Cremimycin, a Novel 19-Membered Macrocyclic Lactam Antibiotic, from *Streptomyces* sp.

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(Received for publication November 7, 1997)

A novel 19-membered macrocyclic lactam antibiotic, cremimycin, was isolated from the culture broth of an actinomycete strain. The producing organism, designated MJ635-86F5, was identified as a member of *Streptomyces*. Cremimycin was isolated from the mycelial cake by extraction with $CHCl_3$-MeOH and precipitation with hexane-MeOH. The structure of cremimycin was determined by spectroscopic study.

Cremimycin showed broad antimicrobial activities against Gram-positive bacteria including MRSA.

In the course of our screening program for new antibiotics, we found that a strain of *Streptomyces* sp. MJ635-86F5 produced a new antibiotic, cremimycin (**1**, Fig. 1). Cremimycin showed broad antimicrobial activities against Gram-positive bacteria including MRSA.

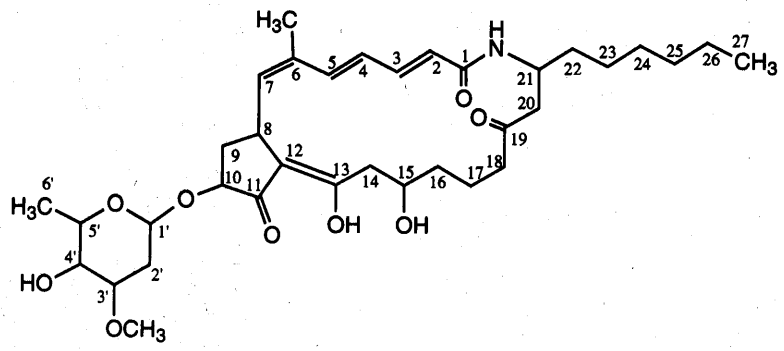
In this paper, we describe the identification of the producing organism together with the isolation, fermentation, physico-chemical properties, structure elucidation and biological activities of cremimycin.

Materials and Methods

General

Optical rotation was measured with a Perkin-Elmer model 241 polarimeter. UV spectra were recorded with a Hitachi 557 spectrophotometer. IR spectrum was recorded with a Horiba FT-210 fourier transform infrared spectrometer. The ^1H and ^{13}C NMR spectra were measured with a JEOL JNM-A500 spectrometer. The mass spectra were recorded with a JEOL JMS-SX102

Fig. 1. Structure of cremimycin (**1**).



(1)

mass spectrometer.

Taxonomy

Cremimycin producing organism, strain MJ635-86F5, was isolated from a soil sample collected at Yokohama, Kanagawa, Japan. Morphological, cultural and physiological properties of the strain MJ635-86F5 were examined according to the methods described by SHIRLING and GOTTLIEB[1], and WAKSMAN[2]. Detailed observation of mycelial morphologies was performed with the use of scanning electron microscope (Model S-570, Hitachi) after the strain MJ635-86F5 was incubated on a sucrose-nitrate agar and inorganic salts-starch agar (ISP No. 4) at 27°C for 10 days. Chemical analyses of cell wall and menaquinones were performed with the methods of STANECK and ROBERTS[3] and TAMAOKA *et al.*[4], respectively.

Fermentation

A slant culture of the cremimycin-producing organism was inoculated into a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of galactose 2%, dextrin 2%, Bacto-soytone (Difco) 1.0%, corn steep liquor (Iwaki Co.) 0.5%, glycerol 1.0%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% in deionized water (pH 7.4 before sterilization). The culture was incubated on a rotary shaker (180 rpm) at 30°C for 3 days. The seed culture (330 ml) of the strain was transferred into a 30-liter jar fermentor containing 15 liters of a producing medium which was consisting of glucose 5%, soy bean meal 1%, polypepton 0.4%, yeast extract 0.1%, meat extract 0.4%, NaCl 0.25% and $CaCO_3$ 0.5% in deionized water (pH 7.4). The fermentation was carried out at 27°C for 9 days with agitation of 200 rpm and aeration of 15 liters/minute.

Analytical Procedure

Content of cremimycin in the fermentation broth and its purification steps were monitored with reversed phase HPLC and silica gel TLC. HPLC was performed with a CAPCELL PAK C_{18} column (4.6 × 150 mm, Shiseido Co., Ltd., Japan; mobile phase, acetonitrile/H_2O/formic acid = 60/40/1; flow rate, 1.0 ml/minute; column temperature, 0°C; detection, UV at 300 nm). It was eluted at 4.6 minutes. TLC was performed with Kieselgel 60 F_{254} (Art. No. 5715, Merck) developed with $CHCl_3$-MeOH-AcOH (90 : 10 : 1). Spot of the antibiotic on a TLC was detected by molybdophosphoric acid-sulfuric acid and UV quenching (254 nm). Rf value of cremimycin was 0.63.

Biological Activity

The minimum inhibitory concentrations (MIC) of cremimycin were examined by serial agar dilution method using Nutrient agar containing 1% glucose for yeast and fungi and Mueller-Hinton agar (Difco) for bacteria. The MIC was observed after an incubation for 42 hours at 27°C against yeast and fungi, and incubations for 18 or 42 hours at 37°C against bacteria, respectively.

The cytotoxicity against murine tumor cell lines was examined by MTT assay method. The tumor cells were incubated in 9-well plate for 24 hours prior to the addition of cremimycin into the culture well at varied concentrations. After 2 days incubated at 37°C, MTT reagent was added and further incubated for 4 hours. Growth inhibition was determined according to the standard MTT assay method[10] and IC_{50} was calculated.

Results and Discussion

Taxonomic Features of Strain MJ635-86F5

Strain MJ635-86F5 produced well-branched vegetative mycelia. This strain formed long aerial hyphae which bore spirals of 2 to 5 turns. Mature spore chain consisted of 10 or more spores. The spore was oval with smooth surface and 0.6~0.8 × 0.5~1.0 μm in size (Photo. 1). No synnemata, sclerotia or sporangia were observed.

The cultural characteristics of strain MJ635-86F5 on various agar media are shown in Table 1. The aerial mycelia were grayish white to light brownish gray. The

Photo. 1. Scanning electoron micrograph of spore chains of *Streptomyces* sp. MJ635-86F5 grown on sucrose-nitrate agar for 10 days at 27°C.

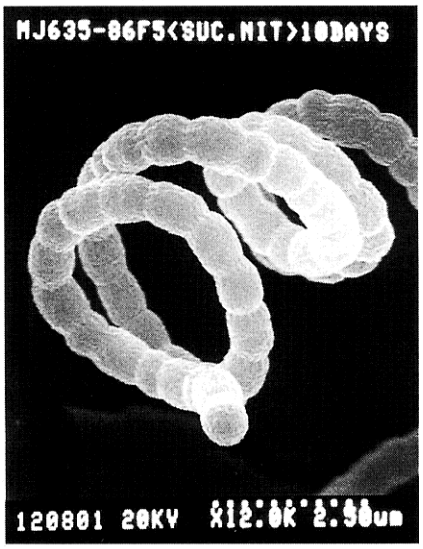


Table 1. Cultural characteristics of strain MJ635-86F5.

Medium	Growth	Aerial mycelium	Soluble pigmemt
Sucrose-nitrate agar	Colorless	Thin, graysh white [2 cb, Ivory Tint]	None
Yeast extract-malt extract agar (ISP No.2)	Pale yellow [2 ea, Lt Wheat ~ 2 gc, Bamboo]	Gray [3 fe, Silver Gray ~ 3 ih, Beige Gray]	None
Oatmeal agar (ISP No.3)	Colorless	Thin, light brownish gray [3 ec, Bisque ~ 3 ig, Beige Brown]	None
Inorganic salts-starch agar (ISP No.4)	Colorless	Tin, brownish white [3 dc, Natural]	None
Glycerol-asparagine agar (ISP No.5)	Poor, colorless	Thin, graysh white [2 cb, Ivory Tint ~ 2 dc, Natural]	None
Tyrosine agar (ISP No.7)	Colorless	Thin, brownish white [3 dc, Natural] ~ light brownish gray [3 ig, Beige Brown]	None
Glucose-asparagine agar	Yellowish gray [2 ba, Pearl ~ 2 ca, Lt Ivory]	Thin, graysh white [2 dc, Natural] ~ light brownish gray [2 ig, Slate Tan]	None
Nutrient agar	Pale yellow [2 ea, Lt Wheat ~ 2 gc, Bamboo]	None	None
Starch agar	Poor, colorless	Thin, graysh white [2 dc, Natural]	None

Observation after incubation at 27 °C for 21 days.
Color names and numbers from Color Harmony Manual, Container Corporation of America.[9]

vegetative mycelia were colorless to pale yellow. The soluble pigments were not formed. Physiological characteristics and carbohydrate utilization are shown in Table 2. Permissive temperature ranges for growth of the strain were 20°C to 37°C. The optimal temperature for growth of the strain was between 27 and 30°C.

Whole-cell hydrolysates of strain MJ635-86F5 contained LL-diaminopimelic acid. The strain has MK-9(H_6) and MK-9(H_8) as the major components of menaquinones.

These taxonomic properties suggested that strain MJ635-86F5 belonged to the genus *Streptomyces*. We searched the data of known *Streptomyces* species. In the result, strain MJ635-86F5 was not closely related to the species. Therefore, strain MJ635-86F5 was designated *Streptomyces* sp. MJ635-86F5. Strain MJ635-86F5 has been deposited in the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, Tsukuba, Japan, under the accession No.FERM P-14696.

Fermentation and Isolation

The antibiotic was monitored by the antibacterial activity against *Staphylococcus aureus* Smith and HPLC analysis during the purification process. A typical time course for production of cremimycin in the 30-liter jar fermenter was shown in Fig. 2. The production of cremimycin in the broth started after 4 days of cultivation and reached a maximum (*ca.* 14 mg/liter) after 8

Table 2. Physiological characteristics of strain MJ635-86F5.

Temperature range for growth(°C)	20 ~37
Optimum temperature(°C)	27 ~30
Formaition 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
Peptonizatin of milk	Negative
Hydrolysis of starch	Negative
Reduction of nitrate	Positive
Utilization of	
L-Arabinose	±
D-Xylose	+
D-Glucose	+
D-Fructose	±
Sucrose	-
Inositol	-
Rhamnose	(+)
Raffinose	-
D-Mannitol	-

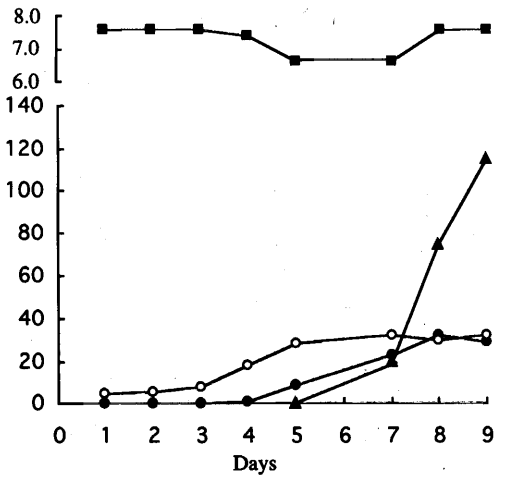
+ : utilization, (+) : probably utilization,
± : doubtful, - : no utilization

days fermentation. The content of cremimycin in the mycelium reached maximum (*ca.* 120 mg/liter) after 9 days or more fermentation.

The antibiotic in the mycelial cake was extracted with $CHCl_3$-MeOH (1:1, 4 liter) and filtered. The extract was concentrated *in vacuo* to yield a brown oily material. The oily material was extracted with a mixture of hexane-MeOH (1:1, 2 liters). The lower layer was collected and concentrated *in vacuo* to dryness yielding a brown material. The material containing cremimycin was dissolved in a mixed solvent of $CHCl_3$-MeOH (4:1, 500 ml) and washed with alkaline water (0.01 N NaOH), acidic water (0.01 N HCl), and deionized water successively. The organic layer was collected and concentrated *in vacuo* to yield a brownish residue. The residue was dissolved in MeOH and cooled for overnight at 5°C, and then cremimycin appeared as light brownish powder (420 mg). The antibiotic was collected and purified by a precipitation from a solution of hexane-MeOH (1:1, 150 ml) as a colorless powder (225 mg).

Fig. 2. Production of cremimycin.

■ pH, ● cremimycin (broth, μg/ml), ▲ cremimycin (mycelium, μg/ml), ○ packed cell volume (%).



Structure Elucidation

The molecular formula of cremimycin (**1**) was established as $C_{35}H_{53}NO_9$ on the basis of HRFAB-MS and NMR spectral analysis. The IR spectrum of **1** suggested the presence of ketone carbonyl (1710 cm^{-1}), α,β-unsaturated amide carbonyl (1650 cm^{-1}) and chelated β-diketone carbonyl (1608 cm^{-1}). Furthermore, the UV absorption maximum at 300 nm (ε 42,000) and positive color reaction with $FeCl_3$ of **1** supported the presence of β-diketone and triene amide chromophore in the molecule. The physico-chemical properties of **1** are summarized in Table 3.

The ^{13}C NMR spectrum of **1** showed 35 carbon signals. The DEPT and HMQC experiments revealed the presence of three methyls, one methoxy, twelve methylenes, three methines, five oxygen bearing methines, five olefinic methines, two quaternary olefinic carbons and four carbonyl carbons. The ^1H and ^{13}C NMR chemical shifts of **1** were similar to those of hitachimycin[11] except for those of a sugar, an alkyl side-chain and a ketone moiety. The ^1H and ^{13}C NMR spectral data of **1** were shown in Table 4.

Cremimycin (**1**) contained five partial structures (**a**, **b**, **c**, **d** and **e**) from analyses of the ^1H-^1H COSY and HMQC spectra as shown in Fig. 3. The presence of 19-membered macrocyclic lactam was revealed by con-

Table 3. Physico-chemical properties of cremimycin.

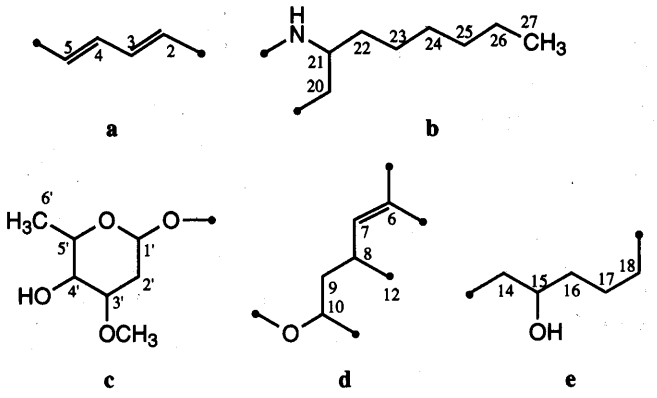
MP (°C)	219~220(dec.)
Molecular formula	$C_{35}H_{53}NO_9$
FAB-MS (*m/z*)	632 $(M+H)^+$ 630 $(M-H)^-$
HRFAB	
Calcd:	632.3799
Found:	632.3785$(M+H)^+$
$[\alpha]_D^{27}$	+52.4(*c* 1, DMSO)
UV λ_{max}^{MeOH} nm(ε)	
MeOH-0.01N HCl	300(42,400)
MeOH-0.01N NaOH	305(40,500)
IR ν_{max}(KBr)cm^{-1}	3430, 3280, 2930, 1710, 1650 1608, 1460, 1090, 1004,
Color reaction	
positive	$FeCl_3$, Rydone-smith reagent, vanilline-H_2SO_4 molybdophosphoric acid-sulfuric acid
negative	ninhydrin

Table 4. ^{13}C and ^{1}H NMR data of cremimycin in DMSO-d_6.

	δ_C[a]	δ_H[b]		δ_C[a]	δ_H[b]
1	166.0		18	44.5	2.26 (2H, dd, 6.6, 6.2)
2	123.9	6.03 (1H, d, 15.0)	19	209.0	
3	140.9	7.07 (1H, dd, 15.0, 11.0)	20	48.4	2.15 (1H, dd,11.0, 3.0)
4	127.0	6.43 (1H, dd, 15.0, 11.0)			2.37 (1H, dd, 13.0, 11.0)
5	136.5	7.21 (1H, d, 15.0)	21	47.3	4.12 (1H, br m)
6	130.5		21-NH		7.84 (1H, d, 9.4)
6-Me	19.3	1.81(3H, s)	22	34.9	1.4 (2H, m)
7	135.4	5.37 (1H, d, 10.5)	23	25.5	1.2~1.3 (2H, m)
8	32.8	4.17 (1H, m)	24	28.3	1.2~1.3 (2H, m)
9	36.0	1.96 (2H, m)	25	31.2	1.2~1.3 (2H, m)
10	77.3	4.81 (1H, dd, 9.0, 8.0)	26	22.0	1.2~1.3 (2H, m)
11	192.3		27	13.9	0.84 (3H, t, 6.2)
12	111.3		1'	97.8	4.98 (1H, dd, 10.0, 2.0)
13	189.5		2'	34.6	1.42 (1H, m)
14	45.2	1.92 (1H, m)			2.09 (1H, m, 13.6, 3.4, 2.0)
		2.26 (1H, m)	3'	77.3	3.50 (1H, m, 3.4, 3.0, 2.8)
15	66.8	3.75 (1H, m)	3'-OMe	57.6	3.36 (3H, s)
15-OH		4.52 (1H, d, 5.4)	4'	72.7	3.08 (1H, m, 8.8, 7.0, 3.0)
16	38.2	0.93 (2H, m)	4'-OH		4.65 (1H, d, 7.0)
17	19.7	1.05 (1H, m)	5'	69.9	3.6 (1H, m)
		1.62 (1H, m)	6'	18.4	1.13 (3H, d, 9.2)

a 125MHz, chemical shifts in ppm, multiplicity.
b 500MHz, chemical shifts in ppm, multiplicity.

Fig. 3. Partial structures in cremimycin.



necting the four partial structures (**a**, **b**, **d** and **e**) which were analyzed by the HMBC spectrum. An amide proton at δ_H 7.84 (21-NH) and olefinic methine proton at δ_H 6.03 (2-H) were coupled to a carbonyl carbon at δ_C 166.0 (C-1). Methyl signal at δ_H 1.81 (6-Me) was coupled to two olefinic carbons at δ_C 136.5 (C-5) and δ_C 135.4 (C-7). A methine proton at δ_H 4.17 (8-H) was coupled to a quaternary olefinic carbon at δ_C 111.3 (C-12). Methylene protons at δ_H 1.92 and 2.26 (14-H_2) were coupled to the quaternary olefinic carbons, C-12 and δ_C 189.5 (C-13). Two methylene protons at δ_H 2.15 and δ_H 2.26 (18-H), and 2.37 (20-H_2) were coupled to a carbonyl carbon at δ_C 209.0 (C-19). The alkyl side chain moiety (**b**) was assigned by the HMBC spectrum and using the chemical shift data for paraffins[12].

The presence of cyclopentanone moiety (**d**) was revealed by the HMBC spectrum. A methine proton at δ_H 4.81 (10-H) was coupled to a carbonyl carbon at δ_C 192.3 (C-11) and the quaternary olefinic carbon, C-12.

The *O*-glycoside linkage of the sugar residue (**c**) was also revealed by the analysis of HMBC spectrum. Two methine protons at δ_H 3.60 (5'-H) and δ_H 4.81 (10-H) were coupled to an anomeric carbon at δ_C 97.8 (C-1'). The above described result of HMBC experiment for **1**

Fig. 4. ^1H-^1H COSY and HMBC experiments of cremimycin in DMSO-d_6.

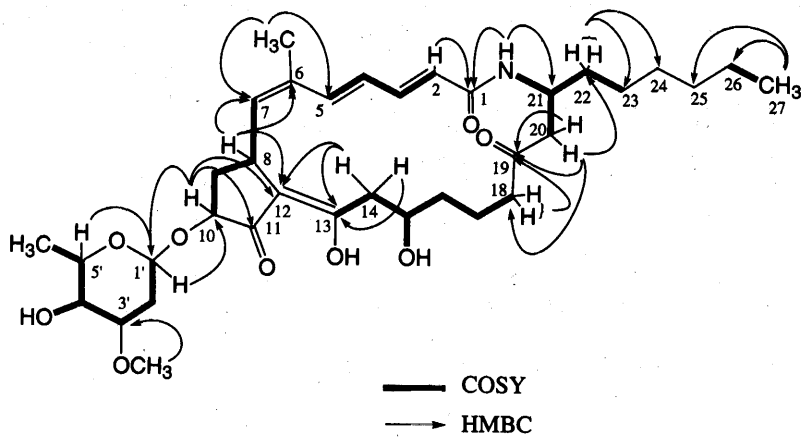


Table 5. Antimicrobial activities of cremimycin.

Test organisms	MIC(μg/ml)
Staphylococcus aureus FDA209P	0.39
S. aureus Smith	< 0.2
S. aureus MS9610	0.78
S. aureus MS16526(MRSA)	0.39
S. aureus TY-04282(MRSA)	0.78
Micrococcus luteus IFO3333	0.39
M.luteus PCI1001	0.39
Bacillus subtilis NRRL B-558	< 0.2
B. cereus ATCC10702	< 0.2
Corynebacterium bovis 1810	0.39
Escherichia coli NIHJ	> 100
Shigella dysenteriae JS11910	> 50
Salmonella enteritidis	> 50
Proteus mirabilis IFM OM-9	> 100
Providencia rettgeri GN466	> 50
Serratia marcescens	> 100
Pseudomonas aeruginosa A3	> 50
Klebsiella pneumonie PCI602	> 50
Mycobacterium smegmatis ATCC607[a]	> 100
Candida albicans 3147[b]	> 100
Saccharomyces cerevisiae [b]	> 50
Pyricularia orizae[b]	> 50

Mueller Hinton agar 37°C 18 hours. a: 37°C 42 hours.
b: Nutrient agar +1% Glucose 27°C 42 hours.

Table 6. Cytotoxicities of cremimycin.

Cell lines	IC_{50}(μg/ml)
L1210 leukemia	3.55
EL4 leukemia	2.92
P388 leukemia	3.25
IMC carcinoma	6.46
S180 sarcoma	16.3
B16 melanoma	8.64
FS3 fibrosarcoma	16.0

is summarized in Fig. 3.

Geometry for the three olefins at C-2, -4 and -6 in the 19-membered lactam moiety were revealed to be 2*E*, 4*E*, 6*Z* by their spin coupling constants ($J_{2,3}=15.0$ and $J_{4,5}=15.0$ Hz) and observing of NOE between 6-CH_3 and 7-H, and 6-CH_3 and 4-H in NOESY spectrum of **1**. The sugar moiety was considered to be cymarose by its NMR data. The configuration of the anomeric center was elucidated to be β by the spin coupling constants ($J_{1',2'eq}=2.0$ and $J_{1',2'ax}=10.0$ Hz, $J_{1',C\text{-}1'}=162.1$ Hz) and observing of NOEs between 1'-H and 5'-H, and 1'-H and 3'-OCH_3 in the NOESY spectrum. From the all above described results, the structure of cremimycin was determined as shown in Fig. 1. Detailed stereochemical studies of cremimycin are now in progress.

Biological Activity

The antimicrobial activities of cremimycin are shown in Table. 5. Cremimycin exhibited broad and strong antibacterial activity against Gram-positive bacteria and no activities against Gram-negative bacteria, yeast and fungi including *Pyricularia orizae*. Cremimycin exhibited weak cytotoxicity against murine tumor cell lines *in vitro*. The results are summarized in Table 6. The acute toxicity of cremimycin was tested by intraperitoneal administrations into mice. The LD_{50} of **1** was over 100 mg/kg.

Cremimycin having strong antibacterial activities is related to hitachimycin which is classified as 19-membered macrocyclic lactam antibiotics. A few 19-mem-

bered macrocyclic lactam antibiotics have been reported as microbial metabolites such as hitachimycin and BE-14106[13]. They were discovered by their antitumor activities. Furthermore, hitachimycin showed both antibacterial and antifungal activities. However, cremimycin did not exhibit any antitumor activity *in vivo* (data not shown) and antifungal activity.

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