ISOLATION OF CEPAFUNGINS I, II AND III FROM *PSEUDOMONAS* SPECIES

JUN'ICHI SHOJI, HIROSHI HINOO, TOSHIYUKI KATO, TERUO HATTORI, KEIICHIRO HIROOKA, KATSUYA TAWARA, OSAMU SHIRATORI and YOSHIHIRO TERUI*

Shionogi Research Laboratories, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553, Japan

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New acylpeptide antibiotics named cepafungins I, II and III were isolated from the culture broth of a strain identified as *Pseudomonas* sp. These antibiotics are neutral substances, soluble in aqueous alcohols and dimethyl sulfoxide, and show UV maxima at 260.5 nm. The IR spectra indicated these to be peptides. Molecular formulas $C_{28}H_{46}N_4O_6$, $C_{27}H_{44}N_4O_6$ and $C_{26}H_{42}N_4O_6$ for cepafungins I, II and III were indicated by elemental analysis and SI-MS. Cepafungins exhibited inhibitory activity against yeast and fungi, and antitumor activity against P388 leukemia in mice.

In the course of our screening work for new antibiotics from bacterial strains, a strain designated CB-3 and identified as *Pseudomonas* sp. was found to produce antibiotics active against fungi and murine leukemia P388. The antibiotics named cepafungins I, II and III were isolated and determined to be acylpeptides having an interesting 12-membered ring. The structures are presented in the following paper[1].

In this paper, the taxonomy of the producing strain, the isolation, physico-chemical properties as well as the biological activities are presented.

Taxonomy

The producing organism numbered CB-3 was isolated from a soil sample collected in Kikaijima, Kagoshima Prefecture, Japan.

The organism is Gram-negative, consisting of non-sporulating rods ($0.8\,\mu m \times 2.0 \sim 4.0\,\mu m$) with rounded ends that are motile by polar multitrichous flagella. Formation of myxospores or sporangia, unlike *Polyngium brachysporum* sp. nov.[2,3],†, was not observed. On nutrient agar, it forms circular, entire, convex, wet and glistening colonies with grayish cream color. Fluorescent pigments are not formed on KING's B medium. Poly-β-hydroxybutyrate is accumulated as an intracellular carbon reserve.

The organism, aerobic, showed good growth at 28°C but not at 5°C and 37°C. Other physiological characteristics are shown in Table 1. On cleavage of carbohydrates, acid formation was observed from D-glucose, D-galactose, D-fructose, D-mannose, D-xylose, maltose, sucrose and lactose. No gas formation was observed from the above carbohydrates.

From comparison of these characteristics with those of bacteria registered in Volume 1 of BERGEY's Manual of Systematic Bacteriology[4], the organism was identified as *Pseudomonas* sp. closely related to *Pseudomonas cepacia.*

Isolation

Cells of strain CB-3 were inoculated into 100 ml of medium, consisting of sucrose 3.0%, Soytone

† As revealed in the following paper[1], this organism had produced the substance identical with cepafungin II.

Table 1. Physiological characteristics of strain CB-3.

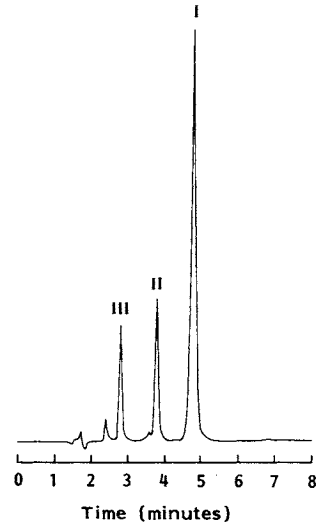
Properties observed	Result	Properties observed	Result
Catalase test	+	Arginine dihydrolase test	−
Oxidase test	+	Lysine decarboxylase test	−
OF-test	Oxidative	Ornithine decarboxylase test	−
Peptonization of milk	+	β-Galactosidase test	+
Coagulation of milk	−	Urease test	−
Haemolysis	−	Acylamidase test	−
Gelatin liquefaction	+	Voges-Proskauer test	−
Esculin hydrolysis	+	Methyl red test	−
Tween 80 hydrolysis	+	Nitrate reduction	−
Starch degradation	−	Denitrification	−
Indole production	−	Citrate utilization	+
H_2S production	−	Malonate utilization	−

1.0%, yeast extract 0.3%, pH 7.0 in a Sakaguchi-flask, that was cultured at 27°C for 3 days in the usual shaking manner.

To the culture broth (10 liters), methanol (5 liters) and butanol (5 liters) were added, and the mixture was filtered. After slight evaporation, the filtrate was extracted twice with butanol. The butanol extract was washed with dilute hydrochloric acid, water, dilute sodium bicarbonate solution and water, successively. Then it was concentrated *in vacuo* to an oily residue, that was triturated with petroleum ether to give a crude powder (3.0 g). The powder was then applied to a silica gel column (Merck Silica gel 60H, 5.4 × 30 cm) and eluted with chloroform - methanol (5 : 1). The active eluate was evaporated to a small volume to give a gel-like precipitate. Trituration with petroleum ether afforded a colorless powder (770 mg) of a complex of cepafungins I, II and III.

Fig. 1. HPLC of cepafungins I, II and III.

Column: Nucleosil $5C_{18}$ (4.6 × 150 mm), mobile phase: 50% acetonitrile, flow rate: 1.125 ml/minute, chart speed: 1.0 cm/minute, monitored at 260 nm.



The complex was not further separable by TLC, but was easily separable by HPLC in reversed phase mode (Fig. 1).

Some 50 mg of the complex was subjected to preparative HPLC using a Nucleosil 10 C_{18} column (2.0 × 25 cm) with 50% acetonitrile, 3 mg being applied for each run. Fractions containing components I, II and III were collected respectively. Each fraction was evaporated and extracted with butanol. The butanol extracts were washed with water and concentrated to dryness, giving colorless powders. Thus, components I (20 mg), II (7 mg) and III (7 mg) were obtained.

Physico-chemical Properties

Cepafungins I, II and III are neutral substances, obtained as colorless amorphous powders. They are soluble in aqueous methanol, aqueous ethanol, aqueous butanol, dimethyl sulfoxide and a mixture of chloroform and methanol, but sparingly soluble or insoluble in water and pure organic solvents such as

methanol, ethanol, butanol, acetone, ethyl acetate and chloroform. They show nearly identical UV and IR spectra; these spectra for component I are illustrated in Figs. 2 and 3. ^{1}H and ^{13}C NMR spectra of component I are shown in Fig. 4. Other physico-chemical properties are listed in Table 2.

The broad and strong absorptions in the IR centered at 1640 and 1530 cm^{-1} suggested that these antibiotics are peptides. By acid hydrolysis of these antibiotics followed by automatic amino acid analysis in the usual manner, only threonine was detected as a usual amino acid.

Biological Properties

Biological activities of cepafungins were measured using the complex of components I, II and III. Cepafungins showed moderate inhibitory activity against pathogenic yeasts and fungi as shown in Table 3. However, a curative effect in mice infected with *Candida albicans* M-9 was not observed. Cepafungins showed no activity against any bacteria.

Cepafungins exhibited a moderate effect on prolonging the survival period of mice in which murine lymphatic leukemia P388 cells were implanted (Table 4). However, it exhibited only a weak effect to mice bearing leukemia L-1210 (T/C 117) or B-16 melanoma (T/C 119).

Fig. 2. UV spectrum of cepafungin I (95% EtOH).

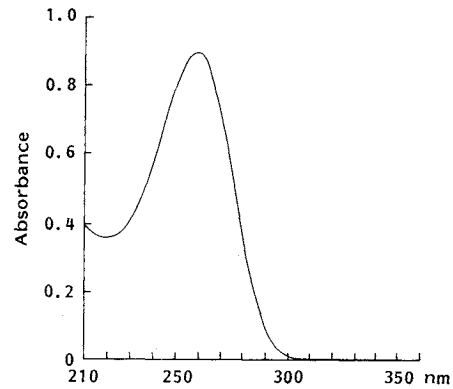


Fig. 3. IR spectrum of cepafungin I (KBr).

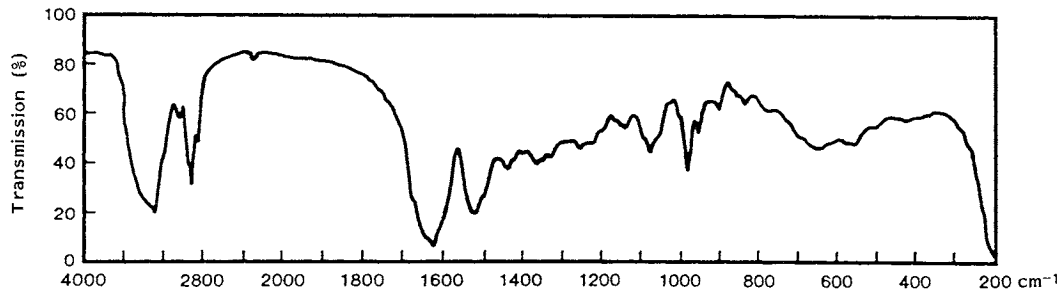


Table 2. Physico-chemical properties of cepafungins I, II and III.

	I	II	III
MP (°C, dec)	235~240	245~250	215~220
Anal Calcd	$C_{28}H_{46}N_4O_6 \cdot H_2O$:	$C_{27}H_{44}N_4O_6 \cdot H_2O$:	$C_{26}H_{42}N_4O_6$:
	C 60.87, H 8.71, N 10.14	C 60.22, H 8.55, N 10.41	C 61.66, H 8.34, N 11.07
Found:	C 61.57, H 8.51, N 10.72	C 60.30, H 8.39, N 10.38	C 61.80, H 8.78, N 11.74
Positive[a] (*m/z*) ($(M+H)^+$)	535	521	507
Negative[b] (*m/z*) ($(M-H)^-$)	533	519	505
$[\alpha]_D^{24}$	$-124\pm2°$	$-127\pm6°$	$-110\pm10°$
	(*c* 0.694, MeOH)	(*c* 0.283, MeOH)	(*c* 0.155, MeOH)
UV $\lambda_{max}^{95\%\ EtOH}$ nm (ε)	260.5 (32,700)	260.5 (33,900)	260.0 (24,200)

[a,b] SI-MS.

Fig. 4. NMR spectra of cepafungin I (DMSO-d_6 at 25°C).

(A) ^{1}H NMR spectrum, (B) ^{13}C NMR spectrum.

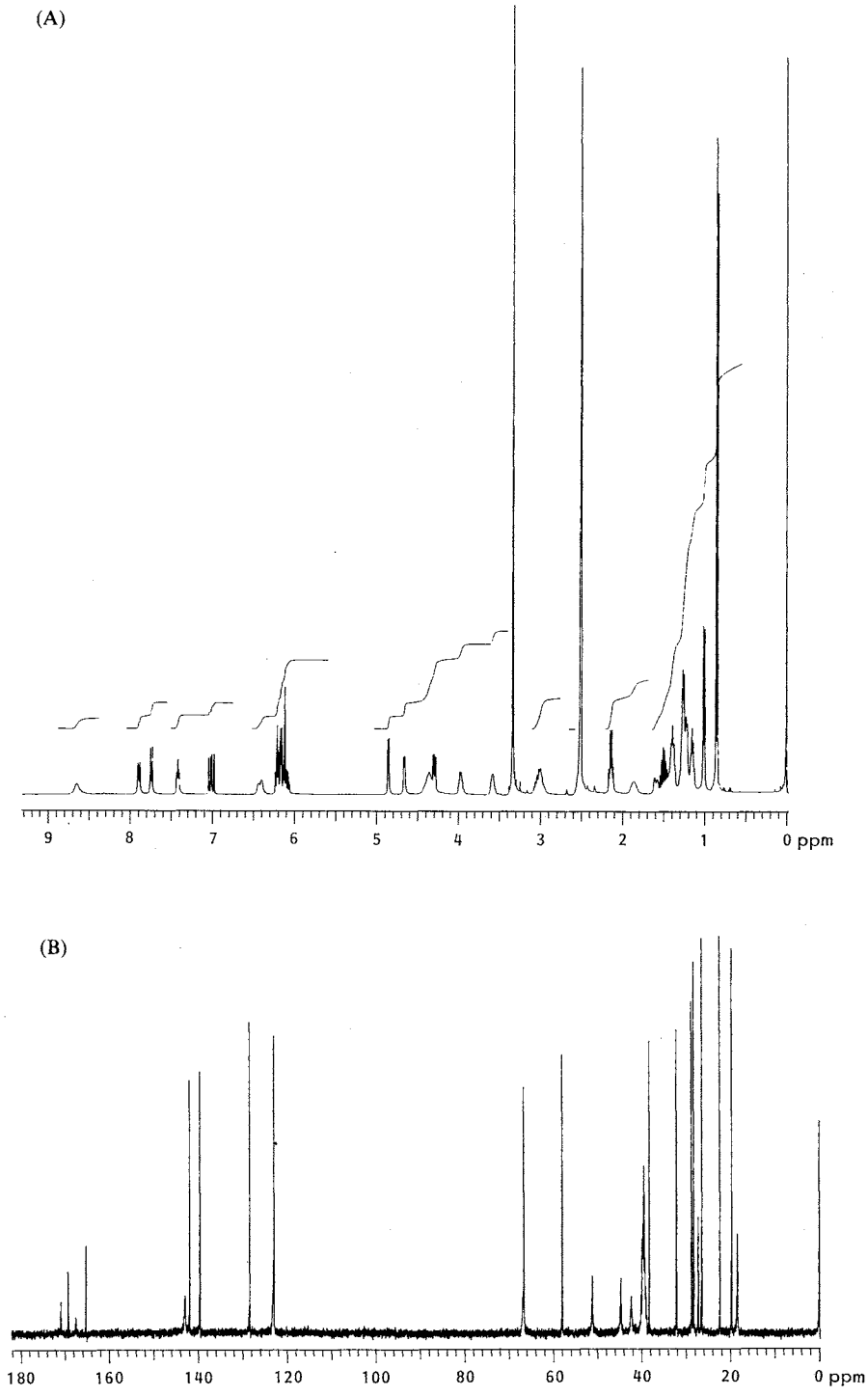


Table 3. Antifungal activity of cepafungin complex.

Organism	MIC (μg/ml)
Candida albicans KE-2	3.1
C. albicans M-9	1.6
C. albicans IFO 0579	3.1
C. krusei IFO 1063	3.1
Aspergillus fumigatus KE	6.3
Penicillium digitatum IFO 7876	>100
Microsporum canis IFO 7863	6.3
Trichophyton rubrum IFO 5467	12.5
T. rubrum IFO 5807	12.5
T. mentagrophytes IFO 5809	6.3
T. mentagrophytes IFO 5810	6.3
T. asteroides AV	12.5

Measured on SABOURAUD's glucose agar.

Table 4. Antitumor activity of cepafungin complex against murine leukemia P388 (ip-ip).

Dose (mg/kg)	T/C (%)
0.2	117
0.5	140
1.0	151
2.0	164
5.0	191
10	131 (toxic)

The drug was administered 1 day after tumor inoculation.

Experimental

General Methodology

The UV absorption spectrum was measured with a Hitachi 323 spectrometer and the IR absorption spectrum with a Jasco DS-403G spectrometer, $[\alpha]_D$ with a Perkin-Elmer 241 polarimeter and SI-MS with a Hitachi M-68 mass spectrometer. Amino acid analysis was carried out with a Hitachi amino acid autoanalyzer 835.

Acknowledgments

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