MULUNDOCANDIN, A NEW LIPOPEPTIDE ANTIBIOTIC

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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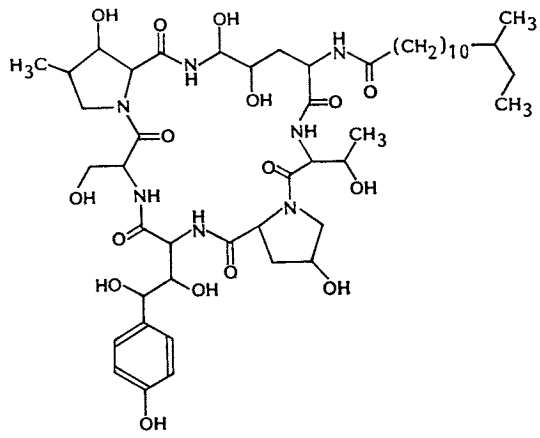
Mulundocandin, a new lipopeptide antibiotic, was isolated from the culture broth of a strain of *Aspergillus sydowi* No. Y-30462. The antibiotic, obtained as a colorless amorphous powder having the molecular formula $C_{48}H_{77}N_7O_{16}$, is an antifungal antibiotic active against yeasts and filamentous fungi.

In the course of our screening for new antibiotics from fungi, a fungal culture No. Y-30462 was isolated from soil collected in Bangladesh. It was identified as *Aspergillus sydowi* (Bainier and Sartory) Thom and Church var. nov. *mulundensis* Roy. It produces a new antibiotic which we have named mulundocandin (Fig. 1). This paper describes the taxonomy of the producing strain and the fermentation, isolation, and physico-chemical and biological properties of mulundocandin.

Taxonomical Studies

Fig. 1. Structure of mulundocandin.



Morphological Characteristics

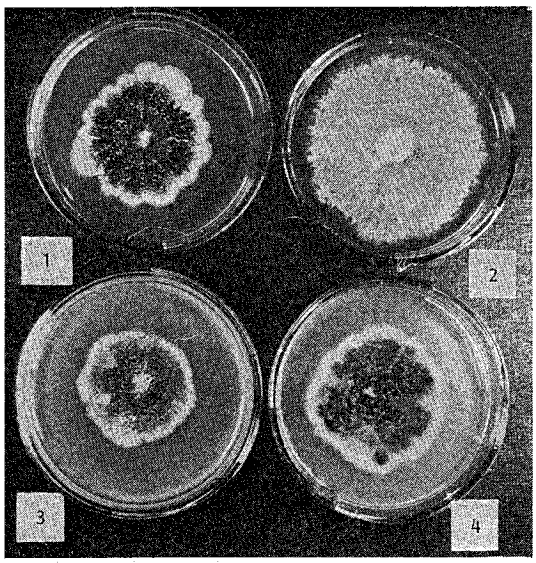
Conidiophores mostly arise from submerged hyphae, smooth and hyaline, thick-walled, 40~125×5~7.5 μm. Generally they are transformed at their tips into a globose vesicle (20 μm i.d.). Metulae are present over 3/4th surface of the vesicle, each having 1~3 phialides. Phialides are bottle shaped, 2.5~4×5~10 μm, bearing chains of conida. The conida are globose, delicately spinulose, usually 2.5~4×2.5~4.5 μm, hyaline when single but appear green in mass. The hulle cells are abundant, thick-walled, colorless at first but become purple with age, scattered unevenly, 18.7~31.2×25~37.5 μm.

Cultural Characteristics

Comparative growth morphology was studied on four different media *viz.* SABOURAUD's agar, potato-glucose agar, YpSs agar[1] and CZAPEK-DOX agar. Comparative growth morphology of Y-30462 after 2 weeks is shown in Fig. 2.

On SABOURAUD's agar, the growth is rapid (colony diameter 5 cm in 2 weeks at 26±1°C). Initially felty, bluish green at places with furrows and ridges, later becomes granular, dark purple with

Fig. 2. Comparative growth morphology of Y-30462 after 2 weeks.
1. SABOURAUD's agar. 2. Potato-glucose agar. 3. YpSs agar. 4. CZAPEK-DOX agar ×1/2.



lots of exudate in small droplets. The purple areas represent the hulle cells. The colony margin is wavy and low growing. Reverse appears yellowish-orange.

Growth on potato-glucose agar is faster (colony diameter 7.5 cm under the same conditions). It appears bluish-green with white margin. Scanty woolly growth appears at the center, otherwise granular. Minute droplets of exudate (purple color) are seen. Reverse appears in shades of brown.

Colony on YpSs agar grows moderately well (colony diameter 4.5 cm in 2 weeks at 26±1°C). The major growth area is purplish and granular due to hulle cells with plenty of dark colored exudates. Sectoring at places is observed. The outer margin of the colony is white. Scanty woolly growth is seen at the center. Reverse appears in shades of brown.

On CZAPEK-DOX agar, the growth is initially slow but later becomes rapid (colony diameter 5 cm under the same conditions). The outer margin is white and wavy followed by a small zone of bluish green and then purplish. Scanty exudates are seen over the purple zone. The reverse is dark brown.

The above morphology and cultural characteristics identify the fungus to be *Aspergillus sydowi* (Bainier and Sartory) Thom and Church. However, since hulle cells are present in abundance and since Y-30462 produces a new antibiotic, mulundocandin, we believe this fungus to be a new variant and have named it *Aspergillus sydowi* (Bainier and Sartory) Thom and Church var. nov. *mulundensis* Roy.

Fermentation

Shake Flask Fermentation

The fungal culture No. Y-30462 was maintained on SABOURAUD's glucose agar. A few loop-fuls from a well sporulated slant culture were inoculated into a 500-ml wide mouth Erlenmeyer flask with 100 ml of the seed medium and grown at 26°C (±1°C) for 60 hours on a rotary shaker at 240 rpm. This was used for inoculating the production medium. The seed medium contains soybean meal 20 g, glucose 30 g, $CaCO_3$ 6 g, NaCl 3 g, NH_4Cl 2.5 g, KH_2PO_4 2 g in 1 liter of demineralized water. The pH of the medium is adjusted to 6.5 prior to autoclaving. The production medium consists of beef extract 3 g, Tryptone 5 g, glucose 10 g, soluble starch 24 g, yeast extract 5 g, $CaCO_3$ 4 g, $MnCl_2 \cdot 4H_2O$ 0.5 mg, $ZnSO_4 \cdot 7H_2O$ 0.22 mg, $CaCl_2$ 0.55 mg, $FeSO_4 \cdot 7H_2O$ 0.5 mg, $CuSO_4 \cdot 5H_2O$ 0.16 mg, $CoCl \cdot {}_2 6H_2O$ 0.16 mg in 1 liter of demineralized water. The pH of the medium is adjusted to 6.5 before sterilization. The fermentation is carried out in 1-liter Erlenmeyer flasks with 200 ml medium inoculated with 1% seed and incubated at 26°C (±1°C) on a rotary shaker for 76 hours at 240 rpm.

Fermentation of Y-30462 was also carried out in stainless steel fermenters of 15 liters, 150 liters

Table 1. Fermentation parameters for the culture No. Y-30462.

Parameters	Fermenters		
	15 liters	150 liters	390 liters
Temperature	26~27°C	26~27°C	26~27°C
Aeration	6~8 l.p.m.	60~80 l.p.m.	160~224 l.p.m.
Agitation	160 rpm	100~110 rpm	100~110 rpm
Harvest time	66 hours	66 hours	66 hours
pH prior to autoclaving of the medium	6.5	6.5	6.5
Desmophen (Bayer AG)	4 ml	40 ml	75 ml

l.p.m.: Liter per minute.

Scheme 1. Isolation and purification of mulundocandin.

Culture filtrate (75 liters)
- adsorption on Diaion HP-20 (0.7 liter)
- elution with MeOH (2 liters)

Concentrated active eluates (75 ml)
- dilution with H_2O to 1 liter
- extraction with EtOAc (300 ml×6)

Concentrated extracts (11 g)
- addition of CH_3CN (160 ml)
- filtration

Residue (280 mg)
- droplet counter-current chromatography (DCCC*)
- solvent system: $CHCl_3$ - MeOH - PrOH - H_2O (9: 12: 1: 8)
- stationary phase; upper layer
- mobile phase; lower layer

Mulundocandin (152 mg)

* Alternatively column chromatography using TLC grade silica gel as the adsorbent (ratio of compound to silica gel, 1: 500) and EtOAc - PrOH - H_2O (5: 3: 1) as eluant may be used.

and 390 liters capacity with 10 liters, 95 liters and 270 liters of the same production medium. Desmophen (Bayer AG) is used as antifoam agent in the fermenters. The fermentation parameters are given in Table 1.

The activity of the fermented broth was determined by agar-diffusion assay using *Candida albicans* and *Saccharomyces cerevisiae* as test strains.

Isolation and Purification of Mulundocandin

Mulundocandin, present both in the culture filtrate and mycelium, was isolated primarily from the former because of higher yield and easier purification. Recovery of mulundocandin was about 2 mg per liter of the culture filtrate. The isolation procedure is shown in Scheme 1.

Physico-chemical Properties

Mulundocandin is a colorless amorphous powder having the molecular formula $C_{48}H_{77}N_7O_{16}$: elemental analysis found C 55.70, H 7.82, N 11.29%, calculated for $C_{48}H_{77}N_7O_{16}$: C 57.19, H 7.64, N 9.72%; mp 225°C and $[\alpha]_D^{25}$ −42.77° (*c* 1.6, MeOH). It is soluble in methanol, *N*,*N*-dimethylformamide and dimethyl sulfoxide but insoluble or sparingly soluble in acetonitrile, chloroform and

Fig. 3. UV spectrum (Kontron, Uvikon 810, 200~400 nm) of mulundocandin in methanol (——) and in alkaline methanol (------).

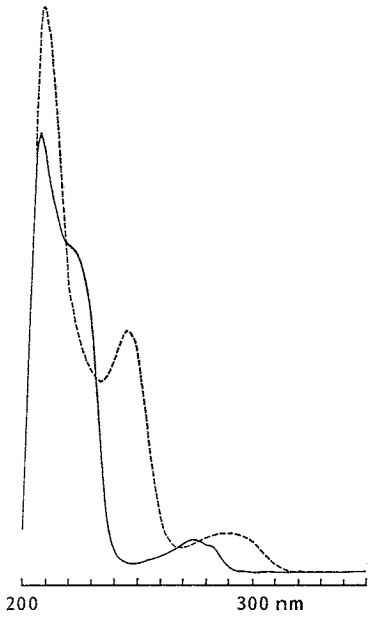


Table 2. Antifungal activity of mulundocandin.

Test organism	MIC (μg/ml)
Candida albicans 200/175	0.97
Saccharomyces cerevisiae	10
Penicillium italicum	>1,000
Aspergillus niger 500/284	31.25
Cercospora beticola	4
Fusarium nivale	>1,000
Botrytis cinerea	125
Trichophyton mentagrophytes 100/25	>125
T. rubrum 100/58	>125
Microsporum gypseum	4
M. canis 150/353	>125

other common organic solvents as well as in water. The compound gives the following color reactions—Pauly test; positive, $KMnO_4$; decolorizes, ninhydrin; negative, $FeCl_3$ spray; negative, benzidine-periodate test; positive, Dragendorf spray; negative, ferric chloride-potassium ferricyanide reagent; positive, PCF test; negative, Ehrlich reagent; negative, iodine (vapour); positive.

The compound gives a UV spectrum (Fig. 3) similar to other echinocandin type of antibiotics: λ_{max} (MeOH) nm 208, 223 (sh), 275, 282 (sh); λ_{max} (0.01 N KOH+MeOH) nm 209, 245, 290. The IR spectrum (Fig. 4) of mulundocandin shows main absorbances at 3333, 2941, 1640, 1613, 1515, 1413, 1220 and 1060 cm^{-1}. The ^1H NMR and ^{13}C NMR spectra are shown in Figs. 5 and 6, respectively. The details of the structure elucidation will be published separately[2].

Biological Properties

Mulundocandin has been found to possess anti-yeast and antifungal properties. The minimum

Fig. 4. IR spectrum (Perkin-Elmer 157, KBr) of mulundocandin.

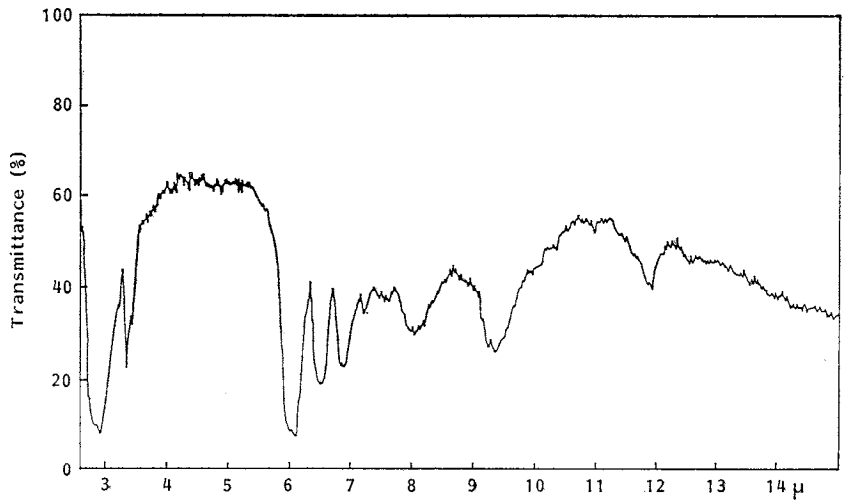


Fig. 5. ^{1}H NMR spectrum (Bruker AM 400 WB, 400 MHz) of mulundocandin in DMSO-d_6. Chemical shift in ppm; internal standard tetramethylsilane.

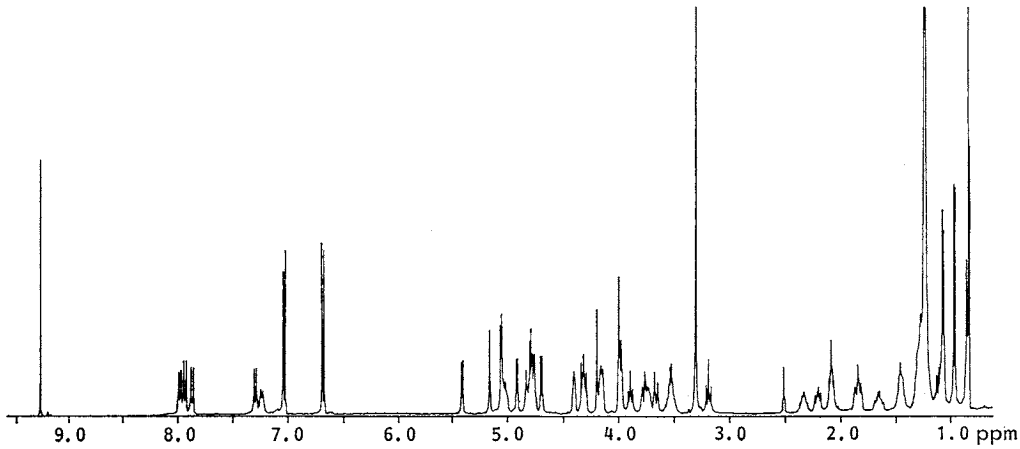
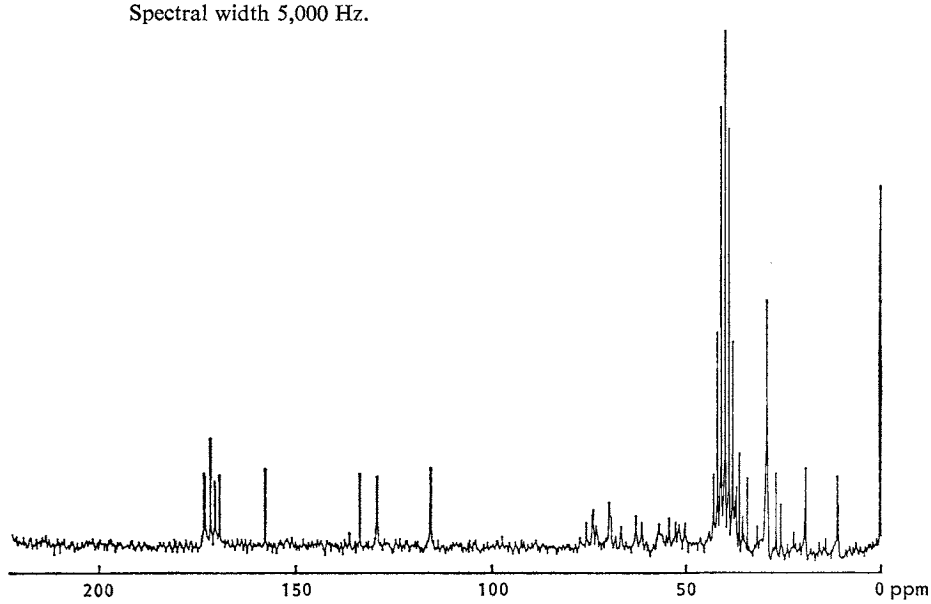


Fig. 6. ^{13}C NMR spectrum (Jeol FX90Q, 22.5 MHz) of mulundocandin in DMSO-d_6. Spectral width 5,000 Hz.



inhibitory concentrations required to inhibit different yeast and fungal strains are shown in Table 2.

Discussion

Mulundocandin is a cyclic peptide with a fatty acid side chain. The UV absorption pattern is similar to the echinocandin[8] type of molecules which are also cyclic peptides with a fatty acid side chain. The molecular weight and the structure of this compound is different from any of the known echinocandin type of antibiotics. Hence mulundocandin is described as a new echinocandin type of antibiotic.

Acknowledgments

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