UDC 615.779.931-011

29. Hyozo Taniyama*1 and Shoji Takemura*2 : Chemical Studies on Antibiotics produced by Actinomycetes. VII.[5] Racemomycin. (4). On Racemomycin-O (i).

(*Pharmaceutical Faculty, University of Osaka*,*1 *and Faculty of Pharmacy, Kinki University**2)

In the previous papers of this series,[1~3] it was reported that three basic antibiotics, racemomycin-A, -B, and -C had been isolated from the fermented broth of an Actinomycetes designated *Streptomyces racemochromogenus novo sp.* One of these antibiotics, racemomycin-B, was purified as its picrate and it was hydrolyzed to obtain two uncommon amino acids, roseonine and β-lysine.

Considering the results of investigations, it was concluded that these antibiotics belong to the streptothricin group. These antibiotics have relatively high antibacterial activity, but show strong toxicity to mice, as do many other streptothricin-like substances.

However, in the early periods of the studies on antimicrobial substances of this species, it had been reported by Sugai[4] that an antibiotic with no toxicity to mice was found in the filter culture. About three years after the beginning of the investigations (1953), this species changed to its mutant by some unknown reasons and it was concluded that after the period of mutation, the species produced entirely different kinds of antibiotics, 229-A, -B, and -C.

On the basis of these reports and present studies, racemomycins-A, -B, and -C are probably identical with the substances 229-A, -B, and -C, respectively.[1]

Unexpectedly, it was found that this species had been stored carefully in a good condition by the discoverer of the species, Prof. R. Shinobu.*3 The seed of this strain (Shinobu-strain) was submitted to test fermentation (Tables I, II, and III). The fermented broth of this strain was examined and slight toxicity was found in the antibacterial substance which was isolated from the broth in accordance with the method described below. In the filter culture of this fermented broth only one antibacterial substance was found by paper chromatography (Rf 0.00 by BuOH:AcOH:H_2O (4:1:5); 0.18 by PhOH:H_2O (1:1); 0.10 by a mixture of 1.5 g. of sodium *p*-hydroxybenzenesulfonate, 38 cc. of BuOH, 50 cc. of H_2O, and 10 cc. pyridine).[1,5] However, with regard to the relationship between this antibiotic and the nontoxic substance 229, which had been reported by Sugai, direct comparison was difficult as substance 229 was not available. The present antibiotic differs from the descriptions of substance 229 on the following points :

(1) The antibiotic was very similar to racemomycin-A, -B, and -C in chemical and physical properties so that it was regarded as a streptothricin-like antibiotic, while substance 229 differs completely from 229-A, -B, and -C. It seemed to be a neomycin-like substance according to Sugai's work.[4]

(2) The antibacterial activity of the antibiotic was relatively lower than was pre-

*1 Hotarugaike, Toyonaka, Osaka (谷山兵三).
*2 Kowakae, Fuse, Osaka-fu (竹村庄司).
*3 Osaka University of Liberal Arts and Education, Nagare-cho, Hirano, Higashisumiyoshi-ku, Osaka.

1) H. Taniyama, S. Takemura : Yakugaku Zasshi, **77**, 1210(1957).
2) *Idem.* : *Ibid.*, **77**, 1215(1957).
3) *Idem.* : *Ibid.*, **78**, 742(1958).
4) T. Sugai : J. Antibiotics (Japan), **9**, Ser. B. 170(1956).
5) Part VI. S. Takemura : Kinki Daigaku Yakugakubu Kiyo, **2**, 15(1958).

sumed from the description of substance 229 even after repeated purifications.

This antibiotic was named Racemomycin-O. The conditions of fermentation of Shinobu-strain and extraction of racemomycin-O are as follows: The seed strain was fermented in the medium shown in Table I for 48 hours with shaking and about 2% of this culture was implanted for tank fermentation.

Table I. Fermentation of Seed Strain of *Streptomyces racemochromogenus* (Shinobu-strain)

	(%)		(%)
Soluble starch	3	Peptone	0.2
Soybean cake	2	NaCl	0.2
$(NH_4)_2SO_4$	0.1	$CaCO_3$	0.2

temp., 28°; shaking amplitude, 6 cm.; stirring speed, 140 r.p.m.

The tank fermentation was carried out in liquid volume of 200 L. in the medium shown in Table II and under conditions listed in Table III.

Table II. Tank Fermentation of *Str. racemochromogenus* (Shinobu-strain)

	(%)		(%)
Soluble starch	3	$(NH_4)_2SO_4$	0.2
Peptone	0.1	$CaCO_3$	0.3
Meat extract	0.1	Soybean Cake	3
NaCl	0.5		

pH, 7.2; bubbled air volume, 50% in volume; stirring speed, 120~130 r.p.m.; foam suppression, silicone resin KM added at the start; time, 70 hr.

Table III. Measurement of pH, Sugar Content, and Nitrogen during Fermentation

Hours	0	24	36	48	60	70	
pH	7.2	7.6	7.6	7.6	7.4	7.7	
Sugar content	3.0	—	2.2	1.6	0.7	0.6	mg./cc.
NH_3-nitrogen	65	58	60	40	11	28	〃
Total nitrogen	250	117	119	96	44	58	〃

The most suitable period was assumed from the time when pH began to rise again.

Extraction and Purification of Racemomycin-O

The filter culture was brought to pH 2.0, filtered, the filtrate was adjusted to pH 7.0, and filtered again to remove a precipitate. The filtered liquor amounted to 165 L. The active substance was adsorbed on 3000 cc. of Amberlite IRC-50 (NH_4-type). Some activity was observed in the washings, so the washings were adsorbed again on 2000 cc. of the same resin. The column was eluted with 1*N* ammonium hydroxide and the eluate was warmed to 30° *in vacuo* to drive off ammonia, adjusted to pH 2.0 with 50% sulfuric acid, decolorized with charcoal, brought to pH 7.0 with sodium hydroxide, and lyophilized. The crude product weighed 34 g. and the potency of this substance was 402 U/mg.,[4] Rf values in various solvents as given above, and no more active component was found on paper. Result of toxicity tests of this crude substance is shown in Table IV.

Table IV. Toxicity Tests of Racemomycin-O (RMO) in Mice

Material	No. of animals	Dose (γ)	Concn. (γ/cc.)	No. of animals	Notes
Crude RMO-HCl	5	0.5	5	5(for five days)	Slight shock was observed in 2 animals.
〃	5	0.5	2.5	5(〃)	Strong shock in 1 and slight shock in 1 animal were observed.
Pure RMO-HCl	5	0.5	5	5(〃)	No shock was observed.
〃	5	0.5	2.5	5(〃)	〃

Aqueous solution was injected intravenously. Body weight: 15~18 g.

For the purification of the crude substance, it was derived to its picrate (syrup), dissolved in acetone-water mixture, precipitated by addition of water, washed with water, and this procedure was repeated to remove inorganic impurities, decomposed with hydrochloric acid in acetone to obtain a white syrupy material. This syrup was treated again with the minimum volume of water and acetone was added to precipitate hygroscopic hydrochloride as a white powder. Further purifications caused no great change in analytical and antibacterial data. Pure free base, picrate, *p*-(*p*-hydroxyphenylazo)benzenesulfonate were also obtained by the methods described in the experimental part. Analytical data and molecular weight determination (Barger method[6]) of the free base support the molecular formula, $C_{25}H_{44}O_{10}N_8$, or its hydrate.

Racemomycin-O was positive to ninhydrin and negative to Elson-Morgan, Sakaguchi, maltol, ferric chloride, biuret, and Fehling reactions, and precipitated with mercuric chloride, phosphotungstic acid, and reineckate. Infrared spectrum of the hydrochloride was similar to that of racemomycin-B sulfate. Racemomycin-O has no acid group but has basic groups in its molecule as observed from the titration curve of its picrate (Fig. 2).

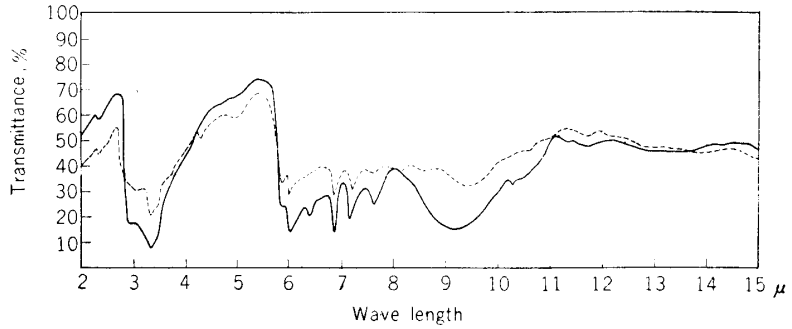

Fig. 1. Infrared Absorption Spectra of Racemomycin-O Hydrochloride(——) and Racemomycin-B Sulfate (------) (Nujol mull)

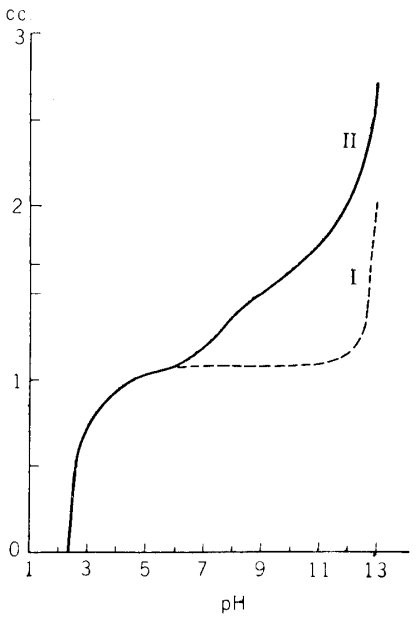

Fig. 2. Titration Curves of Equivalents Racemomycin-O Picrate (II) and Picric Acid (I) (216 mg., 0.1*N* MeOH-NaOH)

6) S. Akiya : Yakugaku Zasshi, **57**, 967(1937).

By acid hydrolysis of racemomycin-O for a long period, three ninhydrin-positive spots and two triphenyltetrazolium-positive spots were detected on paper chromatogram. The two ninhydrin-positive spots were identified with β-lysine and roseonine, respectively. The other ninhydrin-positive spot was identical with one of the tetrazolium spots in Rf values. The isolations and structures of these hydrolysates will be reported in later papers.

Experimental

Purification of Racemomycin-O—To a solution of 5 g. of crude racemomycin-O dissolved in 100 cc. of water, saturated picric acid solution was added at 30°, and stood overnight. The syrupy yellow picrate was decanted from the water layer and washed twice with water. The syrup was dissolved in a mixture of 1 cc. of water and 38 cc. of acetone, insoluble material was filtered off and the filtrate was added to 300 cc. of water. After 14 hr., the precipitated picrate was again dissolved in a mixture of 1 cc. of water and 16 cc. of acetone, and filtered. The filtrate was added to 120 cc. of cold water and allowed to stand overnight. The syrupy precipitate was then dried over P_2O_5 *in vacuo* to obtain yellow hygroscopic powder (yield, 1.6 g.), m.p. 187°; $[\alpha]_D^{19}$ −34°(c=0.5, in acetone-water, 10:1). *Anal.* Calcd. for $C_{25}H_{44}O_{10}N_8 \cdot 3C_6H_3O_7N_3$: C, 39.60; H, 4.07; N, 18.27. Found : C, 39.71; H, 4.38; N, 18.46.

Salts of Racemomycin-O—i) Hydrochloride of the antibiotic was derived from its picrate; 0.5 g. of the picrate was dissolved in 120 cc. of acetone-water (15:1), 2 cc. of conc. HCl was added while stirring, and allowed to stand overnight in a refrigerator. The syrupy material was decanted from acetone layer and the syrup was dissolved again in a small amount of water, to which was added 50 cc. of acetone. After storing in the refrigerator overnight, the white precipitate was treated many times with minimum volumes of water and acetone. The syrupy hydrochloride solidified, m.p. 161~166°(decomp.); $[\alpha]_D^{19}$ −33°(c=0.5, H_2O); yield, 0.11 g. *Anal.* Calcd. for $C_{25}H_{44}O_{10}N_8 \cdot 3HCl \cdot 3H_2O$: C, 38.56; H, 6.81; N, 14.40. Found : C, 38.26, 38.61; H, 7.64, 7.90; N, 13.86, 15.14.

ii) *p*-(*p*-Hydroxyphenylazo)benzenesulfonate was prepared from the hydrochloride. The hydrochloride (0.05 g.) was dissolved in 2 cc. of water and 1 cc. of 10% *p*-(*p*-hydroxyphenylazo)benzenesulfonic acid solution was added. The orange-red precipitate was collected, washed with water, dried over P_2O_5 *in vacuo*; m.p. 217°(decomp.); yield, 0.08 g. *Anal.* Calcd. for $C_{25}H_{44}O_{10}N_3 \cdot 3C_{12}H_{10}O_4N_2S \cdot 3H_2O$: C, 46.87; H, 5.32; N, 13.03. Found : C, 46.49; H, 5.32; N, 12.60.

iii) The helianthate was prepared in a similar manner from the hydrochloride. Dark red amorphous substance of m.p. 212°(decomp.). *Anal.* Calcd. for $C_{25}H_{44}O_{10}N_8 \cdot 3C_{14}H_{15}O_3N_3S \cdot 6H_2O$: C, 49.05; H, 6.16; N, 14.52. Calcd. for $C_{25}H_{44}O_{10}N_8 \cdot 3C_{14}H_{15}O_3N_3S \cdot 5H_2O$: C, 49.66; H, 6.11; N, 14.68. Found: C, 49.36; H, 6.55; N, 14.44.

iv) The flavianate was precipitated from MeOH solution by addition of saturated MeOH-H_2O solution of flavianic acid, m.p. 198°(decomp.). *Anal.* Calcd. for $C_{25}H_{44}O_{10}N_8 \cdot 3C_{10}H_6O_8N_2 \cdot 6H_2O$: C, 39.62; H, 4.44; N, 12.37. Found : C, 39.87; H, 4.71; N, 12.61.

Free Base of Racemomycin-O—The picrate (500 mg.) was dissolved in 20 cc. of *N* HCl and filtered. The filtrate was added to the top of the column of Amberlite IRA-400 (OH-type, 1×30 cm.) and washed with water. Ninhydrin-positive eluate (50 cc.) was collected, evaporated, and triturated with dry acetone to obtain a strongly hygroscopic white powder, m.p. 127~131°(decomp.). *Anal.* Calcd. for $C_{25}H_{44}O_{10}N_8 \cdot 3H_2O$: C, 44.78; H, 7.46; N, 16.72; mol. wt., 670.46. Found : C, 44.51; H, 7.92; N, 17.02; mol. wt.(Barger), 620.

Hydrolysis of Racemomycin-O—The hydrochloride (20 mg.) was heated on a water bath for 32 hr. with 1 cc. of 6*N* HCl in a sealed tube and evaporated *in vacuo*. Paper chromatography of the residue showed that it contains β-lysine (Rf 0.14), roseonine (Rf 0.09), amino sugar ? (Rf 0.12), and an unknown triphenyltetrazolium-reducing substance (Rf 0.05) (BuOH:AcOH:H_2O, 4:1:5).

The authors are greatly indebted to the members of Yodogawa Factory of Meiji Seika Co., Ltd. for the fermentation and to Prof. R. Shinobu for his kindness in offering them the strain and helpful advices, and thank Mr. N. Fukuda, University of Osaka, for the microanalysis.

Summary

A new streptothricin-like antibiotic, racemomycin-O, obtained from the fermented broth of *Streptomyces racemochromogenus novo sp.* (Shinobu-strain), was examined. The isolation and purification of this antibiotic as a picrate is described and some physical and chemical properties are presented. It was shown that analytical and molecular weight determinations of free base and various salts agreed with the molecular formula of $C_{25}H_{44}O_{10}N_8$. Racemomycin-O is a nontoxic antibiotic while racemomycin-B has a strong toxicity.

(Received July 27, 1959)