Carbohydrate Structures of Three Novel Phosphoinositol-Containing Sphingolipids from the Yeast *Histoplasma capsulatum*†

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ABSTRACT: From the yeast phase of the human pathogen *Histoplasma capsulatum*, three novel glycolipids were isolated, shown to react with sera from histoplasmosis patients, and partially characterized: compound V, ceramide–P–inositol–[mannose$_2$]; compound VI, ceramide–P–inositol–[mannose$_2$, galactose]; compound VIII, an isomer of compound VI [Barr, K., & Lester, R. L. (1984) *Biochemistry* (preceding paper in this issue)]. Ammonolysis of these lipids has yielded all the carbohydrate (oligosaccharides V, VI, and VIII) as novel, intact oligosaccharides suitable for characterization. Anomeric configurations were determined by specific glycosidase digestion and by the stability of peracetylated saccharides to CrO_3 oxidation. Linkages were established by methylation analysis. These experiments yielded the following structural assignments:

$man_p(\alpha 1 \rightarrow 3)man_p(\alpha 1 \rightarrow$ 2 or 6)myoinositol

oligosaccharide V

$man_p(\alpha 1 \rightarrow 3)$, $gal_f(\alpha 1 \rightarrow 6)$ $\rightarrow man_p(\alpha 1 \rightarrow$ 2 or 6)myoinositol

oligosaccharide VI

$man_p(\alpha 1 \rightarrow 3)$, $gal_p(\beta 1 \rightarrow 4)$ $\rightarrow man_p(\alpha 1 \rightarrow$ 2 or 6)myoinositol

oligosaccharide VIII

The occurrence of galactofuranose is novel for glycosphingolipids, and it is noteworthy that compound VI is immunoreactive.

Five inositolphosphosphingolipids have been purified from the yeast phase of the human pathogen *Histoplasma capsulatum* (Barr & Lester, 1984). Two of these, designated as compounds II and III, were inositolphosphoceramides, similar to those isolated from *Saccharomyces cerevisiae* (Smith & Lester, 1974), and their composition was established. Compounds V, VI, and VIII were of novel composition, also possessing an identical inositolphosphoceramide core but differing by the glycosyl substitution on the polar head groups: compound V, ceramide–P–inositol–[mannose$_2$]; compound VI, ceramide–P–inositol–[mannose$_2$, galactose]; compound VIII, ceramide–P–inositol–[mannose$_2$, galactose].

Compound VIII, isomeric to compound VI in an unknown manner, was found in both the mycelial and yeast phases, whereas compounds V and VI were unique to the yeast phase (Barr & Lester, 1984). Due to the novel nature of these phosphosphingolipids and the observation that compounds V, VI, and VIII react with sera from patients with histoplasmosis (Barr & Lester, 1984), studies of sequence, linkage, and anomeric configuration were initiated on these lipids and are now reported.

Experimental Procedures

Materials. The solvents used for permethylations, which included chloroform, methanol, toluene, pyridine, absolute ethanol, and acetic anhydride were redistilled before use. NaB^2H_4 was purchased from Stohler Isotope Chemicals, dimethyl sulfoxide was from Burdick and Jackson Laboratories, and the methylsulfinyl carbanion was prepared according to an established procedure of Corey & Chaykovsky (1962). HPLC-grade cyclohexane, lactose, and raffinose were purchased from Fisher Scientific Co., Louisville, KY. Triphenylmethane, Sephadex LH-20, and stachyose were purchased from Sigma Chemical Co., St. Louis, MO. *Cannavalia ensiformis* α-mannosidase was obtained from Boehringer-Mannheim, Indianapolis, IN. Thin-layer chromatography was done on Whatman LK 5 silica gel plates (Fisher Scientific Co., Louisville, KY). AG 1-X2 and AG 50W-X8 were obtained from Bio-Rad Laboratories, Richmond, CA.

Permethylation of Oligosaccharides of Compounds V, VI, and VIII. The oligosaccharides of compounds V, VI, and VIII were isolated from the intact lipids after ammonolysis (Ballou et al., 1963) as described by Barr & Lester (1984) and permethylated according to Hakomori (1964) in the following manner. The oligosaccharides were dried under nitrogen, dissolved in 0.5 mL of dimethyl sulfoxide, and sonicated for 3 h. A 0.25-mL aliquot of freshly prepared 2 M methylsulfinyl carbanion in dimethyl sulfoxide (Corey & Chaykovsky, 1962) was added to the samples, followed by sonication for 4 h. Before the addition of 2 mL of methyl iodide, carbanion excess was checked by reaction of the mixture with triphenylmethane (Rauvala, 1979). Following a 1-h sonication, the excess methyl iodide was evaporated under a stream of nitrogen before another addition of 0.25 mL of carbanion. After 4 h of sonication, 1.0 mL of methyl iodide was added, and the samples were sonicated for 1 h. The samples were applied to a 1 cm × 35 cm Sephadex LH-20 column in acetone. Fractions of 1 mL were collected; 10 μL of each fraction was spotted on silica gel plates and sprayed with orcinol–H_2SO_4 (Skipski & Barclay, 1969). The carbohydrate-containing fractions were pooled and dried under N_2.

The permethylated oligosaccharides were further purified by liquid chromatography on a 0.45 × 30 cm Lichrosorb Si60 (5 μM) column after dissolving the samples in cyclohexane/ethanol (1:1). The permethylated compounds were eluted with a linear gradient, which was provided by a two-pump solvent delivery system (Model 6000A, Waters Associates, Milford, MA) equipped with a Model 600 programmer. The solvent composition changed from 0 to 100% solvent B

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in 20 min. Solvent A was cyclohexane, and solvent B was cyclohexane/ethanol (70:30). The flow rate was 2.0 mL/min, and fractions were collected every 0.5 min. Elution of nonvolatile carbon was monitored with a Pye Unicam moving-wire detector. Fractions were pooled on the basis of the detector profile and gave single spots after silica gel thin-layer chromatography on plates developed with cyclohexane/ethanol (70:30), and sugar detection was by orcinol–H_2SO_4.

The permethylated oligosaccharides of compounds V, VI, and VIII were chromatographed on a 38 cm × 2 mm glass column packed with OV 101 Ultrabond. The column temperature was maintained at 250 °C for 1 min and then increased from 250 to 315 °C at the rate of 10 °C/min. Helium was the carrier gas at a flow rate of 30 mL/min, and ammonia was the reagent gas, supplying 0.5-Torr pressure inside the ion source.

α-Mannosidase Treatment of Oligosaccharide V. Oligosaccharide V (150 nmol) was incubated with 0.6 unit of *Canavalia ensiformis* α-mannosidase in a final volume of 60 μL of 0.05 M sodium citrate buffer, pH 4.5, at 37 °C for 24 h (Li & Li, 1972). The sample was desalted by sequential passage over AG 1X-2 (bicarbonate form, 200–400 mesh,) and AG 50W-X8 (H^+ form, 50–100 mesh). The eluate was dried under N_2 and redissolved in 0.1 mL of H_2O. A 5-μL aliquot was spotted on a silica gel plate, which was developed with CH_3CN/H_2O (2:1) and sprayed with orcinol–H_2SO_4. As a control, oligosaccharide V was incubated with buffer alone under identical conditions. The enzyme preparation, tested for β-mannosidase activity with α and β isomers of *p*-nitrophenyl D-mannoside as substrates, showed β-mannosidase ≤0.7% of the α-mannosidase activity.

The mannosidase digest was assayed for monosaccharides by gas chromatography as follows. The sample was dried and acetylated for 2 h at 100 °C in 1 mL of acetic anhydride/pyridine (1:1). Mannose (200 nmol) and inositol (100 nmol) were acetylated as reference standards. Acetic anhydride was removed by drying in vacuo several times as a toluene azeotrope. The residue was dissolved in 2 mL of $CHCl_3$, and 2 mL of H_2O was added. After the $CHCl_3$ phase was removed, the H_2O layer was extracted with 2 mL of $CHCl_3$. The combined $CHCl_3$ phases were washed with H_2O and dried. The acetylated sugars were dissolved in 50 μL of acetone for analysis by gas–liquid chromatography on a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionization detector, in a 6 ft × 2 mm glass column packed with 3% OV 275 on 100/120 Chromosorb WAW at 210 °C.

Determination of Anomeric Configuration by Chromium Trioxide Oxidation. The oligosaccharides from compounds V, VI, and VIII, lactose, and raffinose were acetylated in 2.0 mL of acetic anhydride/pyridine (1:1) overnight in a 37 °C sonic bath followed by 2 h at 100 °C. CrO_3 oxidation was carried out as previously described (Hoffman et al., 1972; Laine & Renkonen, 1975) under the following conditions. The dried acetylated oligosaccharides were dissolved in glacial acetic acid and divided into 0.5-mL aliquots. After the samples were placed in a 40 °C sonic bath, 50 mg of CrO_3 was added, and samples were removed at 0, 5, 15, and 45 min (oligosaccharide VIII), 0 and 15 min (oligosaccharide V), and 0 and 45 min (oligosaccharide VI). The reaction was terminated with 3 mL of 0.02 M $NaHCO_3$. The aqueous layer was extracted 3 times with 2 mL of $CHCl_3$. The chloroform phases were combined, washed with 1 mL of 0.02 M $NaHCO_3$ to remove color from the aqueous phase (3–4 times), and dried.

The carbohydrate resistant to CrO_3 oxidation was measured by gas chromatography after conversion to alditol acetates (Albersheim et al., 1967). The samples were dissolved in 50 μL of acetone for gas–liquid chromatography on a Hewlett-Packard 5830A gas chromatograph with a 6 ft × 2 mm glass column packed with 3% OV 275 on 100/120 Chromosorb WAW at 215 °C.

Linkage Determination. Oligosaccharides V, VI, and VIII were permethylated, purified by liquid chromatography as described above, and converted to partially methylated alditol and myoinositol acetates (Bjorndal et al., 1967, 1970) in the following manner. The orcinol-positive fractions were pooled and dried under N_2 before adding 0.5 mL of 0.5 N H_2SO_4 in 95% glacial acetic acid. Acetolysis was done at 80 °C for 16 h followed by the addition of 0.5 mL of distilled water and hydrolysis at 80 °C for 4 h. The samples were applied to 0.5-mL columns containing AG 3X-4A equilibrated with methanol. Columns were washed with 4 mL of methanol. After being dried, the residue was treated with 0.5 mL of 1 N NH_4OH containing 10 mg of NaB^2H_4 for 3 h at room temperature. The reaction was stopped with glacial acetic acid, and the borate was removed as described above. After acetylation, the partially methylated alditol and myoinositol acetates were dissolved in 25 μL of acetone for analysis by gas chromatography/mass spectrometry.

Analysis of Substitution Pattern of Oligosaccharide VI. Oligosaccharide VI (150 nmol) was incubated in 0.2 mL of 0.05 M sodium citrate buffer, pH 4.5, with 1.25 units of *C. ensiformis* α-mannosidase for 70 h at 37 °C (Li & Li, 1972). The reaction mixture was desalted by sequential passage over the anion-exchange resin AG 1X-2 (bicarbonate form) and the cation-exchange resin AG 50W-X8 (H^+ form) and dried. α-Mannosidase-treated oligosaccharide VI was permethylated and purified on a Lichrosorb column as described above. After hydrolysis, reduction with NaB^2H_4, and acetylation, the resulting partially methylated alditol and myoinositol acetates were analyzed by mass spectrometry.

Determination of Galactose–Mannose Linkage of Oligosaccharide VIII. Oligosaccharide VIII was acetylated (described above) and redissolved in 2 mL of glacial acetic acid. Half of the sample was removed, treated for 45 min with 50 mg of CrO_3 in a 40 °C sonic bath, and diluted with 3 mL of 0.02 M $NaHCO_3$. The control, or zero time point, was diluted with 3 mL of 0.02 M $NaHCO_3$ and 50 mg of CrO_3. Both samples were extracted 3 times with 2 mL of $CHCl_3$. The combined $CHCl_3$ layers were washed with 0.02 M $NaHCO_3$ until no color remained in the aqueous layer. After the $CHCl_3$ layer was taken to dryness, the residue was dissolved in 0.3 mL of dimethyl sulfoxide. The acetylated oligosaccharides were permethylated as described above. Orcinol-positive fractions from the Sephadex LH-20 column were combined and chromatographed on a Lichrosorb Si60 column. Pooled column fractions were hydrolyzed, reduced with NaB^2H_4, and acetylated as above. The partially methylated alditol acetates were analyzed by chemical ionization mass spectrometry.

Permethylated Alditol Acetate Standards. Methyl β-galactofuranoside was a gift from Dr. John Gander (Rietschel-Berst et al., 1977) and Dr. John Nordin (Bardallaye and Nordin, 1977). Methyl β-D-mannofuranoside was a gift from Dr. S. J. Angyal (Angyal et al., 1980). Methyl 2,3,4-tri-*O*-methyl-α-D-mannoside was obtained from Dr. Clinton E. Ballou. To prepare 2,4-di-*O*-methylmannitol, the mannan from *Pichia mucosa*, NRRL No. YB 1344 (Seymour et al., 1976), obtained from Dr. M. E. Slodki, was permethylated, followed by hydrolysis in 2 N trifluoroacetic acid for 90 min at 120 °C. The products were reduced with $NaBH_4$, borate was removed by methanol distillation, and the sample was

applied to 1 mL of AG 50 (H^+ form) and AG 1-X2 (OH^- form) columns sequentially. The permethylated alditols were separated by reverse-phase liquid chromatography on a 0.94 × 50 cm column of Whatman Partisil M9-1050 ODS-2, in a manner similar to that described by Sadaat & Ballou (1983). The alditols were eluted with a linear gradient for 2 h from 0% solvent B to 100% solvent B. Solvent A was water, and solvent B was water/acetonitrile (90:10). The flow rate was 4.0 mL/min, and the peaks were collected with regard to the response from the moving-wire detector. The peaks corresponding to 2,4-di-*O*-methylmannitol and the standards described above were converted to partially methylated alditol acetates for gas chromatography/mass spectrometry analysis.

Gas Chromatography/Mass Spectrometry. All mass spectrometry was performed on a Finnigan 3300-6110 instrument. In the chemical ionization mode, conditions included 1-Torr methane pressure in the ion source, ionizing electron energy at 150 eV, and source temperature at 60 °C. Spectra were scanned from m/z 100 to 450 at a rate of 2 s/scan. Electron-impact conditions included ionization electron energy of 70 eV and scan rate from m/z 40 to 450 at 2 s/scan.

Results

Analysis of the Permethylated Oligosaccharides. Strong ammonolysis of lipids V, VI, and VIII yielded in each case a single oligosaccharide containing the inositol and the hexoses of the intact lipids (Barr & Lester, 1984). As a verification of the mass and composition of the oligosaccharide moieties of the *Histoplasma* lipids, we examined the permethylated oligosaccharides of compounds V, VI, and VIII by gas chromatography/chemical ionization mass spectrometry (Experimental Procedures). Methane, isobutane, and ammonia were tried as reagent gases, but the molecular ion addition product, $[M + 18]^+$, was detected only with ammonia (Ando et al., 1977).

Permethylated oligosaccharide V gave a single peak in the total ion recording. The ammonium addition product molecular ion, $[M + 18]^+$, retention time 2.1 min, had a m/z at 690, which would be expected for a trisaccharide including two hexoses and inositol. Little information was obtained from the lower mass range of the spectrum.

A single major peak was also observed for permethylated oligosaccharide VIII, and its mass spectrum exhibited an $[M + 18]^+$ ion, at m/z 894 and retention time 6.2 min, consistent with an ammonium addition product composed of three hexoses and inositol. The $[M + 18]^+$ ion of permethylated oligosaccharide VI appeared at m/z 894, identical with the results obtained with oligosaccharide VIII, although it appeared as a single peak at a retention time of 7.0 min, as compared to 6.2 min for oligosaccharide VIII. This confirmed that oligosaccharide VI was also a tetrasaccharide composed of three hexose molecules and one inositol. Examination of the permethylated oligosaccharides by electron-impact mass spectrometry revealed no differences between oligosaccharide VI and oligosaccharide VIII with no ions observed beyond m/z 236. Thus, mass spectrometry of the intact permethylated oligosaccharides confirmed that VI and VIII were isomers, as predicted by the compositional data.

Determination of Anomeric Configuration of Mannoses in Oligosaccharide V. Oligosaccharide V was treated with α-mannosidase, and the products were examined by thin-layer chromatography. Approximately 90% of the mannose of oligosaccharide V was released by α-mannosidase treatment as judged by the appearance of free mannose and the disappearance of orcinol-positive material that migrated with oligosaccharide V.

Table I: Determination of Anomeric Configuration by CrO_3 Oxidation of Peracetylated Oligosaccharides[a]

	mol/mol of myoinositol detected		
	Man	Gal	Ins
oligosaccharide V			
before CrO_3	2.29		1.00
after CrO_3	2.19		1.00
% survival	96		
oligosaccharide VI			
before CrO_3	1.9	0.81	1.00
after CrO_3	1.4	0.09	1.00
% survival	74	11	
oligosaccharide VIII			
before CrO_3	2.17	1.27	1.00
after CrO_3	2.06	0.0	1.00
% survival	95	0	

[a] The oligosaccharides of compounds V, VI, and VIII were peracetylated and subjected to CrO_3 treatment: V, 15 min; VI and VIII, 45 min. Alditol acetates were prepared and analyzed by gas–liquid chromatography.

The products produced by *C. ensiformis* α-mannosidase incubation with oligosaccharide V were quantified by gas–liquid chromatography. The products of the glycosidase digestion were acetylated, and the response factors were compared with authentic standards. Mannose and inositol were liberated in a ratio of 2.06:1, respectively, indicating that both of the mannoses of oligosaccharide V were α-linked.

Determination of Anomeric Configuration of Oligosaccharide VIII. Oligosaccharide VIII was resistant to treatment with various α-mannosidases, α-galactosidases, and β-galactosidases, including single- and double-enzyme incubations. Alternatively, anomeric configuration was established from treatment of acetylated oligosaccharides with CrO_3 in glacial acetic acid. Acetylated hexopyranosides in an α-linkage are resistant to CrO_3 oxidation whereas those in a β-linkage are destroyed by the same treatment (Hoffman et al., 1972; Laine & Renkonen, 1975). No galactose was detected after 45 min of CrO_3 treatment of oligosaccharide VIII, whereas 95% of the mannose of oligosaccharide VIII survived the oxidation (Table I). Both of the mannoses of oligosaccharide V were resistant to 15-min exposure to CrO_3 (Table I), which gave support to the results obtained from glycosidase digestion. These results indicated that both of the mannoses of oligosaccharide VIII were in an α-linkage and the galactose was in the β-configuration.

Anomeric Configuration of Oligosaccharide VI. Oligosaccharide VI was incubated with *C. ensiformis* α-mannosidase for 70 h at 37 °C in 0.05 M sodium citrate buffer, pH 4.5. The reaction mixture was desalted and chromatographed on silica gel thin-layer plates developed twice with acetonitrile/H_2O (2:1), and sugars were detected with orcinol–H_2SO_4. None of the starting oligosaccharide VI (R_f 0.3) remained; the products were free mannose (R_f 0.77) and an orcinol-positive spot (R_f 0.38) that migrated between galactinol (galactosylinositol, R_f 0.41, provided by Dr. C. E. Ballou) and oligosaccharide V (dimannosylinositol, R_f 0.36). Thus, at least one of the mannoses of oligosaccharide VI was α-linked. Galactose was not released by incubating oligosaccharide VI with α-mannosidase in combination with *Aspergillus niger* α- or β-galactosidases.

As an alternate approach, oligosaccharide VI was acetylated and subjected to 45-min CrO_3 oxidation as described for oligosaccharide VIII (Hoffman et al., 1972; Laine & Renkonen, 1975). When compared to the zero time point, 70% of the mannose and 11% of the galactose survived the CrO_3 treatment (Table I). Thus, the galactose did not survive the CrO_3

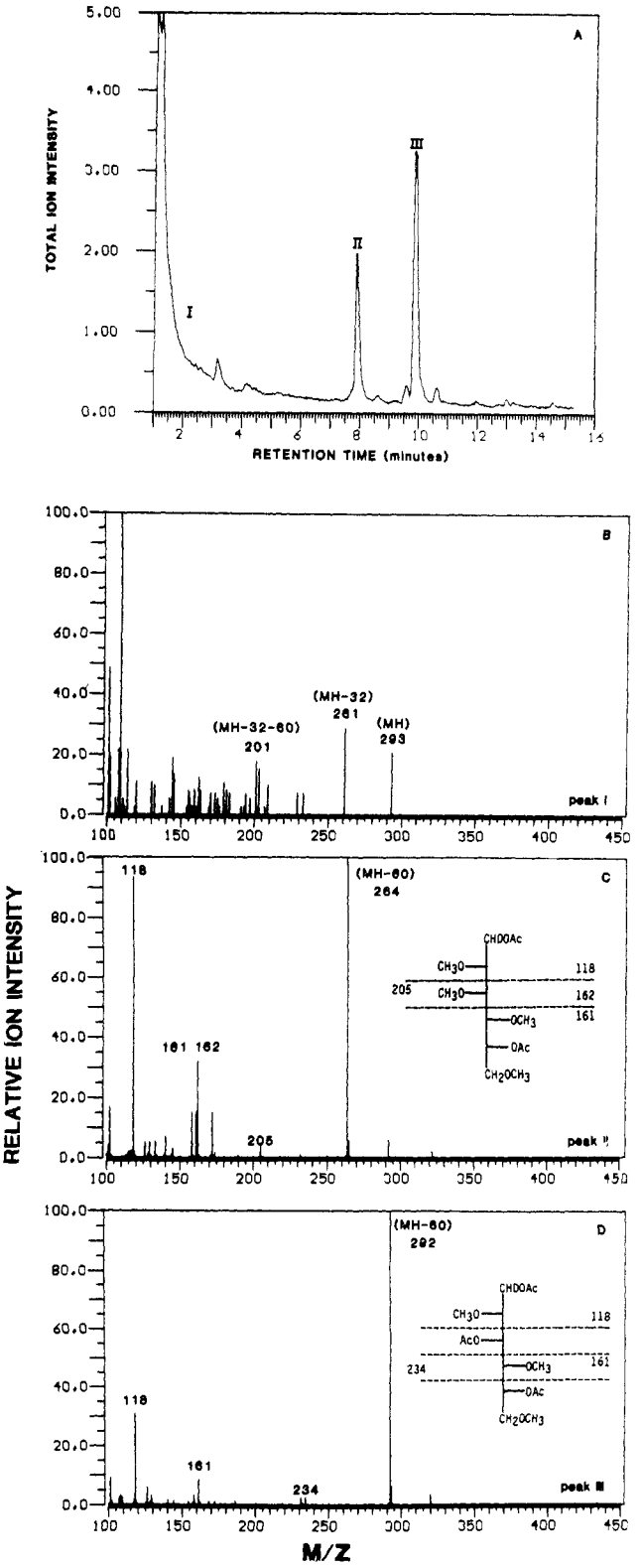


FIGURE 1: Methylation analysis of oligosaccharide V. Oligosaccharide V was permethylated, converted to partially methylated alditol acetates, and analyzed by chemical ionization mass spectrometry. The total ion current is shown as well as the mass spectra of the specified peaks (not corrected for molar response). An aliquot was chromatographed on a 1.5 m × 2 mm glass column packed with 3% OV 210. The column temperature was maintained at 125 °C for 2.5 min and then programmed from 125 to 240 °C at 6 °C/min. The methane pressure was 0.8 Torr inside the ion source, and the helium flow rate was 35 mL/min, giving a partial helium pressure of 200 mTorr in the source.

treatment; at least half of the mannose was in the α-configuration, and the remaining mannose was partially susceptible to CrO_3 oxidation.

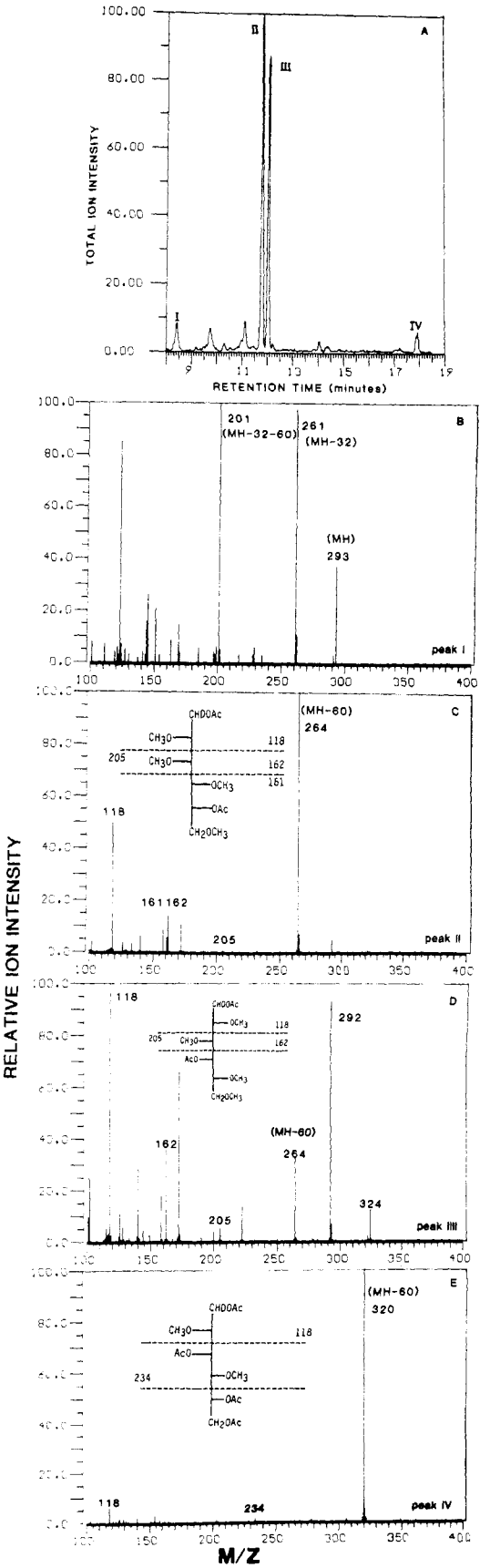


FIGURE 2: Methylation analysis of oligosaccharide VI. Oligosaccharide VI was permethylated, converted to partially methylated alditol acetates, and analyzed by chemical ionization mass spectrometry (not corrected for molar response). An aliquot was chromatographed on a 30 m × 0.25 mm ID fused quartz capillary Carbowax column with 0.25-μm phase coating. The column temperature was maintained at 160 °C for 1 min and then programmed from 160 to 220 °C at 10 °C/min. A splitless injection was used, and the helium flow rate was 0.6 mL/min. The injector temperature was 220 °C.

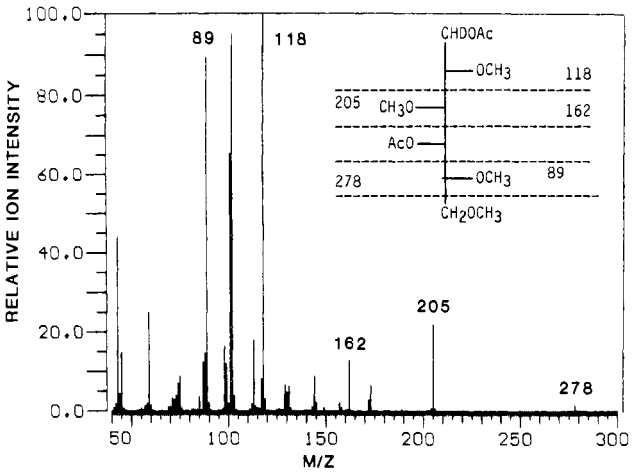


FIGURE 3: Electron-impact mass spectrometry of oligosaccharide VI. Oligosaccharide VI was permethylated, converted to partially methylated alditol acetates, and analyzed by electron impact mass spectrometry (70 eV). The mass spectrum of the peak III (Figure 2) is presented. An aliquot was chromatographed on a 30 m × 0.25 mm ID Carbowax column with 0.25-μm thickness. The column temperature was maintained at 160 °C for 1 min and then programmed from 160 to 220 °C at 10 °C/min.

Linkage Determination. The linkages of the sugars of oligosaccharides V, VI, and VIII were determined by methylation analysis. The total ion current recording of the methylated and acetylated alditols and myoinositol derived from oligosaccharide V is given in Figure 1A, and the mass spectra of peaks I–III are shown in Figure 1B–D. Other minor peaks (Figure 1A) did not contain fragments characteristic of partially methylated alditol acetates. In this particular analysis, peak I, revealed by mass chromatography at m/z 261 and 293, was low and buried in the background, probably owing to its volatility. Peak I (Figure 1A) was identified as mono-*O*-acetylpenta-*O*-methylinositol (Figure 1B) from the following ions: m/z 293, $[MH]^+$; m/z 261, [MH – methanol]$^+$; m/z 201, [MH – methanol – acetic acid] (Hsieh et al., 1978). In Figure 1C, the mass spectrum of peak II (Figure 1A) is shown and was identified as a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol due to the abundance of the ion at m/z 264, $[MH - 60]^+$, and fragment ions at m/z 118, 161, 162, and 205. The mass spectrum (Figure 1D) of peak III, Figure 1A, supports the assignment of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol to this peak since abundant ions at m/z 118, 161, and 234 (Figure 1D) are diagnostic for the derivative of a hexose substituted at the 3-position. Previous work had shown that inositol 1-phosphate was a major product after acid hydrolysis of compounds V, VI, and VIII (Barr & Lester, 1984). Compounds V, VI, and VIII were stable to alkaline hydrolysis (Barr & Lester, 1984), indicating substitution on the vicinal hydroxyl groups of inositol. In accord with these data, we propose that the hexose of all three *Histoplasma* oligosaccharides is substituted at the 2- or 6-position of inositol. Therefore, the structure of oligosaccharide V is proposed as

$$\text{Man}_p(\alpha 1\rightarrow 3)\text{Man}_p(\alpha 1\rightarrow 2 \text{ or } 6)\text{myoinositol}$$

Four peaks, characteristic for hexoses or myoinositol, were identified in the total ion current recording of oligosaccharide VI (Figure 2A, not corrected for molar response). The mass spectra of the other minor peaks were not characteristic of partially methylated alditol acetates. Peak I, Figure 2A, was identified as mono-*O*-acetylpenta-*O*-methylinositol due to the presence of prominent ions at m/z 201, 261, and 293 (Hsieh et al., 1978) in the spectrum of this peak (Figure 2B). The retention time of peak II in Figure 2A was identical with that

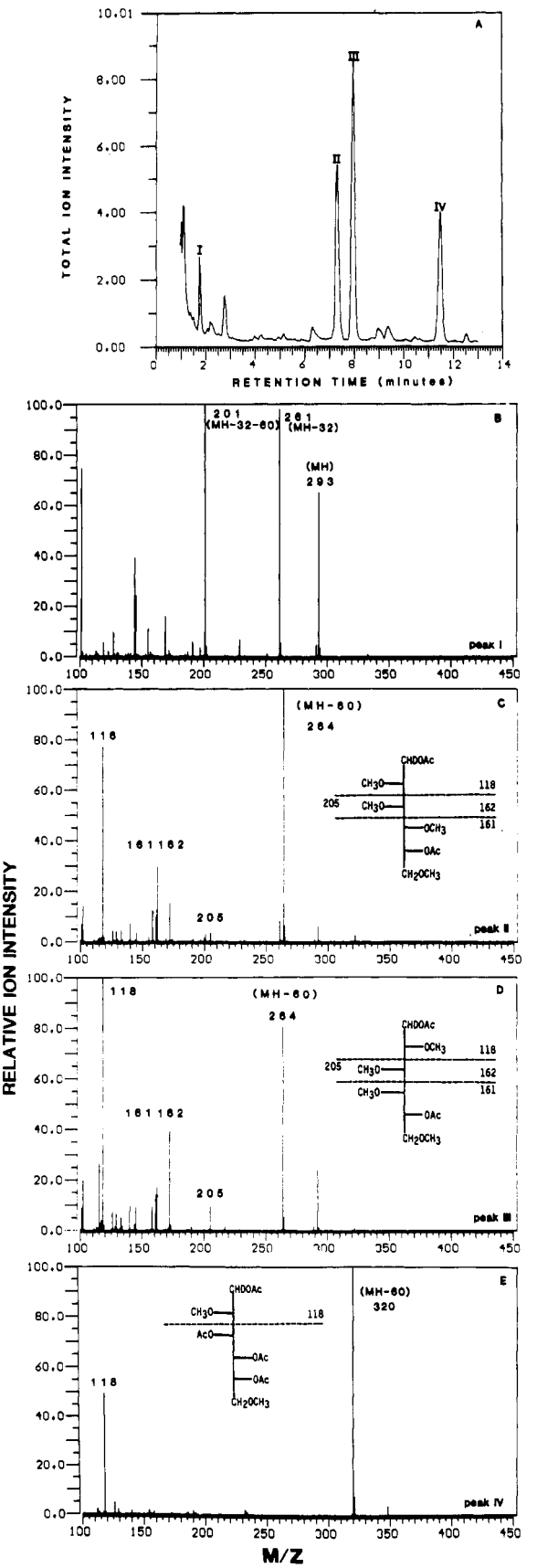


FIGURE 4: Methylation analysis of oligosaccharide VIII. Oligosaccharide VIII was permethylated, converted to partially methylated alditol acetates, and analyzed as described in the legend to Figure 1.

of peak II, Figure 1A, and that of authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol. The mass spectrum of peak II (Figure 2C) exhibited a major ion at m/z 264 and

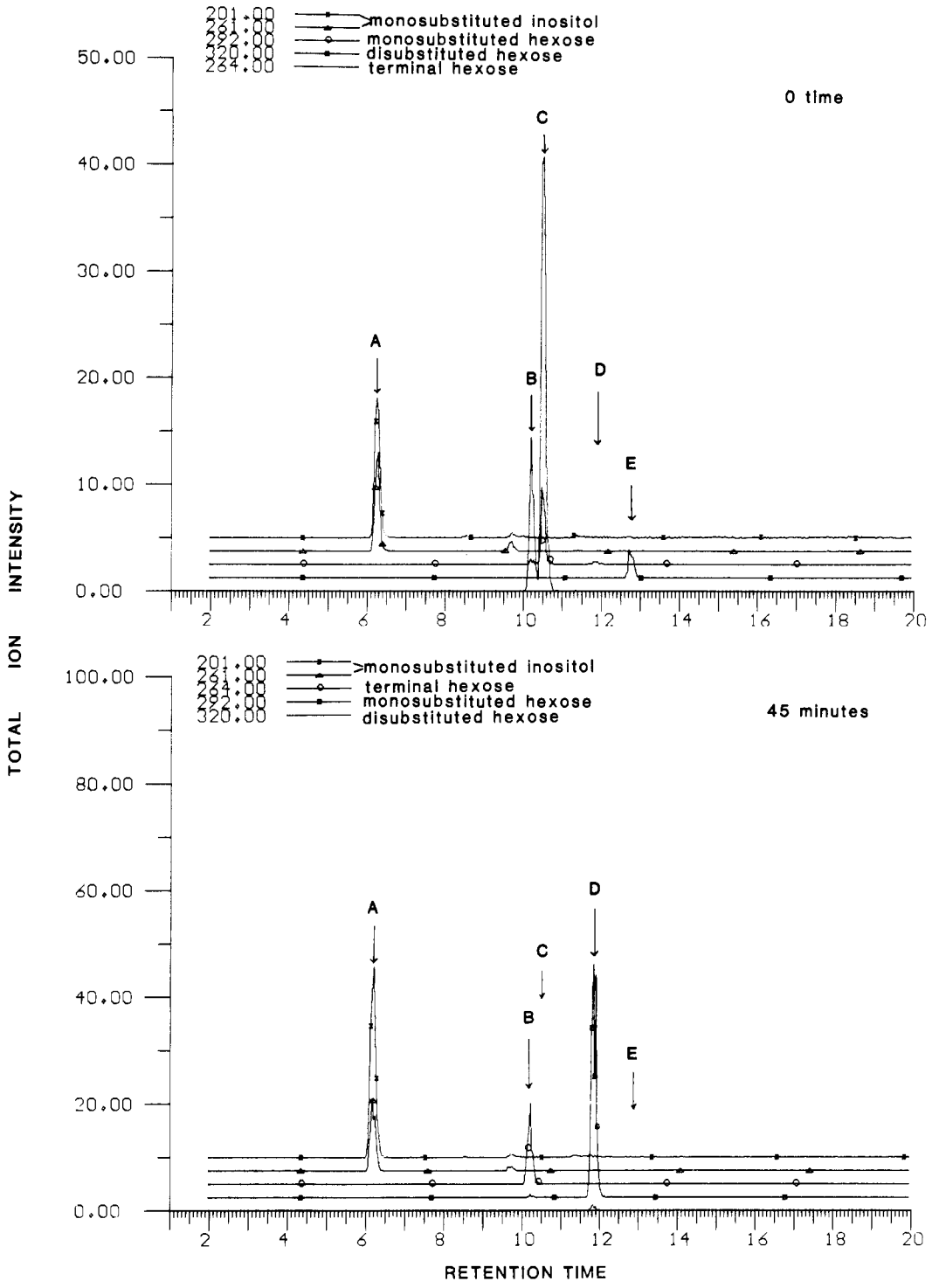


FIGURE 5: Determination of galactose–mannose linkage of oligosaccharide VIII by CrO_3 oxidation. Peracetylated oligosaccharide VIII was treated with CrO_3 for 45 min (lower panel), and the control is shown in the upper panel. The products were permethylated, converted to partially methylated alditol acetates, and analyzed by chemical ionization mass spectrometry. Composite ion tracings are shown, and the specific ions monitored are shown in the upper left-hand corner of each panel. The GC/MS conditions were the same as described in the legend to Figure 1.

fragment ions at m/z 118, 161, 162, and 205, indicating the presence of unsubstituted mannopyranoside in oligosaccharide VI (Laine, 1981).

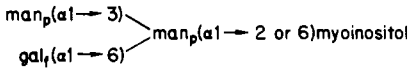
The final hexose peak in the chromatogram (peak IV, Figure 2A) had the same retention time and mass spectrum as an authentic sample of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-mannitol. Ions at m/z 118 and 234 (Figure 2E) support this designation.

Peak III, which eluted very close to peak II, exhibited a very different spectrum. The predominant ion in the spectrum (Figure 2D) of this peak was m/z 292, the usual major ion of the derivative of a monosubstituted hexose (Laine, 1981), yet ions at m/z 264, $[MH^+ - 60]$, and 324, $[MH^+]$, suggested that this was a derivative of a terminal hexose. Ions at m/z 118 and 162, coupled with the absence of an ion at m/z 161 (Figure 2D), indicated that the hexose was unsubstituted at positions 2 and 3 but acetylated at carbon 4. The retention time of peak III matched the retention time of 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylgalactitol prepared from authentic methyl β-galactofuranoside and did not chromatograph with the partially methylated alditol acetate of β-mannofuranoside. These data are consistent with the designation of peak III as

derived from a terminal galactofuranoside.

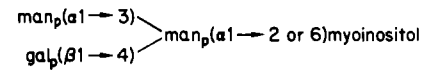
Examination of the partially methylated alditol acetates of oligosaccharide VI by electron-impact mass spectrometry showed several ions in the spectrum of peak III (Figure 2), diagnostic of a galactofuranoside (Figure 3). The large ion at m/z 89, representing cleavage between carbons 4 and 5, as shown in the inset in Figure 3, would only be produced if both carbons 5 and 6 were methylated. Additional proof was the presence of an ion at m/z 278, which results from cleavage between carbons 5 and 6. Both of these ions are unique to the spectrum of a terminal hexofuranoside derivative (Bjorndal et al., 1967) and were not found in the electron-impact spectrum of peak II, which was derived from a terminal mannopyranoside. As indicated in Figure 3, ions at m/z 118, 162, and 205 confirmed that this was a nonreducing terminal hexose. The data obtained from linkage analysis of oligosaccharide VI were consistent with the anomeric configuration data. Treatment of oligosaccharide VI with α-mannosidase appeared to remove only one mannose residue, since only one mannose residue would be accessible to the glycosidase. Acetylated hexofuranosides in an α-linkage are destroyed by CrO_3 treatment whereas those in a β-linkage are stable to such treatment (Oshima & Ariga, 1976). In accord with our observations (Table I), we tentatively conclude that galactofuranose is probably in the α-configuration, although unambiguous assignment would require further experimentation.

The presence of terminal galactofuranoside and mannopyranoside raised the question of the 3-linked or 6-linked substitution of each terminal sugar on the mannose of oligosaccharide VI. Therefore, oligosaccharide VI was treated with α-mannosidase for 70 h to remove the terminal mannopyranoside and the products were permethylated and converted to partially methylated alditol acetates. Analysis by chemical ionization mass spectrometry revealed monosubstituted inositol, terminal galactofuranoside, and a monosubstituted hexose with the same retention time as authentic 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol. Thus, α-mannosidase treatment of oligosaccharide VI generated a mannitol substituted at the 6-position, indicating that the mannopyranose residue was at the 3-position in the original oligosaccharide VI. These results suggest that the structure of oligosaccharide VI is



Four major peaks were also found in the total ion current recording of oligosaccharide VIII (Figure 4A), with mass spectra characteristic of partially methylated alditol and myoinositol acetates. On the basis of its spectrum (Figure 4B), peak I was designated as mono-*O*-acetylpenta-*O*-methylinositol. Peaks II and III, Figure 4A, were identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, respectively, from a comparison with the retention times of standards and due to the presence of a strong ion at m/z 264 in the spectra of both peaks (Figure 4C,D). Peak IV (Figure 4A) was 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylmannitol on the basis of the presence of ions of m/z 118 and 320 and the absence of ions at m/z 161, 162, 189, 190, and 234 (Figure 4E). This indicates the presence of 3,4-disubstituted mannopyranose, terminal mannopyranose, terminal galactopyranose, and monosubstituted inositol in oligosaccharide VIII.

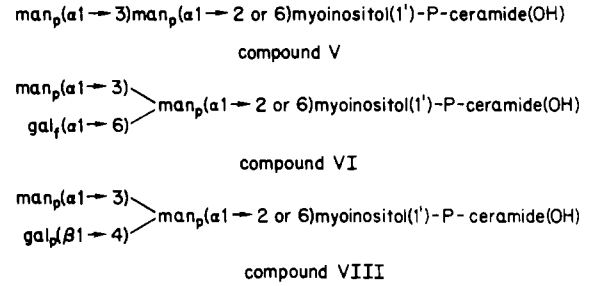
Determination of Galactose–Mannose Linkage of Oligosaccharide VIII. Analysis of the partially methylated alditol acetates of oligosaccharide VIII did not delineate the position of substitution of the terminal mannose and galactose on the disubstituted mannose. We therefore selectively removed the β-linked galactose by CrO_3 oxidation and examined the structure of the resulting dimannosylinositol by methylation analysis. Since 45-min CrO_3 treatment of oligosaccharide VIII had destroyed all of the galactose (Table I), peracetylated oligosaccharide VIII was treated with CrO_3 for 0 and 45 min, followed by permethylation, purification by liquid chromatoghraphy, conversion to partially methylated alditol acetates, and analysis by chemical ionization mass spectrometry. Composite mass chromatograms of specific ions are shown in Figure 5. Four peaks were evident in the zero time point, Figure 5, and included monosubstituted inositol (m/z 201 and 261, peak A), terminal mannose and terminal galactose (m/z 264, peaks B and C, respectively), and disubstituted mannose (m/z 320, peak E). They were not corrected for molar response factors. After 45-min CrO_3 oxidation, peak C (terminal galactose) had disappeared. Also, the ion peak at m/z 320 from disubstituted mannose (peak E) had disappeared and had been replaced by a new ion peak at m/z 292 (peak D), indicative of a monosubstituted mannose. In the mass spectrum of peak D (not shown), ions at m/z 118, 161, 234, and 292 confirmed that the hexose was a pyranoside substituted at the 3-position. The retention time of the 3-linked hexose of the 45-min time point (11.8 min) was identical with the retention time of 3-linked mannopyranose from oligosaccharide V, demonstrating that the internal mannose was substituted with mannose at the 3-position and galactose at the 4-position. This established the structure of oligosaccharide VIII as



Discussion

Fungi and plants uniquely contain a group of sphingolipids possessing the common core structure ceramidephosphoinositol. In plants, a complex variety of structures exist with the inositol substituted with glucuronic acid, glucosamine, arabinose, galactose, and mannose (Carter et al., 1965; Laine et al., 1980). In *S. cerevisiae* and *Neurospora crassa*, simpler structures exist with inositol substituted with mannose and inositol phosphate (Smith & Lester, 1974; Lester et al., 1974).

Compounds V, VI, and VIII, purified from the yeast phase of *H. capsulatum*, represent novel phosphoinositolsphingolipids that are related to the lipids described above. The data acquired to date suggest that the structures of these compounds are



Compound V may represent a biosynthetic precursor to compounds VI and VIII since both contain the same trisaccharide core as V but with the addition of a galactofuranose residue at the 6-position of mannose (compound VI) and the addition of galactopyranose at the 4- position of mannose (compound VIII).

Compound VI appears to be the first reported occurrence of galactofuranose in a glycosphingolipid. The occurrence of terminal galactofuranose residues in cell wall polymers has

been reported, specifically as the constituents of the malonogalactan of *Penicillium citrinium* Thom 1131 (Kohana et al., 1974), the galactomannan of the cell wall of *A. niger* (Bardallaye & Nordin, 1977), and polysaccharides isolated from the mycelial forms of *H. capsulatum*, *Histoplasma duboisii*, *Paracocciodes brasiliensis*, and *Blastomyces dermatitidis* (Azumo et al., 1974). To date, galactofuranose has not been reported in the yeast phase of *H. capsulatum*. It is interesting that compound VI is not found in the mycelial phase (Barr & Lester, 1984).

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Registry No. Oligosaccharide V, 92344-09-7; oligosaccharide VI, 92344-10-0; oligosaccharide VIII, 92344-12-2; galactofuranose, 19217-07-3.

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