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Note

4-Methylmorpholine *N*-oxide–methyl sulfoxide soluble glucan of *Piptoporus betulinus*

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Fungal and yeast cell walls contain a large proportion of (1→3)-β- and (1→6)-β-D-glucopyranans[1–3]. Recently (1→3)-α-D-glucopyranans were identified as common yeast cell-wall components[4–6]. These glucans function as virulence factors[7], storage polysaccharides[8], and contributors to the structural integrity of the cell wall. ^{13}C-N.m.r. spectra of water-insoluble glucans from *Streptococcus mutans*, that contain both (1→3)-α- and (1→6)-α-linkages have been acquired from native polysaccharides and from a dextranase-degraded derivative[9,10]. These spectra were noted for their broad linewidths due, in part, to incomplete digestion of (1→6)-α-linked residues. Linear (1→3)-linked residues and branched (1→3) residues were not distinguishable in these spectra.

The insolubility of these D-glucans in water makes hydrolysis and analysis difficult and extraction of water-insoluble cell-wall components with alkaline reagents can also cause degradation of the polysaccharides. The solvents 4-methylmorpholine *N*-oxide (4-MMNO) and methyl sulfoxide (Me_2SO) are capable of complete solubilization of plant cell walls when used in tandem[11,12]. 4-MMNO, for example, is an excellent solvent for polysaccharides[13,14]. The utility of these reagents in the isolation and characterization of a water-insoluble glucopyranan from *Piptoporus betulinus* (formerly *Polyporus betulinus*) is described herein. *P. betulinus* is a bracket fungus which contains predominantly (1→3)-α-D-glucopyranan[15] and lesser amounts of (1→3)-β-D-glucopyranan, chitin, and a wax-like substance[16]. Included in this study is a description of a practical method for the complete acid-catalyzed hydrolysis of water-insoluble glucopyranans.

EXPERIMENTAL

Analytical methods. — The constituent monosaccharides of the water-insoluble polysaccharide were identified and quantitated as their per-*O*-acetylated alditol[17] and as

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their per-*O*-acetylated aldononitrile (PAAN)[18] derivatives by gas-liquid chromatography (g.l.c.). Acid-catalyzed hydrolysis was carried out by dissolving a sample (5 mg) in formic acid (1 mL, 88%), which also contained 2M TFA (99%), for 1 h at 120°. Water (1 mL) was added, and hydrolysis was continued for another 0.75 h. The acid was evaporated under reduced pressure, water was added to the residue to hydrolyze formates, and the sample was then concentrated to dryness. Quantitative analysis by g.l.c. was done with a Sigma 1 gas chromatograph (Perkin–Elmer) equipped with a flame-ionization detector. The analyzer was fitted with a RSL-300 0.2-μm capillary column (30 m × 0.25 mm, Applied Science). Helium was used as the carrier gas. The oven temperature was increased over the ranges and at the rates described in the following programs: (a) for alditol acetates, 120° → 220° at 15° min^{-1}; (b) for PAAN derivatives of neutral sugars, from 200° → 220° at 7.5° min^{-1}. G.l.c.–m.s. was done with a separate chromatograph (Perkin–Elmer 8420 capillary g.l.c.) equipped with an ion-trap detector (Perkin–Elmer LTD). G.l.c. separation was done with a SE-54 0.25-μm capillary column (30 m × 0.25 mm, Supelco). The oven temperature program for the quantitation of the per-*O*-methylated PAAN derivatives was as follows: 120° → 220° at 15° min^{-1}. Total nitrogen was determined by the ninhydrin-hydrindantin method described by Wilchek *et al.*[19]

Extraction with 4-methylmorpholine N-*oxide and* Me_2SO. — The trama of *P. betulinus* (Provided by Errol Reiss, Centers for Disease Control, Atlanta, GA) was suspended in 3:2 4-MMNO–water at a concentration of 18 $mg.mL^{-1}$ and heated under nitrogen for 30 min at 120°. An equal volume of Me_2SO, warmed to 37°, was added, and the suspension was stirred for an additional 20 min at 23°. The soluble and insoluble residue were recovered by centrifugation. The MMNO–Me_2SO soluble extract was exhaustively dialyzed versus distilled water. The precipitate that formed during dialysis was recovered by centrifugation. The precipitate was suspended in water (4°) and centrifuged and washed with cold water a total of three times in order to remove any water-soluble contaminants. The washed precipitate was suspended in water, shell frozen, and lyophilized.

Reduction of water-insoluble polysaccharide. — The sample was reduced with 0.1M $NaBH_4$ for 16 h at 4°. The product was triturated with small portions of cold water, suspended in water, and finally lyophilized, to give the alkali-soluble, reduced, water-insoluble polysaccharide, RPi.

Gel filtration. — The RPi (10–12 $mg.mL^{-1}$) was dissolved in M NaOH, and the sample was chromatographed on a column (90 × 1.5 cm) of Sepharose (Sigma CL-2B-300), equilibrated with M NaOH. The column was eluted with M NaOH at a flow rate of 18 $mL.h^{-1}$. Fractions were analyzed for neutral carbohydrate by the phenol–sulfuric method[20]. The column was calibrated with polysaccharide molecular-weight standards (Polymer Laboratories, Ltd., Stow, Ohio).

Methylation analysis. — A dried sample (3–5 mg) was methylated according to the Hakamori procedure[21] as modified by Darvill *et al.*[22] Sep-Pak C18 cartridges (Millipore, Waters Associates) were used to purify the methylated derivatives as described by Mort *et al.*[23] The methylated derivatives were hydrolyzed in 88% formic

acid for 1 h at 100°. The formic acid was removed *in vacuo* below 40°, and the residue was then hydrolyzed in M TFA for 1 h at 121°. The sample was dried *in vacuo*, and the per-*O*-methylated, per-*O*-acetylated aldononitrile (PAAN) derivatives were prepared as previously described, except that the reaction temperature was 80°, and the TFA was evaporated under reduced pressure, rather than being extracted with ether[18]. Analysis and identification of the per-*O*-methylated PAAN derivatives were done by g.l.c.–m.s.

N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectrum was recorded with a JEOL-GX 270 n.m.r. spectrometer operated at 67.8 MHz and equipped with a 5-mm, ^{1}H–^{13}C dual probe. The spectrum of the D-glucan (100 mg.mL^{-1}) was recorded at 40°, and the chemical shifts were measured relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). The deuterium resonance of the solvent, deuterium oxide, pD 14, served as internal lock. In other regards, the spectrum was recorded as previously described.[24]

RESULTS AND DISCUSSION

A 0.5-h extraction of *P. betulinus* trama with 3:2 4-MMNO–water, followed by the addition of Me_2SO and stirring for 20 min at 23°, gave a 20% yield of soluble polysaccharide. Greater than 90% of the carbohydrate present in the extract precipitated as a result of dialysis *vs.* water. The precipitate was triturated with cold water, suspended in water, and lyophilized. The residue obtained after extraction of the trama accounted for 80% of the weight of the sample, and it was not analyzed. Prolonged extraction of similar fungal samples have left as little as 5% of the weight of the sample as a residue[25], and similar behavior would be expected for *P. betulinus*.

The formic acid–TFA catalyzed hydrolysis method does not require special apparatus, and the polysaccharide was completely soluble in the reagents. Glucose, determined by g.l.c. as the per-*O*-acetylated alditol, was the only monosaccharide detected in the hydrolysate. A similar analysis using the PAAN derivative revealed a second component that eluted just prior to glucose. It is probably an unknown by-product that resulted from the combination of the hydrolysis conditions and the formation of the PAAN derivatives. The unknown peak was not identified. Since nitrogen was not detected in the polysaccharide, the sample was considered a homoglucopyranan.

TABLE I

G.l.c.–m.s. methylation analysis of a trama polysaccharide of *Piptoporus betulinus*

Methylated PAAN deriatives (mol %)						
Tetra-O-*Me*	*Tri*-O-*Me*		*Di*-O-*Me*			
2,3,4,6-	*2,4,6-*	*2,4,6-*	*2,4-*	*2,6-*	*2,6-*	*4,6-*
1.8	91.7	0.9	1.0	1.7	1.7	1.1

G.l.c.–m.s. of the per-*O*-acetylated aldononitriles derived from the D-glucan gave 2,4,6-tri-*O*-methyl-D-glucopyranose as the predominant derivative (see Table I). Non-reducing termini and disubstituted derivatives (<2%) were also detected. The multiple disubstituted derivatives may indicate microheterogeneity in the glucopyranan structure. A second 2,4,6-tri-*O*-methylhexose (0.9%) that was also detected may be due to a small amount of mannose in the sample which was not detected in the RPi hydrolysate. More likely, it represents an impurity which is identified as a hexose derivative because of its proximity to 2,4,6-tri-*O*-methylglucose in the g.l.c.–m.s. The mol. wt. of the D-glucan was estimated as 9000 daltons, based on the occurrence of 2,3,4,6-tetra-*O*-methylglucose relative to 2,4,6-tri-*O*-methylglucose. As expected, the mol. wt. of the glucan was higher (~15 000 daltons) when it was determined by gel-filtration chromatography. Both of these estimates were several times greater than the one reported by Duff[15].

The ^{13}C-n.m.r. spectrum of the reduced–gel filtered RPi gave six major resonances. The chemical shifts and $^{1}J_{C,H}$ coupling constants for the glucan are given in Table II. They are consistent with an α-(1→3)-linked glucopyranose structure. Minor resonances (less than 1% of the most intense resonance) which appear in the spectrum probably represent alkaline degradation products produced as a result of incomplete reduction of the glucan.

TABLE II

N.m.r. data for *Piptoporus betulinus* trama polysaccharides and related α-(1→3)-glucans

Chemical shifts in p.p.m. and ($^{1}J_{C,H}$) in Hz

C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*Ref. No*
103.11 (171.18)	73.81	85.50	73.31	75.51	63.86	*a*
103.9	74.05	87.21	73.55	76.04	64.16	26
103.43 (170.68)	73.92	86.02	73.44	75.67	63.67	25
102.4	73.60	84.5	73.0	75.1	63.8	9
102.88	73.36	84.89	73.23	75.33	63.79	27

[a] Present data.

Recently, Kiho *et al.*[26] described a (1→3)-α-D-glucan obtained by the extraction of *Agrocybe cylindacea* with NaOH. The mol. wt., based on gel filtration chromatography, was 560 000 daltons, a value which is ~6 times that reported for related glucans[9,16,25]. A ^{13}C-n.m.r. spectrum with a poor signal-to-noise ratio was obtained. This observation may be related to the high mol. wt. of the D-glucan, the temperature (23°) at which the spectrum was acquired, and the sample size (23 mg per 0.5 mL). A (1→3)-α-D-

glucan, synthesized *in vitro* using a glycosyltransferase from *Streptococcus sorbrinus* 6715, was studied by Shimura[27]. The ^{13}C-n.m.r. spectrum obtained under comparable experimental conditions was similar to the one obtained in this study. The ^{13}C-n.m.r. chemical shift data for several (1→3)-α-glucopyranans are compared to RPi in Table II.

The combination of 3:2 4-MMNO–water and Me_2SO extracted a very pure D-glucan from the trama of *P. betulinas*. The polysaccharide was a linear (1→3)-α-D-glucopyranan with a mol. wt. of ~9000 daltons. Small amounts of compounds that are indicative of putative branch points were detected. Whether the occurrence of branch points is real or reflects the limitations of the methods employed was not determined. The ^{13}C-n.m.r. spectrum gave no evidence of branching, and thus there was no need to distinguish between the C-3 of branched or linear residues as was necessary in other studies[9,10]. The minor resonances, which probably arose from the partial degradation of the glucopyranan at pD14, were not assigned.

CONCLUSIONS

A mild method for the isolation of an (1→3)-α-D-glucopyranan from *P. betulinus* by the tandem use of 4-MMNO and Me_2SO is described. The structure of the D-glucopyranan was characterized by ^{13}C-n.m.r. spectroscopy and by g.l.c.–m.s. of its per-*O*-methylated derivative.

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REFERENCES

1 H. J. Phaff, *Ann. Rev. Microbiol.*, 17 (1963) 15–30.
2 J. S. D. Bacon, V. C. Farmer, D. Jones, and I. F. Taylor, *Biochem. J.*, 114 (1969) 557–567.
3 D. J. Manners, A. J. Masson, and J. C. Patterson, *Biochem. J.*, 135 (1973) 19–30.
4 B. J. M. Zonneveld, *Biochim. Biophys. Acta*, 249 (1971) 506–514.
5 I. D. Reid and S. Bartnicki-Garcia, *J. Gen. Microbiol.*, 96 (1976) 35–50.
6 M. Petit and A. Schneider, *Arch. Microbiol.*, 135 (1983) 141–146.
7 G. San-Blas, *Curr. Top. Med. Mycol.*, 1 (1985) 235–257.
8 B. J. M. Zonneveld, *Biochim. Biophys. Acta*, 273 (1972) 174–187.
9 H. M. Davis, W. J. Boyko, and J. R. Edwards, *Carbohydr. Res.*, 152 (1986) 279–282.
10 P. Colson, H. J. Jarrell, B. L. Lamberts, and I. C. P. Smith, *Carbohydr. Res.*, 71 (1979) 265–272.
11 J. -P. Joseleau, G. Chambat, and B. Chumpitazi-Hermoza, *Carbohydr. Res.*, 90 (1981) 339–344.
12 G. Chambat, F. Barnoud, and J. -P. Joseleau, *Plant Physiol.*, 74 (1984) 687–693.
13 H. Chanzy, M. Dube, and R. H. Marchessault, *J. Polym. Sci., Polym. Lett.*, 17 (1979) 219–226.
14 H. Chanzy, B. Chumpitazi, and A. Peguy, *Carbohydr. Polym.*, 2 (1982) 35–42.
15 R. B. Duff, *J. Chem. Soc.*, (1952) 2592–2594.
16 J. Jelsma and D. R. Kreger, *Arch. Microbiol.*, 119 (1978) 249–253.
17 H. Bjorndall, C. G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
18 S. H. Turner and R. Cherniak, *Carbohydr. Res.*, 95 (1981) 137–144.
19 M. Wilchek, T. Miron, and J. Kohn, *Methods Enzymol.*, 104 (1984) 9.

20 M. Dubois, K. A. Giles, J. K. Hamilton, R. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
21 S. Hakamori, *J. Biochem.* (Tokyo), 55 (1964) 205–208.
22 A. G. Darvill, M. McNeill, and P. Albersheim, *Plant Physiol*, 62 (1978) 418–422.
23 A. J. Mort, S. Parker, and M. -S. Kuo, *Anal. Biochem.*, 13 (1983) 380–384.
24 R. Cherniak, R. G. Jones, and M. E. Slodki, *Carbohydr. Res.*, 182 (1988) 227–239.
25 P. G. James, R. Cherniak, R. G. Jones, and E. Reiss, *Carbohydr. Res.*, 198 (1990) 23–38.
26 T. Kiho, I. Yoshida, K. Hagai, S. Ukai, and C. Hara, *Carbohydr. Res.*, 189 (1989) 273–279.
27 A. Shimamura, *Carbohydr. Res.*, 185 (1989) 239–248.