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O-Glycosidically linked oligosaccharides from peptidorhamnomannans of *Sporothrix schenckii*

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β-Elimination of peptidorhamnomannans purified from yeast-like and mycelial phases of *Sporothrix schenckii* released neutral and acidic reduced oligosaccharides that were O linked to serine and/or threonine. Man-(α1-2)Man-ol, Rha(α1-3)Man(α1-2)Man-ol, Rha(α1-4)GlcA(α1-2)Man(α1-2)Man-ol, and Rha(α1-4)[Rha(α1-2)] GlcA(α1-2)Man(α1-2)Man-ol were characterized based on methylation analysis, proton magnetic resonance and fast atom bombardment mass spectrometry.

Keywords: Oligosaccharides, *Sporothrix schenckii*

Abbreviations: FAB, fast atom bombardment; GLC, gas liquid chromatography; GlcA, D-glucopyranosyluronic acid; Man, D-mannopyranose; Man-ol, D-mannitol; MS, mass spectrometry; NMR, nuclear magnetic resonance; Rha, L-rhamnopyranose.

Sporothrix schenckii is a dimorphic pathogenic fungus forming hyphae and conidia at room temperature, and yeast-like cells at 37 °C [1]. Dimorphic fungi are good models for studies of the correlation of the expression of cellular components and morphological differentiation [2–4]. With this purpose, several studies have appeared on the association of cell surface molecules and the cell shape [5], their role in immunogenic aspects [6–8] as well as in fungal penetration of host cells [9].

Previous reports showed that the polysaccharides isolated from *S. schenckii* contain rhamnose and mannose as the major constituents [10–12]. These sugar residues have also been found in several species of *Ceratocystis* [11, 12], *Graphium* [13] and *Europium* [14], and are responsible for the serological crossreactivity among these genera and *S. schenckii* [14]. Structural analysis of rhamnomannans isolated by alkaline extraction from *S. schenckii* cultures, characterized single-unit α-L-rhamnopyranosyl side chains in the yeast-like [11–15] and conidial forms [16], whereas (1-2)-linked di-α-L-rhamnopyranosyl side chains were identified in the mycelial phase [11, 15]. Both side chains are attached to positions 3 on a (1-6)-linked α-D-mannan, and are the main antigenic determinants in such molecules [17]. However, it became clear that some reactivities of the peptidorhamnomannans with antibodies [17] and lectins [18] could involve carbohydrate structures other than the N linked polysaccharide chains in the glycoconjugate. Therefore we started looking for O linked oligosaccharide chains in these molecules.

In the present paper, we report experiments on the β-elimination of purified peptidorhamnomannans isolated from different cell types of *S. schenckii*, and the characterization of the resulting O linked oligosaccharides.

Materials and methods

Microorganism and growth conditions

S. schenckii strain 1099-18 was isolated from a human case of sporotrichosis and was originally obtained from the Mycology Section of the Department of Dermatology, Columbia University, New York, USA. Yeast forms of *S. schenckii* were obtained at 37 °C in brain heart infusion (BHI). Cultures in the mycelial phase were grown at 25 °C in Sabouraud (2% glucose, 0.5% yeast extract, 1% peptone) medium. Fresh cultures in solid Sabouraud were used as inocula. Transfers were initially made to flasks containing 200 ml BHI or Sabouraud media and incubation carried out with shaking for 3–4 days. Once a stable morphological phase was established the entire culture volume was used as inoculum for 3 l Erlenmeyer flasks containing 1 l of the same medium.

Extraction and β-elimination of peptidorhamnomannans

Peptidorhamnomannans were isolated from the early stationary phase of *S. schenckii* cells by citrate buffer extraction, and purified by Cetavlon fractionation [10]. β-Elimination was carried out by dissolving 50 mg peptidorhamnomannan in 100 ml 0.1 N NaOH containing 0.3 M $NaBH_4$ [19]. The mixture was left at room temperature for 24 h and the reaction was stopped by neutralization with 1 N acetic acid. The mixture was then concentrated in a rotatory evaporator at 40 °C. Boric acid was removed by repeated additions of methanol, and evaporation to dryness. The residue was taken up in 0.1 M acetic acid and applied to a Bio-Gel P-2 column (−400 mesh) (0.5 cm × 145 cm), which was eluted with 1–1.5 bed volumes of 0.1 M acetic acid.

For aminoacid analysis, the peptidorhamnomannans before and after (in the void volume of the Bio-Gel P-2 column) β-elimination, were hydrolysed with 5.6 N HCl for 24 h at 110 °C. Amino acids were analysed in an autoanalyser (type 119 CL; Beckman Instruments) by the method of Fauconnet and Rochemont [20].

Analytical procedures

Total carbohydrate was determined by the phenol–sulfuric acid method [21]. For quantitative determination of carbohydrate, the oligosaccharide alditol (about 100 μg) was methanolysed in 1 ml 0.5 M HCl in methanol (with 20 μg inositol as internal standard) for 18 h at 80 °C. After evaporation to dryness with a stream of N_2, the samples were trimethylsilylated with 100 μl of bis(trimethylsilyl)trifluoracetamide–pyridine (1:1 by vol) for 4 h at room temperature [22], and the products analysed by gas liquid chromatography (GLC) in a capillary column of OV-101 (25 m × 0.2 mm inner), with helium as the carrier gas at 0.05 bar. A column temperature program was set at 2 °C min^{-1} from 120 °C to 240 °C.

Methylation analysis

The oligosaccharide alditol was methylated with methyl iodide in the presence of methylsufinyl anion [23]. The permethylated oligosaccharide was recovered by chloroform extraction from the reaction mixture, to which a saturated aqueous solution of sodium thiosulfate was added. The chloroform extract was washed 10 times with equal volumes of water and evaporated under a stream of N_2. The methylated products, reduced or not with lithium borohydride, were methanolysed with 0.5 M HCl in methanol for 18 h at 80 °C. The methanolysates were dried under nitrogen, and the residues treated with acetic anhydride–pyridine (50 μl, 9:1 by vol) for 24 h at 18 °C. The resulting mixture of acetylated, partly *O*-methylated methyl glycosides were examined by GLC on the OV-101 capillary column (25 m × 0.2 mm inner) at 120 °C to 180 °C (2 °C min^{-1}, then hold). The resulting peaks were identified by their typical retention times and GLC/MS, as described by Fournet *et al.* [24], and quantified by their areas.

FAB-MS analysis

FAB-MS of oligosaccharide alditols was recorded with an MS-50 mass spectrometer (Kratos, Manchester, UK). FAB spectra were obtained with glycerol as the matrix and a xenon fast atom source operated at 1.2 mA and 8 kV. The spectra were recorded in negative-ion mode at 7 kV acceleration voltage in a mass-controlled linear scan at a resolution of 300 ppm.

^{1}H-NMR spectroscopy

^{1}H-NMR spectra were obtained at 400 MHz with a Bruker AM-400 WB spectrometer (Bruker, Karlsruhe, Germany), equipped with Aspect 3000 computers operated in the Fourier transform mode. Chemical shifts (δ) at 300 K are expressed relative to sodium 3-[2,2,3,3-$^2H_4$]trimethylsilylpropionate (TSP) as the internal standard. Samples were prepared by passing the aqueous solution through a 1 ml column of Chelex 100 (Bio-Rad) to remove metal ions, exchanging five times with ^2H_2O by lyophilization, and dissolving the product in 99.96% ^2H_2O (Aldrich). The carbohydrate concentration ranged from 4 to 6 mM. Sample tubes of 5 mm were used.

Results

β-Elimination and fractionation of oligosaccharides

Products of β-elimination of the peptidorhamnomannan were separated by gel filtration chromatography to give the patterns shown in Fig. 1(a, b). The carbohydrate-containing peaks, from right to left, are in the di-, tri-, penta- and hexasaccharide regions, according to the elution of a series of (1-4)-linked α-D-glucopyranosyl oligosaccharides used as standards. To confirm that these oligosaccharides were *O*-glycosidically linked to serine and/or threonine, the amino acid composition of peptidorhamnomannans was determined before and after β-elimination. Table 1 shows that in the yeast-like forms, 63% of the serine was destroyed during the reaction, 83% of which was converted to alanine by reduction. The threonine content decreased 50%, and 53% of it was recovered as α-aminobutyric acid. In the β-eliminated peptidorhamnomannan isolated from the mycelial phase, 50% of the serine was destroyed and 69% of it was converted to alanine by reduction. The threonine content decreased 53%, and 33% of it was recovered as α-aminobutyric acid. Thus, in the peptidorhamnomannans isolated from *S. schenckii*, 27% of the amino acid residues were serine and threonine. About 55% of them were substituted by O linked oligosaccharide chains.

Sugar composition and optical rotation of the oligosaccharides

Component sugars of the oligosaccharide fractions were determined after methanolysis as their *O*-trimethylsilyl

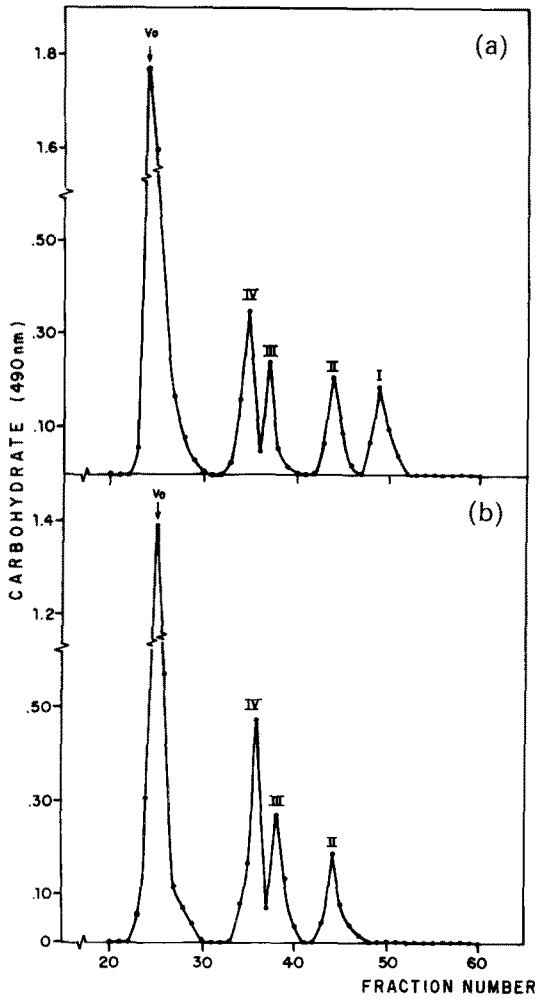

Figure 1. Fractionation on a Bio-Gel P-2 column (0.5 cm × 145 cm) of the oligosaccharide alditols released after β-elimination of *Sporothrix schenckii* peptidorhamnomannans from (a) mycelial, and (b) yeast-like forms. The column was equilibrated and eluted with 0.1 N acetic acid (0.5 ml fractions).

ethers by GLC. The monosaccharide constituents of the oligosaccharides, released from peptidopolysaccharides of yeast-like forms were identical to those from the mycelial phase. Thus, oligosaccharide II (Fig. 1) contained rhamnose, mannose and mannitol (molar ratio 1:1:1), where oligosaccharides III and IV had rhamnose, glucuronic acid, mannose and mannitol in molar ratios of 1:0.2:0.3:1 and 2:0.2:0.3:1, respectively. The low proportion of mannose in oligosaccharides III and IV could be due to incomplete hydrolysis because of the presence of glucuronic acid, which forms a more stable glycosidic bond.

The molecular ions $(M-H)^-$ obtained by FAB-MS, demonstrated that the oligosaccharides II (*m/z* 489), III (*m/z* 665), and IV (*m/z* 811) were the reduced tri-, tetra- and pentasaccharides containing rhamnose, mannose, mannitol; rhamnose, glucuronic acid, mannose, mannitol; and $(\text{rhamnose})_2$, glucuronic acid, mannose, mannitol, respectively. FAB-MS analysis of oligosaccharide alditol I (*m/z* 343), that

Table 1. Amino acid composition of *S. schenckii* peptidorhamnomannans before and after β-elimination.

Amino acid	*Percentage*[a]			
	Yeast		*Mycelium*	
	Before	*After*	*Before*	*After*
Aspartic acid	8.67	11.76	8.24	11.27
Threonine	10.77	5.34	14.30	6.65
Serine	16.59	6.13	13.31	6.61
Glutamic acid	8.47	10.67	8.64	10.30
Proline	5.31	5.47	6.07	6.00
Glycine	8.84	8.45	9.30	11.51
Alanine	13.04	21.77	12.31	16.94
α-Aminobutyric acid	0.0	2.90	0.0	2.57
Valine	5.82	6.00	5.61	6.21
Methionine	0.62	0.56	0.49	0.74
Isoleucine	3.21	3.46	3.01	3.11
Leucine	4.49	4.63	4.18	4.30
Tyrosine	2.17	2.08	2.17	2.55
Phenylalanine	2.51	2.24	2.48	2.29
Histidine	1.63	1.63	1.70	1.91
Lysine	4.36	4.08	5.30	1.61
Arginine	3.48	2.84	2.85	2.41

[a] Residues per 100 residues.

Table 2. Optical rotations of the purified O linked oligosaccharides.

Oligosaccharide	$[\alpha]_D^{20}$
Pentasaccharide	+ 4°
Tetrasaccharide	+20°
Trisaccharide PAH[a]	+61°
Trisaccharide	− 8°
Disaccharide	+43°

[a] Derived from partial acid hydrolysis of the pentasaccharide.

was present only in the mycelium form, showed that it was a reduced disaccharide composed of mannose and mannitol.

The specific optical rotations of the O linked oligosaccharides from *S. schenckii* (Table 2), when compared with the values reported for 2-*O*-α-D-mannopyranosylmannitol [25], and 2-*O*-β-D-glucopyranosyluronic acid-α-L-rhamnose [26], indicated that these oligosaccharides contained only α linkages.

Methylation analysis

Methylated oligosaccharides were methanolysed, and the products converted to *O*-acetylated, partly *O*-methylated methyl glycosides, which were characterized by GLC and MS. The methylation products of oligosaccharides I and II, shown in Fig. 2(a, b), were 2,3,4,6-tetra-*O*-methylmannose; and 1,3,4,5,6-penta-*O*-methylmannitol for the disaccharide

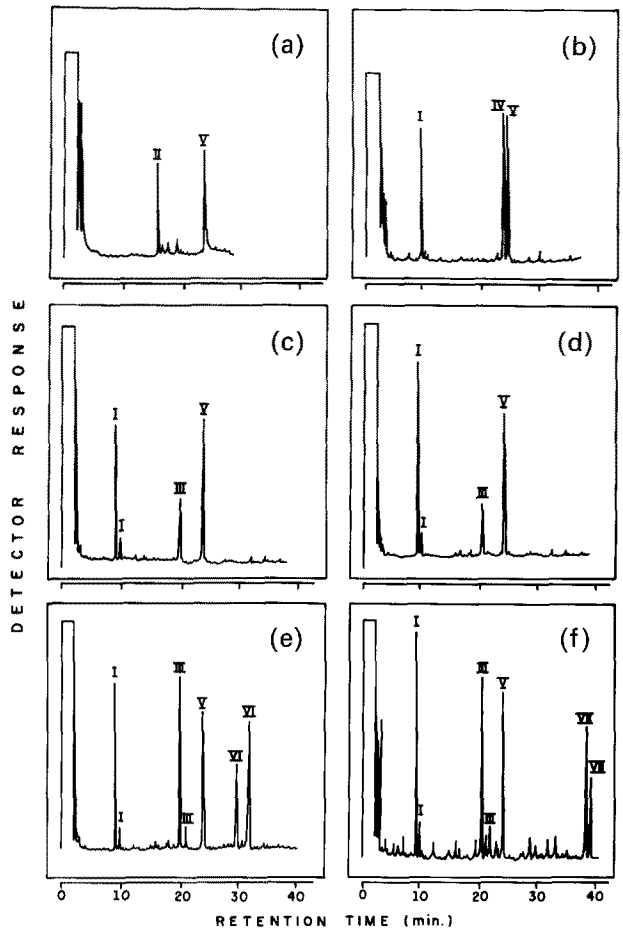

Figure 2. GLC of *O*-acetylated partially *O*-methylated methyl aldosides of (a) oligosaccharide alditol I, (b) oligosaccharide alditol II, (c) oligosaccharide alditol III, (d) oligosaccharide alditol IV, (e) reduced oligosaccharide alditol III, (f) reduced oligosaccharide alditol IV. Derivatives were designated as follows: I, methyl 2,3,4-tri-*O*-methylshamnoside; II, methyl 2,3,4,6-tetra-*O*-methylmannoside; III, methyl 3,4,6-tri-*O*-methyl 2-acetyl mannoside; IV, methyl 2,4,6-tri-*O*-methyl-3-acetyl mannoside; V, 1,3,4,5,6-penta-*O*-methyl-2-acetyl mannitol; VI, methyl 2,3-di-*O*-methyl-4,6-*O*-acetyl glucoside; VII, methyl-3-*O*-methyl-2,4,6-*O*-acetyl glucoside.

and 2,3,4-tri-*O*-methylrhamnose; 2,4,6-tri-*O*-methylmannose; and 1,3,4,5,6-penta-*O*-methylmannitol for the trisaccharide, indicating that both oligosaccharides contained a terminal mannitol residue substituted at position 2 by mannose.

In addition to 1,3,4,5,6-penta-*O*-methylmannitol (1.0 mol) from mannose at the reducing end, the oligosaccharides III and IV gave rise to 2,3,4-tri-*O*-methylrhamnose and 3,4,6-tri-*O*-methylmannose derivatives in a molar ratio of 1:0.54 and 2:0.5, respectively (Fig. 2c, d). Since both oligosaccharides have one glucuronic acid unit, the low proportion of trimethylmannose suggests that this mannosyl residue, due to its linkage to glucuronic acid, is partially released upon acid methanolysis. This was confirmed as follows. Partial acid hydrolysis of reduced tetra- and

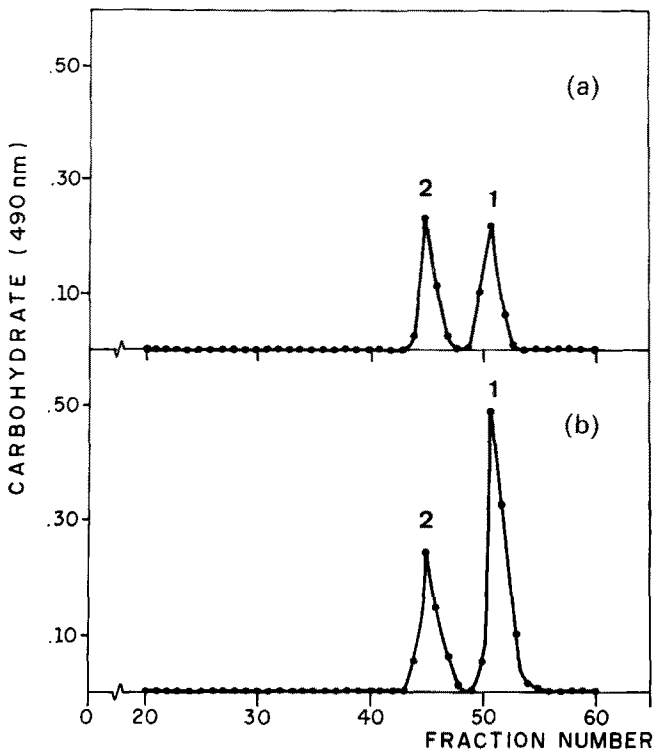

Figure 3. Gel filtration on a Bio-Gel P-2 column (0.5 cm × 145 cm) of the products of partial acid hydrolysis of (a) oligosaccharide alditol III, and (b) oligosaccharide alditol IV.

pentasaccharide with trifluoracetic acid pH 1.1 for 5 h at 100°C, followed by gel filtration (Fig. 3a, b), resulted in the liberation of two fractions (1 and 2). Sugar analysis showed that fraction 1 contained only rhamnose, and fraction 2 contained glucuronic acid, mannose and mannitol. FAB-MS analysis confirmed that fraction 2 was a reduced trisaccharide composed of glucuronic acid, mannose and mannitol (*m/z* 519). Methylation analysis of this fraction gave trimethylmannose (0.5 mol) and pentamethylmannitol (1.0 mol). These are expected results for a disaccharide alditol with glucuronic acid as the nonreducing end unit. Thus, the original tetra- and pentasaccharides contain a glucuronic acid unit linked to the mannosyl residue adjacent to mannitol.

In order to determine in which position(s) the rhamnose residue(s) is/are attached to glucuronic acid, the methylated oligosaccharide alditols III and IV were reduced with lithium borohydride, and then subjected to acid methanolysis. The products, converted to *O*-acetylated, partly *O*-methylated methyl glycosides were analysed by GLC/MS. The following derivatives: 2,3,4-tri-*O*-methylrhamnose (1.0 mol); 3,4,6-tri-*O*-methylmannose (1.0 mol); 1,3,4,5,6-penta-*O*-methylmannitol (1.0 mol), and 2,3-di-*O*-methylglucose (1.0 mol) was obtained from the tetrasaccharide (Fig. 2e) whereas the pentasaccharide gave rise to 2,3,4-tri-*O*-methylrhamnose (2.0 mol); 3,4,6-tri-*O*-methylmannose (1.0 mol); 1,3,4,5,6-penta-*O*-methylmannitol (1.0 mol), and 3-*O*-methylglucose

(1.0 mol) (Fig. 2f). The formation of 2,3-di-*O*-methylglucose and 3-*O*-methylglucose revealed that the glucuronic acid unit was substituted either in position 4 or 2,4 by rhamnose, in the tetra- or pentasaccharide, respectively.

Proton magnetic resonance of oligosaccharides

^{1}H-NMR data for the oligosaccharide alditols are in agreement with the structures suggested by methylation analysis. The ^{1}H-NMR spectrum of oligosaccharide I is identical to that of 2-*O*-α-D-mannopyranosylmannitol, with a single H-1 signal at δ 4.990 ($J_{1,2}$ 1.5 Hz) (Fig. 4a). The ^{1}H-NMR spectrum for oligosaccharide II (Fig. 4b) corresponds to a disaccharide alditol, and differs from structure I mainly by the new signal at δ 5.036 ($J_{1,2}$ 1.5 Hz), which is characteristic of the nonreducing end unit of α-L-rhamnopyranose linked to position 3 of α-D-mannopyranose. Substitution at position 3 of mannopyranose slightly affects its anomeric proton chemical shift: the H-1 signal of the mannopyranosyl unit at δ 4.950 ($J_{1,2}$ 1.5 Hz) in this oligosaccharide is shifted upfield by only 0.04 ppm from that of H-1 of oligosaccharide I.

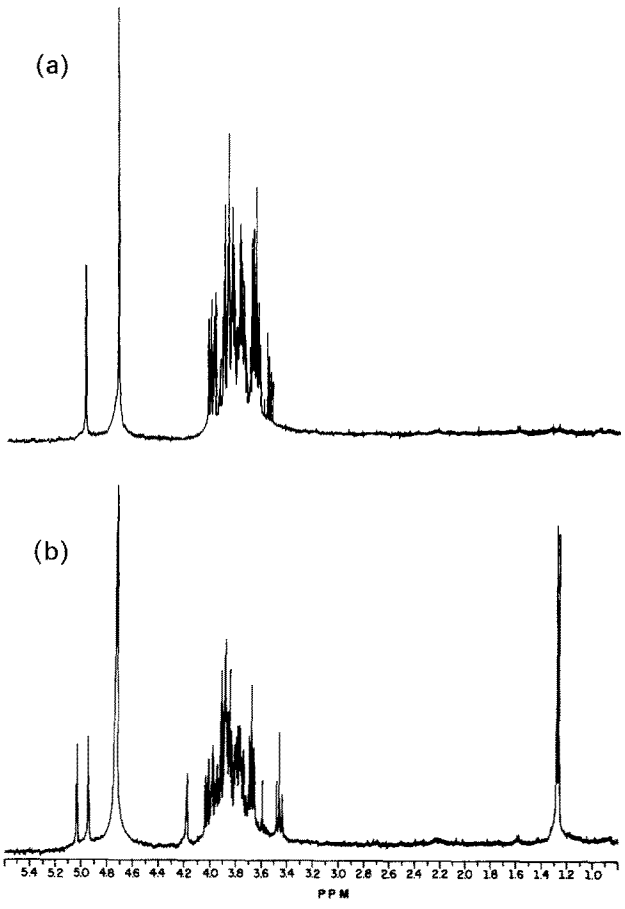

Figure 4. 400 MHz ^{1}H-NMR spectra of (a) oligosaccharide alditol I, and (b) oligosaccharide alditol II.

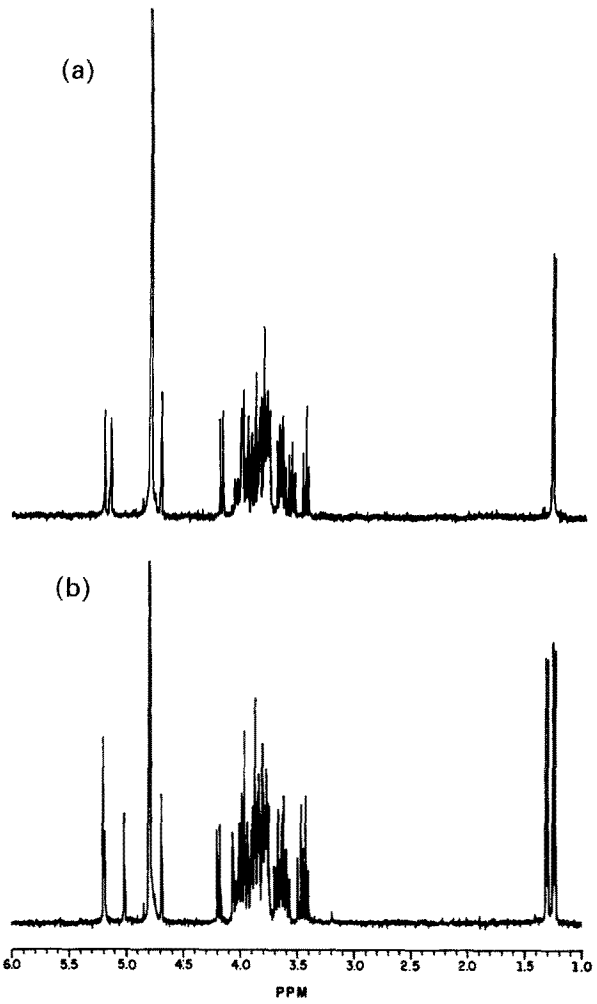

Figure 5. 400 MHz ^{1}H-NMR spectra of (a) oligosaccharide alditol III, and (b) oligosaccharide alditol IV.

As expected from the chemical analysis, the ^{1}H-NMR spectrum of oligosaccharide III (Fig. 5a) showed three anomeric proton signals, at δ 4.697 ($J_{1,2}$ 1.7 Hz), 5.134 ($J_{1,2}$ 4 Hz) and 5.194 ($J_{1,2}$ 1.4 Hz), which were interpreted as follows. From the magnitude of the coupling constant values, it is clear that the signal at δ 5.134 arises from α-D-glucopyranosyluronic acid. The signal at δ 5.194 should arise from an α-D-mannopyranosyl residue linked to position 2 of mannitol, the anomeric hydrogen of which has been deshielded by substitution at position 2 by glucuronic acid. The signal at δ 4.697 therefore arises from the nonreducing end unit of α-L-rhamnopyranose linked to position 4 of α-D-glucopyranosyluronic acid. All three assignments are supported by the methylation analysis (see Fig. 2).

The ^{1}H-NMR spectrum of oligosaccharide IV (Fig. 5b) showed the presence of four anomeric proton signals, at δ 4.691 ($J_{1,2}$ 1.8 Hz), 5.024 ($J_{1,2}$ 1.5 Hz), 5.185, and 5.198. The main characteristics of this spectrum, in comparison with those of oligosaccharide III, are the downfield shift of

Table 3. Structures of reduced oligosaccharides *O*-glycosidically linked in *S. schenckii* peptidorhamnomannans.

Oligosaccharide alditol		*Structure*
I	disaccharide[a]	Man(α1-2)Man-ol
II	trisaccharide[b]	Rha(α1-3)Man(α1-2)Man-ol
III	tetrasaccharide[b]	Rha(α1-4)GlcA(α1-2)Man(α1-2)Man-ol
IV	pentasaccharide[b]	Rha(α1-4)[Rha(α1-2)]GlcA(α1-2) Man(α1-2)Man-ol

[a] Present only in the mycelial component.
[b] Present in both mycelial and yeast phases.

the H-1 of α-D-glucopyranosyluronic acid unit (δ 5.185), due to attachment of an additional α-L-rhamnopyranosyl residue at position 2, and the presence of a new signal at δ 5.024, arising from H-1 of this nonreducing end unit.

A summary of the structures of the *O*-glycosidically linked oligosaccharides from peptidorhamnomannans of *S. schenckii* is given in Table 3.

Discussion

Our results show that mild alkaline borohydride treatment of peptidorhamnomannans from the yeast and mycelial phases of *S. schenckii*, for β-elimination of the substituted serine and threonine residues, released *O* linked neutral and acidic oligosaccharides. The β-elimination carbohydrate products were fractionated by gel filtration, and consisted of reduced di-, tri-, tetra- and pentasaccharides (structures I, II, III and IV).

On the basis of methylation analysis and proton NMR data, oligosaccharide I found only in the mycelial phase, was characterized as Man(α1-2)Man-ol. Oligosaccharide II is the trisaccharide Rha(α1-3)Man(α1-2)Man-ol, which has been identified in the O linked structures of both morphological phases. Lloyd and Travassos [17] determined that the major antigenic epitope in the rhamnomannans of yeast forms was the disaccharide Rha(α1-3)Man. Therefore, antibodies recognizing this structure could bind to both the N linked high molecular weight polysaccharides and the O linked trisaccharides in the peptidorhamnomannan molecules.

Two acidic oligosaccharides were β-eliminated from peptidorhamnomannans of yeast-like and mycelium forms. Acidic oligosaccharide III is a tetrasaccharide with several unusual features. It contains an α-glucopyranosyluronic acid unit attached to mannose which is 4-*O*-substituted by a terminal rhamnopyranosyl residue. A similar structure has been previously characterized in *C. stenoceras* acidic rhamnomannans [26]. Its presence in *S. schenckii* has been suggested by ^{13}C-NMR spectroscopy of rhamnomannans that could be fractionated into neutral and acidic types [27]. However, an important variation in the anomeric configuration of the glucuronic acid unit was observed in the O linked oligosaccharide chains. Substitution of α-glucuronic acid for β-glucuronic acid may be an element in the creation of another antigenic epitope to be recognized by antibodies from sera of patients with sporotrichosis or immunized animals. Also, in the acidic side chains of *C. stenoceras* rhamnomannen, the glucuronic acid units are wedged between two rhamnose units, unlike the present structure in which the glucuronic acid is linked to mannose. It is possible that such a structure is also present in acidic rhamnomannans from *S. schenckii* but this has not been demonstrated so far.

Acidic oligosaccharide IV differs from structure III in that it contains a glucuronic acid residue which is 2,4-disubstituted by rhamnose units. To our knowledge, a disubstituted glucuronic acid unit with two terminal residues of rhamnose has never been described before.

The fact that identical fragments were obtained by β-elimination of peptidopolysaccharides of both yeast and mycelial phases, suggests that the same glycosyltransferases are involved in the biosynthesis of these O linked oligosaccharides in both forms. However, the fact that O linked mannobiose chain is found only in hyphae suggests that either the next glycosylation step is much more effective in the yeast forms, or that glycosyltransferases of different specificity may occur, reflecting typical events related to morphological differentiation or to the temperature shift in the fungal cultures (yeast forms were grown at 37 °C and the mycelial phase was obtained at 25 °C). In a previous study, we have shown that some structural features of *S. schenckii* rhamnomannans were temperature-dependent while others were associated with cell differentiation [15].

A common biosynthetic pathway is suggested for the O linked oligosaccharide chains of both yeast and mycelial peptidorhamnomannans, because the intermediate carbohydrate structures are actually present in the glycoconjugates. Possible biosynthetic sequences are:

A. $Man_2 \rightarrow Rha(1\text{-}3)Man_2$

B. $Man_2 \rightarrow GlcA(1\text{-}2)Man_2 \rightarrow Rha(1\text{-}4)GlcA\text{-}Man_2 \rightarrow$
$\rightarrow Rha(1\text{-}4)GlcA\text{-}Man_2$
2
|
1
Rha

The glycosylation step of pathway A parallels that for the yeast rhamnomannan biosynthesis, whereas the one in pathway B, except for the mannobiose core formation, is unique and typical of the O linked chains of *S. schenckii* glycoconjugates.

The presence of 2-*O*-substituted α-D-mannopyranose units in the O linked chains is compatible with the reactivity of *S. Schenckii* peptidorhamnomannans with concanavalin

A, which has been suggested before [18]. Other biological differences between peptidorhamnomannans and rhamnomannans, such as activation of complement components [28], might also involve O linked chains, but this requires other investigation.

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