Carbohydrate Research, 224 (1992) 209–217
Elsevier Science Publishers B.V., Amsterdam

Structure of a heteroglycan isolated from the fungus *Omphalia lapidescens**

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(Received March 1st, 1991; accepted July 4th, 1991)

ABSTRACT

The major, water-soluble polysaccharide isolated from the sclerotia of *Omphalia lapidescens*, and purified by chromatography on DEAE-Sephadex A-50 and Toyopearl HW-65F, is a heteroglycan (OL-3), $[\alpha]_D^{20}$ − 50° (*c* 1, water), which is composed of D-glucose, 2-acetamido-2-deoxy-D-glucose, and D-glucuronic acid in the molar ratios 1.21:1.00:1.38. Methylation analysis, partial acid hydrolysis, and f.a.b.-m.s. of the hydrolysates indicated OL-3 to have a highly branched structure involving (1→3)-, (1→4)-, (1→6)-, and (1→3,6)-linked, and non-reducing terminal D-glucopyranosyl residues, and also to contain 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyluronic acid and β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyluronic acid segments.

INTRODUCTION

The sclerotia of the fungus *Omphalia lapidescens*, belonging to Agaricales in Tricholomataceae, is a crude drug "Raiguan (Leiwan)" that is used as a vermifuge. We now report on the structure of the major polysaccharide component, a hot water-soluble heteroglycan, OL-3.

RESULTS AND DISCUSSION

Extraction of the sclerotia with water and precipitation with ethanol gave the carbohydrate material which was treated with pronase E, then by the Sevag method, and subjected to chromatography on DEAE-Sephadex A-50 (Fig. 1). The major fraction (eluted with 0.45M sodium chloride) was purified further by elution from a column of Toyopearl HW-65F with water (Fig. 2).

The purified polysaccharide (OL-3) had $[\alpha]_D^{20}$ − 50° (*c* 1, water), gave single spots in electrophoresis on glass-fibre paper in borate buffer (0.026M, pH 9.2) and acetate

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* Studies on Fungal Polysaccharides, Part XXXVII. For Part XXXVI, see ref. 1. A part of this work was presented at the XVth International Carbohydrate Symposium, Yokohama, Japan, August 1990.

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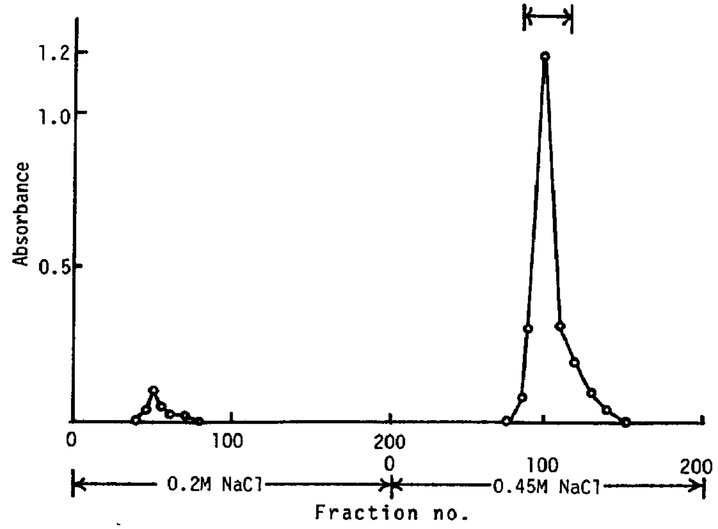


Fig. 1. Elution of the carbohydrate portion of "Raiguan (Leiwan)" from a column (3.2 × 15 cm) of DEAE-Sephadex A-50 with 0.2M and 0.45M NaCl. Each fraction (2.0 mL) was assayed using the phenol–H_2SO_4 reagent (absorbance at 485 nm).

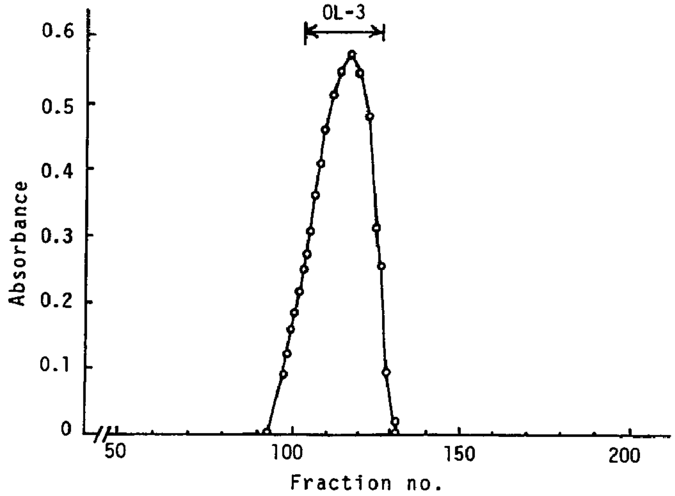


Fig. 2. Gel filtration of OL-3 on a column (2 × 95 cm) of Toyopearl HW-65F by elution with water. Each fraction (2.0 mL) was assayed using the phenol–H_2SO_4 reagent (absorbance at 485 nm).

buffer (0.2M, pH 5.6), and was homogeneous in ultracentrifugation (Fig. 3). Gel filtration of OL-3 on Toyopearl HW-65F gave a narrow, single peak with an apparent molecular weight of 450,000, which contained neutral sugar[2] (28.5% as D-glucose), amino sugar[3] (28.7% as 2-acetamido-2-deoxy-D-glucose), and uronic acid[4] (34.9% as D-glucuronic acid), but no phosphorus (Fiske–SubbaRow method[5]).

The component sugars of OL-3 were identified (t.l.c.) as glucose, 2-acetamido-2-

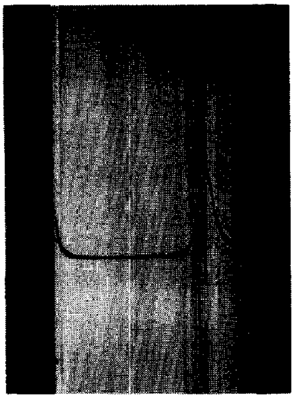


Fig. 3. Sedimentation pattern (Hitachi Model 282 ultracentrifuge) of an aqueous 1.5% solution of OL-3 at 60,000 r.p.m.

deoxyglucose, and glucuronic acid in the molar ratios 1.21:1.00:1.38 (assayed by colorimetry[2-4]), and 2-amino-2-deoxyglucose in the hydrolysate was further identified by an amino acid analyser. No amino acids were detected in the hydrolysate. The components of OL-3 are assumed to be D.

The n.m.r. spectrum of OL-3 contained a signal at δ 2.0 assigned[6] to NAc, and the i.r. spectrum contained bands at 1420, 1620, and 890 cm^{-1} indicative of carboxyl, acetamido[6], and β-glucosidic[7] linkages, respectively.

OL-3 was methylated by the Hakomori method[8] and then hydrolysed. The products were converted into alditol acetates, and analysed by g.l.c. and g.l.c.–m.s. 1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-me-

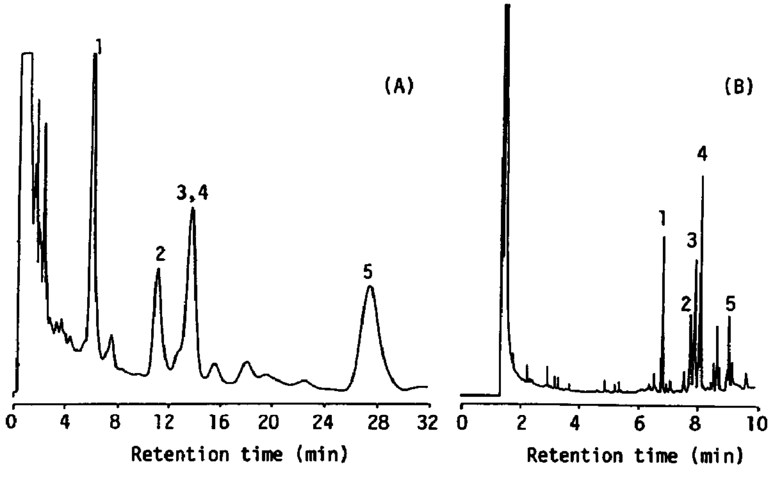


Fig. 4. Gas–liquid chromatograms on *A*, 5% ECNSS-M and *B*, DB-5 of alditol acetates derived from methylated OL-3: 1, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol; 2, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol; 3, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol; 4, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol; 5, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylglucitol.

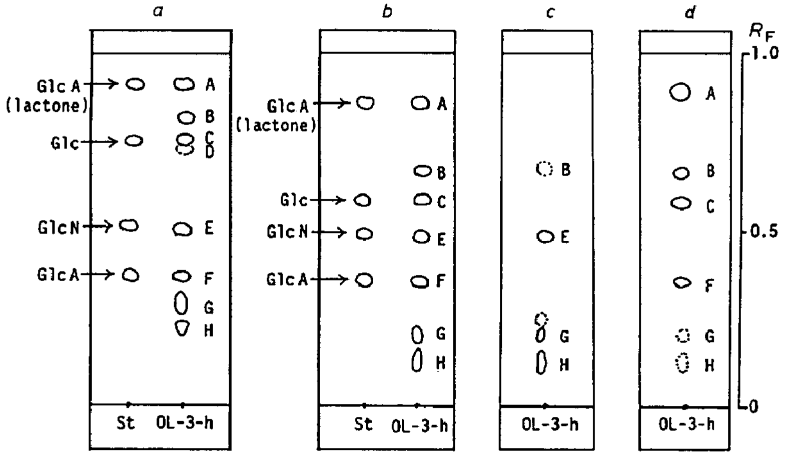


Fig. 5. Thin-layer chromatograms of a partial hydrolysate of OL-3: on (*a*) Si 50000 (detection with $AgNO_3$–NaOH), (*b*) cellulose (detection with $AgNO_3$–NaOH), (*c*) cellulose (detection with ninhydrin), (*d*) cellulose (detection with *p*-anisidine). Each plate was developed with ethyl acetate–pyridine–acetic acid–water (5:5:1:3).

thylglucitol were separated by capillary g.l.c. (Fig. 4). The methylation analysis of OL-3 gave 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylglucitol, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol in the molar ratios[9,10] 1.3:3.3:3.6:1.0:2.7. These results indicated OL-3 to have a highly branched structure involving (1→3)-, (1→4)-, (1→6)-, and (1→3,6)-linked Glc, and non-reducing terminal Glc residues.

OL-3 was hydrolysed with 0.05M trifluoroacetic acid at 100° for 6h and the hydrolysate was dialysed. T.l.c. of the diffusate (OL-3-h) revealed (Fig. 5) glucuronolactone, glucose, 2-amino-2-deoxyglucose, glucuronic acid, and four other products [B, G, and H had R_F values of 0.67, 0.19, and 0.09, respectively, in t.l.c. on cellulose, and B, D, G, and H had R_F values of 0.81, 0.73 (trace), 0.29, and 0.23, respectively, in t.l.c. on Si 50000; detection with alkaline silver nitrate[11]]. Products B, G, and H gave a reddish brown colour with *p*-anisidine[12] and purple spots with ninhydrin[13], but D did not react with these reagents. These results suggested that B, G, and H contained glucuronic acid and 2-amino-2-deoxyglucose moieties.

The products B, G, and H were isolated by t.l.c. and analysed by mass spectrometry. The negative-ion f.a.b.-mass spectrum of G (Fig. 6) contained an $[M - H]^-$ ion (*m/z* 354) presumed to be a disaccharide of 2-amino-2-deoxy-D-glucose and D-glucuronic acid. M.s. of the daughter ions of *m/z* 354 (Fig. 7) confirmed the presence of 2-amino-2-deoxy-D-glucose and D-glucuronic acid by the formation of fragment ions with *m/z* 162.3, and 193.0, respectively. These data are consistent with G being 4-*O*-(2-amino-2-deoxy-*β*-D-glucopyranosyl)-D-glucuronic acid (**1**).

The negative-ion f.a.b.-mass spectrum of H (Fig. 8) contained an $[M - H]^-$ ion (*m/z* 516) consistent with a trisaccharide composed of D-glucose, 2-amino-2-deoxy-D-

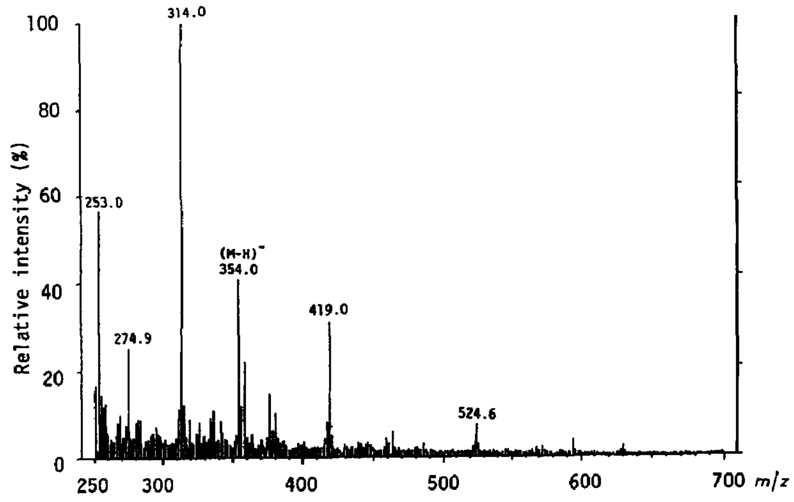


Fig. 6. Negative-ion f.a.b.-mass spectrum of G in Fig. 5.

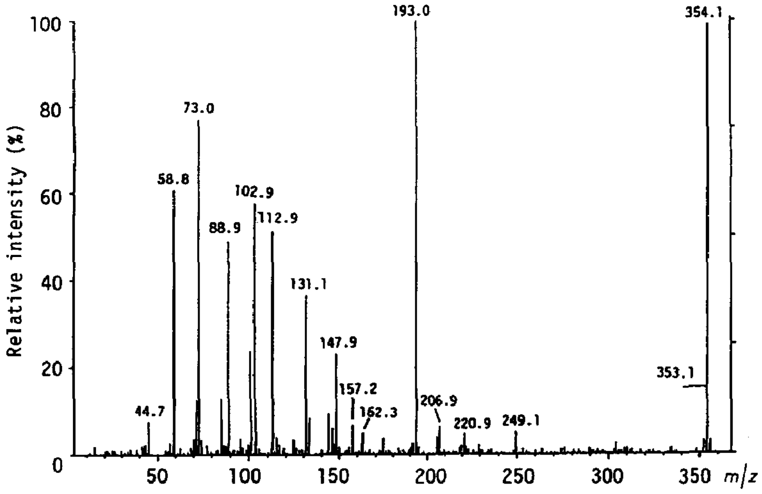
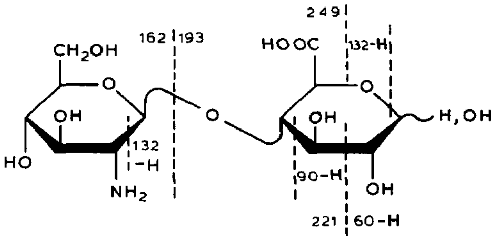


Fig. 7. Mass spectrum of the daughter ions of *m/z* 354 in Fig. 6.



1 (G in Fig. 5)

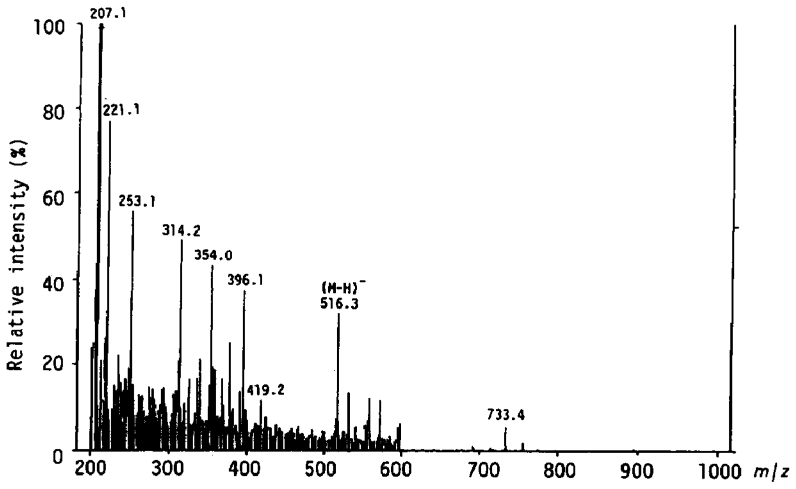


Fig. 8. Negative-ion f.a.b.-mass spectrum of H in Fig. 5.

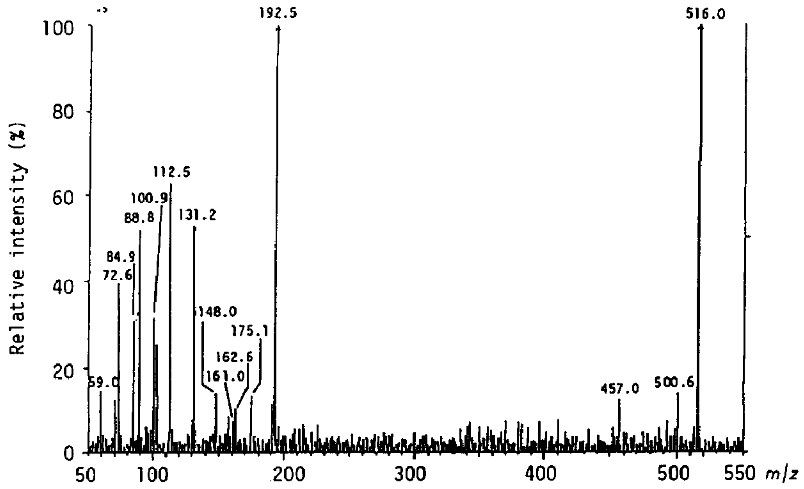
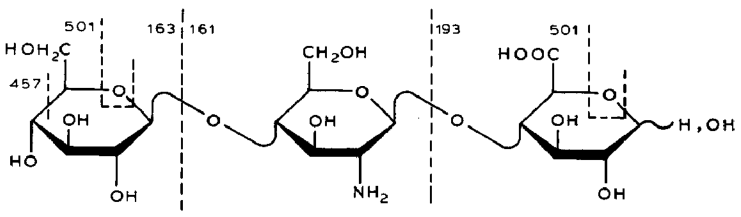


Fig. 9. Mass spectrum of the daughter ions of *m/z* 516 in Fig. 8.



2 (H in Fig. 5)

glucose, and D-glucuronic acid. An $[M - H]^-$ ion with m/z 354 was also present, which suggested that **1** was part of the structure of H. M.s. of the daughter ions of m/z 516 (Fig. 9) confirmed the presence of D-glucose, 2-amino-2-deoxy-D-glucose, and D-glucuronic acid by the formation of the fragment ions with m/z 162.6, 161.0, and 192.5, respectively. These data are consistent with H being *O*-β-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-D-glucuronic acid (**2**). The small quantity of B available precluded f.a.b.-m.s.

Thus, it is concluded that OL-3 has a highly branched structure involving (1→3)-, (1→4)-, (1→6)-, and (1→3,6)-linked, and non-reducing terminal D-glucopyranosyl residues, and also contains 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyluronic acid and β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyluronic acid segments.

EXPERIMENTAL

The glycosaminoglycan from Omphalia lapidescens. — *(a) Isolation.* Crushed, dried sclerotia (900 g) of the fungus were stirred with distilled water for 8 h at 100°. This procedure was repeated until the extract gave a negative phenol–sulfuric acid reaction[2]. After centrifugation, the supernatant solution was dialysed (Visking cellophane tubing) against running water for 2 days, then concentrated to a small volume *in vacuo*. Ethanol (4 vol.) was added, and the precipitate was collected by centrifugation, washed with ethanol, acetone, and ether, and dried *in vacuo* to give the crude glycosaminoglycan (11.1 g).

(b) Digestion with protease. A solution of the crude polysaccharide (2.0 g) in water (300 mL) was adjusted to pH 7.0 with aqueous 1% sodium hydroxide, then pronase E (150 mg, Kaken Kagaku Co. Ltd., Tokyo) was added. The mixture was stirred at 37° for 5 days, dialysed against running water for 3 days, concentrated to 100 mL, and shaken vigorously for 30 min with chloroform–1-butanol (4:1, 30 mL). The procedure was repeated until a gelatinous precipitate was no longer formed. After centrifugation, the supernatant solution was concentrated under reduced pressure at <40°, and ethanol (4 vol.) was added. The precipitate (1.3 g) was collected by centrifugation, and dried *in vacuo*.

(c) Purification. A solution of the crude polysaccharide from (*a*) (1 g) was applied to a column (3.2 × 15 cm) of DEAE-Sephadex A-50 equilibrated in 0.2M sodium chloride. Stepwise elution was performed with 0.2M and 0.45M sodium chloride. The fractionation was monitored by the phenol–sulfuric acid reaction[2]. Appropriate fractions were combined, dialysed (Visking cellophane tubing) against running water for 3 days and against distilled water for 1 day, and concentrated to a small volume *in vacuo*. Ethanol (10 vol.) was added, and the precipitate was collected by centrifugation and treated as described above. The yields were as follows: 0.2M salt eluate, 0.07 g; 0.45M eluate, 0.57 g. A portion (300 mg) of the latter eluate was applied to a column (2 × 95 cm) of Toyopearl HW-65F (Toyo Soda MFG. Co., Ltd., Tokyo) and eluted with water at 20 mL/h. The fractionation was monitored by the phenol–sulfuric acid reac-

tion[2]. Appropriate fractions were combined and concentrated to a small volume *in vacuo*. Ethanol (4 vol.) was added, and the precipitate was collected and treated as in (*a*) to give OL-3 (188 mg) as the major fraction.

Investigation of OL-3. — *(a) Component sugars.* OL-3 (10 mg) was hydrolysed with M trifluoroacetic acid (1 mL) at 100° for 8 h, and the hydrolysate was concentrated to a syrup under reduced pressure at <40°. T.l.c. of the syrup (ethyl acetate–pyridine–acetic acid–water, 5:5:1:3) on cellulose revealed glucose, glucuronic acid, glucuronolactone, and 2-amino-2-deoxyglucose on detection with alkaline silver nitrate[11] and *p*-anisidine hydrochloride[12]. The proportions of glucose, glucuronic acid, and 2-amino-2-deoxyglucose (as 2-acetamido-2-deoxyglucose) were determined by the phenol–sulfuric acid[2], carbazole[4], and Elson–Morgan[3] methods, respectively, and the identity of the 2-amino-2-deoxyglucose was confirmed using a Hitachi 835 amino acid analyser. The constituent sugars of OL-3 are assumed to be D.

(b) Properties. The optical rotation was determined with a JASCO automatic polarimeter. The n.m.r. spectrum was recorded with a JEOL FX-200 spectrometer on a solution in D_2O at room temperature. The i.r. spectrum was recorded with a Hitachi 215 spectrometer. Paper electrophoresis of OL-3 in 0.026M borate buffer (pH 9.2) and 0.2M acetate buffer (pH 5.6) showed a single spot (detected with alkaline silver nitrate[11] and *p*-anisidine hydrochloride[12]). The molecular weight of OL-3 was estimated by elution with water from a column (2 × 95 cm) of Toyopearl HW-65F and comparison with dextran standards (T-500, mol.wt. 500,000; T-250, 250,000; T-110, 110,000) and blue dextran 2000 (Pharmacia LKB Biotechnology).

(c) Methylation. OL-3 was methylated by the method of Hakomori (three times)[8], until there was no significant i.r. absorption of hydroxyl groups at 3500 cm^{-1}. The methylated polysaccharide was heated with aqueous 90% formic acid at 100° for 4 h, the hydrolysate was concentrated, and the residue was hydrolysed with M trifluoroacetic acid at 100° for 8 h followed by concentration to dryness. The products were reduced with sodium borohydride at room temperature for 8 h and then acetylated[14]. The results are given in Table I.

(d) G.l.c. and g.l.c.–m.s. of partially methylated alditol acetates. A Hitachi 163 gas chromatograph equipped with a glass column (0.3 × 200 cm) packed with 5% of ECNSS-M on Chromosorb W (AW-DMCS, 80–100 mesh) was used at 180° with a nitrogen flow rate of 50 mL/min. A Varian 3500 gas chromatograph equipped with a

TABLE I

Molar ratio of glucitol acetates derived from methylated OL-3

Component	*Molar ratio*
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	2.7
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol	3.6
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol	3.3
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol	1.3
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methylglucitol	1.0

DB-5 capillary column (0.25-μm film, 30 m × 0.25 mm i.d., J & W Scientific) was also used, with split injection (ratio 10:1), helium as the carrier gas at 1.8 mL/min, and the temperature program 150° for 1 min, →250° at 5°/min, and 250° for 4 min.

G.l.c.–m.s. (70 eV) was performed on a Finnigan MAT INCOS 50 instrument equipped with a DB-5 capillary column under the above conditions.

(e) Partial acid hydrolysis. OL-3 (80 mg) was hydrolysed with 0.05M trifluoroacetic acid (20 mL) at 100° for 3 h. The hydrolysate was dialysed against distilled water for 48 h (3 times with 200 mL of distilled water), and the diffusate was concentrated to ~1 mL under reduced pressure at <40°. T.l.c. (ethyl acetate–pyridine–acetic acid–water, 5:5:1:3) of the residue was performed on Si 50000 (Merck) and cellulose (Merck), with detection by alkaline silver nitrate[11], *p*-anisidine hydrochloride[12] (100° for 10 min), and ninhydrin[13] (100° for 10 min). The products were extracted conventionally from the plates with water and then analysed by f.a.b.-m.s.

(f) Mass spectrometry. F.a.b.-m.s. and m.s. of daughter ions were performed with a Finnigan MAT TSQ 70 instrument. The copper tip was loaded with diethanolamine (2–3 μL) and the sample solutions (1–1.5 μL; 5 μg/μL of water) were applied. The copper tip was bombarded with Xe atoms (7.0 kV). The mass spectrometer was operated with an accelerating potential of 8 keV. Spectra were recorded in the negative-ion mode. The mass range 200–1000 was scanned at 10 s/decade with a mass resolution of 3500. Collision-activated dissociation experiments were conducted at − 10 and − 20 eV. Ar (0.8–1.0 mtorr) was employed as the collision gas.

ACKNOWLEDGMENT

We thank Dr. N. Ohno for his help with the n.m.r. experiment.

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