Notes

L-681,572—A NEW ANTIFUNGAL AGENT
ISOLATION, CHARACTERIZATION, AND BIOLOGICAL ACTIVITY

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A new antifungal agent L-681,572 that exhibits a moderately broad spectrum of activity skewed towards filamentous fungi has been isolated from a stationary fermentation source. Production of antifungal activity by *Fusarium* sp. ATCC 20883 occurred following a two-stage fermentation protocol. The contents of a preserved source of the culture was inoculated into a liquid seed medium (Medium I). Growth phase was in a 250-ml unbaffled Erlenmeyer flask (44 ml volume) at 28°C with agitation at 220 rpm on a rotary shaker with a 5-cm amplitude. After 2 to 4 days a 2.0-ml portion of this growth was used to inoculate each flask of a production medium (Medium II). Production Medium II was incubated at 25°C with agitation at 220 rpm on the rotary shaker as before. Fermentation broths were assayed for antifungal activity and harvested after 9 days of incubation.

Medium I contains corn steep liquor 5 g, tomato paste 40 g, oat flour 10 g, glucose 10 g, trace elements mix 10 ml, distilled water 1,000 ml, presterile pH adjusted to 6.8 with NaOH. Trace elements mix contains: $FeSO_4 \cdot 7H_2O$ 1 g, $MnSO_4 \cdot 4H_2O$ 1 g, $CuCl_2 \cdot 2H_2O$ 25 mg, $CaCl_2$ 100 mg, H_3BO_3 56 mg, $(NH_4)_6MoO_4 \cdot 4H_2O$ 19 mg, $ZnSo_4 \cdot 7H_2O$ 200 mg, 1,000 ml distilled water. Medium II contains millet 15 g, yeast extract 100 mg, alfalfa meal 100 mg, distilled water 15 ml per 250-ml Erlenmeyer flask. The media was sterilized by autoclaving after which an additional 10 ml per flask of distilled water was added and the flasks were reautoclaved.

The antifungal activity was released from the fermented millet solids with 50% aqueous acetone at room temperature for 20 hours. A 1,200-ml solvent extract was obtained from thirty-five 250-ml Erlenmeyer flasks, each containing 40 ml of solid media. Agar diffusion susceptibility plates containing *Penicillium* sp. MF5014 were used to guide the isolation procedure. The extract was evaporated into 400 ml of water and adsorbed on a 120-ml column of Diaion HP-20 resin. The resin was washed with 120 ml of water and the activity was eluted with 350 ml of methyl alcohol. The alcohol eluate, which contained 2.1 g of solids, was evaporated into 100 ml of water and adjusted to pH 5.7. The resin eluate concentrate was extracted with ethyl acetate. The solvent extract was dried with magnesium sulfate and evaporated to yield 1.1 g of drug powder. The antifungal activity was dissolved in 9.5 ml of methylene chloride and chromatographed on a 30-g column of Silica gel 60 (E. Merck). A methyl alcohol gradient in methylene chloride was used to develop the chromatogram. The activity was eluted in fractions containing 10 to 20% methyl alcohol. The most potent fractions were combined and evaporated to yield 554 mg of dried solids. The dried isolate was taken up in 2.4 ml of methyl alcohol-water (3:1) and chromatographed on a column containing 180 ml of LiChroprep RP-18 (E. Merck). The column was developed with 75% aqueous methyl alcohol and 4 ml fractions were collected. The most active fractions yielded 102 mg of dried product. The physico-chemical properties of L-681,572 are summarized in Table 1 and indicate the presence of an acidic molecule with a formula of $C_{34}H_{54}O_9$.

Disk diffusion assays of L-681,572 were carried out against a panel of 50 filamentous fungi and yeasts to assess both breadth and novelty of spectrum. A comparison of the resultant antifungal spectrum of this product against the spectra of over

Table 1. Physico-chemical properties of L-681,572.

MW	606
Molecular formula	$C_{34}H_{54}O_9$ [a]
IR (KBr)	See Fig. 1
^1H NMR (CD_3OD)	See Fig. 2
^{13}C NMR (CD_3OD)	See Fig. 3
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	206 (897), 238 (897), 282 (273)
TLC (silica gel plate)[b]	Rf 0.46

[a] High-resolution electron impact MS (TMS derivative) calcd for $(C_{34}H_{54}O_9 + (SiC_3H_8)_6 - CH_3)$ 1,023.5905, found 1,023.5860.

[b] Solvent system: CH_2Cl_2-MeOH (7:3).

Fig. 1. KBr pellet IR spectrum of L-681,572; taken on a Nicolet 7199 at 2 cm^{-1} resolution, plotted without smoothing.

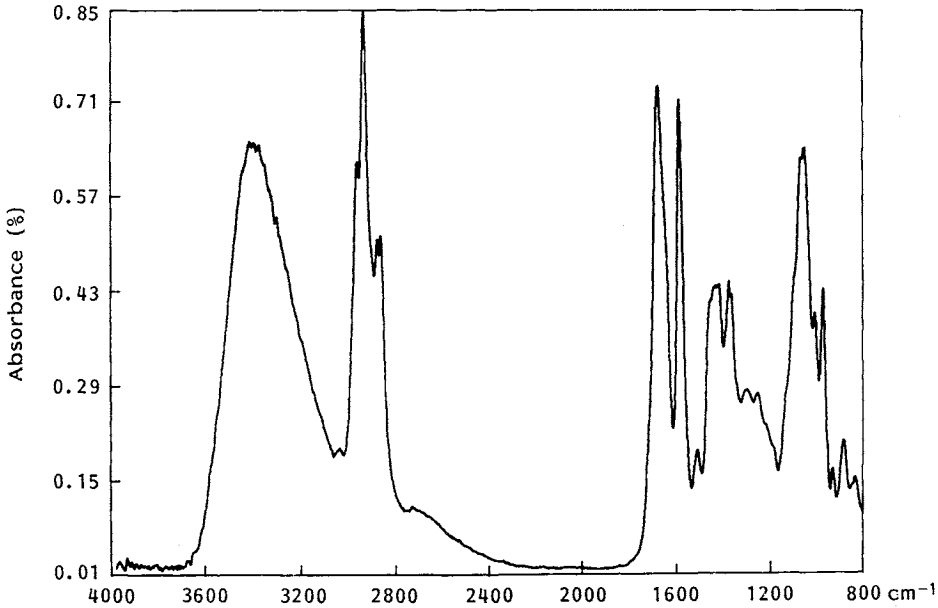
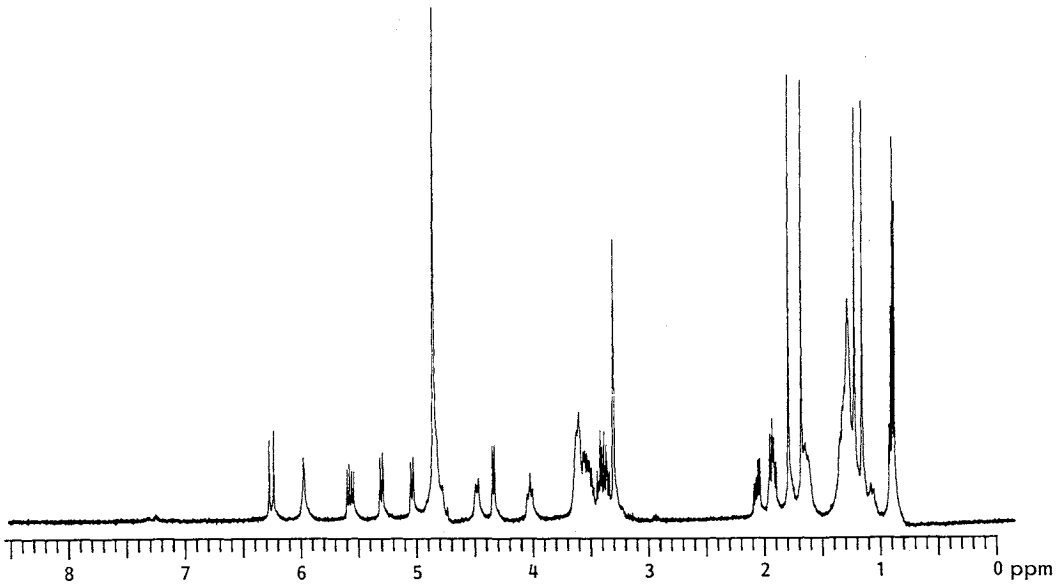


Fig. 2. ^1H NMR spectrum of L-681,572 in CD_3OD[a].

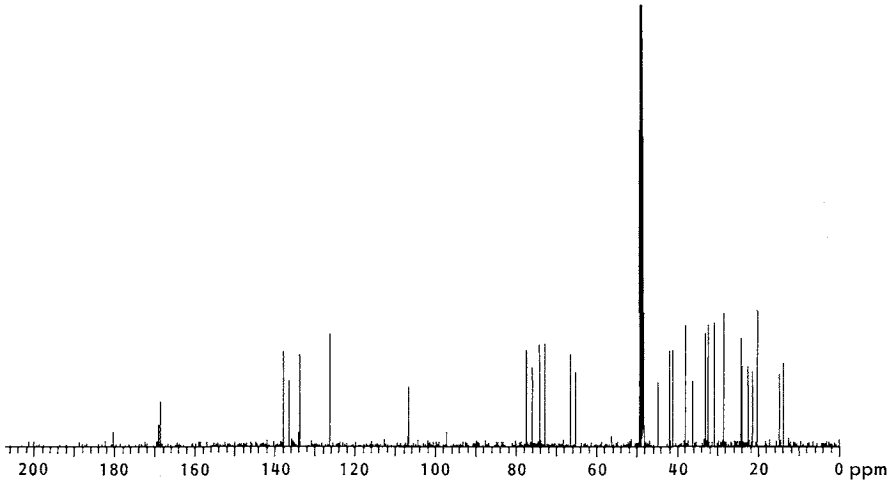


[a] Referenced to the solvent peak at δ 3.30 downfield of TMS.

700 other antifungal agents revealed that this agent exhibited a unique pattern of biological activity. Although the biological spectrum of L-681,572 bore some similarities to polyoxin[1] (an actinomycete product) it was considerably more active vs. *Candida* and *Cryptococcus* sp. in this test. L-681,572 does not, however, appear to be an inhibitor of cell wall biosynthesis.

In vitro studies were done to determine the MICs of product L-681,572 compared to nystatin *versus* a panel of fungi and yeasts. The results of these experiments are summarized in Table 2. The

Fig. 3. ^{13}C NMR spectrum of L-681,572 in CD_3OD[a].



[a] Referenced solvent peak at 49.0 ppm (downfield of TMS). Signals near 24.0, 78.0, 127.0 and 134.5 ppm each correspond to two carbons.

Table 2. Antifungal comparison of L-681,572 and nystatin.

Strain No.[a]	MIC (μg/ml)	
	L-681,572	Nystatin
Aspergillus niger MF442	3.1	1.6
A. niger MF11	1.6	1.6
A. fumigatus MF4839	64.0	0.5
A. flavus MF383	64.0	0.5
Acremonium sp. MF4641	12.5	3.1
Ophiostoma ulmi MF4042	25.0	1.6
Cercospora beticola MF4608	4.0	0.8
Fusarium oxysporum MF4014	25.0	6.2
Penicillium sp. MF5014	3.1	0.4
Penicillium sp. MF5020	1.6	3.1
Phoma sp. MF4332	3.1	0.8
Scopulariopsis acremonium MF376	6.2	12.5
Trichoderma viride MF3560	6.2	3.1
Trichoderma sp. MF4064	1.6	0.4
Ustilago zeae MF1996	8.0	0.8
Verticillium serrae MF3794	25.0	12.5
Candida albicans MY1055	>200.0	1.6
C. pseudotropicalis MY1100	25.0	0.8
C. rugosa MY1022	25.0	1.6
C. tropicalis MY1012	25.0	50.0
Cryptococcus neoformans MY1046	8.0	0.125
C. neoformans MY1050	32.0	0.125
C. neoformans MY1051	128.0	0.125
C. albidus MY1070	8.0	1.6
C. laurentii MY1074	8.0	0.8
C. laurentii MY1073	6.2	0.8
C. laurentii MY1077	12.5	1.6
Debaryomyces guilliermondi MY321	6.2	3.1

[a] Merck microbial resources culture collection.

compound exhibits a moderately broad spectrum of activity which was skewed toward filamentous fungi and *Cryptococcus* species. MICs ranged from 1.6 to 64 μg/ml for filamentous fungi and 6.2 to >200 μg/ml for yeasts. Anti-*Candida* activity observed in disk diffusion assays was found in agar dilution MICs. This may, however, be a result of the solubility of the agent rather than a lack of activity. No acute or chronic toxicity with L-681,572 was observed in Charles River CD-1 female mice at doses of 100 mg/kg ip. When L-681,572 was tested with doses of 100 mg/kg ip for efficacy in mice infected intravenously with the most susceptible strain of *Cryptococcus neoformans* (*e.g.* MIC 8 μg/ml *in vitro*), it did not control the infection which generally yields 100% mortality by 10 days post infection. The reasons for the lack of *in vivo* activity may be rapid metabolism of the compound to an inactive state following injection or sequestering of the compound following injection, leading to plasms levels insufficient to significantly inhibit the infecting strain.

Although L-681,572 does not appear to be suitable for treatment of disease in man or animals, this agent does display considerable activity against a variety of plant pests including the causative agents of Dutch elm disease (*Ceratocystis ulmi*), leaf spot in beets (*Cercospora beticola*), damping off and root rot (*Fusarium oxysporum* and *Phoma* sp.) and corn smut (*Ustilago maydis*). This agent is also active against a variety of storage pests and mycotoxin producers. This activity, coupled with the apparent

lack of toxicity, suggests that L-681,572 may be of some utility in agriculture and industry.

Acknowledgment

We are grateful to Dr. B. KATZ of Mycosearch Inc., P.O. Box 941, Chapel Hill, North Carolina 27514 for offering us his culture which was used to produce the antifungal activity described in this manuscript. The culture has been deposited with the ATCC as a member of the genus *Fusarium* in support of patent applications with the accession No. ATCC 20883.

Reference

1) SUZUKI, S.; K. ISONO, J. NAGATSU, T. MIZUTANI, Y. KAWASHIMA & T. MIZUNO: A new antibiotic, polyoxin A. J. Antibiotics, Ser. A 18: 131, 1965