L-687,781, A NEW MEMBER OF THE PAPULACANDIN FAMILY OF β-1,3-D-GLUCAN SYNTHESIS INHIBITORS

I. FERMENTATION, ISOLATION, AND BIOLOGICAL ACTIVITY

FRANK VANMIDDLESWORTH, MARY NALLIN OMSTEAD, DENNIS SCHMATZ, KEN BARTIZAL, ROBERT FROMTLING, GERALD BILLS, KARL NOLLSTADT, SUSAN HONEYCUTT, MARCIA ZWEERINK, GEORGE GARRITY and KEN WILSON

Merck, Sharp & Dohme Research Laboratories, Rahway, N.J. 07065, U.S.A.
(Received for publication July 16, 1990)

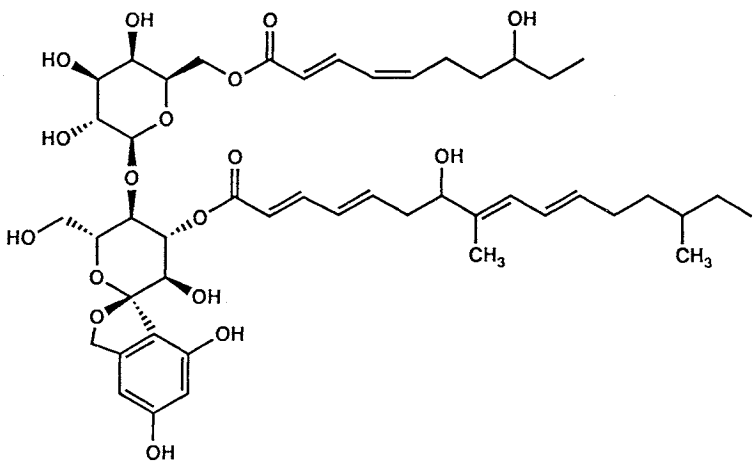
A new β-1,3-D-glucan synthesis inhibitor, L-687,781 is produced by the cultivation of *Dictyochaeta simplex* ATCC 20960. L-687,781 exhibits potent *in vitro* antifungal activity as well as anti-*Pneumocystis* activity in a rat model.

In the course of our efforts to discover novel antifungal agents, we found that cultivation of a strain of the Hyphomycete *Dictyochaeta simplex* produces a new β-1,3-D-glucan synthesis inhibitor, L-687,781. β-1,3-D-Glucan synthesis recently has been identified as an effective target for combatting *Pneumocystis carinii* pneumonia (PCP)[1]. PCP is a common opportunistic infection in AIDS patients which is frequently life threatening. The structure of L-687,781 was determined to be **1**, a new member of the papulacandin family of antibiotics[2,3]. Described in this paper are the production, isolation, and some biological properties of L-687,781. The structure elucidation of L-687,781 will be described elsewhere[4].

Results

Identification of Producing Microorganism

The strain of *D. simplex* (Hughes and Kendrick) Holubova-Jechova (ATCC 20960=MF5247) was

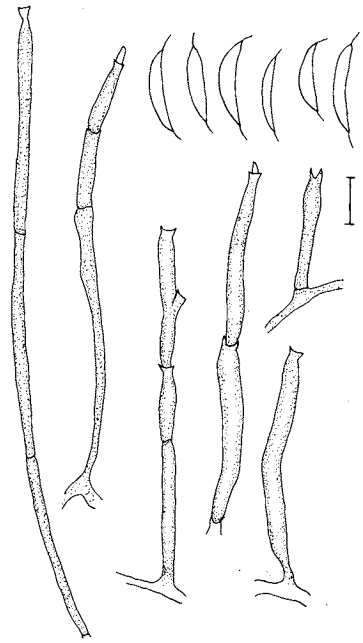


1

recovered from hardwood leaf litter from Fayette Station, Fayette Co., West Virginia, U.S.A. This organism is more widely known as *Codinaea simplex* Hughes and Kendrick. We have chosen to recognize the generic placement of this species in *Dictyochaeta* rather than in *Codinaea* based on the nomenclatural reasons explained by GAMUNDI *et al.*[5]. *D. simplex* is a widely distributed species, being known from deciduous leaf litter and wood in temperate regions of Europe, Asia, North America, and New Zealand. The morphological features of our strain agree well with descriptions published by HUGHES and KENDRICK[6], and by ELLIS and ELLIS[7]. In the following description of colony morphology, the capitalized color names are from RIDGWAY[8]. The diagnostic microscopic features are illustrated in Fig. 1.

Fig. 1. *Dictyochaeta simplex* (ATCC 20960 = MF5247).

Conidiophores and conidia on malt extract agar (standard bar = 10 μm).



Colonies on corn meal agar effuse, appressed, minutely tomentose, velvety, or pruinose, slightly convex in side view, up to 15 mm i.d. in 7 days, olive-gray, olive-black, pale to dark grayish black, dark greenish olive, dark olive, dark grayish olive, olivaceous black, mineral gray, smoke gray.

Mycelium slightly immersed in the agar, septate, branched, with hyphae 1~5 μm wide, hyaline to gray brown or olive gray in KOH. Setae absent. Conidiophores arising directly from the vegetative mycelium, macronematous or mononematous, 23~140 × 3~5 μm, simple or rarely branched, straight to slightly flexuous, 0~4 septate, cylindrical or sometimes tapered at point of attachment to vegetative mycelium, sometimes inflated towards apex, often with successive proliferations, thin-walled to slightly thick-walled, hyaline to pale olive brown in KOH. Conidiogenous cells phialidic, integrated, with terminal or lateral collarettes. Collarettes shallowly cylindrical to nearly hemispherical, up to 4 μm wide × 2.5 μm deep, thin-walled, hyaline. Conidia 13~19.5 × 3~4.5 μm, with two setulae, narrowly crescent-shaped to fusiform with, smooth-walled, aseptate, with finely granular cytoplasm, hyaline to pale grayish yellow in KOH. Setulae at distal ends of conidia, straight to slightly curved, hyaline, 3~6 μm long.

Fermentation

The average time course of L-687,781 production by *D. simplex* grown in 70-liter fermenters is shown in Fig. 2. As is typical with fungi, cell growth continued through 5 days, as evidenced by an increase in packed cell volume until a maximum of 15~17% was attained. The pH of the culture broth decreased gradually over the course of the fermentation. Production of L-687,781 was first determined at 108 hours, at which time an average titer of 20 mg/liter was obtained. Accumulation of L-687,781 continued exponentially over the next 3 to 4 days until the fermentation was terminated at 192 hours with an average titer of 105 mg/liter.

Isolation

The majority of the L-687,781 produced by ATCC 20960 was mycelial bound and could be extracted by treatment of the filtered mycelia with MeOH - H_2O. Polypropylene glycol 2000, used as an antifoaming agent in the fermentation, and lipophilic contaminants were removed by washing the aqueous solution with hexane. The L-687,781 then was extracted into CH_2Cl_2, concentrated, and the crude residue was then subjected to silica gel chromatography followed by reverse phase chromatography. A final reverse phase HPLC purification provided homogeneous L-687,781. The majority of the antifungal activity could be accounted for by the L-687,781 present, however, trace amounts of unidentified antifungal components also were detected in the fermentation.

Antifungal Activity

As shown in Table 1, L-687,781 is a potent antifungal agent against various *Candida* spp. *in vitro*. As was observed for other members of the papulacandin family[2], L-687,781 is inactive against bacteria, filamentous fungi (data not shown) and

Fig. 2. Time course of fermentation of *Dictyochaeta simplex* ATCC 20960.

(A) Packed cell volume, (B) pH profile, (C) production of L-687,781.

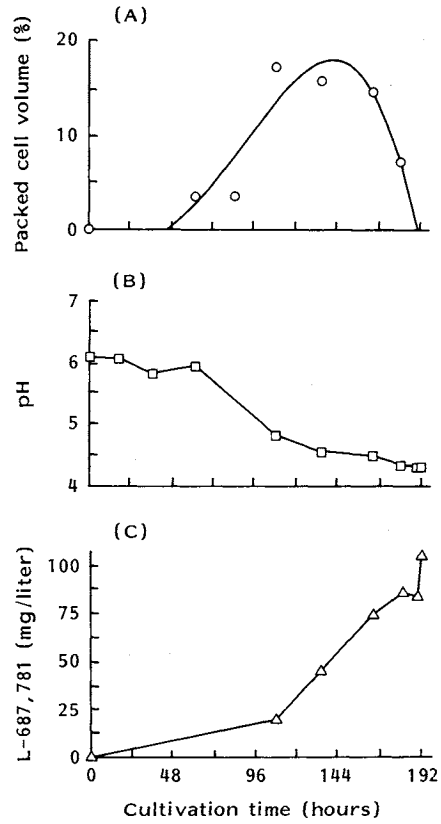


Table 1. Antifungal activity of L-687,781 compared with other antifungal agents.

Test organism	MIC (μg/ml)				
	L-687,781	AMB	5-FC	KTZ	L-671,329
Candida albicans MY1055	1	8	>64	>64	1
C. albicans MY1585	2	4	0.2	>64	1
C. albicans MY1208	2	2	NT	>128	2
C. albicans MY1028	2	1	NT	>128	1
C. albicans MY1750	1	2	>64	>64	1
C. albicans MY1783	2	2	>64	>64	0.5
C. albicans MY1753	2	1	>64	>64	0.5
C. albicans MY1781	2	2	>64	>64	0.5
C. albicans MY1782	2	2	<0.03	>64	1
C. tropicalis MY1012	1	>64	0.5	>64	0.25
C. parapsilosis MY1009	2	4	NT	NT	8
C. parapsilosis MY1010	1	2	NT	0.12	4
C. guilliermondii MY1019	>128	4	NT	NT	>64
Cryptococcus neoformans MY1051	>128	1	8	16	>128
C. neoformans MY1146	>128	1	>64	32	>128

Abbreviations: AMB, Amphotericin B; 5-FC, 5-fluorocytosine; KTZ, ketoconazole; L-671,329, ref 9; NT, not tested.

MIC values (μg/ml) were determined using broth (yeast nitrogen base glucose) microdilution assay.

Cryptococcus neoformans (Table 1). In general the MIC values for L-687,781 are comparable to those observed for amphotericin B and are lower than those observed for 5-fluorocytosine and ketoconazole. The echinocandin β-1,3-D-glucan synthesis inhibitor L-671,329, recently isolated in these labs[9,10], shows comparable MIC values with those observed for L-687,781.

Due to the potent *in vitro* activity exhibited by L-687,781, an *in vivo* efficacy study was undertaken. In this study mice were systemically challenged (iv) with *C. albicans* MY1055 and treated (ip) with L-687,781 (twice-daily). Four days after challenge the mice were sacrificed and the number of viable *C. albicans* colonies recovered from the mouse kidneys was determined. Despite the potent *in vitro* activity, treatment with L-687,781 in this *in vivo* model up to 10 mg/kg body weight produced no significant efficacy compared to infected DMSO sham-treated control mice.

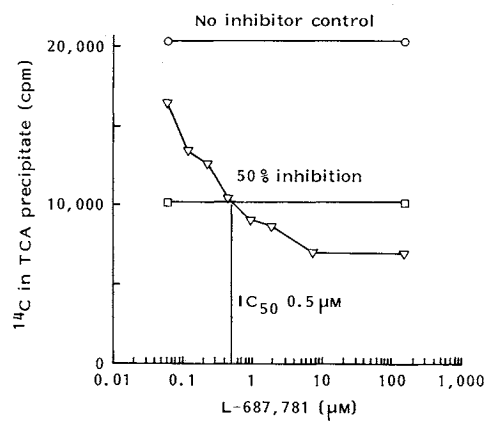
Inhibition of β-1,3-D-Glucan Synthesis

After the structure of L-687,781 was determined to be similar to the papulacandins, mode of action studies centered on inhibition of β-1,3-D-glucan synthesis[11]. As shown in Fig. 3, addition of L-687,781 to a membrane preparation from *C. albicans* MY1208 inhibits the incorporation of UDP-[*U*-^{14}C]glucose into trichloroacetic acid precipitable polymer with an IC_{50} of approximately 0.5 μM. As described previously, the majority of this polymer in *C. albicans* has been identified as β-1,3-D-glucan[12].

Anti-*Pneumocystis carinii* Activity

As described by SCHMATZ *et al.*[1] β-1,3-D-glucan synthesis inhibitors have been shown recently to be effective in PCP infections. Therefore, L-687,781 was evaluated for efficacy in a rat acute PCP model. The results shown in Table 2 show that doses of L-687,781 which are ineffective for treating *C. albicans* infections in mice (*vide supra*), were statistically effective in reducing *P. carinii* cysts in rats.

Fig. 3. Inhibition of *Candida albicans* β-1,3-D-glucan synthesis by L-687,781.



Experimental

Fermentation

Frozen vegetative mycelia (FVM) of *D. simplex*

Table 2. Two titrations of L-687,781 in the acute PCP rat model (4-day treatment period).

Treatment	Total cysts mean $\log_{10}$ (±SEM)	% Reduction of cysts	Survivors/total animals
DMSO control	7.46±0.10	—	5/6
L-687,781 5.0 mg/kg	6.68±0.09	83.5[a]	5/6
L-687,781 2.5 mg/kg	7.15±0.09	51.5	6/6
L-687,781 1.2 mg/kg	7.15±0.12	51.6[a]	6/6
DMSO control	7.46±0.04	—	5/5
L-687,781 10.0 mg/kg	6.77±0.16	79.6[a]	5/5
L-687,781 5.0 mg/kg	6.89±0.14	72.6[a]	5/5
L-687,781 2.5 mg/kg	7.48±0.06	0.0	5/5

The total number of cysts per animal lung is expressed as the mean $\log_{10}$ (±the standard error of the geometric mean, SEM). The lower limit of detection was 2×10^4 cysts per lung ($\log_{10} = 4.26$).

[a] Significantly different from their corresponding controls ($P \leq 0.05$, Student's t-test).

were prepared and used to inoculate flasks of seed medium. The seed medium consisted of (in g/liter): tomato paste 40.0, corn steep liquor 5.0, oat flour 10.0, Cerelose 10.0 and 10.0 ml/liter of a trace element solution No. 2; the pH of this medium was adjusted to 6.8. Trace element solution No. 2 consisted of the following inorganic salts (in g/liter): $FeSO_4 \cdot 7H_2O$ 1.0, $MnSO_4 \cdot 4H_2O$ 1.0, $CuCl_2 \cdot 2H_2O$ 0.025, $CaCl_2 \cdot 2H_2O$ 0.1, H_3BO_3 0.056, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.019, $ZnSO_4 \cdot 7H_2O$ 0.2, dissolved in 1 liter 0.6 N HCl. Complete seed medium was dispensed 50 ml into 250-ml Erlenmeyer flasks or 500 ml into 2-liter Erlenmeyer flasks and sterilized at 121°C, 1.05 kg/cm^2 for 20 minutes. Culture development was always begun by inoculating 2.0 ml from an FVM into 50 ml seed medium and incubating at 26°C, 220 rpm, 85% humidity for 72 hours. The resultant culture was aseptically transferred (2% inoculum) to the same seed medium in 2-liter Erlenmeyer flasks and incubated at 26°C, 180 rpm, 85% humidity for 48 hours. Two 70-liter Chemap fermenters were charged with 50 liters production medium which consisted of (in g/liter): Cerelose 55.0, glycerol 10.0, glycine 2.0, lard water 5.0, soybean meal 5.0, sodium citrate 2.0, KH_2PO_4 2.0, $CoCl_2 \cdot 6H_2O$ 0.01 and P-2000 (antifoam), 2.0 ml/liter; medium pH was adjusted to 7.0 and sterilized at 122°C, 1.05 kg/cm^2 for 25 minutes. The resultant culture was transferred (4% inoculum) to each of the previously prepared fermenters. Fermentations proceeded at 26°C for 192 hours with an air flow of 15 liters per minute and an agitation rate of 400 rpm.

Isolation of L-687,781

Biological activity was followed during the course of the isolation by agar disk diffusion assay *versus* *Candida pseudotropicalis* MY1100.

The mycelia from the above 100-liter fermentation were separated from the broth using a Sharples centrifuge. By HPLC analysis (Whatman ODS-3, 4.6 mm i.d. × 25 cm, 1.0 ml/minute, acetonitrile-10 mM potassium phosphate buffer (46 : 54, pH 6.5), UV 265 nm, Rt 18.5 minutes), the filtered broth contained <5% of the L-687,781 present in the whole broth. The mycelial cake (1.2 kg) was extracted (2×) by stirring overnight with 4 liters of MeOH - H_2O (80 : 20). The extracts were filtered through Celite to give 8 liters of MeOH - H_2O (80 : 20) extract which was calculated to contain 160 g total solids and *ca.* 10 g L-687,781.

Hexane (4 liters) was added to the MeOH-H_2O extract, the layers were separated. A saturated NaCl solution (4 liters) was added to the resulting aqueous methanol layer and the L-687,781 was extracted into CH_2Cl_2 (3 × 6 liters). The CH_2Cl_2 extracts were combined, dried over anhydrous Na_2SO_4, and concentrated to a tar (*ca.* 80 g).

The residue was dissolved in MeOH, adsorbed onto silica gel (400 g), and the MeOH was removed *in vacuo*. The residue coated silica gel was then applied to a silica gel chromatography column (1.5 liters of Silica gel 60, 0.040~0.063 mm) packed in CH_2Cl_2. The column was eluted with CH_2Cl_2 containing increasing amounts of MeOH. L-687,781 was obtained in the MeOH - CH_2Cl_2 (10 : 90) eluate. These fractions were combined and concentrated to yield 6.2 g of a black solid.

This rich cut was dissolved in MeOH (20 ml) and added (with some precipitation) to 50 mM potassium phosphate pH 6.5 buffer (40 ml) at the head of a reverse phase flash chromatography column (50 ml of Baker octadecyl, 0.040 mm). The column was eluted with 50 mM potassium phosphate pH 6.5 buffer containing increasing amounts of MeOH. The L-687,781 was obtained between the 60% and the 70% MeOH fractions. The L-687,781 containing fractions were combined and concentrated to yield 3.1 g of a tan solid.

A portion (39 mg) of the above solid was reconstituted in 2 ml of 46 : 54, acetonitrile - H_2O and applied to a preparative HPLC column (Whatman Magnum 20 C_{18}, 22 mm i.d. × 25 cm, 10 ml/minute, 46 : 54, acetonitrile-H_2O). The fractions eluting between 32 and 39 minutes were combined and the solvents removed *in vacuo* to yield 23 mg of pure L-687,781 as a white solid: MP 168~173°C (with dec). The UV spectrum (MeOH) of L-687,781 exhibited λ_{max} nm ($E^{1\%}$) at 262 (330), 240 (260), and a shoulder at 230 (240). FAB-MS indicated a MW of 902 (observed (M+H) at *m/z* 903)[4].

β-1,3-D-Glucan Synthesis Inhibition Assay

Protoplasts of *C. albicans* MY1208 were prepared as described by TAFT *et al.*[12] with the following modifications: 1.2 M glycerol was substituted for 1.2 M sorbitol and the protoplasts were washed three additional times after Novozyme 234 treatment. Aliquots (1 ml) of washed protoplasts were stored in 1.2 M

glycerol-PBS containing 0.5 mM phenylmethyl sulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) at −80°C. The glucan synthase system was prepared prior to use by sonication, centrifugation (100,000 × *g*, 30 minutes), and resuspension in the storage buffer.

β-1,3-D-Glucan synthesis was measured by an assay similar to that described by CABIB and KANG for *Saccharomyces cerevisiae*[13]. Each reaction vessel contained a total of 80 μl consisting of the following: 4 μl of L-687,781 dissolved in DMSO at various concentrations, 15 μl of the above protoplast suspension, 5 μl of UDP-[*U*-^{14}C]glucose (348 mCi/mmol), α-amylase (4 units), Tris-HCl (pH 7.5) 125 mM, DTT 0.25 mM, PMSF 0.15 mM, glycerol 0.40 M, EDTA 0.75 mM, bovine serum albumin 1.0%, guanosine 5′-*O*-(3-thiotriphosphate) tetralithium salt 40 nM, and laminaribiose 4.0 mM. After incubation for 60 minutes at room temperature, the reactions were stopped by addition of 10% TCA (1 ml). The solutions then were cooled in an ice bath for 15 minutes and filtered. The filtrates were washed with TCA (four times) and ethanol (twice), added to scintillation liquid (10 ml of Aquasol-2), and the radioactivity was measured.

Acute Rat Model *P. carinii* Pneumonia and Evaluation of Infected Lung Tissues

The *P. carinii* pneumonia rat model used in this study[1] is a modification of those originally described by FRENKEL *et al.*[14] and by HUGHES *et al.*[15]. Male Sprague-Dawley rats weighing approximately 200 g, obtained from Sasco, Inc. (Omaha, Nebraska) were maintained on a low protein diet (8.0%) and immunosuppressed with dexamethasone in the drinking water (2 mg/liter) to induce *P. carinii* pneumonia. Tetracycline (1 g/liter) also was added to the drinking water to prevent bacterial infections. After 6 weeks of immunosuppression three rats were selected randomly and sacrificed to confirm the presence of *P. carinii* pneumonia. The remaining rats were distributed into groups of 5~6 and injected ip twice-daily for 4 days with 0.5 ml of various concentrations of L-687,781 in vehicle (10% DMSO) or vehicle alone. At the completion of treatment all rats were sacrificed by exposure to carbon dioxide gas, the lungs were removed and homogenized with a Brinkmann homogenizer in 10.0 ml of saline solution.

The lung homogenates were centrifuged (1,000 × *g*) and the supernatant discarded. The pellet was resuspended in 5.0 ml of 0.85% ammonium chloride solution and incubated for 5 minutes at 37°C to lyse the red cells. The solution was centrifuged (1,000 × *g*) and the resulting pellet was washed two additional times by alternate addition of saline and centrifugation. The final pellet was resuspended in 2.0 ml of saline. A 5.0-μl aliquot was taken from each sample and dried onto teflon coated microscope slides with a fixed surface area (11 mm circles).

The total number of cysts per animal lung was determined by counting the number of cysts per 50 microscope fields (1,000 ×) of homogenized lung tissue on slides fixed with ether-sulfuric acid and stained with toluidine blue[16]. The total number of organisms per rat lung was determined as a function of the surface area on the slide, the volume of the applied sample and the total volume of the processed homogenate.

Acknowledgments

Dr. BARRY KATZ of Mycosearch Inc., Chapel Hill, NC kindly supplied the strain of *Dictyochaeta simplex*. We would also like to thank other Merck researchers for making valuable scientific contributions to this study especially G. ABRUZZO, R. JENKINS, L. A. PITTARELLI, M. A. ROMANCHECK, C. TRAINOR, and M. TURNER.

References

1) SCHMATZ, D. M.; M. A. ROMANCHECK, L. A. PITTARELLI, R. E. SCHWARTZ, R. A. FROMTLING, K. H. NOLLSTADT, F. L. VANMIDDLESWORTH, K. E. WILSON & M. J. TURNER: Treatment of *Pneumocystis carinii* pneumonia with β-1,3-D-glucan synthesis inhibitors. Proc. Natl. Acad. Sci. U.S.A. 87: 5950~5954, 1990
2) TRAXLER, P.; J. GRUNER & J. A. L. AUDEN: Papulacandins, a new family of antibiotics with antifungal activity. I. Fermentation, isolation, chemical and biological characterization of papulacandins A, B, C, D and E. J. Antibiotics 30: 289~296, 1977
3) TRAXLER, P.; H. FRITZ, H. FUHRER & W. J. RICHTER: Papulacandins, a new family of antibiotics with antifungal activity. Structures of papulacandins A, B, C and D. J. Antibiotics 33: 967~978, 1980
4) VANMIDDLESWORTH, F.; C. DUFRESNE, J. SMITH & K. WILSON: Structure Elucidation of L-687,781, a new β-1,3-D-glucan synthesis inhibitor. Tetrahedron, to submitted
5) GAMUNDI, I. J.; A. M. ARAMBARRI & A. GIAIOTTI: Micoflora de la hojarasca de *Nothofagus dombeyi*. Darwiniana 21: 81~114, 1977

6) HUGHES, S. J. & W. B. KENDRICK: New Zealand fungi. 12. *Menispora, Codinaea, Menisporiopsis*. New Zealand J. Bot. 6: 323~375, 1968
7) ELLIS, M. B. & J. P. ELLIS (*Ed.*): Microfungi on Land Plants. MacMillan Publ. Co., 1985
8) RIDGWAY, R. (*Ed.*): Color standards and Nomenclature. R. RIDGWAY, 1912
9) SCHWARTZ, R. E.; R. A. GIACOBBE, J. A. BLAND & R. L. MONAGHAN: L-671,329, a new antifungal agent. I. Fermentation and isolation. J. Antibiotics 42: 163~167, 1989
10) FROMTLING, R. A. & G. K. ABRUZZO: L-671,329, a new antifungal agent. III. *In vitro* activity, toxicity and efficacy in comparison to aculeacin. J. Antibiotics 42: 174~178, 1989
11) PEREZ, P.; R. VARONA, I. GARCIA-ACHA & A. DURAN: Effect of papulacandin B and aculeacin A on β-(1-3)glucan-synthase from *Geotrichum lactis*. FEBS Lett. 129: 249~252, 1981
12) TAFT, C. S.; T. STARK & C. P. SELITRENNIKOFF: Cilofungin (LY121019) inhibits *Candida albicans* (1-3)-β-D-glucan synthase activity. Antimicrob. Agents Chemother. 32: 1901~1903, 1988
13) CABIB, E. & M. KANG: Fungal 1,3-β-glucan synthetase. Methods Enzymol. 138: 637~642, 1987
14) FRENKEL, J.; J. GOOD & J. SCHULTZ: Latent *Pneumocystis* infection in rats, relapse, and chemotherapy. Lab. Invest. 15: 1559~1577, 1966
15) HUGHES, W. T.; P. C. MCNABB, T. D. MAKRES & S. FELDMAN: Efficacy of trimethoprim and sulfamethoxazole in the prevention and treatment of *Pneumocystis carinii* pneumonitis. Antimicrob. Agents Chemother. 5: 289~293, 1974
16) CHALVARDIJIAN, A. & L. GRAWE: A new procedure for the identification of *Pneumocystis carinii*. J. Clin. Pathol. 16: 383~387, 1963