Synthesis, Mechanism of Action, and Antiviral Activity of a New Series of Covalent Mechanism-Based Inhibitors of *S*-Adenosyl-L-Homocysteine Hydrolase

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A direct method for the preparation of 5′-*S*-alkynyl-5′-thioadenosine and 5′-*S*-allenyl-5′-thioadenosine has been developed. Treatment of a protected 5′-acetylthio-5′-deoxyadenosine with sodium methoxide and propargyl bromide followed by deprotection gave the 5′-*S*-propargyl-5′-thioadenosine **4**. Under controlled base-catalysis with sodium *tert*-butoxide in *tert*-butyl alcohol **4** was quantitatively converted into 5′-*S*-allenyl-5′-thioadenosine **5** or 5′-*S*-propynyl-5′-thioadenosine **6**. Incubation of recombinant human placental AdoHcy hydrolase with **4**, **5**, or **6** resulted in time- and concentration-dependent inactivation of the enzyme (K_i: 45 ± 0.5, 16 ± 1, and 15 ± 1 μM, respectively). Compound **4** caused complete conversion of the enzyme from its E-NAD^+ to E-NADH form during the inactivation process. This indicates that **4** is a substrate for the 3′-oxidative activity of AdoHcy hydrolase (type I inhibitor). In contrast, the NAD^+/NADH content of the enzyme was not affected during the inactivation process with **5** and **6**, and their mechanism of inactivation was further investigated. Addition of enzyme-sequestered water on the *S*-allenylthio group of **5** or *S*-propynylthio group of **6** within the active site should lead to the formation of the corresponding thioester **7**. This acylating-intermediate agent could then undergo nucleophilic attack by a protein residue, leading to a type II mechanism-based inactivation. ElectroSpray mass spectra analysis of the inactivated protein by **5** supports this mechanistic proposal. Further studies (MALDI-TOF and ESI/MS^n experiments) of the trypsin and endo-Lys-C proteolytic cleavage of the fragments of inactivated AdoHcy hydrolase by **5** were carried out for localization of the labeling. The antiviral activity of **4**, **5**, and **6** against a large variety of viruses was determined. Significant activity (EC_{50}: 1.9 μM) was noted with **5** against vaccinia virus.

Introduction

S-Adenosylhomocysteine (AdoHcy) is the product of all biological methylation in which *S*-Adenosylmethionine (AdoMet) is utilized as a methyl donor. This important metabolite is reversibly hydrolyzed to L-homocysteine and adenosine (Ado) by *S*-adenosylhomocysteine hydrolase (E.C. 3.3.1.1).[1] Inhibition of this cellular enzyme results in intracellular accumulation of AdoHcy leading to a feedback inhibition of AdoMet-dependent methylation reactions (i.e., viral mRNA methylation) which are essential for viral replication.[2] Therefore, AdoHcy hydrolase has become an attractive target for the molecular design of antiviral agents.[3] During the past decade, the design and synthesis of mechanism-based inhibitors have received considerable attention, particularly since the mechanism of the catalysis of AdoHcy hydrolase was elucidated by Palmer and Abeles.[4] Two classes of potent irreversible inhibitors have been identified for AdoHcy hydrolase. Type I mechanism-based inhibitors are substrates for the C-3′ oxidative action of the enzyme and irreversibly keep the AdoHcy hydrolase in its NADH form, thus disabling the cycle of the overall enzyme reaction.[3a,5] Type II mechanism-based inhibitors utilize the same "oxidative action" of the enzyme or its "hydrolytic activity" to generate an electrophilic site on the inhibitor which can then bind an active site nucleophile.[6,7]

We recently found that fluorinated analogues of 5′-methylthioadenosine are potent inactivators of AdoHcy hydrolase.[8] The interaction of these derivatives with the different catalytic steps of the enzyme was presumed to generate in the enzyme active cavity highly reactive electrophilic species that could bind covalently with active site residue.[9] This result led us to consider that other thionucleosides such as 5′-*S*-allenyl-5′-thioadenosine **5** or 5′-*S*-propynyl-5′-thioadenosine **6** (Figure 1) could also function as alternative substrates for AdoHcy hydrolase and be good candidates as new covalent mechanism-based inhibitors.

Two plausible mechanisms by which **5** and **6** should inactivate AdoHcy hydrolase can be envisioned. We reasoned that enzymatic oxidation of **5** and **6** into their corresponding 3′-ketoderivatives could be accompanied by a β-elimination of an allenyl or propynyl mercaptan.[10] Under their presumed tautomeric thioaldehyde forms these intermediates are quite reactive[11] and might

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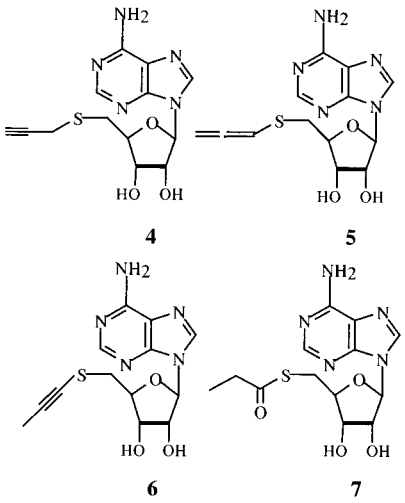


Figure 1.

irreversibly acylate nucleophilic residues involved in the catalytic process within the enzyme cavity (Scheme 1, pathway A). An alternative process involving the hydrolytic activity of AdoHcy hydrolase should not be excluded. Addition of the enzyme's sequestered water to the *S*-allenylthio group of **5** or the *S*-propynylthio group of **6** might generate the formation of the corresponding reactive thioester **7** (Figure 1). Attack of such an intermediate by amino acid functionalities of the active site might also cause type II (covalent binding) inhibition of the enzyme (Scheme 1, pathway B).

We now describe the syntheses of the new 5′-*S*-propargyl-5′-thioadenosine **4**, 5′-*S*-allenyl-5′-thioadenosine **5**, and 5′-*S*-propynyl-5′-thioadenosine **6**, their inhibitory effects on AdoHcy hydrolase, and their antiviral activities. Compounds **4**, **5**, and **6** are irreversible inactivators of the enzyme, and we undertook a mass spectral analysis of the inactivated protein to provide evidence for the formation a covalent adduct induced by mechanism-based inhibitors.

Chemistry

The general synthetic procedure used for the preparation of **4**, **5**, and **6** was as follows (Scheme 2). Tosylation of N^6-benzoyl-2′,3′-isopropylidene adenosine **1**, readily available from adenosine,[12] followed by reaction with potassium thioacetate gave rise to thioacetate **2**. Treatment of **2** with 1 equiv of sodium methoxide in methanol followed by alkylation with propargyl bromide gave the corresponding 5′-*S*-propargyl-5′-thioadenosine derivative. In situ removal of the *N*-benzoyl protecting group of this intermediate can be achieved readily by stirring the resulting solution with a second equivalent of MeONa at 40 °C to afford **3** in a total yield of 72% from **2**. Because of their acid instability, the desired compounds **5** and **6** had to be prepared from the deprotected *S*-propargylthioadenosine **4**. Removal of the isopropylidene protecting group of **3** with aqueous 80% HCOOH resulted in the formation of 5′-*S*-propargyl-5′-thioadenosine **4** in 72% yield. Base-catalyzed isomerization[13] of **4**, using 1 or 3.5 equiv of potassium *tert*-butoxide in *tert*-butyl alcohol at 50 °C afforded the desired target nucleosides 5′-*S*-allenyl-5′-thioadenosine **5** or 5′-*S*-propargyl-5′-thioadenosine **6**, respectively.

Inactivation of *S*-Adenosyl-L-homocysteine Hydrolase

5′-*S*-Allenyl-5′-thioadenosine **5** and 5′-*S*-propynyl-5′-thioadenosine **6** and their precursor the 5′-*S*-propargyl-5′-thioadenosine **4** were evaluated for inhibition of purified recombinant human placental AdoHcy hydrolase.[14] Incubation of the enzyme with **4**, **5**, and **6** resulted in time- and concentration-dependent inactivation of the enzyme. As shown in Figure 2 (panels B and C), the inactivation by **5** and **6** appeared to be biphasic showing pseudo-first-order kinetics only in the first period of inactivation.

Using the Kitz and Wilson method,[15] a double reciprocal plot of the initial pseudo-first-order inactivation rate constant ($1/K_{app}$) versus 1/[I] gave the K_i and K_{inact} values listed Table 1. K_i values for **4**, **5**, and **6** are in the same range (16−40 μM), but **5** and **6** appeared to be better inactivators in terms of their K_{inact} values.

When AdoHcy hydrolase (1 unit) was incubated in assay buffer with **4**, **5**, or **6** (200 μM), total inactivation of the enzyme occurred after 15 min. In each case, the inactivation was irreversible since the enzyme activity lost could not be restored after dialysis (24 h) against assay buffer. We confirmed that the action of each of the nucleosides **4**, **5**, and **6** was active site-directed by means of protection experiments with adenosine.[16]

The effects of **4**, **5**, and **6** on the NAD^+/NADH content of AdoHcy hydrolase were also determined after complete inactivation of the enzyme. The freshly prepared recombinant placental AdoHcy hydrolase was determined to contain 0.75 mol of NAD^+ and 0.25 mol of NADH per enzyme subunit, in accord with reported data.[6b]

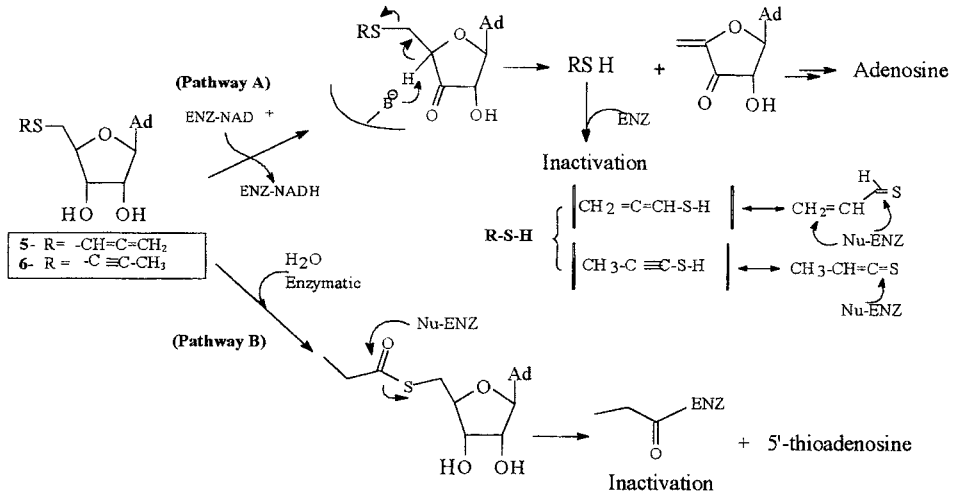
5′-*S*-Propargyl-5′-thioadenosine **4** (600 μM) caused complete conversion of the enzyme (20 μM) from its E-NAD^+ to E-NADH form during the inactivation process. This is indicative that **4** is substrate for the 3′-oxidative activity of AdoHcy hydrolase. Surprisingly, inactivation of AdoHcy hydrolase, carried out under the same conditions, with **5** and **6** resulted in only minor changes in the initial NAD^+/NADH ratio (Table 2).

Upon complete inactivation of the enzyme (25 μM) with 200 μM of **4**, **5**, and **6**, the reaction products were analyzed by liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS). With **4**, no trace of product other than unreacted nucleoside **4** was detected.

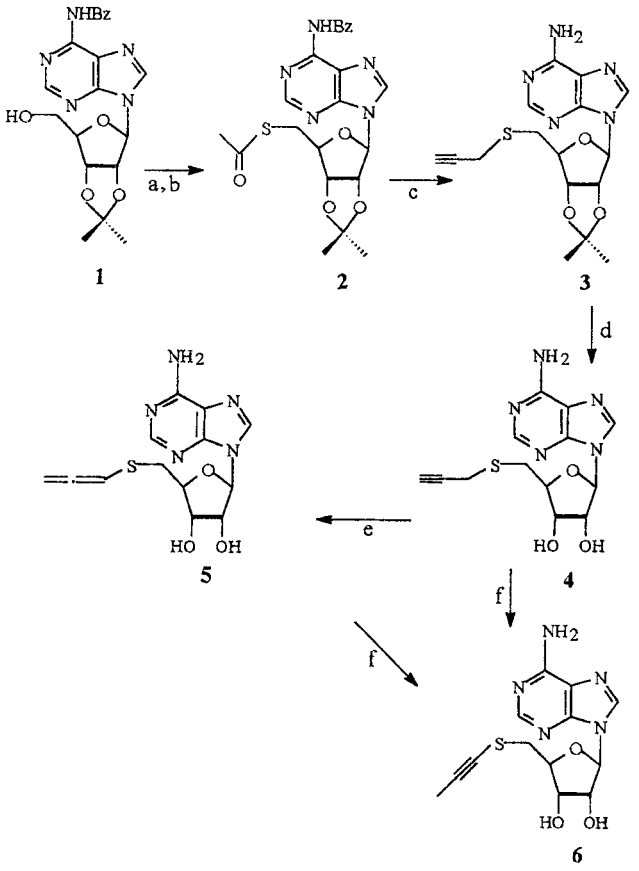
In contrast, in experiments carried out with **5** and **6**, among few other detected compounds, adenosine was identified by its retention time on the column (compared with Ado used as authentic) and its molecular peak $(M + H)^+$ $m/z = 268$. This result indicated that the inhibition process with **5** and **6** is competing with substrate activity. The partition ratio was estimated to be 90/10 in favor of inactivation. This result was confirmed by the inactivation assays of AdoHcy hydrolase carried out with **5** and **6** in the presence of a large excess (2.5 mM) of D,L-homocysteine. In these experiments, AdoHcy was detected and identified by its molecular peak $(M + H)^+$ $m/z = 385$ in the reaction product.

This result is important to explain the biphasic inactivation-time profiles observed for inactivation of AdoHcy hydrolase with **5** and **6**. A progressive formation

Scheme 1. Possible Generation of Active Intermediates from **5** and **6** after Interaction with AdoHcy Hydrolase



Scheme 2[a]



[a] Reagents and conditions: (a)TsCl, pyridine, 95%; (b) CH_3COSK, DMF, 60 °C, 70%; (c) MeONa, Me0H; $HC{\equiv}CCH_2Br$ (72%); (d) 80% HCOOH, 40 °C, 72%; (e) 1 equiv tBuOK , tBuOH, 50 °C, 1H (55%); (f) 3.5 equiv tBuOK, tBuOH, 50 °C, 3H (60%).

of Ado during the interaction of **5** and **6** with the enzyme could protect AdoHcy hydrolase against its irreversible inactivation by **5** and **6**. Similar biphasic kinetics have already been observed when AdoHcy hydrolase was inactivated by 5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine.[6b]

From these initial results it appeared that inactivation of AdoHcy hydrolase by **4** involves a type I mechanism (cofactor depletion).[5]

In contrast, the fact that the interacion of **5** and **6** with AdoHcy hydrolase causes minor changes in the NAD^+/NADH content of the enzyme suggests that the main pathway by which **5** and **6** proceed to inactivate AdoHcy hydrolase do not involve its 3′-oxidative activity.

The tertiary structure of human AdoHcy hydrolase complexed with NAD^+ and the type I mechanism-based inhibitor (1′*R*,2′*S*,3′*R*)-9-(2′,3′-dihydroxycyclopentan-1′-yl)-adenine is presently known.[17] Taking in account the information on the position and interactions of this inhibitor cocrystallized in the AdoHcy hydrolase structure, a computer model of **5**, docked at the active site of the enzyme, was established (Figure 3). This model revealed the proximity of the sequestered water molecule involved in the hydrolytic activity of the enzyme to the C-6′ of the allenylthio group of **5**. This observation, and the fact that **5** and **6** can be easily transformed by acid treatment (aqueous formic acid) into 5′-*S*-propionyl-5′-thioadenosine **7**, strongly suggests the possible reaction of sequestered water with **5** to generate a thioester intermediate. Attack by amino acid functionalities of the enzyme's active site might cause irreversible covalent inactivation accompanied by a covalent linkage of 54 Da on each enzyme subunit.

In the second proposed mechanism of inactivation (Scheme 1, pathway A), each catalytic event with **5** and **6** also produces 3′-keto-4′,5′-dehydroadenosine, an intermediate which is converted by normal enzymatic reaction into adenosine, in the absence of homocysteine. If reactive mercaptans produced in this process are not immediatly quenched by reaction with a nucleophile, adenosine might be formed. Since the formation of adenosine was observed upon incubation of **5** and **6** with the enzyme, the partial involvement of allenyl or propynyl mercaptan groups in the covalent irreversible inactivation of AdoHcy hydrolase cannot be excluded. If occurring, such an inactivation process might be accompanied this time by a covalent linkage of 72 Da.

To discriminate between these two possibilities, the mechanism of inactivation was further investigated using ESI-MS analysis of AdoHcy hydrolase inactivated by **5**.

Accurate molecular weight of AdoHcy hydrolase subunit was determined from the wide distribution of highly charged states observed in ESI-MS analysis under the acidic conditions described in Experimental Section. As shown in Figure 5 (panel A), the freshly

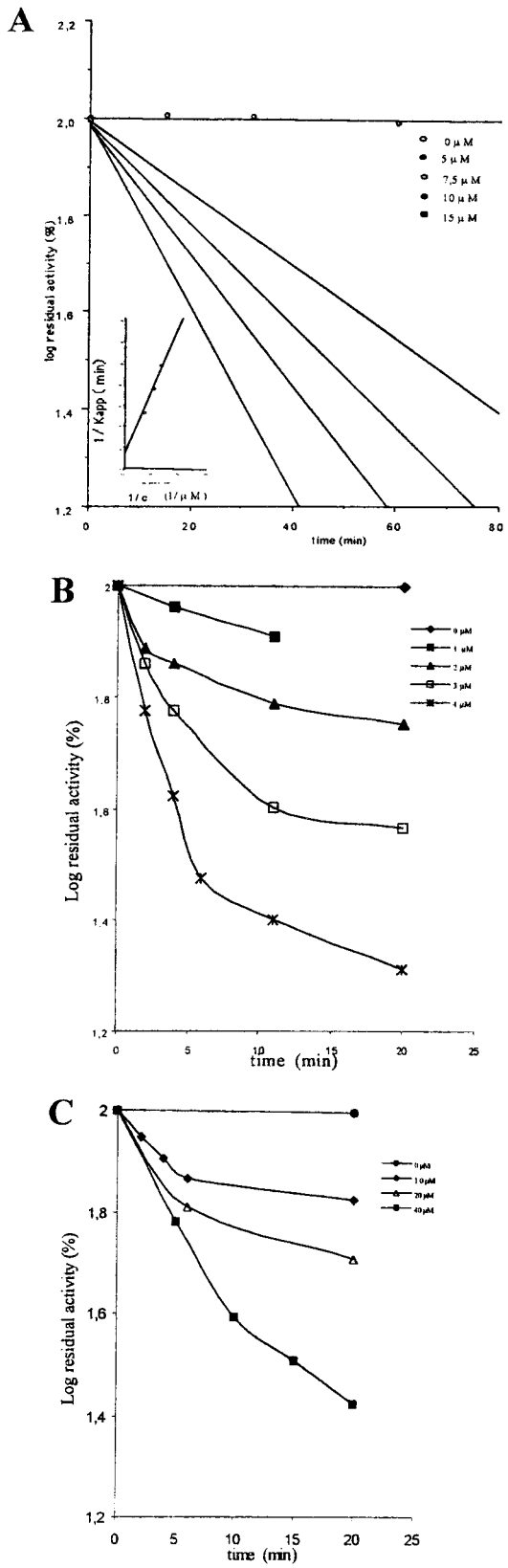


Figure 2. Time-dependent inactivation of AdoHcy hydrolase with **4** (panel A), **5** (panel B), and **6** (panel C). AdoHcy hydrolase was incubated with inhibitors at concentrations of 5−40 μM in assay buffer at 37 °C. At the indicated time points, residual enzyme activity was determined in the synthetic direction as described in the Experimental Section.

purified enzyme was present with a major molecular species being detected at 47604.8 ± 1.5 Da. A singly charged species was detected at 664.2 *m*/*z* which was attributed to NAD^+ and/or NADH, the cofactors present in AdoHcy hydrolase. After incubation with **5**, the inactivated enzyme was detected at a molecular weight of 47659.5 ± 3.0 Da (Figure 4, panel B) showing that inhibition of the enzyme was accompanied by a covalent linkage of 54 ± 4.5 Da.

This result supports the mechanistic proposal described in Scheme 1 (pathway B) via the putative formation of a thioester intermediate which could irreversibly acylate a nucleophilic residue on the enzyme. Further evidence in support of this mechanism comes from inactivation experiments carried out with 5′-*S*-propionyl-5′-thioadenosine **7** prepared in our laboratory.

Table 1. Inhibition Constants for **4**, **5**, and **6** with AdoHcy Hydrolase

compd	type of inhibition	K_i (μM)	k_{inact} (min^{-1})	$t^{1/2}$ (min)
4	irreversible, time-dependent	45	0.0074	19[a]
5	irreversible, time-dependent	16	0.12	7[b]
6	irreversible, time-dependent	16	0.062	7[c]

[a] The half-time ($t_{1/2}$) of enzyme inactivation at 15 μM of **4**. [b] The half-time ($t_{1/2}$) of enzyme inactivation at 3 μM of **5**. [c] The half-time ($t_{1/2}$) of enzyme inactivation at 40 μM of **6**.

Table 2. Effect of **4**, **5**, and **6** on NAD^+/NADH Content of AdoHcy Hydrolase[a]

sample	NAD^+ content (mol/mol of enzyme subunit)	NADH content (mol/mol of enzyme subunit)
purified enzyme	0.75 ± 0.02	0.25 ± 0.04
enzyme + **4**	0	1 ± 0.04
enzyme + **5**	0.70 ± 0.02	0.30 ± 0.04
enzyme + **6**	0.68 ± 0.02	0.32 ± 0.04

[a] AdoHcy hydrolase (20 μM) was incubated with 600 μM **4**, **5**, and **6** in assay buffer at 37 °C until total inactivation. The NAD^+/NADH content was assayed as described in the Experimental Section.

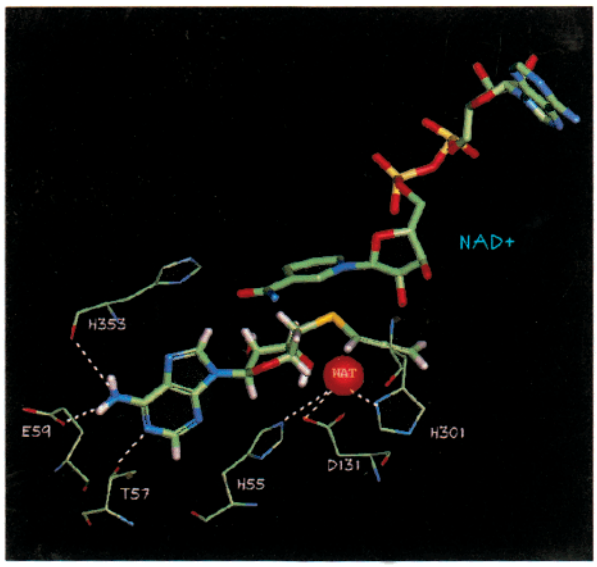


Figure 3. Compound **5** (colored by atom type) docked into the active site of AdoHcy hydrolase. For clarity, only the residues providing the main hydrogen bond interactions and the NAD+ molecule are shown. Hydrogen bonds are represented as dotted lines. The proximity of a water molecule (red sphere) to the allenyl moiety of the inhibitor strongly suggests the possibility of a reaction with water to give the thioester intermediate **7**.

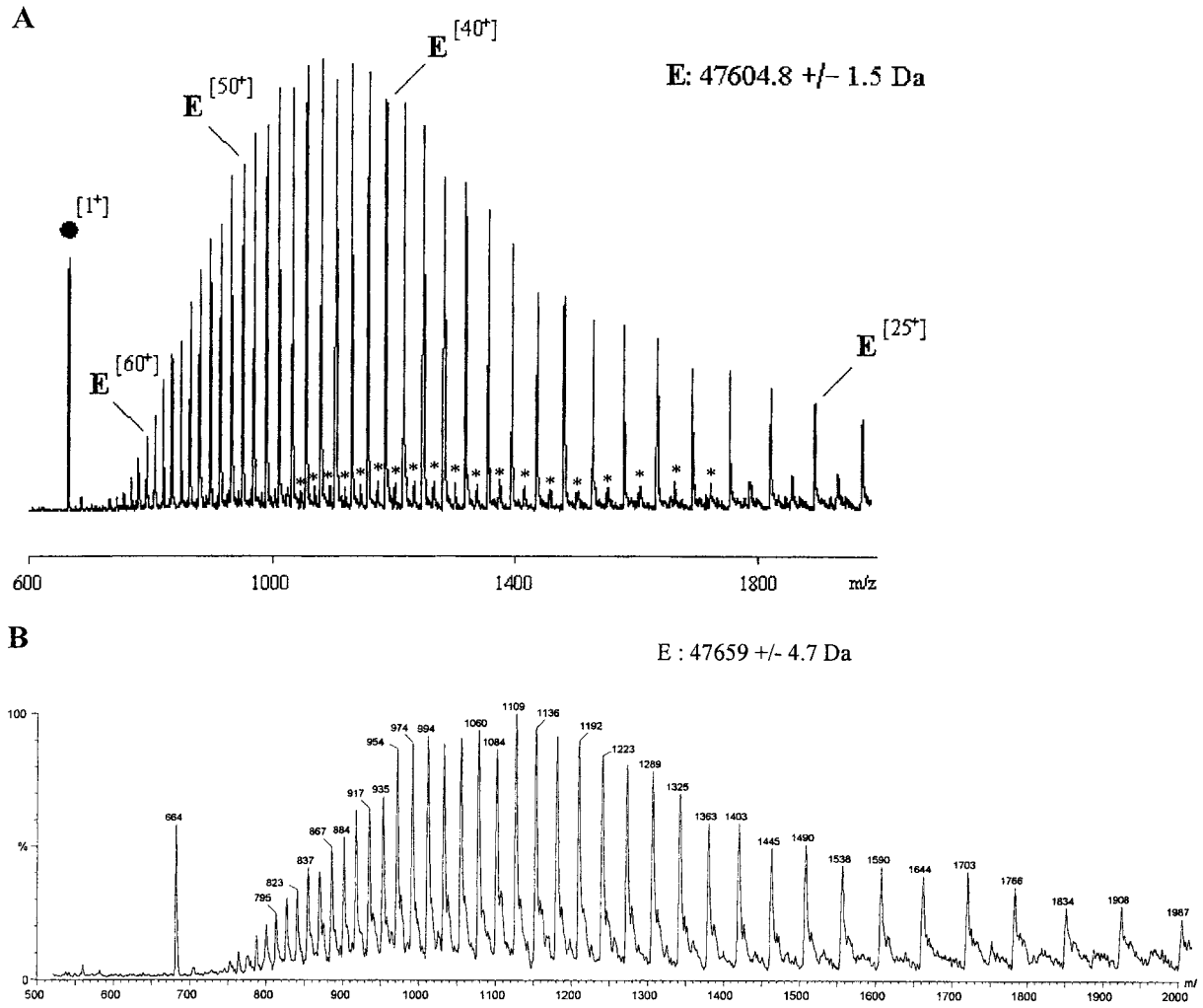


Figure 4. Panel A: Positive ion-ESI mass spectrum recorded for purified AdoHcy hydrolase (5 μM in 49.5:49.5:1 (v/v) H_2O-CH_3-CN-HCOOH). The enzyme displays a wide distribution of highly protonated states, some of which are indicated. The peak labeled with a solid circle (●) corresponds to the singly charged ion produced by NADH (664 Da), the known cofactor of AdoHcy hydrolase. The peaks labeled with a star (*) in which mass exceeds by 665 ± 5 Da may possibly come from residual cofactor associated to the enzyme. Panel B: ESI mass spectrum of Ado Hcy hydrolase inactivated with **5**.

Inactivation of AdoHcy hydrolase with **7** was shown to be both concentration- and time-dependent as predicted (Figure 6), and kinetic analysis of the inactivation process by the Kitz and Wilson method gave kinetic constants ($K_i = 78\ \mu$M; $K_{inact} = 0.155\ min^{-1}$) in the range of those obtained with **5** and **6**.

To localize the amino acid(s) involved in the binding of $\Delta M = 54.8 \pm 4.5$ Da, AdoHcy hydrolase inhibited by *S*-allenylthioadenosine **5** was submitted to proteolytic cleavage by Endo-LysC. The cleavage products (i.e., a mixture of peptides C-terminally cleaved at Lys residues) were analyzed without further separation by MALDI-MS, and compared to those obtained from native AdoHcy hydrolase. This comparison revealed a singly charged ion at 2414.0 *m*/*z* in the MALDI mass spectrum of the inactivated protein, which was absent in the mass spectrum of the native protein (Figure 5). A separation of the proteolysis cleavage peptides was achieved by LC/ ESI-MS for both the native and the inhibited AdoHcy hydrolase. The fraction containing the peptide at 2414.0 *m*/*z* was isolated. This fraction was submitted to further nanoESI-MS^n experiments on an Ion-Trap instrument in order to characterize its primary structure and to determine whether it was responsible for the chemical modification observed on the inactivated protein ($\Delta M = 54.8 \pm 4.5$ Da). These studies (described in Experimental Section) allowed the determination of the whole primary sequence of the peptide detected at 2414.0 *m*/*z*, which was identified as the Endo-LysC cleaved C-terminal peptide of AdoHcy hydrolase **QAQYLGMSCDGPFKPDHYRY** containing an unlocalized adduct of 38 Da. The excess mass (38 Da) recovered by analysis of the cleavage products of inactivated AdoHcy hydrolase is 17 Da lower than expected from the protein subunit before proteolysis. The loss of OH group (−17 Da) from the C-terminal end peptide of the protein, possibly induced by the MALDI process itself, might explain this difference.

Antiviral Activity

Compounds **4**, **5**, and **6** were evaluated for antiviral activity in cell cultures[18] (Table 3). No significant antiviral activity was observed against Parainfluenza-3, Sindbis, Punta Toro, or Coxsackie virus B4 in Vero cells at concentrations that were significantly (>5-fold) lower than the cytotoxicity threshold. No specific antiviral activity was noted against any other viruses, not even vesicular stomatisis virus or reovirus which nor-

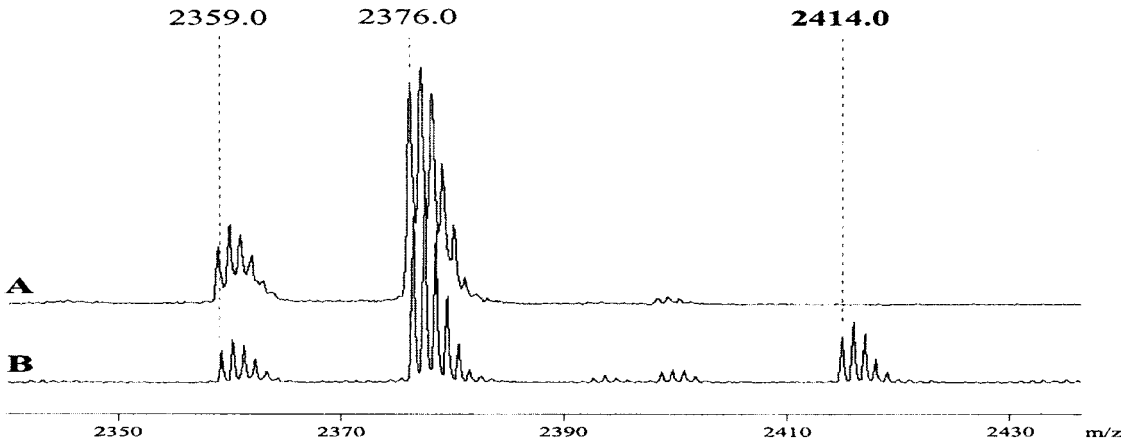


Figure 5. MALDI mass spectra recorded in the reflector positive mode (range 2345−2435 *m*/*z*) for the peptides mixture obtained by enzymatic (Endo-LysC) proteolysis of AdoHcy hydrolase (A) and inhibited AdoHcy hydrolase (B). The singly charged ion detected at 2414.0 *m*/*z* (5B) is not present on the mass spectrum of the purified protein (5A), which may suggest that this peptide carries the modification observed on the entire (inhibited) protein. The ion detected at 2376.0 *m*/*z* for both the purified and the inactivated protein is attributed to the singly charged ion produced by the Endo-LysC cleaved C-terminal peptide of the protein (i.e., 20 last amino acids of the protein: QAQYLGMSCDGPFKPDHYRY). While the singly charged ion detected at 2359.0 *m*/*z* does not correspond to any expected cleavage product and is rather imputed to the loss of an OH group (−17 Da) from the C-terminal-end peptide of the protein. This loss may possibly be induced by the MALDI process itself.

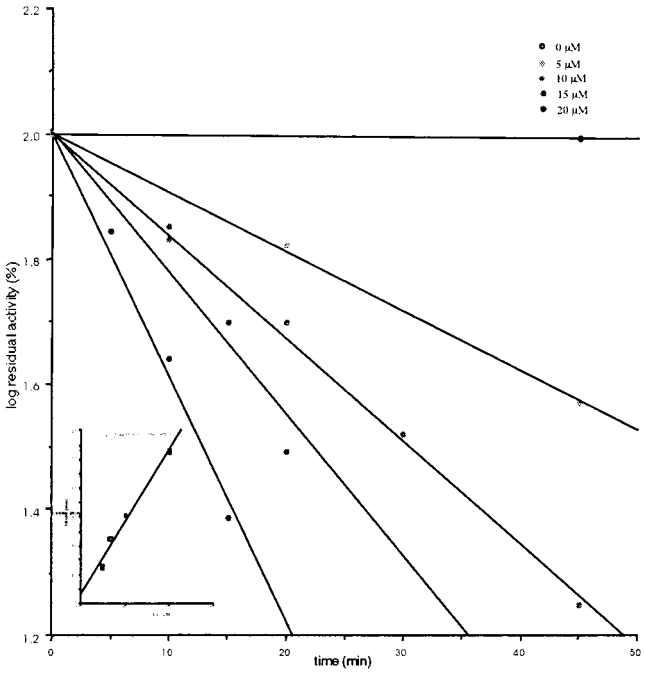


Figure 6. Time-dependent inactivation of AdoHcy hydrolase with 5′-*S*-propionyl-5′-thioadenosine **7**. AdoHcy hydrolase was incubated with this inhibitor at concentrations of 5, 10, 15, and 20 μM in assay buffer at 37° for various times. At the indicated time, residual enzyme activity was determined in the synthetic direction as described in the Experimental Section.

mally fall within the activity spectrum of the AdoHcy hydrolase inhibitors. However, **6** and particularly **5** proved to be specifically active against vaccinia virus in E_6SM cells, at a concentration that was >10-fold lower than the cytotoxic concentration. For **5**, the EC_{50} (50% effective concentration) in inhibiting vaccinia virus replication was 1.9 μM.

Conclusion

In summary, we have synthesized a new series of nucleosides containing a 5′-sulfur atom that produces time- and concentration-dependent inactivation of AdoHcy hydrolase.

Our results reveal that inactivation of AdoHcy hydrolase by **4** involves a type I mechanism. Compounds **5** and **6** inactivate the enzyme in a different manner since these two inactivators do not induce NAD^+ depletion. The main pathways of the inactivation process have been determined with mass spectral analysis of the inactivated protein. ESI-MS analysis of the enzyme inactivated with **5** demonstrates that inhibition of AdoHcy hydrolase is accompanied by covalent modification of each subunit of the enzyme.

Moreover, **5** and **6** are easily transformed by acid hydrolysis into 5′-S-propionyl-5′-thioadenosine, a reactive thioester which can inactivate AdoHcy hydrolase in a time- and concentration-dependent manner. All these results are consistent with the mechanistic proposal depicted in Scheme 1 (pathway B) in which **5** or **6** could be activated by the "hydrolytic activity" of AdoHcy hydrolase. Thus, they represent a new class of type II covalent inhibitors of AdoHcy hydrolase. In addition, we demonstrated that ESI-MS and Nano ESI-MS^n are valuable tools for studying the nature and localization of the covalent adduct formed during covalent mechanism-based inhibition.

Dihalovinyl and dihaloacetylene analogues derived from adenosine have been characterized as first type II mechanism-based inhibitors of human placental AdoHcy hydrolase that relies only on the enzyme's hydrolytic activity".[7b−d] Arg 196 and Lys 318 were identified as amino acid residues that attack an activated intermediate catalytically generated from 6′-bromo-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine[7c] and 5′,5′,6′,6′-tetrahydro-6′-deoxy-6′-chloro- and 6′-iodoadenosine,[7d] respectively, whereas, from our results it appeared that **5** covalently modifies each enzyme's subunit at the C-terminal peptide of the protein (i.e., 20 last amino acids)—a peptide sequence which contains Lys 426 and Tyr 430, two essential amino acids for binding of the NAD cofactor and enzyme activity.[17,19]

Table 3. Antiviral Evaluation of **4**−**6**

				EC_{50}[b] (μM)											
	MIC[a] (μM)			HSV-1 (KOS)	HSV-2 (G)	HSV-1/TK$^-$ B2006 or VMW1837	vaccinia virus	VSV	VSV	Coxsackie virus B4	RSV	RSV	parainfluenza-3 virus	reovirus	Punta Toro virus
compd	E_6SM	HeLa	Vero	(E_6SM)	(E_6SM)	(E_6SM)	(E_6SM)	(E_6SM)	(HeLa)	(HeLa)	(Vero)	(HeLa)	(Vero)	(Vero)	(Vero)
4	1240	1240	1240	>250	>250	>250	>250	>250	>250	>120	>250	>250	>120	>120	>120
5	1240	1240	1240	250	>250	150	1.9	>250	>250	>600	>250	>250	>620	>380	>620
6	1240	>1240	1240	250	620	>250	49	>250	>1240	>120	>1240	>250	>120	>120	>120
c		>2000	>2000						230	230	>2000	2000	230	45	>2000
d	>1600	>1600	>1600	320	320	0.005	65	40	1000	>320	>1600	1.5	196	40	40
e	>1600			0.0012	0.0012		>100	>100							

			EC_{50}[b] (μM)								
			TK$^+$VZV		TK$^-$VZV		CMV				
	MIC[a] (μM)		YS strain	OKA strain	07/01 strain	YS/R strain	AD-169 strain	Davis strain	Sindbis virus	Junin virus	Tacaribe virus
compd	HEL	Vero	(HEL)	(HEL)	(HEL)	(HEL)	(HEL)	(HEL)	(Vero)	(Vero)	(Vero)
4	>200	>200	>200	>200	154	126	>200	>200	>40	>200	>200
5	>200	>200	>200	>200	87	>200	>200	>200	>200	>200	>200
6	>200	>200	>200	>200	200	200	>200	>200	>40	>200	>200
f	>150		4.09	4.53	41	63					
d		>500							240	3.2	3.2
e	>150						7.8	11.8			

[a] Minimal inhibitory concentration required to elicit a microscopically visible alteration of cell morphology. [b] Effective concentration required to inhibit virus-induced cytopathicity by 50%. [c] (*S*)-2,3-Dihydroxypropyladenine [(*S*)-DHPA]. [d] Ribavirin. [e] 9-(1,3-Dihydroxy-2-propoxymethyl)guanine (DHPG, gancyclovir). [f] 9-(2-Hydroxyethoxymethyl) guanine (ACV, acyclovir). Abbreviations: TK$^-$, thymine kinase deficient; HSV, Herpes simplex virus; VSV, vesicular stomatitis virus; RSV, respiratory synaptial virus; E_6SM, embryonic skim-muscle cells; VZV, variecella-zoster virus; CMV cytomegalovirus.

Additional studies are in progress using nano ESI-MS^n techniques to elucidate the exact localization of the covalent linkage induced by AdoHcy with **5**, **6,** and **7** as well as to clarify the mechanism of inactivation of other analogues such as 5′-*S*-ethynyl-5′-thioadenosine and 5′-*S*-vinyl-5′-thioadenosine identified recently as new mechanism-based inhibitors of the enzyme.

Experimental Section

General. NMR spectra (^{1}H, 250 MHz; ^{13}C, 62.5 MHz) were recorded on a Brucker spectrometer. IR spectra were recorded on a FTIR Midac. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Mass spectra were obtained using a VG Instruments AUTOSPEC spectrometer. Protein electrospray ionization were performed on a VQ Bio Q triple quadrupole spectrometer. Flash column chromatography was performed using 9385 Merck Kieselgel 60 and TLC with silica gel plates Merck 60 F_{254}. Purification on hydrophobic resin was performed using a HP20SS Resin from Mitsubishi Fine Chemicals. Analytical reversed-phase HPLC analyses were performed with a C-18 column (ODS2 spherisorb 5 μm; 250 × 4.6 mm).

2′,3′-*O*-Isopropylidene-5′-*S*-propargyl-5′-thioadenosine 3. To a dry oxygen-free methanol solution (15 mL) of thioacetate **2** (2 g, 4.26 mmol) was added 1 equiv of sodium methoxide (226 mg, 4.26 mmol) at 0 °C under argon, and stirring was continued for 8 h at room temperature. To the resulting solution was added propargyl bromide (0.32 mL, 4.26 mmol) in 3 mL of methanol at 0 °C. The reaction mixture was allowed to warm to room temperature, 1 equiv (226 mg) of sodium methoxide was then added, and stirring continued at 40° for 1 h. Methanol was evaporated under reduced pressure, and the crude product obtained was extracted with ethyl acetate/water. The crude material was purified by flash column chromatography on silica gel (ethyl acetate/methanol 9/1) to give pure **3** (1.10 g, 72%) as a white foam. mp: 65 °C. MS (DCI/NH_3): 362 $(MH)^+$. $[\alpha]_D^{20}$ −21° (*c* 0.23, $CHCl_3$). IR(KBr) 21.05; 3156 cm^{-1}. ^{1}H NMR, $CDCl_3$, δ (ppm), *J* (Hz): 1.40 and 1.62 (2 s, 6H, $(CH_3)_2C$); 2.17 (dd, 1H, CC*H*, *J* = 2.67); 3.02 (d, 2H, H-5′, *J* = 6.87); 3.26 (d, 2H, CH_2CC, *J* = 2.67); 4.48 (ddd, 1H, H-4′, *J* = 3.44, 6.87, 6.87); 5.12 (dd, 1H, H-3′, *J* = 6.1, 3.44); 5.51 (dd, 1H, H-2′, *J* = 2.29, 6.1); 5.98 (s, 2H, NH_2); 6.09 (d, 1H, H-1′, *J* = 2.29); 7.93 and 8.35 (2 s, 2H, H-2 and H-8). ^{13}C NMR, $CDCl_3$, δ (ppm): 19.6 (*C*H_2CC); 25.3 and 27.0 (*C*H_3)$_2$C); 33.5 (C-5′); 71.5(*C*CH); 79.4 (C*C*H); 83.1 (C-4′); 83.8 (C-3′); 86.7 (C-2′); 90.7 (C-1′); 114.4 ((CH$_3$)$_2$*C*); 120.2 (C-6); 139.9 and 153.0 (C-2 and C-8); 149.1 and 155.8 (C-4 and C-5).

5′-*S*-Propargyl-5′-thioadenosine 4. A total of 315 mg (0.87 mmol) of **3** in 3 mL of formic acid/water solution (8/2) was heated at 40 °C for 1.5 h. After cooling, formic acid was evaporated under reduced pressure, and traces of acid were removed by coevaporation with water (3 times). The crude material was dissolved in 3 mL of water, and the pH of the solution was adjusted to pH 7 with a few drops of 2 N NH_4-OH. Crude **4** was purified on a column of hydrophobic resin HP20SS (elution: methanol/water 3/7). Evaporation of methanol and lyophilization gave **4** (200 mg, 72%). mp: 87−89 °C. $[\alpha]_D^{20}$ −20.5° (*c* 0.23; $CHCl_3$/MeOH: 4/1). IR (KBr) 2170, 3281 cm^{-1}. ^{1}H NMR, $CDCl_3$, ϵ CD_3OD δ (ppm), *J* (Hz): 2.27 (t, 1H, CC*H*, *J* 2.67); 3.01 (dd, 1H, H5′α, *J* = 14.11, 6.1); 3.12 (dd, 1H, H5′β, *J* = 14 0.11, 4.57); 3.28 (d, 2H, CH_2CC, 2.67); 4.21 (m, 1H, H-4′); 4.29 (dd, 1H, H-3′, *J* = 5.34, 5,72); 4.46 (dd, 1H, H-2′, *J* 5.34, 3.81); 5.81 (d, 1H, H-1′, *J* = 3.81); 8.09 and 8.21 (2 s, 2H, H-2 and H-8). ^{13}C NMR, $CDCl_3$, ϵ CD_3OD, δ (ppm): 20.0 (*C*H_2CC); 33.3 (C-5′); 71.6(*C*CH); 72.3 (C-4′); 74.2 (C-3′); 80.0 (C*C*H); 83.9 (C-2′); 89.6 (C-1′); 120.0 (C-6); 139.3 and 152.8 (C-2 and C-8); 149.1 and 155.9 (C-4 and C-5). HRMS (CI/NH_3) calcd C_{13} $H_{15}O_3S$ + H 322.0973, found 322.0979. Anal. ($C_{13}H_{15}O_3S$) C, H, N.

5′-*S*-Allenyl-5′-thioadenosine 5. A total of 160 mg (0.5 mmol) of **4** was dissolved in 5 mL of anhydrous *tert*-butyl alcohol at 50°, and 1 equiv (56 mg, 0.5 mmol) of potassium *tert*-butoxide was then added. The reaction was followed by analytical reversed-phase HPLC (elution methanol/water 5/5). After complete transformation of **4** into **5**, the reaction mixture was cooled to room temperature, and 3 mL of water was added. *tert*-Butyl alcohol was evaporated under reduced pressure, and the pH of the resulting solution was carefully adjusted to pH 7 at 0 °C. Crude **5** was purified on a column of hydrophobic resin HP20SS (elution: methanol/water 3/7). Evaporation of methanol and lyophilization gave pure **5** (90 mg, 55%). mp: 90−92 °C. $[\alpha]_D^{20}$ −18° (*c* 0.18; $CHCl_3$/MeOH: 4/1). IR (KBr) 1944 cm^{-1} (weak). ^{1}H NMR, $CDCl_3$, ϵ CD_3OD δ (ppm), *J* (Hz): 2.93 (dd, 1H, H-5′α, *J* = 14.5, 4.96); 3.05 (dd, 1H, H-5′β, *J* = 14.5, 4.96); 4.21 (m, 1H, H-4′); 4.22 (dd, 1H, H-3′, *J* = 6.1, 4.95);

4.49 (dd, 1H, H-2′, J = 4.95, 4,2); 4.94 (d, 2H, CH_2═C═CHS, J = 6.48); 5.70 (dd, 1H, CH_2═C═C*H*S, J = 6.48); 5.86 (d, 1H, H-1′, J = 4.2); 8.05 and 8.15 (2 s, 2H, H-2 and H-8). ^{13}C NMR, $CDCl_3$, ϵ CD_3OD, δ (ppm): 34.2 (C-5′); 72.1 (C-4′); 74.2 (C-3′); 81.0 (*C*H_2═C═CHS); 83.0 (C-2′); 87.0 (CH_2═C═*C*HS); 89.3 (C-1′); 119.4 (C-6); 139.1 and 152.2 (C-2 and C-8); 148.7 and 155.3 (C-4 and C-5); 205.6 (CH_2═*C*═CHS). HRMS (CI/NH_3) calcd C_{13} $H_{15}O_3S$ + H 322.0973, found 322.0979. Anal. ($C_{13}H_{15}O_3S$) C, H, N.

5′-*S*-Propynyl-5′-thioadenosine 6. A total of 225 mg (0.62 mmol) of **4** was dissolved in 10 mL of anhydrous *tert*-butyl alcohol at 50 °C, and 3.5 equiv (224 mg, 2.18 mmol) of potassium *tert*-butoxide was added. The reaction mixture was stirred for 2.5 h at 50 °C. After the reaction mixture was cooled to room temperature, 5 mL of water was added. *tert*-Butyl alcohol was evaporated under reduced pressure, and the pH of the resulting solution was carefully adjusted to pH 7 at 0 °C. Crude **6** was purified on a column of hydrophobic resin HP20SS (elution: methanol/water 3/7). Evaporation of methanol and lyophilization gave pure **6** (140 mg, 60%). mp: 95−97 °C. $[\alpha]_D^{20}$ +22.5° (*c* 0.020; $CHCl_3$/MeOH: 4/1). ^{1}H NMR, $CDCl_3$, ϵ CD_3OD, δ (ppm), J (Hz): 1.87 (s, 3H, CCC*H*$_3$); 3.02 (dd, 1H, H-5′α, J = 13.72, 6.5); 3.13 (dd, 1H, H-5′*β*, J = 13.72, 6.1); 4.31 (m, 1H, H-4′); 4.32 (dd, 1H, H-3′, J = 2.27, 5.2) 4.39 (dd, 1H, H-2′, J = 5.2, 3.6); 6.04 (d, 1H, H-1′, J = 3.6); 8.19 and 8.29 (2 s, 2H, H-2 and H-8). ^{13}C NMR, $CDCl_3$, ϵ CD_3OD, δ (ppm): 4.4 (CC*C*H$_3$); 39.2 (C-5′); 68 (*C*CCH$_3$); 73.8 (C-4′); 74.8 (C-3′); 84.8 (C-2′); 89.0 (C*C*CH$_3$); 90 (C-1′); 119.4 (C-6); 141.2 and 153.8 (C-2 and C-8); 150 and 157.3 (C-4 and C-5). HRMS (CI/NH_3) calcd C_{13} $H_{15}O_3S$ + H 322.0973, found 322.0837. Anal. ($C_{13}H_{15}O_3S$) C, H, N.

5′-*S*-Propionyl-5′-thioadenosine 7. To a dry oxygen-free methanol solution (15 mL) of thioacetate **2** (1 g, 2.13 mmol) was added 2 equiv of sodium methoxide (226 mg, 4.26 mmol) at 0° under argon. Stirring was continued for 8 h at room temperature. To the resulting corresponding debenzoylated thiolate, a solution of propionic anhydride (0.38 mL, 3 mmol) in 3 mL of methanol was added at 0 °C. The reaction mixture allowed to warm to room temperature, and stirring was continued for 3 h. Methanol was evaporated under reduced pressure, and the crude product obtained was taken in 3 mL of formic acid/water solution (8/2). After heating at 40° for 1.5 h, formic acid was evaporated and the crude material neutralized (2 N NH_4OH). Crude **7** was purified on a column of hydrophobic resin HP20SS (elution: methanol/water 3/7). Evaporation of methanol and lyophilization gave **7** (430 mg, 60%). mp: 60 °C. MS (DCI/NH_3): 340 $(MH)^+$. $[\alpha]_D^{20}$ −6.1° (*c* 0.392, MeOH). IR (KBr): 1650 cm^{-1}. ^{1}H NMR, DMSO-d_6, δ (ppm), J (Hz): 1.10 (t, 3H, J = 7.6); 2.6 (q, 2H, J = 7.6); 3.15 (dd, 1H, H5′α, J = 13.7, 7.3); 3.40 (dd, 1H, H5′*β*, J = 13.7, 5.2); 3.90 (m, 1H, H-4′); 4.10 (m, 1H, H-3′); 4.80 (ddd, 1H, H-2′, J = 5.6, 5,7, 5.6); 5.40, 5.60 (2d, D_2O exchangeable 3′- and 2′-OH); 5.90 (d, 1H, H-1′, J = 5.7); 7.35 (s, 2H, NH_2); 8.15 and 8.40 (2 s, 2H, H-2 and H-8). ^{13}C NMR, DMSO-d_6, δ (ppm): 9.5 (*C*H_3-CH_2C(O)S); 30.9 (CH_3-*C*H_2C(O)S); 36.9 (C-5′); 72.6 (C-3′ and C-4′); 74.2; 83.0 (C-2′); 87.6 (C-1′); 119.2−156.0 (C-adenine); 199.3 *C*(O). Anal. ($C_{13}H_{17}N_5$ O_4S) C, H, N.

Enzyme Purification. The recombinant human placental AdoHcy hydrolase was purified from cell-free extracts of JM 109 *Escherichia coli* transformed with the plasmid pPROK cd 20 according to the procedure described by Yuan et al.[6a] for the first steps of purification, including ammonium sulfate fractionation, DEAE Cellulose, and Sephacryl S200. The purification was continued by FPLC. Aliquots of S200 purified AdoHcy hydrolase (2 mL) were recycled twice on a Mono QHR 10/10 column equilibrate 1 with 10 mM Tris/HCl, 1 mM dithiothreitol, pH 7.8 and developed with a gradient from 0 to 0.35 M NaCl at a flow rate of 4 mL/min. Active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 100 vol of 50 mM sodium phosphate, pH 7.4, 1 mM dithiothreitol. The homogeneity of the AdoHcy hydrolase preparation was checked by ESI-MS, the major molecular species being detected at 47604.8 ± Da for each enzyme subunit (Figure 5, panel A).

Assay of AdoHcy Hydrolase Activity. AdoHcy hydrolase activity was assayed in the synthetic direction by measuring the rate of formation of [8-^{14}C]-AdoHcy from [8-^{14}C]-Ado and Hcy as described previously by Della Ragione et al.[20]

Enzyme was mixed with 10 μM [8-^{14}C]-Ado (17 800 dmp), 5 mM D,L Hcy, 20 mM potassium phosphate buffer pH 7.5, 1 mg/mL BSA, and 1 mM EDTA in a final volume of 50 μL. The assay mixture was incubated at 37 °C for 10 min and the reaction stopped by addition of 150 μL of 15 mM HCl. A total of 150 μL of the sample was then applied to a microcolumn (0.5 mL) of Cellex P equilibrated with 10 mM HCl. The remaining [8-^{14}C]-Ado was eluted first with 5 mL of 10 mM HCl and then the [8-^{14}C]-AdoHcy formed with 5 mL of 0.5 N HCl. The eluates were directly poured into scintillation vials with 7 mL of Ultima Flow AP (Packard) for counting. Under these conditions, the velocity of AdoHcy hydrolase showed normal Michaelis−Menten kinetics with the K_m value for Ado being 1 μM, in accord with the literature value.[21]

Determination of NAD^+ and NADH Content of AdoHcy Hydrolase. NAD^+ and NADH present in enzyme before and after inactivation with **4**, **5**, and **6** were mesured as previously described[6a,22] by monitoring the intrinsic fluorescence emitted by NADH at 460 nm upon excitation at 340 nm. NADH was measured directly whereas NAD^+ was first converted to NADH by Baker's yeast alcohol dehydrogenase in the presence of ethanol.

(a) Sample Preparation. AdoHcy hydrolase (20 μM) was incubated in 200 μL of assay buffer with (600 μM) of **4**, **5**, or **6** at 37 °C. After total inactivation, both NAD^+ and NADH were released from the enzyme by addition of 500 μL of ethanol and centrifuged (10000*g* for 5 min) to sediment precipitated material. The precipitate was washed twice with 200 μL of ethanol, and the pooled supernatants were lyophilized in the dark. The residue was dissolved in 0.4 mL of H_2O and 0.6 mL of 100 mMphosphate buffer, pH 8.8. The resulting solution was analyzed for NAD^+ and NADH.

(b) Effect of Adenosine on the Inactivation of AdoHcy Hydrolase by 4, 5, and 6. AdoHcy hydrolase (0.1 μg) was preincubated in 50 μL of assay buffer for 20 min at 37 °C with the inactivators **4**, **5**, or **6** alone or in the presence of adenosine (14−28 μM). Remaining activity was assayed by the transfer of a 10 μL portion of the incubation mixture to 40 μL of standard assay mixture.

Analysis of Reaction Products. AdoHcy hydrolase (28 μM) was incubated with **4**, **5**, or **6** (0.2 mM) in 200 μL of 20 mM ammonium acetate, 1 mM EDTA, 1 mg/mL BSA, pH 7.5, without or in the presence of (2.5 mM) D,L-homocysteine. After inactivation of the enzyme was completed, the reaction mixtures were filtered through an ultrafree-MC, 10000 Millipore filter. The filtrates were analyzed by liquid chromatography/electrospray ionization-mass spectrometry. The filtrates were injected into an HPLC column (Spherisorb, C18, 5 μM, 250 × 4.6 mm, Interchrom). The column was eluted at a flow rate of 1 mL/min in gradients of 20−80% (MeOH/H_2O) over 8 min and 40−60% (MeOH/H_2O) over 30 min. The effluent was flow−split with 1/10 of the flow directed toward the ESI mass spectrometer and the residual 9/10 directed toward the UV detector. ESI-MS data were recorded in the positive ion mode. In these conditions, retention time for AdoHcy, Ado, **4**, **5**, and **6** were 5.3, 7.5, 26.4, 28.7, and 30.3 min, respectively, and the compounds were characterized by their molecular peaks (MH^+). In experiments with **5** and **6**, in addition to Ado, another peak of *m*/*z* 271 was detected at 11.4 min retention time. It has been attributed to 5′-thioadenosine.

Chemical Stability of 5 and 6. Compounds **5** and **6** proved to be stable in assay buffer for several days at 37 °C (HPLC control) but were subjected to facile acid hydrolysis. Compound **6** was quantitatively transformed into 5′-*S*-propionyl-5′-thioadenosine **7** when treated with aqueous HCOOH (80%) at 40 °C for 1 h. Under the same conditions, **5** led to the formation of **7** and a substantial amount of 5′-thioadenosine (NMR characterization).

Molecular Modeling. The structure of AdoHcy has been extracted from the Brookhaven Database (PDB entry 1A7A).

Inhibitor **5** has been built with the molecular modeling software QUANTA 98.[23] Its geometry has been optimized using the CHARMm force field.[24,25] The compound has been docked manually from the information on the position and interactions of the inhibitor cocrystallized in the 1A7A structure. When unfavorable steric interactions occurred, the amino acid side chains were manually reoriented. No subsequent geometry optimization has been performed, as we were mainly interested in a visual investigation of the possibility of a reaction between the water molecule present in the enzymatic active site and the C-6′ carbon of **5**.

Mass Spectrometry. Abbreviations: MS (mass spectrometry), ESI (electrospray ionization), MALDI (matrix-assisted laser desorption ionization), LC (liquid chromatography).

Samples of AdoHcy hydrolase were desalted by a five dilution−concentration step dialysis (5 × 60 min) in 10 mM ammonium acetate (pH 7.0) using Centricon PM3 concentrators (Millipore). This desalting procedure allows the removal of the inorganic salts that broaden the signal in mass spectrometric analysis.

Accurate Mass Measurement of AdoHcy Hydrolase. AdoHcy hydrolase (purified enzyme or enzyme inactivated by **5**) was diluted to 5 μM in a 1:1 H_2O-CH_3CN mixture (v/v), acidified with ca. 1% HCOOH. Mass data were collected in the positive ion mode. Scanning was performed in the range 500−2000 *m*/*z* with a declustering voltage (V_c) of 45 V. Calibration of the instrument is performed using the multiply charged ions produced by horse heart myoglobin (2 μM in 49.5:49.5:1 (v/v) $H_2O-CH_3CN-HCOOH$).

Enzymatic Proteolytic Cleavage of AdoHcy Hydrolase. An aliquot of approximately 150 μg of AdoHcy hydrolase (purified enzyme or enzyme inactivated by **5**) was incubated with either 1.2 μg of Endo-LysC (Boehringer-Mannheim) or 5 μg of Trypsin (Promega). Endo-LysC specifically cleaves peptide bonds C-terminally at Lys; Trypsin specifically cleaves peptide bonds C-terminally at Arg and Lys. For both enzymes, the proteolysis was carried out overnight at 35 °C in 25 mM NH_4HCO_3 (pH 8.5); note that the final volume of the sample never did exceed 60 μL. Proteolysis was stopped by freezing the samples at −20 °C.

Matrix-Assisted Laser Desorption Ionization-Mass Spectrometry (MALDI- MS). Cleavage products of AdoHcy hydrolase were mass-analyzed without prior separation on a BIFLEX MALDI-time of flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with the SCOUT high-resolution optics and a gridless reflector. Mass data were collected in the reflector positive mode at a maximum accelerating potential of 20 kV. Ionization was accomplished with a pulsed nitrogen laser beam ($\lambda = 337$ nm) at a repetition rate of 3 Hz. External calibration of the mass spectrometer was performed in the range 1000−2500 *m*/*z* by using the singly charged ions produced by four peptides (Angiotensin, P-Substance, Bombesin and ACTH).

A saturated solution of α-cyano-4-hydroxycinnamic acid in acetone was used as a matrix. Samples were prepared as follows: first a layer of fine crystals of matrix was obtained by spreading and fast evaporating 0.5 μL of matrix solution. A 0.5 μL droplet of aqueous HCOOH (5% in water) was then deposited on this first matrix crystals layer. A droplet (0.5 μL) of the cleavage products obtained by proteolysis of AdoHcy hydrolase was subsequently deposited. Finally, a 0.2 μL droplet of matrix saturated in a 1:1 (v/v) H_2O-CH_3CN mixture was added. The preparation was dried under vacuum and washed three times with 1 μL of aqueous HCOOH.

Liquid Chromatography/Electrospray Ionization-Mass Spectrometry (LC/ESI-MS). The chromatography was carried out on an Alliance HPLC system (Waters 2690) equipped with a diode array detector (Waters 996). In this study, absorbances were monitored at 214 nm with a chromatography manager Millennium 2.15.

After enzymatic proteolytic cleavage of AdoHcy hydrolase, an aliquot of 35 μL of the peptides mixture was loaded on a Nucleosil 300-5C18 column (Macherey Nagel, 2.1 mm × 125 mm). The solvent system used for elution of the peptides consisted of 0.1% TFA in H_2O (solvent A) and 0.08% TFA in CH_3CN (solvent B); elution was performed at a flow rate of 250 μL/min using a biphasic gradient of 2% B (5 min), 2−60% B (60 min), 60−80% B (5 min), and 80% B (5 min), successively. The effluent was flow-split via a stainless steel Valco tee (Supelco) with 1/15 of the flow directed toward the ESI mass spectrometer and the residual 14/15 directed toward the UV-detector. Fractions of the residual effluent were hand-collected.

ESI-MS data were recorded in the positive ion mode using a VG BioQ triple-quadrupole mass spectrometer. Scanning was performed in the range 200−2000 *m*/*z* at a scan rate of 6 s/scan, with a declustering voltage (V_c) of 35 V. Before coupling the chromatography system to the mass spectrometer, an external calibration of the mass spectrometer was performed using the multiply charged ions produced by horse heart myoglobin (2 μM in 49.5:49.5:1 (v/v) $H_2O-CH_3CN-HCOOH$).

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