

# Sphingolipids of the mycopathogen *Sporothrix schenckii*: identification of a glycosylinositol phosphorylceramide with novel core GlcNH<sub>2</sub>α1 → 2Ins motif

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**Abstract** Acidic glycosphingolipid components were extracted from the yeast form of the dimorphic mycopathogen *Sporothrix schenckii*. Two minor and the major fraction from the yeast form (Ss-Y1, -Y2, and -Y6, respectively) have been isolated. By a combination of 1- and 2-D <sup>1</sup>H-nuclear magnetic resonance (NMR) spectroscopy, electrospray ionization mass spectrometry (ESI-MS), and gas chromatography/mass spectrometry (GC/MS), Ss-Y6 was determined to be triglycosylinositol phosphorylceramide with a novel glycan structure, Manα1 → 3Manα1 → 6GlcNH<sub>2</sub>α1 → 2Ins1-P-1Cer (where Ins = *myo*-inositol, P = phosphodiester). While the GlcNH<sub>2</sub>α1 → 6Ins1-P- motif is found widely distributed in eukaryotic GPI anchors, the linkage GlcNH<sub>2</sub>α1 → 2Ins1-P- has not been previously observed in any glycolipid. Ss-Y1 and Ss-Y2 were both found to have the known glycan structure Manα1 → 3Manα1 → 2Ins1-P-1Cer. Together with the results of a prior study [Toledo et al. (2001) *Biochem. Biophys. Res. Commun.* 280, 19–24] which showed that the mycelium form expresses GIPCs with the structures Manα1 → 6Ins1-P-1Cer and Manα1 → 3Manα1 → 6Ins1-P-1Cer, these results demonstrate that *S. schenckii* can synthesize glycosylinositol phosphorylceramides with at least three different core linkages. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Sphingolipid; Glycolipid; Dimorphism; Mycopathogen; Nuclear magnetic resonance; Mass spectrometry

## 1. Introduction

The risk of systemic fungal disease among growing populations of immunosuppressed or -compromised patients [5],

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**Abbreviations:** NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; GC, gas chromatography; ESI, electrospray ionization; MS, mass spectrometry; CID, collision-induced decomposition; DMSO, dimethyl sulfoxide; TMS, trimethylsilyl; FAME, fatty acid methyl ester

along with recent work showing that fungi are vulnerable to inhibitors of sphingolipid biosynthesis (reviewed in [6]), has led to an increased interest in the structure, biosynthesis, and functional roles of fungal sphingolipids as potential targets for antifungal agents. A particularly interesting target is the fungal inositol phosphorylceramide (IPC) synthase [6], inhibitors of which are highly toxic to many mycopathogens, but exhibit low toxicity in mammals [7–10]. IPC synthase catalyzes the transfer of *myo*-inositol-1-phosphate from phosphatidylinositol to ceramide; further processing of IPC by glycosyltransferases yields glycosylinositol phosphorylceramides (GIPCs). A number of studies have pointed to potential interactions of GIPCs of fungi with the mammalian immune system [11–13]. Elucidation of such interactions calls for detailed knowledge of the structures of these compounds, which is still limited in comparison to what is known about glycosphingolipids (GSLs) of animal species. Also, as occurred earlier with mammalian studies, expanding knowledge of fungal GIPC structures can be expected, by extension, to reveal a great deal about the activities and expression patterns of the enzymes responsible for their synthesis. The detection of novel glycosyl linkages, and therefore of novel glycosyltransferases, is an important complement to molecular and cell biological approaches to glycoconjugate function; in addition, specific GIPC processing enzymes could offer further potential targets for development of inhibitors as antifungal agents.

In this paper, we report on the structure elucidation of GIPCs from the dimorphic mycopathogen *Sporothrix schenckii*. Among other observations, these studies revealed that a major component of the yeast form has the structure Manα1 → 3Manα1 → 6GlcNH<sub>2</sub>α1 → 2Ins1-P-1Cer<sup>2</sup>. To our knowledge, this is the first report of any glycolipid with the GlcNH<sub>2</sub>α1 → 2Ins1-P- structural motif, although the isomeric GlcNH<sub>2</sub>α1 → 6Ins1-P- linkage is commonly encountered in eukaryotic GPI anchored proteins and phosphoglycans [14,15].

## 2. Materials and methods

### 2.1. Fungal isolate and growth conditions

The culture of *S. schenckii*, strain 65 (originally obtained from cutaneous footpad lesion of an otherwise healthy individual), was

<sup>2</sup> A preliminary description of these studies has been presented in poster form [1].

provided by Dr. Olga Gompertz, Department of Cellular Biology, Universidade Federal de São Paulo/Escola Paulista de Medicina, São Paulo, SP, Brazil. Mycelium and yeast forms of *S. schenckii* strain 65 were grown in brain-heart infusion (BHI; 37 g/l water), incubated at 25 and 37°C, respectively, and harvested as described [16].

## 2.2. Extraction, purification, and thin layer chromatographic analysis of GSLs

Extraction and purification of GSLs were carried out as described previously [11,17,18]. Briefly, GPIs were extracted by homogenizing yeast or mycelium forms (25–35 g wet weight) in an Omni-mixer (Sorvall Inc. Wilmington, DE, USA), three times with 200 ml of isopropanol/hexane/water (55:20:25, v/v/v, upper phase discarded), and twice with 200 ml of chloroform/methanol (2:1, v/v). The five extracts were pooled, dried on a rotary evaporator, dialyzed against water, lyophilized, resuspended in chloroform/methanol/water (30:60:8, v/v/v; solvent A), and applied to a column of DEAE-Sephadex A-25 (Ac<sup>-</sup> form). Neutral GSLs were eluted with five volumes of solvent A. Acidic GSLs were eluted with five volumes of 0.5 M sodium acetate in MeOH. The acidic fraction was dried, dialyzed exhaustively against deionized water, redried and further purified by repetitive preparative-scale high performance liquid chromatography (HPLC) (50 cm×4.6 mm Sphereclone (Phenomenex, Torrance, CA, USA) 10 μm porous spherical silica; elution with 2-propanol-hexane-water gradient from 55:40:5 to 55:25:20 over 120 min, then isocratic for 40 min; flow rate 0.5 ml/min; 80×1 ml fractions collected). The identity and purity of each fraction was assessed by analytical high performance thin layer chromatography (HPTLC), performed on silica gel 60 plates (E. Merck, Darmstadt, Germany) using chloroform/methanol/water (60:35:10, v/v/v) as mobile phase, with detection by Bial's orcinol reagent.

## 2.3. <sup>1</sup>H-Nuclear magnetic resonance (NMR) spectroscopy

Samples of underivatized GIPC (~0.5–1.0 mg) were deuterium exchanged by repeated evaporation from CDCl<sub>3</sub>/CD<sub>3</sub>OD (2:1, v/v) under N<sub>2</sub> stream at 37°C, and then dissolved in 0.5 ml dimethyl sulfoxide (DMSO)-d<sub>6</sub>/2% D<sub>2</sub>O [19] for NMR analysis. 1-D <sup>1</sup>H-NMR, 2-D <sup>1</sup>H-<sup>1</sup>H-gradient correlation spectroscopy (COSY) [20], -total correlation spectroscopy (TOCSY) [21,22] and -nuclear Overhauser effect spectroscopy (NOESY) [23] experiments were performed at 35°C on Varian Unity Inova 600 or 800 MHz spectrometers using standard acquisition software available in the Varian VNMR software package. Proton chemical shifts are referenced to internal tetramethylsilane (δ=0.000 ppm).

## 2.4. Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS and tandem ESI-MS/collision-induced decomposition (CID)-MS were performed in the positive ion mode with Li<sup>+</sup> adduction on a PE-Sciex (Concord, ON, Canada) API-III spectrometer, using a standard Ionspray source (orifice-to-skimmer voltage (OR), 100–130 V or 160–180 V ('high OR'); Ionspray voltage, 5 kV; interface temperature, 45°C) and sample introduction by direct infusion (3–5 μl/min) of GIPC samples dissolved (~20 ng/μl) in 100% MeOH. For generation of Li<sup>+</sup> adducts, a solution of LiI (10 mM) in MeOH was added [4,24] until the observed ratio of M·Li<sub>2</sub><sup>+</sup> to M·LiNa<sup>+</sup> in <sup>+</sup>ESI-MS profile mode was >95:5 (the final concentration of LiI was generally 3–6 mM). For <sup>+</sup>ESI-MS/CID-MS experiments, precursor ions selected in Q1 were subjected to collision induced dissociation (with argon as collision gas) in Q2, while the useful mass range (starting from *m/z* 100) was scanned in Q3 (0.2 u steps); alternatively, appropriate product ions were selected in Q3 while the useful precursor mass range was scanned in Q1. OR was set to 100–120 V; other parameters were as described previously [24]. In general, spectra represent summations of 10–30 scans for single analyzer profiles, and 30–50 scans for CID experiments. Fragment nomenclature is after Domon and Costello [25,26] as modified for use with GIPCs [27]. Nominal monoisotopic *m/z* values are used in the description of all ESI-MS results.

## 2.5. Miscellaneous procedures

Selective *N*-acetylation was carried out on an aliquot of Ss-Y6: to ~200 μg in 1 ml MeOH was added 50 μl pyridine and 50 μl acetic anhydride; vortexed and incubated for 1 h at room temperature; evaporated to dryness (three times with addition of toluene) under N<sub>2</sub> stream at 37°C. Component analyses were performed by gas chro-

matography (GC)/MS of per-*O*-trimethylsilyl (TMS) derivatives of methyl glycosides, inositol, sphingoids, and fatty acid methyl ester (FAME)s released from GIPCs by methanolysis, using procedures described in detail elsewhere [3].

## 3. Results

### 3.1. HPTLC profile of *S. schenckii* GIPCs

HPTLC profiles of GIPCs from mycelial and yeast forms of *S. schenckii* differed considerably, as shown in Fig. 1. In the mycelial form, five bands were visible with orcinol staining, designated Ss-M1 through Ss-M5 (lane M). Of these, the first three appeared to be major components, with decreasing amounts of Ss-M4 and Ss-M5. Characterization of Ss-M1, -M2, and -M3 has been reported [4]. The appearance of GIPCs from the yeast form was more complex, with five very minor bands, designated Ss-Y1 through Ss-Y5, migrating above a single major band, Ss-Y6, having a very low R<sub>f</sub> (lane Y). Several more closely spaced minor band were visible below this one. Of these yeast form fractions, only Ss-Y1, -Y2, and -Y6 were purified in sufficient quantity and were homogeneous enough for detailed characterization.

### 3.2. Characterization of *S. schenckii* yeast form GIPCs (Ss-Y1, -Y2, and -Y6)

1-D <sup>1</sup>H-NMR spectra of *S. schenckii* yeast form fractions Ss-Y1 and Ss-Y2 (not shown) were both essentially identical to that of Man<sub>3</sub>Man<sub>2</sub>IIPC previously isolated and characterized from *Paracoccidoides brasiliensis* [3]. At least with respect to glycan structure, the identities of Ss-Y1 and -Y2 were therefore considered to be known.

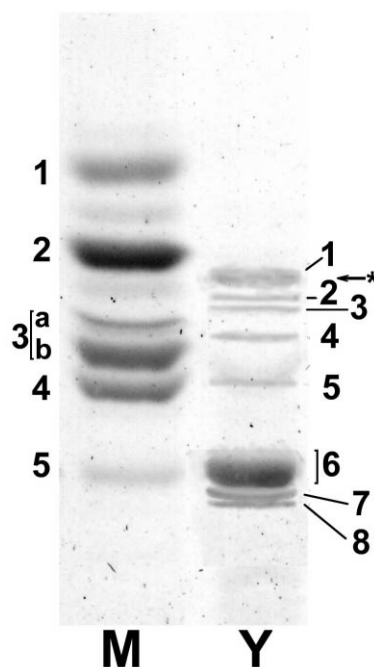


Fig. 1. HPTLC analysis comparing crude *S. schenckii* mycelium and yeast form GIPC fractions (Lanes M and Y, respectively). Top half of plate has been cropped; original image contrast-enhanced using software available with Corel Photopaint 8.0. Orcinol positive components are numbered as shown to left and right of lanes (Ss-M1 through -M5 and Ss-Y1 through -Y8, respectively). The asterisk-arrow (←\*) marks the position of migration of authentic Man<sub>3</sub>Man<sub>2</sub>InsPCer from *P. brasiliensis*.

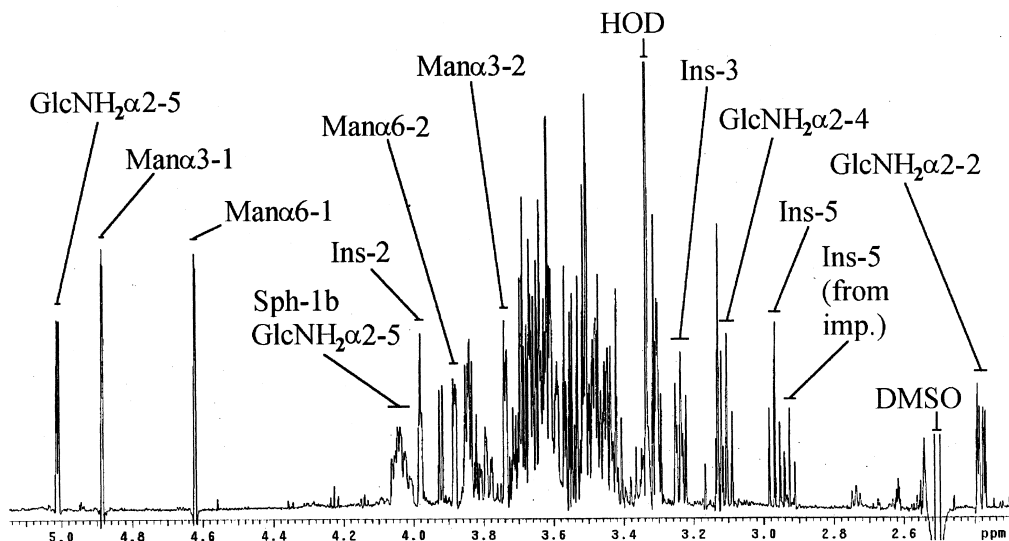


Fig. 2. Downfield sections of 1-D  $^1\text{H}$ -NMR spectra of GIPC fractions Ss-Y6 (A) and Ss-M3 (B). Key reporter signals are labeled.

Component analysis by GC/MS indicated that fraction Ss-Y6 contained mannose, glucosamine (detected as GlcNAc), and *myo*-inositol in ratio 2:1:1; other components detected were t18:0 and t20:0 4-hydroxysphinganine (phytosphingosines) and h24:0 fatty acid (major). The relevant downfield section of a 1-D  $^1\text{H}$ -NMR spectrum of fraction Ss-Y6 is reproduced in Fig. 2; a section of the TOCSY spectrum corresponding to this region is reproduced in Fig. 3A. Chemical shift/connectivity assignments of all  $^1\text{H}$  signals, as well as approximate measurements of  $^3J_{ij}$  coupling constants, in the monosaccharide, inositol, and proximal part of the ceramide were obtained from sequential application of 2-D  $^1\text{H}$ - $^1\text{H}$  gradient-COSY and TOCSY experiments. Complete assignments of  $^1\text{H}$  resonances derived from this analysis are listed in Table 1. The *myo*-inositol residue was recognized as a cyclic spin system in which all  $^3J_{ij}$  are large except for  $^3J_{1,2}$  and  $^3J_{2,3}$ , as H-2 is the only equatorial proton in the 1,2,3,4,5,6-hexahy-

droxy-cyclohexane ring. The three monosaccharide residues were recognized by their connectivity/coupling patterns [28] starting from the most downfield signal (H-1) of each spin system. Aside from the presence of two Man residues recognizable by their signature small values for  $^3J_{1,2}$  and  $^3J_{2,3}$ , the NMR spectrum of Ss-Y6 is characterized in particular as exhibiting a sugar H-2 signal shifted far upfield at 2.380 ppm, part of a spin system, originating from H-1 at 5.011 ppm, having  $^3J_{ij}$  coupling patterns consistent with an  $\alpha$ -glucopyranosyl configuration. The upfield shifted signal is consistent with H-2 attached to a carbon atom bearing a free amino group. The linkage assignments were established by observation in the NOESY spectrum (Fig. 3B) of strong interresidue correlations between H-1 of the GlcNH<sub>2</sub> residue and Ins H-2 (3.982 ppm), between Man H-1 at 4.623 ppm and GlcNH<sub>2</sub> H-6 (3.568 ppm), and between Man H-1 at 4.885 ppm and H-3 of the first Man residue (3.614 ppm). Weak interresidue cor-

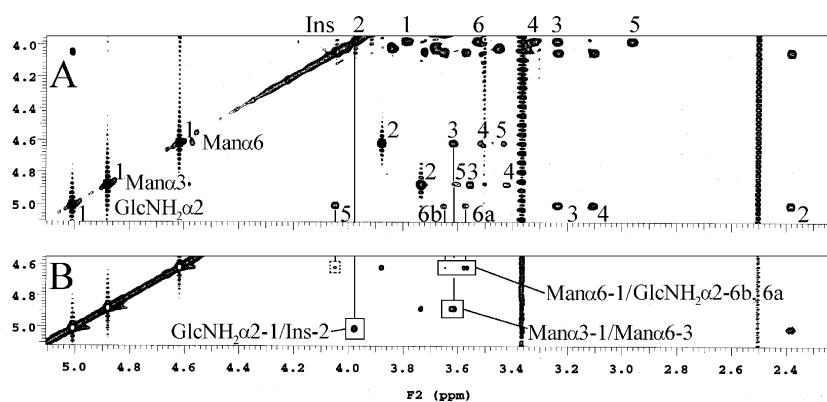


Fig. 3. Sections of 2-D  $^1\text{H}$ -NMR spectra of GIPC fraction Ss-Y6;  $^1\text{H}$ - $^1\text{H}$ -TOCSY (A) and  $^1\text{H}$ - $^1\text{H}$ -NOESY (B). Key through-bond chemical shift correlations for the *myo*-inositol and three monosaccharide residues are labeled in A, key interresidue NOE correlations are boxed and labeled in B.

relations were also observed from Man H-1 to GlcNH<sub>2</sub> H-5 (4.046 ppm) and other H-6 (3.645 ppm). Based on NMR spectroscopic analysis, the structure of Ss-Y6 could be proposed as Man $\alpha$ 3Man $\alpha$ 6GlcNH<sub>2</sub> $\alpha$ 2IPC.

In <sup>+</sup>ESI-MS analysis two major M•Li<sub>2</sub><sup>+</sup> for Ss-Y6 were observed at *m/z* 1423 and 1451, in ~equal abundance (Fig. 4A), consistent with a composition of Hex<sub>2</sub>•HexNH<sub>2</sub>•Ins•P linked to Cers composed of t18:0 and t20:0 phytosphingosines with h24:0 fatty acid. Some minor ceramide variants differing by 14 Th (CH<sub>2</sub>) increments from the major species were also detectable. Product ion mode <sup>+</sup>ESI-MS/CID-MS of the molecular species at both *m/z* 1423 and 1451 (data not shown; designations of fragments are illustrated in Scheme 1) yielded significant fragments at *m/z* 255 [Ins•P•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>1</sub>/B<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), 331 [B<sub>2</sub>•Li]<sup>+</sup>, 416 [HexNH<sub>2</sub>•Ins•P•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>2</sub>/B<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), 434 [HexNH<sub>2</sub>•Ins•P•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>2</sub>/C<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), 492 [B<sub>3</sub>•Li]<sup>+</sup> or [Y<sub>3</sub>/B<sub>4</sub>•Li]<sup>+</sup>, 510 [C<sub>3</sub>•Li]<sup>+</sup> or [Y<sub>3</sub>/C<sub>4</sub>•Li]<sup>+</sup>, 578 [Hex•HexNH<sub>2</sub>•Ins•P•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>3</sub>/B<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), 596 [Hex•HexNH<sub>2</sub>•Ins•P•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>3</sub>/C<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), 654 [B<sub>4</sub>•Li]<sup>+</sup>, 672 [C<sub>4</sub>•Li]<sup>+</sup>, 740 [Hex<sub>2</sub>•HexNH<sub>2</sub>•Ins•P•Li<sub>2</sub>]<sup>+</sup> ([B<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), and 758 [Hex<sub>2</sub>•HexNH<sub>2</sub>•Ins•P•Li<sub>2</sub>]<sup>+</sup> ([C<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>). Ceramide-related ions were observed at *m/z* 690 [Cer•Li]<sup>+</sup> ([Y<sub>0</sub>•Li]<sup>+</sup>) and 776 [CerPO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>0</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>) in the product spectrum from *m/z* 1423. In the product spectrum from *m/z* 1451, these were shifted to *m/z* 718 and 802, respectively.

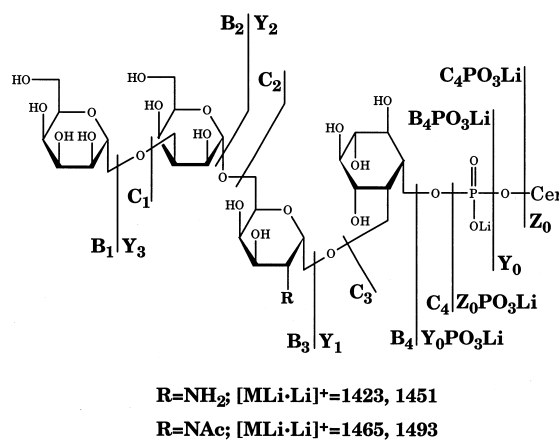
The identity and placement of the HexNH<sub>2</sub> residue in the sequence was confirmed by treating an aliquot of Ss-Y6 with acetic anhydride under conditions known to yield selective *N*-acetylation. Following this treatment, the two major molecular ion species were observed in <sup>+</sup>ESI-MS profile at *m/z* 1465 and 1493, respectively (Fig. 4B), consistent with addition of one acetyl group, i.e. conversion of the HexNH<sub>2</sub> residue to HexNac. Although the high OR <sup>+</sup>ESI-MS spectrum was too noisy to interpret fully, fragments were clearly observable at *m/z* 255 [Ins•P•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>1</sub>/B<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), 690 and 718 [Cer•Li]<sup>+</sup> ([Y<sub>0</sub>•Li]<sup>+</sup>), and 800 [Hex<sub>2</sub>•HexNac•Ins•P•Li<sub>2</sub>]<sup>+</sup> ([C<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>) (data not shown). <sup>+</sup>ESI-MS/CID-MS precursor ion scanning from the *m/z* 800 fragment (Fig. 5A) clearly showed its origins from the major molecular ions at *m/z* 1465 and 1493; minor molecular species at *m/z* 1479, 1507, and 1521 were also detectable (~5–10% the abundance of *m/z* 1493). <sup>+</sup>ESI-MS/CID-MS product ion scanning from the *m/z* 800 fragment (Fig. 5B) yielded abundant fragments characteristic for the glycan sequence, i.e. at *m/z* 111 [H<sub>2</sub>PO<sub>4</sub>•Li<sub>2</sub>]<sup>+</sup>, 169 [B<sub>1</sub>•Li]<sup>+</sup> or [Y<sub>1</sub>/B<sub>4</sub>•Li]<sup>+</sup>, 187 [C<sub>1</sub>•Li]<sup>+</sup> or [Y<sub>1</sub>/C<sub>4</sub>•Li]<sup>+</sup>, 255 [Ins•P•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>1</sub>/B<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), 273 [Ins•P•Li<sub>2</sub>]<sup>+</sup> [Y<sub>1</sub>/C<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>, 331 [B<sub>2</sub>•Li]<sup>+</sup>, 349 [C<sub>2</sub>•Li]<sup>+</sup>, 372 [Y<sub>2</sub>/B<sub>4</sub>•Li]<sup>+</sup>, 390 [Y<sub>2</sub>/C<sub>4</sub>•Li]<sup>+</sup>, 398, 458 [HexNac•Ins•P•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>2</sub>/B<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), 476

Table 1

<sup>1</sup>H chemical shifts (ppm) for monosaccharide, inositol, ceramide sphingoid and fatty-*N*-acyl (in parentheses) residues of Man $\alpha$ 3Man $\alpha$ 6GlcNH<sub>2</sub> $\alpha$ 2IPC (Ss-Y6) from *S. schenckii* in DMSO-*d*<sub>6</sub>/2% D<sub>2</sub>O at 35°C

Ss-Y6	Man $\alpha$ 1	→3Man $\alpha$ 1	→6GlcNH <sub>2</sub> $\alpha$ 1	→2Ins1	←P	→1Cer
H-1	4.885	4.623	5.011	3.795	3.682	4.024
( <sup>3</sup> J <sub>1,2</sub> )	(<2.0)	(1.8)	(3.6)			
H-2	3.738	3.881	2.380	3.980	3.845	(3.838)
H-3	3.545	3.614	3.238	3.241	3.445	
H-4	3.422	3.508	3.105	3.324	3.345	
H-5	3.602	3.432	4.046	2.968		
H-6	3.47	3.47	3.568	3.532		
H-6'	3.63	3.63	3.645			

<sup>3</sup>J<sub>1,2</sub> (Hz) for monosaccharide residues are given in parentheses.



Scheme 1. Molecular ions and fragmentation of Ss-Y6 and *N*-acetylated Ss-Y6 in positive ion mode ESI-MS and -MS/CID-MS.

[HexNac•Ins•P•Li<sub>2</sub>]<sup>+</sup> ([Y<sub>2</sub>/C<sub>4</sub>PO<sub>3</sub>•Li<sub>2</sub>]<sup>+</sup>), 534 [B<sub>3</sub>•Li]<sup>+</sup> or [Y<sub>3</sub>/B<sub>4</sub>•Li]<sup>+</sup>, 552 [C<sub>3</sub>•Li]<sup>+</sup> or [Y<sub>3</sub>/C<sub>4</sub>•Li]<sup>+</sup>, 696 [B<sub>4</sub>•Li]<sup>+</sup>, and 714 [C<sub>4</sub>•Li]<sup>+</sup>. The pairs of fragments 331/349 (Hex<sub>2</sub>), 255/273 (Ins•P), and 458/476 (HexNac•Ins•P) clearly exclude the HexNac residue from any other position in the sequence.

#### 4. Discussion

Analysis of GIPCs in the dimorphic mycopathogen *S. schenckii* (in [4] and the present study) has revealed (i) the expression of a variety of glycan structures, some of which have not been previously reported in fungi; (ii) different patterns of both glycan and ceramide expression in the yeast and mycelial forms of this fungus; and (iii) that, between the two forms, *S. schenckii* is capable of creating at least three different glycosyl linkages with the *myo*-inositol ring of InsPCer. These are Man $\alpha$ 1→2Ins, Man $\alpha$ 1→6Ins, and GlcNH<sub>2</sub> $\alpha$ 1→2Ins; of these, the first has been confirmed to be widely distributed in GIPCs of fungi [2,3,12,29]<sup>3</sup>, but the second has been detected only recently in *S. schenckii* mycelium form GIPCs [4]; the third linkage has not, to our knowledge, been reported in any eukaryotic glycoconjugate or carbohydrate. The isomeric structure, GlcNH<sub>2</sub> $\alpha$ 1→6Ins, is of course well known as the common core linkage found in glycosylinositol phospholipids (GIPLs) of parasites and in the GPI protein membrane anchors widely distributed among eukaryotes [14,30]. The occurrence of non-*N*-acetylated glucosamine in another linkage to *myo*-inositol is particularly interesting, since it is known from studies with mammalian and trypanosomatid cells that two steps are required for formation of GlcNH<sub>2</sub> $\alpha$ 1→6Ins found in GPI anchors, transfer of GlcNac from UDP-GlcNac to Ins of phosphatidylinositol, and subsequent de-*N*-acetylation [30]. That both reactions could occur together in forming an alternate glycosyl linkage is an intriguing possibility.

From the results of this and other studies [2–4,12,29,31], a picture is emerging of GIPC structural diversity which may

<sup>3</sup> Although Barr et al. [2] did not specify precisely the linkage between Man and Ins in their compounds, but characterized it as Man $\alpha$ 1→2/6Ins, NMR spectroscopy of *H. capsulatum* GIPCs shows that the linkage is Man $\alpha$ 1→2Ins1-P-1Cer as reported previously for GIPCs of *P. brasiliensis* [3] and for Ss-Y1 and -Y2 as reported herein.

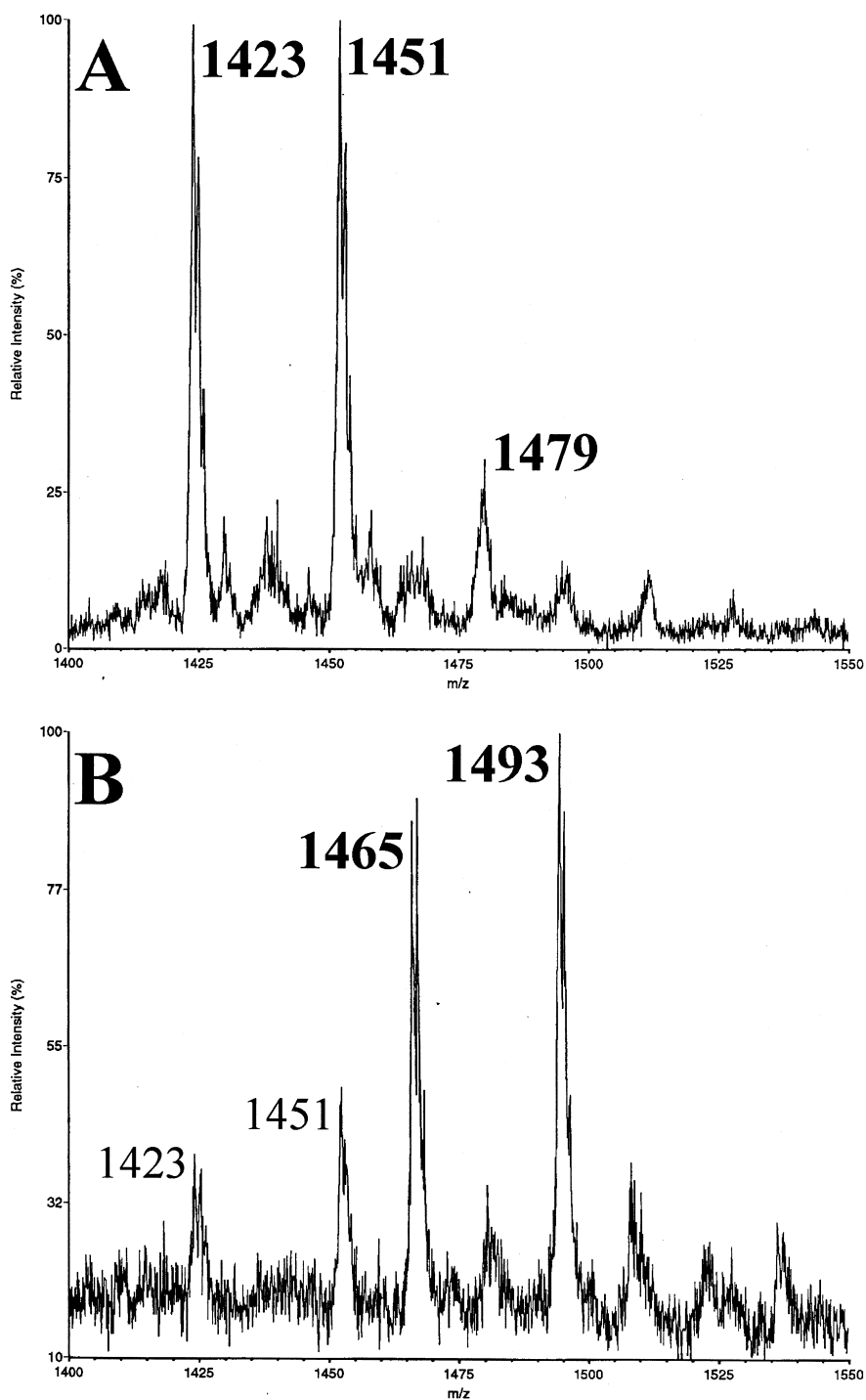


Fig. 4. Singly charged  $\text{Li}^+$  adduct molecular ion profiles of Ss-Y6 (A) and N-acetylated Ss-Y6 (B) in positive ion mode ESI-MS.

ultimately rival that found in animal GSLs. As in the animal kingdom, some structures appear to be shared in common by two or more fungal species, while the overall expression repertoire may be unique for each species. A GIPC having the structure  $\text{Man}\alpha 3(\text{Gal}\beta 4)\text{Man}\alpha 2\text{IPC}$  has been found in both yeast and mycelium forms of *Histoplasma capsulatum* [2,12]<sup>3</sup> and in the mycelium form of *S. schenckii* [4]; and the common intermediate structures  $\text{Man}\alpha 2\text{IPC}$  and  $\text{Man}\alpha 3\text{Man}\alpha 2\text{IPC}$  have been isolated from several species [2,3,12,29]<sup>3</sup>. As with

their mammalian counterparts, patterns of GIPC expression within a particular fungal species can also be expected to depend in a complex manner on a variety of factors, such as strain, culture conditions, and stage of development.

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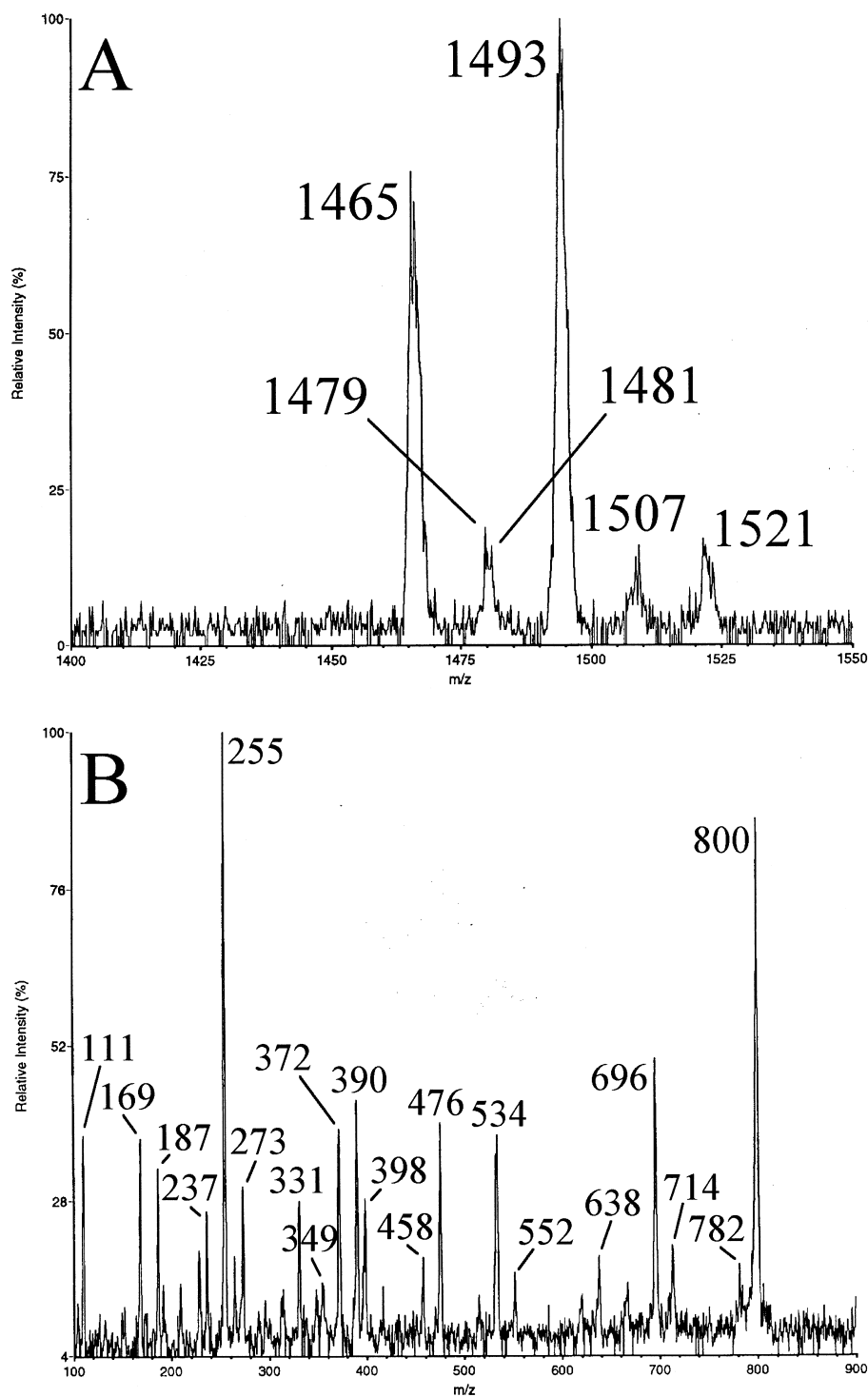


Fig. 5. Positive ion mode ESI-MS/CID-MS of  $m/z$  800 ion in spectrum of *N*-acetylated Ss-Y6. A: Precursor ion scan (molecular ion region). B: Product ion scan.

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