CANDIPLANECIN, A NEW ANTIBIOTIC FROM *AMPULLARIELLA REGULARIS* SUBSP. *MANNITOPHILA* SUBSP. NOV.

II. ISOLATION, PHYSICO-CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITIES

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New antibiotic, candiplanecin, was found in the culture broth of an actinomycete identified as *Ampullariella regularis* subsp. *mannitophila* subsp. nov. Candiplanecin was produced by conventional submerged culture and isolated by column chromatography on Diaion HP 20 followed by extraction with ethyl acetate and further column chromatography on Sephadex LH-20 column and finally on PrepPAK-500/C_{18} column. The antimicrobial spectrum of the antibiotic revealed its activity against yeasts and fungi.

As described in the preceding paper[1], a new antifungal antibiotic, candiplanecin, was produced by a new subspecies of *Ampullariella* designated as *A. regularis* subsp. *mannitophila* subsp. nov. Physico-chemical properties coupled with biological properties of candiplanecin differs from those of known antibiotics produced by *Ampullariella* such as taitomycin[2], neplanocin A[3] and related antibiotics[4,5,6] and as well as those of antibiotics from streptomycete. In the present paper, isolation, physico-chemical properties and biological properties of candiplanecin are described.

Isolation

Three hundred and fifty liters of the culture broth from a 600-liter fermentor was filtered with an aid of infusorial earth (Celite 545, John-Manville Products Corp., U.S.A.). The filtrate (280 liters) thus obtained was adjusted to pH 3.0 with diluted hydrochloric acid and further filtered with an aid of Celite 545. The pH 3.0 filtrate (270 liters) was adsorbed on a column of 27 liters of Diaion HP 20 (Mitsubishi Chemical Industries Ltd.). The column was washed with 54 liters of deionized water and the antibiotic was eluted with 80% alkaline aqueous methanol (pH 12). The active fraction (140 liters) was neutralized to pH 7.0 and concentrated to remove methanol under reduced pressure. The concentrate (40 liters) was adjusted to pH 2.5 with diluted hydrochloric acid and the antibiotic was extracted two times each with 40 liters of ethyl acetate. The extracts were pooled and concentrated to 200 ml under reduced pressure. The concentrate was applied on a column consisting of 1.85 liters of Sephadex LH-20 equilibrated with ethyl acetate and the column was developed with the same solvent. The active fraction (1.1 liters) was concentrated under reduced pressure to dryness, dissolved in 200 ml of chloroform and applied onto a Florisil column (40 g, Kanto Kagaku Co., Japan). The column was developed first with 800 ml of chloroform then ethyl acetate. The eluates were collected in each 100 ml fraction. Fractions No. 9~19 were pooled and concentrated to yield 3 g of oily substance (containing a 234 mg of candiplanecin). The oily substance was dissolved in 13 ml of methanol and injected into one Prep-

PAK-500/C_{18} cartridge (Waters Ltd., U.S.A.) equilibrated with 60% aqueous methanol, then eluted with 85% aqueous methanol at a flow rate of 100 ml/minute and the eluate was collected in each 300 ml fraction. Fractions No. 11~14 were pooled and concentrated to 280 ml under reduced pressure. The concentrate was adjusted to pH 2.5 with diluted hydrochloric acid and extracted two times each with 250 ml of ethyl acetate. The extract was pooled, concentrated to 5 ml *in vacuo* and applied on the Sephadex LH-20 column (900 ml) equilibrated with the solvent mixture consisting of chloroform - ethyl acetate (1: 1 in volume ratio) and developed with the same solvent system. The eluate was collected in 10-ml fractions and fractions No. 55~67 were pooled and concentrated to give 165 mg of pale yellowish oil of candiplanecin.

Physico-chemical Properties

Candiplanecin was obtained as pale yellow oil, soluble in methanol, ethyl acetate and chloroform, but sparingly soluble in water. The antibiotic reacted positively to sulfuric acid, iodine and potassium permanganate-sulfuric acid. It behaved as an acidic substance on high voltage paper electrophoresis (55 V/cm, 0.8 mA/cm) in 0.1 M tris-HCl buffer at pH 7.5 for 30 minutes. The relative mobility was 0.46 when the mobility of bromophenol blue was defined as 1.0. The molecular formula was estimated to be $C_{17}H_{26}O_7$ from the elementary analysis and the number of peaks of CMR spectrum. These results as well as other physical and chemical properties are summarized in Table 1. The IR, UV, PMR and CMR spectra of candiplanecin are shown in Figs. 1, 2, 3 and 4, respectively.

Existence of a carboxylic acid in candiplanecin molecule revealed by its IR spectrum and behavior on high voltage paper electrophoresis was also supported by the fact that candiplanecin gives a methyl ester when treated with ethereal diazomethane. These data coupled with its biological properties described below suggested that candiplanecin is a new antibiotic.

Fig. 1. Infrared absorption spectrum of candiplanecin in $CHCl_3$.

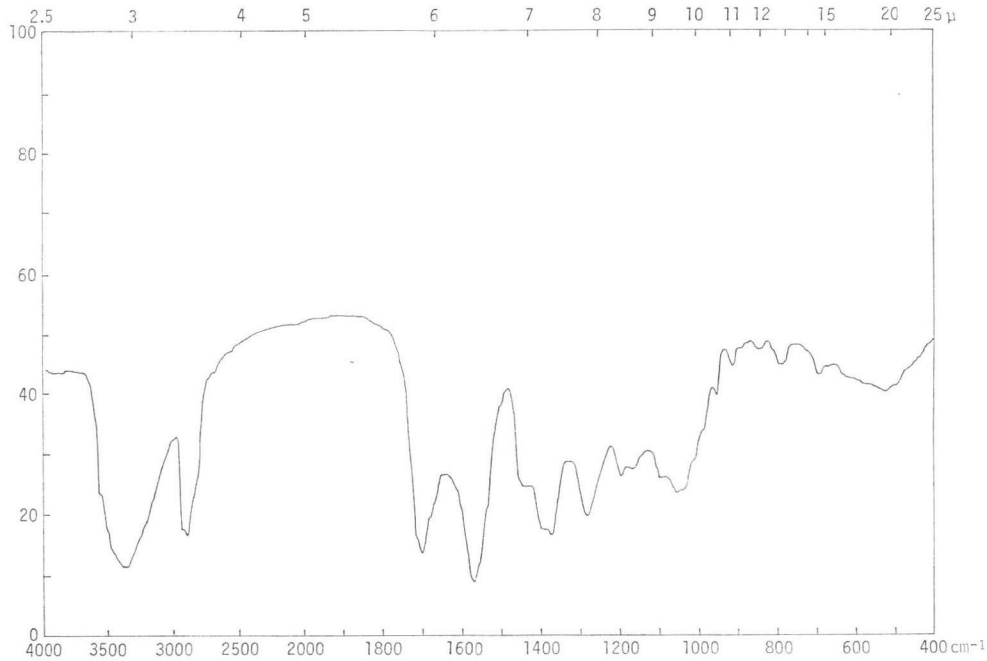


Table 1. Physico-chemical properties of candiplanecin.

Nature	Acidic, pale yellow oil
$[\alpha]_D^{20}$	+26.3° (*c* 0.85, $CHCl_3$)
Elementary analysis (%)	Found C 59.29, H 7.37
	Calcd. for $C_{17}H_{26}O_7$.
	C 59.63, H 7.65
λ_{max}^{MeOH} ($E_{1cm}^{1\%}$)	270 nm (296)
$\nu_{cm^{-1}}$ ($CHCl_3$)	3700~2500, 2950, 2900, 1720, 1700, 1570
Rf*	0.4
Color reaction	
Positive	I_2, $KMnO_4$, H_2SO_4
Negative	Ninhydrin

* Merck silica gel plate F_{254}, Art 5715 ($CHCl_3$-MeOH, 4: 1)

Fig. 2. UV spectrum of candiplanecin in methanol.

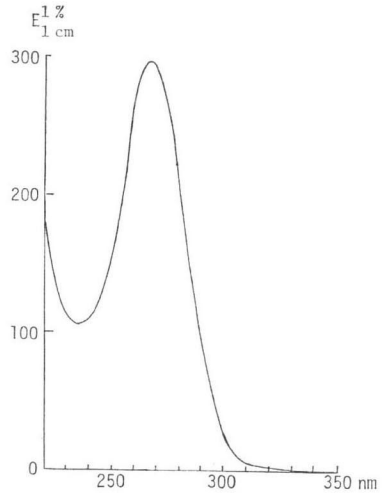


Fig. 3. PMR spectrum of candiplanecin in $CDCl_3$ (60 MHz).

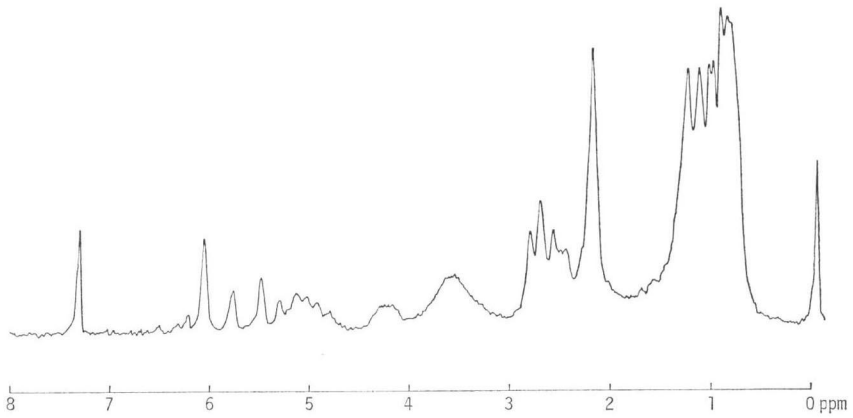
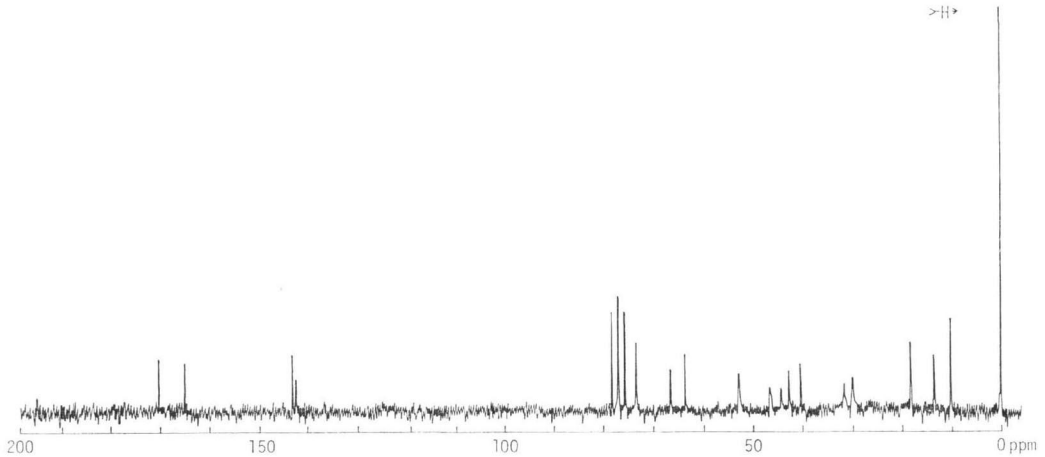


Fig. 4. CMR spectrum of candiplanecin in $CDCl_3$.



Biological Activity

The minimal inhibitory concentrations (MICs) of candiplanecin against bacteria, yeasts and fungi were determined by a serial two-fold agar dilution method. The results are shown in Table 2. The medium used for bacteria was heart infusion agar, that for yeasts and some fungi, such as *Aspergillus, Cryptococcus, Penicillium* and *Trichophyton* was SABOURAUD-dextrose agar and that for the other fungi was potato-dextrose agar. The MICs were determined after 24 or 48 hous at 37°C for bacteria and 2 or 14 days at 26°C for yeasts and fungi. Candiplanecin was weakly active against yeasts and fungi but inactive against bacteria even at a concentration of 100 μg/ml.

The acute toxicity (LD_{50}) of candiplanecin in mice by intravenous administration was 1 mg/kg.

Table 2. Antimicrobial spectrum of candiplanecin.

Test organism	Medium*	MIC (μg/ml)
Staphylococcus aureus FDA 209P JC-1	1	>100
Bacillus subtilis PCI 219	1	>100
Escherichia coli NIHJ JC-2	1	>100
Proteus vulgaris OX19	1	>100
Pseudomonas aeruginosa SANK 73860	1	>100
Aspergillus oryzae	2	12.5
Candida albicans YU 1200	2	50
Cryptococcus neoformans SANK 59863	2	100
Penicillium chrysogenum SANK 12763	2	25
Saccharomyces cerevisiae SANK 50161	2	25
Trichophyton mentagrophytes SANK 20767	2	100
T. rubrum IFO 5467	2	12.5
Cochliobolus miyabeanus SANK 10458	3	12.5
Colletorichum lagenarium	3	12.5
Fusarium oxysporum SANK 21772	3	100
Pellicularia filamentosa SANK 12268	3	12.5
Pyricularia oryzae SANK 14758	3	25

Inoculum level: Bacteria 10^8 CFU/ml.
Candida albicans 10^6 CFU/ml.

*1 Heart infusion agar
2 SABOURAUD-dextrose agar
3 Potato-dextrose agar

References

1) ITOH, Y.; R. ENOKITA, T. OKAZAKI, S. IWADO, A. TORIKATA, T. HANEISHI & M. ARAI: Candiplanecin, a new antibiotic from *Ampullariella regularis* subsp. *mannitophila* subsp. nov. I. Taxonomy of producing organism and fermentation. J. Antibiotics 34: 929~933, 1981
2) TORIKATA, A.; R. ENOKITA, H. IMAI, T. OKAZAKI, Y. ITOH & M. ARAI: Studies on the antibiotics from family *Actinoplaneceae*. II. Taxonomy of the producers and their antibiotics. Ann. Meeting of Agricult. Chem. Soc. Japan Abstr. Paper 3P-19, 1980
3) YAGINUMA, S.; N. MUTO, M. TSUJINO, Y. SUDATE, M. HAYASHI & M. OTANI: Studies on neplanocin A, new antitumor antibiotic. I. Producing organism, isolation and characterization. J. Antibiotics 34: 359~366, 1981
4) Toyo jozo Co. Ltd.: A new antibiotic, A-11079-B_2. Japan Kokai 55-24,157, Feb. 21, 1980
5) Toyo jozo Co. Ltd.: A new antibiotic, neplanocin D and its derivatives. Japan Kokai 55-100,388, July 31, 1980
6) Toyo jozo Co. Ltd.: New antibiotics, neplanocin B and F. Japan Kokai 55-113,786, Sep. 2, 1980