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Inactivation of human *S*-adenosylhomocysteine hydrolase by covalent labeling of cysteine 195 with thionucleoside derivatives

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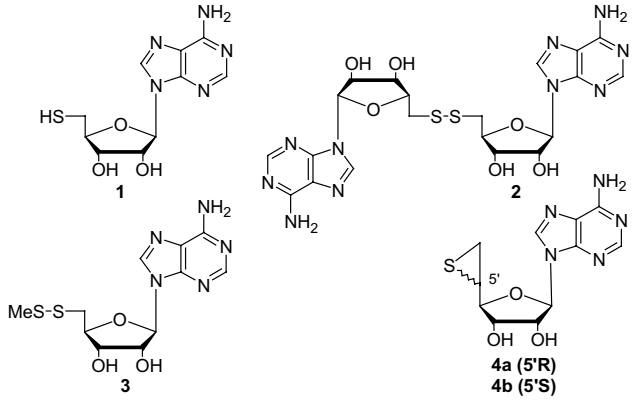
Abstract—A new series of 5′-thioadenosine derivatives **1**–**4** were synthesized for selectively targeting ^{195}Cys of human AdoHcy hydrolase. Their incubation with the enzyme resulted in time- and concentration-dependent inactivation, without major modifications of the NAD^+/NADH ratio. The electrospray mass analysis of the inactivated enzyme with **1**, **2**, **3**, and **4b** showed that inhibition was accompanied by the formation of a specific and covalent labeling of each AdoHcy hydrolase subunit. Proteolytic cleavage (endo-Lys-C) and subsequent peptide characterization of the labeled enzyme revealed that ^{195}Cys was the residue modified during the inactivation process.

1. Introduction

S-Adenosylhomocysteine (AdoHcy) hydrolase catalyses the interconversion of AdoHcy into adenosine (Ado) and L-homocysteine (Hcy).[1] Inhibition of this enzyme results in intracellular accumulation of AdoHcy which in turn provokes feed back inhibition of *S*-adenosylmethionine-dependent methylation reactions (i.e., viral mRNA methylation) which are essential for viral replication.[2] AdoHcy hydrolase also controls levels of Hcy, which appear to be a risk factor for cardiovascular diseases.[3] Therefore, AdoHcy hydrolase has emerged as a target for molecular design of antiviral agents as well as therapeutic inhibitors that may restore normal plasma levels of Hcy.

The recent success in the determination of the X-ray structures of rat liver[4] and human[5] AdoHcy hydrolase has led to the identification of the essential amino acids involved in the different steps of the catalytic cycle. Among them, the ^{194}Cys residue in rat liver enzyme (^{195}Cys in human enzyme) was proposed to modulate the oxidation state of the bound cofactor NAD^+.[4a] This proposal is consistent with the results obtained by Yuan et al. in their pioneer study on chemical modification and site-directed mutagenesis of cysteine residues in AdoHcy hydrolase.[6] These two details led us to consider that specific covalent modification of ^{195}Cys should lead to the inactivation of human AdoHcy hydrolase. This possibility was examined with a series of thionucleosides **1**–**4**.



These sulfur-containing nucleosides fulfill the basic requirements of being active-site-directed inhibitors for selectively targeting ^{195}Cys. Disufide bond formation resulting from their interaction with ^{195}Cys at the active site might provoke covalent inactivation of AdoHcy hydrolase (Fig. 1).

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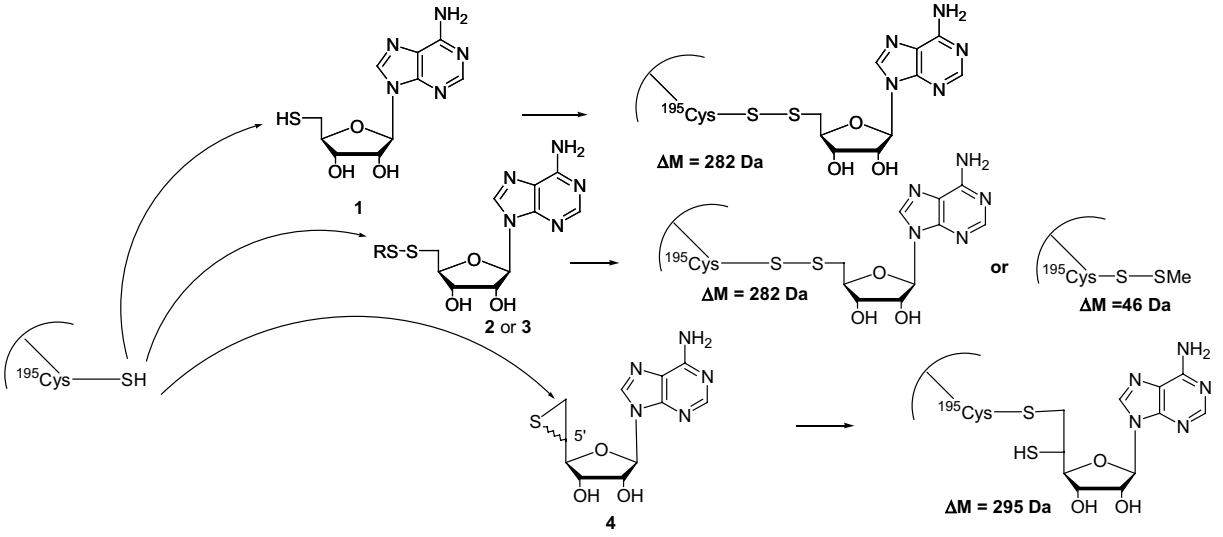


Figure 1. Proposed mechanisms for inactivation of AdoHcy hydrolase by **1**–**4**.

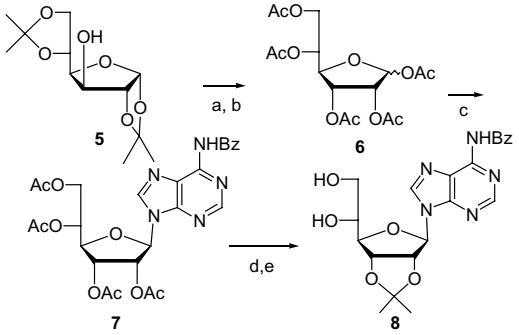
We describe here the synthesis of thionucleosides **1**–**4** and their interactions with human placental AdoHcy hydrolase.

2. Chemistry

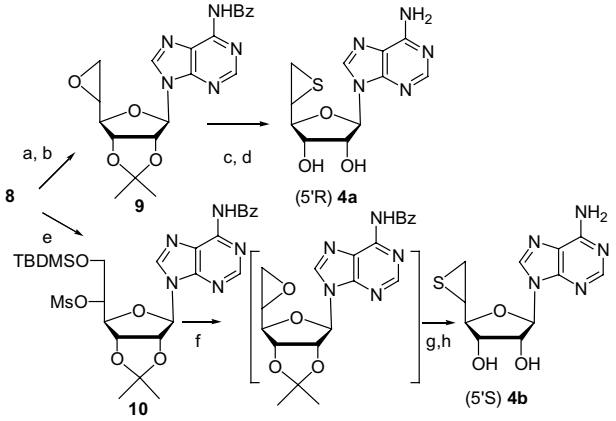
5′-Deoxy-5′-thioadenosine **1** was obtained from Ado by a described procedure.[7] The corresponding disulfide **2** was prepared by iodine oxidation of **1**.[8] The disulfide **3**, which was synthesized according to the general procedure described by Décout and co-workers,[9] was a gift from these authors. The epithionucleosides **4a** and **4b** were obtained from an unique precursor: the N^6-benzoyl-2′,3′-*O*-isopropylidene-9-(β-D-allofuranosyl)adenine **8**, which was prepared from diacetone-D-glucose **5** by the procedure outlined in Scheme 1.

After standard inversion of stereochemistry at C_3 and acidic cleavage of the two 1,2- and 5,6-isopropylidene groups, the D-allofuranoside derivative thus obtained was fully acetylated and condensed with N^6,N^9-bis(trimethylsilyl)adenine.[10] Next, compound **7** was deacetylated and the selective protection of the 2′- and 3′-positions was achieved in two steps to generate the desired allofuranosyladenine derivative **8** in a 22% overall yield. The β-D configuration of **8** was confirmed by ^{1}H NMR ($J_{1',2'}$ = 3 Hz and $\Delta\delta$ = 0.2 ppm between the chemical shifts of the two CH_3's of the 2′,3′-isopropylidenated nucleoside).[11] The epoxynucleoside **9** prepared in two steps from **8** was treated with thiourea leading to the corresponding epithionucleoside with inversion of configuration at $C_{5'}$ (Scheme 2).[12]

Removal of the isopropylidene protecting group led to thiirane **4a**[13] in 15% yield from **8**. Its 5′ epimer **4b**[14] was obtained by the same methodology via the silylated allofuranosyladenine derivative **10** in 19% overall yield from **8**.



Scheme 1. Reagents and conditions: (a) PDC, Ac_2O, CH_2Cl_2, then $NaBH_4$, EtOH, 83%; (b) CH_3CO_2H, Ac_2O, APTS, 79%; (c) N^6,N^9-bis(trimethylsilyl)adenine, $SnCl_4$, dichloroethane, 70%; (d) NaOH 2 M, EtOH, 70%; (e) dimethoxypropane, acetone, APTS then CH_3CO_2H/H_2O 7/3, 70%.



Scheme 2. Reagents and conditions: (a) TsCl, pyridine, 60%; (b) NaH, THF, 89%; (c) thiourea, MeOH, 55%; (d) HCO_2H/H_2O 4/1 then MeONa, MeOH, 50%; (e) TBDMSCl, imidazole, pyridine then MsCl, pyridine, 50%; (f) TBAF, THF; (g) thiourea, MeOH, 77%; (h) HCO_2H/H_2O 4/1 then MeONa, MeOH, 50%.

3. Results and discussion

Recombinant human placental AdoHcy hydrolase purified to homogeneity was used in this study.[15] AdoHcy hydrolase (10 nM) was assayed in the synthetic direction in the presence of [8-^{14}C]-Ado or [2,8-^{3}H]-Ado (15 μM, 300 Bq) and Hcy (5 mM) in 20 mM potassium phosphate buffer pH 7.5, 1 mM EDTA. Incubation of the enzyme with **1**, **2**, **3**, **4a**, and **4b** resulted in time- and concentration-dependent inactivation. Compounds **1**–**4** are stable under assay conditions whereas thiol **1** became contaminated with 15% of disulfide **2** after 6 h at 37 °C. In each case, the inactivation was irreversible since enzyme activity could not be restored after dialysis (24 h) against assay buffer. We confirmed that the action of each of the thionucleosides **1**–**4** was active site directed by means of protection experiments with Ado. Using the Kitz and Wilson method[16] 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 in Table 1.

The effects of **1**–**4** on the NAD$^+$/NADH content of AdoHcy hydrolase were also determined after complete inactivation of the enzyme. Compounds **1**, **2**, **3**, and **4a** led to minor changes in the initial NAD$^+$/NADH ratio whereas 30% depletion of the enzyme's NAD$^+$ was observed with **4b** (Fig. 2).

This is indicative that the redox activity of the enzyme is not involved in the inactivation process with **1**, **2**, **3**, and **4a** but it might partially participate in the pathway by which compound **4b** proceeds to inactivate AdoHcy hydrolase.

The mechanism of inactivation was further investigated using electrospray ionization mass spectrometry (ESI-MS) analysis of the protein. An accurate molecular weight of AdoHcy hydrolase subunit for the native or inactivated protein with **1**–**4** was determined from the wide distribution of highly charge states observed in positive ion ESI-MS analysis under acidic conditions.[18] An algorithm (MaxEnt) based on the maximum entropy method was used to produce true molecular mass spectra from multiply charged electrospray spectra. The native enzyme had a subunit molecular weight of 47,597 ± 6 Da (Fig. 3).

The mass modifications carried by each AdoHcy hydrolase subunit upon interaction of the enzyme with inhibitors **1**–**4** are listed in Table 2 and illustrated in Figure 4 for compound **3**.

Inhibition of the enzyme with thionucleosides **1**, **2**, **3**, and **4b** was accompanied by a specific covalent linkage and no further native enzyme was detected after total inactivation.

These results support the mechanistic proposal described in Figure 1 involving the formation of a covalent disulfide bound between a cysteine of the active site and each of the sulfur-containing inhibitors **1**, **2**, **3**, and **4b**. To confirm the implication of the ^{195}Cys in the inactivation process, AdoHcy hydrolase inhibited by the

Table 1. K_i and k_{inact} values for the inhibitory effect of **1**–**4** on AdoHcy hydrolase

Compd	K_i, μM	k_{inact}, min^{-1}
1	4	0.04
2	96	0.05
3	19	0.05
4a	105	0.10
4b	50	0.03

AdoHcy hydrolase (2 μM) was incubated with inhibitor at various concentrations and various times in 20 mM potassium phosphate buffer pH 7.5, 1 mM EDTA at 37 °C. Residual activity was determined as described and a double reciprocal plot of the initial pseudo-first-order inactivation rate constant versus $1/[I]$ gave the K_i and k_{inact} values.[16]

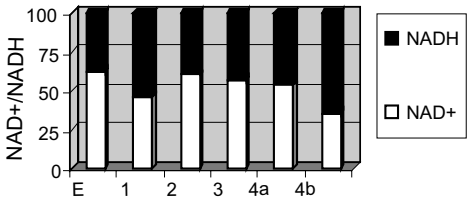


Figure 2. Variation of NAD$^+$/NADH content upon incubation with inhibitors: AdoHcy hydrolase (20 μM) was incubated with 600 μM of **1**–**4** in 20 mM potassium phosphate buffer pH 7.5, 1 mM EDTA at 37 °C until total inactivation. NAD$^+$ and NADH in native AdoHcy hydrolase (E) and after inactivation (**1**, **2**, **3**, **4a**, and **4b**) were measured by a fluorescence method.[17]

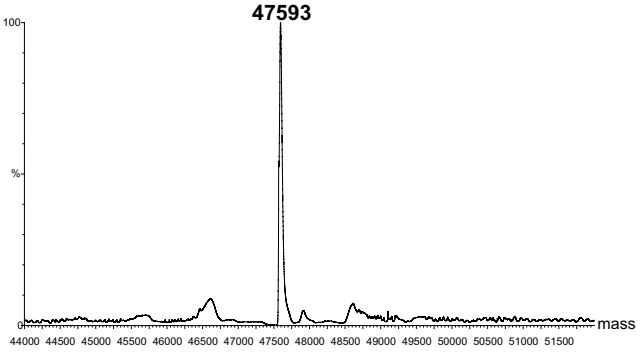


Figure 3. Reconstructed ESI mass spectrum (MaxEnt) obtained for purified AdoHcy hydrolase.

Table 2. Mass excess observed for each subunit of AdoHcy hydrolase inactivated by **1**–**4**

Inhibitor	Δ*M* observed, Da ±2
1	283
2	281
3	46
4a	None
4b	293

Native AdoHcy hydrolase or enzyme inactivated by **1**–**4** was diluted to 1 μM in a H_2O/CH_3CN mixture (v/v) acidified with ca. 1% HCOOH and was analyzed by ESI-MS.[19]

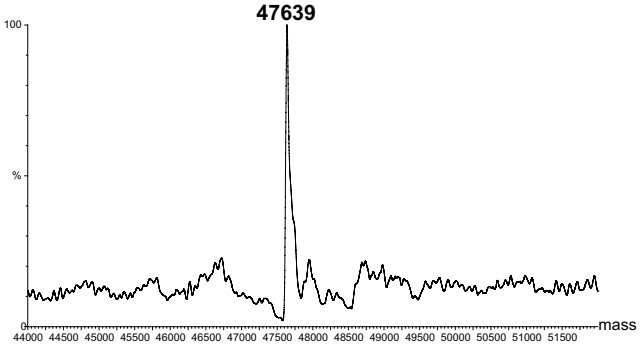


Figure 4. Reconstructed ESI mass spectrum (MaxEnt) obtained for AdoHcy hydrolase inhibited by **3**.

disulfide **2** was submitted to proteolytic cleavage by endo-Lys-C.[20] The cleavage products (i.e., a mixture of peptides C-terminally cleaved at Lys residues) were mass analyzed by LC/ESI-MS[21] and compared to those obtained from native AdoHcy hydrolase.

The ^{195}Cys containing peptide was characterized in the HPLC chromatogram of the digested native AdoHcy hydrolase (FDNLYG^{195}CRESLIDGIK, m/z = 922.01 Da, doubly charged ion, molecular weight 1841.89 Da). This peptide was absent in the peptide map in the experiments carried out with inactivated enzyme, but a new peptide was detected in the corresponding HPLC chromatogram. This peptide was analyzed using on line ESI-MS detection. The peptidic fragment (m/z = 1062.55 Da, doubly charged ion, molecular weight 2123.08 Da) was attributed to the same peptide that bears an adduct of 281 Da: FDNLYG^{195}CRESLIDGIK +281 Da. This result strongly suggests that ^{195}Cys was the residue modified in the inactivation process as illustrated in Figure 1. In similar experiments carried out with the disulfide **3**, we confirmed that an excess mass of 46 Da was bound to the same peptidic fragment (FDNLYG^{195}CRESLIDGIK +46 Da), a result consistent with a chemical modification of ^{195}Cys into a methyl disulfide adduct (Fig. 1).

4. Conclusion

We have identified a new series of inhibitors of AdoHcy hydrolase, which covalently modify the enzyme by interactions with ^{195}Cys. These results confirm the crucial role of ^{195}Cys at the catalytic center of AdoHcy hydrolase. Docking experiments with the epithionucleosides **4a** and **4b** into the active site of AdoHcy hydrolase are under progress to understand their different behaviors.

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13. Compound **4a**: ^{1}H NMR (DMSO-d_6, 250 MHz, δ ppm, JHz, 323 K) 2.40 (d, 1H, $J_{6'a,5'}$ 5.0, $H_{6'a}$); 2.60 (d, 1H, $J_{6'b,5'}$ 5.0, $H_{6'b}$); 3.37 (dt, 1H, $J_{5',4'}$ 8.0, $J_{5',6'}$ 5.0, $H_{5'}$); 3.55 (dd, 1H, $J_{4',5'}$ 8.0, $J_{4',3'}$ 5.0, $H_{4'}$); 4.35 (dd, 1H, $J_{3',2'}$ 10.0, $J_{3',4'}$ 5.0, $H_{3'}$); 4.75 (dd, 1H, $J_{2',3'}$ 10.0, $J_{2',1'}$ 5.0, $H_{2'}$); 5.90 (d, 1H, $J_{1',2'}$ 5.0, $H_{1'}$); 8.15 and 8.40 (s, 2*1H, H_2 and H_8). ^{13}C NMR (DMSO-d_6, 62.5 MHz, δ ppm) 21.6 ($C_{6'}$); 36.2 ($C_{5'}$); 73.5 ($C_{4'}$); 79.2 ($C_{3'}$); 87.5 ($C_{2'}$); 88.1 ($C_{1'}$); 119.2 (C_5); 139.7 (C_6); 149.4 (C_8); 152.7 (C_2); 156.1 (C_4). $[\alpha]_D^{20}$ +22.5 (c 6×10^{-3}, $CHCl_3$/MeOH 4/1). MS m/z 296 (22, MH^+); 264 (60); 157 (55); 136 (100). Anal. Calcd for $C_{11}H_{13}N_5O_3S{\cdot}0.5H_2O$: C, 43.41; H, 4.64; N, 23.01. Found: C, 43.22; H, 4.27; N, 22.46.
14. Compound **4b**: ^{1}H NMR (DMSO-d_6, 250 MHz, δ ppm, J Hz, 323 K) 2.35 (d, 1H, $J_{6'a,5'}$ 5.0, $H_{6'a}$); 2.60 (d, 1H, $J_{6'b,5'}$ 5.0, $H_{6'b}$); 3.47 (m, 1H, $H_{5'}$); 3.57 (dd, 1H, $J_{4',5'}$ 9.0, $J_{4',3'}$ 3.0, $H_{4'}$); 4.25 (dd, 1H, $J_{3',2'}$ 5.0, $J_{3',4'}$ 3.0, $H_{3'}$); 5.00 (dd, 1H, $J_{2',3'} = J_{2',1'} = 5.0$, $H_{2'}$); 5.55 (d, 1H, $J_{1',2'}$ 5.0, $H_{1'}$); 8.15 and 8.40 (s, 2*1H, H_2 and H_8). ^{13}C NMR (DMSO-d_6, 62.5 MHz, δ ppm) 24.1 ($C_{6'}$); 34.4 ($C_{5'}$); 72.2 ($C_{4'}$); 73.3 ($C_{3'}$); 87.2 ($C_{2'}$); 88.7 ($C_{1'}$); 119.2 (C_5); 140.0 (C_6); 149.5 (C_8); 152.6 (C_2); 156.1 (C_4). $[\alpha]_D^{20}$ −50 (c 3×10^{-3}, $CHCl_3$/MeOH 4/1). MS m/z 296 (6, MH^+); 264 (23); 157 (31); 136 (100). Anal. Calcd for $C_{11}H_{13}N_5O_3S{\cdot}0.5H_2O$: C, 43.41; H, 4.64; N, 23.01. Found: C, 43.39; H, 4.36; N, 23.02.
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19. ESI-MS data were obtained in the positive ion mode on a quadrupole instrument (Waters-Micromass ZQ). The source and desolvation temperatures were kept at 80 and 150 °C, respectively. Nitrogen was used as a drying and nebulizing gas at flow rates of 250 and 50 L/h, respectively. The capillary voltage was 3 kV and the cone voltage 40 V. The mass range was 400–2400 Da and the spectra were recorded at 4 s/scan in the profile mode. Calibration of the instrument was performed using the multiply charged ions produced by horse heart myoglobin. Data acquisition and processing were performed with MassLynx V4.0 software.
20. 150 μg of AdoHcy hydrolase, native or inactivated by **2**, was incubated with 1.2 μg of endo-Lys-C (Roche) in 25 mM NH_4HCO_3 buffer pH 8.5 at 35 °C overnight. Proteolysis was stopped by freezing the samples at −20 °C.
21. The chromatography was carried out on an Alliance HPLC system (Waters 2695) equipped with a UV detector. Absorbances were monitored at 210 nm. The peptide mixture was loaded on a Nucleosil 100-5C18 column, 150 × 4.6 mm (Macherey Nagel). Elution was performed at a flow rate of 1 mL/min using a biphasic gradient of A (0.1% TFA in H_2O) and B (0.08% TFA in CH_3CN): 2% B (5 min), 2–60% B (60 min), 60–80% B (5 min), and 80% B (5 min). The effluent was flow-split via a peek tee with 1/5 of the flow directed toward the ESI source of the ZQ mass spectrometer and the residual 4/5 directed toward the UV detector. LC/ESI-MS data were recorded in the positive ion mode with a capillary voltage of 3 kV and a cone voltage of 45 V. Scanning was performed in the range 200–2200 Da at a scan rate of 2 s/scan.