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Biosynthesis of Streptothricin Antibiotics. I. Incorporation of ^{14}C-Labeled Compound into Racemomycin-A and Distribution of Radioactivity

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As a part of the investigation on the biosynthesis of streptothricin antibiotics, the incorporation of ^{14}C-labeled D-glucose, L-lysine, L-arginine, acetic acid, and sodium bicarbonate into racemomycin-A from *Streptomyces lavendulae* ISP 5069 was examined. By the analysis of the degradation products from ^{14}C-labeled racemomycin-A produced by the addition of lysine, a high rate of the isotope was shown to be present in the β-lysine moiety. Incorporation of glucose followed by degradation of racemomycin-A demonstrated that glucose was incorporated into gulosamine moiety. Acetic acid was incorporated into streptolidine moiety and carbonate showed a preferential incorporation into a carbamoyl group.

Racemomycins,[2] which belong to the streptothricin antibiotics, show a growth inhibition against gram-positive and gram-negative bacteria, Mycobacteria, and many clinically resistant strains against other antibiotics. However, streptothricin antibiotics have a typical delayed toxicity which prevents their clinical use.

Racemomycin-A, the simplest representative of the streptothricin antibiotics, consists of a streptolidine moiety, a D-gulosamine moiety, and a β-lysine moiety linked by an N-glycoside, and has an amide bond and a carbamoyl group in its structure as shown in Fig. 1. The structure of the streptolidine moiety suggests that it may be biosynthesized from amino acids, especially from L-arginine. The presence of D-gulosamine and β-lysine suggested that they may be derived from D-glucose and α-lysine, respectively.

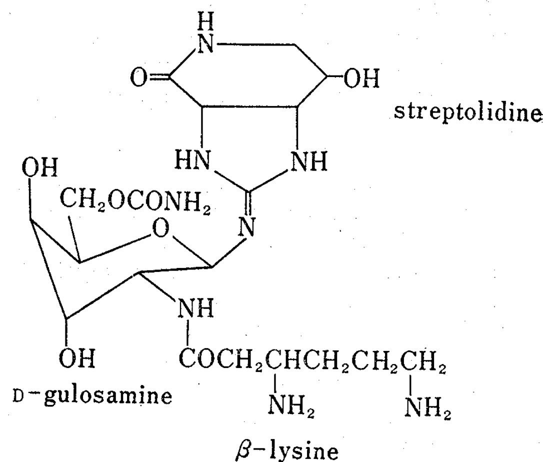

Fig. 1. Structure of Racemomycin-A

Recently, some streptothricin-like antibiotics[3–8] have been isolated and their structural features were elucidated by many investigators. The study on the diversity of chemical structure of streptothricin antibiotics and the studies on their *in vivo* and *in vitro* mode of action urged us the need of radioactive streptothricin antibiotics. In this paper, the incorporations of ^{14}C-labeled compounds, such as D-glucose [U-^{14}C], L-lysine[U-^{14}C], L-arginine-

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[U-^{14}C], acetic[1-^{14}C] acid, and sodium bicarbonate-^{14}C, into racemomycin-A, and the relative distribution of the radioactivity into each of the components are reported.

Materials and Methods

Selection of Racemomycin-producing Organism——*Streptomyces lavendulae* ISP 5069 was selected from 24 strains in our stock. A single spot corresponding to racemomycin-A and a higher potency of antibiotic activity were observed on this strain under various fermentation conditions.

Fermentation——Two ml of the pre-cultivation (medium consisting of 10 g polypeptone, 5 g yeast extract, and 3 g NaCl in 1 liter of H_2O, pH 7.0) of this *S. lavendulae* was inoculated into 100 ml of the fermentation medium (consisting of 5 g glucose, 10 g polypeptone, 5 g yeast extract, and 3 g NaCl in 1 liter of H_2O, pH 7.2) in a 500-ml flask, and this flask was shaken at 160 rpm for 40 hr at 27°. The production of racemomycin-A, and the change of pH during the shaking culture are shown in Fig. 2.

Each of ^{14}C-labeled compounds in H_2O or in 95% EtOH was diluted with 0.5 ml of H_2O containing 1 mg/ml of authentic cold compound sterilized and was added into the culture (100 ml) at 16 and 20 hr after the inoculation. The shaking-culture was continued further for 20 hr.

Determination of Racemomycin-A——The amount of racemomycin-A in broth was determined by a disk method using *Escherichia coli* NIHJ and calculated from the standard curve of racemomycin-A. The cultivation of an indicator strain was made at 37° for 18 hr.

Isolation and Purification of ^{14}C-Labeled racemomycin-A——The culture (100 ml) with the ^{14}C-labeled compound was diluted with 400 ml of another culture without isotope. The culture filtrate (500 ml) was adjusted to pH 7.0 with 2 N HCl and the broth was passed through a column (1.6×10 cm) of Amberlite IRC-50 in H^+ form (Rohm and Hass Co., Philadelphia). The column was washed with H_2O and then developed with 0.3 N HCl. The eluate which showed the inhibitory activity against *E. coli* NIHJ was neutralized with 2 N NaOH. After concentration to 5 ml, it was adsorbed on a column (2.5×140 cm) of Sephadex G-10 (Pharmacia Co., Sweden) and eluted with H_2O. The fractions containing radioactive racemomycin-A were further purified by chromatography on a column (1.5×50 cm) of cellulose powder (Whatman Co., England) eluted with a solvent system of B (BuOH: pyridine: HOAc: H_2O: *t*-BuOH=75: 50: 191: 236: 548). A white powder of radioactive racemomycin-A was obtained by lyophilization as the acetate salt in yield of about 60 mg in each of experiments.

Aqueous solutions in each step were concentrated by a rotary evaporator *in vacuo* with the bath temperature below 40°.

The antibiotic purity was performed by paper chromatography on Toyo Roshi No. 51 UH paper in a solvent system of A (BuOH: pyridine: HOAc: H_2O: *t*-BuOH=15: 10: 3: 12: 4). Detection was made with ninhydrin reaction, bioautography by use of *E. coli* or autoradiography using an X-ray film (Sakura, Type Y, Konishiroku Ind., Co., Tokyo).

Hydrolysis of ^{14}C-Labeled Racemomycin-A——^{14}C-Labeled racemomycin-A was refluxed in 6 N HCl in a sealed tube to give streptolidine and β-lysine at 110—120° for 24 hr and streptolidyl-D-gulosaminide was given when it was hydrolyzed in 3 N HCl at 90—100° for 1 hr. On acid hydrolysis in 3 N H_2SO_4 at 100—110° introducing CO_2 into the saturated solution of $Ba(OH)_2$, CO_2 was yielded.

Preparation of Racemomycin-A Components——Paper chromatography for preparative separation of racemomycin-A components was carried out on Toyo Roshi No. 51 by an ascending system, developed in a solvent system of B. A part of the hydrolyzate from an exact amount of racemomycin-A was spoted on the paper by aid of microsyringe and the total radioactivity spoted was calculated. *Rf* values of streptolidine, β-lysine, and streptolidyl-gulosaminide were 0.27, 0.39, and 0.32, respectively.

Assay of Radioactivity——The radioactivity of racemomycin-A was measured with a liquid scintillation counter (Aloka LSC-601) using H_2O-soluble scintillator consisting of 7 g PPO (2,5-diphenyloxazole), 0.3 g POPOP {1,4-bis[2-(4-methyl-5-phenyloxazoyl]benzene}, and 100 g naphthalene in 1 liter of dioxane. Radioactive racemomycin-A in 1 ml of H_2O to be counted is mixed in 10 ml of the scintillator. For the determination of the radioactivity of each component, a piece of paper strip correspondin gto the component was immersed into 1 ml of H_2O, stood overnight at room temperature, and 10 ml of the scintillator was added into this solution. Radioactivity in the powder of $Ba^{14}CO_3$ was measured with a 2π gas-flow counter (Aloka FC-1E).

Radioactive Chemicals——Acetic [1-^{14}C]acid (spec. act.: 5.0 mCi/mM) was purchased from New England Nuclear, Co., Boston, and D-glucose[U-^{14}C] (spec. act.: 5 mCi/mM), L-lysine[U-^{14}C] (spec. act.: 280 mCi/mM), L-arginine[U-^{14}C] (spec. act.: 175) and sodium bicarbonate-^{14}C (spec. act.: 18.3 mCi/mM) from Daiichi Pure Chemical Co., Tokyo.

Results

The rapid production phase of racemomycin-A by *Streptomyces lavendulae* ISP 5069 is observed during 16—30 hours after the start of the shaking culture as shown in Fig. 2. This production phase indicates the proper time for the addition of labeled compounds as 18—26

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hours of the shaking culture. Therefore, the labeled compounds were added at 20 hours of the shaking culture, and 20 hours thereafter the shaking culture was stopped. Glucose[U-^{14}C] was also added at 16 hours as another experiment of incorporation.

^{14}C-Labeled racemomycin-A was isolated through several steps of purification procedures allowing successive examination of incorporation and distribution of radioactivity. The amount of ^{14}C-racemomycin-A was determined by the disk method. The total radioactivity of ^{14}C-racemomycin-A showed the incorporation of labeled compounds into racemomycin-A during the shaking culture. The results are shown in Table I.

As shown in the Table, the radioactive compounds tested were incorporated into Racemomycin-A. The addition of varied concentrations of glucose[U-^{14}C]resulted in the incorporation of radioactivity into racemomycin-A to almost the same

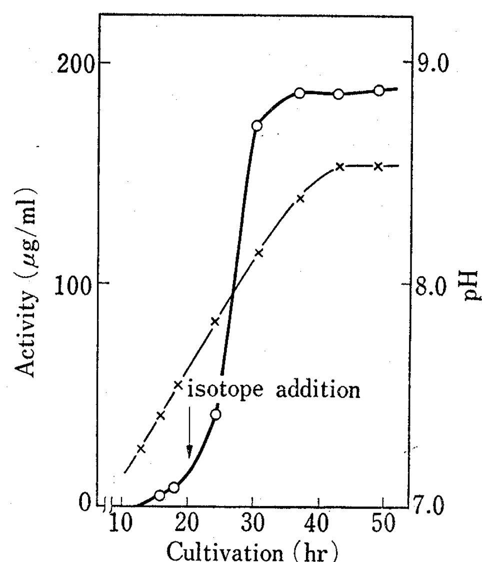

Fig. 2. Fermentation of *Stereptomyces lavendulae* ISP 5069

○: antimicrobial activity, ×: pH

Table I. Incorporation of ^{14}C-Labeled Compounds in Racemomycin-A and Distribution of Carbon-14 in Racemomycin-A Components

^{14}C Compound	Amount of ^{14}C added (μCi)	Racemomycin-A recovered: Spec. act.[a] (dpm/μM)	Racemomycin-A recovered: Total act.[b] (dpm × 10^4)	Incorporation (%)	Spec. act./C atom[c] Racemomycin-A	Spec. act./C atom[c] Gulosamine	Relative incorporation of gulosamine[d]
D-Glucose[U-^{14}C]	100	38880	154.7	0.70	2046	3668	1.79
	50	18350	73.0	0.66	966	1969	2.04
	50[i]	14910	59.5	0.54			
Acetic[1-^{14}C]acid	25	2550	10.2	0.18	134	103	0.77
L-Lysine[U-^{14}C]	50	113310	451.0	4.06	5964	1185	0.20
L-Arginine[U-^{14}C]	50	22190	88.3	0.80	1168	591	0.51
Sodium bicarbonate-^{14}C	50	3340	13.3	0.12	176	32	0.18

^{14}C Compound	Spec. act./C atom[c] Streptolidine	Relative incorporation of streptolidine[e]	Spec. act./C atom[c] β-Lysine	Relative incorporation of β-lysine[f]	Spec. act.[g]/C atom[c] Barium carbonate	Relative incorporation of carbamoyl group[h]
D-Glucose[U-^{14}C]	1015	0.50	1207	0.59		
	346	0.38	278	0.29	1318	1.36
Acetic[1-^{14}C]acid	186	1.38	47	0.35		
L-Lysine[U-^{14}C]	874	0.15	14235	2.39		
L-Arginine[U-^{14}C]	716	0.61	1337	1.14		
Sodium bicarbonate-^{14}C	83	0.47	206	1.17	1288	7.32

a) liquid scintillation counter determination
b) Calculation based on amount of racemomycin-A by disc assay at the stage of culture broth.
c) C atom in gulosamine, streptolidine, and β-lysine: 6, and that in racemomycin-A: 19
d) $\frac{\text{specific activity of gulosamine moiety/C atom}}{\text{specific activity of racemomycin-A/C atom}}$
e) $\frac{\text{specific activity of streptolidine moiety/C atom}}{\text{specific activity of racemomycin-A/C atom}}$
f) $\frac{\text{specific activity of }\beta\text{-lysine moiety/C atom}}{\text{specific activity of racemomycin-A/C atom}}$
g) 2π gas-flow counter determination
h) $\frac{\text{specific activity of carbomoyl group/C atom}}{\text{specific activity of racemomycin-A/C atom}}$
i) addition of isotope at 16 hr

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degree. The radioactivity of racemomycin-A on addition of glucose[U-^{14}C] at 16 hours showed a little low value comparing with the experiment at 20 hours. Lysine[U-^{14}C] was incorporated into racemomycin-A to a very high degree, 4.06%.

The hydrolysis products of racemomycin-A are streptolidine, L-β-lysine, D-gulosamine, carbondioxide, and ammonia. Among these products, the yield of gulosamine is poor because of the lability and resistance of a N-glycoside bond against acid hydrolysis. Therefore, the radioactivity of D-gulosamine moiety was determined as follows: Specific activity of gulosamine=Specific activity of streptolidyl–gulosaminide−Specific activity of streptolidine.

The relative incorporation of each component was obtained from the radioactivities of paper strips corresponding to each component. The clear-cut separation of streptolidine and β-lysine or streptolidyl-gulosaminide and β-lysine was obtained by use of paper chromatography.

As expected, glucose and lysine were incorporated in a high proportion into gulosamine and β-lysine moieties, respectively. However, the incorporation of arginine into streptolidine moiety was low, contrary to expectation. Acetic acid was incorporated more into streptolidine than into other compounds tested. The carbonate was located in a carbonyl group in Racemomycin-A comparing with the sample which was labeled with glucose.

Discussion

Glucose or its catabolites seem to be the precursor of amino-sugar moiety in racemomycin-A, as in the case of some aminoglycoside antibiotics.[9-13] We have investigated a catabolite repression on the production of racemomycin-A by *Streptomyces lavendulae* ISP 5069[14] and found that the addition of glucose into various media markedly suppressed the production of racemomycin-A. Therefore, we used a fermentation medium containing the minimum amount of glucose which provided carbon and energy sources for the mycelial growth. The improvement of the medium composition and the addition period of ^{14}C-labeled glucose into the broth may have led to the higher incorporation of the isotope into Racemomycin-A.

The incorporation of lysine into β-lysine moiety of racemomycin-A was found to be the highest compared with other radioactive compounds. Therefore, it seems more probable that lysine is the direct precursor of β-lysine than other compounds in *Streptomyces lavendulae* strains which produce novel streptothricin antibiotics. This confirms strongly the report of Voronina, *et al.*[15] that, in streptothricin-F produced by *Actinomyces polymycini* in the presence of radioactive lysine, almost all the radioactivity was located in β-lysine moiety while streptolidine and gulosamine moieties were practically unlabeled. The enzymic transamination of lysine to form β-lysine is well known in strains of *Clostridia*,[16] and the same enzyme may be present in the strains producing streptothricin antibiotics.

Streptolidine has a cyclic guanidino group in its molecule. Arginine showed a poor incorporation into streptolidine moiety, while acetic acid precursor was utilized to a larger

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extent. It may be assumed that the biosynthesis of streptolidine moiety may be derived from low-molecular precursors including acetic acid.

The effective incorporation of arginine into viomycidine residue of viomycin, a tuberculostatic antibiotic, was observed.[17] The structure of viomycidine[18] has a similarity to that of streptolidine. However, a scanty incorporation of arginine into streptolidine moiety in racemomycin-A suggests that the precursors for these two antibiotics are different. Further investigations on the exact precursor are clearly required.

Lysine was more effective than other compounds for labeling racemomycin-A under established conditions, and provided ^{14}C-labeled antibiotic with sufficient specific activity for the continued studies on the mode of action of this antibiotic.

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