Isolation and composition of inositolphosphorylceramide-type sphingolipids of hyphal forms of Candida albicans.

G B Wells, R C Dickson and R L Lester
J. Bacteriol. **1996, 178(21):6223.**

JOURNAL OF BACTERIOLOGY, Nov. 1996, p. 6223–6226 Vol. 178, No. 21
0021-9193/96/$04.00+0

Isolation and Composition of Inositolphosphorylceramide-Type Sphingolipids of Hyphal Forms of *Candida albicans*

GERALD B. WELLS, ROBERT C. DICKSON, AND ROBERT L. LESTER*

Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40536

Received 5 July 1996/Accepted 27 August 1996

Hyphal forms of the human pathogen *Candida albicans* have been found to contain substantial quantities of phosphosphingolipids. These lipids were fractionated into three classes by normal-phase high-performance liquid chromatography. The first class contained equimolar amounts of phosphorus, inositol, phytosphingosines, and fatty acids; their composition and chromatographic behavior suggest that these compounds are inositolphosphorylceramides. The second class contained equimolar amounts of phosphorus, mannosylinositol, phytosphingosines, and fatty acids; their composition and chromatographic behavior indicate that these compounds are mannosylinositolphosphorylceramides. The third class of compounds contained phosphorus, mannosylinositol, inositol, phytosphingosines, and fatty acids in a molar ratio of 2:1:1:1:1; their composition and chromatographic behavior indicate that these compounds are mannosyldiinositolphosphorylceramides. Molecular species in each class differ in the composition of long chain bases and fatty acids; the most abundant long chain bases were C_{18} and C_{20} phytosphingosines, and the most abundant fatty acids were hydroxy and nonhydroxy C_{24-26}. The array of sphingolipids in *C. albicans* is similar to that of *Saccharomyces cerevisiae*. Sphingolipids have been shown to be essential in *S. cerevisiae*, thus these lipids, which are not present in animals, offer a potentially unique targets for antifungal chemotherapy against *C. albicans*.

Inositolphosphorylceramides (IPCs) constitute a group of membrane sphingolipids not found in animals but found in various plants, fungi, yeast, and protozoans (6). Biochemical and genetic studies indicate that in *Saccharomyces cerevisiae* these plasma membrane sphingolipids are essential for growth (15), for viability (11), and for resistance to environmental stress (10). The essential nature of yeast sphingolipids is further indicated by the action of sphingofungin and australifungin, fungitoxic antibiotics that act as inhibitors of sphingolipid synthesis in *S. cerevisiae* (17) and *Candida* spp. (8).

Human mycoses are a continuing medical problem, with *Candida* infections being quite significant (2). Since sphingolipids may represent a unique target for antifungal chemotherapy against *Candida* species, we have sought to establish the nature of the IPCs present in *Candida albicans.*

MATERIALS AND METHODS

Culture of *C. albicans*. The hyphal forms of *C. albicans* (ATCC 62342 [A26]) were prepared by first inoculating a liter of Sabouraud dextrose medium with 10^7 cells from an overnight agar slant of Sabouraud dextrose medium incubated at 35°C. The culture was incubated at 35°C for 48 h with shaking at 150 rpm, and the cells were harvested by centrifugation at 2,500 × *g* for 10 min and washed with saline. These cells were resuspended in a liter of RMPI medium without glutamate at pH 7.0 but containing 20% fetal calf serum and incubated for 48 h with slow shaking at ~60 rpm. The cells were harvested as described above, suspended in 5% trichloroacetic acid for 30 min at 4°C, and washed with saline three times; the pellet was weighed and stored at −20°C until lipid extraction. The transformation of *C. albicans* blastospores to hyphal forms was confirmed by direct observation by light microscopy. These cells were generously provided by R. S. Gordee, Eli Lilly and Co.

Extraction and deacylation of lipids. Lipid extraction was carried out essentially as described previously (5). To the cell pellet (~40 ml), 60 ml of ethanol-diethyl ether-pyridine-concentrated NH_4OH (15:5:1:0.018 [vol/vol/vol/vol]) and 80 ml of solvent E {ethanol-water-diethyl ether-pyridine-concentrated NH_4OH [15:15:5:1:0.018 (vol/vol/vol/vol/vol)]} were added, and the mixture was heated for 60 min at 60°C. After centrifugation while still warm, the supernatant was reserved, and the pellet was reextracted with 60 ml of solvent E for 30 min at 60°C. After centrifugation while warm, the supernatant was removed and combined with the first extract.

To 200 ml of the extract, 200 ml of 0.2 N KOH in methanol was added, and

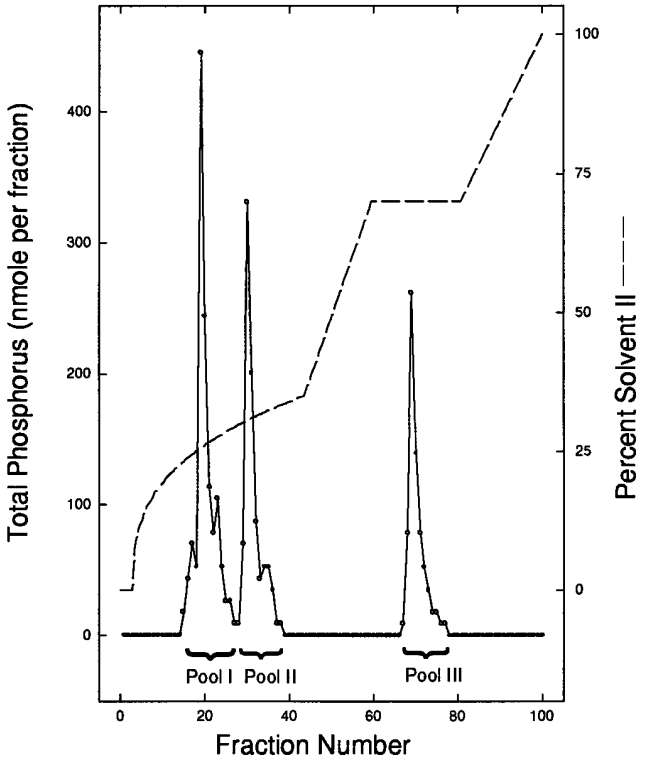

FIG. 1. Preparative HPLC of alkali-stable phospholipids of *C. albicans*. The alkali-stable lipid fraction was resolved by chromatography on a silica gel column, with each fraction assayed for total phosphorus. The indicated fractions were pooled for further analysis.

* Corresponding author. Mailing address: Department of Biochemistry, College of Medicine, University of Kentucky, Lexington, KY 40536-0084. Phone: (606) 323-6046. Fax: (606) 323-1037. Electronic mail address: rlester@pop.uky.edu.

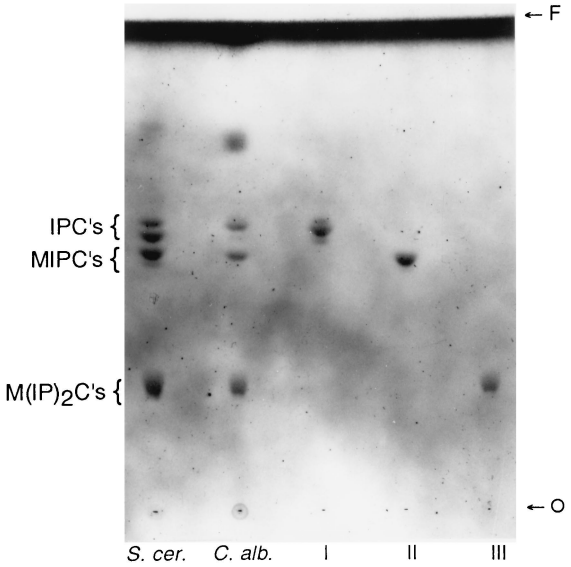

FIG. 2. TLC of lipids purified by preparative HPLC. The pooled fractions (I, II, and III) after preparative HPLC (Fig. 1) and the crude alkali-stable lipid mixtures from *S. cerevisiae* (*S. cer.*) and *C. albicans* (*C. alb.*) were subjected to TLC and detected by charring.

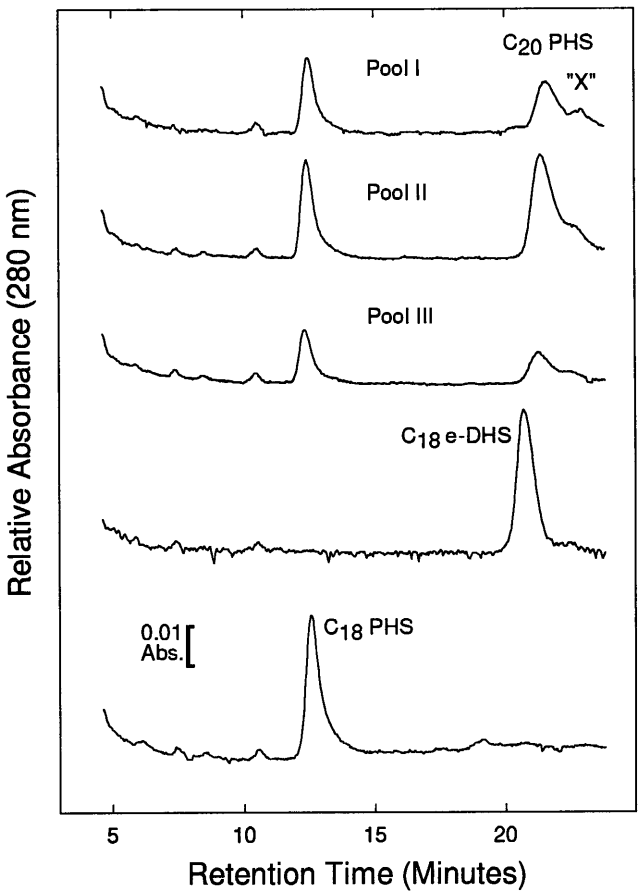

FIG. 3. Long chain base analysis of the purified alkali-stable phospholipids of *C. albicans*. The pooled fractions (Fig. 1) were hydrolyzed with methanolic HCl, converted to *N*-biphenylcarbonyl derivatives, and separated by reverse-phase HPLC, with the eluates monitored at 280 nm. Standards: C_{18}-erythrosphinganine (C_{18} e-DHS); C_{18} and C_{20} phytosphingosines (C_{18} and C_{20} PHS). Abs., absorbance.

after 30 min at room temperature 133 ml of a thick water slurry of Chelex 100 (Na^+, 200/400 mesh; Bio-Rad Laboratories), 266 ml of 0.2 M potassium acetate, and 16 ml of concentrated NH_4OH were added. After being mixed well, 40 ml of 1 N acetic acid was added and well mixed. The resultant mixture of alkali-stable lipids was purified from nonlipid impurities by adsorption to and elution from a C_{18} Celite column. For the total sample, 40 ml of packed C_{18} Celite was used; however, the processing was carried out with smaller volumes. The Celite column was packed in methanol and equilibrated with solvent W {solvent E–0.2 N KOH in methanol–1 N glacial acetic acid–water [1:1:0.2:1.16 (vol/vol/vol/vol) adjusted to pH 8 with concentrated NH_4OH]}. For each 1 ml of C_{18} Celite, a 21-ml sample of the slurry of deacylated lipids plus Chelex resin was added. For elution, the mixture included the sample, 12 ml of solvent W, and 4 ml of methanol-water (1:1 [vol/vol]). The base-stable lipids were eluted with 2 ml of methanol and then with 16 ml of $CHCl_3$-CH_3OH-H_2O (16:16:5 [vol/vol/vol], adjusted to pH 9.5 with NH_4OH). The yield was 1.8 μmol of P per ml of packed cells.

TABLE 1. Composition of purified alkali-stable phospholipids of *C. albicans*

Phospholipid	Amt (mol/mol of P) in:		
	Pool I	Pool II	Pool III
Long-chain bases			
C_{18} phytosphingosine	0.47	0.51	0.31
C_{20} phytosphingosine	0.40	0.77	0.26
Sum	0.87	1.26	0.57
Peak X	0.12	0.10	0.08
Very long-chain fatty acids			
$(OH)_2$ 24:0 (peak 1)[a]	0.068	0.104	0.059
OH 24:0 (peak 2)[a]	0.590	0.671	0.349
OH 25:0 (peak 3)	0.119	0.154	0.063
OH 26:0 (peak 4)	0.112	0.087	0.055
24:0 (peak 5)	0.198	0.024	0.015
26:0 (peak 6)	0.022	0.0	0.0
Total	1.11	1.04	0.54
Ammonolysate products			
Inositol	1.01	0.0	0.47
Mannosylinositol	0.0	0.94	0.42
Glycerol	0.0	0.0	0.0

[a] The presence of some OH 22:0 in peak 1 or some $(OH)_2$ 26:0 in peak 2 cannot be excluded. See Fig. 4.

Preparative HPLC of base-stable lipid fraction. To prepare a concentrated sample for high-performance liquid chromatography (HPLC), an aliquot containing 25 μmol of P of the base-stable lipid mixture was evaporated to dryness, warmed in a sonic bath with 3.0 ml of solvent 2 ($CHCl_3$–95% ethanol–concentrated NH_4OH-H_2O [30:52:6:12] plus 0.6 g of ammonium acetate per liter), and subjected to centrifugation. Approximately half the phospholipid dissolved (12 μmol of P), and thin-layer chromatography (TLC) indicated that more of the less-polar lipids were in solution.

A 1.2-ml aliquot (4.85 μmol of P) was added directly (no precolumn) to a column (1.9 by 30 cm) of 5-μm-diameter Lichrosorb Si60 (E. Merck), and gradient elution was carried out exactly as previously described (7), with the collection of 23-ml fractions. Aliquots (2 ml) of each fraction were dried and digested with perchloric acid, and the P_i was analyzed (1). Fractions (Fig. 1) were pooled (fractions 15 to 27, pool I; 28 to 38, pool II; and 67 to 77, pool III) and desalted as follows. After evaporation to dryness, they were suspended in 3 ml of solvent W, applied to 1-ml columns of C_{18} Celite, and eluted as described above in the deacylation procedure. The eluted lipid fractions were evaporated to dryness and dissolved in $CHCl_3$-CH_3OH-H_2O (16:16:5 [vol/vol/vol]). The yields of desalted lipids in pools I, II, and III were 1.39, 1.20, and 0.87 μmol of P, respectively. The composition of these HPLC-purified lipids was analyzed.

TLC. TLC was carried out on Whatman LK5 plates and detected by charring after being sprayed with 10% (wt/vol) $CuSO_4 \cdot 5H_2O$ in 8% H_3PO_4 followed by heating at 160°C for 30 min (4) with the ninhydrin reagent for long chain bases and with the orcinol reagent for carbohydrates (12). For intact sphingolipids the plates were developed with HPLC solvent 2. Free long-chain bases and fatty acid methyl esters were liberated from the purified sphingolipids by treatment with 1.0 N HCl in methanol-water (82:18 [vol/vol]) for 16 h at 80°C, evaporated to dryness, and dissolved in $CHCl_3$-CH_3OH-H_2O (16:16:5 [vol/vol/vol]). The fatty acid methyl esters were resolved with benzene-chloroform-acetic acid (90:10:1)

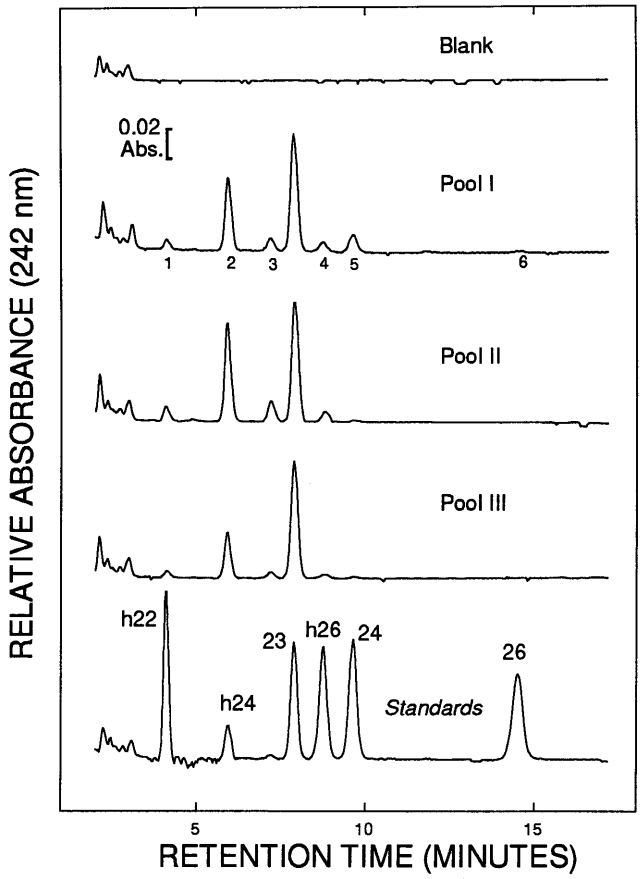

FIG. 4. Very long chain fatty acid analysis of the purified alkali-stable phospholipids of *C. albicans*. The pooled fractions (Fig. 1) were saponified along with an internal C_{23} fatty acid standard, converted to phenacyl derivatives, and resolved by reverse-phase HPLC, with the eluates monitored at 242 nm. See Table 1 for designations of peaks 1 to 6 in pools I, II, and III. Abs., absorbance.

and the long chain bases were resolved with chloroform–methanol–2 N NH_4OH (40:10:1 [vol/vol/vol]).

Compositional analysis of fatty acids, long-chain bases, and head groups. Fatty acids were liberated by saponification and converted to UV-absorbing phenacyl derivatives, which were resolved and quantitated by reverse-phase HPLC (7, 16). Sphingolipid long-chain bases were liberated with methanolic HCl treatment, converted to UV-absorbing biphenylcarbonyl derivatives, and separated and quantitated by reverse-phase HPLC as described previously (3). Inositol, mannosylinositol, and glycerol were measured after strong ammonolysis by conversion to benzoyl derivatives, which were separated and quantitated by reverse-phase HPLC as described previously (7).

RESULTS

Isolation of purified alkali-stable phosphosphingolipids from *C. albicans*. Lipids were extracted from trichloroacetic acid-treated cells, subjected to mild alkali-catalyzed methanolysis, and freed from nonlipid impurities yielding 1.8 μmol of phosphorus per ml of packed cells. This lipid fraction was subjected to preparative HPLC on silica gel, with the eluate monitored for total P. Three major fractions were observed (Fig. 1), with evident inhomogeneities in each. The three major fractions were pooled (pools I, II, and III), desalted, and subjected to qualitative and quantitative analyses. TLC disclosed that each pooled fraction was free of the others and consisted of one major spot with one or two closely migrating spots (Fig. 2); pools II and III, but not pool I, were carbohydrate positive. Pools I, II, and III corresponded in R_fs to IPCs, mannosylinositolphosphorylceramides (MIPCs), and mannosyldiinositolphosphorylceramides [$M(IP)_2Cs$], respectively, from *S. cerevisiae* (Fig. 2).

Long chain base compositions of pools I, II, and III. The purified lipids were hydrolyzed with methanolic-HCl, and the bases were converted to UV-absorbing *N*-biphenylcarbonyl derivatives, which were separated by reverse-phase HPLC (Fig. 3). All three samples gave major peaks with the retention times expected for C_{18} and C_{20} phytosphingosine derivatives. *S. cerevisiae* sphingolipids (data not shown) were used as the sources (13) of the C_{20} phytosphingosine R_f standard. Normal-phase TLC of the methanol-HCl hydrolysates from all three purified lipids gave a single ninhydrin-positive spot at the same R_f as standard phytosphingosine (data not shown); erythrosphinganine as well as sphingosine standards separated well from phytosphingosine, and no ninhydrin-positive signals for these long chain bases were evident in the purified lipids. The HPLC profile (Fig. 3) shows an unidentified peak (X), which is about 10% of the total long-chain-base integration units; since it is not clear that this represents a long chain base it is not included in further calculations. We conclude that the purified phospholipids are all sphingolipids containing C_{18} and C_{20} phytosphingosines as the major long-chain bases in integral amounts (Table 1).

Very long chain fatty acid compositions of pools I, II, and III. The purified lipids were saponified, and the free fatty acids along with 23:0 as the internal standard were converted to

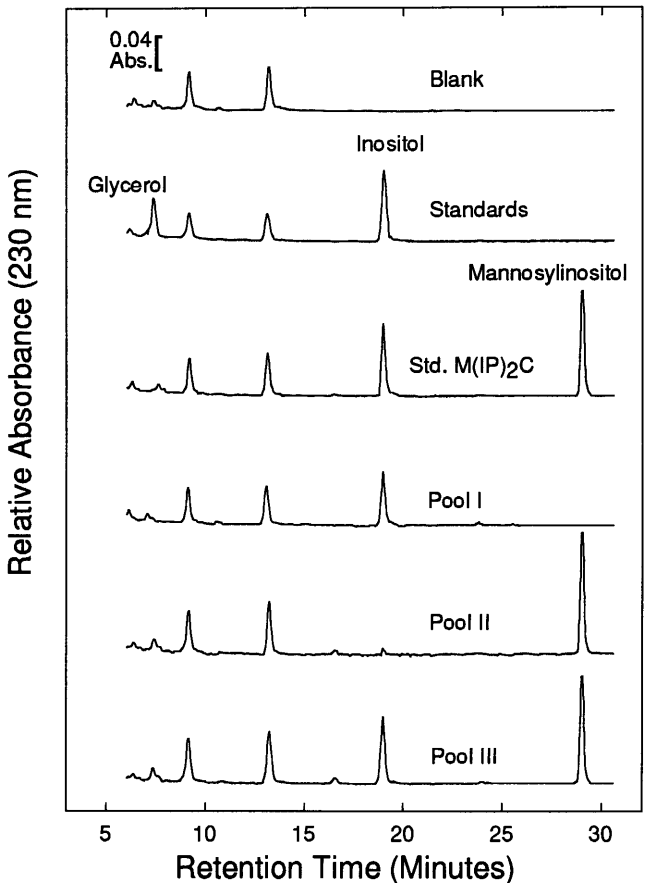

FIG. 5. Analysis of ammonolysates of the purified alkali-stable phospholipids of *C. albicans*. The pooled fractions (Fig. 1) were subjected to strong ammonolysis, the resulting products were perbenzoylated, and the benzoyl derivatives were separated by reverse-phase HPLC, with the eluates monitored at 230 nm. The peaks at approximately 9 and 13 min are due to reagents.

TABLE 2. Summary of analytical data for purified phospholipids of *C. albicans*

Pool	Amt found (amt expected)				
	Fatty acids	Long-chain bases	Inositol	Mannosyl inositol	Phosphorus
I (IPC)	1.11 (1.0)	0.87 (1.0)	1.01 (1.0)	0.0 (0.0)	1.0 (1.0)
II (MIPC)	1.04 (1.0)	1.26 (1.0)	0.0 (0.0)	0.94 (1.0)	1.0 (1.0)
III [$M(IP)_2C$]	1.08 (1.0)	1.14 (1.0)	0.94 (1.0)	0.84 (1.0)	2.0 (2.0)

UV-absorbing phenacyl derivatives, which were separated by reverse-phase HPLC (Fig. 4). The major peaks (no. 2, 3, and 4) appeared to be monohydroxy C_{24}, C_{25}, and C_{26} fatty acids, respectively, for all three lipids. Pool I had significant levels of nonhydroxy C_{24} and lesser levels of 26:0. All three lipids exhibited a peak (no. 1) consistent with the retention time expected for a dihydroxy C_{24} fatty acid or an OH 22:0 fatty acid. Normal-phase TLC was carried out on the methyl esters of the fatty acids derived from pools I, II, and III (data not shown), and the results are fully consistent with the above interpretations. Namely, the major spot in all three samples was a monohydroxy fatty acid, all had lesser amounts at the R_f of the dihydroxy fatty acids, and only pool I had detectable nonhydroxy fatty acids. The sum of the very long-chain fatty acids (Table I) were roughly equal to the amount of long chain base, consistent with the conclusion that these lipids were ceramide derivatives.

Analysis of polar constituents of pools I, II, and III. Each purified lipid was subjected to strong ammonolysis, which hydrolyzes phosphate esters but not glycosides; the products were perbenzoylated and separated by reverse-phase HPLC (Fig. 5). Peaks were detected at the retention times expected for inositol in pools I and III and for mannosylinositol in pools II and III. Quantitative evaluation (Table 1) showed that these derivatives were obtained in stoichiometric relationship to total P; no glycerol was detected.

Identity of pools I, II, and III. The quantitative compositional data (Table 2) of the lipid and polar constituents suggest the following identities: pool I, IPC; pool II, MIPC; and pool III, $M(IP)_2C$. Each lipid is heterogeneous with respect to the lipid constituents, differing in chain length and level of fatty acid hydroxylation. The proposed structures are indicated in Fig. 6.

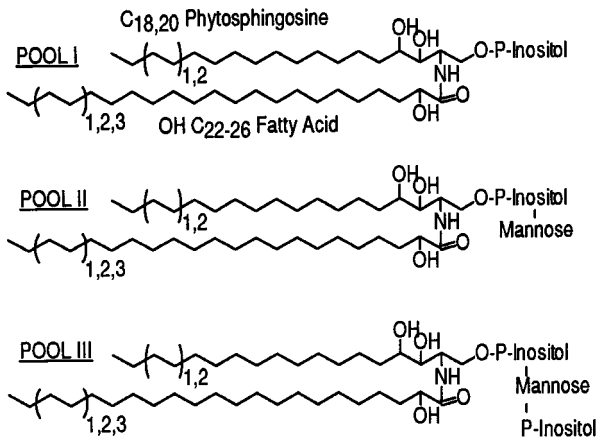

FIG. 6. Proposed structures for predominant sphingolipid species of *C. albicans.*

DISCUSSION

Phosphoinositol-containing sphingolipids have been identified in various yeasts and fungi: *S. cerevisiae*, *Histoplasma capsulatum*, *Neurospora crassa*, *Aspergillus niger*, *Phytophtera capsica* (6), and *Cryptococcus neoformans* (14). This work is direct evidence for the existence of phosphoinositol-containing sphingolipids in *C. albicans*. Their composition and chromatographic behavior by HPLC and TLC suggest that they closely resemble the sphingolipids identified in *S. cerevisiae*. The polar head groups appear to be identical, and they differ slightly in that the sphingolipids of *C. albicans* have predominantly C_{24} fatty acids (as do those of those of *H. capsulatum* and *N. crassa*) whereas *S. cerevisiae* sphingolipids have C_{26} fatty acids (6).

Since *C. albicans* contains lipids shown to be essential in *S. cerevisiae* (11), they may be essential in this pathogen and might serve as a logical target for candidiasis therapy.

ACKNOWLEDGMENTS

This work was supported in part by U.S. Public Health Service grant GM43102 and National Science Foundation grant EHR-9108764.

REFERENCES

1. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234:**466–468.
2. **Bullock, W., T. Kozel, S. Scherer, and D. M. Dixon.** 1993. Medical mycology in the 1990s: involvement of NIH and the wider community. ASM News **59:**182–185.
3. **Dickson, R. C., G. B. Wells, A. Schmidt, and R. L. Lester.** 1990. Isolation of mutant *Saccharomyces cerevisiae* strains that survive without sphingolipid. Mol. Cell. Biol. **10:**2176–2181.
4. **Fewster, M. E., B. J. Burns, and J. F. Mead.** 1969. Quantitative densitometric thin-layer chromatography of lipids using copper acetate reagent. J. Chromatogr. **43:**120–126.
5. **Hanson, B. A., and R. L. Lester.** 1980. The extraction of inositol-containing phospholipids and phosphatidylcholine from *Saccharomyces cerevisiae* and *Neurospora crassa*. J. Lipid Res. **21:**309–315.
6. **Lester, R. L., and R. C. Dickson.** 1993. Sphingolipids with Inositolphosphate-containing head groups. Adv. Lipid Res. **26:**253–274.
7. **Lester, R. L., G. B. Wells, G. Oxford, and R. C. Dickson.** 1993. Mutant strains of *Saccharomyces cerevisiae* lacking sphingolipids synthesize novel inositol glycerophospholipids that mimic sphingolipid structures. J. Biol. Chem. **268:** 845–856.
8. **Mandala, S. M., R. A. Thornton, B. R. Frommer, J. E. Curotto, W. Rozdilsky, M. B. Kurtz, R. A. Giacobbe, G. F. Bills, M. A. Cabello, I. Martin, F. Peláez, and G. H. Harris.** 1995. The discovery of australifungin, a novel inhibitor of sphinganine *N*-acyltransferase from *Sporormiella australis*, producing organism, fermentation, isolation, and biological activity. J. Antibiot. **48:**349–356.
9. **Patton, J. L., and R. L. Lester.** 1991. The phosphoinositol sphingolipids of *Saccharomyces cerevisiae* are highly localized in the plasma membrane. J. Bacteriol. **173:**3101–3108.
10. **Patton, J. L., B. Srinivasan, R. C. Dickson, and R. L. Lester.** 1992. Phenotypes of sphingolipid-dependent strains of *Saccharomyces cerevisiae*. J. Bacteriol. **174:**7180–7184.
11. **Pinto, W., J. B. Srinivasan, S. Shepherd, A. Schmidt, R. C. Dickson, and R. L. Lester.** 1992. Sphingolipid long-chain-base auxotrophs of *Saccharomyces cerevisiae*: genetics, physiology, and a method for their selection. J. Bacteriol. **174:**2565–2574.
12. **Skipski, V. P., and M. Barclay.** 1969. Thin layer chromatography of lipids. Methods Enzymol. **14:**545–546.
13. **Smith, S. W., and R. L. Lester.** 1974. Inositolphosphorylceramide, a novel substance and the chief member of a major group of yeast sphingolipids containing a single inositol phosphate. J. Biol. Chem. **249:**3395–3405.
14. **Vincent, V. L., and L. S. Klig.** 1995. Unusual effect of *myo*-inositol on phospholipid synthesis in *Cryptococcus neoformans*. Microbiology **141:**1829–1837.
15. **Wells, G. B., and R. L. Lester.** 1983. The isolation and characterization of a mutant strain of *Saccharomyces cerevisiae* that requires a long chain base for growth and for synthesis of phosphosphingolipids. J. Biol. Chem. **258:**10200–10203.
16. **Wood, R., and T. Lee.** 1983. High-performance liquid chromatography of fatty acids: quantitative analysis of saturated, monoenoic, polyenoic and geometrical isomers. J. Chromatogr. **254:**237–246.
17. **Zweerink, M. M., A. M. Edison, G. B. Wells, W. Pinto, and R. L. Lester.** 1992. Characterization of a novel, potent, and specific inhibitor of serine palmitoyltransferase. J. Biol. Chem. **267:**25032–25038.