Tetrodecamycin and Dihydrotetrodecamycin, New Antimicrobial Antibiotics against *Pasteurella piscicida* Produced by *Streptomyces nashvillensis* MJ885-mF8

I. Taxonomy, Fermentation, Isolation, Characterization and Biological Activities

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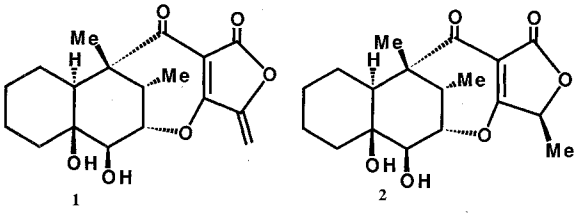
The novel antimicrobial antibiotic against *Pasteurella piscicida*, tetrodecamycin (**1**) and weakly active dihydrotetrodecamycin (**2**) were isolated from the fermentation broth of *Streptomyces nashvillensis* MJ885-mF8. They were purified by adsorption on Diaion HP-20, silica gel column chromatography and crystallization. The MICs of **1** were 6.25~12.5 μg/liter and 1.56~6.25 μg/ml against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and 12 strains of *P. piscicida*, respectively.

Pasteurella piscicida is well known as the causative agent of pseudotuberculosis in cultured yellowtail, *Seriola quinqueradiata*[1]. Pseudotuberculosis is a septicemia. Internally granulomatous-like deposits develop in the kidney and spleen and these deposits comprise many grayish-white bacterial colonies. This disease has become of considerable economic importance, causing significant losses in farmed yellowtail in Japan. Prevention of this disease is therefore considered an important aspect for the successful culture of yellowtail. Various chemotherapeutic agents such as ampicillin, amoxicillin, florfenicol, chloramphenicol, bicozamycin, pyridonecarboxylic acids and tetracycline derivatives have been used to treat bacterial infections in cultured fish. The extensive use has led to an increase in drug resistant strains among fish pathogens, despite the great variety of therapeutic agents in current drug resistant strains among pseudotuberculosis.

In the course of our screening program for novel antimicrobial antibiotics against *P. piscicida*, we have isolated new antibiotics, tetrodecamycin (**1**) and weakly active dihydrotetrodecamycin (**2**) from a culture broth of *Streptomyces nashvillensis* MJ885-mF8 (Fig. 1). A preliminary communication of this work has been reported[2]. In this paper, the details of taxonomy, fermentation, isolation, physico-chemical properties and biological properties of **1** and **2** are described. The structural elucidation study of **1** and **2** will be reported in another paper.[3]

Fig. 1. The structures of tetrodecamycin (**1**) and dihydrotetrodecamycin (**2**).



Materials and Methods

Microorganisms

Streptomyces zaomyceticus[4], IMC S-0663 (ISP 5196) and *Streptomyces nashvillensis*[5,6] IMC S-0726 (ISP 5314) were compared taxonomically with strain MJ885-mF8. Test strains of *P. piscicida* were supplied by Dr. KUSUDA (Fish Disease Laboratory, Faculty of Agriculture, Kochi University). These strains were isolated from kidney and spleen of *Seriola quinqueradiata*.

Taxonomic Studies

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

Time Course of the Production

The time course of antibiotic production was followed in jar fermenter. A slant culture of the strain MJ885-mF8

was inoculated into a 500 ml Erlenmeyer flask containing 110 ml of seed medium composed of galactose 2.0%, dextrin 2.0%, Bacto-Soytone (Difco) 1.0%, corn steep liquor 0.5%, glycerol 1.0%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (adjusted to pH 7.4 before sterilization). The flask was shaken on a rotary shaker (180 rpm) at 27°C for 24 hours. Four hundred forty-ml of this seed culture was transferred to 24-liter jar fermanter containing 12 liters of the same medium. The fermentation was continued at 27°C for 54 hours with aeration of 12 liters per minute and agitation of 200 rpm. Each time 90 ml samples of the fermentation broth were drawn. The pH value and production of tetrodecamycin and dihydrotetrodecamycin were measured with each sample. The antibiotics in the broth supernatant were extracted with AcOEt and the concentrations of tetrodecamycin and dihydrotetrodecamycin were measured by reversed phase HPLC (Capell Pak UG 4.6×150 mm, Shiseido) with a solution of CH_3CN-Water (25:75) at the UV absorption of 270 and 254 nm, respectively.

Measurement of Antimicrobial Activity

The minimum inhibitory concentrations (MIC) of tetrodecamycin and dihydro-tetrodecamycin were examined by serial agar dilution method using Mueller-Hinton agar (Difco) for an antibacterial test which was incubated at 37°C for 18 hours and the treble diluted Brain Heart Infusuin agar containing 2% NaCl for *P. piscicida* test which was incubated at 27°C for 18 hours.

Spectroscopic Methods

UV absorption spectra were measured with a Hitachi U-3210 spectrophotometer. IR absorption spectra were obtained with a Hitachi I-5020 FT-IR spectrometer. FAB-MS and HRFAB-MS were obtained on a Jeol JMS-SX102 mass spectrometer. Optical rotations were taken by a Perkin-Elmer 241 polarimeter using a microcell (light path 10 cm). Melting points were determined in a YANACO apparatus, and are uncorrected.

Results

Taxonomic Studies

The producing microorganism, strain MJ885-mF8, was isolated from a soil sample collected in Suginami-ku, Tokyo, Japan. The strain MJ885-mF8 has branched substrate mycelia. The aerial hyphae are straight. The spiral-formation, whirl-formation, sporangia, flagellated spores and fragmentation of substrate mycelia were not observed. Mature spore chains consisted of 30 or more cylindrical spores. The spores were 0.5~0.6×0.9~1.2 mm in size with rugose surfaces. The cultural characteristics of the strain MJ885-mF8 are summarized in Table 1.

The physiological properties and the utilization of carbon sources were summarized in Table 2.

Analysis of the whole-cell hydrolyzate of the strain showed the presence of LL-diaminopimelic acid. On the basis of morphological and chemical these characteristics, the strain MJ885-mF8 was found to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *S. zaomyceticus*[4] and *S. nashvillensis*[5,6] were selected as similar to strain MJ885-mF8. The comparisons of these strains are summarized in Table 3.

The physiological properties of strain MJ885-mF8 were different from *S. zaomyceticus* in the color of aerial mycelium, soluble pigment and the utilization of L-

Table 1. Cultural characteristics of strain MJ885-mF8.

Medium	Aerial mycelium	Growth	Soluble pigment
Sucrose-nitrate agar	Thin, Grayish white [3dc, Natural]	Colorless	None
Glucose-asparagine agar	Thin, White to grayish white [2dc, Natural]	Pale yellow [2ca, Lt Ivory]	None
Glycerol-asparagine agar (ISP No. 5)	Grayish white [3dc, Natural] to light gray [3fe, Silver Gray]	Pale yellow [2ec, Biscuit] to grayish yellow brown [2ie, Lt Mustard Tan]	None
Inorganic salts-starch agar (ISP No. 4)	Light gray [3fe, Silver Gray to 5fe, Ashes]	Pale yellow [2ec, Biscuit] to grsyish yellow brown [2ie, Lt Mustard Tan]	None
Tyrosine agar (ISP No. 7)	Grayish white [3dc, Natural] to light gray [3fe, Silver Gray]	Pale yellow [2ec, Biscuit] to pale yellowish brown [2lg, Mustard Tan]	None
Nutrient agar	Scant, white	Pale yellow [3ec, Bisque]	Brown
Yeast exstrat-malt extract agar (ISP No. 2)	Light gray [3fe, Silver Gray to 5fe, Ashes]	Pale yellow [2ec, Biscuit] to grayish yellow brown [2ie, Lt Mustard Tan]	None
Oatmeal agar (ISP No. 3)	Light gray [2fe, Covert Gray to 3fe, Silver Gray]	Pale yellow [2ec, Biscuit]	None
Glycerol-nitrate agar	Thin, White	Pale yellow [2cb, Ivory Tint]	None
Starch agar	Thin, White	Pale yellow [2ec, Biscuit]	None
Calcium-malate agar	None	Colorless	None

Observation after incubation at 27°C for 21 days.
The color names used in this table were based on the Color Harmony Manual (Container Corporation of America).

arabinose, rhamnose and lactose. It was closely related to *S. nashvillensis* except the utilization of L-arabinose and rhamnose. We examined the comparison of strain MJ885-mF8 with *S. nashvillensis* on the utilization of carbon sources, nitrogen sources and amino acid sources. (Tables 4 and 5)

From the above results, the strain MJ885-mF8 is closely related to *S. nashvillensis* except for the utilization of ammonium acetate, DL-threonine and DL-methionine. Therefore, it was designated as *S. nashvillensis* MJ885-mF8. The strain was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-13863.

Table 2. Physiological properties of strain MJ885-mF8.

Temperature range for growth (°C)	10~37
Optimum temperature (°C)	27~30
Formation of melanoid pigment	+
Hydrolysis of starch	+
Decomposition of cellulose	−
Utilization of	
L-Arabinose	−
D-Xylose	+
D-Glucose	+
D-Fructose	−
Rhamnose	+
Sucrose	−
Raffinose	−
Inositol	−
D-Mannitol	−
Lactose	+

+: Utilized.
−: Not utilized.

Fermentation

A slant culture of the tetrodecamycin-producing organism was inoculated into a 500 ml Erlenmeyer flask containing 110 ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, Bacto-Soytone (Difco) 1.0%, corn steep liquor 0.5%, glycerol 1.0%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (adjusted to pH 7.4 before sterilization). The inoculated medium was incubated at 27°C for 24 hours on a rotary shaker. Two liters of the seed culture were transferred to a 200-liter jar fermenter containing 100 liters of the same medium. The fermentation was carried out at 27°C for 24 hours with aeration of 100 liters per minute and agitation of 200 rpm.

The time course of the production of tetrodecamycin and dihydrotetrodecamycin is shown in Fig. 2. The amount of tetrodecamycin reached maximum at 25 hours after fermentation and began to decrease drastically after 25 hours. On the other hand, dihydrotetrodecamycin was increased after 24 hours. This result was suggested that tetrodecamycin was converted to dihydrotetrodecamycin in the fermentation.

Table 3. Comparison of taxonomic characterization of strain MJ885-mF8 with *Streptomyces zaomyceticus* and *Streptomyces nashvillensis*.

	MJ885-mF8	*S. zaomyceticus* IMC S-0663 (ISP 5196)	*S. nashvillensis* IMC S-0726 (ISP 5314)
Aerial mycelium morphology	Straight	Straight	Straight or hook
Spore surface	Rugose	Rugose Smooth[4]	Rugose Smooth[5]
Color of aerial mycellium	Grayish-white to light gray	White to grayish white	Grayish white to light gray
Color of growth	Pale yellow to grayish yellow brown	Pale yellow	Pale yellow to grayish yellow brown
Soluble pigment	Faint, brown	Faint, yellow	Faint, brown
Formation of melanoid pigment			
ISP1	+	+	+
ISP6	+	+	+
ISP7	±	±	±
Hydrolysis of starch	+	+	+
Utilization of[a]			
L-Arabinose	(−)	+	+
D-Xylose	(+)	±	+
D-Glucose	+	+	+
D-Fructose	−	−	−
Sucrose	−	−	−
Inositol	−	−	−
Rhamnose	(+)	−	−
Raffinose	−	−	−
D-Mannitol	−	−	−
Lactose	+	−	+

[a] +: Utilized, ±: doutful, (+): probably utilized, (−): probably not utilized, −: not utilized.
[4),5)]: reference.

Table 4. Comparison of strain MJ885-mF8 and *Streptomyces nashvillensis* on the utilization of the carbon sources.

	MJ885-mF8	*S. nashvillensis* IMC S-0726 (ISP 5314)
Ribose[a]	(−)~±	±
d-Mannose	+	±
Galactose	+	+
L-(−)-Sorbose	−	−
Cellobiose	+	+
Maltose	+	+
α-d-Melibiose	+	+
Trehalose	−	−
d-(+)-Melezitose	−	−
Inurin	−	−
Dextrin	+	+
Cellulose	−	−
l-(−)-Arabitol	−	−
Adonitol	−	−
Dulcitol	−	−
Glycerol	+	+
d-Sorbitol	−	−
i-Erythritol	−	−
Tartrate	−	−
Ammonium succinate	(+)	(+)
Acetate	(+)	(−)~±
Citrate	(+)	(+)
Formate	−	−
Lactate	−	−
Malate	(+)	(+)
Pyruvate	(+)	(+)
Sodium succinate	(+)	(+)
Esculin	−	−
Salicin	(+)	(+)
Methyl d-glucoside	−	−

[a] +: Utilized, ±: doutful, (+): probably utilized, (−): probably not utilized, −: not utilized.

Table 5. Comparison of strain MJ885-mF8 and *Streptomyces nashvillensis* on the utilization of the nitrogen sources and amino acids.

	MJ885-mF8	*S. nashvillensis* IMC S-0726 (ISP 5314)
Ammonium carbonate[a]	(+)	(±)
Ammonium chloride	+	+
Ammonium phosphate	+	+
Ammonium sulfate	+	+
Sodium nitrite	−	−
Sodium nitrate	+	+
L-(+)-Glutamic acid	+	+
L-Asparatic acid	+	+
DL-Alanine	+	+
L-Arginine	+	+
L-(+)-Cysteine	(+)	(+)
L-(−)-Cystine	(+)	±
Glycine	+	+
L-Histidine	+	+
L-Hydroxyproline	(+)	+
DL-Isoleucine	+	+
L-Leucine	+	+
L-Lysine	+	+
DL-Methionine	+	−
DL-Norleucine	(−)	−
DL-Threonine	(−)	+
DL-Serine	+	+
L-(−)-Tyrosine	+	+
DL-Tryptophane	(+)	±
L-Phenylalanine	+	+
DL-Valine	+	+
Urea	+	+
L-(+)-Asparagine	+	+
Acetamide	−	−

[a] +: Utilized, ±: doutful, (+): probably utilized, (−): probably not utilized, −: not utilized.

Isolation

A diaion HP-20 (7 liters) was added to the fermentation broth (100 liters) with stirring. The mixture was stirred at room temperature for 30 minutes and stored for 15 minutes. The HP-20 resin was collected by filtration and washed with water (200 liters). The antibiotics were eluted with 80% aqueous MeOH from the resin. The eluate (15 liters) was concentrated under reduced pressure to a volume of about 4 liters, which was extracted with AcOEt (4 liters × 2) and the organic layer was dried over Na_2SO_4. The extract was concentrated *in vacuo* obtaining an oily residue. The residue was applied on a silica gel column (Merck, Kiselgel 60, 450 ml). The column was washed with 1.5 liters of toluene, 1.7 liters of toluene-AcOEt (10:1) and 1.3 liters of toluene-AcOEt (7:1), successively. The active components were eluted with toluene-AcOEt (4:1 and 2:1). The fractions which contained active substance (Rf 0.46 on silica gel TLC Merck, Kieselgel $60F_{254}$, $CHCl_3$-MeOH 10:1) were collected and concentrated under reduced pressure

Fig. 2. Production of tetrodecamycin and dihydrotetrodecamycin.

▲ pH, ○ tetrodecamycin, ● dihydrotetrodecamycin.

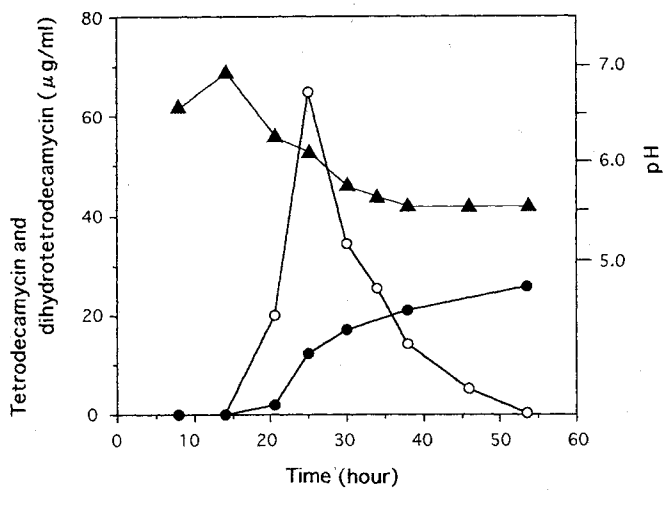


Table 6. Physico-chemical properties of tetrodecamycin (**1**) and dihydrotetrodecamycin (**2**).

	1	**2**
Appearance	Amorphous powder	Colorless prism
Nature	Neutral	Neutral
Molecular formula	$C_{18}H_{22}O_6$	$C_{18}H_{24}O_6$
FAB-MS (*m/z*)	335 $(M+H)^+$	337 $(M+H)^+$
	333 $(M-H)^-$	335 $(M-H)^-$
HRFAB-MS (*m/z*)		
	Calcd for $C_{18}H_{23}O_6$: 335.1459	Calcd for $C_{18}H_{25}O_6$: 337.1651
	Found: 335.1477 $(M+H)^+$	Found: 337.1660 $(M+H)^+$
UV λ_{max} (ε) nm		
in MeOH	271 (12500)	204 (9810), 249 (10400)
in 0.01 N NaOH-MeOH	252 (8230)	249 (10400)
in 0.01 N HCl-MeOH	270 (12600)	207 (8240), 249 (10500)
IR ν_{max} (KBr) cm^{-1}	3460, 2930, 1780, 1670, 1590, 1440, 1280	3530, 2950, 1760, 1650, 1610, 1595, 1440
$[\alpha]_D^{23}$	$-6°$ (*c* 0.5, MeOH)	$+78°$ (*c* 0.52, MeOH)
Rf	0.47[a]	0.40[a]
	0.66[b]	0.49[b]
m.p.	87~94°C	200~203°C (dec.)

[a] Silica gel TLC (Merck Art. No. 5715) chroloform-methanol (10:1).
[b] Silica gel TLC (Merck Art. No. 5715) toluene-acetone (1:1).

Table 7. Antimicrobial activities of tetrodecamycin (**1**) and dihydrotetrodecamycin (**2**).

Test organism	MIC (μg/ml)	
	1	**2**
Staphylococcus aureus FDA209P	6.25	>100
S. aureus Smith	12.5	>100
S. aureus MS9610	12.5	>100
S. aureus No. 5 (MRSA)	12.5	>100
S. aureus No. 17 (MRSA)	12.5	>100
Micrococcus luteus FDA 16	12.5	>100
M. luteus IFO 3333	12.5	>100
M. luteus PCI 1001	12.5	>100
Bacillus anthracis	6.25	100
B. subtilis NRRL B-558	12.5	>100
B. subtilis PCI 219	12.5	>100
B. cereus ATCC 10702	6.25	>100
Corynebacterium bovis 1810	25	>100
Escherichia coli NIHJ	50	>100
E. coli K-12	>100	>100
E. coli K-12 MLI629	>100	>100
E. coli BEM11	>100	>100
E. coli BE1121	>100	>100
E. coli BE1186	100	>100
Shigella dysenteriae JS11910	50	>100
S. flexneri 4b JS11811	100	>100
S. sonnei JS11746	>100	>100
Salmonella typhi T-63	>100	>100
S. enteritidis 1891	>100	>100
Proteus vulgaris OX19	>100	>100
P. mirabilis IFM OM-9	25	>100
Providencia rettgeri GN311	>100	>100
P. rettgeri GN466	>100	>100
Serratia marcescens	>100	>100
Pseudomonas aeruginosa A3	>50	100
P. aeruginosa GN315	>100	>100
Klebsiella pneumoniae PCI602	100	>100

Table 8. Antimicrobial activities against twelve strains of *P. piscicida* of tetrodecamycin (**1**) and dihydrotetrodecamycin (**2**).

Test organism	MIC (mg/ml)	
	1	**2**
Pasteurella piscicida sp. 6395	1.56	50
P. piscicida sp. 6356	1.56	50
P. piscicida p-3340	3.12	
P. piscicida p-3343	3.12	
P. piscicida p-3344	3.12	
P. piscicida p-3346	3.12	
P. piscicida p-3347	3.12	
P. piscicida p-3348	3.12	
P. piscicida p-3349	3.12	
P. piscicida p-3350	1.56	
P. piscicida p-3353	6.25	
P. piscicida p-3354	3.12	

to give an amorphous powder (3.3 g) of pure tetrodecamycin. The fractions which eluted toluene-AcOEt (1:1) were collected and concentrated *in vacuo* to provide a crude powder (0.87 g) of dihydrotetrodecamycin. The pure dihydrotetrodecamycin was crystallized from AcOEt as colorless prism (0.32 g).

Physico-chemical Properties

Physico-chemical properties of tetrodecamycin and dihydrotetrodecamycin are summarized in Table 6. The antibiotics were soluble in MeOH, AcOEt, acetone, acetonitrile, CH_2Cl_2 and insoluble in hexane and water. Tetrodecamycin and dihydrotetrodecamycin gave positive color reactions to iodine and molybdophosphoric acid-sulfuric acid reagent, negative to $FeCl_3$, ninhydrin and Rydon-Smith reagents. The molecular formula of tetrodecamycin and dihydrotetrodecamycin were established as $C_{18}H_{22}O_6$ and $C_{18}H_{24}O_6$ by HRFAB-MS, respectively. The UV spectra of tetrodecamycin and dihydrotetrodecamycin showed the characteristic ab-

sorption maximum at 270 and 249 nm in MeOH, respectively.

Biological Activities

Antimicrobial activities of tetrodecamycin and dihydrotetrodecamycin are shown in Tables 7 and 8. The activities of dihydrotetrodecamycin were weak against Gram-positive bacteria and *P. piscicioda*. Tetrodecamycin showed potent inhibitory activity against Gram-positive bacteria and 12 strains of *P. piscicida*. Tetrodecamycin and dihydrotetrodecamycin did not show any toxicity in mice at a dose of 100 mg/kg when administered intraperitoneally, respectively.

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