ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1994, p. 2750–2757
0066-4804/94/$04.00+0

Vol. 38, No. 12

Increased Antifungal Activity of L-733,560, a Water-Soluble, Semisynthetic Pneumocandin, Is Due to Enhanced Inhibition of Cell Wall Synthesis

M. B. KURTZ,[1]* C. DOUGLAS,[1] J. MARRINAN,[1] K. NOLLSTADT,[2] J. ONISHI,[1] S. DREIKORN,[1] J. MILLIGAN,[1] S. MANDALA,[1] J. THOMPSON,[1] J. M. BALKOVEC,[3] F. A. BOUFFARD,[3] J. F. DROPINSKI,[3] M. L. HAMMOND,[3] R. A. ZAMBIAS,[3] G. ABRUZZO,[4] K. BARTIZAL,[4] O. B. McMANUS,[5] AND M. L. GARCIA[5]

Infectious Disease Research,[1] Antibiotic Evaluation,[4] Basic Animal Sciences Research,[2] Synthetic Chemical Research,[3] and Membrane Biochemistry & Biophysics,[5] Merck Research Laboratories, Rahway, New Jersey 07065

Received 2 May 1994/Returned for modification 8 July 1994/Accepted 19 September 1994

The pneumocandins are natural lipopeptide products of the echinocandin class which inhibit the synthesis of 1,3-β-D-glucan in susceptible fungi. The lack of a corresponding pathway in mammalian hosts makes this mode of action an attractive one for treating systemic infections. Substitution by an aminoethyl ether at the hemiaminal and dehydration and reduction of the glutamine of pneumocandin B_0 produced a semisynthetic compound (L-733,560) with intrinsic water solubility, significantly increased potency, and a broader antifungal spectrum. To evaluate the mechanism for the improved antifungal efficacy, we determined that L-733,560 was a more potent inhibitor of glucan synthase activity in vitro, did not affect the other membrane-bound enzymes tested, conferred susceptibility to lysis in the absence of osmotic support, and did not disrupt currents in liposomal bilayers or $^{86}Rb^+$ fluxes from liposomes. In *Aspergillus* species L-733,560 also produced the same morphological alterations as pneumocandin B_0. A stereoisomer of L-733,560 with poor antifungal activity was a weak inhibitor of glucan synthase. All of these results support the notion that the enhanced antifungal activity of L-733,560 is achieved by superior inhibition of glucan synthesis and not by nonspecific membrane effects or a second mode of action.

The echinocandins, pneumocandins, and papulacandins are antifungal agents which inhibit the synthesis of 1,3-β-D-glucan in susceptible organisms (3, 26, 27, 35, 38–40, 42). Because the likelihood of mechanism-based toxicity is reduced, inhibition of the synthesis of a fungus-specific structure is an attractive mode of action for antifungal drug candidates. In addition to inhibition of in vitro glucan synthase activity, several observations on whole cells support the notion that these compounds act to inhibit cell wall synthesis in intact fungi. First, osmotic support prevents the lysis of cells treated with drug at the MICs (13, 45). Since the intact fungal cell wall counteracts the high turgor pressure of the protoplast, an agent which disrupts cell wall integrity produces membrane swelling and ultimately causes lysis in the absence of osmotic support. Second, susceptible organisms undergo gross morphological changes after treatment with these agents, consistent with cell wall alterations (10, 13, 16, 22, 28, 33, 44). Third, whole-cell labeling experiments which monitor macromolecular synthesis demonstrate the preferential inhibition of cell wall synthesis (3, 13). Lastly, Douglas et al. (21) recently showed that a mutation responsible for pneumocandin resistance in *Saccharomyces cerevisiae* cosegregates with enzyme activity resistant to inhibition by the pneumocandins.

Originally, semisynthetic derivatives of the natural products were screened for reduced erythrocyte lysis, and this led to the development of the echinocandin B-based analog cilofungin (26, 27). The fatty acid side chain modification of the echinocandin B nucleus that resulted in cilofungin did not significantly alter the in vitro potency of the lipopeptide against *Candida* species compared with that of the natural product (17). Newer side chain analogs of the echinocandin B nucleus, such as LY303366 and LY307853, have improved antifungal spectra and potencies (14, 15, 18). Unlike cilofungin, the pneumocandin B_0 analog L-693,989 was optimized for improved water solubility (4). Recently, pneumocandin B_0 derivatives such as L-733,560, L-731,373, and L-705,589 have been shown to be significantly more potent antifungal agents than the corresponding natural product and have broader antifungal spectra (1, 7). The aminopneumocandins have the advantage of intrinsic water solubility (8). Given the potential of these new derivatives as broad-spectrum therapies for systemic fungal disease, we sought to demonstrate that one of these analogs, L-733,560, like pneumocandin B_0, is a specific inhibitor of glucan synthesis. This report shows that L-733,560 is a potent, specific inhibitor of fungal glucan synthesis, as determined by the standard tests for an inhibitor of cell wall synthesis.

MATERIALS AND METHODS

Antifungal compounds. Pneumocandin A_0 (43), pneumocandin B_0 (36), the semisynthetic pneumocandin L-733,560 (8), its stereoisomer L-734,735, and zaragozic acid A (6) were provided by scientists at Merck Research Laboratories, Rahway, N.J. Figure 1 shows the structures of selected pneumocandins. All compounds were shown by high-performance liquid chromatography (HPLC) to be >95% pure. Fluconazole was obtained from Pfizer Central Research, Groton, Conn., 5-flucytosine was obtained from Hoffmann-La Roche, Nutley, N.J., and amphotericin B was obtained from Bristol Myers-Squibb, Princeton, N.J. Tyrocidin and tunicamycin were from

* Corresponding author. Mailing address: Department of Infectious Disease Research, Merck Research Laboratories, RY80Y-220, P.O. Box 2000, Rahway, NJ 07065-0900. Phone: (908) 594-5124. Fax: (908) 594-1399. Electronic mail address: Myra_Kurtz@Merck.com.

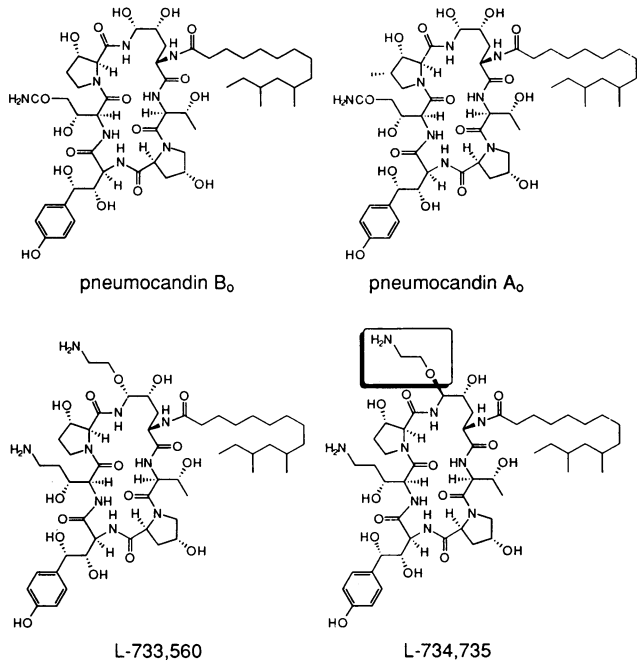

FIG. 1. Chemical structures of 1,3-β-D-glucan synthesis inhibitors.

Sigma (St. Louis, Mo.), and nikkomycin Z was from Calbiochem (La Jolla, Calif.).

In vitro antifungal activity. The in vitro antifungal susceptibilities of *Candida albicans*, *S. cerevisiae*, and *Aspergillus fumigatus* to the pneumocandins were determined (see Table 1). Yeast Nitrogen Base (Difco) with 2% glucose was used for MIC and minimum fungicidal concentration (MFC) determinations by a previously described broth microdilution assay (5). Briefly, 10^4 yeast or conidia were inoculated into 0.15 ml of medium containing twofold serial dilutions of test compound. Growth was monitored visually after incubation for 48 h at 30°C for the MIC assay. For MFC determinations, aliquots were taken from the microtiter dishes after 24 h at 30°C and were inoculated onto solidified Sabouraud's medium with a Dynatech 2000 inoculator (Dynatech, Inc.). The MFC was defined as the well with the lowest concentration of compound that showed fewer than four colonies. As a result of pneumocandin treatment, the mycelia of *Aspergillus* species are severely altered in their growth, producing short, highly branched filaments which appear as very compact clumps in microtiter dilution wells (33). The morphological effect can be scored macroscopically and quantitated as a minimum effective concentration (MEC). For the assay for MEC determination the same medium, inoculum, and growth conditions used for the standard MIC determination were used, and end points were determined visually as described by Kurtz et al. (33).

In vivo antifungal activity. An in vivo mouse model of disseminated candidiasis which measures the efficacy of experimental antifungal agents by the clearance of CFU from the kidney (target organ kidney assay [5]) was used to evaluate pneumocandin analogs. In brief, complement component C5-deficient DBA/2N female mice (average weight, 19 to 21 g; Taconic Farms, Germantown, N.Y.) were challenged intravenously with 1×10^4 to 5×10^4 CFU of *C. albicans* MY1055. The compounds were administered twice daily via intraperitoneal injection at 0.001 to 0.375 mg/kg of body weight for 4 days beginning immediately after infection (5). At day 7 after infection, animals were euthanized, the kidneys were excised and homogenized, and serial dilutions were plated to enumerate the CFU per gram of kidneys. The analysis of variance test of dose-response relationships with single degree of freedom contrasts was used. The mean $\log_{10}$ number of yeast CFU per organ was compared with those in the organs of sham-treated controls by the multiple comparison procedure of Dunnett (23). Inverse regression was used to estimate the dose which reduced CFU-per-organ counts to 90% lower than those in controls. All animal procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee. The care and use of research animals at Merck meet or exceed all applicable local, national, and international laws and regulations.

Erythrocyte lysis assay. The potentials for the compounds to lyse erythrocytes were tested in a microtiter assay against freshly drawn heparinized whole blood from humans or DBA/2 mice. The test compounds were dissolved in distilled water and were tested at final concentrations of 0.20 to 400 μg/ml. The 4% erythrocyte suspension was added to each well, and the results were determined after 2 h of incubation at room temperature. The minimum lytic concentration was determined after visual observation as the lowest concentration of a test compound that produced either complete (red) or partial (pink) hemolysis, as determined by the colors of the supernatants of wells containing erythrocytes. Amphotericin B was used as a positive control, producing a pinkish red color in the supernatant when amphotericin B was present at 6.25 μg/ml, while 5-flucytosine showed a clear supernatant at the highest concentration tested (400 μg/ml).

Inhibition of 1,3-β-D-glucan synthase. Glucan synthase activity was measured in microsomal membranes prepared from *A. fumigatus* MF4839, *C. albicans* MY1055, and *S. cerevisiae* W303-1A as described previously (5, 20, 33). The assay measures the synthesis of radiolabeled trichloroacetic acid (TCA)-precipitable material from [^{3}H]UDP-glucose. The reaction mixtures were harvested onto glass fiber filter mats, washed with water, blown dry, and counted. Each assay was run in duplicate. Protein determinations were performed by the dye-binding method (11). Activity was expressed as nanomoles of product formed · minute^{-1} · milligram of crude protein^{-1}.

In vitro enzyme assays. The inhibition of various membrane-associated enzymes (chitin synthase [CHS], squalene synthase, serine palmitoyltransferase) from fungal and mammalian sources by L-733,560 was tested at L-733,560 concentrations of up to 10 μM (11.7 μg/ml). The compound was tested at concentrations of up to 2 μM in the myristoyltransferase assay, which produces a membrane-associated product. CHS I and squalene synthase activities were measured in microsomal membranes prepared from *S. cerevisiae* W303-1A and *C. albicans* MY1055 as described by Cabib et al. (12) and Bergstrom et al. (6), respectively. The CHS I assay measures the formation of the TCA-precipitable material formed from [^{3}H]UDP-*N*-acetylglucosamine under conditions in which CHS I activity predominates over CHS II and III activities (37). Squalene synthase activity was measured by the incorporation of the radiolabeled water-soluble substrate (farnesyl pyrophosphate) into organic solvent-extractable product (squalene). For the measurement of myristoyltransferase activity in cell-free extracts of *S. cerevisiae* and *C. albicans*, the formation of radiolabeled *N*-myristoylated peptide from [^{3}H]myristoyl coenzyme A (CoA) and an octapeptide substrate (GNAAAARR-NH_2) was monitored by HPLC as described

previously (41). Serine palmitoyltransferase activity in HeLa cells and *C. albicans* microsomes was quantitated by measuring the formation of radioactive ketodihydrosphinogine from palmitoyl CoA and radiolabeled serine (30).

Whole-cell macromolecular synthesis. The effect of L-733,560 on macromolecular synthesis in *C. albicans* MY1055 was determined by whole-cell labeling experiments. Exponential-phase yeast cells growing in CMS (1% Bacto Peptone, 0.5% yeast extract, 1 μM adenine, 1 μM leucine, 0.05% glucose, 0.8 M sorbitol) were treated with the test compounds at their MICs. During the treatment period, the cells were radiolabeled with 0.2 μCi of [8-^{14}C]adenine per ml, 1.0 μCi of L-[4,5-^{3}H(N)]leucine per ml, or 0.2 μCi of D-[1-^{14}C]glucose per ml. The amount of adenine and leucine incorporated into RNA and protein, respectively, was determined by adding 1 volume of 10% TCA to cells labeled for 30 min and collecting the TCA-insoluble pellet on glass fiber filters (Whatman GF/A). Adenine incorporation into DNA was determined following a 60-min treatment period with alkaline hydrolysis as described previously (29). Cells treated and labeled for 30 min with glucose were precipitated with TCA, washed, and fractionated into alkali-soluble and alkali-insoluble fractions by the procedures described by Bowers et al. (9). The alkali-insoluble residue was extracted with 0.5 N acetic acid at 90°C for 2 h, and the residue was washed with water. The acetic acid extract, which consists of either glycogen or β-(1,6)-glucan, contained less than 10% of the total counts in the alkali-insoluble pellet. The acetic acid extract was not characterized further. The pellet remaining after acetic acid extraction was treated with either α-amylase (A-2643; Sigma) or laminarinase (L-9259; Sigma) for 24 h at 30°C in 0.05 M acetate buffer (pH 5.4). The radioactivity associated with the alkali-insoluble, acetic acid-insoluble pellet from control and L-733,560-treated cells was quantitatively solubilized by laminarinase digestion, but the pellet was resistant to α-amylase. Mannoprotein was recovered from the alkali-soluble fraction by precipitation with Fehling's reagent as described by Baguley et al. (3). No radioactive material was detected in the alkali-soluble polysaccharide extract, which was recovered as an ethanol precipitate of the alkali-soluble extract.

Lipid bilayer experiments. Artificial lipid bilayers were formed by painting a solution of egg yolk lecithin (60% phosphatidylcholine) at 50 mg/ml across a small hole separating two aqueous compartments. An electrochemical gradient was established across the bilayer either by applying a voltage to one side or by elevating the ion concentrations on one side. The currents across the bilayer were measured by conventional voltage clamp techniques. The lipids readily formed bilayers of 100 to 200 pF. Experiments were done either with 150 mM NaCl–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2) on both sides of the bilayer or with 150 mM NaCl–10 mM HEPES (pH 7.2) on one side and 25 mM NaCl–10 mM HEPES (pH 7.2) on the other side to determine the ion selectivities of the currents induced by pneumocandins. Control measurements of membrane currents were done for about 1 h before drug application to ensure that the bilayer was stable. Because no currents were observed in the control, the currents observed after the addition of the drugs were attributed to the effects of the drugs.

Preparation of liposomes. L-α-Phosphatidylcholine (P-5394; Sigma) liposomes were prepared in 50 mM potassium phosphate (pH 7.4) at a concentration of 50 mg of lipid per ml. The suspension of lipids and buffer was sonicated until clarity under N_2 in a water bath sonicator. Liposomes were frozen in liquid N_2 in the presence of 10 mM RbCl and were stored at −70°C. An aliquot of thawed liposomes was sonicated until clarity in a water bath sonicator. Then, valinomycin was added at a final concentration of 20 μM; this was followed by the addition of 0.5 mCi of ^{86}RbCl per ml. $^{86}Rb^{+}$ efflux was performed in the following manner. An aliquot (2 μl) of liposomes was diluted 200-fold into 50 mM sodium phosphate (pH 7.4) in the presence or absence of other compounds. The suspension was immediately mixed on a vortex mixer and was incubated at room temperature. At a given time, 3.5 ml of ice-cold sodium phosphate (pH 7.4) was added, the sample was immediately filtered (0.22-μm-pore-size GSWP 02500 filter; Millipore), and the filter was washed once with the same cold buffer. The radioactivity retained on the filters was determined by liquid scintillation spectrometry.

TABLE 1. Comparison of MICs and IC_{50}s of L-733,560 and pneumocandin B_0 for glucan synthase in three fungi

Compound	*C. albicans* MY1055		*S. cerevisiae* W303-1A		*A. fumigatus* MF4839	
	MIC (μg/ml)	IC_{50} (μg/ml)	MIC (μg/ml)	IC_{50} (μg/ml)	MEC (μg/ml)	IC_{50} (μg/ml)
Pneumocandin B_0	1.0	0.070	8.0	>10.6	0.71	0.067
L-733,560	0.15	0.001	0.25	1.3	0.05	0.027

RESULTS

In vitro antifungal activity and inhibition of glucan synthase by L-733,560. A direct comparison of the antifungal activities of L-733,560 and pneumocandin B_0 against *C. albicans*, *S. cerevisiae*, and *A. fumigatus* showed that L-733,560 is 15-, 32-, and 12-fold more potent than pneumocandin B_0, respectively, as determined by measuring the MIC and the MEC (Table 1). In view of the known activities of the pneumocandins against the glucan synthases of *C. albicans* and *A. fumigatus*, the possibility that the semisynthetic analog is a more potent inhibitor of the enzyme was tested. Table 1 shows that the 50% inhibitory concentration (IC_{50}) of L-733,560 is 70-fold lower than that of pneumocandin B_0 for *C. albicans*, at least 8-fold lower for *S. cerevisiae*, and ~2-fold lower for *A. fumigatus*. The analog is not nearly as potent against the *S. cerevisiae* enzyme as it is against the glucan synthase activity from the pathogens. The enhanced potency of L-733,560 against the *Aspergillus* enzyme was much smaller than that against the other fungi. Inhibition of the *S. cerevisiae* enzyme by pneumocandin B_0 was barely detectable, and IC_{50} determinations may have been complicated by the insolubility or micelle formation of the compound at the high concentrations required. For *C. albicans* MY1055 and *A. fumigatus* MF4839, the IC_{50}s of L-733,560 against the crude enzyme preparation were 15- and 2-fold lower, respectively, than the MIC or MEC for the whole cell. Thus, the enhanced inhibition of glucan synthase is sufficient to account for the growth-inhibitory effects seen in drug-treated cells of these organisms. In contrast, the MIC for *S. cerevisiae* was fivefold lower than the observed IC_{50} for the in vitro enzyme preparation.

L-733,560 does not inhibit other membrane-bound enzymes. The increased potency of L-733,560 against the in vitro glucan synthase activities from the yeasts raised the possibility that the analog has the ability to inhibit membrane-associated enzymes in a general fashion. To test this hypothesis, inhibition of fungal membrane-associated activities, CHS I, serine palmitoyltransferase, and squalene synthase was tested (Table 2). There was no detectable inhibition of the CHS at concentrations as high as 10 μM (11.7 μg/ml), which is 10,000-fold higher

TABLE 2. Effects of L-733,560 and pneumocandin B_0 on selected yeast and mammalian in vitro enzyme activities

Assay	Membrane associated	Highest concn (μM [μg/ml]) tested	% Inhibition
CHS I (*S. cerevisiae*)	Yes	10 (11.7)	0
CHS I (*C. albicans*)	Yes	10 (11.7)	0
Serine palmitoyltransferase (*C. albicans*)	Yes	10 (11.7)	0
Serine palmitoyltransferase (HeLa cells)	Yes	1 (1.2)	0
Squalene synthase (*S. cerevisiae*)	Yes	10 (11.7)	12
Myristoyltransferase (*S. cerevisiae*)	No	2 (2.3)	41

than the IC_{50} for *C. albicans* glucan synthase and 10-fold higher than that for the *S. cerevisiae* enzyme (Table 1). Under the conditions used, nikkomycin Z, a known competitive inhibitor of CHS, has an IC_{50} of 0.6 μM. L-733,560 at 10 μM was a very poor inhibitor of *S. cerevisiae* squalene synthase and a very weak inhibitor of the soluble myristoyltransferase activity. Zaragozic acid A has an IC_{50} of 0.3 nM for the yeast squalene synthase in this assay. The weak inhibition of the myristoyltransferase activity was 100-fold less than the inhibition of the glucan synthase activity of *S. cerevisiae*.

Stereoisomer of L-733,560 is not a potent glucan synthase inhibitor. Polycationic compounds have the potential to interact with anionic sites on cell membranes and may thus affect the activities of membrane-associated enzymes (34). Exogenously supplied polyamines have been reported to activate Ca^{2+}-dependent glucan synthase activity from soybeans in vitro (31, 32). Spermine and spermidine treatments result in the production of abnormal septa and elongated, malformed cell walls in *S. cerevisiae* (34). The fact that L-733,560 has been modified by the addition of two charged amines suggests that perhaps the increased potency of L-733,560 is due to a structurally nonspecific membrane-perturbing effect. Thus, it is noteworthy that L-734,735, a stereoisomer of L-733,560, unexpectedly inhibits glucan synthase only weakly (Table 3). L-734,735 was 26-fold less potent than L-733,560 in inhibiting the in vitro enzyme. The MFCs and efficacies of L-733,560 and L-734,735 in the animal model of candidiasis mirrored the difference in IC_{50} for the glucan synthase of *C. albicans*. Neither compound was significantly lytic for human erythrocytes at concentrations (200 to 400 μg/ml) many times the concentration needed to kill *C. albicans* in vitro (Table 3).

Effect of L-733,560 on currents in lipid bilayers and $^{86}Rb^+$ fluxes from liposomes. To test if L-733,560 has an enhanced membrane-perturbing effect compared with pneumocandin B_0, both compounds were assayed for their abilities to induce currents in lipid bilayers and affect $^{86}Rb^+$ fluxes from liposomes. Lipid bilayers that had high resistances ($>10^{10}$ Ω) and that were stable under control conditions for more than 1 h were formed. The membrane potential of the lipid bilayer was pulsed from 0 to −40 mV, and pneumocandin B_0 or L-733,560 was added to the solution on one side of the membrane. The addition of 10 μg of pneumocandin B_0 per ml had no discernible effect, while the addition of 20 μg of pneumocandin B_0 per ml caused the appearance of small irregular currents during steps to −40 mV, and the addition of 50 μg of pneumocandin B_0 per ml caused the appearance of larger irregular currents and often broke the bilayer. L-733,560 at 10 μg/ml induced currents equal to or greater than those observed with 20 μg of pneumocandin B_0 per ml, while L-733,560 at 20 μg/ml caused larger irregular currents, and 50 μg of L-733,560 per ml caused large leaky currents and typically broke the bilayer. L-734,735, a stereoisomer of L-733,560 which is a less potent enzyme inhibitor and antifungal agent than L-733,560, was as effective as L-733,560 in causing leaks in bilayer experiments. Experiments were done with an asymmetric gradient of NaCl (150 mM on one side and 25 mM on the other side) in order to assess the ionic selectivities of the currents induced by these compounds. Under these conditions, at 0 mV the currents flowed from the side with 150 mM NaCl to the side with 25 mM NaCl, demonstrating that the current was predominantly carried by cations rather than anions.

When liposomes equilibrated with $^{86}Rb^+$ are diluted into sodium phosphate in the presence of valinomycin, efflux of the cation occurs very slowly (i.e., after 20 min more than 95% is still retained by the liposomes). This phenomenon is due to the formation of a membrane potential, which is negative inside, that is maintained for long periods of time because of the impermeability of the liposomes to counterions (25). The addition of carbonyl cyanide *m*-chlorophenylhydrazone, which increases the permeability of the membrane to protons and results in the dissipation of the membrane potential, causes a marked increase in the rate of $^{86}Rb^+$ efflux (half-life of 1 min). Moreover, even more rapid rates of efflux (half-lives of $\ll 1$ min) are observed when potassium is added because of the electroneutral exchange of $^{86}Rb^+$ with potassium via valinomycin or when nigericin is added because of the electroneutral exchange of Rb^+ with protons. These results demonstrate that the liposomes are highly impermeable to the ions present in the reaction mixture. Therefore, any agent that causes an increase in membrane permeability should enhance the rates of $^{86}Rb^+$ fluxes. When Rb^+ efflux is monitored in the presence of either pneumocandin B_0 or L-733,560 at 50 μM, the rate of disappearance of the cation from the liposomes is identical to that seen under control conditions. Thus, under these experimental conditions, neither compound appears to affect the permeabilities of the liposome membranes. Other experiments in which the liposomes were either pretreated with 50 μM L-733,560 or diluted in the presence of up to 150 μM L-733,560 gave identical results. As another test of the reliability of the assay for detecting membrane-disruptive agents, different compounds were included in the dilution buffer at 20 μg/ml; these included cetyltrimethylammonium bromide, polymyxin, tyrocidin, gramicidin S, amphotericin B, moenomycin, vancomycin, and tunicamycin. Of all of these compounds, only tyrocidin, gramicidin S, and amphotericin B caused a marked increase in the rates of $^{86}Rb^+$ efflux, suggesting that these compounds can alter the permeabilities of the liposomes.

Preferential inhibition of glucan synthesis in whole cells. In order to show that the potent in vitro enzyme inhibition

TABLE 3. Biological properties of L-733,560 and its stereoisomer L-734,735

Compound	MLC (μg/ml)[a]	IC_{50} (μg/ml)[b]	MFC (μg/ml)[c]	TOKA ED_{90} (mg/kg)[d]
L-733,560	400	0.001	0.15	0.03
L-734,735	200	0.028	2.0	>0.38

[a] MLC, minimum lytic concentration for erythrocytes.
[b] Glucan synthase of *C. albicans* MY1055.
[c] *C. albicans* MY1055 was used.
[d] TOKA ED_{90}, target organ kidney assay for estimation of the dose that reduced the CFU-per-organ counts to 90% lower than those in controls. *C. albicans* MY1055 was used.

TABLE 4. Effects of inhibitors on precursor incorporation into macromolecules of *C. albicans* MY1055

Macromolecule	% Control incorporation				
	L-733,560 (0.10 μg/ml [0.09 μM])	L-733,560 (29 μg/ml [25 μM])	Amphotericin B (2.5 μg/ml)	Tyrocidin (25 μg/ml)	Tunicamycin (25 μg/ml)
Protein	100	55	52	29	184
DNA	80	28	15	<0.1	82
RNA	84	17	34	0.9	67
β-1,3-Glucan	46	18	43	2.1	74
Glycoprotein	94	29	35	1.7	29

observed with L-733,560 correlates with the specific inhibition of glucan biosynthesis in whole cells, the effects of inhibitors on precursor incorporation into macromolecules were determined. The synthesis of protein, DNA, RNA, glucan, and glycoprotein by *C. albicans* MY1055 cells treated with amphotericin B (a membrane perturbant), tyrocidin (a membrane lytic agent), tunicamycin (inhibitor of glycoprotein synthesis), and L-733,560 at their MICs was determined and compared with synthesis in untreated cultures (Table 4). L-733,560 preferentially inhibited glucan synthesis. The inhibition pattern is unlike that of the membrane-active agents amphotericin B and tyrocidin or like that of tunicamycin, which showed specific inhibition of glycoprotein synthesis. At 250 times the MIC, L-733,560 no longer specifically inhibited glucan synthesis.

Protection by osmotically supported media. One of the characteristics of the known glucan synthase inhibitors (echinocandins, papulacandins, and pneumocandins) is the protection against cell lysis by osmotic stabilization (13, 45). The effects of pneumocandin B_0 and L-733,560 on the viability of *C. albicans* MY1055 and *C. albicans* MY1208 after 12 h of drug treatment with and without 1.0 M sorbitol are given in Table 5. In osmotically supported Yeast Nitrogen Base glucose medium, a significant portion of the yeast cells survived drug treatment which caused a 1,000-fold decrease in the CFUs in medium without osmotic support. The degree of protection afforded by 1.0 M sorbitol is similar for pneumocandin B_0 and L-733,560, consistent with the model that interference with cell wall integrity is the mode of action of both compounds. This observation was confirmed in an experiment designed to determine the optimal concentration of drug for whole-cell labeling of macromolecules. In that experiment, rich medium with a reduced level of glucose (see Materials and Methods) was used. The concentration of L-733,560 was varied from 1 times the MFC (0.1 μg/ml) to 250 times the MFC (25 μg/ml), and the reversibility of the fungicidal effects on viability was determined after 16 h of drug treatment. Protection by sorbitol was most evident at the MFC (0.1 μg/ml), with greater than 2 log units of reversal (data not shown). Cell death was not prevented by the presence of sorbitol in the medium at very high concentrations (25 μg/ml) of L-733,560 (data not shown).

Morphological alterations induced by L-733,560. Previous studies showed that pneumocandin B_0 causes the hyphae of *A. fumigatus* to grow abnormally, with highly branched tips, swollen germ tubes, and many distended, balloon-like cells (33). Figure 2 shows that L-733,560 has similar morphological effects on *A. fumigatus* cells. The gross appearance of the hyphae made during treatment is the same whether pneumocandin B_0 or its semisynthetic analog is used. L-733,560 induces the effect at lower concentrations, consistent with the MEC ranking above.

DISCUSSION

The data in this report support the hypothesis that the new semisynthetic derivatives of pneumocandin B_0, with increased potencies and spectra of activity compared with those of the natural product (2, 8), achieve their enhanced antifungal activities by their superior inhibition of glucan synthesis and not by nonspecific membrane effects or a second mode of action. As expected for an inhibitor of cell wall synthesis, L-733,560 conferred susceptibility to lysis in the absence of osmotic support. Inhibition of glucan synthase was demonstrated at the in vitro enzyme level (Table 1) and in whole-cell labeling studies (Table 4). The IC_{50}s were low enough to account for the MICs of both the natural product and the semisynthetic analog for *A. fumigatus* and *C. albicans*. The ratios of MIC-MEC/IC_{50} for the natural product were quite similar for *A. fumigatus* and *C. albicans*: 11- and 14-fold, respectively (Table 1). We would expect that if the inhibition of the enzyme were the sole limiting factor in antifungal activity, the ratio of enzyme inhibition to whole-cell activity would remain the same with the more potent analog. In fact, the ratio

TABLE 5. Viabilities of *Candida* strains treated with antifungal agents with and without sorbitol

Strain and compound	Concn (μg/ml)	CFU/ml in		Fold protection
		Medium alone	Medium + sorbitol	
C. albicans MY1208				
Dimethyl sulfoxide control		7.5×10^5	1.0×10^6	1
Pneumocandin B_0	4.0	3.3×10^2	1.2×10^4	36
L-733,560	0.3	4.1×10^2	6.5×10^3	16
Amphotericin B	2.0	7.0×10^1	1.5×10^2	2
C. albicans MY1055				
Dimethyl sulfoxide control		3.0×10^4	7.7×10^4	
Pneumocandin B_0	4.0	1.0×10^2	1.1×10^3	11
L-733,560	0.3	0.6×10^2	1.6×10^3	26
Amphotericin B	2.0	2.1×10^2	0.4×10^2	<1

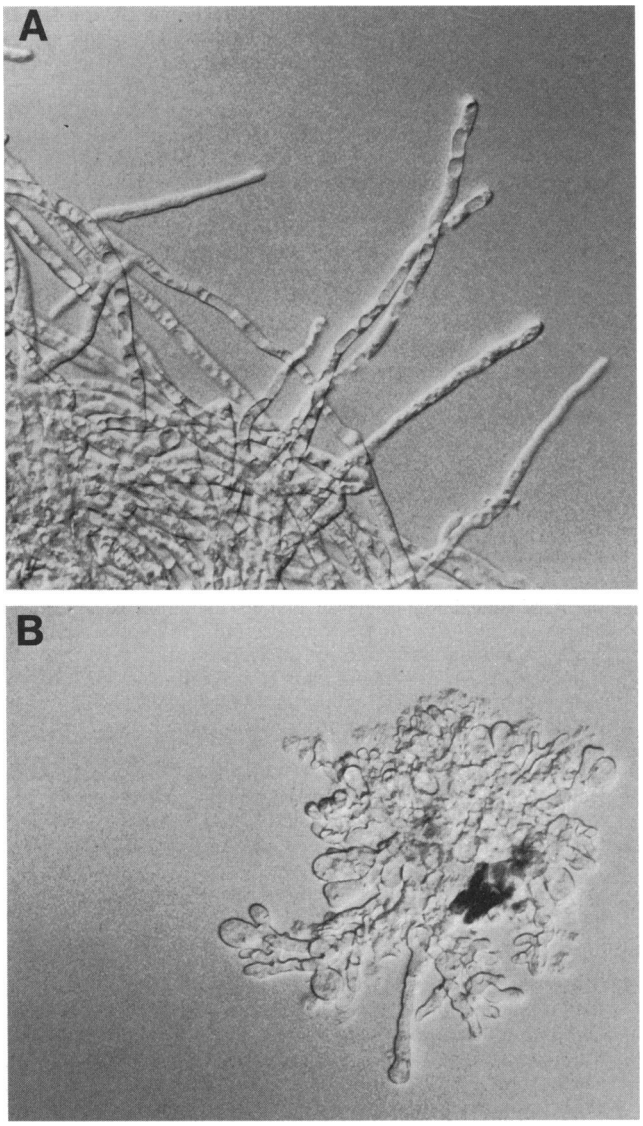

FIG. 2. Light micrograph of drug-induced morphology changes in *A. fumigatus* MF4839. (A) Control; (B) treatment with 2 μg of L-733,560 per ml. Magnification, ×284.

for the semisynthetic analog was down to 2-fold for *A. fumigatus* and increased to 150-fold for *C. albicans* (Table 1). These results suggest that another factor such as net charge may also be important in determining the in vitro activity of the pneumocandin class of compounds. A better comparison can be made with the L-733,560 and its stereoisomer (Table 3). In this case, in which both compounds would be expected to have the identical charge, the ratio for *C. albicans* is 76-fold, which is much closer to the 150-fold seen for L-733,560 and within the 2-fold accuracy of the MIC determination.

The IC_{50}s of the pneumocandins for the *S. cerevisiae* crude microsomal enzyme, however, appear to be too high to account for the MIC for this organism (Table 1). Nonetheless, the more potent compound, L-733,560, was a more potent inhibitor of the *S. cerevisiae* enzyme, suggesting that at least some of the enhanced anti-*S. cerevisiae* activity might be due to the inhibition of glucan synthesis. Characterization of the enzyme activity from an *S. cerevisiae* wild-type strain and a mutant resistant to L-733,560 provides genetic evidence that glucan synthesis is the target in this yeast (21). Enzymatic activities from both strains were inhibited by L-733,560 in a dose-dependent fashion, but much higher concentrations were required to achieve 50% inhibition with crude microsomes from the mutant (IC_{50} of 50 μM for mutant versus 1 μM for the wild type [21]). The 50-fold differential in IC_{50} matches the 50-fold difference in MIC (8 μg/ml for the mutant versus 0.25 μg/ml for the wild type). The relatively poor inhibition of in vitro enzyme activity by pneumocandin B_0 may reflect the crude state of the enzyme preparation rather than the actual inhibition of the activity in vivo. In fact, most investigators have reported difficulty in demonstrating that this yeast enzyme is susceptible to echinocandin-like compounds, despite obvious fungicidal activity against the whole organism (19, 24). On the other hand, Yamaguchi et al. (44) reported on the inhibition (70%) of *S. cerevisiae* enzyme activity with aculeacin A at 1 μg/ml. They could not achieve a complete inhibition and, in fact, saw reduced levels of inhibition at higher concentrations which they attributed to the formation of insoluble micelles. We also observed a lack of complete inhibition with the *S. cerevisiae* enzyme when the sparingly soluble natural product pneumocandin B_0 was used. These different observations are similar to observations of echinocandin enzyme inhibition with *C. albicans* membrane preparations (28, 35). Here, too, the IC_{50} of the enzyme did not account for the growth inhibition of the yeast. A more accurate assessment of the in vitro inhibition awaits purification and characterization of the enzyme.

Enzyme inhibition by L-733,560 appears to be specific for glucan synthase; the compound did not affect other fungal membrane-bound enzymes such as CHS I or squalene synthase, although an exhaustive survey of potential enzymes might demonstrate the inhibition of some enzymes. In fact, some weak inhibition of myristoyltransferase activity was seen (Table 2), but at concentrations that were much too high to account for the inhibition of growth of whole cells. The effects on myristoylation were not specific to the more potent pneumocandin analogs. Pneumocandin B_0 produced nearly the same level of inhibition of the enzyme as the semisynthetic compound at 2 μM (data not shown). In addition, attempts to show the membrane-perturbing effects of L-733,560 in lipid bilayers or by the production of ^{86}Rb flux in liposomes failed to show appreciable differences among L-733,560, its isomer, and pneumocandin B_0. The abilities of these compounds to cause currents in bilayer experiments at high concentrations (50 μM) may explain the inability of osmotic support to prevent cell lysis at similar drug concentrations.

In summary, the present study demonstrates that L-733,560 is a potent, specific inhibitor of fungal glucan synthesis. The enhanced whole-cell activity of this pneumocandin derivative against *C. albicans* and *A. fumigatus* can be attributed to the increased level of inhibition of the glucan synthase. Furthermore, L-733,560 and its relatively inactive stereoisomer L-734,735 show promise for elucidating the interactions of pneumocandins with an essential cell wall biosynthetic process in a genetically tractable organism, *S. cerevisiae*, as well as the clinically important pathogens.

ACKNOWLEDGMENTS

We thank our colleagues at Merck, J. Heck and R. Schwartz, for critical reading of the manuscript.

REFERENCES

1. **Abruzzo, G. K., A. M. Flattery, C. J. Gill, J. G. Smith, H. Kropp, and K. F. Bartizal.** 1993. Evaluation of water soluble lipopeptides L-733,560, L-705,589 and L-731,373 in a mouse model of dissem-

inated aspergillosis. Program Abstr. 33rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 355.
2. **Abruzzo, G. K., A. M. Flattery, C. J. Gill, L. Kong, J. G. Smith, D. Krupa, V. B. Pikounis, H. Kropp, and K. Bartizal.** Unpublished data.
3. **Baguley, B., G. Rommele, J. Gruner, and W. Wehrli.** 1979. Papulacandin B: an inhibitor of glucan synthesis in yeast spheroplasts. Eur. J. Biochem. **97**:345–351.
4. **Balkovec, J. M., R. M. Black, M. L. Hammond, J. V. Heck, R. A. Zambias, G. Abruzzo, K. Bartizal, H. Kropp, C. Trainor, R. E. Schwartz, D. C. McFadden, K. H. Nollstadt, L. A. Pittarelli, M. A. Powles, and D. M. Schmatz.** 1992. Synthesis, stability, and biological evaluation of water-soluble prodrugs of a new echinocandin lipopeptide. Discovery of a potential clinical agent for the treatment of systemic candidiasis and *Pneumocystis carinii* pneumonia (PCP). J. Med. Chem. **35**:194–198.
5. **Bartizal, K., G. Abruzzo, C. Trainor, D. Krupa, K. Nollstadt, D. Schmatz, R. Schwartz, M. Hammond, J. Balkovec, and F. Vanmiddlesworth.** 1992. In vitro antifungal activities and in vivo efficacies of 1,3-β-D-glucan synthesis inhibitors L-671,329, L-646,991, tetrahydroechinocandin B, and L-687,781, a papulacandin. Antimicrob. Agents Chemother. **36**:1648–1657.
6. **Bergstrom, J. D., M. M. Kurtz, D. J. Rew, A. M. Amend, J. D. Karkas, R. G. Bostedor, V. S. Bansal, C. Dufresne, F. L. Vanmiddlesworth, O. D. Hensens, J. M. Liesch, D. L. Zink, K. E. Wilson, J. Onishi, J. A. Milligan, G. Bills, L. Kaplan, O. M. Nallin, R. G. Jenkins, L. Huang, M. S. Meinz, L. Quinn, R. W. Burg, and Y. L. Kong.** 1993. Zaragozic acids: a family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase. Proc. Natl. Acad. Sci. USA **90**:80–84.
7. **Bernard, E. M., F. F. Edwards, D. Armstrong, and M. B. Kurtz.** 1993. Activity of three pneumocandins in an animal model of pulmonary aspergillosis. Program Abstr. 33rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 354.
8. **Bouffard, F. A., R. A. Zambias, J. F. Dropinski, J. M. Balkovec, M. L. Hammond, K. H. Nollstadt, J. A. Marrinan, M. B. Kurtz, D. C. McFadden, K. H. Nollstadt, M. Powles, and D. M. Schmatz.** 1994. Synthesis and antifungal activity of novel cationic pneumocandin B_0 derivatives: L-705,589, L-731,373, and L-733,560. J. Med. Chem. **37**:222–225.
9. **Bowers, B. G., G. Levin, and E. Cabib.** 1974. Effect of polyoxin B on chitin synthesis and septum formation in *Saccharomyces cerevisiae*. J. Bacteriol. **119**:564–575.
10. **Bozzola, J. J., R. J. Mehta, L. H. Nisbet, and J. R. Valent.** 1984. The effect of aculeacin A and papulacandin B on morphology and cell wall structure in *Candida albicans*. Can. J. Microbiol. **30**:857–863.
11. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72**:248–254.
12. **Cabib, E., M. S. Kang, and J. Au-Young.** 1987. Chitin synthase from *Saccharomyces cerevisiae*. Methods Enzymol. **138**:643–649.
13. **Cassone, A., R. E. Mason, and D. Kerridge.** 1981. Lysis of growing yeast-form cells of *Candida albicans* by echinocandin: a cytological study. Sabourandia **19**:97–110.
14. **Current, W., C. Boylan, J. Radding, P. Raab, D. Zeckner, T. Butler, R. Gordee, L. Petersen, W. Green, W. Turner, M. Debono, L. LaGrandeur, M. Rodriguez, and M. Zweifel.** 1994. Activity and efficacy of LY307853 and related echinocandin agents against pathogenic fungi, including *Pneumocystis carinii*. Abstr. 12 Congress Int. Soc. Hum. Animal Mycology, abstr. P7.2.
15. **Current, W. L., C. J. Boylan, and P. A. Raab.** 1993. Anti-*Pneumocystis* activity of LY303366 and other echinocandin B analogs. Program Abstr. 33rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 368.
16. **Davila, T., B. G. San, and B. F. San.** 1986. Effect of papulacandin B on glucan synthesis in *Paracoccidioides brasiliensis*. J. Med. Vet. Mycol. **24**:193–202.
17. **Debono, M., B. J. Abbott, J. R. Turner, L. C. Howard, R. S. Gordee, A. S. Hunt, M. Barnhart, R. M. Molloy, K. E. Willard, D. Fukuda, T. F. Butler, and D. J. Zeckner.** 1988. Synthesis and evaluation of LY121019, a member of a series of semisynthetic analogues of the antifungal lipopeptide echinocandin B. Ann. N. Y. Acad. Sci. **554**:152–167.
18. **Debono, M., W. W. Turner, L. LaGrandeur, F. Burkhardt, M. Rodrigues, M. Zweifel, J. Nissen, K. Clingerman, R. Gordee, D. Zeckner, T. Butler, T. Parr, and J. Tang.** 1993. LY303366—a new semi-synthetic lipopeptide antifungal agent related to echinocandin B. Synthetic and structure-activity studies. Program Abstr. 33rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 359.
19. **de Mora, J. F., E. Herrero, and R. Sentandreu.** 1993. A kinetic study on the regeneration of *Candida albicans* protoplasts in the presence of cell wall synthesis inhibitors. FEMS Microbiol. Lett. **111**:43–48.
20. **Douglas, C., J. Marrinan, J. Curotto, J. Onishi, and M. Kurtz.** 1992. Activity of a new echinocandin, L-688,786, against filamentous fungi, abstr. A-41, p. 7. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, D.C.
21. **Douglas, C. M., J. A. Marrinan, W. Li, and M. B. Kurtz.** A *Saccharomyces cerevisiae* mutant with echinocandin resistant 1,3-β-D glucan synthase activity. J. Bacteriol. **176**:5686–5696.
22. **Drouhet, E., B. Dupont, L. Improvisi, M. Lesourd, and M. C. Prevost.** 1990. Activity of cilofungin (LY-121019), a new lipopeptide antibiotic, on the cell wall and cytoplasmic membrane of *Candida albicans*. Structural modifications in scanning and transmission electron microscopy. J. Med. Vet. Mycol. **28**:425–436.
23. **Dunnett, C. W.** 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. **50**:1096–1121.
24. **Duran, A., R. Varona, P. Perez, and I. Garcia-Acha.** 1984. Biosynthesis of fungal wall β-glucan and its inhibition by antifungal agents., p. 297–305. *In* C. Nombela (ed.), Microbial cell wall synthesis and autolysis. Elsevier, Amsterdam.
25. **Garcia, M. L., A. A. Guffanti, and T. A. Krulwich.** 1983. Characterization of the Na^+/H^+ antiporter of alkalophilic bacilli in vivo: $\Delta\Psi$-dependent $^{22}Na^+$ efflux from whole cells. J. Bacteriol. **156**: 1151–1157.
26. **Gordee, R. S., D. J. Zeckner, L. F. Ellis, A. L. Thakkar, and L. C. Howard.** 1984. *In vitro* and *in vivo* anti-*Candida* activity and toxicology of LY121019. J. Antibiot. **37**:1054–1065.
27. **Gordee, R. S., D. J. Zeckner, L. C. Howard, W. E. Alborn, Jr., William E., and M. DeBono.** 1988. Anti-*Candida* activity and toxicology of LY121019, a novel semisynthetic polypeptide antifungal antibiotic. Ann. N. Y. Acad. Sci. **544**:294–309.
28. **Gruner, J., and P. Traxler.** 1977. Papulacandin, a new antibiotic active specially against yeasts. Experientia **33**:137.
29. **Henry, S. A., K. D. Atkinson, A. I. Kolat, and M. R. Culbertson.** 1977. Growth and metabolism of inositol-starved *Saccharomyces cerevisiae*. J. Bacteriol. **130**:472–484.
30. **Horn, W. S., J. L. Smith, G. Bills, S. Raghoobar, G. Helms, M. Kurtz, J. Marrinan, B. Frommer, R. Thornton, and S. Mandala.** 1992. Sphingofungins E and F: novel serine palmitoyltransferase inhibitors from *Paecilomyces variotii*. J. Antibiot. **45**:1692–1696.
31. **Kauss, H., and W. Jeblick.** 1985. Activation by polyamines, polycations, and ruthenium red of the Ca^{2+}-dependent glucan synthase from soybean cells. FEBS Lett. **185**:226–230.
32. **Kauss, H., and W. Jeblick.** 1987. Solubilization, affinity chromatography and Ca^{2+}/polyamine activation of the plasma membrane-located 1,3-β-glucan synthase. Plant Sci. **48**:63–69.
33. **Kurtz, M. B., I. B. Heath, J. Marrinan, S. Dreikorn, J. Onishi, and C. Douglas.** 1994. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activity against (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. **38**:1480–1489.
34. **Poli, F., C. Romagnoli, G. Dallolio, and M. P. Fasulo.** 1993. Effects of spermine and spermidine on cell-division and wall morphogenesis in *Saccharomyces cerevisiae*. Microbios **73**:261–267.
35. **Schmatz, D. M., M. A. Romancheck, L. A. Pittarelli, R. E. Schwartz, R. A. Fromtling, K. H. Nollstadt, F. L. Vanmiddlesworth, K. E. Wilson, and M. J. Turner.** 1990. Treatment of *Pneumocystis carinii* pneumonia with 1,3-β-glucan synthesis inhibitors. Proc. Natl. Acad. Sci. USA **87**:5950–5954.
36. **Schwartz, R. E., D. F. Sesin, H. Joshua, K. E. Wilson, A. J. Kempf, K. A. Goklen, D. Kuehner, P. Gailliot, C. Gleason, R. White, E. Inamine, G. Bills, P. Salmon, and L. Zitano.** 1992. Pneumocandins from *Zalarion arboricola*. I. Discovery and isolation. J. Antibiot. **45**:1853–1866.
37. **Shaw, J. A., P. C. Mol, B. Bowers, S. J. Silverman, M. H. Valdivieso, A. Durán, and E. Cabib.** 1991. The function of chitin

synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol. **114:**111–123.

38. **Taft, C. S., and C. P. Selitrennikoff.** 1988. LY121019 inhibits *Neurospora crassa* growth and 1,3-β-D-glucan synthase. J. Antibiot. **41:**697–701.
39. **Taft, C. S., T. Stark, and C. P. Selitrennikoff.** 1988. Cilofungin (LY121019) inhibits *Candida albicans* (1-3)-β-D-glucan synthase activity. Antimicrob. Agents Chemother. **32:**1901–1903.
40. **Tkacz, J. S.** 1992. Glucan biosynthesis in fungi and its inhibition, p. 495–523. *In* J. A. Sutcliffe and N. H. Georgopapdakou (ed.), Emerging targets in antibacterial and antifungal chemotherapy. Chapman & Hall, New York.
41. **Towler, D. A., S. P. Adams, S. R. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser, and J. I. Gordon.** 1988. Myristoyl CoA:protein N-myristoyltransferase activites from rat liver and yeast possess overlapping yet distinct peptide substrate specificities. J. Biol. Chem. **263:**1784–1790.
42. **VanMiddlesworth, F., M. N. Omstead, D. Schmatz, K. Bartizal, R. Fromtling, K. Nollstadt, S. Honeycut, M. Zweerink, G. Garrity, and G. Bills.** 1991. L-687,781, a new member of the papulacandin family of β-1,3-D-glucan synthesis inhibitors. I. Fermentation, isolation, and biological activity. J. Antibiot. **44:**45–51.
43. **Wichmann, C. F., J. M. Liesch and R. E. Schwartz.** 1989. L-671,329, a new antifungal agent. II. Structure determination. J. Antibiot. **42:**168–173.
44. **Yamaguchi, H., T. Hiratani, M. Baba, and M. Osumi.** 1985. Effect of aculeacin A, a wall-active antibiotic, on synthesis of the yeast cell wall. Microbiol. Immunol. **29:**609–623.
45. **Yamaguchi, H., T. Hiratani, K. Iwata, and Y. Yamamoto.** 1982. Studies on the mechanism of antifungal action of aculeacin A. J. Antibiot. **35:**210–219.