SULPHATED POLYSACCHARIDES OF THE *Grateloupiaceae* FAMILY

PART VII[1]. INVESTIGATION OF THE ACETOLYSIS PRODUCTS OF A PARTIALLY DESULPHATED SAMPLE OF THE POLYSACCHARIDE OF *Pachymenia carnosa*

A. J. FARRANT, J. R. NUNN, AND H. PAROLIS*

Chemistry Department, Rhodes University, Grahamstown (South Africa)

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ABSTRACT

Investigation of the acetolysis products of a partially desulphated sample of the polysaccharide isolated from *Pachymenia carnosa* led to the isolation and characterization of the following oligosaccharides: 3-*O*-α-D-galactopyranosyl-D-galactose (**1**), 4-*O*-β-D-galactopyranosyl-D-galactose (**2**), 3-*O*-(2-*O*-methyl-α-D-galactopyranosyl)-D-galactose (**3**), a 4-*O*-galactopyranosyl-2-*O*-methylgalactose (**4**), 3-*O*-α-D-galactopyranosyl-6-*O*-methyl-D-galactose (**5**), 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose (**6**), 2-*O*-methyl-4-*O*-(6-*O*-methyl-β-D-galactopyranosyl)-D-galactose (**14**), *O*-β-D-galactopyranosyl-(1→4)-*O*-α D-galactopyranosyl-(1→3)-D-galactose (**8**), *O*-α-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-galactose (**9**), *O*-β-D-galactopyranosyl-(1→4)-*O*-α-(2-*O*-methyl-D-galactopyranosyl)-(1→3)-D-galactose (**11**), *O*-α-(2-*O*-methyl-D-galactopyranosyl)-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-galactose (**12**), *O*-α-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-2-*O*-methyl-D-galactose (**13**), *O*-α-(2-*O*-methyl-D-galactopyranosyl)-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-2-*O*-methyl-D-galactose (**16**), and *O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-galactose (**10**). In addition, evidence was obtained for the presence of 4-*O*-(6-*O*-methyl-β-D-galactopyranosyl)-D-galactose (**7**) and *O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-galactopyranosyl-(1→3)-6-*O*-methyl-D-galactose (**15**).

INTRODUCTION

Pachymenia carnosa, a red seaweed of the *Grateloupiaceae* family, yields a methylated, sulphated galactan[1], which on acid hydrolysis affords D-galactose, 2-*O*-methyl-D-galactose, 6-*O*-methyl-D-galactose, 4-*O*-methylgalactose, xylose, and sulphate in the molar ratios 6.15:1.0:0.52:0.21:0.03:6.59. The majority of the ester sulphate groups present in the polymer were shown[1] to be alkali-stable. Partial hydrolysis[1] of the polysaccharide resulted in the isolation of 4-*O*-β-D-galactopyranosyl-D-galactose (**2**), 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose (**6**), 3-*O*-(2-*O*-methyl-D-galactopyranosyl)-D-galactose, 4-*O*-(6-*O*-methyl-β-D-galactopyrano-

*To whom correspondence should be addressed.

TABLE I

OLIGOSACCHARIDES OBTAINED BY ACETOLYSIS

	Oligosaccharide[a]	*Total hydrolysis product*	*Partial hydrolysis products*	*Reducing partial hydrolysis products (after reduction)*	*Hydrolysis or methanolysis products of methylated oligosaccharide*[d]	*D.p.*	*Reaction with triphenyltetrazolium chloride*
1	α-D-Gal-(1→3)-D-Gal (syrup)	Gal			A, B	1.9	
2	β-D-Gal-(1→4)-D-Gal (cryst.)	Gal			A, C 1:1		
3	α-D-2Gal-(1→3)-D-Gal (syrup)[b]	Gal, 2Gal	Gal, 2Gal	2Gal	A, B	2.0	+
4	D-Gal-(1→4)-D-2Gal (syrup)	Gal, 2Gal	Gal, 2Gal	Gal	A, C	2.2	−
5	α-D-Gal-(1→3)-D-6Gal (syrup)		Gal, 6Gal	Gal	A, B	2.1	
6	β-D-Gal-(1→4)-D-2Gal (cryst.)	Gal, 2Gal	Gal, 2Gal	Gal	A, C 1:1.1		
7	β D-6Gal-(1→4)-D-Gal (paper chromatog.)	Gal, 6Gal	Gal, 6Gal	6Gal	A, C 1:1		
8	β-D-Gal-(1→4)-α-D-Gal-(1→3)-D-Gal (cryst.)	Gal	Gal, 1, 2	Gal, 2	A, B, C	3.0	
9	α-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Gal (cryst.)		Gal, 1, 2	Gal, 1			
10	β-D-Gal-(1→4)-α-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Gal (syrup)	Gal	Gal, 1, 2, 8, 9	Gal, 1, 2, 8		4.0	
11	β-D-Gal-(1→4)-α-D-2Gal-(1→3)-D-Gal (syrup)		Gal, 2Gal, 3, 6	Gal, 2Gal, 6			+
12	α-D-2Gal-(1→3)-β-D-Gal-(1→4)-D-Gal (cryst.)		Gal, 2Gal, 2, 3	Gal, 2Gal, 3	A, B, C	3.0	+
13	α-D-Gal-(1→3)-β-D-Gal-(1→4)-D-2Gal (syrup)	Gal, 2Gal	Gal, 2Gal, 1, 6	Gal, 1	A, B. C,	2.9	−
14	β-D-6Gal-(1→4)-D-2Gal (syrup)[c]		2Gal, 6Gal	6Gal	A, C 1:1.1		
15	β-D-Gal-(1→4)-α-D-Gal-(1→3)-D-6Gal	Gal, 6Gal	Gal, 6Gal, 5, 2	Gal, 2			
16	α-D-2Gal-(1→3)-β-D-Gal-(1→4)-D-2Gal (syrup)		Gal, 2Gal, 3, 6	Gal, 2Gal, 3	A, B, C	2.8	−

[a]2Gal, 2-*O*-methylgalactose; 6-Gal, 6-*O*-methylgalactose. [b]Also obtained on partial hydrolysis of the polysaccharide. [c]Chromatographically identical with 2-*O*-methyl-4-*O*-(6-*O*-methyl-β-D-galactopyranosyl)-D-galactose. [d]A, 2,3,4,6-tetra-*O*-methylgalactose; B, 2,4,6-tri-*O*-methylgalactose; C, 2,3,6-tri-*O*-methylgalactose.

syl)-D-galactose (**7**), 2-*O*-methyl-4-*O*-(6-*O*-methyl-β-D-galactopyranosyl)-D-galactose and a 6-*O*-methyl-(2-*O*-methyl-D-galactopyranosyl)-D-galactose (**17**).

The partial-hydrolysis study suggests that the polymer contains mainly β-(1→4) links with a smaller number of (1→3) links. However, since (1→3)-linked galactose residues are more readily hydrolysed with aqueous mineral acid than are (1→4)-linked residues, it is possible that the proportion of (1→3)-linked units present in the polymer is greater than indicated by this study. We now report the results of an acetolysis study carried out on the partially desulphated polysaccharide in order to obtain a better estimate of the structural significance of (1→3)-linked units in the polymer, since it is known that (1→4)-linked galactose residues are acetolysed preferentially[5].

RESULTS AND DISCUSSION

A partially desulphated sample of the polysaccharide was used for this investigation, because of the possible effect of the large number of sulphate groups present in the native polymer on the cleavage pattern. After acetolysis and deacetylation, acidic material was removed from the mixture, and the neutral oligosaccharides were fractionated by a combination of gradient elution from a charcoal–Celite column and paper chromatography. The oligosaccharides isolated are listed in Table I, together with data essential for the determination of their structures.

Oligosaccharide **4** was chromatographically distinguishable from the crystalline 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose. The low, positive value for the specific rotation of the disaccharide suggests that one of the monomers occurs as the L-isomer, but insufficient material was available for this to be verified. Although neither 2-*O*-methyl-L-galactose nor L-galactose was isolated from hydrolysates of this polysaccharide, 2-*O*-methyl-L-galactose has been isolated from the polysaccharide of *Grateloupia elliptica*[2], and the polysaccharides of *Aeodes orbitosa*[3] and *Phyllymenia cornea*[4] (all *Grateloupiaceae*) contain minor proportions of L-galactose.

Oligosaccharides **1**, **8**, and **9** have been isolated[5] from the acetolysis products of λ-carragenan, and **2** and **6** have been previously isolated[4] from the partial hydrolysis products of phyllymenan.

G.l.c. investigation of the methanolysate of the methylated, (1→3)-linked disaccharides revealed, in addition to the peaks due to the methyl glycosides of 2,4,6-tri- and 2,3,4,6-tetra-*O*-methylgalactose, a peak at *T* 4.41 (column *1*). This latter peak appears to be due to the presence of a non-reducing degradation product formed during the methylation of the oligosaccharides, since (*a*) no additional products were revealed on paper or thin-layer chromatograms of an acid hydrolysate of the methylated oligosaccharides when sprayed with *p*-anisidine hydrochloride reagent, and (*b*) methylation of a methanolysed sample of methylated **1**, followed by g.l.c., revealed a peak at *T* 1.48 (column *1*) in addition to a peak with the retention time of methyl 2,3,4,6-tetra-*O*-methylgalactosides. It is not yet certain whether the degradation product is produced during the methylation of those trisaccharides having (1→3)-

linkages on their reducing end, since T 4.41 coincides with one of the peaks given by methyl 2,3,6-tri-*O*-methylgalactosides.

Significantly, a large number of the oligosaccharides from the acetolysate contain α-(1→3)-glycosidic links. All the oligosaccharides previously obtained on partial hydrolysis of the polysaccharide, with the exception of **17**, were isolated from the acetolysis products. All the trisaccharides obtained were found to contain both α-(1→3) and β-(1→4) linkages, and that these probably occur in an alternating sequence is supported by the isolation of the tetrasaccharide **10**. This type of alternating structure is well established in several galactans isolated from red seaweeds[6].

At present, no unique repeating-unit can be proposed for the polysaccharide. However, it is fairly clear that (*a*) all the D-galactose residues occur linked either through position 3 or position 4, and that these linkages occur in an alternating sequence, (*b*) all the 6-*O*-methyl-D-galactose residues appear to be linked through position 3, and can be linked to either position 4 of a 2-*O*-methyl-D-galactose or D-galactose residue, (*c*) the 2-*O*-methyl-D-galactose residues appear to be linked exclusively through position 4, and may be linked to position 3 of either a 6-*O*-methyl-D-galactose or D-galactose residue. No information is at present available concerning the structural significance of the 4-*O*-methylgalactose residues in the polymer. All these results apply to the unfractionated polymer.

EXPERIMENTAL

The analytical methods used were described in Part VI[1]. In addition, the following spray (*iv*) was used: 2% *p*-anisidine hydrochloride in butyl alcohol containing 5% of 0.1M hydrochloric acid. Paper electrophoresis was performed on Whatman No. 1 paper with 0.4M borate buffer (pH 10) at 50 mamp (solvent *f*). M_{GAL} values refer to rates of movement of sugars relative to D-galactose on pherograms. The degree of polymerisation (d.p.) of oligosaccharides was determined by the phenol–sulphuric acid method[7]. G.l.c. separations of methyl glycosides were made on columns containing (*1*) 15% by weight of poly(butane-1,4-diol succinate) on acid-washed Celite (80–100 mesh) at 175° and (*2*) 5% of neopentylglycol adipate on Chromasorb W (80–100 mesh; acid-washed) at 160°.

Acetolysis of partially desulphated polysaccharide. — Polysaccharide (39.6 g; SO_4^{2-}, 30.3%) (dried in *vacuo* over P_2O_5 for 48 h) was shaken with 0.15M methanolic hydrogen chloride (2 l) for 60 h at room temperature. The insoluble material was removed by centrifugation, dissolved in water, and dialysed against distilled water for 7 days. The partially desulphated material (28.0 g; SO_4^{2-}, 11.5%) was isolated by freeze-drying.

Dry, partially desulphated polysaccharide (28.0 g) was added to a mixture of acetic anhydride (155 ml), acetic acid (112 ml), and sulphuric acid (15.5 ml) with continual stirring, over a period of 0.5 h[5]. The resultant solution was shaken for 96 h at room temperature, clarified by centrifugation, mixed with ice-cold water (1.6 l), and neutralised ($NaHCO_3$) to Congo Red. The precipitated oligosaccharide acetates

were removed by filtration, and the solution was extracted with chloroform (3 × 500 ml). The extracts were combined with a solution of the precipitate in chloroform, dried (sodium sulphate), filtered, and evaporated to a syrup. Addition of water and freeze-drying gave a mixture of oligosaccharide acetates (31.2 g).

Deacetylation[8] was carried out by vigorously stirring the mixture of oligosaccharide acetates (31.2 g) with sodium methoxide (0.2M; 372 ml) for 1 h at 25–30°. The suspension was made slightly acid with acetic acid, and water was added to dissolve the precipitated oligosaccharides. The solution was deionized, by passage first through a column (3 × 36 cm) of Amberlite IR-120(H^+) resin and then through a column (3.7 × 30 cm) of Amberlite IRA-400 (AcO^-) resin, and concentrated, and the mixture of oligosaccharides was isolated by freeze-drying (19.75 g). Paper chromatography (solvent *a*) revealed numerous products with $R_{GAL} < 1$, in addition to substances with the mobilities of galactose and monomethylgalactoses. The infrared spectrum showed no ester carbonyl peak.

A portion (10.6 g) of the oligosaccharide mixture was applied to a charcoal–Celite column (5.4 × 61 cm; 1:1 w/w), which was eluted with water (8 l) and then with a linear gradient of aqueous ethanol (0→35%) over 58 l. Fractions were collected, and, on the basis of paper chromatography (solvents *a* and *d*), were recombined into the following 13 fractions. The column was finally washed with 10% butanone (5 l).

Fraction 1. The partially crystalline syrup (2.46 g), eluted with water (8 l) and aqueous ethanol (0→6.2%; 10.5 l), was shown by paper chromatography to be a mixture of galactose, xylose (trace), and 2-, 4-, and 6-*O*-methylgalactose.

Fraction 2. The chromatographically and electrophoretically pure syrup (239 mg); R_{GAL} 0.23 (solvent *d*), 0.36 (solvent *a*); M_{GAL} 0.66 (solvent *f*); d.p. 1.9; eluted with 6.2→6.9% aqueous ethanol (1.2 l), had $[\alpha]_D^{20}$ +150° (*c* 0.80). It gave only galactose on hydrolysis. The derived phenylosazone[9], after recrystallisation from water, had m.p. 233–234°; (lit:[5] 234–236° for 3-*O*-α-D-galactopyranosyl-D-galactose phenylosazone). A solution of the disaccharide (8 mg) in methyl sulphoxide (0.6 ml) and *N*,*N*-dimethylformamide (0.6 ml), was stirred with barium oxide (0.2 g) and barium hydroxide octahydrate[10] (0.1 g) under nitrogen with cooling. Methyl sulphate (0.3 ml) was added slowly and the mixture stirred for 24 h at room temperature. Excess methyl sulphate was destroyed with ammonia (0.3 ml), and the gel extracted with chloroform (5 × 3 ml). The extracts were washed with water (10-ml portions) until neutral and dried ($MgSO_4$). Methylation was found to be complete (t.l.c. spray *iii*). Examination by paper chromatography (solvent *d*, spray *i*) of an acid hydrolysate (0.5M H_2SO_4, 2 h, 100°) of the methylated oligosaccharide revealed 2,3,4,6-tetra-*O*-methylgalactose and 2,4,6-tri-*O*-methylgalactose as the only reducing products. The methylated product was refluxed with 3% methanolic hydrogen chloride for 4 h and the derived methyl glycosides were examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-*O*-methylgalactose [*T* 1.79 (column *1*); 1.52, 1.58 (column *2*)] and 2,4,6-tri-*O*-methylgalactose [*T* 4.26, 4.65 (column *1*); 2.97, 3.35 (column *2*)] were observed. A further peak [*T* 4.41 (column *1*); 3.24 (column *2*)] was also present. This latter peak is considered to be a degradation product, arising as a result of the alkaline methylation

conditions, since if the methylated, methanolysed product is treated with Purdie's reagents[11] and then examined by g.l.c. (column *1*) a peak with *T* 1.48 is obtained in addition to that of 2,3,4,6-tetra-*O*-methylgalactose (*T* 1.79). The oligosaccharide was methylated by a variety of methods: *viz.* by the procedure of Haq and Percival[12], completed by treatment with Purdie's reagents[11]; formation of the methyl glycosides by treatment with methanolic hydrogen chloride before methylation; and methylation by the Kuhn and Trischmann[10] procedure, but without *N*,*N*-dimethylformamide. In all cases, the peak at *T* 4.41 occurred, but to a varying extent. The above evidence indicates that this disaccharide is 3-*O*-α-D-galactopyranosyl-D-galactose (**1**). The α-D configuration is assumed from the specific rotation of the compound.

Fraction 3. The syrup (421 mg), eluted with 6.9→9.2% aqueous ethanol (3.6 l), was shown by electrophoresis (solvent *f*) to be a mixture of 3-*O*-α-D-galactopyranosyl-D-galactose and a sugar electrophoretically equivalent to 4-*O*-β-D-galactopyranosyl-D-galactose (**2**). The latter, which crystallised from the mixture when triturated with aqueous methanol, had m.p. 211–212° alone and in admixture with 4-*O*-β-D-galactopyranosyl-D-galactose, $[\alpha]_D^{20}$ +79° (3 min) → +66° (equil., *c* 0.53), R_{GAL} 0.39 (solvent *a*), 0.26 (solvent *d*), M_{GAL} 0.53 (solvent *f*). The infrared spectrum was identical to that obtained from an authentic sample of 4-*O*-β-D-galactopyranosyl-D-galactose[3].

Fraction 4. The syrup (186 mg), eluted with 9.2→10.2% aqueous ethanol (2.0 l), contained two major components in addition to 4-*O*-β-D-galactopyranosyl-D-galactose. Separation of the syrup on Whatman No. 1 paper (solvent *a*, 3 days) and extraction of the appropriate portions of the papers with 50% aqueous methanol afforded each component chromatographically pure.

Oligosaccharide **3**. The syrup (56 mg), R_{GAL} 0.62 (solvent *a*), 0.36 (solvent *d*), d.p. 2.0, $[\alpha]_D^{20}$ +175° (*c* 0.67), gave galactose and 2-*O*-methylgalactose on partial, acid hydrolysis (0.5M H_2SO_4, 15 min, 100°), and was revealed with spray (*ii*). It was chromatographically identical (solvents *a*–*d*) to the 3-*O*-(2-*O*-methylgalactopyranosyl)-galactose obtained on partial hydrolysis of the polysaccharide[1]. An α-D configuration is assumed from the optical rotation of the compound, and the oligosaccharide is thus 3-*O*-(2-*O*-methyl-α-D-galactopyranosyl)-D-galactose.

Oligosaccharide **4**. The syrup (12 mg), which had R_{GAL} 0.80 (solvent *a*), 0.44 (solvent *d*), d.p. 2.2, $[\alpha]_D^{20}$ +7° (*c* 0.58), failed to react with spray (*ii*). Partial, acid hydrolysis revealed (paper chromatography) the presence of galactose and 2-*O*-methylgalactose in addition to the original material, and total hydrolysis gave only galactose and 2-*O*-methylgalactose, in approximately equal amounts. Disaccharide (1 mg) in water (1 ml) was reduced with sodium borohydride (5 mg) for 16 h. The solution was acidified with Amberlite IR-120(H^+) resin, and the boric acid was removed by distillation with methanol. Partial hydrolysis of the non-reducing syrup, followed by paper chromatography, revealed galactose as the only reducing sugar (spray *i*). A portion (2 mg) was methylated with methyl sulphate and barium oxide/hydroxide[10]. Hydrolysis with 0.5M sulphuric acid for 2 h, followed by paper chromatography (solvent *d*), showed 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylgalactose only. Methanolysis, followed by neutralisation with silver carbonate

and analysis by g.l.c., showed peaks corresponding to 2,3,4,6-tetra-*O*-methylgalactose [*T* 1.79 (column *1*); 1.53, 1.60 (column *2*)] and 2,3,6-tri-*O*-methylgalactose (*T* 3.32, 4.40, 4.72 (column *1*); 2.40, 2.95, 3.04, 3.33 (column *2*)]. The above results indicate that the oligosaccharide is a 4-*O*-galactopyranosyl-2-*O*-methylgalactose. The disaccharide is chromatographically distinguishable (solvents *a* and *d*) from 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose. It is tentatively suggested that one of the monomers occurs as the L isomer.

Fraction 5. The syrup (146 mg), eluted with aqueous ethanol (10.2→10.8%; 1.1 l), was found by paper chromatography to be a mixture of Fractions 4 and 6.

Fraction 6. The syrup (401 mg), eluted with 10.8→12.2% aqueous ethanol (2.3 l), was shown (paper chromatography) to contain 2 major oligosaccharides. Separation of a portion (202 mg) of the syrup on Whatman No. 1 paper (solvent *d*, 4 days), followed by extraction of the relevant portions of the paper with methanol–water (1:1), yielded the following two products.

Oligosaccharide **5.** The chromatographically pure syrup (80 mg), R_{GAL} 0.75 (solvent *a*), 0.52 (solvent *d*), d.p. 2.1, had $[\alpha]_D^{20}$ +188° (*c* 0.50). Galactose and 6-*O*-methylgalactose were produced on partial, acid hydrolysis of a sample, whereas reduction followed by hydrolysis yielded galactose as the only reducing sugar. A portion (4.4 mg) was methylated by using the procedure of Kuhn and Trischmann[10]. Paper-chromatographic examination of a hydrolysate of the methylated product revealed 2,3,4,6-tetra-*O*-methylgalactose and 2,4,6-tri-*O*-methylgalactose. This was confirmed by t.l.c. (spray *iii*). The methanolysed product gave peaks on g.l.c. equivalent to 2,3,4,6-tetra-*O*-methylgalactose [*T* 1.78 (column *1*); 1.53, 1.59 (column *2*)] and 2,4,6-tri-*O*-methylgalactose [*T* 4.18, 4.66 (column *1*); 2.98, 3.36 (column *2*)]. The peak at *T* 4.41 (column *1*), obtained on methylation of 3-*O*-α-D-galactopyranosyl-D-galactose, was also present. The oligosaccharide is assigned the structure 3-*O*-α-D-galactopyranosyl-6-*O*-methyl-D-galactose, the α-D configuration being assumed from the optical rotation.

Oligosaccharide **6.** The crystalline disaccharide (48 mg), R_{GAL} 0.96 (solvent *a*), 0.76 (solvent *d*), $[\alpha]_D^{20}$ +85° (4 min) → +70° (equil., *c* 0.47), had m.p. 217–218° alone and in admixture with 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose. The two compounds had identical infrared spectra.

Fraction 7. The fraction (412 mg), eluted with aqueous ethanol (12.2→14.2%; 3.2 l), was shown by paper chromatography to contain the oligosaccharides of fraction 8 and a substance, R_{GAL} 0.96 (solvent *a*), 0.68 (solvent *d*), chromatographically identical with 4-*O*-(6-*O*-methyl-β-D-galactopyranosyl)-D-galactose[1] (7).

Fraction 8. The syrup (361 mg), eluted with 14.2→15.7% aqueous ethanol (2.9 l), crystallised from methanol on standing. The oligosaccharide **8** (143 mg), R_{GAL} 0.09 (solvent *a*), 0.06 (solvent *d*), d.p. 3.0, had $[\alpha]_D^{20}$ +130° (*c* 0.60), m.p. 233–236°, after recrystallisation from methanol. On partial, acid hydrolysis (0.5M H_2SO_4, 15 min, 100°), the oligosaccharide gave products electrophoretically identical with oligosaccharides **1** and **2**, in addition to galactose, whereas total hydrolysis yielded galactose only. Partial hydrolysis of a sample (2 mg) after borohydride reduction

gave oligosaccharide **2** as the only reducing disaccharide. Methylation[10] and methanolysis of a further reduced sample (5 mg) showed, on g.l.c., peaks corresponding to 2,3,4,6-tetra-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, and a penta-*O*-methylgalactitol (*T* 0.54, column *1*). The oligosaccharide (5 mg) was methylated with barium oxide/hydroxide[10] under nitrogen, hydrolysed, and examined by paper chromatography (solvent *d*) and t.l.c. In both cases, spots having the mobilities of 2,3,4,6-tetra-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, and 2,4,6-tri-*O*-methylgalactose were obtained. A methanolysed sample gave, on g.l.c. analysis, peaks corresponding to 2,3,4,6-tetra-*O*-methylgalactose [*T* 1.78 (column *1*); 1.53, 1.59 (column *2*)] and the mixture of tri-*O*-methylgalactoses [*T* 3.32, 4.41, 4.70 (column *1*); 2.39, 3.01, 3.32 (column *2*)]. The oligosaccharide is thus assigned the structure *O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-galactopyranosyl-(1→3)-D-galactose.

The supernatant solution was concentrated to a syrup (132 mg) and separated by paper chromatography (Whatman No. 1, solvent *a*, 10 days). Extraction of the relevant portions of paper with 50% aqueous methanol yielded pure oligosaccharide **9** (14 mg), R_{GAL} 0.14 (solvent *a*), 0.06 (solvent *d*), $[\alpha]_D^{20}$ +126° (3 min) → +146° (equil., *c* 0.38), which readily crystallised from aqueous ethanol, and after recrystallisation from the same solvent had m.p. 232–233°. On partial hydrolysis, galactose and oligosaccharides **1** and **2** were obtained (solvent *f*), whereas partial hydrolysis of a reduced sample gave oligosaccharide **1** as the only reducing disaccharide. Thus, the oligosaccharide is *O*-α-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-galactose.

Fraction 9. The syrup (277 mg), eluted with 15.7→17.3% aqueous ethanol (2.8 l), contained 4 major oligosaccharides. Separation in solvent *a* (7 days) yielded the following chromatographically pure products.

Oligosaccharide **10.** The syrup (6 mg) had R_{GAL} 0.05 (solvent *a*), 0.01 (solvent *d*), d.p. 4.0, and $[\alpha]_D^{20}$ +115° (*c* 0.71). Paper chromatography of a total hydrolysate gave a product with the mobility of galactose. On partial hydrolysis, products corresponding to oligosaccharides **1**, **2**, **8**, and **9** were obtained in addition to the original sugar (solvents *a* and *f*). Reduction of a portion, followed by partial hydrolysis and paper chromatography, showed the presence of oligosaccharides **1**, **2**, and **8**. The above evidence indicates that **10** is *O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-galactose.

Oligosaccharide **11.** The syrup (6.5 mg), R_{GAL} 0.22 (solvent *a*), 0.05 (solvent *d*), $[\alpha]_D^{20}$ +100° (*c* 0.50), which was revealed with spray (*ii*), gave oligosaccharides **3** and **6**, in addition to galactose and 2-*O*-methylgalactose, on partial hydrolysis. On reduction, followed by partial hydrolysis, galactose, 2-*O*-methylgalactose, and oligosaccharide **6** were obtained. Thus, the oligosaccharide is assigned the structure *O*-β-D-galactopyranosyl-(1→4)-*O*-α-(2-*O*-methyl-D-galactopyranosyl)-(1→3)-D-galactose.

Oligosaccharide **12.** The sugar (14 mg), m.p. 263–264° (from methanol), had R_{GAL} 0.25 (solvent *a*), 0.11 (solvent *d*), $[\alpha]_D^{20}$ +142° (*c* 0.55), d.p. 3.0, and was revealed with spray (*ii*). Partial, acid hydrolysis produced oligosaccharides **2** and **3**, galactose, and 2-*O*-methylgalactose, and hydrolysis of the derived oligosaccharide alcohol gave galactose, 2-*O*-methylgalactose, and oligosaccharide **3** as the reducing products.

A portion (3 mg) was methylated by the method of Kuhn and Trischmann[10]. Paper-chromatographic (solvent *d*) and t.l.c. examination of the acid hydrolysate revealed spots with the mobilities of 2,3,4,6-tetra-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, and 2,4,6-tri-*O*-methylgalactose. This trisaccharide is thus *O*-(2-*O*-methyl-α-D-galactopyranosyl)-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-galactose.

Oligosaccharide **13**. The syrup (56 mg), which was not revealed with spray (*ii*), had R_{GAL} 0.34 (solvent *a*), 0.16 (solvent *d*), $[\alpha]_D^{20}$ +153° (*c* 0.45), and d.p. 2.9. On partial, acid hydrolysis, it gave oligosaccharides **1** and **6**, galactose, and 2-*O*-methylgalactose; total hydrolysis gave galactose and 2-*O*-methylgalactose in the approximate ratio 2:1. Treatment of a portion (2 mg) with sodium borohydride, followed by partial, acid hydrolysis, gave oligosaccharide **1** and galactose as the only reducing products. Methylation, followed by hydrolysis and paper chromatography (solvent *d*) and by methanolysis and g.l.c., showed 2,3,4,6-tetra-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, and 2,4,6-tri-*O*-methylgalactose or their glycosides, respectively. Thus, the oligosaccharide is assigned the structure *O*-α-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-2-*O*-methyl-D-galactose.

Fraction 10. The syrup (305 mg), eluted with aqueous ethanol (17.3→18.7%; 2.1 l), was shown by chromatography to be a mixture of several oligosaccharides. The two major components were obtained by paper-chromatographic separation (3 days) of a portion (94 mg) in solvent (*d*).

Oligosaccharide **14**. The syrup (4 mg), which had R_{GAL} 1.97 (solvent *a*), 1.70 (solvent *d*), gave 2-*O*-methylgalactose and 6-*O*-methylgalactose on partial, acid hydrolysis, and only 6-*O*-methylgalactose on reduction followed by hydrolysis. This oligosaccharide is chromatographically identical to 2-*O*-methyl-4-*O*-(6-*O*-methyl-β-D-galactopyranosyl)-D-galactose obtained from the partial hydrolysis studies[1].

The syrup (47 mg), R_{GAL} 0.34 (solvent *a*), 0.16 (solvent *d*), was chromatographically and electrophoretically homogeneous in all the solvents used. Acid hydrolysis of the fraction produced galactose, 2-*O*-methylgalactose, and 6-*O*-methylgalactose; partial hydrolysis gave the oligosaccharides **1**, **2**, **5**, and **6**, in addition to the above monomers. Reduction, followed by partial, acid hydrolysis and examination by electrophoresis (solvent *f*), gave oligosaccharides **1** and **2** and galactose (spray *iv*). The above results can be explained by assuming the fraction to be a mixture of oligosaccharide **13**, *O*-α-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-2-*O*-methyl-D-galactose, and *O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-galactopyranosyl-(1→3)-6-*O*-methyl-D-galactose (**15**).

Fraction 11. The syrup (357 mg), eluted with aqueous ethanol (18.7→20.4%; 2.8 l), contained several slow-moving products (paper chromatography) and was not further investigated.

Fraction 12. The syrup (292 mg) was eluted with 20.4→22.1% aqueous ethanol (2.9 l). The major component (26 mg) was obtained by fractionation of a portion (136 mg) on Whatman No. 1 paper (solvent *a*, 6 days) and had R_{GAL} 0.53 (solvent *a*), 0.28 (solvent *d*), d.p. 2.8, $[\alpha]_D^{20}$ +126° (*c* 0.46); it was not revealed with spray (*ii*). Partial, acid hydrolysis of a portion yielded oligosaccharides **3** and **6**, galactose, and

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REFERENCES

1 A. LINKER AND R. S. JONES, *Nature* (London), 204 (1964) 187; *J. Biol. Chem.*, 241 (1966) 3845.
2 P. A. J. GORIN AND J. F. T. SPENCER, *Can. J. Chem.*, 44 (1966) 993.
3 E. L. HIRST AND D. A. REES, *J. Chem. Soc.*, (1965) 1182.
4 D. A. REES AND J. W. B. SAMUEL, *J. Chem. Soc.*, (1967) 2295.
5 A. HAUG, B. LARSEN, AND O. SMIDSRØD, *Acta Chem. Scand.*, 20 (1966) 183.
6 A. HAUG, B. LARSEN, AND O. SMIDSRØD, *Acta Chem. Scand.*, 21 (1967) 691.
7 A. PENMAN, M. J. TAIT, F. W. WOOD, AND R. A. JONES, unpublished results.
8 E. R. MORRIS, D. A. REES, AND D. THOM, to be published; for a preliminary account, see D. A. REES, *Biochem. J.*, in press.
9 A. HAUG, S. MYKLESTAD, B. LARSEN, AND O. SMIDSRØD, *Acta. Chem. Scand.*, 21 (1967) 768.
10 Z. DISCHE, *J. Biol. Chem.*, 167 (1947) 189.
11 C. A. KNUTSON AND A. JEANES, *Anal. Biochem.*, 24 (1968) 470.
12 C. A. KNUTSON AND A. JEANES, *Anal. Biochem.*, 24 (1968) 482.
13 A. PENMAN AND A. J. INGRAM, unpublished results.
14 A. HAUG AND B. LARSEN, *Acta Chem. Scand.*, 16 (1962) 1908.
15 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
16 E. R. MORRIS, D. A. REES, AND G. R. SANDERSON, unpublished results.
17 E. D. T. ATKINS, W. MACKIE, AND E. E. SMOLKO, *Nature* (London), 225 (1970) 626; *Polymer Lett.*, 9 (1971) 311.
18 E. L. HIRST, E. PERCIVAL, AND J. K. WOLD, *J. Chem. Soc.*, (1964) 1493.
19 A. S. PERLIN, B. CASU, G. R. SANDERSON, AND J. TSE, *Carbohyd. Res.*, 21 (1972) 123.
20 A. S. PERLIN, B. CASU, G. R. SANDERSON, AND L. F. JOHNSON, *Can. J. Chem.*, 48 (1970) 2260.
21 A. S. PERLIN, D. M. MACKIE, AND C. P. DIETRICH, *Carbohyd. Res.*, 18 (1971) 185.
22 O. SMIDSRØD, A. HAUG, AND B. LARSEN, *Acta Chem. Scand.*, 17 (1963) 2628.
23 A. HAUG AND B. LARSEN, *Carbohyd. Res.*, 17 (1971) 297.