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THE STRUCTURE OF AN α-D-GLUCAN FROM *Cyttaria harioti* FISCHER

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ABSTRACT

A homogeneous glucan has been isolated from the fruiting bodies of *Cyttaria harioti* Fischer. Partial acid hydrolysis produced major amounts of isomaltose, whereas acetolysis gave maltose and maltotriose. Enzymic hydrolysis with amyloglucosidase and pullulanase indicated a structure based on maltotriose residues connected by (1→6)-α-D linkages. This conclusion was supported by periodate-oxidation data which also showed that 3–7% of the glucose resisted oxidation. Methylation analysis confirmed the presence of (1→6) and (1→4) linkages in the ratio 1:2.4.

INTRODUCTION

Cyttaria harioti Fischer is a fungus which grows as a parasite of Nothofagus, producing a characteristic tumour that causes death of the tree. It is found in forests on the slopes of the Andes from a latitude of 30° to Cape Horn in the southern part of Argentina and Chile, and is known under the name "dihueñe del cohiue".

Lederkremer and Ranalli[1] studied the simple sugars and polyols extracted from the fruiting bodies of the fungus, and Cirelli and Lederkremer described the structure of two polysaccharides, a water-soluble heteropolysaccharide composed mainly of D-glucose[2,3], and a gel-forming, (1→3)-linked β-D-glucan, highly branched through position 6 and isolated by alkaline extraction[4].

We now report the isolation from the aqueous extract of the fruiting bodies of this fungus of a new glucan which was characterized as a pullulan.

RESULTS AND DISCUSSION

Dried, ground, fruiting bodies of *Cyttaria harioti* Fischer were extracted with water at room temperature. Addition of ethanol (to 40%) to the aqueous extract

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precipitated the heteropolysaccharide which was previously studied[2,3]. On increasing the ethanol concentration to 52%, a new polysaccharide material (1.5%) precipitated having $[\alpha]_D^{20}$ +157° (water).

When this material was fractionally precipitated with ethanol from solution in water, the first precipitate (47%, 41–46% ethanol) had $[\alpha]_D^{20}$ +171° (water). Two further reprecipitations did not change the $[\alpha]_D$ value of the polysaccharide which appeared to be homogeneous, as it gave a single, symmetrical sedimentation peak on ultracentrifugation and it was precipitated (95%) from aqueous solution over a narrow range (4%) of ethanol concentration.

The pure polysaccharide, containing <0.8% of nitrogen, gave no ash on combustion and had an i.r. band at 850 cm^{-1} characteristic of the α-D configuration[5]. When the polysaccharide was incubated for 24 h with amyloglucosidase, a quantitative yield (102%) of glucose was obtained, suggesting the presence of (1→4)- and (1→6)-α-D linkages. Partial acid hydrolysis of the glucan produced major amounts of isomaltose together with maltose, maltotriose, and higher oligosaccharides, whereas acetolysis gave maltose and maltotriose.

The results confirm the presence of (1→6)- and (1→4)-α-D linkages in the glucan, and indicate that two (1→4) linkages are consecutive. The fact that only 8% of hydrolysis was obtained with bacterial alpha-amylase suggests that no more than three of those linkages are consecutive[6].

Hydrolysis of the glucan with pullulanase produced a maximum reducing-power corresponding to 32–33% of hydrolysis, and p.c. of samples after 24-h hydrolysis showed maltotriose to be the sole product. When the glucan was hydrolysed with pullulanase under dialysing conditions[7], the material which dialysed was maltotriose, isolable as a pure syrup (p.c., gel c., optical rotation) having an n.m.r. spectrum in D_2O that contained a signal at δ 5.35 for the anomeric proton of the non-reducing glucosyl units and which was characterised as the hendeca-acetate.

The non-dialysing material, which contained major amounts of maltotriose together with small amounts of higher oligosaccharides and traces of maltose, gave three fractions when subjected to preparative p.c. Fraction *1* contained maltotriose and maltose. Fraction *2* was chromatographically homogeneous and contained an oligosaccharide having R_{XYL} 0.09. On the basis of a (1→4) linkage involving the reducing group, the d.p. was 6, and the $[\alpha]_D^{20}$ value of +170° (water) agrees with that expected for 6^3-α-maltotriosylmaltotriose[9]. If the linkage of the reducing group was (1→6), then the reducing power indicates a d.p. of 3, but the more probable trisaccharide, isopanose, has $[\alpha]_D$ +128° (water)[10]. Fraction *3* consisted of a mixture of oligosaccharides of very low mobility which were converted by pullulanase into maltotriose.

The d.p. of the glucan determined chemically was 248, on the assumption that the terminal reducing group was (1→4)-linked[11].

The glucan consumed 1.28 mol. of periodic acid, and produced 0.33 mol. of formic acid, per glucosyl residue; 0.03 mol. of glucosyl residue resisted oxidation.

These results indicate that 33% of the linkages in the glucan are (1→6) and 62%

are (1→4), and the remaining sugar residues are periodate-resistant. This resistance could be due to the presence of (1→3) linkages or branch points, and also to HO-3 groups blocked because of hemi-acetal formation with aldehydo groups formed by periodate oxidation[12]. However, the polyalcohol obtained by reduction of the oxopolysaccharide was resistant to periodate, indicating the absence of masked hydroxyl groups in the latter. Total hydrolysis of the polyalcohol yielded glucose, erythritol, and glycerol in molar ratios 7:29:64. Small proportions of periodate-resistant glucosyl residues have been found in other pullulans[13–15] and have been attributed to the presence of (1→3) linkages.

Methylation (Hakomori[16]) of the glucan yielded a product (OMe, 36.5%) which could not be methylated further by this procedure. Further methylation by the Purdie method[17] gave a product (OMe, 41.6%) extraction of which with benzene–light petroleum (b.p. 60–70°) gave a main fraction (OMe, 43.8%) which did not show i.r. absorption for hydroxyl groups.

Hydrolysis of the methylated glucan under conditions that cause minimum degradation[18] yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, and (possibly) 2,6-di-*O*-methyl-D-glucose in molar ratios of 2:63:26:5:4 (g.l.c. of trimethylsilylated alditols). The *O*-methyl derivatives were separated by column chromatography on cellulose, and the tetra- and tri-methylated glucoses were identified by p.c., g.l.c., and m.s. The 2,3-di-*O*-methylglucose was identified by p.c. and g.l.c., and the 2,6-di-*O*-methylglucose was tentatively identified by its chromatographic and electrophoretic behaviour.

From the methylation results and the d.p. value, it follows that some of the di-*O*-methylglucoses must be associated with branch points. The excess (7%) is probably due to undermethylation.

This conclusion is suggested by the results shown in Table I, which gives the molar percentages of the methylated sugars obtained from the glucan with different degrees of methylation.

The glucan described herein is similar to the extracellular pullulans isolated from various strains of *Pullularia pullulans* and from *Tremella mesenterica* (Table II). It contains a backbone of maltotriose units attached by (1→6)-α linkages, together with a small proportion of (1→3) linkages, as in other pullulans[13–15], which might explain the presence of glucose after periodate oxidation. This fact can be explained by the occurrence of branched points.

Traces of 2,3-di-*O*-methylglucose isolated after methylation of the glucan were found also by Wallenfels *et al.*[19] for a pullulan from *Pullularia pullulans*. As far as we know, there are no previous reports of the identification of 2,6-di-*O*-methylglucose in the products of hydrolysis of methylated pullulans.

The α-D-glucan described herein appears to be the first intracellular pullulan-type glucan isolated from a fungus. However, the possibility cannot be excluded that the polysaccharide is elaborated by an internal yeast, as whole stroma were used for the study. The presence of free glucose and sucrose in *Cyttaria harioti* has been

TABLE I

MOLAR PERCENTAGES OF PARTIALLY METHYLATED GLUCOSES FROM "PULLULAN" WITH DIFFERENT DEGREES OF METHYLATION

Methylated glucoses	*Degree of methylation of "pullulan" (% OMe)*		
	39.7[a]	*41.6*[b]	*43.8*[c]
2,3,4,6-Tetra	—	1	2
2,3,6-Tri	48	56	63
2,3,4-Tri	24	27	26
2,3-Di	17	7	5
2,6-Di	8	8	4
Monomethyl?	3	1	—

[a]Obtained by successive methylation by the Hakomori and Purdie methods. [b]Fractions of the methylated derivative obtained by the Hakomori method remethylated by the Purdie method. [c]Selected fraction of the methylated derivative obtained by the Hakomori method remethylated by the Purdie method.

TABLE II

PROPERTIES OF "PULLULAN" FROM DIFFERENT SOURCES

Source	$[\alpha]_D$ (*degrees*)	*D.p.*	*Ratio* (*1→4*)/(*1→6*)	*Observations*
Pullularia pullulans[19]	+192	300	1.5	
Pullularia pullulans[13]	+190	250	2.0	Glucose detected after periodate oxidation
Pullularia pullulans (De Bary) Berkhout	+189		2.2	6% of (1→3) linkages
Tremella mesenterica[31]	+185	200	2.0	
Cyttaria harioti	+172	248	2.4	4% of residual glucose after periodate oxidation

reported[1], and these sugars have been used as substrates for the synthesis of pullulans. Gamundi[20] observed that yeasts of the *Saccharomyces* type inhabit the stroma, but these yeasts have not been reported as producing pullulans.

EXPERIMENTAL

Material. — Fruiting bodies of *Cyttaria harioti* Fischer were collected in San Martin de Los Andes (Provincia de Neuquén, Argentina) in January 1969.

General. — All concentrations were performed under diminished pressure at 40–50° (bath). Descending p.c. was conducted on Whatman No. 1 paper with *A*, 1-butanol–ethanol–water (5:2:2); *B*, 1-butanol–pyridine–water (6:4:3); *C*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); *D*, pyridine–ethyl acetate–acetic acid–water (5:5:1:3); *E*, butanone–water azeotrope; *F*, ethyl acetate–benzene (1:1):

and *G*, 1-butanol–ethanol–water (4:1:5, upper layer). Detection was effected with *A*, silver nitrate–sodium hydroxide; *B*, aniline hydrogen phthalate; and *C*, *p*-anisidine hydrochloride.

Melting points are uncorrected. Optical rotations were recorded with a Perkin–Elmer 141 polarimeter, n.m.r. spectra with a Varian A-60 spectrometer, and mass spectra with a Varian Mat Ch-7 mass spectrometer at an ionizing potential of 70 eV, ionizing current of 100 μA, and various inlet temperatures. G.l.c. was effected with a Hewlett–Packard 5750 gas chromatograph equipped with stainless-steel columns (183 × 0.3 cm) packed with *A*, 3% of OV-17 on Chromosorb W (HP, 80–100 mesh); *B*, 10% of NPGS on Chromosorb G (HP, 100–120 mesh); *C*, 3% of OV-101 on Chromosorb W (AW/DMCS, 60–80 mesh); and *D*, 3% of SE-30 on Chromosorb W (AW/DMCS, 60–80 mesh); with a nitrogen flow-rate of 30 ml/min. The trimethylsilyl ethers were prepared as described by Sweeley and coworkers[21]. The acetates of the partially methylated alditols were prepared according to the procedure of Björndal *et al.*[22]. Quantitative determinations of glucose were performed by the D-glucose oxidase–peroxidase procedure[23], reducing power was determined by the method of Somogyi–Nelson[24], and total sugars with the phenol–sulphuric acid reagent[25]. Room temperature implies 20–25°. Paper electrophoresis was carried out in borate buffer (pH 10).

Isolation and purification of the polysaccharide. — Powdered, dried, fruiting bodies of *Cyttaria harioti* Fischer (260 g) were processed as described in previous work[4]. Ethanol (to 40%) was added to the cold-water extract to precipitate the heteropolysaccharide[2,3]. On increasing the ethanol concentration to 52%, new material began to precipitate. Precipitation was complete between 53–55% ethanol concentration, and the insoluble product (3.9 g, 1.5%), collected by centrifugation and dried by solvent exchange, had $[\alpha]_D^{20}$ +157° (*c* 0.4, water).

A solution of the crude product in water (800 ml) was treated with charcoal and filtered through Celite. Portionwise addition of ethanol resulted in a first precipitation at 41–46% ethanol (1.76 g, 47%). Further addition of ethanol, up to 63% concentration, gave another product (0.7 g, 18%). Each polysaccharide was dried by solvent exchange.

The $[\alpha]_D$ value (+171°, water) of the first precipitate did not change when the material (1 g) was twice reprecipitated from solution in water (930 ml) with ethanol. At 52,460 r.p.m. in a Spinco Model E ultracentrifuge at 20°, a 0.5% solution of the purified polysaccharide in 0.1M NaCl showed a single peak with a sedimentation coefficient ($S_{w,20}$) of 3.1×10^{-13}.

Investigation of the polysaccharide. — (*a*) *Determination of homogeneity by fractional precipitation.* To a solution of the pure polysaccharide (0.4 g) in water (200 ml), ethanol was added dropwise, with stirring, to turbidity (ethanol concentration, 35%). The ethanol concentration was then increased in steps of 4%, and the fractions were collected by centrifugation and dried by solvent exchange; 95% of the polysaccharide was recovered at an ethanol concentration of 40–44%, and had

$[\alpha]_D^{20}$ +172° (*c* 0.7, water) and gave a slight violet–brown colour with iodine (Found: N, <0.8%; ash, ~0%).

(*b*) *Acid hydrolysis.* A solution of the pure glucan (60 mg) in 0.5M sulphuric acid (10 ml) was kept in a sealed tube for 18 h at 110°, and then neutralized ($BaCO_3$), filtered through Celite, stirred with Dowex 50 (H^+) resin, and concentrated to dryness. P.c. (solvents *A*–*C*) of the syrupy residue (50 mg) and g.l.c. of a reduced and silylated sample showed glucose as the only component. Reaction with *p*-nitrophenylamine gave *N*-(*p*-nitrophenyl)-D-glucopyranosylamine[26], m.p. and mixture m.p. 183–184°.

(*c*) *Partial acid hydrolysis.* The glucan (63 mg) was treated with 0.5M sulphuric acid (15 ml) for 3 h in a boiling water-bath. The solution was neutralized ($BaCO_3$), filtered, and concentrated to dryness. P.c. (solvents *A* and *B*) of the residue revealed mainly glucose and isomaltose together with smaller amounts of maltose. Three other spots of R_{GLC} 0.33, 0.22, and 0.1 (solvent *A*) were also detected.

The mixture, when subjected to preparative p.c. (solvent *A*), gave (*a*) glucose (15 mg), (*b*) a syrup (15 mg) containing mainly isomaltose together with maltose and higher oligosaccharides, and (*c*) material (10 mg) containing isomaltose and higher oligosaccharides.

Fractions (*b*) and (*c*) were separately chromatographed on columns (30 × 1 cm) of Sephadex G-10 (10 g) which had been calibrated previously with glucose, maltose, and the glucan. Aliquots (0.3 ml) of the eluate were analyzed by the phenol–sulphuric acid reaction, and those with higher absorptions were analyzed by p.c. (solvents *A* and *B*). Maltose, isomaltose, and maltotriose were identified in fraction (*b*), whereas fraction (*c*) was composed of isomaltose and higher oligosaccharides with R_{GLC} 0.11, 0.22, 0.31, and 0.43 (p.c., solvent *A*).

Further identification of maltose and isomaltose as the silylated alditols was effected by g.l.c. [column *B*: temperature programme, 150–250° (15°/min)].

(*d*) *Acetolysis.* The polysaccharide (400 mg) was added to a mixture of acetic anhydride (5.5 ml), acetic acid (4 ml), and sulphuric acid (0.7 ml) at 0°. The mixture was kept for 7 days at room temperature and then for 1 h at 60°, poured into ice–water (250 ml), neutralized ($NaHCO_3$), and extracted with dichloromethane (5 × 50 ml). Concentration of the extract gave a syrup (350 mg), $[\alpha]_D$ +73° (*c* 1, dichloromethane).

A solution of the mixture of acetates in methanol was deacetylated overnight at room temperature with 0.1M sodium methoxide (10 ml). The resulting precipitate was dissolved by the addition of water, and the solution was passed through Dowex 50 (H^+) resin and concentrated to a syrup (300 mg). P.c. (solvents *A* and *B*) showed the main components to be glucose and maltose together with a small proportion of maltotriose. Glucose was separated by preparative p.c. (solvent *A*), and the fraction containing the oligosaccharides was fractionated on a column (30 × 1 cm) of Sephadex G-10 (10 g) previously calibrated with maltose, glucose, and the glucan. Aliquots (0.3 ml) of the eluate were analysed by the phenol–sulphuric acid reaction. Fractions were appropriately combined and submitted to p.c. Maltose and maltotriose were detected in the eluates (solvent *A*).

Enzymic hydrolysis of the glucan. — (*a*) *With amyloglucosidase.* The polysaccharide was incubated with 0.2 mg of amyloglucosidase (50 U/mg) in 5 ml of sodium acetate–acetic acid buffer (pH 4.8) at 37°. Determinations of glucose after 2 and 24 h gave 29% and 102%, respectively.

(*b*) *With bacterial alpha-amylase.* The polysaccharide (6.6 mg) was incubated with bacterial alpha-amylase (10 mg, 170 U/mg) in 2 ml of 15mM sodium glycerophosphate buffer (pH 5.7) at 37°. Aliquots (0.2 ml) were taken at different times and the reducing power was determined. A maximum value of 8% of hydrolysis was obtained.

(*c*) *With pullulanase.* The polysaccharide (5.2 mg) was incubated at 37° with pullulanase (0.6 mg, 0.8 U/mg) in 2.5 ml of 0.01M sodium citrate–citric acid buffer (pH 5.0) containing 1 mg of human serum albumin. Aliquots were taken at different times and the reducing power was determined. A maximum of 32–33% hydrolysis was obtained. P.c. of aliquots taken at 4, 8, and 24 h showed only one spot [R_{GLC} 0.45 (solvent *A*), 0.32 (solvent *B*)] which was identified as maltotriose. Aliquots taken at 30 h and 48 h showed also traces of glucose and maltose (p.c., same solvents).

Isolation of maltotriose. — Enzymic hydrolysis (*c*) was repeated by using 400 mg of glucan and 2 mg of pullulanase in 200 ml of buffer contained in a dialysis bag. After 8 h, the solutions outside (*A*) and inside the bag (*B*) were processed separately. Each was heated for 5 min in a boiling water-bath to inactivate the enzyme, and then filtered, deionized by stirring with Dowex 50 (H^+) and Amberlite IR-45 (HO^-) resins, and concentrated. *A* gave maltotriose (154 mg, homogeneous in p.c. and column chromatography on Sephadex G-10), $[\alpha]_D^{20}$ +159° (*c* 1.5, water); lit.[27] $[\alpha]_D^{20}$ +157° (*c* 1, water). The n.m.r. spectrum in D_2O showed a signal for anomeric protons at δ 5.35[8]. The sugar was acetylated by treatment with anhydrous sodium acetate (50 mg) and acetic anhydride (2 ml) at 120° for 10 min, followed by 1 h at 100°, to give a syrup which, after crystallization and recrystallization from ethanol, was chromatographically pure (t.l.c. on silica gel), R_F 0.42 (solvent *F*), and had m.p. 134–137°, $[\alpha]_D^{20}$ +92° (*c* 1, dichloromethane); lit.[27] for maltotriose hendeca-acetate, m.p. 134–136°, $[\alpha]_D^{20}$ +86° (*c* 1, chloroform).

B gave a syrup (100 mg) consisting mainly of maltotriose with traces of maltose and higher oligosaccharides (p.c., solvents *A* and *B*). It was fractionated by preparative p.c. (solvent *B*) into three fractions.

Fraction *1* contained maltotriose and traces of maltose.

Fraction *2* (6.5 mg) was chromatographically pure [R_{GLC} 0.13 (solvent *B*)] and had $[\alpha]_D^{20}$ +168° (*c* 1, water). A solution of 3.1 mg in water (5 ml) was reduced with sodium borohydride (10 mg) for 20 h. The excess of borohydride was decomposed by dropwise addition of acetic acid and the pH was adjusted to 7.5 with sodium hydrogen carbonate. Sodium periodate (25 mg) was added and the solution was kept for 4 days in the dark at room temperature. The excess of oxidant was decomposed, after acidification with 0.5M sulphuric acid (7.5 ml), by the addition of M sodium arsenite (2.5 ml), and the formaldehyde produced was determined by the chromotropic acid

method[28]. The amount of formaldehyde corresponded to d.p. 3 if the terminal reducing-group was (1→6)-linked, but 6 if it was (1→4)-linked.

The oligosaccharide (1 mg) was hydrolyzed with pullulanase (1.8 mg/5 ml) for 24 h at 37° in 1 ml of acetate buffer. After hydrolysis, p.c. (solvent *B*) showed maltotriose to be the only product. When the oligosaccharide (1 mg) was treated with alpha-amylase for 48 h at 37° in glycerophosphate buffer, no significant hydrolysis occurred (p.c., solvent *B*).

Fraction *3* was a syrup (20 mg) composed of oligosaccharides of low mobility [R_{GLC} 0.08 (solvent *B*)]. On hydrolysis with pullulanase, they gave only maltotriose (p.c., solvent *B*).

Periodate oxidation of the polysaccharide. — A solution of the glucan (123 mg) in 0.1M sodium periodate (50 ml) was diluted to 100 ml and kept at room temperature in the dark. The consumption of periodate was monitored by the arsenite method[29], and the production of formic acid by titration with 10mM sodium hydroxide. After extrapolating the data to zero time, the periodate consumption was 1.28 mol per mol of glucosyl residue, with the production of 0.33 mol of formic acid per mol of glucosyl residue. At intervals during the oxidation, aliquots (1 ml) were reduced with sodium borohydride, the polyalcohol was hydrolyzed (0.5M sulphuric acid, 15 h, 100°), and glucose was determined; 0.03 mol of glucose per mol of glucosyl residue remained after oxidation of the polysaccharide.

After the oxidation was complete, the excess of periodate was reduced with ethylene glycol (2 ml), and the solution was dialysed. The polyaldehyde was reduced with sodium borohydride (100 mg, twice with 24-h interval), and after a further 24 h the borohydride was decomposed by the careful addition of acetic acid to pH 5. The polyalcohol was desalted by dialysis, and recovered by freeze-drying. The polyalcohol (10 mg), when treated with sodium periodate as described above, consumed no oxidant during 24 h.

The polyalcohol (10 mg) was hydrolyzed with 0.5M sulphuric acid (15 h, 100°). P.c. (solvents *A–D*, reagents *A* and *C*) of the neutralized ($BaCO_3$) solution, after removal of cations with Dowex 50 (H^+) resin, revealed erythritol and glycerol together with a small proportion of glucose. G.l.c. of trimethylsilylated products on column *D* (temperature programme from 70–200° at 6°/min) showed the presence of glucose, erythritol, and glycerol in the molar proportions 29:64:7.

Determination of the d.p. of the glucan. — The glucan (27 mg) was suspended in water (10 ml) and stirred with sodium borohydride (25 mg) for 20 h. The excess of borohydride was decomposed by the dropwise addition of acetic acid and the pH was adjusted to 7.5 by addition of saturated, aqueous sodium hydrogen carbonate. Sodium periodate (25 mg) was added and the solution was kept in the dark, at room temperature, for 4 days. The excess oxidant was decomposed with 0.5M sulphuric acid (7.5 ml) and M sodium arsenite (2.5 ml). The solution was dialysed against an equal volume of distilled water for 48 h, and the formaldehyde was determined in the dialysate by the chromotropic acid method[28]. It corresponded to a d.p. of 248, assuming that the terminal reducing-group was (1→4)-linked.

Methylation analysis of the glucan. — The polysaccharide was methylated by the Hakomori method[16]. Dried glucan (300 mg) was dissolved in dry methyl sulphoxide (10 ml), and the sodium methylsulphinylmethanide reagent[30] (10 ml) was added. The mixture was stirred at room temperature for 6 h under nitrogen and then cooled in an ice–water bath, and methyl iodide was added dropwise keeping the temperature below 25°. The solution was left overnight at room temperature, and then dialyzed and freeze-dried. The methylated product showed a small i.r. hydroxyl band (Found: OMe, 36.5%).

This product (300 mg) was subjected to four Purdie methylations with methyl iodide (10 ml) and freshly prepared silver oxide (1 g) at 60° for 10 h. The solids were removed by filtration, and washed with hot dichloromethane, and the combined filtrates and washings were evaporated to a syrup (290 mg) (Found: OMe, 39.7%).

Fractional extraction of the methylated glucan with mixtures of benzene–light petroleum (b.p. 60–70°) furnished a main fraction, $[\alpha]_D^{25}$ +148° (*c* 1, dichloromethane), which did not show i.r. absorption for hydroxyl (Found: OMe, 43.8%). A solution of the methylated glucan (140 mg) in 66% sulphuric acid (5 ml) was kept at room temperature for 3 h, and then diluted to 12% and heated at 100° for 8 h. The solution was neutralized ($BaCO_3$), decationized with Dowex 50 (H^+) resin, and concentrated to dryness.

P.c. (solvent *E*, reagent *C*) of the syrup showed spots of R_F 0.84 (pink, 2,3,4,6-tetra-*O*-methylglucose), 0.67 (yellow, 2,3,4-tri-*O*-methylglucose), 0.62 and 0.57 (pink, probably anomers of 2,3,6-tri-*O*-methylglucose), 0.23 (yellow, 2,3-di-*O*-methylglucose), and 0.20 (brown, possibly 2,6-di-*O*-methylglucose).

The mixture of partially methylated glucoses (10 mg) was reduced with sodium borohydride (20 mg) for 12 h, and the alditols were analyzed as their trimethylsilyl derivatives and as the acetates. G.l.c. of the silyl derivatives and acetates on column *C* (temperature programmed from 130–220° at 4°/min) and of the acetates on column *B* (at 190°) revealed 2,3,4,6-tetra-*O*-methylglucose, 2,3,6- and 2,3,4-tri-*O*-methylglucoses, and 2,3-di-*O*-methylglucose (identified by comparison with standards), and a peak attributed to 2,6-di-*O*-methylglucose, in molar ratios 2:63:26:5:4, measured directly from the peak areas (column *C*, trimethylsilyl derivatives).

The mixture of partially methylated glucoses (60 mg) was fractionated by using a column (15 × 1.5 cm) of cellulose and elution with solvent *E*. Fractions (3 ml) were monitored by p.c. (solvent *E*), and the appropriate fractions were combined.

Fraction *1* (2 mg) contained (p.c. and g.l.c.) 2,3,4,6-tetra-*O*-methylglucose which was identified by m.s. of its acetylated alditol.

Fraction *2* (5 mg), R_F 0.67 (solvent *E*, yellow spot with reagent *C*), contained (g.l.c. on column *C*) 2,3,4-tri-*O*-methylglucose with traces of 2,3,6-tri-*O*-methylglucose.

Fraction *3* (30 mg) was a syrupy mixture of 2,3,4- and 2,3,6-tri-*O*-methylglucose (p.c. and g.l.c.).

Fraction *4* (6 mg) contained (p.c., solvent *E*, reagent *C*; and g.l.c.) 2,3,6-tri-*O*-methylglucose. Its identity was confirmed by m.s. of the acetylated alditol.

Fraction *5* (6 mg) contained a mixture of 2,3,6-tri-*O*-methylglucose and a substance of R_F 0.23 (solvent *E*, reagent *C*) which was identical (p.c. and g.l.c., column *B*) with 2,3-di-*O*-methylglucose.

Fraction *6* (5 mg), when subjected to p.c.[31] and paper electrophoresis[32], showed two spots of R_F 0.23 and M_G 0.12 (yellow, 2,3-di-*O*-methylglucose) and R_F 0.21 and M_G 0.18 (brown, tentatively identified as 2,6-di-*O*-methylglucose[31,32]).

Fraction *3* (26 mg) was refractionated on the same column of cellulose. Only one substance appeared in the first fraction and was identical (p.c., solvent *E*, reagent *C*; and g.l.c., column *C*) to 2,3,4-tri-*O*-methylglucose. The identification was confirmed by m.s. of the acetylated alditol.

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