CEPACIDINE A, A NOVEL ANTIFUNGAL ANTIBIOTIC PRODUCED BY *Pseudomonas cepacia*

I. TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL ACTIVITY

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Cepacidine A is a potent antifungal antibiotic produced by *Pseudomonas cepacia* AF 2001. The compound was isolated from the fermentation broth with 1 vol isopropyl alcohol, followed by the collection of the precipitation formed upon concentration of the extract. Purification was effected by chromatography on Diaion HP-20, alumina and reversed phase C_{18} followed by TLC on silica gel. These techniques afforded the two closely related compounds, cepacidine A_1 and cepacidine A_2. A mixture of these two compounds called cepacidine A, showed high *in vitro* antifungal activity against the various animal and plant pathogenic fungi. The activity was diminished by the presence of serum. No antibacterial activity was demonstrable.

In the course of our screening for new antifungal substances from the various microorganisms, a novel antifungal antibiotic, named cepacidine A, was found in the fermentation broth of a strain of *Pseudomonas cepacia* AF 2001, deposited in the Korean Federation of Culture Collections, Seoul, Korea, with the registration number KFCC 10773. Cepacidine A is a mixture of two closely related compounds, cepacidine A_1 and cepacidine A_2. The primary structures of these two compounds are shown in Fig. 1. The producing organism, *Pseudomonas cepacia* AF 2001, was isolated from the soil samples collected in Munchon, Kyunggi-Do, Korea.

In this paper we describe the taxonomy of the producing microorganism, the producing and isolation procedures of cepacidine A, and their biological activity. The results of physico-chemical properties and structural elucidation are presented in the accompanying report.[1)]

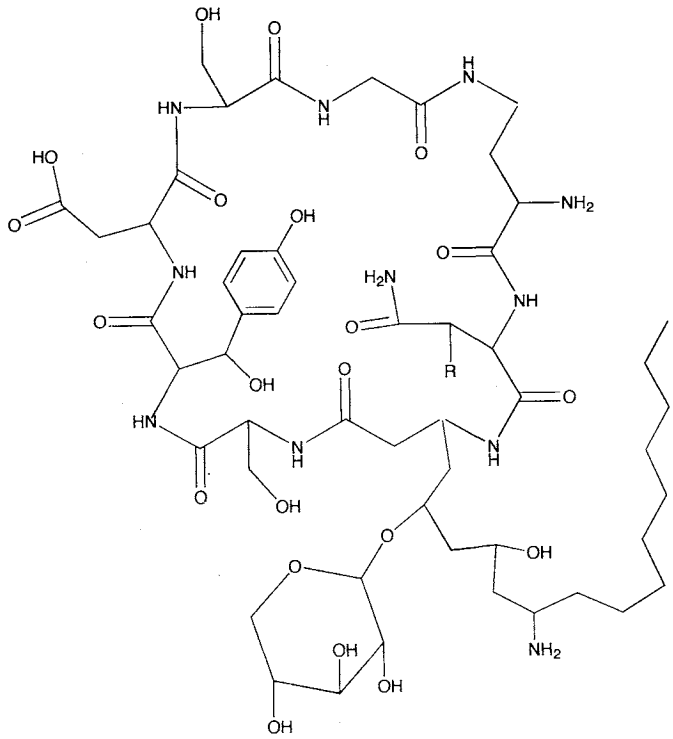
Taxonomy

The strain AF 2001, producing cepacidine A, is a Gram-negative rod sized $0.4 \sim 0.6 \times 1.0 \sim 1.3\,\mu m$ and motile by means of polar flagella. It is oxidative and cytochrome positive. These characteristics serve to identify the strain as a *Pseudomonas*. A summary of the key characteristics and the carbon utilization pattern for the growths is shown in Table 1. These characteristics are very similar to the description for *Pseudomonas cepacia* given by BALLARD *et al.*[2)] However, strain AF 2001 is different from *Pseudomonas cepacia* as mentioned above in reference to maltose, sucrose and adonitol utilization. It can grow on maltose and sucrose as a sole carbon source but it cannot grow on adonitol. In these points, strain AF 2001 is thought to be new *Pseudomonas cepacia*. Therefore, we designated the strain *Pseudomonas cepacia* AF 2001.

Production

Cultures of *Pseudomonas cepacia* AF 2001 were maintained at $-85°C$. When needed, working stock

Fig. 1. The structures of cepacidine A.



Cepacidine A_1 R=OH
Cepacidine A_2 R=H

Table 1. Characteristics of *Pseudomonas cepacia* AF 2001.

Cell type	Short rod	Benzene ring cleavage	Ortho
Gram stain	Negative	Growth at 4°C	Negative
Motility	Positive	Growth at 41°C	Positive
Biochemical characteristics:		Growth at pH 3	Negative
Oxidative	Positive	Growth at pH 9	Positive
Oxidase	Positive	Carbon utilization:	
Catalase	Positive	Positive: maltose, sucrose, L-arabinose, glucose, mannose, xylose, fructose, tartarate, dulcitol, salicin, cellobiose, fumaric acid, caprate, thereonine, lysine, arginine	
Urease	Negative		
DNase	Negative		
Lysine decarboxylase	Negative		
Nitrate reduction	Negative	Negative: adonitol, D-raffinose, lactose, maleic acid, inulin, ethylene glycol, phthalic acid, L-isoleucine, starch, melibiose	
Poly β-hydroxybutyrate accumulation	Positive		
Fluorescence	Negative		
Diffusible pigment	Positive		
Esculin hydrolysis	Positive		

cultures were prepared on agar slants composed of glucose 3%, peptone 1.5% and agar 2%. The slants were incubated at 28°C for 24 hours and used to inoculate germinator flasks containing 100 ml of medium in 500-ml Erlenmeyer flasks. This medium consisted of bactopeptone 1%, yeast extract 1% and glucose 3%. The germinator was incubated for 24 hours at 28°C on a rotary shaker at 300 rpm, and then used to inoculate (1%) the same medium (10 liters). The fermentation was run for 72 hours at 28°C with an agitation rate of 300 rpm. Progress of the fermentation and the subsequent isolation steps were monitored by

paper-disc-agar diffusion assay, with *Candida albicans* ATCC 38245 as the assay microorganism.

Isolation Procedure

Cepacidine A was isolated and purified by the procedure outlined in Fig. 2. The fermentation broth, 10 liters, was mixed with equal volumes of isopropanol (10 liters), and the mixture was adjusted to pH 4.0 with conc HCl. The mixture was centrifuged and the cell mass was discarded. The supernatant was concentrated then under reduced pressure to remove isopropanol. The precipitate that formed during the concentration process contained the bioactivity as shown by conventional agar diffusion assay. The concentrate and the accompanying precipitate were stored at 4°C for 6 hours to allow the precipitation to proceed to completion. The precipitate was collected by filtration with diatomaceous earth, washed with water and then eluted with 50% isopropanol in water. This solution was concentrated *in vacuo* to remove isopropanol. After extraction of this concentrate with iso-butanol - methanol (8 : 2), the solvent extract was then concentrated under reduced pressure. The precipitate that formed during the concentration was collected by centrifugation and dissolved in 50% isopropanol adjusted to pH 10.0 with NaOH. This solution was diluted with 10 vol distilled water and applied to Diaion HP-20 resin column chromatography. After elution with 50% isopropanol, the eluent was concentrated *in vacuo* into a small volume. The precipitate formed during the concentration was dissolved in 50% isopropanol and applied to alumina column chromatography. The pass through that contained the bioactivity was concentrated into a small volume and stored at 4°C for 24 hours to allow precipitation. After washing with water, the precipitate was dissolved in 50% isopropanol and applied on a preparative ODS-silica gel column of Waters μ-Bondapak C_{18} (3×30 cm, 10 μm) and developed with acetonitrile - water (6 : 4, pH 3.5). The fraction containing anti-candidal activity was collected and concentrated *in vacuo* in order to give residues of pure cepacidine A (100 mg). Cepacidine A was further resolved into two closely related components, *i.e.*, A_1 and A_2, by TLC on silica gel (Merck Silica gel 60 F_{254}), eluting with isopropanol - conc ammonia - water (4 : 2 : 1). Visualization of the components was accomplished either by charring, after spraying with a solution containing 5% ammonium molibdate and 0.1% ceric ammonium sulfate in 10% sulfuric acid, or by spraying conventional ninhydrin reagent. The Rf value of A_1 and A_2 is 0.53 and 0.58, respectively. The producing ratio of A_1 and A_2 was determined to 9 : 1 according to the quantitative analysis of the amounts of purified A_1 and A_2. Unfortunately, it was very difficult to isolate large amount of pure A_1 and A_2, because cepacidine A was not separated into A_1

Fig. 2. Isolation procedures of cepacidine A.

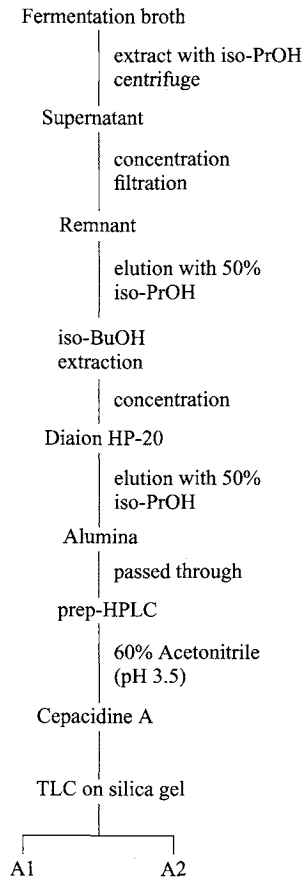


Table 2. Antifungal spectrum of cepacidine A and amphotericin B.

Organism	KCTC No*	MIC (μg/ml)	
		Cepacidine A**	Amphotericin B
Candida albicans	1940	0.391	0.782
Candida albicans	38245	0.391	0.782
Candida glabrata	1714	0.013	0.196
Cryptococcus neoformans	1197	0.025	0.098
Saccharomyces cerevisiae	1213	0.049	0.098
Aspergillus niger	2119	0.098	0.196
Microsporum gypseum	1252	0.196	0.196
Microsporum canis	11621	0.025	0.391
Epidermophyton floccosum	1246	0.049	3.125
Trichophyton mentagrophyte	6085	0.049	3.125
Trichophyton rubrum	38484	0.049	0.782
Fusarium oxysporum	6084	0.196	0.196
Rhizopus stolonifer	6062	0.391	0.391

* Korean Collection for Type Cultures.
** Two component mixture.

and A_2 using the HPLC system described above. Therefore, the mixture of A_1 and A_2 was used for the following studies, *i.e.*, biological activity, physicochemical properties and structural elucidation.

Biological Activity

The *in vitro* antifungal activity of cepacidine A and amphotericin B against a wide range of medically important fungi are shown in Table 2. The activity was determined by the two fold agar dilution method on potato dextrose agar medium. Cepacidine A exhibited a broad antifungal spectrum against all strains tested. In particular, cepacidine A was highly active against dermatophytes, namely *Microsporum canis*, *Trichophyton* spp., and *Epidermophyton* spp., and true yeast at concentrations lower than 0.049 μg/ml. The activities of cepacidine A were greater than those of amphotericin B in almost all strains. However, no antibacterial activity was detected (MIC > 100 μg/ml) when cepacidine A was assayed against the bacteria *i.e.*: *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Table 3. Effects of serum on the activity of cepacidine A*.

Organism	Treatment	MIC (μg/ml)
Candida albicans	Control	0.08
	+50% human serum	1.28
Cryptococcus neoformans	Control	0.02
	+50% human serum	0.16

* Two component mixture.

The effects of human serum upon the antifungal activity of cepacidine A was determined by a standard microtiter dilution assay prepared in the presence of 50% human serum. Controls consisted of wells without serum. The results are shown in Table 3. It is evident that the activity is diminished about eight fold in the case of *Cryptococcus neoformans* and sixteen fold in *Candida albicans* by the presence of serum. Until now, it has not been determined if the loss of activity is due to serum binding or degradation of cepacidine A.

References

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