ORYZOXYMYCIN, A NEW ANTIBIOTIC

TOSHIHIRO HASHIMOTO, SHINICHI KONDO, TOMOHISA TAKITA,
MASA HAMADA, TOMIO TAKEUCHI, YOSHIRO OKAMI
and HAMAO UMEZAWA

Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan

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A new antibiotic, oryzoxymycin ($C_{21}H_{30}N_2O_{10}$, m. w. 470.47; λ_{max} 285 mμ in 0.1 N HCl; $[\alpha]_D^{25}$ +240°, c=1, in H_2O), inhibiting *Xanthomonas oryzae* was isolated. The producing strain was classified as *Streptomyces venezuelae* var. *oryzoxymyceticus*. Production, isolation, purification and physico-chemical preperties of this antibiotic are described.

During the course of screening for antibiotics active against *X. oryzae in vitro* and in green house tests, a potent broth was obtained from a culture of an *Actinomycetes* isolated from soil. The active component was purified, differentiated from all known anti-xanthomonas antibiotics, and was named oryzoxymycin. It inhibited the growth of *X. oryzae in vitro* at 1~2 mcg/ml but was not effective against bacterial leaf blight in a pot test, when applied in solution at 1,000 mcg/ml.

Oryzoxymycin-Producing Organism

A streptomyces was isolated on glycerol-ammonium salt agar from a soil sample collected at Karuizawa, Nagano Prefecture, Japan, in October 1964, and designated M979-M1. The strain grew well with many branching mycelia in agar medium and developed straight aerial mycelium. Whorl structure was not observed in the aerial mycelium, but spiral structure, with open loops was observed on the tips of aerial mycelium when grown on KRAINSKY's glucose-asparagine agar. The surface of the spore was smooth under the electron-microscope as shown in Plate 1.

Characteristics of this producing organism on various media are listed in Table 1. The utilization of carbon sources on PRIDHAM-GOTTLIEB basal medium was as follows: Good growth with glycerol, glucose, mannose, dextrin, starch; doubtful growth with maltose; no growth with arabinose, xylose, rhamnose, fructose, galactose, lactose, sucrose, dulcitol, inositol, mannitol, sorbitol, raffinose, inulin and salicin. This strain belongs to genus *Streptomyces* and the characteristics of this strain can be summarized as follows: no whorl formation but open loops at tip of aerial mycelium; the surface of conidial spore is smooth; growth on various media is generally pale yellowish brown to light

Plate 1. *S. venezuelae* var. *oryzoxymyceticus* ×2,500

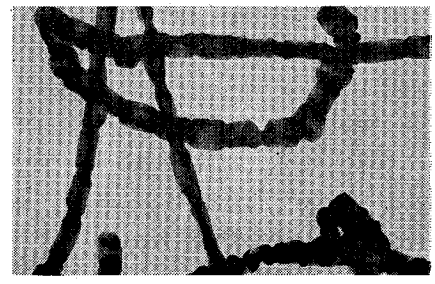


Table 1. Characteristics of the strain M979-M1

	Growth	Aerial mycelium	Soluble pigment	
Glycerol CZAPEK-KRAINSKY's agar (27°C)	good growth, pale yellowish brown	none or scant white	yellowish	
Glucose-asparagine agar (27°C)	pale yellow~pale yellowish brown	white~light gray with tinge of faint pink	none	
Calcium malate agar (27°C)	pale yellow	thin, white	pale yellow	Strong solubilization of Ca-malate
Peptone solution (containing 1.0 % $NaNO_3$) (27°C)	colorless	none	none	Positive nitrate reduction
Starch plate (27°C)	pale yellow~dark yellow-orange~light brown	white~brownish white	brownish~light brown	Positive hydrolysis of starch
Tyrosine agar (27°C)	coloress~brownish	none	faint-blackish	Probably negative tyrosinase reaction
Potato plug	pale yellowish brown~light brown	white	yellowish brown	
Nutrient agar (27°C)	colorless	none	faint brownish	
Nutrient agar (37°C)	colorless	none	faint brownish	
LOEFFLER'S coagulated serum (37°C)	pale yellow~pale brownish yellow~grayish yellow brown	none	light brownish gray	Negative liquefaction of coagulated serum
Gelatin stab (20°C)	colorless~pale yellowish brown ~yellowish brown	later becoming thin, light gray	brown	Positive liquefaction of gelatin
Skimmed milk (37°C)	colorless	none	yellowish	Coagulation and rapid peptonization
Cellulose (27°C)	none or very poor			

brown; white aerial mycelium turns to light gray with tinge of pink; soluble pigment is yellowish to light brown; on tyrosine agar, soluble pigment is slightly blackish; a brown soluble pigment is produced on gelatin but slightly brown soluble pigment on nutrient agar; strong proteolytic action is observed on gelatin and milk media; hydrolysis of starch is strong. Among known species, *Streptomyces venezuelae* has many characteristics in common with this strain. As shown in Table 2, the strain M979-M1 and *S. venezuelae* resemble each other except spiral formation of the former and chromogenecity of the latter. GAUZE *et al.*[1] have reported a variant designated *Actinomyces venezuelae* var. *spiralis* forming spirals and MORAIS *et al.*[2] have reported another variant *Streptomyces venezuelae* var. *roseospori* showing chro-

Table 2. Comparison of the strain M979-M1 with *S. venezuelae*

Properties	M979-M 1	*S. venezuelae*
Spore surface	smooth	smooth
Whorl	−	−
Spiral	+	−
Color of aerial mycelium	white~light gray with pinkish tinge	gray or light lavender
Color of growth	pale yellowish brown~light brown	yellow~brown
Proteolysis	relatively strong	strong
Hydrolysis of starch	relatively strong	positive
Chromogenecity	probably not chromogenic	chromogenic type
Antibiotic produced	oryzoxymycin	chloramphenicol

Table 3. Production of oryzoxymycin in media containing various carbon sources and pH at the highest production during the shaking culture

Carbon sources	%	3rd day		4th day		5th day		6th day	
		pH	mcg/ml	pH	mcg/ml	pH	mcg/ml	pH	mcg/ml
Glucose Starch	2.0 2.0	7.0	<10.0	7.0	33.3	7.0	80.8	8.4	44.2
Glucose	3.0	5.2	<10.0	5.2	<10.0	5.2	<10.0	5.6	<10.0
Starch	3.0	8.6	<10.0	8.6	<10.0	8.6	<10.0	8.6	<10.0
Glycerin	3.0	5.6	<10.0	5.6	<10.0	5.6	<10.0	5.6	<10.0
Maltose	3.0	8.4	<10.0	8.6	<10.0	8.6	<10.0	8.8	<10.0
Dextrin	3.0	8.6	<10.0	8.6	<10.0	9.0	<10.0	8.8	<10.0
Lactose	3.0	8.4	<10.0	8.6	<10.0	9.0	<10.0	8.6	<10.0
Sucrose	3.0	8.6	<10.0	8.6	<10.0	8.8	<10.0	8.8	<10.0
Wheat gluten	3.0	8.4	<10.0	8.8	<10.0	8.8	<10.0	8.8	<10.0
Glucose Starch Soybean oil	2.0 2.0 1.0	6.8	10.0	6.8	245.8	7.2	291.7	8.2	180.0

The basal medium : 2.0 % soybean meal, 0.5 % yeast extract, 0.25 % NaCl, 0.35 % $CaCO_3$(ppt), 0.0005 % $CuSO_4 \cdot 5H_2O$, 0.0005 % $MnCl_2 \cdot 4H_2O$, 0.005 % $ZnSO_4 \cdot 7H_2O$ and the initial pH was adjusted to pH 7.0.

Table 4. Production of oryzoxymycin in media containing various nitrogen sources and pH at the highest production

Nitrogen sources	%	3rd day		4th day		5th day		6th day	
		pH	mcg/ml	pH	mcg/ml	pH	mcg/ml	pH	mcg/ml
Soybean meal Yeast extract	2.0 0.5	6.4	32.5	6.8	39.1	7.8	80.0	8.2	54.0
S. V. P.	2.0	4.4	<10.0	5.0	<10.0	5.0	<10.0	5.2	<10.0
S. V. P. Yeast extract	2.0 0.5	4.2	<10.0	5.0	<10.0	5.0	<10.0	5.0	<10.0
C. S. L.	2.0	5.4	<10.0	6.4	<10.0	6.4	<10.0	6.4	<10.0
C. S. L. Yeast extract	2.0 0.5	4.6	<10.0	5.2	<10.0	5.2	<10.0	5.2	<10.0
Peanut meal	2.0	5.6	<10.0	5.6	<10.0	5.6	<10.0	5.6	<10.0
Peanut meal Yeast extract	2.0 0.5	5.4	<10.0	5.2	<10.0	5.6	<10.0	5.6	<10.0
Cotton seed meal	2.0	6.8	27.5	6.0	<10.0	6.0	64.1	6.2	53.5
Cotton seed meal Yeast extract	2.0 0.5	5.6	20.0	5.0	<10.0	5.0	<10.0	5.0	<10.0
*Soybean meal	3.0	6.4	308.0	7.4	650.0	7.4	575.0	8.4	400.0
*Soybean meal Yeast extract	3.0 1.0	4.6	20.8	5.8	27.5	5.8	43.3	6.8	40.5
†Soybean meal Yeast extract	3.0 1.0	5.4	<10.0	5.4	<10.0	5.8	16.1	5.8	15.0

The basal medium : 2.0 % glucose, 2.0 % starch, 0.25 % NaCl, 0.35 % $CaCO_3$, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.0005 % $CuSO_4 \cdot 5H_2O$, 0.0005 % $MnCl_2 \cdot 4H_2O$, 0.005 % $ZnSO_4 \cdot 7H_2O$ and adjusted pH to 7.0.

* The media contained 2.0 % glucose, 2.0 % starch, as the carbon source.

† $CaCO_3$ was omitted. S. V. P.: Soluble vegetable protein. C. S. L.: Corn steep liquor.

mogenic characteristics. Therefore, it is considered proper to designate strain M 979-M1 as a variant of *Streptomyces venezuelae* named *Streptomyces venezuelae* var. *oryzoxymyceticus* HAMADA *et* OKAMI.

Production of Oryzoxymycin

Strain M979-M1 was shake-cultured (140 strokes/min., 8 cm amplitude) in 125 ml of various media in 500-ml flasks at 27°C. The production and pH change are shown in Tables 3 and 4. The production in 30-liter stainless steel jar fermentor was tested in 15 liters of the following medium: glucose 2.0 %, starch 2.0 %, soybean meal 3.0 %, $CaCO_3$ (ppt) 0.35 %, K_2HPO_4 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, NaCl 0.3 %, soybean oil 1.0 %, $CuSO_4 \cdot 5H_2O$ 0.0005 %, $MnCl_2 \cdot 4H_2O$ 0.0005 %, and $ZnSO_4 \cdot 7H_2O$ 0.005 %. The temperature was 27°C and the rate of aeration was 15 liters per minute under stirring at 250 r.p.m.

The pH of cultured broth on the second and the third day was 6.4 and 7.4, and 98 and 200 mcg/ml of the antibiotic were produced respectively.

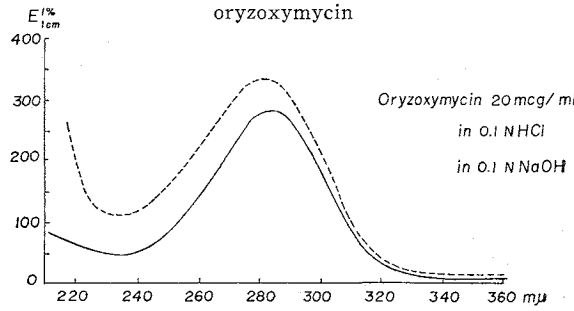
Isolation and Purification of Oryzoxymycin

Oryzoxymycin was obtained from the cultured liquid. One hundred g of oxalic acid and 500 g of diatomaceous earth (Hyflo Supercel) were added to 30 liters of the cultured broth and filtered. The antibiotic (200 mcg/ml) in the filtrate (26.5 liters) was adsorbed on a column of cation-exchange resin, Amberlite RI-120 (H form). After washed with water, the antibiotic was eluted with 0.5 N NH_4OH. The active eluate was concentrated under reduced pressure, yielding 18.1 g of brownish solid. The crude powder thus obtained was dissolved in 100 ml of water and purified by carbon column chromatography. The column was washed with water and 0.1 N HCl and then eluted with 1.0 liter of 10 % methanol (Fraction 1), 3.0 liters of 10 % methanol and 3.0 liters of 30 % methanol (Fraction 2) successively. Each fraction was neutralized with anion exchange resin, Amberlite IR-45 (OH form), and evaporated *in vacuo*. Fraction 1 gave 1.34 g (purity 82.1 %) of the antibiotic and Fraction 2 gave 3.82 g (purity 37.6 %). These crude powders were further purified by silica gel chromatography. The crude powder (1.34 g) of fraction 1 was suspended in 80 ml of a mixture of methanol and ethyl acetate (1 : 4 by volume) and placed on the top of the column (3.2 cm × 100 cm) of silicic acid (Mallinckrodt). After washing with 800 ml of the same solvent, the antibiotic was eluted with a mixture of methanol and ethyl acetate (2 : 3 by volume). The eluate was collected in fraction which were 20 ml tested for antimicrobial activity against *X. oryzae* and examined by thin-layer chromatography. The chromatograms were examined for antimicrobial activity or with 0.5 % $KMnO_4$. Oryzoxymycin showed Rf value 0.32 in the solvent system of *n*-butanol - acetic acid - water (4 : 2 : 1), and Rf 0.42 in methanol. The fractions which gave single spots on thin-layer chromatography were combined and evaporated to dryness under reduced pressure to yield 219 mg of the purified colorless powder of the antibiotic. Using 3.82 g of the crude powder from fraction 2 described above, 98.0 mg of the purified antibiotic was obtained by a similar method.

Physical and Chemical Properties of Oryzoxymycin

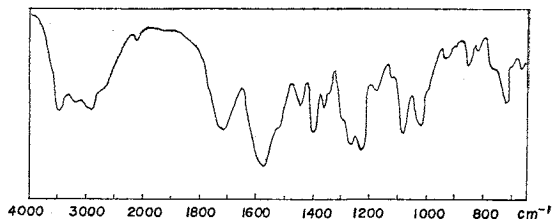
Oryzoxymycin is obtained as an amphoteric colorless powder, m.p. 155°C (decomp.), $[\alpha]_D^{25}$ +240° (c=1, H_2O). It is soluble in methanol and water, but insoluble in most organic solvents. As shown in Fig. 1, oryzoxymycin shows a maximum at 285 mμ ($E_{1cm}^{1\%}$ 285) in 0.1 N HCl or at 282 mμ ($E_{1cm}^{1\%}$ 335) in 0.1 N NaOH. The infrared spectrum is shown in Fig. 2.

Fig. 1. The ultraviolet spectra of oryzoxymycin



Potentiometric titration shows the presence of four dissociable groups at pK'a 2.2, 3.1, 7.6 and 8.6 and a titration equivalent of 448. Molecular weight estimated by vapor pressure osmometer using water as the solvent is 200. *Anal.* Found: C, 53.45, 53.11; H, 6.25, 6.25; N, 6.07, 5.79; O, 34.45, 34.70. Calculated for $C_{21}H_{30}N_2O_{10}$ (m.w. 470.47); C, 53.61; H, 6.45; N, 5.95; O, 34.07.

Fig. 2. The infrared of spectrum of oryzoxymycin (KBr)



It decolorizes permanganate solution and gives positive FOLIN-LOWRY and xanthoproteic reactions. Ninhydrin reaction is weakly positive, while MOLISCH, anthrone, SAKAGUCHI and biuret reactions are negative.

Rf values of oryzoxymycin and the solvent systems employed are shown below:

Solvent	Rf
Paper chromatography (Toyo No. 51 filter paper)	
1) 80 % ethanol	0.66
2) *n*-butanol – acetic acid – water (4 : 1 : 2)	0.13
Thin-layer chromatography (Kieselgel G (Merck))	
1) methanol	0.42
2) *n*-butanol – acetic acid – water (4 : 2 : 1)	0.32
3) *n*-propanol – acetic acid – pyridine – water (50 : 6 : 20 : 24)	0.64
4) methanol – ethyl acetate (1 : 1)	0.07

On high voltage paper electrophoresis (3,300 V/42 cm, 65 mA/20 cm, 15 min.), using a buffer of formic acid – acetic acid – water (25 : 75 : 900 in volume), oryzoxymycin moves 7 cm toward the cathode from the starting point.

The properties above described differentiate oryzoxymycin from known antibiotics active against *X. oryzae*[3].

Biological Activities of Oryzoxymycin

Oryzoxymycin inhibits *Xanthomonas oryzae* at 1.56 mcg/ml on glucose nutrient agar containing 1.0 % glucose and at 0.78 mcg/ml on 1.5 % sucrose agar containing rice straw extract (10 %) at pH 7.0 and 0.39 mcg/ml at pH 5.0. On the medium containing 1.5 %

sucrose and rice straw extract at pH 7.0, oryzoxymycin inhibits *Xanthomonas citri* and *X. phaseoli* at 1.56 and 25.0 mcg/ml respectively but shows no inhibition against *X. pruni* and *X. campestris* at 50 mcg/ml. It does not inhibit growth of the following plant pathogens at more than 100 mcg/ml: *Absidia spinosa*, *Alternaria kikuchiana*, *Cladosporium spherosporum*, *Colletotrichum phomoides*, *Fusarium lini*, *Fusarium oxysporum*, *Fusarium roseum*, *Gibberella saubinetii*, *Gibberella fujikuroi*, *Gloeosporium kaki*, *Glomerella laginarium*, *Helminthosporium sesamum*, *Ophiobolus miyabeanus*, *Piricularia grisea*, *Piricularia oryzae*, *Pellicularia filamentosa*, *Sclerotium rolfsii*.

Oryzoxymycin shows no preventive and curative effects in the pot test against *X. oryzae*, when sprayed in solution at 1,000 mcg/ml. When administered intravenously, mice tolerated a single dose of 50~100 mg/kg.

References

1, 2) Waksman, S. A. : The actinomycetes. Vol. II, p. 280, The Williams & Wilkins Co. (Baltimore) 1961

3) Umezawa, H.; S. Kondo, K. Maeda, Y. Okami, T. Okuda & K. Takeda : Index of antibiotics from actinomycetes. University of Tokyo Press, 1967