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TYPE-SPECIFIC POLYSACCHARIDES OF *Cryptococcus neoformans*. N.M.R.-SPECTRAL STUDY OF A GLUCURONOMANNAN CHEMICALLY DERIVED FROM A *Tremella mesenterica* EXOPOLYSACCHARIDE

ROBERT CHERNIAK, RONALD G. JONES*,
Department of Chemistry and Laboratory of Microbial and Biochemical Sciences, Georgia State University, Atlanta, Georgia 30303 (U.S.A.)

AND MOREY E. SLODKI
Northern Regional Research Laboratory, Peoria, Illinois 61604 (U.S.A.)

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ABSTRACT

A glucuronomannan (GM) was derived by removal, through Smith degradation, of xylose from the native (3-*O*-acetylglucurono)xylomannan exopolysaccharide isolated from *Tremella mesenterica*. ^{13}C-N.m.r. chemical shifts measured at various pD values were compared for *p*-nitrophenyl β-D-glucopyranosiduronic acid (**1**) and two GMs (**2** and **3**) differing in GlcA content (Man:GlcA; **2**, 10:1; and **3**, 5:1). Also measured and compared were pK_a values for **1** and **2**. One-dimensional and two-dimensional (COSY and HETCOR) n.m.r. data allowed unambiguous assignments of pD-sensitive chemical shifts due to 2-*O*-β-D-Glc*p*A substituents attached to a (1→3)-linked α-D-Man*p* backbone. The pK_a and n.m.r. data indicated that the CO_2H groups in either GM are independent of each other, and are similar in behavior to those of *p*-nitrophenyl β-D-glucopyranosiduronic acid molecules. The n.m.r. data confirmed the previous, chemically deduced, structural role of Glc*p*A in the native polysaccharide from *T. mesenterica*, and indicated that significant pD-induced changes occur in the stabilities of the glycosidic orientations in the GM. Previous ^{13}C-n.m.r. assignments for 2-*O*-β-D-Glc*p*A in polysaccharides derived from *Cryptococcus neoformans* serotype A-variant were confirmed, except for the signal due to the anomeric carbon atom. This signal is now known to be pD-sensitive. In acidic solutions, it is coincident with the signal (104.5 p.p.m.) due to the anomeric carbon atoms of the unsubstituted α-D-Man*p* backbone residues. In basic solutions, the 2-*O*-β-D-Glc*p*A anomeric carbon resonance is shifted upfield by ~0.2 p.p.m., and is observed as a separate signal.

*To whom correspondence should be addressed.

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INTRODUCTION

As part of an investigation of the structural heterogeneity in *Cryptococcus neoformans* polysaccharides, a combination of chemical degradations and n.m.r.-spectroscopic techniques is being applied to permit faster and more-detailed determination of the primary structure in the native polymer than has previously been possible. The *C. neoformans* capsular polysaccharides in general consist of a linear backbone of (1→3)-linked α-D-Man*p* residues bearing β-D-Xyl*p*, β-D-Glc*p*A, and 6-*O*-acetyl substituents, and are commonly known[1,2] as glucuronoxylomannans (GXM). Chemical methods have been reported for derivation of the mannan (M) backbone and the corresponding xylomannan (XM) from GXM of the A-variant serotype. These derivatives were studied[3] by ^{13}C-n.m.r. spectroscopy. The chemical-shift assignments proved useful in defining the structure of the native GXM. The ^{13}C-n.m.r. data permitted the determination of the location of the 2-*O*-β-D-Glc*p*A residues on the M backbone, but the spectral complexity left in doubt the assignment of the resonances attributable to Glc*p*A and the Man*p* units influenced by attachment or proximity to the uronic moieties. There was no convenient method, other than partial hydrolysis with acid, for obtaining the corresponding glucuronomannan (GM) directly from *C. neoformans* GXM in order to serve as a model, but this procedure did not provide the quantity or purity of GM required for n.m.r. analysis.

Tremella mesenteria NRRL Y-6151 produces extracellular polysaccharides that are similar in composition and structure to those elaborated by *C. neoformans*, except that β-D-Xyl*p* is present as 2-linked side-chains that are two and three Xyl*p* units long, and β-D-Glc*p*A occurs as individual, 2-linked 3-*O*-acetyl-Glc*p*A residues[4–6]. Because of the fortuitous location of the *O*-acetyl substituents on Glc*p*A residues, it is possible to remove, selectively, the Xyl*p* components by Smith degradation, and to isolate[4,5] GM. We now report the results from a n.m.r.-spectroscopic study over a range of pD values for two GMs differing in Glc*p*A content, and of *p*-nitrophenyl β-D-glucopyranosiduronic acid used as a model monosaccharide to facilitate the spectral assignments. The results provided chemical-shift assignments for β-D-Glc*p*A 2-linked to a (1→3)-α-D-Man*p* backbone, and new evidence for the detailed structural specification of this polysaccharide. They also allowed confirmation, and one correction, of previous tentative assignments for the polysaccharides derived from *C. neoformans* serotype A-variant[3].

EXPERIMENTAL

Native and modified glucuronoxylomannan. — (a) (O-Acetylglucurono)xylomannan (AGXM). Extracellular polysaccharide was recovered from filtrates of 96-h cultures of *Tremella mesenterica* NRRL Y-6151 by precipitation with methanol in the presence of potassium acetate, as previously described[6]. AGXM was deproteinized by four cycles of denaturation by solvent by the Sevag method[7]. The

polysaccharide was further purified by selective precipitation with hexadecyltrimethylammonium bromide[8].

(b) Sonicated AGXM. The AGXM (200 mg in 50 ml of de-ionized water) was sonicated for 15 min at 10° at a power setting of 8 and a 40% pulse (Cell Disruptor, Heat Systems-Ultrasonics, Inc., Model R225R). The rate of heat-transfer from the sample vessel to the surrounding cooling medium was not sufficient to maintain the 10° initial temperature, but that of the polysaccharide solution did not rise above 20° during the experiment. This procedure was repeated until 2 g of the AGXM had been processed. The sonicated AGXM was exhaustively dialyzed, and recovered by lyophilization.

(c) Glucuronomannan (GM) by Smith degradation of AGXM. AGXM (1.79 g) was dissolved in 750 mL of 0.15M $NaIO_4$ and kept for 96 h at 4° in the dark. Ethylene glycol (35 mL) was added, and after 3 h, $NaBH_4$ (21 g) was added slowly. The mixture was kept in a refrigerator for 24 h, and the pH finally adjusted to 5 with acetic acid. Soluble polyalcohol was recovered by lyophilization after exhaustive dialysis (yield, 947 mg; 53%). The polyalcohol was dissolved in 95 mL of H_2O, and then 5 mL of 2M trifluoroacetic acid was added. The mixture was incubated overnight at 37° and then for 1 h at 95°. GM was recovered by lyophilization after exhaustive dialysis (yield, 468 mg; 26% overall).

(d) Free acid of GM. Sonicated GM (110 mg per mL of H_2O) was applied to a column (1 × 8 cm) of Dowex-50 X8 (H^+) ion-exchange resin (200–400 mesh), and the column was eluted with 25 mL of H_2O. The wash and eluate were combined, and GM was recovered by lyophilization (yield, 92 mg; 84%).

Ionization-constant measurements. — Titrations were conducted in a 2-mL cell that was designed to minimize exposure of the sample to the atmosphere. The pH was measured with a Digital Ionalyzer (model 70141, Orion) and a combination electrode (12 mm o.d.) fitted with a 14/20 standard-taper male joint (Orion Research 91-61). The electrode was fitted with a 15-mm (i.d.) glass tube that had a 14/20 standard-taper female joint. The tube was sealed with a flat bottom sufficiently below the tip of the electrode to accommodate the volume of the sample, the volume of the titrant, and a micro magnetic stirring-bar. A sidearm (3 × 40 mm) was connected 5 mm above the bottom of the tube, at an inclined angle of 45°, and sealed with a rubber septum at its flared top. The cell temperature was controlled at 25°. The system produced stable measurement of buffers to 0.001 pH unit. Saccharide was dissolved in 0.2M KCl (2.00 mL) in order to simulate the ionic strength of the phosphate-buffered solutions used for n.m.r. spectroscopy. The saccharide solution was added to the cell, the electrode positioned, the sample mixed continuously by means of a water-driven magnetic stirrer, and the cell temperature equilibrated. When the initial pH had been stabilized, the septum was penetrated with the syringe needle, which was positioned near the stirring bar. The titrant (501.6mM KOH in CO_2-free water) was contained in a 250-μL syringe (725, Hamilton) equipped with a calibrated manual injector (PB-600, Hamilton) that delivered 5.0-μL increments. Near the equivalence point, a 10-μL syringe (701,

Hamilton) was substituted, and the titration was completed by the addition of titrant in 1-μL increments.

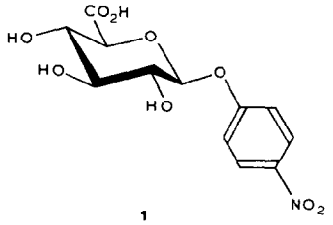
N.m.r. spectroscopy. — All spectra were recorded at 70°. Chemical shifts were measured relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) taken as 0.00 p.p.m. The deuterium resonance of the D_2O solvent (normally potassium phosphate-buffered or pD-adjusted) served as an internal lock.

(a) One-dimensional, ^{13}C-n.m.r. spectra. The spectra were recorded with a JEOL-GX270 n.m.r. spectrometer operated at 67.80 MHz and equipped with a 5-mm, ^1H and ^{13}C dual probe. The spectral width was 16,025 kHz and 16-k data points were collected by use of a 45-degree pulse repeated at 1.51-s intervals. Spectra were zero filled to 128-k data points before fast Fourier transformation (f.f.t.) with 0.77-Hz line-broadening. The resulting digital resolution in the frequency-domain spectra was 0.0036 p.p.m. Proton decoupling was accomplished by application of a 3-kHz noise-band at 270.05 MHz with an offset frequency of 118.333 kHz. Carbon–hydrogen coupling constants were determined with gated decoupling providing retention of the nuclear Overhauser effect (n.O.e.). The typical sample contained 50 mg of saccharide in 0.7 mL of solution.

(b) Proton and two-dimensional spectra. The ^1H-homonuclear correlated spectroscopy (COSY) and ^1H–^{13}C-heteronuclear correlated spectroscopy (HETCOR) spectra were recorded with a Varian VXR-400 n.m.r. spectrometer equipped with a 5-mm ^1H probe or a 10-mm multinuclear probe. For the ^1H-n.m.r. spectra, the operating frequency was 399.952 MHz, and the spectral width was 4 kHz, with 32-k data points collected by use of a 45-degree pulse repeated at 5.75-s intervals. A line-broadening factor of 0.1 Hz was used in the f.f.t. In the COSY experiment, the spectral width was 786.7 Hz. A 90-degree pulse repeated at 1.65-s intervals was employed for collection of 128 transients of 1024 data points each, and 256 increments were collected for transformation in the second dimension. Resolution enhancement was used in both dimensions during data processing. The ^1H–^{13}C HETCOR experiments were performed with an operating frequency of 100.577 MHz. The ^{13}C spectral-width was 4,522.8 Hz with 2048 data points collected for each of 320 transients. In the ^1H dimension, the spectral width was 815.4 Hz, and 256 increments were collected with 2048 data points, and resolution enhancement was used in both dimensions during transformation.

RESULTS AND DISCUSSION

The results of this investigation are based on three saccharide species. *p*-Nitrophenyl β-D-glucopyranosiduronic acid (**1**) was chosen as a model monosaccharide for comparison of ^{13}C chemical shifts. Two GMs (**2** and **3**) were prepared by identical procedures, but from samples of AGXM differing in *O*-acetyl content. Consequently, the two GMs differed in their content of β-D-Glc*p*A. The native AGXM having the smaller proportion of *O*-acetyl groups was aged, had become partially *O*-deacetylated, and yielded GM **2**, with a Man:GlcA ratio of



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10:1, as fewer of the original AGXM Glc*p*A moieties were protected from Smith degradation by 3-*O*-acetyl substituents. The AGXM richer in these protective groups was freshly prepared, and gave GM **3**, having a correspondingly higher Glc*p*A content and a Man:GlcA ratio of 5:1.

The pK_a values measured for **1** and **2** (see Table I), were computed from pH titration curves through use of the complete cubic equation[9]. In the computations, hydrogen-ion activities were calculated by means of activity coefficients (f_i) estimated from ionic strength (I) values through the following relationship[10].

$$-\log f_i = \frac{0.512\sqrt{I}}{1 + 1.6\sqrt{I}}.$$

Both saccharides were treated as simple, monobasic carboxylic acids in the computations. The titration curves for **1** and **2** in H_2O are given in Fig. 1. The similarity in the pH curves shows that the carboxyl groups in **2** act independently, and this indicates that they are not in close proximity to each other. As expected, calculated pK_a values (see Table I) were more consistent over wider ranges of pH and percent neutralization for H_2O solutions titrated with KOH in H_2O than for

TABLE I

ACID IONIZATION CONSTANTS (pK_a VALUES) FROM POTENTIOMETRIC TITRATIONS

Saccharide	*Solvent*	pK_a[a]	*Std. dev.*	*Range*[b]		
				pK_a	*pH*	*% neut.*[c]
1	H_2O	3.00	0.02	3.06	2.50	5
				2.97	4.67	97
	D_2O	2.98	0.05	3.03	2.55	11
				2.86	4.18	92
GM **2**[d]	H_2O	3.00	0.03	3.07	2.45	0
				2.98	4.16	91

[a]Average value; from a single titration at 25°, a pK_a value was calculated from each data point observed within a selected pH range; the values were averaged. [b]Range of parameter values related to the average pK_a value; for GM **2**, the average value (3.00) was derived from 17 data points (see Fig. 1) within the selected pH range (2.45–4.16), with a corresponding range in % neut. (0–91%); the extreme range of values averaged was from pK_a 3.07 to pK_a 2.98. [c]Percentage of the acidic saccharide neutralized. [d]Man:GlcA, 10:1.

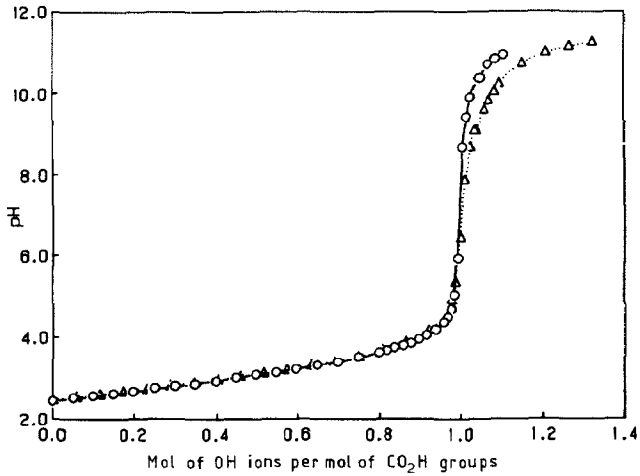


Fig. 1. pH Curves from the titration, with 0.5M KOH, of saccharides in aqueous 0.2M KCl solutions at 25°. Key: ○, *p*-nitrophenyl β-D-glucopyranosiduronic acid (**1**); △, glucuronomannan **2**.

similarly titrated D_2O solutions. There is no difference in ionization constants (K_a) for the two saccharides titrated in H_2O. Furthermore, the K_a values measured for **1** in the two solvents differ by only 2%. On the basis of these findings, the use of the pD-sensitive ^{13}C-n.m.r. resonances of **1** is reasonable for comparison with those of **2**, for assignment of the latter to carbon atoms of constituent β-D-Glc*p*A and directly associated α-D-Man*p* residues in **2**.

The effect of a change in pD on the ^{13}C-n.m.r. chemical-shifts of **1** is

TABLE II

^{13}C-N.M.R. DATA FOR *p*-NITROPHENYL β-D-GLUCOPYRANOSIDURONIC ACID IN POTASSIUM PHOSPHATE SOLUTIONS IN D_2O AT 70°

pD[a]	*Chemical shifts*[b] *and shift differences* [Δδ][c] *in p.p.m.*									
	Glucosyluronic carbon atoms						*Aglycon carbon atoms*			
	1	*2*	*3*	*4*	*5*	*6*	*1*	o	m	p
2.2	102.13	75.14	77.72	73.73	77.44	174.66	164.13	128.45	119.28	145.00
3.0	102.17	75.21	77.82	73.92	77.97	175.71	164.24	128.47	119.28	145.00
3.9	102.22	75.26	77.92	74.14	78.54	176.90	164.37	128.52	119.32	144.99
4.9	102.24	75.29	77.96	74.23	78.74	177.32	164.40	128.53	119.33	144.98
6.6	102.29	75.34	78.00	74.27	78.81	177.38	164.46	128.55	119.38	145.03
9.1	102.32	75.35	78.01	74.27	78.79	177.39	164.48	128.57	119.39	145.07
	[0.19]	[0.21]	[0.29]	[0.54]	[1.35]	[2.73]	[0.35]	[0.12]	[0.11]	[0.07]

[a]All solutions contained 0.1M K^+ (as KCl) or higher concentrations (from pD adjustments with potassium phosphates). [b]Relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. [c]The difference in δ at pD 9.1 and at pD 2.2.

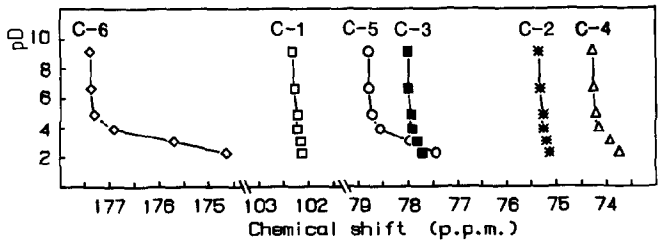


Fig. 2. Chemical shifts of the pyranoside carbon atoms of *p*-nitrophenyl β-D-Glc*p*A (**1**) at various pD values.

presented in Table II and Fig. 2. The largest pD-produced [Δδ] was for the carbonyl carbon atom, as expected, and as reported for the corresponding pyranose[11]. A progressively diminishing effect of pD on the chemical shift of carbon atoms increasingly removed by distance from the carboxylic acid group was observed for **1**, as was previously reported for simple aliphatic monobasic carboxylic acids[12]. The second-largest [Δδ] was for C-5, which had a chemical shift similar to that of C-3. Consequently, the relative chemical-shift values for C-3 and C-5 became reversed at pD ~2.7. On the basis of theoretical considerations[11] for D-Glc*p*A, similarity in [Δδ] values would have been anticipated for C-1 and C-3 of **1**; both values are small, but C-1 has the lesser value. Apparently, the charge-field effect of the carboxylate anion in **1** is attenuated by the intervening O-5 atom. The *ipso* carbon atom of the aryl ring exhibited a larger [Δδ] value than did C-1, C-2, or C-3. This effect may be due either to closer proximity of the *ipso* carbon atom to the carboxylate anion than to the latter carbon atoms, or to a charge-field-induced change in the degree, or mechanism, of electronic interaction between O-1 and the attached aryl group.

The ^{13}C resonances of appreciable intensity observed for **2** are given in Table III. Additional signals of very minor intensity were observed which are attributable to terminal residues. For example, signals for the anomeric carbon atoms of the reducing Man*p* terminus are at ~96 p.p.m. (see Fig. 3). Six major ^{13}C resonances were observed for **2**. These signals were five to seven times as intense as another group of 18 signals. Five of the major signals are shown truncated in Fig. 3. These signals were insensitive to pD changes, and are due to the (1→3)-α-D-Man*p* backbone residues in **2** that are unsubstituted. The chemical shifts for these five signals are close to those of signals previously reported for related polysaccharides[3,13,14], and they are assigned to α-D-Man-a residues (see Table III and structures **2** and **3**).

The five minor resonances due to C-2 through C-6 of β-D-Glc*p*A residues were assigned by comparison of the pD-dependent data for **1** (see Table II) with those for **2**. The C=O resonance was observed at ~178 p.p.m. at pD 7.7, and was assigned to C-6 of the pyranoside residue β-D-Glc*p*A-b (see Table III). Sensitivity to change in pD (Δδ) was monitored for the resonances of C-2 through C-5 of this residue. These four resonances are shown in Fig. 3 as three minor signals at pD 2.2

TABLE III

N.M.R. DATA FOR A 2-*O*-β-D-GLUCOPYRANOSYLURONO-(1→3)-α-D-MANNOPYRANAN IN POTASSIUM PHOSPHATE SOLUTIONS IN D_2O AT 70°

Sugar residue	*pD*	*Chemical shifts[a] and shift differences, [Δδ][b] and {Δδ}[c] in p.p.m., and coupling constants ($^1J_{CH}$) in Hz*											
		C-1	*H-1*	*C-2*	*H-2*	*C-3*	*H-3*	*C-4*	*H-4*	*C-5*	*H-5*	*C-6*	*H-6*
Man-a	2.2	104.51	5.13	72.30		80.80		68.85		76.13		63.70	
	7.7	104.56	5.13	72.32	4.22	80.85	4.03	68.86	3.79	76.18	3.83	63.72	3.80
		(174)		(151)		(144)		(146)		(147)		(144)	
GlcA-b	2.2		4.57	75.11	3.39	77.73	3.53	73.9	3.64	77.67	>3.75	174.84	
	7.7	104.32	4.49	75.32	3.38	78.13	3.50	74.35	3.58	79.47	3.65	178.03	
		[~−0.1]	[−0.08]	[0.21]	[−0.01]	[0.40]	[−0.03]	[0.42]	[−0.06]	[1.80]	[<−0.10]	[3.19]	
		(~160)[d]		(148)				(148)					
Man-c	2.2	102.25	5.26	80.18		79.27		69.03		75.97		63.31	
	7.7	102.41	5.25	79.77[e]	4.28	79.30	4.09	69.08	3.84	76.06	3.98	63.31	3.84
		[0.16]	[−0.01]	[−0.41]		[0.03]		[0.05]		[0.09]		[0.00]	
		(173)										(141)	
		{−2.15}		{7.45}		{−1.55}		{0.22}					
Man-d	2.2	105.04				81.07							
	7.7	105.19	5.11			81.07	4.02						
		[0.15]	[−0.04]			[0.00]							
		(176)											
Man-e	2.2	104.67[f]	5.15			81.30							
	7.7	104.72[f]	5.19			81.12	3.96						
		[0.15]	[0.04]			[−0.18]							
Man-d or -e	2.2									76.32			
	7.7			72.54	4.22			69.28	3.68	76.30		63.65	4.42
				[>0.08]				[>0.24]		[−0.02]		[~−0.08]	
				(151)									

[a]Relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. [b]Difference in δ at pD 7.7 and pD 2.2. [c]Difference in δ as a result of 2-*O*-β-D-Glc*p*A substitution, *i.e.*, $\delta_{Man\text{-}e} - \delta_{Man\text{-}a}$. [d]Estimated from the lower-field half of the doublet and the higher-field component visible as a shoulder with a maximum on the high-field side of the unsubstituted Man doublet. The estimated value is the largest value compatible with the data. [e]This signal had an unassigned, lower intensity companion at 79.86. [f]Seen only as a shoulder on the low-field side of the unsubstituted Man-a C-1 resonance at δ 104.5.

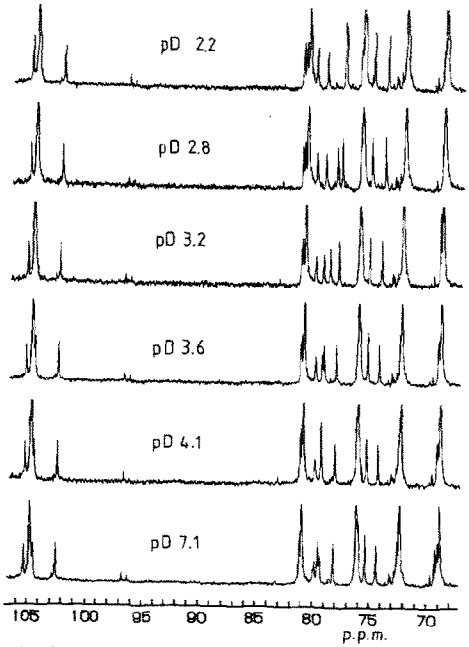
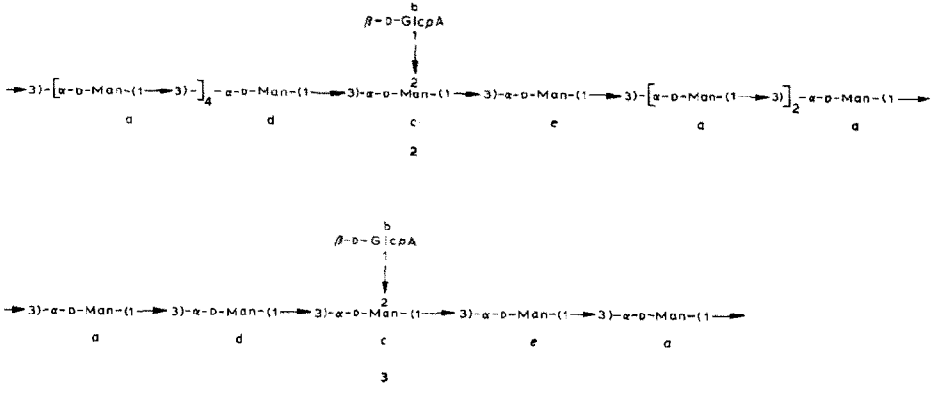


Fig. 3. Proton-decoupled ^{13}C-n.m.r. spectra of glucuronomannan **2** at various pD values.



occurring at ~74, ~75, and ~78 (a doubled signal) p.p.m., where the last consists of the resonances for C-3 and C-5 with almost coincident chemical shifts at this pD value. At slightly lower acidity (pD 2.8), this doubled signal separates (see Fig. 3) as the resonance for C-5 moves downfield, exhibiting its large pD-induced [$\Delta\delta$] effect. At pD 4.1, the C-5 resonance is superimposed on the pD-insensitive Man-c signal at ~79 p.p.m. At pD 7.1, it is observed downfield from the pD-insensitive signal. Comparison of the δ and [$\Delta\delta$] values for these five signals with the analogous

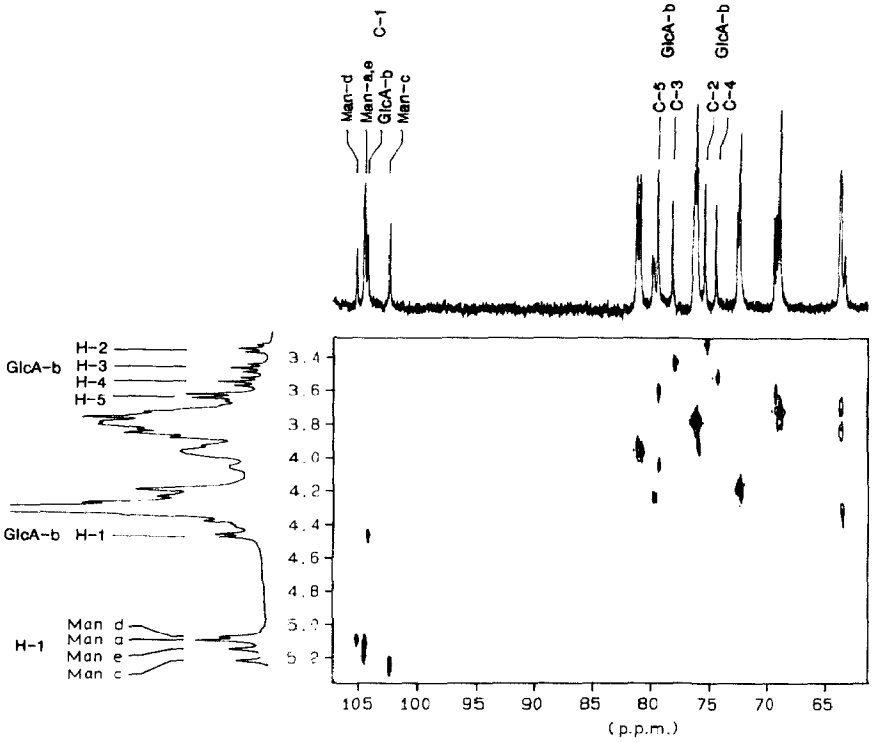


Fig. 4. HETCOR spectrum of glucuronomannan **3** at pD 7.7 and 70°. ^1H δ Values on the ordinate; ^{13}C δ values on the abscissa.

data for **1** indicated excellent agreement, considering the difference in the aglycons attached to β-D-Glc*p*A in **1** and **2** (see Tables II and III).

The ^{13}C resonance for C-1 of β-D-Glc*p*A in **2** was not clearly discernible in one-dimensional spectra at pD values below 3.6. At the higher pD values, however, it is a minor signal (104.3 p.p.m.) immediately upfield of the Man-a C-1 signal at 104.5 p.p.m. (see Fig. 3). This resonance was assigned by means of two-dimensional, ^1H–^{13}C HETCOR experiments (see Fig. 4). The H-1 resonance of β-D-GlcA was easily identified as a doublet ($^3J_{HH}$, 7.70 Hz) at 4.49 p.p.m. at pD 7.7, well removed from the Man*p* H-1 signals at ~5.1 p.p.m. A second ^1H signal of low intensity (owing to β-D-Glc*p*A) and adjacent to the major signal was observed at ~4.50 p.p.m. This low-intensity signal may be due to a Glc*p*A substituent that is attached to the mannan backbone near a terminal Man residue.

The ^{13}C resonances of the Man*p* residue substituted at O-2 by β-D-Glc*p*A are given in Table III (for pyranoside Man-c). The values assigned for C-5 and C-6 of Man-c are tentative, but the assignments for the remaining carbon atoms are supported by the interrelationships between ^1H and ^{13}C signals revealed through the 2-D HETCOR (see Fig. 4) and COSY (see Fig. 5) experiments, and are keyed to the C-1 signal at ~102 p.p.m. This anomeric resonance, appropriately shifted

upfield[15] from the anomeric-carbon resonance of the unsubstituted backbone (see Table III, Man-a) at ~104.5 p.p.m., is analogous to the similar resonance previously assigned[13] to the same type of α-D-Man*p* residue substituted at O-2 by β-D-Xyl*p*. At pD 7.7, this most-upfield Man*p* anomeric ^{13}C resonance correlated with the most-downfield ^{1}H resonance (see Fig. 4). (As observed for H-1 of GlcA already noted, the most-downfield ^{1}H resonance had associated with it a lower-intensity analog. Another unassigned ^{1}H resonance of lower intensity was observed at 5.16 p.p.m. These signals were estimated to be comparable in intensity to the reducing terminal anomeric ^{1}H signal, and are ignored in the following discussion.) Identification of the H-1 resonance of Glc*p*A in the COSY experiment led to assignment of the signals corresponding to H-2, H-3, H-4, and H-5 for this residue (see Fig. 5). These minor ^{1}H signals are correlated with corresponding ^{13}C signals in the HETCOR experiment (see Fig. 4). The ^{1}H–^{1}H and ^{1}H–^{13}C correlations from the 2-D n.m.r. experiments were confirmed through spectrometer-screen display of individual slices in the second dimension of the COSY or HETCOR data. The H-2–C-2 and H-3–C-3 correlations assigned to Man-c were confirmed by selective decoupling experiments at lower field-strength, as was the H-1–C-1 correlation of GlcA-b.

All of the ^{1}H–^{13}C correlations given in Table III for GlcA-b were clearly correlated by the 2-D COSY and HETCOR results (see Figs. 4 and 5). The

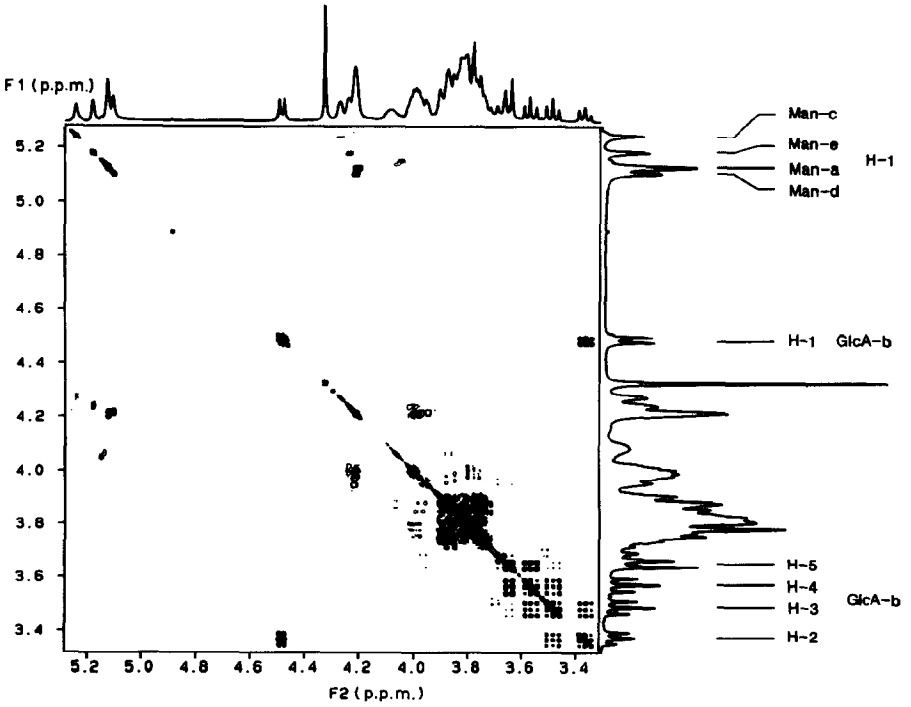


Fig. 5. COSY spectrum of glucuronomannan **3** at pD 7.7 and 70°. ^{1}H δ Values relative to DSS.

remaining ^{1}H–^{13}C correlations in Table III are based on the 2-D n.m.r. data, although some of the assignments to specific atoms are tentative.

Structures **2** and **3** show the relationships between the GlcA-b and Man-c, -d, and -e residues noted in Table III. The pD sensitivities of some of the ^{13}C signals assigned to these Man*p* residues ([$\Delta\delta$] in Table III) were surprisingly large. For Man-c, C-2 exhibited a large upfield shift (negative [$\Delta\delta$]) on increase in pD, while C-1 of the same residue gave a corresponding but smaller positive value, and C-3 was pD-insensitive. These data indicate occurrence of significant, pD-induced, orientational changes in the three glycosidic linkages associated with the Man-c moiety. Two other pD-sensitive signals tend to support this interpretation, namely, the signals for C-1 of Man-d and C-3 of Man-e. These atoms would experience significant changes in their environments upon alteration of normal glycosidic orientations. Only one other resonance displayed a relatively large pD shift; C-4 of either Man-d or Man-e has [$\Delta\delta$] larger than 0.24 p.p.m. Because this signal cannot be that of Man-c, it is undoubtedly due to one of the Man*p* backbone residues adjacent to the substituted moiety. Examination of molecular models suggested that this pD sensitivity may be caused by a change in hydrogen bonding between the analogous OH-4 and the carboxylic acid group in the acidic and anionic forms.

Even though the two samples of GM differed in GlcA content by a factor of two, the chemical shifts observed in all the spectra, and the correlations for them from 2-D experiments, were essentially identical; only the relative intensities of the resonances differed significantly. The most notable differences were for the six resonances of the unsubstituted Man-a residues (see Table III), which had averaged, normalized, relative intensities 3.4 times as large in the ^{13}C-n.m.r. spectrum of **2** as in that of **3**. The chemical shifts and coupling constants observed for GM were consistent with the previous chemically deduced anomeric configurations and with the order of attachment of the Man*p* and Glc*p*A residues in AGXM. For both **2** and **3**, the data also showed that, although there is only one major environment for the β-D-Glc*p*A residues, there are at least four unique major environments for backbone α-D-Man*p* residues. Three of these Man*p* environments have equal frequencies of occurrence that are also equal to the frequency of occurrence of Glc*p*A. The fourth Man*p* environment was found to be more abundant in both samples, and was clearly associated with the Man*p* backbone residues least influenced by the Glc*p*A side-chains. These observations indicated that the structure of GM in general consists of repeating units wherein β-D-Glc*p*A residues are attached to every fifth Man*p* residue of the α-(1→3)-D-mannopyranan backbone (see formulas **2** and **3**). Half of the Glc*p*A sidechains are absent in **2**, because they were not *O*-acetylated in the aged AGXM sample and were lost in the Smith degradation, effectively increasing the relative number of Man-a residues in the derived GM.

The tentative chemical shifts previously reported for *C. neoformans* (serotype A-variant) native glucuronoxylmannan, and polysaccharides derived therefrom[3], agree with the assignments in the present study, except for that of the

anomeric carbon atom of β-D-Glc*p*A. Based largely on an estimated value of $^1J_{CH}$, the Glc*p*A C-1 atom was tentatively assigned to the signal at 104.9–105.0 p.p.m. observed for the polysaccharides reported in the previous study (see Table II, and ref. 3). Based on the data given in Table III, this signal is in fact due to an unsubstituted α-D-Man*p* residue adjacent to the Man*p* backbone moiety to which β-D-Glc*p*A is attached, and the signal of the anomeric carbon atom of Glc*p*A is hidden under the more-intense signal of the unsubstituted Man*p* residues in the *C. neoformans* GXM derivatives.

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