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Galactoxylomannans of *Cryptococcus neoformans*

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Galactoxylomannans (GalXMs) from single isolates of *Cryptococcus neoformans* serotypes A, B, and D were isolated from culture supernatants and then purified by affinity, ion-exchange, and gel-filtration chromatography. GalXMs are a group of closely related complex polysaccharides. GalXMs from serotypes A (9759 A) and C (3183 C) and an acapsular mutant of serotype D (Cap67 D) have similar galactose, xylose, and mannose molar ratios, but each has some unique structural features. GalXM 9759 A and GalXM 3183 C were associated with a starchlike glucan that was removed during purification. Only a trace of glucose was detected in the Cap67 D GalXM. Gas-liquid chromatography-mass spectroscopy of per-*O*-methylated polysaccharides and ^{13}C nuclear magnetic resonance spectroscopy showed that GalXM is a complex branched polysaccharide. The main chain consists of mannose or galactose or alternating mannose and galactose residues. Xylose is present only as nonreducing termini. Galactofuranose occurs only in 3183 C and Cap67 D, and it is always present as nonreducing termini.

People are frequently exposed to *Cryptococcus neoformans* by the inhalation of desiccated cells found in the environment. This natural exposure rarely results in the clinical symptoms associated with cryptococcosis because the innate cellular resistance mechanisms present in the lungs effectively clear the invaders. In the few cases where the organism does cause infection, it is self-limiting (15). In patients whose immune system is debilitated by chemotherapy or by an illness, it is more likely that exposure to *C. neoformans* will lead to disease. Cryptococcosis has emerged as a major opportunistic disease in patients diagnosed with AIDS (16). It is the fourth most common life-threatening infection in AIDS patients after *Pneumocystis carinii*, cytomegalovirus, and *Mycobacterium avium-M. intercellulare* (3). As many as 10% of patients diagnosed with AIDS are also suffering from cryptococcosis (42). If the progression of the disease is not curtailed, then dissemination to other tissues results in a life-threatening situation (15). The yeast has a predilection for the central nervous system, where it causes cryptococcal meningoencephalitis (15). The cell envelope of *C. neoformans* is composed of the following: a rigid cell wall, constituted mainly of glucans (22); a capsular polysaccharide, glucuronoxylomannan (GXM), composed of mannose (Man), xylose (Xyl), glucuronic acid, and *O*-acetyl (2, 8, 37); and at least two minor carbohydrate antigens, galactoxylomannan (GalXM) and mannoprotein (MP) (13, 38). GXM, a viscous polysaccharide, composes about 88% of the capsule mass, and GalXM and MP together compose approximately 12% (13). GXM, GalXM, and MP are isolated from growth medium by selective precipitation with ethanol and differential complexation with hexadecyltrimethylammonium bromide (11–13). GalXM and MP can be fractionated by concanavalin A (ConA) chromatography because of the affinity that the nonreducing and (*O*-2)-linked mannopyranosyl residues of MP (38) have for ConA. GXM is a prominent virulence factor because it is tolerogenic and antiphagocytic (5, 6, 25, 26, 31, 33); acapsular mutants are less virulent (7, 18, 21, 24). AIDS patients have a severely restricted ability to invoke cell-mediated immunity. This additional factor makes AIDS patients particularly vulnerable to cryptococcosis, because cell-mediated immunity is a primary mechanism for clearing the yeast from the lungs (15). The cell-mediated immune response to the soluble antigens of *C. neoformans* has been studied (30). The MP modulates delayed-type hypersensitivity (32), whereas GalXM elicits little immune response (34). The role of GalXM as a virulence factor has not been studied. Recently van de Moer et al. (40) produced monoclonal antibodies (MAbs) by immunizing mice with a *C. neoformans* serotype A spheroplast lysate. One of these MAbs (CN6, an immunoglobulin M) reacts strongly with the purified GalXM described herein (39, 40). Hamilton et al. (20) described MAbs that were specific to a noncapsular exoantigen of *C. neoformans*. This exoantigen has not been characterized chemically. However, screening patient serum samples for this MAb for diagnostic purposes has been suggested. GXM is responsible for the serotype specificity of *C. neoformans* (1, 2, 8, 37), but the role of GalXM and MP in serotype specificity and pathogenicity is unknown. Herein we describe the purification and partial chemical characterization of GalXM from the following three strains of *C. neoformans*: 9759 (serotype A), 3183 (serotype C), and Cap67 (acapsular serotype D).

MATERIALS AND METHODS

Analytical methods. Neutral carbohydrate was determined by the phenol sulfuric acid method of Dubois et al. (17). The constituent monosaccharides of the polysaccharides were identified and quantified as their per-*O*-acetylated aldononitrile derivatives by gas-liquid chromatography (GLC). Samples were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120°C as previously described (36), except that the trifluoroacetic acid was removed by extraction with ether (9). Quantitative analysis by GLC was done with a Sigma 1 gas chromatograph (Perkin-Elmer) equipped with a flame ionization detector. The analyzer was fitted with an RSL-300 0.2-μm-pore-size capillary column (30 m by 0.25 mm; Applied Sciences). Helium was used as the carrier gas. The temperature program for the quantitation of per-*O*-acety-

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lated aldononitrile derivatives was 200 to 220°C at 7.5°C per min.

Methylation analysis. Dried samples (3 to 5 mg) were per-*O* methylated by the Hakomori procedure (19) as modified by Darvill et al. (14). Sep-Pak C18 Millipore cartridges (Waters Associates) were used to purify the derivatives as described by Mort et al. (29). The purified derivatives were hydrolyzed in 88% formic acid (0.5 ml) for 1 h at 100°C. The formic acid was removed in vacuo below 40°C, and the residue was then hydrolyzed in 2 M trifluoroacetic acid for 1 h at 120°C. The samples were dried in vacuo, and the per-*O*-acetylated alditol derivatives (35) were prepared. GLC-mass spectrometry (MS) was done with a separate chromatograph (Perkin-Elmer 8420) equipped with an ion-trap detector (Perkin-Elmer, GC/ITD and associated computer software). The GLC-MS analyzer was fitted with an SE-54 0.20-μm-pore-size capillary column (30 m by 0.25 mm; Supelco). Helium was used as the carrier gas. The temperature program was 120 to 220°C at 15°C per min. Methylated per-*O*-acetylated derivatives were identified by their relative elution compared with that of 2,3,4,6-tetra-*O*-methyl glucopyranose and by comparison with published mass fragments (23, 28).

NMR spectroscopy. ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a Varian VXR 400 NMR spectrometer equipped with a 10-mm multinuclear probe and operated at 100.577 MHz. Chemical shifts were measured relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate at 70°C. The deuterium resonance of the solvent, deuterium oxide, served as an internal lock. Otherwise, the spectra were recorded as previously described (10).

Cultures. Three *C. neoformans* isolates were used in this study: 9759 A (serotype A) and 3183 C (serotype C) were from E. Reiss (Centers for Disease Control, Atlanta, Ga.), and the acapsular mutant Cap67 D (serotype D parent) was from E. S. Jacobson (Veterans Administration Hospital).

Antigen purification. The procedures used for preparation and partial purification of GalXM and MP were described previously (13, 38).

(i) Ion-exchange chromatography. GalXM obtained by affinity chromatography (38) was dissolved in 0.01 M 2-amino-2-hydroxymethyl-1,3-propandiol (Tris) buffer (pH 7.6) at a concentration of 50 to 100 mg/ml, and the sample was applied to a DEAE cellulose DE 52 column (4 by 15 cm; Whatman Chemical Separations, Ltd.) equilibrated with the same buffer. The column was washed with Tris buffer, and then bound polysaccharide was eluted with 800 ml of eluent with a linear concentration gradient of 0.01 M Tris to 0.01 M Tris–1 M NaCl (pH 7.6) at a flow rate of 22 ml/h. The eluate was monitored by the phenol sulfuric acid reaction, and the fractions containing carbohydrate were appropriately pooled, dialyzed, and lyophilized.

(ii) Gel filtration. The major GalXM fraction (F-2) obtained by ion-exchange chromatography was dissolved (70 mg/ml) in 0.01 M Tris–0.15 M NaCl (pH 7.6), and the sample was chromatographed on a Sephacryl S-300 high-resolution column (1.5 by 167 cm; Pharmacia) previously equilibrated with the same buffer. The column was eluted at a flow rate of 19 ml/h, and fractions were analyzed for neutral carbohydrate. Appropriate fractions were pooled, dialyzed, and lyophilized.

RESULTS

Antigen purification. ConA affinity chromatography of GalXM-N (38) from three different strains of *C. neoformans* gave a major component (GalXM, which does not bind to ConA) and a minor component (MP, which binds to ConA and is eluted with methyl-α-D-mannopyranoside); the relative mass percentages of GalXM and MP are shown in Table 1. GalXM was fractionated by ion-exchange chromatography on DE 52 into two fractions, F-1 and F-2 (Fig. 1). The more strongly bound fraction (F-2) was the major component (Table 1). Fraction F-1 from 9759 A and Cap67 D eluted with the wash buffer, and F-2 was eluted by the linear NaCl gradient (Fig. 1a and b). GalXM from 3183 C was chromatographed similarly. However, here all the material was bound by the DE 52. Two fractions, F-1 and F-2, were eluted by the linear NaCl gradient (Fig. 1c). Since the F-2 fractions represented the majority of polysaccharide applied to the columns (Table 1) and had the lower glucose concentration (Table 2), they were chosen for additional purification and analysis. The F-2 fractions derived from 3183 C and 9759 A were purified by gel filtration column chromatography. F-2 from 3183 C was partially resolved into three fractions, P-1, P-2, and P-3 (Fig. 2a); F-2 from 9759 A was partially resolved into two fractions, P-2 and P-3 (Fig. 2b).

TABLE 1. Chromatographic purification of *C. neoformans* GalXM

Fraction	% Recovery (by wt) of GalXM from strain:		
	3183 C	9759 A	Cap67 D
ConA[a]			
MP	17.6	9.4	4.9
GalXM	45.8	67.0	51.5
DEAE[b]			
F-1	26.5	33.4	8.0
F-2	64.5	55.5	65.7
Sephacryl[c]			
P-1	17.6	0	ND[d]
P-2	33.7	21.1	ND[d]
P-3	29.9	45.6	ND[d]

[a] Fractionation by affinity chromatography on ConA-agarose.
[b] Anion-exchange column chromatography of GalXM on DE 52.
[c] Gel filtration column chromatography of F-2 on Sephacryl S-300 HR.
[d] ND, not done.

Identification and quantitation of the carbohydrate constituents. The F-1 fractions of 9759 A and 3183 C contained substantial amounts of Glc and lesser amounts of Xyl, Man, and Gal (Table 2). The major monosaccharide residues present in all three F-2 fractions were Xyl, Man, and Gal. Glc, probably present as a separate glucan, was present in the F-2 fractions of 3183 C and 9759 A. The F-1 fraction of Cap67 D contained much less Glc than was found in the corresponding peaks of 9759 A and 3183 C; no Glc was detected in the F-2 peak of Cap67 D.

Gel filtration column chromatography of the F-2 fractions from 9759 A and 3183 C removed most of the Glc, but in most cases a small quantity of a Glc-containing component copurified with GalXM (Table 3). For example, GLC analysis showed that the P-2 fractions of 9759 A and 3183 C (Table 3) were substantially lower in Glc than were the parent F-2 fractions (Table 2). The P-1, P-2, and P-3 fractions of 3183 C were also considered separate fractions because of minor differences in the molar ratios of the constituent sugars and the skewed appearance of the gel filtration elution profile (Fig. 2b).

Methylation analysis and ^{13}C NMR. The GLC-MS results (Table 4) show that GalXM contains a large variety of

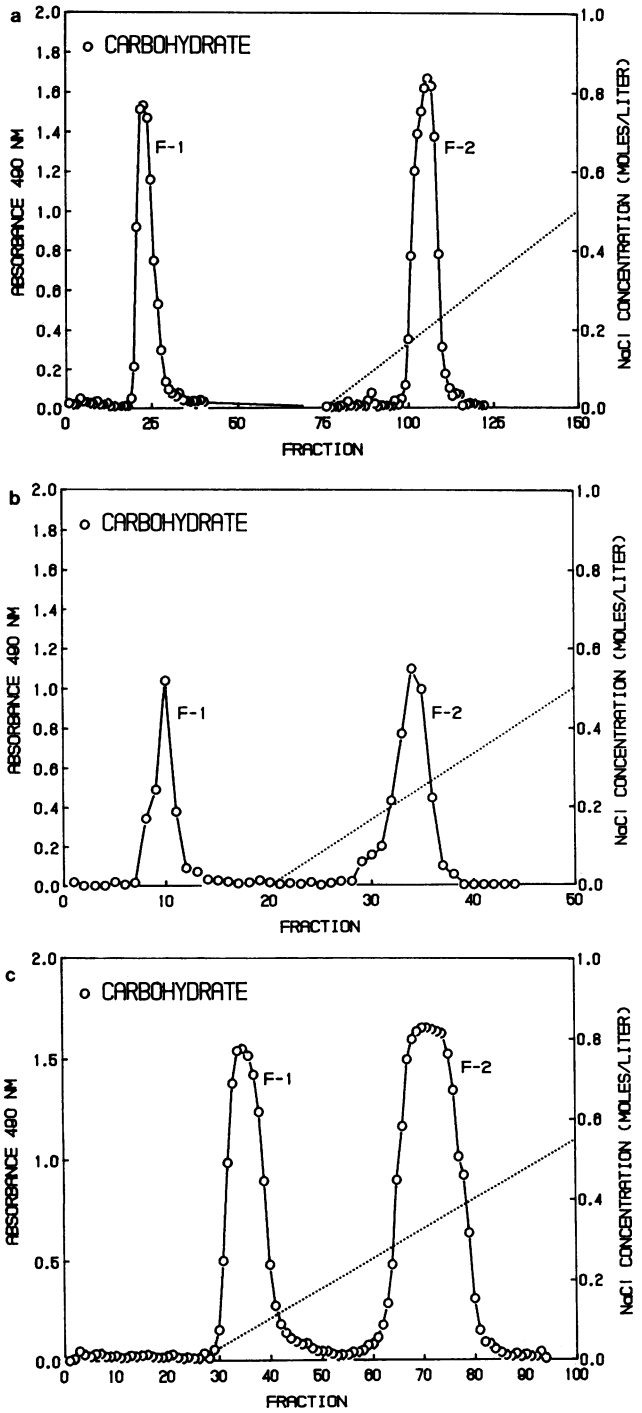

FIG. 1. Ion-exchange chromatography of GalXMs of *C. neoformans* on a column of DE 52. Carbohydrates were determined as the A_{490} in the phenol-sulfuric acid assay. (a) GalXM of 9759 A; (b) GalXM of Cap67; (c) GalXM of 3183 C.

glycosidic linkages. It is a branched structure, as evidenced by the number and amount of disubstituted methyl derivatives. A prominent distinction between the GalXM from 9759 A (P-2) and those from 3183 C (P-2) and Cap67 D (F-2) was the absence of 2,3,5,6-tetra-*O*-methylgalactofuranose in the former and the presence of 2,3,4,6-tetra-*O*-methylgalactopyranose in the latter.

TABLE 2. Monosaccharide molar ratios[a] of GalXM from *C. neoformans* after DEAE 52 chromatography

Strain	Fraction	Molar ratio			
		Xyl	Man	Glc	Gal
3183 C	F-1	1.0	1.4	35.4	1.8
	F-2	1.0	1.6	1.0	1.4
9759 A	F-1	–[b]	1.0	72.9	2.1
	F-2	1.0	1.7	2.4	1.5
Cap67 D	F-1	1.0	2.1	1.6	2.4
	F-2	1.0	1.7	–	1.6

[a] Molar ratios were determined by using GLC.
[b] –, not detected.

^{13}C NMR spectroscopy of GalXM revealed 10 to 15 different anomeric centers for each serotype. The anomeric regions of the spectra (Fig. 3) illustrated the complex nature of the molecules and their structural relationships. The anomeric regions also showed the presence of the galactofuranose anomeric centers (4) for 3183 C and Cap67 D, which have chemical shifts of approximately 112 ppm, and the absence of this derivative in 9759 A GalXM.

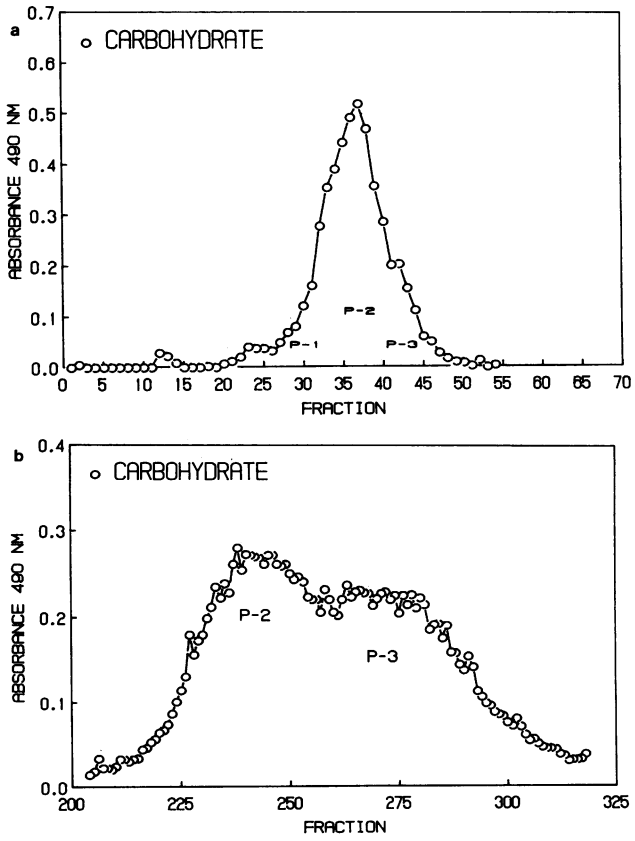

FIG. 2. Gel filtration chromatography of GalXM DE 52 F-2 fractions on a column of Sephacryl S-300 HR. (a) 3183 C (column a, 1.5 by 167 cm) (P-1, tubes 24 through 34; P-2, tubes 35 through 40; P-3, tubes 41 through 47); (b) 9759 A (column b, 4.0 by 170 cm) (P-2, tubes 218 through 252; P-3, tubes 253 through 310).

TABLE 3. Monosaccharide molar ratios[a] of GalXM from *C. neoformans* after gel filtration column chromatography on Sephacryl S-300 HR

Strain	Fraction	Molar ratio			
		Xyl	Man	Glc	Gal
3183 C	P-1	1.0	1.2	0.1	0.5
	P-2	1.0	1.6	0.3	1.9
	P-3	1.0	1.3	6.7	1.1
9759 A	P-2	1.0	1.4	0.0	1.5
	P-3	1.0	1.4	0.1	1.7

[a] Molar ratios were determined by using GLC.

DISCUSSION

Turner et al. (38) partially characterized a GalXM from *C. neoformans* Cap67 D and a strain 371 serotype A variant. The full characterization of GalXM is difficult, because it is a minor polysaccharide that is available in limited quantities. However, if it could be demonstrated that GalXM is a common antigenic component of *C. neoformans*, then the GalXM-containing fractions reserved during previous studies of GXM could be combined and analyzed as a unit. Therefore, purified GalXMs from three isolates of *C. neoformans* were investigated independently, and their compositions and structures were studied by using GLC, GLC-MS, and ^{13}C NMR.

The analyses of the three serotypes indicated that GalXM (3183 C [P-2], 9759 A [P-2], Cap67 D [F-1]) is not a single molecular entity common to all isolates of *C. neoformans*, but rather that the GalXMs are a group of complex closely related polysaccharides composed of Xyl, Man, and Gal. The analysis of 9759 A fraction P-3 was similar to that obtained for fraction P-2. Glc in a starchlike molecule was present in all of the primary samples; most of it was removed by DE 52 chromatography (fraction F-1). Xyl, present in all three isolates, and galactofuranose, present in 3183 C and Cap67 D, were present only as nonreducing side chains. Methylation analysis and ^{13}C NMR showed that Man and Gal are substituted in a variety of ways; typically, GalXM is composed of Xyl, Man, and Gal linked 15 different ways. The complexity of these data indicates that the purified GalXM fractions may still be a composite of several closely related antigens. Reiss et al. (34) reported that the GalXM of *C. neoformans* 371A gave borderline positive results in two of six sera from patients with cryptococcosis. Recently, van de Moer et al. (40) showed that MAb CN6, produced against a spheroplast lysate of *C. neoformans* serotype A, was reactive with GalXM. The binding of the MAb to whole cells is blocked by the presence of GXM, but the acapsular mutant binds the MAb well. van de Moer et al. (39) showed that MAb CN6 has a high affinity for the three GalXM polysaccharides (9759 A [P-2], 3183 C [P-2], and Cap67 [F-2]) described herein. James et al. (22) showed that GalXM and MP occur in the supernatant fraction after homogenization and purification of cell walls derived from Cap67 D. Therefore, GalXM and MP are not part of the cell wall matrix because they are not covalently bound to the glucans. Vartivarian et al. (41) suggested that the MP is generated on the cytosol side of the cell wall and diffuses slowly through the wall to the various extracellular environments, cell membrane, cell wall, and capsule. It has been demonstrated that GalXM originates and migrates similarly (39). Kozel et al. (27) suggested that MP, GalXM, and the cell wall glucans of *C. neoformans* are activators of the alternative complement pathway. It is apparent that GalXM will need to be isolated in quantity from selected strains of *C. neoformans* in order to apply modern analytical methods for the elucidation of its complex structure. The availability of chemically defined GalXM is necessary if we are to define the role GalXM plays in the immunobiology of *C. neoformans*.

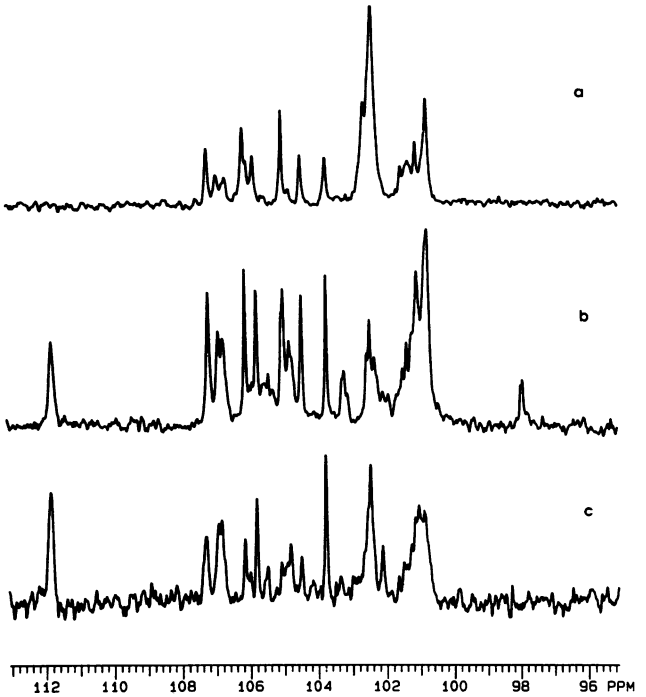

FIG. 3. Anomeric region of proton-decoupled ^{13}C NMR spectra of GalXM. (a) GalXM fraction P-2 of 9759 A; (b) GalXM fraction F-2 of Cap67 D; (c) GalXM fraction P-2 of 3183 C.

TABLE 4. GLC-MS methylation analysis of GalXM from *C. neoformans*

O-Methyl derivative[a]	mol% of derivative from the following fractions:		
	3183 C P-2	9759 A P-2	Cap67 D F-2
2,3,4-Me_3-Xyl	17.4	31.3	14.5
2,3,4,6-Me_4-Man	8.3	21.9	14.5
2,3,4,6-Me_4-Gal	–[b]	3.7	–
2,3,5,6-Me_4-Gal*f*	8.3	–	7.2
3,4,6-Me_3-Man	–	–	10.1
2,3,6-Me_3-Glc	6.6	–	–
2,3,6-Me_3-Hex[c]	7.4	6.3	8.7
2,4,6-Me_3-Hex	–	6.3	5.8
2,3,4-Me_3-Man	5.8	3.1	4.3
2,6-Me_2-Hex	4.1	3.1	4.3
4,6-Me_2-Man	14.1	9.4	14.5
2,4-Me_2-Man	7.4	–	–
2,4-Me_2-Gal	14.9	9.4	13.0
3-Me-Gal	5.8	6.3	1.4
3- or 4-Me-Hex	–	–	1.4

[a] 2,3,4-Me_3-Xyl, 2,3,4-tri-*O*-methyl-D-xylose, etc.
[b] –, derivative not present.
[c] We were unable to identify which hexose is present.

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