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Diperamycin, a New Antimicrobial Antibiotic Produced by *Streptomyces griseoaurantiacus* MK393-AF2

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

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Antibacterial antibiotics, diperamycin (**1**) was produced in the culture broth of *Streptomyces griseoaurantiacus* MK393-AF2. Various spectroscopic analyses of **1** suggested that **1** belonged to a member of cyclic hexadepsipeptide antibiotic.

Antibiotic **1** had potent inhibitory activity against various Gram-positive bacteria including *Enterococcus seriolicida* and methicillin-resistant *Staphylococcus aureus*.

During the course of our screening for antibiotics from microbial metabolites, a new antibiotic diperamycin (**1**) was obtained from the fermentation broth of *Streptomyces* sp. MK393-AF2. This strain was isolated from a soil sample collected in Yokohama City, Kanagawa prefecture, Japan and was classified to *Streptomyces griseoaurantiacus*. The NMR and MS spectroscpic analyses suggested that **1** belonged to a member of cyclic hexadepsipeptide antibiotic. Antibiotic **1** had potent inhibitory activities against various Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) (MIC 0.1～0.39 μg/ml) and exhibited strong growth inhibition activities on various tumor cell lines.

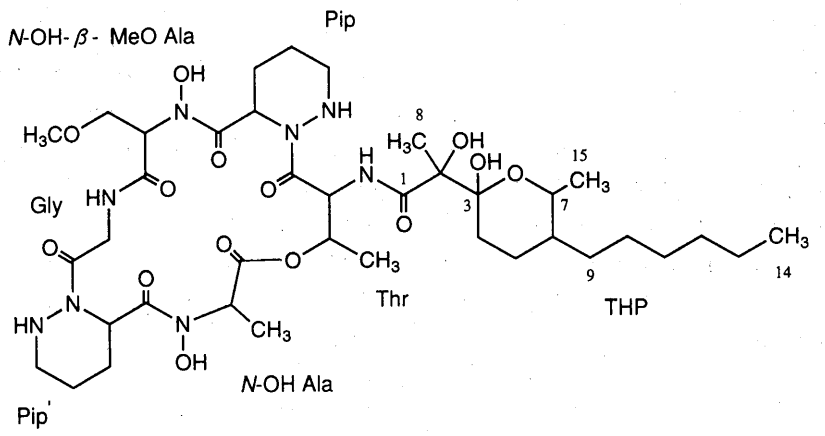
In this paper, we report on the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and biological activities of **1**.

Materials and Methods

Microorganisms

Streptomyces griseoaurantiacus[1] IMC S-0709[T] (ISP 5430) was compared taxonomically with strain MK393-AF2.

Fig. 1. Structure of diperamycin.



Taxonomic Studies

Culture and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB[2] and by the methods of WAKSMAN[3]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[4]. The substrate and aerial mass color including soluble pigment were assigned by Color Harmony Manual, 1958[5] (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 hydrolysate of the culture growth according to the method of STANECK and ROBERTS[6]. Menaquinone was analyzed by the methods of TAMAOKA *et al.*[7].

Spectroscopic Methods

^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-A500 and a JEOL JNM-EX400 spectrometers. Chemical sifts are given in ppm from TMS as an internal standard. FAB-MS and HRFAB-MS were obtained on a JEOL JMS-SX102 mass spectrometer. The UV absorption spectrum was measured with a Hitachi U-3210 spectrophotometer. The IR spectrum was obtained with a Hitachi I-5020 FT-IR spectrometer. Optical rotation was taken by a Perkin-Elmer 241 polarimeter using a micro-cell (light path 10 cm). The melting point was determined with a Yanaco MP-S3 micro melting point apparatus which were uncorrected.

Measurement of Antimicrobial Activity

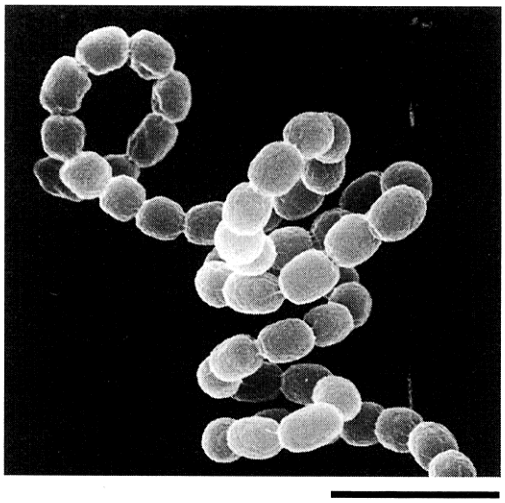
The minimum inhibitory concentrations (MIC) of **1** were examined by serial agar dilution method using Mueller-Hinton agar (Difco) for antibacterial test which was incubated at 37°C for 18 hours, a nutrient agar containing 1% glycerol for mycobacteria test which was incubated at 37°C for 42 hours, and brain heart infusion agar containing 2% sodium chloride for *Pasteurella piscicida* and *Enterococcus seriolicida* tests which was incubated at 27°C for 18 hours.

Anititumor Activity

Tumor cells were incubated in 96-well plate for 24 hours prior to the addition of diperamycin 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[8] and IC_{50} was calculated.

Fig. 2. Scanning electron micrograph of spore chains of *Streptomyces* sp. MK393-AF2.

Bar represents 2.50 μm.



Results and Discussion

Taxonomy of the Producing Strain

The strain MK393-AF2 formed well-branched substrate long mycelia and aerial hyphae bore spirals. Mature spore chains consisted of 10 to 50 or more oval spores. The spores were $0.5\sim0.6\times0.7\sim0.9\,\mu$m in size with smooth surface. No whirl-formation, sporangia, motile spores or synnemata were observed (Fig. 2).

The cultural characteristics of the strain MK393-AF2 on various agar media are summarized in Table 1. The vegetative growth color was dull yellow orange to light brown or pink on various media tested. The aerial mycelium color was light brownish gray to light gray.

The physiological characteristics and carbohydrate utilization of strain MK393-AF2 were shown in Table 2. Optimum temperature for growth was 27~30°C. Formation of melanoid pigment was negative and hydrolysis of starch was positive. Liquefaction of gelatin and coagulation of milk were both negative.

Analysis of whole-cell hydrolysate of the strain showed the presence of LL-diaminopimelic acid. The predominant menaquinone was MK-9(H_8) and MK-9(H_6). Based on these characteristics, strain MK393-AF2 was found to belong to the genus *Streptomyces*. For comparing the strain MK393-AF2, *S. griseoaurantiacus* was selected as representative strain of known species of *Streptomyces*. The comparison of taxonomical characteristics between strain MK393-AF2 and *S. griseoaurantiacus* is shown

Table 1. Cultural characteristics of strain MK393-AF2.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Colorless~yellow [1ic, Citron Yellow]	Light brownish gray [3ig, Beige Brown]	Faint, yellowish
Yeast extract-malt extract agar (ISP No.2)	Dull yellow orange [3ne, Topaz]~ dark yellow orange [4ne, Luggage Tan]	Light gray [3fe, Silver Gray]	Faint, brownish
Oatmeal agar (ISP No.3)	Dull yellow orange [3ne, Topaz]	Light gray [3fe, Silver Gray]	Yellowish
Inorganic salts-starch agar (ISP No.4)	Pink [6ic, Coral Rose~7ic, Colonial Rose]	Light gray [2fe, Covert Gray]	Shade of pale pink
Glycerol-asparagine agar (ISP No.5)	Light brown [4ng, Lt Brown]	Brownish white [3cb, Sand]~ light gray [2fe, Covert Gray]	Shade of pale orange
Tyrosine agar (ISP No.7)	Pale yellowish brown [2le, Mustard]~ yellowish brown [3ng, Yellow Maple]	Light gray [2fe, Covert Gray]	Shade of yellowish brown

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

Table 2. Physiological characteristics of strain MK393-AF2.

Temperature range for growth (℃)	20~37
Optimum temperature (℃)	27~30
Formation of melanoid pigment	
ISP No.1	Negative
ISP No.6	Negative
ISP No.7	Negative
Liquefaction of	
gelatin	Negative
glucose peptone gelatin	Negative
Coagulation of milk	Negative
Peptonization of milk	Negative
Hydrolysis of starch	Positive
Reduction of nitrate	Variable
Utilization of*	
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	d
Inositol	d
Rhamnose	+
Raffinose	-
D-Mannitol	+

* +, utilization; −, no utilization; d, doubtful utilization

in Table 3. As a result, strain MK393-AF2 is closely related to *S. griseoaurantiacus*.

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-16079.

Fermentation

A slant culture of the diperamycin-producing organism was inoculated into a 500 ml Erlenmeyer flask containing 110 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 27°C for 48 hours on a rotary shaker (220 rpm). This seed culture was transferred into a 30-liter jar fermenter containing 12 liters of 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 1.5 ml of silicon oil (adjusted to pH 7.4 before sterilization). Fermentation was carried out at 27°C for 7 days with 12 liters/minute aeration and agitation at 200 rpm.

Isolation and Purification

The isolation procedure of **1** is outlined in Fig. 3. The fermentation broth was centrifuged, and the mycelial cake was extracted with acetone (3 liters × 2) and concentrated under reduced pressure until acetone was removed. The resultant aqueous solution (2.5 liters) was

Table 3. Taxonomical characteristic comparison of strain MK393-AF2 and *Streptomyces griseoaurantiacus*.

	Strain MK393-AF2	*S. griseoaurantiacus* IMC S-0709^T(ISP 5430)
Spore chain	Spiral	Spiral
Spore surface	Smooth	Smooth
Aerial mycelium	Light gray	Grayish white~light gray
Growth	Dull yellow orange~light brown, pink	Pale yellow~pink
Soluble pigment	Yellowish~brownish, pinkish	Yellowish~brownish, pinkish
Formation of melanoid pigment	Negative	Negative
Liquefaction of gelatine	Negative	Negative
Coagulation of milk	Negative	Weakly positive
Peptonization of milk	Negative	Negative
Hydrolysis of starch	Positive	Positive
Reduction of nitrate	Variable	Positive
Utilization of*		
L-Arabinose	+	+
D-Xylose	+	+
D-Glucose	+	+
D-Fructose	+	+
Sucrose	d	-
Inositol	d	(+)
Rhamnose	+	+
Raffinose	-	-
D-Mannitol	+	+

* +, positive utilization; −, no utilization; (+), probably utilization; d, doubtful utilization

extracted with 2.5 liters of ethyl acetate. The organic phase layer was dried over anhydrous sodium sulfate, then concentrated *in vacuo* to dryness. The residue was chromatographed on a silica gel column (Merck Kieselgel 60, 25 g) using chloroform-methanol (50:1) as eluant. The fractions active against *Staphylococcus aureus* Smith were collected and concentrated under reduced pressure to afford pale red syrup (1.32 g). This crude material was subjected to a Sephadex LH-20 column and eluted with methanol. The fractions indicating Rf value of 0.41 on silica gel TLC (Merck Kieselgel $60F_{254}$, chloroform-methanol, 10:1) were collected and concentrated *in vacuo* to afford pale red syrup (450 mg), which was further purified by preparative HPLC (mobile phase: 65% aq. CH_3CN, flow rate: 10 ml/minute) using an ODS column (SHISEIDO, CAPCELL PAK UG120Å 20 mm i.d. × 250 mm) to give pure **1** (100.9 mg) as a white powder.

Physico-chemical Properties

The physico-chemical properties of **1** are summarized in Table 4. Antibiotic **1** was soluble in methanol, acetone, chloroform and ethyl acetate, and insoluble in water, toluene and *n*-hexane. The molecular formula of **1** was established as being $C_{38}H_{64}N_8O_{14}$ on the basis of HRFAB-MS and ^{13}C NMR spectroscopic information. Antibiotic **1** showed only an end absorption in the UV spectrum. The IR spectrum of **1** showed characteristic absorption bands of the hydroxy and/or amino group at 3409 cm^{-1}, the amide carbonyl group at 1643 cm^{-1} and the other carbonyl group at 1749 cm^{-1}. Antibiotic **1** showed positive color reactions to Rydon-Smith and $FeCl_3$. These results suggested that **1** belonged to a member of the cyclic hexadepsipeptide antibiotics. And furthermore, the ^1H and ^{13}C NMR spectral pattern of **1** were similar to those of antibiotic L-156,602[9], IC101[10], azinothricin[11], GE3[12], and so on. The ^1H and ^{13}C NMR

Fig. 3. Isolation procedure of diperamycin.

Fermentation broth 10 L
↓ centrifuged at 4000 rpm
Mycelial cake
↓ extracted with acetone 3 L x 2
concentrated under reduced pressure
Aqueous solution 2.5 L
↓ extracted with ethyl acetate 2.5 L
Ethyl acetate extract
↓ concentrated under reduced pressure
Brown residue
↓ silica gel column chromatography
eluted with chloroform-methanol (50 : 1)
Pale red syrup 1.32 g
↓ Sephadex LH-20 column
eluted with methanol
Pale red syrup 450 mg
↓ prep. HPLC (CAPCELL PAK C_{18})
eluted with 65 % aq. acetonitrile
Diperamycin (100.9 mg)

data are shown in Table 5. Details of structural study will be reported in another paper.

Biological Activities

As shown in Table 6, the antimicrobial activity of **1** against various Gram-positive bacteria including methi-

Table 4. Physico-chemical properties of diperamycin.

Appearance	White powder
Nature	Neutral
Molecular formula	$C_{38}H_{64}N_8O_{14}$
FAB-MS (*m/z*)	$(M + Na)^+$ 879 $(M - H)^-$ 855
HRFAB-MS (*m/z*)	Calcd : 879.4474 (as $C_{38}H_{64}N_8O_{14}Na$) Found : 879.4440 $(M - H)^-$
UV λ max(ε) in MeOH	203 (34220)
$[\alpha]_D^{20}$ (*c* 1.0, MeOH)	+ 25.7 °
IR ν max (KBr) cm^{-1}	3409, 2933, 1749, 1643, 1523, 1444, 1414, 1252, 1120, 1092
Rf value [a]	0.41
mp (℃)	152～154 (dec.)

[a] Silica gel TLC
(Merck Art. 1.05715, $CHCl_3$: MeOH = 10 : 1)

Table 5. ^1H and ^{13}C NMR data of diperamycin in $CDCl_3$.

		^{13}C (ppm)[a]	^1H (ppm)[b]	*J* (Hz)
Thr:	NH		7.37 br d	9.4
	Cα	48.5 d	5.87 br d	9.4
	Cβ	71.8 d	4.96 m	
	Cγ	15.9 q	1.33 d	6.0
	C=O	170.8 s		
Gly:	NH		7.01 br s	
	Cα	41.86 t	3.92 dd	3.8, 18.3
			4.72 dd	3.8, 18.3
	C=O	172.6 s		
Pip:	NH		4.79 br d	12.4
	Cα	49.2 d	5.43 dd	1.9, 6.5
	Cβ	24.5 t	1.94 m	
			2.21 br d	13.8
	Cγ	21.1 t	1.62 m (2H)	
	Cδ	46.84	2.75 m, 3.16 m	
	C=O	172.9 s		
Pip':	NH		4.91 br d	12.5
	Cα	48.8 d	5.36 m	
	Cβ	23.6 t	2.01 m	
			2.12 br d	13.5
	Cγ	20.5 t	1.62 m (2H)	
	Cδ	46.9 t	2.75 m	
			3.16 br t	12.6
	C=O	172.3 s		
N -OH Ala:	Cα	53.1 d	5.36 m	
	Cβ	12.6 q	1.39 d	6.6
	C=O	169.1 s		
N -OH-MeO Ala:	Cα	57.2 d	5.20 dd	5.4, 7.7
	Cβ	67.8 t	3.86 dd	10.4, 5.4
			3.98 dd	10.4, 7.7
	CH_3O	59.1 q	3.39 s	
	C=O	168.3 s		
THP:				
	1	176 s		
	2	76.9 s		
	3	99.2 s		
	4	27.3 t	1.69 br d	10.4
			1.78 t	12.0, 3.0
	5	24.1 t	1.47 m	
			1.69 br d	
	6	41.93	1.01 m	
	7	71.2 d	3.76 m	
	8	21.6 q	1.56 s	
	9	26.2 t	1.18 m, 1.33 m	
	10	31.7 t	1.01 m, 1.33 m	
	11[c]	29.8 t	1.23 m (2H)	
	12[c]	31.8 t	1.20 m (2H)	
	13[c]	22.7 t	1.28 m (2H)	
	14	14.1 q	0.88 t	7.0
	15	19.5 q	1.06 d	7.0
	6-OH		6.10 br s	
	7-OH		6.18 br	

[a] Measured at 125 MHz, [b] Measured at 500 MHz, [c] These carbons were deduced from empirical data.

Table 6. Antimicrobial activity of diperamycin.

Test organism	MIC (μ g/ml)
Staphylococcus aureus FDA209P	0.10
S. aureus Smith	0.10
S. aureus MS9610	0.20
S. aureus MRSA No. 5	0.10
S. aureus MRSA No. 17	0.10
S. aureus MRSA 16526	0.10
S. aureus MRSA 04282	0.20
Micrococcus luteus FDA16	0.10
M. luteus IFO3333	0.10
M. luteus PCI1001	0.10
Bacillus anthracis	0.10
B. subtilis NRRL B-558	0.39
B. subtilis PCI219	0.20
B. cereus ATCC 10702	0.39
Corynebacterium bovis 1810	0.20
Enterococcus seriolicida 4038	0.20
Escherichia coli NIHJ	>100
E. coli BE1121	>100
E. coli BE1186	>100
Shigella dysenteriae JS11910	>100
Pseudomonas aeruginosa A3	>100
Mycobacterium smegmatis ATCC 607	12.5
Candida albicans 3147	12.5

Table 7. Growth inhibition of cultured cell lines by diperamycin.

Cell line	Origin	IC_{50} (μ g/ml)
L1210	Leukemia	0.018
P388	Leukemia	0.014
EL-4	Leukemia	0.013
HL-60	Leukemia	0.069
K562	Leukemia	0.085
LS180	Colon cancer	0.098
KB	Nasopharynx cancer	0.015
HeLaS3	Uterus cancer	0.009
FM3A	Carcinoma	0.018
Meth A	Fibrosarcoma	0.018
B16-BL6	melanoma	0.023

cillin-resistant *Staphylococcus aureus* and *Enterococcus seriolicida* were potent, while **1** exhibited no antimicrobial activity against Gram-negative bacteria and *Pasteurella piscicida* even at the concentration of 100 μg/ml. Antibiotic **1** showed moderate inhibitory activity against *Mycobacterium smegmatis* and *Candida albicans*.

The antitumor activity of **1** against various tumor cell lines *in vitro* are listed in Table 7. Antibiotic **1** exhibited strong growth inhibitory effect on tumor cell lines tested. As for the acute toxicity, 9.4 mg/kg dose of **1** caused death in female ICR mice when administrated intravenously.

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