Carbohydrate Research, 225 (1992) 331–337
Elsevier Science Publishers B.V., Amsterdam

Note

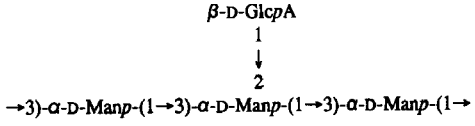
Glucuronoxylomannan of *Cryptococcus neoformans* serotype C: structural analysis by gas–liquid chromatography–mass spectrometry and ^{13}C-nuclear magnetic resonance spectroscopy

Robert Cherniak*, Laura C. Morris, and Sally A. Meyer
Department of Chemistry and Laboratory for Biological and Chemical Sciences, Georgia State University, Atlanta, GA 30303 (U.S.A.)

(Received July 26th, 1991; accepted September 24th, 1991)

INTRODUCTION

Cryptococcus neoformans has emerged as a primary cause of opportunistic infections associated with acquired immunodeficiency syndrome (AIDS)[1,2]. This yeast is the major etiological agent of cryptococcosis. Although *C. neoformans* is usually a pulmonary pathogen, it frequently disseminates to the central nervous system where it causes meningoencephalitis[3]. A prominent virulence factor of *C. neoformans* is its capsular polysaccharide. The major capsular antigen is a high mol. wt. D-glucurono-D-xylo-D-mannan (GXM) that is partially 6-*O*-acetylated on mannose. Four antigenic types–serotypes A, B, C, and D—have been specified, based on a set of structurally related GXM molecules[4–6]. The occurrence of a fifth type, A-D, has been recently postulated[7]. Current models of GXM depict a general structure consisting 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[4,5]. A simple structural relationship between the polysaccharides of the four serotypes exists. They are all comprised of a core repeating unit,



to which 2-*O*-β-D-Xyl*p* and 4-*O*-β-D-Xyl*p* units are added in increments of one to four residues. Serotypes A and D GXM are generally substituted at *O*-2 only, whereas serotypes B and C GXM are substituted with Xyl*p* at *O*-2 and at *O*-4. However,

* Author to whom correspondence should be addressed.

0008-6215/92/$ 05.00

structural elements thought to be characteristic of one serotype have been identified in others[6,8,9]. The initial use of gas–liquid chromatography–mass spectrometry of per-*O*-methylated sugar derivatives of GXM was first applied to the analysis of serotype C GXM by Bhattacharjee *et al.*[10,11]. The data show the molar ratio of xylose:mannose:glucuronic acid to be 4:3:1; the mannose bearing 2-*O*-β-D-Glc*p*A also has a 4-*O*-β-D-Xyl*p* substituent. This data, on a single isolate, is the only report concerning the structure of serotype C GXM. Because structural heterogeneity exists within isolates of serotype A[6,8] and within isolates of serotype D[9] we decided to reinvestigate the GXM of *C. neoformans* serotype C. Herein, we present evidence that shows the presence of structural heterogeneity within *C. neoformans* serotype C GXM obtained from five isolates.

EXPERIMENTAL

Native and modified O-*acetyl*-D-*glucuronxylomannan (GXM).* — NIH isolates of *C. neoformans* used in this study were as follows: Nos. 18, 34, 298, and 401 (from J. K. Kwong-Chung, NIH); CDC No. 3183 (NIH 257) (from E. Reiss, The Centers for Disease Control, Atlanta, GA). All isolates were grown in a chemically defined medium, and the GXMs were isolated and purified as previously described[8,12,13]. The mol. wt. of all the purified GXM samples was reduced by ultrasonic irradiation (GXM-S), and then a portion of each GXM-S was chemically *O*-deacetylated at pH 11 (NH_4OH) for 24 h at 23° (refs. 8, 13).

Analytical methods. — The experimental details of the following methods are either described or appropriately cited in ref. 12. Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen[14]. *O*-Acetyl content was quantitated by the procedure of Hestrin[15] using D-mannitol hexaacetate as standard. GXM-S was analyzed by ion-exchange column chromatography using DEAE Sepharose CL-6B (Pharmacia) and a linear elution gradient of 0.01M Na_2HPO_4 to 0.01M Na_2HPO_4–01.0M NaCl, pH 7.1. The constituent monosaccharides of GXM-S were identified and quantitated as their per-*O*-acetylated aldononitrile (PAAN) derivatives by gas–liquid chromatography after hydrolysis of the polysaccharide in 2M trifluoroacetic acid for 1 h at 120°. Per-*O*-methylation of GXM-S was done by the method of Hakomori[16] as modified by Darvill *et al.*[17] G.l.c.–m.s. analyses of per-*O*-methyl PAAN derivatives were carried out with a capillary gas chromatograph equipped with ion-trap detector (Perkin–Elmer GC/ITD) as previously described[12], except the g.l.c. was done using a SPB-5 0.25 μm capillary column (30 m × 0.25 mm, Supelco). ^{13}C-N.m.r. spectra were recorded at 70° with a Varian VXR-400 n.m.r. spectrometer equipped with a 10-mm multinuclear probe operated at 100.58 MHz. All other parameters were exactly as previously[12] described.

RESULTS AND DISCUSSION

The yield of GXM from the serotype C isolates was generally the same as that observed in comparable studies with serotypes A and B[8,12]. The viscosity of the medium increased markedly during the growing period, and the yield of polysaccharide was high

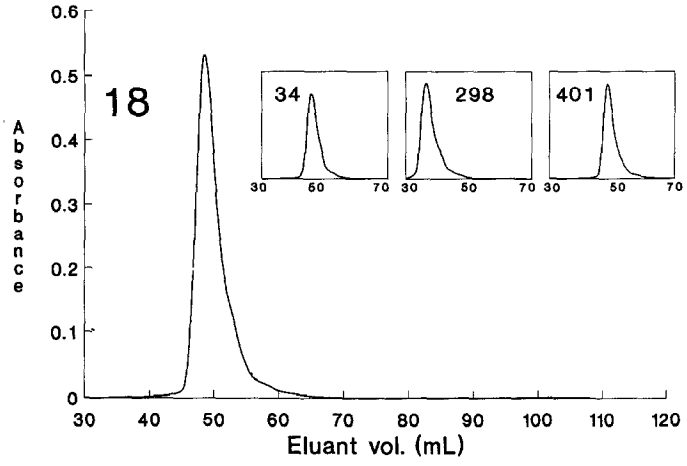


Fig. 1. Ion-exchange column chromatography of GXM-S on DEAE-Sepharose CL-6B using a linear gradient of 0.01 M Na_2HPO_4 to 0.01 M Na_2HPO_4–1.0 M NaCl. The eluant was assayed for neutral carbohydrate by the phenol–sulfuric acid method and absorbance measurements were obtained at 490 nm.

upon ethanol precipitation of the unconcentrated cell-free medium. Purification was done by selective precipitation with hexadecyltrimethylammonium bromide in the usual manner (an average yield of >1 g of purified GXM per liter of medium was obtained)[8,12]. GXM-S from each isolate eluted as a single peak by ion-exchange chromatography using DEAE Sepharose CL-6B (Fig. 1). The elution of GXM 298 at an NaCl concentration lower than that observed for the other polysaccharides cannot be explained at this time. In 27 previous cases, analyses of GXM-S and GXM-S that were further purified by ion-exchange chromatography and gel-filtration chromatography did not produce analytical data that differed significantly within a particular isolate[6,8,9,12]. Therefore, in this study all the data were derived from the analyses of GXM-S and *O*-deacetylated GXM-S. The molar ratios of the substituents, Man, Xyl, GlcA, and *O*-acetyl, calculated relative to Man taken as 3.00, are presented in Table I. Inspection of the data shows that these serotype C isolates consist of two separate groups. Group I

TABLE I

Molar ratios of GXM from *C. neoformans*, serotype C

Strain	D-*Man*	D-*Xyl*	D-*GlcA*	O-*Acetyl*
34	3.00	3.62	0.84	0.59 (2.2%)
298	3.00	3.42	0.89	0.30 (1.2%)
401	3.00	2.88	0.82	0.87 (3.6%)
3183	3.00	2.95	0.86	0.75 (3.0%)
18	3.00	2.89	0.83	0.79 (3.3%)
3939[a]	3.00	3.65	0.79	2.70 (9.5%)

[a] Serotype B[12], for comparison.

TABLE II

G.l.c.–m.s. methylation analysis of GXM from *C. neoformans*, serotype C

Strain	*Methylated PAAN derivatives (mol. ratios)*			
	Tri-O-*Me*		*Di*-O-*Me*	O-*Me*
	2,3,4-D-*Xyl*[a]	*2,4,6*-D-*Man*	*4,6*-D-*Man*	*6*-D-*Man*
34	4.08	n.d.[b]	1.08	1.92
298	3.51	0.06	1.48	1.46
401	3.02	0.10	1.96	0.94
3183	2.99	n.d.[b]	2.15	0.85
18	2.86	n.d.[b]	2.31	0.69
3939[c]	3.55(3.08)[d]	n.d.[b]	2.13	0.87

[a] Identified by g.l.c–m.s. and molar ratios calculated based on the degree of substitution of mannose. [b] Not detected. [c] Serotype B[12], for comparison. [d] The value in parenthesis is calculated as described in footnote a.

is made up of 34 and 298, and group II consists of 18, 3183, and 401. This grouping remains the same based on: (*i*) molar composition (Table I), (*ii*) methylation analysis (Table II), and (*iii*) ^{13}C-n.m.r. spectroscopy (Fig. 2). The quantitative data in Tables I and II have a large degree of uncertainty because of the of the requirement for total acid-catalyzed hydrolysis and the nature of the polysaccharides investigated[12]. The data can be improved if additional analytical strategies, as invoked in our previous studies, are performed[5,6,12]. However, we can now reliably deduce the structure of *O*-deacetylated GXM by comparison of its ^{13}C-n.m.r. spectrum to our data base of spectra obtained from GXM and GXM derivatives[5,6,12,18,19]. Most, if not all, of the data for the *O*-deacetylated GXM of the group II isolates correspond to the data obtained previously for serotype B GXM[12]. The B isolates have an average of 8% *O*-acetyl substituents (see ref. 12), whereas the C isolates have an average of 3% *O*-acetyl (Table I). The observed serological differences between serotypes B and C may depend on the difference in the molar ratios of the *O*-acetyl substituent. This is particularly evident for the group II GXM of serotype C since the carbohydrate backbones of these polysaccharides are similar to those reported for serotype B[12]. The ^{13}C-n.m.r. spectra of the *O*-deacetylated GXM-S of group II are identical to those reported previously for analogous serotype B polysaccharides[12], except for 401 which has one additional unassigned anomeric resonance (101.94 p.p.m.). The complete assignment of the ^{1}H- and ^{13}C-n.m.r. spectra and the structure of a typical *O*-deacetylated GXM, *C. neoformans* serotype B 409, was recently determined by 2-D n.m.r. spectroscopy[19]. The assignment of the anomeric resonances to particular structural elements for group II GXM (**1**) is based on this previous study. The assignment of M^A and M^C are the reverse of that reported in ref. 12. The ^{13}C-n.m.r. spectra of the *O*-deacetylated GXM of group I are complicated by the addition of a second 4-*O*-*β*-D-Xyl*p* (**2**). The relative dispositions of this additional residue (indicated by the parentheses in **2**) and how it effects the chemical shifts of the anomeric carbons has not been determined. The absence of discrete anomeric reso-

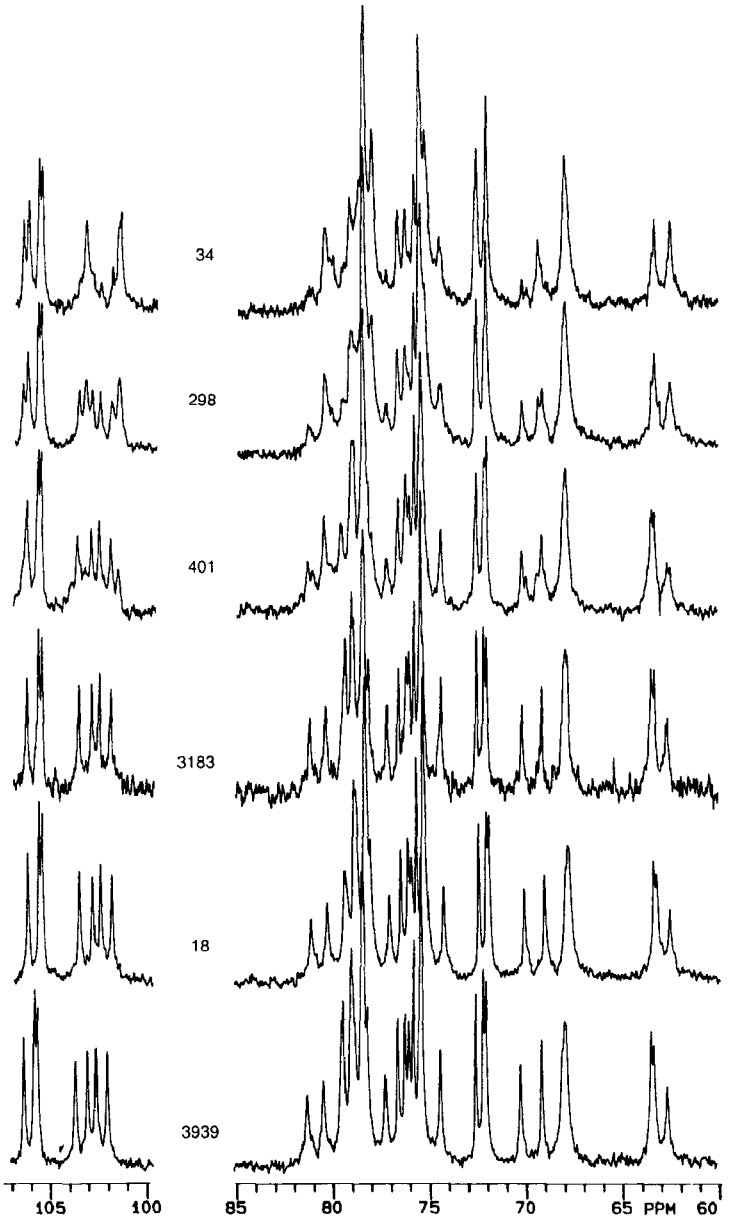
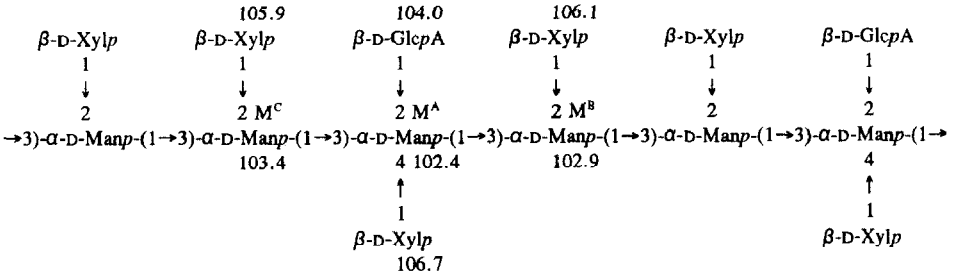
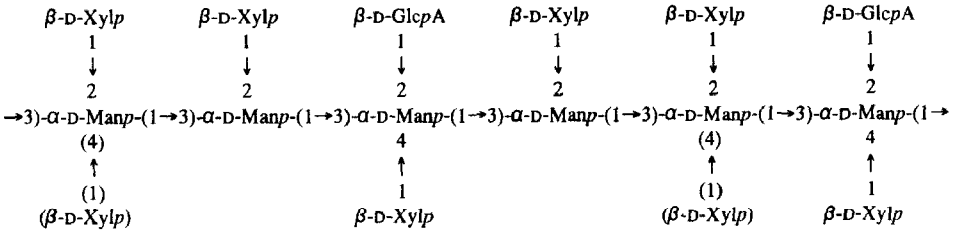


Fig. 2. Proton-decoupled ^{13}C-n.m.r. spectra (70°, 100 MHz) of *O*-deacetylated GXM-S. Serotype C isolates are Nos. 34, 298, 401, 3183, and 18. The spectrum of serotype B isolate 3939 is taken from ref. 12.

nances for the Man*p* residue ^{13}C-n.m.r. spectrum of strain 34 shows that rotation about the glycosidic linkages of the mannan backbone of this highly substituted polysaccharide is restricted. The same rotational restriction is less evident (see most upfield signal of the anomeric region in Fig. 2) in the ^{13}C-n.m.r. spectrum of strain 298. Work is in progress to resolve the disposition of the sugars in the GXM of strain 34 and 298.



1



2

CONCLUSIONS

The sonicated, *O*-deacetylated GXMs of serotype C studied herein fall into two groups. Group I fits the data of isolate 191 previously reported by Bhattacharjee *et al.*[10,11] The carbohydrate backbone of group II is similar to that of serotype B[12,18], based on molar ratios of Xyl:Man:GlcA, methylation analysis, and ^{13}C-n.m.r. spectroscopy. However, group I is more heterogeneous.

ACKNOWLEDGMENTS

The authors acknowledge the support of this investigation by Public Health Service Grant AI 31769, and National Science Foundation Grant CHE 8409599 for the purchase of the Varian VXR-400 n.m.r. spectrometer. They also thank Dr. K. J. Kwong-Chung, National Institutes of Health, for the donation of the NIH cultures.

REFERENCES

1 Centers for Disease Control, *Morbid. Mortal. Weekly Rep.*, 39 (1989) 110–119.
2 W. E. Dismukes, *J. Infect. Dis.*, 157 (1988) 624–628.
3 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.
4 A. K. Bhattacharjee, J. E. Bennett, and C. P. J. Glaudemans, *Rev. Infect. Dis.*, 6 (1984) 619–624.
5 R. Cherniak, *Curr. Top. Med. Mycol.*, 2 (1988) 40–54.

6 S. H. Turner and R. Cherniak, in J. P. Latagé and D. Boucias (Eds.), *Fungal Cell Walls and Immune Response*, Nato ASI Ser., Ser. H, Vol. 53, Springer-Verlag, New York, 1991, pp. 123–142.
7 R. Ikeda, A. Nishikawa, T. Shinoda, and Y. Fukazawa, *Microbiol. Immunol.*, 29 (1985) 981–991.
8 R. Cherniak, L. C. Morris, B. C. Anderson, and S. A. Meyer, *Infect. Immun.*, 59 (1991) 59–64.
9 R. Cherniak, L. C. Morris, and S. H. Turner, *Carbohydr. Res.*, 223 (1992) 263–269.
10 A. K. Bhattacharjee, K. J. Kwong-Chung, and C. P. J. Glaudemans, *Mol. Immunol.*, 16 (1978) 531–532.
11 A. K. Bhattacharjee, K. J. Kwong-Chung, and C. P. J. Glaudemans, *Immunochemistry*, 15 (1978) 673–679.
12 S. H. Turner and R. Cherniak, *Carbohydr. Res.*, 211 (1991) 103–116.
13 R. Cherniak, E. Reiss, and S. H. Turner, *Carbohydr. Res.*, 103 (1982) 239–250.
14 N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
15 S. Hestrin, *J. Biol. Chem.*, 180 (1949) 249–261.
16 S. I. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
17 A. G. Darvill, M. McNeil, and P. Albersheim, *Plant Physiol.*, 62 (1978) 418–422.
18 M. A. Skelton, R. Cherniak, L. Poppe, and H. van Halbeek, *Magn. Reson. Chem.*, 29 (1991) 786–793.
19 M. A. Skelton, H. van Halbeek, and R. Cherniak, *Carbohydr. Res.*, 221 (1991) 259–268.