CISPENTACIN†, A NEW ANTIFUNGAL ANTIBIOTIC

I. PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE

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A new antibiotic, cispentacin, was isolated from the culture broth of a *Bacillus cereus* strain, L450-B2. The antibiotic is water-soluble and amphoteric; its structure was determined by spectroscopic analysis and chemical synthesis to be (1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid. Cispentacin demonstrated only weak *in vitro* activity against certain fungi but strong protection of mice from lethal infection of *Candida albicans* A9540.

In our search of the microbial metabolites effective against systemic *Candida* infection in mice, a bacterial strain L450-B2 was discovered to produce a new active substance named cispentacin. The producing strain was identified as *Bacillus cereus* by our taxonomical studies. The antibiotic was extracted from the culture broth and purified by ion exchange chromatographies. The structure of cispentacin was determined to be (1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid by spectral analysis. It showed weak inhibitory activity against *Candida albicans* A9540 and *Cryptococcus neoformans* IAM 4514 only in certain media and no activity against other fungi and bacteria. In a murine systemic infection model with *C. albicans*, the antibiotic exhibited strong protection of the mice from the lethal infection by intravenous, intramuscular, or oral administration.

This report describes the taxonomy of the producing organism and the production, isolation, physico-chemical properties and structure of cispentacin. The biological activities of the antibiotic are reported in a companion paper[1].

Taxonomy

Strain L450-B2 was isolated from a soil sample collected around *Berberis* (barbering) tree at Cuenca, Providence Azuay, Ecuador. The strain is an endospore-forming, Gram-positive rod bacterium. As shown in Table 1, the cultural and physiological characteristics of strain L450-B2 correspond to the genus *Bacillus*[2]. The characteristics of strain L450-B2 are similar to those of *Bacillus megaterium* or *B. cereus*. It was, however, differentiated from *B. megaterium* in the colonial morphology, V-P reaction, growth in anaerobic agar, acid production of V-P reaction and resistance to lysozyme, while it could not be differentiated from *B. cereus* in any way. Thus, strain L450-B2 is assigned to *B. cereus*.

Antibiotic Production

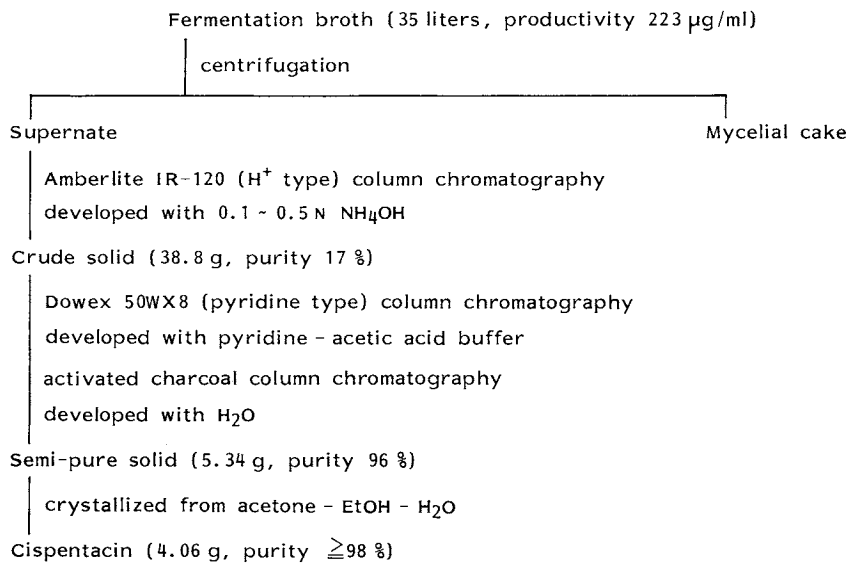
A loopful of the slant culture of *B. cereus* strain L450-B2, was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of vegetative medium composed of glycerol 3%, distiller's solubles (Sungrowth)

† Cispentacin was originally cited as BU-3201 or BMY-28795.

Table 1. Cultural and physiological characteristics of strain L450-B2.

Cell mass growth in Tryptosoy broth	Floccose, sedimented and white; not viscous	Catalase	+
		V-P reaction	+
		Growth in anaerobic agar	+
Colony on nutrient agar (28°C, 6 days):		Growth at 50 and 65°C	−
		Growth in 7% NaCl	+
Color	Whitish	Gas from glucose	−
Extreme	Rhizoid outgrowth	NO_3 reduced to NO_2	+
Surface	Dull, frosted glass appearance	Starch hydrolyzed	+
		pH in V-P medium, <6	+
Size (mm)	14~18	Acid from:	
Microcolony[8]	Parallel rows of straight chains of rods	Glucose	+
		Xylose	−
		Arabinose	−
		Mannose	−
		Hydrolysis of casein	−
		Growth with lysozyme (0.001%)	+

Scheme 1. Isolation and purification of cispentacin.



1.5%, Pharmamedia (Traders Protein) 1%, fish meal (Hokuyo Suisan) 1% and $CaCO_3$ 0.6% (the pH being adjusted to 7.0 prior to sterilization).

The seed flask was shaken on a rotary shaker (200 rpm) at 28°C for 4 days and 5 ml of the culture was transferred into a 500-ml Erlenmeyer flask containing 100 ml of the production medium having the same composition as the vegetative medium. The fermentation was carried out at 28°C for 5 to 7 days on a rotary shaker. The antibiotic activity in the fermentation broth was monitored by HPLC analysis using a modified PICO-TAG method (Waters) and also by a broth dilution assay using *C. albicans* A9540[1]. The maximal antibiotic production was approximately 175 μg/ml.

A high-producing strain, L450-B2-C20, obtained by the repeated single colony isolation technique, yielded 660 μg/ml of the antibiotic.

Isolation

The isolation of cispentacin from the fermentation broth is outlined in Scheme 1. The activity was monitored by bioassay using *C. albicans* A9540 and also by TLC (silica gel, CH_3CN-H_2O-28% NH_4OH (20:6:1), ninhydrin). The purified cispentacin was crystallized from acetone-EtOH-H_2O mixture as colorless prisms.

Physico-chemical Properties

Cispentacin exhibited the physico-chemical properties summarized in Table 2. It was readily soluble in water, dimethylformamide and dimethyl sulfoxide, slightly soluble in methanol and insoluble in acetone and ethyl acetate. It gave positive response to ninhydrin reagent. The antibiotic did not exhibit absorption above 210 nm in water. The IR, ^1H and ^{13}C NMR spectra of cispentacin are shown in Figs. 1, 2 and 3, respectively.

Structural Studies

Cispentacin was shown to have a molecular formula of $C_6H_{11}NO_2$ based on its mass spectrum and ^{13}C NMR. Broad IR absorption at around 3000~2100 and 1660~1500 cm^{-1} together with positive

Table 2. Physico-chemical properties of cispentacin.

Nature	Colorless prisms
MP	204~206°C
$[\alpha]_D^{25}$	$-10.7°$ (c 1.0, H_2O)
SI-MS (m/z)	168 $(M+K)^+$, 152 $(M+Na)^+$, 130 $(M+H)^+$
Formula	$C_6H_{11}NO_2$
UV	End absorption
IR (KBr) cm^{-1}	2950, 2870, 2680, 2550, 2200, 1645, 1550, 1415, 1335, 1310, 1170, 1070, 840
^1H NMR (400 MHz, D_2O) δ	1.70~1.89 (4H, m), 2.04~2.15 (2H, m), 2.87 (1H, br q, J=*ca.* 6.2 Hz), 3.73 (1H, br q, J=*ca.* 6.2 Hz)
^{13}C NMR (100 MHz, D_2O) δ	22.0 (t), 28.8 (t), 30.2 (t), 48.4 (d), 53.7 (d), 181.7 (s)

Fig. 1. IR spectrum of cispentacin (KBr).

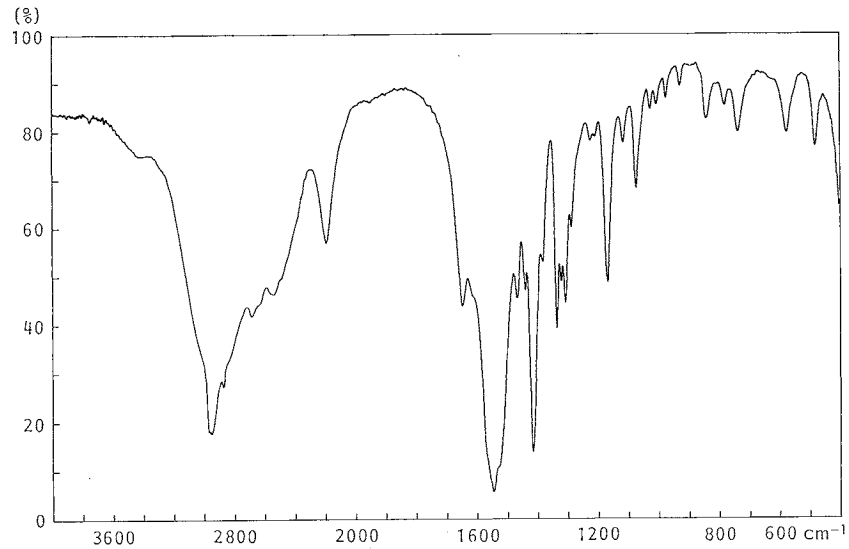


Fig. 2. ^{1}H NMR spectrum of cispentacin (400 MHz, in D_2O).

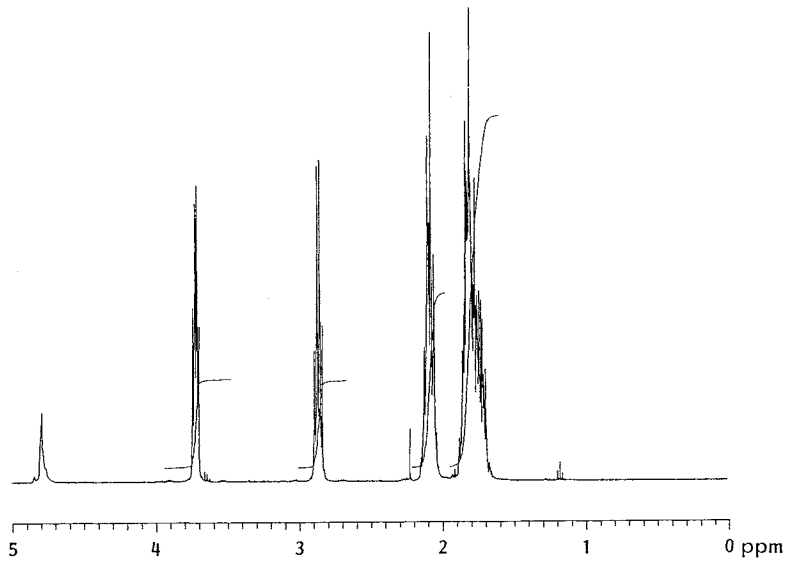
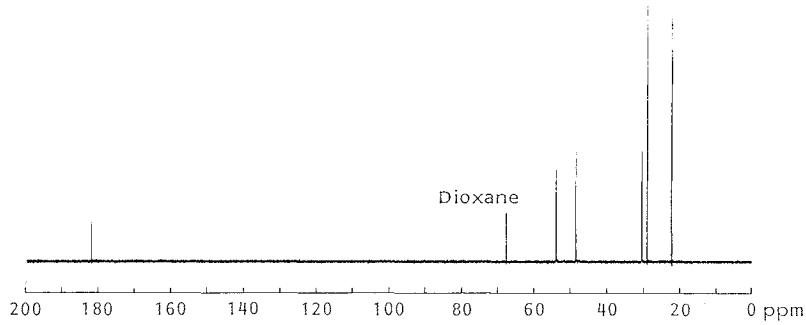
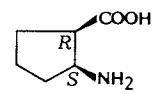


Fig. 3. ^{13}C NMR spectrum of cispentacin (100 MHz, in D_2O).



response to ninhydrin indicated that the antibiotic is an amino acid. The ^{13}C NMR spectrum (Fig. 3) showed signals assignable to a carboxylic acid (181.7 ppm), three methylenes (22.0, 28.8 and 30.2) and two methines (48.4 and 53.7). The ^{1}H NMR (Fig. 2) displayed four protons at δ 1.70~1.89 (m), two protons at δ 2.04~2.15 (m) and one proton each at δ 2.87 and 3.73 (both br q, J=*ca.* 6.2 Hz). These NMR data suggested that cispentacin is 2-aminocyclopentane-1-carboxylic acid. The mp of the antibiotic (204~206°C) is consistent with that reported for the *cis* isomer[3] but significantly different from that of the *trans* isomer. When treated with 1-(3-methylaminopropyl)-3-ethylcarbodiimide in water, cispentacin cyclized to its β-lactam. The CD spectrum of the lactam exhibited a positive Cotton effect (θ +1,300°) at 214 nm which indicated 1*R*,2*S* configuration of the antibiotic[4].

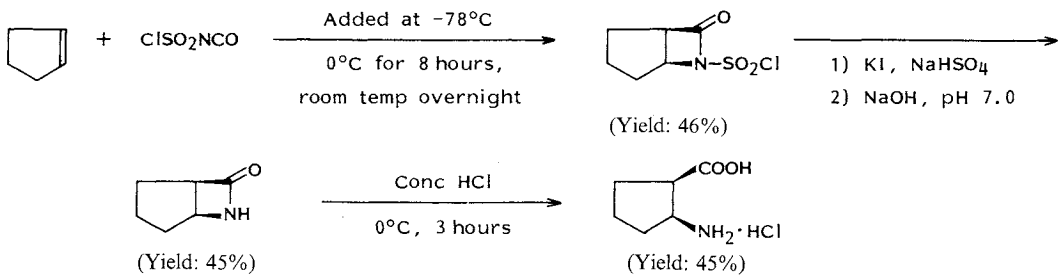


(1*R*,2*S*)-2-Aminocyclopentane-1-carboxylic acid

Synthesis of (±)-*cis*-2-Aminocyclopentane-1-carboxylic Acid ((±)-*cis*-ACPA) and Its Optical Resolution

In order to confirm the structure of cispentacin, (±)-*cis*-ACPA was synthesized by the procedure of

Scheme 2. Preparation of (±)-*cis*-ACPA.



NATIV and RONA[5]. Coupling of chlorosulfonyl isocyanate and cyclopentene at −78°C yielded 6-chlorosulfonyl-6-azabicyclo[3.2.0]heptane-7-one which was treated with potassium iodide and sodium bisulfite followed by sodium hydroxide to remove the chlorosulfonyl group. The resulting β-lactam derivative was hydrolyzed with concentrated hydrochloric acid to give (±)-*cis*-ACPA in *ca.* 10% overall yield. The synthesized (±)-*cis*-ACPA was identical with cispentacin in physico-chemical properties, although the optical rotational value of the (±)-*cis*-ACPA was zero while that of the natural antibiotic was −10.7°.

Optical resolution of (±)-*cis*-ACPA was carried out by fractional crystallization with (+)-dehydroabietylamine (DAA). Treatment of (±)-*cis*-ACPA with benzyl chloroformate afforded (±)-benzyloxycarbonyl(Cbz)-*cis*-ACPA in *ca.* 60% yield after crystallization. The acid was mixed with DAA and the resulting salt was repeatedly crystallized from acetonitrile. Cbz-*cis*-ACPA was recovered from the crystals by alkali treatment and deblocked by catalytic hydrogenation to yield (+)-*cis*-ACPA ($[\alpha]_D^{25}$ +8.9°). The salt contained in the filtrate of the crystallization was deblocked similarly to give (−)-*cis*-ACPA ($[\alpha]_D^{25}$ −7.3°).

The synthetic (±)-*cis*-ACPA was about half as active as cispentacin against *C. albicans* A9540 by the broth dilution assay (MIC 25.0 μg/ml; *cf.* MIC of cispentacin 12.5 μg/ml). The optically resolved (−)-*cis*-ACPA (optical purity 88%) showed comparable antifungal activity (MIC 12.5 μg/ml) to that of cispentacin, while (+)-*cis*-ACPA (optical purity 93%) was nearly inactive (MIC >400 μg/ml).

Discussion

Our antifungal screening with emphasis on systemic *in vivo* activity has discovered a new simple amino acid-type antibiotic, cispentacin. The antibiotic exhibits only weak *in vitro* activity against *C. albicans* and *C. neoformans* but potent and selective *in vivo* activity against *C. albicans* and a few other yeasts as described in the accompany paper[1].

cis-ACPA has been synthesized[5,6] and also isolated from the hydrolysate of the antibiotic amipurimycin[7]. However, optical resolution of the synthetic (±)-*cis*-ACPA has not been attempted and the absolute configuration of the amino acid obtained from amipurimycin was not reported.

Cispentacin represents the first *cis*-ACPA, optically identified. Also, we report in this paper the first optical resolution of the synthetic amino acid.

It is of particular interest that, cispentacin with a (1*R*,2*S*)-2-aminocylopentane-1-carboxylic acid structure has prominent *in vivo* antifungal activity[1] while its antipode, (1*S*,2*R*)-isomer, is inactive against fungi.

Experimental

Isolation and Purification of Cispentacin

The harvested broth (35 liters) was separated to supernate and mycelial cake by use of a Sharples

centrifuge (Kokusan No. 4A). The supernate was charged on a column of Amberlite IR-120 (H^+ type, 5 liters) and the column was developed with water (10 liters), 0.1 N NH_4OH (33 liters) and 0.5 N NH_4OH (8 liters). The eluates were collected in fractions which were examined by bioassay using *C. albicans* A9540 and TLC (silica gel, CH_3CN-H_2O-28% NH_4OH (20:6:1), detection; ninhydrin reagent, Rf 0.21). The appropriate fractions were pooled and concentrated *in vacuo* to give crude solid cispentacin (38.8 g, purity 17%). The solid (38.0 g) was dissolved in water and charged on a column of Dowex 50WX8 (pyridine type, 1.2 liters) which had been equilibrated with 0.1 M pyridine-formic acid buffer (pH 3.1). Elution was performed first with the same buffer (2 liters) and then with a linear gradient from 0.1 M pyridine-acetic acid buffer (pH 4.4, 2 liters) to 0.6 M pyridine-acetic acid buffer (pH 5.1, 2 liters). After monitoring by the bioassay and TLC, the active fractions were combined and concentrated *in vacuo*. The solid obtained (7.61 g, purity 75%) was chromatographed on a column of activated charcoal (300 ml) developed with water. The active eluates were combined and concentrated *in vacuo* to afford a white homogeneous solid of cispentacin (5.34 g, purity 96%).

Crystallization of the solid from acetone - EtOH - H_2O gave colorless prisms of pure cispentacin (4.06 g).

Synthesis of (±)-*cis*-ACPA

The synthesis of (±)-*cis*-ACPA was carried out by the procedure reported by NATIV and RONA[5]. Chlorosulfonyl isocyanate (49 g, 0.35 mol) was added to cyclopentene (25 g, 0.37 mol) at −78°C under gentle stirring. The solution was stirred at 0°C for 8 hours and then at room temperature overnight. The reaction solution was poured into ice-water and extracted with ethyl acetate (500 ml). The extract was washed with saturated sodium chloride solution and dried over anhydrous sodium sulfate. Evaporation of the solvent gave 33 g of *N*-chlorosulfonyl β-lactam. The β-lactam (33 g, 0.16 mol) was poured into cold potassium iodide solution (200 ml, 1.5 mol %) containing sodium bisulfite (2.5 g) and the solution was neutralized with 12 N sodium hydroxide. The resulting solution was extracted with ethyl acetate (300 ml). The organic phase was washed with saturated sodium chloride solution and dried over anhydrous sodium sulfate. Evaporation of the solution yielded 6-azabicyclo[3.2.0]heptan-7-one (7.8 g). The product (7.7 g, 69 mmol) was dissolved in concentrated hydrochloric acid (150 ml) at 0°C. An exothermic reaction took place and white crystals deposited. After addition of acetone (150 ml), the product was collected and recrystallized from aqueous acetone to give pure (±)-*cis*-ACPA hydrochloride (5.2 g, 31 mmol).

The hydrochloride (150 mg, 0.91 mmol) was dissolved in water (2 ml) and applied on a column of Amberlite IR-120 (H^+, 30 ml). The resin was washed with water (200 ml) and eluted with 1 N NH_4OH (100 ml). The eluate was evaporated to dryness to leave a white crystalline powder. Recrystallization of the solid from aqueous acetone afforded pure zwitterionic (±)-*cis*-ACPA (80 mg, 0.62 mmol).

Optical Resolution of (±)-*cis*-ACPA

Cbz-Cl (3 ml) was added dropwise to a stirred mixture of (±)-*cis*-ACPA hydrochloride (2 g, 12 mmol) and $NaHCO_3$ (5 g) in H_2O (50 ml) and diethyl ether (30 ml) at 0°C. The mixture was stirred vigorously for 5 hours at 0°C and then the aqueous layer was collected. The solution was adjusted to pH 2.0 by 2 N HCl and extracted with ethyl acetate (50 ml). The extract was concentrated *in vacuo* to dryness, and the residue was crystallized from benzene to yield (±)-Cbz-*cis*-ACPA (1.9 g, 7.2 mmol).

To a solution of 1.4 g (5.2 mmol) of the acid in 60 ml of methanol was added a solution of 1.5 g (5.2 mmol) of DAA in 20 ml of methanol. The mixture was shaken vigorously for 5 minutes and concentrated *in vacuo* to dryness. The resultant solid was dissolved in 100 ml of hot acetonitrile and left to stand at room temperature for 1 day. The precipitate was collected by filtration, again dissolved in 100 ml of hot acetonitrile and left to stand at room temperature for 2 days, to yield fine needles of the DAA salt of Cbz-*cis*-ACPA. The crystalline salt was collected by filtration and recrystallized from 100 ml of hot acetonitrile (458 mg).

(+)-*cis*-ACPA: To a suspension of 220 mg of the above recrystallized salt in 30 ml of 0.1 N NaOH was added 30 ml of diethyl ether with stirring. The stirring was continued until all of the solid dissolved. The separated aqueous layer was washed with 30 ml of diethyl ether, and then adjusted to pH 2.0 by 1 N HCl, and extracted with ethyl acetate (30 ml × 2). The extract was concentrated *in vacuo* to dryness. The residue was dissolved in 25 ml of 60% aqueous ethanol and hydrogenated over 5% palladium on carbon (60 mg) at atmospheric pressure for 15 hours. After removal of the catalyst by filtration, the filtrate was

evaporated *in vacuo* to yield 35 mg of (+)-*cis*-ACPA.

MP 182~184°C; $[\alpha]_D^{25}$ +8.9° (*c* 0.5, H_2O); optical purity by HPLC, (1*R*,2*S*)-(1*S*,2*R*), 7:93; condition, column: TSK gel ENANTIO L1 (4.6 mm i.d. × 150 mm, Tosoh Co.), mobile phase: 1 mM $CuSO_4$, temperature: 50°C, flow rate: 0.5 ml/minute, detection: UV absorption at 254 nm, retention time: (−)-*cis*-ACPA, 7.7 minutes and (+)-*cis*-ACPA, 6.5 minutes.

(−)-*cis*-ACPA: The filtrate in the above crystallization was evaporated *in vacuo* to dryness (1.3 g). The residue was dissolved in a hot mixture of benzene (20 ml) and *n*-hexane (20 ml) and left to stand at room temperature for 2 days. The crystals deposited (780 mg) were filtered and the filtrate was evaporated to yield 474 mg of solid which contained (−)-*cis*-ACPA rich DAA salt. The salt (220 mg) was deblocked as for the above (+)-*cis*-isomer to give 48 mg of (−)-*cis*-ACPA.

MP 179~182°C; $[\alpha]_D^{25}$ −7.3° (*c* 0.5, H_2O); optical purity: (1*R*,2*S*)-(1*S*,2*R*), 88:12 by chiral HPLC.

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