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Molecular analysis of a novel family of complex glycoinositolphosphoryl ceramides from *Cryptococcus neoformans:* structural differences between encapsulated and acapsular yeast forms

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Complex glycoinositolphosphoryl ceramides (GIPCs) have been purified from a pathogenic encapsulated wild-type (WT) strain of *Cryptococcus neoformans* var. *neoformans* and from an acapsular mutant (Cap67). The structures of the GIPCs were determined by a combination of tandem mass spectrometry, nuclear magnetic resonance spectroscopy, methylation analysis, gas chromatography–mass spectrometry, and chemical degradation. The main GIPC from the WT strain had the structure Man*p*(α1-3)[Xyl*p*(β1-2)]Man*p*(α1-4)Gal*p*(β1-6)Man*p*(α1-2)Ins-1-phosphoryl ceramide (GIPC A), whereas the compounds from the acapsular mutant were more heterogeneous in their glycan chains, and variants with Man*p*(α1-6) (GIPC B), Man*p*(α1-6)Man*p*(α1-6) (GIPC C), and Man*p*(α1-2)Man*p*(α1-6)Man*p*(α1-6) (GIPC D) substituents linked to the nonreducing terminal mannose residue found in the WT GIPC A were abundant. The ceramide moieties of *C. neoformans* GIPCs were composed of a C_{18} phytosphingosine long-chain base mainly *N*-acylated with 2-hydroxy-tetracosanoic acid in the WT GIPC while in the acapsular Cap67 mutant GIPCs, as well as 2-hydroxy-tetracosanoic acid, the unusual 2,3-dihydroxy-tetracosanoic acid was characterized. In addition, structural analysis revealed that the amount of GIPC in the WT cells was fourfold less of that in the acapsular mutant.

Key words: C. neoformans/glycophosphosphingolipids/mass spectrometry/NMR spectroscopy

Introduction

Cryptococcus neoformans var. *neoformans* is an encapsulated fungus that causes a systemic mycosis, the most common clinical manifestation of which is meningitis (Mitchell and Perfect, 1995). Those most susceptible to infection have T lymphocyte deficiencies resulting from HIV infection, lymphoproliferative diseases, or immunosuppressive therapy (Mitchell and Perfect, 1995; Buchanan and Murphy, 1998). Of several known virulence factors, production of a large polysaccharide capsule is considered the most important determinant of pathogenicity. Both glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) components of the capsule are detectable in body fluids of patients, and aid diagnosis (Buchanan and Murphy, 1998). The GXMs confer serotype specificity and are composed of a (1→3)-linked linear α-D-mannopyranan laterally substituted by β-xylopyranosyl and β-glucopyranosyluronic acid residues with variable degrees of 6-*O*-acetylation (Cherniak and Sundstrom, 1994). GalXMs are also heterogeneous and consist of an (1→6)-linked α-D-galactopyranan backbone, alternate galactose residues of which are substituted on *O*-3 with complex branched side chains composed of β-D-galactopyranosyl, α-D-mannopyranosyl and β-D-xylopyranosyl residues (James and Cherniak, 1992; Vaishnav *et al.*, 1998).

Some fungal glycans are linked to sphingolipid or sphingophospholipid anchors. Glycoinositolphosphoryl ceramides (GIPCs) are membrane glycolipids containing a phosphodiester linkage between inositol and ceramide, inositol-(1-*O*)-phosphoryl-(*O*-1) ceramide constituting a common structural element (Laine and Hsieh, 1987). Phosphorylinositol-containing sphingolipids are not present in animals but have been detected in protozoa (including the soil amoeba *Acanthamoeba castellanii*), plants, fungi (Lester and Dickson, 1993), and in the parasitic nematode *Ascaris suum* (Sugita *et al.*, 1996). In yeast, phosphorylinositol-containing sphingolipids are essential for growth and viability, despite constituting a low percentage of total cell membrane phospholipids (Dickson and Lester, 1999). In addition, pathogenic fungi such as *C. neoformans*, *Candida albicans*, *Aspergillus fumigatus*, and *Histoplasma capsulatum* are killed by inhibitors of inositolphosphoryl ceramide (IPC) synthase, an essential glycosphingolipid biosynthetic enzyme (Takesako *et al.*, 1993; Mandala *et al.*, 1997, 1998). Therefore, the biosynthesis of phosphorylinositol-containing sphingolipids is a promising target for the development of more effective antifungal agents (Dickson and Lester, 1999; Nagiec *et al.*, 1997).

Ceramide-(phosphorylinositol)$_2$-mannose, ceramide-phosphorylinositol-mannose and ceramide-phosphorylinositol have been identified in *C. neoformans* (Vincent and Klig, 1995). Similar compounds have been described in *Saccharomyces cerevisae* (Smith and Lester, 1974), *Neurospora crassa* (Lester *et al.*, 1974), *H. capsulatum* (Barr *et al.*, 1984), *Phytophthora capsici* (Lhomme *et al.*, 1990), and *C. albicans* (Wells *et al.*,

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1996). However, more complex GIPC structures have only been found in the mycopathogens *Aspergillus niger, H. capsulatum* (Lester and Dickson, 1993), *Paracoccidioides brasiliensis* (Levery *et al.*, 1998), and more recently in *Sporothrix schenckii* (Penha *et al.*, 2000; Toledo *et al.*, 2001). In the present study we have isolated, purified, and characterized members of an unusual series of complex GIPCs from *C. neoformans*, and structural differences between wild-type (WT) encapsulated and mutant acapsular yeast cells are described.

Results

Isolation of GIPCs from C. neoformans

After removal of capsular polysaccharides with citrate buffer, GIPCs were isolated from the residual cells by aqueous phenol extraction and solubilization in chloroform/methanol/water. Assuming 1 mol of inositol/mol of GIPC, the amount of these complex sphingophospholipids in the acapsular Cap67 mutant was fourfold higher than in the encapsulated WT strain.

Carbohydrate and lipid composition of WT- and Cap67-GIPCs from C. neoformans

Compositional analysis showed that the isolated GIPCs contained mannose, galactose, xylose, and inositol. The Cap67-GIPCs contained a higher molar ratio of Man compared to the WT (designated here GIPC A) isolate. Glucuronic acid (GlcA), hexosamine, or sialic acid were not detected in any of the preparations (Table I). Gas chromatography (GC) and GC–mass spectrometry (GC-MS) analyses of acid methanolysates of the crude GIPCs show the presence of C_{18} phytosphingosine *N*-acylated with 2-hydroxy tetra-, penta-, or hexacosanoic fatty acids, and with 2-hydroxy and 2,3-dihydroxy tetra- or pentacosanoic acids for WT-GIPC A and Cap67-GIPCs respectively (Table I). Due to the absence of authentic standards, these two unusual 2,3-dihydroxy fatty acids were identified by electron ionization mass spectrometry (EI-MS). The EI-MS of 2,3-dihydroxytetracosanoic acid and 2,3-dihydroxypentacosanoic acid was similar to that of 2,3-dihydroxy-12-methyltridecanoate isolated from *Legionella pneumophila* (Mayberry, 1981), except that the fragments (M-15), (M-59), and (M-161) were shifted up in mass by 140 and 154 units, respectively.

In agreement with the chemical composition, the negative-ion matrix-assisted laser desorption ionization and time-of-flight (MALDI-TOF) mass spectrum of WT-GIPC A contained a major signal $[M-H]^-$ at *m/z* 1705.3 consistent with one pentose and four hexose residues linked to 2-hydroxy tetracosanoyl-C18:0-phytosphingosine-phosphoryl inositol (calculated value 1704.9). Minor signals were observed at *m/z* 1719.4 and 1733.4. The increment of 14 mass units between the consecutive deprotonated molecules confirmed that the ceramide of WT-GIPC A contains homologous fatty acids differing by one carbon (Figure 1A). The MALDI-TOF mass spectrum of crude Cap67-GIPCs (Figure 1B), however, was more complex with five triplet groups of deprotonated molecules (two major and three minor) at *m/z* (1705.3, 1721.2, 1735.2), (1867.3, 1883.4, 1897.4), (2029.4, 2045.3, 2059.5), (2191.4, 2207.5, 2221.5), and (2353.6, 2369.5, 2383.5). Each triplet group is separated by 162 mass units, consistent with the presence of three series of inositolphosphorylceramides, differing in their ceramide moieties, containing 1 Pen and 4, 5, 6, 7 or 8 Hex residues. The difference of 16 units between the two most abundant ^{12}C peaks within each triplet cluster is consistent with the replacement of the monohydroxyl *N*-acyl substituents on C_{18} phytosphingosine by the dihydroxy fatty acids detected in the GC-MS analysis (Table I).

Table I. Chemical composition of crude GIPCs isolated from WT and Cap67 mutant of *C. neoformans*

Compound	WT	Cap67
Myo-inositol*	1.0	1.0
Mannose*	3.0	5.2
Galactose*	1.0	1.0
Xylose*	1.0	1.0
Long chain base†		
C_{18}-phytosphingosine	100	100
Fatty acids‡		
C24:0 2-OH	62	48
C24:0 2,3-diOH	—	37
C25:0 2-OH	20	—
C25:0 2,3-diOH	—	15
C26:0 2-OH	18	—

*Mol/mol determined by GC-MS as trimethylsilyl derivatives of *myo*-inositol and methylglycosides.
†mol% determined by GC-MS after *N*-acetylation and trimethylsilylation.
‡% of peak integration determined by GC-MS as fatty acid methyl esters.

Chromatography of the crude Cap67-GIPCs on a Florisil column gave three partially purified glycolipid fractions, analyzed with high-perfomance thin-layer chromatography (HPTLC) (Figure 2) and designated Cap67-GIPC B, C, and D, of which fractions B and D were abundant enough for detailed structural characterization. Carbohydrate analysis revealed that Cap67-GIPCs B, C, and D contain Man, Gal, and Xyl in a molar ratio of 3.9:1.0:1.0, 5.2:1.0:1.0, and 5.8:1:0:1:0 respectively. MALDI-TOF MS analysis showed that the major signals in the main triplet of deprotonated molecules of Cap67-GIPC B (at *m/z* 1867.3, 1883.1, 1897.4) (Figure 3A) differ from the corresponding peaks in Cap67-GIPC D (*m/z* 2191.2, 2207.1, and 2221.1) (Figure 3B) by the mass of two Hex residues (324). The ratio of the deprotonated molecules at *m/z* 2191.2 and 2207.1 is approximately 1:1 in the latter fraction, suggesting that a higher proportion of the C_{18} phytosphingosine of Cap67-GIPC D is acylated with 2,3-dihydroxytetracosanoic acid than is the major GIPC species of Cap67-GIPC B, which has predominantly 2-hydroxytetracosanoic acid.

Structural characterization

Methylation linkage analysis. The *O*-acetylated partially *O*-methylated methyl glycosides obtained after permethylation, methanolysis, and acetylation of WT-GIPC A, Cap67-GIPCs B, C, and D were identified and quantified by GC and GC-MS (Table II). The methylation analysis of GIPCs A, B, and C showed the presence of methyl 2,3,4-tri-*O*-methyl xylopyranoside; 2,3,4,6-tetra-, 2,3,4-tri-, and 4,6-di-*O*-methyl mannopyranoside; and 2,3,6-tri-*O*-methyl galactopyranoside in molar ratios of

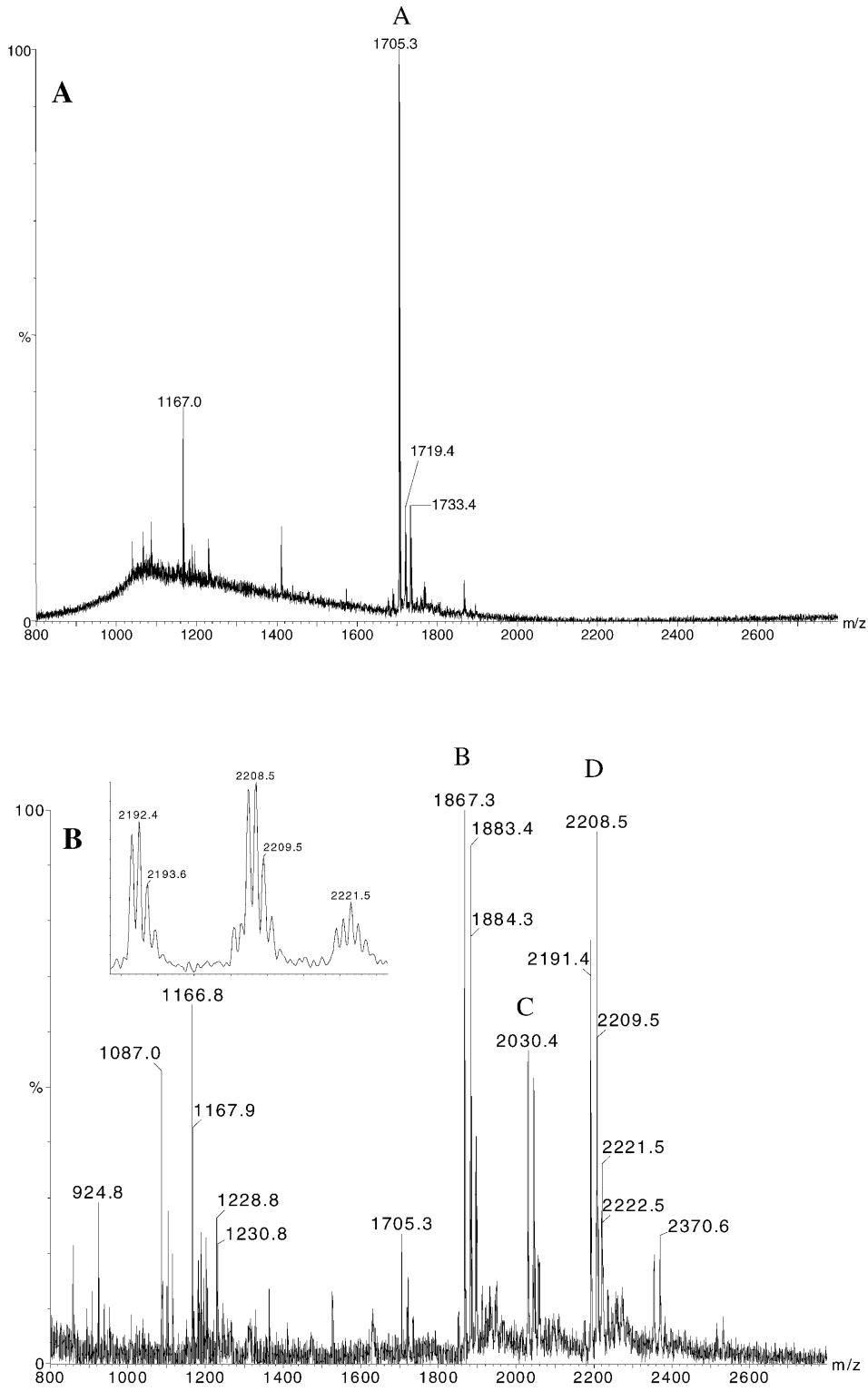

Fig. 1. Negative mode MALDI-TOF-MS spectra of crude GIPCs isolated from capsular WT (**A**) and acapsular mutant (**B**) cells of *C. neoformans*. The peaks signaled by A, B, C, and D correspond to a homologous series of C_{18} phytoceramide-phosphoryl-inositol, containing 1 Pen residue plus 4, 5, 6, and 7 Hex residues at *m/z* 1705.3, 1867.3, 2029.4, and 2191.4, respectively. The inset in **B** represents the parent ion region at *m/z* from 2190 to 2224.

0.8:1.0:1.0:1.0:1.0, 0.9:1.0:2.0:1.0:1.0 and 0.8:0.9:3.0:1.0:1.0, respectively. The methylation products from Cap67-GIPC D differed from those of the Cap67-GIPC C only by the presence of methyl 3,4,6-tri-*O*-methyl mannopyranoside. Thus the methylation analysis results suggest that the GIPCs from *C. neoformans* form a homologous series of branched

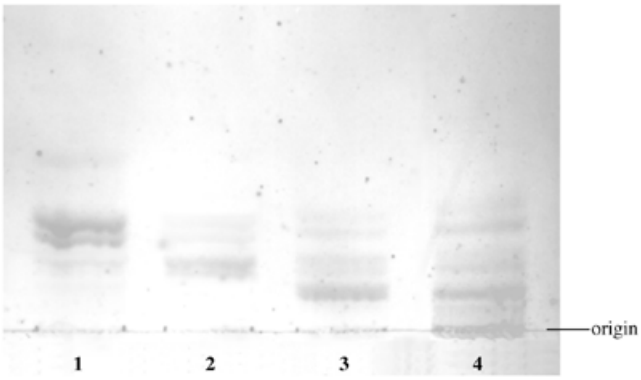

Fig. 2. HPTLC analysis of GIPCs from acapsular mutant (Cap67) of *C. neoformans*. Lane 1, Cap67-GIPC B; lane 2, Cap67-GIPC C; lane 3, Cap67-GIPC D; lane 4, crude Cap67-GIPC.

oligosaccharides. The phosphorylated Ins derivative was not detected but was characterized in the methylation analysis of the ammonolysis products of WT-GIPC A and Cap67-GIPC B and D as 1,3,4,5,6-penta-*O*-methyl-2-*O*-acetyl inositol (Table II). As expected, the other methylated derivatives obtained after ammonolysis were identical to those from intact GIPCs.

Tandem electrospray ionization mass spectrometry (ESI–MS/MS) of the GIPCs after permethylation enabled their partial characterization. Because the ceramide groups were lost during the methylation procedure, the size heterogeneity attributable to the *N*-acyl groups present in the native samples was abolished. This loss of ceramide was also observed when methylation was performed using the procedure of Ciucanu and Kerek (1984). The daughter ion spectrum (Figure 4) of the sodium cationized permethylated oligosaccharide from the WT-GIPC A at *m/z* 1343.5 contained a complete series of Y-type fragment ions (Domon and Costello, 1988). The signals at *m/z* 1125.5 ($Y_{4\alpha}$), 1169.5 ($Y_{4\beta}$), and 951.4 ($Y_{4\beta}/Y_{4\alpha}$) were consistent with a Hex-[Pen]-Hex-Hex-Hex-Ins-PO_4 structure where the branching point is located at the third Hex unit distal from the Ins residue. Sodium cationized B-type ions were observed down to B_2, B_4 at *m/z* 1013.5 being particularly abundant. In addition a ring cleavage $^{0,3}A_4$ was detected at *m/z* 897.5 with secondary fragments at *m/z* 679.4 ($Y_{4\alpha}/^{0,3}A_4$), 505.2 ($Y_{4\beta}/Y_{4\alpha}/^{0,3}A_4$), and 315.2 ($Y_3/^{0,3}A_4$). The fragmentation patterns of the sodium cationized permethylated GIPC-derived Ins- oligosaccharides (precursor ions at *m/z* 1547.6, 1751.7, and 1955.9) from the Cap67 mutant were similar (spectra not shown). The major difference was the appearance of $^{0,3}A_5$, $^{0,3}A_6$, and $^{0,3}A_7$ fragments at *m/z* 1101.6, 1305.6, and 1509.8, respectively, consistent with more complex structures where additional permethylated Hex units (increments of 204 mass units) were linked to the nonreducing terminal Hex residue found in the WT-GIPC A.

Smith degradation. The methylation analysis results (Table II) and the observed fragmentation pattern (Figure 4) are consistent with two alternative core structures:

(Structure **I**) [Xyl*p*(1-2,3)]Man*p*(1-4)Gal*p*(1-6)Man*p*(1-2)Ins

(Structure **II**) [Xyl*p*(1-2,3)]Man*p*(1-6)Man*p*(1-4)Gal*p*(1-2)Ins

To discriminate between these possibilities, the GIPCs were subjected to Smith degradation and the products fractionated on Bio-Gel P-2. The main carbohydrate fraction, eluting at a volume consistent with a Hex disaccharide, was hydrolyzed, and the derivatized products when analyzed by GC and GC-MS showed the presence of Man and threitol in a molar ratio of 1.0:1.0. The ESI mass spectrum of this carbohydrate fraction contained abundant signals at *m/z* 285 and 307, which correspond to the molecular masses of protonated and sodium cationized mannosyl-threitol, respectively. Because the threitol derives from Gal C-3, 4, 5, and 6 we conclude that the Ins-oligosaccharide structure **I** is the common core structure of GIPCs from WT and Cap67, consistent with a glycan sequence [Xyl*p*(1-2,3)]Man*p*(1-4)Gal*p*(1-6)Man*p*(1-2)Ins.

Linkage of the phosphate group to Ins. The phosphorylation position was deduced by a combination of periodate oxidation and methylation analysis. Oxidation of WT-GIPC A and Cap67-GIPCs (B and D) with $NaIO_4$ and subsequent reduction and hydrolysis yielded a phospho-alcohol that, after treatment with alkaline phosphatase and acetylation, yielded a product that was identified as erythritol tetra-acetate by GC and GC-MS. Because the erythritol is derived from carbons 1, 2, 3, and 6 of the inositol, and methylation analysis showed that the oligosaccharide chain is linked to the Ins *O*-2, *O*-1 must carry the phosphate group.

NMR spectroscopy of the Ins-oligosaccharides

Ins-oligosaccharides released from WT-GIPC A and Cap67-GIPCs B and D by ammonolysis were analyzed by nuclear magnetic resonance (NMR) spectroscopy. Proton NMR spectra were assigned from total correlation spectroscopy (TOCSY) experiments, and the ^{13}C spectra through hetero-nuclear correlation. Information on the linkage and sequence of the sugar residues was obtained from rotating frame nuclear Overhauser enhancement spetroscopy (ROESY) spectra and long-range ^{1}H-^{13}C correlations. Additional ^{1}H and ^{13}C assignments were derived from a two-dimensional heteronuclear single-quantum correlation (HSQC)-TOCSY experiment. Figure 5 shows the 1D spectra, and assignments are summarized in Table III. The sequence of and linkage between the sugar residues was established from interresidue nuclear Overhause effects (NOEs) and long-range transglycosidic ^{1}H–^{13}C correlations; these data are summarized in Figure 6. The β-anomeric configuration of the Gal and Xyl residues was established from the magnitudes of $^{3}J_{H1,H2}$, from the chemical shifts of the H-1 and C-1, and from the chemical shifts of H-3 and H-5 (or H-5a), when identified. The α-anomeric configurations of the Man residues were established from the chemical shifts of H-1 and C-1 and of H-2, H-3, and H-5. The Ins residue was identified from its lack of an anomeric resonance and by the presence of a low-field H-2 at 4.13 ppm. In the ROESY spectrum, this correlated with Man(1) H-1 at 5.122 or 5.127 ppm, establishing the α-Man*p*-(1→2)-Ins linkage.

The spectra of all Ins-oligosaccharides contained a spin system for a β-Gal*p* residue, and inter-residue NOEs were observed between the β-Gal H-1 and the α-Man(1) H-6 at 3.91 ppm and the H-6′ or H-5 at 4.19 ppm. The low-field chemical shift of the Man(1) C-6 at 70.5 ppm was in agreement with this; in α-Man*p*-(1→6)-Man*p* the C-6 is typically at 67 ppm (Forsgren *et al.*, 1985; Jansson *et al.*, 1994). The H-1

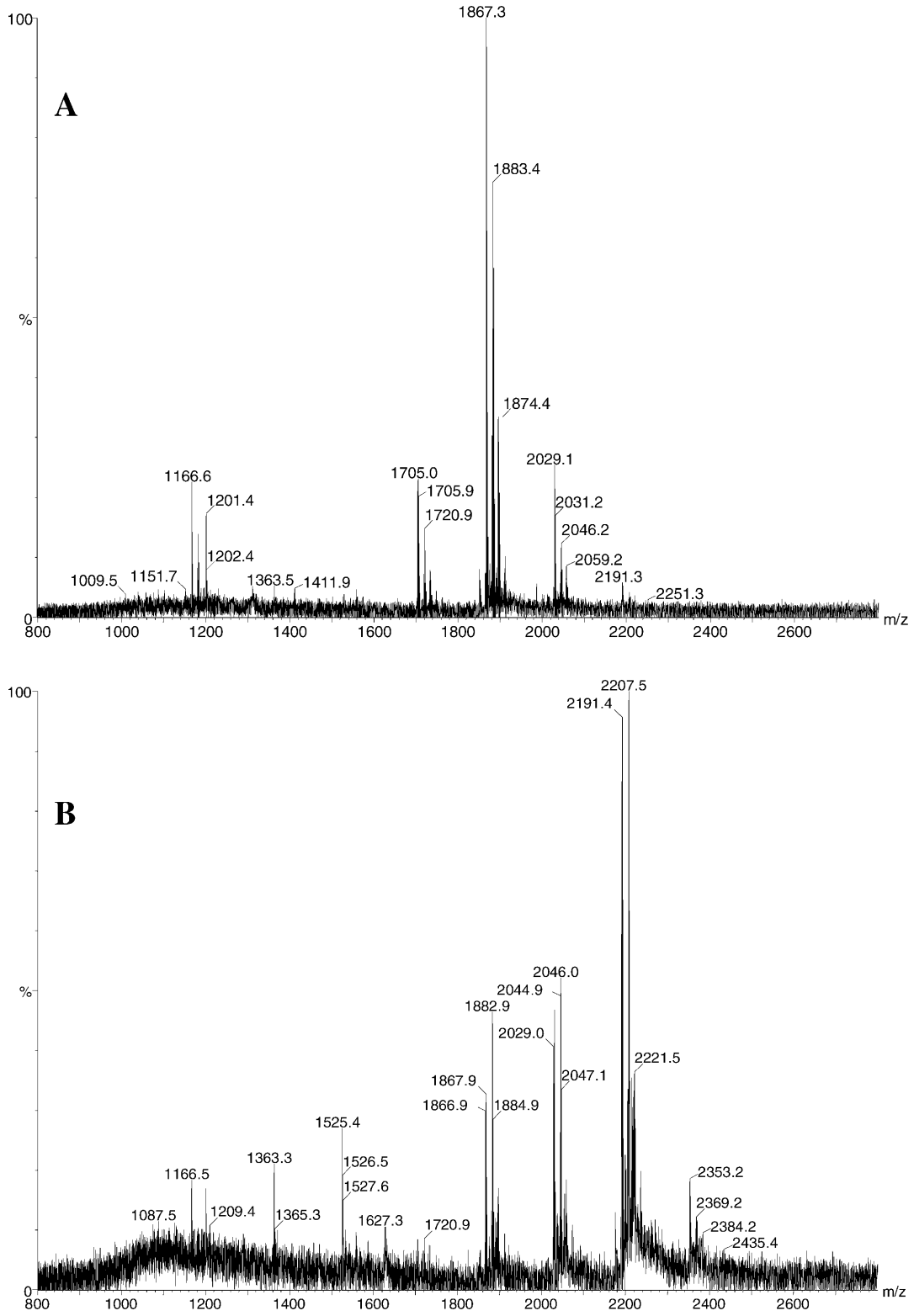

Fig. 3. Negative mode MALDI-TOF MS spectra of GIPCs obtained by fractionation of crude Cap67-GIPCs on Florisil column. (**A**) Cap67-GIPC B; (**B**) Cap67- GIPC D.

of the branch point Man(2) showed an interresidue NOE to the β-Gal H-4 at 4.08–4.10 ppm. The TOCSY spectra of all Ins-oligosaccharides contained a spin system from a terminal β-Xyl*p* residue, and the anomeric resonance of this residue showed interresidue NOEs to Man(2) H-1 and H-2, consistent with a β-Xyl*p*-(1→2)-Man*p*(2) linkage. The low-field chemical shift of the Man(2) C-3 shows that this is also a site of substitution, and an interresidue NOE is observed between the

Table II. Methylation analysis of partially purified GIPC species isolated from WT (GIPC A) and Cap67 mutant (GIPC B, C, and D) of *C. neoformans*

	WT GIPC		Cap67 GIPC	
Compound[a]	A	B	C	D
Methyl 2,3,4-tri-*O*-methyl xylopyranoside	0.8	0.9	0.8	0.7
Methyl 2,3,4,6-tetra-*O*-methyl mannopyranoside	1.0	1.0	0.9	0.9
Methyl 2,3,4-tri-*O*-methyl-6-*O*-acetyl mannopyranoside	1.0	2.0	3.0	2.9
Methyl 2,3,6-tri-*O*-methyl-4-*O*-acetyl galactopyranoside	1.0	1.0	1.0	1.0
Methyl 3,4,6-tri-*O*-methyl-2-*O*-acetyl mannopyranoside	0.1	0.1	0.1	0.9
Methyl 4,6-di-*O*-methyl-2,3-di-*O*-acetyl mannopyranoside	1.0	1.0	1.0	1.0
1,3,4,5,6-Penta-*O*-methyl-2-*O*-acetyl *myo*-inositol[b]	1.0	1.0	1.0	1.0

[a]Identified and quantified by GC-MS.
[b]Results from GIPC-derived Ins-oligosaccharides.

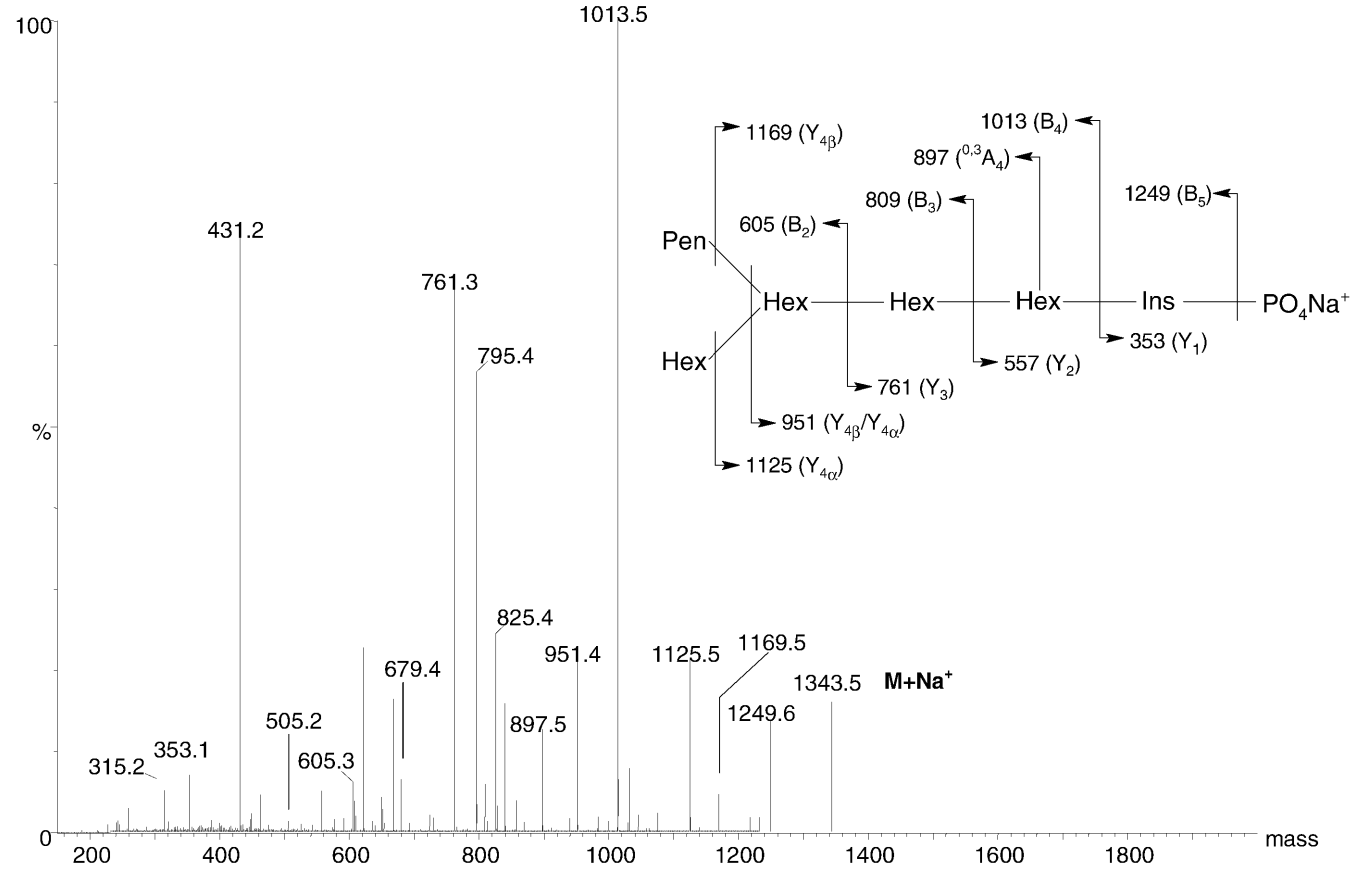

Fig. 4. Positive-ion ESI MS/MS spectrum of permethylated WT-GIPC A. The sodium cationized molecule at *m/z* 1343.63 was selected with a quadrupole analyzer, fragmented by collision with argon and its daughter ion spectrum recorded with the time-of-flight analyzer. The spectrum was deisotoped by a maximum entropy method (MaxEnt 3, Micromass, UK). All sugar-fragment ions are singly charged and are described using the nomenclature introduced by Domon and Costello (1988).

Man(3) H-1 and the Man(2) H-2 and H-3 at 4.170 and 4.055 ppm, respectively. The long-range $^{1}H–^{13}C$ correlation spectrum of the Ins-hexasaccharide (Cap67-GIPC B) showed correlations between the Man(1) H-1 and the Ins C-2, between the Man(2) H-1 and the Gal C-4 and between the Man(3) H-1 and the Man(2) C-3 confirmed the core structure as [Xyl*p*(β1-2)] Man*p*(α1-4)Gal*p*(β1-6)Man*p*(α1-2)Ins (structure **I**).

Compared to the Ins-pentasaccharide from the WT-GIPC A, the Ins-hexasaccharide from the Cap67-GIPC B contained an additional α-Man residue with both H-1 and C-1 resonating at relatively high field (4.930 and 101.52 ppm respectively), consistent with the presence of an additional α-Man*p*-(1→6) residue, and supported by the presence of a low-field methylene resonance at 67.03 ppm, the downfield shift of the Man(3) C-5 (73.24 ppm), and a small change in the chemical shift of the Man(3) H-1. An interresidue NOE is observed between the Man(4) H-1 and a resonance at 3.68 ppm, which in turn correlates with the C-6 at 67.03 ppm in the HSQC, assigned as

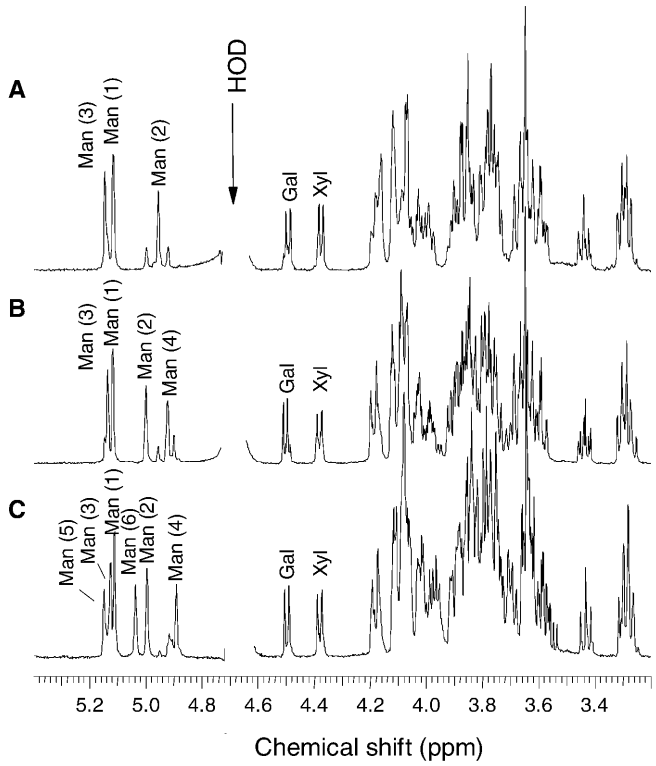

Fig. 5. Partial 500 MHz ^{1}H NMR spectra of Ins-oligosaccharides of GIPC s from *C. neoformans*. (**A**) Ins-pentasaccharide derived from WT-GIPC A; (**B**) Ins-hexasaccharide derived from the Cap67-GIPC B; (**C**) Ins-octasaccharide derived from the Cap67-GIPC D. The spectra were collected at 30°C. The anomeric proton resonances are indicated. Numbers in parentheses represents the position of the Man residues distal to Ins.

the Man(3) H-6. This is consistent with extension of the Man arm with an additional α-Man*p*(1→6)-linked residue.

The Ins-octasaccharide (Cap67-GIPC D) contained two more α-Man residues with H-1s at relatively low field (δ_H/δ_C 5.161/100.08 and δ_H/δ_C 5.045/104.13 ppm). These chemical shifts are consistent with a α-Man*p*-(1→2)-α-Man*p*(1→ system (Carreira *et al.*, 1996), and interresidue NOEs were observed between Man(6) H-1 (5.045) and the Man(5) H-1 and H-2. The Man(5) H-1 showed an interresidue NOE to a resonance at 3.773 ppm, assigned as the Man(4) H-6, and consistent with the Ins-octasaccharide being the same as the Ins-hexasaccharide but with an extension of α-Man*p*-(1→2)-α-Man*p*-(1→6) sequence (Figure 6).

Discussion

The polysaccharide capsule of the pathogenic fungus *C. neoformans* is an established virulence factor (Buchanan and Murphy, 1998). Although several capsular polysaccharides have been characterized, much less is known about the structures of other complex glycoconjugates from this pathogen. In the current study we show that encapsulated and acapsular *C. neoformans* (WT and Cap67) synthesize a novel family of complex structurally related GIPCs: Man*p*(α1-3)[Xyl*p*(β1-2)]Man*p*(α1-4)Gal*p*(β1-6)Man*p*(α1-2)Ins1→ (WT-GIPC A), Man*p*(α1-6)Man*p*(α1-3)[Xyl*p*(β1-2)] Man*p*(α1-4) Gal*p*(β1-6)Man*p*(α1-2)Ins1→ (GIPC B), Man*p*(α1-6) Man*p*(α1-6) Man*p*(α1-3)[Xyl*p*(β1-2)]Man*p*(α1-4) Gal*p*(β1-6) Man*p*(α1-2) Ins1→ (GIPC C), and Man*p*(α1-2) Man*p*(α1-6)Man*p*(α1-6) Man*p*(α1-3)[Xyl*p*(β1-2)]Man*p*(α1-4) Gal*p*(β1-6)Man*p*(α1-2) Ins1→ (GIPC D) (Table IV). GIPC B, C, and D are found only in the acapsular mutant Cap67, whereas that designated WT-GIPC A is present in both yeast forms. All are Xyl-branched glycans chains based on a Gal*p*(β1-6)Man*p*(α1-2)Ins core, which in the native glycolipid is linked to phosphoryl ceramide. Previously, only ceramide-(phosphoryl-Ins)$_2$-Man and ceramide-phosphoryl-Ins-Man, also found in *S. cerevisiae* (Smith and Lester, 1974) and *C. albicans* (Wells *et al.*, 1996), have been identified in *C. neoformans* (Vincent and Klig, 1995).

Until recently, the most complex GIPCs characterized in pathogenic fungi contained an α1→3 mannobiose chain with α/βGal*f* or β-Gal*p* branches (Barr *et al.*, 1984). However, unbranched inositol-tetra- and -penta-mannosides have been reported in GIPCs from *Sporothrix schenckii* (Penha *et al.*, 2000, 2001). As demonstrated in the present study, *C. neoformans* WT and Cap67 synthesize more elaborate GIPCs bearing a Xyl sidechain linked to *O*-2 of the second Man distal to Ins. Moreover, the oligosaccharide can be extended by addition of a further Man to this residue (in the WT-GIPC A) or up to four Man in the Cap67-GIPCs. Interestingly, the oligosaccharide moiety of these sphingophospholipids is structurally related to the saccharide side chains of the capsular GalXM of *C. neoformans* (Vaishnav *et al.*, 1998). The outer portion of the oligosaccharide chain of WT-GIPC A differs from the GalXM side chain (Vaishnav *et al.*, 1998) in that the β1→2-linked xylopyranose is always present, whereas xylopyranose β1→3 residues are not. The structural relation between the Ins-oligosaccharide of GIPCs and the GalXM side chains suggests that some common Man and Xyl transferases could be involved in the assembly of both polymers. It is probably significant that all basidiomycetes known to synthesize GIPCs containing the core Gal*p*(β1-6)Man*p*(α1-2)Ins (*Cryptococcus* and the higher mushrooms *Agaricus*, *Amanita*, *Calvatia*, *Leccinum*, *Lentinus*, and *Pleurotus*; Jennemann *et al.*, 1999, 2001) also elaborate complex glycans.

Another interesting structural feature is the extension of the Ins-pentasaccharide by additional Man units in Cap67-GIPCs, but not in the WT. The reason for this is not clear, because identical Ins-pentasaccharide precursors are found in both strains. One possible explanation is that the putative α1→6 mannosyl transferase, which adds the first Man residue, may be inactive in the WT cells, or possibly insufficient levels of GDP-Man are available for its activity, because of depletion by extensive GXM capsular polysaccharide production (Doering, 2000). Additional structural diversity is apparent in the ceramide domain. In common with the majority of fungi studied so far, the WT synthesizes GIPC based on C_{18} phytosphingosine predominantly *N*-acylated with 2-hydroxylignoceric acid, whereas the Cap67 mutant synthesizes GIPCs, which are *N*-acylated with 2,3-dihydrolignoceric acid or with 2-hydroxy-lignoceric acid.

The biological reason for the interstrain differences in *C. neoformans* ceramide composition is unclear. The fact that the in the Cap67 mutant the amount of GIPC is increased four-fold compared with WT-cells is suggestive of an adaptation to compensate for the absence of the GXM capsule. The presence of a higher percentage of 2,3-dihydroxy acids

Table III. ^{1}H and ^{13}C NMR chemical shifts of Ins-oligosaccharides of GIPC from *C. neoformans*

GIPC-derived Ins-oligosaccharide	H-1	H-2	H-3	H-4	H-5	H-6 or H-5′	H-6′	C-1	C-2	C-3	C-4	C-5	C-6
Ins-pentasaccharide (GIPC A)													
Man (3)*	5.151	4.080	3.860	3.670	[4.005]†	3.895	3.764	104.31	71.90	72.25	68.65	[75.09]	62.95
Xyl	4.389	3.303	3.446	3.644	4.006	3.296		105.44	74.55	77.40	[73.31]	66.97	
Man (2)	4.973	4.179	4.054	3.869	[4.105]	[3.763]	[3.781]	101.53	80.40	78.10	67.96	[74.98]	[62.22]
Gal	4.495	3.620	3.765	4.085	3.786	3.865	3.815	105.19	72.30	73.86	79.20	76.68	61.87
Man (1)	5.122	4.124	3.876	3.798	4.192	4.182	3.904	103.48	72.00	72.25	68.31	73.65	70.48
Ins	[3.670]	4.136	3.590	3.657	3.300	[3.670]		74.47	82.19	72.23	71.09	74.60	[74.47]
Ins-hexasaccharide (GIPC B)													
Man (4)	4.930	4.032	3.888	[3.677]	[3.658]	[3.910]	[3.774]	101.52	71.82	[72.44]	[68.63]	74.62	62.82
Man (3)	5.145	4.091	3.852	[3.876	[4.099]	4.088	3.660	104.86	72.00	72.60	67.84	73.24	67.03
Xyl	4.391	3.309	3.438	3.634	3.998	3.286		104.95	74.59	77.41	73.35	67.02	
Man (2)	5.011	4.081	4.042	[3.857]	[4.087]	[3.800]	[3.772]	101.10	79.90	79.15	67.81	75.18	[62.33]
Gal	4.503	3.619	3.765	4.103	3.802	3.851	3.851	105.41	72.71	73.95	78.92	76.76	61.97
Man (1)	5.127	4.118	3.870	3.799	4.188	4.196	3.910	103.49	72.04	72.38	68.35	73.68	70.55
Ins	[3.662]	4.134	3.584	3.651	3.292	[3.712]		74.45	82.18	72.25	71.11	76.67	[74.51]
Ins-octasaccharide (GIPC D)													
Man (6)	5.045	4.079	3.850	[3.657]	[4.091]			104.13	71.86	[73.01]	[68.71]	[75.10]	
Man (5)	5.161	4.018	[3.970]	[3.636]				100.08	80.63	[72.06]	[68.75]		
Man (4)	4.900	4.036	3.876	[3.872]				103.67	71.77	[72.65]	[68.33]		
Man (3)	5.135	4.089	[3.847]	[3.898]	4.101	[4.093]	[3.655]	104.91	72.00	[72.62]	67.82	73.25	[67.03]
Xyl	4.391	3.307	3.440	3.645	3.992	3.289		104.90	74.51	77.37	73.3	66.94	
Man (2)	5.009	4.073	4.038	[3.860]				101.17	79.88	79.31	[67.74]	[74.95]	
Gal	4.503	3.612	3.760	4.097	3.800			105.37	72.70	73.95	78.97	76.71	
Man (1)	5.122	4.116	3.867	3.794	4.192	4.196	3.902	103.44	71.92	72.21	68.30	73.51	70.80
Ins		4.132	3.581	3.670	3.289				82.19	72.23		76.59	

* Numbers in parentheses represent the position of the Man residues distal to Ins.
†Numbers in brackets indicate tentative assignment.

in the acapsular mutant would confer enhanced hydrogen-bonding capacity promoting formation of a thicker and less permeable bilayer (Levine *et al.*, 2000). Studies with *S. cerevisiae* have shown that plasma membrane sphingophospholipids are essential for growth (Wells and Lester, 1983), viability (Pinto *et al.*, 1992), and resistance to environmental stress (Patton *et al.*, 1992); this is likely to be the case in other fungal species, including *C. neoformans*. Thus, Luberto *et al.* (2001) recently showed that the down-regulation of the IPC synthase gene (*IPC1*) results in defective growth of *Cryptococcus* at acidic pH and that the biosynthesis of complex sphingolipids is essential for intracellular growth, suggesting that GIPC could function as virulence factors in *C. neoformans.*

Materials and methods

C. neoformans

An encapsulated form of *C. neoformans* (strain 444, here designated WT) was kindly provided by Prof. Luiz R. Travassos from the culture collection of the Laboratory of Mycology of the Universidade Federal de São Paulo, Brazil. This strain, originally isolated from a patient with AIDS who developed cryptococcal meningitis, is serotype A. The serotype D acapsular mutant strain Cap67 (Jacobson *et al.*, 1982) was kindly provided by Dr. Robert Cherniak, Georgia State University. Yeast forms were grown in a defined liquid medium (Cherniak *et al.*, 1982) at 37°C in a shaker at 100 rpm. After 5 days, cells were collected by centrifugation (7,000 × *g*, 15 min at 4°C) and washed three times with cold 150 mM NaCl, pH 7. Stock cultures of *C. neoformans* cells were grown on Sabouraud dextrose agar medium at 37°C and maintained at 4°C.

Extraction and purification of GIPCs from C. neoformans

GIPCs were extracted with aqueous phenol as previously described (Previato *et al.*, 1990). Briefly, after removal of capsular GXM and GalXM from *C. neoformans* (250 g wet weight) with 450 ml of 20 mM citrate buffer, pH 7.0 (90 min at 121°C) (Lloyd and Bitoon, 1971) the residual cells were recovered by centrifugation, (6,000 × *g*, 15 min) and extracted with 45% (v/v) aqueous phenol at 75°C for 20 min. After cooling

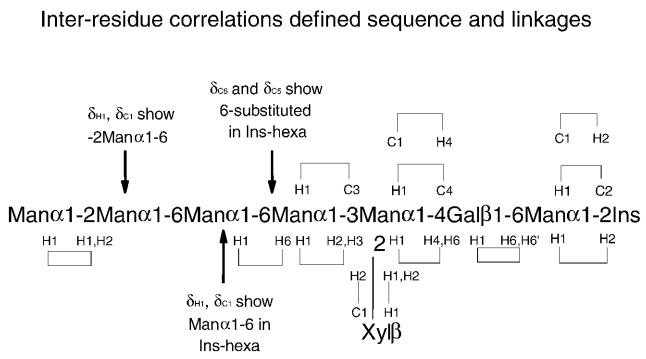

Fig. 6. Schematic compilation of the NMR data from experiments of the Ins-oligosaccharide derived from Cap67-GIPCs. The figure shows the inter-residue NOE data, long-range interresidue $^{1}H–^{13}C$ correlations, and chemical shift arguments used to determine the linkages between and sequence of the sugar residues.

and centrifugation (3,000 × *g*, 30 min) the aqueous layer was dialyzed, freeze-dried, dissolved in water, and applied to a column (2 × 80 cm) of Bio-Gel P-6DG. The excluded material was lyophilized, and the GIPCs recovered by three extractions with 200 ml chloroform/methanol/water (10:10:3, v/v/v). After compositional and structural analyses this was further purified on a Florisil column (1.2 × 40 cm) eluted with chloroform/methanol/water (10:10:3, v/v/v). Fractions of 1 ml were collected and screened for homogeneity by negative-ion MALDI-TOF MS (see following procedures) and HPTLC on Silica gel 60 plates (Merck) using chloroform/methanol/1 M NH_4OH (10:10:3, v/v/v) as mobile phase. GIPCs were detected by spraying with orcinol-sulfuric acid (Humbel and Collaert, 1975).

Carbohydrate and inositol analyses

The monosaccharide composition of GIPCs was determined according to Sweeley *et al.* (1963)). After methanolysis with 0.5 M HCl in methanol (18 h at 80°C), the mixture was extracted three times with hexane and the methanolic phase neutralized with Ag_2CO_3. The products were *N*-acetylated with acetic anhydride (overnight at room temperature in dark), dried under a stream of nitrogen, and treated with bis-(trimethylsilyl) trifluoroacetamide (BSTFA)/pyridine (1:1, v/v, 1 h at room temperature). Trimethylsilyl derivatives were analyzed by GC using a DB-5 fused silica capillary column (25 m × 0.25 mm ID) with hydrogen (10 psi) as the carrier gas. The column temperature was programmed from 120°C to 240°C at 2°C/min. For the analysis of Ins, GIPCs were hydrolyzed with 6 M HCl (18 h at 105°C), dried under reduced pressure, treated with BSTFA/pyridine (1:1, v/v, 1 h at room temperature), and analyzed by GC as described. Peaks were identified by comparison of their retention times to authentic standards and by GC-MS.

Lipid analysis

After methanolysis of the GIPCs with methanolic-HCl (18 h at 80 °C), fatty acid methyl esters were extracted with hexane. The extracts were combined, concentrated under a stream of nitrogen, and analyzed by GC after derivatization with BSTFA/pyridine as described. The column temperature was programmed from 180°C to 280°C at 3°C/min. Peaks were identified by their retention times compared to authentic standards and by GC-MS. For the analysis of long-chain bases, GIPCs were methanolyzed (1 M methanol-HCl made 10 M with respect to water) (Carter and Gaver, 1967) for 18 h at 80 °C. After adjusting the pH to about 11 with aqueous NaOH,

Table IV. Structures of Ins-oligosaccharides of GIPCs from *C. neoformans*

GIPC-derived Ins-oligosaccharide	Structure
Ins-pentasaccharide (GIPC A)	β-Xyl*p* 1 ↓ 2 α-Man*p*-(1→3)-α-Man*p*-(1→4)-β-Gal*p*-(1→6)-α-Man*p*-(1→2)Ins-P
Ins-hexasaccharide (GIPC B)	β-Xyl*p* 1 ↓ 2 α-Man*p*-(1→6)-α-Man*p*-(1→3)-α-Man*p*-(1→4)-β-Gal*p*-(1→6)-α-Man*p*-(1→2)Ins-P
Ins-heptasaccharide (GIPC C)*	β-Xyl*p* 1 ↓ 2 α-Man*p*-(1→6)-α-Man*p*-(1→6)-α-Man*p*-(1→3)-α-Man*p*-(1→4)-β-Gal*p*-(1→6)-α-Man*p*-(1→2)Ins-P
Ins-octasaccharide (GIPC D)	β-Xyl*p* 1 ↓ 2 α-Man*p*-(1→2)-α-Man*p*-(1→6)-α-Man*p*-(1→6)-α-Man*p*-(1→3)-α-Man*p*-(1→4)-β-Gal*p*-(1→6)-α-Man*p*-(1→2)Ins-P

*Deduced by methylation analysis and negative mode MALDI-TOF.

long-chain bases were extracted with diethyl ether. The combined extracts were washed with water, dried with anhydrous sodium sulfate, evaporated to dryness, dissolved in methanol, and *N*-acetylated with acetic anhydride (18 h at room temperature in the dark). The product was treated with BSTFA/pyridine and analyzed by GC and GC-MS as described for the fatty acid methyl esters.

Isolation of Ins-oligosaccharides from GIPCs by ammonolysis

GIPCs were hydrolyzed with 10 M ammonium hydroxide (18 h at 150°C) (Barr and Lester, 1984). After cooling, the hydrolysate was evaporated, dissolved in water, and ultra-centrifuged (40,000 × *g*, 6 h). The supernatant was passed through a reversed-phase LC-18 SPE column (Supelco); the Ins-oligosaccharides was eluted with water and further fractionated on a Bio-Gel P-4 (extra-fine) column (1 × 120 cm). Carbohydrate-containing fractions were pooled, lyophilized, and characterized by methylation analysis and NMR spectroscopy.

Methylation analysis

GIPCs and Ins-oligosaccharides were permethylated by the procedure of Paz Parente *et al.* (1985) methanolyzed (0.5 M HCl in methanol, 18 h at 80°C), acetylated (acetic anhydride/pyridine 9:1, v/v, 18 h at room temperature) and analyzed by GC and GC-MS on DB-1 fused silica column using a temperature programme from 110°C to 200°C at 2°C/min. The acetylated partially methylated methyl glycosides were initially identified by comparison of their retention times to those of authentic standards and confirmed by GC-MS as described by Fournet *et al.* (1981). The methylated GIPC was also analyzed by positive-ion ESI-MS/MS.

Determination of phosphorylation position of inositol

A modification of the procedure of Sugita *et al.* (1996) was used to determine the substitution pattern of Ins. GIPCs were oxidized (20 mM $NaIO_4$ in 0.2 M sodium acetate buffer, pH 5, 72 h at 4°C in the dark), reduced with $NaBH_4$, and hydrolyzed (2 M HCl, 2 h at 100°C). The products were chromatographed on Dowex 1X8 column (100–200 mesh, Cl^- form). The phospho-alcohol was eluted with 0.1 M HCl and freeze-dried. After dephosphorylation by incubation with 1 U of alkaline phosphatase from *Escherichia coli* (type III, Sigma), the product was peracetylated and identified by GC-MS as described. A peracetylated threitol (prepared by periodate oxidation of a purified (1→4)-linked β-D-galactopyranan kindly provided by Prof. José P. Parente of Núcleo de Pesquisas de Produtos Naturais, UFRJ, Brazil) and erythritol (prepared by acetylation of erythritol from Sigma) were used as standards.

Smith degradation of GIPCs

GIPCs were oxidized with $NaIO_4$ and reduced with $NaBH_4$ as described. The products were hydrolyzed (0.02 M trifluoroacetic acid, 40 min at 100°C), fractionated on a Bio Gel P-2 (extra-fine) column (1.0 × 100 cm) eluted with water. Fractions of 1.0 ml were collected and assayed for carbohydrate (Dubois *et al.*, 1956). A fraction eluting at a volume consistent with that of a Hex disaccharide was isolated, hydrolyzed (2 M trifluoroacetic acid, 1 h at 100°C), reduced with $NaBH_4$, and analyzed by GC after peracetylation (as described for methylation analysis). Derivatives were identified by comparison of their retention times with those of mannitol, threitol, erythritol, and glycerol and by GC-MS.

Other analytical methods

Total phosphorus was determined by the method of Ames (1966). The procedure of Lauter and Trams (1962) was used for the quantitative analysis of the long-chain bases in the methanolysates of GIPCs, using C_{18} phytosphingosine as standard.

MALDI-TOF-MS and ESI-QTOF-MS

MALDI mass spectra were recorded with a Micromass TofSpec 2E spectrometer, equipped with a 337-nm nitrogen laser. The instrument was operated in the positive ion reflectron mode at 20 kV accelerating voltage with time-lag focusing enabled. Samples were dissolved in 5% formic acid, and 1 µl was mixed with an equal volume of norharmane matrix solution (10 mg/ml in 50% acetonitrile) (Nonami *et al.*, 1998) and air-dried on the stainless steel target. Spectra were externally calibrated using deprotonated molecules of angiotensin I (*m/z* 1294.670), and ACTH clip 18–34 (*m/z* 2463.183) as references.

For nano-ESI-MS samples were dissolved in 50% (v/v) methanol/0.1% (v/v) aqueous formic acid, loaded into palladium coated borosilicate nanoelectrospray needles (Protana, Odense, Denmark), and mounted in the source of a Micromass Q-Tof hybrid quadrupole/orthogonal acceleration time of flight spectrometer. Stable electrospray was obtained at capillary voltage between 1200 and 1800 V. The collision gas was argon, and collision energies and argon pressure were tuned to optimise the fragmentation pattern of individual precursor ions.

NMR spectroscopy

NMR spectra were obtained on a Varian Unity 500 spectrometer equipped with pulsed field gradients and a 5-mm pulsed field gradient triple resonance probe, at a probe temperature of 30°C, as previously described (Todeschini *et al.*, 2001). Standard pulse sequences were used for 1D proton, TOCSY, ROESY spectra, except for the inclusion of spin-echo sequences into the TOCSY and ROESY pulse programs. Mixing times were 80 ms in TOCSY and 150 ms in ROESY spectra. HSQC spectra were obtained using the sequence of Wider and Wuthrich (1993), employing pulsed field gradients to suppress unwanted signals. These were optimized for $^1J_{C,H}$ of 150 Hz (delay = 3.3 ms) or for $^nJ_{C,H}$ of 20 Hz (delay = 25 ms). A 2D HSQC-TOCSY spectrum, related to the pulse sequence described by Crouch *et al.* (1990) but using the Wider and Wuthrich sequence for heteronuclear correlation (optimized for $^1J_{C,H}$ = 150 Hz and with a 20 msec mixing time), was also performed. Proton and ^{13}C chemical shifts were referenced to internal acetone at 2.225 ppm and 31.50 pm respectively.

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Abbreviations

BSTFA, bis-(trimethylsilyl)trifluoroacetamide; EI, electron ionization; ESI, electrospray ionization; GalXM, galactoxylomannan; GC, gas chromatography; GIPC, glycoinositolphosphoryl ceramide; GXM, glucuronoxylomannan; HPTLC, high-performance thin-layer chromatography; HSQC, heteronuclear single-quantum correlation; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; WT, wild type.

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