

Alternate Substrates and Inhibitors of 1-Aminocyclopropane-1-carboxylic Acid Synthase

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Structural analogs of (-)-*S*-adenosyl-L-methionine (SAM), in which the heterocyclic base was modified, were used to initiate studies to elucidate the active site conformation of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which was partially purified from *Lycopersicon esculentum* (tomato). These potential substrate analogs were screened for activity both as substrates and/or as inhibitors of ACC synthase. In general, ACC synthase was found to have a rather rigid specificity for the structural features of the natural substrate (SAM) in that only the purine base adenosine and adenosine analogs in which the *N*⁶ nitrogen was modified were substrates. © 1987 Academic Press, Inc.

INTRODUCTION

The conversion of (-)-*S*-adenosyl-L-methionine (SAM)¹ to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase, a pyridoxal phosphate (PLP)-containing enzyme, has recently been shown to be the rate-limiting step in the biosynthesis of the important plant hormone ethylene (*I*) in extracts of tomato fruits (2-5).

Preliminary studies on the crude enzyme preparation have shown an apparent *K_m* of 13 μM (4) and of 20 μM (5) for SAM and a strict requirement for PLP (5). It is interesting to note that the previously reported (6) active-site-directed irreversible inhibitors of enzymes requiring PLP as a cofactor, such as aminoethoxyvinylglycine (AVG), aminoxyacetic acid (AOA), and rhizobitoxine, inhibit ACC synthase, but only in a reversible manner. The *K_i* value for AOA was estimated to be 0.8 μM (4) and for AVG 0.2 μM (5). Several analogs of SAM, such as *S*-adenosyl-L-ethionine (SAE), methionine methylsulfonium salt, *S*-pentosyl-L-methionine, and *S*-adenosyl-L-homocysteine (SAH), have been tested as both substrate and

¹ Abbreviations used: SAM, (-)-*S*-adenosyl-L-methionine; ACC, 1-aminocyclopropane-1-carboxylic acid; *N*⁶(Me)SAM, *S*-*N*⁶-methyladenosyl-L-methionine; SIM, *S*-inosyl-L-methionine; SGM, *S*-guanosyl-L-methionine; SCM, *S*-cytidyl-L-methionine; SUM, *S*-uridyl-L-methionine; *N*⁶(Me)₂-SAM, *S*-*N*⁶,*N*⁶ dimethyladenosyl-L-methionine; *N*⁶(Bz)SAM, *S*-*N*⁶-benzyladenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; PLP, pyridoxal phosphate; AVG, aminoethoxyvinylglycine; AOA, aminoxyacetic acid; SAE, *S*-adenosyl-L-ethionine; HMPA, hexamethyl phosphoramide.

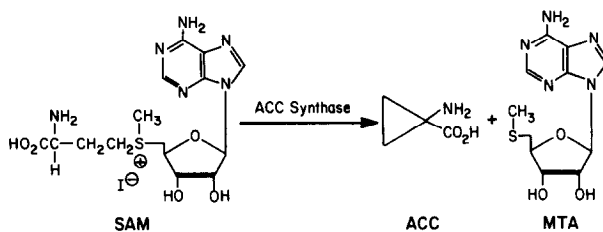


FIG. 1. Reaction catalyzed by 1-aminocyclopropane-1-carboxylate synthase.

inhibitor (4, 5). Of the analogs, only SAE served as an alternate substrate, with an apparent K_m value of $6 \mu\text{M}$ and a V_{max} 30% that of SAM (4). The best SAM-like substrate inhibitor, SAH, had an $I_{50} = 50 \mu\text{M}$ (4). Recently, the naturally occurring antifungal antibiotics, Sinefungin, A9145C, and the synthetic nucleoside 5'-*S*-isobutyl-5'-deoxythioadenosine, have been shown (7) to be inhibitors of ACC synthase. Sinefungin, the best inhibitor of the three, had an $I_{50} = 25 \mu\text{M}$ for ACC synthase isolated from red tomatoes.

Because the above data suggest that both the sulfonium function and the adenosine moiety (or maybe only an aromatic base) of the SAM molecule are essential components of an enzymatically active substrate for ACC synthase and since no studies to date have involved the testing of compounds which might serve as alternate substrates for ACC synthase, thereby allowing for the possibility of enhancing the ripening process, we decided to synthesize and study the effects of various analogs of SAM in which the base portion (adenine) of SAM is varied on the synthesis of ACC by ACC synthase.

RESULTS AND DISCUSSION

(-)-*S*-Adenosyl-L-methionine is a biological, ubiquitous molecule which may be viewed as being composed of three parts: an amino acid portion (methionine), a sugar portion (ribose), and an aromatic heterocyclic base portion (adenine). The study described here will present the results obtained when the adenine base portion was either modified or replaced with a different heterocycle.

The purine and pyrimidine analogs listed in Fig. 2 were synthesized by the routes outlined in Scheme 1. These analogs were tested as alternate substrates and/or inhibitors of ACC synthase. The appropriate biological data are summarized in Table 1.

Of the base modifications reported in this paper, only derivatization of the adenine ring's N^6 nitrogen led to compounds with catalytic activity. The replacement of the adenine ring with a pyrimidine base or the purine bases inosine or guanosine gave compounds with neither catalytic nor inhibitory activity.

Since the sulfonium center was generated by chemical alkylation of the SAH analogs with methyl iodide, the stereochemistry at the sulfur center should be racemic. We have recently shown (8) that the apparent K_m for synthetic (\pm)-L-SAM (supposedly racemic at the sulfur atom but actually 55:45 (+)-L-SAM to

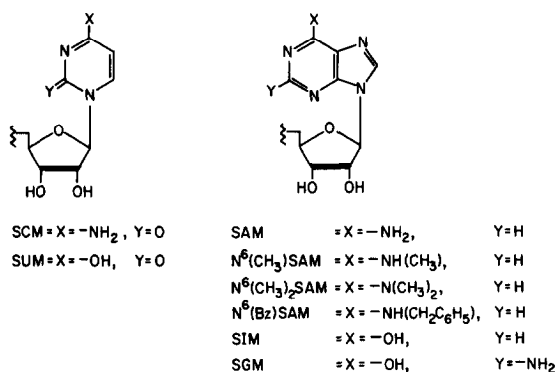
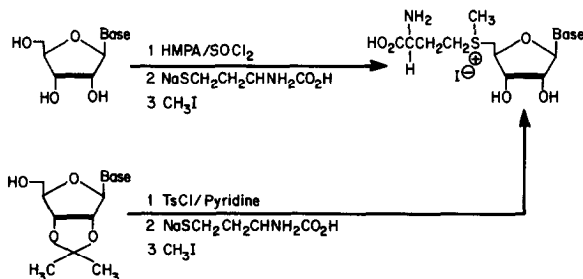


FIG. 2. Substitution pattern of various purines and pyrimidines tested.

(-)-L-SAM as determined by ¹H NMR is $40 \pm 10 \mu\text{M}$ and that the apparent K_m for (+)-L-SAM (the unnatural stereoisomer at the sulfur center) is $15 \pm 5 \mu\text{M}$. Therefore each analog tested contained, in a ratio of $\sim 55:45$ unnatural to natural stereoisomer at the sulfur center, both a potentially enzymatically active isomer and a potentially enzymatically inactive isomer which may be inhibitory. It is, therefore, unlikely that the failure of the purine and pyrimidine SAM analogs to show any catalytic or inhibitory activity is simply due to the presence of a racemic sulfonium center.

The replacement of the 6-amino group of adenosine with a 6-hydroxyl group, inosine, led to a compound (SIM) inactive both as a substrate and as an inhibitor. This is rather surprising since both the amino and hydroxyl functions are capable of acting as hydrogen bond acceptors at the active site and both compounds are known to exist in solution in dynamic equilibrium between the preferred *anti* and *syn* conformation (9). Adding an amino function to the 2-position of the inosine portion of the SIM molecule, guanosine, generates a compound (SGM) inactive both as a substrate and as an inhibitor. Guanosine is also known to exist in solution in the favored *anti* conformation, as are most pyrimidines and purines other than those substituted in the 6- or 8-position, respectively, which generally prefer the *syn* conformation. Thus the lack of activity between SAM and SIM/SGM toward ACC synthase cannot be explained simply on the basis of potential



SCHEME 1. Synthetic route to the various (\pm)-S-nucleosyl-L-methionines.

TABLE I
 BIOLOGICAL DATA FOR VARIOUS *S*-NUCLEOSYL-L-METHIONINES

Compound	V_{\max} (relative)	K_m (μM)	K_i (μM)
(-)-L-SAM	1	20 ± 5	—
(\pm)-L-SAM	1	40 ± 10	—
(+)-L-SAM	—	—	15 ± 5
(\pm)-L-SAM	—	—	70 ± 20
(\pm)- <i>S</i> -Inosyl-L-methionine	—	—	>500
(\pm)- <i>S</i> -Guanosyl-L-methionine	—	—	>500
(\pm)- <i>S</i> - <i>N</i> ⁶ -Methyladenosyl-L-methionine	0.8	60 ± 10	—
(\pm)- <i>S</i> - <i>N</i> ⁶ , <i>N</i> ⁶ -Dimethyladenosyl-L-methionine	0.1	30 ± 10	—
(\pm)- <i>S</i> - <i>N</i> ⁶ -Benzyladenosyl-L-methionine	0.25	5 ± 2	—
(\pm)- <i>S</i> -Uridyl-L-methionine	—	—	>500
(\pm)- <i>S</i> -Cytidyl-L-methionine	—	—	>500

changes in the base–sugar conformation since there are apparently no significant conformational differences among the three. The major difference, other than the obvious one between oxygen and nitrogen, among the three compounds is that in the case of inosine (SIM) and guanosine (SGM) the 6-hydroxyl group exists predominantly in the keto form (i.e., no hydrogen atom on the oxygen atom) and therefore can only serve as a hydrogen bond acceptor and not a hydrogen donor. In order to further explore the role of the hydrogen atoms attached to the hetero atom at position 6 in the purines, we synthesized the three nitrogen-substituted SAM analogs, *N*⁶(Me)SAM, *S*-*N*⁶-methyladenosyl-L-methionine; *N*⁶(Me)₂SAM, *S*-*N*⁶,*N*⁶-dimethyladenosyl-L-methionine, and *N*⁶(Bz)SAM, *S*-*N*⁶-benzyladenosyl-L-methionine.

As can be seen from Table 1, the V_{\max} tends to diminish as the *N*⁶ of the adenine ring becomes more substituted. The differences in the V_{\max} values among SAM (1), *N*⁶(Me)SAM (0.8), and *N*⁶(Me)₂SAM (0.1) are particularly interesting. These data along with those presented above suggest that the hydrogen atoms which are covalently bonded to the 6-amino group of the adenine portion may play an important role in the catalytic activity of SAM in the active site. This catalytic activity could presumably be through hydrogen bond donation of the hydrogen atoms at the *N*⁶-position to an appropriate group in the active site. This seems feasible since in *N*⁶(Me)₂SAM the nitrogen has no hydrogen atoms of its own to donate. In contrast, the inosine analog, SIM, which exists predominantly in the keto form, also could not act as a donor of hydrogens in hydrogen bond formations. However, both *N*⁶(Me)₂SAM and SIM could both act as hydrogen bond acceptors. This could also explain some of the potential differences in the ability of SAM, SGM, and SIM to bind to the enzyme active site. One could argue that the differences in V_{\max} occur with increasing substitution on the 6-amino group (i.e., increase in steric bulk at the *N*⁶ nitrogen); however, *N*⁶(Bz)SAM, monosubstituted at the *N*⁶-position with a large group, appears to have a greater V_{\max} than the disubstituted *N*⁶(Me)₂SAM, indicating that the rate of catalysis may be inde-

pendent of steric factors when comparing the analogs at hand. The K_m values of the N^6 -substituted SAM analogs, with the possible exception of $N^6(\text{Bz})\text{SAM}$, are all equivalent, indicating little difference in their binding affinities.

Based on the ring specificity observed above it is not surprising that the replacement of the purine base with the pyrimidine bases uridine or cytidine led to compounds which were totally devoid of both catalytic or inhibitory activity.

It is interesting to note that AVG, AOA, and *S*-ribosyl-L-methionine are inhibitors of ACC synthase although the first two lack both the aromatic ring and the sugar portion and the latter one lacks the heterocyclic base. Apparently these inhibitors bind only to the amino acid-binding portion of the active site, which potentially could be separated from the binding site of the sugar and base moiety, or some other phenomenon is causing the lack of activity of ACC synthase.

Of the potential substrate analogs screened for activity both as substrates and/or as inhibitors of ACC synthase only the purine base adenosine and adenosine analogs in which the N^6 nitrogen was modified were substrates. We therefore conclude that ACC synthase has a rather rigid specificity for the structural features of the natural substrate (SAM).

Studies are currently underway to evaluate the effect of changes in the nitrogen substitution pattern within the adenine ring system (i.e., various deazaadenosine analogs).

EXPERIMENTAL PROCEDURE

Materials. Organic, inorganic, and biological chemicals were procured from Aldrich Chemical Company, Alfa-Ventron Corporation, and Sigma Chemical Company, respectively. Tomatoes (*Lycopersicon esculentum*) in the "light pink" stage, vine-picked or purchased from a local vendor, were used to obtain the pericarp tissue for enzyme preparation.

Enzyme purification. The unpeeled pericarp tissue of tomatoes was chopped into small pieces and allowed to stand for 3 h at 25°C. The tissue was homogenized in a glass-Teflon homogenizer with an equal volume of 100 mM Hepes (pH 8.5), 4 mM dithiothreitol, 0.5 μM pyridoxal phosphate, and 5% polyvinylpyrrolidone (w/v). The preparation was pressed through several layers of cheesecloth and centrifuged at 15,000g for 30 min. The supernatant was fractionated by precipitation with solid ammonium sulfate. The fraction precipitating between 40 and 90% (pH 7.2) was dialyzed overnight against 2 mM Hepes (pH 8.5) containing 0.1 mM dithiothreitol and 0.2 μM pyridoxal phosphate, and the dialyzed enzyme was used without further purification.

Enzyme assay. A modification of the assay previously described was employed to analyze the ACC synthase activity (10). The assay consisted of 50 nmol of SAM or increasing concentrations of alternate substrates, 30 μmol of K-Hepes, pH 8.5, and 700 μl of the 40–90% $(\text{NH}_4)_2\text{SO}_4$ ppt dialyzed enzyme preparation in a total volume of 800 μl . After 15 min to 3 h at 30°C the reaction mixtures were cooled in ice to 4°C, 100 μl of 10 mM HgCl_2 was added to each tube, the volumes were adjusted to 1 ml for each mixture, and the tubes were capped with rubber septa,

vortexed, and returned to the ice bath. To analyze the amount of ACC formed in each tube, the ACC was converted to ethylene by the addition of 100 μ l of NaOCl (5%)–NaOH (10 N) mixture (2:1 v/v). After the addition of the bleach solution, the tube was vortexed for 30 s and a 1-ml sample from the gas phase was withdrawn through the septum cap for the determination of ethylene by a gas chromatograph equipped with a flame ionization detector. A column (45 \times 0.64 cm) of 60–80 mesh of Al₂O₃, at 90°C, with helium as carrier gave a retention time of 25 s for ethylene. To determine the efficiency of the conversion of ACC to ethylene, a known amount of ACC (10 nmol) was added as an internal standard to another similarly incubated reaction mixture and converted to ethylene as described above. Yields of ethylene were consistently 80–90%, and ethylene obtained from blank samples containing no substrate or enzyme was less than 1% of that obtained with enzyme. Protein was determined according to Bradford (11).

Enzyme inhibition. ACC synthase prepared from light pink tomatoes with a specific activity of 1.8 nmol/mg protein/h was incubated with various amounts of inhibitor and the enzyme activity assayed as described above with (–)-L-SAM.

Syntheses

5'-Chloro-5'-deoxynucleoside. These derivatives of the adenosine analogs were prepared according to the procedure of Kikugawa and Ichino (12) as follows: To a stirred solution of 10 ml of thionyl chloride in 10 ml of hexamethyl phosphoramide (HMPA) was added the solid unprotected nucleoside (4.1 mmol). After stirring at room temperature for 18 h, the solution was loaded directly onto a Dowex 50-X 4-200 column (NH₄⁺ form) (125 ml) and washed with water to remove the HMPA. The products were eluted with 1.0 M NH₄OH and the fractions containing products were neutralized with 1.0 N HCl and the water was removed under reduced pressure. The solid residues were recrystallized from the appropriate solvent and all physical data are in agreement with literature values.

2',3'-Isopropylidene-5'-O-toluene-p-sulfonylnucleoside. These derivatives of guanosine, inosine, and uridine were prepared by the procedure of Borchardt *et al.* (13) as follows: To a stirred solution of 2.33 mmol of the 2',3'-isopropylidene nucleoside in 20 ml of dry pyridine, freshly distilled from barium oxide, at 0–5°C (ice–salt bath), was added all at once 2.5 mmol of freshly recrystallized *p*-toluene-sulfonyl chloride. The mixture was stirred over night (12 h) at room temperature. The pyridine was removed under reduced pressure and the residue taken up in water. The solution was extracted with CHCl₃ (3 \times 50 ml) and the combined CHCl₃ extracts were extracted with H₂O and then 5% NaHCO₃. The organic layer was dried over MgSO₄ and the CHCl₃ removed under reduced pressure (water aspirator) to give a tacky solid which was crystallized from CHCl₃ hexanes. All physical data for these compounds are in agreement with literature values.

S-Nucleosyl-L-homocysteine. A suspension of the 5'-chloro-5'-deoxynucleoside or the 2',3'-isopropylidene-5'-O-toluene-*p*-sulfonyl nucleoside (2.0 mmol), potassium iodide (10 mg), and the sodium salt of L-homocysteine (2.5 mmol) was heated to reflux in water (7 ml) for 2 h. The reaction mixture became clear, and TLC (Avicel F, 250 μ m, CHCl₃–EtOH–H₂O, 3:2:0.5) showed the absence of any 5'-

chloro-5'-deoxynucleoside or 5'-tosylate-5'-deoxynucleoside. The mixture was cooled and made acidic with 1 N HCl. In addition to adjusting the pH of the reaction mixture this also serves to remove the isopropylidene-protecting group. The solution was then applied directly to a column of Dowex 50-X 4-200 (NH₄⁺ form) (125 ml). The column was washed thoroughly with water (200 ml) and then eluted with 1 N NH₄OH. The eluate was evaporated to a small volume *in vacuo* and lyophilized. The products were recrystallized from the appropriate solvents. Yields averaged 45–80% (14).

(±)-*S*-Nucleosyl-L-methionine. *S*-Nucleosyl-L-homocysteine (25 mg) was added to a stirred solution of a 1:1 (v/v) mixture of acetic acid:formic acid (1 ml). To this mixture was added 0.1 ml of methyl iodide and then 25 mg of silver acetate. The mixture was stirred in a foil-wrapped flask at room temperature for 4 h. The mixture was centrifuged and the supernatant extracted with diethyl ether (15). The aqueous layer was then applied to a C-18 reversed-phased, low-pressure column. The (±)-*S*-nucleosyl-L-methionines eluted immediately after the void volume. The appropriate fractions were lyophilized. The purity was checked by HPLC (Whatman Particil PXS 10/25 SCX); buffer 0.1 M NH₄PO₄, pH = 3.5; flow rate 3.5 ml/min; uv detector (260 nm wavelength monitor); sample retention times (ml) were as follows: MTA 11.3, SAH 7.5, and SAM 50. In all cases the (±)-*S*-nucleosyl-L-methionines eluted last. ¹H NMR spectra taken at 270 MHz on an IBM WP 270 SY showed two methyl signals at $\delta \cong 2.9$ (sulfonium methyl group resonance for the unnatural stereoisomer at the sulfur center) and 3.0 (sulfonium methyl group resonance for the natural stereoisomer at the sulfur center) for each of the SAM analogs prepared (16).

These synthetic sequences are summarized in Scheme 1 and all compounds exhibited the physical and spectral characteristics in accordance with the reported values.

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