OKILACTOMYCIN, A NOVEL ANTIBIOTIC PRODUCED BY A *STREPTOMYCES* SPECIES

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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Okilactomycin, a novel antibiotic, was isolated from the culture filtrate of a strain of actinomycetes. The producing organism, strain YP-02908L, was identified as *Streptomyces griseoflavus* subsp. *zamamiensis* subsp. nov. The antibiotic was extracted with ethyl acetate and purified by silica gel column chromatography. It was obtained as colorless prisms from a dichloromethane solution. It exhibited weak antimicrobial activity against Gram-positive organisms *in vitro*. It also exhibited antitumor activity against Ehrlich ascites carcinoma *in vivo*. The apparent molecular formula of okilactomycin was determined as $C_{24}H_{32}O_6$. It is a new member of the lactone group antibiotics.

In the course of our screening for new antibiotics, a novel antibiotic, okilactomycin, was found. The antibiotic was produced by *Streptomyces griseoflavus* subsp. *zamamiensis* subsp. nov. It exhibited weak antimicrobial activity against Gram-positive organisms and antitumor activity agaist Ehrlich ascites carcinoma. In this paper, we describe the taxonomy of the producing organism, the fermentation, isolation and characterization of the antibiotic.

Materials and Methods

Chemicals

Chemicals employed were as follows: Kieselgel 60 and TLC plates Silica gel 60 F_{254} (0.25 mm thickness) from E. Merck, Darmstadt, FRG; Sephadex LH-20 from Pharmacia Fine Chemicals, Sweden; mitomycin C from Kyowa Hakko Kogyo Co., Ltd., Japan. All other chemicals were of analytical grade.

Components for Media

Components for media employed were as follows: Polypeptone (PP) from Daigo Eiyo Kagaku Co., Ltd., Japan; yeast extract (YE) from Oriental Yeast Co., Ltd., Japan; corn steep liquor (CSL) from Hounen Seiyu Co., Ltd., Japan; meat extract (ME) from Kyokuto Seiyaku Kogyo Co., Ltd., Japan; soy bean meal (SBM) from Ajinomoto Co., Inc., Japan; Pharmamedia (PM) from Procter & Gamble Co., U.S.A.; brain heart infusion (BHI) from Eiken Kagaku Co., Ltd., Japan; Mueller-Hinton medium from Difco Laboratories, U.S.A.

Animals

*dd*Y-SLC mice, 4 to 6 weeks of age, were purchased from Shizuoka Laboratory Animal Center, Japan. Experiments were started when mice were 8 weeks old.

Microorganism

Strain YP-02908L was isolated from a soil sample collected at Zamami island, Okinawa Prefecture, Japan and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession number FERM P-8610.

Taxonomic Studies

For taxonomic studies, most cultures were grown in accordance with methods adopted by the International Streptomyces Project (ISP).[1] For experiments on cultural properties, all cultures were incubated at 28°C and were observed for 14~21 days. The color was described according to the "Guide to Color Standard".† Physiological properties including utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB.[2] Cell wall composition was analyzed by the method of BECKER *et al.*[3]

Media and Fermentation

To prepare vegetative inoculum for the production of okilactomycin, the strain was grown in a 500-ml Erlenmeyer flask containing 60 ml of seed medium with following composition (g/liter): Dextrin 20.0, glucose 5.0, PP 5.0, YE 5.0, CSL 5.0, ME 3.0, BHI 5.2 and $CaCO_3$ 2.0. The pH of the medium was adjusted to 8.0 before sterilization. After 72 hours of growth at 28°C on a rotary shaker, 1.2 liters of the seed culture broth were inoculated into a 50-liter jar fermentor containing 35 liters of production medium with following composition (g/liter): Dextrin 30.0, glucose 30.0, SBM 15.0, PM 15.0, KH_2PO_4 0.6, K_2HPO_4 0.25 and $CoCl_2 \cdot 6H_2O$ 0.004. The pH of the medium was adjusted to 7.0 before sterilization. The fermentation was carried out 27°C for 72 hours (aeration; 40 liters/minute, agitation; 300 rpm). The antibiotic production was monitored by a paper-disk assay using *Bacillus subtilis* ATCC 6633 as test organism.

Isolation

The antibiotic in the culture filtrate (30 liters, pH 6.8) was extracted with EtOAc (30 liters). The solvent layer was concentrated *in vacuo* to dryness. The residue (3 g, brownish powder) was dissolved in MeOH and filtered. After evaporation of the filtrate, the crude substance (2 g, a pale brownish powder) was chromatographed on Silica gel column (E. Merck, Kieselgel 60, 50 g) using an eluent of $CHCl_3$ - MeOH (90 : 1). The active fractions were combined and then concentrated *in vacuo* to afford a pale yellowish powder (620 mg). The powder was rechromatographed on Sephadex LH-20 column (300 ml bed volume) with MeOH. The active eluate was concentrated to give a pure colorless powder (510 mg). Okilactomycin was obtained as colorless prisms from dichloromethane solution.

Antimicrobial Activity

The antimicrobial spectra of okilactomycin was determined by a conventional agar dilution method using Mueller-Hinton medium for Gram-positive and Gram-negative organisms. MIC were expressed in term of μg/ml after overnight incubation at 37°C for bacteria. Due to the poor solubility in H_2O, okilactomycin was initially dissolved in DMSO and subsequently diluted with medium.

Antitumor Activity

The antitumor activity of okilactomycin was determined in experimental tumor system in mice. Ehrlich ascites carcinoma was implanted intraperitoneally into *dd*Y-SLC mice (female, 8 weeks old) at an inoculum size of 1×10^6 cells per mouse. Drug treatments were given intraperitoneally once daily from day 1 to day 5 (qd 1-5) starting 24 hours after the tumor implantation. Ten mice were used in each test group. The injection volume was 0.2 ml in all experiments. Control animals received intraperitoneal dose of physiological saline solution. Okilactomycin was initially dissolved in ethanol then suspended in physiological saline solution. Mitomycin C was suspended in physiological saline solution and comparatively tested simultaneously as a reference compound. Death or survival of the treated and non-treated animals was recorded daily during the observation period of

† Guide to Color Standard., Nihon Shikisai Sha, 1954.

30 days after the tumor implantation, and the median survival time (MST) was calculated for each of the test and control groups. Antitumor activity was evaluated by the MST of group of mice and also expressed as T/C % value [MST of treated group/MST of non-treated group, ×100].

TLC/Bioautography

Okilactomycin was monitored with a TLC system using Silica gel 60 F_{254} plate (Merck 5744) developed in a solvent system containing $CHCl_3$ - MeOH (9 : 1). Antimicrobial activity of the antibiotic on TLC plate was detected by bioautography against *B. subtilis* ATCC 6633.

Physico-chemical Properties

Melting point was taken using a Yanaco MP-3 aparatus and is uncorrected. UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and IR spectra on a Hitachi 260-50 spectrophotometer. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. Fast atom bombardment mass spectra (FAB-MS) were obtained on a Jeol JMS-DX300 mass spectrometer. The elementary analysis was carried out with a Yanagimoto MT-3 coder.

Results and Discussion

Taxonomic Studies of the Producing Strain

Strain YP-02908L was isolated from a soil sample collected at Zamami island, Okinawa Prefecture, Japan. On the basis of morphological observation with light and electron microscopes on various cultures grown at 28°C for 14~21 days, mature spores occured in chains of more than 10 spores forming loose spirals. The spores were cylindrical and 0.5~0.7×1.0~1.4 μm in size and had spiny surfaces. Sclerotic granules, sporangia and flagellated spores were not observed (Fig. 1).

Cultural characteristics were observed on various kind of media described by SHIRLING and GOTTLIEB.[4] The vegatative mycelia grew abundantly on yeast extract - malt extract agar, tyrosine agar and BENNETT's agar.

Aerial mass color is in the gray series when grown on yeast extract - malt extract agar, glycerol - asparagine agar, inorganic salts - starch agar, peptone - yeast extract - iron agar and BENNETT's agar. Results are shown in Table 1.

The cell wall analysis of strain YP-02908L showed that it was containing LL-diaminopimelic acid and glycine. Accordingly, the cell wall of the strain was type I.

Physiological properties of the strain were shown in Table 2. Temperature range for growth was from 5 to 33°C with the optimum from 20 to 27°C. Gelatin liquefaction, milk peptonization and milk coagulation were positive.

Utilization of carbon sources by the strain was examined according to the methods of PRIDHAM and GOTTLIEB.[2] The results are summarized in Table 3.

Microscopic studies and cell wall analysis of

Fig. 1. Scanning electron micrograph of spore chain of strain YP-02908L.

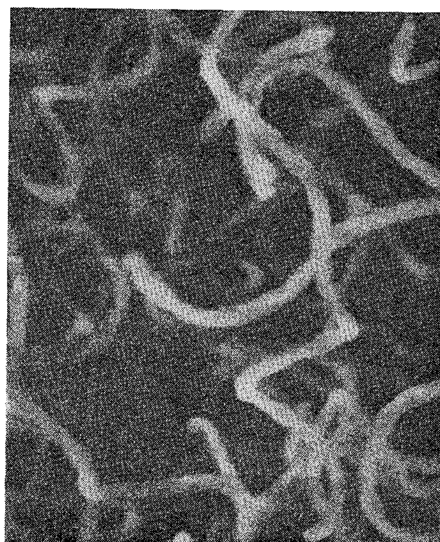


Table 1. Cultural characteristics of strain YP-02908L on various media.

Medium		
Yeast extract - malt extract agar (ISP 2)	G:	Good, pale yellowish brown
	R:	Pale yellowish brown
	AM:	Abundant, light gray
	SP:	None
Oatmeal agar (ISP 3)	G:	Moderate, yellowish gray
	R:	Light brownish gray
	AM:	Moderate, light gray - light brownish gray
	SP:	None
Inorganic salts - starch agar (ISP 4)	G:	Poor, grayish white
	R:	Grayish white
	AM:	Poor, grayish white
	SP:	None
Glycerol - asparagine agar (ISP 5)	G:	Poor, grayish white
	R:	Grayish white
	AM:	Poor, grayish white
	SP:	None
Peptone - yeast extract - iron agar (ISP 6)	G:	Moderate, pale yellowish brown
	R:	Pale yellowish brown
	AM:	Moderate, light gray
	SP:	None
Tyrosine agar (ISP 7)	G:	Good, yellowish gray
	R:	Grayish white
	AM:	Abundant, light gray
	SP:	None
Sucrose - nitrate agar	G:	None
	R:	None
	AM:	None
	SP:	None
Nutrient agar	G:	Moderate, yellowish gray
	R:	Yellowish gray
	AM:	None
	SP:	None
BENNETT's agar	G:	Good, pale yellowish brown
	R:	Pale yellowish brown
	AM:	Abundant, light gray
	SP:	None

Abbreviations: G; Growth of vegetative mycelium, R; reverse side of color, AM; aerial mycelium, SP; soluble pigment.

strain YP-02908L indicated that the strain was classified in the genus *Streptomyces* Waksman and Henrici 1943, 339. Accordingly, the strain was compared with the published descriptions[4~8] of various *Streptomyces* species and the results showed that strain YP-02908L was considered to resemble *Streptomyces rubiginosus* and *S. griseoflavus.*

On the basis of the comparison of strain YP-02908L with the two strains in several discriptions, strain YP-02908L was different from *S. rubiginosus* with the following properties: The former did not produce soluble pigment, while the latter produced red pigment on several agar media.

On the other hand, strain YP-02908L resembled *S. griseoflavus* in many aspects. Therefore, the cultural characteristics of strain YP-02908L was directly compared with *S. griseoflavus* JCM-4040.

The results were shown in Table 4. Strain YP-02908L differed from *S. griseoflavus* JCM-4040

Table 2. Physiological properties of strain YP-02908L.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Liquefaction of gelatin (21°C)	+
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	+
Cellulolytic activity	−
Starch hydrolysis	−
Temperature range for growth	5～33°C

+: Active, −: not active.

Table 3. Utilization of carbohydrates by strain YP-02908L.

L-Arabinose	−
D-Xylose	±
D-Glucose	+
D-Fructose	±
D-Mannitol	−
Sucrose	−
Inositol	+
Raffinose	−
L-Rhamnose	±
Starch	−

+: Utilized, ±: weakly utilized, −: not utilized.

Table 4. Comparison of YP-02908L with *Streptomyces griseoflavus.*

	YP-02908L	*S. griseoflavus* JCM-4040
Spore shape	Cylindrical	Cylindrical
Spore surface	Spiny	Spiny
Aerial mycelium	Grayish white-light gray	Grayish white
Vegetative mycelium	Grayish white-pale yellowish brown	Grayish white-pale yellowish brown
Soluble pigment	None	None
Temperature range for growth	5～33°C	10～33°C
Tyrosinase reaction	−	−
Starch hydrolysis	−	+
Peptonization of milk	+	+
Coagulation of milk	+	+
Liquefaction of gelatin	+	+
Utilizable carbon sources	D-Glucose, D-xylose, D-fructose, inositol, L-rhamnose	D-Glucose, D-xylose, D-fructose, D-mannitol, L-arabinose
Antibiotic produced	Okilactomycin	None

+: Utilized, −: not utilized.

for starch hydrolysis, utilization of L-arabinose and D-mannitol and the antibiotic productivity, but otherwise, strain YP-02908L resembled to *S. griseoflavus.* Therefore, strain YP-02908L is considered to be a new subspecies of *S. griseoflavus* and the name *S. griseoflavus* subsp. *zamamiensis* subsp. nov. is proposed.

The type strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. YP-02908L with the accession No. FERM P-8610.

Production and Isolation

Strain YP-02908L was cultured in a 50-liter jar fermentor at 27°C for 72 hours. A typical time course for the fermentation is shown in Fig. 2. Okilactomycin production started at 40 hours after inoculation, then gradually increased and reached a maximum (56 μg/ml) at 72～80 hours. From the culture filtrate (30 liters), the antibiotic was isolated as shown in Fig. 3. The total yield of okilactomycin was 510 mg.

Fig. 2. Time course of okilactomycin production.
□ pH, ○ growth, ● okilactomycin.

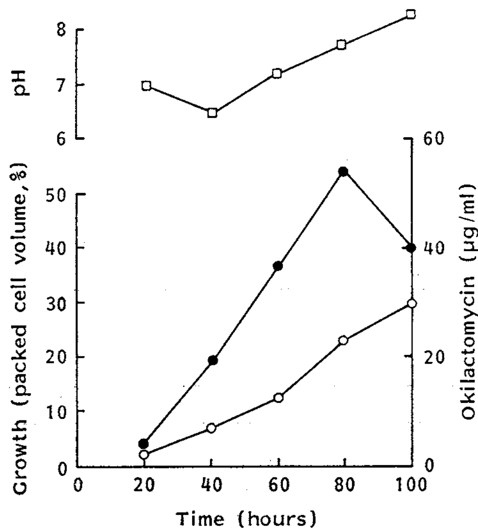


Fig. 3. The procedure for isolation of okilactomycin.

Fermentation broth
| filtration
Filtrate (30 liters)
| adjusted to pH 6.8
EtOAc extract (30 liters)
| removed EtOAc by evaporation
Silica gel column chromatography
| eluted with $CHCl_3$ - MeOH (90: 1)
| evaporated to dryness
A pale yellowish powder (620 mg)
|
Sephadex LH-20 column chromatography
| developed with MeOH
| concd to dryness
A pure colorless powder (510 mg)
| crystallized from CH_2Cl_2
Colorless prisms (470 mg)

Biological Properties

Antimicrobial Activity

The antimicrobial activity of okilactomycin is shown in Table 5. The antibiotic exhibited weak antimicrobial activity against Gram-positive organisms with MIC ranging from 12.5 to 50 μg/ml. However, no activity was observed against Gram-negative organisms and acid-fast *Mycobacterium smegmatis*.

Antitumor Activity

The cytotoxic activity *in vitro* of okilactomycin was determined as follows. Concentration of the antibiotic required for 50% inhibition of cell growth (IC_{50}; μg/ml) was examined by plotting the logarithms of the concentration versus the growth rate (percentage of control) of the treated cells. It possessed cytotoxic activity against lymphoid leukemia L1210 and leukemia P388 with IC_{50} value of 0.09 and 0.037

Table 5. Antimicrobial spectra of okilactomycin.

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P JC-1	25
S. epidermidis IID 866	12.5
Streptococcus pyogenes Cook	12.5
Enterococcus faecalis IID 682	50
E. faecium CAY 09-1	50
Mycobacterium smegmatis ATCC 607	>100
Escherichia coli 0-1	>100
Citrobacter freundii CAY 17-1	>100
Klebsiella pneumoniae ATCC 10031	>100
Proteus vulgaris OXK US	>100
Pseudomonas aeruginosa NCTC 10490	>100

Inoculum size; 10^6 cfu/ml.

Table 6. Antitumor activity of okilactomycin against Ehrlich ascites carcinoma.

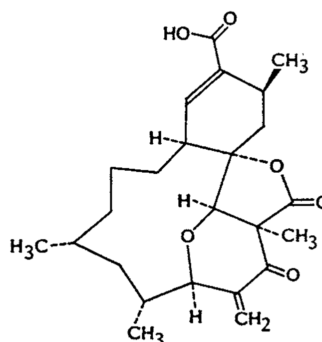
Antibiotic	Dose (mg/kg/day)	MST (days)	T/C (%)	Survival (30 days)
Okilactomycin	0.625×5 ip	16.0	91.4	0/10
	1.25	22	128.6	1/10
	2.5	25.5	145.7	4/10
	5.0	20.5	117.1	0/10
Mitomycin C	0.5×5 ip	>30	>170	7/10
	1.0	>30	>170	9/10
Control	—	17.5	100	0/10

Table 7. Physico-chemical properties of okilactomycin.

	Okilactomycin
Appearance	Colorless powder
$[\alpha]_D^{20}$	+34° (*c* 1, MeOH)
MP (°C)	161
UV λ_{max}^{MeOH} nm (ε)	No characteristic absorption
IR ν_{max}^{KBr} cm^{-1}	2960, 1790, 1760, 1715, 1690, 1630, 1460, 1260, 1190, 1130
FAB-MS (*m/z*)	417 $(M+H)^+$
Molecular formula	$C_{24}H_{32}O_6$
Elemental analysis	
Calcd for $C_{24}H_{32}O_6$:	C 69.21, H 7.74.
Found:	C 68.86, H 7.70.
TLC (Rf)*	0.4

* Silica gel plate $CHCl_3$ - MeOH (9 : 1).

Fig. 4. The structure of okilactomycin.



μg/ml, respectively. Additionally, the antitumor activity of the antibiotic was determined in mice. The results are shown in Table 6. Okilactomycin exhibited antitumor activity against Ehrlich ascites carcinoma. However, the antibiotic did not have antitumor activity against leukemia P388.

The acute toxicity of okilactomycin in mice was LD_{50} >300 mg/kg (po) and 25 mg/kg (ip).

Physico-chemical Properties

The physico-chemical properties of okilactomycin are summarized in Table 7. The antibiotic is readily soluble in methanol, ethanol, acetone and ethyl acetate, soluble in chloroform and benzene, slightly soluble in hexane, and insoluble in water. It gave a positive reaction to $KMnO_4$ and iodine, though negative to ninhydrin, Molisch and Ehrlich reagent. From the physico-chemical properties mentioned above, it can be concluded that okilactomycin is a new member of the lactone group antibiotics. Studies in our laboratories have shown that okilactomycin has the chemical structure shown in Fig. 4. Details of structure elucidation will be reported in a separate paper.[9]

Addendum in Proof

Okilactomycin is identical to compound YP-02908L-A.

Acknowledgments

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