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Cedarmycins A and B, New Antimicrobial Antibiotics from *Streptomyces* sp. TP-A0456

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Two novel butyrolactone antibiotics, cedarmycins A and B, were isolated from the cultured broth of the actinomycete *Streptomyces* sp. TP-A0456. The new compounds were purified by HP-20 resin, silica gel, ODS column chromatographies and preparative HPLC, consecutively. The structure of cedarmycin was determined by spectroscopic methods as an α,β-unsaturated butyrolactone with a fatty acid side chain. These compounds showed antibiotic activity against Gram-positive and -negative bacteria and yeasts. Among the tested organisms, cedarmycins potently inhibited the growth of *Candida glabrata* IFO 0622 with the MIC of 0.4 μg/ml, comparable to that of amphotericin B.

In the course of screening for new bioactive compounds from plant-associated actinomycetes, new antibiotics cedarmycins A (**1**) and B (**2**) (Fig. 1) were found to be produced in the fermentation broth of an actinomycete strain TP-A0456. The producing strain was isolated from the stem of *Cryptomeria japonica* and identified as *Streptomyces* sp. on the basis of taxonomic studies. Spectroscopic analyses revealed that these compounds belonged to the butyrolactone class of secondary metabolites from *Streptomyces*.

We herein report on the taxonomy and fermentation of the cedarmycin-producing strain, physico-chemical properties, structure elucidation and biological properties of cedarmycins A and B.

Materials and Methods

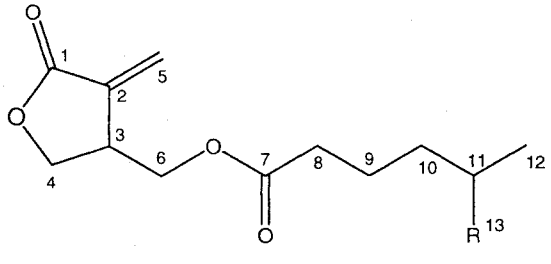
Spectral Analysis

NMR experiments were performed on a JEOL JNM-LA400 NMR spectrometer in $CDCl_3$ at 24°C. Chemical shifts are given in ppm using TMS as an internal standard. The MS spectra were measured on a JEOL JMS-HX110A spectrometer. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were recorded on a Shimadzu FT IR-300 spectrophotometer. Optical rotations were measured on a Horiba SEPA-300 polarimeter.

Producing Strain

The producing microorganism, strain TP-A0456, was isolated from the wild plant of *Cryptomeria japonica* collected in Kosugi-machi, Toyama, Japan. The stem of the

Fig. 1. Structure of cedarmycins A (**1**) and B (**2**).



Cedarmycin A (**1**) R = CH_3
B (**2**) R = H

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plant was cut into pieces of *ca.* 4 cm in length. They were successively immersed in 70% ethanol and 1% NaClO solution for 3 minutes, respectively. Then, they were rinsed with sterilized water and incubated on an agar plate consisting of agar 1.5%, amphotericin B 0.005% and methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate 0.02% at 32°C for 30 days. A colony of TP-A0456 that grew out of a piece of the plant was isolated and purified on an agar plate consisting of glucose 0.5%, soluble starch 0.5%, meat extract (Kyokuto) 0.1%, yeast extract (Difco Laboratories) 0.1%, NZ-case (Humco Scheffield Chemical Co.) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.5%. A pure culture of strain TP-A0456 was preserved in 20% glycerol at −80°C. It was also maintained at 10°C for laboratory use as a slant on Bennett's agar.

Taxonomy

Taxonomic characteristics of strain TP-A0456 were determined by cultivation on various media described by SHIRLING and GOTTLIEB[1], WAKSMAN[2] and ARAI[3]. Morphological characteristics were observed after incubation of the culture at 30°C for 14 days on oatmeal agar (ISP med. 3). Cultural and physiological characteristics were determined after growth at 30°C for 14 days. The color names and hue numbers were assigned using the Manual of Color Names (Japan Color Enterprises Co., Ltd., 1987). The carbon utilization was determined by the method of SHIRLING and GOTTLIEB[1]. Cell wall composition was analyzed by the method of LECHEVALIER and LECHEVALIER[4], using thin layer chromatography plates as described by STANECK *et al.*[5].

Biological Assay

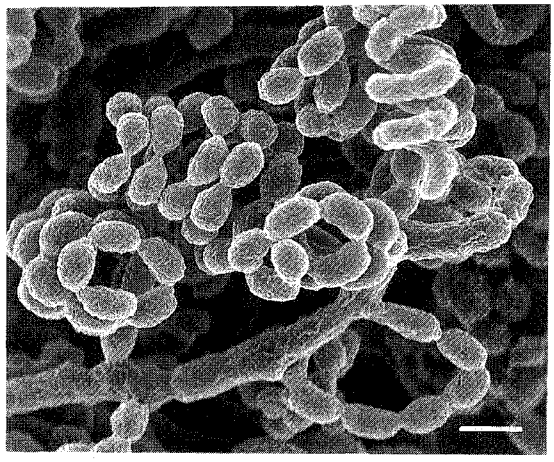
Antibiotic acitivity in fermentation broths and purification samples were monitored by the conventional paper disc assay using *Saccharomyces cerevisiae* S-100 as the test microorganism. MIC values were determined by the two-fold serial agar dilution method on Sabouraud's agar medium against laboratory strains.

Results and Discussion

Taxonomic Studies of the Producing Strain

By scanning electron microscope, the strain TP-A0456 formed spiral type spore chain and each had more than ten spores per chain. The spores were cylindrical, 0.5×0.9 μm in size, and had smooth surface (Fig. 2). The cultural characteristics of TP-A0456 are summarized in Table 1.

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. TP-A0456.



Bar represents 1.00 μm.

Table 1. Cultural characteristics of strain TP-A0456.

Medium	Growth	Aerial mycelium	Reverse side	Diffusible pigment
Yeast extract - malt extract agar (ISP med. 2)	good	White (388)	Brownish black (428)	None
Oatmeal agar (ISP med. 3)	good	Brownish black (428)	Beige gray (401)	None
Inorganic salts - starch agar (ISP med. 4)	good	Olive gray (410)	Medium gray (406)	None
Glycerol asparagine agar (ISP med. 5)	poor	White (389)	Yellowish white (393)	None
Tyrosine agar (ISP med. 7)	moderate	White (389)	Pale beige (84)	None

The aerial mass color showed brownish black and color of reverse side showed beige gray. Diffusible pigments were not formed. The strain TP-A0456 utilized sucrose, D-xylose, D-glucose, inositol, D-mannitol, D-fructose, L-rhamnose and raffinose for growth. Analysis of the whole-cell hydrolysates showed the presence of L,L-diaminopimelic acid, galactose, glucose and mannose. On the basis of these morphological and chemical characteristics, strain TP-A0456 was assigned to the genus *Streptomyces*.

Fermentation

Streptomyces sp. TP-A0456 cultured on a slant agar medium was inoculated into five 500-ml K-1 flasks each containing 100 ml of the seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case (Humco Scheffield Chemical Co.) 0.3%, yeast extract (Difco Laboratories) 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The inoculated flasks were cultivated on a rotary shaker (200 rpm) at 30°C for 4 days. Three-ml of the seed culture was transferred into a hundred 500-ml K-1 flasks each containing 100 ml of the production medium consisting of glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia (Traders Protein) 1.5%, yeast extract (Difco Laboratories) 0.3% and Diaion HP-20 (Mitsubishi Chemical Co.) 1%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were cultured on a rotary shaker (200 rpm) at 30°C for 6 days.

Isolation

A six-day old fermentation broth of *Streptomyces* sp. TP-A0456 (10 liters) was added to acetone (10 liters) and stirred for 2 hours. This mixture was separated into filtrate and mycelia by centrifugation (8,000 rpm, 15 minutes). The mycelia were extracted with acetone (5 liters) and filtered on Celite. The filtrates were combined and concentrated *in vacuo* to an aqueous solution (10 liters). The pH of the aqueous solution was adjusted to 7.0 with 0.1 N NaOH and applied to a Diaion HP-20 column (1 liter). The column was washed with water (2 liters) and eluted with 10% methanol (2 liters), 80% methanol (3 liters) and acetone (2 liters). The fraction eluted with 80% methanol, in which the activity was found, was concentrated *in vacuo* and the pH of the aqueous solution was adjusted to 8.0 with 0.1 N NaOH. The aqueous solution was extracted twice with *n*-butanol (400 ml). The organic layer was concentrated *in vacuo* to give a dark brown oil (4.68 g) and the oily substance was applied to a column of silica gel (Wakogel C-200, 200 g) with the eluent of $CHCl_3$-MeOH (100 : 1~5 : 1). The active fractions were combined and concentrated *in vacuo* to give a brown oil (170 mg). This crude material was applied to a column of silica gel (Wakogel C-200, 8 g) with the eluent of ethyl acetate-*n*-hexane (10 : 1~1 : 1). The active fractions were combined and concentrated *in vacuo* to give a mixture of cedarmycins A and B (55 mg). The mixture was purified by reversed phase HPLC using Cosmosil 5C18-AR-II (Nacalai Tesque Inc., 20×250 mm) column with the eluent of acetonitrile-0.15% KH_2PO_4, pH 3.5 (70 : 30) at a flow rate of 8.0 ml/minute. The active fractions containing cedarmycins A and B were combined separately and concentrated *in vacuo*. The pH of the resultant aqueous solutions were adjusted to 8.0 and extracted with ethyl acetate (20 ml). These organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give cedarmycins A (18.4 mg) and B (6.3 mg) as a colorless oil, respectively.

Physico-chemical Properties and Structure

Physico-chemical properties of cedarmycins A (**1**) and B (**2**) are summarized in Table 2. These compounds were obtained as colorless oil soluble in most organic solvents except for *n*-hexane. The $[\alpha]_D$ values in $CHCl_3$ of **1** and **2** were +29.2 (*c* 1.00) and +11.7 (*c* 0.30), respectively. The IR spectra suggested the presence of two carbonyl groups (1720 and 1740 cm^{-1}).

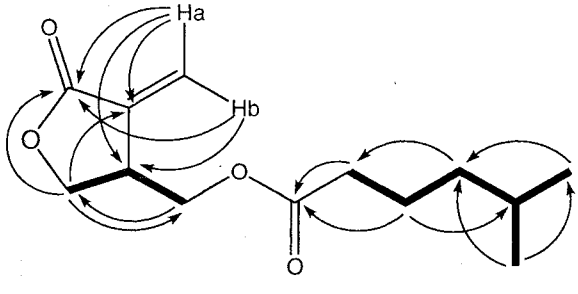
The structure determination of **1** and **2** was carried out by NMR spectroscopic analyses. In the ^{13}C-NMR spectrum of **1**, twelve carbon signals were observed. The DEPT and HMQC experiments confirmed the presence of two methyl groups, six sp^3 and one *exo*-methylenes, one methine and two quaternary sp^2 carbons. Taking this result into account, the molecular formula of **1** was determined to be $C_{13}H_{20}O_4$ on the basis of the high resolution FAB-MS (*m/z* 241.1439 $[M+H]^+$, Δ−0.1 mmu). The DQF-COSY and proton-decoupling experiments revealed two spin systems H-4/H-3/H-6 and H-8/H-9/H-10/H-11/H-12/H-13 as shown in Fig. 3. Chemical shifts of C-4 (68.1 ppm) and C-6 (64.7 ppm) indicated the substitution of oxygen atoms at their positions. HMBC correlations were detected from the *exo*-methylene protons to a quaternary sp^2 carbon at 134.6 ppm, carbonyl carbon at 169.8 ppm and C-3. The long range couplings were also observed from H-4 to the sp^2 and carbonyl carbons. Thus, the presence of α,β-unsaturated butyrolactone moiety was established. Long range couplings from H-8 and H-9 to another carbonyl carbon at 173.5 ppm indicated the presence of 5-methylcaproyl side

Table 2. Physico-chemical properties of cedarmycins A (**1**) and B (**2**).

	1	**2**
Appearance	Colorless oil	Colorless oil
$[\alpha]_D^{28}$	+29.2 (*c* 1.00, $CHCl_3$)	+11.7 (*c* 0.30, $CHCl_3$)
HRFAB-MS		
Found:	241.1439 $[M+H]^+$	227.1283 $[M+H]^+$
Calcd:	241.1440 (for $C_{13}H_{21}O_4$)	227.1283 (for $C_{12}H_{19}O_4$)
Molecular formula	$C_{13}H_{20}O_4$	$C_{12}H_{18}O_4$
UV λ_{max}^{MeOH} nm (log ε)	212 (4.06)	214 (3.72)
IR ν_{max} (KBr) cm^{-1}	1740, 1770	1740, 1770
TLC (Rf)[a]	0.41	0.41
HPLC (Rt)[b]	7.6 min	6.6 min

[a] Silica gel TLC (Merck Art 5715): (*n*-hexane:AcOEt=2:1).
[b] HPLC conditions: Cosmosil AR-II (250 x 4.6 mm, i.d.), Mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (70:30), Flow rate: 0.7 ml/min, Detection: UV-210 nm.

Fig. 3. NMR analysis of cedarmycin A.



: ^1H-^1H homodecouplings
: ^1H-^{13}C long range couplings

chain. Although the HMBC correlation from H-6 to the carbon at 173.5 ppm was not detected, the structure of **1** was determined in consideration of the molecular formula as shown in Fig. 1. The two protons of the *exo*-methylene were assigned based on the consideration of the deshielding effect on Ha by the adjacent lactone carbonyl group.

The ^{13}C NMR spectrum of **2** showed eleven carbon signals. In the ^1H NMR spectrum of **2**, two doublet methyls at 0.89 ppm due to H-12 and H-13 in **1** had disappeared and a triplet methyl at 0.90 ppm appeared. The methyl group was identified to be located at terminus of the aliphatic side chain by the ^1H–^1H homodecoupling experiments. NMR spectral patterns around the butyrolactone moiety were almost identical with those of **1** as summarized in Table 3. Thus the structure of **2** was concluded to be a demethyl analog of **1** as shown in Fig. 1. The high resolution FAB-MS gave a $[M+H]^+$ ion at *m/z* 227.1283 which was exactly identical with the theoretical value for $C_{12}H_{19}O_4$. The absolute configuration at C-3 of **1** and **2** was not determined.

Biological Properties

The antimicrobial activities of cedarmycins A (**1**) and B (**2**) are shown in Table 4 in comparison with amphotericin B. **1** showed weak activity against Gram-positive and -negative bacteria and moderate activity against yeasts. **1** exhibited the most potent activity against *Candida glabrata* IFO 0622, the emerging cause of nosocominal bloodstream infection in hospitalized patients[6], with the MIC of 0.40 μg/ml, comparable to that of amphotericin B. The antimicrobial spectrum of **2** was similar to that of **1**. **2** showed very weak activity against Gram-positive and -negative bacteria but potent activity against *C. glabrata* with the IC_{50} of 1.60 μg/ml.

The cytotoxicity of **1** and **2** was tested against several tumor cell lines (data not shown). Both compounds showed weak activity with the IC_{50} of 10~100 μM and the apoptotic cell death was observed in U937 cells at 50 μM.

Table 3. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for cedarmycins A (**1**) and B (**2**) in $CDCl_3$.

Position	1		2	
	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
1	169.8		169.9	
2	134.6		134.6	
3	38.1	3.43 (1H, m)	38.1	3.43 (1H, m)
4	68.1	4.19 (1H, dd, *J*=9.4, 5.1 Hz)	68.1	4.19 (1H, dd, *J*=9.4, 5.2 Hz)
		4.48 (1H, dd, *J*=9.6, 8.5 Hz)		4.48 (1H, dd, *J*=9.4, 8.3 Hz)
5	124.1	6.39 (1H, d, *J*=2.7 Hz)*	124.1	6.39 (1H, d, *J*=2.7 Hz)*
		5.76 (1H, d, *J*=2.4 Hz)**		5.76 (1H, d, *J*=2.2 Hz)**
6	64.7	4.18 (1H, dd, *J*=11.2, 7.3 Hz)	64.7	4.18 (1H, dd, *J*=11.2, 7.3 Hz)
		4.25 (1H, dd, *J*=10.9, 5.6 Hz)		4.25 (1H, dd, *J*=11.2, 5.6 Hz)
7	173.5		173.5	
8	34.3	2.31 (2H, t, *J*=7.6 Hz)	34.0	2.34 (2H, t, *J*=7.8 Hz)
9	22.8	1.62 (2H, m)	24.6	1.62 (2H, quint, *J*=7.6 Hz)
10	38.3	1.19 (2H, m)	31.3	1.32 (2H, m)
11	27.7	1.55 (1H, m)	22.3	1.32 (2H, m)
12	22.5	0.89 (3H, d, *J*=6.6 Hz)	13.9	0.90 (3H, t, *J*=7.1 Hz)
13	22.5	0.89 (3H, d, *J*=6.6 Hz)		

*Ha, **Hb

Table 4. *In vitro* antibacterial activities of cedarmycin A (**1**) and B (**2**), and amphotericin B.

Organism	MIC (μg/ml)		
	1	2	amphotericin B
Staphylococcus aureus 209P JC-1	50	>100	>100
Staphylococcus aureus F-507 MRSA	100	>100	>100
Bacillus subtilis ATCC 6633	25.0	100	>100
Escherichia coli RFM 795	100	>100	>100
Escherichia coli NIHJ JC-2	50	100	>100
Proteus vulgaris IFO 3851	>100	>100	>100
Proteus mirabilis ATCC 21100	>100	>100	>100
Pseudomonas aeruginosa A3	>100	>100	>100
Saccharomyces cerevisiae S-100	12.5	25.0	0.40
Candida albicans A9540	50	50	1.60
Candida tropicalis IFO 1400	50	100	1.60
Candida glabrata IFO 0622	0.40	1.60	0.40
Cryptococcus neoformans ATCC 90112	12.5	25.0	0.40
Aspergillus fumigatus IFO 8866	25	50	1.60

Cedarmycins biosynthetically belong to the butyrolactone class of metabolites. Whereas some butyrolactones including A-factor[7] have been found to exert the hormonal response in *Streptomyces*, their antimicrobial activity has not been described so far. The antifungal and cytotoxic activity of cedarmycins may be attributed to the unsaturated lactone structure: α,β-unsaturated carbonyl compounds often serve as the Michael acceptor for the thiol residue of proteins. Though the molecular target is not elucidated yet, the selective and potent activity of cedarmycins against *C. glabrata* with less cytotoxicity than amphotericin B should be noted.

Acknowledgments

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