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Lactonamycin, a New Antimicrobial Antibiotic Produced by *Streptomyces rishiriensis* MJ773-88K4

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

NAOKI MATSUMOTO, TOSHIO TSUCHIDA, MASATO MARUYAMA, NAOKO KINOSHITA, YOSHIKO HOMMA, HIRONOBU IINUMA, TSUTOMU SAWA, MASA HAMADA and TOMIO TAKEUCHI

Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

NAOHIKO HEIDA and TAKEO YOSHIOKA

Mercian Corporation, Central Reserch Laboratories, 4-9-1 Jhonan, Fujisawa-shi, Kanagawa 251-0057, Japan

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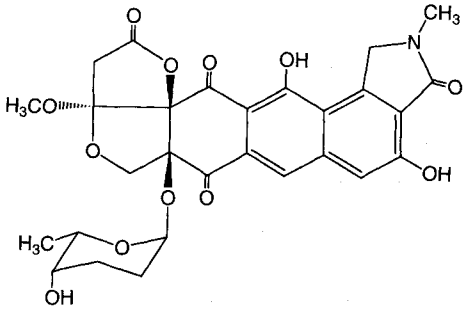
Lactonamycin (**1**) was isolated from a culture broth of *Streptomyces rishiriensis* MJ773-88K4. Antibiotic **1** exhibited antimicrobial activities against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE).

Methicillin-resistant *Staphylococcus aureus* (MRSA) has caused a serious problem in clinics since 1980s. Two anti-MRSA agents, vancomycin and arbekacin are used clinically in Japan. But emergence of vancomycin-resistant *Enterococcus* (VRE) reported recently threatened those engaged in medical treatment. So we are devoted to discover antibiotics effective against various bacteria including MRSA and VRE from microbial sources.

In the course of our screening from soil microorganisms for new antibiotics, we have isolated a new antibiotic, lactonamycin (**1**)[1] (Fig. 1) from a culture broth of *Streptomyces rishiriensis* MJ773-88K4 which was isolated from a soil sample collected at Yokohama City, Kanagawa prefecture, Japan. Antibiotic **1** showed antimicrobial activities against Gram-positive bacteria including MRSA and VRE. In this paper, we report the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and biological activities of **1** in detail. Structural determination with absolute configuration of **1** is reported in the succeeding paper.

Fig. 1. Absolute structure of lactonamycin (**1**).



Materials and Methods

Microorganisms

Streptomyces rishiriensis[2] IMC S-0775^T (ISP 5489), *Streptomyces galilaeus*[3] IMC S-0853^T (ISP 5481), *Streptomyces diastatochromogenes*[4] IMC S-0712^T (ISP 5449) and *Streptomyces aurantiogriseus*[5] IMC S-0069 (ISP 5138) were compared taxonomically with strain MJ773-88K4.

Taxonomic Studies

Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB[6] and by the method of WAKSMAN[7]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[8]. The substrate and aerial mass color including soluble pigment were assigned by Color Harmony Manual, 1958 (Container Corporation of America, Chicago)[9]. Morphological characteristics were observed with a scanning electron microscope (Hitachi S-570). 2,6-Diaminopimelic acid in the cell wall was analyzed from the hydrolysate of the culture growth according to the method of BECKER *et al.*[10].

Measurement of Antimicrobial Activity

The minimum inhibitory concentrations (MIC) of **1** were examined by serial agar dilution method using Mueller-Hinton agar (Difco) for antibacterial test which was incubated at 37°C for 18 hours and a nutrient agar containing 1% glycerol for mycobacteria test which was incubated at 37°C for 42 hours.

Anititumor Activity

Tumor cells were incubated in 96-well plate for 24 hours prior to the addition of **1** into culture well at varied concentrations. After 2 to 3 days incubation at 37°C, MTT reagent was added and further incubated for 4 hours. Growth inhibition activity was determined according to the standard MTT assay method[11] and method[11] and IC_{50} was calculated.

Table 1. Cultural characteristics of strain MJ773-88K4.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Pale yellow [1 1/2ga, butter yellow]	None	None
Glucose-asparagine agar	Pale yellow [2gc, bamboo]~pale yellowish brown [2le, mustard]	Light brownish gray [3dc, natural]	None
Yeast extract-malt extract agar (ISP No. 2)	Pale yellowish brown [3ne, topaz]	White~light brownish gray [3dc, natural] ~light gray [3fe, silver gray]	Brownish
Oatmeal agar (ISP No. 3)	Pale yellow [2ca, lt ivory~1 1/2ea, lt yellow]	Light gray [3fe, silver gray]	Faint, yellowish
Inorganic salts-starch agar (ISP No. 4)	Colorless~pale yellowish brown [2ne, mustard gold]	Light gray [3fe, silver gray~5fe, ashes]	None
Glycerol-asparagine agar (ISP No. 5)	Pale yellow [2ea, lt wheat]~dull yellow [2lc, gold]	Light brownish gray [3dc, natural] ~light gray [3fe, silver gray]	Faint, brownish
Tyrosine agar (ISP No. 7)	Yellowish brown [3ng, yellow maple ~3ni, clove brown]	Yellowish gray [2ba, pearl]~light brownish gray [3dc, natural]	Dark brown
Nutrient agar	Pale yellowish brown [2le, mustard ~3le, cinnamon]	None	Brown
Starch agar	Dull yellow [1 1/2lc, gold]~pale yellowish brown [3ne, topaz]	Scant, white	None
Calcium-malate agar	Colorless~pale yellow [1 1/2ca, cream]	None	None

Observation after incubation at 27°C, for 21 days.
Color names and numbers from Color Harmony Manual, Container Corporation of America.

Results

Taxonomic Studies

The producing microorganism, strain MJ773-88K4, was isolated from a soil sample collected in Yokohama City, Kanagawa prefecture, Japan.

The strain MJ773-88K4 has branched substrate mycelia and aerial hyphae bore spirals. Mature spore chains consisted of 10 to 50 cylindrical spores. The spores were $0.5 \sim 0.6 \times 1.0 \sim 1.4\,\mu m$ in size with smooth or rugose surfaces. No whirl-formation, synnemata and sporangia was observed. The cultural characteristics of the strain MJ773-88K4 on various agar media are summarized in Table 1. The vegetative growth color was pale yellow to pale yellowish brown on various media tested. The aerial mycelium color was light brownish gray to light gray.

The physiological properties and the utilization of carbon sources are shown in Table 2. Optimum temperature for growth was 27°C. Formation of melanoid pigment was positive and hydrolysis of starch was positive. Liquefaction of gelatin was weakly positive and coagulation of milk was negative.

Analysis of whole-cell hydrolysate of the strain showed the presence of LL-diaminopimelic acid. On the basis of morphological and chemical characteristics, strain MJ773-88K4 was found to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *Streptomyces rishiriensis*, *Streptomyces galilaeus*, *Streptomyces diastatochromogenes* and *Streptomyces aurantiogriseus* were selected as similar to the strain MJ773-88K4. The taxonomical properties of strain MJ773-88K4 were different from *Streptomyces galilaeus* in the color of growth, and soluble pigment. They were different from *Streptomyces aurantiogriseus* in the color of soluble pigment and utilization of D-mannitol. They were different from *Streptomyces diastatochromogenes* in the coagulation of milk and utilization of D-mannitol. The comparison of strain MJ773-88K4 and *Streptomyces rishiriensis* is shown in Table 3.

From these results, strain MJ773-88K4 is closely related to *Streptomyces rishiriensis*. Therefore, it was designated as *Streptomyces rishiriensis* MJ773-88K4. This strain has been deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-14906.

Table 2. Physiological characteristics of strain MJ773-88K4.

Temperature range for growth (°C)	20～37
Optimum temperature (°C)	27
Formation of melanoid pigment	
ISP No. 1	Positive
ISP No. 6	Positive
ISP No. 7	Positive
Liquefaction of	
gelatine (20°C)	Weakly positive
glucose peptone gelatin (27°C)	Weakly positive
Coagulation of milk (37°C)	Negative
Peptonization of milk (37°C)	Positive
Hydrolysis of starch	Positive
Reduction of nitrate	Positive
Utilization of[a]	
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	+
myo-Inositol	+
Rhamnose	+
Raffinose	+
D-Mannitol	−

[a] +, Utilization; −, no utilization.

Fermentation

A slant culture of the lactonamycin-producing organism was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glycerol 2%, dextrin 2%, Bacto-soytone (Difco) 1%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and one drop of silicon oil (adjusted to pH 7.0 before sterilization). The inoculated medium was incubated at 28°C for 48 hours on a rotary shaker (220 rpm). One mililiter aliquots of this seed culture were transferred into seventy of 500 ml Erlenmeyer flasks each containing 100 ml of a producing medium consisting of glycerol 2%, dextrin 2%, Bacto-soytone (Difco) 1%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and one drop of silicon oil (adjusted to pH 7.4 before sterilization). The fermentation was carried out at 28°C for 120 hours on rotary shaker (220 rpm).

Table 3. Comparison of strain MJ773-88K4 and *Streptomyces rishiriensis*.

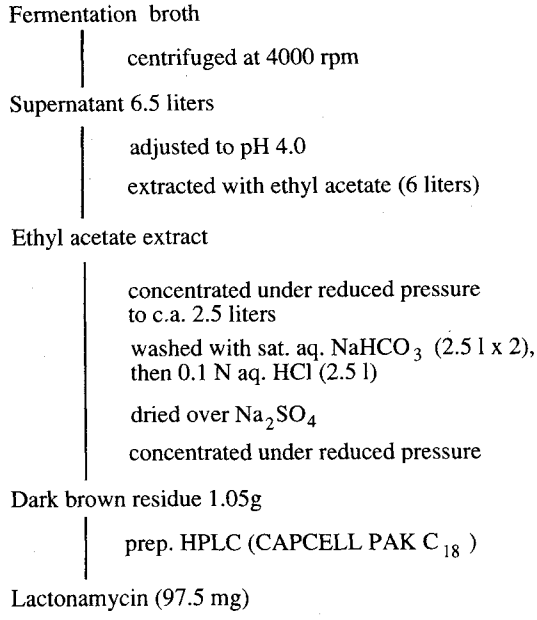
	Strain MJ773-88K4	*S. rishiriensis* IMC S-0775^T (ISP 5489)
Spore chain	Spiral	Spiral
Spore surface	Smooth	Smooth
Aerial mycelium (27°C)	Light gray	Light gray
Growth (27°C)	Pale yellow ~ pale yellowish Brown ~ dull yellow orange	Pale yellow ~ pale yellowish Brown
Soluble pigment (27°C)	None ~ brownish	None ~ brownish
Formation of melanoid pigment	Positive	Positive
Liquefaction of gelatin	Weakly positive	Weakly positive
Coagulation of milk (37°C)	Negative	Negative
Peptonization of milk (37°C)	Positive	Positive
Hydrolysis of starch	Positive	Positive
Reduction of nitrate	Positive	Positive
Utilization of[a]		
D-Glucose	+	+
L-Arabinose	+	+
D-Xylose	+	+
D-Fructose	+	+
Sucrose	+	+
myo-Inositol	+	+
Rhamnose	+	+
Raffinose	+	+
D-Mannitol	−	−

[a] +, Utilization; −, no utilization.

Isolation and Purification

The isolation procedure of **1** is outlined in Fig. 2. The fermentation broth was centrifuged, and the supernatant (6.5 liters) was extracted with ethyl acetate (6 liters) at pH 4.0. The ethyl acetate layer was concentrated under reduced pressure to *ca.* 2.5 liters, which was washed succesively with saturated aq. $NaHCO_3$ (2.5 liters × 2) and 0.1 N aq. HCl (2.5 liters). The organic layer was dried over anhydrous sodium sulfate, and concentrated *in vacuo* to give a dark brown residue (1.05 g). The crude material was subjected to preparative HPLC (mobile phase: CH_3CN-0.1% aq. AcOH (40:60), flow rate: 10 ml/minute) using an ODS column (SHISEIDO, CAPCELL PAK UG 120 Å 20 mm i.d. × 250 mm). Active fractions against *Bacillus stearothermophilus* were collected and concentrated to give **1** (97.5 mg) as a pale yellow powder.

Fig. 2. Isolation procedure of lactonamycin.



Physico-chemical Properties

Physico-chemical properties of **1** are summarized in

Table 4. Physico-chemical properties of lactonamycin.

Appearance	Pale yellow powder
Nature	Acidic
Molecular formula	$C_{28}H_{27}NO_{12}$
FAB-MS (*m/z*)	$(M+H)^+$ 570
	$(M-H)^-$ 568
HRFAB-MS (*m/z*)	Calcd: 570.1612 (as $C_{28}H_{28}NO_{12}$)
	Found : 570.1613 $(M+H)^+$
UV λ_{max} (ε) in MeOH	228 (11300), 257 (13200), 300 (20000), 395 (6700), 412 (7500)
in 0.01 N NaOH-MeOH	232 (11600), 255 (12900), 287 (12200), 323 (11300), 367 (10700), 398 sh, 448 sh
$[\alpha]_D^{25}$	+34° (*c* 0.27, MeOH)
IR ν_{max} (KBr) cm^{-1}	3410, 2950, 1810, 1690, 1640 sh, 1620, 1250
Rf[a]	0.25
HPLC Rt (min)[b]	7.4
mp	168~171°C

[a] HPTLC, Merck Art 13124, RP-18 $W_{254}S$, solvent: CH_3CN-H_2O (1:1).
[b] Column: Siseido CAPCELL PAK C_{18} (UG120 Å, 4.6 i.d. × 150 mm), solvent: AcCN-0.1% aq AcOH (40:60), flow rate: 1.0 ml/minute, detection: UV absorbance at 300 nm.

Table 5. Antimicrobial activities of lactonamycin against various bacteria.

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA209P	0.39
S. aureus Smith	0.39
S. aureus MS9610	0.78
Micrococcus luteus FDA16	0.78
Bacillus anthracis	0.39
B. subtilis NRRL B-558	0.39
B. cereus ATCC10702	0.20
Corynebacterium bovis 1810	0.78
Escherichia coli NIHJ	>100
Shigella dysenteriae JS11910	>100
Salmonella typhi T-63	>100
Proteus vulgaris OX19	>100
Providencia rettgeri GN311	>100
Serratia marcescens	>100
Pseudomonas aeruginosa GN315	>100
Klebsiella pneumoniae PCI602	>100
Mycobacterium smegmatis ATCC607	>100
Candida albicans 3147	>100

Table 6. Antimicrobial activities of lactonamycin against *Streptococcus* species.

Test organism	MIC (μg/ml)
Streptococcus faecalis 37787	0.39
S. pyogenes Cook	0.39
S. pyogenes group A St-92TC	0.78
S. pyogenes group A St-107TC	0.78
S. pyogenes group A St-108TC	0.39
S. pyogenes group A St-56·188SM	0.39
S. pyogenes TY-5727	0.20
S. pyogenes TY-5740	0.78
S. pyogenes TY-5914	0.39
S. pyogenes TY-5708	>100
S. pyogenes TY-5745	0.78
S. pyogenes TY-5834	0.78
S. pyogenes TY-5840	0.39

Table 4. Antibiotic **1** is readily soluble in acetone and DMSO, slightly soluble in methanol, chloroform and ethyl acetate and practically insoluble in water, toluene and *n*-hexane. Antibiotic **1** was isolated as a yellowish powder with melting point of 168~171°C, and optically active with $[\alpha]_D^{27}$ +34° (*c* 0.27, MeOH). The molecular formula of **1** was established as $C_{28}H_{27}NO_{12}$ by combination of HRFAB-MS and ^{13}C NMR spectral data. Antibiotic **1** showed absorption bands of a few

Table 7. Antimicrobial activities of lactonamycin against various MRSA.

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)
Staphylococcus aureus TY-00930	0.78	*S. aureus* TY-01847	0.78
S. aureus TY-00932	0.78	*S. aureus* TY-01852	0.78
S. aureus TY-00933	0.78	*S. aureus* TY-01856	0.78
S. aureus TY-00934	0.78	*S. aureus* TY-01857	0.78
S. aureus TY-00936	0.78	*S. aureus* TY-01859	0.78
S. aureus TY-01022	0.78	*S. aureus* TY-03450	0.78
S. aureus TY-01033	0.78	*S. aureus* TY-03454	0.39
S. aureus TY-01058	0.78	*S. aureus* TY-03456	0.78
S. aureus TY-01759	1.56	*S. aureus* TY-03460	0.78
S. aureus TY-01760	0.78	*S. aureus* TY-03463	0.78
S. aureus TY-01796	0.78	*S. aureus* TY-03466	0.78
S. aureus TY-01798	0.78	*S. aureus* TY-03467	0.78
S. aureus TY-01800	0.78	*S. aureus* TY-03468	0.78
S. aureus TY-01806	0.39	*S. aureus* TY-03470	0.39
S. aureus TY-01809	0.78		

Table 8. Antimicrobial activities of lactonamycin against various VRE.

Test organism	MIC (μg/ml)
Enterococcus faecalis NCTC 12201 VCM R	0.20
E. faecium NCTC 12202 VCM R	0.39
E. faecalis NCTC 12203 VCM R	0.78
E. faecium NCTC 12204 VCM R	0.20
E. faecalis 5803	0.39
E. faecium 5804	0.78

Table 9. Growth inhibition of cultured cell lines by lactonamycin.

Cell line	Origin	IC_{50} (μg/ml)
L1210	Leukemia	0.087
P388	Leukemia	0.123
EL-4	Leukemia	0.064
Ehrlich	Carcinoma	1.290
S180	Sarcoma	3.300
IMC carcinoma	Carcinoma	1.970
FS-3	Fibrosarcoma	2.220
Meth A	Fibrosarcoma	0.150
B16-BL6	Melanoma	0.860

different carbonyl groups in the IR spectrum, and bathochromic shift in alkaline solution was observed in the UV spectra. Antibiotic **1** gave positive color reactions to molybdophosphoric acid-sulfuric acid, $FeCl_3$, Rydon-Smith reagent and negative to ninhydrin reagent.

Biological Activities

As shown in Table 5, the antimicrobial activities of **1** against various Gram-positive bacteria such as *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus anthracis*, *B. subtilis*, *B. cereus* and *Corynebacterium bovis* were strong, while **1** exhibited no antimicrobial activity against Gram-negative bacteria. The antimicrobial activities against *Pasteurella piscicida* and *Enterococcus seriolicida* were very weak (data not shown).

Antibiotic **1** was effective against various *Streptococcus* including streptomycin-resistant strains, tetracycline-resistant strains and clinically isolated strains (Table 6).

Remarkably, **1** also showed potent antimicrobial activities against clinically isolated methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) (Table 7, 8).

Antibiotic **1** showed cytotoxicity at $0.06 \sim 3.3$ μg/ml against various tumor cell lines (Table 9). Single intraperitoneal injection of 100 mg/kg of **1** did not cause death in female ICR mice (4-weeks old).

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