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Characterization of novel structures of mannosylinositolphosphoryl-ceramides from the yeast forms of *Sporothrix schenckii*

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Novel structures of glycoinositolphosphorylceramide (GIPC) from the infective yeast form of *Sporothrix schenckii* were determined by methylation analysis, mass spectrometry and NMR spectroscopy. The lipid portion was characterized as a ceramide composed of C-18 phytosphingosine N-acylated by either 2-hydroxylignoceric acid (80%), lignoceric (15%) or 2,3-dihydroxylignoceric acids (5%). The ceramide was linked through a phosphodiester to *myo*-inositol (Ins) which is substituted on position O-6 by an oligomannose chain. GIPC-derived Ins oligomannosides were liberated by ammonolysis and characterized as:

Man*p*α1→6Ins
Man*p*α1→3Man*p*α1→6Ins
Man*p*α1→6Man*p*α1→3Man*p*α1→3Man*p*α1→6Ins
Man*p*α1→2Man*p*α1→6Man*p*α1→3Man*p*α1→3Man*p*α1→6Ins

These structures comprise a novel family of fungal GIPC, as they contain the Man*p*α1→6Ins substructure, which has not previously been characterized unambigously, and may be acylated with a 2,3 dihydroxylignoceric fatty acid, a feature hitherto undescribed in fungal lipids.

Keywords: *Sporothrix schenckii*; glycoinositolphosphorylceramide; NMR; mass spectrometry.

Sporothrix schenckii is a dimorphic fungus that can infect healthy hosts when conidial forms or hyphal fragments penetrate broken skin. Within infected tissue, the fungus differentiates into yeast forms and proliferates leading to cutaneous and lymphocutaneous sporotrichosis [1]. This mycosis is frequently seen in immunocompromised patients who develop disseminated and difficult to treat forms of sporotrichosis [1–4]. The increased incidence of fungal infections in the past decade, particularly in immunocompromised individuals, and the emergence of drug resistant strains, prompted a search for new chemotherapeutic agents. Several of these novel antifungal compounds such as khafrefungin [5], aureobasidin A [6,7] and rustmicin [8] inhibit enzymes involved in the synthesis of inositolphosphorylceramide (IPC). Although IPCs comprise a relatively small proportion of fungal membrane phospholipids, they are essential for cell viability [9], and as they are not expressed by mammalian cells, may thus represent a specific target for antifungal chemotherapy.

In yeasts and fungi, plasma membrane IPC is initially modified by the addition of α-Man*p* residues giving rise to a class of glycolipids named glycoinositolphosphorylceramide (GIPC), which is not related to the glycosylphosphatidylinositol (GPI) family [10]. For example, in *Saccharomyces cerevisiae* [11] and *Candida albicans* [12] the α-Man*p*-Ins core structure is further modified by addition of Ins-PO_4, while in *Histoplasma capsulatum* [13] and *Paracoccidioides brasiliensis* [14] α-Man*p*, α/β-Gal*f* or β-Gal*p* are added to this core, forming species-specific epitopes which may be useful for diagnosis or as taxonomic markers.

To better understand the expression and distribution of GIPC on fungal cellular envelope, we have characterized the GIPC from *S. schenckii* yeast forms and report here the structure of a novel GIPC family based on Man*p*α1–6Ins-1PO_4-ceramide substructure.

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Abbreviations: FAME, fatty acid methyl ester; GIPC, glycoinositolphosphorylceramide; GIPL, glycoinositolphospholipid; Ins, *myo*-inositol; Ins-Man, Ins-mannoside; Ins-Man_2, Ins-dimannoside; Ins-Man_4, Ins-tetramannoside; Ins-Man_5, Ins-pentamannoside; IPC, inositolphosphorylceramide; Man*p*, mannopyranose; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; GPI, glycosylphosphatidylinositol; BHI, brain heart infusion.

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MATERIALS AND METHODS

Sporothrix schenckii strain and growth conditions

S. schenckii, strain 1099–18, isolated from a case of human sporotrichosis was obtained from the Mycology Section,

Department of Dermatology, Columbia University, New York, USA. The culture was maintained in solid Sabouraud medium and used as initial inoculum. Transfer was made to flasks containing 200 mL of brain heart infusion (BHI) incubated for 4 days at 37 °C with shaking. Once a stable yeast morphology was established, the culture was used as inoculum for 3 L conical flasks containing 1 L BHI, and incubated for 7 days under the conditions described above. Cells were harvested by centrifugation at 2500 ***g*** for 10 min and washed three times with cold 0.9% NaCl.

Isolation of GIPC

S. schenckii yeast cells (50 g wet weight) were extracted twice with 200 mL of 20 mM citrate buffer pH 7.0 for 90 min at 121 °C [15]. The cell debris recovered by centrifugation (3000 ***g***) was re-extracted with 45% (v/v) aqueous phenol for 20 min at 80 °C [16]. The aqueous layer was dialyzed, freeze dried, dissolved in water and applied to a column (2.5 × 100 cm) of Bio-Gel P-60 (100–200 mesh). Carbohydrate-containing material in the excluded volume was lyophilized and GIPC extracted by two treatments with 300 mL chloroform/methanol/water (10 : 10 : 3, v/v/v). The extracts were combined, evaporated to dryness, dissolved in water and freeze dried.

Isolation of inositol oligosaccharides

GIPC (25 mg) were subjected to hydrolysis in 10 M NH_4OH (4 mL) for 18 h at 150 °C. After cooling, the hydrolysate was concentrated by evaporation under N_2, lyophilized, dissolved in water and ultracentrifuged (40 000 ***g***, 6 h). The supernatant was passed through reverse-phase LC-18 SPE column (Supelco), the oligosaccharides eluted with water, freeze-dried, dissolved in water, and fractionated on a Bio-Gel P-4 (extra fine) column (1 × 120 cm). Fractions of 1.0 mL were collected and assayed with phenol/sulfuric acid [17]. Four carbohydrate fractions were isolated and analysed on silica gel HPTLC plates developed in ethanol/water/*n*-butanol/pyridine/acetic acid (100 : 30 : 10 : 10 : 3, v/v/v/v/v). Spots were detected after spraying the HPTLC plates with orcinol–sulfuric acid (110 °C for 5 min).

Carbohydrate and inositol analyses

For monosaccharide analysis, GIPC (200 μg) and Ins oligosaccharides (40 μg) were methanolyzed (0.5 M HCl in methanol for 18 h at 80 °C), using mannitol as internal standard. Fatty acid methyl esters (FAMEs) were extracted with heptane and the methyl glycosides O-trimethylsilylated with bis-(trimethylsilyl)trifluoroacetamide (BSTFA)/pyridine (1 : 1, v/v) for 2 h at room temperature. The products were analyzed by gas-liquid chromatography on a fused silica column of DB-1 (30 m × 0.25 mm i.d.) using hydrogen (0.7×10^5 Pa inlet pressure) as the carrier gas. The column temperature was programmed to increase from 120 to 240 °C at 2 °C·min^{-1}. For Ins analysis two derivatives were obtained: (a) the GIPC (500 μg) was methanolyzed (3 M HCl in methanol for 18 h at 80 °C), hydrolyzed (6 M HCl for 18 h at 100 °C), peracetylated as described by Barr *et al*. [18], and analyzed by GC under the conditions described above for monosaccharides; (b) Ins oligosaccharides (40 μg) were methanolyzed, O-trimethylsilylated and analysed as described for monosaccharides. The absolute configurations of the mannose (Man) residues of GIPC were determined by GC of their trimethylsilylated (–)-2-butylglycosides [19].

Lipid analysis

For analysis of fatty acids, GIPC (500 μg) was methanolyzed (0.5 M methanolic HCl for 18 h at 80 °C). FAMEs were extracted with heptane and analyzed by GC, before and after trimethylsilylation, on a DB-5 fused silica column (30 m × 0.25 mm i.d.). The column temperature was programmed to increase from 180 to 300 °C at 3 °C·min^{-1}. Peaks were identified by their retention times and by GC-MS. For analysis of long chain sphingosine base, GIPC (500 μg) was treated with 1 M HCl in methanol (made up to 10 M with respect to water) for 18 h at 80 °C [20]. The pH was adjusted to 11 with aqueous NaOH, and the methanolysate extracted three times with 2 vol. of diethyl ether. The combined extracts were dried under N_2, dissolved in methanol and N-acetylated with Ac_2O. The N-acetyl derivative was O-trimethylsilylated and analyzed by GC as described for FAMEs, and by GC-MS.

Methylation analysis

Ins oligosaccharides were permethylated by the method of Ciucanu & Kerek [21], methanolyzed (0.5 M HCl in methanol for 18 h at 80 °C), dried under N_2 and acetylated with Ac_2O/pyridine (9 : 1, v/v) for 18 h at room temperature. Gas chromatography was as described for monosaccharides. Peaks were quantified by integration and identified by retention time and GC-MS. Authenic standard of 1,2,3,4,5-penta-*O*-methyl-6-*O*-acetyl-Ins was prepared from Ins oligosaccharides derived from glycoinositolphospholipids (GIPLs) of *Trypanosoma cruzi* Tulahuen strain [22], according to procedures described above. The Ins oligosaccharide derived from *T. cruzi* GIPL was subjected to N-acetylation prior to methylation as described previously [23]. The retention time of 1,2,3,4,5-penta-*O*-methyl-6-*O*-acetyl-Ins relative to methyl-2,3,4,6-tetra-*O*-methyl-*manno*-pyranoside was 1.81.

Linkage between phosphorylceramide and inositol

To determine the substitution position of the Ins, GIPC (2 mg) was oxidized with 20 mM $NaIO_4$ in 0.2 M sodium acetate buffer (pH 5; 2 mL) for 72 h at 4 °C in the dark. The oxidized products were reduced with $NaBH_4$, dialyzed, lyophilized, hydrolyzed with 2 M HCl at 100 °C for 2 h, lyophilized, dissolved in water and chromatographed on an anion exchange column of Dowex 1 x 8 (100–200 mesh, Cl^- form). The phosphorylester eluted with 0.1 M HCl was recovered by lyophilization, dissolved in 0.2 M ammonium acetate buffer (pH 8.5) and incubated with 1 unit of alkaline phosphatase (from *Escherichia coli*; Sigma) for 24 h at 37 °C. The reaction mixture was lyophilized and the products peracetylated and analyzed by GC as described for monosaccharides. The resulting peaks were identified by their retention times and GC-MS.

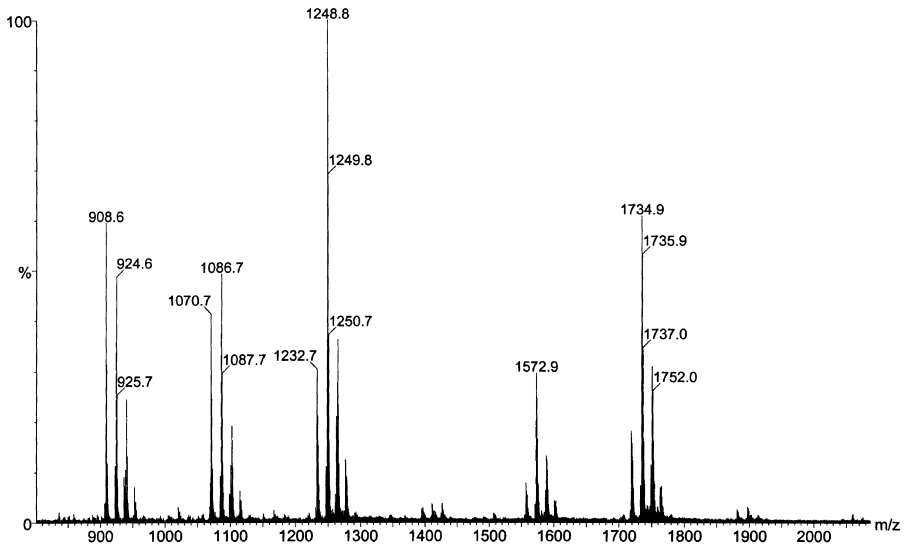

Fig. 1. MALDI-TOF MS of GIPC from *S. schenckii*. Mass spectrum was recorded in the negative ion mode.

GC-MS

GC-MS was performed with a Shimadzu GC-MS-QP5050 quadrupole mass spectrometer, interfaced with a Shimadzu GC 17A gas chromatograph equipped with a DB-1 capillary column, using an ionization energy of 70 eV and an ionizing current of 0.2 mA. The column temperature used was programmed to raise from 120 to 240 °C at 2 °C·min^{-1} or 180–300 °C at 3 °C·min^{-1} for methylation and lipid analyses, respectively.

Other analytical methods

Total phosphorus was determined by the method of Ames [24]. The procedure of Lauter and Trams [25] was used for the quantitative analysis of the long-chain bases in methanolysates of GIPC, using C18-phytosphingosine as standard.

Matrix assisted laser desorption mass spectrometry

MALDI mass spectra were recorded with a Micromass TofSpec 2E spectrometer, equipped with a 337 nm nitrogen laser. The instrument was operated in negative ion reflectron mode at 20 kV accelerating voltage with time-lag focussing enabled. The matrix was *nor*-harmane [26], prepared as a 10 mg·mL^{-1} solution in 50% aqueous acetonitrile. Equal volumes of matrix and sample (in 50% acetonitrile) were mixed, and 1.5 μL volumes were applied to the stainless steel target and allowed to dry.

NMR spectroscopy

NMR spectra were obtained on a Bruker Avance DRX 400 with a 5-mm broadband probe. Samples for NMR spectroscopy were deuterium exchanged by repeated lyophilization from deuterium oxide and dissolved in 0.5 mL of D_2O before analysis. Proton NMR spectra were assigned through a TOCSY spectra, with some additional assignments and information on the sequence and linkage of the sugar residues derived from NOESY spectra collected with mixing times between 400 and 800 ms. Carbon chemical shifts were assigned from the HMQC spectrum recorded with carbon decoupling and by long range correlation observed in an HMBC experiment. Proton spectra were referenced to internal acetate anion at 1.908 p.p.m. and ^{13}C spectra were referenced to external methanol at 50 p.p.m.

RESULTS

Isolation and chemical composition of purified GIPC of *S. schenckii*

Extraction of *S. schenckii* yeast forms with hot phenol released into the aqueous layer a glycolipid fraction that was soluble in chloroform/methanol/water (10 : 10 : 3). Compositional analysis showed that this fraction contained Man (2.7 mol), Ins (1 mol), phosphorus (1 mol), C18-phytosphingosine (0.9 mol) predominantly N-acylated with 2-hydroxylignoceric acid (80%), or with lignoceric (15%) or 2,3-dihydroxylignoceric acids (5%). The C18-phytosphingosine and the fatty acids were characterized by comparision of their GC retention times and EI-MS with those of authentic standards, whereas the 2,3-dihydroxylignoceric acid, a novel fatty acid, was identified only by EI-MS. The EI-MS of this compound was similar to that of 2,3-dihydroxy-12-methyltridecanoate isolated from *Legionella pneumophila* [27], except that the fragments (M-15) (M-59) and (M-161) shifted up in mass by 140 units.

The negative ion MALDI-TOF mass spectrum (Fig. 1) of the GIPC consisted of five triplets of deprotonated molecules at *m/z* (908.6, 924.6 and 940.6) (1070.7, 1086.7 and 1102.7) (1232.7, 1248.7 and 1264.7) (1556.8, 1572.8 and 1588.8) and (1718.9, 1734.9 and 1750.9). Each group of triplets is separated by a multiple of 162, suggesting the presence of glycan chains containing 0, 1, 2, 4 or 5 hexose residues. The space of 16 between the ^{12}C peaks within each triplet cluster is consistent with the presence of a IPC moiety composed of C-18 phytosphingosine N-acylated with either lignoceric acid, 2-hydroxylignoceric acid (major), or 2,3-dihydroxylignoceric acid.

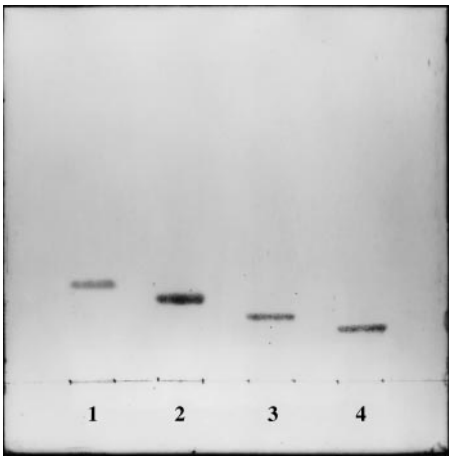

Fig. 2. HPTLC of Ins oligomannosides isolated from *S. schenckii* GIPC. The Ins oligomannosides purified by gel filtration chromatography on Bio Gel P-4 column were analysed by silica-gel 60 HPTLC. Lanes (1) Ins-Man; (2) Ins-Man_2 (3); Ins-Man_4; (4) Ins-Man_5. The relative concentration of component applied in lane (3) was ≈ threefold that in lanes (1) (2) and (4).

Analysis of inositol oligomannosides from the GIPC of *S. schenckii*

Ins oligosaccharides were released from *S. schenckii* GIPC by ammonolysis, separated by gel-filtration chromatography, and analysed by HPTLC (Fig. 2). Four included carbohydrate containing fractions were obtained, methanolyzed, and their compositions determined by GC after trimethylsilylation. The fractions containing Man*p* and Ins in the molar ratio 0.9 : 1, 1.9 : 1, 4 : 1, and 4.8 : 1 were designated as Ins-Man, Ins-Man_2, Ins-Man_4 and Ins-Man_5, respectively.

Methylation analysis

Permethylated Ins-Man, Ins-Man_2, Ins-Man_4 and Ins-Man_5 were methanolysed and the products converted to O-acetylated partially O-methylated methyl glycosides which were characterized by GC and GC-MS. The methylation products showed the presence of 1,2,3,4,5-penta-*O*-methyl-6-*O*-Ins from all the Ins oligomannosides, which was identified by its retention time after comparison with an authentic standard prepared from a GIPL of *T. cruzi* [22], establishing that in the GIPC of *S. schenckii* the mannosyl chain is linked to the Ins *O*-6. The methylation products of Ins-Man_2 revealed that the Man*p* adjacent to Ins is 3-O substituted. The other derivatives showed that the Ins-Man_4 contains terminal Man*p*, 3-O and 6-O substituted Man*p* in molar ratio 1 : 2 : 1, whereas the Ins-Man_5 contains an additional Man*p* residue substituted on O-2. These results show that both Ins-Man_4 and Ins-Man_5 are linear oligosaccharides.

Location of phosphorylceramide group on the inositol

The position on the Ins to which the phosphorylceramide group is attached was deduced by a combination of the methylation and periodate oxidation studies. Exhaustive oxidation of GIPC with $NaIO_4$, followed by $NaBH_4$ reduction and hydrolysis, yielded a tetraolphosphate which after digestion with alkaline phosphatase and acetylation, gave rise to threitol tetraacetate which was characterized by GC and GC-MS. As the threitol is derived from carbons 1, 2, 5 and 6 of the Ins ring and the methylation analysis established that the mannoside-chain of GIPC is attached to position 6 of the Ins, we conclude that the ceramide moiety is linked to position 1 via a phosphodiester bridge.

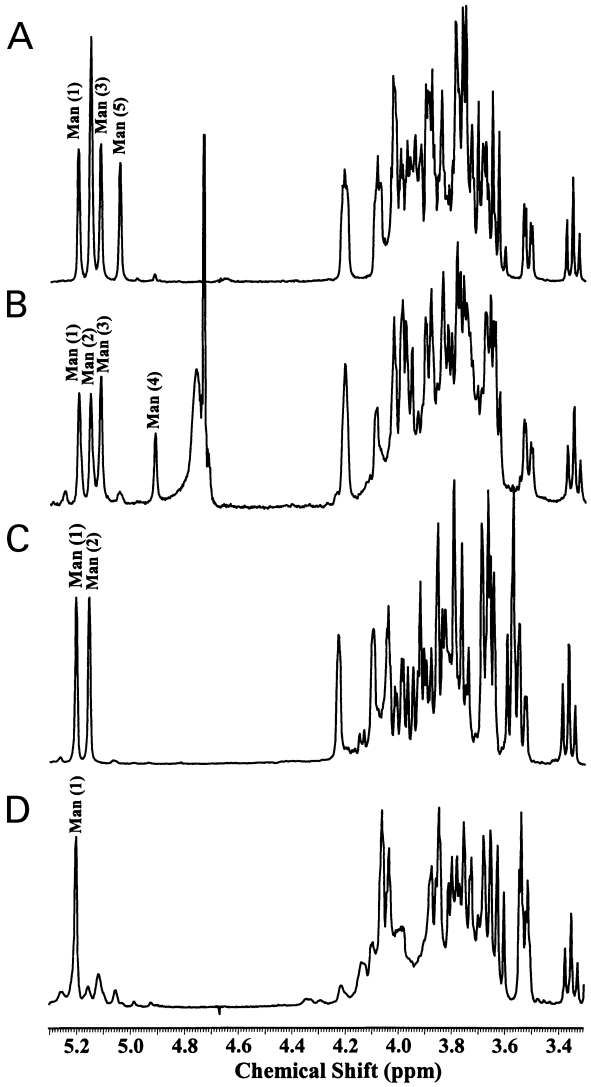

Fig. 3. Partial ^1H NMR spectra of Ins oligomannosides isolated from *S. schenckii* GIPC. 400 MHz spectra of Ins-Man_5 (A); Ins-Man_4 (B); Ins-Man_2 (C); (D) Ins-Man. The labels indicate the resonance of the anomeric protons.

NMR spectroscopy of the inositol oligomannosides

NMR data for the Ins oligomannosides are in agreement with the methylation analysis. Proton spectra were assigned from TOCSY spectra with 80 ms mixing times, NOESY, and the ^{13}C spectrum of Ins-Man_5 through proton-detected heteronuclear correlation. The assignments are listed in

Table 1. NMR chemical shifts (p.p.m.) of Ins oligomannosides from *S. schenckii*. Values in square brackets represent tentative assignments.

	Manpα1→2Manpα1→6Manpα1→3Manpα1→3Manpα1→6Ins					
	(5)	(4)	(3)	(2)	(1)	
H-1	5.042	5.156	5.114	5.154	5.196	
C-1	102.53	98.85	103.36	102.53	101.42	
H-2	4.078	4.016	4.086	4.204	4.210	4.022
C-2	70.59	79.33	70.61	70.52	70.53	73.21
H-3	3.844	3.950	3.883	3.984	4.003	3.514
C-3	70.95	70.88	71.10	79.85	78.21	71.56
H-4	3.621			3.835	3.878	3.644
C-4	67.54				66.72	75.13
H-5	3.834	[3.783]	[3.953]		3.688	3.348
C-5	73.70	[73.90]	72.33		73.40	73.53
H-6	3.903	3.903	3.962			3.749
C-6	61.80	61.70	66.70			80.20
H-6′	3.719	3.719	3.759			

	Manpα1→6	Manpα1→3	Manpα1→3	Manpα1→6	Ins
	(4)	(3)	(2)	(1)	
H-1	4.912	5.113	5.152	5.196	
H-2	3.991	4.084	4.201	4.212	4.022
H-3	3.880	3.890	3.989	4.010	3.526
H-4	3.668		3.834	3.855	3.667
H-5					3.343
H-6		3.952			3.761
H-6′		3.759			

	Manpα1→3	Manpα1→6	Ins
	(2)	(1)	
H-1	5.154	5.203	
H-2	4.088	4.227	4.034
H-3	3.891	3.999	3.535
H-4	3.782	3.852	3.659
H-5	3.700	3.898	3.365
H-6	3.849		3.755
H-6′	[3.767]		

	Manpα1→6	Ins
	(1)	
H-1	5.202	
H-2	4.062	4.034
H-3	3.855	3.520
H-4	3.271	3.653
H-5	3.623	3.350
H-6	3.868	3.754
H-6′	3.524	

Table 1 and the 1D ^{1}H are shown in Fig. 3. The sequence and linkages between the sugar residues were established from NOESY spectra and, for the Ins-Man$_5$, a long-range ^{1}H-^{13}C correlation. The anomeric configurations of the Man*p* residues were established from the chemical shifts of H-1, C-1, C-2, H-3 and H-5. As Ins lacks an anomeric proton the starting point for the assignments was the H-6, known to be glycosylated from the chemical degradation studies. An nOe was observed in all the NOESY spectra between the α-Man*p*(1) H-1 and a well-resolved 10-Hz triplet at 3.7 p.p.m. assigned as the Ins H-6, which correlated with a lowfield Ins C-6 at 80.20 p.p.m. These data are consistent with the Manpα1→6Ins linkage. The *myo* configuration of the Ins ring was proven by the $^3J_{H,H}$-values, estimated from the fine structure of the TOCSY cross peaks. Whilst the H-6, H-5 and H-4 resonances are 10 Hz triplets, H-3 appears as a 10-Hz, 2-Hz double doublet showing that H-2 is equatorial. The highfield position of the H-2 resonance (4.022 p.p.m.) is inconsistent with a Manpα1→2Ins linkage, when it is typically close to 4.1 p.p.m. [28]. H-4 resonates at lowfield (3.644 p.p.m.) due to a 1,3 diaxial interaction with O-2, whilst the H-5 (at 3.348 p.p.m.) lacks such an interaction. The 1D ^{1}H NMR spectrum of Ins-Man$_2$ (Fig. 3C) differs from that of Ins-Man$_1$ (Fig. 3D) by the presence of an additional α-Man*p* spin system (H-1 at 5.151 p.p.m.) designated Man*p*(2) (Table 1), consistent with a terminal Manpα1→3Man*p* sequence [28] in agreement with the methylation analysis and confirmed by the observation of an nOe between Man*p*(2) H-1 and Man*p*(1) H-3 at 3.999 p.p.m.

The 1D ^{1}H NMR spectrum of the Ins-Man$_4$ (Fig. 3B) contains two additional α-Man*p* spin systems (H-1 s at 5.113 and 4.912 p.p.m.) designated Man*p*(3) and Man*p*(4), respectively (Table 1). An interresidue nOe was observed between Man*p*(3) H-1 and Man*p*(2) H-3 at 3.989, and the Man*p*(2) H-2 is shifted downfield compared to its position in In-Man$_2$ (Fig. 3C), showing a Man*p*(3)α1→3Man*p*(2) linkage. The chemical shift of Man*p*(4) H-1 is characteristic of a Manpα1→6Man*p* sequence (28). An nOe was observed between Man*p*(4) H-1 and a resonance at 3.759 p.p.m. In the HMQC spectrum of Ins-Man$_5$ (Table 1) a lowfield C-6 (66.70 p.p.m.) correlates to H-6 s at 3.759 and 3.962 p.p.m., and together with the methylation analysis data, this indicates a Man*p*(4)α1→6Man*p*(3) motif. As the methylation analysis showed the presence of a terminal Man*p* and Man*p* O-6 and O-3 substituted, in molar ratio of 1 : 1 : 2, the Manpα1→6Manpα1→3Manpα1→3Manpα1→ substructure was suggested for Ins-Man$_4$. In the 1D ^{1}H NMR spectrum of Ins-Man$_5$ (Fig. 3A) the Man*p*(4) H-1 resonance has been shifted dowfield to 5.156 p.p.m., overlapping with the Man*p*(2) H-1, whilst the Man*p*(4) C-1 is at highfield (98.85 p.p.m.), due to the attachment of α-Man*p*(5) at O-2. The terminal Man*p*(5)α1→2Man*p*(4) sequence was confirmed by the lowfield position of Man*p*(4) C-2 at 79.33 p.p.m., which correlates to Man*p*(5) H-1 in the HMBC spectrum (Fig. 4), as well as by the presence of an additional interresidue nOe between Man*p*(5) H-1 and Man*p*(4) H-2. The Man*p*(4)α1→6Man*p*(3) linkage was confirmed by the lowfield Man*p*(3) C-6 at 66.70 p.p.m., which shows a long-range ^{1}H-^{13}C correlation to Man*p*(4) H-1. Other long-range ^{1}H-^{13}C correlations and the ^{13}C assignments are fully in accord with the structure Manpα1→2Manpα1→6Manpα1→3Manpα1→3Manpα1→6Ins for the Ins-Man$_5$ (Fig. 4).

DISCUSSION

In the present work Ins- mono, di, tetra, and pentamannosides were released from GIPC of yeast forms of *Sporothrix*

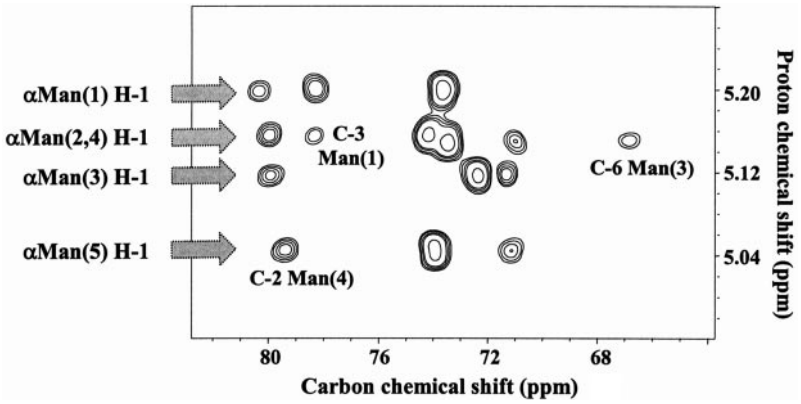

Fig. 4. Partial 400 MHz HMBC spectrum of the Ins-Man$_5$ from GIPC of *S. schenckii* showing the long-range ^{1}H-^{13}C connectivities of the anomeric protons. Labeled cross peaks indicate three-bound couplings ($^3J_{CH}$) across the glycosidic bonds. Cross peaks that are not marked refer to couplings within the glycosyl ring.

schenckii by ammonolysis and separated by gel filtration chromatography. Their structures were determined by a combination of compositional analysis, NMR spectroscopy and methylation analysis. All are unbranched *manno*-oligosaccharides containing a core sequence Man*p*α1→6Ins which, in the intact GIPC, is linked via a phosphodiester bridge to a ceramide moiety composed of C-18 phytosphingosine mainly N-acylated with 2-hydroxylignoceric acid or with lignoceric acid or 2,3-dihydroxylignoceric acid. It is noteworthy that the Ins head group of *S. schenckii*-derived GIPCs are substituted at position O-6 by an α-D-Man*p*, a feature that has not been reported in the glycolipids of eukaryotic cells. It has, however, been described in the phosphatidylinositolmannosides which anchor the lipomannans and lipoarabinomannans of *Mycobacterium,* though in these compounds the Ins is also substituted at position O-2 by a unmodified α-D-Man*p* unit [29,30]. The Man*p*α1→2-Ins motif has been identified as a common core structure of all GIPC expressed by *Saccharomyces cerevisae* [11], *Neurospora crassa* [11], *Histoplasma capsulatum* [13], *Paracoccidioides brasiliensis* [14] and *Cryptococcus neoformans* ([31] and N. Heise, A. L. Gutierrez, C. Jones, R. Wait, K. A. Mattos, J. O. Previato and L. Mendonça-Previato, unpublished results). Recently, the occurence of GIPC oligosaccharides chains attached to Ins either at O-2 or O-4 positions was described previously [32]. Although relatively few fungal GIPC oligosaccharides glycan chains have been characterized, the currently known structures can be classified into two groups (independent of phylogenetic affinity), according to the nature of the second sugar residue distal to Ins (Table 2). Either Man*p*α1→3Man*p*-Ins or Man*p*α1–6Man*p*-Ins motifs characterize the first group. The former motif can be modified by addition of up to three Man*p* units, as in *S. schenckii* yeast form; or may be decorated by addition of α/β-Gal*f* or β-Gal*p* residues to the Man*p* linked to Ins, as in *H. capsulatum* [13] and *P. brasiliensis* [14] and *S. schenckii* mycelium form [33]. The latter motif can also be modified by addition of a single

Table 2. Complex structures of glycoinositolphosphorylceramides from fungi. The positions of the motifs (groups 1 and 2) in the structures are shown in bold types.

Species	Structure	Ref.
Group 1: Man*p*α(1→3)Man*p*-Ins motif		
S. schenckii	**Man*p*α(1→3)Man*p*α(1→6)Ins**	[33,34],
	Man*p*α(1→6)Man*p*α(1→3)**Man*p*α(1→3)Man*p*α(1→6)Ins**	this work
	Man*p*α(1→2)Man*p*α(1→6)Man*p*α(1→3)**Man*p*α(1→3)Man*p*α(1→6)Ins**	
H. capsulatum	**Man*p*α(1→3)Man*p*α(1→2 or 6)Ins**	[13]
	Man*p*α(1→3)[Gal*f*α(1→6)]**Man*p*α(1→2 or 6)Ins**	
	Man*p*α(1→3)[Gal*f*β(1→4)]**Man*p*α(1→2 or 6)Ins**	
P. brasiliensis	**Man*p*α(1→3)Man*p*α(1→2)Ins**	[14]
	Man*p*α(1→3)[Gal*f*β(1→6)]**Man*p*α(1→2)Ins**	
Group 2: Gal*p*β(1→6)Man*p*-Ins motif		
Agaricus spp.	Gal*p*α(1→6)[Fuc*p*α(1→2)]**Gal*p*β(1→6)Man*p*α(1→2)Ins**	[32]
	Gal*p*α(1→6)Gal*p*α(1→6)[Fuc*p*α(1→2)]**Gal*p*β(1→6)Man*p*α(1→2)Ins**	
	Gal*p*α(1→6)Gal*p*α(1→6)Gal*p*α(1→6)[Fuc*p*α(1→2)]**Gal*p*β(1→6)Man*p*α(1→2)Ins**	
C. neoformans	Man*p*α(1→3)[Xyl*p*β(1→2)]Man*p*α(1→4)**Gal*p*β(1→6)Man*p*α(1→2)Ins**	[31][a]
	Man*p*α(1→6)Man*p*α(1→3)[Xyl*p*β(1→2)]Man*p*α(1→4)**Gal*p*β(1→6)Man*p*α(1→2)Ins**	
	Man*p*α(1→6)Man*p*α(1→6)Man*p*α(1→3)[Xyl*p*β(1→2)]Man*p*α(1→4)**Gal*p*β(1→6)Man*p*α(1→2)Ins**	
	Man*p*α(1→2)Man*p*α(1→6)Man*p*α(1→6)Man*p*α(1→3)[Xyl*p*β(1→2)]Man*p*α(1→4)**Gal*p*β(1→6)Man*p*α(1→2)Ins**	

[a] N. Heise, A. L. Gutierrez, C. Jones, R. Wait, K.A. Mattos, J. O. Previato & L. Mendonça-Previato, unpublished results.

α-Man*p* unit, as in *Cantharellus cibarius* [32]. The second group contains a Gal*p*β1→6Man*p*-Ins motif, which can be modified either by addition of up to three α-Gal*p* units (with an α-Fuc*p* branch on the Man*p*-linked β-Gal*p*), for example as in *Agaricus* spp. [32], or alternatively by addition of up to five α-Man*p* units (with a β-Xyl*p* branch on the β-Gal*p*-linked α-Man*p*) as in *C. neoformans* ([31] and N. Heise, A. L. Gutierrez, C. Jones, R. Wait, K. A. Mattos, J. O. Previato & L. Mendonça-Previato, unpublished results). The biological significance of the structural diversity of the GIPC chains are unknown, however, several functions have been ascribed to *S. cerevisiae* IPC, a GIPC-related molecule [9], and it has recently been hypothesized by Levery *et al*. [14] that the expression of more complex GIPC-glycan chains could be a characteristic of fungi exhibiting mycelial forms or thermal dimorphism. In this context, *S. schenckii* would be a new example of a dimorphic fungus, expressing a GIPC-derived complex glycan. However, clarification of the role of glycan structural diversity of fungal GIPC must await characterization glycans from fungi belonging to a broader range of divisions, classes and subclasses.

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