ISOLATION OF CB-25-I, AN ANTIFUNGAL ANTIBIOTIC, FROM *SERRATIA PLYMUTHICA*

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A new antifungal antibiotic, CB-25-I, was isolated from the culture broth of a strain of *Serratia plymuthica*. The antibiotic, a water-soluble dipeptide, is structurally related to Sch 37137 and A 19009, both produced by strains of Actinomycetales. The antibiotic exhibits inhibitory activity against *Candida albicans* in YNB medium (a synthetic medium), but the activity is significantly reduced in Sabouroud dextrose medium.

In the course of our screening work for new antifungal antibiotics of bacterial origin, a strain numbered CB-25 identified as *Serratia plymuthica* was found to produce antibiotics showing inhibitory activity against *Candida albicans*. The major product named CB-25-I was isolated and characterized. The structure and biological properties of the antibiotic are related to Sch 37137[1] isolated from a *Micromonospora* sp. and also to A 19009[2~4] from a *Streptomyces* sp.

In this paper, the taxonomy of the producing organism, production, isolation, structure and biological properties of CB-25-I are presented.

Taxonomy

The producing organism numbered CB-25 was isolated from a soil sample collected in Fukiagecho, Kagoshima Prefecture, Japan.

The organism is Gram-negative, non-sporulating rods ($0.8 \times 1.0 \sim 2.0$ μm) with rounded ends, motile by one lateral or polar flagellum. On heart infusion agar, it forms circular, entire, convex, opaque, wet and glistening colonies with yellowish cream color.

The organism, facultatively anaerobic, showed good growth at 28°C. Other physiological char-

Table 1. Physiological characteristics of strain CB-25.

Properties observed	Result	Properties observed	Result
Catalase test	+	Ornithine decarboxylase test	−
Oxidase test	−	β-Galactosidase test	+
OF-test	Fermentative	Urease test	−
Peptonization of milk	+	Deoxyribonuclease test	+
Coagulation of milk	+	Tween 80 hydrolysis	+
Hemolysis	+	Voges-Proskauer test	+
Gelatin liquefaction	+	Methyl red test	−
Indole production	−	Nitrate reduction	+
H_2S production	−	Citrate utilization	+
Arginine dihydrolase test	−	Malonate utilization	−
Lysine decarboxylase test	−	Gluconate oxidation	+

acteristics are shown in Table 1. On cleavage of carbohydrates, acid and gas were formed from DL-arabinose, D-xylose, D-glucose, D-fructose, sucrose, maltose, trehalose, and D-mannitol. Delayed acid formation without gas was observed on lactose.

From comparison of these characteristics with those of bacteria registered in the Volume 1 of BERGEY's Manual of Systematic Bacteriology[5], the organism was identified as non-pigmented *Serratia plymuthica*.

Fermentation

A cell suspension of strain CB-25 was inoculated into 250 liters of a medium consisting of CA-1 (Chow diet, Japan Clea Co., Ltd.) 2.0% and starch 2.0% (pH not adjusted) in a 500-liter jar fermenter. Fermentation was carried out for 1 day at 28°C under aeration of 300 liters per minute and agitation of 250 rpm.

A pulp disk agar diffusion method using YNB agar medium (the ingredients of the medium are cited later) seeded with *C. albicans* M-9 and a pulp disk (6 mm diameter, Toyo Seisakusho, Japan) was applied for monitoring antifungal activity throughout this experiment. A culture broth obtained as above showed pH 7.3 and an inhibitory zone of 25.0 mm diameter.

Isolation

The culture broth (250 liters) was centrifuged at pH 2.5. Fifteen liters of an ion-exchange resin, Dowex 50-X8 (NH_4^+), was added to the supernatant, which was stirred for 1 hour. The resin was then separated by decantation and packed into a glass column, and the antibiotic adsorbed on the resin was eluted with 2% aqueous pyridine. Active eluate fractions were collected and passed through a Dowex 1-X2 (Cl^-) column (3 liters) at pH 8.5. The column was eluted with 5% NaCl in 50 mM phosphate buffer, pH 4.5. The antibiotics in the eluate were adsorbed on an active carbon (110 g, Wako Chemicals), and were eluted with 50% aqueous acetone at pH 4.5. Concentration followed by freeze-drying the active eluate gave a crude powder (17 g).

The crude powder was subjected to chromatography on a column (4.1×60 cm) of MCI gel CHP-20P (150~300 μm) with 20 mM phosphate buffer, pH 7.0, resulting in the rough separation into a major component, CB-25-I (faster moving) and a minor component, CB-25-II[†].

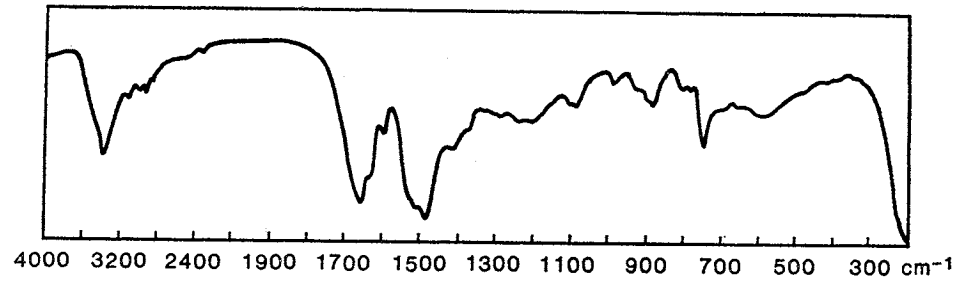
The fraction containing the major component was further purified by chromatography on a MCI gel CHP-20P column (200~400 mesh) with 20 mM phosphate buffer, pH 7.0. Finally, the fraction containing only the antibiotic (by HPLC) was charged on the same column and developed with water. The active eluate fraction was adjusted to pH 4.5 with dilute HCl and freeze-dried to give CB-25-I hydrochloride as a colorless powder (200 mg).

Physico-chemical Properties and Structure

CB-25-I is a water-soluble amphoteric substance. The hydrochloride is obtained as a colorless amorphous powder. It is positive to ninhydrin reaction, but not to Sakaguchi's reagent. It shows a single spot on TLC giving an Rf 0.29 on an Avicel cellulose plate with 70% propanol, and a single peak on HPLC giving a retention volume of 4.65 ml on a Nucleosil 5 C_{18} column (4.6×150 mm) with 50 mM phosphate buffer, pH 7.0, monitored at 220 nm.

† CB-25-II was purified only in a small amount. Characterization of this substance was inadequate, but it was confirmed to be a congener of CB-25-I differing only in the replacement of the valine residue in I by isoleucine residue.

Fig. 1. IR spectrum of CB-25-I hydrochloride (KBr).



The UV spectrum measured in water shows only end absorption. In the IR spectrum (Fig. 1), absorptions attributable to peptide bonds (1675 and 1580 cm^{-1}) are dominant.

A molecular formula, $C_{12}H_{20}N_4O_6$, was indicated by elemental analysis and secondary ion mass spectrometry (SI-MS): *m/z* 317 (M+H) and 339 (M+Na).

Anal Calcd for $C_{12}H_{20}N_4O_6 \cdot 6HCl$: C 40.85, H 5.95, N 15.88.
Found: C 40.27, H 5.65, N 15.47.

CB-25-I gave valine and α,β-diaminopropionic acid (Dap) by acid hydrolysis. These amino acids were L-leucylated and compared with respective authentic dipeptides by HPLC[6], indicating both the amino acids to be in the L-form.

When CB-25-I was 2,4-dinitrophenylated and then hydrolyzed with 6 N HCl in the usual manner, N^{α}-(2,4-dinitrophenyl) (DNP)-Dap was produced. This meant that the α-amino group of the Dap residue was free and the β-amino group was blocked. Edman degradation reaction was applied to CB-25-I and the residual part of the first reaction was subjected to amino acid analysis without further acid hydrolysis, resulting in the detection of valine. This meant that the carboxy group of the Dap residue was linked to the amino group of valine residue. Thus, a partial structure of CB-25-I was shown to be *N*-(α,β-diaminopropionyl)valine whose β-amino group was blocked.

^1H NMR experiments including spin decoupling and ^{13}C NMR spectroscopy including SFORD showed the signals assignable to the valine and Dap residues as shown in Table 2. Further, the presence of an epoxide with two protons (the *trans* form was deduced from their coupling constants) was indicated, and signals for four carbonyl carbons, two of which arise from the valine and Dap residues, were observed.

From these NMR data and the molecular formula, it was deduced that the moiety that blocks the β-amino group of the Dap residue is $-CO-\underset{\diagdown\,O\,\diagup}{CH-CH}-CONH_2$. Accordingly, it was concluded that the total structure of CB-25-I is **1** in Fig. 2. These NMR data are similar to those of Sch 37137, especially for the epoxide part[1].

The antibiotic is named (*trans*)-*N*-[3-[[[3-(aminocarbonyl)oxiranyl]carbonyl]amino]-L-alanyl]-L-valine or can be called N^{β}-[(*trans*)-3-carbamoyl-2,3-epoxypropionyl]-L-α,β-diaminopropionyl-L-valine as a derivative of Dap→Val.

Biological Properties

CB-25-I is inactive against Gram-positive and Gram-negative bacteria. It shows inhibitory activity against *C. albicans* M-9 in a medium consisting of Yeast nitrogen base (Difco) 0.7%, L-asparagine 0.15% and glucose 1.0% (abbreviated as YNB medium) as shown in Table 3. However,

Table 2. ^{1}H and ^{13}C NMR data for CB-25-I hydrochloride[a].

Assignment	^{1}H[b]	^{13}C[c]
Val		
$2\times CH_3$	0.90 (3H, d, J=6.8)	18.1 (q)
	0.94 (3H, d, J=6.8)	19.1 (q)
β-CH	2.13 (H, m)	30.9 (d)
α-CH	4.07 (H, d, J=5.8)	61.9 (d)
Dap		
β-CH_2	3.74 (H, dd, J=6.0, 15.0)	40.5 (t)
	3.82 (H, dd, J=5.4, 15.0)	
α-CH	4.24 (H, t-like, J=6.0)	53.5 (d)
O / CH–CH	3.68 (H, d, J=1.8)	54.3 (d)
	3.70 (H, d, J=1.8)	54.4 (d)
$4\times CO$		167.9 (s)
		170.4 (s)
		171.6 (s)
		178.2 (s)

[a] The spectra were measured in D_2O at 25°C.
[b] TMS was used as an external reference (δ 0.662). J in Hz.
[c] Dioxane was used as an internal reference (δ 67.4).

Table 3. Antimicrobial properties of CB-25-I.

Organism	Medium	MIC (μg/ml)
Staphylococcus aureus	I	>100
Streptococcus pyogenes	II	>100
Escherichia coli	I	>100
Klebsiella pneumoniae	I	>100
Candida albicans M-9	III	0.2
Aspergillus fumigatus	III	>100
Trichophyton asteroides	II	>100

Medium I: Mueller-Hinton broth (Difco), medium II: Trypto-soy broth (Difco), medium III: YNB broth (the ingredients are cited in the text).

Fig. 2. Structures of CB-25-I, Sch 37137, A 19009 and its isomer.

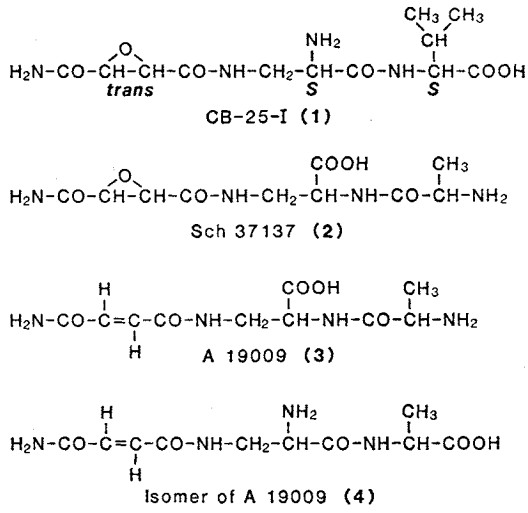


Table 4. Antifungal activity of CB-25-I on Sabouroud dextrose agar.

Organism	MIC (μg/ml)
Candida albicans KE-2	>100
C. albicans M-9	100
C. albicans IFO 0579	>100
C. krusei IFO 1063	3.1
C. guilliermondii IFO 0454	>100
Aspergillus fumigatus KE	>100
Penicillium digitatum IFO 7876	>100
Microsporum canis IFO 7863	>100
Trichophyton rubrum IFO 5467	>100
T. rubrum IFO 5808	>100
T. mentagrophytes IFO 5809	>100
T. mentagrophytes IFO 5810	>100
T. asteroides AV	>100

the activity against *C. albicans* is significantly reduced, when measured in Sabouroud dextrose agar (Table 4).

CB-25-I showed no toxicity by ip administration at a dose of 100 mg/kg to mice.

Discussion

An antibiotic substance CB-25-I which is active against *C. albicans* in YNB medium was isolated from a strain of *S. plymuthica*.

The structure **1** is partly related to an antifungal compound Sch 37137 (**2**) produced by a *Micromonospora* sp.[1]. A partial structure, H_2N–CO–CH(O)–CH–CO–NH–CH_2–CH(NH)–CO–, is common to both the compounds. The partial structure is linked through the carboxy group to valine in **1**, whereas it is linked through the amino group to alanine in **2**.

A related antibiotic A 19009 has been previously isolated from a *Streptomyces* sp. The structure was originally reported to be **4** in Fig. 2[2]. Later, compounds **3** and **4** in Fig. 2 were synthesized and detailed NMR experiments revealed that the real structure of A19009 is **3** and, therefore, **4** is an isomer of A 19009[3,4]. The only difference between compounds **2** and **3** is replacement of an epoxide by a double bond.

The antifungal activity of **2** has been examined in comparison with **3** and **4**. These three compounds showed similar activities, suggesting a common mechanism in their antifungal action. They were active against *Candida* and dermatophytes in MA medium (a glucose - salt medium containing biotin), but essentially inactive in Sabouroud dextrose and modified Eagle's minimum essential media[1].

The antibiotic CB-25-I, reported here, is related to these three compounds in structure and antifungal activity.

Experimental

The UV absorption spectrum was measured with a Hitachi 323 spectrometer, the IR absorption spectrum with a Jasco DS-403G spectrometer and the SI-MS with a Hitachi M-90 mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with a Varian XL-200 spectrometer. Amino acid analysis was carried out with a Hitachi amino acid autoanalyzer 835.

The Constituent Amino Acids

CB-25-I hydrochloride was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. When the hydrolysate was analyzed by an amino acid analyzer, Val (1.94 μmol/mg) was detected and the peak for Dap overlapped with that of ammonia. These amino acids were confirmed by chromatography on paper (Toyo Roshi No. 51) with butanol - acetic acid - water (4 : 1 : 2) in comparison with authentic specimens (Dap: Rf 0.16, Val: Rf 0.52).

The hydrolysate was adsorbed on a short column of Dowex 50-X8 (NH_4^+) and eluted with 0.3 N NH_4OH. Concentration of the eluate gave a mixture of Dap and Val. It was L-leucylated in the usual manner[6] and the resultant dipeptides were analyzed by HPLC on a Nucleosil 10 C_{18} column (4.6×150 mm) with acetonitrile - 20 mM KH_2PO_4 (5 : 95). Authentic dipeptides showed the following retention volumes: L-Leu - L-Val (2.1 ml), L-Leu - D-Val (>15 ml), L-Leu - L-Dap (3.0 ml) and L-Leu - D-Dap (8.0 ml). The dipeptides derived from CB-25-I coincided with L-Leu - L-Val and L-Leu - L-Dap.

2,4-Dinitrophenylation

A 2-mg portion of CB-25-I hydrochloride was 2,4-dinitrophenylated in the usual manner. The DNP-CB-25-I was hydrolyzed with constant boiling hydrochloric acid at 110°C for 16 hours, and the DNP-amino acid produced was analyzed by TLC on an Avicel cellulose plate with 0.5 M phosphate buffer, pH 6.0. Authentic specimens of α-DNP-Dap and β-DNP-Dap showed Rf values of 0.65 and 0.57, respectively. The DNP-amino acid from CB-25-I coincided with α-DNP-Dap.

The authentic specimen of β-DNP-Dap was prepared by dinitrophenylation of cupric complex of Dap. The α-DNP-Dap was prepared through β-*Z*-Dap prepared by benzyloxycarbonylation of the cupric complex.

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