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Structure of the ^{13}C-enriched *O*-deacetylated glucuronoxylomannan of *Cryptococcus neoformans* serotype A determined by NMR spectroscopy

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Abstract

The complete assignment of ^{1}H and ^{13}C chemical shifts for 99% uniformly ^{13}C-labeled *O*-deacetylated glucuronoxylomannan (GXM) of *Cryptococcus neoformans* serotype A isolate 9759-Mu-1 was accomplished by the analysis of HCCH-TOCSY and HCCH-COSY spectra. The sequence of the glycosyl residues was determined by a GHMBC experiment using 20% uniformly ^{13}C-labeled GXM; GXM was prepared by a novel procedure that insured the virtual exclusion of adjacent ^{13}C-labeled carbon atoms. For each residue in the GXM of 9759-Mu-1 we determined its linkage position, its anomeric configuration, and its position in the repeating sequence as follows:

β-D-Glc*p*A	β-D-Xyl*p*	β-D-Xyl*p*
1	1	1
↓	↓	↓
2	2	2

[→3)-α-D-Man*p*-α-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1]$_n$

Keywords: 2D NMR spectroscopy; HCCH–TOCSY; HCCH–COSY; GHMBC; HSQC; Polysaccharide; ^{13}C Labeling, Structure; Antigen

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1. Introduction

Cryptococcus neoformans is an opportunistic pathogenic yeast that causes a life-threatening meningoencephalitis in individuals with an impaired immune system [1]. The incidence of cryptococcosis has increased dramatically in recent years as a consequence of the AIDS epidemic, and it is a leading cause of death in patients with AIDS [2–5]. *C. neoformans* is unusual among pathogenic fungi in that it has a polysaccharide capsule, glucuronoxylomannan (GXM). GXM is antiphagocytic and poorly immunogenic, and acapsular strains have significantly reduced virulence [6,7]. In vitro, GXM inhibits leukocyte migration [8], enhances HIV infection in human lymphocytes [9] and promotes L-selectin shedding from neutrophils [10]. The typical GXM is composed of a linear (1 → 3)-α-D-mannopyranan bearing β-D-xylopyranosyl (Xyl*p*), β-D-glucopyranosyluronic acid (Glc*p*A), and 6-*O*-acetyl substituents [11–13]. Differences in the extent and location of the Xyl*p* substitutients in GXM provide antigenic variability that has led to the classification of *C. neoformans* strains into five serotypes (A, B, C, D, and A–D) [14–17]. *O*-Acetylation is an indispensable component of the epitope structure responsible for the serological activity observed with type specific antibodies [18] and monoclonal antibodies [19]. The disposition of the *O*-acetyl substituents contributes to the antigenic multiplicity observed among GXMs obtained from all serotypes with the exception of highly substituted GXMs obtained from serotype C. The *O*-acetyl may be part of the epitope; it may serve only to help fix the correct conformation of the epitope; or it may serve in both capacities.

Two distinct varieties of *C. neoformans* have been described; *C. neoformans* var *neoformans*, comprised of serotypes A and D, and *C. neoformans* var *gattii*, comprised of serotypes B and C, [20]. Most cryptococcal infections in AIDS patients are due to *C. neoformans* var *neoformans*, with serotype A comprising the majority of isolates [21,22]. GXMs from several serotype A isolates were investigated by methylation analysis [23,24]. Identical structures based on the data were proposed based on repeating triad structure comprised of three (1 → 3)-α-D-mannosyl residues substituted with two residues of 2-*O*-β-D-xylosyl and one residue of 2-*O*-β-D-glucopyranosyluronic acid. However, the reported data allows only the postulation of an average structure. In addition, the data does not allow for the detection of structural microheterogeneity within the polysaccharide chain. Recent studies of several serotype A isolates, using high-field NMR spectroscopy fingerprinting, have documented the presence of several structural elements usually found in other serotypes [25,26]. However, the complete assignment of the carbon and proton spectra of a serotype A GXM of a reference strain of *C. neoformans* has not been published. This data is a prerequisite for determining the *O*-acetyl disposition and solution conformation of serotype A GXM.

The primary sequences of *O*-deacetylated GXMs from serotypes B, C, and D were determined previously by 2D NMR spectroscopy [27–29]. Assignments of the ^{1}H and ^{13}C chemical shifts were accomplished using polysaccharides isolated from culture filtrates containing glucose having natural abundance carbon-13. A similar approach for assigning the primary sequence of *O*-deacetylated GXM from serotype A was only partially successful [30]. TOCSY cross sections through Man H-2 and/or Man H-3 signals did not yield unambiguous assignments of H-4, H-5, H-6, and H-6′. The Man H-4, H-5, H-6, and H-6′, and C-4, C-5, and C-6 signals could not be attributed unequivocally to a particular Man residue (**1**). In the present study, isotopically enriched (20 and 99% carbon-13) serotype A GXMs were prepared. The use of carbon-13 labeled GXMs facilitated the complete assignment of the carbon and proton spectra using recently developed heteronuclear 2D NMR experiments. The strategy used for the sequential assignment of the ^{1}H and ^{13}C spectra of the serotype A GXM consisted of the following two steps: (i) complete ^{1}H and ^{13}C assignments using HCCH–TOCSY and HCCH–COSY experiments with 99% uniformly labeled polysaccharide [31]; (ii) sequencing the structure using a gradient modification of HMBC [32,33]. The GHMBC experiment was done using a 20% uniformly labeled GXM prepared by a novel procedure that insured the virtual exclusion of adjacent ^{13}C labeled carbon atoms.

2. Experimental

C. neoformans *isolate.*—The parent isolate used in this study was serotype A 9759 (H. Jean Shadomy, Medical College of Virginia). The parent isolate was cultured on Sabouraud agar and selected mucoid colonies were expanded in 50 mL of a chemically defined liquid medium as previously described [34]. Purified GXMs were obtained by selective precipitation with hexadecyltrimethylammonium bromide

(CTAB) as previously described, except the preliminary treatments by ultrasonic irradiation and EtOH were omitted [34]. After five days the culture was autoclaved for 25 min at 121 °C, and the cells were removed by centrifugation at 18 000 × *g* for 1 h. The culture supernatant (~ 50 mL) was adjusted to 0.2 M NaCl and 1.5 g of CTAB was added to the stirred solution at 23 °C. A 0.05% solution of CTAB (100 mL) was added slowly and with stirring. The precipitate was recovered by centrifugation at 5000 × *g* for 15 min at 23 °C. The pellet was triturated with 10% EtOH, and the suspension was centrifuged as above. The pellet was dissolved in M NaCl (25 mL) by stirring overnight or until the precipitate was completely dissolved. GXM was precipitated by the slow addition of three volumes of 95% EtOH, and the flask was placed at 4 °C. The GXM was recovered by centrifugation at 5000 × *g* for 15 min at 4 °C. The GXM was dissolved in 2 M NaCl (~ 25 mL) with stirring until it was completely dissolved. The solution was treated by ultrasonic irradiation (Branson Sonifier, Model 450) for 2 h at 80% power and 40% pulse below 20 °C. A portion of the GXM was *O*-deacetylated at pH 11 (NH_4OH) for 24 h at 23 °C [35], dialyzed, and lyophilized.

Carbon-13 enriched GXM.—A stock culture (10 mL) of *C. neoformans* was prepared using the standard liquid medium containing natural abundance glucose. This culture was used to inoculate (5%) a second aliquot (10 mL) of defined medium. After 48 h the culture was centrifuged (30 000 × *g* for 1 h) and the cells were resuspended in glucose-free medium and the centrifugation repeated. This procedure was repeated three times in order to remove as much of the natural-abundance glucose as feasible. The washed cells were suspended in fresh medium (50 mL) containing 2% w/v glucose (U–$^{13}C_6$, ^{13}C, 99% +, Cambridge Isotope Labs.). The culture was incubated for 5 d, autoclaved, and centrifuged. The supernatant was lyophilized and redissolved in 10 mL of 0.2 M NaCl. The labeled GXM was isolated as described above. A variation of this procedure was used to prepare 20% enriched polysaccharides. A mixture of five unique carbon-13 labeled glucose (99% enriched) samples were used in equal proportions to give the usual 2% glucose in the media. The glucose was labeled as follows: (C-1,6–$^{13}C_2$, 99%, 0.33 g; C-2–^{13}C, 99%, 0.166 g; C-3–^{13}C, 99%, 0.166 g; C-4–^{13}C, 99%, 0.166 g; C-5–^{13}C, 99%, 0.166 g). The GXM was isolated in the usual manner. This resulted in the overall presence of 20% carbon-13 in the isolated product. The most important fact is that this method avoids the complication of carbon coupling when adjacent carbons are also labeled with carbon-13. The yield of GXM was 10–13% based on the glucose added.

Nuclear magnetic resonance spectroscopy.—*O*-Deacetylated GXM (~ 10 mg) was exchanged twice in 99.96% D_2O with intermediate lyophilization. The sample was dissolved in 0.70 mL of 99.96% D_2O and transferred into a 5-mm NMR tube (Wilmad 535-PP). All NMR experiments were performed at 60 °C using a Varian UnityPlus 600 spectrometer equipped with a 5-mm inverse probe (^1H, X). The proton spectral width was 2500 Hz and the carbon spectral width was 11,000 Hz in all experiments. Measurements of 2 × 440 × 1024 data points were recorded for the constant-time HCCH–COSY, HCCH–TOCSY, [31] and HSQC [36–38] experiments. The isotropic mixing time in the HCCH–TOCSY experiment was 25 ms. All the other parameters used in HCCH–TOCSY and HCCH–COSY [31] experiments were as reported in the literature. Quadrature detection in the F1 dimension was achieved by the States–TPPI method [39]. ^{13}C decoupling was accomplished using the Garp pulse sequence [40]. The pulse sequence used for the GHMBC experiment is depicted in Fig. 1. The sequence uses gradient pulses to select coherence transfer pathways and allows phase-sensitive GHMBC spectra to be recorded without changing the sign of gradient pulses.

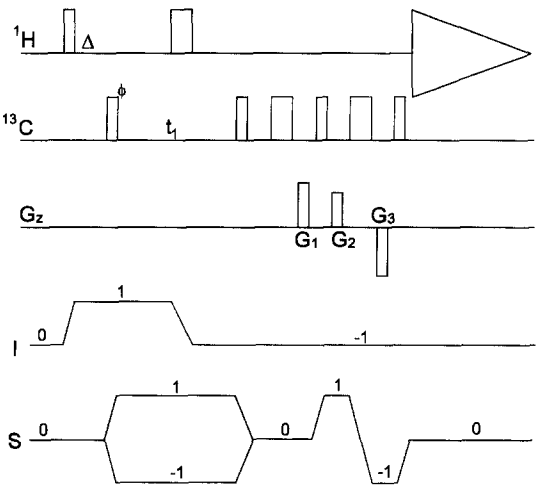

Fig. 1. Pulse sequence and coherence-transfer pathway of the gradient-enhanced HMBC experiment. Thin and thick bars denote 90° and 180° pulses, respectively. G1, G2 and G3 denote the three gradient pulses used for coherence pathway selection. Quadrature detection in t_1 is achieved by using the States-TPPI method [39] with the incrementing of the phase, ϕ. In the coherence transfer scheme, I stands for spin ^1H and S for spin ^{13}C.

Separation of the gradient pulses from the evolution period is the key for recording spectra in phase-sensitive mode. The rephasing condition for the desired coherence transfer pathway is given in Fig. 1. The combination of G1:G2:G3 = 4:3:−5 satisfies this condition. Squared gradient pulses of 1.5 ms duration were used; z gradients G1, G2 and G3 were 15, 11.25 and 18.75 G/cm respectively. The delay, Δ, used to allow for the evolution of heteronuclear long-range couplings was 45 ms. Measurements of $2 \times 256 \times 4096$ data points were made; 50 scans were accumulated for each t_1 increment.

The data were processed off-line by using the FELIX 95.0 software package (Biosym/Molecular Simulations, San Diego) with a Silicon Graphics Indy workstation. A Lorentzian-to-Gaussian weighting function (lb = −0.5, gb = 0.05) was applied in the t_2 domain and a squared cosine-bell in the t_1 domain with the first data point multiplied by 0.5 to reduce t_1 noise. Zero-filling by an equal number of data points was applied in both dimensions. ^{1}H and ^{13}C Chemical shifts were measured relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate taken as 0.00 ppm.

3. Results

Carbon 13-enriched GXM.—The isolate 9759 MU-1 was selected based on the presence of three — and only three — characteristic resonances observed

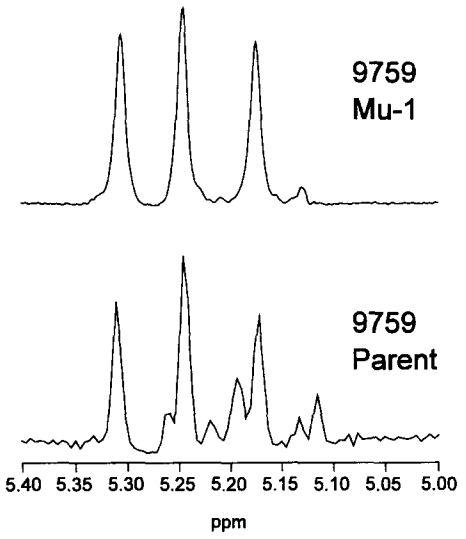

Fig. 2. Resolution-enhanced annomeric region of the mannose reporter groups for the *O*-deacetylated GXM of 9759-Mu-1 (top) and 9759 parent isolate (bottom). ^{1}H NMR Spectra were recorded at 500 MHz and 80 °C.

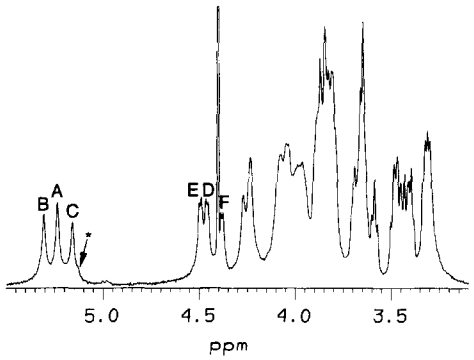

Fig. 3. 1D ^{1}H NMR spectrum of ^{13}C-labeled GXM 9759-Mu-1 recorded at 600 MHz and 60 °C.

in 1D ^{1}H nuclear magnetic resonance spectroscopy (5.244, 5.306, and 5.175 ppm) in the region of the spectrum where the anomeric protons of mannosyl residues are located (Fig. 2) [41]. These chemical shifts are characteristic of the presence of the serotype A triad structure as defined by Bhattacharjee et al. [11] Isolates derived from the parent culture that gave GXMs exhibiting ^{1}H chemical shifts characteristic of the mannosyl triads of other serotypes, serotype D [29,42], serotype B [27], serotype C [28], were discarded.

NMR analysis.—The 1D ^{1}H NMR spectrum of GXM 9759-Mu-1 (99% enriched with carbon-13) has six anomeric proton signals (A–F) of approximately equal intensity (Fig. 3). This data supports the previous model hexasaccharide structure for the GXM obtained from serotype A (**1**) [11].

β-D-Glc*p*A^D	β-D-Xyl*p*^{E}	β-D-Xyl*p*^{F}	
1	1	1	
↓	↓	↓	(1)
2	2	2	

[→3)-α-D-Man*p*^{A}α-(1→3)-α-D-Man*p*^{B}-(1→3)-α-D-Man*p*^{C}-(1]$_n$

The anomeric signals labeled as A–C were assigned to the Man residues based on their shapes and chemical shifts. The well resolved doublets labeled as D–F were assigned to Xyl and GlcA residues. The complete assignment of ^{1}H and ^{13}C resonances started with the identification of spin system of each monosaccharide residue based on the HCCH–COSY

and HCCH–TOCSY spectra [31]. As illustrated for residue C in Fig. 4a, H-1 showed a one bond correlation to C-1 (5.16–103.81 ppm). H-1 also showed a correlation to C-2 (5.16–80.68 ppm). H-2 had correlations to C-1, C-2 and C-3 as indicated by a the vertical dashed line in Fig. 4a. C-2 showed correla-

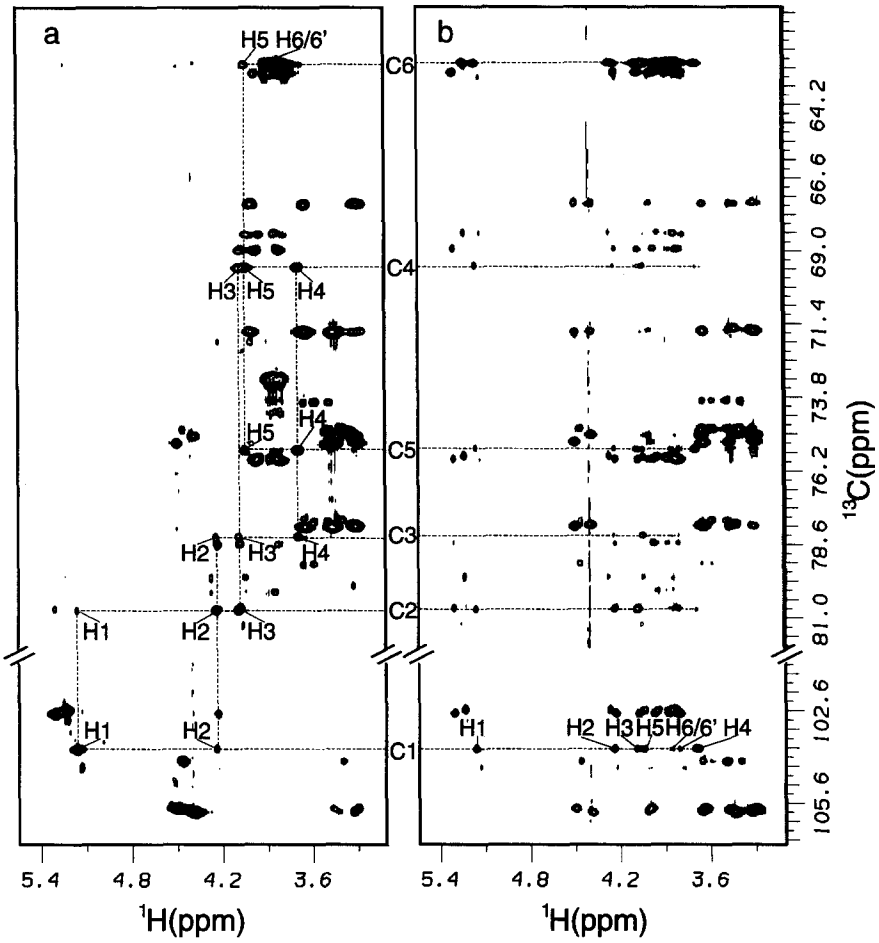

Fig. 4. (a) 2D constant-time HCCH–COSY spectrum of ^{13}C-labeled GXM 9759-Mu-1. (b) 2D constant-time HCCH–TOCSY spectrum of ^{13}C-labeled GXM 9759-Mu-1 recorded at 600 MHz and 60 °C.

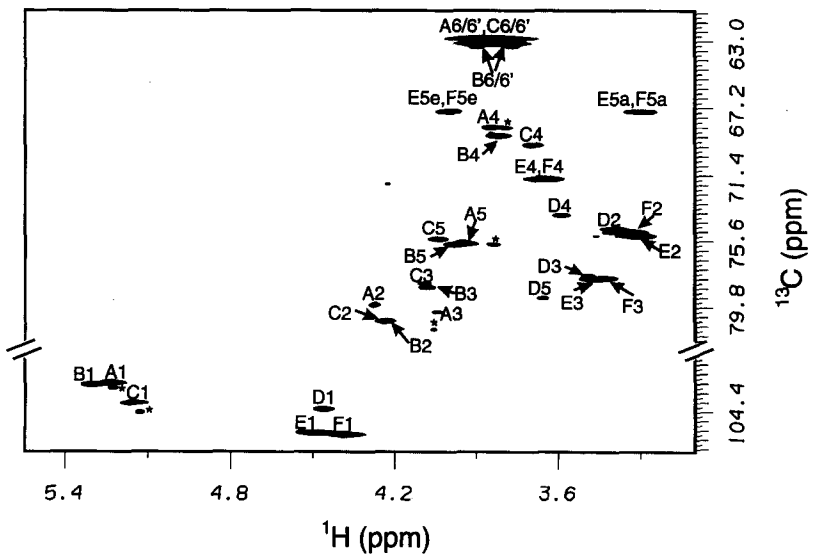

Fig. 5. 2D constant-time HSQC spectrum of GXM 9759-Mu-1 recorded at 600 MHz and 60 °C

tion to H-1, H-2 and H-3 indicated by horizontal dashed line in Fig. 4a. In general, each H-n was correlated to C-($n-1$), C-n and C-($n+1$). Similarly, each C-n was correlated to H-($n-1$), H-n and H-($n+1$). The spin system of residue C was identified by following this correlation pattern. The one ambiguity for this residue was the chemical shifts for H-6 and H-6′, due to the severe overlap in the H-6, H-6′/C-6 region. The HCCH–TOCSY spectrum provided the necessary data to resolve the remaining assignments (Fig. 4b). All the ^{1}H signals of residue C were observed in the HCCH-TOCSY spectrum at C-1. Since H-1–H-5 were already assigned from the HCCH–COSY spectrum, the remaining two peaks located at 3.86 and 3.80 ppm were assigned to H-6 and H-6′. This completed the assignment of the ^{1}H and ^{13}C signals of residue C. The ^{1}H and ^{13}C resonances were assigned for the other five residues in similar fashion. Residue D was identified as GlcA as it only showed one H-5 signal. Residues E and F were assigned to Xyl as they both showed two H-5 signals.

The 2D constant-time HSQC experiment [36–38] gave a simplified spectrum of GXM that was used to confirm all the chemical shift assignments (Fig. 5). The spectral region between 82 ppm and 101 ppm has been deleted since there were no signals in that area. The low intensity peaks, marked by asterisk in the HSQC spectrum, indicated that a slight heterogeneity existed in the GXM of 9759-Mu-1 (Fig. 5). We were not able to identify the origin of these peaks since their intensities were low compared to those of the major signals (< 5%). Table 1 contains the ^{1}H and ^{13}C chemical shift assignments for the serotype A GXM of 9759-Mu-1.

Table 1
^{1}H and ^{13}C chemical shifts [a] of the GXM-A polysaccharide measured at 60 °C

	A Man	B Man	C Man	D GlcA	E Xyl	F Xyl
H-1	5.24	5.31	5.16	4.64	4.50	4.39
H-2	4.27	4.23	4.24	3.40	3.30	3.33
H-3	4.05	4.08	4.09	3.49	3.46	3.44
H-4	3.85	3.83	3.70	3.59	3.65	3.65
H-5a	3.95	3.97	4.04	3.66	3.31	3.28
H-5e	–	–	–	–	4.00	4.00
H-6	3.85	3.88	3.86	–	–	–
H-6′	3.83	3.80	3.80	–	–	–
C-1	102.59	102.73	103.81	104.24	105.74	105.88
C-2	79.67	80.68	80.68	74.80	75.30	75.02
C-3	80.10	78.61	78.31	77.81	78.02	77.95
C-4	68.43	68.93	69.50	73.94	71.58	71.58
C-5	75.73	75.80	75.46	79.23	67.43	67.43
C-6	62.84	63.13	62.84	177.86	–	–

[a] In ppm relative to internal DSS at δ 0.0.

The next step was the assignment of the sequence of the glycosyl residues. The GHMBC spectrum, recorded according to the pulse sequence of Fig. 1, was used to determine the long-range H-1–C-x' and C-1–H-x' connectivities (Fig. 6). The 99% ^{13}C uniformly labeled GXM was unsuitable for this experiment because of the poor resolution in the ^{13}C dimen-

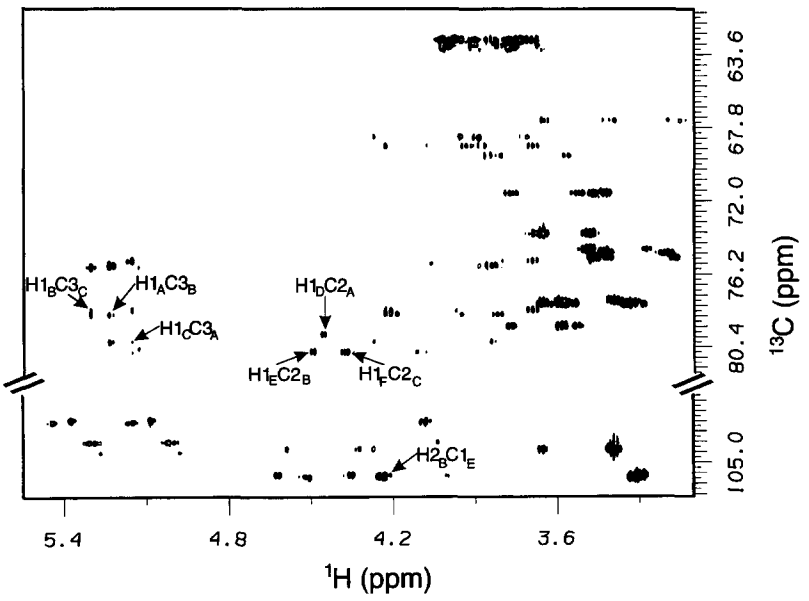

Fig. 6. 2D gradient-enhanced HMBC spectrum recorded at 600 MHz and 60 °C according to the pulse sequence of Fig. 1.

sion caused by $^{13}C-^{13}C$ splitting that is difficult to remove in multiple quantum coherence experiments. Therefore, 20% labeled GXM that contained virtually no adjacent ^{13}C-carbon atoms was used for this experiment. The observation of a cross peak between H-1 of residue B and C-3 of residue C shows that residue B was linked to residue C through a (1 → 3)-linkage (Fig. 6). Similarly, the cross peaks $H\text{-}1_A\text{–}C\text{-}3_B$ and $H\text{-}1_C\text{–}C\text{-}3_A$ indicate that residue A is linked to residue B through a (1 → 3)-linkage and that residue C is linked to residue A through a (1 → 3)-linkage. It is clear that the Man residues comprise the (1 → 3)-mannan backbone of GXM (**1**). The cross peak between H-1 of residue D and C-2 of residue A indicates that residue D is linked to residue A through a (1 → 2)-linkage. The other two cross peaks located at (4.50/80.68) and (4.39/80.68) cannot be unequivocally assigned since the C-2 chemical shifts of residues B and C are degenerate. Fortunately, the C-1 chemical shifts of residues E and F were slightly different and this permitted the differentiation of residues E and F. The cross peak between H-2 of residue B and C-1 of residue E indicated that residue E is linked to residue B through a (1 → 2)-linkage. The remaining residue, F, must be (1 → 2)-linked to residue C.

The GHMBC spectrum (Fig. 6) also provided the $^1J_{CH}$ couplings for the anomeric protons. The $^1J_{CH}$ coupling of ~ 170 Hz observed for residues A, B, and C indicates that the three mannose residues were of the α configuration [43]. The $^1J_{CH}$ couplings of ~ 162 Hz for residues D, E and F indicated that the Xyl and GlcA residues have the β configuration. This completed the determination of the primary sequence of the GXM obtained from serotype A 9759-Mu-1 (**1**).

4. Discussion

The ^1H and ^{13}C chemical shifts of GXM 9759-Mu-1 encompass relatively small regions of their respective spectra, ~ 2 ppm for ^1H and ~ 40 ppm for ^{13}C. It is difficult to assign the ^1H and ^{13}C spectra completely by using conventional NMR methods due to the severe overlap of the resonances. The ability to prepare uniformly ^{13}C-labeled polysaccharide enabled us to apply NMR experiments involving $^{13}C-^{13}C$ coherence transfer. Coherence transfer is much more efficient in this case since the $^1J_{HC}$ (~ 150 Hz) and $^1J_{CC}$ (~ 40 Hz) coupling constants are much larger than the $^3J_{HH}$ (1–12 Hz) coupling constants. The identification of the Man spin system in $\{^1H,^1H\}$ correlation spectroscopy is difficult since no appreciable magnetization transfer occurs through H-2. This is expected because the $^3J_{H\text{-}1\text{–}H\text{-}2}$ coupling constant for Man is < 2 Hz.

An HMBC experiment provides an unequivocal way to assign glycosyl linkages. However, low sensitivity is observed in HMBC for high mol. wt. biomolecules due to short T_2 and the long time delays required for heteronuclear long-range couplings to evolve. Therefore, an impracticable acquisition time is required to record an HMBC spectrum with these molecules. This problem can be ameliorated by using ^{13}C-enriched polysaccharides. The labeling needs to be selective to insure that only negligible quantities of adjacent ^{13}C labeled carbon atoms occur in the polysaccharide. This avoids multiplicity of the signal caused by one bond $^{13}C-^{13}C$ couplings that causes lower digital resolution in the ^{13}C dimension. This is particular troublesome in cases where the ^{13}C signals are not well separated as observed for the serotype A GXM studied herein. The selective labeling of GXM was done by using five different ^{13}C-labeled Glc (C-1,6-$^{13}C_2$; C-2-^{13}C; C-3-^{13}C; C-4-^{13}C;) during the growth of *C. neoformans*. This permitted the isolation of GXM that was 20% enriched in the ^{13}C and that possessed virtually no adjacent ^{13}C-labeled carbon atoms. Additional improvement in the digital resolution in ^{13}C dimension was achieved by recording the gradient-enhanced HMBC spectrum in the phase-sensitive mode (Fig. 1 and Fig. 6).

The method used to prepare the 20% ^{13}C labeled polysaccharide produced a sample that minimized the effect of $^{13}C-^{13}C$ dipolar relaxation. The ^{13}C-labeled polysaccharide is ideal for determining T_1, T_2, $T_{1\rho}$ and heteronuclear NOEs [44–46] because the interpretation of the relaxation data is simplified. T_1, T_2, $T_{1\rho}$ and heteronuclear NOEs are the key parameters for probing dynamic properties of polysaccharides.

5. Conclusions

The assignment of ^1H and ^{13}C chemical shifts for the ^{13}C labeled GXM, of *C. neoformans* serotype A isolate 9759-Mu-1 was achieved. For each residue in GXM we determined its linkage position, its anomeric configuration, and its position in the repeating sequence (**1**). A novel procedure was used to prepare a ^{13}C-labeled polysaccharide that has virtually no adjacent ^{13}C atoms. The primary structure of the ^{13}C-

labeled GXM was determined by 2D NMR using a new gradient enhanced, phase-sensitive HMBC pulse sequence.

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