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Letters

Inactivation of *S*-Adenosyl-L-homocysteine Hydrolase by 6′-Cyano-5′,6′-didehydro-6′-deoxyhomoadenosine and 6′-Chloro-6′-cyano-5′,6′-didehydro-6′-deoxyhomoadenosine. Antiviral and Cytotoxic Effects

Georges Guillerm,[§] Murielle Muzard,[§,*] Cédric Glapski,[§] Serge Pilard,[†] and Erik De Clercq[‡]

Laboratoire de Chimie bioorganique, UMR 6519, UFR Sciences, B.P. 1039, 51687 Reims Cedex 2, France and Plate-Forme Analytique, Université de Picardie Jules Verne, 33 rue Saint-Leu, 80039 Amiens, France, and Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

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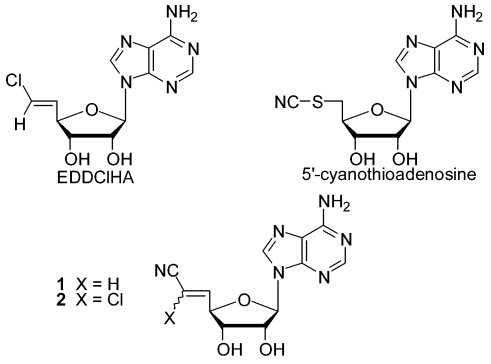
Abstract: 6′-Cyano-5′,6′-didehydro-6′-deoxyhomoadenosine (*E*)-**1**, (*Z*)-**1**, and 6′-chloro-6′-cyano-5′,6′-didehydro-6′-deoxyhomoadenosine (*E*)-**2** were synthesized and tested as new mechanism-based inhibitors of AdoHcy hydrolase. Nucleoside (*E*)-**1** was identified as a type I inhibitor of the enzyme, whereas inactivation of the enzyme by nucleosides (*Z*)-**1** and (*E*)-**2** was accompanied by the formation of a covalent labeling of AdoHcy hydrolase.

S-Adenosyl-L-homocysteine (AdoHcy) hydrolase (EC 3.3.1.1) catalyzes the reversible hydrolysis of AdoHcy into adenosine (Ado) and homocysteine (Hcy) and thus controls intracellular levels of the two esssential metabolites, AdoHcy and Hcy. Intracellular accumulation of AdoHcy provokes feedback inhibition of *S*-adenosylmethionine-dependent methylation reactions which are essential for viral replication,[1] whereas Hcy levels appear to be a risk factor for cardiovascular diseases.[2]

A number of irreversible inhibitors of the enzyme, analogues of Ado, have been identified: they act as substrates for the 3′-oxidative activity of AdoHcy hydrolase and keep the enzyme in its inactive NADH form (type I inhibitor)[3] and/or they use its 5′/6′- hydrolytic activity (type II inhibitor).[4]

Chart 1. Structures of Type II Irreversible Inhibitors of AdoHcy Hydrolase and Structures of the Targeted Compounds **1** and **2**.



Among them, AdoHcy hydrolase was irreversibly inactivated by the (*E*)-5′,6′-didehydro-6′-chlorohomoadenosine (EDDClHA, Chart 1).[5] The type II inactivation observed in this case involved a rapid addition of water to the 5′,6′ double bond of EDDClHA and was concomitant with the reduction of the enzyme-bound NAD^+ to NADH. Dihalogenated analogues of EDDClHA were also recognized as substrates for the hydrolytic activity of the enzyme.[6] On the other hand, we have shown that 5′-*S*-cyano-5′-thioadenosine[7] (Chart 1) caused inactivation of the enzyme with only partial depletion of the enzyme's cofactor NAD^+. The proposed mechanism consisted of enzyme-mediated addition of water to the thiocyano group and subsequent formation of 5′-thioadenosine, a nucleoside which is known to induce a specific covalent linkage of the enzyme.[8]

These results led us to consider that nucleosides **1** and **2** (Chart 1) might also function as type II inhibitors by affecting the 5′/6′-hydrolytic activity of AdoHcy hydrolase. In particular, the 2-cyanovinyl conjugated group, which bears multiple electrophilic centers, might be easily susceptible to nucleophilic attack by the activated water molecule, or a nucleophilic residue, at the active site of the enzyme.

Synthesis of 6′-cyano-5′,6′-didehydro-6′-deoxyhomoadenosine **1** and 6′-chloro-6′-cyano-5′,6′-didehydro-6′-deoxyhomoadenosine **2** was accomplished via Wittig reaction of 5′-aldehyde

* To whom correspondence should be addressed. Phone: +33 3 26 91 32 38. Fax: +33 3 26 91 31 66. E-mail: murielle.muzard@univ-reims.fr.
[§] Laboratoire de Chimie bioorganique, UMR 6519, UFR Sciences, Reims, France.
[†] Plate-Forme Analytique, Université de Picardie Jules Verne, Amiens, France.
[‡] Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium.

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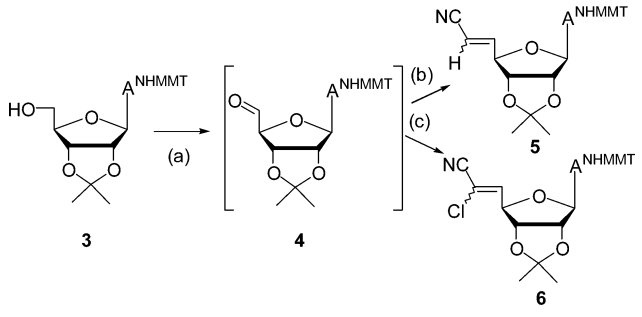
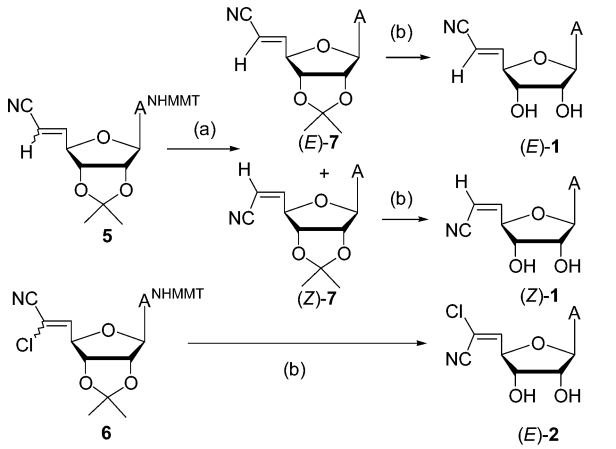
Scheme 1[a]



[a] Reagents: (a) DCC, DMSO, $CHCl_2COOH$; (b) $(CN)HC{=}PPh_3$; (c) $(CN)ClC{=}PPh_3$.

Scheme 2[a]



[a] Reagents: (a) acetic acid/water 4/1 (b) formic acid/water 1/1.

4 (Scheme 1). Key intermediate **4** resulted from Moffatt oxidation[9] of compound **3**, which was obtained by N^6 protection of 2′,3′-*O*-isopropylideneadenosine[10] by a monomethoxytrityl group. **4** was used without purification in the next step. Treatement of **4** with commercial cyanomethylenetriphenylphosphorane gave **5** in good yield (91%). Analogue **6** was obtained by the same procedure using cyanochloromethylenetriphenylphosphorane[11] (87%).

Compounds **5** and **6** were obtained as diastereoisomeric *Z*/*E* mixtures, with *E* as the major diastereoisomer. Configuration of the double bond for compound **6** was determined after deprotection of a (*Z*+*E*) mixture and comparison of $\delta_{H-5'}$ for each isomer, in agreement with Tronchet et al. results for the analogous thymidine[12a] and carbohydrate[12b] derivatives: $\delta_{H-5'}$ (*Z*-**2**) = 7.41 ppm > $\delta_{H-5'}$ (*E*-**2**) = 7.18 ppm.

Table 1. K_i and k_{inact} Values and $t_{1/2}$ for the Inhibitory Effect of **1** and **2** on AdoHcy Hydrolase

compound	K_i (μM)	k_{inact} (min^{-1})	$t_{1/2}$ (min)
(*E*)-**1**	–	–	10 [I][a] = 3 μM
(*Z*)-**1**	–	–	6 [I][a] = 100 μM
(*E*)-**2**	17	0.007	164 [I][a] = 5 μM

[a] Concentrations of inhibitor used to determine $t_{1/2}$.

Table 2. Effect of (*E*)-**1**, (*Z*)-**1**, and (*E*)-**2** on NAD^+ Content of AdoHcy Hydrolase

	% NAD^+ [a]
native enzyme	100
enzyme inactivated by (*E*)-**1** (100)[b]	34
enzyme inactivated by (*Z*)-**1** (60)[b]	73
enzyme inactivated by (*E*)-**2** (100)[b]	72

[a] AdoHcy hydrolase (10 μM) was incubated with 4 mM of inhibitor in assay buffer at 37 °C until significant or total inactivation was observed. The NAD^+ content was determined by HPLC as described in Supporting Information. [b] % inactivation.

Table 3. Mass Excess Observed for Each Subunit of AdoHcy Hydrolase

	ΔM observed, Da ± 2
enzyme inactivated by (*E*)-**1**, FW = 288	none
enzyme inactivated by (*Z*)-**1**, FW = 288	290
enzyme inactivated by (*E*)-**2**, FW = 322.5	321.5

The nonseparable mixture of (*E*)-**5** and (*Z*)-**5** was treated with acetic acid/water (4/1) to remove the methoxytrityl protecting group (Scheme 2). Isomers (*E*)-**7** and (Z)-**7** thus obtained were separated by silica gel chromatography.

Final deprotection of the isopropylidene group was achieved with formic acid/water (1/1) and afforded the (*E*)-6′-cyano-5′,6′-didehydro-6′-deoxyhomoadenosine (*E*)-**1** and (*Z*)-6′-cyano-5′,6′-didehydro-6′-deoxyhomoadenosine (*Z*)-**1**.

Accordingly, the nucleoside (*E*)-**6** was isolated in a pure form by careful chromatography after the Wittig reaction and then deprotected in one step to obtain (*E*)-6′-chloro-6′-cyano-5′,6′-didehydro-6′-deoxyhomoadenosine (*E*)-**2**. However, all our attemps to isolate pure (*Z*)-**6** failed, even after N^6 deprotection. In consequence, (*Z*)-**2** could not be obtained as a pure compound and thus was not tested.

Incubation of purified recombinant placental AdoHcy hydrolase[13] with nucleosides (*E*)-**1**, (*Z*)-**1**, and (*E*)-**2** resulted in time- and concentration-dependent inactivation of the enzyme (Figure 1).

For each compound, the inactivation observed was irreversible since enzyme activity could not be restored after dialysis (24 h) against assay buffer.

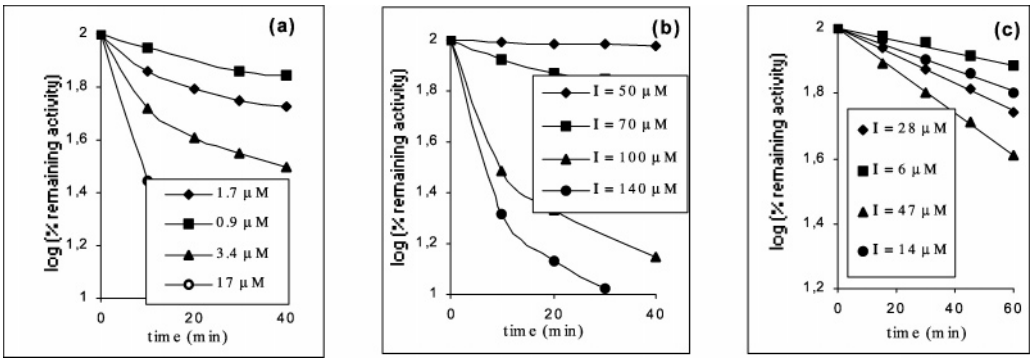


Figure 1. Time-dependent inactivation of AdoHcy hydrolase with (*E*)-1 (panel a), (*Z*)-1 (panel b), and (*E*)-2 (panel c). AdoHcy hydrolase was incubated with inhibitors at indicated concentrations in assay buffer at 37 °C, and remaining activity was determined in the synthetic direction as described in Supporting Information.

Table 4. Antiviral Activity and Cytotoxicity for Compounds (*E*)-**1**, (*Z*)-**1**, and (*E*)-**2** and Controls

	antiviral activity EC_{50} (μg/mL)[a]				cytotoxicity (μg/mL)	
compound	AD-169 strain	Davis strain	TK^+ VZV OKA strain	TK^- VZV 07/10 strain	cell morphology (MCC)[b]	cell growth (CC_{50})[c]
(*E*)-**1**	>20	>20	>20	>20	≥20	11
(Z)-**1**	>20	>20	>20	>20	100	>20
(*E*)-**2**	>20	>20	11	17	100	1.2
ganciclovir	3.2	3.2			≥400	34
cidofovir	0.49	0.64			≥400	23
acyclovir			0.22	14	>400	118
brivudin			0.0051	149	>400	>200

[a] Effective concentration required to reduce virus plaque formation by 50%. [b] Minimun cytotoxic concentration that causes a microscopically detectable alteration of cell morphology. [c] Cytotoxic concentration required to reduce cell growth by 50%.

Inactivation of AdoHcy hydrolase by (*E*)-**2** followed classical pseudo-first-order kinetics (Figure 1, panel c); K_i and k_{inact} values were determined using the Kitz and Wilson method (Table 1).[14]

(*E*)-**2** proved to be a potent AdoHcy hydrolase inhibitor displaying $K_i = 17$ μM but with a low value of 0.007 min^{-1} for k_{inact}. Compounds (*E*)-**1** and (*Z*)-**1** led to a biphasic system (Figure 1, panels a and b), showing pseudo-first-order kinetics only during the first period of inactivation ($t < 10$ min). For these two compounds, a $t_{1/2}$ was determined which allowed comparison of the inhibitory potency of each inhibitor (Table 1).

Experiments were then conducted in order to investigate the mechanism of inactivation of the enzyme by inhibitors **1** and **2**. In a first set of experiments, compounds (*E*)-**1**, (*Z*)-**1**, and (*E*)-**2** were incubated with reconstituted NAD^+ form AdoHcy hydrolase (100% NAD^+).[13] Significant inhibition of the enzyme was observed with only partial depletion of its NAD^+ content (Table 2).

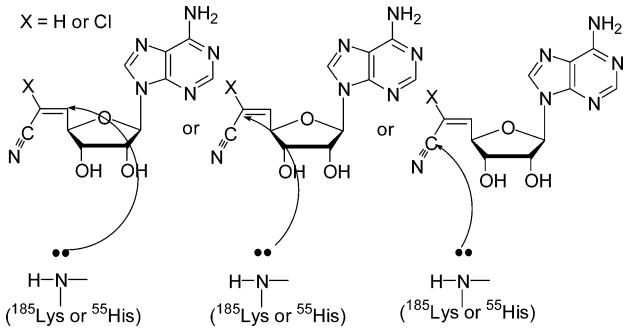
These results suggest that the enzyme's oxidative activity was not the only pathway by which compounds **1** and **2** inactivated AdoHcy hydrolase. Indeed nucleoside (*E*)-**1** caused an important conversion of the enzyme from its E-NAD^+ to the E-NADH form and appeared to be mainly a type I inhibitor. However, there was clearly another way of inactivation which could use the hydrolytic activity of the enzyme since the inactivated AdoHcy hydrolase was not completely reduced to its E-NADH form. Similar results were previously obtained by Wnuk et al. with sugar-modified diene[15a] and enyne[15b] analogues of adenosine. Inactivation of AdoHcy hydrolase with (*Z*)-**1** and (*E*)-**2** led to more than 70% NAD^+ unchanged.

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 (*E*)-**1**, (*Z*)-**1**, and (*E*)-**2** was determined from the wide distribution of highly charged states observed in positive ion ESI-MS analysis under acidic conditions. 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 47586.7 ± 0.4 Da. The mass modifications (ΔM) obtained after total inactivation of enzyme by inhibitors are listed in Table 3.

After total inactivation of AdoHcy hydrolase by compound (*E*)-**1** only unchanged native enzyme was detected in the mass spectrum. Thus inhibition of AdoHcy hydrolase occurred without covalent bonding of (*E*)-**1** or a corresponding metabolite. This result reinforces the above hypothesis suggesting a type I inhibitor behavior for (*E*)-**1**.

On the other hand, inactivation of enzyme with cyanovinyl nucleosides (*Z*)-**1** and (*E*)-**2** was accompanied by a specific covalent linkage, the observed ΔM corresponding to the molecular weight of the inhibitors concerned. The mechanism of inactivation seemed to proceed via a direct nucleophilic addition of an amino acid residue on the inhibitor at the active site of the enzyme. Crystallographic data on rat liver[16] and human[17] AdoHcy hydrolase and site-directed mutagenesis experiments[18] allowed the identification of the amino acids present at the active site. In particular, ^{55}His and ^{186}Lys (^{54}His and ^{185}Lys in rat liver AdoHcy hydrolase) were identified as essential amino acids involved in the substrate binding.[18] They were also assumed to participate as a base in their neutral form to the catalytic process.[19] Consequently, they could add to the C-5′/C-6′ double bond of inhibitors or to the CN group (Scheme 3).

Scheme 3. Proposed Mechanisms for Irreversible Inactivation of AdoHcy Hydrolase by Compounds (*Z*)-**1** and (*E*)-**2**



Interestingly, the reaction occurred only with (*Z*)-**1** and (*E*)-**2** featuring the CN group and nucleoside moiety on the same side of the double bond. The CN group's position might be essential to induce the appropriate binding interactions. It might also act as hydrogen-bond acceptor and become more reactive toward enzymatic nucleophiles.

Compounds (*E*)-**1**, (*Z*)-**1**, and (*E*)-**2** were evaluated for antiviral activity against cytomegalovirus (CMV) and varicella-zoster virus (VZV) in human embryonic lung cells (Table 4). No specific activity antiviral effects (i.e. minimal antivirally effective concentration ≥ 5-fold lower than minimal cytotoxic concentration) were noted for compounds against the tested CMV or VZV strains.

In conclusion, we have identified three new irreversible inhibitors of AdoHcy hydrolase: nucleoside (*E*)-**1** is a type I inhibitor, whereas nucleosides (*E*)-**2** and (*Z*)-**1** act as active-site directed irreversible inhibitors and covalently modify the enzyme without previous activation (3′-oxidation or 5′/6′-hydrolytic activity).

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Supporting Information Available: Experimental procedures for synthesis of targeted compounds, AdoHcy hydrolase assay,

inactivation of enzyme, determination of NAD+ content, ESI-MS of native and inactivated enzyme, HRMS and HPLC analyses of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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