Table 2. Incorporation of [^{3}H]proline into collagen and noncollagen proteins during incubation *in vitro* of minced granulation tissue from rats treated with CaEDTA*

	14-Day control	CaEDTA treatment
Net body wt (g)	199 ± 5	172 ± 8†
Exudate (g)	31.9 ± 3.6	36.2 ± 5.0
Granulation tissue, wet wt (g)	5.87 ± 0.43	3.81 ± 0.26‡
Collagen hydroxyproline (mg) in the whole tissue	17.212 ± 1.288	16.158 ± 1.590
Noncollagen protein (mg) in the whole tissue	263.07 ± 21.17	153.19 ± 11.53‡
Incorporation of [^{3}H]proline		
Radioactivity of collagen hydroxyproline (dis./min/μg DNA)	3.396 ± 0.627	3.017 ± 0.367
Radioactivity of noncollagen protein (dis./min × 10^{-2}/μg DNA)	2.154 ± 0.243	2.127 ± 0.133

* Data are shown as means ± S. E. There were eight rats in each group.
† Values are significantly different from 14-day control, $P < 0.05$.
‡ Values are significantly different from 14-day control, $P < 0.01$.

steriod enhanced the resorption of pre-existing granulation tissue through a strong inhibitory action on protein synthesis without apparently affecting the degradation of noncollagen protein. CaEDTA also enhanced the resorption of pre-existing granulation tissue. However, the mode of action of CaEDTA is different from that of the steriod; CaEDTA enhances the resorption of pre-existing granulation tissue as a result of the increased degradation of noncollagen protein without apparently affecting the syntheses of collagen and noncollagen proteins.

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Biochemical Pharmacology, Vol. 27, pp. 1981–1983

0006-2952/78/0801-1981$02.00/0

Inhibition of methyltransferases by some new analogs of *S*-adenosylhomocysteine

(*Received* 16 *December* 1977; *accepted* 25 *January* 1978)

S-adenosylmethionine (SAMe)-dependent methyltransferases are inhibited by the reaction product *S*-adenosylhomocysteine (SAH). Numerous synthetic derivatives of SAH have been prepared and studied as inhibitors of methyltransferases (see, for example, Ref. 1–3). We are reporting here the inhibition of three mammalian SAMe-dependent methyltransferases by a group of novel antibiotics of microbial origin that are chemically related to SAH. The enzymes studied were norepinephrine *N*-methyltransferase (EC 2.1.1.28), the epinephrine-forming enzyme; histamine *N*-methyltransferase (EC 2.1.1.8), which methylates histamine on the aromatic ring; and catechol *O*-methyltransferase (EC 2.1.1.6), which *O*-methylates catecholamines and other catechols. The inhibitors studied are shown in Fig. 1. Compound I (known as sinefungin[4]) differs from SAH only in having the sulfur replaced with an amino-substituted methylene unit. In compound II, an amide linkage between that amino group and the carboxyl group forms a six-membered ring. Compound III is like I except that it has a double bond at the 4′, 5′-position. The lactam IV has the same relationship to III as II does to I. These compounds are all metabolites of *Streptomyces griseolus* (the lactams II and IV may have been formed during the extraction and purification process) and were isolated because of the antifungal activity of compound I (sinefungin)[5]. Their structural similarity to SAH led us to study their effects on these methyltransferases.

Norepinephrine *N*-methyltransferase was purified from rabbit adrenal glands and assayed, with L-norepinephrine as substrate, by previously described methods[6]. Histamine *N*-methyltransferase was purified from guinea pig brain through the stage of ammonium sulfate fractionation and assayed with histamine as the methyl acceptor by the methods of Brown *et al.*[7]. Catechol *O*-methyltransferase was prepared from rat liver through the stage of ammonium sulfate fractionation and assayed with L-norepinephrine as the methyl

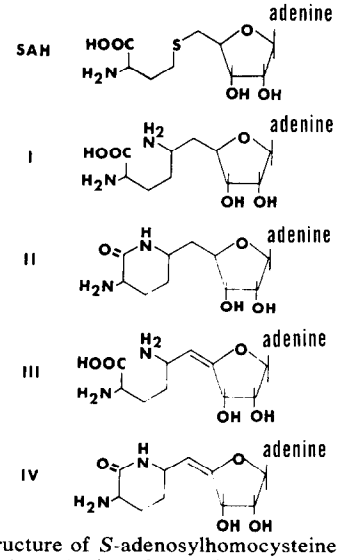

Fig. 1. Structure of *S*-adenosylhomocysteine (SAH) and four analogs obtained from microbial fermentation.

acceptor by the methods of Axelrod and Tomchick[8]. All of the radiometric assays used methyl-[^{14}C]-labeled SAMe, obtained from New England Nuclear (Boston, Mass.) (NEC-363), as the methyl donor.

The effects of the compounds whose structures are given in Fig. 1, on these three methyltransferases, are shown in Fig. 2. The lactams (II and IV) were less effective inhibitors than the corresponding open-chain compounds (I and III) in all cases, and some of the activity of the lactams might have resulted from ring opening through hydrolysis during incubation in the enzyme assay mixture. Both compounds I and III were more effective as inhibitors of histamine *N*-methyltransferase than of norepinephrine *N*-methyltransferase and were comparatively ineffective against catechol *O*-methyltransferase. Compound III inhibited histamine *N*-methyltransferase by 50 per cent at 2.5×10^{-7} M, making it one of the most effective known inhibitors of this enzyme.

Table 1 relates the effects of these new compounds to those of SAH itself. With norepinephrine *N*-methyltransferase, only one of the derivatives was a more effective inhibitor than SAH; compound III was ten times more effective than SAH. With histamine *N*-methyltransferase, all except II were more effective than SAH; compound III was more than 1000 times as active as SAH. SAH itself was slightly better as an inhibitor of catechol *O*-methyltransferase than of histamine *N*-methyltransferase, and none of the analogs were even one-tenth as active as SAH in inhibiting this enzyme.

The specificity of inhibition that can result from a

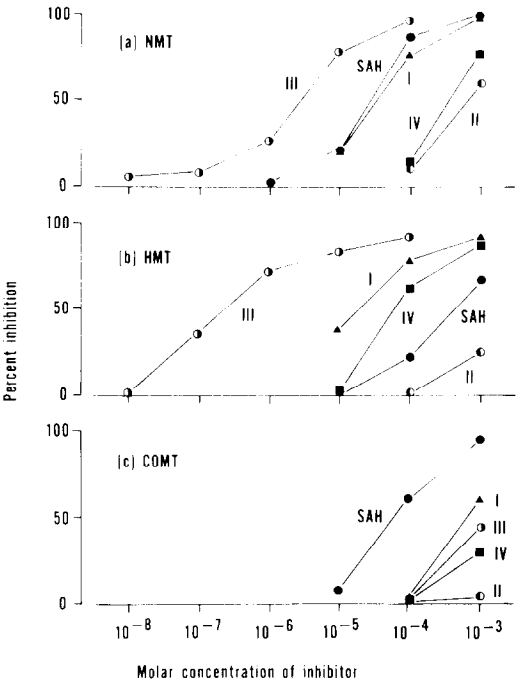

Fig. 2. Inhibition of (a) norepinephrine *N*-methyltransferase (NMT) from rabbit adrenal, (b) histamine *N*-methyltransferase (HMT) from guinea pig brain, and (c) catechol *O*-methyltransferase (COMT) from rat liver. The concentration of SAMe in the enzyme assay mixtures was 20 μM for NMT, 8 μM for HMT, and 20 μM for COMT.

compound that is a structural analog of SAH is remarkable. For example, the ratios listed in Table 1 covered a 244-fold range with I and a 43,000-fold range with III. Selectivity with some other SAH analogs has been reported; for instance, Borchardt and Wu[9] found that D-SAH was more effective as a histamine *N*-methyltransferase inhibitor than L-SAH but was much less effective as an inhibitor of norepinephrine *N*-methyltransferase and catechol *O*-methyltransferase. However, the 1750-fold increase in activity compared to SAH (compound III against histamine *N*-methyltransferase) apparently is larger than has been previously reported with other SAH analogs. The fact that such a high degree of selectivity is achievable lends some encouragement to the search for useful analogs that will block a specific methyltransferase without appreciably affecting methyl transfer processes generally. The possibility that the antifungal activity of compound I may be due to inhibition of some key methyltransferase in sensitive micro-organisms deserves consideration.

Table 1. Relative inhibitory activity

Test compound	Ratio of SAH activity to analog activity [I_{50} (SAH)/I_{50} (test compound)]*		
	Norepinephrine *N*-methyltransferase	Histamine *N*-methyltransferase	Catechol *O*-methyltransferase
I (sinefungin)	0.8	22	0.09
II	0.04	Inactive†	Inactive†
III	10	1750	0.04
IV	0.06	7	Inactive†

* I_{50} is the molar concentration producing 50 per cent inhibition.
† The term "inactive" is used when there was less than 50 per cent inhibition at 10^{-3} M.

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Biochemical Pharmacology, Vol. 27, pp. 1983–1985

0006-2952/78/0801-1983$02.00/0

Role of cytochrome P-450 in CCl_4-induced microsomal lipid peroxidation

(*Received* 3 *November* 1977; *accepted* 8 *February* 1978)

Although CCl_4 is considered to be activated to free radicals in the microsomal NADPH-dependent electron transport pathway before it acts as a stimulant of lipid peroxidation[1–3], no definite proof exists on which step—NADPH–cytochrome *c* reductase or cytochrome P-450—is involved in the activation. Slater and Sawyer[4] suggested that the former is involved, while Recknagel and Glende[3] pointed out the latter. Recently, Sipes *et al.*[5] specified cytochrome P-450 as a bioactivation step by examining the covalent binding of ^{14}C-metabolites of $^{14}CCl_4$ to microsomal proteins as a measure of the conversion of CCl_4 to reactive intermediates.

This study was begun to determine which step of the electron transport pathway is concerned with the activation of CCl_4, by using sonicated microsomes and a reconstituted microsomal enzyme system. Lipid peroxidation was measured as an indicator of CCl_4 bioactivation.

Materials and methods

Seven- to eight-week-old female Wistar strain rats were used. Male rats of the same strain and age were used for purification of the enzymes.

Microsomes were prepared from livers well-perfused with a cold 0.15 M KCl solution. The livers were homogenized with 4 vol. of 0.15 M KCl–10 mM EDTA (pH 7.5) and centrifuged at 15,000 *g* for 15 min. The supernatant fraction was then centrifuged at 125,000 *g* for 30 min, and the precipitated microsomes, after washing with 0.1 M potassium phosphate buffer (pH 7.5), were suspended in the same buffer (40–50 mg protein/ml).

Cytochrome P-450 and NADPH–cytochrome *c* reductase were purified from liver microsomes isolated from rats treated with phenobarbital (80 mg/kg/day, i.p., 5 days) according to the methods described by Imai and Sato[6] and Imai[7]. Our final cytochrome P-450 preparation (7.9 nmoles/mg of protein) was only partially purified, if judged from the reported value of 16.8 nmoles/mg of protein[6], but no NADPH–cytochrome *c* reductase was detected. The specific activity of the NADPH–cytochrome *c* reductase preparation was 1.3 μmoles cytochrome *c* reduced/mg of protein/min (6.52 μmoles cytochrome *c* reduced/ml/min) at 25°, using horse heart cytochrome *c* as an electron acceptor.

Microsomal lipids were extracted by the method described by Folch *et al.*[8] and lipid phosphorous was determined by the method of Bartlett[9]. Liposomes (20 μmoles P_i/ml) were prepared just before use by sonicating the solvent-free lipids in 0.1 M potassium phosphate buffer (pH 7.5) in an ice bath using a Branson micro-tip for a total of 40 sec, with several interruptions, with a power of 40 W. The microsomal suspension (20 mg protein/ml) was sonicated similarly.

The degree of lipid peroxidation was assessed by measuring malondialdehyde (MDA) in the reaction mixture. The regular method is as follows: sonicated microsomes (1 mg protein/50 μl) or liposomes (1 μmole P_i/50 μl) were mixed with NADPH–cytochrome *c* reductase (activity: 0.33 μmoles cytochrome *c* reduced/min/50 μl, which was omitted in the case of sonicated microsomes, and cytochrome P-450 (2 nmoles/50 μl) in a final volume of 0.15 ml. After a preincubation period of 20 min at 37°, the mixture was cooled in an ice bath and brought to 1.0 ml with 0.1 M potassium phosphate buffer (pH 7.5), and then NADPH (400 μM) and 0.5 μl CCl_4 were added (complete system). The test tube was capped, mixed vigorously for about 10 sec using a mixer, and incubated for 20 min at 37°. The MDA produced in the reaction mixture was determined by a thiobarbituric acid method[10] using the extinction coefficient $1.56 \times 10^5\ cm^{-1}\ M^{-1}$ for the colored product. Endogenous MDA content was subtracted from the value obtained. Any variations of the procedure are given in the legends.

Protein was determined by the method of Lowry *et al.*[11].

Results and discussion

Initially, the effect of added cytochrome P-450 on microsomal lipid peroxidation was examined. Sonicated microsomes were mixed with cytochrome P-450 in a small volume and preincubated in order to enhance the incorporation of the hemoprotein into microsomal vesicles[12], and were then peroxidized after dilution. As shown in Fig. 1, MDA production in the complete system