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ANTIFUNGAL ANTIBIOTICS AND SIBA INHIBIT 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE ACTIVITY

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Summary. The antifungal antibiotics Sinefungin and A9145C isolated from *Streptomyces griseolus* and the synthetic nucleoside Siba, which are analogs of S-adenosylmethionine, inhibit the activity of 1-aminocyclopropane 1-carboxylic acid synthase from tomato fruits. Sinefungin and Siba were shown to be more potent inhibitors than A9145C. In extracts of green fruits, the enzyme activity was inhibited by Sinefungin with an I_{50} of 1 μM, which was similar to that caused by aminoethoxyvinylglycine, and by Siba with an I_{50} of 100 μM; in extracts from red tomatoes, the I_{50}'s were 25 μM and 100 μM, respectively. The inhibition of ACC synthase by these analogs could be reversed by gel filtration chromatography.

Ethylene has long been known to have a profound effect on plants (1). The pathway of ethylene biosynthesis has been gradually elucidated and can be summarized in the sequence: Methionine —> S-adenosylmethionine (SAM)[1] —> 1-aminocyclopropane-1-carboxylic acid (ACC)[1] —> ethylene (2). The rate-limiting enzyme of this process was shown to be ACC synthase (2,3). This enzyme, which converts SAM to ACC, was described by Boller *et al.* (4) and by Yu *et al.* (5) in extracts of tomato fruit.

ACC synthase showed a Km between 13 μM (4) and 20 μM (5) with respect to its substrate, SAM, and it showed a strict requirement for pyridoxal phosphate. Thus, inhibitors of enzymes requiring pyridoxal phosphate as a cofactor, such as aminoxyacetic acid and aminoethoxyvinylglycine (AVG),[1] were shown to inhibit efficiently ACC synthase (2). Some analogs of SAM, like S-adenosylethionine and S-adenosylhomocysteine (SAH),[1] were also shown

Abbreviations: SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; Siba, 5'-deoxy-5'-5-isobutyl-thioadenosine; SAH, S-adenosylhomocysteine; MES, (2-[N-morpholino]ethanesulfonic acid); Epps, (4-[2-hydroxyethyl]-1-piperazine-propanesulfonic acid).

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to act as inhibitors in the ACC synthase reaction, although 50 μM SAH was needed to bring about 50% inhibition (5).

The naturally occurring antifungal antibiotics Sinefungin and A9145C, and the synthetic nucleoside Siba (5'deoxy-5'-5-S-isobutylthioadenosine), form part of a family of compounds which are analogs to SAM and SAH (Fig. 1). These compounds have been shown to inhibit *in vivo* the proliferation of a tumor virus (6) and parasites (7), and to inhibit *in vitro* a number of tRNA methylases, N-methyl transferases and O-methyl transferases (6,8). More recently, another type of transmethylation reaction involving the creation of a cyclopropane ring at a site of unsaturation of a membrane phospholipid fatty acid has been shown to be strongly inhibited by Sinefungin and A9145C (9).

Since the conversion of SAM to ACC and methylthioadenosine involves an elimination of an α-H along with a γ-substituent (1-3 elimination) giving rise to a cyclopropane ring (5), it was of interest to study the effect of analogs of SAM and SAH on the synthesis of ACC.

Experimental. Sinefungin and A9145C were a gift from Dr. R. Nagarajan, E. Lilly Research Laboratories (Indianapolis, IN 46285, USA). Siba was a gift from Prof. E. Lederer, Institut de Chimie des Substances Naturelles (CNRS), 92290 Gif Sur Yvette, France.

Enzyme extractions. Red or green tomatoes (Faculty 16 variety) were sliced to 0.5 cm width and were incubated overnight in 10 mM MES[1] buffer, pH 6.1, 0.6 M sorbitol and 10 μg/ml chloramphenicol. The slices were then washed twice with cold water and homogenized in a Sorvall Omnimixer Blender at 2°C in the presence of 2 mM DTT, 1 μM pyridoxal phosphate, 0.1 M Na phosphate buffer, pH 7.9. The homogenate was pressed through four layers of cheese-cloth, brought to 0.1 M $MgCl_2$ to precipitate pectins, and centrifuged at 27000 x *g* for 20 min. The supernatant fluid was concentrated under pressure by ultrafiltration through an Amicon YM-10 membrane. The concentrated enzyme was then passed through a Sephadex G-25 column equilibrated with 20 mM Na phosphate buffer, pH 7.9, 0.4 mM DTT, 2.5 μM pyridoxal phosphate. The protein containing fraction was used as the source of enzyme. Protein was determined according to Bradford (11).

Enzyme assay. The standard reaction mixture consisted of 50 mM Epps[1] buffer, pH 8.5, 150 μM SAM, 200 μl enzyme (70-100 μg protein) and equilibration buffer in a total volume of 600 μl. Reactions were incubated at 30°C for 1 h and were terminated by the addition of 0.1 ml of cold 10 mM $HgCl_2$. Tubes were then sealed with serum caps and ACC formed was determined by conversion to ethylene according to Lizada and Yang (12). The efficiency of conversion of ACC to ethylene was 70 to 80% in all our experiments. A sample of gas was taken to determine the concentration of ethylene by gas chromatography.

Results and Discussion

Sinefungin, Siba or A9145C, which are structural analogs of SAM (Fig. 1), inhibited the activity of ACC synthase from tomato fruit when assayed in the

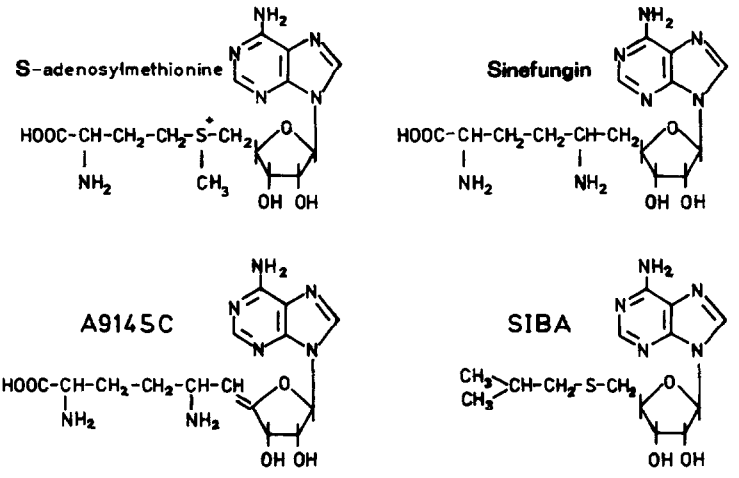

Fig. 1. Structural formulas of SAM and some analogs.

presence of SAM as substrate. These compounds, however, were not converted to ACC by either extract from red or green tomatoes. The inhibitory effect of these SAM analogs on the activity of ACC synthase isolated from red tomatoes is shown in Fig. 2, as a function of their molar concentration. For comparison we have included in this study treatments with AVG, which is a

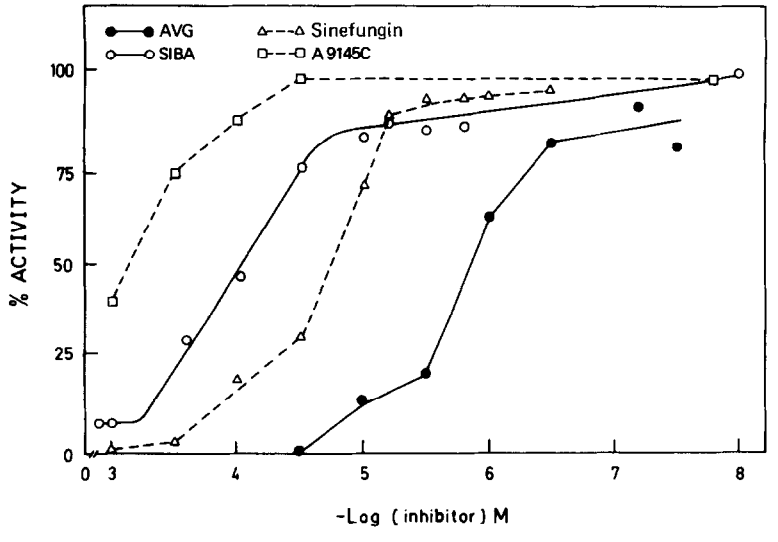

Fig. 2. Effect of SAM structural analogs and AVG on ACC synthase. ACC synthase from a red tomato preparation with a specific activity of 15 mnmoles/mg protein/h protein was incubated with or without the indicated concentrations of inhibitors as described under Experimental.

Table I

Sensitivity to AVG and Sinefungin of ACC synthase from red and green tomatoes

	I_{50} (μM)	
	Green	Red
AVG	1.0	1.7
Sinefungin	1.0	25.0
Siba	100.0	95.0

ACC synthase preparation from green tomatoes with a specific activity of 1.4 nmoles/mg protein/h, or ACC synthase preparation from red tomatoes (15 nmoles/mg protein/h) were incubated with increasing concentrations of inhibitors as described under Experimental.

known potent inhibitor of ACC synthase (4). Among the analogues tested, Sinefungin was found to be the most potent inhibitor of ACC synthase. It showed an I_{50} of 25 μM. Siba was less potent with an I_{50} of 95 μM, and A9145C was the least effective showing an I_{50} of 0.85 mM. In extracts from red tomatoes, AVG was a more potent inhibitor of ACC synthase than the SAM structural analogs tested showing an I_{50} of 1.7 μM. However, ACC synthase extracted from green tomato fruits was far more sensitive to Sinefungin than that extracted from red tomatoes. In the case of enzyme from green tomatoes, Sinefungin was as effective as AVG, showing an I_{50} of 1.0 μM, which is identical to that for AVG (Table I). Siba had the same I_{50} for ACC synthase obtained from green or red tomatoes.

The inhibition produced by Sinefungin or Siba, as well as AVG, can be reversed almost completely by passing the inhibited enzyme through a G-25 column (Table II). Thus indicating that the inhibitors tested are not covalently bound to ACC synthase.

The fact that the structural analogs of SAM did not serve as a substrate for ACC synthase is in accordance with the results obtained by Boller *et al.* (4) and Yu *et al.* (5) indicating that both the sulfonium function and the

Table II

Reversibility of the inhibition of ACC synthase caused by various inhibitors

Inhibitor	Enzyme activity (% control)	
	Directly	After G-25 Sephadex chromatography
None	100.0	100.0
AVG, 2.5 μM	50.7	91.8
Sinefungin, 100 μM	29.5	86.3
Siba, 208 μM	68.3	119.0

ACC synthase (21.6 nmoles/mg protein/h) obtained from red tomatoes was preincubated with the indicated inhibitors and assayed directly or after G-25 Sephadex chromatography

adenosine moiety of the SAM molecule are essential components of an active substrate for ACC synthase.

When tested for their ability to inhibit ACC synthase activity, Sinefungin was found to be the most potent and A9145C the least potent. In contrast, the unsaturated molecule, A9145C, was reported to be the most potent inhibitor of transmethylation reactions (9). Yu *et al*, (5) have shown that several sulfur-containing SAM analogs are capable of inhibiting ACC synthase. The data presented herein indicated that the presence of a sulfur atom in the molecule is not a prerequisite for the inhibition, since Sinefungin which lacks a sulfur atom is a more potent inhibitor than Siba which has one.

Differences in sensitivity to AVG between red and green tomatoes have been reported by Baker *et al*. (10), where the production of ethylene by green tomato tissue was 1.6 to 2.2-fold more sensitive to AVG than that of red tomato tissue. Our results showed that Sinefungin inhibition of ACC synthase activity followed a similar pattern, where a 25-fold higher molar concentration was required to achieve 50% inhibition in red tomatoes as compared with green tomatoes.

In conclusion, we report here that the naturally occurring antibiotic Sinefungin is a new and potent inhibitor of ACC synthase that is at least twice as effective as SAH (5). Siba and especially Sinefungin may prove of importance in further understanding the mechanism of ethylene biosynthesis.

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