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Sterol Composition of *Cryptococcus neoformans* in the Presence and Absence of Fluconazole

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Analysis of the sterol compositions of 13 clinical isolates of the pathogenic yeast *Cryptococcus neoformans* obtained from five patients with recurring cryptococcal meningitis showed that, unlike *Candida albicans*, the major sterols synthesized by this yeast were obtusifoliol (range, 21.1 to 68.2%) and ergosterol (range, 0.0 to 46.5%). There was considerable variation in the sterol contents among the 13 isolates, with total sterol contents ranging from 0.31 to 5.9% of dry weight. The isolates from the five patients who had relapses had dif ferent total sterol contents and compositions in comparison with those of the pretreatment isolates, indicating either that the sterols had been changed by therapy or that the patients were infected with new isolates with different sterol compositions. Growth of the cryptococcal isolates in the presence of subinhibitory concentrations of fluconazole (0.25× the MIC) significantly altered the sterol content and pattern. The total sterol content decreased in nine isolates and increased in four isolates in response to pretreatment with fluconazole. Fluconazole had no consistent effect on ergosterol levels. In contrast, fluconazole caused a decrease in obtusifoliol levels and an increase in 4,14-dimethylzymosterol levels in all isolates. These results indicate extensive diversity in sterol content, sterol composition, and sterol synthesis in response to subinhibitory concentrations of fluconazole in *C. neoformans* strains. We propose that fluconazole inhibits the sterol synthesis of *C. neoformans* by interfering with both 14α-demethylase-dependent and -independent pathways. No correlation between the sterol compositions of *C. neoformans* isolates and their susceptibilities to fluconazole was found.

Cryptococcosis is a disseminated infection caused by the ubiquitous yeast *Cryptococcus neoformans* (10). This fungus is primarily a pathogen for immunocompromised patients, and individuals with AIDS are at high risk of cryptococcosis. Currie and Casadevall (4) have estimated that the prevalence of cryptococcosis among AIDS patients is between 7.4 and 13.8%. In the setting of AIDS, cryptococcal infections are particularly difficult to treat because antifungal therapy does not usually eradicate the infection (13). Given the high incidence of relapse after initial antifungal therapy, current management of *C. neoformans* infections in patients with AIDS includes lifelong suppressive therapy with antifungal drugs. In recent years, fluconazole has become the drug of choice for suppressive therapy because it is effective, can be given orally, and has few side effects (12).

Fluconazole is a triazole derivative that is believed to exert its antifungal activity by interfering with ergosterol synthesis, leading to membrane disorganization, leakage of essential cytoplasmic materials, and growth arrest (1). The mechanism of action of fluconazole has been studied primarily in *Candida albicans*, in which the drug interacts selectively with cytochrome P-450-dependent 14α-demethylase (11), leading to the accumulation of 14-methyl sterols and the inhibition of ergosterol synthesis.

Despite the enormous increase in the number of *C. neoformans* infections in recent years and the widespread use of fluconazole therapy in patients with cryptococcal meningitis, relatively little is known about the sterol content of this yeast. Only two studies have dealt with the sterol composition of *C. neoformans*. In 1975 Kim et al. (8) reported that the principal sterol of two strains of *C. neoformans* was Δ^7-ergosten-3β-ol (epifungisterol) rather than ergosterol. Recently, Vanden Bossche et al. (14) characterized sterol synthesis in one isolate of *C. neoformans* and reported that ergosterol was the major sterol. The discordance of these findings may reflect strain differences or differences in the conditions used to grow the fungus. *C. neoformans* clinical isolates are characterized by extensive genetic diversity (13, 14), which may also be associated with variation in the sterol composition of the cell membrane.

Because of the limited available information on cryptococcal sterols, reported differences in their predominant sterols, and studies demonstrating extensive genetic diversity (2, 17) among clinical isolates, and since sterol composition may be an important factor in the effectiveness of azoles, we undertook a characterization of 13 clinical isolates of *C. neoformans* that represented the initial and relapse isolates from five patients (3, 13). The results indicate that the predominant sterols in this pathogenic yeast are obtusifoliol and ergosterol. Furthermore, variations in sterol content, sterol composition, and sterol synthesis in response to fluconazole among different strains were observed. On the basis of our results we suggest that fluconazole inhibits *C. neoformans* by blocking sterol biosynthesis of this organism at two different sites.

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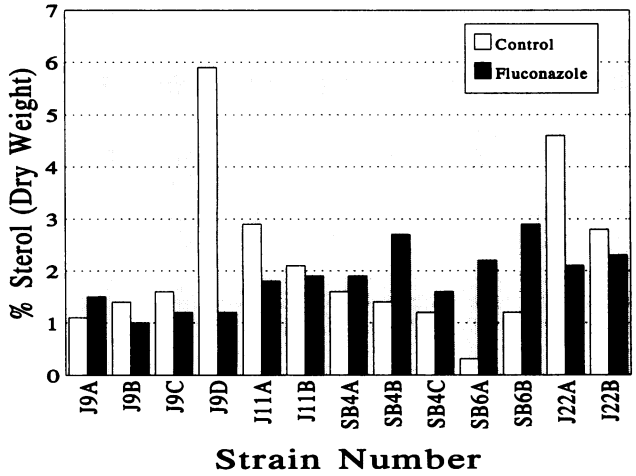

FIG. 1. Total sterol content extracted from the initial and relapse isolates of *C. neoformans* in the absence (control) or presence of fluconazole (MIC_{25}). Data are percentage of the cell dry weight and varied by <0.5% in duplicate runs.

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MATERIALS AND METHODS

Organisms. *C. neoformans* J9, J11, J22, SB4, and SB6 have been described previously, and each was isolated from a different patient (7, 11). For each strain the A isolate was recovered from a patient with cryptococcal meningitis before antifungal therapy was begun (3). The B, C, and D isolates were recovered during clinical recurrence, and all isolates have been exposed to fluconazole and/or amphotericin B in vivo (for details of the clinical course, see reference 3). For each strain the initial and relapse isolates have been shown to be genetically related by DNA typing methods (3, 13).

Sterol extraction and analysis. Prior to sterol extraction all cryptococcal strains were grown as a shake culture (200 rpm) in Yeast Nitrogen Base broth with amino acids (Difco, Detroit, Mich.) and enriched with 0.5% (wt/vol) glucose (YNB) at 37°C for 22 to 24 h (late exponential growth phase, corresponding to an optical density at 420 nm of 0.9 for all isolates), ensuring that the observed differences in sterols among the strains were not due to sampling at various stages of growth. Sterols were extracted by the method of Ghannoum et al. (6). Briefly, KOH (1.5 g) in 2 ml of distilled water was added to 0.2 g (wet weight) of intact cells, and the volume of the suspension was adjusted to 10 ml by the addition of ethanol. The solution was refluxed under nitrogen for 3 h, diluted with an equal amount of water, and extracted twice with four volumes of heptane. The extract was washed once with water, dried over anhydrous Na_2SO_4, and evaporated in vacuo to give the total sterols.

The sterols were fractionated by thin-layer chromatography by using silica gel G plates (0.25 mm) and 40 to 60°C petroleum ether-diethyl ether (3:1; vol/vol) as a developing solvent mixture. The Lifschutz reagent (concentrated sulfuric acid-glacial acetic acid [1:1]) was used to detect sterols as red or purple spots. The fractions separated by thin-layer chromatography were identified by comparing their R_f values with commercially available standards.

Silylated sterols were prepared by a modification of the method of Vandenheuvel and Court (16). Up to 1 mg of extracted sterol was reacted in a glass-stoppered flask with 50 μl of hexamethyl disilazane and 50 μl of 10% trimethyl chlorosilane in chloroform (vol/vol), with the reagents being added in that order. Brief mixing by stirring or vibration was applied after each addition. The reaction mixture was left at room temperature for at least 4 h. Excess solvent and reagents were removed by evaporation under N_2. Fifty microliters of hexane was then added to the flask to dissolve the reaction mixture. Half-microliter portions were analyzed by gas chromatography by using an OV-1 column (3% on 100/120 gaschrome Q) in a Vista 6000 (Varian, Sugarland, Tex.) gas chromatograph. Comparison of the retention times of the peaks with those of reference standards by using polydimethylsiloxane (JXR) as a second column was also performed. The samples were eluted at 230°C with helium as the carrier gas (30 ml/min) and an injection temperature of 250°C, and the nonsaponifiable lipids were detected with a flame ionization detector at 300°C. Individual components were identified by comparison of the retention times relative to that of ergosterol and with those of commercially available standards. Gas-liquid chromatography (GLC) peaks were quantified by using a Varian Vesta 402 integrator. To minimize quantitative errors, cholesterol was included in the GLC analyses as an internal standard. Unless otherwise specified all chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Susceptibility testing. The MICs of fluconazole for the inhibition of *C. neoformans* were determined by the microdilution technique described elsewhere (5). Briefly, fluconazole was diluted in YNB to provide a stock solution. YNB was buffered to pH 7.0 with 0.05 M morpholinepropanesulfonic acid (MOPS). Drug-free medium was dispensed into all wells of microtiter assay plates. Ten twofold dilutions of fluconazole stock solution were made by adding fluconazole to wells numbered 2 through 10 (concentration ranges, 64 to 0.06 μg/ml). Well 1 served as a sterility control and a blank for the spectrophotometric assays; well 12 served as a growth control. The microtiter plate wells were inoculated with 10 μl of yeast

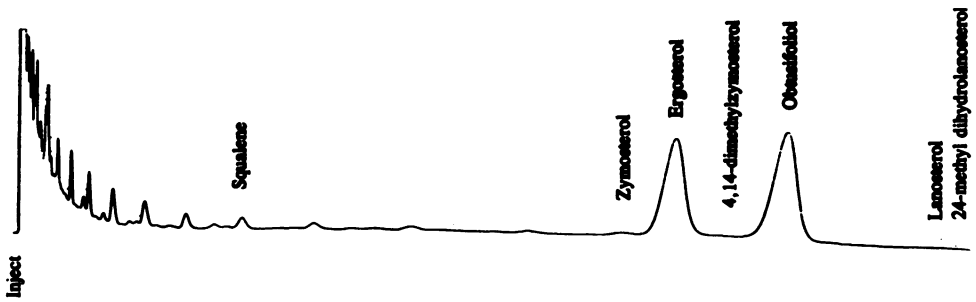

FIG. 2. Typical gas-liquid chromatogram of sterols extracted from *C. neoformans* by using a 3% OV-1 column.

TABLE 1. GLC analysis of sterols of *C. neoformans* grown in the absence or presence of subinhibitory concentration (MIC_{25}) of fluconazole

Strain	% (wt/wt) of total amount of sterols[a]															
	24-Methylenedihydrolanosterol		Squalene		Lanosterol		4,14-Dimethylzymosterol		Zymosterol		Obtusifoliol		Calciferol		Ergosterol	
	−F	+F	−F	+F	−F	+F	−F	+F	−F	+F	−F	+F	−F	+F	−F	+F
J9A	8.9	ND	16.0	4.4	ND	5.3	14.3	39.4	ND	ND	29.4	8.3	13.4	4.4	14.9	38.3
J9B	ND	ND	1.7	4.1	ND	2.9	ND	69.2	1.6	1.6	52.7	17.7	0.8	2.4	43.2	2.1
J9C	ND	ND	8.5	9.2	1.6	ND	3.0	40.4	ND	2.7	36.2	35.1	4.1	9.0	46.5	3.5
J9D	ND	ND	14.9	34.2	25.7	21.8	5.2	8.0	ND	ND	35.3	14.6	15.7	19.0	3.2	2.4
J11A	4.2	ND	6.2	8.0	2.4	11.0	2.6	16.8	2.7	ND	34.6	15.6	3.2	9.7	44.1	38.8
J11B	6.9	ND	6.3	3.8	16.3	9.0	5.1	58.9	3.6	ND	21.1	5.9	5.2	4.9	35.4	17.2
SB4A	ND	ND	2.1	12.5	ND	9.7	2.5	31.0	2.8	ND	68.2	23.2	1.2	11.3	23.2	12.2
SB4B	ND	ND	5.4	13.0	ND	10.6	ND	26.2	3.1	ND	58.8	12.7	1.9	12.0	38.8	25.4
SB4C	6.0	ND	4.9	11.4	17.9	24.8	9.4	24.2	ND	ND	55.3	31.1	6.5	8.5	ND	ND
SB6A	ND	ND	8.0	7.3	ND	14.9	4.6	31.1	2.0	ND	50.4	10.2	4.3	8.6	30.7	28.0
SB6B	ND	ND	11.8	24.1	7.6	ND	21.0	33.2	ND	ND	24.7	11.1	11.4	15.0	23.6	16.6
J22A	ND	ND	14.6	6.3	ND	12.8	ND	34.1	5.4	ND	56	8.2	14.4	4.0	9.7	33.6
J22B	ND	ND	1.9	17.1	ND	ND	ND	36.2	3.2	ND	51.4	10.6	1.6	12.1	43.0	24.0

[a] The values varied by < 10%. The relative retention time for different runs varied by <0.006. Abbreviations: −F, no drug (control); +F, fluconazole; ND, not detected.

cells to achieve a final inoculum of approximately 10^4 CFU/ml. The inoculated plates were incubated for 48 h at 35°C without shaking. Yeast cell growth was determined by measurements of the optical density by using an automatic microplate reader (Dupont Instruments, Wilmington, Del.) after agitation of the plates for 15 s with a vortex shaker (Vortex-Genie 2 Mixer; Scientific Industries, Inc. Bohemia, N.Y.). The MIC endpoint was defined as the lowest drug concentration that exhibited 50% (or more) reduction of the optical density at 420 nm in comparison with that of the control well (MIC_{50}).

RESULTS

Sterol content of *C. neoformans*. In the absence of fluconazole the total sterol content of 13 clinical cryptococcal isolates studied ranged from 0.31% (isolate SB6A) to 5.9% (isolate J9D) of the dry weight. There was considerable variation in the total sterol contents among the initial and relapse isolates from individual patients (Fig. 1). The sterol contents of the relapse isolates were higher than those of the original isolates for strains J9 and SB6, whereas they were lower than those of the original isolates for strains J11, SB4, and J22. Thin-layer chromatographic analysis showed that the major sterols present in the 13 cryptococcal isolates tested were 4-desmethyl sterols (such as ergosterol), 4-methyl sterols, and 4,4-dimethyl sterols (such as lanosterol). These sterols were subsequently resolved and quantitated by GLC analysis (Fig. 2). The major sterols in this set of isolates were obtusifoliol (range, 21.1 to 68.2%) and ergosterol (range, 0.0 to 46.5%) (Table 1). In only two isolates was another sterol present at more than 20%. Ergosterol was the predominant sterol in only three isolates (range, 35.4 to 46.5%). Obtusifoliol was the predominant sterol in nine isolates (range, 29.4 to 68.2%); however, five of these isolates also contained >20% ergosterol. One isolate (SB6B) contained nearly equal amounts of ergosterol and obtusifoliol; this was also the only isolate that contained a large amount of 4,14-dimethylzymosterol (21.0%). Two isolates (SB4C and J9D) contained very little ergosterol (0.0 and 3.2%, respectively) and relatively large amounts of lanosterol.

As with the total sterol content, there was considerable variation in the types and quantities of sterols present in the initial and relapse isolates from individual patients. Two trends were noted: the obtusifoliol content was higher in the initial isolate than in the relapse isolates for all strains except J9, and the lanosterol content increased in relapse isolates in comparison with that in the initial isolates (except for J22, in which it was not detected in either isolate). For the other sterols there were no obvious patterns in the compositions of the initial and relapse isolates. For three strains (J9D, J22A, J11A) measurement of total sterols and sterol composition were performed on two separate occasions, and less than 10% variation was revealed among duplicate measurements.

Sterol content following exposure to fluconazole. Exposure to subinhibitory concentrations of fluconazole, defined as the concentration that caused 25% growth inhibition in comparison with the growth of the control (MIC_{25}), produced both increases and decreases in the total sterol content, depending on the isolate (Fig. 1). The initial and relapse isolates of strains J11 and J22 showed a decrease in their sterol contents following exposure to fluconazole. In contrast, strains SB4 and SB6 exhibited an increase in their sterol contents when grown in the presence of fluconazole. The sterol content of the initial isolate of strain J9 following exposure to fluconazole increased, while those of the relapse isolates decreased upon fluconazole treatment.

TABLE 2. Effect of subinhibitory concentration of fluconazole on sterol compositions of 13 cryptococcal isolates

Sterol	% of total sterols[a]		*P* value[b]
	Control	Fluconazole	
Squalene	7.9 ± 5.1	12.0 ± 8.8	1.24
24-Methylenedihydrolanosterol	2.0 ± 3.3	0.0 ± 0.0	0.43
Lanosterol	4.6 ± 8.0	10.7 ± 8.2	0.742
4,14-Dimethylzymosterol	5.2 ± 6.0	34.5 ± 15.4	0.001
Zymosterol	4.4 ± 9.3	0.4 ± 0.82	0.23
Obtusifoliol	44.2 ± 14.1	16.1 ± 8.9	<0.001
Calciferol	5.7 ± 4.6	9.3 ± 4.5	1.26
Ergosterol	27.4 ± 15.6	18.6 ± 13.4	1.23

[a] Data are means ± standard deviations for 13 isolates.
[b] *P* values were calculated by the paired *t* test with the Bonferroni correction for multiple comparisons.

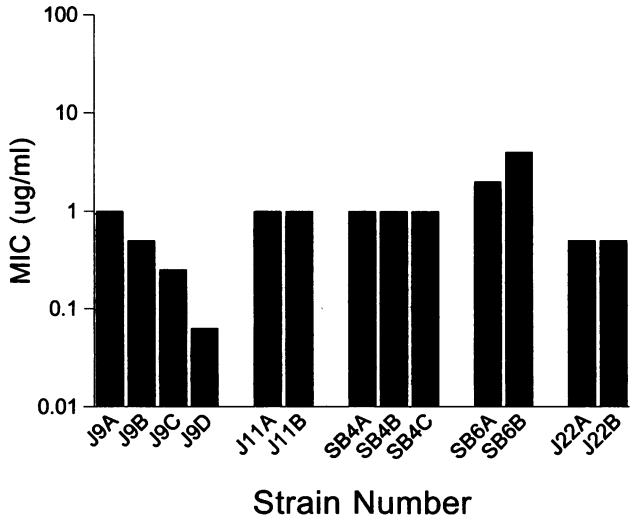

FIG. 3. Susceptibilities of 13 *C. neoformans* strains to fluconazole. Values are MICs of fluconazole for 10^4 cells per ml measured in YNB after 48 h.

GLC analysis revealed profound changes in the sterol profiles in response to fluconazole (Table 1). Table 2 summarizes the average contents of the different sterols resolved from the 13 clinical isolates tested. Growth in the presence of fluconazole resulted in large decreases in obtusifoliol and increases in 4,14-dimethylzymosterol ($P < 0.001$). Additionally, fluconazole caused a decrease in the ergosterol contents in 10 of the 13 isolates and an increase in the ergosterol contents in 2 of the 13 isolates (in isolate SB4C ergosterol was not detectable in the presence or absence of fluconazole). However, when data for all isolates were analyzed together, fluconazole had no significant effect on ergosterol levels ($P > 0.5$). 24-Methylenedihydrolanosterol was not detected in any isolate following exposure to fluconazole, while low levels were detected in four strains in the absence of fluconazole (Table 2).

MIC. The MIC_{50}s of fluconazole for the 13 *C. neoformans* clinical isolates were determined by the microdilution technique (Fig. 3). Fluconazole MIC_{50}s ranged from 0.06 μg/ml (isolate J9D) to 4 μg/ml (isolate SB6B). In none of the strains was there a significant increase in the MIC_{50} for the relapse isolates.

DISCUSSION

Analysis of sterols from 13 clinical isolates of *C. neoformans* showed that, for the majority of isolates, the predominant sterol was obtusifoliol, not ergosterol. Obtusifoliol is an important intermediate in the synthesis of sterols (18) and has been observed in several fungal species following treatment with azoles (15). The differences in the sterol patterns that we observed among the isolates studied under standardized conditions strongly suggest that there are innate (sometimes large) differences in sterol metabolism among natural isolates of *C. neoformans*. In addition, we showed that serial, clonally related isolates from patients with recurrent cryptococcal meningitis can exhibit marked differences in their individual sterol compositions as well as total sterol contents. These results suggest that selection of sterol variants occurred in vivo or that the sterol pattern changed because of the effect of antifungal therapy. In contrast to *C. neoformans*, *C. albicans* does not show significant strain-to-strain variation in sterol patterns; moreover, ergosterol is the predominant sterol in *C. albicans*. These results suggest that the sterol compositions of patho-

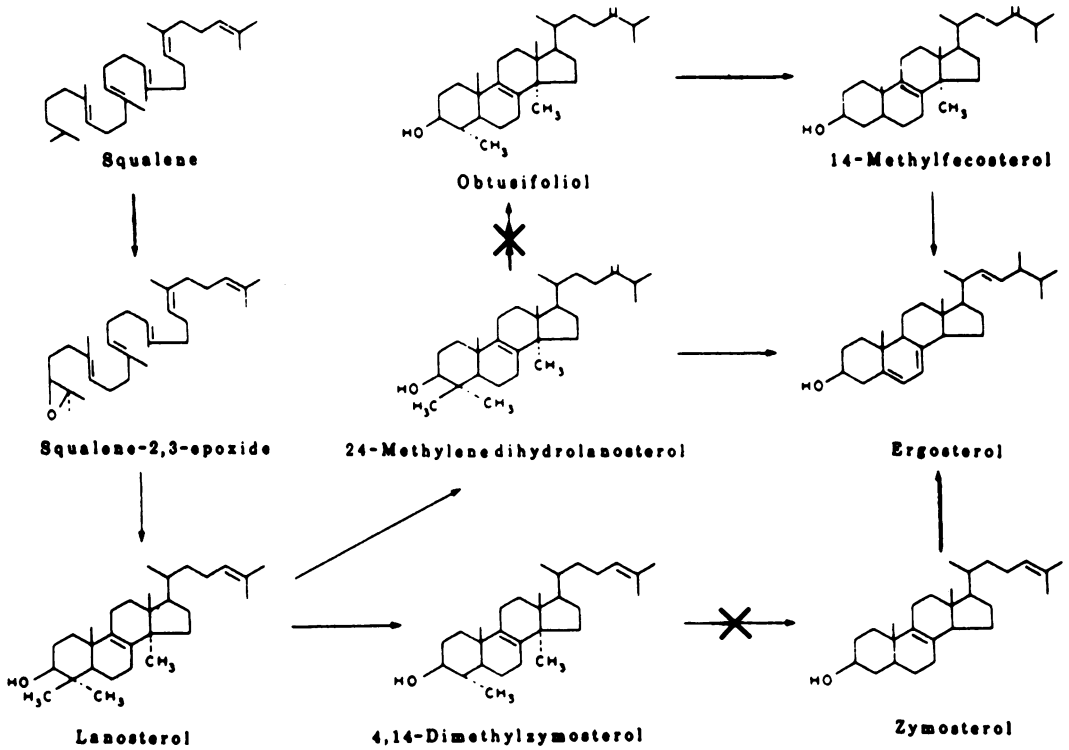

FIG. 4. Pathways of metabolic conversion of sterols in yeasts. The X's indicate possible sites where fluconazole affects ergosterol synthesis.

genic fungi should be carefully studied since generalizations concerning membrane sterol composition on the basis of previous data for a few species (e.g., *C. albicans* and *Saccharomyces cerevisiae*) may not be applicable to other yeast genera.

Growth of *C. neoformans* in the presence of subinhibitory concentrations of fluconazole resulted in altered sterol contents and compositions in all of the isolates tested. Fluconazole caused a significant decrease in the amount of obtusifoliol and a significant increase in the amount of 4,14-dimethylzymosterol in all isolates tested. The increase in 4,14-dimethylzymosterol levels is consistent with previous studies showing that triazole antifungal agents inhibit cytochrome P-450-dependent 14α-demethylase (1, 11). It is well recognized that sterols serve as bioregulators of membrane fluidity, asymmetry, and consequently, membrane integrity in eukaryotic cells (9). Membrane integrity requires inserted sterol components that lack C-4 methyl groups (14) and our data demonstrate that fluconazole mediates the blockage of the conversion of 4,14-dimethylzymosterol to zymosterol, resulting in the accumulation of the former (Fig. 4). Thus, one possible fungistatic effect of fluconazole may be due to the accumulation of 4,14-dimethylzymosterol.

The decrease in the amount of obtusifoliol in the presence of fluconazole is not readily explained. Since the synthesis of this compound does not involve a 14α-demethylation step, there is probably an additional site of fluconazole inhibition in the cryptococcal sterol biosynthetic pathway. Vanden Bossche et al. (14) demonstrated that, in addition to inhibiting the 14α-demethylase in *C. neoformans*, itraconazole affects the reduction of obtusifolione to obtusifoliol. Fluconazole may have a similar mode of action (Fig. 4). In addition, inhibition of one branch of the sterol biosynthetic pathway may have major effects on the other branches.

Fluconazole MICs for the 13 clinical isolates obtained by the microdilution method were lower than the values obtained by the macrodilution method (3). Nevertheless, the MICs obtained by both antifungal susceptibility methodologies reflect the same pattern of fluconazole susceptibility, and it is likely that the different MICs reflect differences in the methods. It is important that the microdilution test results showed no increase (within 1 well dilution) in the MICs for the five sets of initial and relapse isolates, thus supporting the previous conclusion that the relapses of cryptococcal meningitis in these patients were not due to the development of fluconazole resistance (3).

In the current study we attempted to determine the possible relationship between the sterol compositions of cryptococcal strains and their susceptibilities to fluconazole. Despite the wide variation in sterol contents and compositions seen in our isolates, they exhibited a relatively narrow range of susceptibility to fluconazole. Therefore, although we observed that organisms for which MICs were higher tended to have a lower sterol content, and vice versa, it was not possible to establish a definite correlation. Examination of large numbers of resistant isolates (when they become available) would help resolve this issue. The present study revealed an unexpected complexity in the sterol profiles of *C. neoformans* strains and strongly suggests the need for further investigations to elucidate the mechanisms responsible for such diversity and their clinical implications.

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