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Potential Inhibitors of *S*-Adenosylmethionine-Dependent Methyltransferases. 2. Modification of the Base Portion of *S*-Adenosylhomocysteine

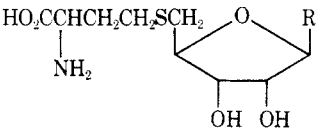
Ronald T. Borchardt,* Joan A. Huber, and Yih Shiong Wu

Department of Biochemistry, McCollum Laboratories, University of Kansas, Lawrence, Kansas 66044. *Received January 28, 1974*

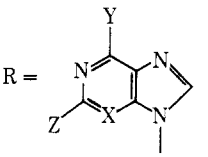
The specificity of *S*-adenosyl-L-homocysteine (SAH) inhibition of enzymatic transmethylation was explored by preparing structural analogs of SAH in which the base portion of the molecule was modified. The various SAH analogs have been evaluated as inhibitors of catechol *O*-methyltransferase, phenylethanolamine *N*-methyltransferase, histamine *N*-methyltransferase, and hydroxyindole *O*-methyltransferase. Inhibition studies indicated that there exists a specificity by these enzymes for the adenine portion of SAH, with an absolute requirement of the 6-amino group for maximum activity. Substitution of other pyrimidine and purine bases in place of adenine resulted in complete loss of activity. However, minor modifications of the adenine moiety of SAH could be tolerated at the enzymatic binding sites. For example, *S*-3-deazaadenosyl-L-homocysteine was found to have inhibitory activity similar to SAH itself. Some differences in the binding requirements of these methyltransferases have been observed so that differential inhibition may be possible.

A general feature of most *S*-adenosylmethionine (SAM)† dependent methyltransferases is the inhibition produced by the demethylated product *S*-adenosyl-L-homocysteine (SAH). This product inhibition by SAH suggests a possible biological regulatory mechanism for transmethylation reactions,[1] as well as a new class of potential inhibitors of methyltransferases.[2-10] In the preceding paper of this series,[2] we described the synthesis and enzymatic evaluation of a series of compounds with modification only in the homocysteine portion of the SAH molecule. It was concluded from this study that all of the functional groups of the homocysteine portion of SAH are required for maximum binding of SAH to these enzymes. Furthermore, it was proposed[2] that there exists three points of attachment of the homocysteine portion of SAH to the enzyme site. These points of attachment are the terminal amino group, the terminal carboxyl group, and the sulfur atom. In order to further study the nature of the intermolecular forces involved in the binding of SAH, we have synthesized a series of SAH derivatives with modification only in the base portion of the SAH molecule (Chart I). The methyltransferases of interest in this study were catechol *O*-methyltransferase (COMT), phenylethanolamine *N*-methyltransferase (PNMT), histamine *N*-methyltransferase (HMT), and hydroxyindole *O*-methyltransferase (HIOMT). Using the base-modified SAH analogs (Chart I) as probes of the active sites of COMT, PNMT, HMT, and HIOMT, we have delineated the contributions of the adenine moiety in the enzymatic binding of SAH. The present paper reports the results of this investigation.

Chart I. Derivatives of SAH Synthesized to Probe the Binding Sites of COMT, PNMT, HMT, and HIOMT

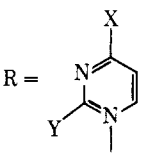


purine derivatives



compd	X	Y	Z
SAH	N	NH_2	H
SGH	N	OH	NH_2
SIH	N	OH	H
1	N	$NHCH_3$	H
2	CH	NH_2	H
3	CH	$NHCH_3$	H
4	CH	$N(CH_3)_2$	H
5	CH	Cl	H

pyrimidine derivatives



compd	X	Y
SUH	OH	OH
SCH	NH_2	OH

Experimental Section

The general experimental techniques and equipment used in this study were described in detail in the preceding paper in this series.[2] The nmr assignments for the 3-deazaadenosine derivatives are given in terms of the purine numbering system, rather than the imidazo[4,5-*c*]pyridine numbering system.

The following compounds were commercially available from the indicated sources: 3,4-dihydroxybenzoate (Aldrich); DL-β-phenylethanolamine, histamine dihydrochloride, *N*-acetylserotonin, SAH, 2′,3′-isopropylideneuridine, 2′,3′-isopropylidenecytidine, 2′,3′-isopropylideneguanosine, 2′,3′-isopropylideneinosine, and *S*-benzyl-L-homocysteine (Sigma). N^6-Methyl-SAH (1) was a gift from Dr. J. Hildesheim of the Institut de Chimie des Substances Naturelles, C.N.R.S., Gif-sur-Yvette, France. SUH and SGH were prepared by Hildesheim, *et al.*[6,7]

S-Inosyl-L-homocysteine (SIH). 2′,3′-Isopropylideneinosine (2.0 g, 6.2 mmol) was dissolved in 20 ml of dry pyridine, freshly distilled from barium oxide, and the resulting solution was cooled to *ca.* 0–5° in an ice–salt bath. Freshly recrystallized *p*-toluenesulfonyl chloride (1.30 g, 6.8 mmol) was added to the cooled solution in one portion and the reaction mixture was stirred for 12 hr at ambient temperature. The pyridine was removed under reduced pressure and H_2O was added to the residue. The resulting aqueous solution was extracted several times with $CHCl_3$. The $CHCl_3$ layer was then washed with H_2O and 5% $NaHCO_3$ and

†Abbreviations used are: SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; SGH, *S*-guanosyl-L-homocysteine; SIH, *S*-inosyl-L-homocysteine; SCH, *S*-cytidyl-L-homocysteine; SUH, *S*-uridyl-L-homocysteine; N^6-methyl-SAH, *S*-N^6-methyladenosyl-L-homocysteine; 3-deaza-SAH, *S*-3-deazaadenosyl-L-homocysteine; N^6-methyl-3-deaza-SAH, N^6-methyl-3-deazaadenosyl-L-homocysteine; N^6-dimethyl-3-deaza-SAH, N^6-dimethyl-3-deazaadenosyl-L-homocysteine; COMT, catechol *O*-methyltransferase (E.C. 2.1.1.6); PNMT, phenylethanolamine *N*-methyltransferase (E.C. 2.1.1); HMT, histamine *N*-methyltransferase (E.C. 2.1.1.8); HIOMT, hydroxyindole *O*-methyltransferase (E.C. 2.1.1.4); K_{is}, inhibition constant for the slope; K_{ii}, inhibition constant for the intercept.

dried ($MgSO_4$). The $CHCl_3$ was removed under reduced pressure and the residue crystallized ($CHCl_3$–hexane) to yield 1.85 g (65%) of the desired tosylate, mp 149–152°. *Anal.* ($C_{20}H_{22}N_4O_7S$) C, H, N.

To S-benzyl-L-homocysteine (1.04 g, 5 mmol) in 45 ml of liquid NH_3 was added Na in small pieces until a blue color was maintained for 20 min. To this solution of L-homocysteine anion was added 2′,3′-isopropylideneinosine 5′-tosylate (1.85 g, 4.0 mmol) and the resulting mixture stirred vigorously at −70° for 6 hr. The NH_3 was allowed to slowly evaporate. The solid residue was dissolved in 20 ml of H_2O, and the resulting aqueous solution was neutralized to pH 7.0 with 1.0 *N* HCl. Extraction with $CHCl_3$, followed by lyophilization of the aqueous layer, gave a crude product which was further purified by chromatography on cellulose (Whatman CF-11) eluting with EtOH–H_2O (3:1). The desired fractions were combined and the solvent was removed under reduced pressure to yield a colorless solid, 670 mg (41%). The 2′,3′-isopropylidene-SIH was homogeneous on tlc [cellulose, EtOH–H_2O (3:2)] and was not further purified.

2′,3′-Isopropylidene-SIH (650 mg, 1.54 mmol) was dissolved in 15 ml of 0.5 *N* H_2SO_4 and the solution allowed to stir for 24 hr. The reaction mixture was neutralized to pH 6.0 with lead carbonate and filtered, and the H_2O was removed by lyophilization. The crude SIH was crystallized (EtOH–H_2O) to yield 525 mg (85%), mp 182–184° [lit.[10] mp 186–188° (prepared by a different route)]. *Anal.* ($C_{14}H_{19}N_5O_6S$) C, H, N.

S-Cytidyl-L-homocysteine (SCH). 2′,3′-Isopropylidenecytidine (0.75 g, 2.33 mmol) was converted to the corresponding 5′-tosylate by reaction with *p*-toluenesulfonyl chloride (0.48 g, 2.5 mmol) in pyridine using a procedure similar to that described above for the preparation of SIH. The desired 2′,3′-isopropylidenecytidine 5′-tosylate was purified by crystallization ($CHCl_3$–hexane) to yield 540 mg (54%), mp 245–248° dec. *Anal.* ($C_{19}H_{24}N_3O_7S$) C, H, N.

The 5′-tosylate (0.44 g, 1.0 mmol) was condensed with S-benzyl-L-homocysteine (0.26 g, 1.1 mmol) in Na and liquid NH_3 as described above for SIH. The crude 2′,3′-isopropylidene-SCH was purified by thick-layer chromatography on cellulose (Analtech, 1000 μ) eluting with EtOH–H_2O (3:2). The product was removed from the cellulose by extraction with H_2O followed by lypholization to yield 220 mg (55%). The 2′,3′-isopropylidene-SCH was homogeneous on tlc [cellulose, EtOH–H_2O (3:2)].

2′,3′-Isopropylidene-SCH (0.15 g, 0.38 mmol) was converted to SCH using 0.5 *N* H_2SO_4 followed by chromatography on cellulose (Whatman CF-11) eluting with EtOH–H_2O (3:2). The crude SCH was crystallized (EtOH–H_2O) to yield 94 mg (69%), mp 174–176° [lit.[10] mp 184–186° (prepared by a different procedure)]. *Anal.* ($C_{13}H_{21}N_4O_6S \cdot 2H_2O$) C, H, N.

4-Amino-1-(β-D-ribofuranosyl)imidazo[4,5-*c*]pyridyl-5′-*S*-L-homocysteine (3-Deaza-SAH, 2). 3-Deazaadenosine was prepared according to the procedures of Rousseau, *et al.*[11] 4-Chloro-1-(β-D-ribofuranosyl)imidazo[4,5-*c*]pyridine[11-15] (4.99 g, 17.5 mmol) was heated at 100° in 100 ml of anhydrous hydrazine under N_2. The resulting 4-hydrazino derivative was reduced using W-5 Raney nickel. Recrystallization of the product afforded 3.92 g (85%), mp 145–147°. 3-Deazaadenosine (3.1 g, 11.7 mmol) was converted to the corresponding 2′,3′-isopropylidene derivative using 330 ml of acetone, 2,2-dimethoxypropane (1.21 g, 11.6 mmol), and *p*-toluenesulfonic acid (20 g) according to the procedure of Mizuno, *et al.*[14] Recrystallization (EtOH) afforded 3.0 g (84%), mp 169–170°. *Anal.* ($C_{14}H_{18}N_4O_4$) C, H, N.

In a manner exactly analogous to that described for SIH, 2′,3′-isopropylidene-3-deazaadenosine (0.57 g, 1.86 mmol) was converted to its corresponding 5′-tosylate (0.90 g, 98%) which was isolated as a glassy solid and by tlc was shown to be sufficiently pure to be utilized in subsequent conversions. The 5′-tosylate (0.86 g, 1.87 mmol) was condensed with S-benzyl-L-homocysteine (0.47 g, 2.08 mmol) in Na and liquid NH_3 using the procedure described above for SIH. The crude 2′,3′-isopropylidene-3-deaza-SAH was purified by chromatography on 25 g of cellulose (Whatman CF-11) using EtOH–H_2O (3:2) as eluent. The product-containing fractions were evaporated to dryness to yield 280 mg (36%) of a colorless solid. The product was homogeneous on tlc using cellulose (EtOH–H_2O, 3:2) with an R_f of 0.60. Ir, nmr, and uv were consistent with the proposed structure.

2′,3′-Isopropylidene-3-deaza-SAH (260 mg, 0.61 mmol) was hydrolyzed using 0.5 *N* H_2SO_4 at ambient temperature for 24 hr, followed by neutralization, filtration, and lyophilization. The crude 3-deaza-SAH was purified by chromatography on a cellulose column (EtOH–H_2O, eluent). Fractions containing pure product, as determined by tlc, were combined and evaporated to yield 100 mg (42%): softened at 165–170° and melted at 213°; nmr (D_2O) δ 8.80 (s, 1 H, C_8-H), 8.10 (d, 1 H, C_2-H), 7.35 (d, 1 H, C_3-H), 6.20 (d, 1 H, $C_{1'}$-H), 4.90–4.40 (m, 3 H, $C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H), 4.00 (m, 1 H, C_α-H), 3.20–2.50 (m, 4 H, C_γ-H_2, $C_{5'}$-H_2), and 2.20 (m, 2 H, C_β-H_2). *Anal.* ($C_{15}H_{21}N_5O_5S \cdot H_2O$) C, H, N.

4-Methylamino-1-(β-D-ribofuranosyl)imidazo[4,5-*c*]pyridyl-5′-*S*-L-homocysteine (*N*⁶-Methyl-3-deaza-SAH, 3). *N*⁶-Methyl-3-deazaadenosine was prepared by a modification of the procedure of Mizuno, *et al.*[14] 4-Chloro-1-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)imidazo[4,5-*c*]pyridine[14] (3.0 g, 7.3 mmol) was added to a glass-lined reaction vessel and under nitrogen 40 ml of methylamine was collected from a Dry Ice–acetone condenser. The vessel was sealed and the system heated at 115–125° for 5 hr. The vessel was cooled to 0° and opened and the methylamine allowed to evaporate. The residue was crystallized (EtOH) to afford 0.82 g (82%), mp 242°. *N*⁶-Methyl-3-deazaadenosine was converted to the corresponding 2′,3′-isopropylidene derivative using the same procedure described above in the synthesis of 3-deaza-SAH. The 2′,3′-isopropylidene-*N*⁶-methyl-3-deazaadenosine was purified by chromatography on cellulose (Whatman C-4) eluting with EtOH, followed by rechromatography on silica gel eluting with 20% EtOH–$CHCl_3$ to yield 500 mg (22%), mp 156–158°. The product was homogeneous on tlc with silica gel (20% EtOH–$CHCl_3$) with an R_f of 0.55. *Anal.* ($C_{15}H_{20}O_4N_4$) C, H, N.

Using procedures completely analogous to those described above for 3-deaza-SAH, the 2′,3′-isopropylidene-*N*⁶-methyl-3-deazaadenosine (0.50 g, 1.56 mmol) was converted to the corresponding 5′-tosylate to yield a glassy solid (390 mg, 53%), which contained only one minor impurity by tlc on silica gel (10% EtOH–$CHCl_3$). This 5′-tosylate was condensed with S-benzyl-L-homocysteine to yield the corresponding 2′,3′-isopropylidene-*N*⁶-methyl-3-deaza-SAH, which was hydrolyzed to *N*⁶-methyl-3-deaza-SAH using 1 *N* H_2SO_4. The crude product was purified using thick-layer chromatography on cellulose (EtOH–H_2O, 3:2), followed by crystallization (H_2O): mp 214–215° dec; nmr (D_2O) δ 8.20 (s, 1 H, C_8-H), 7.70 (d, J = 6 Hz, 1 H, C_2-H), 6.90 (d, J = 6 Hz, 1 H, C_3-H), 5.95 (d, J = 5 Hz, 1 H, $C_{1'}$-H), 4.40 (m, 1 H, $C_{4'}$-H), 2.95 (s, 3 H, N-CH_3); uv λ_{max} (H_2O) 268 nm (ε 8700). Ir, nmr, and uv data were consistent with the proposed structure, but an accurate analysis could not be obtained.

4-Dimethylamino-1-(β-D-ribofuranosyl)imidazo[4,5-*c*]pyridyl-5′-*S*-L-homocysteine (*N*⁶-Dimethyl-3-deaza-SAH, 4). *N*⁶-Dimethyl-3-deazaadenosine was prepared by a procedure similar to that described above for the preparation of *N*⁶-methyl-3-deazaadenosine. 4-Chloro-1-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)-imidazo[4,5-*c*]pyridine[14] (3.0 g, 7.3 mmol) was heated to 125–130° for 5.5 hr in 40 ml of dimethylamine in a sealed vessel. The residue was crystallized (EtOH) to yield 2.5 g (85%), mp 129–132°. Since the product was very hygroscopic, it was immediately converted to the corresponding 2′,3′-isopropylidene derivative using the procedure outlined above for 3-deazaadenosine. From 2.0 g (6.8 mmol) of *N*⁶-dimethylamino-3-deazaadenosine was isolated 1.20 g (53%) of 2′,3′-isopropylidene-*N*⁶-dimethyl-3-deazaadenosine. The sample was recrystallized (CH_3OH) to yield white needles, mp 179–180°. *Anal.* ($C_{16}H_{22}N_4O_4$) C, H, N.

2′,3′-Isopropylidene-*N*⁶-dimethyl-3-deazaadenosine was converted to the *N*⁶-dimethyl-3-deaza-SAH using the general route described above for the synthesis of 3-deaza-SAH. Synthesis of the 5′-tosylate was accomplished as described above for SIH and the product was shown to be homogeneous on tlc with silica gel (10% EtOH in $CHCl_3$, R_f 0.54). The 5′-tosylate was condensed in Na and liquid NH_3 with S-benzyl-L-homocysteine to afford the corresponding 2′,3′-isopropylidene-*N*⁶-dimethyl-3-deaza-SAH, which was hydrolyzed to *N*⁶-dimethyl-3-deaza-SAH using 1 *N* H_2SO_4. The crude product was purified by ion-exchange chromatography in Dowex 50 W-X8, eluting with H_2O to remove sodium tosylate and Na_2SO_4, followed by increasing concentrations of NH_4OH until the desired product was eluted with 0.1 *N* NH_4OH. The product-containing fractions were concentrated by lyophilization. The *N*⁶-dimethyl-3-deaza-SAH crystallized (H_2O): mp 180° dec; nmr (D_2O) δ 8.40 (s, 1 H, C_8-H), 7.55 (d, J = 7 Hz, 1 H, C_2-H), 7.00 (d, J = 7 Hz, 1 H, C_3-H), 5.85 (d, J = 6 Hz, 1 H, $C_{1'}$-H), 4.50 (m, 2 H, $C_{2'}$-H, $C_{3'}$-H), 4.30 (m, 1 H, $C_{4'}$-H), 3.80 (t, 1 H, C_α-H), 3.50 [s, 6 H, N(CH_3)₂], 3.20–2.50 (m, 4 H, $C_{5'}$-H_2, C_γ-H_2), and 2.40–2.00 (m, 2 H, C_β-H_2). *Anal.* ($C_{17}H_{25}N_5O_5S \cdot H_2O$) C, H, N.

4-Chloro-1-(β-D-ribofurnasoyl)imidazo[4,5-*c*]pyridyl-5′-*S*-L-homocysteine (5). 4-Chloro-1-(2′,3′-isopropylidene-β-D-ribofuranosyl)[4,5-*c*]pyridine (0.50 g, 1.54 mmol), which was prepared by a previously described procedure,[14] was converted to its corre-

Table I. Inhibition of COMT, PNMT, HMT, and HIOMT by SAH Analogs[a]

Compd	Inhibitor[b] concn, m*M*	% inhibition COMT[c]	PNMT[d]	HMT[e]	HIOMT[f]
SAH	0.2	39	49	40	71
	2.0	87	92	89	94
SGH	0.2	0	0	0	6
	2.0	0	0	7	14
SIH	0.2	2	0	5	7
	2.0	16	7	19	17
SUH	0.2	0	0	0	8
	2.0	10	4	5	15
SCH	0.2	0	0	0	5
	2.0	5	0	9	10
1	0.2	15	11	11	7
	2.0	55	42	28	13
2	0.2	25	29	25	11
	2.0	68	91	79	50
3	0.2	9	5	0	4
	2.0	18	20	17	17
4	0.2	1	0	0	3
	2.0	5	0	0	10
5	0.2	0	0	0	0
	2.0	0	0	0	0

[a] COMT, PNMT, HMT, and HIOMT were purified as described in the Experimental Section. [b] The inhibitors were prepared as aqueous stock solutions (10.0 μmol/ml). [c] Assay conditions:[2] SAM concentration, 1.0 m*M*; dihydroxybenzoic acid concentration, 2.0 m*M*; Mg^{2+} concentration, 1.2 m*M*; TES buffer, pH 7.60. [d] Assay conditions:[2] SAM concentration, 1.0 m*M*; DL-β-phenylethanolamine concentration, 1.0 m*M*; phosphate buffer, pH 7.80. [e] Assay conditions:[2] SAM concentration, 1.0 m*M*; histamine concentration, 1.0 m*M*; phosphate buffer, pH 7.40. [f] Assay conditions:[2] SAM concentration, 1.0 m*M*; *N*-acetylserotonin concentration, 1.0 m*M*; phosphate buffer, pH 7.90.

sponding 5′-tosylate using the procedures described for the preparation of SIH. The 5′-tosylate was isolated as a glassy product affording 0.66 g (80%) and was homogeneous on tlc with silica gel (10% EtOH in $CHCl_3$, R_f 0.65).

The 5′-tosylate (400 mg, 0.857 mmol) was condensed with *S*-benzyl-L-homocysteine (0.244 g, 1.03 mmol) in Na and liquid NH_3 as described above for SIH. The crude 2′,3′-isopropylidene derivative of **5** was purified by thick-layer chromatography on Avicel F (1000 μ) eluting with EtOH-H_2O (3:2) and then hydrolyzed using 0.5 *N* H_2SO_4. The crude **5** was purified by thick-layer chromatography on Avicel F eluting with EtOH-H_2O (3:1). The product was isolated by washing the cellulose with H_2O, filtration, and lyophilization to yield 23 mg of a fluffy powder, which could not be induced to crystallize. However, the uv, ir, and nmr spectral data support the proposed structure of **5**: nmr (D_2O) δ 8.50 (s, 1 H, C_8-H), 8.10 (d, J = 6 Hz, 1 H, C_2-H), 7.65 (d, J = 6 Hz, 1 H, C_3-H), 5.90 (d, J = 6 Hz, 1 H, C_1'-H), 4.50 (m, 2 H, C_2'-H, C_3'-H), 4.20 (m, 1 H, C_4'-H), 3.60 (m, 2 H, C_α-H), 3.05–2.60 (m, 4 H, C_5'-H_2, C_γ-H_2), and 2.20 (m, 2 H, C_β-H_2).

Enzyme Purification and Assay. The enzymes used in this study were purified from the following sources according to previously described procedures: COMT,[16,17] rat liver (male, Sprague-Dawley, 180–200 g); PNMT,[18] bovine adrenal medulla (Pel-Freez Biologicals); HMT,[19] guinea pig brain (Pel-Freez Biologicals); and HIOMT,[20] bovine pineal glands (Pel-Freez Biologicals). COMT, PNMT, HMT, and HIOMT were assayed using radiochemical techniques as described in the preceding paper of this series.[2] Processing of the kinetic data was achieved as previously described.[2,17,21,22]

Results and Discussion

Chemistry. The various structural analogs of SAH synthesized to probe the specificity of the SAH enzymatic binding sites are listed in Chart I. These derivatives and their synthetic intermediates were characterized by their ir, nmr, and uv spectral properties, their chromatographic properties, and elemental analyses. The general route used to prepare these base-modified SAH derivatives is shown in Scheme I. This involved first the synthesis, if not commercially available, of the parent nucleoside, followed by protection of the 2′,3′-hydroxyl groups, activation of the 5′ position by formation of the corresponding 5′-tosylate, condensation of the tosylate with *S*-benzyl-L-homocysteine, and finally removal of the isopropylidene protecting group to yield the desired base-modified SAH derivative.

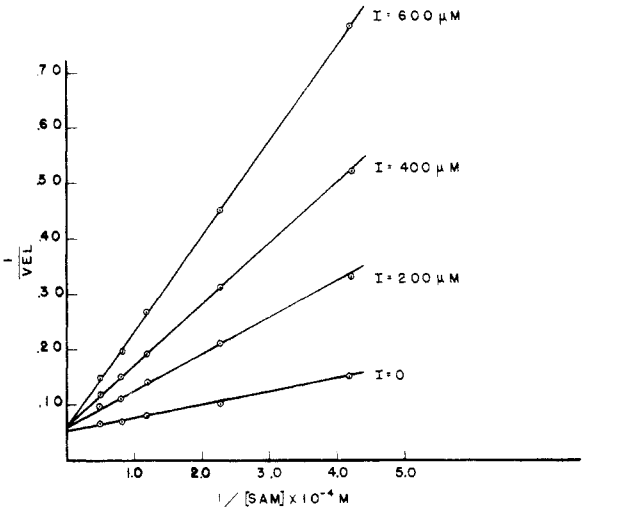
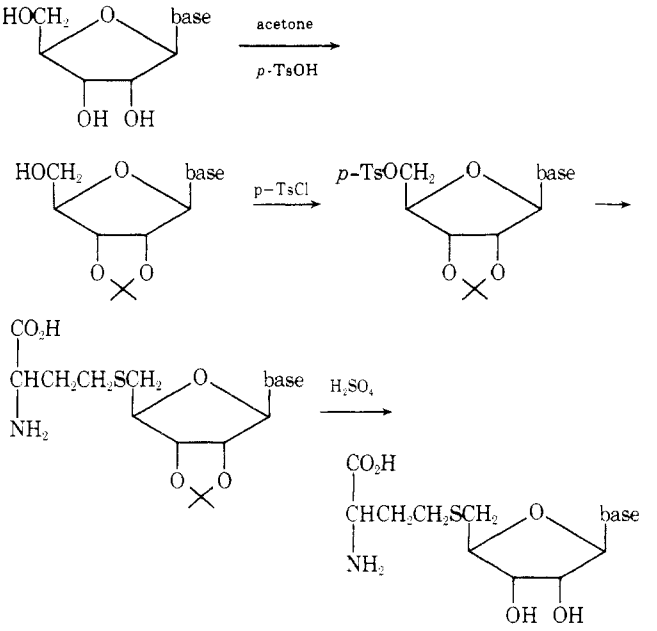


Figure 1. Reciprocal plots with SAM as the variable substrate and 3-deaza-SAH (**2**) as the inhibitor. Assay conditions are outlined in the Experimental Section. SAM concentrations, 24–210 μ*M*. 3,4-Dihydroxybenzoate concentration, 2.0 m*M*. Vel = nmol of product/mg of protein/min.

Scheme I



In Vitro **Inhibition Studies.** Table I shows the degree of inhibition of COMT, PNMT, HMT, and HIOMT activities produced by the various structural analogs of SAH which were synthesized as part of this study. Replacement of the adenine moiety of SAH with pyrimidine bases (*e.g.*, uracil, cytosine) resulted in nearly complete loss of inhibitory activity, as shown by the low inhibitory activity of SUH and SCH. Similarly, replacement of the adenine moiety with other purine bases, which lack a 6-amino group (*e.g.*, SGH, SIH), resulted in complete loss of activ-

Table II. Kinetic Constants for SAH, N^6-Methyl-SAH, and 3-Deaza-SAH Inhibition of COMT

Inhibitor	Substrate concn,[a] mM DHB	Substrate concn,[a] mM SAM	Inhibition[b] pattern	Inhibition constants, μM[c] K_{is} ± S.E.M.	Inhibition constants, μM[c] K_{ii} ± S.E.M.
SAH	2.0	Var	C	36.3 ± 2.20	
	Var	1.0	NC	754 ± 15.9	585 ± 111
N^6-Methyl-SAH (1)	2.0	Var	C	867 ± 112	
	Var	1.0	NC	2040 ± 680	1650 ± 420
3-Deaza-SAH (2)	2.0	Var	C	80.6 ± 5.02	
	Var	1.0	NC	322 ± 139	771 ± 34

[a] Var indicates the variable substrate; DHB concentration, 40–400 μM; SAM concentration, 24–210 μM; TES buffer, pH 7.60; Mg^{2+} concentration, 1.2 mM. [b] C indicates linear competitive kinetics; NC indicates linear noncompetitive kinetics. [c] The inhibition constants were calculated as previously described.[2,17,21,22]

ity. Weak inhibitory activity of tRNA methyltransferase by SUH, SIH, and SGH has previously been reported by Hildesheim, *et al.*[8] Similarly, SIH was previously shown to be a poor inhibitor of COMT by Coward, *et al.*[5] In contrast, replacement of the adenine moiety of SAH with 3-deazaadenine (analog 2) resulted in only a slight decrease in inhibitory activity with each of the enzymes tested. The corresponding 6-chloro-3-deazapurine derivative 5 was completely inactive as an inhibitor. These preliminary data would point to a strict requirement for the adenine moiety of SAH, particularly the 6-amino group of adenine, in binding to methyltransferases. Further support for the importance of the 6-amino group of adenine can be seen by the sharp reduction in activity observed for the corresponding N^6-methyl-SAH (1) relative to SAH. With COMT and PNMT, N^6-methyl-SAH (1) still exhibited some inhibitory activity, but with HMT and HIOMT a substantial reduction in activity was observed for this analog. A similar trend was observed in the activities of 3-deaza-SAH (2), N^6-methyl-3-deaza-SAH (3), and N^6-dimethyl-3-deaza-SAH (4).

These results suggest that the 6-amino group of adenine contributes significantly to the binding of SAH at the enzymatic sites. Since the pK_a value of the 6-amino group of SAH would be approximately 4.1 (adenine, pK_a = 4.12),[23] it should be in the un-ionized state under the normal assay conditions (pH 7.0–8.0). Therefore, it is reasonable to assume that a hydrogen bond is responsible for the interaction between the amino group and the enzyme, rather than an electrostatic binding. Since it can be expected that pK_a values for the 6-amino group of SAH and the 6-methylamino group of N^6-methyl-SAH (1) would be similar (N^6-methyladenine, pK_a = 4.15),[23] the difference in biological activity must be the result of increased steric bulk rather than a change in pK_a. This would be further substantiated by the order of inhibitory activity of 3-deaza-SAH (2), N^6-methyl-3-deaza-SAH (3), and N^6-dimethyl-3-deaza-SAH (4), where large differences in the pK_a values of the 6-amino groups would not be expected, but yet substantial differences in biological activities were observed.

The N-3 position of the adenine moiety of SAH does not appear to be essential for binding with COMT, PNMT, and HMT, since 3-deaza-SAH (2) exhibited potent inhibitory activity on these enzymes. However, with HIOMT the nitrogen at the 3 position of SAH must be important in binding since 3-deaza-SAH was much less effective as an inhibitor with this enzyme than SAH itself.

Using reciprocal velocity *vs.* reciprocal substrate plots, the kinetic patterns for the inhibition of COMT, PNMT, HMT, and HIOMT by the active SAH analogs [SAH, N^6-methyl-SAH (1), 3-deaza-SAH (2), and N^6-methyl-3-deaza-SAH (3)] were determined. A rather extensive study of the inhibition of COMT was carried out in order to establish the similarities in the binding and inhibition

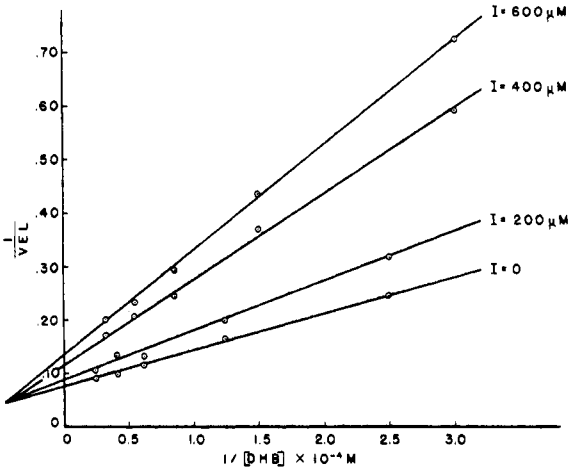


Figure 2. Reciprocal plots with 3,4-dihydroxybenzoate as the variable substrate and 3-deaza-SAH (2) as the inhibitor. 3,4-Dihydroxybenzoate concentrations, 40–400 μM. SAM concentration, 1.0 mM. Vel = nmol of product/mg of protein/min.

characteristics of SAH, N^6-methyl-SAH (1), and 3-deaza-SAH (2). As shown in Figure 1, when SAM was the variable substrate and 3-deaza-SAH (2) the inhibitor, a linear competitive type of inhibition of COMT was observed. A similar inhibition pattern was observed for N^6-methyl-SAH (1) and SAH. N^6-Methyl-SAH (1) has previously been shown to be a competitive inhibitor of tRNA methyltransferase by Hildesheim, *et al.*[8] This competitive pattern for SAH inhibition of COMT has been previously reported.[2,24] Using the appropriate equation for linear competitive kinetics, the K_{is}'s for these inhibitors were calculated and are given in Table II. When dihydroxybenzoate was the variable substrate, a linear noncompetitive pattern of inhibition was observed for SAH, 1, and 2. The kinetic plot for 3-deaza-SAH (2) inhibition of COMT is shown in Figure 2. This noncompetitive pattern for SAH with varying dihydroxybenzoate is different from that observed by Coward, *et al.*,[24] who have previously reported competitive kinetics using epinephrine as the variable substrate. The difference in the kinetic patterns appears to be the result of the catechol substrate used, since with epinephrine and norepinephrine as the variable substrate and SAH as the inhibitor we have also observed competitive kinetics in agreement with Coward, *et al.*[24] In our study the noncompetitive pattern with varying dihydroxybenzoate was consistent for SAH, 1, and 2, and the K_{is}'s and K_{ii}'s for these inhibitors are given in Table II.

To provide further evidence that SAH, 1, and 2 are all binding to the same site on COMT, a study of the kinetics of multiple inhibition of COMT by SAH and 3-deaza-SAH (2) was conducted using the procedures of Yonetani and Theorell.[25] These studies were carried out at pH 7.60 where the two inhibitors show competitive kinetics with

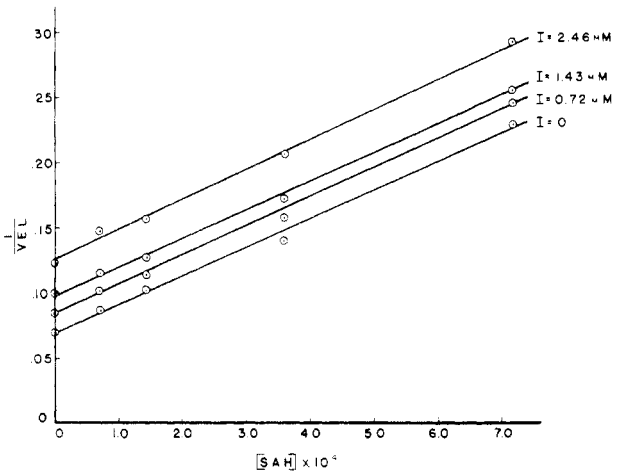


Figure 3. Reciprocal velocity *vs.* SAH concentration with varying 3-deaza-SAH concentration. 3,4-Dihydroxybenzoate concentration, 2.0 m*M*. SAM concentration, 1.0 m*M*. Vel = nmol of product/mg of protein/min.

respect to SAM. As shown in Figure 3, a series of parallel straight lines was obtained when reciprocal velocities were plotted *vs.* SAH concentrations at varying concentrations of 3-deaza-SAH (**2**). The slope of the lines remained constant with varying concentrations of **2**; however, the intercepts were a linear function of the concentration of **2**. This provides evidence that SAH and 3-deaza-SAH (**2**) are competing for the same site on COMT.

The kinetic patterns for inhibition of PNMT, HMT, and HIOMT by SAH, N^6-methyl-SAH (**1**), 3-deaza-SAH (**2**), and N^6-methyl-3-deaza-SAH (**3**) were also determined and the results are given in Table III. For each enzyme and inhibitor studied, varying the SAM concentrations resulted in a competitive pattern of inhibition. With PNMT and HMT the inhibition constants for 3-deaza-SAH (**2**) were of similar magnitude to those found for COMT (Table II) and were within the same order of magnitude as that for SAH, indicating that the nitrogen in the 3 position of SAH is not an absolute requirement in binding to these enzymes. For HIOMT, the K_{is} for 3-deaza-SAH was about 12 times greater than that for SAH, further illustrating the high specificity of this enzyme for the structural features of SAH. With all of the enzymes studied the methylated derivatives **1** and **3** had inhibition constants 10–100 times greater than that for SAH, again indicating the importance of the 6-amino group in binding.

Multiple inhibition experiments, similar to those described above for COMT, were carried out for PNMT, HMT, and HIOMT. Graphic plots, similar to those shown in Figure 3 for the inhibition produced by SAH and 3-deaza-SAH (**2**), were obtained for each of the enzymes. The conclusion drawn from these experiments is that the inhibitory sites to which SAH and 3-deaza-SAH (**2**) are bound on PNMT, HMT, and HIOMT are similar.

Conclusions

In this study we have attempted to delineate the importance of the adenine moiety in binding of SAH to the enzymes COMT, PNMT, HMT, and HIOMT. This objective has been achieved by making structural changes in the base portion of SAH but not altering the amino acid or sugar moieties of this molecule. These changes have included the substitution of other purine and pyrimidine bases for the adenine base, as well as derivatization of the 6-amino functionality of adenine. It was concluded that for each of the methyltransferases studied there exists a very strict requirement for the adenine moiety of SAH, particularly the 6-amino group, in binding to the enzyme. This conclusion was derived from the fact that substitution of uracil, cytosine, guanine, or hypoxanthine in place of adenine in SAH resulted in almost complete loss of inhibitory activity. Methylation of the 6-amino group of SAH generally resulted in a marked reduction in activity. This reduction in activity probably results from increased steric bulk at the 6 position rather than an electronic effect. It was observed, however, that replacement of the adenine moiety in SAH with modified adenine bases (*e.g.*, 3-deazaadenine) resulted in retention of the majority of the inhibitory activity. Therefore, it appears that the nitrogen in the 3 position of adenine is not an absolute requirement for binding. In fact, there may exist a general lack of importance of the adenine ring nitrogens in binding, since Coward‡ has recently observed that the tubercidin analog of SAH retained substantial inhibitory activity of COMT, tRNA methylase, and indolethylamine *N*-methyltransferase. It, therefore, can be concluded from these results that minor structural modifications of the adenine portion of SAH can be tolerated by methyltransferases as long as the modified adenine base still retains the 6-amino group. This conclusion is similar to that previously proposed by Coward, *et al.*[5]

Table III. Inhibition Constants for SAH, N^6-Methyl-SAH (**1**), 3-Deaza-SAH (**2**), and N^6-Methyl-3-deaza-SAH (**3**) toward PNMT, HMT, and HIOMT[a]

Inhibitor	Inhibition constants, μM, K_{is} ± S.E.M. PNMT	HMT	HIOMT
SAH	29.0 ± 2.84	18.1 ± 2.19	18.5 ± 1.9
3-Deaza-SAH (**2**)	81.1 ± 14.9	59.2 ± 6.12	229 ± 11
N^6-Methyl-SAH (**1**)	264 ± 63	1541 ± 205	
N^6-Methyl-3-deaza-SAH (**3**)	1243 ± 141		

[a] PNMT, HMT, and HIOMT were purified and assayed as described in Table II except in each case SAM concentration, 24–210 μ*M*.

The importance of the 6-amino group in the enzymatic binding of SAM has previously been reported.[26] Coupled with the data reported in this paper on SAH analogs, it appears that the 6-amino group of these molecules probably represents a primary point of attachment to the enzyme. Together with the data published in the preceding paper,[2] we propose four major points of attachment of SAH to these enzymes. These would be the terminal carboxyl, the terminal amino, the sulfur atom, and the 6-amino group of the adenine moiety. Whether the sugar moiety of SAH is directly involved in binding has yet to be determined. However, the data of Coward, *et al.*,[4,5] indicates that the ribose moiety plays a primary role either in binding or in maintaining the correct conformation. This latter point has yet to be resolved.

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‡ James Coward, personal communication.

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Four Cyclic Disulfide Pentapeptides Possessing the Ring of Isotocin and Glumitocin†

Clark W. Smith, Victor J. Hruby,*

Department of Chemistry, The University of Arizona, Tucson, Arizona 85721

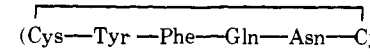
and Martha F. Ferger

Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received January 16, 1974*

[4-Serine]tocinoic acid and [4-serine]tocinamide (the 20-membered disulfide pentapeptide and pentapeptide amide ring of isotocin and glumitocin), as well as the two deamino analogs [4-serine]deaminotocinoic acid and [4-serine]deaminotocinamide, were synthesized from four protected polypeptide precursors which had been prepared by the stepwise active ester method. All four precursors were prepared from the same intermediate Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (1). For the preparation of the two-ring compounds containing C-terminal amides, 1 was treated with MeOH saturated with NH_3 *prior* to condensation with either Boc-Cys(Bzl)-ONSu or β-Mpa(Bzl)-ONp. For the preparation of [4-serine]tocinoic acid and [4-serine]deaminotocinoic acid, 1 was condensed with either Z-Cys(Bzl)-ONp or β-Mpa(Bzl)-ONp. The resulting four protected precursors were then converted to the corresponding ring compounds by deprotection with Na in NH_3, followed by oxidative cyclization. None of the ring compounds showed any significant oxytocic, avian vasodepressor, or rat pressor activity. All showed a slight degree of antioxytocic and antiavian vasodepressor activity but no antipressor activity. Both [4-serine]tocinoic acid and [4-serine]tocinamide showed some milk-ejecting activity.

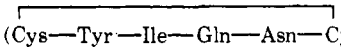
At the present time nine chemically related polypeptide hormones have been isolated from the posterior pituitary gland of mammals, birds, amphibians, and fish: oxytocin,[2] arginine-vasopressin,[3] lysine-vasopressin,[4] vasotocin,[5] mesotocin,[6] isotocin,[7] glumitocin,[8] valitocin,[9] and aspartocin.[9] All of these hormones contain a 20-membered disulfide ring.

The four cyclic polypeptides corresponding to the ring moiety of arginine- and lysine-vasopressin



pressinoic acid[10] and pressinamide,[10-13] and their deamino analogs, deaminopressinoic acid,[10] and deaminopressinamide,[10,14] have been synthesized. None of these four compounds showed any pressor[10,13,14] or avian vasodepressor (AVD)[10] activities. Ferger, *et al.*, found that all except pressinoic acid showed a slight degree of oxytocic activity in the range of 0.05–0.5 unit/mg.[10] Under comparable conditions of oxytocic assay, Zaoral and Flegel[13,14] found 0.26 and 1.03 units/mg for pressinamide and deaminopressinamide, respectively. They also reported antidiuretic and milk-ejecting activity for both compounds.

Four polypeptides corresponding to the ring moiety of oxytocin, vasotocin, valitocin, and mesotocin



have also been synthesized. Tocinoic acid[15] and tocinamide[13,16-18] have oxytocic potencies of 0.2–0.3 unit/mg[15] and about 3 units/mg,[13,16,18] respectively. Deaminotocinoic acid[15] has an oxytocic potency of about 3.7 units/mg, while deaminotocinamide was found by Hruby, *et al.*,[18] to have 34 units/mg and by Zaoral and Flegel[14] to have 16 units/mg of this activity. Zaoral and Flegel have also re-

†All optically active amino acids are of the L variety. The symbols for the amino acid residues follow the recommendations (1971) of the IUPAC-IUB Commission on Biochemical Nomenclature;[1] β-Mpa represents the β-mercaptopropionic acid residue. Chemical synthesis and purification, as well as the milk ejecting assays, were done at The University of Arizona. The other bioassays were performed at Cornell University.