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Structural characterization of the galactoxylomannan of *Cryptococcus neoformans* Cap67

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Abstract

The galactoxylomannan (GalXM) obtained from the culture supernatant of an acapsular mutant of *Cryptococcus neoformans* Cap67 was purified by Concanavalin A affinity, ion-exchange, and gel-filtration chromatographies. The structure of GalXM was determined by methylation analysis and by 1D and 2D NMR spectroscopic studies of the intact polysaccharide and of the oligosaccharide fragments generated by Smith degradation and by acetolysis. GalXM is a complex polysaccharide with an α-(1 → 6)-galactan backbone. The polysaccharide is branched at C-3 of alternate Gal units of the backbone. C-3 is the point of attachment of the oligosaccharide side chains comprised of α-D-Man-(1 → 3)-α-D-Man-(1 →

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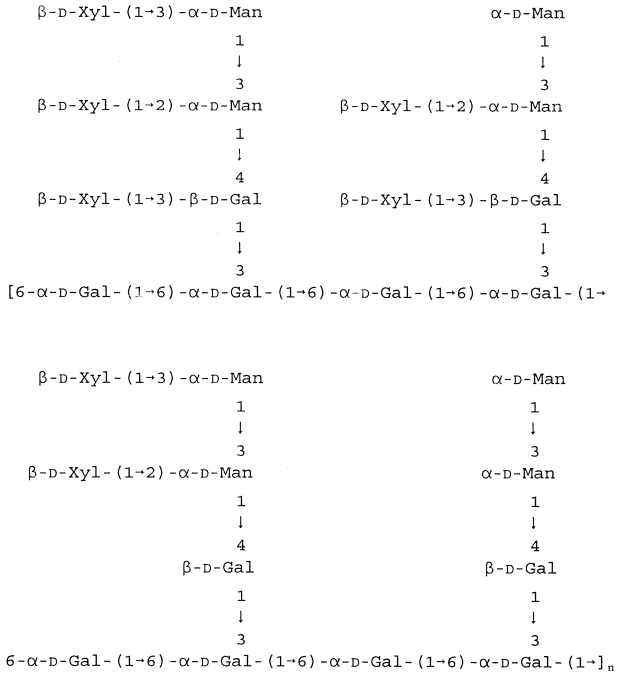
4)-β-D-Gal-substituted with zero to three terminal β-Xyl residues as shown in the following structure:



Keywords: Galactoxylomannan (GalXM); 2D NMR spectroscopy; *Cryptococcus neoformans*; Polysaccharide structure

1. Introduction

Cryptococcus neoformans is an encapsulated pathogenic yeast that is responsible for meningoencephalitis in individuals whose immune system is debilitated by chemotherapy or by illness. Cryptococcosis has emerged as a common life-threatening disease in patients with AIDS [1,2].

The cell envelope of *C. neoformans* is composed of cell-wall glucans, a major capsular glucuronoxylomannan (GXM), and two minor polysaccharides, galactoxylomannan (GalXM), and mannoprotein (MP) [3–5]. All three antigens are present in cryptococcal culture supernatants. GXM determines the serotype of the yeast, and it is considered to be a major virulence factor [6].

In an early study, Xyl, Man, Gal, and GlcA were identified by paper chromatography as components of the soluble antigen of *C. neoformans* serotypes A, B, and C [7]. A serotype A capsular polysaccharide was precipitated with hexadecyltrimethylammonium bromide (CTAB) and shown to be composed of Xyl, Man, and Gal. The polysaccharide cross-reacted with *Streptococcus pneumoniae* type XIV antiserum [8]. The specificity of this antiserum depends on terminal, (1 → 3)-, (1 → 6)-, and (1 → 3,6)-linked galactosyl residues [8]. The Gal content of the immunoprecipitated serotype A polysaccharide increased because of the reaction with the type XIV antiserum [8]. The results suggested that the capsular polysaccharide was comprised of a mixture of heteroglycans. In later studies, the capsular polysaccharide of *C. neoformans* serotype B was partially purified from the culture supernate by precipitation with EtOH [9]. The precipitate was comprised of Xyl, Man, Gal, and GlcA in the molar ratios of 2:3:0.5:1, respectively. In recent studies, the capsular polysaccharides from several serotypes of *C. neoformans* were isolated and purified [10–13]. The purified polysaccharides were comprised of Xyl, Man, and GlcA; Gal was not present in any of the purified GXMs. This suggested that Gal was present in the *C. neoformans* capsule as a separate glycan. The high viscosity of solutions containing GXM impeded the resolution of the mix-

ture of polysaccharides into individual components in earlier studies.

GalXM was first isolated as a distinct entity from the culture supernate of *C. neoformans* serotype A [3]. It is believed to be loosely associated with the cell-wall rather than covalently linked to it [14]. A monoclonal antibody specific for GalXM was used in conjunction with immunocytochemical analysis to localize GalXM on the surface of the acapsular mutant *C. neoformans* Cap67 [15]. Immunogold transmission electron microscopy and micro agglutination assay indicate that the epitopes recognized by the monoclonal antibody are located within the cytoplasm and the cell-wall of *C. neoformans* [A. van de Moer, R. Cherniak, S.L. Salhi, N. Schnoy, S. Jouvert, M. Bastide, and J.M. Bastide, unpublished results].

Purified GalXM was obtained from three different strains (serotype A, C and Cap67) of *C. neoformans* [16]. GalXM from all three strains were comprised of Gal, Man, and Xyl in similar, but not identical, molar ratios. Methylation analysis of all the isolated GalXM indicated that some of the glycosyl residues are branched and that all of the Xyl and Gal*f* are present as nonreducing terminal residues.

Herein, we report the structural characterization of the GalXM of *C. neoformans* Cap67. The isolation of GalXM and MP is complicated by the presence of GXM. Therefore, an acapsular mutant, *C. neoformans* Cap67, was used for the analysis of the primary structure of GalXM.

2. Experimental

Analytical methods.—The neutral carbohydrates were analyzed by the phenol–sulfuric acid method [17]. Protein was determined by the bicinchoninic acid (BCA) (Pierce, Rockford, IL) analysis method using BSA as a standard [18]. The 2-amino-2-deoxyhexoses were analyzed by the method of Smith and Gilkerson [19]. The constituent monosaccharides of GalXM were analyzed as their per-*O*-acetylated aldononitrile derivatives by GLC [16,20]. Underivatized monosaccharides were determined by HPAEC with pulsed amperometric detection (Dionex Bio LC) [21,22]. The pulse potential and duration were $E_1 =$ 0.05 V ($t_1 = 300$ ms); $E_2 = 0.6$ V ($t_2 = 120$ ms); and $E_3 = -0.6$ V ($t_3 = 60$ ms). Fucose (12 μg mL^{-1}) was used as an internal standard, and the monosaccharides were separated on a CarboPac MA1 analytical column (4×250 mm) with a CarboPac MA1 guard column (4×35 mm) (Dionex). The monosaccharides were eluted with 600 mM NaOH at 0.5 mL min^{-1}. Per-*O*-methylation was performed by the method of Ciucanu and Kerek [23] as described by Carpita and Shea [24]. *myo*-Inositol (30 μg) was added to the samples as an internal standard. The partially methylated alditol acetates were analyzed with a Shimadzu GC 17A MS 5000 spectrometer equipped with an electron-impact detector and an HP-5 capillary column. The absolute configuration of the glycoses was determined by GLC–MS analysis of the per-*O*-acetylated (+)-(*S*)-2-butyl glycosides [25,26].

Electrospray ionization mass spectrometry (ESIMS).—(ESIMS) was performed with a PE-Sciex API III biomolecular analyzer operated in the positive-ion mode. Solutions of oligosaccharides or oligoglycosyl alditols (100 μg) in aqueous 30% MeOH containing 0.75% HCl (100 μL) were infused into the electrospray source at 4 μL min^{-1} using a Harvard 22 syringe pump. The ionspray was operated at 5000 V with an orifice potential of 35 V. At least 10 scans (100–1500 amu) were collected and averaged.

NMR spectroscopy.—Spectra were recorded using a Varian VXR 400, a Varian UnityPlus 500, and a Varian UnityPlus 600 spectrometers. ^{13}C spectra were recorded with a dual (^{1}H, ^{13}C) probe. ^{1}H spectra were recorded using either 5-mm (^{1}H, ^{19}F) probe or a triple resonance (^{1}H, ^{13}C, ^{15}N) pulsed-field gradient probe. The sample (10–40 mg) was exchanged twice with 99.9% deuterium oxide with intermediate lyophilization, and the sample was dissolved in 99.96% deuterium oxide (0.8 mL) and transferred to a 5-mm NMR tube (Wilmad 535-PP) for analysis. The ^{1}H chemical shifts of the external standards, sodium 4,4-dimethyl-4-silapentanesulfonate and acetone, were calibrated at several temperatures between 25 °C and 60 °C. Acetone was used as an internal reference at 31.05 ppm, and Waltz decoupling [27] was used in ^{13}C NMR experiments. Quadrature detection in the F1 dimension was achieved by States et al.'s method [28].

DQF-COSY [29], TOCSY [30] and NOESY [31] experiments were performed in the phase-sensitive mode [28]. The data were processed off line using Felix 230 (Biosym Software, San Diego, CA) on a Silicon Graphics personal Iris workstation. Typically, $2 \times 256 \times 1024$ data points were collected and zero filled in t_1 to give a final data matrix of 1 K × 1 K. Sine-bell apodization with 45° phase shifts were used in t_1 dimensions for DQF-COSY, TOCSY and NOESY. The squared sine-bell multiplication function was applied in the t_2 dimension with a 90° phase

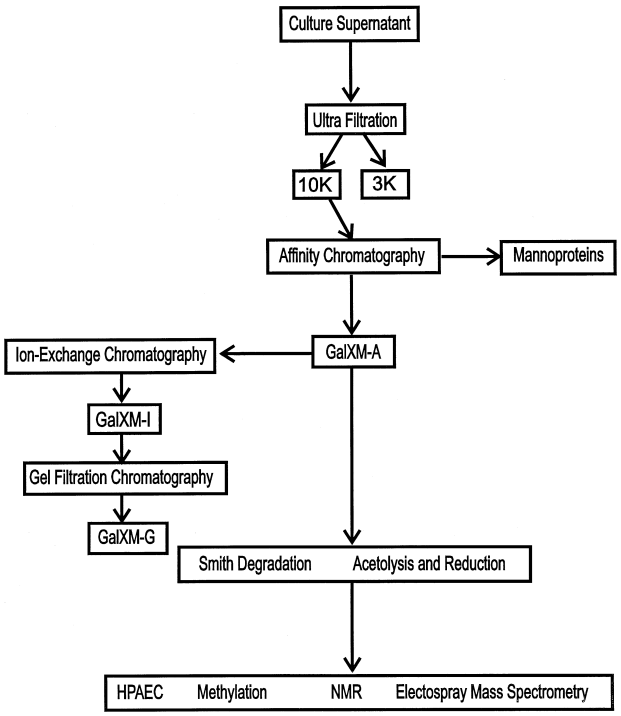


Fig. 1. Purification scheme for GalXM.

shift for TOCSY and NOESY. The HOD signal was presaturated during the relaxation delay and during the mixing time for NOESY experiments. Two-dimensional GHSQC [32] and GHMBC [33,34] experiments were performed with Varian UnityPlus 500 spectrometer. The ^{13}C decoupling during acquisition was accomplished by the Garp-1 [35] sequence. The sweep width was 1400 Hz in the t_2 (^1H) dimension and 11,000 Hz in the t_1 (^{13}C) dimension. The data matrix was $2 \times 256 \times 2$ K complex data points. A Lorentzian-to-Gaussian weighting function (lb = -0.5, gb = 0.05) was applied in the t_2 domain and squared sine-bell in the t_1 domain with the first data point multiplied by 0.5 to reduce t_1 noise.

Growth and culture conditions.—An acapsular mutant of *C. neoformans* Cap67 (Jacobson et al., Medical College of Virginia) [36] was grown on chemically defined media as previously described [3]. The culture filtrate was concentrated by ultrafiltration using a 10,000 amu mol wt. cutoff filter (Spiral Cartridge Concentrator S1Y10, Amicon). The retentate (10 K) was lyophilized and reserved.

GalXM.—GalXM was purified by a modification of previously described procedures [16,37] (Fig. 1). The 10 K retentate (700 mg) was dissolved in 0.01 M 2-amino-2-hydroxymethyl-1,3-propandiol (Tris), 0.5 M NaCl, pH 7.2, 1 mM each $CaCl_2$ and manganese(II) chloride, (start buffer) and filtered through a 0.22-μm filter (Millex-GS, Millipore). The filtrate was applied to a Concanavalin A (Con A) agarose column (2.5×11 cm, Sigma, type VI C-7555) at 4 °C. The sample was recirculated through the column for 16 h at a flow rate of 18 mL h^{-1}. The effluent was collected, and the column was washed with start buffer (300 mL) until the effluent was negative for carbohydrates. The effluent and washes were combined, dialyzed, and lyophilized (GalXM-A).

Ion - exchange chromatography of GalXM - A.—GalXM-A (1 mg) in 15 mL of 0.01 M Tris buffer, pH 7.6 was applied to a DEAE Sephadex column (4×25 cm, DE 52 Whatman Chemical Separations,) equilibrated with 0.01 M Tris, pH 7.6. The column was washed with 0.01 M Tris, pH 7.6, until the effluent was negative for neutral sugars, ($\sim$ 500 mL). The column was then eluted with a 2000-mL linear gradient of 0.0–1.0 M NaCl in 0.01 M Tris, pH 7.6, at a flow rate of 21 mL h^{-1}. Appropriate fractions containing carbohydrate [17] were pooled, dialyzed, and lyophilized (GalXM-I).

Gel-filtration chromatography of GalXM-I.—The GalXM-I from the ion-exchange chromatography (5.2 mg in 1 mL of 0.01 M Tris, 0.1 M NaCl, pH 7.6) was applied to a Sepharose CL 6B column (1.5×85 cm, Sigma Chemical) that was calibrated using dextran mol wt. standards (Polymer Laboratories). The appropriate fractions containing carbohydrate [17] were pooled, dialyzed, and lyophilized (GalXM-G).

Smith degradation.—GalXM-A (508 mg) was oxidized with 0.04 M $NaIO_4$ (500 mL) for 96 h at 4 °C [38], reduced with $NaBH_4$ (7.4 g) for 18 h at 4 °C, dialyzed and lyophilized. The oxidized and reduced polysaccharide (353 mg) was hydrolyzed with 0.1 M TFA (25 mL) for 16 h at 37 °C. The mixture of oligosaccharides was lyophilized and then applied to a calibrated Bio-Gel P2 column (186×2.5 cm, Bio-Rad). The oligosaccharides were eluted with deionized water, and the fractions were pooled according to the carbohydrate profile of the column, and then lyophilized.

Acetolysis and reduction.—GalXM-A (400 mg) was acetylated [39,40] and then treated for 9 h at 40 °C with a 100:100:1 mixture of CH_3COOH–Ac_2O–concentrated H_2SO_4 [39,40]. The acetolysate was *O*-deacetylated, and the solution of oligosaccharides (4 mL) was deionized with mixed-bed resin (Bio-Rex 501-X8, 20–50 mesh, 10 g, Bio-Rad Laboratories). The oligosaccharides were reduced with $NaBH_4$ (0.16 g per 40 mL of 0.1 M ammonium hydroxide) for 18 h at 4 °C. The reaction was quenched by adding glacial

CH_3COOH, and the reduced mixture of oligosaccharides was evaporated to dryness in vacuo. The dry product was dissolved in water, passed through an OnGuard-H cartridge (Dionex), and pre-evaporated with MeOH three times. The solution was concentrated to dryness, dissolved in deionized water (2 mL), filtered through a 0.22-μm filter (Millex-GS, Millipore), and applied to a Bio-Gel P2 column (186×2.5 cm, Bio-Rad Laboratories). The column was eluted with deionized water (20 mL h^{-1}), and 4.5 mL fractions were collected. The appropriate carbohydrate containing fractions were combined according to the carbohydrate profile of the column, dialyzed, and lyophilized.

3. Results

Antigen purification.—The 10 K retentate obtained by ultrafiltration was used for the isolation of GalXM and MP (Fig. 1). Con A affinity chromatography yielded three fractions: (1) GalXM-A, the effluent that did not bind to the Con A column (38%); (2) 0.2 MP, a glycoprotein that was eluted with 0.2 M methyl α-D-mannopyranoside (Man*p*) (24%); and (3) 0.4 MP, a glycoprotein that was eluted with 0.4 M Man*p* (2%). The fractions eluted with Man*p* were reserved for future analysis. GalXM-A was resolved by anion-exchange chromatography into a minor peak (8%) that was eluted with the 0.01 M Tris, pH 7.6; and a major peak (GalXM-I, 92%) that was eluted at $\sim$0.2 M NaCl. Gel-filtration chromatography of GalXM-I gave a single symmetrical peak (GalXM-G) with an apparent mol mass of 22 kDa.

GalXM was comprised of Man, Xyl and Gal in molar ratio of 10:6:10. The molar ratios of the sugars in GalXM-A, GalXM-I, and GalXM-G were similar (Fig. 1). Each monosaccharide had the D-configuration.

Methylation analysis.—Methylation analysis of GalXM-I showed 13 unique monosaccharide derivatives, eight major (5 to 22 mol %) and five minor (less than 2 mol %) (Table 1). The identification of di-*O*-methyl and mono-*O*-methyl glycosyl residues indicated that GalXM-I was a branched polymer (Table 1). Xyl (22%) accounted for most of the nonreducing terminal residues, although some terminal nonreducing Man*p* (7%) and Gal*f* ($< 2\%$) were present. GLC–MS analysis showed that Man was present in three major unique linkages, (terminal, 7 mol %; 3-*O*-linked, 8 mol %; and 2,3-*O*-linked, 12 mol %) and that Gal*p* was present in four major unique linkages (4-*O*-linked, 13 mol %; 6-*O*-linked, 11 mol %; 3,4-di-*O*-linked, 5 mol %; and 3,6-di-*O*-linked, 17 mol %). Methylation analysis indicated that 31% of the total linkages involved Gal at carbon 6 and 44% of the total linkages involved Man and Gal at carbon 3 (Table 1).

Table 1
Methylation analysis of GalXM-I[a]

Derivative identified	Mol %	Deduced linkage
2,3,4-Xyl[b]	22	T-Xyl[c]
2,3,4,6-Man	7	T-Man*p*
2,3,5,6-Gal	2	T-Gal*f*
2,4,6-Man	8	3-*O*-Man
2,4,6-Gal	1	3-*O*-Gal
2,3,6-Gal	13	4-*O*-Gal
2,3,4-Man	1	6-*O*-Man
2,3,4-Gal	11	6-*O*-Gal
4,6-Man	12	2,3-di-*O*-Man
2,6-Gal	5	3,4-di-*O*-Gal
2,4-Gal	17	3,6-di-*O*-Gal
4-Man	1	2,3,6-tri-*O*-Man
4-Gal	1	2,3,6-tri-*O*-Gal

[a] The methylated alditol acetates were identified by their retention times relative to the internal standard, *myo*-inositol. The retention times were obtained by using partially methylated, alditol acetate standards for each of the monosaccharides.
[b] 1,5-Di-*O*-acetyl-1-deuterio-2,3,4-tri-*O*-methyl-D-xylitol.
[c] Nonreducing terminus.

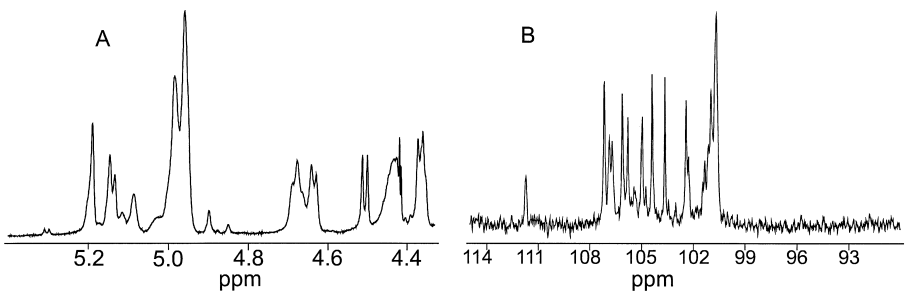


Fig. 2. (A) Anomeric region (5.4–4.3 ppm) of the 1D ^1H spectrum of GalXM recorded at 600 MHz and 56 °C. (B) Anomeric region (110–90 ppm) of the 1D ^{13}C spectrum of GalXM at 100 MHz and 62 °C.

NMR spectroscopy.—The ^{1}H NMR spectrum of GalXM-I showed at least 15 resonances in the anomeric region between 4.4 and 5.4 ppm (Fig. 2A). At least 16 signals for anomeric carbons were present in the ^{13}C NMR spectrum of GalXM-I (90–115 ppm) (Fig. 2B). A signal observed at 111.76 ppm was consistent with occurrence of a β-Gal*f* residue [41]. GalXM could not be structurally characterized directly by NMR spectroscopy since both the ^{1}H and ^{13}C spectra are complex.

Partial depolymerization of GalXM-A.—Due to the complexity of the GalXM structure, a scheme for the partial depolymerization of GalXM was developed based on two specific chemical methods: (1) Smith degradation and (2) acetolysis and reduction (Fig. 1). (1) *Smith degradation*: Seven oligosaccharide fractions were obtained by gel-filtration chromatography after Smith degradation of GalXM-A (Fig. 3, 1S–7S). The structures of the oligosaccharides in each fraction were determined by HPAEC, methylation analysis, electrospray–ionization MS (ESIMS), and 1D and 2D NMR spectroscopy. The broad and poorly resolved ^{1}H NMR spectra for fractions 1S and 2S indicated that these fractions were a complex mixture of oligosaccharides, and they were not studied (data not shown).

Fraction 3S. The elution volume observed by gel-filtration chromatography suggested that fraction 3S was a pentasaccharide. Fraction 3S was comprised of Man, Gal, and glycerol. The ESIMS spectrum of fraction 3S contained an ion at *m/z* 741 that corresponds to $[M + 1]^{+}$ of an oligosaccharide composed of four hexosyl residues and glycerol. The ESIMS also contained an ion at *m/z* 579 corresponding to $[M + 1]^{+}$ of an oligosaccharide comprised of three hexosyl residues and glycerol. This fragment is the major component of fraction 4S (see below). Its presence did not interfere with the characterization of the tetraglycosyl-glycerol since it accounted for only a small portion of fraction 3S. Four major uniquely linked sugar residues were identified by methylation analysis: (1) terminal Man, (2) 3-*O*-linked Man, (3) 4-*O*-linked Gal, and (4) 3-*O*-linked Gal (Table 2). The linkage of the glycerol cannot be determined by this method since the methylated derivative is very volatile and is lost during sample work-up. The data suggested that fraction 3S was a linear tetraglycosyl-glycerol.

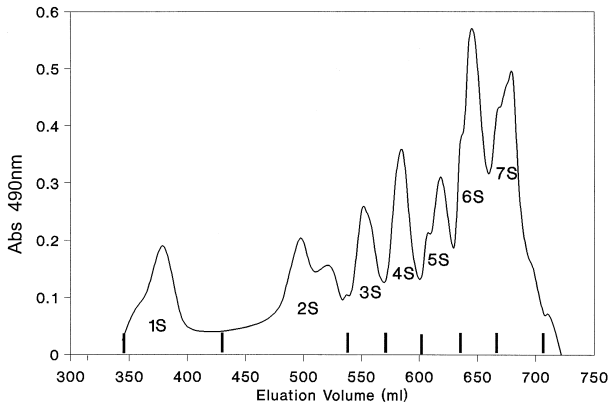


Fig. 3. Gel-filtration chromatography (Bio-Gel P2, 186× 1.5 cm) of the oligosaccharides generated by Smith degradation of GalXM-A. Seven fractions (1S–7S) were pooled as shown.

Table 2
Methylation analysis of oligosaccharides obtained after Smith degradation[a]

Derivative identified	Fraction mol %					Deduced linkage
	3S	4S	5S	6S	7S	
2,3,4,6-Man[b]	30	36	46	21	51	T-Man[c]
2,3,4,6-Gal	3	0	5	61	13	T-Gal
2,4,6-Man	24	9	36	12	33	3-*O*-Man
2,4,6-Gal	19	25	4	1	0	3-*O*-Gal
2,3,6-Gal	18	23	3	1	0	4-*O*-Gal
2,3,4-Man	2	3	2	1	1	6-*O*-Man
2,3,4-Gal	3	3	3	1	<1	6-*O*-Gal
4,6-Gal	0	0	1	2	1	2,3-di-*O*-Gal

[a]The methylated alditol acetates were identified by their relative retention time with the internal standard, *myo*-inositol. The retention times were obtained by using partially methylated, alditol acetate standards for each of the monosaccharides.
[b]1,5-Di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methyl-D-mannitol.
[c]Nonreducing terminus.

Four anomeric signals were observed by ^{1}H spectroscopy (Fig. 4A). The residues were labeled A–D, in order of their chemical shifts from low field to high field in the ^{1}H anomeric region, to facilitate the description of the subsequent 2D NMR experiments. The signals at 5.13 ppm ($^{3}J_{1,2} < 1$ Hz), 4.97 ppm ($^{3}J_{1,2}$ 3.2 Hz), 4.85 ppm ($^{3}J_{1,2}$ 1.5 Hz), and 4.64 ppm ($^{3}J_{1,2}$ 7.9 Hz) were assigned to α-Man (A), α-Gal (B), and α-Man (C), and to β-Gal (D), respectively, based on their coupling constants ($^{3}J_{1,2}$) (Fig. 4A) [41].

The protons of the α-Man residues (A and C), H-1 through H-4 of the α- and β-Gal residues (B and D), and the five protons of the terminal glycerol (E) were identified by the DQF-COSY experiment (Fig. 5, Table 3). The cross peaks for Man (A) are traced in

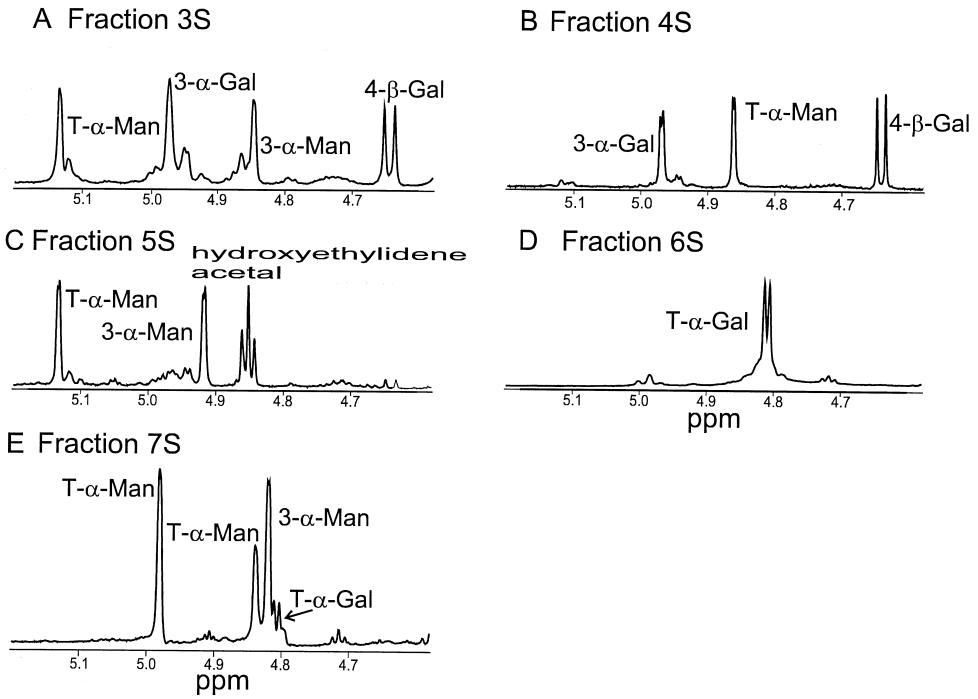


Fig. 4. 1D ^{1}H NMR spectra recorded at 500 MHz and 43 °C of the fractions 3S–7S (A–E) obtained after Bio-Gel P2 chromatography of the fragments generated by Smith degradation of GalXM-A.

Fig. 5 and the proton chemical shifts are given in Table 3. Similarly, all the protons of Man (C) were assigned (Table 3).

A combination of DQF-COSY, TOCSY, and GHSQC experiments was required to assign all the chemical shifts for Gal (B). The cross peak Gal B (2,1) was identified in the DQF-COSY spectrum (Fig. 5). Examination of the GHSQC spectrum (Fig. 6) showed that the proton chemical shifts of H-2 and H-3 of Gal (B) were degenerate at 4.02 ppm. Due to this degeneracy, the cross peak B (2,3) in DQF-COSY (Fig. 5) was obscured; it was on the diagonal. The cross peak of Gal B (3,4) was traced from the Gal B (2,3) cross peak (Fig. 5). We identified protons H-5 and H-6 of Gal (B) from the TOCSY trace through H-4 (spectrum not shown).

Despite the overlap in the chemical shifts of protons H-5 and H-6 of Gal (D), their chemical shifts values were obtained from the GHSQC spectrum (Fig. 6) because they were separated in the ^{13}C dimension. The ^{13}C chemical shifts of the tetraglycosyl-glycerol were assigned by correlation to the attached proton(s) ($^{1}J_{CH}$) observed in the GHSQC experiment (Fig. 6). For example, the cross peak A1 showed an H-1 signal at 5.13 ppm and a corresponding C-1 signal at 101.39 ppm for Man (A). Similarly, the cross peaks B1, C1 and D1 were identified by the H-1/C-1 connectivities of the Gal (B), Man (C) and Gal (D), respectively (Fig. 6, Table 3). The same process was used to identify the remaining carbons for the four hexoses and the three carbons of glycerol.

The ^{1}H and ^{13}C assignments of fraction 3S (Table 3), were used in conjunction with the data from the

Table 3
^{1}H and ^{13}C chemical shifts (ppm) of fraction 3S obtained after Smith degradation of GalXM-A

	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	C-1	C-2	C-3	C-4	C-5	C-6	$^{1}J_{C-1,H-1}$ (Hz)
α-Man (A)	5.13	4.07	3.88	3.65	3.79	3.89	3.75	101.39	69.30	69.53	66.18	72.50	60.37	171
α-Gal (B)	4.97	4.02	4.02	4.22	3.95	3.73[a]	3.73[a]	97.63	66.69	78.82	68.24	69.77	60.20	171
α-Man (C)	4.85	4.16	3.95	3.83	4.06	3.80[a]	3.80[a]	100.96	69.00	77.46	65.07	72.51	59.71	170
β-Gal (D)	4.64	3.61	3.73	4.04	3.74	3.72[a]	3.72[a]	103.70	70.37	71.31	76.86	74.23	59.46	164
Gro (E)	3.75, 3.59	3.95	3.68, 3.62					67.98	69.58	61.67				

[a]The chemical shifts for H-6/H-6′ are degenerate.

HMBC experiment (Fig. 7) to assign the glycosidic linkage, the anomeric configuration, and the sequence of the tetraglycosyl-glycerol. For example, the cross peak C H-3/A1 at 3.95/101.39 ppm showed the connectivity across the glycosidic linkage between the Man (A) and Man (C); (Fig. 7). Similarly, the cross peaks D H-4/C1, B H-3/D1, suggested that the α-Man (C) was linked to position 4 of β-Gal (D) and that β-Gal (D) was linked to position 3 of α-Gal (B). The cross peaks E H1/B1 and E H1′/B1 were assigned to the glycosidic linkage between α-Gal (B) and glycerol (E) (Fig. 7). The $^{1}J_{C-1,H-1}$ coupling constants for the anomeric resonances were 171 Hz for the α-Man (A and C), 170 Hz for α-Gal (B), and 164 Hz for β-Gal (D) (Fig. 7). The accepted $^{1}J_{C-1,H-1}$ coupling constants are 170 ± 2 Hz and 161 ± 1 Hz for α and β anomers, respectively [42]. Taken together, these results establish that the tetraglycosyl-glycerol in fraction 3S has the sequence **1**:

α-Man (A)-(1 → 3)-α-Man (C)-(1 → 4)
-β-Gal-(D)-(1 → 3)-α-Gal (B)-(1 → 1)
-glycerol (E) (3S)

1

Fraction 4S. Gel-filtration chromatography suggested that fraction 4S was a trisaccharide. Fraction 4S was comprised of Man, Gal, and glycerol. Three major residues were identified by methylation analysis of fraction 4S: (1) T-Man (C), (2) 4-*O*-linked Gal (D), and (3) 3-*O*-linked Gal (C) (Table 2). The ^{1}H NMR spectrum of fraction 4S contained three anomeric protons resonances at 4.97 ppm ($^{3}J_{1,2}$ 2.5 Hz), 4.85 ppm ($^{3}J_{1,2}$ 1.7 Hz), and 4.64 ppm ($^{3}J_{1,2}$ 7.8 Hz) that were assigned to α-Gal (B), α-Man (C), and β-Gal

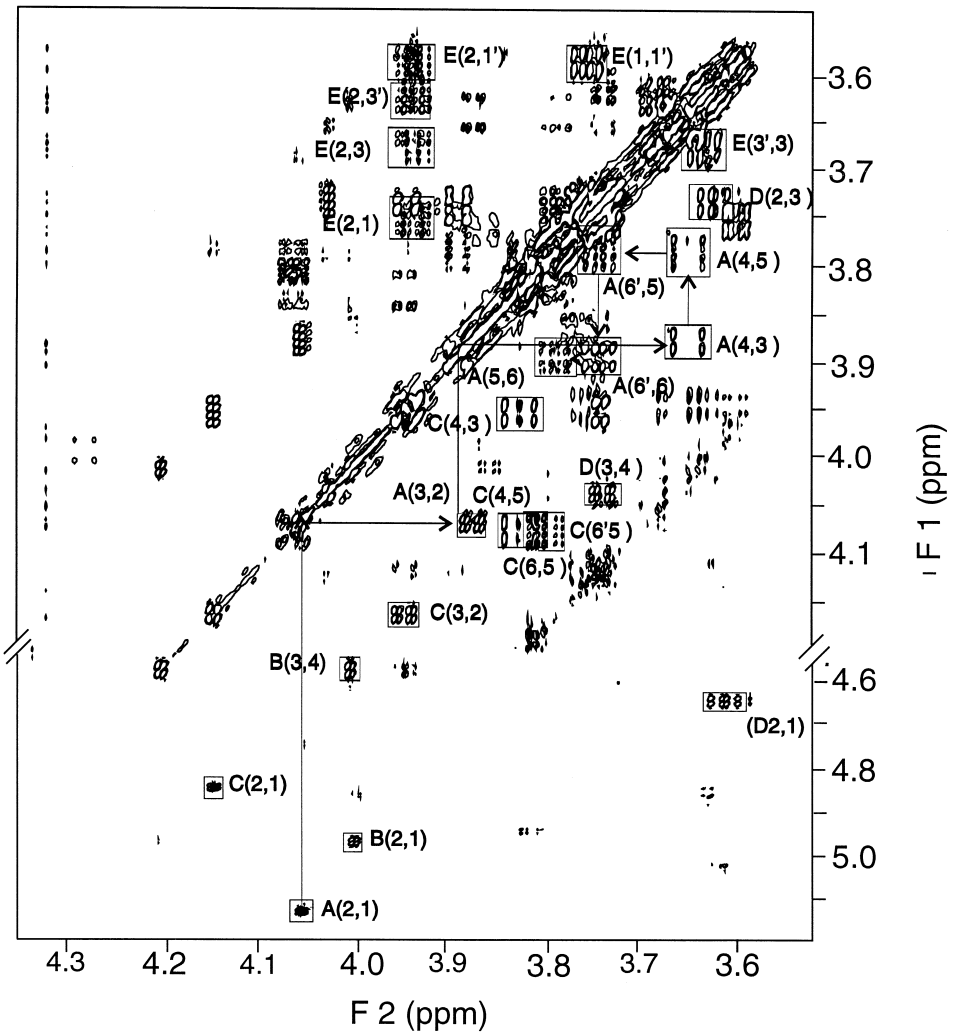


Fig. 5. Phase sensitive DQF-COSY spectrum recorded at 600 MHz and 43 °C of fraction 3S obtained after Smith degradation of GalXM-A.

(D), respectively (Fig. 4B, Table 4). The ^{1}H NMR spectrum of the anomeric region of fraction 4S was similar to that of fraction 3S except for the fact that the spectrum of 4S lacked the signal for α-Man (A) at 5.13 ppm. All ^{1}H and ^{13}C NMR signals for fraction 4S were assigned as discussed above for fraction 4S. The sequence of the glycosyl residues in fraction 4S was determined by an HMBC experiment. Methylation analysis (Table 2) also indicated the absence of a second Man residue. Therefore, fraction 4S may have evolved by the loss of the terminal Man (A) of **1**. These results establish that the triglycosylglycerol in fraction 4S has the sequence **2**:

α-Man (C)-(1 → 4)-β-Gal (D)-(1 → 3)

-α-Gal (B)-(1 → 1)- glycerol (E) (4S)

2

Fraction 5S. The elution volume observed by gel-filtration chromatography suggested that fraction 5S was either a tri- or a tetrasaccharide. Fraction 5S was comprised of Man, Gal, and threitol. The ESIMS spectrum of fraction 5S contained an ion at *m/z* 489

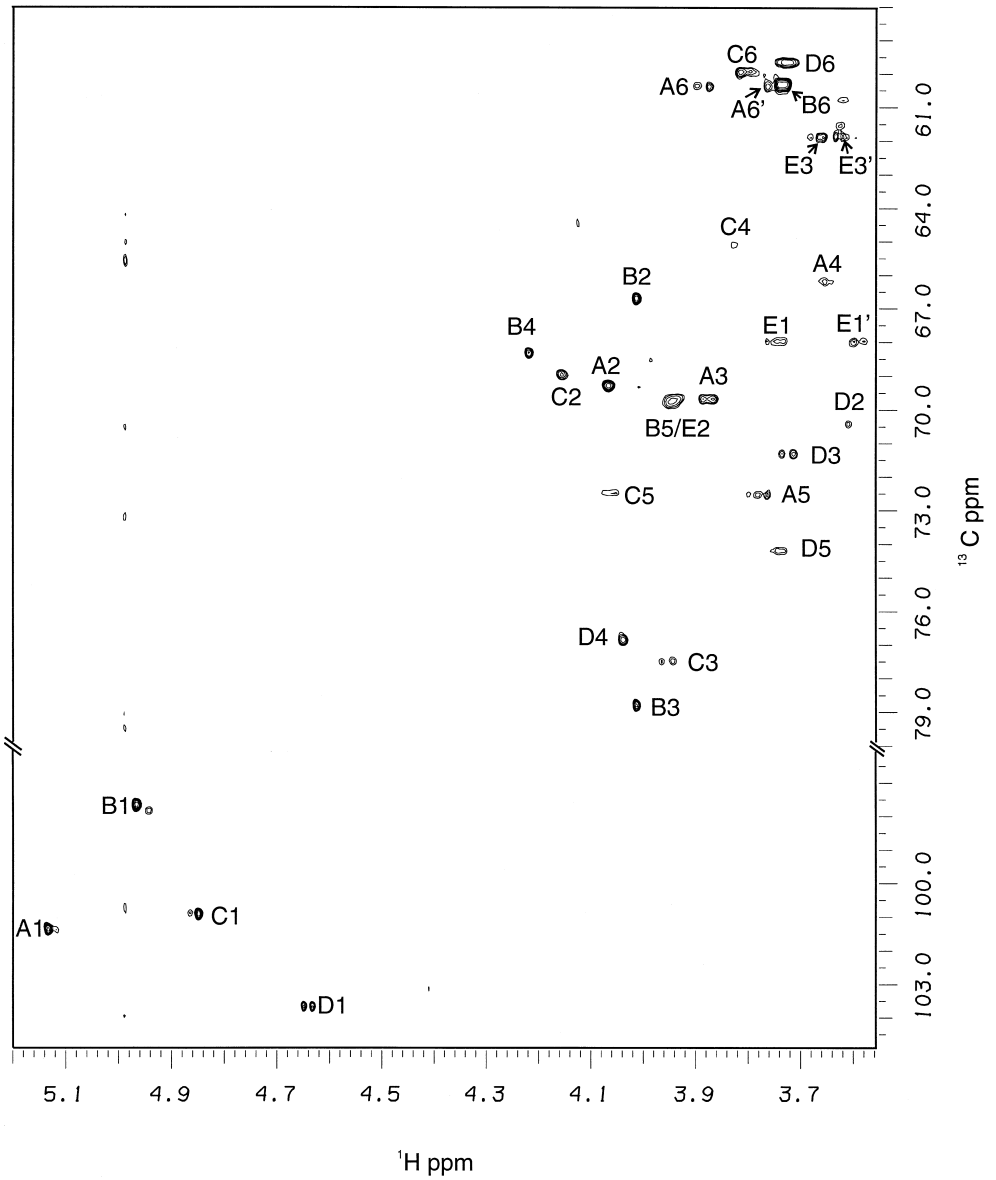


Fig. 6. 2D gradient-enhanced HSQC spectrum recorded 500 MHz of fraction 3S obtained after Smith degradation of GalXM-A.

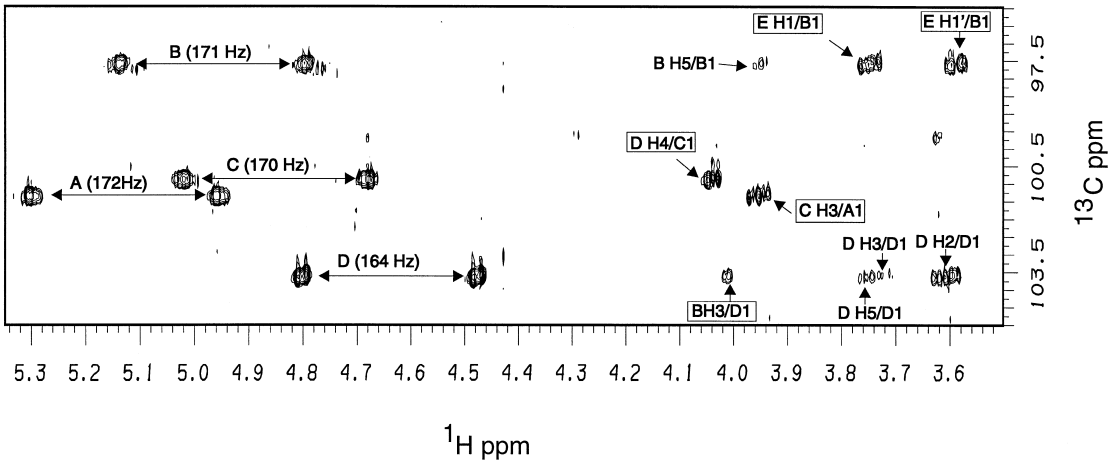
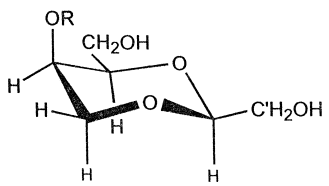


Fig. 7. 2D gradient-enhanced HMBC spectrum recorded 500 MHz of fraction 3S obtained after Smith degradation of GalXM-A.

that corresponds to $[M + 1]^+$ of an oligosaccharide composed of two hexosyl residues and hydroxyethylidene acetal, **3**:



3

The hydroxyethylidene acetal is believed to originate by acid-catalyzed transacetalation of the glycoaldehyde moiety formed by oxidation of the 4-linked Gal [43,44]. Fraction 5S was comprised of Man, Gal, glycerol, and threitol. The threitol is generated by acid hydrolysis of the *O*-2-hydroxyethylidene acetal. Two major residues were identified by methylation analysis: (1) T-Man (A) and (2) 3-*O*-linked Man (C) (Table 2). Small amounts of other oligosaccharides comprised of Man, Gal, glycerol, and threitol were also present in fraction 5S. The presence of these oligosaccharides did not interfere with the structural analysis of fraction 5S.

Three resonances were observed in the anomeric regions of both the ^{1}H (Fig. 4C) and ^{13}C (not shown) NMR spectra. The signals at 5.13 ppm ($^3J_{1,2}$ 1.3 Hz) and at 4.92 ppm ($^3J_{1,2}$ 1.5 Hz) were assigned to the T-Man (A) and the 3-*O*-linked Man (C), respectively (Table 4). The triplet observed in the proton spectrum at 4.85 ppm ($^3J_{1,2}$ 9.2 Hz) was assigned to the proton (H-1) nested between the two oxygens in the six-membered ring coupled to the protons of C′H$_2$OH of the hydroxyethylidene acetal, **3** [43,44]. All ^{1}H and ^{13}C NMR signals for fraction 5S were assigned as discussed above. The sequence of the glycosyl residues in fraction 5S was determined by an HMBC experiment. These result established that the diglycosyl-hydroxyethylidene acetal in fraction 5S has the sequence **4**:

α-Man (A)-(1 → 3)-α-Man (C)-(1 → 2)

-hydroxyethylidene acetal (5S)

4

Table 4
Chemical shifts (ppm) of anomeric proton of fractions 3S–7S oligosaccharides obtained after Smith degradation of GalXM-A

	T-Man	3-*O*-Man	4-*O*-Gal	3-*O*-Gal	Acetal[a]
Fraction 3S	5.13	4.85	4.65	4.97	–
Fraction 4S	4.86	–	4.64	4.97	–
Fraction 5S	5.13	4.92	–	–	4.85
Fraction 6S	–	–	–	4.81	–
Fraction 7S	4.98, 4.84	4.82	–	4.81	–

[a]Acetal: six-membered *O*-2′-hydroxyethylidene acetal.

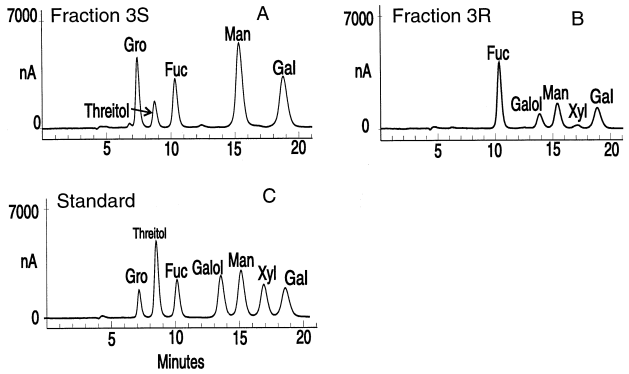


Fig. 8. HPAEC-PAD of the monosaccharides and alditols released by acid hydrolysis of the oligosaccharides obtained after Smith degradation, acetolysis and reduction of GalXM-A. (A) Fraction 3S. (B) Fraction 3R. (C) Authentic standards.

Fraction 6S. The elution volume observed by gel-filtration chromatography suggested that fraction 6S was a disaccharide. Fraction 6S was comprised of Gal and glycerol. One major residue, T-Gal (B), was identified by methylation analysis of fraction 6S (Table 2). The monoglycosyl-glycerol in fraction 6S was identified by ^{1}H and ^{13}C NMR spectroscopy. The ^{1}H NMR spectrum for fraction 6S had one anomeric proton at 4.81 ppm ($^{3}J_{1,2}$ 3.7 Hz) (Fig. 5D). Similarly, the ^{13}C NMR spectrum showed one anomeric signal at 98.04 ppm (spectrum not shown). These results established that the monoglycosyl-glycerol in fraction 6S has the sequence **5**:

$$\alpha\text{-Gal}\,(B)\text{-}(1\rightarrow 1)\text{-glycerol}\,(E) \qquad (6S)$$
5

Fraction 7S. Fraction 7S was comprised of Man, Gal, glycerol, and threitol. Three major residues were identified by methylation analysis: (1) T-Man (A and C), (2) 3-*O*-linked Man (C), and (3) T-Gal (B) (Table 2). The ^{1}H (Fig. 4E, Table 4) and ^{13}C NMR (spectrum not shown) spectroscopy of fraction 7S was consistent with the presence of three α-linked Man residues and an α-linked Gal residue (B). Based on these results and the structures **1–4** assigned previously, we propose that fraction 7S contains one diglycosyl-threitol **6**, one monoglycosyl-glycerol **7**, and one monoglycosyl-threitol **8**:

$$\alpha\text{-Man}\,(A)\text{-}(1\rightarrow 3)\text{-}\alpha\text{-Man}\,(C)\text{-}(1\rightarrow 2)$$
$$\text{-threitol}\,(D) \qquad (7.1S)$$
6

$$\alpha\text{-Gal}\,(B)\text{-}(1\rightarrow 1)\text{-glycerol}\,(E) \qquad (7.2S)$$
7

$$\alpha\text{-Man}\,(C)\text{-}(1\rightarrow 2)\text{-threitol}\,(D) \qquad (7.3S)$$
8

Selective fragmentation by acetolysis and reduction.—The degradation products resulting from acetolysis and reduction of GalXM-A were separated into six oligosaccharides by gel-filtration chromatography (fractions 1R–6R). Fractions 1R and 2R were complex mixtures of oligosaccharides, and they were not studied. Fractions 3R–6R were comprised of Man, Gal, Xyl(trace), and galactitol. Peak 6R was a mixture of monosaccharides produced by random cleavage of GalXM-A. This fraction was not analyzed because pertinent information about the primary structure of GalXM would not be obtained. The structures of the oligosaccharides from fractions 3R–5R were determined using the analytical strategies applied previously for the products of Smith degradation.

Fraction 3R. The elution volume observed by gel-filtration chromatography suggested that fraction 3R was a pentasaccharide. Fraction 3R was comprised of Man, Gal, and galactitol Fig. 8. The ESIMS spectrum of fraction 3R contained an ion at *m/z* 831 that corresponds to $[M + 1]^{+}$ of an oligosaccharide composed of four hexosyl residues and an hexitol. Four

Table 5
Methylation analysis of oligosaccharides obtained after acetolysis and reduction[a]

Derivative identified	Mol %		Deduced linkage
	3R	5R	
2,3,4,6-Xyl[b]	4	2	T-Xyl[c]
2,3,4,6-Man	23	24	T-Man
2,3,5,6-Gal*f*	3	2	T-Gal*f*
2,3,4,6-Gal	8	2	T-Gal
2,4,6-Man	19	36	3-*O*-Man
2,4,6-Gal	12	0	3-*O*-Gal
2,3,6-Gal	19	34	4-*O*-Gal
2,3,4-Man	2	2	6-*O*-Man
2,3,4-Gal	5	0	6-*O*-Gal
4,6-Gal	3	0	2,3-di-*O*-Man

[a]The methylated alditol acetates were identified by their retention times relative to *myo*-inositol. The retention times were obtained by using authentic standards for each of the monosaccharides. Peaks corresponding to 6-*O*-galactitol (3R) or 3-*O*-galactitol (5R) were detected, but their identities could not be confirmed by GLC-MS because lack of appropriate standards.
[b]1,5-Di-*O*-acetyl-1-deuterio-2,3,4-tri-*O*-methyl-D-xylitol.
[c]Nonreducing terminus.

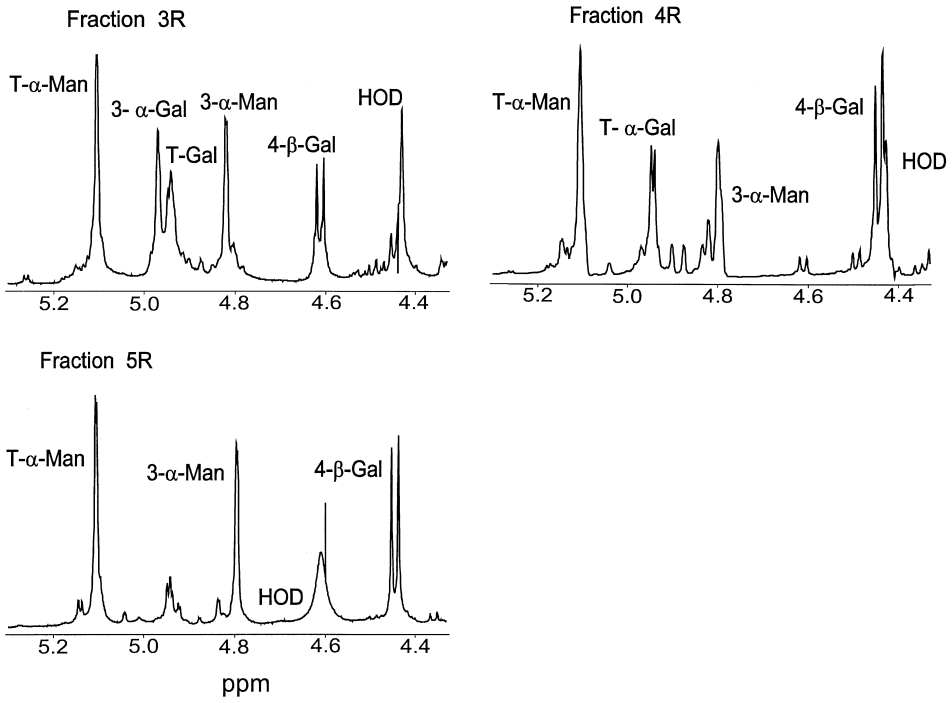


Fig. 9. 1D ^{1}H NMR spectra recorded at 600 MHz of fractions 3R–5R obtained after acetolysis and reduction of GalXM-A.

major residues were identified by methylation analysis: (1) T-Man (A); (2) 3-*O*-linked Man (C); (3) 3-*O*-linked Gal (B); and (4) 4-*O*-linked Gal (D) (Table 5). A derivative probably due to 6-*O*-galactitol (E) was detected, but its identity could not be confirmed by GLC–MS because of the lack of an appropriate standard. Five major anomeric signals were observed by ^{1}H NMR spectroscopy (Fig. 9 and Table 6, 3R). These signals were assigned to specific residues based on the observed coupling constants and the similarities of the NMR signals to those present in **1**. The glycosyl sequence of the tetraglycosyl-galactitol was obtained by DQF-COSY, TOCSY, GHSQC, and HMBC experiments (data not shown). An additional α-Gal with a chemical shift at 4.94 ppm was detected but no connectivity to any other residues was observed (Fig. 9). These results established that the tetraglycosyl-galactitol in fraction 3R has the sequence **9**:

α-Man (A)-(1 → 3)-α-Man (C)-(1 → 4)-β-Gal (D)
-(1 → 3)-α-Gal (B)-(1 → 6)- galactitol (E) (3R)

9

Fraction 4R. The elution volume observed by gel-filtration chromatography suggested that fraction 4R was a pentasaccharide. Fraction 4R was comprised of Man, Gal, and galactitol. Terminal α-Man (A), 3-*O*-linked α-Man (C), 4-*O*-linked β-Gal (D), and a terminal Gal (E) were the four major anomeric signals observed by 1D ^{1}H NMR spectroscopy (Fig. 9 and Table 6, 4R). The ^{1}H NMR spectra of peaks 3R and 4R were similar except for the absence of the signal for the 3-*O*-linked α-Gal (B) and the upfield shift for β-Gal (D) in fraction 4R. The difference in

Table 6
Chemical shifts (ppm) of anomeric proton of fractions 3R–5R obtained after acetolysis and reduction

	T-Man	3-*O*-Man	4-*O*-Gal	3-*O*-Gal	Gal	T-Gal
Fraction 3R	5.11	4.82	4.61	4.97	4.94	–
Fraction 4R	5.11	4.80	4.49	–	–	4.94
Fraction 5R	5.11	4.80	4.44	–	–	–

the chemical shift values of the β-Gal (D) residues of oligosaccharides of fractions 3R and 4R suggested that these fragments were homologous but not identical. An HMBC experiment established that in fraction 4R the β-Gal (D) was attached to position 3 of galactitol. Thus, in this fragment, the galactitol is linked at positions 3 and 6. We propose that the tetraglycosyl-galactitol has the structure **10**:

α-Man(A)-(1→3)-α-Man(C)-(1→4)-β-Gal(D)-(1→3)-galactitol (B) (4R)

6

↑

1

α-Gal(E)

10

Fraction 5R. The elution volume obtained by gel-filtration chromatography indicated that peak 5R was a tetrasaccharide. Fraction 5R was comprised of Man, Gal, and galactitol. The ESIMS spectrum of fraction 5R contained an ion at *m/z* 669 that corresponds to $[M + 1]^+$ of an oligosaccharide composed of three hexosyl residues and hexitol. Four major uniquely linked sugar residues were identified by methylation analysis: (1) T-Man (A), (2) 3-*O*-linked Man (C), (3) 4-*O*-linked Gal (D), and (4) Galactitol (B) (Table 5). Three major anomeric signals of approximately equal intensity were observed by 1D ^{1}H NMR (Fig. 9). They were assigned as terminal α-Man (A), 3-*O*-linked α-Man (C), and 4-*O*-linked β-Gal (D) (Table 5). These results establish the sequence of triglycosyl-galactitol as **11**:

α-Man (A)-(1 → 3)-α-Man (C)-(1 → 4)

- β-Gal (D)-(1 → 3)-galactitol (B) (5R)

11

4. Discussion

Methylation analysis of GalXM-I indicated that 44% of the Man and Gal residues were 3-*O*-linked, and that 31% of the Gal were 6-*O*-linked. Based on these data, two degradation procedures were selected:

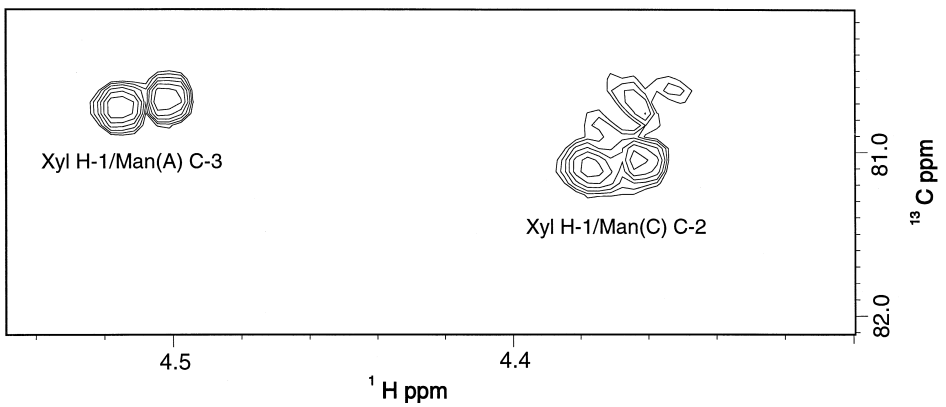


Fig. 10. Putative structure of GalXM. The exact sequence and frequency of occurrence of the oligosaccharides branches 1–4 could not be determined.

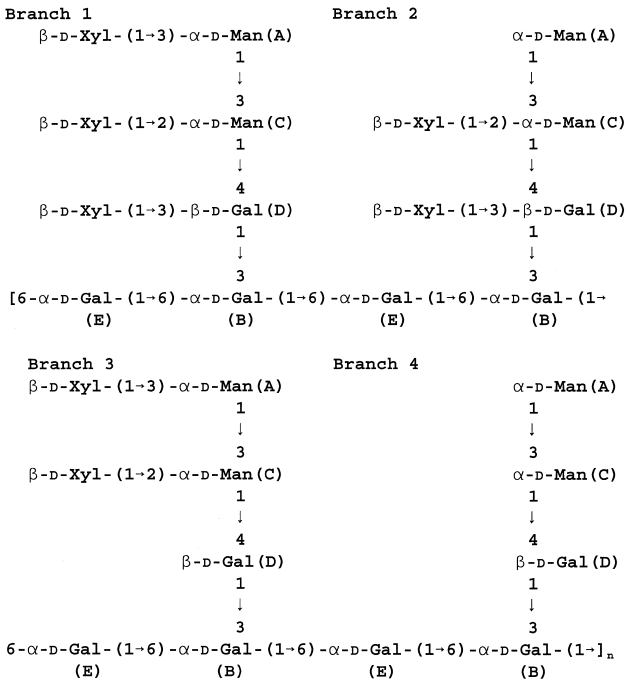


Fig. 11. 2D gradient-enhnaced HMBC spectrum recorded at 600 MHz of GalXM-I.

(1) Smith degradation [to take advantage of the resistance of (1 → 3)-linked residues to oxidation by sodium periodate], and (2) acetolysis [to take advantage of the selective cleavage of (1 → 6)-linked Gal residues]. GalXM-A was used as starting material for the fragmentation experiments since the small amount of residual protein and glucan did not affect the composition of the oligosaccharides resolved later by gel-filtration chromatography.

Smith degradation resulted in the formation of an homologous series of oligosaccharides that arose from the variation in the substitution of Man and Gal by Xyl residues (Fig. 10). Xyl was not present as a component of any of the Smith degradation fragments since it occurred only as a nonreducing residue. Methylation analysis of GalXM-I showed 3-*O*-linked Man, 2,3-di-*O*-linked Man, and nonreducing T-Man. Therefore, the retention of Man (A) in fraction 3S and fraction 5S was due to its substitution with Xyl at O-3 or its disubstitution with Xyl at *O*-2 and *O*-3. The identity of the disubstituted Man, Man (A) or Man (C), could not be resolved by methylation analysis or by NMR spectroscopy of the oligosaccharide fragments. However, an HMBC experiment of intact GalXM-I showed connectivity between H-1 of Xyl and C-3 of Man (Man A) at 4.51/80.76 ppm and between H-1 of Xyl and the C-2 of Man (Man C) at 4.37/81.12 ppm. This portion of the HMBC experiment is presented in (Fig. 11). No connectivity was detected for a Man disubstituted at H-2 and H-3 with Xyl. The NOESY spectrum (not shown) is consistent with the HMBC data since intact GalXM-I showed NOEs from Xyl H-1 to Man (A) H-3 (4.51/4.01 ppm) and from Xyl H-1 to Man (C) H-2 (4.37/4.10 ppm). It is likely that Man (A) was 3-*O*-linked and Man (C) was 2,3-di-*O*-linked in GalXM. Man (C) will always survive oxidation regardless of further substitution by Xyl since it is 3-*O*-linked (Fig. 10, branches 1 and 3). The 4-*O*-linked Gal (D) must be substituted with Xyl at O-2 or O-3, since it survived sodium periodate oxidation. Methylation analysis of GalXM-I indicated the presence of 3,4-di-*O*-linked Gal. Therefore, the retention of Gal (D) in 3S was due to the presence of Xyl linked at O-3. Based on these data, the primary sequence that gave rise to oligosaccharide **1** was formulated as depicted in Fig. 10, branch 1. Evidence for the occurrence of other sequences or block structures at low frequency due to microheterogeneity was not observed; however, they cannot be ruled out unequivocally.

Methylation analysis of GalXM-I showed a terminal Man residue that would be susceptible to periodate oxidation. The oligosaccharide **2** is homologous to the oligosaccharide **1** except for the absence of Man (A). Based on these data, the primary sequence that gave rise to oligosaccharide **2** was formulated as depicted in Fig. 10, branch 2.

The oligosaccharide **4** arose from a pentasaccharide sequence homologous to branch 1, Fig. 10, that was substituted on terminal Man (A) but lacked a Xyl linked O-3 to Gal (D). The glycolaldehyde moiety formed by oxidation of Gal (D) underwent acid-catalyzed transacetalation to give a six-membered *O*-2′-hydroxyethylidene acetal that was detected in the 1D ^{1}H NMR spectrum (Fig. 4C). Acid hydrolysis of **4** resulted in the cleavage of the six-membered *O*-2′-hydroxyethylidene acetal **3** with the concomitant release of threitol. Threitol could only arise from 4-*O*-linked Gal present in GalXM. Based on these data, the primary sequence that gave rise to the diglycosyl-hydroxyethylidene acetal **4** was formulated as depicted in Fig. 10, branch 3. The ratios of the oligosaccharides **1**, **2**, and **4** were approximately 1:1:1. These structures represent 92% of the GalXM structure. Therefore, *β*-4-*O*-Gal is substituted 57% of the time (branches 1 and 2) and unsubstituted 43% of the time (branches 3 and 4).

The monoglycosyl-threitol **8** would arise due to the microheterogeneity in the Xyl substitution patterns of the putative GalXM structure other than those de-

picted in Fig. 10. The presence of α-Gal (B)-(1 → 1′)-glycerol (E) sequence in **1** and **2** suggested that terminal Gal (B) residues of **1** and **2** were linked α-(1 → 6). The presence of α-Gal (B)-(1 → 1)-glycerol (E) **7** would be generated by cleavage of the galactan backbone of the putative GalXM structure where Man (A), Man (C) and Gal (D) were not substituted with Xyl (Fig. 10, branch 4).

Six different oligosaccharides produced by acetolysis and reduction reflected the disposition of the (1 → 6) linkages in the parent GalXM structure. Three of these oligosaccharides, **9**, **10**, and **11**, were an homologous series of oligosaccharides terminating in galactitol. Since all the oligosaccharides ended in galactitol, and considering the chemical specificity of acetolysis, we formulated a putative model of GalXM as a galactan backbone comprised of α-(1 → 6)-linked residues (Fig. 10). Evidence for a Gal-mannitol linkage was not obtained; this suggested that Man was not a component of the GalXM backbone. Oligosaccharides **1** and **9** were identical in all respects except for residue (E). The structural identity of residue (E) was related to the type of fragmentation used: it was glycerol in the case of Smith degradation and galactitol in the case of acetolysis and reduction. The generation of oligosaccharides **9** and **10** suggested that Gal (B) was sandwiched between Gal (E)-linked (1 → 6) and that Gal (B) was the point of origination of branches 1–4.

The only apparent difference between **9** and **10** was in the disposition of the terminal Gal and galactitol. The terminal Gal (B) in **10** was nested between two Gal residues, and each residue was linked α-(1 → 6). This supports the presence of the proposed α-(1 → 6)-galactan backbone which was cleaved to form oligosaccharides **9**, **10**, and **11** by cleavage of the α-(1 → 6)-linked Gal residues by acetolysis.

The putative structure given in Fig. 10 agrees with the data obtained by methylation analysis and by NMR spectroscopy of GalXM and of the degradation products derived from it.

We were unable to confirm the presence of a substituent on Gal (D) by a 2D NMR NOESY experiment of GalXM-I. However, the substituent had to be Xyl based on the sequence data available for all the oligosaccharides. We were unable to determine the linkage of the small quantity of terminal Gal*f* due to the complexity of the NMR spectra of GalXM.

In summary, GalXM of *C. neoformans* is a complex polysaccharide with an α-(1 → 6) galactan backbone. It is a branched polysaccharide in which every other Gal of the backbone is substituted at O-3. This is the point of origination of the oligosaccharide branches comprised of α-Man-(1 → 3)-α-Man-(1 → 4)-β-Gal-substituted with zero to three terminal β-Xyl residues (Fig. 10). The exact sequence and frequency of occurrence of the oligosaccharides branches 1–4 could not be determined.

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