Analogs of S-Adenosylhomocysteine as Potential Inhibitors of Biological Transmethylation. Inhibition of Several Methylases by S-Tubercidinylhomocysteine†

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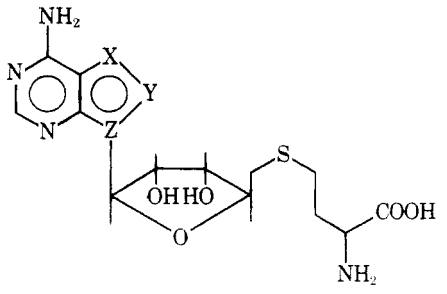
The synthesis of S-adenosylhomocysteine analogs, in which the adenosine portion is replaced by the nucleosidase-resistant antibiotics formycin and tubercidin, has been investigated. Attempts to prepare the formycin analog failed due to the predominance of undesired reactions attributed to ionization of the acidic pyrazole N-H. The tubercidin analog was synthesized and found to be a potent inhibitor of catechol O-methyltransferase, indoleethylamine N-methyltransferase, and tRNA methylases.

In previous communications from this laboratory, the synthesis and biological activity of several analogs of S-adenosylhomocysteine (SAH)‡ have been reported.[1,2] We have demonstrated a strict requirement for the ribose and homocysteine portions of SAH in order to maintain activity as an inhibitor of catechol O-methyltransferase (COMT, E.C. 2.1.1.3). These data, together with similar data obtained with tRNA methylases of rat liver,[3,4] lead to the conclusion that only minor modifications of the SAH molecule can be made without drastically reducing its potency as a methylase inhibitor. Our goal from the outset of this work has been to develop an analog of SAH which would be stable to nucleosidases[5] *in vivo* and which would retain the potent inhibitory activity of SAH. Some of our initial work[1,2] involved the synthesis of analogs in which the ribose moiety of SAH was replaced by a pentyl or cyclopentylmethyl group; *i.e.*, the oxygen atom of the labile ribose-purine (-O-C-N-) linkage was replaced by carbon to give a stable 9-alkylated adenine. None of these compounds were potent inhibitors of COMT. However, certain modifications on the adenine ring which left the planar, aromatic purine intact led to active SAH analogs.[2] Based on the latter observation, we set out to synthesize SAH analogs modified in the heterocyclic base so that a stable ribose-base bond was incorporated. The synthesis and biological activity of this type of compound are the subject of this paper.

Chemistry. We chose to attempt the synthesis of 1 by methods previously employed by ourselves[1,2] and others[3,4,6] for the synthesis of SAH analogs. Both of the parent nucleosides, formycin and tubercidin, have been thoroughly investigated in numerous chemical and biochemical studies and are known to be stable to hydrolytic cleavage of the base-ribose bond under mild conditions.[7] Following the nomenclature employed for SAH, we refer to the formycin derivative **1a** as S-formycinylhomocysteine (SFH) and the tubercidin derivative **1b** as S-tubercidinylhomocysteine (STH).



1a, X = NH; Y = N; Z = C (SFH)
b, X = CH; Y = CH; Z = N (STH)

There are no data in the literature concerning the synthesis of 5′-substituted formycin derivatives other than the work of Ward, *et al.*,[8] on the synthesis of formycin 5′-mono-, -di-, and -triphosphates. These nucleotides were synthesized under acidic or neutral conditions, whereas the synthesis of SAH analogs involves the displacement of a chloride or tosylate by homocysteine anion in liquid NH_3.[1-4,6] The pK_a of 9.5 for the pyrazole N-H of formycin[9] caused many problems in our efforts to synthesize **1a**. Initially, we attempted to tosylate the 5′-hydroxyl group of 2′,3′-isopropylideneformycin by standard procedures but were able to obtain only low (<20%) yields of the desired tosylate. In addition, the isolated tosylate could not be converted into the 2′,3′-isopropylidene precursor of **1a** by methods previously employed for similar nucleoside analogs.[1,2] Chromatographic and spectral analysis of the final product mixture indicated that the homocysteine moiety was not coupled to formycin. The low yield of isolable tosylate, together with the inability to convert the tosylate to **1a**, indicated that perhaps cyclization of the intermediate tosylate was occurring *via* ionization of the acidic pyrazole N-H.

The use of the *tert*-butoxycarbonyl group to block the N-H of pyrazole has been described previously,[10] and this appeared to have the properties required for use in the proposed synthetic route, *i.e.*, base stable and acid labile. Treatment of **3** with sodium hydride and *tert*-butyloxycarbonyl azide gave an oily product which appeared to be homogeneous by tlc but did not have the spectral properties expected of **4** (Scheme I). The ultraviolet spectrum of the isolated product exhibited a bathochromic shift in base which is generally attributed to the ionization of the pyrazole N-H bond.[9] This type of shift is not observed when N^1 is alkylated, as in 1-methylformycin.[11],§ In addition, the N-acetyl methyl resonance of **3** was absent in the pmr spectra of the isolated material. Solvolysis of this crude material in methanolic NH_3 gave a mixture of two products: **2** and a mono-Boc derivative **6**. Spectral data indicate that the Boc group of **6** is on N^7 rather than N^1, thus precluding the use of the reaction sequence in Scheme I for the synthesis of **1a**. The exact chronology of events in the conversion of **3** to **6** has not been investigated further. It should be noted that deacetylation at N^7 occurred under the extremely mild conditions of an aqueous extraction during work-up of the basic reaction solution (see Experimental Section). This facile deacetylation is in contrast to the conditions required for deacylation of N^6-acyladenosines[12] and suggests participation of the anion generated at N^1 under the reaction conditions. This could lead to an intramolecular acyl shift ($N^7 \rightarrow N^1$) and rapid hydrolysis of the resulting N^1-acylformycin on work-up.

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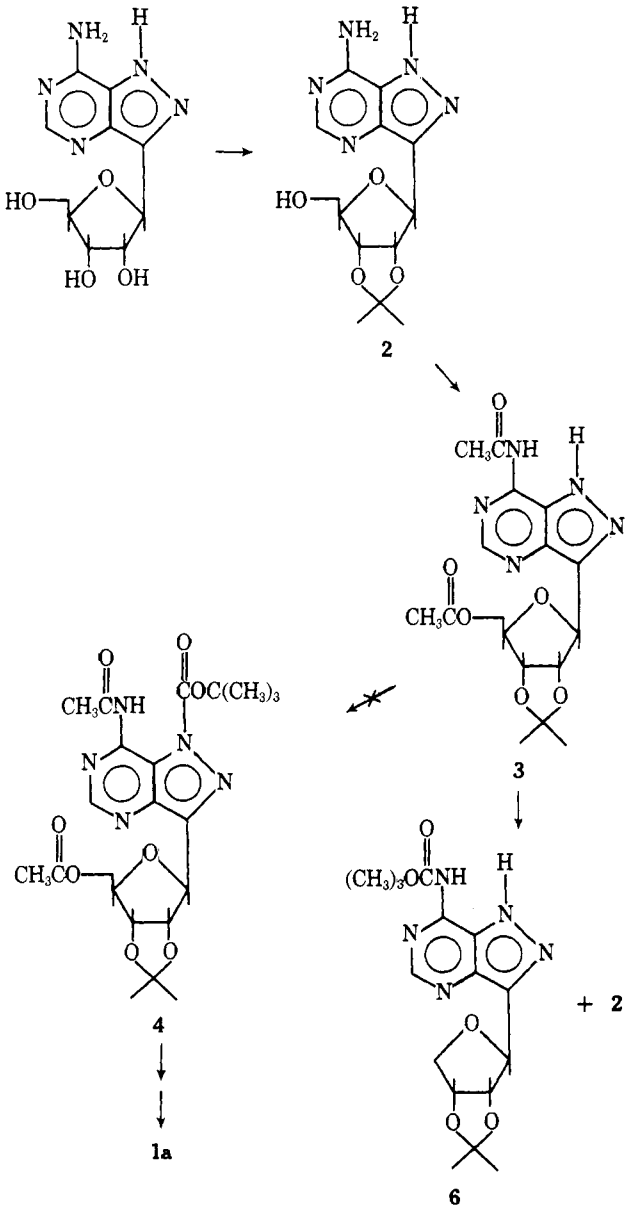
‡Abbreviations used: SAH, S-adenosylhomocysteine; SFH, S-formycinylhomocysteine (**1a**); STH, S-tubercidinylhomocysteine (**1b**); Boc, *tert*-butoxycarbonyl; COMT, catechol O-methyltransferase; INMT, indoleethylamine N-methyltransferase; tRNA MT, tRNA methyltransferases.

§The numbering system is that of a pyrazolo[4,3-*d*]pyrimidine, not a purine.

The limited supply of formycin available to us, coupled with the success obtained with the tubercidin derivative **1b** described below, led us to abandon any further effort to synthesize **1a**.

Scheme I



The synthesis of **1b** followed the procedures employed previously.[1-4] Tubercidin was converted to the 2′,3′-isopropylidine 5′-*O*-tosyl derivative by standard procedures. Treatment of this tosylate with homocysteine anion in liquid NH_3, followed by acid hydrolysis of the isopropylidene group and chromatography on Dowex-1, led to the desired analog, STH (**1b**).

Results and Discussion

Our initial kinetic studies into the effect of STH on the COMT reaction indicated that STH was a very potent inhibitor of that reaction. These inhibitors have been designed for use *in vivo*, and since product inhibition by SAH seems to be a general feature of most biological transmethylation reactions,[13] we also investigated the effectiveness of STH against two other methylases which appear to have altered activity in specific diseases. Indoleethylamine *N*-methyltransferase (INMT, E.C. 2.1.1.) has been implicated in certain mental diseases.[14] The

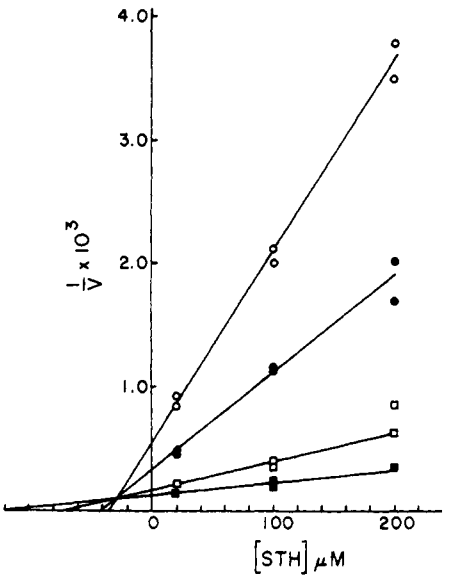


Figure 1. Inhibition of COMT by STH at SAM concentrations of 0.1 (○), 0.2 (●), 0.5 (□), and 1.0 m*M* (■). Specific activity of SAM in the assay was 0.2 μCi/μmol.

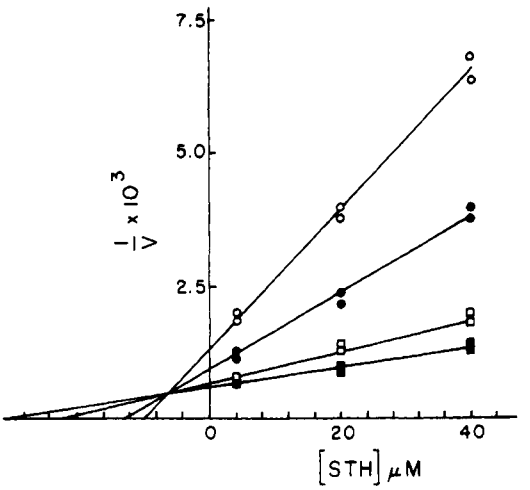


Figure 2. Inhibition of INMT by STH. SAM concentrations are the same as in Figure 1. Specific activity of SAM in the assay was 0.4 μCi/μmol.

tRNA methylases (tRNA MT) from several tumors have been shown by numerous workers to have elevated activity *in vitro*.[15] The partially purified enzymes were assayed as described in the Experimental Section, and the data are presented as Dixon plots[16] in Figures 1-3. Similar data (not shown) have been obtained with the natural product inhibitor, SAH. The values of K_i compiled from these data, together with values reported in the literature for SAH,[2,3,17] are tabulated in Table I. It is clear from these data that STH is a very potent inhibitor of the three methylases investigated; its efficacy closely parallels that of the natural inhibitor, SAH. In addition, we have studied three SAH analogs previously prepared in this laboratory[2] as inhibitors of INMT and tRNA MT. In agreement with the results obtained with COMT,[2] only 2-fluoro-*S*-adenosylhomocysteine (2-FSAH) has appreciable activity as an inhibitor of these two enzymes. These data are summarized in Table II.

Previous work[1-4] demonstrated that the ribosyl and homocysteine residues of SAH are required for full inhibitory activity against at least two methylases. The present work supports our previous suggestion[2] that modifications in the base portion of SAH can lead to active methylase

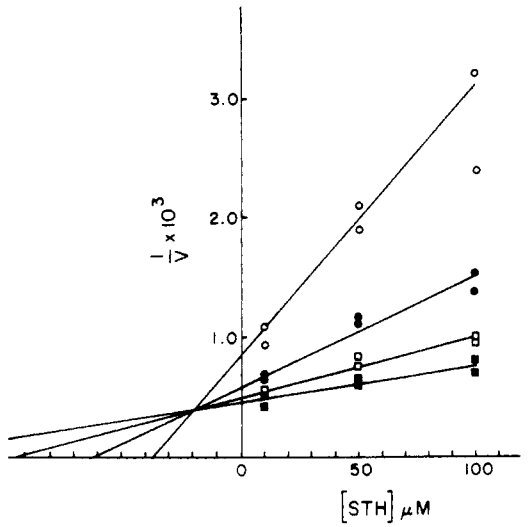


Figure 3. Inhibition of tRNA MT by STH at SAM concentrations of 10 (○), 25 (●), 50 (□), and 100 μM (■). Specific activity of SAM in the assay was 5.0 μCi/μmol.

inhibitors, as long as the planar, aromatic nature of the base is maintained. In this regard, it is of interest to note that 3-deaza-SAH has recently been synthesized, and it is a good inhibitor of COMT (K_i = 80 μM).[18] It is also known that N^6-methyl-SAH is an inhibitor of tRNA methylases from rat liver (K_i = 60 μM),[4] and in preliminary studies, we found this compound to inhibit COMPT (K_i = ~0.2 mM). These data are further support for the hypothesis that the heterocyclic base is one of the few areas of SAH which may be altered with the maintenance of significant inhibitory activity.

Data available in the literature indicate that the drugs currently known to inhibit these methylases, such as tropolones for COMT[19] or cytokinins for tRNA methylases,[20] have certain limitations. Tropolones have been shown to inhibit other enzymes of catecholamine metabolism, notably dopamine β-hydroxylase,[21] and cytokinins must be utilized in high concentrations relative to *S*-adenosylmethionine (SAM) in order to obtain even modest inhibition.[20] Therefore, the potent inhibition of the methylases by STH would appear to satisfy the deficiencies mentioned above; it should be specific for methylases, and it is effective at low doses. However, as is clear from the data presented in Table I, STH is effective against several methylases and therefore is not specific within the family of SAM-dependent methylases. This does not detract from the fact that STH is a very potent inhibitor of biological methylation. It should be useful as a tool for studying cells in culture, in order to assess the consequences of inhibiting selected methylases known to be localize in specific cells. Further work is now in progress in order to develop potent inhibitors which are more specific for particular methylases than is STH.

Experimental Section

All melting points were taken in capillary tubes on a Mel-temp block and are uncorrected. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements or functions were within 0.4% of the theoretical values. Ir spectra were run on a Perkin-Elmer Model 21 spectrophotometer, uv spectra on a Cary 15 spectrophotometer, and nmr spectra were obtained in $CDCl_3$ on a Varian T-60A spectrophotometer. Chemical shifts are in parts per million downfield from TMS. Tlc were run and spots detected by standard techniques.[1,2] *S*-Adenosylmethionine and *S*-adenosylhomocysteine were obtained from Boehringer-Mannheim. [Methyl-^{14}C]-SAM, specific activity 58 mCi/mmol, was from New England Nuclear Corp. and was diluted as appropriate for each enzyme assayed. Formycin was purchased from Meija Seika Kaisha Ltd., and tubercidin was a gift from Dr. John Whitfield, Upjohn Co. All other chemicals were reagent grade.

Table I. Inhibition of Methylases by *S*-Adenosylhomocysteine and Its 7-Deaza Analog, STH[a]

Methylase	SAH, K_i, μM	STH, K_i, μM
COMT	n.d. (20)	30
INMT	5 (10)	7
tRNA MT	25 (10)	20

[a] For SAH, the values in parentheses are from the literature as cited in the text.

Table II. % Inhibition of Methylases by SAH Analogs

Compound[a]	INMT[b]	tRNA MT[c]
SAH	40	65
STH	37	51
2-F-SAH	20	29
SIH	0	9
dC-SAH	0	8

[a] Abbreviations not used in text are SIH, *S*-inosylhomocysteine, and dC-SAH, cyclopentyl analog of SAH (compound 1 of ref 2). [b] All inhibitors at a final concentration of 20 μM, [SAM] = 1 mM (0.2 μCi), and other conditions as in the Experimental Section. [c] All inhibitors at a final concentration of 100 μM, [SAM] = 100 μM (0.4 μCi), and other conditions as in the Experimental Section.

COMT, isolated fom rat liver, was purified and assayed as previously described[1] except that [methyl-^{14}C]-SAM was varied as shown in Figure 1. Indoleethylamine *N*-methyltransferase (INMT), isolated from rabbit lung, was purified through one $(NH_4)_2SO_4$ fractionation and assayed as described by Axelrod,[22] except that [methyl-^{14}C]-SAM was varied as shown in Figure 2. The tRNA methylases (tRNA MT) of rat liver were purified through one $(NH_4)_2SO_4$ fractionation as described by Pegg as a "source of methylases."[23] The activity of the tRNA methylases was assayed by the method of Baguley and Stahelin[24] except that [methyl-^{14}C]-SAM was varied as shown in Figure 3. The acceptor substrates used for the three enzymes were 3,4-dihydroxybenzoic acid (Sigma) for COMT, *N*-methyltryptamine (Sigma) for INMT, and unfractionated *Escherichia coli* tRNA (General Biochemicals) for tRNA MT. All velocities are expressed in terms of cpm per aliquot from the standard assays.

N^7,5′-Diacetyl-2′,3′-isopropylideneformycin (3). Formycin was converted to the 2′,3′-isopropylidine derivative **2** by standard procedures.[25] The oily product was homogeneous by tlc on cellulose (BuOH-HOAc-H_2O, 12:3:5), R_f 0.90, and had the expected spectral characteristics: uv λ_{max} (0.1 *N* HCl) 235, 294, 303 nm (s); uv λ_{max} (0.1 *N* OH^-) 234, 303 nm; nmr 1.33, 1.57 [pair of s, 6, $>C(CH_3)_2$], 4.4-5.4 (6, ribose ring protons[24]), 8.13 (s, 1, C_5H[26]). The isopropylideneformycin (1.149 g, 3.78 mmol) was dissolved in 10 ml of anhydrous pyridine and the mixture was cooled to *ca.* 5° in a cold room. Cold acetic anhydride (3.42 ml, 37.8 mmol) was added to the mixture and stirring continued overnight at 5°. EtOH (11 ml) was then added and the resulting mixture stirred at ambient temperature fo 90 min, after which time the solvent was evaporated at *ca.* 30° with the aid of a vacuum pump. The resulting residue was dissolved in 30 ml of $CHCl_3$ and washed successively with 5% aqueous $NaHCO_3$ (3 × 20 ml), water (2 × 30 ml), and saturated NaCl solution. The dried $CHCl_3$ layer was then evaporated to dryness at ambient temperature to give an amorphous material, net wt 1.01 g (88%). A portion of this residue was crystallized from $CHCl_3$-Et_2O to give the product as a white powder: mp 164–169°; tlc R_f 0.61 on cellulose in BuOH-HOAc-H_2O (12:3:5); uv λ_{max} (0.1 *N* HCl) 239, 272, 312, 322 nm (s); uv λ_{max} (0.1 *N* OH^-) 277, 322 nm; ir 1715 cm^{-1} (amide C=O), 1740 cm^{-1} (ester C=O); nmr 1.37, 1.57 [pair of s, 6, $>C(CH_3)_2$], 1.97 [s, 3, $-OC(=O)CH_3$], 2.30 [s, 3, $-NC(=O)CH_3$], 4.04–4.53 (m, H, C^2H, C^3H, C^4H), 4.92 (m, 1, C^1H), 5.50 (m, 2, C^5H_2), 8.58 (s, 1, C^5H), 9.43 (br m, 1, –NHC–), 12.25 (br m, 1,

N^1-H). An analytical sample was obtained by an additional recrystallization from $CHCl_3$-Et_2O: mp 178–179°. *Anal.* ($C_{17}H_{21}N_5O_6$) C, H, N.

Reaction of 3 with NaH and *tert*-Butyloxycarbonyl Azide. A solution of 717 mg (1.83 mmol) of 3 in *ca.* 20 ml of anhydrous THF under N_2 was cooled to 0° and a solution of 315 mg (2.2 mmol) of *tert*-butyloxycarbonyl azide in 3 ml of anhydrous THF was added slowly while maintaining the temperature at 0°. The progress of the reaction was monitored by tlc on silica gel in EtOAc, and after 2 hr no further increase in the product spots (R_f 0.75) was observed; however, it appeared that only *ca.* 30% of 3 (R_f 0.66) had been converted to the new material. This procedure was repeated several times until tlc indicated that nearly all of 3 had been converted to the new material (R_f 0.76). After the final reaction mixture was partitioned between $CHCl_3$ and H_2O, the $CHCl_3$ layer was washed with H_2O and saturated NaCl. The dried $CHCl_3$ solution was evaporated *in vacuo* to give the crude product; yield 928 mg. The nmr spectrum of this product clearly showed the loss of the *N*-acetyl group, the presence of the *O*-acetyl group, and a complex series of peaks in the high-field region which supported the presence of a *tert*-butyl group in the oily residue. Therefore, a 682-mg portion of this crude product was treated with methanolic NH_3 overnight to effect complete O-deacetylation of the intermediate(s). The product obtained from this ammonolysis contained two materials as shown by tlc on silica gel in EtOAc. Treatment of the oily residue with cold MeOH afforded 116 mg of the higher moving material (R_f 0.66) as a white solid. A similar procedure afforded an analytical sample: mp 186° dec with preliminary softening; uv λ_{max} (0.1 *N* HCl) 238, 265, 312 nm; uv λ_{max} (0.1 *N* OH^-) 235, 321 nm; ir 1750 cm^{-1} (urethane C=O); nmr 1.40, 1.63 [overlapping s + d, 16, $>C(CH_3)_2$, $-C(CH_3)_3$, $0.5H_2O$], 3.88 (br, 2, $-CH_2OH$), 4.52 (br s, 1, C⁴′H), 5.08 (m, 2, C²′H, C³′H), 5.48 (m, 1, C¹′H), 8.71 (s, 1, C⁵′H). *Anal.* ($C_{18}H_{25}N_5O_6 \cdot 0.5H_2O$) C, H, N. The spectral and chromatographic data, as well as the elemental analysis, support the structural assignment of this material as N^7-*tert*-butyloxycarbonyl-2′,3′-isopropylideneformycin (6). The MeOH-soluble fraction of the reaction product contained additional 6 and a lower moving material (R_f 0.14). These two products were separated by preparative tlc on silica gel in EtOAc, and the material with R_f 0.14 was shown to be 2′,3′-isopropylideneformycin (2) by spectral and chromatographic comparisons with authentic 2; net wt 102 mg (18%). Additional 6 was obtained from the preparative tlc plate for a total of 175 mg (23%) of 6 obtained from 3.

***S*-Tubercidinylhomocysteine (1b).** 2′,3′-Isopropylidinetubercidin[27] [tlc on cellulose (BuOH–HOAc–H_2O, 12:3:5), R_f 0.89] was converted directly to the 5′-*O*-tosyl derivative:[27] one major spot on silica gel (EtOAc), R_f 0.43, along with two minor spots at R_f 0.70 and 0.81. After standing overnight at −20°, an increase in intensity of the two high-running spots was observed. Because of this instability, the crude tosylate residue was routinely used directly for the next step. *S*-Benzyl-DL-homocysteine[28] (433 mg, 1.92 mmol) was dissolved in *ca.* 15 ml of liquid NH_3 in a three-necked round-bottom flask fitted with a trubore stirrer, cooled in a Dry Ice–acetone bath and flushed with nitrogen. Sodium metal (114 mg, 5 mg-atoms) was added in small chunks over a 30-min period until a blue color persisted for at least 15 min. The tosylate (885 mg, 1.92 mmol) dissolved in *ca.* 5 ml of liquid NH_3 was added in one portion. The reaction mixture was allowed to stir for 15 min, the stirrer and bath were removed, and the reaction vessel was wrapped with glass wool and towels and the NH_3 evaporated slowly. The last traces of NH_3 were removed under vacuum and the residue was taken up in *ca.* 10 ml of water. After neutralizing with 1 *N* H_2SO_4 to pH 6, the solution was filtered and washed with $CHCl_3$ (5 × 5 ml) and then lyophilized to give 944 mg of crude product: uv λ_{max} (0.1 *N* HCl) 270, 226 nm; uv λ_{max} (0.1 *N* NaOH) 270 nm. Tlc on cellulose (*n*-BuOH–HOAc–H_2O, 12:3:5) showed one major spot (ninhydrin positive and uv absorbing), R_f 0.72. The 2′,3′-isopropylidene group was hydrolyzed with 1 *N* H_2SO_4. The residue after work-up (340 mg) showed one major spot (ninhydrin positive, uv absorbing), R_f 0.39 on cellulose (*n*-BUOH–HOAc–H_2O, 12:3:5). The crude product, dissolved in *ca.* 3 ml of 0.01 *M* $HCOONH_4$ buffer (pH 10.6), was applied to a Bio-Rad AG 1-X8 ($HCOO^-$ form) column (280 × 20 mm) and eluted in a pH gradient (0.01 *M* $HCOONH_4$) as previously described for similar SAH analogs.[1,2] The purified product (R_f 0.38) was eluted at pH 4.7 and the pooled fractions were lyophilized to give a white glassy material (88 mg) which could not be crystallized: uv λ_{max} (0.1 *N* HCl) 270, 226 nm; uv λ_{max} (0.1 *N* NaOH) 270 nm. An analytical sample was obtained from the center portion of the product peak. *Anal.* ($C_{11}H_{21}N_5O_5S$) C, H, N.

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