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Structure of the *O*-deacetylated glucuronoxylomannan from *Cryptococcus neoformans* Cap70 as determined by 2D NMR spectroscopy

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Abstract

Cryptococcus neoformans, an opportunistic pathogen, is the fourth leading cause of death among AIDS patients. The yeast's capsule is a major virulence factor, and serotype is related to the chemical structure of glucuronoxylomannan (GXM), its capsular polysaccharide. The GXM from Cap70, a hypocapsular mutant of serotype D isolate B-3501, was investigated by chemical analysis and 2D NMR spectroscopy. The assignment of ^{1}H and ^{13}C chemical shifts for the *O*-deacetylated polysaccharide was accomplished from the analysis of DQF-COSY, TOCSY, and gradient-enhanced HSQC spectra. The sequence and linkage positions of glycosyl residues were determined by NOESY and ROESY spectra. Two repeating polysaccharide components were identified as having the following structures in approximately equal proportions:

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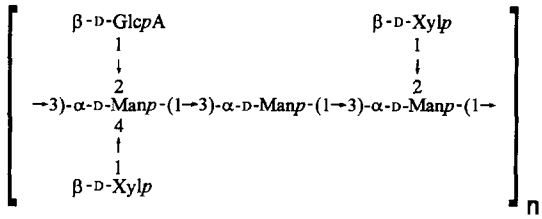

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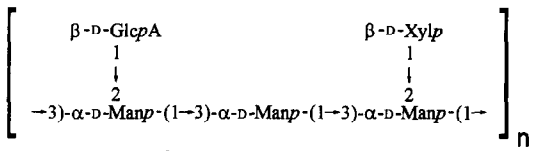

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It is not known if these repeating units comprise a single or two separate polymer chains. Pentasaccharide **2** has been known to be the major GXM polymer of B-3501 and other serotype D isolates. Hexasaccharide **1** is identified for the first time although it has subsequently been identified in other *C. neoformans* isolates. The presence of **1** in the GXM of Cap70 is consistent with the extra xylose found relative to that in isolate B-3501. The mannose:xylose:glucuronic acid:*O*-acetyl molar ratio of Cap70 GXM is 3.00:1.73:0.78:1.75, while the same ratio for B-3501 and other serotype D isolates is approximately 3.00:1.00:0.80:1.75. Methylation analysis confirmed that the GXM of Cap70 contains unsubstituted, monosubstituted (2-linked), and disubstituted (2- and 4-linked) mannose in a ratio of 0.87:1.75:0.38. Dot blot immunoassay indicates that Cap70 is a serotype D isolate like its parent strain.

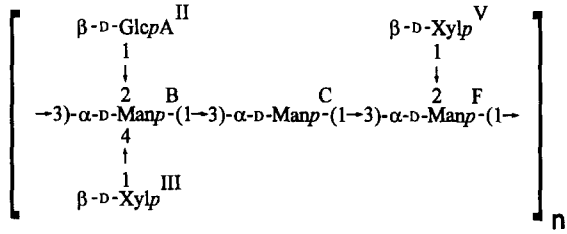
Keywords: *Cryptococcus neoformans*; 2D NMR spectroscopy; Capsular polysaccharide

1. Introduction

Cryptococcus neoformans is an encapsulated yeast responsible for life-threatening meningoencephalitis in up to 10% of patients diagnosed with AIDS [1–3]. The yeast's virulence is partially due to its polysaccharide capsule [4]. The major capsular polysaccharide antigen is a high molecular weight glucuronoxylomannan (GXM) that is partially 6-*O*-acetylated on mannose [4–6]. Acapsular mutants lose virulence, and hypocapsular mutants exhibit reduced virulence [7]. GXM may actually be responsible for enhancing HIV-1 infection in immunocompromised individuals [8].

Serological reactivity of the capsular polysaccharide has traditionally been used to define 4 serotypes (A, B, C, and D) [9,10] which have been classified into two varieties based on biochemical differences: var. *neoformans* (serotypes A and D) and var. *gattii* (serotypes B and C) [11–13]. The existence of a fifth serotype, A–D, has been described

more recently [14]. Homogeneous structural models were originally proposed for serotypes D, A, B, and C: structures **2**, **3**, **4**, and **5**, respectively [5]. However, structural heterogeneity has since been found for all serotypes except serotype B [15]. Structural elements believed to be characteristic of one serotype have been identified in others [5,16–20]. Additionally, glucuronomannan (GM) **6** [16,18,21] and unsubstituted (1 → 3)-linked mannan (M) **7** [19] have been detected in some isolates.



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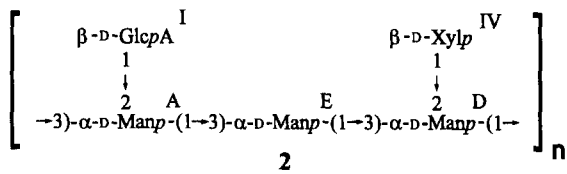

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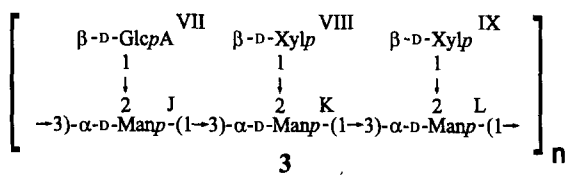

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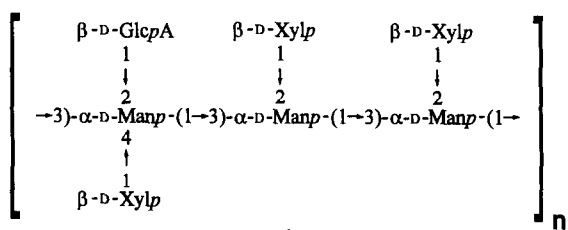

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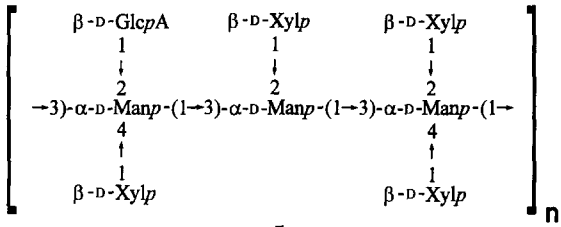

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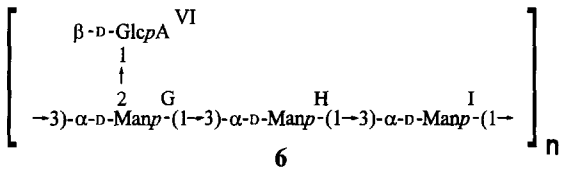

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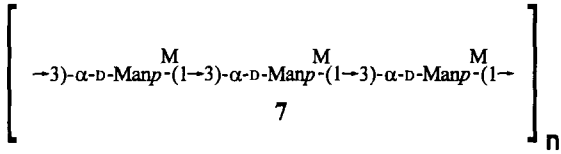

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The *O*-deacetylated GXMs from serotype D [22], B [23], A [24], and C [19] isolates have previously been characterized by 2D NMR spectroscopy [25]. Herein we report the complete assignments of the ^{1}H and ^{13}C NMR spectra of *O*-deacetylated GXM from Cap70, a mutant of serotype D isolate B-3501. Although Cap70 was originally reported to be acapsular [26–28], it has been found to be thinly encapsulated upon reexamination several years after its original description, and we now characterize the strain as hypocapsular.

2. Experimental

C.neoformans *strains.*—Isolates of *C. neoformans* used in this study were as follows: Cap70 (E.S. Jacobson, Veterans Administration Hospital, Richmond, VA), B-3501 (K.J. Kwon-Chung, National Institutes of Health, Bethesda, MD), and 9375 (H.J. Shadomy, The Medical College of Virginia, Richmond, VA).

Antigen production.—Cap70 [26–28], a hypocapsular isolate of *C. neoformans*, was grown in 1 L of chemically defined broth containing 2% glucose for 4 days at 37 °C as described previously [29]. After growth the cultures were autoclaved, and the cells were removed by centrifugation. The culture supernatant was filtered through a glass wool pad and concentrated by ultrafiltration (Amicon RA2000, spiral cartridge S1Y10, 10000

molecular weight cutoff). The retentate was dialyzed exhaustively versus running tap water and deionized water, centrifuged (5000 *g*), and lyophilized. The lyophilized retentate was dissolved in deionized water, and the purified polysaccharide antigen (GXM) was obtained by selective precipitation with hexadecyltrimethylammonium bromide as previously described [29], except that the initial 15 min sonication step was omitted. A 50-mg portion of the purified GXM was dissolved in 10 mL of H_2O and *O*-deacetylated (GXM-D) by adjusting the pH to 11.25 with NH_4OH and stirring the resulting solution for 24 h at 23 °C. The GXM of isolate 9375 was available from a previous study [16,22].

Dot enzyme assay (DEA).—Cryptococcal factor serum 1, pan specific, and factor sera 5, 6, 7, and 8 (lot 910610), specific for serotypes B, C, A, and D, respectively, were purchased from Iatron Laboratories, Inc., Tokyo, Japan. Sera were stored at −20 °C except for short-term storage (4 °C). Assays were done by the method of Tsang et al. [30] as modified by Belay et al. [31]. Strips of nitrocellulose transfer membrane (0.5 by 5.0 cm) were spotted every centimeter with 1 μL of a freshly prepared solution of GXM (1 mg/mL in 0.06 M sodium carbonate buffer, pH 9.6) and processed as described. GXM solutions were used immediately after their preparation and then discarded since the carbonate buffer, pH 9.6, causes the slow saponification of the *O*-acetyl esters.

Analytical methods.—Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen [32]. *O*-Acetyl was estimated by the Hestrin procedure with mannitol hexaacetate as the standard [33]. The constituent monosaccharides of the polysaccharides were identified and quantified as their per-*O*-acetylated aldononitrile derivatives (PAAN) by GLC as previously described [29]. Per-*O*-methylation of GXM was done by the method of Hakamori [34] as modified by Darvill et al. [35].

Nuclear magnetic resonance spectroscopy.—Most spectra were recorded at 73 °C with a Varian VXR-400 NMR spectrometer equipped with a 5-mm $^{1}H/^{19}F$ probe and operating at 399.952 MHz for ^{1}H observation. 2D {^{1}H, ^{13}C} NMR spectra were recorded at 56.5 °C with a Varian UnityPlus NMR spectrometer using a 5-mm PFG (pulsed field gradient) {^{1}H, ^{13}C, ^{15}N} triple resonance probe operating at 499.895 and 125.707 MHz for ^{1}H and ^{13}C, respectively.

O-Deacetylated polysaccharide samples (20–30 mg) were exchanged twice in 99.9% D_2O with intermediate lyophilization and were each dissolved in 0.70 mL of 99.96% D_2O. Initially prepared Cap70 and B-3501 samples (which were only partially soluble) were adjusted to pD 10 with NH_4OH and were dissolved in 0.70 mL of 99.96% D_2O after intermediate lyophilization. The pD 10 Cap70 sample was later subjected to exhaustive dialysis versus deionized water, lyophilized, exchanged three times with 99.9% D_2O, and dissolved in 0.70 mL 99.96% D_2O. Prior to NMR analysis, this pD 5 Cap70 sample was filtered through a Millipore MILLEX-GS 0.22 μm filter unit.

All 2D {^{1}H, ^{1}H} NMR spectra were acquired in the phase-sensitive mode according to the method of States et al. [36]. The spectral width was 1200 Hz in both dimensions, and two sets of from 200 to 256 t_1 measurements of 2048 data points were acquired. Zero filling in the F1 dimension resulted in a 1K × 1K data matrix. DQF-COSY [37,38], TOCSY [39], ROESY [40–42] (mixing time of 75 ms), and NOESY [43] (mixing time of 50 ms) spectra were acquired for both the pD 5 and pD 10 Cap70 GXM samples.

A gradient enhanced {^{1}H, ^{13}C} HSQC spectrum was recorded on the pD 10 Cap70

Table 1
Molar ratios of GXM from *C. neoformans* isolates Cap70 and B-3501

Strain	D-Man	D-Xyl	D-GlcA	*O*-Acetyl
B-3501	3.00	1.0	n.d. [b]	n.d.
Cap70	3.00	1.73	0.78	1.75
9375 [a]	3.00	0.85	0.75	1.74

[a] Molar ratio of a typical serotype D isolate, from ref. [16]; 1D NMR spectra of *O*-deacetylated GXMs from B-3501 and 9375 are essentially identical.
[b] Not determined.

Table 2
GLC–MS methylation analysis of GXM from *C. neoformans* isolate Cap70

Strain	Methylated PAAN derivatives (molar ratio)			
	2,3,4-Me_3-Xyl	2,4,6-Me_3-Man	4,6-Me_2-Man	6-Me-Man
Cap70	1.86	0.87	1.75	0.38
9375 [a]	1.30	0.99	1.95	0.05

[a] Typical serotype D isolate, from ref. [16].

GXM sample according to ref. [44]. The ^{1}H and ^{13}C spectral widths were 3400 and 10 000 Hz, respectively. ^{13}C decoupling during acquisition was achieved by the GARP-1 scheme [45]. A data matrix of $2 \times 256 \times 2048$ complex points was acquired using States-TPPI acquisition [46].

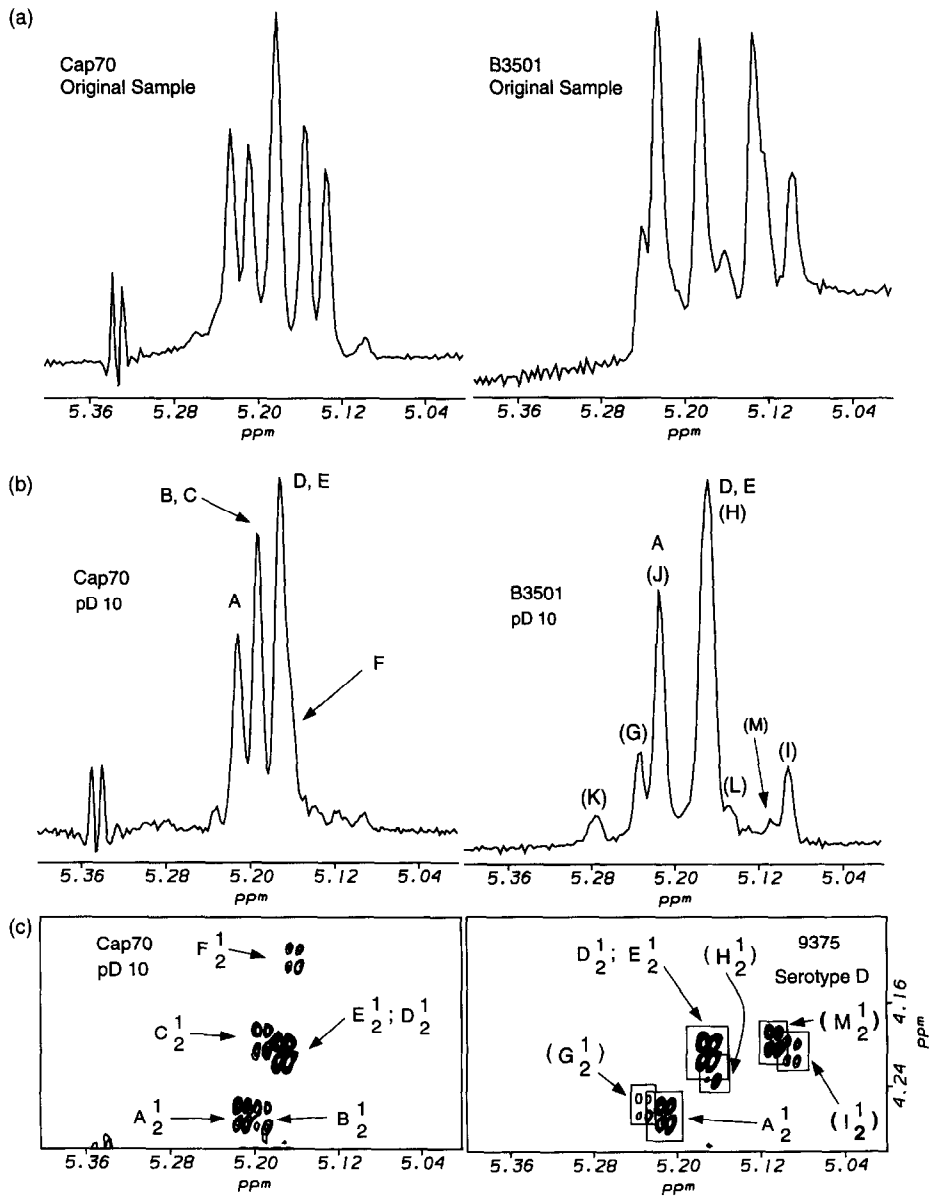
3. Results and discussion

Purification.—The average yield of purified GXM from a 1-L culture was 0.115 g for Cap70 and 1.53 g for B-3501.

Fig. 1. (a) Resolution enhanced portions of the 400-MHz ^{1}H NMR spectra of *O*-deacetylated GXM from Cap70 and from its parent strain, B-3501, as obtained on initial isolated samples. The region shown contains Man anomeric proton signals. The sharp doublet at 5.33 ppm is an impurity that is made to look more prominent with the resolution enhancement used. (b) Resolution-enhanced NMR spectra of the same region as in (a) for pD 10 adjusted samples of GXM from Cap70 and B-3501. The Man H-1 resonances of GXM Cap70 in this region are labeled with uppercase letters A, B, C, D, E, and F. Minor Man H-1 signals of GXM B-3501 are labeled with uppercase letters presented in parentheses (G, H, I, J, K, L, and M). (c) Man H-1–H-2 cross peak region of the phase sensitive DQF-COSY spectra of GXM from Cap70 (pD 10) and 9375 [a serotype D isolate which gives a 1D ^{1}H NMR spectrum essentially identical to that shown for B-3501 in (b)]. Arabic numerals denote proton positions in the given residue [specified by uppercase letters as in (b)]. In a cross peak label, the superscript denotes the proton whose chemical shift is given on the F2 (horizontal) axis, and the subscript denotes the proton whose chemical shift is given on the F1 (vertical) axis. Cross peaks for Man residues belonging to the most prominent GXM repeating structural unit found in serotype D isolates (pentasaccharide **2**) are clearly labeled in the portion of the spectrum shown for GXM 9375 (residues A, D, and E; the H-1 and H-2 chemical shifts for residues D and E are identical).

Carbohydrate composition.—The substituent sugars and *O*-acetyl of GXMs were determined by GLC and colorimetry. The molar compositions of Xyl, Man, GlcA, and *O*-acetyl, calculated relative to Man taken as 3.00, are presented in Table 1.

Methylation analysis.—The *O*-deacetylated GXM of Cap70 was methylated and analyzed by GLC–MS. The derivatives obtained were 2,3,4-tri-*O*-methyl-D-Xyl, 2,4,6-tri-*O*-methyl-D-Man, 4,6-di-*O*-methyl-D-Man, and 6-*O*-methyl-D-Man. The molar ratios



of the methylated derivatives for Cap70 are compared to the molar ratios for the reference serotype D isolate 9375 (as determined in a previous study [16,22]) in Table 2. The presence of a significant amount of 6-*O*-methyl-D-Man is consistent with the structural motif typical of serotype B GXM: GlcA and Xyl are *O*-2 and *O*-4 linked to a (1 → 3)-linked Man, respectively.

DEA.—Cap70 GXM gives a strong dot with pan specific factor serum 1 and a strong dot with serotype D specific factor serum 8 (data not shown). There is a weak reaction with serotype A specific factor serum 7 and no reaction with serotype B and C specific factor sera 5 and 6.

NMR spectroscopy.—The solubility of the *O*-deacetylated GXMs from Cap70 and B-3501 was poor. One-dimensional ^{1}H NMR spectral analysis of incompletely dissolved D_2O solutions of the two samples showed mannose H-1 resonances that had not been identified in previous studies using a variety of GXMs obtained from different serotypes (Fig. 1a) [19,20,22–24]. Both samples became fully soluble once the pD of the solutions was adjusted to pD 10 with NH_4OH. The pD adjustment simplified the mannose H-1 regions of the resolution enhanced ^{1}H NMR spectra (Fig. 1b). The spectrum of GXM B-3501 is essentially identical to that obtained for GXM 9375, a typical serotype D isolate [20,22]. Portions of the phase sensitive DQF-COSY spectra of GXMs Cap70 and 9375 are shown in Fig. 1c; these regions show the fingerprint mannose H-1–H-2 cross peaks for the respective isolates. Two cross peaks in the Cap70 DQF-COSY spectrum (A and D=E) are due to the constituent mannose residues of GXM structure **2**, the predominant repeating structural unit of serotype D isolates, while three additional cross peaks (B, C, and F) have not been observed previously. The remainder of the DQF-COSY spectrum of GXM Cap70 is shown in Fig. 2. The chemical shifts of the cross peaks labeled above the diagonal are consistent with those ^{1}H assignments previously reported for the three Man (residues A, D, and E), 1 GlcA (residue I), and 1 Xyl (residue IV) residues of **2** [22]. (It should be noted that 2 chemical shift assignments in ref. [22] are reversed: the H-5ax assignments for chemical shifts at 4.03 and 3.85 ppm as reported in this reference.)

Cross peaks for residues other than those present in **2** are labeled below the diagonal in Fig. 2. H-2–H-3 and H-3–H-4 cross peaks are observed for residues B, C, and F, and reflect coupling constants consistent with $^4C_1$ Man*p* residues ($J_{2,3} < 5$ Hz, $J_{3,4}$ and $J_{4,5} > 5$ Hz). Two newly identified Xyl residues (III and V) and one newly identified GlcA residue (GlcA-II) are identified by their cross peaks as marked. Complete cross peak connectivities are observed for the Xyl residues while the connectivity from H-1 to H-4 is observed for GlcA-II; the H-4–H-5 cross peak is too close to the diagonal to be assigned unequivocally. H-1–H-5 relay cross peaks were observed for GlcA residues I and II in the TOCSY spectra recorded with mixing times of both 50 and 100 ms (data not shown). Coupling constants are consistent with Xyl*p* and Glc*p*A residues having the $^4C_1$ conformation ($J_{1,2}$, $J_{2,3}$, $J_{3,4}$, and $J_{4,5a} > 5$ Hz).

Chemical shift assignments for Man, Xyl, and GlcA residues in Cap70 GXM, pD 10, are presented in Table 3. Tentative linkage assignments of these residues were obtained from a 50 ms NOESY spectrum; however, there was some ambiguity in these linkage assignments due to the similarity of Man H-1 chemical shifts. Therefore, the Cap70 GXM sample was dialyzed in water to pH 5 and analyzed as the pD 5 solution in D_2O.

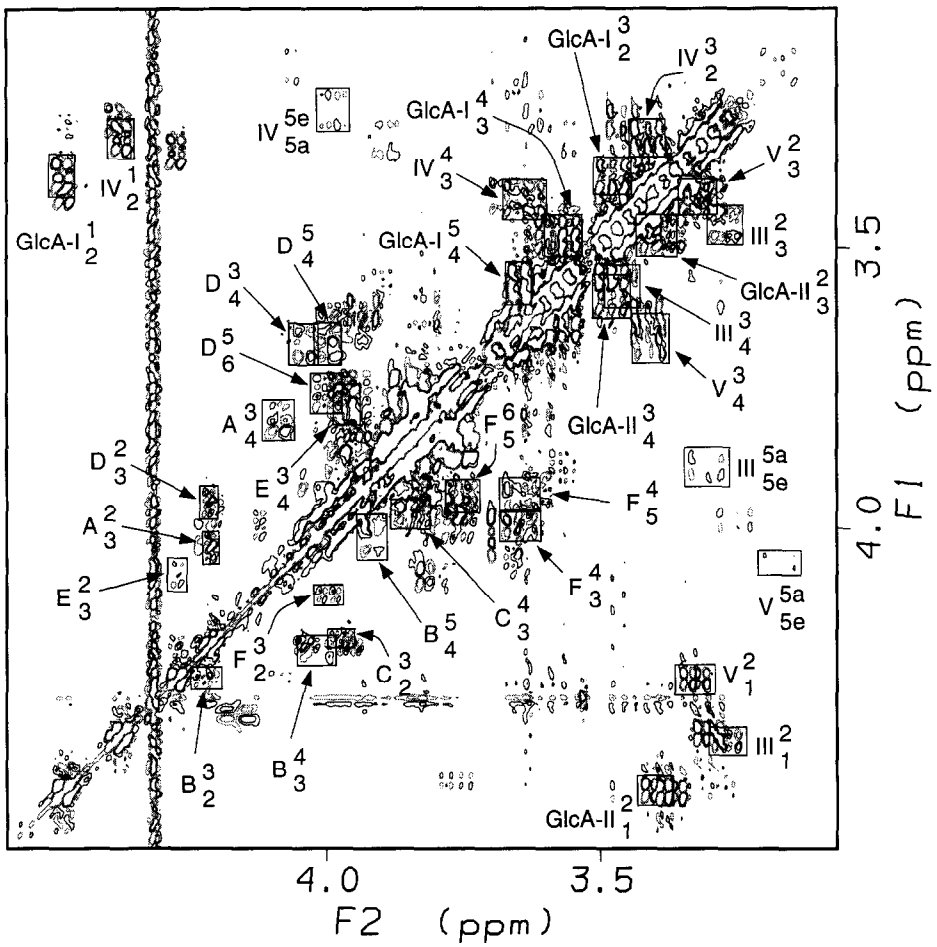


Fig. 2. Portion of the phase sensitive DQF-COSY spectrum of *O*-deacetylated GXM Cap70 (pD 10) recorded at 400 MHz; the corresponding Man H-1 region is shown in Fig. 1c. Roman numerals refer to Xyl and GlcA residues; the GlcA residues are marked explicitly as, for example, GlcA-II. Otherwise, cross peaks are labeled as described for Fig. 1. To avoid crowding, the cross peaks for residues A, D, E, GlcA-I, and IV (structure **2**) are labeled above the diagonal, and cross peaks for residues B, C, F, GlcA-II, III, and V (structure **1**) are labeled below the diagonal.

Analogous to the mannose H-1 1D NMR spectral region and to the Man H-1–H-2 DQF-COSY cross peak region shown in Fig. 1b and 1c for the pD 10 Cap70 GXM sample, these same regions are shown for the pD 5 sample in Fig. 3a. The H-1 chemical shifts of Man residues A, B, C, D, E, and F at pD 5 are more dispersed than in the pD 10 sample, and the Man residue DQF-COSY cross peaks (as well as the Xyl and GlcA residue cross peaks) are shifted in position relative to those of the pD 10 sample (full DQF-COSY spectrum not shown). Most notably, GlcA H-5 protons of the pD 5 Cap70 GXM are shifted downfield relative to the pD 10 sample.

Table 3
^{1}H NMR chemical shifts of the ring protons of the constituent carbohydrate residues of **1** and **2**, as identified in the *O*-deacetylated GXM of *C. neoformans* isolate Cap70, pD 10

	Chemical shift [a]							
	H-1	H-2	H-3	H-4	H-5a	H-5e	H-6	H-6′
GXM 1								
Man-B [b]	5.194 [c]	4.270	4.217	4.018	3.917	n.a. [d]	3.923	n.d. [e]
Man-C	5.193	4.197	3.972	3.842	3.907	n.a.	n.d.	n.d.
Man-F	5.164	4.121	3.995	3.644	3.946	n.a.	3.752	3.860
GlcA-II	4.472	3.397	3.478	3.589	3.643	n.a.	n.a.	n.a.
Xyl-III	4.382	3.270	3.458	3.578	3.306	3.891	n.a.	n.a.
Xyl-V	4.270	3.328	3.408	3.666	3.174	4.060	n.a.	n.a.
GXM 2								
Man-A	5.212	4.268	4.084	3.808	3.820	n.a.	3.879	3.807
Man-D	5.173	4.210	4.034	3.674	3.998	n.a.	3.760	3.840
Man-E	5.173	4.210	3.956	3.781	3.971	n.a.	n.d.	n.d.
GlcA-I	4.475	3.370	3.476	3.566	3.648	n.a.	n.a.	n.a.
Xyl-IV	4.369	3.302	3.414	3.638	3.254	3.985	n.a.	n.a.

[a] In ppm relative to HOD as externally referenced according to temperature with DSS (δ 0.0) as internal standard.
[b] For residue labeling, see the formulae.
[c] Unless otherwise noted, chemical shifts were assigned from DQF-COSY spectrum recorded at 73 °C and are reported to three decimal places with an accuracy of ± 0.003 ppm.
[d] Not applicable.
[e] Not determined.

Complete chemical shift assignments for Man, Xyl, and GlcA residues in Cap70 GXM, pD 5, are presented in Table 4. TOCSY relay peaks (Xyl and GlcA H-1–H-3, H-1–H-4 and GlcA H-1–H-5) were used to confirm overlapping (but identifiable) Xyl and GlcA DQF-COSY cross peaks (TOCSY spectra not shown). The TOCSY spectra were additionally useful in identifying one (or both) H-6 proton(s) of Man residue B. The H-3–H-5 relay cross peak of Man-B was observed in the 50 ms TOCSY spectrum, while the H-3–H-6 relay cross peak was visible at about the same place in the 100 ms TOCSY spectrum (data not shown).

Once the ^{1}H NMR spectrum was virtually completely assigned, the linkages of glycosyl residues were determined from interresidue ROESY and/or NOESY correlations. Portions of the ROESY and NOESY spectra of GXM Cap70 (pD 5) are shown in Fig. 3b and 3c, respectively. The portion of the 75 ms ROESY spectrum in Fig. 3b shows rotating frame NOE correlations to the H-1 anomeric protons of Man residues. Intraresidue Man H-1–H-2 cross peaks are visible; the absence of intraresidue Man H-1–H-3 or H-1–H-5 cross peaks confirms the α-configuration for all the Man residues. Labeled interresidue cross peaks correlate a particular Man H-1 proton with either (1) H-3 of the Man residue to which it is (1 → 3) linked or (2) H-1 of the Xyl or GlcA residue substituting the 2-position. Of the interresidue Man H-1–Man H-3 cross peaks, Man-A H-1–Man-E H-3, Man-B H-1–Man-C H-3, and Man-E H-1–Man-D H-3 are easily assigned. Due to the similar chemical shifts of Man-C H-1 and Man-D H-1, the

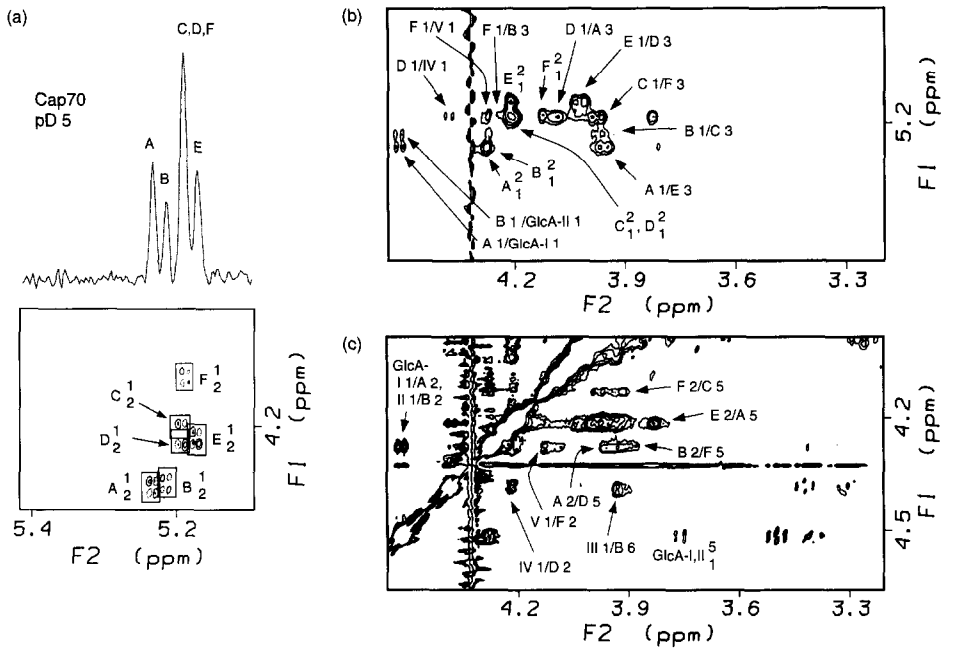


Fig. 3. (a) Man H-1–H-2 cross peak region of the phase sensitve DQF-COSY spectrum of *O*-deacetylated GXM Cap70 (pD 5), with resolution enhanced 1D NMR spectrum of Man H-1 region at top. (b) The region of the 75 ms ROESY spectrum of GXM Cap70 (pD 5) showing cross peaks involving Man H-1 protons. The uppercase letters refer to Man residues, and the Roman numerals refer to Xyl and GlcA residues. The labeling for intraresidue NOE cross peaks is the same as for the DQF-COSY cross peaks in Fig. 1c and Fig. 2. Interresidue NOEs are marked as, for example, E 1/D 3 to indicate through-space connectivity between Man-E H-1 and Man-D H-3. (c) Part of the 50 ms NOESY spectrum of GXM Cap70 (pD 5). This region contains cross peaks due to through space interactions of Xyl and GlcA H-1 protons with Man H-2 and H-6 protons. Interresidue Man H-2–Man H-5 NOE cross peaks also appear in this region as do unlabeled intraresidue Man cross peaks (e.g., H-2–H-3).

assignments of the Man-D H-1–Man-A H-3 and Man-C H-l–Man-F H-3 cross peaks are uncertain. Assignment of the Man-F H-1–Man-B H-3 cross peak is uncertain since it is largely obscured by overlapping intraresidue cross peaks (Man-C H-1–H-2 and Man-D H-1–H-2). Of the interresidue Man H-1–GlcA (or Xyl) H-1 cross peaks labeled in Fig. 3b, Man-A H-1–GlcA-I H-1, Man-B H-1–GlcA-II H-1, and Man-F H-1–Xyl-V H-1 are easily assigned, whereas there is some question concerning the Man-D H-1–Xyl-IV H-1 cross peak — again due to the similarity of chemical shifts for the H-1 protons of Man-C and Man-D.

The portion of the 50 ms NOESY spectrum shown in Fig. 3c shows Man H-5–Man H-2 interresidue NOE cross peaks for each (1 → 3) linkage showing a H-1–H-3 NOE cross peak in Fig. 3b [47]. Although the Man-B H-5–Man-C H-2 and Man-E H-5–Man-D H-2 cross peaks overlap with unmarked intraresidue Man H-2–H-3 cross peaks, the Man-C H-5–Man-F H-2, Man-D H-5–Man-A H-2, and Man-F H-5–Man-B H-2 cross peaks remove the ambiguities of the Man H-1–Man H-3 assignments, which were made

Table 4
^{1}H chemical shifts of the ring protons of the constituent carbohydrate residues of **1** and **2**, as identified in the *O*-deacetylated GXM of *C. neoformans* isolate Cap70, pD 5

	Chemical shift [a]							
	H-1	H-2	H-3	H-4	H-5a	H-5e	H-6	H-6′
GXM 1								
Man-B [b]	5.214 [c]	4.278	4.240	4.032	3.924	n.a. [d]	3.932	n.d. [e]
Man-C	5.192	4.204	3.980	3.850	n.d.	n.a.	n.d.	n.d.
Man-F	5.187	4.132	3.984	3.691	3.899	n.a.	3.760	3.862
GlcA-II	4.520	3.408	3.503	3.619	3.766	n.a.	n.a.	n.a.
Xyl-III	4.392	3.280	3.468	3.588	3.318	3.905	n.a.	n.a.
Xyl-V	4.284	3.333	3.418	3.670	3.189	4.065	n.a.	n.a.
GXM 2								
Man-A	5.232	4.283	4.096	3.832	n.d.	n.a.	n.d.	n.d.
Man-D	5.192	4.219	4.030	3.716	3.951	n.a.	3.766	n.d.
Man-E	5.172	4.216	3.966	3.794	3.982	n.a.	3.764	3.873
GlcA-I	4.516	3.384	3.501	3.600	3.767	n.a.	n.a.	n.a.
Xyl-IV	4.382	3.308	3.424	3.645	3.267	3.993	n.a.	n.a.

[a] In ppm relative to HOD as externally referenced according to temperature with DSS (δ 0.0) as internal standard.
[b] For residue labeling, see the formulae.
[c] Unless otherwise noted, chemical shifts were assigned from DQF-COSY spectrum recorded at 73 °C and are reported to three decimal places with an accuracy of ± 0.003 ppm.
[d] Not applicable.
[e] Not determined.

from the ROESY spectrum in Fig. 3b. Similarly, the presence of the Man-D H-2–Xyl-IV H-1 cross peak confirms the Man-D H-1–Xyl-IV H-1 cross peak assignment. Additionally, the presence of the Man-B H-6–Xyl-III H-1 cross peak is consistent with the presence of a β-Xyl-(1 → 4)-Man linkage as suggested by the results of methylation analysis since the Man H-6–Xyl H-1 NOE has previously been found to be predominant for this same linkage as found in serotype B [23] and C [19] GXM (structures **4** and **5**, respectively).

The observed NOE cross peaks are consistent with the two repeating polysaccharide units in pentasaccharide **2** and hexasaccharide **1**. It is not known if these repeating units comprise a single or two separate polymer chains. Structure **1** had not been confirmed in any GXM isolate prior to this work. Independent of the methylation analysis (which was necessary for establishing the β-Xyl-(1 → 4)-Man linkage in **1**), an attempt to confirm this linkage was made by performing a HMBC experiment on GXM Cap70 (pD 10). However, since the high molecular weight of the polysaccharide results in a short t_2 relaxation for the polymer, most of the ^{1}H signal was lost during the low-pass *j*-filter delay (55 ms) [22]. However, a gradient-enhanced HSQC spectrum [44] was successfully obtained. This spectrum shows one-bond connectivities between carbon atoms and directly bonded protons (spectrum not shown). The assignment of the HSQC signals was straightforward since the ^{1}H NMR spectrum had already been almost completely

Table 5
^{13}C chemical shifts of the constituent carbohydrate residues of **1** and **2**, as identified in the *O*-deacetylated GXM of *C. neoformans* isolate Cap70, pD 10

	Chemical shift [a]					
	C-1	C-2	C-3	C-4	C-5	C-6
GXM 1						
Man-B [b]	102.27 [c]	79.27	76.43	77.47	74.90	62.00
Man-C	104.56	72.20	81.22	68.18	75.81	n.d. [d]
Man-F	102.80	80.10	78.57	69.89	75.73	63.60
GlcA-II	103.82	74.97	77.84	74.00	79.13	n.d.
Xyl-III	106.36	76.05	78.26	72.23	67.59	n.a. [e]
Xyl-V	105.714	75.000	78.069	71.658	67.710	n.a.
GXM 2						
Man-A	102.63	79.87	79.80	68.64	75.88	n.d.
Man-D	103.54	80.61	78.49	69.55	75.68	n.d.
Man-E	104.54	72.20	81.25	68.56	75.79	n.d.
GlcA-I	104.28	74.97	77.84	74.06	79.27	n.d.
Xyl-IV	105.63	75.12	78.07	71.68	67.54	n.a.

[a] In ppm relative to DSS (δ 0.0) as internal standard.
[b] For residue labeling, see the formulae.
[c] Chemical shifts are reported to two decimal places with an accuracy of ± 0.078 ppm.
[d] Not determined.
[e] Not applicable.

assigned. The ^{13}C NMR chemical shifts obtained by analysis of the HSQC spectrum are tabulated in Table 5.

The ^{13}C NMR chemical shift assignments for residues in structure **2** are consistent with those previously reported [22]. It is noteworthy that the C-2 and C-3 chemical shifts of Man-C (structure **1**) are essentially identical to the C-2 and C-3 chemical shifts of Man-E (structure **2**); this result is consistent with the conclusion that both of these (1 → 3)-linked Man residues are not substituted at C-2. Substitution of the other Man residues at C-2 results in a large downfield shift of C-2 (according to the known glycosylation effect at the site of substitution [48]) and in a small upfield shift of C-3 (according to the glycosylation effect of a neighboring gamma substituent [48]). The large downfield shift of Man-B C-4 (relative to the C-4 chemical shifts of the other Man residues) supports the β-Xyl-IV-(1 → 4)-Man-B linkage in the newly identified structure **1**. Small upfield shifts in Man-B C-3 and C-5 (relative to C-3 and C-5 of Man-A) further support this argument. The extreme downfield position of the C-1 chemical shift of the β-Xyl-III-(1 → 4)-linked residue (relative to the C-1 chemical shifts of the β-Xyl-III-(1 → 2)-linked residues (Man-IV and Man-V) is consistent with previous work on the *O*-4 linked mannose structural motif typical of serotype B GXM [23].

The β-Xyl-(1 → 4)-Man linkage has previously been identified in certain serotype A isolates using ^{13}C NMR spectroscopy [18]. Further work is required to determine if a triad structure **1** accounts for the *O*-4 linked Xyl found for these isolates rather than a serotype B triad structure. Preliminary work shows the presence of a small amount of

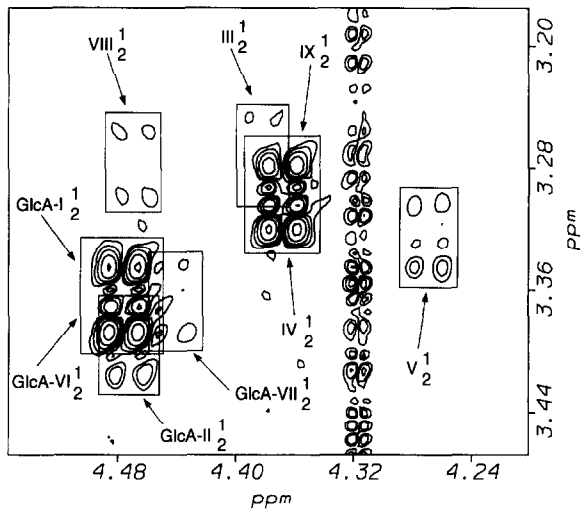


Fig. 4. Portion of the DQF-COSY spectrum of serotype D GXM 9375 showing GlcA and Xyl H-1–H-2 cross peaks. Cross peaks are labeled in the manner described for Fig. 1c and Fig. 2.

triad structure **1** in *O*-deacetylated GXM of a serotype A–D isolate (CBS132) [21]. Furthermore, a significant amount of triad structure **1** has recently been found in the J9 *C. neoformans* strain isolated from a patient with recurrent cryptococcal meningitis, as reported by Cherniak et al. [20].

While **1** was present in strain J9b [20] in sufficient quantity so that confirmation of its presence could be accomplished from the identification of the appropriate cross peaks in a DQF-COSY spectrum (B.E. Bacon and R. Cherniak, unpublished results), the minor presence of **1** can sometimes be overlooked due to the absence of Man H-1–H- 2 cross peaks in such a DQF-COSY spectrum. Thus, while Man H-1–H-2 cross peaks for **1** are not visible in the portion of the DQF-COSY spectrum shown for serotype D GXM 9375 in Fig. 2c, it is likely that a small amount of **1** may be present in this isolate as can be seen from the GlcA/Xyl H-1–H-2 cross peak region shown in Fig. 4. Whereas the Man H-1–H-2 cross peak region in Fig. 1c shows the presence of GM **6** (Man residues G, H, and I) — and the presence of unsubstituted mannan **7** (Man residue M) — in addition to the presence of serotype D triad **1** (Man residues A, D, and E), the cross peak region in Fig. 4 additionally shows cross peaks which can be assigned to a minor amount of **1** (GlcA residue II and Xyl residues III and V), as well as to a minor amount of serotype A triad **3** (GlcA residue VII and Xyl residues VIII and IX).

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