing semicarbazide (0.5%)]. NADH was measured directly while NAD^+ was first converted to NADH by adding 1% solution of baker's yeast alcohol dehydrogenase (10 μL) and 95% ethanol (20 μL). Detection of NADH was accomplished by excitation at 340 nm and measurement of emission at 460 nm.

HPLC Analysis of Possible Enzymatically Generated Products from 5a, 5b, and 6. AdoHcy hydrolase (∼100 μM) was incubated with 200 μM **5a**, **5b**, and **6** for 2 h at 37 °C. Then $HClO_4$ (5 M, 25 μL) was added to denature the enzyme followed by centrifugation at 10000*g* for 15 min. Pooled supernatants were analyzed by RP-HPLC as described.[4b]

Antiviral, Cytotoxic, and Cytostatic Activity Assays. The antiviral, cytotoxic, and cytostatic activity assays were performed according to previously published procedures.[21] For all antiviral assays, except for HIV-1 and HIV-2, antiviral activity measurements were based on the inhibition of virus-induced cytopathicity, which was scored microscopically when the cytopathic effect (CPE) had reached 100% in the control (untreated) virus-infected cell cultures. For the anti-HIV activity assays, CEM cell cultures were suspended at 4×10^5 cells/mL of culture medium and infected with HIV-1(III_B) or HIV-2(ROD) strains at 100 $CCID_{50}$/mL. Then, 100 μL of the infected cell suspension was transferred to 200-μL plate wells containing 100 μL of serially diluted test compound solutions. After 4 days of incubation at 37 °C, all cultures were scored for syncytium formation.

Cytotoxicity tests were based on microscopic evaluation of cell morphology [confluent E_6SM (human embryo fibroblasts), HeLa (human epithelial carcinoma), Vero (African green monkey kidney) and HEL (human embryonic lung fibroblast) cells] and determination of cell growth [HEL and CEM (human T-lymphocytes) cells]. The inhibitory effects of the test compounds on CEM, Molt4/C8 (human lymphocyte), FM3A (murine mammary carcinoma) and L1210 (murine leukemia) cells were determined as follows: 100 μL of the cell cultures containing $4-7 \times 10^4$ cells were added to 200-μL plate wells containing 100 μL of serially (5-fold) diluted test compound solutions. After 3 days (L1210, FM3A) or 4 days (CEM, Molt4/C8) of incubation at 37 °C, the number of viable cells was counted with the automated coulter counter (Coulter Electronics, Harpenden Hertz, U.K.).

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