Table VI. Regression Analysis for Assay of Siomycin A in the Presence of Siomycin B and D_1[a]

Sample No.	Components in 10 μl of mixed sample solution (ng)			Found (ng)
	A(x)	B	D_1	A(y)
1	5	5	50	7
2	5	5	50	7
3	10	5	40	11
4	10	5	40	12
5	21	5	30	21
6	21	5	30	23
7	31	5	20	32
8	31	5	20	33
9	41	5	10	38
10	41	5	10	42

a) regression equation: $y=0.935x+2.40$, s=1.36, c.v.=6.00

Advantages of This Method

This method is 6—40 times more sensitive than previous method,[2] and is also simpler.

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Biosynthesis of Streptothricin Antibiotics. III.[1] Incorporation of D-Glucosamine into D-Gulosamine Moiety of Racemomycin-A

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When D-glucosamine-1-^{14}C was added to a growing culture of *Streptomyces lavendulae* ISP-5069, a high incorporation of radioactivity into racemomycin-A was observed. Radioactivity was exclusively located in D-gulosamine moiety of the molecule.

Keywords——antibiotic biosynthesis; *Streptomyces lavendulae* ISP-5069; incorporation of D-glucosamine-1-^{14}C; D-glucose-U-^{14}C; D-glycerol-U-^{14}C; radioactive racemomycin-A; direct precursor; radioactivity scanning; cellulose partition chromatography

As reported in the previous paper,[3] D-glucose-U-^{14}C, which was added at the early production stage of racemomycin-A by *Streptomyces lavendulae* ISP-5069, was incorporated into D-gulosamine moiety in racemomycin-A. Further studies on the biosynthesis of aminosugar moiety in racemomycin-A molecule made it clear that D-glucosamine was incorporated into

1) Y. Sawada, H. Sakamoto, T. Kubo, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **24**, 2480 (1976).
2) Location: a) *1–14, Bunkyo-machi, Nagasaki, 852, Japan*; b) *2–10–65, Kawai, Matsubara-shi, Osaka, 580, Japan.*
3) Y. Sawada, T. Kubo, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **24**, 2163 (1976).

racemomycin-A to a greater extent than glucose and glycerol, and almost exclusively into D-gulosamine moiety. In this paper, incorporation of D-glucosamine-1-^{14}C is reported, together with the incorporation rates of D-glucose and glycerol.

Materials and Methods

1. **Shaking Culture of *S. lavendulae* ISP-5069**——One ml of the pre-cultivation medium (containing 1% glucose, 1% yeast extract, 1% polypepton, 0.2% NaCl, 0.01% $(NH_4)_2SO_4$, 0.01% KH_2PO_4, 0.01% K_2HPO_4, 0.01% $MgSO_4$, pH 7.2) of *S. lavendulae* was inoculated into 100 ml of the fermentation medium (consisting of 2% maltose, 0.5% polypepton, 0.5% meat extract, 0.3% yeast extract, 0.3% NaCl, 0.1% $MgSO_4$, 1 ml of trace salts solution[4] per 100 ml, pH 7.4) in a 500 ml flask, and this flask was shaken at 160 rpm for 40 hr at 27°. This strain produced about 200 μg/ml of racemomycin-A when it was shake-cultured under the same conditions.

2. **Addition of Labeled Compounds**——Labeled compounds (45 μCi) were dissolved in 0.5 ml (D-glucosamine-1-^{14}C and glycerol-U-^{14}C) and 1 ml (D-glucose-U-^{14}C) of H_2O, respectively. The solution was added to the shake-cultured broth (100 ml), in a 500 ml flask after 20 hr growth of *S. lavendulae*.

The shaking culture was continued for further 20 hr.

3. **^{14}C-Labeled Compound**——D-Glucose-U-^{14}C (spec. act.: 5 mCi/mM) was purchased from Daiichi Pure Chemical Co., Ltd., Tokyo and D-glucosamine-1-^{14}C (spec. act.: 51.5 mCi/mM in 0.5 ml of EtOH–H_2O (7 : 3)) and glycerol-U-^{14}C (spec. act.: 133.4 mCi/mM in 0.5 ml of EtOH–H_2O (1 : 1)) from New England Nuclear Co., Ltd., Boston.

4. **Determination of Racemomycin-A**——The amount of racemomycin-A in broth was determined by a disk assay method using *Bacillus subtilis* PCI-219 and calculated from the standard line of racemomycin-A.

5. **Isolation and Purification of ^{14}C-Labeled Racemomycin-A**——The culture (100 ml) with the ^{14}C-labeled compound was diluted with 900 ml of another culture without isotope. To the culture filtrate (1.0 liter), active carbon (20 g, Wako Pure Chemical Co., Ltd., Osaka) was added, then stirred overnight at room temperature. The queous layer of the suspension exhibited no antibacterial activity against *B. subtilis*. The carbon was filtered, washed with H_2O (500 ml) and then eluted with 50% aqueous acetone (2.0 liters, pH 2.0 by HCl). The eluate was neutralized with 0.1N NaOH, and concentrated to a small volume at 30°. To this solution, MeOH (100 ml) was added and filtered. The filtrate was evaporated to 5 ml, and applied on a column (2.5×140 cm) of Sephadex LH-20 (Pharmacia Co., Uppsala) and developed with H_2O. Active fractions against *B. subtilis* were pooled and evaporated to dryness *in vacuo*. The residue was suspended in 2 ml of a solvent system (BuOH–pyridine–AcOH–H_2O–*tert*-BuOH=75 : 50 : 191 : 236 : 548) and applied on a column (2.0×45 cm) of cellulose (Whatman Co., England), which was eluted with the same solvent system. The fractions with *Rf* 0.36—0.39 on paper chromatography (Toyo Roshi No. 51 with the same solvent system, detection: ninhydrin and Rydon-Smith reagents) were pooled and treated twice with ether. The aqueous layer was evaporated to dryness and the residue was dissolved in H_2O and then lyophilized to give a hygroscopic powder of ^{14}C-labeled racemomycin-A as the acetate salt. Yields of radioactive racemomycin-A in the preparations of D-glucosamine-1-^{14}C, D-glucose-U-^{14}C and glycerol-U-^{14}C were 34.1, 13.8 and 36.1 mg, respectively.

Tests of antibiotic purity were performed as described previously.[3]

6. **Assay of Radioactivity**——The methods were similar to those described in the previous paper.[3]

7. **Degradation of ^{14}C-Racemomycin-A and Preparative Separation of Its Components**——^{14}C-Racemomycin-A (10 mg) was dissolved in 3N or 6N HCl, heated at 110—120° in a sealed tube. The hydrolysate was evaporated to dryness, dissolved in 1.0 ml of H_2O, and 10 μl of the solution was spoted on a paper (Whatman No. 1 paper) and developed with the solvent system described above.

Results

In this study of antibiotic biosynthesis, the glucose in the fermentation medium was replaced by maltose in order to avoid the inhibiting effect by glucose on racemomycin-A production and the dilution of radioactive glucose. Maltose[1] have been found to stimulate the production of racemomycin-A. Fermentation result shown in Fig. 1 indicated that the time course of racemomycin-A production was almost similar to that of the previous data.[3]

In isolation procedures, ^{14}C-labeled racemomycin-A was absorbed by active carbon in the place of ion exchange resin, Amberlite IRC-50. A chromatographycally pure radio-

4) T.G. Pridham and D. Gottlieb, *J. Bacteriol.*, **56**, 107 (1948).

active racemomycin-A was obtained on treatment with active carbon, gel-filtration by Sephadex LH-20 and cellulose partition chromatography.

The incorporation rates of radioactivity from ^{14}C-labeled compounds into racemomycin-A were shown in Table I. Compared to the incorporation of D-glucose-U-^{14}C and glycerol-U-^{14}C into racemomycin-A, the incorporation of D-glucosamine-1-^{14}C was high, 4.46%. The incorporation of D-glucose-U-^{14}C was similar to that previously reported (0.66%). Glycerol-U-^{14}C was also incorporated into racemomycin-A to some extent.

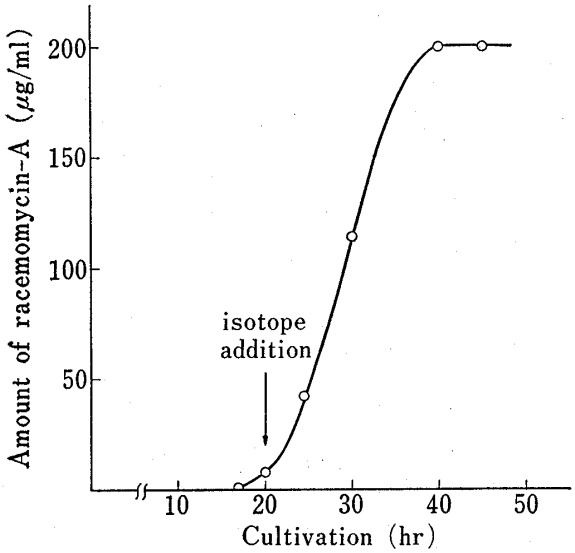

Fig. 1. Fermentation Pattern of *S. lavendulae* ISP-5069

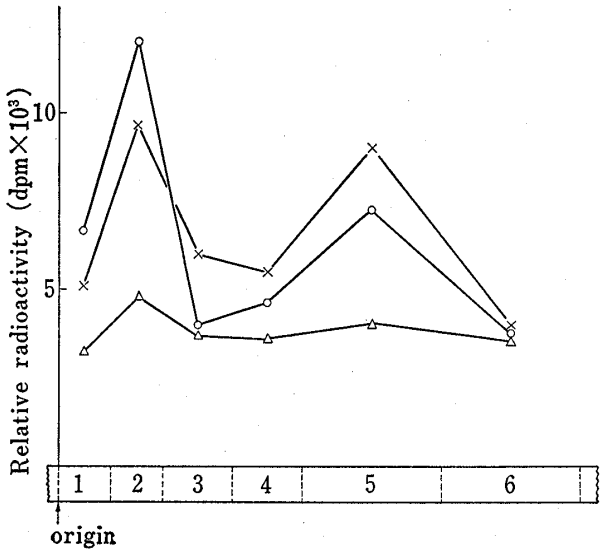

Fig. 2. Radioactivity Scanning of Racemomycin–A Components from D-Glucosamine-1-^{14}C on Paper Chromatograms

2: streptolidyl-D-gulosaminide
3: streptolidine
4: β-lysine
5: D-gulosamine
1,6: unidentified

—○—: 3N HCl, 1 hr
—×—: 3N HCl, 6 hr
—△—: 6N HCl, 72 hr

TABLE I. Incorporation of ^{14}C into Racemomycin–A from D-Glucosamine, D-Glucose and Glycerol

^{14}C Compound	Amount of ^{14}C added (μCi)	Racemomycin–A recovered		Incorporation (%)
		Spec. act. (dpm/μM)	Toral act.[a] (dpm × 10^5)	
D-Glucosamine-1-^{14}C	45	140158	44.5	4.46
D-Glucose-U-^{14}C	45	22345	7.1	0.71
Glycerol-U-^{14}C	45	18372	5.8	0.54

a) Calculation based on the amount of racemomycin -A by a disk assay at the stage of culture broth.

The distribution of radioactivity in racemomycin-A components, D-gulosamine, streptolidine and β-lysine, was examined. Radioactive racemomycin-A was hydrolyzed in 3 N and 6 N hydrochloric acid. Each component of racemomycin-A was separated by paper chromatography. Fig. 2 showed the radioactive patterns of the components obtained from the fermentation with D-glucosamine-1-^{14}C. Streptolidyl-gulosaminide and D-gulosamine fractions on the hydrolysate by 3 N hydrochloric acid showed peaks of radioactivity (recovery of radioactivity: 69—71%), whereas both fractions showed no radioactivity for further degradation by 6 N hydrochloric acid (recovery: 23%). Streptolidine and β-lysine have originally low rate of radioactivity. These facts suggest that D-glucosamine is radioactive

compared with other components and it gradually destrolyed for further hydrolysis. ^{14}C-Racemomycin-A obtained from the fermentation in the presence of D-glucose-U-^{14}C showed the localization of radioactivity in D-gulosamine moiety as previously reported.[3] In ^{14}C-racemomycin-A obtained from the fermentation in the presence of glycerol-U-^{14}C, radioactivity in each component showed no localization. Glycerol was taken equally into three components, though its total incorporation into racemomycin-A was lower to some extent than that of glucose. It may be considered that glycerol is used as a convenient carbon source to balance the production of three moieties of racemomycin-A.

From these results, it is considered that D-glucosamine seemed to be a more direct precursor for D-gulosamine moiety in racemomycin-A than glucose and glycerol.

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