THE HEMICELLULOSES OF BRACKEN

PART II. A GALACTOGLUCOMANNAN

I. BREMNER* AND K. C. B. WILKIE

Chemistry Department, University of Aberdeen, Old Aberdeen, AB9 2UE (Great Britain)

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ABSTRACT

A galactoglucomannan has been isolated from the stem tissues of a fern, bracken (*Pteridium aquilinum*). It had $[\alpha]_D^{20}$ −35° and, on hydrolysis, yielded mannose, glucose, and galactose in the molar ratios of 60:15:1. Mild hydrolysis with acid released galactose only. The d.p., determined by periodate-oxidation and Smith-degradation studies, was 43–45 for a doubly branched molecule. The methylated galactoglucomannan had a d.p. of 25–30 by ebulliometry. Methylation analysis, in combination with other evidence, indicated that the hemicellulose had β-(1→4)-linked D-glucopyranose and D-mannopyranose residues in the ratio of 1 to 4, with residues of the latter contiguous. D-Mannopyranosidic and D-galactopyranosidic residues were present as non-reducing end-groups in the ratio of 7 to 1, but there was no evidence of non-reducing D-glucopyranosidic residues. The branching was to 6-positions on the main glucomannan chain and was probably α-(1→6). The galactoglucomannan is similar to hemicelluloses isolated from softwoods, but the former has a lower d.p.

INTRODUCTION

There have been many structural studies of the non-cellulosic polysaccharides from the lignified tissues of the *Spermatophytae*[1,2] (the *Gymnospermae* and the *Angiospermae*), but very little work on the polysaccharides in the *Bryophytae* and *Pteridophytae*. Such studies should be of chemotaxonomic interest. A galactoglucomannan[3] and an arabinogalactan[3,4] from the apparently lignified stem-tissues of the aquatic moss *Fontinalis antipyretica* have been studied, as have the galactoglucomannan and acidic xylan from cinnamon fern[5] (*Osmunda cinnamonea*) and the acidic xylan from the lignified stem-tissues of bracken[6] (*Pteridium aquilinum*) . The present paper reports on a galactoglucomannan isolated from bracken, which is similar to galactoglucomannans found in softwoods[2].

RESULTS AND DISCUSSION

Stems of bracken, *Pteridium aquilinum*, were dried, milled, and delignified, and the resultant holocellulose was treated with 4% and then with 10% aqueous sodium

*Present address: Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland.

hydroxide under nitrogen, as described earlier. The treatments with 4% alkali extracted hemicellulosic materials accounting for 18.5% of the holocellulose and a further 10.4% was extracted by the 10% alkali. The material extracted by 4% alkali had $[\alpha]_D$ −29.5° and was the source of the acidic xylan studied earlier[6]; the material extracted by 10% alkali had $[\alpha]_D$ −26° and was the source of the galactoglucomannan studied in the work now reported. The latter material, on hydrolysis, gave xylose, mannose, and glucose in the molar ratios of 5:5:2, together with acidic sugars and traces of arabinose and galactose. Partial fractionation could be achieved with Cetavlon[7], but the method introduced by Meier, using barium hydroxide[8], was more successful. The barium hydroxide complex that precipitated was treated with dilute acetic acid, and the free polysaccharide was recovered. During this fractionation, there was a progressive removal of polysaccharides containing xylose and galactose residues. From the hemicellulosic material, 52% of a hemicellulose was isolated that had $[\alpha]_D^{20}$ −35°; on hydrolysis, it gave mannose, glucose, and galactose in the molar ratios of 60:15:1. No further fractionation of this material could be achieved by repeated use of Fehling's solution, and it is referred to as the galactoglucomannan.

Mild, acid hydrolysis (0.025M oxalic acid at 100°) of a sample of the galactoglucomannan released traces of galactose only; this is compatible with the methylation evidence which indicates that the galactose residues in the galactoglucomannan are solely present as non-reducing, terminal, pyranosidic residues.

The galactoglucomannan was methylated and then sub-fractionated by dissolution in various mixtures of light petroleum (b.p. 60–80°) and chloroform. The two most fully methylated fractions (OMe, *A* 45.2% and *B* 42.4%) were examined. On methanolysis, followed by hydrolysis, *A* and *B* gave 2,3,4,6-tetra-*O*-methyl-D-mannopyranose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-mannose, 2,3,6-tri-*O*-methyl-D-glucose, and di-*O*-methylhexoses in the molar ratios *A* 20:3:96:20:14 and *B* 20:3:110:43:16. One of the di-*O*-methylhexoses was chromatographically indistinguishable from 2,3-di-*O*-methyl-D-mannose. The galactoglucomannan reduced 690 mmoles of metaperiodate and released 89 mmoles of formic acid per hexose residue. The galactoglucomannan, when subjected to Smith degradation[9], gave erythritol, glycerol, and hexoses (glucose and mannose) in the molar ratios 54:4: *ca.* 1. After borohydride reduction, the galactoglucomannan released 68 mmoles of formaldehyde per hexose residue on periodate oxidation. The hemicellulose was partially hydrolysed by the enzyme mixture "Hemicellulase". From the hydrolysate, seven oligosaccharides were isolated, four of which [the β-(1→4)-linked D-mannose di-, tri-, and tetra-saccharides, and 4-*O*-β-D-mannopyranosyl-D-glucose] were fully characterised; two others [*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-D-mannopyranose and *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-D-glucopyranose] were identified tentatively.

The amount of formic acid released on periodate oxidation of the galactoglucomannan is compatible with a d.p. of 45 and the presence of two branch-points. The results of the Smith-degradation studies are in accord; the ratio of erythritol to glycerol indicated a d.p. of 43 for a doubly branched molecule. On periodate oxida-

tion, the reduced galactoglucomannan released formaldehyde. Assuming that two molecules of formaldehyde are released per molecule (from the former reducing-end residue), this indicates a d.p. of *ca.* 29. The methylated galactoglucomannan was found to have a d.p. of 25–30 by ebulliometry[10] and, because the molar ratios of tetra-*O*-methyl-D-hexoses, tri-*O*-methyl-D-hexoses, and di-*O*-methyl-D-hexoses were *ca.* 11:66:7, it appears that the methylated hemicellulose molecules have two or three branch-points. The amount of periodate reduced by the galactoglucomannan is compatible with (1→4)-linked residues and (1→6) branch-points, features supported by methylation analysis.

The methylation analysis and periodate-oxidation values are in good agreement. The slight differences are almost certainly due to the unavoidable and normal selective losses that take place during the isolation of any methylated polysaccharide. It is evident from the methylation analysis and partial hydrolysis results that the galactoglucomannan has β-(1→4)-linked D-mannopyranosyl and D-glucopyranosyl residues in the ratio of *ca.* 4 to 1. The partial hydrolysis studies showed that D-mannose residues occurred contiguously, but there was no evidence for contiguous D-glucose residues. The isolation of 2,3,4,6-tetra-*O*-methyl-D-mannopyranose and of 2,3,4,6-tetra-*O*-methyl-D-galactopyranose from the hydrolysed, methylated galactoglucomannan showed that D-mannose and D-galactose were present as non-reducing end-residues, in the ratio of *ca.* 7:1, in the methylated polysaccharide. There was no evidence for the presence of non-reducing, terminal D-glucose residues. That the non-reducing, terminal residues were linked (1→6), and by analogy with other galactoglucomannans and glucomannans probably linked α-(1→6), was indicated by the periodate-oxidation studies and by the isolation of a compound tentatively identified as 2,3-di-*O*-methyl-D-mannose. The slightly low value for the periodate reduced and the survival of a low proportion of hexose residues after periodate oxidation could be due to branch-points at C-2 or C-3 or to the uncertainty that always relates to values obtained by extrapolation to zero time.

Hemicelluloses similar, but not identical, to those in bracken have been isolated by Timell from cinnamon fern[5]. The galactoglucomannan from cinnamon fern had a higher d.p. (103), a higher proportion of glucose and of galactose, some contiguous glucose residues, and some non-reducing, terminal glucopyranosidic residues. The isolation of the galactoglucomannan and of the acidic xylan from bracken is chemotaxonomically interesting. In bracken, the two hemicelluloses occur in approximately equal amounts. In the hardwoods, there are glucomannans, but they normally lack D-galactose residues, and these polysaccharides are present in lower proportion; acidic xylans are the dominant hemicelluloses. By contrast, the softwoods have a lower proportion of acidic xylans (with non-reducing, terminal L-arabinofuranosyl residues) and a higher proportion of glucomannans than in the hardwoods[2]. There are two types of softwood glucomannans. The members of one group of hemicelluloses are soluble in water and contain mannose, glucose, and terminal galactose residues in the molar ratios of 3:1:1. The members of the other group of hemicelluloses are soluble in alkali and contain the above residues in the molar ratios of 30:10:1. The

latter galactoglucomannans are similar to that from bracken which contains these residues in the ratios of 60:15:1. The specific rotations of softwood galactoglucomannans are $-35\pm5°$ and of the bracken galactoglucomannan $-35°$. The softwood, alkali-soluble galactoglucomannans have d.p. values of *ca.* 100, and it is possible that every molecule carries at least one galactose residue. On the other hand, the bracken galactoglucomannan, although having a comparable proportion of galactose and other residues, has a lower d.p. and so some molecules *must* lack D-galactose residues. In spite of this, it seems appropriate to avoid chemical semantics and for consistency to name the bracken hemicellulose also as a galactoglucomannan, although appreciating that some of the material is glucomannan. The stem of the aquatic moss *F. antipyretica* yields a galactoglucomannan having a d.p. similar to that of the bracken hemicellulose, but having terminal, non-reducing galactose and mannose residues in the ratio of 4 to 1. Whereas there is a similarity in the alkali-soluble galactoglucomannans from softwoods, bracken, and cinnamon fern, the xylans are rather different. The bracken xylan has a very low proportion of arabinofuranosyl residues and in this respect is dissimilar to softwood xylans. Timell[11] also reported that bracken xylan has a lower proportion of arabinose residues than has the xylan from cinnamon fern.

EXPERIMENTAL

Details of the extraction of the hemicelluloses from the bracken holocellulose are given in an earlier paper[6].

Paper chromatography was performed on Whatman No. 1 and 3 MM papers, and thin-layer chromatography (t.l.c.) on Kieselgel G (Merck, Darmstadt). R_C values are mobilities relative to that of cellobiose. The irrigants used were (*1*) ethyl acetate–pyridine–water (3.6:1.15:1); (*2*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*3*) ethyl acetate–acetic acid–water (3:1:3); (*4*) butyl alcohol–ethanol–water (4:1:5); (*5*) butyl alcohol–benzene–pyridine–water (5:1:3:3); (*6*) butanone–water (2:1): (*7*) ethyl acetate–pyridine–water (2:1:2); (*8*) butyl alcohol–pyridine–water (6:4:3); (*9*) ethyl acetate–acetic acid–water (3:1:1); (*10*) ethyl acetate–pyridine–water (10:4:3). Chromatographic spray reagents were (*1*) 5% *p*-anisidine hydrochloride in methanol and (*2*) alkaline silver nitrate. Gas–liquid chromatography[12] (g.l.c.) was performed on a Pye–Argon gas chromatograph. The columns (120×0.5 cm) contained as liquid phase: *A* 20% Apiezon M; *B* 20% Carbowax 20 M; *C* poly(butane-1,4-diol succinate)[12]. The support was either alkali-washed (*A*) or acid-washed (*B* and *C*) Celite (mesh 80–120). The argon flow-rate was 30–60 ml/min. Electrophoretic examination of monosaccharides and of oligosaccharides was carried out in either 0.1M borax or in 50mM sodium germanate buffer[13] on Whatman No. 1 or 3MM paper at 3–7 volts/cm. Polysaccharides were examined on Whatman GF/A glass-paper at 8–12 volts/cm, and were detected with alkaline potassium permanganate. Sugars in hydrolysates were determined by the phenol–sulphuric acid method[14], and uronic acid was determined by the method of Anderson *et al.*[15]. Optical rotations were observed at $18\pm3°$.

Polysaccharide samples were hydrolysed in sealed tubes with 0.5M sulphuric acid for 12–16 h at 100°; the hydrolysates were neutralised with barium carbonate.

The hemicellulosic material (49.6 g), used in the present study and extracted from the holocellulose by 10% aqueous sodium hydroxide, had $[\alpha]_D^{20}$ −26° (*c* 0.76, M sodium hydroxide). Paper chromatography (irrigants *1* and *2*) of a hydrolysate of this material showed components indistinguishable from xylose, mannose, glucose, galactose, and arabinose. The last two sugars were present in trace amounts, and the first three sugars were in the molar ratios of 5:5:1. A small amount of a uronic acid was also present.

Isolation of the galactoglucomannan. — Saturated barium hydroxide (400 ml) was added during 3 h to a solution of the hemicellulosic material (20 g) in 10% aqueous sodium hydroxide (1.6 l). The precipitate was collected, dissolved in dilute acetic acid, and treated with ethanol (2 vol.). The precipitate was collected by centrifugation, redissolved in sodium hydroxide, and subjected to three further precipitations using barium hydroxide.

Samples of each precipitate were hydrolysed and examined chromatographically (irrigant 1). There was a progressive decrease in the amount of xylose, galactose, arabinose, and uronic acid relative to mannose and glucose. Hydrolysates of the last two precipitates were indistinguishable. The final precipitate (10.4 g), hereafter referred to as the galactoglucomannan, had $[\alpha]_D^{20}$ −35° (*c* 1.00, 2.5M sodium hydroxide). The acid hydrolysate contained mannose, glucose, and galactose in the molar ratios of 60:15:1. No alteration in the proportion of the residues was obtained on attempted fractionation using Fehling's solution.

Treatment of the galactoglucomannan with dilute acid. — A sample (10 mg) of the hemicellulose was treated with 25mM oxalic acid for 18 h at 100°. After neutralisation with barium carbonate and filtration, the solution was concentrated and examined by paper chromatography (irrigant *1*). A trace of galactose only was detected.

Periodate oxidation of the galactoglucomannan. — A sample (150 mg) of the hemicellulose was oxidised in the dark at 5° with sodium metaperiodate. The periodate consumed (moles per $C_6H_{10}O_5$ residue) was: 0.76 (24 h), 0.92 (54), 0.95 (75.5), 1.02 (120), 1.06 (168), and 1.12 (264), corresponding to an extrapolated value of 0.96 at zero time. The formic acid released (mmoles per $C_6H_{10}O_5$ residue) was: 51 (14.5 h), 87 (43), 90 (64.5), 91 (86), 94 (184), and 98 (304), corresponding to an extrapolated value of 89 at zero time.

Periodate oxidation of the reduced galactoglucomannan. — A solution of the hemicellulose (104 mg) in 5% aqueous sodium hydroxide (10 ml) was neutralised with dilute acetic acid. Sodium borohydride (50 mg) was added and, after 18 h, dilute acetic acid to destroy the excess of reductant. On the addition of ethanol (2 vol.), a precipitate (90 mg) of the reduced hemicellulose was obtained. It was oxidised with 0.1M sodium metaperiodate (20 ml) at 5° in the dark. A sample of non-reduced galactoglucomannan was treated in the same way. Periodically, aliquots (2 ml) were withdrawn from each solution, and to each was added saturated, aqueous lead formate (7 ml). The resultant precipitate was removed by centrifugation, and to

samples (5 ml) of each centrifugate M sulphuric acid (1 ml) was added and the precipitate was removed. Samples (5 ml) of each solution were dialysed against distilled water (10 ml) for 2 h. Chromotropic acid solution (10 ml) was added to samples (2 ml) of the diffusate, and the mixtures were heated for 30 min at 100°. The absorbances were measured at 570 nm, and the formaldehyde was determined by reference to a standard curve obtained by the periodate oxidation of erythritol. Control experiments showed that the dialysis system attained equilibrium within 30 min. The formaldehyde (mmoles) produced per $C_6H_{10}O_5$ residue was, for the non-reduced hemicellulose: 111 (17.5 h), 130 (42), 150 (69), 163 (90), 176 (135), 191 (186), and 202 (214) and, for the reduced hemicellulose: 111 (17.5 h), 224 (28.5), 218 (42), 230 (90), 246 (135), 266 (186), and 276 (214). The difference in the extrapolated values at zero time corresponded to a release of 68 mmoles of formaldehyde per hexose residue.

Smith-degradation of the galactoglucomannan. — A sample (426 mg) of the hemicellulose was oxidised with 0.1M sodium metaperiodate (100 ml) in the dark for 21 days. 0.5M Barium acetate (50 ml) was then added, and the insoluble salts were removed. The solution was deionized with Amberlite IR-45 (OH^-) and IR-120 (H^+) resins. The concentrated eluate was treated with sodium borohydride (500 mg) for 3 days and, after destruction of the excess of borohydride with Amberlite IR-120 (H^+) resin, the solution was evaporated, and borate was removed as methyl borate by distillation of methanol from the residue. The polyalcohol (258 mg) was treated with 0.5M sulphuric acid (15 ml; 6 h at 100°) and, after neutralisation with barium carbonate, the hydrolysate was concentrated and examined by paper chromatography (irrigants *1*, *2*, and *10*; spray *2*). The main component appeared to be erythritol, and there were smaller amounts of glycerol, mannose, and glucose. Glycolaldehyde was detected on most chromatograms and also components found to be derivable from glycolaldehyde on treatment with acid.

Samples of the polyalcohol hydrolysate were quantitatively separated by paper chromatography with irrigants *4*, *9*, or *10*. Each component was eluted from the paper with water (15 ml), and M sulphuric acid (1 ml) and 0.1M sodium metaperiodate (2 ml) were added to the eluate. After 10 min, M sodium arsenite (2 ml) was added and, after a similar period, the solutions were diluted to 25 ml and the formaldehyde was determined as described above. The molar ratios of erythritol, glycerol, and hexoses were 27:2:0.4–0.5. Another sample (5 mg) of the polyalcohol hydrolysate was esterified in the usual way with acetic anhydride–sodium acetate. Examination of the product by g.l.c. (column *A*) revealed components having retention times corresponding to those of glycerol triacetate and erythritol tetra-acetate in a molar ratio of 136:10. A sample of one of the components from the acetylated hydrolysate of the polyalcohol was crystallised from ether–light petroleum (b.p. 60–80°) and gave erythritol tetra-acetate, m.p. 84°.

Partial hydrolysis of the galactoglucomannan. — Hemicellulase (Koch–Light) (250 mg) was added to a dispersion of the hemicellulose (2.5 g) in water (125 ml), and the mixture was dialysed at 32° for 5 days against several volumes (2 l) of distilled water[16]. The non-diffusate solution was heated with ethanol to inactivate the enzymes

and then evaporated. The residue was hydrolysed with M sulphuric acid. Paper chromatography (irrigant *1*) of the neutralised hydrolysate revealed mannose, glucose, galactose, and xylose in the molar ratios of *ca.* 4:1:1:1. The diffusate was treated with charcoal and on evaporation to dryness yielded a syrup (1.83 g) which was dissolved in water and added to a column of Darco G-60 charcoal and Celite 545. Elution with water gave mannose, glucose, and traces of galactose (total, 525 mg). Subsequent elution with an aqueous ethanol gradient (2→40%) gave seven chromatographically homogeneous components (Table I).

TABLE I

DATA ON THE OLIGOSACCHARIDES OBTAINED BY GRADED, ACID HYDROLYSIS OF THE GALACTOGLUCOMANNAN

Oligosaccharides	*Yield (mg)*	M_{Glc}[a]	R_C *Irrigants*			
			5	7	8	10
A *O*-*β*-D-Man*p*-(1→4)-D-Man	265	0.55	1.10	1.00	1.05	1.00
B *O*-*β*-D-Man*p*-(1→4)-*O*-*β*-D-Man*p*-(1→4)-D-Man	146	0.54	0.45	0.47	0.51	0.44
C *O*-*β*-D-Man*p*-(1→4)-*O*-*β*-D-Man*p*-(1→4)-*O*-*β*-D-Man*p*-(1→4)-D-Man	24	0.52	0.12	0.17	0.19	0.17
D *O*-*β*-D-Man*p*-(1→4)-D-Glc	13	0.44	0.65	0.74	0.77	0.71
E *O*-*β*-D-Glc*p*-(1→4)-*O*-*β*-D-Man*p*-(1→4)-D-Man	3	0.41	0.50	0.65	—	0.54
F *O*-*β*-D-Man*p*-(1→4)-*O*-*β*-D-Man*p*-(1→4)-D-Glc	2	0.40	0.17	0.39	—	0.29
G (2 Man+Glc)	7	0.41	0.12	0.32	0.19	0.21

[a]Electrophoretic mobility in 0.1M sodium borate buffer (corrected for electroendosmotic flow).

Samples of the oligosaccharides *A*–*G* were hydrolysed before and after reduction with sodium borohydride. Neutralised and concentrated hydrolysates were compared by paper chromatography (irrigant *1*). The d.p. values of the oligosaccharides were determined by the method of Timell[17]. Samples (≤ 5 mg) of the oligosaccharides were thrice methylated by the Kuhn procedure[18] and then treated with boiling, 3% methanolic hydrogen chloride (1 ml for 12 h). The neutralised methanolysates were examined by g.l.c. (column *A* at 150°)[20] and also hydrolysed with 0.5M sulphuric acid followed by paper chromatography (irrigants *4* and *6*) after neutralisation.

Oligosaccharide *A*, which crystallised from aqueous ethanol, had a d.p. of 2.0, m.p. 203° alone and in admixture with 4-*O*-*β*-D-mannopyranosyl-D-mannose[19], and $[\alpha]_D^{20}$ −7.0° (*c* 2.65, water). 2,3,4-Tri-*O*-methyl-D-mannose and 2,3,4,6-tetra-*O*-methyl-D-mannose and their methyl glycosides were found in equal amount by paper chromatography and g.l.c. as described above.

Oligosaccharide *B*, which crystallised from aqueous ethanol, had a d.p. of 3.0, m.p. 168–170° alone or in admixture with the *β*-(1→4)-linked D-mannose trisaccharide[19], and $[\alpha]_D^{20}$ −17.2° (*c* 1.46, water). The two methylated sugars obtained from oligosaccharide *A* were obtained in the ratio 2:1.

Oligosaccharide *C* was chromatographically and electrophoretically homo-

geneous and had a d.p. of 4.0 and $[\alpha]_D^{20}$ −22.6° (*c* 0.60, water). Methylation analysis gave the same methylated sugars as above, in the ratio 3:1.

Oligosaccharide *D* had a d.p. of 2.0 and $[\alpha]_D^{20}$ +15° (*c* 0.25, water) and on hydrolysis gave mannose and glucose (1:1), whereas the reduced sugar gave mannose but no glucose. Methylation analysis gave equimolar amounts of 2,3,6-tri-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-D-mannose. The R_C values (irrigants *7*, *8*, and *10*) were similar to those reported [19,22,23] for 4-*O*-*β*-D-mannopyranosyl-D-glucose.

Oligosaccharide *E* was chromatographically and electrophoretically homogeneous. On hydrolysis, it gave mannose and glucose in the ratio 2:1, whereas the reduced oligosaccharide gave them in the ratio 1:1. Methylation analysis (g.l.c.) gave methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside and methyl 2,3,6-tri-*O*-methyl-D-mannopyranoside in the ratio 1:2. The sugars had R_C values (irrigants *5*, *7*, and *10*) similar to those reported[19,22,23] for *O*-*β*-D-glucopyranosyl-(1→4)-*O*-*β*-D-mannopyranosyl-(1→4)-D-mannopyranose.

Oligosaccharide *F* was chromatographically and electrophoretically homogeneous and had an R_C value (irrigant *7*) similar to that reported[22] for *O*-*β*-D-mannopyranosyl-(1→4)-*O*-*β*-D-mannopyranosyl-(1→4)-D-glucopyranose. On hydrolysis it gave glucose and mannose in the ratio 1:2, whereas the reduced oligosaccharide gave mannose but no glucose.

Oligosaccharide *G* was chromatographically and electrophoretically homogeneous. On hydrolysis, it gave mannose and glucose in the ratio 2:1, whereas the ratio for the reduced derivative was 1:1. Its R_C values were lower than those reported[22] for *O*-*β*-D-Man*p*-(1→4)-*O*-*β*-D-Glc*p*-(1→4)-D-Man and *O*-*β*-D-Glc*p*-(1→4)-*O*-*β*-D-Man*p*-(1→4)-D-Man.

Methylation of the galactoglucomannan. — A sample (4 g) of the galactoglucomannan was methylated under nitrogen by seven treatments with methyl sulphate and 40% aqueous sodium hydroxide. The partially methylated product (OMe, 32.1%), dissolved in *N*,*N*-dimethylformamide (50 ml), was treated[18] in the dark with methyl iodide (5 × 25 ml) and silver oxide (5 × 20 g). The product (OMe, 42.3%) was again methylated and gave a methylated galactoglucomannan (3.6 g) having OMe 44.2% (theoretical maximum, 45.6%). A sample was methanolysed and then hydrolysed. T.l.c. (irrigant *6*) of the hydrolysate revealed tri-*O*-methylhexoses as the main components and smaller amounts of tetra- and di-*O*-methylhexoses. The methylated polysaccharide was fractionated by graded dissolution in boiling mixtures of chloroform and light petroleum (b.p. 60–80°) and gave the fractions shown in Table II.

Hydrolysis of methylated galactoglucomannan. — A sample of fraction 1 was boiled under reflux for 12 h with 2.5% anhydrous methanolic hydrogen chloride, and the resultant glycosides were hydrolysed with M hydrochloric acid (50 ml, 100° for 12 h). The plasticizer, which floated as a brown liquid on the hydrolysate, was removed; no sugars or methylated sugars were found on examination of this upper layer by t.l.c. The hydrolysate was neutralised with silver carbonate, and silver ions were removed as the sulphide. Concentration of the filtrate gave a yellow syrup (1.60 g) which was eluted from a column of a mixture of equal weights of Darco

TABLE II

FRACTIONATION OF THE METHYLATED GALACTOGLUCOMANNAN

Fraction	*Light petroleum–chloroform*	*Yield (mg)*	*OMe (%)*
1[a]	100/0	*ca.* 2,800	45.2
1′[a]		135	44.6
2	90/10	*ca.* 600	42.4
2′[b]	90/10	266	44.6
3	80/20	120	41.9
4	60/40	37	—

[a]Fraction 1 became contaminated by a plasticizer from a short length of tubing on a rotary evaporator. When an aliquot was boiled in methanol, the insoluble materials contained only traces of the methylated polysaccharide. Addition of an excess of light petroleum (b.p. 100–120°) to the methanolic solution gave fraction 1′ (OMe, 43.1%). The absence of plasticizer from Fraction 1′ was attested by the failure to detect any floating or suspended droplets in an aqueous hydrolysate; the plasticizer was highly insoluble under these conditions. [b]Fraction 2, on further methylation, gave fraction 2′.

G-60 charcoal and Celite 545 (prewashed with concentrated hydrochloric acid and then thoroughly with water) by a 10→30% aqueous ethanol gradient (5 l) followed by 30% aqueous acetone (2 l). The fractionation was monitored by t.l.c. (irrigant *6*). Sub-fractionation was carried out, where necessary, by chromatography (irrigant *4*) and electrophoresis (0.1M borate, 8 volts/cm, 5 h) on Whatman No. 3MM paper.

Fraction *i* (13.5 mg) was a chromatographically and electrophoretically homogeneous syrup. On demethylation, it gave mannose only. It had R_{TMG} values with irrigants *4*, *6*, and *9* of 0.54, 0.30, and 0.70, respectively, and an M_{GLC} value of 0.17 on electrophoresis in 0.1M borate, indicating it to be 2,3-di-*O*-methyl-D-mannose.

Fraction *ii* (5 mg) was a syrup which gave only mannose on demethylation. It had R_{TMG} values with irrigants *4*, *6*, and *9* of 0.54, 0.20, and 0.56, respectively, and an M_{GLC} value of 0.27.

Fraction *iii* (2 mg) appeared to be pure on borate electrophoresis and had an M_{GLC} value (0.09) indicating that it was neither 2,3-di-*O*-methyl-D-glucose nor 2,3-di-*O*-methyl-D-mannose. On demethylation, it gave glucose and mannose (paper chromatography).

Fraction *iv* (269 mg) had $[\alpha]_D^{20}$ −11° (*c* 2.69, water) and gave only mannose on demethylation. The sugar was indistinguishable from 2,3,6-tri-*O*-methyl-D-mannose on paper chromatography (irrigants *4*, *6*, and *9*) and on t.l.c. (irrigant *6*); the di-*p*-nitrobenzoate had m.p. and mixed m.p. 188°. A sample (5 mg) of the free sugar was converted into the methyl glycosides and examined by g.l.c. (columns, *A* at 150°, and *C* at 175°). The retention times (*T*) were measured relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. They were 2.10 (*A*) and 5.00 (*C*), in agreement with the values for methyl 2,3,6-tri-*O*-methyl-D-mannopyranoside.

Fraction *v* (~49 mg) had $[\alpha]_D^{20}$ +60° (*c* 1.5, chloroform) and yielded glucose and a trace of mannose on demethylation. Paper chromatography (irrigants *4*, *6*, and *9*) revealed one component indistinguishable from 2,3,6-tri-*O*-methyl-D-glucose. A second, and minor, component, which was indistinguishable from 2,3,6-tri-*O*-

methyl-D-mannose, was detected by t.l.c. (irrigant *6*). Methyl glycosidation, followed by g.l.c., gave three peaks having *T* values 1.51. 1.84, and 2.10 on column *A* and 3.44, 4.70, and 5.00 on column B^{20}. The first two peaks corresponded to methyl 2,3,6-tri-*O*-methyl-D-glucosides and the last, very minor, peak to a methyl 2,3,6-tri-*O*-methyl-D-mannopyranoside. The presence of 2,3,6-tri-*O*-methyl-D-glucose was confirmed by formation of the di-*p*-nitrobenzoate, m.p. 188–189°.

Fraction *vi* gave galactose (paper chromatography) on demethylation and was chromatographically similar to 2,3,4,6-tetra-*O*-methyl-D-galactose. Its methyl glycoside gave a single peak on g.l.c. (*T*, 1.35 and 1.75 on columns *A* and *C*, respectively). The latter value agrees with that (1.80) reported[20] for methyl 2,3,4,6-tetra-*O*-methyl-D-galactopyranoside.

Fraction *vii* was a syrup (~59 mg), $[\alpha]_D^{20}$ +20° (*c* 0.46, methanol), which was indistinguishable by paper chromatography (irrigants *4*, *6*, and *9*) and t.l.c. (irrigant *6*) from both 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,3,4,6-tetra-*O*-methyl-D-glucose. Demethylation gave mannose only on paper chromatography. Reverse-phase chromatography on sheets impregnated with methyl sulphoxide and irrigated with isopropyl ether[21] revealed two spots (R_{TMG} 0.39 and 0.24) indistinguishable from those given by 2,3,4,6-tetra-*O*-methyl-D-mannose. Methyl glycosides derived from the syrup had *T* values (1.17 and 1.39 on columns *A* and *C*) on g.l.c. similar to those given by methyl 2,3,4,6-tetra-*O*-methyl-D-mannopyranoside[20]. Fraction *vii* gave an aniline derivative, m.p. 126–127°.

Fraction 1′ (Table II) was methanolysed, and the methyl glycoside mixture was examined by g.l.c. (columns *A* and *C*). The molar ratios of the components (determined from the peak areas) were: 2,3,4,6-tetra-*O*-methyl-D-mannopyranose, 2,3,4,6-tetra-*O*-methyl-D-galactopyranose, 2,3,6-tri-*O*-methyl-D-mannose, 2,3,6-tri-*O*-methyl-D-glucose, and di-*O*-methyl-D-hexoses, 1.0:0.15:4.8:1.8:0.7.

Fraction 2 was further methylated[18] by five treatments with *N*,*N*-dimethylformamide (25 ml), methyl iodide (10 ml), and silver oxide (10 g). The product was fractionated by dissolution in light petroleum (b.p. 60–80°)–chloroform (1:9). The solution was decanted and, on removal of the solvents, a syrupy fraction 2′ (166 mg) was obtained having $[\alpha]_D^{20}$ −2.0° (*c* 1.5, chloroform) (Found: OMe, 44.6%). The molecular weight (ebulliometry in benzene)[10] was 4850–5850, equivalent to d.p. 24–28.

Methanolysis of fraction 2′ (Table II) and g.l.c. of the methyl glycosides gave a molar ratio of the methylated sugars of 1.0:0.15:5.50:2.15:0.8, similar to those of fraction 1′. Another sample (150 mg) of fraction 2′ was methanolysed and then hydrolysed with M hydrochloric acid (10 ml, 100°, 12 h). After neutralisation and removal of the silver ions as sulphide, the hydrolysate was evaporated and the product was fractionated by chromatography on Whatman No. 3MM paper (irrigant *4*). The three main fractions were 2,3,4,6-tetra-*O*-methyl-D-mannopyranose (15.3 mg), 2,3,6-tri-*O*-methyl-D-mannose and 2,3,6-tri-*O*-methyl-D-glucose (108 mg), and di-*O*-methyl-D-hexoses (10.6 mg). The molar ratio of 1.0:7.5:0.78 was in good agreement with that given above.

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