[Chem. Pharm. Bull. 26(3) 885—892 (1978)]

UDC 615.332.011.5 : 576.852.098

Biosynthesis of Streptothricin Antibiotics. VII.[1] Origin of Streptolidine Moiety in Antibiotics from *Streptomyces* species

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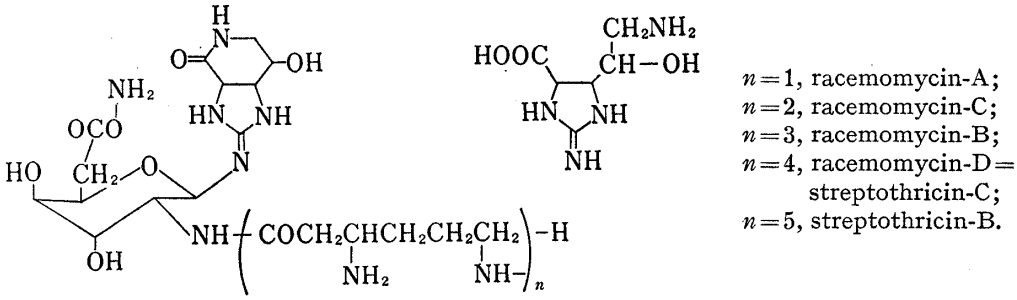
(Received August 15, 1977)

The effective production of streptothricin antibiotics by the addition of various amino acids (chemistry defined medium C) and the incorporation of ^{14}C-labeled amino acids into streptothricin-C molecule (organic medium E) were studied with a strain of *Streptomyces lavendulae* OP-2. It was confirmed that proline, alanine, leucine, and arginine stimulated the production of antibiotics, and arginine and glycine promoted the mycelial growth. Of the amino acids tested, arginine was incorporated preferentially into streptothricin-C, but not tartaric acid or streptolidine itself. Approximately a one-half of radioactivity from arginine incorporated into the antibiotic was located in the streptolidine moiety.

Furthermore, from the localization of ^{14}C from arginine in the antibiotic molecule from four strains, a different pattern from that described above was observed. Localization of radioactivity in streptolidine moiety of racemomycin-A from *S. lavendulae* ISP-5069 was lower than that of β-lysine moiety, suggesting that biogenesis of streptolidine in streptothricins from *S. lavendulae* strains follows two pathways.

Keywords——*Streptomyces lavendulae* OP-2; *Streptomyces lavendulae* ISP-5069; stimulatative effect; biosynthesis of streptolidine; incorporation of arginine; streptothricin-C; racemomycin-A; β-lysine; D-gulosamine; multienzyme system

Two of the present authors previously reported physicochemical and biological properties of racemomycin-A (streptothricin-F), racemomycin-C (streptothricin-E), and racemomycin-B (streptothricin-D),[3] and streptothricin-B.[4] At that time, characteristics of streptothricin-C (racemomycin-D)[5] still remained to be studied.[6] In order to obtain streptothricin-



$n=1$, racemomycin-A;
$n=2$, racemomycin-C;
$n=3$, racemomycin-B;
$n=4$, racemomycin-D= streptothricin-C;
$n=5$, streptothricin-B.

Chart 1. Structures of Streptothricins and Streptolidine

1) Part VI: Y. Sawada, S. Nakashima, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **25**, 3210, (1977).
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3) H. Taniyama, Y. Sawada, and T. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 1627 (1971).
4) Y. Sawada, H. Taniyama, and A. Seino, *J. Ferment. Technol.*, **55**, 290 (1977).
5) The name streptothricin-C was used for racemomycin-D in this paper.
6) Physicochemical and biological properties of streptothricin-C will be published elsewhere.

C in a good yield, a strain OP-2 which belongs to *Streptomyces lavendulae* was isolated from soil samples.[7] As shown in Chart 1, these antibiotics give three components in their principal hydrolysis products, β-lysine, D-gulosamine, and streptolidine (Chart 1). Voronina *et al.*[8] and Sawada *et al.*[1,9] clarified independently that β-lysine moiety in streptothricins produced by streptomycetes is preferentially biosynthesized from lysine.[10] D-Gulosamine moiety in the antibiotics is formed from D-glucose[9] or D-glucosamine,[11] most probably as a direct precursor. The third guanidino compound was reported to be produced biosynthetically from carbons of acetate in a strain of *S. lavendulae* ISP-5069.[9] This result was also confirmed by the incorporation of ^{13}C-labeled acetate compounds.[12] Meanwhile, Gräfe, *et al.*[13] reported the specific incorporation of arginine into streptolidine moiety in nourseothricin with a strain of *S. noursei*. These results concerning the biosynthesis of streptolidine suggest that this amino acid is likely to be produced, at least, in two different pathways.

Studies on the efficiency of amino acids in the fermentative production of streptothricin-C with *S. lavendulae* OP-2, showed that arginine stimulated both the production of the antibiotic and growth of the organism. Furthermore, we found that arginine was incorporated efficiently into streptolidine moiety. A part of this result was reported in the preliminary communication.[14] This paper reports results of experiments to determine the origin of streptolidine molecule in streptothricins in streptomycetes.

Materials and Methods

Microorganisms——For *S. lavendulae* OP-2: Medium A: 2% glucose, 0.5% meat extract, 0.5% peptone, 0.5% NaCl, pH 7.0. Medium B: 3% glucose, 0.5% NaCl, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.04% $CaSO_4 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.6 mM thiamine hydrochloride, 0.6 mM riboflavine, 0.6 mM pyridoxal phosphate, and 0.6 mM nicotinamide, pH 7.0. Medium C: B+1% ammonium tartarate. Medium D: B+1% ammonium citrate. Medium E: 3.5% glycerol, 0.5% meat extract, 0.5% peptone,

culture broth (500 ml)
↓
Amberlite IRC-50 (Na^+) (2.2×10 cm)
↓ elution with 0.2 N HCl
fraction active against *B. subtilis* PCI-219
↓ concentration
Sephadex LH-20 (2.9×140 cm)
↓ elution with H_2O
active fraction
↓
paper chromatography[a]
↓
lyophilization
↓
^{14}C-Streptothricin-C

Chart 2. Isolation and Purification of Streptothricin-C-^{14}C

a) Solvent system: I.

Table I. Yield of ^{14}C-Streptothricin-C purified

^{14}C-Compound tested	Yield of ^{14}C-streptothricin-C (mg)
Arginine	2.3
Alanine	12.2
Proline	1.7
Leucine	18.6
Phenylalanine	16.0
Tyrosine	2.5

7) Y. Inamori, S. Sunagawa, Y. Sawada, and H. Taniyama, *J. Ferment, Technol.*, **54**, 795 (1976).
8) O.I. Voronina, I.I. Tovarova, and A.S. Khoklov, *Izv. Akad. Nauk SSSR, Ser. Biol.*, **2**, 228 (1973).
9) Y. Sawada, T. Kubo, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **24**, 2163 (1976).
10) Amino acids are of L-form, unless otherwise specified.
11) Y. Sawada, S. Nakashima, H. Taniyama, and Y. Inamori, *Chem. Pharm. Bull.* (Tokyo), **25**, 1478 (1977).
12) Y. Sawada, S. Kawakami, H. Taniyama, and Y. Inamori, *J. Antibiot.*, **30**, 630 (1977).
13) U. Gräfe, G. Reinhardt, H. Bocker, and H. Thrum, *J. Antibiot.*, **30**, 106 (1977).
14) Y. Sawada, S. Nakashima, H. Taniyama, Y. Inamori, S. Sunagawa, and M. Tsuruga, *Chem. Pharm. Bull.* (Tokyo), **25**, 1161 (1977).

0.5% NaCl, pH 7.0. For other Streptomyces strains: Medium F: 1% glucose, 1% yeast extract, 1% peptone, 0.2% NaCl, 0.01% $(NH_4)_2SO_4$, 0.01% KH_2PO_4, 0.01% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, pH 7.0. Medium G: 1% maltose, 1% peptone, 0.5% meat extract, 0.5% NaCl, 0.25% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, pH 7.0.

Cultivation——A loopful of the spores of *S. lavendulae* OP-2 was transferred to a 500 ml flask containing 100 ml of medium A and this flask was incubated at 30° with reciprocal shaking (120 spm, amplitude 7 cm) for 24 hr. Two-ml aliquots of the culture were inoculated in a flask containing 100 ml of medium C, D, or E. The flask was then shaken under the same conditions. Radioactive compounds (Tables IV and V) (0.1 ml of aqueous solution per flask) were added to medium E after 4 hr of cultivation. The flask was then shaken for further 44 hr. Amino acid (1.0 ml of aqueous solution, pH 7.0) sterilized in an autoclave at 120° for 15 min was added at the beginning of the fermentation. Medium F was used for the pre-cultivation of other streptomycetes (Table VI). A 1 ml aliquot of the cultures was added to the flask containing 100 ml of medium G. The flask was shaken (120 spm) at 27°. Radioactive compounds were added to 6 hr cultures and antibiotics each were isolated after 72 hr of fermentation.

Isolation of Streptothricin-C——As shown in Chart 2, streptothricin-C samples labeled with ^{14}C were isolated from the corresponding culture filtrate (500 ml) by means of column (2×15 cm) chromatography over Amberlite IRC-50 (Na^+ form), and following Sephadex LH-20 (3×140 cm). Fractions containing streptothricin-C were determined by paper chromatography, and the antibiotic was detected by its reactions with ninhydrin and Rydon-Smith reagents. Yield of the purified antibiotic hydrochloride is given in Table I.

Paper Chromatography——Paper chromatography was carried out with a solvent system I of BuOH–pyridine–AcOH–H_2O–*tert*-BuOH (15: 10: 3: 12: 4, v/v) on Toyo Roshi No. 51 UH paper. To separate the acid-hydrolysis products of the antibiotics, a solvent system II of BuOH–pyridine–AcOH–H_2O–*tert*-BuOH (75: 50: 191: 236: 548, v/v) and Toyo Roshi No. 50 paper were used.

Antimicrobial Activity——Antimicrobial activity was determined by a standard disk method using *Bacillus subtilis* PCI-219 (1×10^6 spores/ml) as the test organism. Heart infusion agar (HIA) was used for the medium. Potency was expressed as the amount (μg/ml) of streptothricin-C hydrochloride.

Determination of Mycelial Weight——The culture broth (100 ml) was filtered through a filter paper (Toyo Roshi No. 51) and the paper was washed with H_2O. After being dried, its increased weight was determined. In the non-supplemented control in Table III, mycelial weight was 428.66 mg.

Chemicals——HIA was purchased from the Eiken Kagaku Co., Tokyo, meat extract from the Mikuni Chemical Co., Tokyo. Alanine [U-^{14}C] (specific activity: 10 mCi/mM), phenylalanine [U-^{14}C] (10 mCi/mM), arginine [U-^{14}C] (10 mCi/mM), proline [U-^{14}C] (10 mCi/mM), tyrosine [U-^{14}C] (10 mCi/mM), leucine [U-^{14}C] (10 mCi/mM), and DL-tartaric acid [1,4-^{14}C] (9.2 mCi/mM) were obtained from The Radiochemical Centre (Amersham, England), DL-arginine [1-^{14}C] (44.3 mCi/mM) and DL-arginine [5-^{14}C] (12.3 mCi/mM) from CIS, and DL-arginine [amidino-^{14}C] (55 mCi/mM) from ICN Pharmaceuticals, and other chemicals from Nakarai Chemicals Ltd., Kyoto. Streptolidine-^{14}C (6450 dpm/mg, 8 mg), was isolated from the acid hydrolysate of streptothricin-C-^{14}C hydrochloride (60 mg) from cultures (*S. lavendulae* OP-2) grown in the presence of arginine [U-^{14}C] by column (2×30 cm) chromatography over cellulose (Whatman Co., England).

Others——Methods for the determination of radioactivity, for the acid-degradation of the antibiotics, and for the separation of the hydrolyzed products have been described previously.[9]

Results

Selection of the Basal Medium

As reported previously,[7] streptothricin antibiotics were efficiently produced by *S. lavendulae* OP-2[15] in a synthetic medium containing an amino acid mixture. This fact suggested that some of these amino acids are responsible for production of the antibiotics. Furthermore, it was found that the catabolite repression on racemomycin-A production by *S. lavendulae* ISP-5069 was restored by the addition of both Pharmamedia (nutrient complex, Traders Oil Mill Co., Texas) or amino acid mixture,[16] suggesting the possibility of biogenesis of racemomycin-A from certain amino acid (s) in both supplements. These results led us to study the effect of adding various amino acids to the basal medium on the antibiotic production.

The basal medium for *S. lavendulae* OP-2 was decided to be medium C with reference to the medium used in the experiment for formycin biogenesis.[17] As shown in Table II, addition

15) The isolation number for *S. lavendulae* OP-2 is S-2. (IFO-13709).
16) Y. Sawada, H. Sakamoto, T. Kubo, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **24**, 2480 (1976).
17) K. Ochi, S. Iwamoto, E. Hayase, S. Yashima, and Y. Okami, *J. Antibiot.*, **27**, 909 (1974).

of ammonium tartarate in 1% to medium B produced a small amount (2 μg/ml) of the antibiotic but that of ammonium citrate did not.

Table II. Levels of Antibiotic Production in Three Media by *S. lavendulae* OP-2

Medium	Antimicrobial activity[a] (μg/ml)
B	0
C	2
D	0

a) Antibiotic content after 48 hr of fermentation was assayed by the standard disk method, using *B. subtilis* PCI-219.

Effect of Amino Acids on the Production of Streptothricins

A chemically defined medium was used to investigate the effect of amino acids on the antibiotic production by *S. lavendulae* OP-2. Amino acids were each added to medium C at the beginning of the fermentation, and antimicrobial activity was assayed after 24, 48, and 72 hr of incubation. As shown in Table III, stimulative effect was observed with several amino acids compared with non-supplemented control, in the experiments of adding proline, alanine, leucine, or arginine. Streptothricin-C was the main product in the antibiotic mixture produced under these conditions. This suggests that there is marked effect on the formation of the antibiotic molecule by the addition of these amino acids. Tyrosine, isoleucine, hydroxyproline, lysine, phenylalanine, aspartic acid, and threonine stimulated the production to some

Table III. Effect of Certain Amino Acids on the Antibiotic Production in Synthetic Medium C

Amino acid added[a] (10^{-2}M)	Antibiotic potency (μg/ml) incubation hr			Mycelial weight[b] (ratio)
	24	48	72	
Proline	30	53	34	1.07
Alanine	18	50	31	1.06
Leucine	8	17	7	0.83
Arginine	8	16	7	1.93
Tyrosine	9	8	15	0.63
Isoleucine	2	8	7	0.96
Hydroxyproline	7	8	8	1.04
Lysine	8	8	8	0.97
Phenylalanine	7	7	7	0.91
Aspartic acid	3	7	3	1.24
Threonine	1	4	9	0.87
Glutamic acid	3	3	3	1.23
Tryptophan	3	3	3	0.74
Histidine	2	2	2	1.16
Serine	1	2	1	1.17
Glycine	1	1	1	1.79
Methionine	1	1	1	0.86
Cysteine	0	0	0	0.29
Cystine	0	0	0	0.38
Valine	0	0	0	0.32
Control	1	2	2	1.00

a) Amino acid was added to the medium at the biginning of the fermentation.
b) Mycelial weight of control experiment at 48 hr was taken as 1.00.

extent, whereas glutamic acid, tryptophan, histidine, serine, glycine, and methionine showed almost no effect, and cysteine, cystine, and valine decreased the antibiotic production.

When arginine or glycine was added, abundant growth of mycelia was observed, while cysteine, cystine, and valine depressed the growth. It is considered that biogenesis of streptothricins in this organism is closely related to arginine metabolism.

Incorporation of Radioactivity into Streptolidine Moiety from ^{14}C-labeled Amino Acids

In order to examine the incorporation of amino acids that stimulate the antibiotic production into streptothricin-C, uniformly ^{14}C-labeled amino acids were added to the fermentation medium E, an organic medium in which *S. lavendulae* OP-2 was growing. The culture was shaken further and streptothricin-C was isolated (Chart 2). As shown in Table IV, arginine was incorporated efficiently into the antibiotic, and alanine, proline, or leucine was incorporated to some extent. Phenylalanine and tyrosine were not.

Table IV. Incorporation of Uniformly Labeled Amino Acids into Streptothricin-C

^{14}C compound	Amount of ^{14}C added (μCi)	Streptothricin-C recovered		
		Spec. act.[a] (dpm/μM)	Total act.[b] ($\times 10^4$ dpm)	Incorp. rate (%)
Arginine	25	15004	260.1	4.68
Alanine	25	2629	45.6	0.82
Proline	25	1303	23.8	0.43
Leucine	25	1111	20.3	0.37
Phenylalanine	25	410	7.5	0.13
Tyrosine	25	246	4.3	0.08

a) Calculation based on the molecular weight 1178 for streptothricin-C hexahydrochloride.
b) Antibacterial potency of about 250 μg/ml was obtained.

Streptothricin-C samples labeled with ^{14}C from ^{14}C-arginine, ^{14}C-alanine, and ^{14}C-proline were each acid-hydrolyzed and their components were separated into three ninhydrin-positive zones by paper chromatography. In ^{14}C-arginine experiment, about 51% of the total radioactivity was located in the streptolidine moiety, while about 11% in the β-lysine moiety, as shown in Fig. 1. On the other hand, radioactivity in alanine and proline experiments did not show apparent localization in either streptolidine or β-lysine. These result indicated that arginine was the main precursor of streptolidine molecule in the fermentative production of *S. lavendulae* OP-2.

To confirm this result that arginine might be involved in the biosynthesis of the portion of streptothricin-C from which streptolidine moiety is derived, cultures of this strain were grown in the presence of arginine specifically labeled with ^{14}C in C-1 and C-5, and in the amidino group. Three arginine compounds were incorporated well into streptothricin-C and arginine labeled in C-5 showed the greatest specific activity and incorporation rate as shown in Table V. Streptolidine moiety was labeled with ^{14}C more than β-lysine moiety in three experiments as well as in the control experiment (arg-U-^{14}C). This fact indicates that arginine molecule is not split into fragments to produce the streptolidine skeleton.

Although the contribution of tartaric acid to the biogenesis of streptothricin-C was tested with the ^{14}C-labeled compound, the precursor gave a product with low specific activity, implying almost no effect. A small amount of radioactivity was also incorporated into the antibiotic from ^{14}C-streptolidine added exogenously.

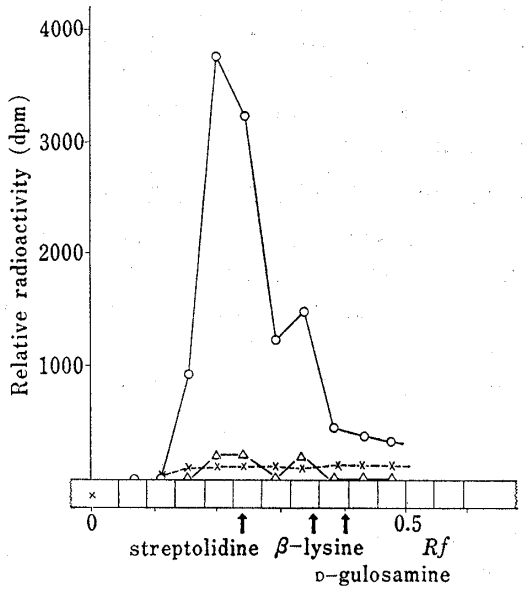

Fig. 1. Radiochromatogram of Principal Components of Streptothricin-C from ^{14}C-labeled Arginine, Alanine, and Proline

—○—: arginine, ---×---: alanine, —△—: proline.

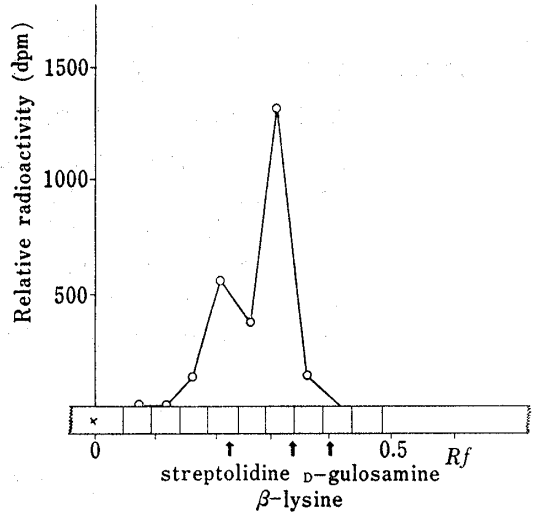

Fig. 2. Radiochromatogram of Principal Components of Racemomycin-A from Arginine-U-^{14}C

TABLE V. Incorporation of ^{14}C-laveled Arginine, Tartaric Acid, or Streptolidine into Streptothricin-C

^{14}C compound	Amount of Streptothricin-C recovered			Incorp. rate (%)	Localization of radioactivity[a] (%)	
	^{14}C added (μCi)	Spec. act. (dpm/μM)	Total act. (×10³ dpm)		streptolidine	β-lysine
DL-Arginine[amidino-^{14}C]	5	3565	189.0	1.7	41	18
DL-Arginine[5-^{14}C]	5	5433	288.0	2.6	45	20
DL-Arginine[1-^{14}C]	5	4669	247.5	2.2	48	22
Arginine-U-^{14}C	5	4329	229.5	2.1	47	16
DL-Tartaric acid-1,4-^{14}C	5	85	4.5	<0.1	—	—
Streptolidine-^{14}C	51600 dpm	8	0.5	<0.1	—	—

a) Molar ratio of streptolidine to β-lysine is 1: 4.
—: Not determined.

TABLE VI. Incorporation of Arginine into Racemomycins by Four *Streptomyces* Strains

Strain	Amount of ^{14}C added (μCi)	Antibiotic tested[a]	Spec. act.[b] (dpm/μM)	Total act. (×10⁴ dpm)	Incorp. rate (%)	Localization of radioactivity (%)	
						streptolidine	β-lysine
S. racemochromogenus 229	25	RM-B	484	4.8	0.1	55	18
S. lavendulae NT-1008	25	RM-B	1887	18.9	0.3	56	22
S. albidoflavus KCC S-0003	25	RM-B	3696	64.7	1.2	37	26
S. lavendulae ISP-5069	25	RM-A	3542	44.6	0.8	22	55

a) RM=Racemomycin.
b) Molecular weight of 630 was used for racemomycin-A trihydrochloride and 995 for racemomycin-B tetrahydrochloride.

Incorporation of Arginine into Streptothricin Antibiotics from Streptomycetes

The incorporation of arginine into streptothricin antibiotics by the fermentative production of four *Streptomyces* strains was then investigated. As shown in Table VI, the incorporation of arginine was the highest in *S. albidoflavus* (1.2%), while that in *S. racemochromogenus* was 0.1%. In the cultural conditions, *S. lavendulae* OP-2 produced the antibiotics in too little yield to compare the radioactivity and its localization. Streptolidine moiety in the antibiotics produced by the three strains was labeled highly with ^{14}C more than β-lysine. The radioactivity profile of components of racemomycin-A from *S. lavendulae* ISP-5069 showed that arginine was incorporated more into β-lysine moiety (55%) than into streptolidine (22%), as shown in Fig. 2. From these results, the biosynthesis of streptolidine moiety in streptothricins by *S. lavendulae* strains seemed, at least, to go through two different pathway.

Discussion

The biosynthesis of β-lysine and D-gulosamine fragments of the streptothricins has already been reported.[1,9,11] The biosynthesis of streptolidine, a guanidino compound (Chart 1), is described in this paper. For this purpose, an organic medium C was used for the experiments on the uptake of ^{14}C-precursor for the understanding of the true production of the antibiotics. Labeling data indicated that the carbon skeleton of streptolidine moiety is mainly derived from arginine, with minor uptake into β-lysine moiety. Arginine was also found to be incorporated appreciably into streptolidine by other streptomycetes except for *S. lavendulae* ISP-5069. This indicates that arginine may be the direct precursor of streptolidine. This consideration is compatible with the hypothesis[18,19] that streptolidine may be formed *via* the dehydroarginine pathway. Gräfe *et al.*[13] published the same result in neurseothricin from *S. neursei* which produced an antibiotic mixture when *o*-aminobenzoic acid was added to the cultures. Putting these results together, streptolidine in streptothricins may be derived from arginine as the main pathway. On the other hand, streptolidine in racemomycin-A from *S. lavedulae* ISP-5069 was found to be biosynthesized from carbons of acetate by preliminary experiments.[9,12] Although streptolidine was labeled to some extent with ^{14}C from arginine (22%, Table VI), main part of the radioactivity (55%) was located rather in β-lysine moiety. In this strain, the biosynthesis of streptolidine seemed to be a minor pathway of arginine. Therefore, we can consider the presence of two pathways for the biosynthesis of streptolidine moiety in streptomycetes.

^{14}C-Labeled streptolidine was scarcely incorporated into streptothricin-C, indicating that this amino acid is not present in the biosynthetic steps as such in streptothricins. In other words, this subunit of the antibiotics may be formed from arginine or its precursors *via* the multienzyme system.[20] Another one of the possibilities is that streptolidine added exogenously is unable to be incorporated from the cells in which the antibiotic molecules are constructed. Details concerning this point remain to be studied further.

Of the amino acids tested with *S. lavendulae* OP-2, arginine stimulated both the antibiotic production and the growth of organisms. Although glycine promoted mycelial growth, incorporation of this amino acid into streptothricin-C was not observed, while carbons from glycine were efficiently incorporated only into the streptolidine moiety in racemomycin-A from *S. lavendulae* ISP-5069.[21] On the other hand, lysine showed almost no stimulating effect. As reported previously,[1,9] however, lysine was found to be converted specifically to β-lysine *via* α,ε-diaminopimelic acid pathway. Therefore, we cannot consider, at present,

18) B.Y. Bycroft, *Nature* (London), **24**, 595 (1969).
19) B.Y. Bycroft and T.J. King, *Chem. Commun.*, 652 (1972).
20) F. Lipmann, *Sciences*, **173**, 875 (1971).
21) Unpublished data.

a relationship between stimulative production of the antibiotics by addition of amino acids and their effective contribution as a precursor. In fact, five amino acids which showed a stimulative activity for the antibiotic production were not incorporated into any components of the antibiotic molecule. These amino acids may contribute to the promotion of certain enzyme system(s) related directly to the biosynthesis.