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RESTRICTED ROTATION ANALOGS OF S-ADENOSYLMETHIONINE: SYNTHESIS, EVALUATION AS INHIBITORS OF S-ADENOSYLMETHIONINE DECARBOXYLASE, AND POTENTIAL USE AS SELECTIVE ANTITRYPANOSOMAL AGENTS

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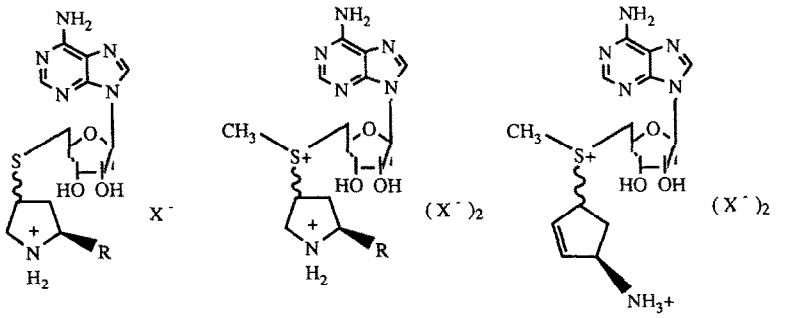
Abstract: A series of restricted rotation analogs of S-adenosylmethionine were synthesized and evaluated as inhibitors of S-adenosylmethionine decarboxylase from *Escherichia coli*. These analogs were also evaluated for inhibitory activity against cultured mammalian cells, and against the parasite *Trypanosoma brucei brucei*. All analogs tested appeared to selectively inhibit trypanosomal growth. In particular, compound **5** (AdoMac) inhibits the growth of *Trypanosoma brucei brucei* with an IC_{50} of 5.2 μM.

The enzyme S-adenosylmethionine decarboxylase (AdoMet-DC) is a controlling enzyme in the biosynthesis of polyamines in both mammalian and non-mammalian systems.[1] Because polyamines are absolutely required for rapid cell growth,[2] this enzyme has become an important target for the design of novel antitumor or antiparasitic agents. Our laboratories have been involved in the synthesis of restricted rotation analog inhibitors of S-adenosylmethionine for use as conformational active site probes. We recently reported the synthesis and preliminary evaluation of the conformationally restricted analogs **1** and **2**,[3] shown in the figure below. The



1 R = H
3 R = COOH

2 R = H
4 R = COOH

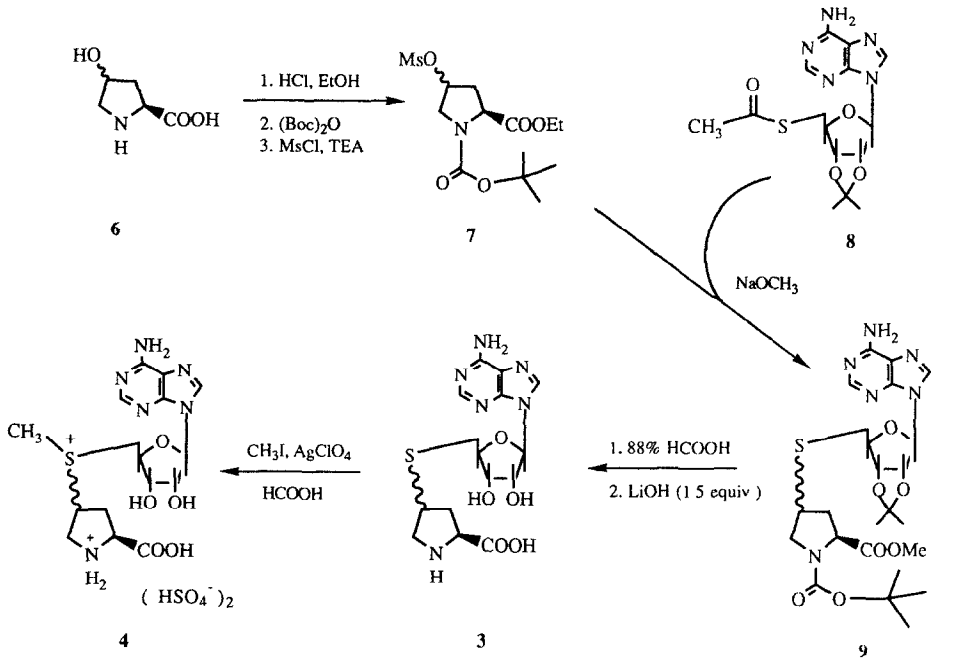
5

synthesis of the carboxyl-containing derivatives **3** and **4** in their pure diastereomeric forms, and of derivative **5** as a mixture of two diastereoisomers, has now been completed. Compound **5**, also known as AdoMac, has been shown to act as a potent, enzyme activated, irreversible inhibitor of the form of AdoMet-DC isolated from *Escherichia coli*.[4] We now report the synthesis of the pure diastereomers of **3** and **4**, the evaluation of analogs **1-4** as inhibitors of AdoMet-DC, and the preliminary evaluation of **1-5** as potential antitrypanosomal agents.

Compounds **1** and **2** were synthesized as a 1:1 mixture of the 4R and 4S diastereomers (with respect to the pyrrolidine ring) as previously described.[3] AdoMac, **5**, was synthesized as a mixture of the 1R,4S and 1R,4R

diastereomers using a chemoenzymatic synthetic route.[4] The synthetic route leading to the pure diastereomeric forms of **3** and **4** is depicted in Scheme 1. Both the 2S,4S and 2S,4R diastereomers of hydroxyproline, **6**, are

Scheme 1



commercially available, and were used individually to synthesize the pure diastereomeric forms of **3** and **4**. Thus the appropriate form of **6** was converted to the ethyl ester[5] (ethanol, HCl), and then to the corresponding N-Boc-protected[6] 4-hydroxy amino acid ester. NMR analysis of this product showed that the desired diastereomer was present in >98% e.e. in each case. Mesylation of the free hydroxyl group[7] then afforded synthon **7**. N^6-formyl-2',3'-isopropylidene-5'-deoxy-5'-thioacetyladenosine **8** was synthesized from 2',3'-isopropylideneadenosine according to a literature procedure.[8,9] Formation of the fully protected thionucleoside **9** was then accomplished by coupling **7** and **8** in the presence of sodium methoxide in a 50:50 mixture of DMF and methanol as previously described.[3] Following this procedure, compound **9** and unreacted **7** were isolated as the methyl ester, since complete transesterification occurred under the reaction conditions employed. Simultaneous removal of the N-Boc and isopropylidene protecting groups (88% HCOOH) followed by hydrolysis of the methyl ester (1.5 equiv. LiOH in 75% ethanol) then afforded the thioether **3**, isolated as the ammonium salt following flash chromatography on silica gel ($CHCl_3$:MeOH:NH_4OH 2:2:1). Methylation of **3** according to the method of Samejima[10] (CH_3I, $AgClO_4$) then yielded the corresponding methylsulfonium analog **4** as the disulfate salt.

AdoMet-DC was isolated from E. coli using an MGBG-Sepharose affinity column prepared by a modification of the method of Anton and Kutny.[11] The column was prepared by incubating MGBG with epoxy-activated Sepharose 4B at pH 11 as described. E. coli (3/4 log phase, Grain Processing Co., Muscatine, IA) were lysed in

5 volumes of 10 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, pH 8.0, by a single pass through a French press. A 5% solution of streptomycin sulfate was then added to give a final concentration of 1%, and the cell debris was removed by centrifugation at 20,000 X g for 2 hours. AdoMet-DC was allowed to adsorb to the gel by stirring the gel and the lysate supernatant together for 1 hour after bringing the $MgCl_2$ concentration to 10 mM. Binding was considered complete when residual AdoMet-DC activity in the supernatant was determined to be 1-3% of the original value. The gel was then packed into a column, and washed (20 mM Tris-HCl, 10 mM $MgCl_2$, 0.6 M KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, pH 8.0) until UV absorbtion reached baseline. AdoMet-DC was then eluted using 20 mM potassium phosphate, 0.6 M KCl, 0.5 mM EDTA and 0.5 mM dithiothreitol, pH 7.0, and the fractions of highest activity were pooled and concentrated (Amicon ultrafiltration cell, PM-30 membrane). Protein was measured by the method of Bradford.[12]

AdoMet-DC activity was monitored by following the evolution of ^{14}C-CO_2 from S-adenosyl-L-[^{14}C-COOH]-methionine according to the method of Markham.[13] A typical reaction mixture contained the desired concentration of inhibitor, 50 µg of AdoMet-DC, 40 µl of S-adenosyl-L-[^{14}C-COOH]-methionine (0.9 mCi/mmol, 20 µM final concentration) in 62.5 mM Tris-HCl/100 mM $MgSO_4$, pH 7.4, with a final volume of 2 ml. Labelled CO_2 was trapped on a filter disk in the vial cap soaked with 200 µl of hyamine. The disk was placed in a scintillation vial with 10 mL of scintillation cocktail and counted. Each data point collected represents the average of two determinations which in each case differed by less than 5%. K_I values for reversible inhibitors **1**-**4** were determined using Dixon plot analyses of the resulting data (not shown), while the K_I for irreversible inhibitor **5** was determined by the method of Kitz and Wilson.[4,14] Mammalian cell IC_{50} values were determined from a growth curve generated by exposing the indicated cell line to varying concentrations (1 - 1000 µM) of each inhibitor.

Antitrypanosomal activity was determined on blood forms of *Trypanosoma brucei brucei* grown *in vitro* using a ^3H-hypoxanthine incorporation assay, or by direct cell count. Continuous cultures of bloodforms were initiated in a feeder layer-free system by inoculating wells of a 24 well culture dish (Falcon 3047) containing 1 ml of modified Iscove's medium[15] with 10^5 trypanosomes from mouse blood. Plates were incubated in 3% CO_2 in air at 37°C. One half the volume of medium was replaced daily, and trypanosomes achieved peak densities of 5 X 10^6 ml^{-1} in 4 days. Inhibitor sensitivity tests were done by dissolving the agent in sterile medium and replacing one half the volume daily with medium containing double strength inhibitor. Cell counts were made daily and IC_{50} values were calculated after 48 hour exposure. Alternatively, trypanosomes were incubated in modified Iscove's medium for 24 hours with varying concentrations of inhibitor, washed and then incubated for an additional 24 hours with medium containing 1 µCi of [^3H]-hypoxanthine. The cells (5 X 10^6 per well) were then harvested by filtration, and the filters were scintillation counted. The results of the biological studies for AdoMet-DC inhibitors **1**-**5** are summarized in Table I.

With the exception of compound **5**, the AdoMet analogs described above proved to be moderate to poor inhibitors of AdoMet-DC. It is interesting to note that thioether derivatives **1** and **3** are more potent inhibitors than the corresponding methylsulfonium analogs **2** and **4**, and that the decarboxylated AdoMet analogs **1** and **2** are more potent than AdoMet analogs **3** and **4**. Prior to decarboxylation, the natural substrate AdoMet must form an imine

linkage with a terminal pyruvate residue which has been shown to be present in the catalytic site of AdoMet-DC.[16] The poor affinity of compounds **1-4** for the active site, therefore, may in part be due to their inability to form such an imine linkage. By contrast, compound **5** is a potent irreversible inactivator of AdoMet-DC, exhibiting a K_I of 18 μM and a k_{inact} value of 0.133 min^{-1}.[4] Since the amino group in this case is exocyclic to the five membered ring, we have postulated that this compound is able to form an imine linkage with the terminal pyruvate, followed by generation of a latent electrophile and subsequent inactivation of the enzyme.

Table I. Comparison of the Inhibitory Activity of Inhibitors 1-5 Against Isolated AdoMet-DC, Mammalian Cells and *Trypanosoma brucei brucei.*

Cmpd.	K_I for AdoMet-DC, μM	Mammalian IC_{50} μM (cell line)	% inhibition (concentration) Trypanosoma brucei brucei
1	415	100[a]	40% (50 μM)[e]
2	669	1000[a]	49% (100 μM)[e]
2S,4S - **3**	478	>1000[b,d]	30% (100 μM)[f]
2S,4R - **3**	960	>1000[b,d]	41% (100 μM)[f]
2S,4S - **4**	1207	>1000[b,d]	64% (100 μM)[f]
2S,4R - **4**	1070	>1000[b,d]	34% (100 μM)[e]
5	18	inactive[a,c,d]	50% (5.2 μM)[e]

[a]L1210 murine lymphocytic leukemia. [b]HL60 human promyelocytic leukemia. [c]HT29 human colon carcinoma [d]IC_{50} value well above concentrations tested. [e] Growth curve method. [f][^{3}H]-hypoxanthine assay.

Compounds **1-5** were next evaluated as cytostatic agents in one of four mammalian cultured cell preparations in order to determine their ability to traverse cell membranes and to alter cellular polyamine metabolism. Compounds **1** and **2** showed moderate to weak inhibitory activity against cultured L1210 cells, but caused significant changes in the cellular ratio of AdoMet to S-adenosylhomocysteine (AdoHcy).[3] However, only compound **2** caused any significant change in intracellular polyamine levels, reducing spermine levels by 50%. Each of the two possible diastereomers of **3** and **4** were evaluated in the human promyelocytic leukemia cell line HL60. This cell line offers distinct advantages to the L1210 cell line, since it is differentiation-capable, is more sensitive to cytotoxic agents, and is a human tumor cell line. However, in each case, there were no discernable effects on cell growth, and the IC_{50} values appeared to be well outside of the concentration range tested. Similarly, compound **5** did not have any effect on cell growth or intracellular polyamine levels in both L1210 and HT29 cultured cell preparations. It is probable that compounds **3-5**, unlike the related thionucleoside analogs **1** and **2**, are not able to pass through the mammalian cell membranes in sufficient quantity to result in any intracellular effects. Because of their relatively poor effects on mammalian cell growth, the intracellular ratio of AdoMet to AdoHcy was not determined for compounds **3-5**. However, the possibility exists that **1-5** may act as inhibitors of enzymes that affect this ratio, such as S-adenosylhomocysteine hydrolase, and these studies are now being conducted.

By contrast, AdoMet analogs **1-5** were markedly more effective against the parasite *Trypanosoma brucei brucei*. Compounds **1** and **2**, as well as both diastereomeric forms of **3** and **4**, showed good inhibitory activity against cultured trypanosomes at concentrations of either 50 or 100 μM, as shown in Table I. The most promising agent

in this regard was compound **5**, AdoMac, which exhibited an IC_{50} concentration of 5.2 μM against cultured *Trypanosoma brucei brucei*. Compound **5** was also active against three drug-resistant clinical isolates of *Trypanosoma brucei rhodesiense*, and gave IC_{50} values of 1.0, 16.0 and 25.0 μM, respectively, in the *in vitro* growth system. Since they are incapable of purine biosynthesis, African trypanosomes possess a specific transport system for purine nucleosides which has been shown to import unnatural nucleosides.[17]

It is likely that the observed selectivity of analogs **1-5** for *Trypanosoma brucei brucei* over the mammalian cell systems described above results from these inhibitors acting as substrates for the trypanosomal purine uptake system. This effect is especially pronounced for analog **5**, AdoMac, which appears to be an effective trypanocide *in vitro* while having virtually no effect in two mammalian cell lines. Further experiments are required in order to determine whether analogs **1-5** are indeed substrates for this purine transport system. Since little is known about the mechanism of trypanosomal purine import, the pure diastereomers of **5** could prove valuable as conformational probes into the spatial requirements of this transport system. Resolution of the pure diastereomers of **5**, assessment of the ability of **1-5** to act as substrates for the trypanosomal purine transport system, and the synthesis of additional analogs are currently underway in our laboratories.

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