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Note

Complete assignment of the ^{1}H- and ^{13}C-n.m.r. spectra of the *O*-deacetylated glucuronoxylomannan from *Cryptococcus neoformans* serotype B

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Cryptococcus neoformans is an opportunistic pathogen with world-wide distribution. The organism can cause fatal *Cryptococcosis*, which is a frequent complication in immuno-suppressed patients, especially those with acquired immunodeficiency syndrome (AIDS)[1–3]. The major capsular polysaccharide of *C. neoformans* is a high-molecular-weight glucuronoxylomannan (GXM) composed of mannose, xylose, glucuronic acid, and *O*-acetyl groups[4,5]. At least four different serotypes of *C. neoformans* are known (A–D)[6]. Primary structures for the repeating units of GXM-A and GXM-B have been proposed[7,8], chiefly based on glycosyl composition and linkage analyses. The epitopes which determine the serotype are believed to reside on GXM, but details of their structures remain unknown.

We now report 2D ^{1}H- and ^{13}C-n.m.r. data for the *O*-deacetylated GXM isolated and purified from *Cryptococcus neoformans* serotype B, NIH strain 409 (denoted GXM-B). The present work provides pertinent ^{1}H and ^{13}C resonance assignments for typing *C. neoformans*. N.m.r. measurements performed on intact GXM and ultimately on the pathogenic yeast cells may eventually reveal the serotype of the organism. Moreover, the assignments are a prerequisite for solution conformational analysis of GXM epitopes. Assignment of the ^{1}H resonances in a polysaccharide the size of GXM-B (1.20×10^{5} Da) represents a considerable challenge. We have explored the applicability of total correlation/rotating-frame n.O.e. spectroscopy (TOCSY–ROESY) (developed for providing the ^{1}H assignments for smaller oligosaccharides[9–13]) to completely assign the ^{1}H-n.m.r. spectrum of GXM-B, and heteronuclear multiple quantum coherence (HMQC) spectroscopy to re-assign the ^{13}C-n.m.r. spectrum reported[8].

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GXM-B has been proposed[8] to be a polymer of mainly the heptasaccharide repeating unit

β-D-Xyl*p*-(1→4)┐
[→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→]$_n$
β-D-Glc*p*A-(1→2)┘ β-D-Xyl*p*-(1→2)┘ β-D-Xyl*p*-(1→2)┘

Accordingly, the high-resolution 1D ^{1}H-n.m.r. spectrum of GXM-B contained seven H-1 signals (see Table I). Based on their shapes (see Fig. 1a), the signals (3 bs) at δ 5.319, 5.197, and 5.138 were assigned to H-1 of the three Man residues ($J_{1,2} < 1.5$ Hz). The well-resolved signals (4 d) at δ 4.489, 4.454, 4.401, and 4.275 ($J_{1,2}$ 7.3 Hz) were attributed to the H-1 of the three Xyl residues and the GlcA residue. The magnitudes of the $J_{1,2}$ values of the Xyl and GlcA residues confirm the β-pyranoid forms.

The resolution in the ^{1}H-n.m.r. spectrum at 57° was sufficient to permit COSY and TOCSY experiments to be performed. Despite the relatively high viscosity of the solution, couplings of >4 Hz were resolved. Virtually all of the ^{1}H resonances were assigned (Table I) by combining these 2D techniques. Fig. 1b shows the TOCSY spectrum of GXM-B in contourplot. Magnetization transfer from H-1 to H-5 for the β-Xyl and β-GlcA residues is virtually unhampered and complete subspectra were obtained as cross-sections through their H-1 resonances (Fig. 1b). The assignment of each of the signals was based on its *J*-signature alone, or in combination with the information from the COSY spectrum. For example, the trace through H-1 at δ 4.489 was identified readily as the subspectrum of a Xyl*p* residue, by virtue of the appearance of the signals for H-2 (t), H-3 (t), H-4 (m), H-5*ax* (bt), and H-5*eq* (bdd). Similarly, the traces through the H-1 signals at δ 4.401 and 4.275 were identified as Xyl*p* subspectra. For each of the three traces, the assignment of the H-4, H-5*ax*, and H-5*eq* signals was straightforward, whereas the signals for H-2 and H-3 (2 t) were distinguished with aid of the connectivity observed between H-1 and H-2 in the COSY spectrum (not shown). The remaining trace of signals through that for H-1 at δ 4.454 was thus attributed to the GlcA residue. The signals of all protons on this trace were virtual triplets with coupling constants of similar magnitudes, except for H-5 (d at δ 3.63). The COSY spectrum showed vicinal-coupling connectivities between H-1 and H-2 (at δ 3.41), H-2 and H-3 (at δ 3.48), and H-3 and H-4 (at δ 3.61), thus completing the assignment of the GlcA protons (Fig. 1b).

The TOCSY subspectra, through the H-1 signals of the Man residues (at δ 5.319, 5.197, and 5.138), showed clearly observable connectivities only to the H-2 resonance for each Man residue (Fig. 1b). Apparently, the small $J_{1,2}$ value of Man hampered propagation of magnetization transfer beyond H-2. Therefore, no complete subspectra for the three Man residues were obtained from cross-sections through the H-1 signals in the TOCSY spectrum. However, the cross-sections through the respective Man H-2 signals clearly showed connectivities between the H-2 and H-1, and H-2 and H-3 up to H-6a,6b for each Man residue (Fig. 1c). The patterns (*i.e.*, the sets of ^3J values) observed for the Man H-2/5 confirmed that each Man residue was pyranoid.

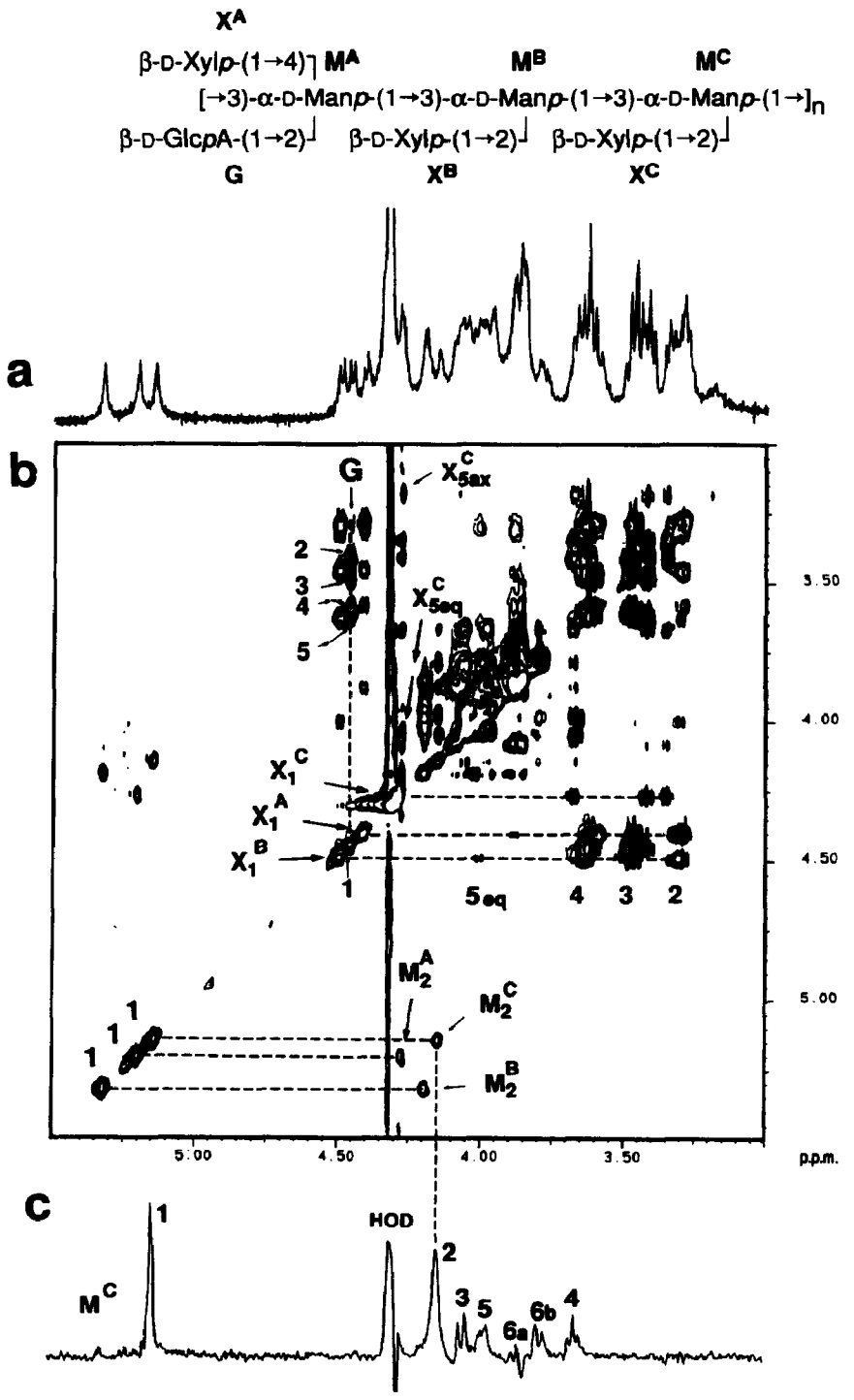

Fig. 1. (a) High-resolution 1D ^{1}H-n.m.r. spectrum of GXM-B recorded for a solution in D_2O–$(CD_3)_2CO$ (9:1) at 500 MHz and 57°. (b) {^{1}H,^{1}H} TOCSY spectrum. The horizontal, dashed lines indicate Xyl subspectra through their respective H-1 signals, and partial subspectra through the Man H-1 signals. The GlcA subspectrum is marked in a vertical column through its H-1 signal. Residues are designated as in the formula and the numbers refer to the protons. (c) Subspectrum of Man^C taken from a row through its H-2 resonance at δ 4.145.

TABLE I

N.m.r. chemical shift data for GXM-B

		Chemical shift[a] (p.p.m.)						
	Assignment	*β-Xyl^A*	*β-Xyl^B*	*β-Xyl^C*	*β-GlcA*	*α-Man^A*	*α-Man^B*	*α-Man^C*
$^1H^b$	H-1	4.401	4.489	4.275	4.454	5.197	5.319	5.138
	H-2	3.28	3.29	3.35	3.41	4.274	4.195	4.147
	H-3	3.46	3.46	3.42	3.48	4.19	4.09	4.05
	H-4	3.58	3.63	3.67	3.61	4.01	3.85	3.67
	H-5*ax*	3.32	3.31	3.19	3.63	4.04	3.88	3.96
	H-5*eq*	3.87	4.00	4.08				
	H-6a,6b					3.96[d]	3.84[d]	3.87,3.78
$^{13}C^c$	C-1	106.47	105.93	105.81	103.83	102.20	102.72	103.14
	C-2	76.29	75.46	75.21	75.09	79.08	81.04	80.19
	C-3	78.71	78.09	78.09	78.09	76.83	78.63	78.63
	C-4	72.27	71.87	71.71	74.09	77.87	68.80	69.95
	C-5	67.62	67.62	67.80	79.08	75.09	75.91	75.70
	C-6				177.78	62.29	62.96	63.15

[a] Data were acquired for solutions in D_2O–$(CD_3)_2CO$ (9:1) at pD 7–8 and 57°. For designation of residues, see Fig. 1. [b] Referenced to internal DSS, with acetone-d_5 as the internal standard. The chemical shifts given to three decimal places were measured from the resolution-enhanced 1D ^{1}H-n.m.r. spectrum. Shifts given to two decimal places were inferred from TOCSY, ROESY, and/or HMQC spectra. [c] Referenced to DSS, with acetone-d_6 as the internal standard. Although the C–H connectivities were established by HMQC (±0.1 p.p.m.), accurate chemical shifts (±0.005 p.p.m.) were obtained from the 1D ^{13}C spectrum at 125 MHz. [d] The chemical shifts of Man^A H-6a and H-6b are within 0.01 p.p.m. from each other, centered at δ ~3.96. Similarly, Man^B H-6a and H-6b are at δ 3.84 ±0.01.

Thus, the ^{1}H-n.m.r. spectrum of the heptasaccharide repeating unit of GXM-B was resolved into seven subspectra that were assigned to particular residues (α-Man*p*, β-Xyl*p*, or β-Glc*p*A). However, the TOCSY/COSY spectra did not reveal any information on sequence. Given the linkage positions, the sequence was readily derived from a 2D ROESY experiment. The ROESY spectrum (Fig. 2) showed connectivities between protons in close proximity (<5 Å). The appearance of intra-residue n.O.e. contacts confirmed several of the assignments made on the basis of the TOCSY spectrum. For example, GlcA H-1 shows intra-residue n.O.e.s to the GlcA H-3 and H-5 that are in accordance with the axial orientation of H-1,3,5. More importantly, the ROESY spectrum showed interglycosidic contacts between each H-1 and the proton on the opposite side of the linkage. For example, the GlcA H-1 signal showed interglycosidic n.O.e. contacts to the signals at δ 5.197 and 4.274, which were identified in the TOCSY experiment as due to H-1 and H-2 of one of the Man residues (Table I). The two inter-residue n.O.e.s support the (1→2) linkage between β-GlcA and one of the Man residues; the Man residue with H-1 at δ 5.197 and H-2 at 4.274 is denoted as Man^A (Table I and Fig. 1).

The assignment of the two remaining Man subspectra (Fig. 1b) and sets of chemical shifts (Table I) to Man^B and Man^C was made by examining the n.O.e. contacts

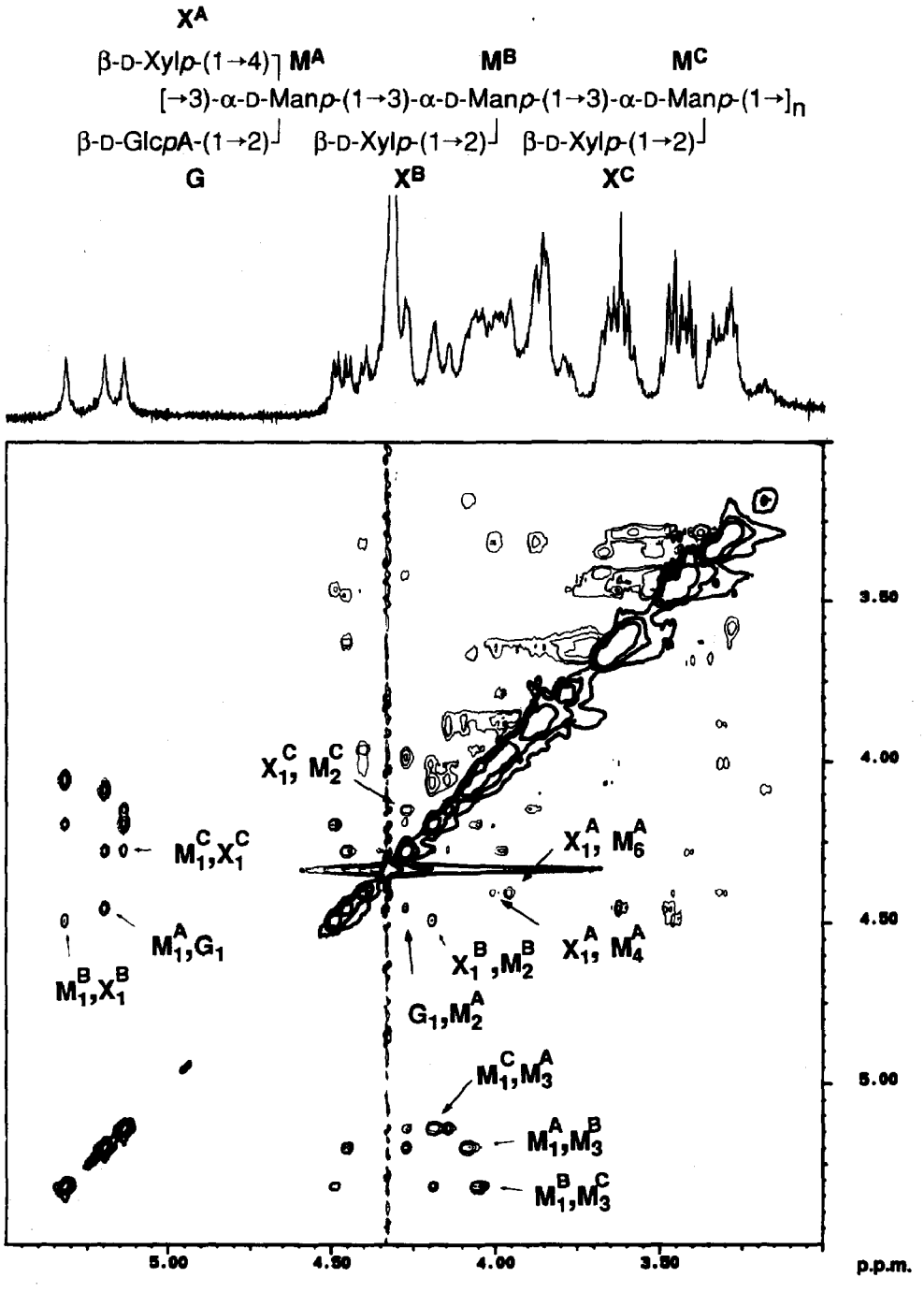

Fig. 2. {^{1}H,^{1}H} ROESY spectrum of GXM-B recorded for a solution in D_2O–$(CD_3)_2CO$ (9:1) at 500 MHz and 57°, with high-resolution 1D projection on top. Only inter-residue n.O.e.s are marked. Negative signals are along the diagonal and depicted with thick-lined contours. The residues are denoted by letters as in Fig. 1.

(Fig. 2) between the Man H-1 signals and their interglycosidic neighbors, bearing in mind the backbone sequence →3)-α-Man^{A}-(1→3)-α-Man^{B}-(1→3)-α-Man^{C}-(1→. The H-1 signal of the Man^{A} residue at δ 5.197 showed an interglycosidic n.O.e. to a signal at δ 4.09, identified in the TOCSY spectrum as due to Man H-3. This identifies the Man residue with H-3 at δ 4.09 as Man^{B} (Table I). In addition, H-1 of Man^{A} showed an intra-residue n.O.e. to Man^{A} H-2, and an inter-residue n.O.e. to GlcA H-1, in keeping with the (1→2) linkage between GlcA and Man^{A}. The absence of intra-residue n.O.e.s between Man^{A} H-1 and its H-3 and/or H-5 confirms the structure α-Man-(1→3)-Man; had H-1 of Man^{A} C-1 been axial, an intra-residue n.O.e. to H-3 and H-5 would have been observed, as with GlcA. Analogously, H-1 (δ 5.319) of the newly identified Man^{B} residue showed an interglycosidic n.O.e. to a Man H-3 with a signal at δ 4.05 (Fig. 2), thus positively identifying the third Man residue as Man^{C} (Table I). The H-1 signal of Man^{C} (δ 5.138) showed an intra-residue n.O.e. to H-3 (δ 4.19) of Man^{A} (Fig. 2), thereby confirming the repeating backbone structure of GXM-B to be a *tri*-mannosyl moiety.

The β-Xyl residues that are (1→2)-linked to Man^{B} and Man^{C} (*i.e.*, Xyl^{B} and Xyl^{C}, respectively) were identified readily by virtue of the interglycosidic n.O.e.s between the Man^{B} and Man^{C} H-1 signals, and the Xyl H-1 signals (see Fig. 2). In addition, the Xyl^{B} H-1 signal (δ 4.489) showed an n.O.e. to Man^{B} H-2 (δ 4.195), whereas Xyl^{C} H-1 showed an n.O.e. to Man^{C} H-2 (δ 4.147). This leaves the third Xyl to be assigned as β-Xyl^{A}, (1→4)-linked to Man^{A}. Indeed, an interglycosidic n.O.e. between β-Xyl^{A} H-1 (δ 4.401) and Man^{A} H-4 (δ 4.01) is observed (Fig. 2), confirming the (1→4) linkage between those residues. In addition, a strong inter-residue n.O.e. was observed between H-1 of Xyl^{A} and H-6 of Man^{A} (Fig. 2); such n.O.e.s may prove to be valuable for future conformational studies of the GXM heptasaccharide.

The assigned ^{1}H-n.m.r. spectrum was used to assign the ^{13}C-n.m.r. spectrum of GXM-B by employing the results of an HMQC experiment. The HMQC spectrum (Fig. 3) showed one-bond ^{1}H,^{13}C connectivities. The major advantage over the conventional (^{13}C-detected) HETCOR experiment[8] is in the much-improved digital resolution in the ^{1}H dimension of the spectrum. The ^{13}C chemical shifts are included in Table I. The only assignments that were not straightforward are those of the two ^{13}C signals connected to the ^{1}H signals of Man^{B} H-2 and Man^{A} H-3 that coincide at δ 4.19 (Fig. 3). The signal at δ 81.04 was attributed to Man^{B} C-2 and that at δ 76.83 to Man^{A} C-3. These conclusions were based on comparison with the chemical shifts of the other Man C-2 and C-3 signals (Table I), and taking into account the well established glycosylation shifts (α- and β-effects)[8,14]. Thus, it is rationalized easily that the Man^{A} C-3 signal is 1.8 p.p.m. upfield from those of C-3 of Man^{B} and Man^{C}, due to Man^{A} being 4-glycosylated (β-effect).

This work is the first detailed ^{1}H-n.m.r. study of the glucuronoxylomannan purified from a *C. neoformans* serotype B strain. With the use of COSY, TOCSY, and ROESY {^{1}H,^{1}H} correlated spectroscopy, in combination with HMQC {^{1}H,^{13}C} correlated spectroscopy, *de novo* signal assignments were made for all non-exchangeable protons and then for the carbon atoms. The {^{1}H,^{1}H} ROESY connectivities provide independent evidence for the linkage positions in the GXM-B previously deduced by chemical methods[8]. Previous ^{13}C signal assignments[8], made by comparing the 1D

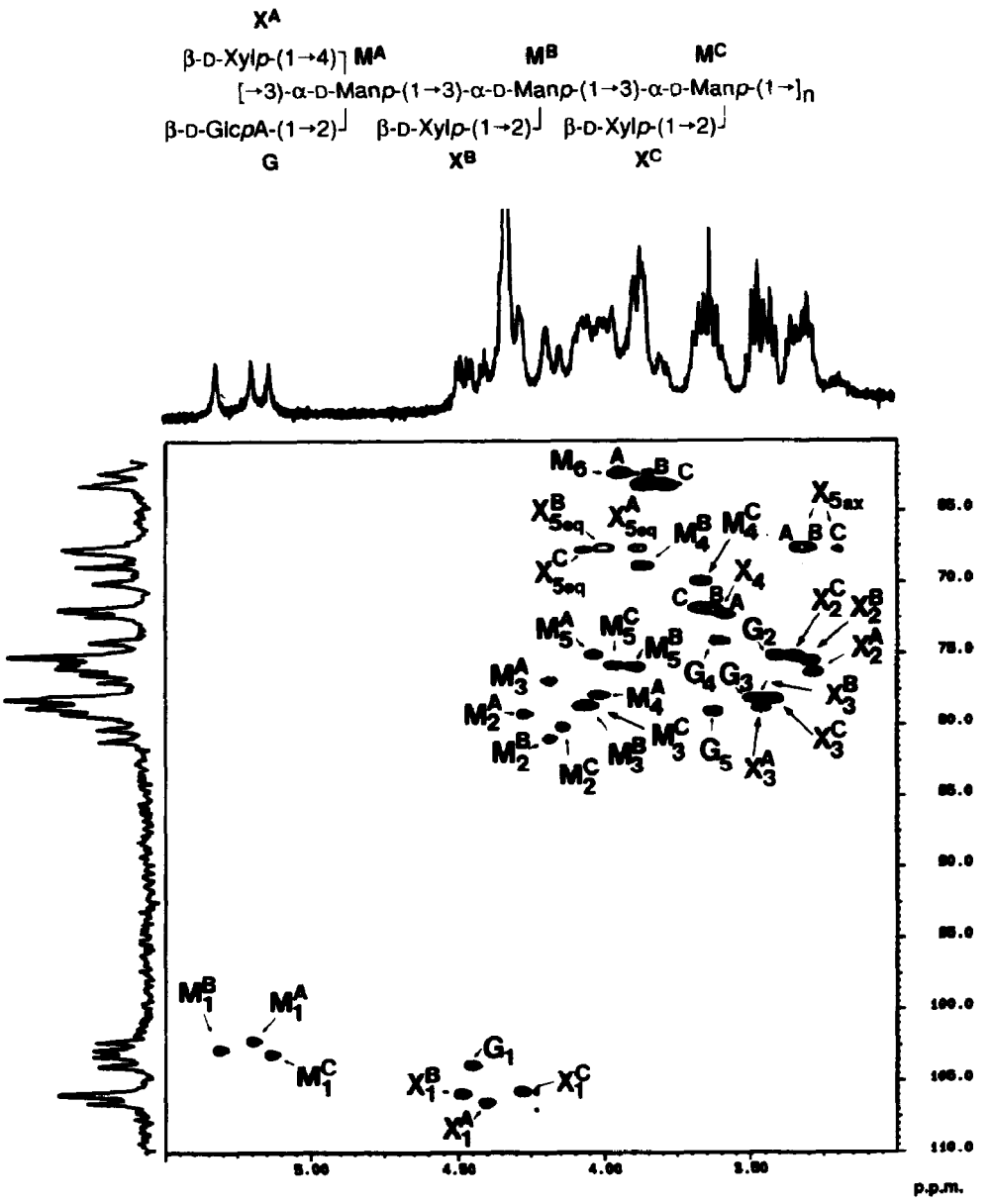

Fig. 3. {^{1}H,^{13}C} One-bond correlation (HMQC) spectrum of GXM-B recorded for a solution in D_2O–$(CD_3)_2CO$ (9:1) at 500 MHz and 57°, with high-resolution 1D ^{1}H and ^{13}C projections. The residues and protons are designated as in Fig. 1.

^{13}C-n.m.r. spectrum of GXM-B with existing n.m.r. databases for carbohydrates[14], are revised based on the current ^{13}C-n.m.r. data that were obtained by HMQC spectroscopy. Specifically, this study provides, for the first time, unambiguous sequence-specific assignments of the ^{13}C signals of the mannosyl and xylosyl residues. The ^{1}H- and

^{13}C-n.m.r. assignments should prove valuable in future n.m.r. studies of native glucuronoxylomannans as well as of *O*-deacetylated glucuronoxylomannans from *C. neoformans* strains with other serotypes.

EXPERIMENTAL

GXM was isolated from the culture supernatant solution of *C. neoformans* serotype B NIH strain 409 (provided by Dr. K. J. Kwon-Chung, National Institutes of Health, Bethesda, MD) and purified as described[8]. The purified GXM was treated with ultrasonic irradiation for 2 h in order to reduce the molecular weight of the polysaccharide (GXM-S)[8] to 1.20×10^5. A portion of GXM-S was *O*-deacetylated by incubation with concentrated NH_4OH at pH 11.0 for 24 h at room temperature, and the product GXM-B was dialyzed and lyophilized. This preparation was used for structural studies. The glycosyl residue and linkage composition, determined by g.l.c.–m.s., has been described[8].

GXM-B (~14 mg) was repeatedly exchanged with D_2O, with intermediate lyophilization. During the first and second exchange, the pD of the solution was adjusted with potassium bis(phosphate) to 7–8. Finally, the sample was dissolved in 0.5 mL of D_2O (99.96% D from Cambridge Isotope Laboratories) and transferred into a 5-mm n.m.r. tube (Wilmad 535-PP). Acetone-d_6 (50 μL) was added as the lock solvent. Spectra were recorded at 57° with a Bruker AM-500 spectrometer interfaced with an Aspect 3000 computer, using the DISR88 software package. The homonuclear experiments were conducted with a 5-mm dedicated ^{1}H probe. The heteronuclear $\{^1H,^{13}C\}$ spectra were acquired using a 5-mm $\{^1H,^{13}C\}$ dual frequency probe. Chemical shifts were referenced to internal acetone-d_6 (^{1}H 2.167 p.p.m.; ^{13}C 31.9 p.p.m.), which, in turn, were referenced to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) taken as 0 p.p.m.

A double-quantum-filtered (DQF) $\{^1H,^1H\}$ shift correlation (COSY) experiment[15] was performed in the phase-sensitive mode, using time-proportional phase incrementation (TPPI)[16] at a spectral width of 2500 Hz. The evolution time (t_1) was incremented in steps of 200 μs in order to obtain 512 free induction decays (f.i.d.s), each acquired in 2K data points in 48 scans. The acquisition (t_2) time was 410 ms and the relaxation delay was 1.1 s. A squared sine-bell function shifted by $\pi/4$ was applied for processing both in the t_1 and the t_2 dimension. Zero filling was used to expand the data matrix to 1K in the t_1 dimension, before Fourier transformation.

A 2D TOCSY experiment was conducted in the phase-sensitive mode (TPPI), using the DIPSI-2 sequence for isotropic mixing[17–19] with the ^{1}H decoupler used as the sole source of r.f. pulses. The mixing time was 150 ms. The spectral width was 2500 Hz; 512 experiments of 32 scans and 2K data points were acquired with an acquisition time of 410 ms. The relaxation delay was 1.1 s and the t_1 increment was 200 μs. A squared sine-bell function shifted by $\pi/2$ was applied for processing in both dimensions. The data matrix was expanded to a 2K by 1K data file before Fourier transformation.

A 2D rotating-frame n.O.e. (ROESY) spectrum was recorded in the phase-sensitive mode (TPPI). The pulse sequence proposed by Rance[20] was applied, employing

the ^1H decoupler as the sole source of r.f. pulses. The mixing time of 72 ms consisted of a train of short (~20°) pulses sandwiched between two 170° pulses and two *z*-filters[21,22]. The r.f. carrier was centered in the spectrum during evolution and acquisition times, and placed 1000 Hz downfield from the center during the mixing time. A total of 512 f.i.d.s, 32 scans each, were collected with an acquisition time of 342 ms. The spectral width was 3000 Hz. A squared sine-bell function shifted by $\pi/2$ was applied for processing in the t_2 dimension. A non-shifted sine-bell window function was applied in the t_1 dimension. The data matrix was expanded to a 2K by 1K data file before Fourier transformation.

A ^1H-detected $\{^1H,^{13}C\}$ one-bond shift correlation (HMQC) spectrum was recorded as described[9,10,13,23–26], using a ^1H spectral width of 2500 Hz and a ^{13}C spectral width of 10 000 Hz. The evolution time t_1 was incremented in steps of 25 μs to obtain 256 f.i.d.s, consisting of 2K data points each. The relaxation delay was 1.1 s. A sine-bell function shifted by $\pi/2$ was applied in the t_2 dimension and a Gaussian window (line broadening 5 Hz) was applied in the t_1 dimension. Zero filling to 1K was used in the t_1 dimension before Fourier transformation.

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REFERENCES

1 R. D. Diamond, in G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (Eds.), *Principles of Infectious Diseases*, Churchill Livingstone, New York, 1990, pp. 1980–1989.
2 W. E. Dismukes, *J. Infect. Dis.*, 157 (1988) 624–628.
3 F. W. Chandler, in M. R. McGuinnes (Ed.), *Current Topics in Medical Mycology*, Vol. 1, Springer, New York, 1985, pp. 1–23.
4 A. K. Bhattacharjee, J. E. Bennett, and C. P. J. Glaudemans, *Rev. Infect. Dis.*, 6 (1984) 619–624.
5 R. Cherniak, in M. R. McGuinnes (Ed.), *Current Topics in Medical Mycology*, Vol. 2, Springer, New York, 1988, pp. 40–54.
6 D. E. Wilson, J. E. Bennett, and J. W. Bailey, *Proc. Soc. Exp. Biol. Med.*, 127 (1968) 820–823.
7 R. Cherniak, R. G. Jones, and E. Reiss, *Carbohydr. Res.*, 172 (1988) 113–138.
8 S. H. Turner and R. Cherniak, *Carbohydr. Res.*, 211 (1991) 103–116.
9 L. Lerner and A. Bax, *Carbohydr. Res.*, 166 (1987) 35–46.
10 H. van Halbeek, in D. Live, I. M. Armitage, and D. Patel (Eds.), *Frontiers of NMR in Molecular Biology*, Liss, New York, 1990, pp. 195–213.
11 D. S. Himmelsbach, H. van Halbeek, R. F. Arrendale, and R. F. Severson, *Magn. Reson. Chem.*, 28 (1990) 682–687.
12 D. A. Powell, W. S. York, H. van Halbeek, J. T. Etse, A. I. Gray, and P. G. Waterman, *Can. J. Chem.*, 68 (1990) 1044–1050.
13 F. J. Cassels, H. M. Fales, J. London, R. W. Carlson, and H. van Halbeek, *J. Biol. Chem.*, 265 (1990) 14127–14135.
14 K. Bock, C. Pedersen, and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193–224.
15 U. Piantini, O. W. Sørensen, and R. R. Ernst, *J. Am. Chem. Soc.*, 104 (1982) 6800–6801.
16 D. Marion and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 113 (1983) 967–974.
17 L. Braunschweiler and R. R. Ernst, *J. Magn. Reson.*, 53 (1983) 521–528.
18 S. P. Rucker and A. J. Shaka, *Mol. Phys.*, 68 (1989) 509–517.
19 L. Poppe and H. van Halbeek, *Magn. Reson. Chem.*, 29 (1991) 355–361.
20 M. Rance, *J. Magn. Reson.*, 74 (1987) 557–564.
21 H. Kessler, C. Griesinger, R. Kerssebaum, K. Wagner, and R. R. Ernst, *J. Am. Chem. Soc.*, 109 (1987) 607–609.

22 Z. Dezheng, T. Fujiwara, and K. Nagayama, *J. Magn. Reson.*, 81 (1989) 628–630.
23 A. Bax and S. Subramanian, *J. Magn. Reson.*, 67 (1986) 565–569.
24 J. Cavanagh and J. Keeler, *J. Magn. Reson.*, 77 (1988) 356–362.
25 R. A. Byrd, W. Egan, M. F. Summers, and A. Bax, *Carbohydr. Res.*, 166 (1987) 47–58.
26 F. P. Tsui, W. Egan, M. F. Summers, R. A. Byrd, R. Schneerson, and J. B. Robbins, *Carbohydr. Res.*, 173 (1988) 65–74.